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SUSTAINING *GNETUM* L. (GNETACEAE) IN AFRICA THROUGH IMPROVED TAXONOMY AND DOMESTICATION

Elvire Hortense Biye

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Doctor of Philosophy

Johannesburg, 2013

DECLARATION

I hereby declare that this thesis is my personal work. The articles are the results of my original work and writing; and I received guidance and assistance from my supervisors to a reasonable extent. The thesis is submitted for the fulfillment of the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg and I attest that it has not been submitted before for any other degree or examination at any other University.

_____

The 30th day of August 2013 in Johannesburg

ABSTRACT

Gnetum L. (Gnetales) plants are useful gymnosperms in Africa that alleviate poverty in the local community as they are widely sold and used as vegetable ('*eru*'). The African species of this genus are however difficult to distinguish from one another, are threatened due to over-exploitation and therefore face the risk of disappearing before they are adequately known. This study aims to clarify the taxonomy of the vulnerable *Gnetum* species in Africa and to promote sustainable utilization and conservation of the species. Thus, sustainable strategies involve, on the one hand, the identification of various species suitable for conservation and, on the other hand, the supply of management guidelines for cultivation based on taste data. The specific objectives undertaken in the genus *Gnetum* in Africa are to:

- investigate the various species to be recognized in the genus in Africa and the important features that distinguish them.
- determine how the cultivated material relates to the naturally occurring taxa.
- elucidate species limits based on genetic patterns.
- identify anatomical features useful to distinguish species in general and to match female plants to corresponding males in particular.
- evaluate any variation in taste between *Gnetum* populations.
- examine the mechanism involved in seed germination in order to promote their sustainable utilization in cultivation.

To address the taxonomic problems in the genus *Gnetum* in Africa, the types and the original descriptions were used to identify and help attribute names to the various forms; numerical analyses of morphological characters were carried out to illustrate the distribution of any variation among various specimens; molecular and anatomical tools were also used to reveal additional characters to differentiate and recognize species and to match male and female specimens. In an attempt to sustain *Gnetum* plants in Africa so that the lives of those who depend on it are not affected in the near future, several strategies are undertaken. Taste analysis was applied to explore species with the best taste

for promotion and the type of leaves to harvest thus limiting mass destruction of the entire *Gnetum* plant. So variation in taste of the leaves of *Gnetum* ('eru' meal) with respect to species, provenance and age of leaf was investigated using both questionnaire survey and the gustatory approach. Seed germination trials were carried out as a possible means to selective cultivation that could result in the bulk production of *Gnetum* plants and so relieve pressure on natural populations.

Gnetum species in Africa present different morphological structures useful for taxonomic clarification. These species are better distinguished morphologically by their reproductive structures; the vegetative features vary within species and even on the same specimen. The original applications of the names *Gnetum africanum* Welw. and *Gnetum buchholzianum* Engl. have been clarified and two new African species have been identified: *Gnetum interruptum* E.H. Biye *sp. nov*. and *Gnetum latispicum* E.H. Biye *sp. nov*. A key to these species is provided and useful characters to recognize species in the genus *Gnetum* in Africa are discussed.

Taste analysis permitted the identification of specific factors for horticulturalists to know which species to propagate for leaves of *Gnetum.* For example in the course of the investigation, it was found that the leaves of *G. buchholzianum* taste best in 'eru' meals, that good *Gnetum* leaves for 'eru' were collected from every region but only the young leaves were best for 'eru' meals. In addition, seeds of *Gnetum* species in Africa are orthodox i.e. they exhibit dormancy but nevertheless they germinate under artificial or nursery conditions when exposed to the appropriate treatments. The study clarified the taxonomic status of *Gnetum* species in Africa and also provided information to identify the forms with good agronomic traits and the conservation strategy to explore in order to sustain *Gnetum* in Africa for future uses.

DEDICATION

To God, the Almighty, be the glory and praise for ever. He was always near and in control of all circumstances. It was through His divine guidance that this study is accomplished.

To
My daughter Athina and my son Yannick,
For the patience, endurance and perseverance of your hardship without parental affection and support during my absence from home.

And
To you, Roger,
Here are the results of your encouragement and confidence in me; may your soul rest in peace.

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I owe debts of gratitude to you, Hermine Ngalle and Tjol Lucie, for having encouraged me not to give up and helped manage my personal and my children's affairs in my absence. You are friends in need and indeed.

To my children, Yannick and Athina to whom I dedicate this thesis and to my dear Gilda, I am particularly indebted for always remembering me in prayers and for your constant loving support, encouragement and understanding. My mom Madeleine, my sisters, brothers and their families, Marie Madeleine Ngneba, Laure Mefan, looking after the children in my absence, Serge and Emile, you all gave me courage and determination to meet any challenge that came my way, I express my thanks.

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TABLE OF CONTENTS

CONTENTS			PAGE
TITLE			
DECLARATION			i
ABSTRACT			ii
DEDICATION			iv
ACKNOWLEDGEMENTS			v
TABLE OF CONTENTS			vi
LIST OF FIGURES			xi
LIST OF TABLES			xiv
LIST OF APPENDICES			xvi
CHAPTER ONE – INTRODUCTION			1
1.1	*Gnetum* Species: A 'Limitless Wealthy Resource in Africa		2
1.2	"What Then is a Species"		7
1.3	Approaches Used in This Study		14
1.4	The Contribution of This Study to Sustainable Use of *Gnetum* species		18
1.5	The Importance of Systematics for Biodiversity and Conservation		18
1.6	Motivation for this Study		19
1.7	The Aims, Objectives and Key Questions		22
1.8	Thesis Structure		24
1.9	References		25
CHAPTER TWO – SPECIES DELIMITATION IN THE GENUS *GNETUM* L. USING A PHENETIC APPROACH			34
2.0	Abstract		36
2.1	Introduction		37
	2.1.1	Importance of the Genus *Gnetum*	37
	2.1.2	Problem Statement	38
	2.1.3	Species/Infraspecies Recognition	39
	2.1.4	The Taxonomic Approach	42
	2.1.5	Purpose of this Research and Key Questions	44

2.2 Materials and Methods 45
2.2.1 Plant material 45
2.2.2 Morphometry 46
2.2.3 Data analysis 49
2.3 Results 51
2.3.1 Results Obtained Using the Male Specimens of *Gnetum* 51
2.3.2 Results Obtained Using the Female Specimens of *Gnetum* 65
2.4 Discussion 78
2.4.1 Taxonomic Implications and Useful Characters for Distinguishing Species. 78
2.5 Conclusion 85
2.6 References 86
2.7 Appendix 93

CHAPTER THREE – A CLARIFICATION OF *GNETUM* L. (GNETACEAE) IN AFRICA AND THE DESCRIPTION OF TWO NEW SPECIES 100

3.0 Abstract 101
3.1 Introduction 102
3.2 Materials and Methods 104
3.3 Results 105
3.3.1 Ecology, Distribution and Habitat 108
3.3.2 Conservation 110
3.4 Taxonomic Treatment 110
3.4.1 Key to Species of *Gnetum* in Africa 111
3.4.2 Description of Species of *Gnetum* in Africa 112
3.5 Acknowledgements 123
3.6 References 123

CHAPTER FOUR – MORPHOLOGICAL CORRELATION BETWEEN THE CULTIVATED AND WILD POPULATIONS OF *GNETUM* L. IN AFRICA 125

4.0 Abstract 127
4.1 Introduction 128

	4.1.1	Comparison between Wild and Cultivated Plants	129
	4.1.2	Problem Statement	130
	4.1.3	The Taxonomy of the Genus *Gnetum*	131
	4.1.4	Purpose of this Research and Key Questions	131
4.2	Materials and Methods		132
	4.2.1	Plant Material	132
	4.2.3	Morphological character examinrd and data Analysis	134
4.3	Results		135
	4.3.1	Phenetic Analysis using Male Specimens of *Gnetum*	135
	4.3.2	Phenetic Analysis using Female Specimens of *Gnetum*	146
4.4	Discussion		156
	4.4.1	Taxonomic Implications	156
4.5	Conclusion		158
4.6	References		158
4.7	Appendix		162

CHAPTER FIVE – THE ASSESSMENT OF GENETIC VARIATION IN *GNETUM* SPECIES IN AFRICA REVEALED BY AFLP ANALYSIS 166

5.0	Abstract		168
5.1	Introduction		169
	5.1.1	The Limitation of Vegetative Morphology in *Gnetum*	169
	5.1.2	Significance of Molecular Analysis to Taxonomy	170
	5.1.3	Efficacy of the AFLP technique	171
	5.1.4	Problem Statement	172
	5.1.5	Knowledge Gap	172
	5.1.6	Purpose of and Key Questions for this Research	173
5.2	Materials and Methods		174
	5.2.1	Plant Materials	174
	5.2.2	DNA Extraction	174
	5.2.3	AFLP Analysis	176
	5.2.4	Data Analysis	179
5.3	Results		180

5.3.1 Band Observations 180
5.3.2 Quantifying Genetic Diversity 181
5.3.3 Phenetic Analysis 183
5.4 Discussion 186
5.5 Conclusion 191
5.6 References 191
5.7 Appendices 200

CHAPTER SIX – UTILITY OF ANATOMICAL FEATURES IN DISTINGUISHING AFRICAN *GNETUM* SPECIES 203
6.0 Abstract 205
6.1 Introduction 206
6.1.1 Importance of Anatomical Features to taxonomy 206
6.1.2 Contribution of Anatomical Features to Taxonomy of *Gnetum* 208
6.1.3 Problem Statement 210
6.2 Materials and methods 210
6.2.1 Leaf Stomata Preparation 210
6.2.2 Petiole Preparation 211
6.3 Results 212
6.3.1 Characteristics of the Leaves of Four Forms of *Gnetum* in Africa 212
6.3.2 Characteristics of the Petiole of Four Forms of *Gnetum* in Africa 220
6.4 Discussion 225
6.5 Conclusion 230
6.5.1 Evidence for Interspecific Relationships in the Genus 230
6.6 References 231
6.7 Appendix 236

CHAPTER SEVEN – EVALUATION OF TASTE VARIATION BETWEEN VARIOUS POPULATIONS OF *GNETUM* L. IN AFRICA: A CONTRIBUTION TO SELECTIVE CULTIVATION 237
7.0 Abstract 239
7.1 Introduction 240
7.1.1 Significance of African Nutritional Resources 240

	7.1.2	Conservation Strategy	240
	7.1.3	Evaluation of Taste Ability	242
	7.1.4	Factors Influencing Plant Taste	243
	7.1.5	Problem Statement	245
	7.1.6	Purpose of this Research	246
7.2	Materials and Methods		246
7.3	Results		250
	7.3.1	Respondent Identification and Questionnaire Survey Data	250
	7.3.2	Taste Analysis of 'Eru' Leaves	258
7.4	Discussion		262
	7.4.1	Correlation of Taste with Genetic Data	262
	7.4.2	The Influence of Ecological Interaction on the Taste of 'Eru'	263
	7.4.3	The Effect of Physiological Condition on the Taste of 'Eru'	266
	7.4.4	Implications of the Effect of Tasting 'Eru' for Selective Cultivation and Conservation of *Gnetum*	267
7.5	Conclusion		271
7.6	References		271
7.7	Appendices		278

CHAPTER EIGHT – SEED GERMINATION TRIALS FOR SUSTAINABLE PROPAGATION OF *GNETUM* L. PLANTS 281

8.0	Abstract		283
8.1	Introduction		284
	8.1.1	Conservation through Cultivation	284
	8.1.2	Sustainable Utilization of *Gnetum* Species	290
	8.1.3	Propagation of *Gnetum* using Seeds	291
	8.1.4	Purpose of this Research and Key Questions	292
8.2	Materials and methods		293
	8.2.1	Seed Collection	293
	8.2.2	Collection Data	293
	8.2.3	Seed Viability Test	294
	8.2.4	Trials of Untreated Seeds	295
	8.2.5	Preconditioning Seeds to Stimulate Germination	296

8.3 Results 298
8.3.1 Seed Viability Test 298
8.3.2 Germination Rate 300
8.4 Discussion 302
8.4.1 The Implication of Seed Viability in two in *Gnetum* Species 302
8.4.2 Factors Affecting Seed Germination in Species of *Gnetum* 306
8.4.3 Implication for Conservation 308
8.5 Conclusion 311
8.6 References 312

CHAPTER NINE – GENERAL DISCUSSION AND CONCLUSION 321

9.1 An Eru-dite Exploration of African *Gnetum* Species 323
9.1.1 The Taxonomy of *Gnetum* Species in Africa 324
9.1.2 Implications of the Taste Tests and Germination Trials for Conservation of African *Gnetum* Species 328
9.2 Conclusion and Future Studies/Recommendation 332
9.3 References 335

LIST OF FIGURES

FIGURE		PAGE
Figure 1.1:	Thesis structure: summary of methods used	21
Figure 2.1:	Some morphological characters analysed in the male spike	47
Figure 2.2:	UPGMA phenograms using average taxonomic distance of the male specimens of *Gnetum* species in Africa	53
Figure 2.3:	Scattergram and 3D diagram generated by Principal Components Analysis (PCA) of male specimens of *Gnetum* species	56
Figure 2.4:	Scattergram (i) and 3D diagram (ii) generated by Principal Coordinates Analysis based on the combined qualitative and quantitative data using the male specimens of *Gnetum* species.	58
Figure 2.5:	Variation in quantitative characters (a. vegetative and b. reproductive) in the male specimens of *Gnetum*	62
Figure 2.6:	UPGMA phenogram using average taxonomic distance of female specimens of *Gnetum* species in Africa	66
Figure 2.7:	UPGMA phenogram using average taxonomic distance of female specimens of *Gnetum* species in Africa	67
Figure 2.8:	Scattergram and 3D diagram generated by Principal Components Analysis (PCA) of the female specimens of *Gnetum.*	69
Figure 2.9:	Scattergram and 3D diagram generated by Principal Coordinates Analysis (PCO) of female specimens of *Gnetum*	71
Figure 2.10:	Variation in quantitative characters in female specimens of *Gnetum*	75
Figure 3.1:	The morphological diversity of *Gnetum* spikes	105
Figure 3.2:	Distribution map of *Gnetum* species in Africa	109
Figure 3.3:	*Gnetum africanum* Welw	113
Figure 3.4:	*Gnetum buchholzianum* Engl.	116
Figure 3.5:	*G. interruptum* E.H.Biye	119

Figure 3.6:	*G. latispicum* E.H.Biye	122
Figure 4.1:	UPGMA phenograms using average taxonomic distance of the male specimens of *Gnetum* species in Africa	136
Figure 4.2:	UPGMA phenograms using average taxonomic distance of the male specimens of *Gnetum* species in Africa	137
Figure 4.3:	Scattergram and 3D diagram generated by Principal Components Analysis based on quantitative data of male specimens of *Gnetum* species.	140
Figure 4.4:	Scattergram and 3D diagram generated by Principal Coordinates Analysis (PCO) based on the combined qualitative and quantitative data of the male specimens of *Gnetum* species	143
Figure 4.5:	Comparison of variation in selected morphological characters of male plants	145
Figure 4.6:	UPGMA phenograms using average taxonomic distance of the female specimens of *Gnetum* species in Africa	147
Figure 4.7:	UPGMA phenograms using average taxonomic distance of the female specimens of *Gnetum* species in Africa based on combined quantitative and qualitative data matrix	148
Figure 4.8:	Scattergram and 3D diagram generated by principal components analysis (PCA) based on quantitative data of female specimens of *Gnetum* species	150
Figure 4.9:	Scattergrams and 3D diagram generated by Principal Coordinates Analysis **(**PCO) based on the combined qualitative and quantitative data of the male specimens of *Gnetum* species	153
Figure 4.10:	Comparison of variation in selected morphological characters of female plants	155
Figure 5.1:	Location of collection sites of Gnetum species in Cameroon	175
Figure 5.2:	Sequi-Gen GT nucleic acid electrophoresis cell	177
Figure 5.3:	An agarose gel showing genomic DNA for selected *Gnetum*	181
Figure 5.4:	Agarose gel show products from the digestion-ligation reactions	181
Figure 5.5:	Band pattern across species of *Gnetum* using GenAIEx	182

Figure 5.6:	Dendrogram based on Jaccard's coefficient of similarity	185
Figure 5.7:	3D diagram recovered from Principal Coordinates Analysis	186
Figure 6.1:	Light micrographs of the abaxial epidermis of *Gnetum* species in Africa	214
Figure 6.2:	Light micrographs of the adaxial epidermis of *Gnetum* species in Africa	215
Figure 6.3:	Fibre distribution in leaves of African *Gnetum* species	217
Figure 6.4:	Light microscopy of adaxial leaf surface of *Gnetum* species showing astrosclereids	219
Figure 6.5:	Transverse sections of the petioles of various species of *Gnetum* in Africa	222
Figure 6.6:	Vascular bundle structure of various species of *Gnetum* in Africa	223
Figure 7.1:	Ten localities in five regions of Cameroon where *Gnetum* was collected	248
Figure 7.2:	Comparison of various respondents' identification themes used in the questionnaire focused on 'eru' (*Gnetum*) taste	251
Figure 7.3:	Comparison between gender of the respondents and their response scores to ten questions on 'eru' (*Gnetum*)	252
Figure 7.4:	Comparison between respondents' age in years and their response scores to ten questions on 'eru' (*Gnetum*)	254
Figure 7.5:	Comparison between respondents' region of origin and their response scores to the questions on eating frequency and leaf age used for 'eru' (*Gnetum*)	256
Figure 7.6:	Distribution of taste score according to various species of *Gnetum* used in 'eru' meal	259
Figure 7.7:	Taste correlation with provenance of *Gnetum* cooked leaves	260
Figure 7.8:	Taste appreciation of different ages of *Gnetum* leaves used in cooking 'eru'	262

Figure 8.1:	Mature seeds of *G. latispicum* and *G. buchholzianum*	294
Figure 8.2:	Longitudinal section of equal halves of a seed of *G. buchholzianum* used for viability tests	295
Figure 8.3:	Third germination trial transferred to the greenhouse after 9 months	298
Figure 8.4:	Viability percentages of 20 seeds/test from each *Gnetum* species	299
Figure 8.5:	Seed coat of *G. buchholzianum* observed after the endosperm was eaten	301
Figure 8.6:	Seedlings of two species of *Gnetum* that emerged from acid treated seeds	302
Figure 9.1:	Synthesis of the eru-dite exploration of *Gnetum* species in Africa.	322

LIST OF TABLES

		PAGE
Table 2.1:	Descriptive List of qualitative and quantitative characters used in numerical analyses	48
Table 2.2:	Eigenvalues obtained from Principal Component Analysis of the male specimens of *Gnetum* species	55
Table 2.3:	A list of the first three 'eigenvalues' of the various characters loaded for Principal Component Analysis of the male specimens	59
Table 2.4:	Eigen values obtained from Principal Component Analysis of the combined data matrix for the female specimens of *Gnetum* species	69
Table 2.5:	Eigenvalues for the various characters loaded for PCO analysis of the female specimens on the first three axes.	72
Table 2.6:	Morphological characters used in recognizing species of *Gnetum*	78
Table 4.1:	Eigenvalues obtained from principal components analysis of the data matrix for the wild and cultivated male specimens of *Gnetum* species	139
Table 4.2:	Taxonomic values of qualitative and quantitative characters of males	142
Table 4.3:	Eigenvalues obtained from PCA of the quantitative data	149
Table 4.4:	Taxonomic values of qualitative and quantative characters of females	151
Table 5.1:	Primer and adapter sequences used for AFLP analysi	176
Table 5.2:	PCR profile for selective amplification	177
Table 5.3:	Silver staining protocol adapted from Promega manual	179
Table 5.4:	Total band pattern for binary data by *Gnetum* species	182
Table 6.1:	Stomata characters examined in the leaves of male and female specimens of four forms of *Gnetum* in Africa	216
Table 6.2:	Sizes of fibres as seen on leaf surfaces of various *Gnetum* species	217

Table 6.3:	Distribution and number of sclereids found in the leaves of *Gnetum* species in Africa	218
Table 6.4:	Size, shape and arrangement or layout of vascular bundles (VBs) in the transverse section of petioles in *Gnetum* species	221
Table 6.5:	Summary of Anatomical Characteristics in the African species of *Gnetum*	224
Table 7.1:	Sample layout of tasters for 'eru' gustatory analysis for five meals	259
Table 7.2:	Comparison between respondents' region of origin and their response scores	264
Table 7.3:	Significance test in survey questionnaire variables	266
Table 7.4:	Distribution of taste scores for various species of *Gnetum* with respect to region of collection	267
Table 7.5:	Taste scores for cooked *Gnetum* leaves in ten localities in Cameroon	268
Table 7.6:	Distribution of taste scores with respect to age of cooked *Gnetum* leaves	270
Table 8.1:	Categories of seed dormancy adapted after Hartmann *et al*. (2002)	298
Table 8.2:	Layout of variously treated seeds of *Gnetum* species	306
Table 8.3:	Viability percentages of 20 seeds/test from each *Gnetum* species	308
Table 8.4:	Seedling emergence percentages observed in *Gnetum* species	310

CHAPTER ONE

INTRODUCTION

1.1 *Gnetum* Species: A 'Limitless' Wealthy Resource in Africa?

Gnetum L. is the only genus of the family Gnetaceae in the gymnosperm order, Gnetales, an extant group whose closest relatives are the flowering plants according to phylogenetic analysis of the seed plants (Crane, 1985; Gillespie & Nowicke, 1994). The plants of this genus are woody trees, shrubs and canopy lianas with their stem partly below ground (Martens 1971). The genus *Gnetum* is of interest because it is more similar to the angiosperm than any other gymnosperm. This is due to the presence of different vessels in the secondary wood with varying degree of fusion that are seen in some primitive angiosperms (Sporne, 1965).

The genus *Gnetum* comprises about 40 species that occur in rainforest or occasionally seasonal forest, and are restricted to tropical and subtropical Asia, South China, Malaysia, the Amazon basin, and two poorly known species described from the African humid tropical forests (Pearson, 1917; Hutchinson & Dalziel, 1954; Maheshwari and Vasil 1964; Fu *et al*., 1999; Shiembo, 1999; Earle 2001; Won and Renner 2006). Only two species, *Gnetum gnemon* L. and *Gnetum costatum* K. Schum., are trees and there are also a few shrubs, but most *Gnetum* plants are lianas. Within the genus, two sections, *Gnetum* and *Cylindrostachys* Markgr., were established based on strobilus morphology (Markgraf, 1930) and the section *Gnetum* was further divided into three allopatric subsections occuring in the Neotropics (*Araeognemones* Markgr.), Africa *(Micrognemones* Markgr.), and tropical Asia (*Gnetum*). Meanwhile, the section *Cylindrostachys* incorporated two subsections, *Stipitati* Markgr. and *Sessiles* Markgr., both Asian (Gillespie & Nowicke, 1994).

The leaves of *Gnetum* are strikingly similar to those of dicotyledonous angiosperms, but they vary so much in size and shape that their features are of little value in its taxonomy; (Chamberlain, 1966). All the leaves are opposite and decussate with a short petiole and no stipules; on climbing species, they are found only on shoots of limited growth. The reproductive organs, often termed 'flowers', are borne in whorls on spike-like inflorescence axes; some species are invariably dioecious while in others such as *G.*

gnemon, the male inflorescences bear sterile ovules which may occasionally be fertile (Haycraft & Carmichael, 2001).

The pollen of *Gnetum* is small (12–20 pm in diameter) and of three types: spheroidal or subspheroidal (sometimes with a flat or concave pore-like area), inaperturate, and spinose or spinulose (Gillespie & Nowicke, 1994). The Asian and African species are characterized by 'spinulose' pollen and the neotropical species by pollen with a 'pilate' or 'probably pilate' sexine (Erdtman, 1954; 1965; Gillespie & Nowicke, 1994). At pollination, the pollen is drawn down the micropyle by means of a drop-mechanism and the lower end of the micropyle becomes plugged (Kato *et al.*, 1995).

In various ways, dioecious *Gnetum* plants resemble many angiosperms, for instance, insect pollination and seed dispersal by fruit-eating birds. Species of *Gnetum* produce nectar, which enhances the efficiency of pollination (Kato *et al.*, 1995; Corlett, 2001). A fleshy integument layer covers the *Gnetum* seeds or 'pseudofruits', and the mechanism underlying seed dispersal has been specifically studied in *Gnetum leyboldii* Tul. (Margulis & Chapman, 2009). It was found that the sweet flesh entices the chestnut-mandible toucan to feed on the pseudofruit; later on the toucan regurgitates and disperses the *Gnetum* seed (Margulis & Chapman, 2009). These observations are more convincing than the seed dispersal by water assumed to occur in several species of *Gnetum* to fit the morphological similarities among apparently young species (Won & Renner, 2006).

The bark of *Gnetum* species provides a strong fibre used for making gunny bags, ropes and fishing nets (*G. montanum* Markgraf; *G. pavifolium* (Warburg) Chun.); and the sap that flows very freely from cut stems can be drunk to quench thirst (*G. montanum*). Some species are widely cultivated in SE Asia for their young leaves which are eaten as a green vegetable, e.g. *G. gnemon* locally called 'bago' in the Philippines, 'belingo' or 'melingo' in Indonesia, 'two leaf' or 'joint fir' in English, 'maninjo' in Malaysia or 'melingo' in Singapore (Manner & Elevitch, 2006). Its seeds are also roasted and eaten although the outer, fleshy layer contains irritant, needlelike crystals and is not generally eaten. The seeds of *G. montanum* and *G. pavifolium* yield an edible oil, eaten fried and used for making wine (Cheng, 1999).

In Africa, the genus consists of lianas possessing strong upright branches and a profusion of pendant shoots upon which most of the foliage is borne. They are multi-branched with nodular stems and opposite-decussate leaves. Generally, these climbers are found in the forest, but grow equally well in a wide range of habitats such as farm fallows, or abandoned farmland (Shiembo, 1999). The vines climb supported by dead or living trees of various sizes and on other climbers such as rattan palms, and produce great quantities of leaf biomass (Fondoun & Manga, 2000). During unfavorable conditions, the underground tubers produced as nutrient reserves and reported to be edible, remain viable many years after the surrounding vegetation and the aerial portions of *Gnetum* plants have been cleared and the soil laid bare (Shiembo, 1999).

Gnetum contributes to the diversity of foods of high economic and nutritional value in Africa. Similar to the Asian *Gnetum*, the leaves of the African species are sold and used as a vegetable, called 'eru' or 'okok' in Cameroon; 'Mfumbwa' in Congo; 'Fumbua' in Angola and 'afang' or 'Ukazi' in Nigeria (Clark *et al.*, 2004). *Gnetum* is thus a source of food and economic stability to humans and like any other plant plays a role in maintaining the equilibrium of the biosphere. The leaves which are rich in proteins and minerals, contain the eight essential amino acids (Sunderland & Clark, 2001). Food made from these leaves is consumed at all levels of society and not only in Africa but in most parts of the world. *Gnetum* leaves are the most exploited and commercialized vegetable in Cameroon (Besong *et al*., 2001) and therefore contribute to alleviating poverty among the common people. The leaves are shipped in reasonable quantities from Cameroon to other African countries, especially Nigeria, to be used locally and also exported to Europe and the USA (Lowe, 1984; Fuashi *et al*., 2010; Ali *et al.*, 2011).

Gnetum is also exploited for traditional medicines (though with no report of scientific evidence): in addition to treating enlarged spleen, sore throat, piles and reducing high blood pressure, it is used to treat nausea, ease childbirth and as an antidote to some poisoning substances (Burkill, 1994). The leaves of *G. africanum* Welw. are also used in alcoholic fermentation in Cameroon (Edissegue, 1990) and noted to be important in the production of a local commonly exported gin in Nigeria (Mbahand, 2001). Consequently,

a lot of *Gnetum* is harvested from the wild in tropical Africa and these plants are threatened because of the method and extent of harvest.

The African species of *Gnetum,* moreover, are under threat in the wild because of the disappearance of their natural humid forest habitat. There is widespread felling of forest trees as well as extensive clearing of vegetation. Due to the destruction of the forest (which is unprotected), there is a high possibility that the number of mature individuals, the number of locations and the area of suitable habitat will continue to be reduced, resulting in the rarity and endangerment of these species.

With the disappearance or growing rarity of *Gnetum* in the tropical forests of Africa, their sustainability therefore becomes critical. The application of biodiversity conservation strategies is now, more than ever, necessary due to an increase in demand for *Gnetum* proportional to an obvious increase in human population number. In some provinces of Cameroon, *Gnetum* plants have become uncommon, therefore the community forest management, research institutes and conservation projects are increasing efforts to conserve *Gnetum* (Lowe, 1984; Ndam *et al*., 2001). There is great need to apply conservation measures, such as the cultivation of the strains with the best taste for the local meal, 'eru' made from *Gnetum* leaves.

Introduction of *Gnetum* into farmland systems is vital to facilitate bulk production and so reduce impact on natural populations of the species in tropical Africa as a whole as *Gnetum* is found neither in gardens, farms nor in plantations (Fuashi *et al*., 2010). Therefore, the identification of species or forms of *Gnetum* with favourable traits for cultivation (e.g. best tasting leaves, ease of seed germination or of growth of cuttings) and recognition of current distributions will be useful in prioritizing conservation approaches.

Prior to this study, very little was known of the diversity of *Gnetum* in Africa and conservation strategies may only be effectively applied after species have been well defined taxonomically. As noted above, the two species previously described from the African forests were not well known. The *Gnetum* species were: *G. africanum,* for which

the type specimen was collected from Angola by *Welwitsch No. 1224* (B, K, PRE) and *G. buchholzianum* Engl., with syntypes collected by *Buchholz, s.n*. in February 1874, *Zenker, No. 522* in 1890/1892, and *Staudt No. 543*, all from Cameroon (B, K), www.aluka.org.

The identities of these two species of *Gnetum* recognized in Africa by Hutchinson and Dalziel (1954) were in dire need of taxonomic clarification. In *G. africanum*, the internodes of the male spike were described as slender, whereas for *G. buchholzianum*, they were described as much thicker at the base than higher up. Upon examination of the types, *G. africanum* presented internodes that were much thicker at the base than higher up but more often, the internodes were fairly even throughout, whereas those of *G. buchholzianum* were much thicker in the upper region than at the base (Chapter 2). Therefore, the two species might have been confused in the Flora of West Tropical Africa (Hutchinson & Dalziel, 1954).

Authors like Won and Renner (2003), Earle (2001), Onguene and Kuyper (2001), Isong *et al*. (1999), and Carlquist (1996) have been uncertain about the number of species to recognize in the genus *Gnetum* in Africa. For example, Isong *et al*. (1999) noted the existence of three varieties of *G. africanum* in Nigeria based on specific collection localities, but no descriptions were provided. In contrast, Won and Renner (2003) concluded that there was just one species occurring in Africa. The identification of the species of *Gnetum* has therefore been confusing. It requires elucidation using updated morphological characterization (Chapters 2, 3 and 4), molecular (Chapter 5) and anatomical (Chapter 6) approaches to clarify their taxonomy. Taxonomy encompasses description, identification, nomenclature and classification. As noted by Simpson (2006), a change of definition and delimitation of a taxon requires several approaches which are fundamental to a real understanding of speciation, some of which have therefore been used in this study.

In a classification based on reproductive morphology and pollen types in particular, Doweld (2000) transferred all species of the genus *Gnetum* with a dimerous system of seed coats into an old available genus *Thoa* Aubelt. *Gnetum* was thus restricted to two

species only: *G. gnemon* and *G. costatum* thought to possess a more primitive, trimerous system of seed coat. However, the results of a phylogenetic study (maximum likelihood analysis) based on nuclear and plastid sequences showed that Doweld's proposed genus *Gnetum* made up of only *G. gnemon* and *G. costatum* nested within the species transferred to *Thoa* (Won & Renner, 2003). This resulted in *Thoa* being a paraphyletic genus, and therefore the Linnaean name *Gnetum* is retained for the entire genus, including the African species in this study.

Species are crucial in numerous biodiversity issues. Studies on conservation, biodiversity, ecology and even legislation concern this taxonomic level (Mallet, 2007). Despite the phylogenetic work on *Gnetum* by Won and Renner (2003; 2004; 2006) and studies on the unique physiological and anatomical adaptations of *Gnetum* (Jauregui *et al*., 2005), several questions remain unanswered as far as the diversity and delimitation of species of this genus in Africa are concerned. There is an uncertainty concerning the diversity of *Gnetum* in Africa, and it is clearly a priority to improve the taxonomy of this group before any useful research on the sustainability of its species would be possible. The key to what is known about a plant is its name (van Steenis, 1957). If conservation strategies such as cultivation are to be applied to *Gnetum*, the questions of which 'member' or 'species' of this genus will be suitable for domestication and how it can be recognized in nature arise. To answer these questions, the species constituting the genus ought to be accurately identified using the appropriate species concepts or species definition.

1.2 "What then is a Species?"

The basic element for any natural classification is the species and it is therefore important to recognize all the species in a given taxon. Species are vital in taxonomy as essential units to store knowledge compiled from individual specimens (Kornet & McAllister, 1993). Morphological diversity among and within populations has led to a series of species definitions, consequently the recognition of a species can only be based on a particular species concept (Mallet, 2007). That there are several species concepts has led to numerous species definitions. If species are considered as a classification unit, then an acceptable definition for all biodiversity studies ought to be established and this seems

impossible (Wheeler & Meier, 2000). Ultimately, all species concepts, for example, morphological, biological and molecular, ought to deal with defining discontinuity in the traits of organisms whereby those that are similar to each other are members of the same species and reflect a common ancestry i.e. a natural classification system.

One of these concepts is the morphological species concept that uses the interpretation of reliable morphological characters to define a species. Morphological differences have been a traditional key criterion for species identification. A morphological species usually possesses vegetative and reproductive features that contribute significantly in distinguishing it from its closest relatives. Some of these structures are consistent within a species and vary between species and, hence are taxonomically important. Others are either invariant or do not correlate with identified species and/or present intraspecific variation, they are therefore less valuable in differentiating between species (Davis & Heywood, 1963). The choice of characters is thus important and their pattern of variation within and between species changes from group to group. The morphological species is mostly based on the description of the type specimen, which is a specimen to which the name of the species is tied. The species is thus defined by the description of the type and mostly deals with external structures of plant organs (van den Berg *et al*., 1998).

The morphological species concept could only be used when there is a gap in morphology in order to differentiate species (Mallet, 2007); nevertheless, morphological characters are often useful criteria in other research fields such as ecology. Morphological analysis forms the basis for taxonomic descriptions and provides an important source of data in the delimitation and circumscription of taxa. The morphology of a plant includes its basic structural components and these organs are delimited by their precise functions. The leaves of the gymnosperms *Ephedra* L., *Gnetum*, and *Welwitschia* Hook. are strikingly different, both in form and venation pattern and these provide distinct morphological characters for each genus (Carlquist, 1996). *Gnetum* presents specialized features, such as the shape and structure of the strobili, the net-veined leaves, lignin composition (more typical of angiosperms than gymnosperms), and a climbing habit, reported unusual among extant gymnosperms (Carlquist, 1996), that differentiate its species from those of *Ephedra*, for example. Moreover, the Venezuelan *Gnetum*

species were distinguished based on leaf size, the length of internodes and characteristics of the bark on the stems (Jauregui *et al.,* 2005).

In order to identify essential characters to be used in taxonomic delimitation, it was possible morphologically to identify six features (bark of stems, length of internodes, size of leaves, colour of seed and number and shape of cotyledons) that distinguish between species of *Gnetum* in Venezuela and a key was produced using the character sets studied (Jauregui *et al*., 2005). The key to the African *Gnetum* species was also constructed based on morphological features (Hutchinson & Dalziel, 1954), although this is in need of clarification and refinement. Morphological examination has been applied in this study in an attempt to clarify *Gnetum* species in Africa based on existing types, previous descriptions, and additional studies using new features and assessing a broad range of specimens.

All concepts use character data to judge species boundaries and often these are morphological features. The application of the morphological species concept in the recognition of organisms of the same species that look different due to environmental adaptation is often difficult and this sometimes results in splitting members of the same species, especially when distinct populations are allopatric. The splitting could be based on a small degree of variation, it is subjective and new tools or more characters usually substantiate the difference in the species.

Due to this drawback, the biological species concept (BSC) has been considered to be more suitable to address the species problem. Based on this concept, a species is defined as a set of organisms that actually or potentially interbreed in nature and are reproductively isolated from other populations (Mayr, 1970; Futuyma, 2006). This implies that a species is the biggest pool of genes possible in nature. Interbreeding, ability to sexually reproduce permitting the flow of genes within a population is identified as a useful criterion in this species concept. The concept emphasizes that the recognition of natural species be placed within a population genetics context (Kottler, 1978).

Hybridization is a measure of close relationship between organisms and thus becomes taxonomically valuable where taxa that interbreed to form fertile hybrids are considered as a single species and those that do not as separate species (Stace, 1989; Simpson, 2006). It usually assumes a crossing between plants belonging to different species or taxa and even between reproductively isolated species (Davis & Heywood, 1963; Brooks & Mclennan, 2002). In the application of the biological species concept to the genus *Gnetum* in Africa, it would be essential to establish whether there is or not gene flow between the two dioecious species (*G. africanum* and *G. buchholzianum*) recognized by Hutchinson & Dalziel (1954), Isong *et al*. (1999) and Onguene & Kuyper (2001).

Reproductive isolation between groups is regarded as the basis of the biological species concept. This concept is however only applicable when sexually reproducing organisms are involved and are sympatric but poses difficulties in plants where asexual reproduction and hybridization naturally occur, resulting in new individuals that might be considered as species (Dobzhansky, 1970; Mallet, 2004; 2007) and also where interfertility between organisms is observed. Nevertheless, the biological species concept does answer the questions of 'why' and 'how' of species (Poulton, 1904; Mayr, 1982). It can also be tested, even in allopatric species; and thus has a more scientific basis than the morphological species concept and relies on the notion of isolating mechanisms.

The BSC is inapplicable to herbarium and museum specimens (Sebola & Balkwill, 2009), but this concept can be implied in the present research; for molecular tools could provide some indication regarding gene flow between species of *Gnetum* in Africa. Moreover, these species are distinguishable on the basis of their reproductive features, thus gaps are perceived in the distribution of morphological characters and could reflect differences in modes of reproduction. The BSC could therefore be theoretically applied within *Gnetum* species, and knowledge of specific factors such as their geographic and ecological distribution, chromosomes number, pollination syndromes is important.

The recognition species concept elaborated by Paterson in 1985 is yet another species concept that underlines the biological significance of species. Species are characterized here by a specific mate recognition system (SMRS), as compared to the reproductive

isolating mechanisms of the biological species concept (Mallet, 2007; Coyne & Orr, 2004). A species is recognized as populations of organisms that share the same SMRS (which is often effected in plants via pollinators); it is thus similar to the biological species concept except that there is evolution of new species when a new SMRS evolves. However this concept faces the same problem as BSC regarding asexual organisms. The recognition system usually involves several sexual signals, which vary according to organisms and are an aid in identification of partners.

The ecological species concept developed by Van Valen (1976) very often refers to 'niche' and 'habitat' in which organisms cluster into groups (Rejmanek & Jenik, 1975; Coyne & Orr, 2004). An ecological species can therefore be defined as a set of organisms exploiting the same niche. This concept is based on the relationship between living organisms and their surroundings, and moreover, an organism may also vary during different life stages or due to changes in environmental conditions (Raxworthy *et al*., 2007). This implies that habitat also affects the genotypes of the organism and therefore speciation could occur due to ecological changes acting as the essential driving force (Jonsell, 1984). Thus, species could be affected by drastic ecological shifts causing a rapid modification under intra- and inter-specific competition, and when coupled with hybridization, can result in new species adapting to new conditions, but might be ecologically inferior (Raxworthy *et al*., 2007). In other words, the concept suggests that species should mostly cluster together ecologically, implying that different niche occupations would mean different species and vice versa (Wheeler & Meier, 2000) which is obviously not always the case. Mallet (2007) pointed that distinct forms of *Quercus* L. (oaks) with a high potential of hybridization retained their distinctness even when co-occurring in an area.

Among the numerous concepts involved with species definition, such as cohesion species concept, genotypic cluster species concept, the composite species concept and evolutionary species concept (Kornet & Mcallister, 1993; Coyne & Orr, 2004; Mallet, 2007), are the phylogenetic and phenetic species concepts. Both concepts allow for species recognition from the use of multiple morphological or genetic characteristics to

sort individual specimens into discreet clustering patterns with few intermediates, and the phylogenetic concept provides knowledge of their evolutionary trends.

The phylogenetic concept considers species to exist as a monophyletic group of common ancestry and as self-perpetuating organisms, bearing unique character sets (Cracraft, 1989). The concept, which infers relationship by common ancestry, focuses on elucidating relationships using branching patterns in cladograms and is involved in examining the evolutionary history of the species (Davis, 1997). The phylogenies indicate the relationship between groups in terms of time expressed topologically in a number of nodes and internodes or evolutionary changes (Sneath, 1988). In applying the phylogenetic concept to both sexual and asexual organisms, species are defined as a group of organisms sharing unique derived or apomorphic characteristics which are not present in their relatives or ancestors. The process of speciation viewed under this concept might be measured as a divergence of the population depending on the character selected.

The phylogenetic concept is now frequently used in taxonomy (e.g. Cron *et al*. 2008; Nakkuntod *et al*., 2009; Bruyns *et al*., 2010) although there is often no agreement on what the taxon-level indicated on the cladograms might be (Wheeler & Meier, 2000). This concept, which deals with historical relationship, is of little help in understanding discreteness of nature; evolutionary history and reproductive compatibility might not always agree (Coyne & Orr, 2004). The regular use of this concept could be the ease with which slightly different geographic or morphological forms are treated, i.e. as a group that descends from one ancestral species and viewed as separate species (Huang *et al.*, 2005). Although not included in this study, a phylogenetic approach could be applied using qualitative morphological and/or DNA sequence characters for the African species of *Gnetum* using outgroups from America to polarize the character states.

The phenetic species concept, in contrast, provides a classification based on grouping patterns with high information content permitting predictive statements that are widely useful and beneficial to many branches of science (Sneath, 1988). This species concept refers to a species as the smallest distinct cluster of organisms (Sokal & Crovello, 1970).

It uses numerous observed qualities (characters) of the organisms, of more or less equal weight, and groups similar specimens together, but without implication of evolutionary direction. This concept, based on grouping organisms to maximize their within-group similarity, is highly applicable to the use of multiple herbarium and museum specimens (Moss & Webster, 1970; Sebola & Balkwill, 2009). The high statistical correlation among individuals usually indicates membership to the same species. It expresses the extent of phenetic similarity between members of the same group and is considered as an aid to objectivity, attempting to eliminate the arbitrary nature of application of the morphological species concept (Gower, 1988).

Although the phenetic approach aims to objectively delimit clusters (species) based on the phenotypic similarity, it sometimes also necessitates subjective and arbitrary decisions regarding the level at which one recognizes groups. Phenetics provides a classification of organisms with similar appearance and is based on various macro-morphological (e.g. leaf, stem), micro-morphological (anatomy) and molecular characters e.g. DNA differences (Chapters 2; 4; 5; 6).

With so many species concepts, a common cohesive view seems complicated or difficult to reach and could be attributed to individual interpretation based on various research fields and also on the complexity of biodiversity. The debate on species' definitions could persist as long as people continue to think freely in different fields; furthermore, there might not be a single concept that applies to all biodiversity studies. In sum, biologists could aim at a complete and well defined species concept or definition that will satisfy all opinions, in order to solve the species problem and address all biological issues (Pigliucci, 2003). This is yet to occur; meanwhile, any research involving overall similarities based on numerous attributes and not focusing on evolutionary traits would adopt the phenetic concept of species and speciation, even though the phylogenetic concept might be preferred when historical relations are considered. Any study interested in ecology or reproductive isolation of organisms will value the ecological or biological concepts respectively. Morphological examination (Chapter 2) and phenetic investigation (Chapters 2, 4 & 5) of the variability of the African species as well as the similarities

(Chapters 5 & 6) between various forms provided enough information as to the number of species to recognise in the classification of *Gnetum*.

1.3 Approaches Used in This Study

1.3.1 Phenetic, molecular and anatomical methods

The phenetic approach, often considered as numerical taxonomy (Rohlf, 2000), is primarily concerned with quantitative taxonomy which uses multivariate analysis of character states, usually morphology or other observable qualities (McNeill, 1984). Phenetics uses distance measures if data is mainly quantitative (or similarity if data is mainly qualitative) producing matrices on which cluster and ordination analyses can be based. It thus compares the degree of similarity between individuals based on character description and relies on a considerable number of characters to provide information about the members studied thus limiting sampling error (Davis & Heywood, 1963). The approach identifies discrete groups that (in this study) may be classified as species in the genus *Gnetum* and assist in the taxonomic revision of the genus in Africa.

Phenetics ideally exploits a great number of characters of equal weight from several approaches to identify species and indicates the similarity between species that may reflect their relationship (Sokal & Crovello, 1970)**.** The identification could be inferred from, for example, genomic or chromosome similarities or differences, anatomical, morphological, and ecological or a combination of the above with no clear evidence of links to common ancestry. The need for species clarification in the genus *Gnetum* in Africa for conservation purposes has prompted the application of methods appropriate to both herbarium and fresh materials that facilitate recognition of taxa on the basis of correlations of character differences. Therefore, in addition to the morphological character descriptions, molecular (Chapter 5) and anatomical (Chapter 6) approaches were also included here because interspecific and intraspecific relationships might not be completely elucidated using external morphological characters alone (Chapters 2, 3, & 4). Moreover, these approaches provided sufficient characters to interpret the similarities and differences among the African species of *Gnetum* and delimit the species.

Various molecular approaches may be used to investigate characters essential in the recognition of species (Hillis *et al*., 1996), and include data from DNA sequences, DNA restriction sites, Random amplified polymorphic DNA (RAPD) and Amplified fragment length polymorphisms (AFLPs) to name a few (Blears *et al*., 1998). The data obtained from DNA-based studies might allow the understanding of molecular events that underlie character and species differences, although molecular characters used in systematics at the DNA level are often selectively unbiased and can only indirectly affect the phenotypes (Schlichting & Smith, 2002)**.** Previous delimitations of *Gnetum* species have been based mostly on variations in macro-morphological features, most notably the prominent male inflorescences (Duthie, 1912; Hutchinson & Dalziel, 1954), as well as on leaf anatomy in the Venezuelan species of *Gnetum* (Jauregui *et al*., 2005) and microspore differences (Pearson, 1912).

Character descriptions based on genetic markers and leaf anatomical features have seldom been used on *Gnetum* species in Africa (Won & Renner, 2003) or on most gymnosperms in general (Jacobson & Lester, 2003). These characters have been helpful in several angiosperms to draw meaningful conclusions on population processes and patterns of relationship between individuals (e.g. Hillis *et al*., 1996; Nakkuntod *et al.*, 2009; Alejandro *et al.*, 2010; Parolly *et al*., 2010). Analyses using AFLPs (Vos *et al*., 1995) have been used here in fingerprinting the DNA of the various species of *Gnetum* in Africa (Chapter 5). Besides that AFLPs can identify higher number of variations in a single assay compared to either RFLPs (Restriction Fragment Length Polymorphism), RAPDs or SSRs (Ajmone-Marsan *et al*., 1998; Pejic *et al*., 1998; Cai *et al*., 2005); it seems to be a promising approach to independently assess relationship among the African species of *Gnetum*.

Anatomy involves the study of the internal structures of organisms which includes the analysis of tissues and cell structure of plant organs using either a high-powered light microscope or an electron microscope to examine these features. Anatomical investigations have provided valuable characters and character states in the assessment of some taxa, e.g. *Chionanthus* L., *Olea* L. (Kiew & Su, 1982) and *Pavetta* L. (Kok & Grobbelaar, 1984). A study carried out by Jauregui *et al*. (2005) revealed the usefulness

of anatomical features to distinguish *Gnetum* species in Venezuela. The leaf anatomy, especially the structure of the epidermal cells and the type of stomata of various species present distinct characters for species recognition in *Gnetum*. The nodal anatomy of *Gnetum ula* Brongn. from India showed that the arrangement of vascular bundles was unique to this species in the genus (Rodin & Paliwal, 1970).

Several other studies have portrayed the usefulness of anatomy in differentiating taxa, for example, calcium oxalate crystals inside the chloroplast, the presence of uniseriate epidermal cells coupled with sunken stomata observed in the leaves of variants of *Viscum* L. were useful anatomical features that established their intervariant relationship (Mbagwu *et al*., 2009). The epidermal cells, stomata, nonglandular hairs, mesophyll and main veins were described and compared for potential taxonomic value in *Pavetta* species and *Boerhaevia* L. (Herman *et al*., 1986; Edeoga & Ikem, 2001). Anatomical characters of the stomata of cotyledons have successfully been used to determine taxonomic value of cuticular characters in the identification of plant species of *Ipomoea* L. at the seedling stage (Ogunwenmo & Ugborogho, 2003). In this study, apart from comparing the African *Gnetum* species based on their anatomy, the female plants of each species must be matched with their respective male plants using these features (Chapter 6).

Three approaches (phenetic, molecular and anatomical) are used in the present investigation to contribute to species recognition in *Gnetum* in Africa; nonetheless, conservation measures are still needed since these species face a major threat of disappearing from their natural habitat.

1.3.2 Biodiversity and conservation strategies

Over-exploitation and destruction of habitats are some of the direct impact and indirect effects of human activities on their surroundings and these represent a threat to the persistence of biodiversity (Lévêque & Mounolou, 2003). Generally, plants that are rare might even disappear without their valuable genetic resources and knowledge of their various uses identified, and the food security of those depending on them is affected

(Tekwe *et al*., 2003). Accurate identification of plant species offers the necessary basis for implementing strategies to manage wild populations in a given area and this is a prerequisite for any conservation strategy to be successful (Callmander *et al*., 2005).

The management and restoration of vulnerable plants for bulk production is essential for their conservation and the domestication of wild and semi-wild food plants is thought to be a viable strategy for any endangered resource (Leakey *et al*., 1996). Although domestication does not always lead to species conservation as there are missing or destroyed 'species types' of most domesticated plants, it nevertheless is an approach that conserves genes and especially those species attributes that are appealing to people (Van Tassel *et al*., 2010). As such, any research contributing to in situ conservation of the wild forms or to optimize the development of the propagated forms of *Gnetum* using seeds for example, is promoted (FAO 2001). There are several effective approaches to identifying individuals or species to actively target for conservation, two of these, taste appreciation (chapter 7) and cultivation trials using seeds (chapter 8), relevant to the sustainable use of biodiversity, have been considered in this study.

Taste appreciation attempts to identify those species that should be conserved *in situ* and through cultivation (Mukai *et al*., 2004). This may include different ecological types with taxonomic variants that characterize a geographical area. This method aims at identifying and establishing a viable population of species in the wild with the best taste that could survive for long. The outcomes from taste analyses of leaves at different stages of development and from various regions could positively influence the harvesting of *Gnetum* species and thereby promote its sustainable utilization (Chapter 7).

Many conservation decisions are based on availability and use of limited funds, but species ought to be managed in order to maintain yield for a long period of time. Modifying production methods and improving consumer habits are means of sustaining species of *Gnetum*. Cultivation of the plants in order to achieve a considerable increase in productivity using cuttings of *Gnetum* stems are ongoing in research centres in Cameroon, although these have almost been abandoned due to shortage of funds. The mechanisms required to break seed dormancy of African *Gnetum* species to enhance

germination of viable seeds are investigated (Chapter 8). This could promote the propagation of *Gnetum* via seeds, thus resulting in a greater availability of *Gnetum* leaves for the local populations (Chapter 8). The production rate and the improved cost to benefit ratio for farmers could therefore be maximized; moreover there will be a relief of pressure on natural populations.

1.4 The contribution of this study to sustainable use of *Gnetum* species and conservation of their habitat

Currently, in Africa, *Gnetum* is harvested from the wild at an unsustainable. In order to slow the rate of harvesting from the wild, it will be desirable to promote the use of cultivated rather than wild material. The cultivated material should be the best tasting *Gnetum* so that the people will want to use it by choice. There is then the need to ascertain the most effective way of propagating the material. In brief, the right entity for propagation needs to be identified (Chapter 7), which requires taxonomic clarity among the species, and finally the most successful means of increasing its bulk production (Chapter 8) and the system of propagation should be determined.

1.5 The Importance of Systematics for Biodiversity and Conservation

The role of systematics in the conservation of biodiversity is to provide a natural classification of organisms and to establish principles of priority for their conservation (Vane-Wright *et al*., 1991). Species are the key elements of biodiversity and their diversity is of interest when considered in time and space, for example, larger areas often have more species and diversity is particularly high at the equator (Preston, 1960; Mallet, 2007). There are several criteria for setting conservation priorities depending not only on the abundance of the species but also on the endemism level in a particular area or the determination of species relationships, resulting in identifying and preserving maximum genetic variation (Bevill & Louda, 1999; Coyne & Orr, 2004).

The number of endemics is essential in the quantification of biodiversity (Vane-Wright *et al.*, 1991). In this respect, questions arising are generally focused on finding the best measures of biodiversity value (genes), the best estimates of its importance (species) and the practical and efficient assessment of this value plus their pattern of relationship (Leadlay & Jury, 2006). While taxonomic measures of diversity are essential for the estimation of plant richness in terms of genes or characters inherited, classification specifically reflects the pattern of gene flow and how this flow influences variation in plants (Endler, 1977; Ellstrand, 1992). Plant diversity is thus considered as an essential economic and social resource for the environment and can be expressed at different levels (Henry, 2005). For example, the phenotypic variation could be useful in the role of plants in an environment and examination of genotypic variation provides an understanding of the genetic origin of this variation.

Diversity is considered meaningful only when species are counted and known. The process of counting species can be done without difficulty when their identity is directly linked to characters that are easily measured (Butlin *et al*., 2008). Most projects concerned with biodiversity distribution are done in well-known and better documented groups of organisms such as angiosperms. Even within these groups, more literature is obtainable on those found in the temperate than tropical areas (Schemske, 2008). *Gnetum* is not only a gymnosperm, one of the groups rarely investigated, but it also grows only in tropical regions where little research attention has been focused and very few publications are known.

1.6 Motivation for this Study

Gnetum species in Africa are reported to be endangered due to unsustainable harvesting and the gradual disappearance of their natural habitat, the humid tropical forest. In order to ensure and promote sustainability of the species, conservation efforts are needed. An insufficiently known taxon is a problem in terms of its biodiversity assessment (Cracraft, 2002); consequently, the knowledge of such a taxon, for example *Gnetum,* needs to be improved. A taxonomic study of this genus is clearly needed because of the method of

harvest and export of huge amounts of biomass whose diversity is insufficiently known, even though the plants are of economic importance, with high nutritional value.

This study, summarised in Fig. 1.1, thus contributes to taxonomic clarification of the genus *Gnetum* in Africa using morphological, anatomical, molecular and biogeographical data to describe differences and similarities among species; thus characterizing and evaluating the diversity exhibited by species of *Gnetum* for their agronomic and gustatory value. The strategy adopted here is the use of various experimental approaches with fresh material and herbarium specimens where applicable to distinguish between various *Gnetum* populations and species and relate them to observed variation in taste, as well as attempts to propagate them via seed germination for sustainable use.

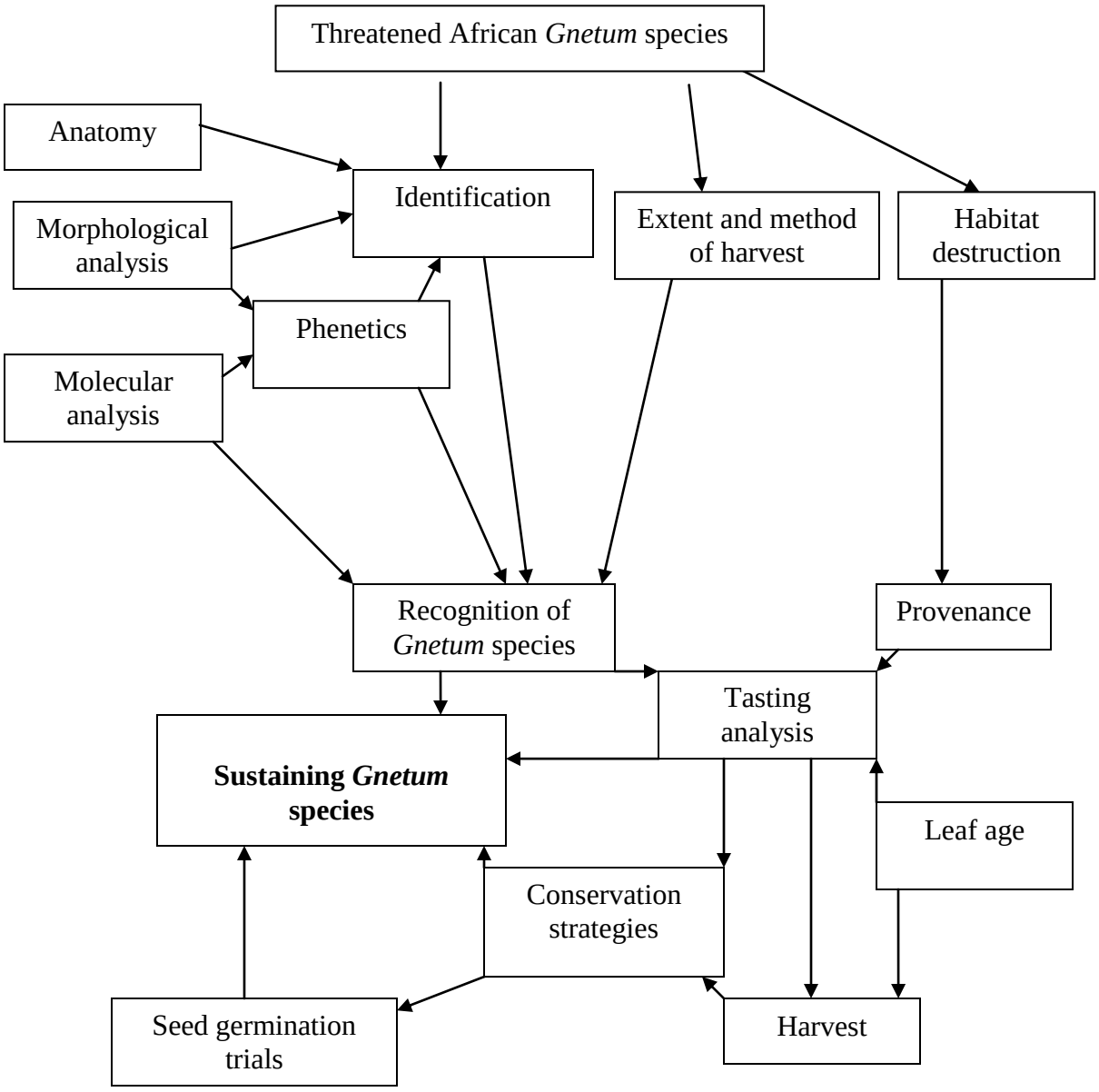


Figure 1.1 Summary of Introduction indicating the rationale for the study of the genus *Gnetum* in Africa and the planned approaches to be used. The arrows indicate uni-directional steps of research procedures.

1.7 The Main Aim of the Research

This study, summarized in Fig. 1.1, promotes the sustainability of the African species of *Gnetum* in general and those with the most popular taste in particular; it is established on accurate species recognition and conservation strategies for these plants and also makes recommendations to reduce impacts on the natural population. It therefore aims not only to clarify taxonomically the genus *Gnetum* in Africa on the basis of its morphological, anatomical, molecular and biogeographical data by delimiting and describing the species, but also to evaluate the diversity exhibited by *Gnetum* for agronomic and gustatory purposes**.** The conservation strategies addressed include the identification of a suitable or appreciated variant for promotion by cultivation through seed germination, in order to reduce the impact on natural populations and redefine sustainable methods of harvesting the plants.

1.7.1 The aims, objectives and key questions

Aim 1: To ascertain species limits and infraspecific variation of natural wild plants in the genus *Gnetum* in Africa.

Objective I: To investigate the various species to be recognized in the genus in Africa and the important features to use in order to distinguish them.

The Key Questions this objective attempts to answer are:

i) How many species should be recognised in the genus in Africa?

ii) What features distinguish them?

Aim 2: To illustrate the distribution of variation between and within the cultivated and wild populations of *Gnetum* in Africa based on morphological features.

Objective II: To determine how the cultivated material from various geographical regions relates to the naturally occurring taxa.

The Key Questions this objective attempts to answer are:

i) Are all the cultivated forms the same species?

ii) What is the distribution of morphological variability between the wild and cultivated populations?

Aim 3: To distinguish among species within the genus *Gnetum* in Africa and elucidate the genetic similarities between and within wild and cultivated members of the genus.

Objective III: To determine species limits based on genetic patterns, and to determine the relatedness between wild and cultivated populations in various geographical regions.

The Key Questions this objective attempts to answer are:

i) Can the genetic pattern clearly define *Gnetum* species in Africa?

ii) What is the distribution of genetic variability among the wild and cultivated populations?

iii) To what extent are the wild varieties reflected genetically among cultivated populations?

Aim 4: To provide anatomical diagnostic characters as additional evidence towards the delimitation of taxa in the genus *Gnetum* in Africa.

Objective IV: To investigate whether anatomical features can be used to distinguish between African species of *Gnetum* in general and if female plants of a particular species present distinguishing anatomical characters similar to male plants of that species.

The Key Questions this objective attempts to answer are:

i) Can anatomical features be used to distinguish between the species?

ii) Does a female plant of a particular species present anatomical characters similar to a male plant of that species but distinct from other species?

Aim 5: To identify factors influencing tastes variation to promote sustainable utilization of *Gnetum* species in Africa.

Objective V: To investigate any variation in taste between *Gnetum* populations with respect to their geographical distribution and the leaf developmental age.

The Key Questions this objective attempts to answer are:

i) Does 'eru' taste differently based on provenance, the leaf age or both?

ii) Does taste distinguish species or populations of *Gnetum*?

Aim 6: To facilitate domestication of *Gnetum* for sustainable utilization.

Objective VI: To investigate *Gnetum* seed coat and endosperm resistance to germination in order to promote sustainable utilization of *Gnetum* via cultivation.

The Key Questions this objective attempts to answer are:

i) Can seeds of *Gnetum* species in Africa be germinated readily under artificial or nursery conditions? If not, how can the resistance be broken?

ii) What mechanisms are involved in seed germination of the African species of *Gnetum*?

iii) Are the seeds viviparous or recalcitrant?

1.8 Thesis Structure

The various chapters in this study are presented as a series of papers for publication, and all aim to resolve the issues around the recognition and sustainable utilization of *Gnetum* species in Africa. The thesis is made up of nine chapters:

Chapter 1: The general introduction sets an overview of the subject title on the taxonomic and genetic variability of the species of *Gnetum* in Africa. It also states the problem and rationale for the investigation, the objectives of the study and key questions. The next five chapters are concerned with taxonomic issues, whereas chapters 7 and 8 deal with the conservation strategies.

Chapter 2: Species delimitation in the genus *Gnetum* L. in Africa using the phenetic approach.

Chapter 3: A clarification of *Gnetum* L. (Gnetaceae) in Africa and the description of two new species.

Chapter 4: Morphological correlation between the cultivated and wild populations of *Gnetum* in Africa.

Chapter 5: The assessment of *Gnetum* species in Africa and their similarities based on the genetic pattern revealed by AFLP analysis.

Chapter 6: Utility of anatomical features in distinguishing African *Gnetum* species.

Chapter 7: Evaluation of taste variation among various populations of *Gnetum* in Africa: a contribution to selective cultivation.

Chapter 8: Seed germination trials for sustainable propagation of *Gnetum*.

Chapter 9: An 'erudite' exploration of *Gnetum.*

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CHAPTER TWO

SPECIES DELIMITATION IN THE GENUS *GNETUM* L. IN AFRICA USING A PHENETIC APPROACH

CONTENT

2.1 Introduction

- Background knowledge:
  Importance of this genus

- Problem statement:
  Taxonomic confusion related to herbarium specimens and previous authors' descriptions

- Knowledge gap:
  Species/infraspecies recognition
  Taxonomic approach

- Purpose of this research and key questions
  Aim

2.2 Materials and Methods

Plant material
Morphometry
Data analysis

2.3 Results

Male results
Phenetic analysis using male specimens
Comparisons of means and ranges of quantitative characters

Female results
Phenetic analysis with female specimens
Comparisons of means and ranges of quantitative characters

2.4 Discussion

Taxonomic implications and useful morphological characters
Comparison of male and female results

2.5 Conclusion

REFERENCES

APPENDIX

Abstract

The economically important gymnosperm *Gnetum* L. of the family Gnetaceae is distributed in the humid tropical forests of Africa. The leaves are used as commercialized vegetables and thus contribute greatly to poverty alleviation for local people. In Africa, only two species have been recognized previously and these are threatened because of the disappearance of their habitat. From the present investigations, it emerges that different names have been variously applied on specimens that apparently represent similar forms of *Gnetum* in Africa. The application of these names interchangeably on herbarium specimens has resulted in taxonomic confusion in this genus. Further, little research attention has focused on the delimitation of species of *Gnetum*, so the number of species to recognize in this genus has been uncertain for over fifty years. To address the delimitation and identification of the species in this genus, morphological characterization using Cluster Analysis, Principal Components Analysis and Principal Coordinates Analysis has been explored to clarify the taxonomic treatment of *Gnetum* species in Africa. Two new African species have been distinguished based on the reproductive structures examined on herbarium specimens and on the clustering produced using the UPGMA and ordination analyses of data. Thus, the delimitation of the various species to recognize in this genus has resolved the identification and number of *Gnetum* species in Africa.

Key-words: *Gnetum*, Taxonomy, Gnetaceae, Africa, Delimitation

2.1 Introduction

2.1.1 Importance of the genus *Gnetum*

The genus *Gnetum* L. is an important economic gymnosperm in regions where it grows naturally in Africa. The genus in Africa comprises over-exploited and commercialized wild vegetable species distributed in the humid tropical forest zone. The sale of leaves of *Gnetum* is one of the important factors that promotes cross border trade not only between African countries but also between Africa and the rest of the world. *Gnetum* leaves are known to be rich in protein and minerals, and contain eight essential amino acids (Sunderland & Clark, 2001), therefore, they can prevent malnutrition. *Gnetum* contributes to poverty alleviation for local women and children who collect and sell it all year round. *Gnetum* leaves also have traditional medicinal values (Burkill, 1994) and are used in the production of a local gin in Nigeria (Mbahand, 2001).

Gnetum is one of the three genera of the family Gnetaceae, order Gnetales and was thought to comprise about 40 species, of which about 7 occur in South America, two in Africa, and c. 30 in tropical Asia (Maheshwari & Vasil, 1964; Earle, 2001). The species of *Gnetum* have opposite leaves with net venation and are generally dioecious. The males produce catkins of stamens and the females have catkins of ovules that are not protected by an envelope, as is characteristic of gymnosperms. *Gnetum* in Africa can be considered an insufficiently known taxon and it has been emphasized that such taxa present difficulties when assessing their contribution to biodiversity (Cracraft, 2002).

The motivations for studying this genus include that its species are of practical economic importance, and that the genus is the only gymnosperm found in the African primary and semi-deciduous forests (an aspect of interest in phytogeography). In addition, a large number of biological processes are unclear or imprecise in the life cycle of the species and their clarification is possible only after the species are taxonomically well identified. One component of taxonomy is the natural classification of organisms which is a step-by-step ranking of organisms from the smallest unit to the more inclusive sets or vice versa. The smallest compulsory ‘unit’ used in natural classification is the ‘species’ and it is therefore important to recognize the various species making up a genus and the probable

intraspecific variation within a given population. It is thought that the difference in the number of species in any particular genus could also be linked to the 'species problem' (Pigliucci, 2003). In other words, species delimitation is not possible unless the term 'species' is well defined and understood.

2.1.2 Problem statement:

Taxonomic confusion related to herbarium specimens and previous authors' description

Hutchinson & Dalziel (1954) described two species of *Gnetum* in Africa based on vegetative structures and on the shape of the internodes of spikes, however, neither their description nor their key to the species allows for a clear distinction between these two species. Since Prain's species descriptions in 1917 and Hutchinson & Dalziel's revision of the genus in 1954, the classification and identification of *Gnetum* species in Africa by various authors has resulted in taxonomic confusion; the members of the genus in Africa have remained insufficiently delimited. Examination of herbarium specimens shows that there has been misapplication of names to species of *Gnetum* in Africa (Biye *et al.*, 2013). Specimens of different morphological forms bore the same name, and in some cases different names were applied to similar forms or even duplicate *Gnetum* specimens. The morphological similarity between individuals of this genus has led to a 'species problem' and the difficulty in identifying species in this genus can be partly attributed to the different forms of *Gnetum* having similar vegetative features.

It seems that several authors (Markgraf, 1930; Gillespie & Nowicke, 1994; Won and Renner, 2003; Earle, 2001; Onguene and Kuyper, 2001; Isong *et al*., 1999; Carlquist, 1996; Hutchinson & Dalziel, 1954) have been uncertain about the number of species to recognize in the genus in Africa. For example, Markgraf (1930) and Gillespie & Nowicke (1994) referred to two species in Africa in their study of the pollen morphology of *Gnetum*; Hutchinson & Dalziel (1954) and Earle (2001) briefly also described the two species of *Gnetum* but Carlquist (1996) noted that these two species, *G. africanum* Welw. and *G. buchholzianum* Engl. are distinguishable based on the circular bordered pits of

vessel elements but might not be taxonomically distinct from each other because of little or no variation in vegetative morphology.

Won & Renner (2003) while studying the distribution of gymnosperm and angiosperm-type introns on the likelihood tree obtained from *Gnetum* nuclear and plastid sequences, also identified only one species in Africa. However, Onguene & Kuyper (2001) working on the mycorrhizal association of some plants in the rainforest of Southern Cameroon, and Isong *et al*. (1999) assaying the nutritional substances and fibre characters in relation to the leaf age of *Gnetum*, reported the existence of three species in the genus in Africa. It is therefore necessary to improve taxonomic knowledge of *Gnetum*. This includes the delimitation of *Gnetum* species in Africa by determining if there are one, two or more species and if distinct groups can be recognized below the species level using the phenetic approach. In brief, there is a great need for species clarification in the genus *Gnetum* in Africa using updated morphological characterization, although interspecific and intraspecific relationships might not be completely elucidated using morphological characters alone.

2.1.3 Species/infraspecies recognition

The lack of sufficient taxonomic research on the African species of *Gnetum* has led to diverse opinion on species identity in this taxon. These plants might not be morphologically distinguishable from their closest relatives (Carlquist, 1996); however, the choice of characters and how much they vary within and between groups plus the methods applied are important to differentiate very similar plants. Morphological variation among and within organisms appears to have motivated a series of debates around the so-called 'species problem' that led to various species concepts. If species are considered as a classification unit, then an acceptable definition for all biodiversity studies ought to be established and this seems impossible (Wheeler & Meier, 2000). Distinct aspects of the species question can be addressed based on the nature or type of data available and also on the main aim of each research project. Among the numerous species concepts established to highlight the origin of some species definitions and

applications (Minelli, 1993; Wheeler & Meier, 2000; Futuyma, 2006), one has proven useful in this study in order to recognize species in the genus *Gnetum* in Africa.

Several species concepts with various species definitions have been established since the time Darwin first revealed his idea of the origin of species in 1859, and this seems to be an indication that species are real and are the fundamental unit of evolution in time and in space (Coyne & Orr, 2004; Mallet, 2007). The morphological species concept seeks the interpretation of shared sets of distinguishing characters to define a species; each species is therefore morphologically different from its closest relatives and the choice of characters varies from group to group and between and within species (Fagerlind, 1941; Futuyma, 2006). Useful morphological characters to carry out, for example, a revision of a group should include features involved in the description of the type i.e. the original material on which the name of a taxon is based. The morphological species concept is therefore based on character interpretation and presents difficulties of identification for organisms of the same species that appear different due to environmental adaptation and genetic drift in allopatric populations.

The application of the biological species concept to species recognition has been relevant in elucidating taxonomic relationships; this concept often considers a species as a group of individuals that actually or potentially interbreed in nature (Futuyma, 2006). The biological species concept, widely adopted in the 1940's (Mayr, 1996), defines a species by its biological function instead of on the basis of morphological differences alone. However, it does not take into consideration the asexually reproducing organisms or those reproducing by parthenogenesis, the case of species living separately and the use of herbarium or museum specimens (Templeton, 1987; Stern *et al*., 2008).

The recognition species concept developed by Paterson defines a species based on its adaptive mating ability (Paterson, 1985; http://evolution.berkeley.edu). This definition emphasizes that species are a set of organisms that share the same specific mate recognition system (SMRS), can recognize each other as potential mates and focuses more on pre-zygotic ability to do so, which occurs in plants via pollination (Grant, 1949; Coyne & Orr, 2004). Organisms therefore use various kinds of features (signals and

responses) to recognize each other as mates (Coyne & Orr, 2004; Mallet, 2007). SMRS presents some advantages for it can sometimes be observed, measured, identified and the testing of hypotheses is possible (Templeton, 1987; Mallet, 2007).

Another concept is the ecological species concept developed by Van Valen (1976) based on the habitation of an ecological niche in which there is clustering of organisms into groups; including asexually reproducing organisms (Coyne & Orr, 2004). This concept involves factors that influence the phenotypes such as the flow of genes and also the habitat in which the organism lives whereby members of a species exploit the same resources (Wallace, 1865; Futuyma, 2006). Similar species therefore must be ecologically distinct to coexist and their conservation in a local area actually preserves its ecological diversity.

The phylogenetic and phenetic species concepts are useful tools in taxonomy. The phylogenetic concept considers a species as a self-perpetuating group of organisms sharing unique characters, not present in their relatives (Cracraft, 1989; Davis, 1997). A species is thus considered to be the smallest set of organisms of common ancestry distinguished from other such sets. This concept, dealing with evolutionary relationships among organisms, seems to be highly accepted among systematists (Davis, 1997). It is similar to the morphological species concept in that unique distinguishing characters are used to identify species but differs in that it tends to reconstruct the parental pattern of ancestry and descent. The phylogenetic species concept is now very often used in species delimitation and recognition (Jacobson & Lester, 2003; Liu *et al*., 2010; Grundmann *et al*., 2010).

The phenetic concept considers the species or other taxa like infraspecies as the lowest level at which distinct phenetic clusters are observed, i.e. the taxon is therefore defined as a set of organisms that are phenotypically similar and look different from other sets on the basis of multivariate statistics (Sokal & Crovello, 1970). This concept traditionally used as many characters as possible to produce clusters based on multivariate statistics. Phenetics is thus a necessary tool for examining the overall patterns of variation in order to identify discrete groups to classify as species (McNeill, 1979; 1984). This approach,

although based on the analysis of morphological characters, attempts to improve on the arbitrary nature of the morphological species concept (Duncan & Baum, 1981; Jensen, 2009).

Phenetics is applicable to herbarium specimens (Radford *et al*., 1974; Sebola & Balkwill, 2009) and has several advantages over conventional taxonomy, but is often faced with the problem of various different patterns of groups resulting from different analyses and delimiting clusters arbitrarily based on personal decision (Sneath & Sokal 1973; Stace 1980; Gower 1988). Nevertheless, many systematists (Sneath, 1976; Hill, 1980; Thorpe, 1983; Herman *et al*., 1987; Krauss, 1994; Chandler & Crisp, 1998; Rohlf, 1998; Brysting & Elven, 2000; Cron *et al.* 2007; Sebola & Balkwill, 2009) continue to use phenetic methods, particularly in addressing species- level or infraspecific questions as is the case in this study. With limited fieldwork and a complete dependence on herbarium specimens for a taxonomic revision, the best method to apply in the recognition of species is the phenetic approach (Sebola & Balkwill, 2009). The criterion here is the phenotypic similarity in recognizing separate species; practically, various morphological characters are measured and presented in the form of cluster diagrams or two or three dimensional plots. This approach, also known as numerical taxonomy (Sokal & Sneath, 1963; Sokal & Crovello, 1970; Romesburg, 1984), based on multivariate analysis of characters (usually morphology or other observable traits), produces a classification based on the overall patterns of similarity in various individual specimens and establishes the identification of discrete groups as a taxon.

2.1.4 The taxonomic approach

Taxonomy is a theory and a practice of classifying organisms into taxa and it deals with the problem by using hierarchical order (Mayr, 1969). A taxon is considered to be a distinct taxonomic group of any rank worthy of being assigned a definite category. Thus the delimitation of a taxon from other taxa of the same rank is a taxonomic decision. For the diversity of organisms to be meaningful, a taxonomic hierarchy is important to illustrate that various species making up a genus are more similar to one another than to

those of other genera in a family. Species are important in taxonomy for they are used to order and store knowledge on particular taxa (Kornet, 1993).

The difficulty in identifying species of *Gnetum* caused confusion in its taxonomy and can be resolved using an appropriate species identification approach, regrettably, there is no standard species concept applicable to all biodiversity studies (Mallet, 2007). Nevertheless, taxonomic measures of diversity are essential for the estimation of plant richness in terms of genes or characters inherited; classification generally reflects the pattern of gene flow and the level of this flow influences variation in plants (Endler, 1977; Ellstrand, 1992).

Ultimately, all species concepts should deal with defining discontinuity in particular traits of organisms, such that those that are similar to each other are considered to be members of the same species. Variation may reflect the interactions between the environment and the genotype and the pattern of variation generally gives some measure of gene flow or breaks in gene flow and could either be continuous or discontinuous with respect to gene flow (Schonewald-Cox *et al*., 1985; Rieseberg & Burke, 2001; Ellstrand, 2003; Araujo *et al*., 2010). Both qualitative and quantitative characters contribute to variation observed within a population. The morphological variations that are observed on both herbarium and fresh specimens of *Gnetum* lead to the establishment of an appropriate classification and eventually nomenclature. It is hoped that the application of the numerical approach to taxonomy will provide an objective and stable classification for species of *Gnetum* in Africa.

Phenetic analyses are employed here as taxonomic tools to address species-level questions, within species variations and in the establishment of a natural classification for the genus *Gnetum* in Africa. This approach presents the pattern of phenotypic similarity that can be used to recognize separate species but usually a range of acceptable patterns results from various analyses; character description seems to be the most intricate aspect for the objectivity and repeatability of these analyses (Gift & Stevens, 1997). Although the phenetic species concept is not necessarily the best as it does not aim to reflect evolutionary relationships, phenetics as a tool allows the investigation of variation at

species and population levels and thus enables the evaluation of discontinuities among herbarium specimens or taxa. This should lead to the recognition of unique diagnostic characters which might be equivalent to apomorphies but might also be plesiomorphies and then do not necessarily reflect recent common ancestry (Snow *et al*., 2003).

A phenetic approach is adopted in the present investigation of *Gnetum*, for it is an aid to objectivity (Gower, 1988); it examines the geographical patterns of variation (Thorpe, 1983); it is applicable to both fresh and herbarium specimens, unlike most other species concepts; and it has produced convincing results in various studies (e.g. Herman *et al*., 1987; Stuessy, 1994; Cron *et al*., 2007). In order to investigate species limits and infraspecific variation in the genus *Gnetum* in Africa, both cluster analyses (Unweighted Pair-Group Method using Arithmetic Averages, UPGMA) and 2-dimensional/3-dimensional plots to view the results of Principal Coordinates Analysis (PCO) or Principal Components Analysis (PCA) are performed to separate specimens into groups. Cluster analysis (CA) generally produces a hierarchical pattern on any data (Thorpe, 1983) showing groupings among the specimens under investigation while the principal components analysis (PCA) is a widely used ordination technique producing independent axes showing proportion of variation. It is most useful for continuous data and when the first few components result in a greater proportion of the variance (Dunn & Everitt, 1982).

2.1.5 Purpose of this research and key questions

Forty species of *Gnetum* are known throughout the world and only two have been reported in Africa (Hutchinson & Dalziel, 1954; Earle, 2001). The African species are threatened due to the disappearance of their habitat and the method and extent of harvesting (Besong *et al*., 2001). These species might disappear before the knowledge of their diversity is totally revealed; moreover the two African species described in the Flora of West Tropical Africa are easily confused and in need of further taxonomic treatment. It seems, then, that despite the last revision in 1954 (Hutchinson & Dalziel, 1954), several questions still remain unanswered as far as the biological diversity and the delimitation of the species of *Gnetum* in Africa are concerned. The phenetic approach is used to assess

the amount of phenotypic plasticity (i.e. the property of a genotype to produce different observable characteristics when exposed to diverse environments), expressed by different morphological characters observed in *Gnetum* in order to carry out a taxonomic revision of the genus in Africa. The intention of this study is not only to produce a key for identification at the species level (or below) but also to provide a description of morphological characters to use in recognizing the taxa. In sum, phenetics is required to address the key questions of 'how many species should be recognised in the genus in Africa' and 'what features should be used to distinguish them'?

The aim of this study is to ascertain species limits and infraspecific variation of natural wild plants in the genus *Gnetum* in Africa and the important features to use in order to distinguish them.

2.2 Materials and Methods

To address the uncertainty of the number of species in the genus *Gnetum* in Africa, the phenetic approach using morphological characters as a tool for data exploration based on herbarium specimens has been applied.

2.2.1 Plant material

The analysis is based on 345 specimens borrowed from the following herbaria: B, BR, FHI, J, K, LISC, MO, P, PRE, US, WAG and YA (herbarium abbreviations according to Holmgren *et al.*, 1990). The specimens were sorted into hypothetical groups irrespective of the names on the labels, and were grouped based on the similarity of morphological characters. Ninety-three male and 231 female specimens were recognized based on their reproductive structures. Sixty male and 130 female specimens with sufficiently mature reproductive structures were measured and twenty-one sterile specimens were difficult to allocate as either males or females. Sixteen specimens (5 males and 11 females) of four species, *Gnetum leyboldii* Tul., *G. nodiflorum* Brongn., *G. paniculatum* Spruce and

Gnetum sp. from South America, borrowed from Missouri Botanical Garden (MO) were used as standard taxa to calibrate the character list and character state definitions.

The specimens used in the analysis were matched to both the types from PRE, K and B (www.aluka.org), and descriptions of *Gnetum* species in Africa (Welwitsch, 1858; Engler, 1902; Hutchinson & Dalziel, 1954). Therefore, identification of the material was partially done by comparison with the type specimens and with the original descriptions (Biye *et al*., 2013). During the sorting of the specimens into groups, it was difficult to assign most females and some of the male plants to a particular group because of the similarities in the morphology. Specimens were selected to cover the overall geographic range and the morphological variability observed in *Gnetum* species in Africa. Only mature or fully developed structures on the herbarium specimens were examined. Specimens used in this study are listed in Appendix 1 and individual herbarium gatherings are treated as OTUs (Operational Taxonomic Units) (Sneath & Sokal, 1973; Stace, 1980; Radford, 1986).

2.2.2 Morphometry

Measurements were carried out on mature branches with spikes. A character list (Table 2.1), including both vegetative and reproductive characters considered to be of taxonomic importance, was based on published keys and descriptions (Welwitsch, 1858; Engler, 1908; Hutchinson & Dalziel, 1954; Jauregui *et al*., 2005), plus new observations as seen in Figure 1 using character terminology as in Radford *et al*. (1974). A total of 67 morphological characters (including 15 ratios derived from the basic characters) thought to be taxonomically helpful were initially selected and analyzed for variation: 53 quantitative and 14 qualitative characters were scored as binary characters (Table 2.1). Characters listed in Figure 2.1 are reported for the first time and are found to be of taxonomic value in this study, the various terms used for these characters describe the features concerned.

For leaf measurements, the lengths and widths of five leaves found at the node of the spike and another five mature leaves adjacent to or at the second node of the fertile

branches on each sampled specimen were measured and the length:width ratio calculated. These two sets of leaf measurements were carried out because the leaves at the inflorescence node were observed to be generally larger in some specimens than those below. All materials were studied using a WILD HEERBRUGG Light Microscope M7A but the reproductive structures were observed and measurements carried out with a ZEISS Stereo Discovery V12 Microscope using AxioVision Release 4.6.3

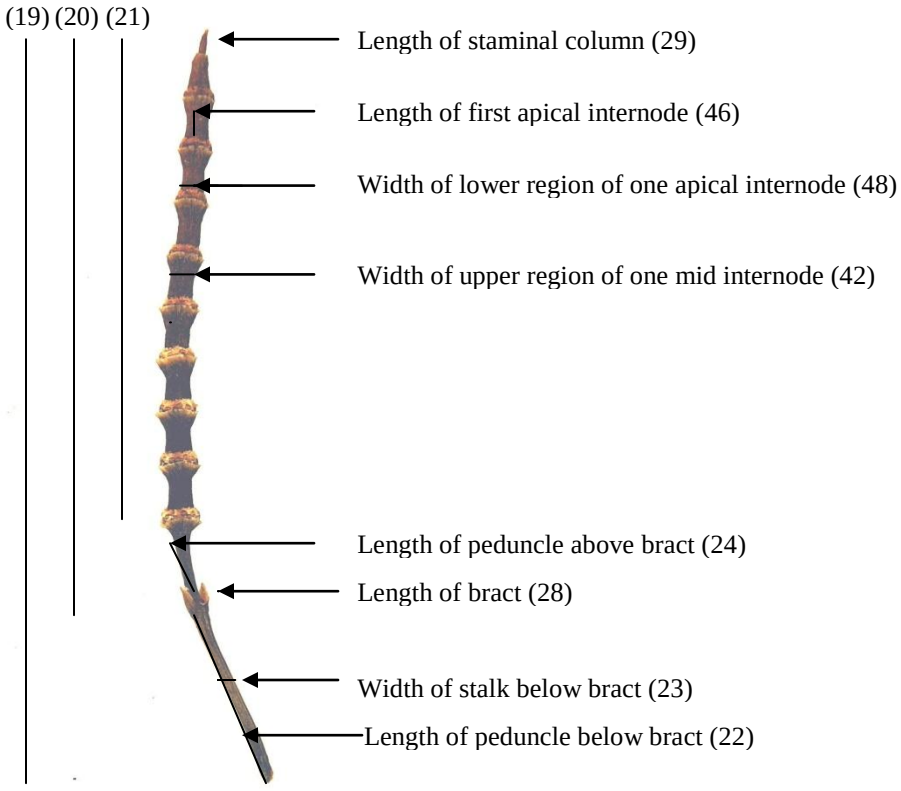


Figure 2.1 Some morphological characters analysed in the male spike. (19) Length of entire catkin; (20) Length of inflorescence axis; (21) Length of all whorls. Numbers in parentheses correspond to character numbers on Table 2.1.

Table 2.1 Descriptive list of qualitative and quantitative characters used in numerical analyses.

	Character
Leaf at first node	1. Maximum lamina length 2. Width at widest point 3. Maximum length/width ratio 4. Length of apex or acumen 5. Petiole length 6. Length from base to widest point 7. Ratio total length/length from base to widest point
Leaf at 2nd node	8. Maximum lamina length 9. Width at widest point 10. Maximum length/width ratio 11. Length of apex or acumen 12. Petiole length 13. Length from base to widest point 14. Ratio total length/length from base to widest point 15. Apex acute-acuminate to caudate (1) or obtuse to acute (2) 16. Secondary veins loop once (1) or twice before margins (2) 17. Secondary veins camptodromous (1) or brochidodromous (2) 18. Lamina ovate-oblong (1) or elliptic oblong (2)
Inflorescence	19. Length of entire catkin 20. Length of inflorescence axis 21. Length of all whorls 22. Length of peduncle below bracts 23. Width of peduncle below bracts 24. Length of peduncle above bracts 25. Width at lower part of peduncle above bracts 26. Width at upper part of peduncle above bracts 27. Ratio of lengths of peduncle below and above bracts 28. Length of bract 29. Length of staminal column 30. Width of staminal column 31. Length of basal internode 32. Width of upper region of basal internode 33. Width of lower region of basal internode 34. Ratio upper/lower width of basal internode 35. Ratio length/width of basal internode 36. Average length of two lower internodes 37. Average width of upper region of two lower internodes 38. Average width of lower region of two lower internodes 39. Average ratio upper/lower width of two lower internodes 40. Ratio length/average width of two lower internodes 41. Average length of two mid internodes 42. Average width of upper region of two mid internodes 43. Average width of lower region of two mid internodes

	44. Average ratio upper/lower width of two mid internodes 45. Ratio length/average width of two mid internodes 46. Average length of two apical internodes 47. Average width of upper region of two apical internodes 48. Average width of lower region of two apical internodes 49. Average ratio upper/lower width of two apical internodes 50. Ratio length/average width of two apical internodes 51. Number of verticils on axis 52. Bracts present (1) or absent (2) 53. Bracts caducous (1) or persistent (2) 54. Number of spikes at each node equal to 1 (1) or at least 1 (2) 55. Arrangements of spikes all terminal (1) or at least some axillary (2) 56. Staminal column apex sharp (1) or blunt (2) 57. Space between seed insertion points large (1) or narrow (2) 58. Ratio peduncle:pedicel length longer (1) equal or shorter (2) 59. Mid internodes diameter even (1) wider than either at top or lower (2) 60. Stamen rows ≤ 2 (1) or at least 2 (2) 61. Stamen alternating with fibres (1) or above row of fibres (2)
Seed	62. Length 63. Width at widest point 64. Ratio length/width 65. Length of apex 66. Maximum number of seeds attached to whorl per spike
Side stem	67. Diameter

2.2.3 Data analysis

Characters were scored either as binary (for qualitative characters) or as means of 5 – 10 measurements on fertile branches on each specimen for each collection (for quantitative characters). The 67 selected characters were not all used in the analyses, because they were not all applicable to both male and female plants and only characters that vary significantly among two or more taxa were mostly selected. Ratios and one of their component basic characters were generally applied to recover better results and several combinations of characters were investigated to carry out the analysis. In total, 58 and 61 characters were relevant to males and females respectively. These characters were assessed in terms of their usefulness in grouping the specimens of *Gnetum*. Tentative grouping of specimens was established by sorting and matching to types but some groups had no types and so were created based on their similarities. Invariant characters were not

included in the qualitative data matrix but those that differ or vary between groups were used. The data matrix produced was first standardized to eliminate the effects of scales of measurement of different characters (Rohlf, 2000).

Numerical analyses were used to analyse and describe morphological relationships among the investigated taxa. To compare and evaluate diagnostic characters and relationships within the genus, three data matrices were used in the analyses, the qualitative, quantitative and combined data matrices. Cluster analysis (CA) was applied to the distance/similarity matrices in order to examine the morphological similarity or dissimilarity within and between groups and also if the groupings match the various forms of specimens. The standardized data matrix was transformed to a distance (or similarity) matrix of OTUs by applying the average taxonomic distance coefficient. To calculate the cophenetic correlation coefficient (r) for the tree to show how well the tree fits the data, the cophenetic values of the tree were compared with the distance or similarity matrix.

The male and female data sets comprised both quantitative and qualitative characters; and these were used to perform Principal Coordinates Analysis (PCO), but only the quantitative data were used in Principal Components Analysis (PCA) as recommended by Sneath & Sokal (1973) and Legendre & Legendre (2003) and the pattern of relationships between OTU's was analysed. For the PCA, the standardised matrix was used to compute correlations among characters and the eigenvector values for each axis extracted. Regarding the PCO, with the standardized data matrix, a distance matrix for the OUT's was computed, double centered and the eigenvectors corresponding to projections of objects were extracted. A similar ordination across the characters (based on the standardised matrix) for the PCO was performed in order to give some indication of which variables were most important in explaining the distribution of the data in 3D space (i.e. in lieu of eigen values/vectors). These scaling techniques permit the visual interpretation of the relationships between taxa, implying that the distances between points will reflect the similarities between OTUs (Dunn & Everitt, 1982).

The level at which various quantitative characters overlapped was compared by computing the means, ranges and standard deviations of the various measurements using the software program STATISTICA (Statsoft, Inc., 2002). Ordination analysis sets coordinate axes at right angles, the points projected producing maximum variance (Rohlf, 2000) and the characters that load heavily are considered as true indicators of taxonomic relationship. Separate CA, PCA and PCO analyses were performed to verify distinct groupings within the OTUs. CA was further performed within each putative group to examine the possible pattern of intraspecific correlation between members of the group and to compute the means and standard deviations for quantitative characters used.

2.3 Results

Generally, the numerical analyses resulted in patterns that corresponded well with the presumed putative groups. The analyses carried out separately using male and female specimens produced similar results i.e. four distinct groups of specimens.

2.3.1 Results obtained using the male specimens

Cluster analysis

A total of 61 quantitative and qualitative characters combined were applicable to the male specimens. When all the identified combined characters were used in the analysis of 61 male specimens, the resulting clusters comprised indistinguishable groupings of various OTU's. Several combinations of characters were then undertaken for the analysis to determine which data revealed the best pattern of groupings. Therefore, 54 characters selected produced suitable results and these included 13 ratios derived from the basic characters plus one of its component (the width) values. Consequently, distinct clusters were formed in the analyses of the males based on qualitative (Figure 2.2a), quantitative (not shown) and combined data matrices (Figure 2.2b) using 15, 39 and 54 characters respectively. These analyses yielded dendrograms with correlation coefficients (r) of 0.99, 0.86 and 0.93 respectively and these values indicated relatively good fit of the data to the tree (Rohlf, 1998).

Separate quantitative (not shown), qualitative data matrices and the combined quantitative and qualitative data set (Figure 2.2) all yielded clusters of specimens representing different taxa. The OTUs of *G. buchholzianum* (b) and *Gnetum sp. nov.1* (i) were nested within those of *G. africanum* (a) indicating a degree of similarity between the specimens, also two or more distinct clusters within *G. buchholzianum* and *Gnetum sp. nov.1* (i) were recovered with no clear geographical pattern. Two specimens *Biye 603* and *Biye 617*, identified as l.C55 and l.C56 respectively clustered together and distinctly from the other three groupings in all the analyses using qualitative, quantitative (not shown) combined qualitative and quantitative data matrices (Figure 2.2a & b).

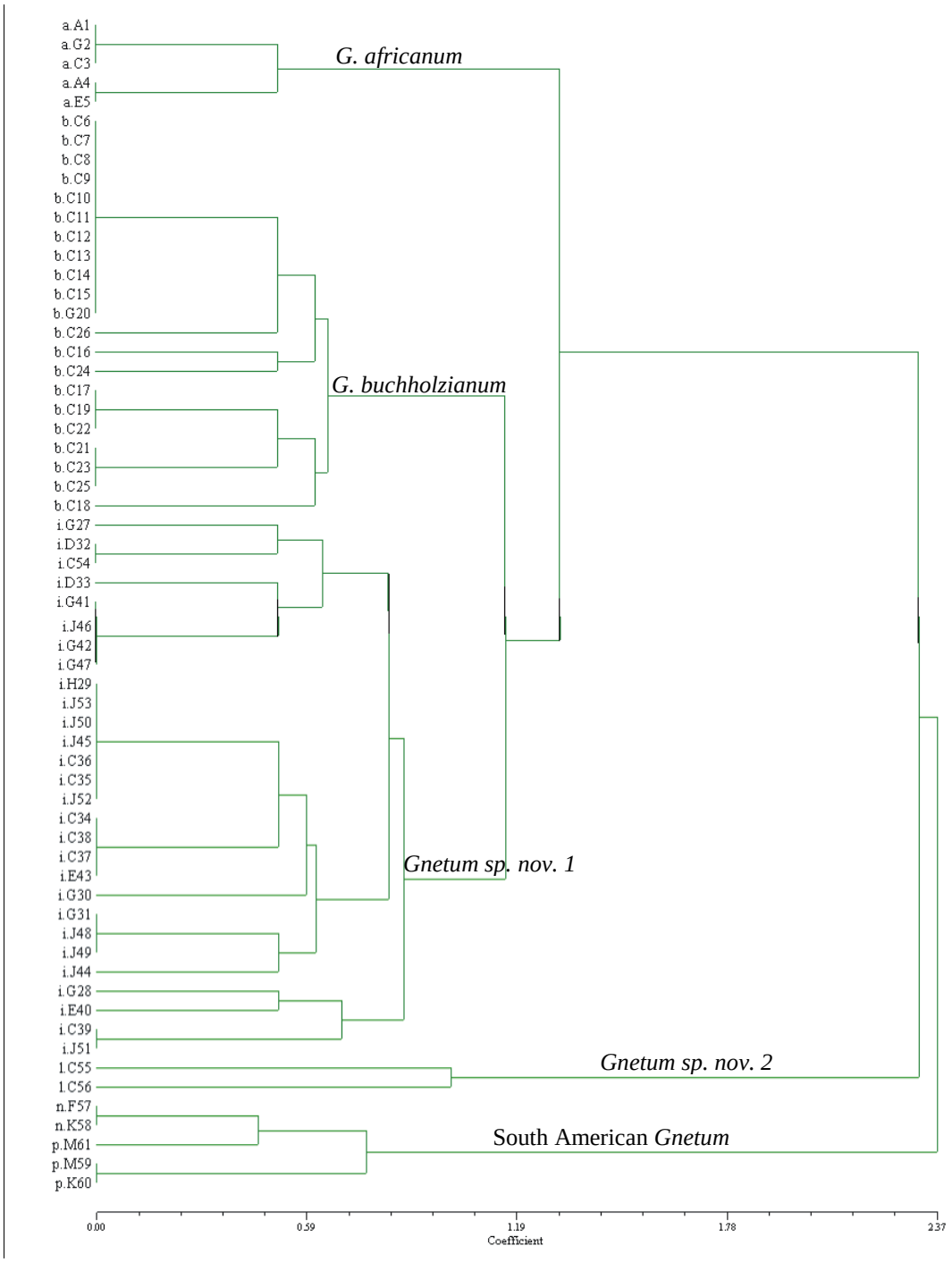


Figure 2.2i UPGMA phenograms using average taxonomic distance of the male specimens of *Gnetum* species in Africa based on qualitative data matrix. Abbreviations, a = *G. africanum*; b = *G. buchholzianum*; i = *Gnetum sp. nov. 1*; *l* = *Gnetum sp. nov. 2*; n = *G. nodiflorum*; p = *G. paniculatum*; A = Angola; B = Amazon; C = Cameroon; D = Central African Republic; E = Congo; F = Colombia; G = Democratic Republic of Congo (DRC); H = Equatorial Guinea; I = Ecuador; J = Gabon; K = Guyana; L = Peru; M = Venezuela + identification number) are used to identify specimens as in Appendix 1.

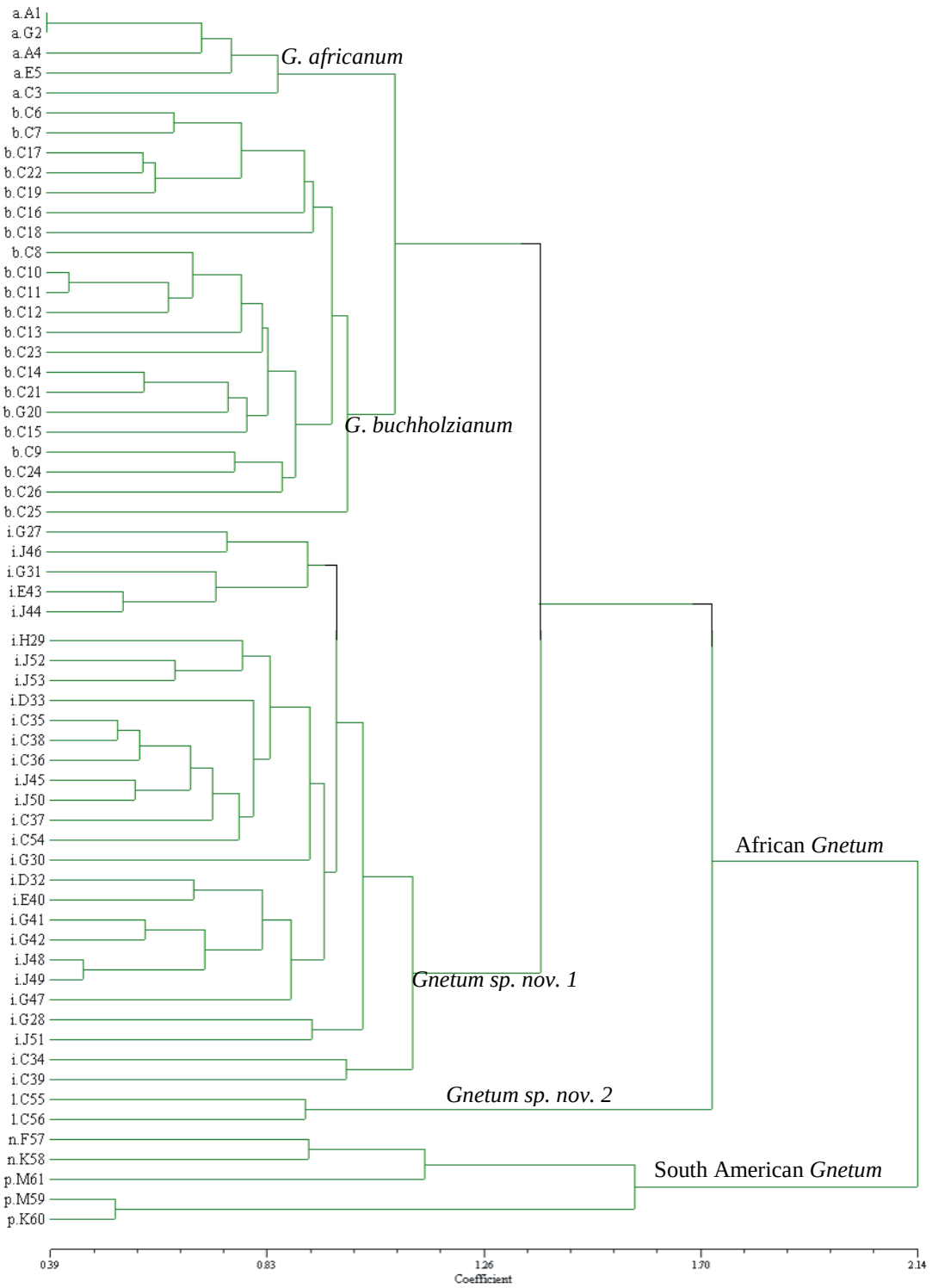


Figure 2.2ii UPGMA phenograms using average taxonomic distance of the male specimens of *Gnetum* species in Africa based on combined quantitative and qualitative data matrices. Abbreviations, a = *G. africanum*; b = *G. buchholzianum*; i = *Gnetum sp. nov. 1*; *l* = *Gnetum sp. nov. 2*; n = *G. nodiflorum*; p = *G. paniculatum*; A = Angola; B = Amazon; C = Cameroon; D = Central African Republic; E = Congo; F = Colombia; G = Democratic Republic of Congo (DRC); H = Equatorial Guinea; I = Ecuador; J = Gabon; K = Guyana; L = Peru; M = Venezuela + identification number) are used to identify specimens as in Appendix 1.

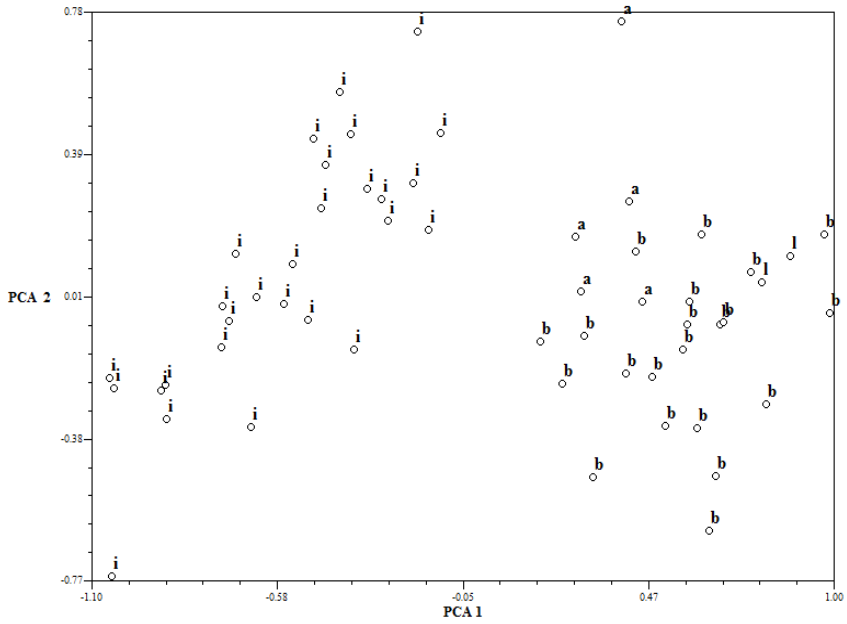
Ordination analyses

Principal Components Analysis (PCA) was applied to quantitative data only. Analysis of the male OTUs based on thirty-nine characters showed two distinct groupings representing *G. africanum* (a) and *G. buchholzianum* (b) at the right side of the plot on the one hand, and the specimens of the new species designated as *Gnetum sp. nov. 1* were placed at the left side of the plot on the other hand. The two specimens l.C55 and l.C56 were found among the plants in the first grouping at the right side of the scattergram.

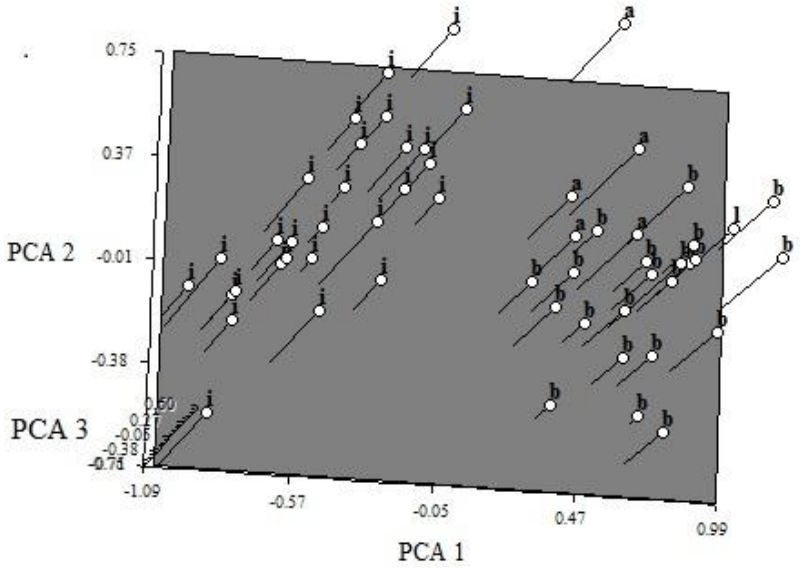
The PCA did not produce distinct clusters for *G. africanum, G. buchholzianum* or *Gnetum sp. nov. 2* (l.C55, l.C56) specimens in either of the 2-dimensional and 3-dimensional scatter diagrams produced in this analysis (Figure 2.3). OTUs of *G. africanum, G. buchholzianum* and *Gnetum sp. nov. 2* were therefore mixed while those of *Gnetum sp. nov. 1* formed a distinct cloud in both scatter diagrams. The standard taxa from South America were were excluded in both PCA and PCO as they were not part of the study group and inclusion of the South American taxa simply confounded the results. The first three axes accounted for 56.77% of the variation as shown by the eigen values summed up to 59.78 for the axes (Table 2.2).

Table 2.2 Eigen values obtained from Principal Components Analysis of the quantitative data matrix for the male specimens of *Gnetum* species in Africa. Variation is concentrated in the first 14 axes (91.79%, with the first 3 axes accounting for 56.77%).

Axis	Eigenvalues	% Total variance contribution	Cumulative %
1	14.77900541	37.8949	37.8949
2	3.75405671	9.6258	47.5207
3	3.60629346	9.2469	56.7676
4	2.25452235	5.7808	62.5484
5	2.16742137	5.5575	68.1059
6	1.87379183	4.8046	72.9105
7	1.42143064	3.6447	76.5552
8	1.24216132	3.1850	79.7402
9	1.04433342	2.6778	82.4180
10	0.95332630	2.4444	84.8624
11	0.77324282	1.9827	86.8451
12	0.73206278	1.8771	88.7222
13	0.65086121	1.6689	90.3911
14	0.54669344	1.4018	91.7928



(i)



(ii)

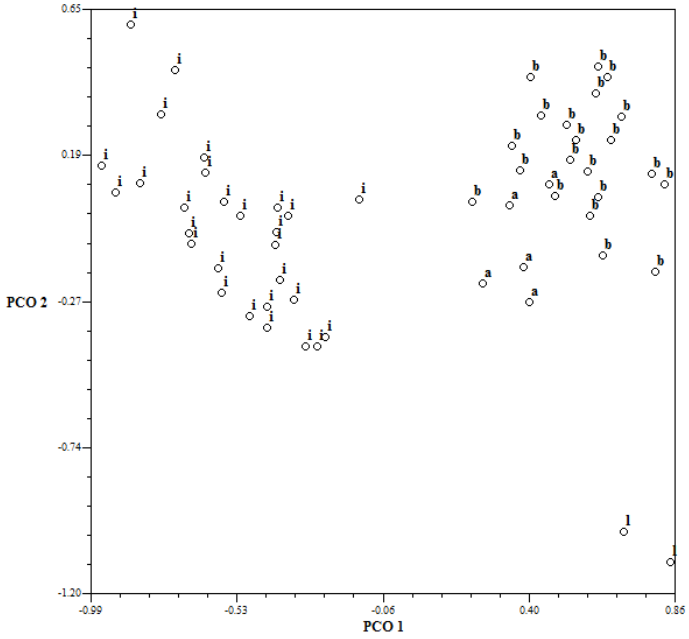
Figure 2.3 Scattergram (i) and 3D diagram (ii) generated by Principal Components Analysis based on quantitative data of male specimens of *Gnetum* species. *Gnetum sp. nov. 1* (i) forms a distinct cloud of OTUs while *G. africanum* (a), *G. buchholzianum* (b) and two specimens l.C55 and l.C56 distinguished in the clustering are overlapping in the 3-dimensional plot.

Principal Coordinates Analysis (PCO) was used to analyse the data of fifty-four quantitative and qualitative characters for the males. Three distinct groupings of the African *Gnetum* species were recovered: one group comprised *G. africanum* (a) and *G. buchholzianum* (b); the second group consists of the two specimens (l.C55 and l.C56) of *Gnetum sp. nov. 2* (l) and the third grouping of *Gnetum sp. nov. 1* (i) (Figure 2.4). The PCO analysis grouped specimens of *G. buchholzianum* (b) at the right side of the plot assuming a more or less intermediate position along the second PCO axis, although overlapping to some extent with *G. africanum* (a) while the two elements of *Gnetum sp. nov. 2* (l) were found towards the bottom right corner of the plot. OTUs of *Gnetum sp. nov. 1* (i) differed from those of *G. africanum* (a), *G. buchholzianum* (b) and *Gnetum sp. nov. 2* (l)) and were found at the lower left side of the plot (Figure 2.4a & b).. The standard taxa were also excluded from the PCO analysis in order to ensure that the first few synthetic axes effectively separate only the study group, i.e. the African plants, into distinct groupings.

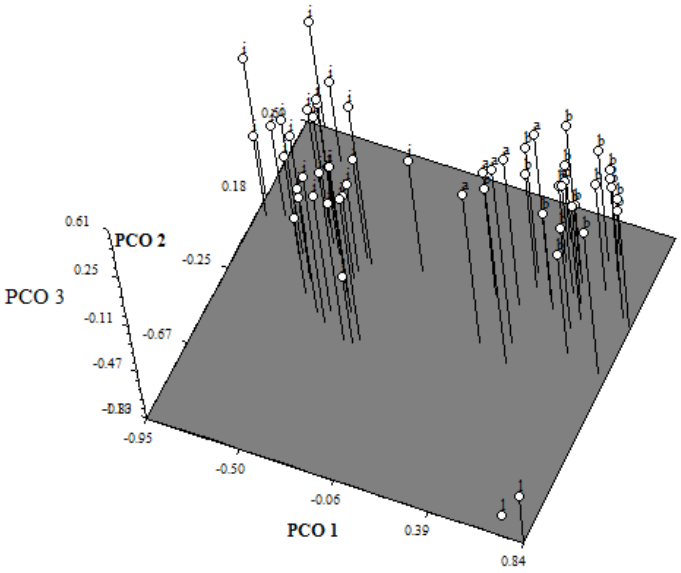
Characters that most influenced the distribution of specimens along the first three principal coordinate axes in the 2-dimensional and 3-dimensional scatter diagrams were those with a positive value ≥ 0.6 (or -6) such as the length and width of peduncle, the ratio of length / width and width of various internodes of spikes, and the presence and shape of the staminal column. Characters that most strongly correlated with the first PCO axis, in addition to those listed above, and thus separating the four groups were ratios of length of peduncle below to above bracts, length of bracts, the presence of staminal column and the position of spikes at the nodes of the stem.

Plants of *Gnetum interruptum* (i) generally had lower scores along the second PCO axis than the complex of the other three putative groups. The characters that negatively correlated with this axis with highest values were the leaf shape characters, the length of catkins and whorls (Figure 2.1), the number of nodes per spike, the ratio of length to width of internodes and the diameter of the side stem. Characters that positively correlated with the third PCO axis and had relatively low scores were the leaf characters, therefore the OTUs were spread out by this axis but there was no apparent regional pattern.

In both PCA and PCO scattergrams (Figures 2.3 & 2.4), the pattern of distribution was similar; the OTUs of *G. africanum* (a), *Gnetum sp. nov. 2* (l) and *G. buchholzianum* (b) clustered together while those of *Gnetum sp. nov. 1* (i) were distinctly grouped.



(i)



(ii)

Figure 2.4 Scattergram (i) and 3D diagram (ii) generated by Principal Coordinates Analysis based on the combined qualitative and quantitative data using the male specimens of *Gnetum* species. *Gnetum sp. nov. 1* (i) and *Gnetum sp. nov. 2* (l) each forms a distinct cloud of OTUs while *G. africanum (a)*, *G. buchholzianum* (b)) are not clearly distinguished from one another in the 3-dimensional plot.

Table 2.3 A listing of the first three 'eigen values' of the various characters loaded for PCO analysis of the male specimens.

Characters generated	1st axis	2nd Axis	3rd axis
Width of Leaf a	0.9674	0.1694	0.0826
Length/width ratio of a	0.9059	-0.0384	0.2609
Length of Petiole a	-0.9812	-0.1333	0.0506
Length of apex a	0.6113	0.5519	0.3856
HL/Length ratio of a	0.9611	0.1288	0.0402
Width of Leaf b	0.9531	0.1186	0.1591
Length/width ratio of b	0.9432	0.1129	0.0985
Length of petiole b	-0.9747	0.1544	0.0771
Length of apex b	-0.0368	0.1133	0.0385
HL/Length ratio of b	0.9587	0.1881	-0.0291
Length of Catkin unit	0.7195	0.0026	0.3606
Length of inflorescence axis	0.9091	0.1139	0.3016
Length of all whorls	0.9091	0.1138	0.3016
Diameter of stalk	-0.9340	0.0864	-0.1323
Length of penduncle	0.8943	0.1034	0.0169
Diameter b penduncle	-0.9878	0.0014	0.0699
# of inflorescence	0.9053	0.3144	-0.2322
Basal Inflorescence width a	-0.3423	0.8980	0.1525
Basal inflorescence width b	-0.7825	0.4944	0.2472
Basal inflorescence width a/b	-0.8036	0.3026	0.0309
Basal inflorescence length/width	0.4523	-0.7925	0.2322
Lower inflorescence width b	-0.0391	0.9398	0.1694
Lower inflorescence width a	-0.6717	0.3937	0.5550
Lower inflorescence width a/b	-0.7003	0.5521	-0.2755
Lower inflorescence length / width	0.0538	-0.8965	-0.1957
Mid inflorescence width a	0.4839	0.8417	-0.1657
Mid inflorescence width b	-0.7407	0.5586	0.2545
Mid inflorescence width a/b	0.3814	0.7561	-0.4358
Mid inflorescence length/width	0.0297	-0.8753	-0.0910
Upper inflorescence width a	0.5744	0.7852	-0.1356
Upper inflorescence width b	-0.8708	0.2036	0.3980
Upper inflorescence width a/b	0.7100	0.5641	-0.3674
Upper inflorescence length/width	-0.0488	-0.8257	0.1847
Length of staminal column	0.7476	-0.0131	0.5398
Width of stamina column	0.7476	-0.0131	0.5398
Length of bracts	0.9352	0.2815	-0.0204
Length of stalk/length of pedicel	0.0107	0.6700	-0.2819
Diameter of secondary stem	-0.0026	0.1372	0.4952

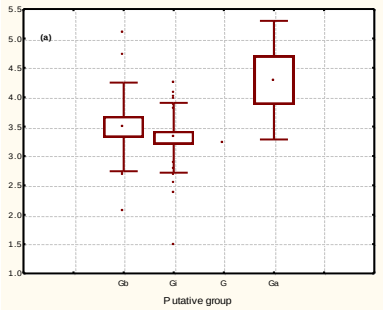
Comparisons of means and ranges of quantitative characters between groups

Thirteen vegetative and 33 reproductive characters were selected and examined in order to identify the most influential characters that distinguish the various forms of male specimens of *Gnetum*. The combinations of characters were obtained from correlations between characters and PCO axes plus the descriptive statistics of single characters (Figure 2.5a1 & b1). The results summarized in Figure 2.5a – f and 2.5g – x respectively show the state of variation of six selected vegetative and 18 reproductive characters examined for the four male groups. Most if not all vegetative characters exhibited continuities among the four putative groups. There were thus overlaps in vegetative characters such as the leaf blade shape and size, petiole length and the diameter of side stem. Notwithstanding, the leaf blade width overlapped between *G. buchholzianum, Gnetum sp. nov. 1* and *Gnetum sp. nov. 2* but was distinct in *G. africanum (*Figure 2.5a) and there was a considerable difference in the means of the apex length (Figure 2.5d) between the groups. Most specimens of *Gnetum sp. nov. 1* were different with respect to the apex length which was usually longer than in members of the other putative groups

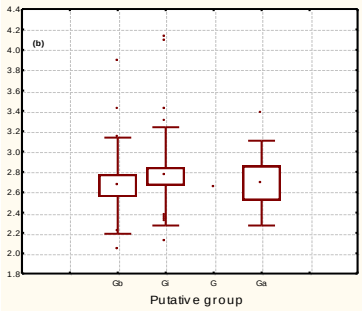
Characters that separated the four presumed groups were similar and consisted mostly of the reproductive features which included widths at lower and upper regions of the various internodes of the spike and ratio of lengths of peduncle below to above bracts (Figure 2.5j, k, l, m, n, o, and v). The ratio of upper to lower regions of internodes was a relatively good character distinguishing between putative groups although it overlapped between *G. africanum* and *Gnetum sp. nov. 1* (Figure 2.5q); the length of each internode and the ratio of length to average width of various internodes of spike (base, lower, mid or upper internodes) overlapped in *G. buchholzianum, G. africanum* and *Gnetum sp. nov. 2* (mean 1.7 – 1.9 mm) while *Gnetum sp. nov. 1* was well demarcated with a mean value of 5 mm (Figures 2.6 r & t). The width at mid internodes of *Gnetum sp. nov. 2* was different and usually greater than either at the lower or upper regions, not observed in the other three members. All other reproductive characters overlapped between putative groups, *G. buchholzianum, G. africanum, Gnetum sp. nov. 2* and *Gnetum sp. nov. 1* however in combination with other characters, they are still useful separating characters.

The length of peduncle above bracts (Figure 2.5k) overlapped mostly between *G. buchholzianum,* and *G. africanum* and then between *Gnetum sp. nov. 2* and *Gnetum sp. nov. 1;* the lengths of inflorescence axis and all the whorls showed distinct structuring between the putative groups. Although most vegetative features and some reproductive characters overlapped, the lengths of peduncle (Figure 2.5j) and their ratios (Figure 2.5v), the widths of various regions of internodes of spikes (Figures 2.5l, m, n & o) and their ratios (Figures 2.5q & p), the number and arrangement of spikes at each node and the sharpness of the tip of the staminal column were found to be highly influential characters in separating between putative groups, *G. buchholzianum, G. africanum, Gnetum sp. nov. 2* and *Gnetum sp. nov. 1* . The specimens, l.55 and l.56, representing *Gnetum sp. nov. 2* had thick catkins, their internodes at mid-point were often wider than at either top or lower regions, their length to width ratio of internodes was ± 1 especially when considering the width at mid-internodes while this was greater than 1 in *Gnetum africanum, G. buchholzianum* and *Gnetum sp. nov. 1*.

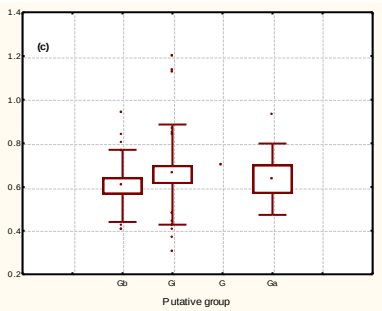
a. Width of leaf at first node



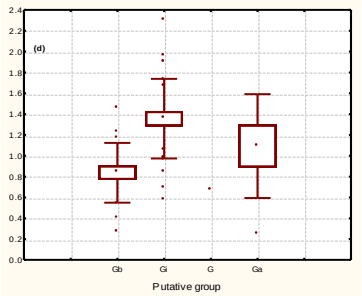
b. Ratio length/width of leaf



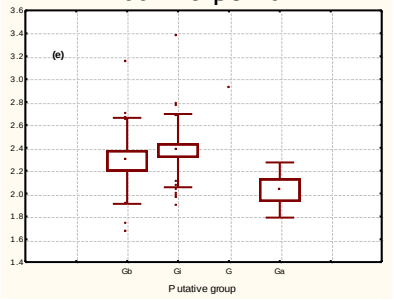
c. Length of petiole



d. Length of leaf apex



e. Ratio entire length/ length at mid point



f. Length of leaf

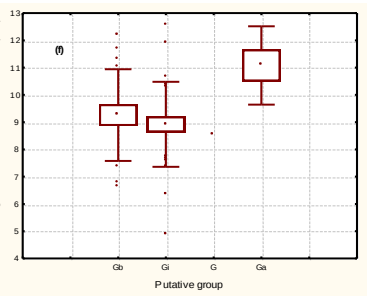
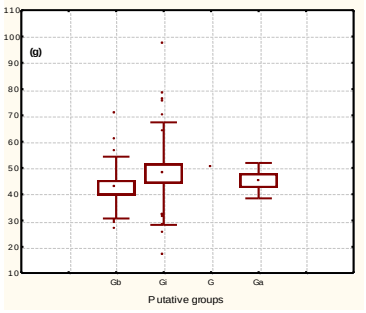
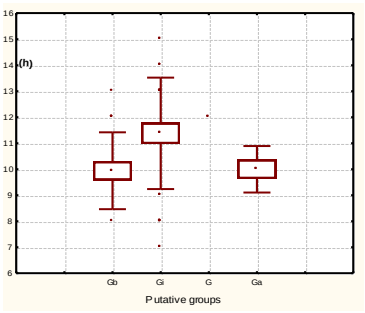


Figure 2.5 Variation in quantitative characters (vegetative) with respect to the putative groups (x-axis) of the males, *Gnetum buchholzianum* (Gb), *G. africanum* (Ga,) *Gnetum sp. nov. 1* (Gi), and the specimens l.C55 and l.C56 designated as (G). Whiskers = deviation indicating the maximum and minimum variation of the sampled values and the dots above or below the whiskers represent extreme values of the range (where applicable y-axis in cm).

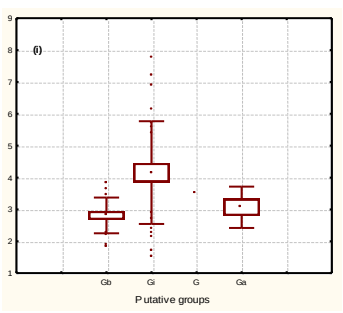
a. Length of all whorls



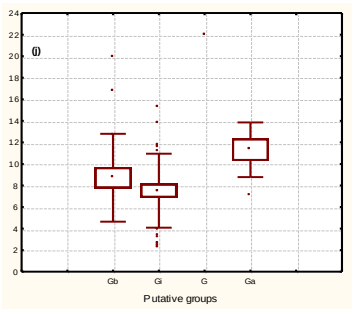
b. Number of whorls per axis



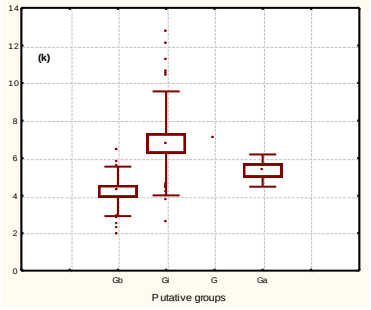
c. Length of basal interrnodes



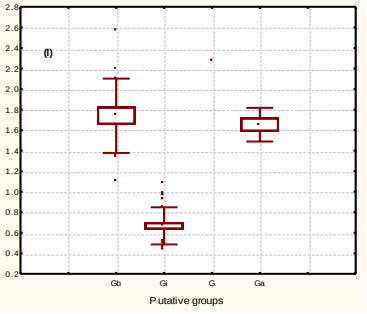
d. Length of peduncle below bracts



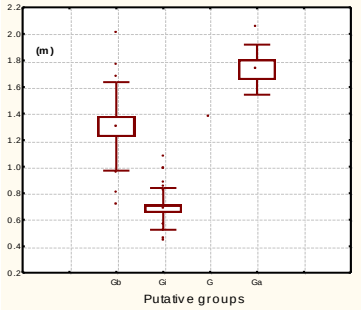
Length of peduncle above bracts



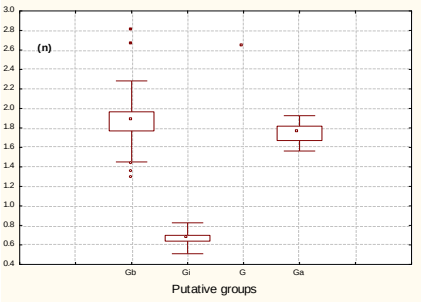
f. Width of upper internodes



g. Width of lower internodes



h. Width of upper internodes



i. Width of lower internodes

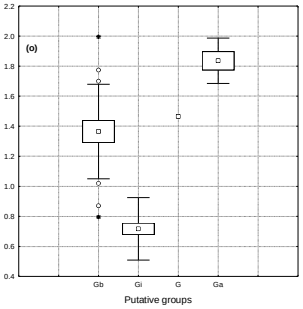
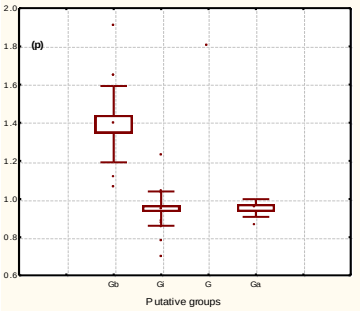
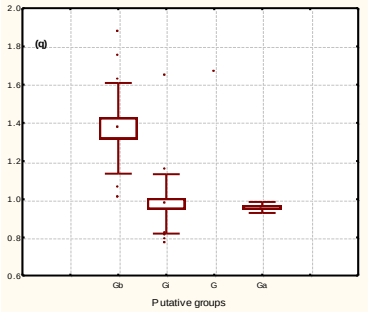


Figure 2.5 (continued) Variation in quantitative characters (reproductive) with respect to the putative groups (x-axis) of the males, *Gnetum buchholzianum* (Gb), *G. africanum* (Ga), *Gnetum sp. nov. 1* (Gi), and the specimens l.C55 and l.C56 designated as (G). whiskers = deviation indicating the maximum and minimum variation of the sampled values and the dots above or below the whiskers represent extreme values of the range (where applicable y-axis in mm).

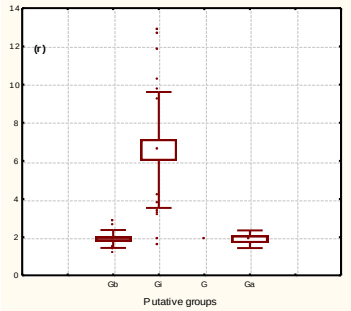
j. Ratio length/width lower internodes



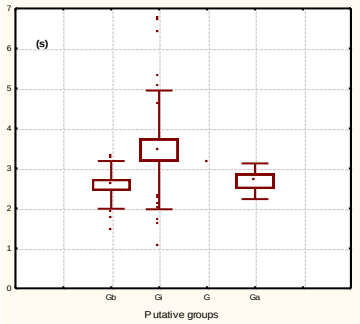
k. Ratio upper/ lower width of internodes



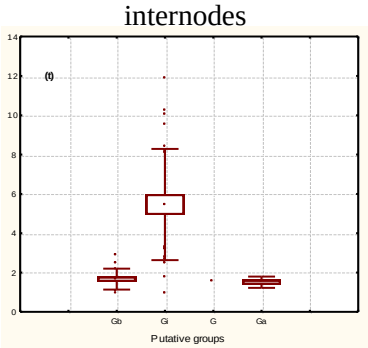
l. Ratio length/ width of internodes



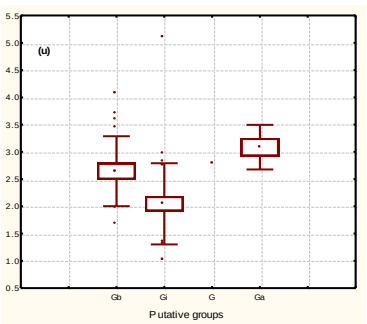
m. Length of lower internodes



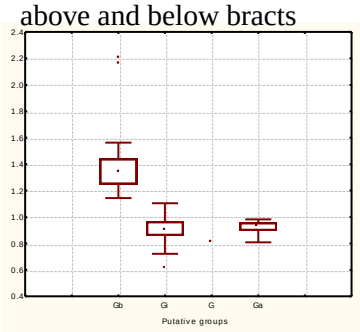
n. Ratio length/ width of internodes



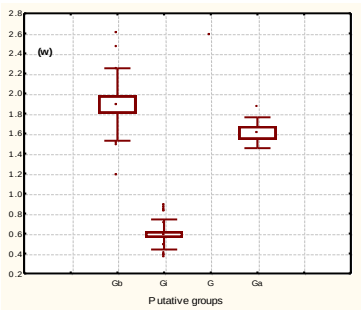
o. Length of bracts



p. Ratio lengths of peduncle above and below bracts



q. Width of upper mid internodes



r. Width of lower apical internodes

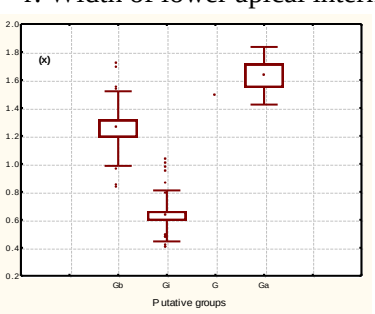


Figure 2.5 (continued) Variation in quantitative characters (b1. reproductive) with respect to the putative groups (x-axis) of the males, *Gnetum buchholzianum* (Gb), *G. africanum* (Ga), *Gnetum sp. nov. 1* (Gi), and the specimens l.C55 and l.C56 designated as (G). whiskers = deviation indicating the maximum and minimum variation of the sampled values and the dots above or below the whiskers represent extreme values of the range (where applicable y-axis in mm).

2.3.2 Results obtained using the female specimens of *Gnetum*

Cluster analysis

UPGMA analysis of the female specimens using 14, 43 and 57 characters for qualitative (Figure 2.6), quantitative (not shown) and combined quantitative and qualitative (Figure 2.7) data yielded phenograms with r = 0.98, 0.83 and 0.92 respectively and these values indicate a good fit of the data to the tree. Cluster analysis performed using 130 OTUs and 14 qualitative characters yielded a phenogram with four distinct clusters of *G. africanum* (a), *Gnetum sp. nov. 1* (i), *G. buchholzianum* (b) and *Gnetum sp. nov. 2* (l) respectively showing different degrees of clustering at which potential infraspecific taxa might be recognized (Figure 2.7). Six specimens (i.G53, i.G70, i.C55, i.J59, i.G62, i.E69) allocated to the set of *Gnetum sp. nov. 1* based on the morphology did not group with the rest. These specimens presented more than two axillary and/or terminal spikes per node, a character state also commonly observed with the male specimens of *Gnetum sp. nov. 1* **.**

The same clustering pattern was obtained with the phenogram using combined qualitative and quantitative characters except that all the specimens fitted clearly into their respective putative groups. Cluster analysis with continuous data only, yielded a phenogram (not shown) with a few overlapping OTUs of various groupings but, overall, four distinct groups, *G. africanum* (a), *G. buchholzianum* (b), *Gnetum sp. nov. 1* and *Gnetum sp. nov. 2* could be differentiated. The occurrence of the type specimens among the clusters on the phenogram permitted the attribution of the names to the various clusters thus groupings were recognized, and consequently, the characters that distinguished them. The phenogram also showed various mixing of the standard OTUs from South America although they clustered together and some of the specimens were without identification to the species level. In both phenograms recovered (Figures 2.6 & 2.7) no geographically based pattern was observed.

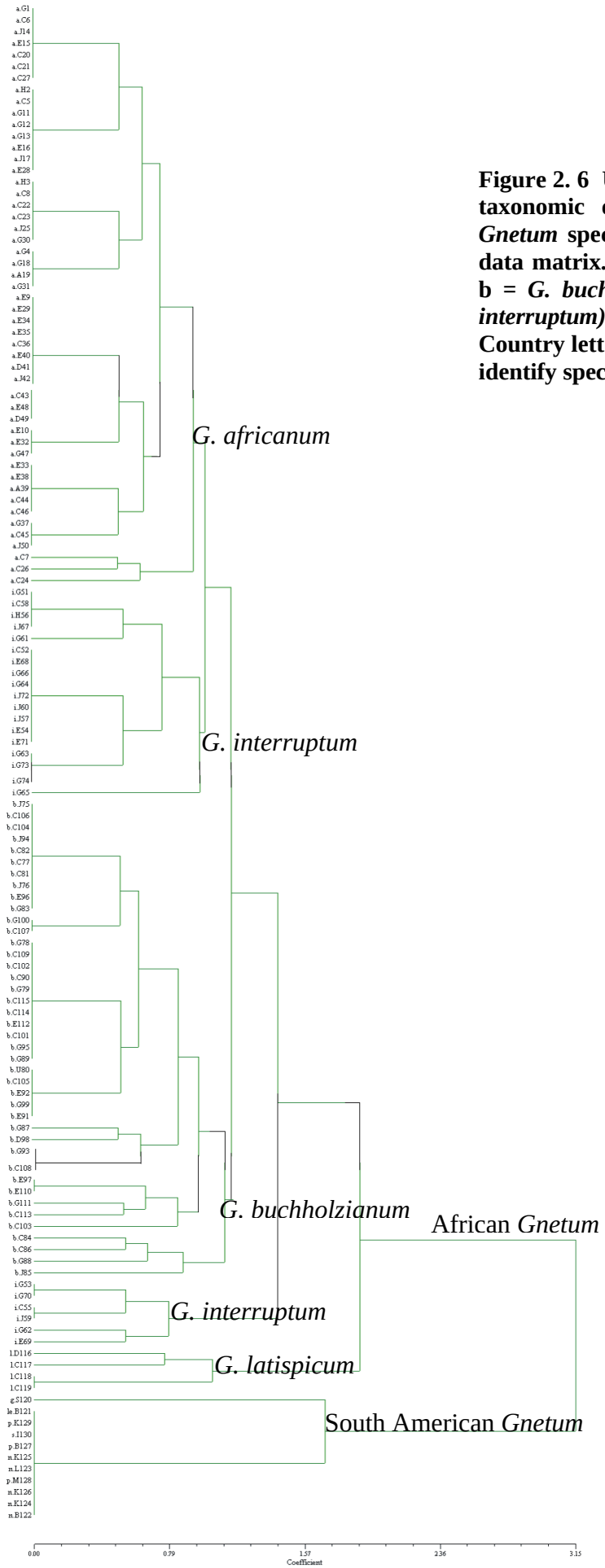


Figure 2. 6 UPGMA phenogram using average taxonomic distance of female specimens of *Gnetum* species in Africa based on qualitative data matrix. Abbreviations (a = *G. africanum*; b = *G. buchholzianum*; i = G. sp. nov. 1 (*G. interruptum)*; l = G. sp. nov. 2 (*G. latispicum*) + Country letter + identification number) used to identify specimens as in Appendix 1.

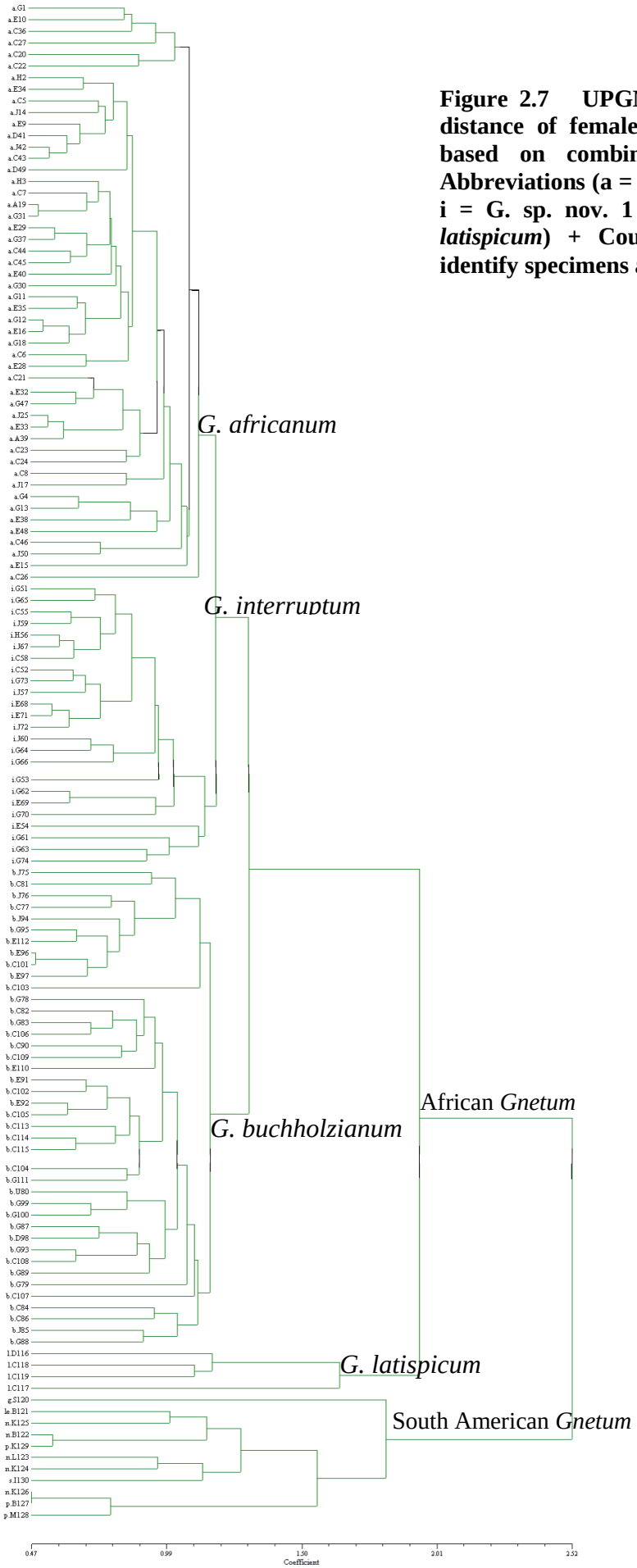


Figure 2.7 UPGMA phenogram using average taxonomic distance of female specimens of *Gnetum* species in Africa based on combined quantitative and qualitative data. Abbreviations (a = *G. africanum*; b = *G. buchholzianum* (Gb); i = G. sp. nov. 1 (*G. interruptum)*; l = G. sp. nov. 2 (*G. latispicum*) + Country + identification number) used to identify specimens as in Appendix 1.

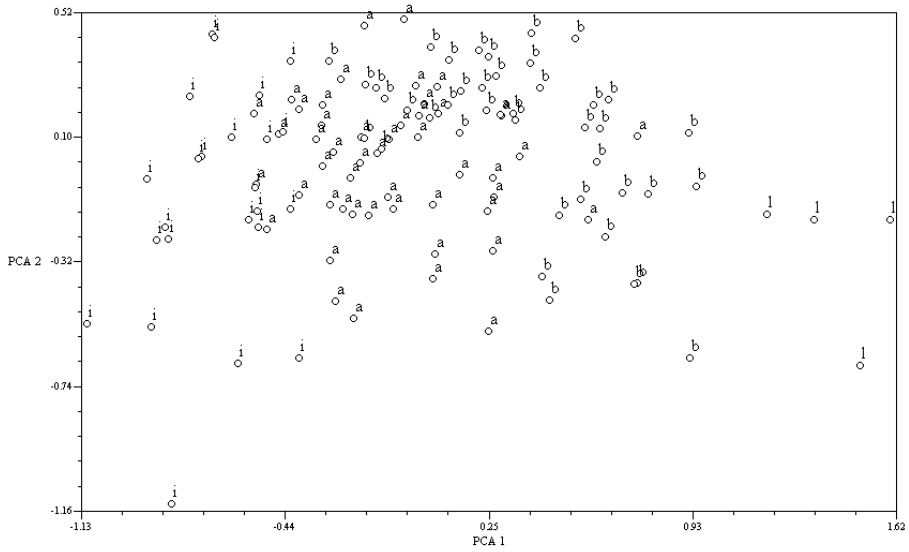
Ordination

Principal Component Analysis (PCA)

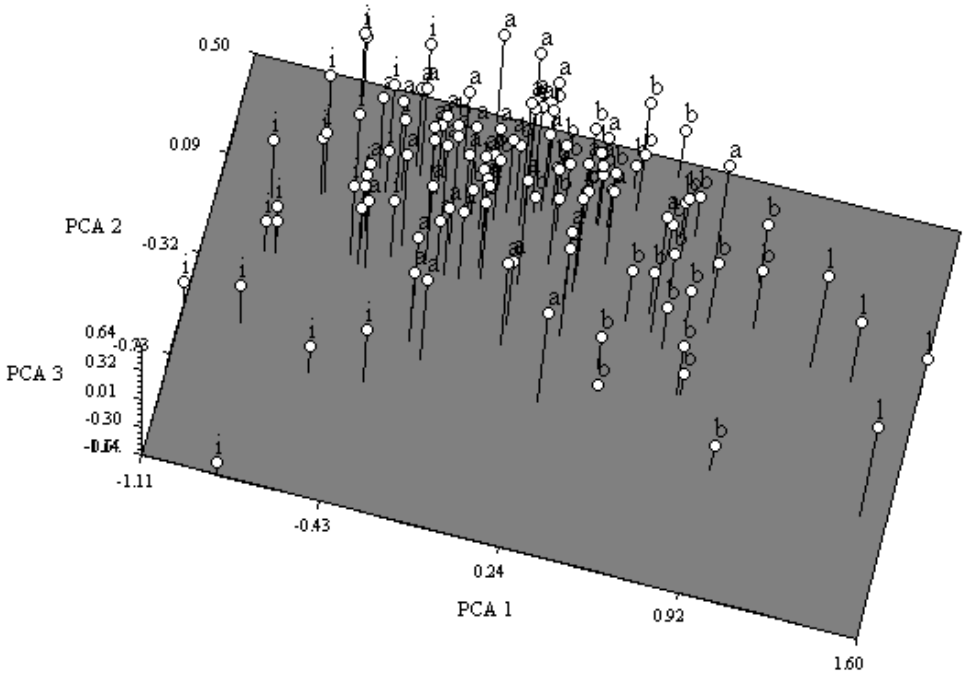
The 2D and 3D plots resulting from the PCA analysis for females using 130 specimens including the standard taxa (11 specimens from South America) produced one mixed cluster in which it is hard to recognize the distribution pattern of the different putative groups of the African *Gnetum* specimens (not shown). The various African specimens were grouped at the right side of the plot, *Gnetum sp. nov. 1* placed at the lower region with the lowest values according to the second axis; *G. africanum* was found overlapping at the intermediate position with some *G. buchholzianum* specimens. The OTUs of *Gnetum sp. nov. 2* were positioned at the uppermost region of the plot with the highest positive values based on the second axis.

The standard specimens were excluded in order to acquire scattergrams with a clearer pattern of grouping among the African *Gnetum* specimens. Thus, in PCA analysis of the female specimens based on quantitative data only, 119 OTUs and 43 characters were used and distinct agglomerations of the OTUs representing *G. africanum* (a), *G. buchholzianum* (b), and two new species *Gnetum sp. nov. 1* (i) and *Gnetum sp. nov. 2* (l) respectively were recovered in both 2D and 3D scatter diagrams (Figure 2.8a & b).

Most plants of *G. africanum* were more or less placed at an intermediate position while those of *G. buchholzianum* covered the lower right side of the plot although a few plants of *G. buchholzianum* overlapped with *G. africanum*. The plants of *Gnetum sp. nov. 1* and *Gnetum sp. nov. 2* were both placed at the opposite extremes with specimens of *Gnetum sp. nov. 1* spreading along the left side while those of *Gnetum sp. nov. 2* were confined at the top right side of the plot. *Gnetum sp. nov. 1* and *Gnetum sp. nov. 2* differed morphologically from *G. buchholzianum* while *G. africanum* included elements with somewhat deviating morphology, close to *G. buchholzianum*. The first three axes accounted for 52.42% of the variation as indicated by the eigenvalues generated during the analysis.



(i)



(ii)

Figure 2.8 Scattergrams (i) and 3D diagram (ii) generated from Principal Components Analysis based on quantitative data of female specimens of *Gnetum* show the pattern of grouping of female specimens of *G. buchholzianum* (b), *Gnetum sp. nov. 1* (i), *Gnetum sp. nov. 2* (l) and *G. africanum* in both 2- and 3-dimensional plots.

Table 2.4 Eigen values obtained from Principal Component Analysis of the combined data matrix for the female specimens of *Gnetum* species in Africa. Variation is concentrated in the first 12 components (89.12%, with the first three accounting for 52.42%).

Axis	Eigen values	% total variance contribution	Cumulative %
1	9.371	32.31	32.313
2	3.217	11.093	43.406
3	2.905	10.018	52.424
4	2.042	7.042	60.466
5	1.776	6.124	66.590
6	1.433	4.942	71.532
7	1.419	4.894	76.426
8	0.946	3.263	79.689
9	0.800	2.759	82.448
10	0.735	2.535	84.983
11	0.625	2.155	87.138
12	0.575	1.984	89.122

Principal Coordinates Analysis (PCO)

From the 2D and 3D plots, four groupings *G. africanum* (a), *G. buchholzianum* (b), and two new species *Gnetum sp. nov. 1* (i) and *Gnetum sp. nov. 2* (l), respectively were distinguished although the distance between some OTUs was rather close (Figure 2.9a & b**).** The Principal Coordinates analysis was performed using 119 specimens of various *Gnetum* species in Africa and 52 characters to clearly define the groupings.

Plants of *G. africanum* and *Gnetum sp. nov. 1* were distinctly grouped at the left side of the plot with *G. africanum* more on the top right and those of *Gnetum sp. nov. 1* were placed more or less at the intermediate position closer to the second axis, though a few of these specimens overlapped with those of *G. africanum*. Specimens of *G. buchholzianum* and *G. sp nov. 2* were placed at the right side of the plot; *G. sp nov. 2* was found at the extreme right of the scattergram with no overlapping. Most plants of *G. africanum* and *G. buchholzianum* had a more or less intermediate position with respect to the first two axes while plants of *Gnetum sp. nov. 2* were found more to the top right. Each putative group could be recognized separately.

Fifty-three qualitative and quantitative characters were used because the invariant qualitative characters were omitted. 41% of characters which most influenced the distribution of specimens along the first three principal coordinate axes in the 2-

dimensional and 3-dimensional scatter diagrams had values ≥ 0.6, for example, the length/width ratio of leaf, length of apex, the width at different regions of internodes of spikes, the length of staminal column, the number and width and the length of apex of seeds.

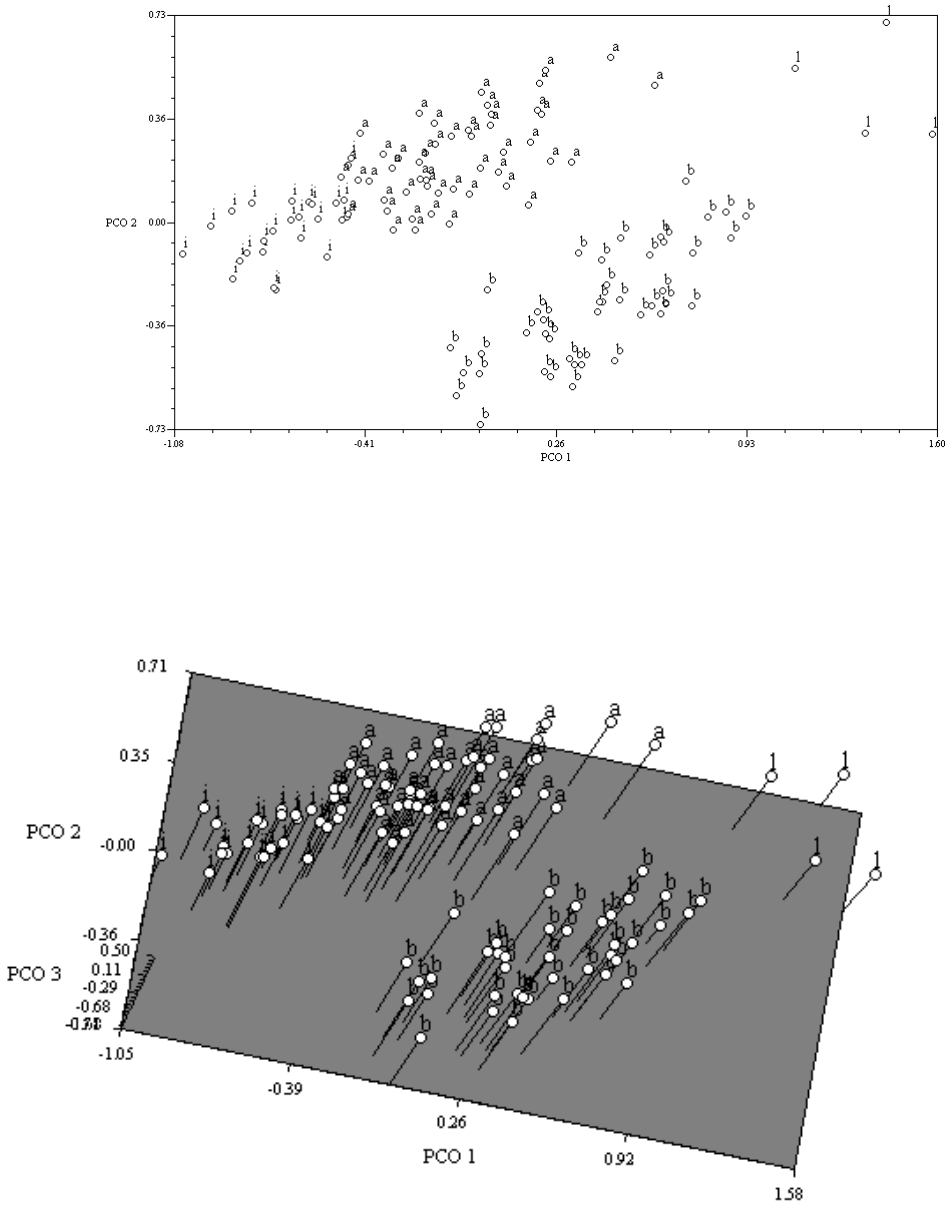


Figure 2.9 Scattergram and 3-dimensional diagrams generated by Principal Coordinates Analysis based on fifty-three combined qualitative and quantitative data of female specimens of *Gnetum africanum* (a), *G. buchholzianum* (b), *Gnetum sp. nov. 1* (i) and *Gnetum sp. nov. 2* (l).

Table 2.5 Eigenvalues for the various characters loaded for PCO analysis of the female specimens on the first three axes.

Characters generated	1st axis	2nd axis	3rd axis
Width of Leaf a	0.5202	0.2265	0.0081
Length/width ratio of a	-0.3274	0.3372	0.2208
Length of Petiole a	-2.3119	-0.3010	0.0737
Length of Hl a	-1.8569	-0.1032	0.2032
HL/Length ratio of a	-0.8267	0.1145	0.2354
Width of Leaf b	0.5148	0.3405	0.1213
Length/width ratio of b	-0.5431	0.2496	0.2030
Length of Catkin unit	8.5181	-1.0573	0.5265
Length of stalk	6.6647	-0.5501	-3.6255
Diameter of stalk	-2.2812	-0.3380	0.0601
Length of penduncle	5.4994	2.1173	-0.2740
Diameter a penduncle	-2.0506	-0.3695	0.0348
Diameter b penduncle	-2.2422	-0.3402	0.0716
# of inflorescence	6.4591	2.5057	0.6725
Basal Inflorescence width a	-1.4722	-0.3610	-0.1046
Length of petiole b	-2.3491	-0.3474	0.0426
Length of apex b	-1.9487	-0.1352	0.1308
Length HL b	0.8708	0.5293	0.1308
HL/Length ratio of a	-0.7416	0.1141	0.1934
Lower inflorescence width b	-1.3922	-0.3103	-0.0431
Lower inflorescence width a/b	-2.0751	-0.2641	0.0802
Lower inflorescence length / width	-2.1419	-0.3020	0.1315
Mid inflorescence width a	-1.4395	-0.3428	-0.0945
Mid inflorescence width b	-1.4705	-0.2990	-0.0454
Mid inflorescence width a/b	-2.0727	-0.2734	0.0742
Mid inflorescence length/width	-2.2160	-0.3556	0.1218
Upper inflorescence width a	-1.5852	-0.3629	-0.1000
Upper inflorescence width b	-1.6057	-0.3185	-0.0590
Upper inflorescence width a/b	-2.0758	-0.2807	0.0760
Upper inflorescence length/width	-2.2343	-0.3266	0.1077
Length of staminal column	-2.6075	-0.5671	0.1120

Width of stamina column	-2.8836	-0.5259	0.0091
Length of bracts	-1.3118	0.1532	-0.0975
Basal inflorescence width b	-1.5085	-0.3069	-0.0323
Basal inflorescence width a/b	-2.0749	-0.2774	0.0623
Basal inflorescence length/width	-1.4795	-0.0634	0.3421
Lower inflorescence width a	-1.3819	-0.3313	-0.0900
Diameter of secondary stem	-0.7860	-0.3866	-0.1131
Width of stalk	3.1988	2.9679	-0.5418
Length/width ratio	-1.6081	0.4423	-0.1916
Length of stalk	-3.0526	0.2392	-0.4915
Length of stalk/length of pedicel	-1.8797	-0.4928	-0.2866
# of seeds	0.4048	0.5779	0.3815
Leaf apex type	-2.0043	-0.2828	0.0780
# of inflorescence	-1.9137	-0.1663	0.1511
Arrangement of spikes	-2.3309	0.4602	-0.3983
Lamina ovate to oblong or elliptic	-1.5312	-0.0555	0.1170
Staminal column apex	-1.0808	-0.8912	1.0466
Space of seed insertion	-1.4969	0.0582	0.2807
Pedicel:penducle length ratio	-1.5848	0.0750	0.3922
Diameter of internode	-2.0124	-0.2997	0.080

Comparisons of means and ranges of quantitative characters between groups

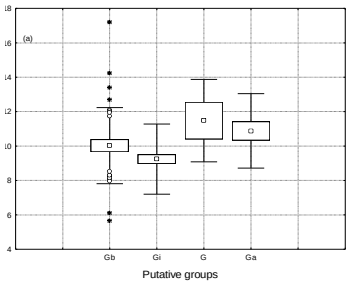
The female plants unlike the males presented greater difficulties when assigning them into different groups based on the shape and size of internodes of spikes because these were usually thicker compared to the internodes of the corresponding male. It could be argued that females bore seeds causing the whorls to thicken and therefore needed to be supported by even thicker internodes. Moreover, they also last longer, so have a longer growing period and also have to transport more nutrients to more new materials. The thick whorls and the enlarged seeds sometimes covered the internodes thus complicating measurements. However, reproductive features permitted a clear distinction between species and these include the characters that were found useful across the different axes in PCO analysis, and also the descriptive statistics of single characters (Figure 2.10a1 & b1).

Fourteen vegetative and thirty-nine reproductive characters were examined to distinguish between the four putative groups, *G africanum* (Ga), *G. buchholzianum* (Gb), *Gnetum sp. nov. 1* (Gi) and *Gnetum sp. nov. 2* (G). The results, Figure 2.10a – g and 2.10h – af respectively show the state of variation of seven of the vegetative and 25 of the reproductive characters examined for the four female groupings. There were overlaps in vegetative characters as seen in the males, nevertheless, the width of leaf showed a degree of variation between the four groups with *Gnetum sp. nov. 2* having the highest mean values followed by *Gnetum sp. nov. 1*. Characters that distinguished between the four groups based on the reproductive features included lengths of peduncle below and above bracts and their ratios, and widths at lower and upper regions of the various internodes of spikes (Figure 2.10j, k, n, r, s, t, v, w, x, z, aa, ae, af). The ratios of upper to lower ends of internodes of spikes were good characters to distinguish between groupings although there was overlap between *Gnetum sp. nov. 1* and *G africanum* then *G. buchholzianum* and *Gnetum sp. nov. 2* (Table 2.4). The lengths of catkin, inflorescence axis, diameter of the whorls, the length of each internode and the ratio of length to width of internodes overlapped between Gb, Gi and Ga but G remained quite distinct with the highest value. Reproductive characters such as ratio of length:width of internodes, the length of internodes, the diameters of peduncle below and above bracts, length and width of staminal column, length of bracts and the number of whorls or internodes overlapped between the putative groups.

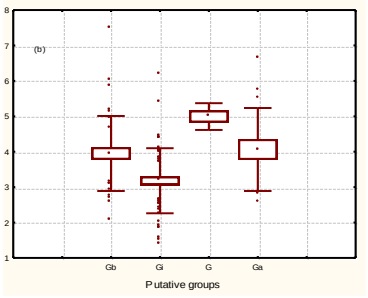
The length of peduncle (Figure 2.10k) overlapped between Gb and Ga and between Gi and G, the length of the entire catkin (Figure 2.10h), the lengths of inflorescence axis and all the whorls, the length, width at widest part and ratio of length to width of seed showed overlapping variation between Gb, Gi, and Ga but G was distinct with respect to these characters. The ratio of length to average width (Figure 2.10u) at various internode levels (base, lower, mid or upper internodes) overlapped in Gb, G and Ga (mean 1.7 – 1.9) while Gi had a mean value between 5 and 7.5. The number of seeds and the ratio of upper to lower widths of various internodes overlapped between Gb and G (generally >1) and between Gi and Ga (generally ≤ 1).

Characters such as length of peduncle (Figure 2.10j), the widths at the upper and lower regions at the various internode levels (Figures 2.10n, o, r, s), ratio of upper to lower regions of internodes (Figures 2.10p & t), the number and arrangement of spikes at each node, the shape of the tip of the staminal column and ratio of lengths of peduncle below to above bracts (Figure 2.10ae) were useful separating characters between putative groups.

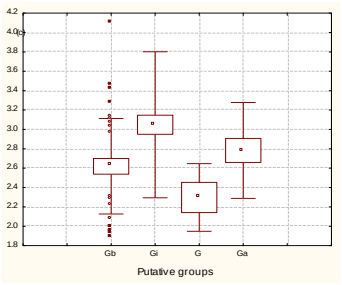
a. Length of leaf at first node



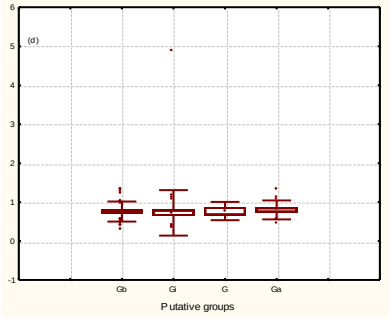
b. Width of leaf at first node



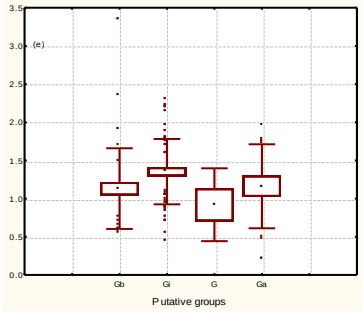
c. Ratio length:width of leaf



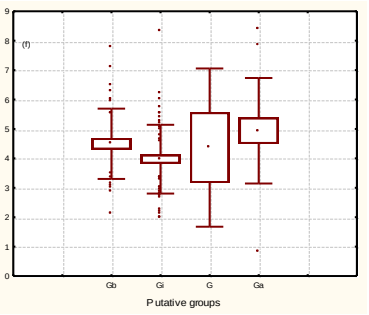
d. Length of petiole at first node



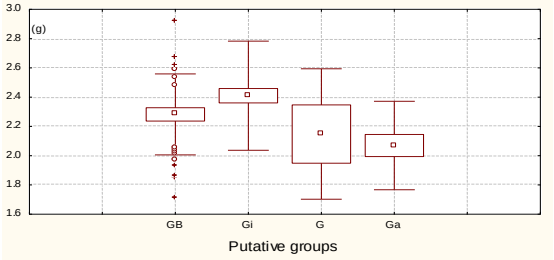
e. Length of leaf apex first node



f. Length at leaf widest point

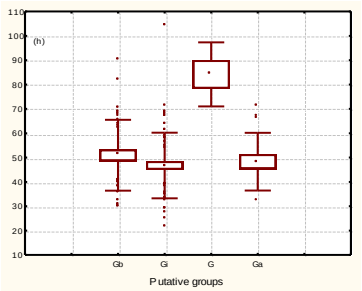


g. Ratio of entire length:length at widest point of leaf

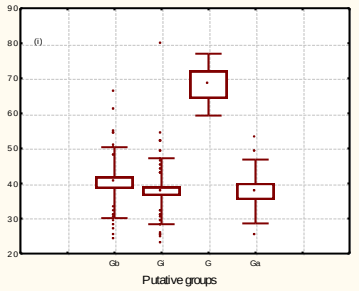


h. Length of catkin **i.** Length of inflorenscence **j.** Length of peduncle below bracts

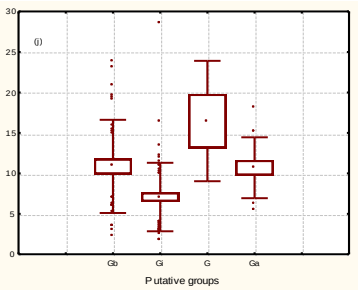
Figure 2.10 Variation in quantitative characters (a1. vegetative) with respect to the putative groups (x-axis) of the females, *Gnetum buchholzianum* (Gb), *Gnetum sp. nov. 1* (Gi), *Gnetum sp. nov. 2* (G) and *G. africanum* (Ga) respectively. Whiskers = deviation indicating the maximum and minimum variation of the sampled values and the dots above or below the whiskers represent extreme values of the range (where applicable y-axis in cm).



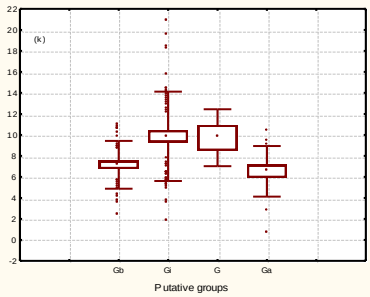
k. Length of peduncle above bract



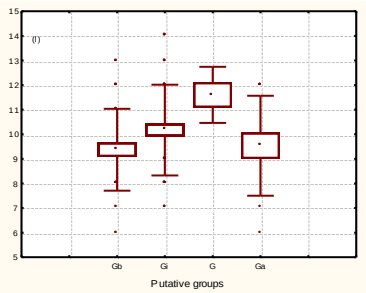
l. Number of whorls



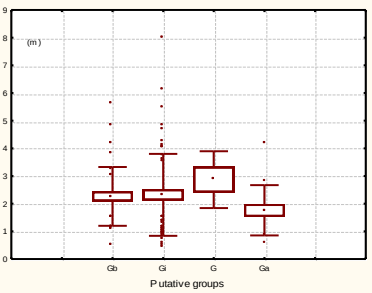
m. Length of basal internode



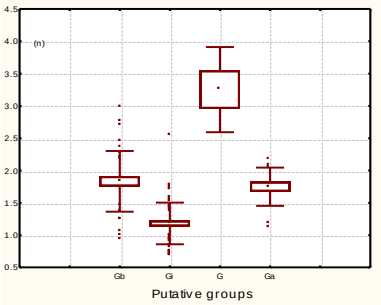
n. Width of upper b. internode



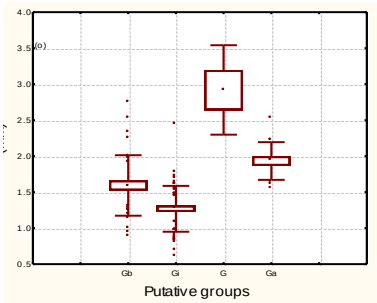
o. Width of lower b. internode



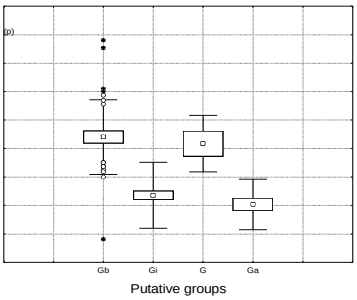
p. Ratio upper/lower internode



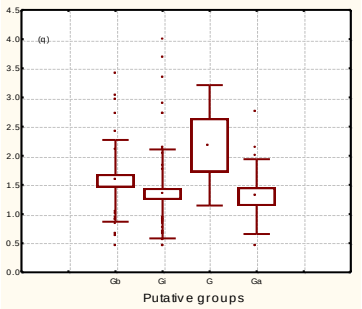
q. Length of lower internode

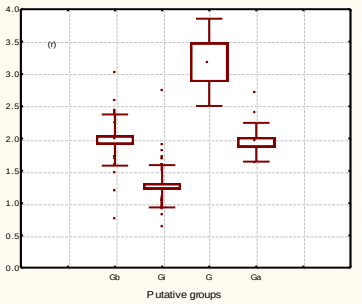


r. Width of upper l. internode



s. Width of lower l. internode





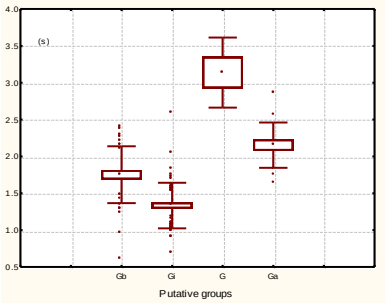
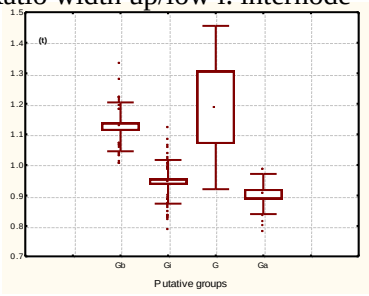
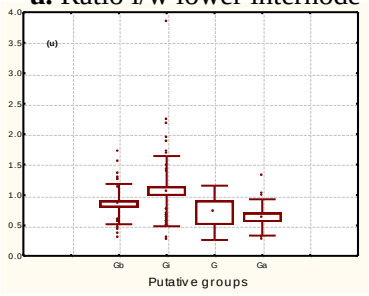


Figure 2.10 (continued) Variation in quantitative characters (b1. reproductive) with respect to the putative groups (x-axis) of the females, *Gnetum buchholzianum* (Gb), *Gnetum sp. nov. 1* (Gi), *Gnetum sp. nov. 2* (G) and *G. africanum* (Ga) respectively, (where applicable y-axis in mm),

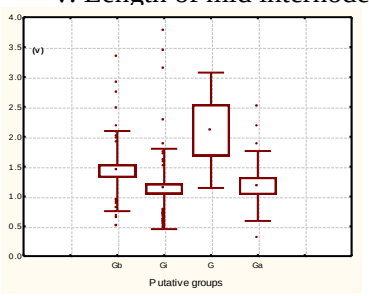
t. Ratio width up/low l. internode



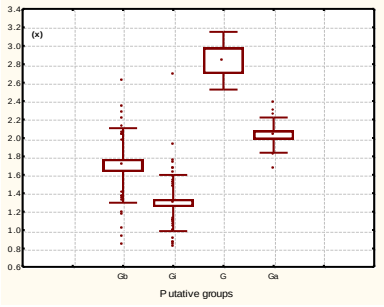
u. Ratio l/w lower internode



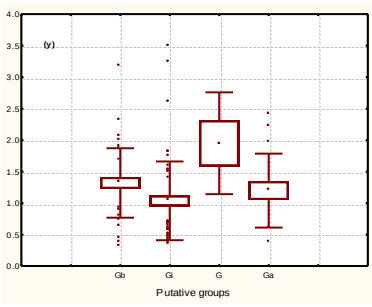
v. Length of mid internode



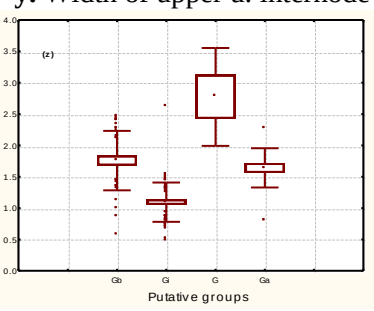
w. Width of lower m. internode



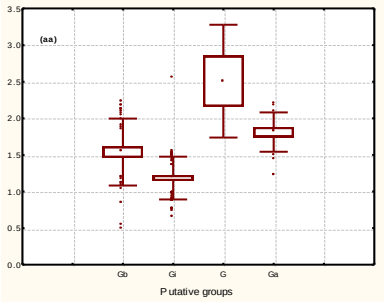
x. Length of upper internode



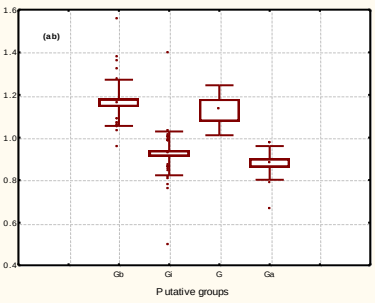
y. Width of upper u. internode



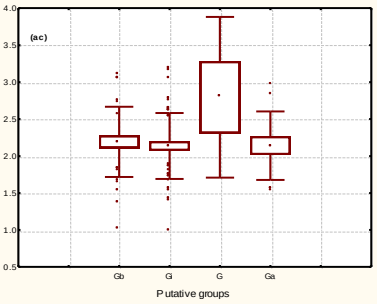
z. Width of lower u. internode



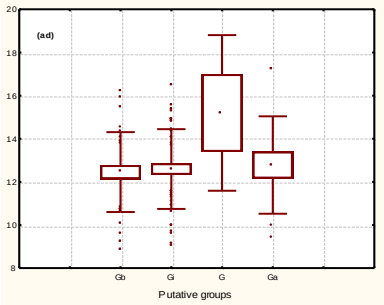
aa. Ratio width upper/lower u. internode



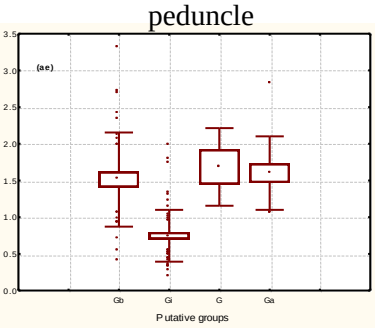
ab. Diameter of side stem



ac. Length of seeds



ad. Ratio lengths 1: 2 of peduncle



ae. Max. number of seeds/mid node

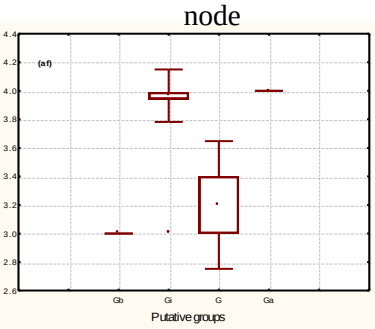


Figure 2.10 (continued) Variation in quantitative characters (b1. reproductive) with respect to the putative groups (x-axis) of the females, *Gnetum buchholzianum* (Gb), *Gnetum sp. nov. 1* (Gi), *Gnetum sp. nov. 2* (G) and *G. africanum* (Ga) respectively. Whiskers = deviation indicating the maximum and minimum variation of the sampled values and the dots above or below the whiskers represent extreme values of the range (where applicable y-axis in mm).

Table 2.6 Morphological characters used in recognizing species of *Gnetum*.

Character (in mm)	*G. africanum*		*G. buchholzianum*		*Gnetum sp. nov. 1*		*Gnetum sp. nov. 2*	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Leaf length width	60 – 170 20 –70	50 – 180 25 – 70	60 – 130 20 – 60	50 – 160 18 – 70	40 – 140 10 – 50	40 – 135 12 – 50	84 – 100 27 – 35	50 – 130 20 – 60
Spike arrangement number at each node	terminal 1	terminal 1	terminal &/or lateral 1 – 2	terminal &/or lateral 1 – 2	terminal &/or lateral 1 – 4	terminal &/or lateral 1 - 3	terminal 1 - 2	terminal 1
Pedicel length width	4 – 21 0.4 – 0.7	5 – 16 0.8 –1.5	5 – 13 0.4 – 0.9	2.4 – 23 0.6 – 1.5	2 – 15 0.3 – 0.8	3 – 24 0.5 – 0.9	2 – 15 0.7 – 2	7 – 15 0.7 – 3
Internode length width diameter ratio length: width	1 – 3.5 1– 3 even or slightly wider at base 1 – 2×	1 – 3.5 0.9 – 4 slightly wider at base 1/2 – 2×	2 – 4 0.5 – 3 wider towards top 1 – 3×	0.7 – 3.5 1– 3 wider at the top ½ – 2×	1– 10 0.3 – <1 even throughout 1 – 10×	0.4 – 10 0.3 –1.6 even throughout 1/2 – ≤7 ×	2.5 – 4 > 2 slightly thicker at mid region 1/2 – 1x	1 – < 4.5 > 2.3 even throughout 1/2 – 1x
Shape of staminal column tip	blunt	blunt	sharp	sharp	blunt	blunt	blunt	blunt
Arrangement of stamen rows at each node	≥ 2 rows alternating with fibres		< 2 rows alternating with fibres		≤ 2 with little fibres present		1 row above fibres row	
Maximun number of seeds at each whorl Seed shape		4 oblong-obovoid		2 – 3 ellipsoid		≥ 4 obovoid		3 or 4 ellipsoid-ovoid

2.4 Discussion

2.4.1 Taxonomic implications and useful characters for distinguishing species

Cluster analyses of the morphological variation in *Gnetum* species in Africa and the ordination analyses support the existence of previously described species *G. africanum* and *G. buchholzianum* (Welwitsch, 1858; Engler, 1908; Hutchinson & Dalziel, 1954; Earle, 2001), but also establish two other distinct groupings in the genus *Gnetum* in Africa, *Gnetum sp. nov. 1* and *Gnetum sp. nov. 2* as species (Biye *et al*., 2013). Therefore, four discrete groups and useful characters to distinguish them have been identified using the clustering and ordination analyses. In the various analyses, specimens of *G.*

africanum appeared to be more similar to those of *G. buchholzianum* in the males than they are to any of *Gnetum sp. nov. 1* and *Gnetum sp. nov. 2.* Meanwhile the female analyses showed that plants of *G. africanum* and *Gnetum sp. nov. 1* were phenetically much closer than they were to either *G. buchholzianum* or *Gnetum sp. nov. 2.*

Six female OTUs of *Gnetum sp. nov. 1* (i) (i.G53, i.G70, i.C55, i.J59, i.G62 and i.E69) were isolated from the *Gnetum sp. nov. 1* grouping; this could be attributed to their possessing some distinct characters, such as the widths at the upper and lower regions at various internode levels being less than 1 mm, the number of spikes per node more than two and the arrangement of these spikes both terminal and axillary. These characters were not generally useful in distinguishing the females, but played a major role in distinguishing between the males (specifically the group *Gnetum sp. nov. 1* from the others). The internodes of the spike of the female were generally thicker (to accommodate the seed-bearing whorls) than in corresponding males, and more than one spike per node was seldom observed in females. As noted, some variation within species is inherited, and some variation is due to the environment (Travis, 1996).

Individual specimens matching *G. africanum*, *G. buchholzianum*, *Gnetum sp. nov. 1* and *Gnetum sp. nov. 2* clustered into putative groups according to the multivariate analyses. On average, results from the cluster analyses and ordination analyses indicated a degree of phenetic similarity between specimens and some taxonomic stability in the groupings was obtained, thus supporting their recognition as distinct taxa (Sokal & Crovello, 1970; McNeill, 1979; 1984). The clustering of the standard taxa is an indication that the data set allowed the groupings of known species together; *G. africanum*, *G. buchholzianum*, *Gnetum sp. nov. 1* and *Gnetum sp. nov. 2* can therefore be recognized at species level.

From the literature, several authors (Won and Renner, 2003; Earle, 2001; Carlquist, 1996; Hutchinson & Dalziel, 1954) had been convinced that one or two previously described species, *G. africanum* by Welwitsch (1858) and *G. buchholzianum* by Engler (1908) existed in Africa. The description of these two species by Hutchinson and Dalziel (1954), was based on the shape of the internodes of spikes, but additional characters such as the thickness, the number and position of spikes per node, the shape of the staminal column

and the ratio of the various lengths of peduncle above and below bracts, have permitted two other forms *Gnetum sp. nov. 1* and *Gnetum sp. nov. 2* to be identified. This study of morphological variation of *Gnetum* species supports the indication by some other authors (Onguene and Kuyper, 2001; Isong *et al*., 1999) that there were three or more species in Africa.

Subspecies can be identified relative to their specific habitat and ecological preferences (Sebola & Balkwill, 2009) and can be defined with respect to their phylogenetic reliability (Cron *et al*., 2007). The humid tropical forest is continuous across West and Central Africa, covering the south eastern part of Nigeria and extending through the southern parts of Cameroon and Central African Republic, the entire Equatorial Guinea, Gabon, Congo, DRC to the north western region of Angola. The delimitation of the vegetation type is independent of the political boundaries designating countries. The specimens of *G. africanum*, *G. buchholzianum* and *Gnetum sp. nov. 1* were distributed evenly throughout the forest zone although *G. buchholzianum* has not been reported in Equatorial Guinea, neither has *Gnetum sp. nov. 1* been collected in Angola. *Gnetum sp. nov. 2* has only been collected in Cameroon and Central African Republic.

The OTUs of the species of *Gnetum* in Africa did not cluster with respect to their geographical range of distribution to produce a pattern to be identified at the subspecies level, even though Isong *et al.* (1999) had mentioned the use of three 'varieties' of *G. africanum* that were attributed names based on collection localities and were thought to be either distinct species or subspecies. The countries are large and may not reflect regional variation and their boundaries may not correspond to biologically meaningful areas, so analyzing by country or locality might mask a pattern that exists. However, the specimens of *G. buchholzianum* (b) and *Gnetum sp. nov. 1* (i) showed some level of infraspecific geographical differentiation, but that also does not clearly support infraspecific delimitation (Travis, 1996). More than half of the specimens generally originated from the same country in both species; the number and an appropriate sampling strategy is crucial in infraspecific delimitation to avoid misleading conclusions due to inadequate sampling (Davis & Heywood, 1963). Fifty-seven and 61 morphological characters identified for males and females respectively to assess the various *Gnetum*

species were based on the reproductive and vegetative features and this is a sufficient number of taxonomically valuable characters (Krell, 2004); characters that were invariant between the groups were eliminated.

The results from PCO analyses of both males and females show that reproductive rather than vegetative characters, were useful for the delimitation of *Gnetum* species in Africa and varied mostly between taxa; the reproductive characters have been recognized to be conservative and inherent (Stuessy, 1990). Main reproductive characters that distinguished *Gnetum* species were the unbranched catkins that were either axillary or terminal on a short branch, solitary or grouped in two to four; some of the male catkins had either slender or slightly thickened internodes of equal width from base to tip or thicker internodes widening from base towards the upper end.

Not all the reproductive characters studied separated species of *Gnetum*, there was some overlap in some of the characters among some groups, e.g. the number of whorls, length and width of staminal column, the length of bracts, length of pedicel, the length of the entire catkin, the lengths of inflorescence axis and all the whorls, and ratio of length to width of seed. Certain features were useful to various degrees. Out of 33 and 46 reproductive characters examined respectively in males and females, 13 were taxonomically valuable, i.e. useful in the delimitation of *Gnetum* species in Africa. In addition, eleven and nine other characters for males and females respectively, offered reliable data for taxonomic purposes and contribute to segregating between at least two putative groups. Although pollen morphology has not been investigated in this study, it has however, been shown that there are usable differences in the dimensions and ornamentation of the pollen grains among the taxa of the African group (Gillespie & Nowicke, 1994) and this could be exploited for a possible additional source of distinguishing characters in the systematic evaluation of *Gnetum*.

Vegetative characters, which are generally reported to be plastic and easily influenced by environmental conditions (Davis & Heywood, 1963; Heywood, 1967; Grant, 1984; Stuessy, 1990), were found to contribute less in the recognition of specific taxa in *Gnetum* in Africa. These characters (leaf size and shape, ratio of length to width of leaf,

length from base to the widest point of leaf, ratio of total leaf length to length from base to widest point, type of venation and length of petiole) varied considerably from one group to another and within members of the same group. The diameter of the side stem (not shown) overlapped between *G. africanum*, *G. buchholzianum* and *Gnetum sp. nov. 1* but differed from that of *Gnetum sp. nov. 2*. The variation in vegetative features overlapped within and between groups of *Gnetum* and this might be because of environmental factors such as soil nutrients, rainfall, and climate (Travis, 1996). The variant characters with little or no taxonomic significance were therefore mostly vegetative and consequently, they were not useful in the delimitation of *Gnetum* species in Africa.

The overlap observed between *G. africanum*, and *G. buchholzianum* in both males and females in several quantitative characters limited the use of a single diagnostic character (Brysting & Elven, 2000), therefore the use of more characters combined satisfactorily differentiated between the groups. Some 41.66% of the reproductive and 100% of the vegetative characters showed overlapping variation between putative groups. Thus, there could be a greater influence exerted on the groupings of the OTUs if not for the application of a combination of several characters in the analyses. The exclusion of some of these characters with a high degree of overlap subsequently produced clear groupings of OTUs, i.e. separated clusters of *G. africanum*, *G. buchholzianum*, and two new species of *Gnetum* (*Gnetum sp. nov. 1* and *Gnetum sp. nov. 2*) respectively in both the male and female analyses. In other words, the distinct clustering of the specimens of *G. africanum*, *G. buchholzianum*, *Gnetum sp. nov. 1* and *Gnetum sp. nov. 2* respectively based on the different analyses performed can be attributed to the influence the valuable characters had on the grouping of the OTUs using the numerical approach.

The intention of a taxonomist is to produce an unbiased classification wherein the fundamental unit is the species (Chapter One). In this present investigation, the Phenetic Species Concept has been applied not only to attempt to remove the bias regarding the classification of this genus by using an objective numerical approach but most especially to clarify the confusion observed in species recognition in the genus *Gnetum*. This numerical approach has enabled the distinction of four species of *Gnetum* in Africa,

drawing on a wide spectrum of morphological characters used in taxonomic elucidation. The most useful of these characters that distinguished between the species are based on the reproductive features (Chapter Three), including differences in both shape and size of the various spikes.

Differences in the reproductive features of these species of *Gnetum* might reflect differences in pollinators, which could lead to reproductive isolation and enable the application of the Biological Species Concept (BSC). However, little is known of the pollinators of the African species of *Gnetum,* beyond that some morphological indications do exist pointing to entomophily (Van der Pijl, 1953), especially to nocturnal, osmatic insect pollinators (Kato *et al.,* 1995). Moreover, the species are found growing and producing fertile structures on various branches along the stem in the still forest understory, which does not favour anemophily (Kato *et al*., 1995). Very often, the plants climb shrubs that are shaded by very tall trees in the forest. Insects are therefore thought to be the pollinators of several species of *Gnetum* (Kubitzki, 1990). For instance, the stinking strobili of both Malaysian *Gnetum gnemon* and *G. cuspidatum* have been visited frequently in the evening and have been shown to be pollinated by nectar-seeking moths of the orders Pyralidae and Geometridae and small flies of Lauxaniidae (Diptera) respectively (Kato *et al.,* 1995). Also nocturnal moth visits to *G. luofuense* coincided with nectar secretion (Corlett, 2001).

Pollination droplets secreted by the male strobili of *G. gnemon* in U.S.A. were found to have common protein amino acids, while these compounds were difficult to detect in the droplets of the female strobili (Murch & Tomlinson, 2011). The presence of these amino acids might attract a pollinator to visit a male structure much longer than a female strobilus. Strong odour secretions by species of *Gnetum* such as the Malaysian *G. gnemon* and *G. cuspidatum* attract moths to their flowers even though they are not typical moth flowers because deep flower tubes are absent and the nectar reward is exposed (Nilsson *et al*., 1990; Kato *et al.,* 1995). However, the nocturnal pattern, the low sugar concentration and fragrance of droplet secretion are pollinator specific and coincide with the moth-pollinated flower syndrome (Kato *et al.,* 1995). These *Gnetum* species are thus characterized by a specific recognition system.

Pollination studies carried out on *Gnetum* species thus far have shown that they exhibit an entomophilous pollination syndrome, but these investigations were based on non–African *Gnetum* species. The presence of nectar (Kato *et al.,* 1995) and the stiff erect catkins are characteristics of insect pollination; moreover, the nocturnal pattern and heavy scent will mostly attract insects like moths. As *Gnetum* in Africa has similar morphology and a specific smell that might be similar to these Asian species, it is likely that they are pollinated by similar organisms. Testing the mutualism between pollinators and catkins of the African *Gnetum* species is a challenge for future studies in order to test whether or not the morphology correlates to pollination syndromes in these species. Pollination studies are therefore needed for these *Gnetum* species before the BSC could be applied here.

The Ecological Species Concept has established that members of a species will cluster into groups exploiting the same habitat (Van Valen, 1976). In other words, the concept emphasizes that species occupy different niches (Chapter One), possibly due to natural selection acting on varying genotypes and resulting in speciation (Jonsell, 1984). *Gnetum* species are known to grow in isolated patches of forest and this might limit their gene flow. Moreover, they are dioecious and outcrossing which guarantees a high degree of genetic variation within populations and the ability to colonize new habitats (Chapter Five). However, the African species of *Gnetum* have remained forest dwelling species implying no distinction in the niches they occupy. The Ecological Species Concept is therefore not useful in species recognition in the genus *Gnetum* in Africa.

Previously, the Morphological Species Concept was apparently used to distinguish discrete taxonomic groups as belonging to different species of *Gnetum* in Africa (Hutchinson & Dalziel, 1954; Earle, 2001; Onguene & Kuyper, 2001). However, the error in identification of the type specimen and the lack of both male and female type specimens essential in the recognition of these species on a morphological basis and the highly variable phenotypes (especially when referring to the vegetative features) led to inadequate distinction among these species (Chapter Three). The intensive morphological investigation carried out in this study has enabled a more stable and satisfactory

classification compared to previous authors**.** Nevertheless, the clustering of specimens revealed through these phenetic analyses seems to be a true reflection of the taxonomic relationship and the stable characters identified in the study are not influenced by environmental variation. Phylogenetic analyses of these species would reveal if they reflect the evolutionary relationships (e.g. are monophyletic, sharing a common ancestor) with diagnosable apomorphies and thus would meet the requirements of the Phylogenetic Species Concepts (Cracraft, 1989; Price, 1996; Wheeler & Meier, 2000).

2.5 Conclusion

Four distinct clusters of plants of *G. africanum, G. buchholzianum, Gnetum sp. nov. 1* and *Gnetum sp. nov. 2* respectively were identified to be recognized at the species level based on numerical analyses. This study has explored the use of a numerical approach to the taxonomy of *Gnetum* species in Africa. The splitting of *Gnetum* therefore owes its interpretative debt to phenetics supported by morphological species description. The morphological species concept that was previously applied, had permitted the various specimens of *Gnetum* in Africa to be easily sorted into three putative groups, (two groups were based on the types and the third not matching any of the types or descriptions), and these were considered as three different species. On applying the phenetic concept, based on the notion that species are distinct phenetic clusters with unique sets of characters that distinguish them from other related species, and that, subspecies can be recognized in relation to their specific habitat or ecological niche, four taxa were identified at the species level. The three morphological forms were confirmed and a fourth taxon was based on the spike being thicker especially at mid-region than at either upper or lower regions and the internodes of spikes were as long as broad, with the female plants producing the largest seed.

The phenetic approach has the ability to examine the overall patterns of variation in various individual specimens establishing the identification of a discrete group as a taxon. More importantly, it can calculate similarities based on many factors simultaneously, whereas the human mind can only deal with a few comparisons simultaneously. This method applied to herbarium specimens has allowed the identification on one hand, of

four discrete groups to be classified as species, thus improving the classification and characterizing more comprehensively the genus *Gnetum* in Africa and on the other hand, useful characters to distinguish between these species. The numerical approach has resolved the taxonomic uncertainty by delimiting the various groupings of *Gnetum* as species. This method thus reduces the level of subjectivity in making taxonomic decisions and provides an objective classification.

G. buchholzianum, *G. africanum*, *Gnetum sp. nov. 1* and *Gnetum sp. nov. 2* are therefore considered as distinct species. Nevertheless, to further explore the taxonomy of the genus *Gnetum*, investigations by means of other tools such as genetic variability using molecular techniques on species in Africa are deemed necessary in order to provide confirmative information on these putative species and construct an appropriate classification reflecting evolutionary (phylogenetic) relationships between the groups. In addition, the extent of correlation between these plants collected from the wild and their cultivated counterparts requires examination as the cultivated variants were previously misidentified. In addition, the two new species, *Gnetum sp. nov.1* and *Gnetum sp. nov. 2* are in need of not only taxonomic descriptions but also names for their identification. A key to species recognition using discrete characters will be valuable to recognize the African species of the genus *Gnetum*. The next chapter provides details that further clarify the species problem in the genus *Gnetum* in Africa.

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APPENDIX

List of voucher specimens examined in the phenetic study arranged in order of identification number for male and then female specimens.

The first letter of the code represents the species (a = *Gnetum africanum*; b = *G. buchholzianum*; i = *Gnetum sp. nov. 1*; l = *Gnetum sp. nov. 2*; n = *G. nodiflorum*; p = *G. paniculatum*; le = *G. leyyoldii*; g = *G. gnemon*; s = *Gnetum* sp. from South America); next letter = country of collection (A = Angola; B = Amazonia; C =Cameroon; D =Central African Republic; E = Congo; F = Colombia; G =Democratic Republic of Congo; H = Equatorial Giunea; I = Ecuador; J = Gabon; K = Guyana; L = Peru; M= Venezuela) followed by identification number.

Code	Collector, No. of specimens & herbarium	Collection Locality
Males		
a.A1	Welwitsch 1224; PRE	Golungo, Angola
a.G2	Wellens 449a; BR	Mayombe, RDC
a.C3	Zenker s.n; BR	Bipindi, Cameroon
a.A4	Gossweiler 5580; LISC	Faremla, Angola
a.E5	Devred 1009, BR	Mbwami, Congo
b.C6	De Bruijn 2181; WAG	Cameroon
b.C7	Setten 346; WAG	Cameroon
b.C8	Bos 5242; WAG, YA	Cameroon

b.C9	Satabie 576; P, YA	Ndikinimeki, Cameroon
b.C10	Breteler 2002; P, WAG	Yaounde, Cameroon
b.C11	Breteler 1553; WAG	Yaounde, Cameroon
b.C12	Sersman 2061; K	Mbunge, Cameroon
b.C13	Bos 7195; K, WAG	Kribi, Cameroon
b.C14	Bos 3885; WAG	Kribi, Cameroon
b.C15	Biye 217; J, YA	Sa'a, Cameroon
b.C16	s.l.; BER	Victoria, Cameroon
b.C17	Zenker 12; BER	Bipindi, Cameroon
b.C18	Thomas *et a*l. 5227; P, YA	Kumba, Cameroon
b.C19	Letouzey 9754; P, WAG, YA	Ntui, Cameroon
b.G20	Devred 913; BR, LISC	Leopoldville, RDC
b.C21	Biye 216b; J, YA	Lekie, Cameroon
b.C22	Bos 3250; K, WAG, YA	Kribi, Cameroon
b.C23	Biholong 258; YA	Yaounde, Cameroon
b.C24	Staudt 543; BER	Cameroon
b.C25	Biye 216a, J, YA	Sa'a Cameroon
b.C26	Zenker 522; BER	Cameroon
i.G27	Wellens 449b; BR	RDC
i.G28	Szafranski 1584; BR, K	Kisangani, Zaire = RDC
i.H29	Carvalho 5100; WAG	Bata-Bome, Equatorial Guinea
i.G30	Corbisier 936; BR	Eala, RDC
i.G31	Breyne 665; BR	Kinshasa, RDC
i.D32	Fay 2181; K	Banbari, Central African Rep.
i.D33	Tisserant 197; P	Bessow, Central African Rep.
i.C34	Zenker 41; P	Bipindi, Cameroon
i.C35	Rudatis 42; K	Mamfe, Cameroon
i.C36	Dinhlage 882; BER	Lokundje, Cameroon
i.C37	Manning 2002; K, YA	Libamba, Cameroon
i.C38	De Wilde 3835; BR, K, WAG	Eseka, Cameroon
i.C39	Bos 5465; BR, WAG, YA	Kribi, Cameroon
i.E40	Louis 12038; BR	Congo
i.G41	Evrard 3893; K	Boende, RDC

i.G42	Louis 14947; BR	Yangambi, RDC
i.J43	Reitsma 2749; WAG	Malibe, Gabon
i.J44	Klaine 1029; BER	Libreville, Gabon
i.J45	Soyaux 87; K, P	Munda, Gabon
i.J46	Deweure 592; BR	Gabon
i.G47	Luja 222; BR	Sankuru, RDC
i.J48	Reitsma 639; WAG	Malibe, Gabon
i.J49	Louis 1765; WAG	Malibe, Gabon
i.J50	Klaine 348; P	Libreville, Gabon
i.J51	Klaine 774; P	Libreville, Gabon
i.J52	s.l;. P	Gabon
i.J53	Klaine 170; P	Gabon
i.C54	Bos 5501;LISC, PRE, WAG, YA	Kribi, Cameroon
l.C55	Biye 603; J	Kumba, Cameroon
l.C56	Biye 617; J, YA	Kribi, Cameroon
n.F57	Rudas *et al*. 2536; MO	Leticia, Colombia
n.K58	Clarke 6954; MO	Takutu-U, Guyana
p.M59	Stergios et al. 9498; MO	Rio Siapa, Venezuela
p.K60	Hoffman et al. 2801; MO	Pomeroon-Supenaam, Guyana
p.M61	Holst *et al*. 2972; MO	Edo. Bolivar, Venezuela
Females		
a.G1	Louis 1698; WAG	Santa Clara, Gabon
a.H2	Carvalho 5036; WAG	Bata-Pembe Equatorial Guinea
a.H3	Wilde et al. 11993; WAG	Rio Muni, Equatorial Guinea
a.G4	Renier 81; BR	Kikwit, RDC
a.C5	Harris & Fay 1516; PRE	Sanaga, Cameroon
a.C6	Zenker 1061; BER	Cameroon
a.C7	Raynal 10247; P, YA	Ma'an, Cameroon
a.C8	Letouzey 11061; P, YA	Massangui, Cameroon
a.E9	Gerard 3198; BR	Madabu, Congo
a.E10	Sita 4079; P	Chaillu, Congo
a.G11	Szafranski 1124;BR	Kisangani, RDC

a.G12	Louis 1165; BR	Yangambi, RDC
a.G13	Louis 3444; BR	Yangambi, RDC
a.J14	Klaine 1131; P	Gabon
a.E15	Dupub II; BR	Bingila, Congo
a.E16	Seret 1005; BR	
a.J17	Reistma 2887; WAG	Libreville, Gabon
a.G18	Mosango 507; BR	Lubutu, RDC
a.A19	Gossweiler 8191; LISC	Belize- Naiombe, Angola
a.C20	Westphal 10075; BR, WAG, YA	Douala, Cameroon
a.C21	Zenker 4380; BR, P	Bipindi, Cameroon
a.C22	Letouzey 14714; P. YA	Miang, Cameroon
a.C23	Westphal 9832; BR, K, WAG	Yaounde, Cameroon
a.C24	Etuge *et al*. 54; YA	Ngusi, Cameroon
a.J25	Breteler 12336; WAG	Ogouoe-Lolo, Gabon
a.C26	Nkongmeneck 697; YA	Ndom, Cameroon
a.C27	Manning 1843; K, YA	Yaounde, Cameroon
a.E28	Dhetchuvi 508; BR	Botsima, Congo
a.E29	Decoulf 316; BR	Congo
a.G30	Germain 375; BR, J	Yangambi, RDC
a.G31	Luja 222; BR	Sankuru, RDC
a.E32	Devred 1187; BR	M'Vuazi-Lemba, Congo
a.E33	Devred 3126; BR	Luki, Congo
a.E34	Pauwels 6747; BR	Bawi
a.E35	Hulstaert 769; BR	Bokuma, Congo
a.C36	Sonke 2764; BR	Nkolfep, Cameroon
a.G37	Bolema 1170; K	Yangambi, RDC
a.E38	Louis 1102; BR	Yaselia, Congo
a.A39	Gossweiler 5255; LISC	Angola
a.E40	Farron 4829; P	Diosso, Congo
a.D41	Tisserant 1518; P	Boukoko, Central African Rep.
a.J42	Wilde & Louis 787; WAG	Libreville, Gabon
a.C43	Bos 4144; WAG	Longii, Cameroon
a.C44	Wilde 3819; WAG	Eseka, Cameroon

a.C45	Letouzey 12435, P	Batoutouk, Cameroon
a.C46	Surville 482; YA	Cameroon
a.G47	Evrard 5821; BR	Mompono, RDC
a.E48	Evrard 3009; BR	Lombiolo, Congo
a.D49	Gutzwiller 1727; BR	Kembe, CAR
a.J50	Wieringa 4818	Estuaire, Gabon
i.G51	Louis 831; BR	Yaosuka, RDC
i.C52	Zenker 4134; BER, BR	Bipindi, Cameroon
i.G53	Pynaert 1385; BR	Eala, RDC
i.E54	Evrard 1733	Boketa, Congo
i.C55	Bos 3721; WAG, YA	Kribi, Cameroon
i.H56	Carvalho 5226; WAG	Bata-Mbini, Equatorial Guinea
i.J57	Simons 422; WAG	Ovingombe, Gabon
i.C58	Bos 4068; K, WAG	Kribi, Cameroon
i.J59	Wieringa 6460; WAG	Haut Ogouoe, Gabon
i.J60	Jongkind 5936; WAG	Cap Esterias, Gabon
i.G61	Vermoesen 321; BR	Mobwasa, RDC
i.G62	Robyns 511; BR	Eala, RDC
i.G63	Gerard 858; BR	Monga, RDC
i.G64	Vermoesen 2338; BR	Eala, RDC
i.G65	Louis 1019; BR	Yangambi, RDC
i.G66	Leonard 149; BR, K, PRE	Eala, RDC
i.J67	Klaine 787; P	Gabon
i.E68	Hulstaert 488; BR	Bolima, Congo
i.E69	Hulstaert 758; BR	Bokuma, Congo
i.G70	Pauwels 724; BR	Kipindi, RDC
i.E71	Deweure 221; BR	Congo
i.J72	Louis 2028; WAG	Bongolo, Gabon
i.G73	Louis 10826; BR	Lilanda, RDC
i.G74	Lisowski 17686; K	Uele, RDC
b.J75	Breteler & Jongkin10688; WAG	Lastoursville, Gabon
b.J76	Breteler 15713; WAF	Ogoue-Ivindo, Gabon
b.C77	Westphal 8763; WAG, YA	Yaounde, Cameroon

b.G78	Devred 4024; BR	Yangambi, RDC
b.G79	Louis 11294; BR	Yangambi, RDC
b.U80	Jans 649; BR	Bokoro
b.J81	Asonganyi 756; P, YA	Mandiba, Cameroon
b.C82	Nemba *et al*. 808; YA	Dibombari, Cameroon
b.G83	Pynaert 1441; BR	Eala, RDC
b.C84	Wilde 2179; WAG, YA	Eseka, Cameroon
b.J85	Wieringa 356; WAG	Tchimbele, Gabon
b.C86	Zenker 2279; WAG	Bipindi, Cameroon
b.G87	Liben 1716; BR	Dibaya, RDC
b.G88	Louis 4217; BR	Yangambi, RDC
b.G89	Hulstaert 942; BR	Bokote, RDC
b.C90	Binuyo & Daramola ; K, WAG	Kumba, Cameroon
b.E91	Evrard 290	Bodangabo, Congo
b.E92	Klaine 1819	Gabania, Congo
b.G93	Corbisier 938; BR	Eala, RDC
b.J94	Florence 698; P	Ipassa, Gabon
b.G95	Vermoesen 1739; BR	Temvo, RDC
b.E96	Maudoux 284; BR	Luki, Congo
b.E97	Donis 2317; BR	Luki, Congo
b.D98	Tisserant 1517; P	Boukoko, Central African Rep.
b.G99	Ndjele 463; BR	Lubutu, RDC
b.G100	Louis 7773; BR	Yangambi, RDC
b.C101	Bos 7192; LISC, K, WAG, YA	Kribi, Cameroon
b.C102	Bos 5700; WAG	Kribi, Cameroon
b.C103	Bos 6434; BR, WAG	Kribi, Cameroon
b.C104	Bos 2993; BR, WAG YA	Kribi, Cameroon
b.C105	Huber 1018; P	Kribi, Cameroon
b.C106	Zenker 1180; P	Bipindi, Cameroon
b.C107	Nkongmeneck 325; YA	Nyandingi, Cameroon
b.C108	Leeuwenberg 9893; BER, LICS, K, PRE, WAG, YA	Nkam, Cameroon
b.C109	Atangana 10; YA	Nkol Awana, Cameroon

b.E110	Toussaint 248; BR	Luki, Congo
b.G111	Compere 1577, BR	Kinkosi, RDC
b.E112	Donis 2447; BR	Luki, Congo
b.C113	Biye 302; J, YA	Nkolo II, Cameroon
b.C114	Biye 303; J, YA	Nkolmebenga, Cameroon
b.C115	Biye 304; J, YA	Ovoabang, Cameroon
l.D116	Tisserant 1984; BR, P	Boukoko, Central African Rep.
l.C117	Bos 4267; WAG	Kribi, Cameroon
l.C 118	Letouzey 5249; K, P, YA	Mopwo, Cameroon
l.C119	Biye 301; J, YA	Sa'a – Lekie, Cameroon
g.S120	s.n.; J	Garden, Durban, South Africa
le.B121	Nascimento 396; MO	Rio Acara, Amazonia
n.B122	Encarnacion 1064; MO	Punto Negrococha, Amazonia
n.L123	Vasquez 19321; MO	Madre de Dios, Peru
n.K124	Hoffman 909; MO	Essequibo, Guyana
n.K125	Mutchnick 828; MO	Potaro-Sipatuni, Guyana
n.K126	Henkel 153; MO	Cuyuni-Mazaruni, Guyana
p.B127	Castill 4454; MO	Rio Orinoco, Amazonia
p.M128	Castillo 5740; MO	Rio Sipapo, Venezuela
p.K129	Clarke 9203; MO	Potaro-Siparuni, Guyana
s.I130	Neil & Iguago 9046; MO	Canton Archidona, Ecuador

CHAPTER THREE

A CLARIFICATION OF *GNETUM* L. (GNETACEAE) IN AFRICA AND THE DESCRIPTION OF TWO NEW SPECIES

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Abstract

Gnetum L. is a genus of economic importance distributed in the humid rainforests of Central Africa. The African species of *Gnetum* are dioecious climbers and only two species (*Gnetum africanum* Welw. and *G. buchholzianum* Engl.) have been described previously. The names *G. africanum* and *G. buchholzianum* have been applied interchangeably to similar morphological forms of *Gnetum* specimens in Africa or on duplicates of specimens causing taxonomic confusion. The nomenclature of *Gnetum* species in Africa is thus in need of reappraisal.

Morphological descriptions are based on examination of over 300 specimens from various herbaria, mainly B, BR, FHI, J, K, LISC, P, PRE, WAG and YA.

The original applications of the names *Gnetum africanum* and *Gnetum buchholzianum* are clarified and two new African species (*Gnetum interruptum* E.H. Biye *sp. nov*. and *Gnetum latispicum* E.H. Biye *sp. nov*.) are described on the basis of morphological features. A key to the African species is provided and the main characters, such as the shape and size of spike that are essential in the recognition of species in the genus *Gnetum* in Africa as well as the importance of conservation are discussed. These species are assessed as threatened and at a high risk of global extinction according to the IUCN Red List categories and criteria, and are in need of conservation measures for their protection.

Key-words – Africa, Gnetaceae, *Gnetum*, Morphology, New species, Taxonomic delimitation

3.1 Introduction

The phylum Gnetophyta, class Gnetopsida, comprises three genera *Ephedra* L., *Gnetum* L. and *Welwitschia* J.D.Hooker. The leaves of these gymnosperms are strikingly different, both in form and venation pattern and provide distinct morphological characters for each genus (Carlquist 1996). *Gnetum* presents specialized features such as decussate, pinnately veined leaves, lignin composition typical of angiosperms and a climbing habit that are all unusual among extant gymnosperms (Carlquist 1996).

Gnetum consists of an estimated forty species, of which most are found in tropical and subtropical Asia, with nine in China, about 10 in north-western South America, and the two previously reported in Africa (Maheshwari & Vasil 1964; Fu et al. 1999; Earle 2001; Won & Renner 2006); all but two are canopy lianas. Won & Renner (2003) suggested that there is just one African species, probably with different forms, although Hutchinson & Dalziel (1954) recognized and described two species of *Gnetum* in Africa. The male spikes of *G. africanum* Welw. were reported to have internodes of more or less the same diameter throughout their length with a staminal column protruding at the mouth of a tubular covering (Hutchinson & Dalziel 1954). The leaves of this species were noted to be 10–13 × 3.5–5 cm, ovate-oblong or elliptic-oblong, rarely lanceolate, attenuate at the base and abruptly acuminate at the tip (Hutchinson & Dalziel 1954). In contrast, the internodes of the male spikes of *G. buchholzianum* Engl. were described as being thicker at the base than in the upper region with the staminal column at the tip hardly existing. The leaves are 9–14 × 4–7 cm and were described as ovate-elliptic or broadly elliptic, with a round or slightly cuneate base, and an abruptly acuminate apex (Hutchinson & Dalziel 1954). From these descriptions and based on the vegetative features, the two species do not seem very distinct from one another.

Carlquist (1996) was convinced that the two African species, *G. africanum* and *G. buchholzianum*, recognized by Hutchinson & Dalziel (1954) and by Earle (2001) were anatomically distinguishable based only on the circular bordered pits of vessel elements, but not necessarily taxonomically distinct from each other. In addition, vernacular names used for *Gnetum* species in Africa (eaten as a vegetable) vary according to locality but

not according to species. This could be because of the high level of vegetative similarity observed between the species, so that recognition is difficult without the reproductive structures. For example, English speaking Cameroonians term *Gnetum* 'eru' while the French speaking call it 'okok', and there are tribes who use particular names, for instance, the Douala use the word 'ikoko'. The Ibo tribe in Nigeria refers to *Gnetum* as 'Ukasi' and in Congo, the expressions 'Mfumbwa' or 'Ekumba esomba' are used (Clark et al. 2004). There is thus a need to clarify the number of indigenous species of *Gnetum* in Africa.

Gnetum is among the three top priority African indigenous vegetables identified for improvement and promotion through research (Shiembo 1999, Clark et al. 2004), but a major constraint that challenges this objective is the lack of taxonomic knowledge of *Gnetum* species in Africa, resulting in confusion around their identities. Hutchinson & Dalziel (1954) did not resolve the nomenclatural problems but added to the confusion as is evident on the examination of the original specimens, *Markgraf s.n.* (B) and *Rudatis 42* (K), mentioned in the Flora of West Tropical Africa, that they based their description of *G. africanum* on two morphologically different specimens. The misapplication of this name could be attributed to the authors not examining the type specimen of *G. africanum* (*Welwitsch 1224*). Consequently, morphologically similar specimens (sometimes duplicate specimens) from many herbaria bore the names *G. africanum* and *G. buchholzianum* interchangeably depending on a particular collector or determinant, and occasionally there was no specific name attributed to a particular variant.

Little attention has been focused on delimiting the species of *Gnetum* in Africa and there are very few publications that deal with their taxonomy (Welwitsch 1858, Engler 1908, Hutchinson & Dalziel 1954, Carlquist & Robinson 1995, Carlquist 1996). National herbaria in countries in West and Central Africa periodically publish accounts of various plant families in the local Flora, e.g. 'La Flore du Cameroun' and 'La Flore du Gabon' reserved for the description of plants growing in those regions respectively, but no information on *Gnetum* is available in any of these. No national flora or checklist of Gnetaceae in Africa is known, and while creating the gymnosperm database web page, Earle (2001) indicated that very little was known about the African species, *G.*

buchholzianum. Isong et al. (1999) noted the use of three 'varieties' of *G. africanum* when carrying out a nutritional and fibre characteristic study. These 'varieties' were attributed names based on collection localities, and could reflect either distinct species or subspecies. Onguene & Kuyper (2001) also carried out a study on mycorrhizal associations based on a hundred species in the rain forest of South Cameroon and three species of *Gnetum* (*G. africanum*, *G. buchholzianum* and *Gnetum* sp.) were included. No description of the *Gnetum* plants used in either case was available. Thus, it seems the genus might comprise one, two or more species in Africa. The lack of adequate publications, the range of opinions on the number of species and the uncertainty surrounding *Gnetum* species in Africa are an indication of the need for a taxonomic revision of the genus.

The purpose of this revision is to clarify the taxonomy of the African species at two levels: namely, to re-examine the current morphological species delimitation and to identify, name and describe all species in the genus *Gnetum* in Africa. The type specimen of *G. africanum* described by Welwitsch (1858) comprised only the male plant, thus morphological features to identify the female were unknown. Engler (1908) described alongside the male, a type specimen for the female plant making it easier to recognize both males and females of *G. buchholzianum*. This study also investigates whether or not the females of *Gnetum* species are morphologically distinct as are the males.

3.2 Materials and Method

Detailed morphological studies were based on the examination of 300 herbarium specimens housed at the following herbaria: B, BR, FHI, J, K, LISC, P, PRE, WAG and YA (herbarium abbreviations according to Holmgren et al. 1990). Type specimens, their photographs (indicated 'e' for digital images) and original descriptions were assessed to determine to which group which name belongs. Measurements of the reproductive structures were carried out using a Stereo Discovery V12 ZEISS Microscope and only mature or fully developed structures on the herbarium specimens were examined. Multivariate analyses of the specimens confirmed the groups (species) recognized and

described here (Biye 2013). Morphological descriptions follow Radford et al. (1974) and preliminary conservation assessments follow the IUCN (2001) guidelines.

3.3 Results

Gnetum species in Africa present various morphological features useful in nomenclatural clarification and four species are recognized here, two newly described. The current study concurs with Hutchinson & Dalziel's (1954) observation that the vegetative features overlap and are therefore less helpful than reproductive ones for the accurate recognition of *Gnetum* species in Africa. The leaf size (6–18 × 2–8 cm) and length/width ratio (up to 4) vary considerably among members within a species and sometimes on an individual specimen of a species. The leaf outline is ovate-oblong to elliptic-oblong, or obovate to broadly elliptic, rarely lanceolate with mostly brochidodromus veining and the midrib is channeled on the ventral surface. The leaf base is shortly attenuate and the apex abruptly acuminate and apiculate, the margin is undulate. The species of *Gnetum* in Africa are thus better distinguished by their reproductive structures. Differences in both shape and size of the internodes of the jointed spikes of the various species (as summarized in Table 2.4) are most striking and permit a clear distinction between the four species described below (Figure 3.1).

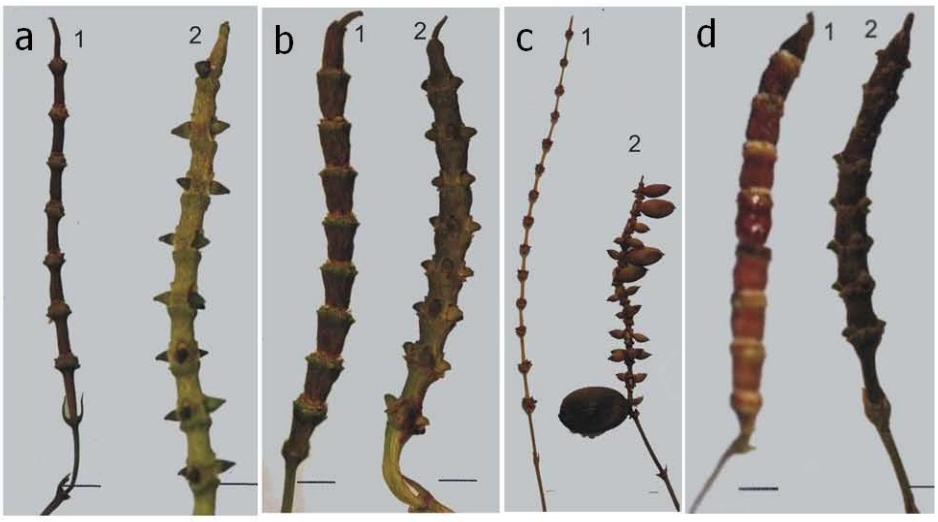


Figure 3.1 The morphological diversity of *Gnetum* spikes in Africa (1, male; 2, female). a, *Gnetum africanum*; b, *Gnetum buchholzianum*; c, *Gnetum interruptum*; d, *Gnetum latispicum*. Vouchers: a1, *Biye* 610; a2, *Biye* 619; b1, *Biye* 612; b2, *Biye* 620; c1, *Boss* 5501; c2, *Bos* 4068; d1, *Biye* 603; d2, *Biye* 590. Scale bar: 10 mm.

In the species matching the type *Welwitsch* 1224 (B, K, PRE) representing *G. africanum* (Figures 3.1a & 3.3), the male spikes are terminal and usually one per branch node. The internodes, less than thrice as long as wide, have a similar diameter of 1.0 mm or more throughout their length or often narrow distally (Figure 3.1a1). The mature spike comprises 8–13 involucrate whorls, stamens are arranged in at least two rows alternating with supporting brownish hairs, the peduncle is up to 21 mm long and a blunt staminal column of less than 1 mm protrudes from a sterile node.

The second species matching the types, *Buchholz* s.n., *Zenker* 522 and *Staudt* 543 (B, K) and named *G. buchholzianum* (Figures 3.1b & 3.4), usually has two male spikes at the terminal and/or axillary positions but occasionally one spike per branch node is observed. The turbinate internodes of the spike are 2–3 times as long as broad, with a diameter of at least 1.0 mm and slightly wider at the top than at the base (Figures 3.1b1). In a mature axis, there are 8–11 involucrate whorls per spike, the stamens are inserted in a row, rarely two alternating with brownish hairy structures, the peduncle seldom exceeds 12 mm long and the staminal column is hardly present but an acuminate tip protrudes.

In *G. interruptum* sp. nov. **(**Figures 3.1c & 3.5), the male spikes are both terminal and axillary, and very often there is more than one spike at a node (sometimes up to 4 spikes per node). The diameter of the internodes of spikes is very narrow (rarely exceeding 0.7 mm) and consistently even along their length. The internodes are between 3 and 10 mm long and sometimes 7 to 10 times longer than broad so that the whorls are well demarcated (Figure 3.1c1). There are up to 15 whorls in fully developed spikes, the stamens are inserted in at least one row alternating with very few hairy structures, the peduncle is as long as 15 mm and the staminal column, blunt at the tip, is more than 0.5 mm long protruding from a sterile node.

G. latispicum sp. nov. **(**Figures 3.1d & 3.6) presents one, or rarely two, terminal male spikes per branch node. The internodes of spikes are equal in diameter throughout their length and as long as broad with a diameter greater at the mid-point than at either end. There are up to 12 whorls along the mature axis, the stamens are inserted in one row or

rarely two above a row of brownish hairy structures, the peduncle is usually less than 5 mm long and the blunt staminal column may or may not protrude from a sterile node.

Like the males, female plants have variable vegetative structures within species but the same distinct morphology of the reproductive structures as the male plants (Figures 3.1a2–d2), i.e. differences in the shape of the spikes, thus facilitating the matching of both sexes of species. The internodes of spikes of all the females are nevertheless slightly thicker and often shorter than those of the corresponding male. The peduncle is thickest in *G. buchholzianum* (Figure 3.4) and thinnest in *G. interruptum* **(**Figure 3.5).

Morpho-anatomical analysis proved successful in the delimitation of the Venezuelan species of *Gnetum* (Jauregui & Benitez de Rojas, 2005). Further investigation carried out using anatomical variation has revealed that the epidermal cells of the leaf on the adaxial surface appear wider compared to those on the abaxial surface in all African *Gnetum* specimens studied and cell walls are either sinuous as in *G. africanum, G. buchholzianum* and *G. interruptum* or the cells have polygonal perimeters as in *G. latispicum* (Biye, 2013).

The seeds also vary in number and shape between species. For example, in *G. africanum* (Figure 3.3) and *G. interruptum* (Figure 3.5), there are four (or sometimes five in the latter case) seeds per whorl and each seed is inserted horizontally at the side of the whorl, diverging from the spike. In contrast, *G. buchholzianum* (Figure 3.4), has mostly three seeds per whorl and *G. latispicum* (Figure 3.6h) 3–4 seeds spreading at about 90° from the axis with a wider space between their insertion points. The seeds of *G. africanum* are narrowly oblong to obovoid, acute and deeply inserted into the whorl while those of *G. buchholzianum* are ellipsoid, acuminate to apiculate and not deeply inserted into the whorl. The seeds of *G. interruptum* **(**Figure 3.5h) are obovoid, apiculate to slighty acuminate at the tip and shallowly inserted into the whorls whereas those of *G. latispicum* (Figure 3.6h) are ovoid to ellipsoid, obtuse to acute at the tip and also not deeply inserted into the whorls.

3.1.1 Ecology, distribution and habitat

Most *Gnetum* species are fairly widespread in the humid tropical rainforest of Central Africa with overlapping geographical distributions. *Gnetum africanum* and *G. interruptum* are distributed in the forests of Cameroon, Central African Republic, Congo and Democratic Republic of Congo, Equatorial Guinea and Gabon (Figure 3.2). The specimens of *G. africanum*, *G. buchholzianum* and *G. interruptum* are widespread throughout the forest zone, although *G. buchholzianum* has not been reported in Equatorial Guinea and *G. interruptum* has not been collected in Angola. *Gnetum latispicum* is only known from Cameroon and the Central African Republic.

The African species are frequently found in the rain forest, growing at sea level to an elevation of 1200 m with an annual rainfall of 900–1500 mm and a temperature range of 25–30°C. Although species of *Gnetum* in Africa occur naturally in the forest, they do well in a wide range of habitats such as abandoned farmland, plantations, mangroves, swamps and dry land. *G. africanum* has been collected along river banks in Cameroon and in mountainous areas in Angola, whereas *G. buchholzianum* is found in semi-deciduous, disturbed, secondary and primary forests at an elevation of about 700 m. *Gnetum interruptum* occurs in primary, secondary, riverside and gallery forests, mangrove, and rubber plantations, usually at an elevation of about 600 m. It is noted specifically to grow in a secondary forest with much *Aucoumea klaineana* Pierre surrounded by mangrove around Malibe in Gabon [*Reitsma* 639 (WAG)]. *Gnetum latispicum* has been collected in anthropogenically modified forests exposed to sunlight as well as in primary forests.

Gnetum vines climb supported by large and small trees, dead trees, saplings, shrubs and other climbers such as rattan, and their orientation is influenced by light. They twine around surrounding trees and are thus capable of reaching the upper strata of the forest and creep when there is no support. The species of this genus generally grow on acidic soils, that are poor in nutrients but with some humus, red or yellowish, clayey or very porous, loose and permeable.

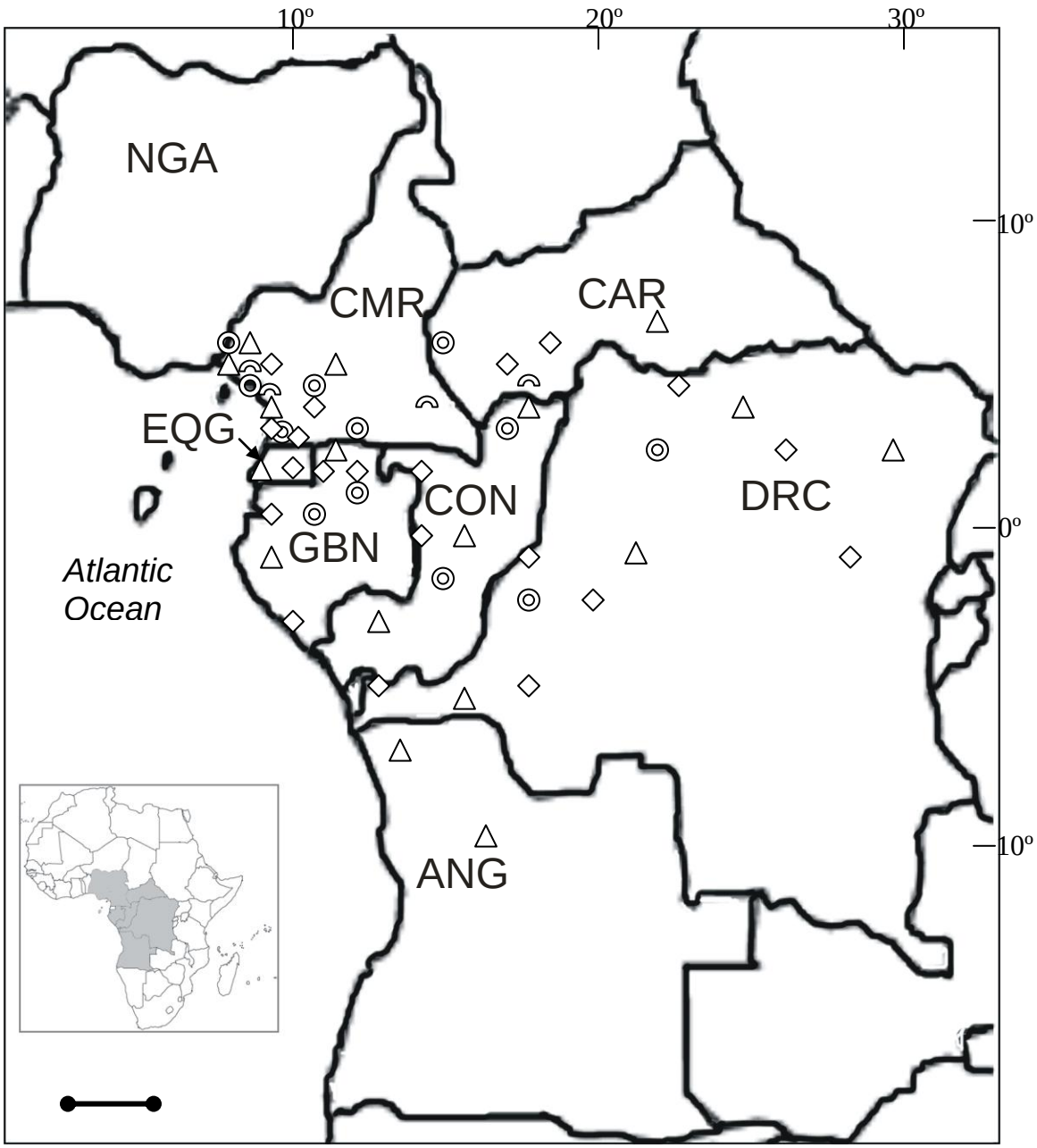


Figure 3.2 Distribution map of *Gnetum* species in Africa.
G. africanum △ ***G. buchholzianum*** ◎ ***G. interruptum*** ◇ ***G. latispicum*** ◠
ANG Angola, CMR Cameroon, CAR Central Africa Republic, CON Congo, DRC Democratic Republic of Congo, EQG Equatorial Guinea, GBN Gabon, NGA Nigeria. Scale 200 km.

3.1.2 Conservation

In the humid forest region where species of *Gnetum* occur, there is widespread felling of canopy, emergent and smaller trees as well as extensive clearing of vegetation by farmers (Clark *et al.,* 2004; Hance 2009). The forest is therefore under threat with no protection from such activities. In addition, an increase in human population and the consequent high demands for land for crop farming and wood for fuel has resulted in forest burning and fragmentation; thus a large portion of the forest is disappearing (Hance 2009). Therefore, uncontrolled deforestation and the harvesting of wild material of *Gnetum* species for food might continue. It is then likely that the number of mature individuals, the number of locations and the extent of suitable habitat will continue to be reduced for all four African species of *Gnetum*. The extent of occurrence of each of the four *Gnetum* species within the various countries is less than 20 000 km² and their area of occupancy less than 2 000 km². Therefore, except for *G. interruptum*, considered here as Vulnerable (VU A4 +B1a-c), the other three African species of *Gnetum* are assessed as Critically Endangered (CR A4 + B1bc) under the criteria of IUCN (2001). These species thus have a high risk of extinction in the wild.

3.4 Taxonomic Treatment

Both vegetative and reproductive features contribute significantly to the delimitation of species (Radford et al. 1974). Some of these structures are consistent within a species and vary between species and hence are taxonomically important whereas others are either invariant or do not correlate with identified species and/or present intraspecific variation and therefore are less valuable in distinguishing between species. The choice of characters is thus important and may or may not be based on the description of a type specimen which determines the application of the name and may be very unrepresentative of the variation of the species in question (Radford et al. 1974; Jeffrey 1977). However, the type specimens have facilitated the correct application of names of *Gnetum* species in Africa.

Morphological investigation of the genus has revealed that there are four species of *Gnetum* in Africa, *G. africanum* (Figure 3.3), *G. buchholzianum* (Figure 3.4) and two new distinct species described here; namely *G. interruptum* (Figure 3.5) and *G. latispicum* (Figure 3.6). The two new species are identified and provided with names thus contributing to the resolution of taxonomic confusion evident in the identification of *Gnetum* species in Africa for over fifty years. In addition, the problem of how to match the female specimens to the corresponding male specimens based on morphology has been addressed. These observations have been supported by phenetic analyses and anatomical techniques and are being further investigated using molecular tools (Biye, 2013).

3.4.1 Key to species of *Gnetum* in Africa (both male and female)

1a. Internode of male spike ≥ 3 times longer than broad, of female spike usually ≥ 2 times longer than broad, both markedly constricted, diameter ≤ 1.0 mm; leaves ovate-oblong or elliptic-oblong, acute ……..*Gnetum interruptum*

1b. Internode of male spike < 3 times longer than broad, of female spike < 2 times longer than broad, thick, diameter >1.0 mm; leaves obovate-elliptic or ovate to broadly oblong, abruptly acuminate.....2

2a. Whorl of male spike in two superimposed rows above sheath, lower row with brownish hairs, upper row with stamens inserted amongst hairs; bracts close to peduncle leaving little or no separation, seeds ovoid…...……..*Gnetum latispicum*

2b. Whorl of male spike in 1 ring or close to sheath, stamens and hairs alternating, bracts c. 1 mm away from peduncle, oblong-obovoid or ellipsoid ……..…….....3

3a. Male and female spikes terminal; internode either of the same diameter throughout length or slightly wider at base than towards apex; staminal column protruding at the tip of a sterile node; stamens in at least two rows, number per row decreasing away from whorl sheath; seeds usually 4, oblong-obovoid ….*Gnetum africanum*

3b. Male and female spikes usually both terminal and axillary; internode wider towards the top than at the base; staminal column hardly protruding at the tip of a sterile node; stamens often in one row rarely two, close to whorl sheath; seeds 3, ellipsoid ……..……*Gnetum buchholzianum*

3.4.2 Descriptions of species of *Gnetum* in Africa

Gnetum africanum Welw. (Welwitsch 1858: 27). -–– Type: *Welwitsch 1224* (Holotype: B!(e); isotypes: K!(e), PRE!).

Plants dioecious, lianes of varying heights in mostly shaded areas of forest, glabrous; *Stem* circular in cross section, woody, glabrous; *branches* both long and short but only short, lateral branches bear leaves. *Leaves* simple, opposite; *lamina* narrowly ovate-oblong, elliptic or elliptic-oblong, more rarely lanceolate 5−18 x 2–7 cm, glabrous, 5–9 pairs of brochidromus secondary veins extending to the channeled midrib; base attenuate; apex acute to abruptly acuminate, occasionally attenuate, distinct acumen < 2 cm long**;** margin slightly undulate to entire; *petiole* up to 1 cm long, channelled, more or less rigid, woody and glabrous. Spike unisexual, jointed, elongate with nodes and internodes, terminal, often one per branch node with two opposite, sterile free bracts with triangular tips, connate at base, the most apical forming blunt sterile staminal column of less than 1 mm; *internodes* same width throughout their length or slightly wider at base along spike; *cupuli* above bracts, series of 8 – 13, superimposed and involucrate whorls(what does this mean?), sterile at apex. *Male spike* numerous male structures arranged in at least 2 layers alternating with brownish fibres in each cupulus; *internodes* 1–3.5 x 1–3 mm. *Female spike* a strobilus, at least 2.5 cm long; cupuli terminating with an almost obtuse, sterile column; *internodes* 0.5–2 times longer than broad; *ovules* perpendicular to the axis; often 2–4 per whorl; *seeds* oblong-obovoid**,** subapiculate, up to 5 mm long on average, 3–5 mm in diameter, with fleshy external envelope red to orange when ripe (Figure 3.3).

Additional specimens examined: **Angola**: Golungo Alto, *Welwitsch 1224* (PRE); Monte Lau, Farenda, *Gossweiler 5580* (LISC); *Gossweiler 5255* (LISC); **Cameroon**: Kumba, *Swarbwick 445* (YA); Massangui Hills, 16 km NE Ngambe, *Satabie 576* (P, YA); Mandiba, 16 km ESS of Mouanko, *Asonganyi 756* (P, YA); Barombi Kang, 5 km East of Kumba, *Biye 610* (J, YA); Kumba main market, *Biye 619*; Bipindi, *Zenker 2279* (WAG); **Central African Republic:** Oubangui Plateau – Ouaka, *Tisserant 2094* (P*);* **Democratic Republic of Congo:** Mbwami forest, *Devred 1009* (BR); Mayombe, *Wellens 449a* (BR); Kisangani-Kilanga, *Szafranski 1124* (BR); Mbie Ile, 5 km from Kisangani, *Mandango*

3072 (BR); **Equatorial Guinea:** South of Bata, along coastal road, *Wilde 11993* (WAG); **Gabon:** About 30 km N of Lastoursville, *Breteler 10688* (WAG).

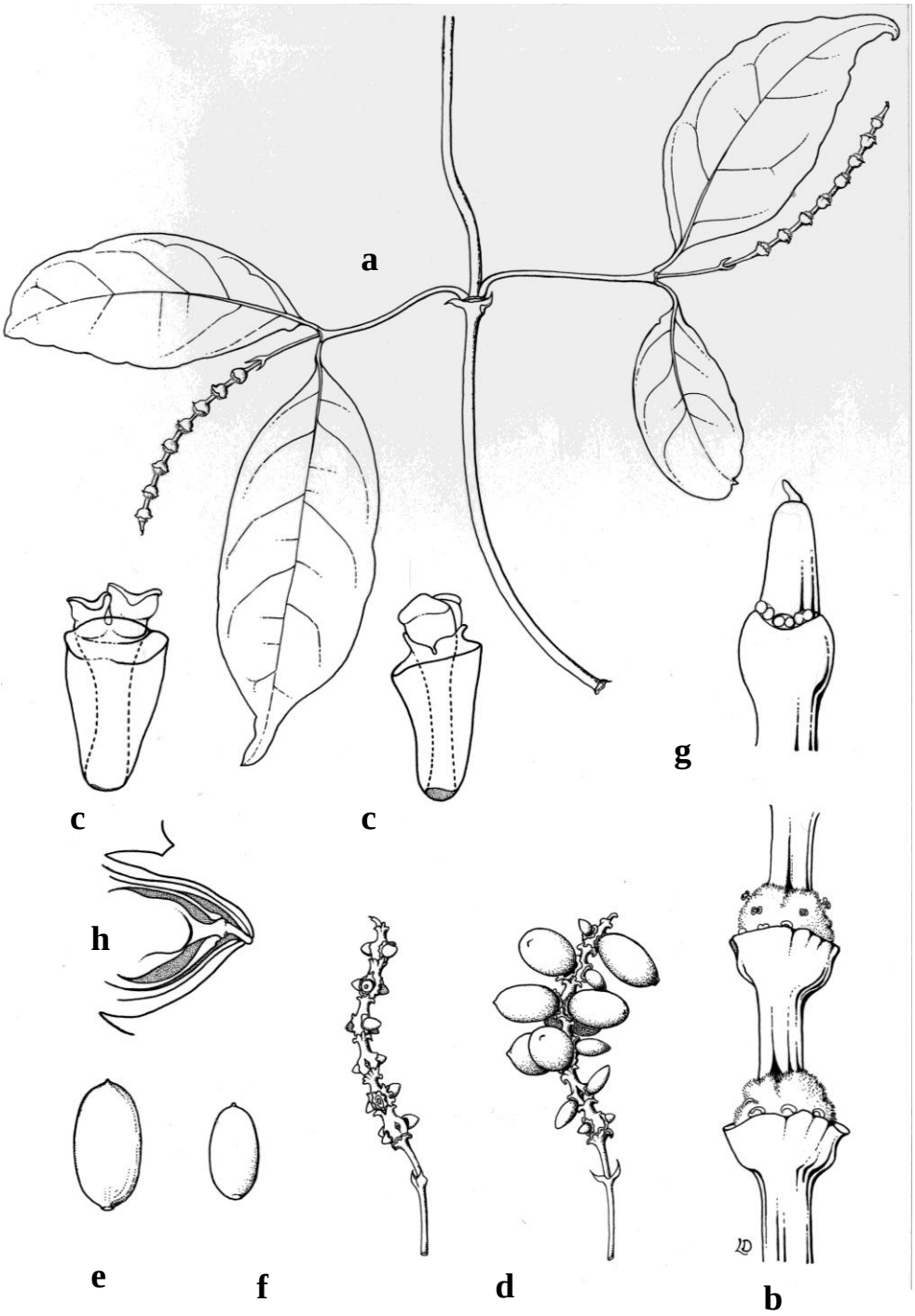


Figure 3.3 ***Gnetum africanum*** **Welw. a, habit; b, male spike; c, from left to right, male flower front and side views; d, from left to right, female spike young and mature; e, fruit; f, seed; G, staminal column; h, c.s. of female flower. (a – c & g, from *Welwitsch 1224*; d - f & h, from *Biye 619*). Scale 1 mm.**

Gnetum buchholzianum Engl. (Engler 1908: 40) – *Types Buchholz* s.n. (Lectotype designated here: B!(e); isolectotype: BR!(e)); syntypes *Staudt 543* (B!(e); K!); *Zenker 522* (BR!(e)).

Plants dioecious, lianes of undetermined height in shaded areas of forest, glabrous; *Stem* round in cross section; *branches* both long and short but only short, lateral branches bear leaves. *Leaves* simple, opposite; *lamina* ovate, elliptic-oblong, 5−16 x 2–7 cm, glabrous, 5–9 pairs of brochidromus secondary veins extending to the channeled midrib; *base* truncate, rounded or slightly cuneate; apex acute to acuminate, occasionally distinct acumen up to 2 cm long, with abrupt change in angle; margin slightly undulate; *petiole* up to 1 cm long, channelled, more or less rigid, woody and glabrous. *Spike* unisexual, jointed, elongate, made of nodes and internodes, terminal and/or axillary or both, not more than 2 per node with two opposite, free bracts with triangular tips, connate at base, terminates with 1 or 2 sterile nodes, the most apical node sharp, sterile with acuminate tip protruding, staminal column hardly exserted at the apex, internodes turbinate, wider at the top than at base; *cupuli* involucrate above bracts, series of 8–11, superimposed. *Male spike* internode 2–4 x 0.5–3 mm; *nodes* circular cupuli surrounded by a regular, thin sheath; numerous male structures in 1 or rarely 2 superimposed layers close to or covered with the whorl sheath**.** *Female spike* a strobilus, at least 2 cm long; cupuli at apex generally sterile, axis terminating with an almost obtuse, sterile column; *internodes* 0.5–2 times as long as broad; *ovules* perpendicular to the axis; often 2–3 per whorl; *seeds* ellipsoid, subapiculate**,** up to 10 mm long on average, 3–5 mm in diameter, with fleshy external envelope red to orange when ripe (Figure 3.4).

Additional specimens examined: **Cameroon**: Abo, *Buchholz s.n*. (B); Jaunde-Station, *Zenker 522* (BR); Johann-Albrechtshöhe, *Staudt 543* (B, K); Nkol Awana, Ekekam road near Yaounde, Atangana *10* (YA); Melen – Yaounde, *Breteler 1553* (WAG); Barombi Kang, 3 km East of Kumba, *Biye 604, 612* (J, YA); Melen – Yaounde, *Biholong 258* (YA); Nkolmeb – Nkolmebenga, *Biye 216, 217* (J, YA); Melen village, Yaounde, *Breteler 2002* (P, WAG); Victoria, *Maitland 749* (FHI); Mvog Mbaiti, *Sersman 2061* (K); Bipindi, *Zenker 12* (B); S.W. Province, forest around Kumba, *Thomas et al. 5227* (P, YA); Nguila, 30 km N of Ntui, *Letouzey 9754* (P, YA); 6 km S of Kribi, *Bos 3250* (K,

WAG, YA); 6 km S of Kribi, *Bos 3885* (WAG); 2 km S of Kribi, *Bos 7195* (K, WAG); Chutes Ekom, River Nkam, *Leeuwenberg 9893* (B, BR, K, LISC, PRE, WAG); Melen, Yaounde, *Biholong 258* (YA); *Setten 346* (WAG); Netherlands, Greenhouse, Botanic Garden, Wageningen, *Bruijn 2181* (WAG); **Central African Republic:** Station Boukoko, Oubangui, *Tisserant 1517* (P); **Democratic Republic of Congo:** Luki valley**,** *Donis 2447* (BR); Leopoldville *Devred 913* (LISC, BR); Badibanga-Dibaya, *Liben 1716* (BR); Beui-Buta, *Pauwels 6747* (BR); **Gabon:** 1.5 km NE of Tchimbele, *Wieringa 356* (WAG); Station d'Ipassa, 10 km de Makokou, *Florence 698* (P); Libreville, *Klaine 1819* (P).

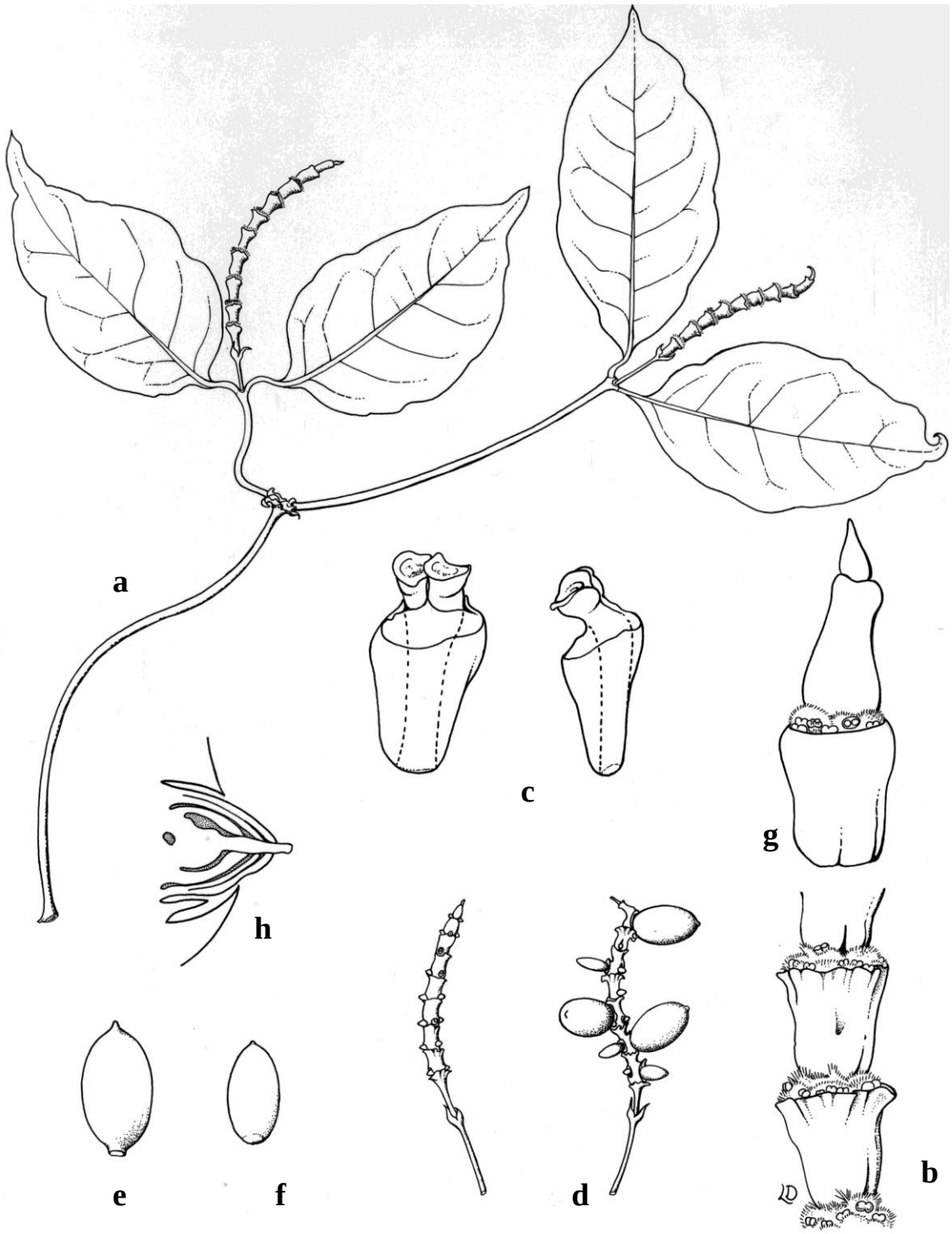


Figure 3.4 ***Gnetum buchholzianum*** **Engl. a, habit; b, male spike; c, from left to right, male flower front and side views; d, from left to right, female spike young and mature; e, fruit; f, seed; g, staminal column; h, c.s. of female flower. (a – c & g, from *Biye 217*; d - f & h, from *Biye 604*). Scale 1 mm.**

Gnetum interruptum E.H.Biye**, sp. nov.**

-- Type**:** Cameroon, Kribi, Lolodorf road, *Bos* 5501 (holo-: WAG; iso-: LISC, PRE, YA).

Plantae dioeciae; caules cylindrici, volubiles, graciles, scandentes; rami flexibiles; internodia valde elongata, nodi leviter incrassati. Folia opposita petiolis brevibus gracilibus, glabri, supra canaliculatis; laminae pallidae viridis aliquantum rigidae, ovatae vel oblongae ad ellipticae; basis leviter truncata; apex acutus vel acuminatus; margines leviter undulati, venae brochidodromae. Spicae singulares vel plus in quoque nodum, glabrae, laterales vel/et terminales; pedunculus 3–24 mm longus; bracteae duo, triangulares, acutae; internodia et nodi alternans, nodi verticillis, 8–15, involucrati cyathiformes, internodiis diametre < 1 mm; internodia ex spica mascula gracilesusque ad 10 plo longior quam lata, filiformia; internodia ex spica femineus multo breviore et incrassatus; semen ellipsoidalis, viridis ubi juvenis, ruber-aurantiacus ubi maturus.

Plants dioecious, slender, glabrous, of indeterminate height, climbing in fully shaded areas of forest; *Stem* round in cross section, internodes 7–15 cm long with diameter up to 3 cm; *branches* both long and short but only short, lateral branches bear leaves. *Leaves* simple, opposite; *lamina* narrowly-ovate, elliptic or elliptic-oblong, 4−14 × 2–7 cm, glabrous, 6–9 pairs of brochidromus secondary veins extending to the channeled midrib; base truncate; apex acute to acuminate, occasionally attenuate, distinct acumen up to 2 cm long, in most cases, with abrupt change in angle; margin slightly undulate; *petiole* up to 1 cm long, channelled, more or less rigid, woody and glabrous. *Spike* unisexual, jointed, elongate, made of bead-like nodes linked by thread-like internodes, terminal or axillary or both, often one or more per node with two opposite, pointed, sterile free bracts with triangular tips, connate at base, terminating with 1 or 2 sterile nodes, the most apical node forming blunt sterile column for both male and female; *cupuli* above bracts, series of 8–15, superimposed and circular (diameter between 1–3 mm); younger strobili 1 mm long entirely protected by 2 basal bracts. *Male spike* 1–10 × 0.2–0.7 mm; *internodes* thread-like, more than 10 times longer than broad, basal internode up to 1.5 cm long; *nodes* 7–15 circular cupuli surrounded by a regular, thin sheath; numerous male structures > 25 in 1 or 2 superimposed layers at axil in each cupulus. *Female spike* a strobilus, at least 2.5 cm long; cupuli at apex generally sterile, axis terminating with an

almost obtuse, sterile column; *internodes* 3–7 times longer than broad; *ovules* perpendicular to the axis; often 2–5 per whorl; *seeds* ellipsoid or obovoid, subapiculate, up to 10 mm long, 3–5 mm in diameter, with fleshy external envelope red to orange when ripe (Figure 3.5).

Etymology – This species is named '*interruptum*' to draw attention to the threadlike internodes of the spikes that are up to 10 times longer than broad, forming constrictions between whorls in the males (the internodes are twice as thick and much shorter in the females). These interruptions between successive whorls are considered here as the main distinguishing feature of the species.

Additional specimens examined: **Cameroon**: Bipindi Plateau, *Zenker 41* (P); Br. Mamfe, *Rudatis 42* (K); Lokoundje, *Dinhlage 882* (B); Central Province, 1 km E of Libamba College, Makak, *Manning 2002* (K, YA); 70 km SSW of Eseka, 10 km SW of Njong River bridge, *de Wilde 3835* (BR, K, WAG); 15 km N of Kribi, Mpolongwe Forest, *Bos 5465* (BR, WAG, YA); 18 km from Kribi, Lolodorf road, *Bos 4068* (K, WAG); 2 km S of Longii, *Bos 4144* (WAG); Maan, 24 km ESE of Nyabesan, *Raynal 10247* (P); **Central African Republic**: 8 km SE of Bambari, Alindao road, S of Liwa Creek, *Fay 2181* (K); Oubangui, *Tisserant 297* (P); Republic of **Congo**: Madabu – Zobia, *Gerard 3198* (BR); Boukoko, *Tisserant 1518* (P); **D.R.Congo**: Kisangani, Tsopo zone, Kandolo village, *Szafranski 1584* (BR, K); Coquilhatville, *Deweure 592* (BR); Maluku, Kinshasa, *Breyne 665* (BR); Eala, *Corbisier 936* (BR); Kingana–Kipindi, *Pauwels* 724 (BR); Haut-Zaire, Bas-Uele, 130 km NW of Likati–Muma, *Lisowski 17686* (K); Eala–Ruki, *Robyns 511* (BR); Yaosuka, Lusambila River, *Louis 831* (BR); Mobwasa, *Vermoesen 321* (BR); Mayombe, *Wellens 449b* (BR); Yangole, 20 km W of Yangambi, *Louis 12038* (BR); Equateur Province, Boende, *Evrard 3893* (K); Yangambi, Isalowe Reserve, *Louis 14947* (BR); Monga, *Gerard 858* (BR); Eala, *Pynaert 1385* (BR); **Equatorial Guinea**: Bata-Bome, *Carvalho 5100* (WAG); Bata-Mbini, 18 km from Estrada, *Carvalho 5226* (WAG); **Gabon**: 6 km NE of Malibe, *Reitsma 2749* (WAG); *Luja 222* (BR); Malibe border, *Reitsma 639* (WAG); Malibe border, Estuaire, *Louis 1765*; Libreville, *Klaine 348* (P); around Libreville, *Klaine 774* (P); *Klaine 170* (P); Ngounie, Bongolo, 100 km from Chutes Louetsi, *Louis 2028* (WAG); 24 km on road

from Okondja to Akieni, *Wieringa 6460* (WAG); Ditione Munda, Sibange Farm; *Soyaux 87* (K, P).

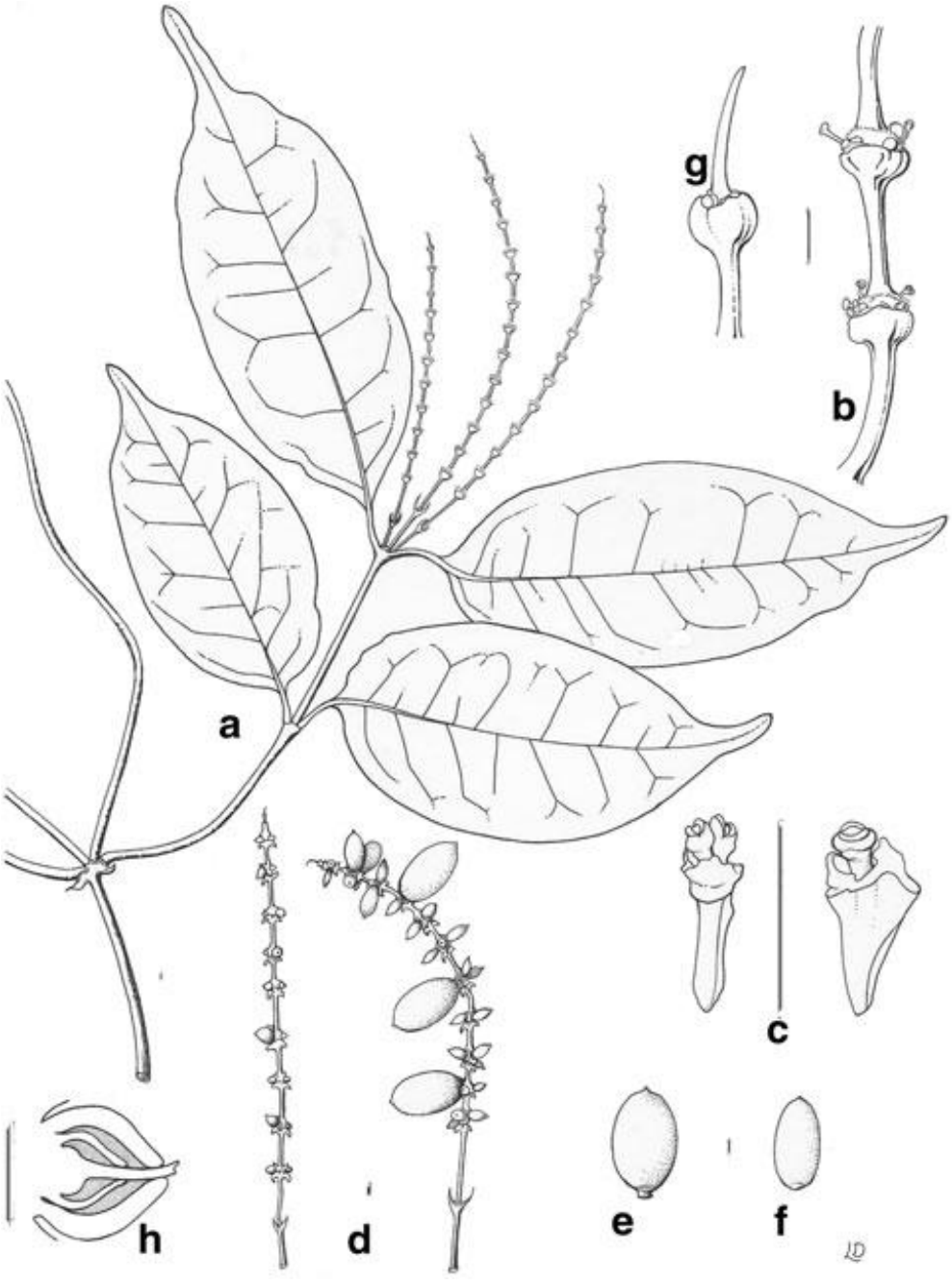


Figure 3.5 ***Gnetum interruptum*****. a, habit; b, male spike with stamens; c, from left to right, male flower front and side views; d, from left to right, female spike young and mature; e, fruit; f, seed; g, staminal column; h, c.s. of female flower. a–c & g, from *Bos* 5501; d–f& h, from *Bos* 4068. Scale bar: 1 mm.**

Gnetum latispicum E.H.Biye**, sp. nov.**

-- Type: Cameroon, Barombi Kang, 5 km from Kumba Park, *Biye* 603 (holo-: J; iso-: YA).

*Plantae dioeciae; caules volubiles, scandentes; rami flexibiles, internodia elongati, nodi tumidi. Folia opposita, petiolus supra canaliculatis; laminae pallidae virides, subtis, rigidae, ovatae-ellipticae, vel obovatae-oblongae, basis breviter attenuata, anguste sensim in petiolum; apex acuminatus; margines leviter undulati vel integrae; venae brochidodromae. Spica saepe una vel duo per nodus, terminalis, lata et aequata, interdum moniliformis, glabra; pedunculus 5 plo longior quam latus; bracteae duo, triangulares, connatae, acutas; internodia et nodi alternans, nodi verticillis, 8–13, conspicuus involucrorum, cylindracorum; internodia ex spica mascula aeque longa ac diametrum vel breviora, >1 mm diametre**,** utiliter crassa**;** internodia in spica femineus breviore et incrassatus; semen ovoideus, obtusus, viridis ubi juvenis, rubellum ubi maturus.*

Plants dioecious; slender climbers of indefinite height, glabrous. *Stem* round in cross section, internodes 5–15 cm long with diameter of 4–5 cm; *branches* varying in length, only lateral ones bear leaves. *Leaves* simple, opposite; *lamina* ovate to broadly oblong or broadly elliptic, 5−10 × 2–6 cm, glabrous, 4–6 pairs of brochidromus secondary veins extending to the channeled midrib; apex abruptly acuminate, distinct acumen < 1 cm long, in most cases, with abrupt change in angle; base shortly attenuate, blade at base gradually attenuate into petiole; *petiole* up to 1 cm long, channelled, more or less rigid and glabrous. *Spikes* jointed, elongate with nodes and internodes, unisexual, terminal, thickened, often one or two per node with two opposite, concave, free bracts with triangular tips, connate at base, terminating with 1 sterile node, the most apical forming a sterile ± obtuse, blunt column; *internodes* slightly thickened, as long as broad, 2.5–4.5 × >2.5 mm; *nodes* above bracts, fused, series of 8–13, superimposed and circular; *female spike* thicker than male spike; *ovules* perpendicular to the axis; often 3–4 per whorl; *seeds* ovoid, blunt, up to 10 mm long, 3–7 mm in diameter, with fleshy external envelope reddish when ripe (Figure 3.6).

Etymology –This species is named *latispicum* because of the bulky spikes with wide, regular internodes bulging between whorls. The main distinguishing feature is that the internodes are broadest in the middle.

Other specimens examined: **Cameroon**: W of Lobe Falls, 5 km from Kribi, *Biye 617* (J, YA); 10 km N from Sa'a, Lekie, *Biye 301* (J, YA); IRAD Station, Barombi Kang 5 km E from Kumba, *Biye 590* (J, YA); Batoke, 4 km NE from Limbe, *Biye 416b* (J, YA); N bank of Kienke River, Kribi, *Bos 4267* (WAG); 26 km W of Mopwo situated on Yokadouma-Batouri road, *Letouzey 5249* (K, P, YA); **Central African Republic:** Boukoko, *Tisserant 1984* (BR, P).

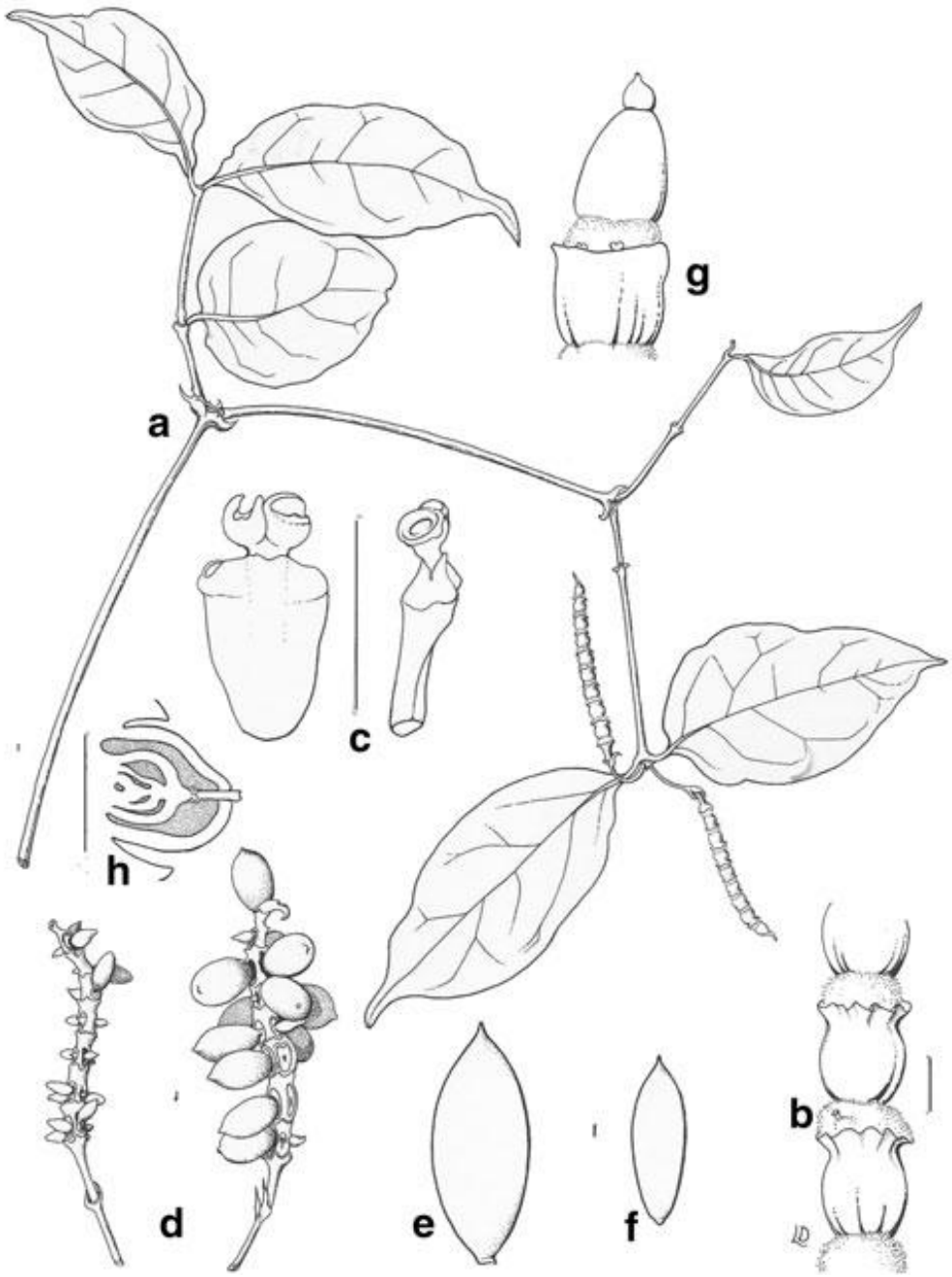


Figure 3.6 ***Gnetum latispicum*****. a, habit; b, male spike; c, from left to right, male flower front and side views; d, from left to right, female spike young and mature; e, fruit; f, seed; g, staminal column; h, c.s. of female flower. (a–c & g, from *Biye* 603; d–f & h, from *Biye* 416b). Scale bar: 1 mm**

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CHAPTER FOUR

MORPHOLOGICAL CORRELATION BETWEEN CULTIVATED AND WILD POPULATIONS OF *GNETUM* L. IN AFRICA

CONTENT

4.1 Introduction

- Background knowledge:
  Comparison between wild and cultivated plants

- Problem statement:
  The extent of correlation between the cultivated materials from various geographical regions and the naturally occurring taxa.

- Knowledge gap:
  The taxonomy of the genus *Gnetum* in Africa

- Purpose of this research and key questions
  Aim

4.2 Materials and Methods

Plant material
Morphological characters and data analysis

4.3 Results

Phenetic analysis using male specimens of *Gnetum*
Phenetic analysis with female specimens of *Gnetum*

4.4 Discussion

Taxonomic implication

4.5 Conclusion

REFERENCES

APPENDIX

Abstract

Gnetum species in Africa are threatened and face the possibility of disappearing because of the method and the extent of their harvest and due to intensive forest exploitation occurring where they grow. The plants of this genus are therefore in need of conservation strategies. Domestication trials have been explored in three research centres in Cameroon based on vegetative propagation using stem cuttings obtained from the naturally occurring plant materials. Prior to this study, the wild species of *Gnetum* were insufficiently known and easily confused; consequently the cultivated variants were also misidentified. The main aim of this chapter is to determine the relationship among cultivated plants and their relationship to the wild plants based on various morphological characters observed on *Gnetum* specimens. Cluster Analysis, Principal Components Analysis and Principal Coordinates Analysis have been used to infer similarities among cultivated plants and between cultivated plants and wild populations of *Gnetum* in Africa. The study reveals that all but one of the species identified in the wild have been used in the domestication trials. The plants undergoing cultivation trials cluster together but next to their wild counterparts for various putative groupings. The extent of correlation between the cultivated and the field collected plants is discussed.

Keyword: Taxonomy, Multivariate Analysis, Cultivation, Relationship.

4.1 Introduction

Gnetum L. of the phylum Gnetophyta consists of about 40 species, of which about seven occur in South America, four in Africa (Chapters 2 & 3), and about 30 in tropical Asia (Hutchinson & Dalziel, 1954; Maheshwari & Vasil, 1964; Earle, 2001; Won and Renner 2006; Biye *et al*., 2013). The African species are lianas that were originally abundant in the wild, grow well in a wide range of habitats, including farm fallows, abandoned farmland, secondary forest and closed forests (Shiembo, 1999). The vines climb supported by various sizes of dead or living trees and on other climbers, and generally produce great quantities of leaf biomass (Fondoun & Manga, 2000).

Gnetum leaves, rich in essential nutrients (Sunderland & Clark, 2001), do not only prevent malnutrition, but have traditional medicinal values (Burkill, 1994) and are used in the production of alcohol (Mbahand, 2001). *Gnetum* seed samples are nutritionally richer with a higher caloric value than other edible vegetables even though the eating of their seeds is unknown in Africa (Ekop, 2007). In brief, *Gnetum* not only contributes to poverty alleviation for most indigenes who collect and sell it throughout the year but it is also a source of food at all levels of society, and has several other uses (Leakey *et al*., 1996; Tekwe *et al*., 2003). Because of the extent and unnecessarily destructive harvesting process to meet its several uses, *Gnetum* is likely to be at risk of extinction.

Due to their threatened status, *Gnetum* species are in need of conservation but it is difficult to conserve the unknown. The plants undergoing domestication trials in three regions of Cameroon were obtained from naturally occurring and easily confused species that were previously misidentified; their diversity is unknown due to nomenclatural uncertainty (Biye *et al.,* 2013). The morphological characters used for species recognition in *Gnetum* in Africa (Chapters 2 and 3) are applied here to differentiate species in cultivation, an essential step for setting priorities for conservation. The abundance of species, their endemism level in an area or their degree of relatedness are some of the factors that usually influence conservation strategies (Bevill and Louda, 1999; Coyne & Orr, 2004). The number of endemic plants is essential in the quantification of biodiversity (Vane-Wright *et al.,* 1991).

Correct identification of plants is vital not only for the assessment and for conservation of biodiversity, but also in cultivation, agriculture as a whole, and forestry (Randler, 2008). Systematic studies ensure identification as well as preservation of genetic variation. When identifying and naming cultivated or cultivated wild plants, reference is often made to records of the plant's wild origin to provide necessary information and comparison for their description (Davis & Heywood, 1963). Plants in cultivation often differ considerably in size, although they generally show little internal variation from their wild counterparts. They are apparently separable such that they are sometimes viewed as distinct taxa by agronomists (Sharsmith, 1961). All available materials are essential when classifying a group; therefore both wild (Chapter 2 & 3) and cultivated plants of *Gnetum* are necessary for accurate species recognition to assess relationship or delimit the taxa, (Davis & Heywood, 1963).

4.1.1 Comparison between wild and cultivated plants

Interest in the distinction between wild and cultivated plants has a history as far back as 370 BC when the ancient Greek botanist Theophrastus attempted to discuss this distinction (Bailey, 1918). Basically, there is no absolute distinction between the systematics of these plants (Jeffrey, 1968), however, they are classified, on one hand, as those that are of known origin or habitat and are referred to as indigens, i.e. discovered in the wild. On the other hand, those cultivated with unknown origin and with characters separating them from known indigens, without any type specimen or description implying no taxonomic knowledge, these are referred to as cultigens, i.e. they arise somehow under the influence of man (Bailey, 1918). These are thus considered as species that appeared due to domestication.

Unlike the wild populations that occur in nature with no human assistance or intervention, cultivated plants are known to be biologically 'weaker'. Most plant organs increase in size in cultivation, however they retain the relative differences observed in the wild forms (Brysting & Elven, 2000). Various biological systems have been investigated in order to compare wild versus cultivated plants (Billings, 1997). It has been observed that the evolutionary varietal selection of wild plants was often determined by species

survival (i.e. natural selection) and that their propagation was usually by either seeds or natural vegetative propagation (Billings, 1997). On the contrary, in plants cultivated for food, the varietal selection was human-directed towards taste and the method of preservation and vegetative propagation is usually artificially mediated (Billings 1997; Kumar *et al*., 2007). Very few studies have been carried out on wild versus cultivated plants especially on specific genera, such as *Gnetum*, so as to correlate the distribution pattern of any variation for the purpose of natural classification.

4.1.2 Problem statement:

The extent of correlation between the various cultivated materials and the naturally occurring taxa.

Gnetum is a useful vegetable in Africa but its cultivation is still experimental (Ndam *et al*., 2001; Besong *et al*., 2001). Prior to addressing the bulk production problem and conservation strategies, it is essential to identify the various forms or species of *Gnetum* under cultivation. This can be achieved by determining the morphological correlation between the cultivated materials collected from various regions and the naturally occurring taxa. Methods to reduce production period and to multiply *Gnetum* can then be developed for the realization of benefits in a relatively short time (see Chapters 7 & 8 for discussion on *Gnetum* in Africa).

The examination of herbarium specimens of the wild plants revealed that various forms of *Gnetum* have been attributed the same name and the similarity in vegetative features between individuals of this genus has led to nomenclatural misunderstanding (Chapters 2 & 3). There is a high possibility that the taxonomic confusion observed in the wild plants of *Gnetum* leads to the misidentification of the cultivated variants. It is therefore necessary to understand the correlation within cultivated members and to match them to the wild plants of *Gnetum* in order to identify the forms involved in cultivation. In brief, there is a great need for species clarification in the cultivated forms identified in this study based on the wild forms of *Gnetum* in Africa using updated morphological characterization (Chapters 2 & 3). Also, by identifying the various species accurately and

analysing their taste (Chapter 7) for nutritional purposes, the species with a good taste could be conserved by cultivation. Moreover, the general destruction of the natural population during harvesting could be reduced.

4.1.3 The taxonomy of the genus *Gnetum* in Africa.

To establish a natural classification, correlation of characters, discontinuity of variation and only genetically fixed variation need to be firmly applied (Davis & Heywood, 1963). Both qualitative and quantitative characters have been used to understand the variation observed within the taxon (Chapter 3). Therefore, the morphological variations that are observed on both wild and cultivated specimens of *Gnetum* will assist with the correlation between various forms and eventually their nomenclature.

The phenetic approach used here will illustrate the distribution of variation among the wild and cultivated populations of the African *Gnetum* species. Phenetics generally leads to the recognition of sets of diagnostic characters which might be equivalent to apomorphies thus reflecting common ancestry (Snow *et al*., 2003). This approach is adopted here as it also enables the examination of the genetically fixed variation and correlation of characters (Radford *et al*., 1974; Thorpe, 1983; Davis & Heywood, 1963) and is applicable to both fresh and dried specimens of various origins (Sebola & Balkwill, 2009). The phenetic approach also greatly contributes in the assessment of the amount of phenotypic plasticity expressed by various morphological characters.

4.1.4 Purpose of this research and key questions

This investigation aims to illustrate the distribution of any variation among the wild and cultivated plants of the genus *Gnetum* in Africa in order to understand the morphological correlation between them. Hence, the taxonomic confusion resulting from vegetative similarities in the cultivated forms and their incorrect identification will be eliminated. Moreover, it is essential to accurately identify for cultivation the species with a good taste (Chapter 7). In sum, the key questions this objective attempts to answer are: (i), 'Are all the cultivated forms the same species?' (ii), 'What is the morphological variability

between the wild and cultivated populations'? And 'Does the morphological variation if any in cultivated plants show geographical pattern of distribution?

The main aim of this study therefore is to identify which species are currently in cultivation and from which regions, and whether there is phenotypic plasticity among them compared to wild populations.

4.2 Materials and Methods

To compare wild versus cultivated specimens of *Gnetum* species in Africa, the methods applied in this investigation are similar to those used in Chapter 2 in analysing the naturally occurring taxa (Biye, 2013), except that fewer wild specimens are included and cultivated materials are added in this analysis.

4.2.1 Plant materials

The analysis was based on data collected from some of the specimens included in the analysis of the naturally occurring *Gnetum* discussed in Chapter 2. A total of 103 pressed herbarium specimens was used, of which 68 were specimens from the wild, borrowed from the following herbaria: B, BR, FHI, J, K, LISC, MO, P, PRE, US, WAG and YA (herbarium abbreviation according to Holmgren *et al*., 1990). Thirty-five cultivated *Gnetum* specimens were collected and pressed from three research centres, (Limbe Botanical Garden, IRAD Barombi Kang, Kumba and IRAD Nkolbisson, Yaounde), involved in domestication trials of *Gnetum* obtained from various natural habitat. However, the origin of the cultivated specimens collected from Kumba Research Centre was unknown.

These plants have been cultivated at the three research centres for the past 12 years at least (note: the plants are younger than 12 years at IRAD, Yaounde due to the trial being restarted after problems with the watering system). However, the plants have not been genetically modified , but they have been propagated via cuttings and are therefore

essentially clones of the original wild material, similar to most crops cultivated in the region. The term ‘cultivated’ is used in this sense here.

Sampling strategy for the cultivated plants included collecting specimens that were at least 2 m apart to eliminate the possibility that apparently separate plants might be originating from a single rooting stem. To obtain a large sample size, the initial intention was to collect six to ten samples of cultivated male and female plant materials from various localities (either in farms or in research centres) in the five provinces of Cameroon, and in neighbouring countries: Central African Republic, Congo, Equatorial Guinea, Gabon, Nigeria and probably Angola. In the course of fieldwork and based on information received from different institutions in various countries plus literature, it appeared that the domestication of *Gnetum* was unusual in most African countries where it grows naturally, and even in Cameroon where trials were undertaken, domestication is still limited to research centres for experimental purposes. Consequently, the study was narrowed to 35 mature specimens obtained from cultivated plants in the three Research Institutes in Cameroon. Fifty-two wild specimens of *Gnetum africanum*, *G. buchholzianum*, *G. interruptum* and *G. latispicum* were selected from the analysis in Chapter 2, of which 23 were males and 29 females.

The wild specimens were selected from various clusters representing the putative species recovered in the phenograms (Figures 2.2; 2.6 & 2.7) taking into account factors like the collection locality if possible, the number of standard specimens and the total number of cultivated materials available for males and females respectively (13 male and 22 female cultivated plants). *G. latispicum* had the lowest number of specimens therefore these were all included in the analyses. Both wild and cultivated materials used here originated from Cameroon although four of the wild specimens of *G. africanum* included were from Angola, Gabon and Equatorial Guinea for lack of an adequate number of specimens from Cameroon.

The cultivated specimens were sorted into hypothetical groups according to the diagnostic morpho-features noted in Table 2.6 (Chapter 2) and keys for the different naturally occurring plants previously identified (Chapter 3). In total, 41 male and 62

female specimens with sufficiently mature reproductive features were measured. Sixteen specimens (5 males and 11 females) of three species, *Gnetum nodiflorum* Brongn., *G. paniculatum* Spruce and *Gnetum* sp. from South America were used as standard taxa to calibrate the character list and character state definitions. They were used only in the cluster analyses and not the ordinations for the same reasons presented in Chapter 2. Specimens used in this study are listed in Appendix 4.7.1, and these are referred to as Operational Taxonomic Units, OTUs (Sneath & Sokal, 1973; Stace, 1980; Radford, 1986).

Measurements of morphological features of the cultivated specimens were carried out on mature branches with spikes using the same character list as for the wild materials (Table 2.1, i.e. 68 characters). The vegetative features were measured using a WILD HEERBRUGG Light Microscope M7A, while a ZEISS Stereo Discovery V12 Microscope using AxioVision Release 4.6.3 was used for the reproductive structures.

4.2.2 Morphological characters examined and data analysis

Ratios and one of their component basic characters were applied to recover meaningful phenograms and several combinations of characters were investigated to carry out the analysis. Of the 68 selected characters on Table 2.1, 60 and 65 characters were relevant to males and females respectively and their usefulness in grouping the specimens of the wild plants of *Gnetum* was assessed in Chapter 2. Numerical analyses were used to describe morphological relationships among the investigated taxa and these were based on two data matrices: the qualitative and combined data matrices.

In order to carry out the numerical analysis, cluster analyses (Unweighted Pair-Group Method using Arithmetic Averages, UPGMA) were performed with NTSYS-PC Version 2.20 software (Rohlf, 2000) to separate specimens into groups and also 2- or 3-dimensional plots to view the results of Principal Coordinates Analysis (PCO) or Principal Component Analysis (PCA) in order to analyse the pattern of similarity between OTU's as recommended by Sneath & Sokal (1973) and Legendre & Legendre (2003). These scaling techniques provide the visual interpretation of the relationships

between taxa and the distances between points reflects the similarities between OTUs (Dunn & Everitt, 1982). In cluster analysis (CA), the standardized data matrix was transformed to a distance matrix of OTUs by applying the average taxonomic distance coefficient and then using the unpaired group method of arithmetic averages (UPGMA) to cluster them. To calculate the cophenetic correlation coefficient (r) for the tree to show how well the tree fits the data, the cophenetic values of the tree were compared with the similarity matrix. Separate male and female CA, PCA and PCO analyses were performed to verify distinct groupings within the OTUs.

4.3 Results

4.3.1 Phenetic analysis using male specimens of *Gnetum*

Cluster analysis

Distinct groupings of the various OTUs were established based on the qualitative (Figure 4.1) and combined data matrices (Figure 4.2). Fourteen and 53 characters were used and phenograms with correlation coefficients (r) equaling 0.97 and 0.93 respectively were obtained. These values signify a relatively good fit of the data to the various trees. Based on the qualitative analysis (Figure 4.1), all the cultivated specimens formed distinct clusters with their wild counterparts. With *G. latispicum*, the lone cultivated specimen clustered neatly with the wild plants. *G. interruptum* was not found in cultivation and the wild specimens formed a distinct cluster in all three analyses. The standard OTUs from South America also formed a distinct group in all the analyses.

Cluster analysis based on the quantitative characters (not shown here) only showed distinct clusters formed by cultivated specimens of *G. buchholzianum* but those of *G. africanum* and *G. latispicum* were found with either the wild or the cultivated specimens of *G. buchholzianum*. In the analysis of the combined qualitative and quantitative characters (Figure 4.2), the specimens of the cultivated *G. africanum*, although forming a distinct cluster, were grouped with some of the cultivated *G. buchholzianum* specimens. The cultivated *G. buchholzianum* specimens formed two distinct clusters that could be

seen as two putative groupings (as in Chapter 2). The wild specimens of *G. latispicum* were grouped with the single cultivated specimen.

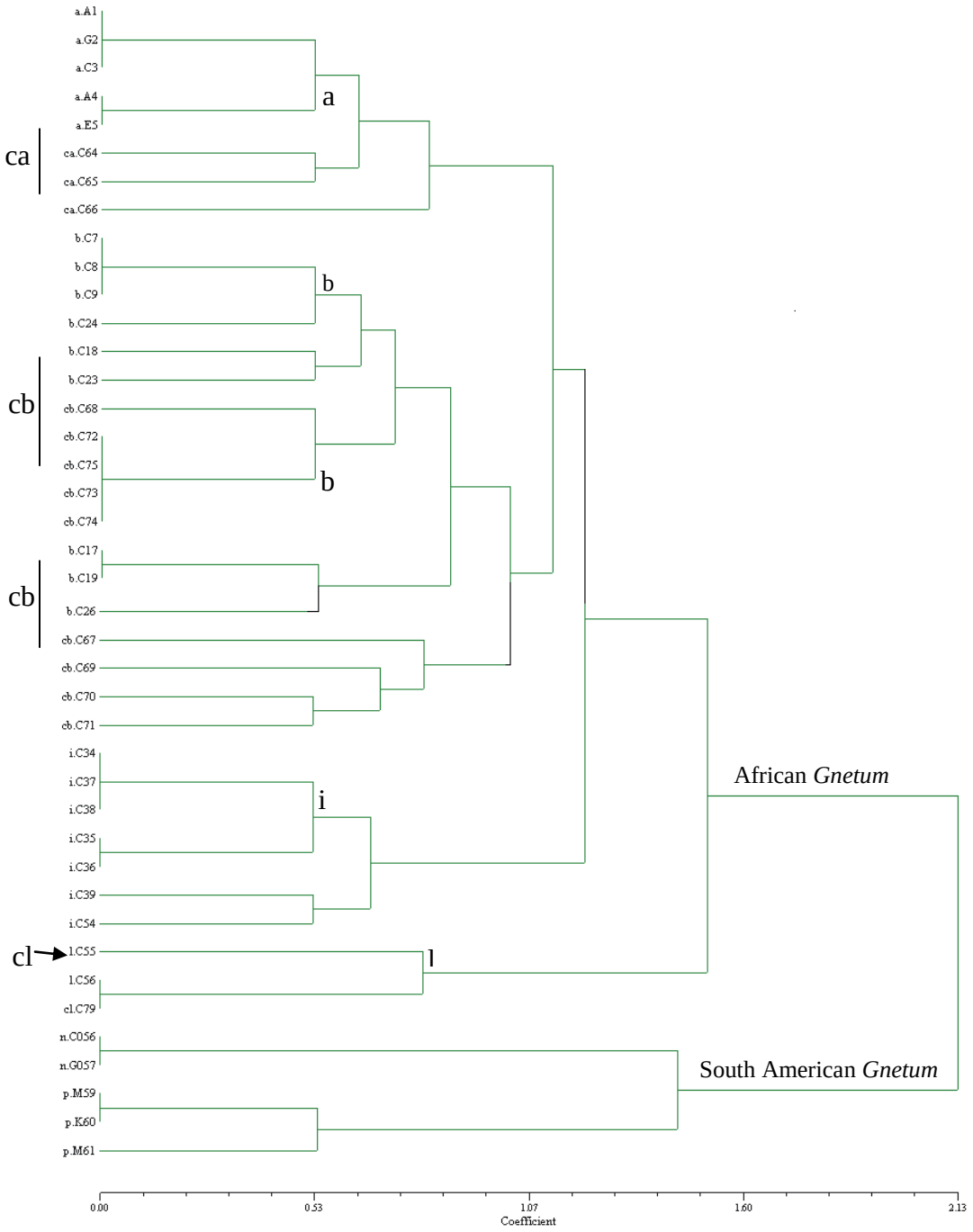


Figure 4.1 UPGMA phenograms using average taxonomic distance of the wild and cultivated male specimens of *Gnetum* species in Africa based on qualitative data matrix. *G. africanum* (wild = a; cultivated = ca), *G. buchholzianum* (wild =b; cultivated = cb), *G. interruptum* (wild = i), *G. latispicum* (wild = l; cultivated = cl). Abbreviations used to identify specimens as in Appendix 4.7.1.

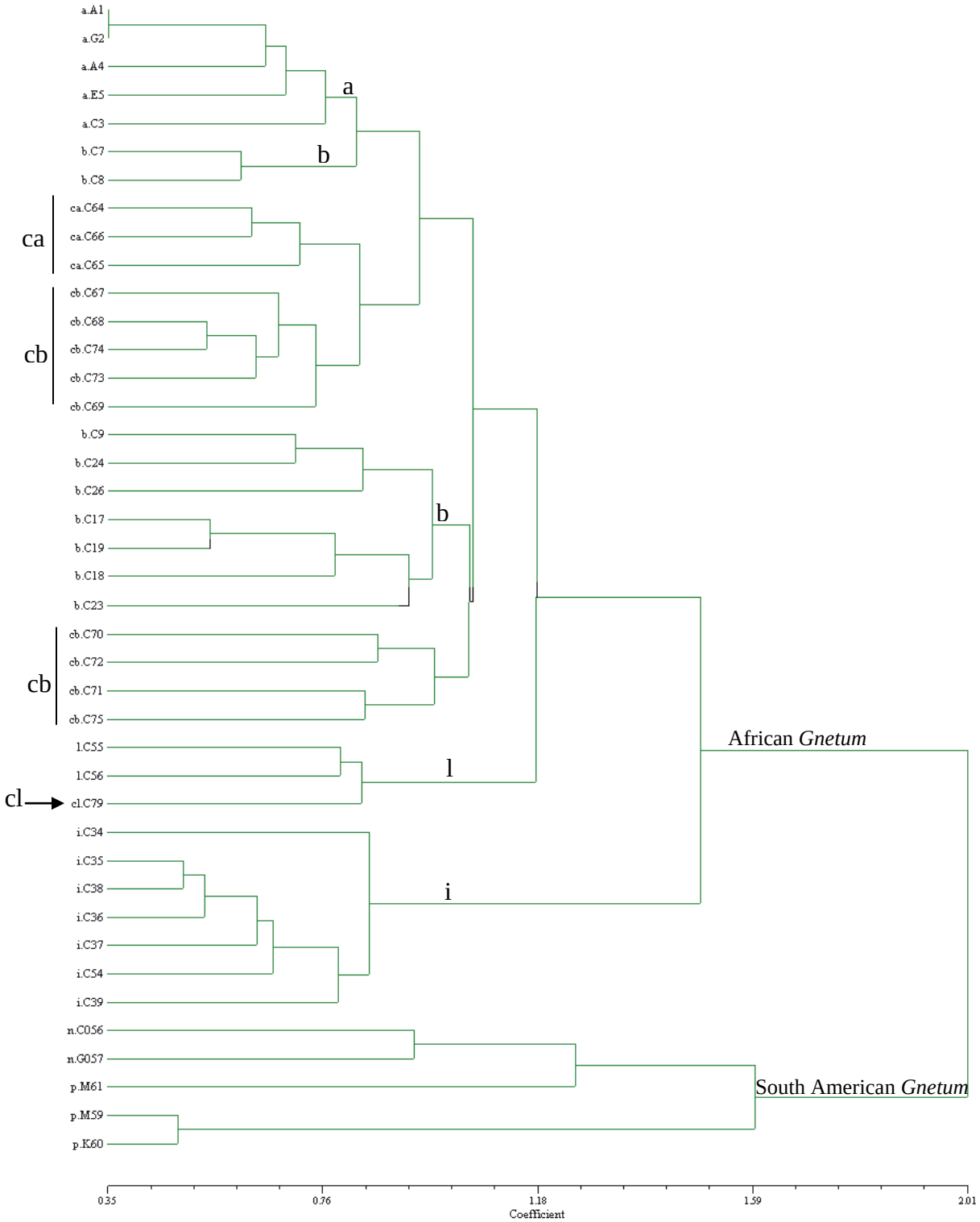


Figure 4.2 UPGMA phenograms using average taxonomic distance of the wild and cultivated (cultivated) male specimens of *Gnetum* species in Africa based on combined quantitative and qualitative data matrix. *G. africanum* (wild = a; cultivated = ca), *G. buchholzianum* (wild =b; cultivated = cb), *G. interruptum* (wild = i), *G. latispicum* (wild = l; cultivated = cl). Abbreviations used to identify specimens as in Appendix 4.7.1.

Ordination

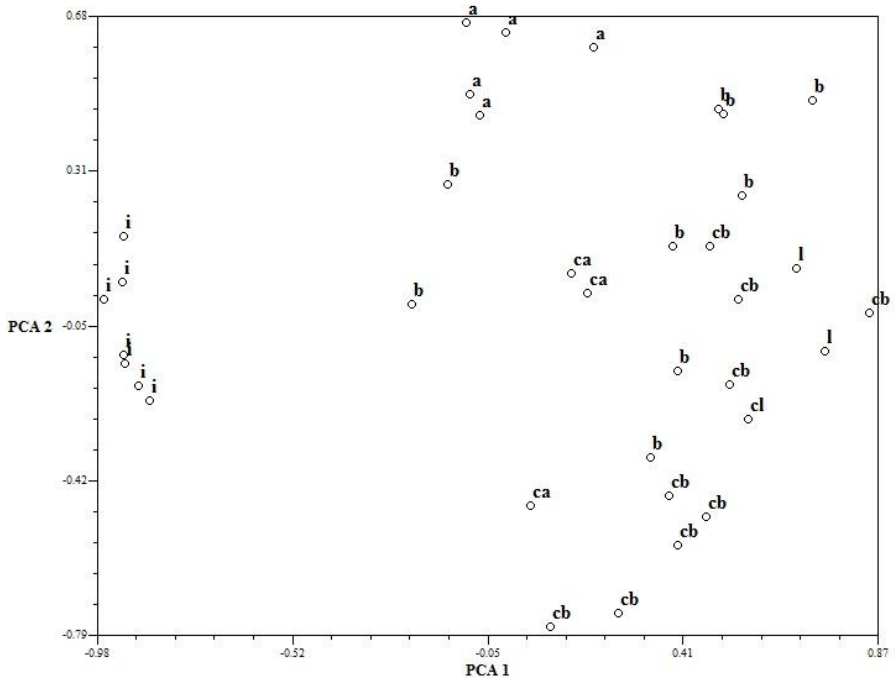
In both Principal Components (PCA) and Principal Coordinates (PCO) analyses, the pattern of distribution of specimens was similar and two distinct groups of specimens of both wild and cultivated plants of *Gnetum* are evident (Figures 4.3 & 4.4). One well distinguished grouping consists of *G. interruptum*, the second is a complex made up of all the specimens belonging to the three putative groups in cultivation, namely *G. africanum*, *G. buchholzianum* and *G. latispicum*.

Principal Components Analysis (PCA) was applied to quantitative data only. Analysis of the male OTUs based on 39 characters (Figure 4.3) shows a distinct group representing a complex of both wild and cultivated *G. africanum* (a), *G. buchholzianum* (b) and *G. latispicum* (l) spreading acoss the right side of the plot. However, although both cultivated and naturally occurring specimens of *G. buchholzianum* spread across the grouping, those of *G. africanum* are found more to the centre and above intermediate position of the plot while those of *G. latispicum* are confined at the right side of the plot. At the intermediate right side of the scattergram, are found the specimens of *G. interruptum*.

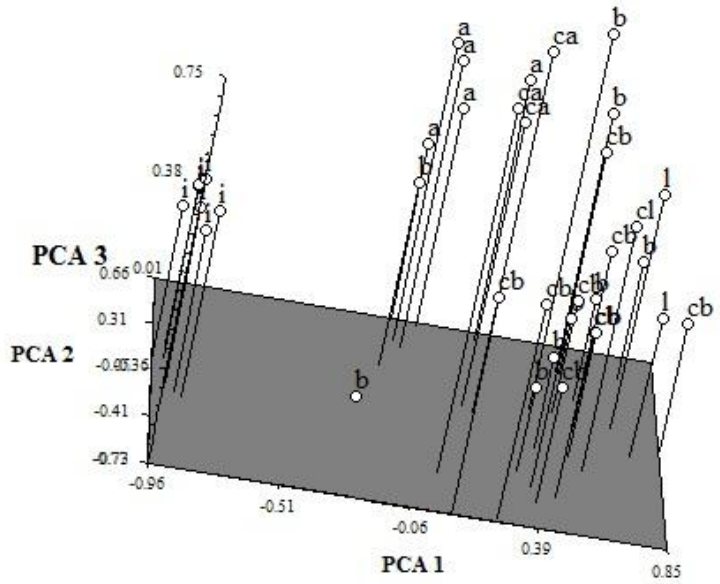
The PCA did not produce distinct clusters for *G. africanum*, *G. buchholzianum* or *G. latispicum* in either of the 2- or 3-dimensional scatter diagrams produced in this analysis (Figure 4.3). A similar cluster pattern was recovered in the analysis with the quantitative data of the naturally occurring specimens in chapter 2. OTUs of *G. africanum, G. buchholzianum* and *G. latispicum* are therefore more similar to one another than they are to those of *G. interruptum* which formed a distinct group in both scatter diagrams. The first three axes accounted for 57.4% of the variation as shown by the eigenvalues for the 39 axes (Table 4.1).

Table 4.1 Eigenvalues obtained from principal components analysis of the quantitative data matrix for the wild and cultivated male specimens of *Gnetum* species in Africa. Variation is concentrated in the first 12 axes (94.54%, with the first 3 axes accounting for 57.4%)

Axis	Eigenvalues	% total variance contribution	Cumulative %
1	11.12522414	30.9034	30.9034
2	5.10431910	14.1787	45.0821
3	4.44485160	12.3468	57.4289
4	2.43700508	6.7695	64.1983
5	2.27223866	6.3118	70.5101
6	1.89649600	5.2680	75.7782
7	1.24483605	3.4579	79.2360
8	1.15797423	3.2166	82.4526
9	0.93856329	2.6071	85.0597
10	0.90845860	2.5235	87.5832
11	0.75818851	2.1061	89.6893
12	0.61116292	1.6977	91.3870
13	0.54957148	1.5266	92.9136
14	0.44153743	1.2265	94.1401
15	0.39233453	1.0898	95.2299



(i)



(i)

Figure 4.3 Scattergram (i) and 3D diagram (ii) generated by Principal Components Analysis based on quantitative data of male specimens of *Gnetum* species. The OTUs of the various African specimens, except for *G. interruptum* (i), do not form distinct clusters in both 2- and 3-dimensional plots. Wild and cultivated specimens identified by lettering as in Figure 4.2.

Principal Coordinates Analysis (PCO) was based on fifty-three quantitative and qualitative characters for both the wild and cultivated males. Two distinct groupings of the African *Gnetum* specimens (wild and cultivated) were recovered: one group comprises *G. africanum* (a & ca), *G. buchholzianum* (b & cb) and *G. latispicum* (l & cl) towards the right side of the plot and the other consists of *Gnetum interruptum* (i) at the left central side of the plot (Figure 4.4). The OTUs of *G. africanum* are found mostly towards the middle of the second axis and at the right side of the first axis while those of *G. latispicum* are placed at the lower right side of the scattergram Figures 4.4a & 4.4b).

The PCO analysis grouped all the wild specimens of *G. buchholzianum* and *G. africanum* at the top right side of the plot with the latter towards the centre. Meanwhile their cultivated counterparts spread at the lower right side of the plot assuming a more or less intermediate position along the second PCO axis, overlapping with *G. latispicum* (l & cl) found further down along this axis. (Figures 4.4a & b).

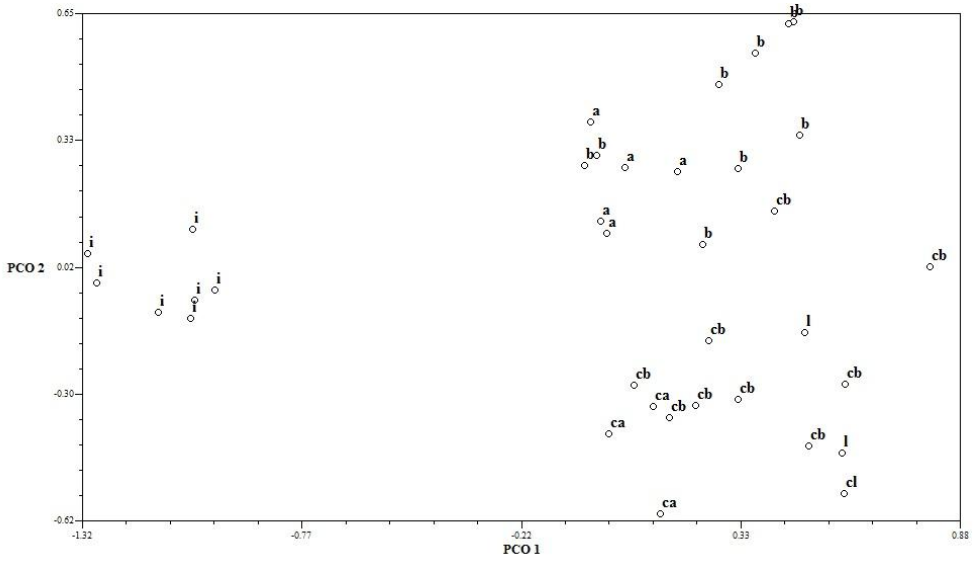
Twenty-eight characters that most influenced the distribution of specimens along the first three principal coordinate axes in the 2-dimensional and 3-dimensional scatter diagrams were those with a value ≥ 0.6 or - 0.6 (Table 4.2) such as the length of leaf at first node, the length of the apex, the length of petiole at second node, the length of spike and that of the whorls, the length and width of peduncle before and after bracts, the number of whorls per spike, the ratio of length / width and width of various internodes of spikes, and the presence and shape of the staminal column. Fifty-seven percent of these characters were strongly correlated with the first PCO axis.

Plants of *Gnetum interruptum* (i) generally had lower scores along the first and second PCO axes than the complex of the other three putative groups. The characters that were strongly negatively correlated with these axes were the length of leaf apex, the length of catkins and whorls (Figure 2.1), the number of nodes per spike, the ratio of length to width of internodes and the diameter of the side stem. All the characters, except for the length/width ratios of the leaf and the length of peduncle below bracts, correlated positively on the third PCO axis (Figure 4.4b) but were very similar, consequently the

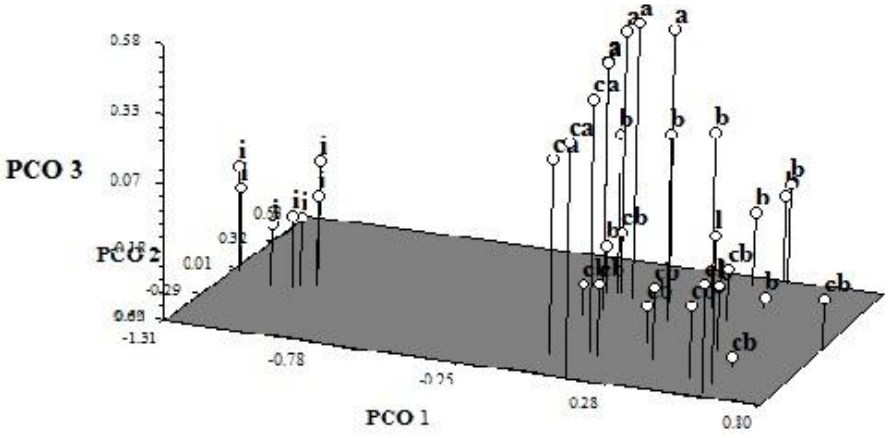
OTUs were fairly concentrated along this axis and there was no apparent distribution based on their collection areas.

Table 4.2 Taxonomic values of quantitative and qualitative characters as loaded on the first three PCO axes.

Characters generated	1st axis	2st axis	3nd axis
Width of Leaf a	0.0460	0.4712	0.1104
Length/width ratio of a	0.2672	-0.2032	0.0930
Length of Petiole a	0.2476	0.0767	0.3050
Length of apex a	0.6637	0.0154	0.0773
HL/Length ratio of a	0.1253	-0.1727	-0.1946
Width of Leaf b	0.1253	-0.1727	-0.1946
Length/width ratio of b	0.1694	0.5913	0.0378
Length of petiole b	0.2471	-0.0035	-0.0812
Length of apex b	0.2667	0.4973	0.1439
HL/Length ratio of b	0.7774	0.0142	-0.0699
Length of Catkin unit	0.1872	0.1346	0.2495
Length of inflorescence axis	0.6314	-0.1328	-0.0564
Length of stalk	0.7048	0.0283	0.0707
Diameter of stalk	-0.0837	-0.4360	0.0216
Length of penduncle	-0.4412	0.3101	0.2119
Diameter a penduncle	0.6864	0.0389	0.2073
Diameter b penduncle	-0.7917	0.1305	0.0424
# of inflorescence	-0.6512	0.0691	0.0223
Basal Inflorescence width a	0.6144	0.0853	0.0441
Basal inflorescence width b	-0.9160	0.0472	0.0137
Basal inflorescence width a/b	-0.8388	-0.1398	0.4051
Basal inflorescence length/width	-0.4273	0.2836	-0.5561
Lower inflorescence width a	0.9129	0.1113	-0.1049
Lower inflorescence width b	-0.8891	0.1410	0.0098
Lower inflorescence width a/b	-0.8585	0.0014	0.4185
Lower inflorescence length / width	-0.4960	0.2577	0.6447
Mid inflorescence width a	0.9053	-0.1370	-0.1108
Mid inflorescence width b	-0.9092	0.1792	-0.0379
Mid inflorescence width a/b	-0.8471	0.0476	0.3595
Mid inflorescence length/width	-0.6171	0.1729	-0.6501
Upper inflorescence width a	0.8695	-0.1943	-0.1308
Upper inflorescence width b	-0.9183	0.1165	-0.0895
Upper inflorescence width a/b	-0.8575	-0.0181	0.3606
Upper inflorescence length/width	-0.4850	0.1946	-0.6536
Length of staminal column	0.8853	-0.1815	-0.1091
Width of stamina column	0.3415	0.4787	0.2994
Length of bracts	0.2342	0.5246	0.1242
Length of stalk/length of pedicel	-0.0724	0.0357	0.5432
Diameter of secondary stem	0.3385	0.6382	0.3182
Space of seed insertion	-0.2478	-0.3106	-0.2482
Lamina ovate to oblong or elliptic	0.2407	-0.8771	-0.2432
Leaf apex type	0.0546	0.1599	0.0837
Length of stalk/length of pedicel	0.5533	0.1537	-0.3312
# of inflorescence	0.4536	0.1836	-0.2911
Arrangement of spikes	0.5251	-0.3348	0.5874
Staminal column apex	0.9054	-0.1457	-0.1687
Diameter of internode	-0.2946	-0.5595	0.0861
Pedicel:penducle length ratio	-0.0527	0.0306	0.8318
Seed shape	-0.2946	-0.5595	0.0861



(i)



(ii)

Figure 4.4 Scattergram (i) and 3- dimensional diagram (ii) generated by Principal Coordinates Analysis based on the combined qualitative and quantitative data of the male specimens of *Gnetum* species. The OTUs of the various African specimens, except for *G. interruptum*, do not form distinct clusters in both 2- and 3-dimensional plots. Wild and cultivated specimens identified by lettering as in Figure 4.2.

Phenotypic plasticity: The relative differences between the wild specimens were retained in the cultivated material despite a change in overall size in some characters. Most characters overlapped between the naturally occurring and the cultivated specimens, as shown for instance in Figure 4.5b & c. In the leaf size characters, ratio of peduncle lengths before and after bracts, and the number of whorls (except in *G. latispicum*), the difference between the plants collected in nature and cultivated materials was sometimes larger than that between taxa (Figure 4.5a, b, e & f). Except for leaf size where length seems to shorten and the width broadens in the cultivated specimens, most plant structures became larger in cultivation and this suggests that these characters are phenotypically plastic. The difference between species was reduced in the cultivated forms as compared to the naturally occurring plants for most characters that overlapped like the length of catkin (Figure 4.5d), length of bracts, length and width of internodes of spikes. Nevertheless, the shape and size of the internodes of spike, the ratio of their upper to lower widths still distinguished the three species (Chapters 2 & 3).

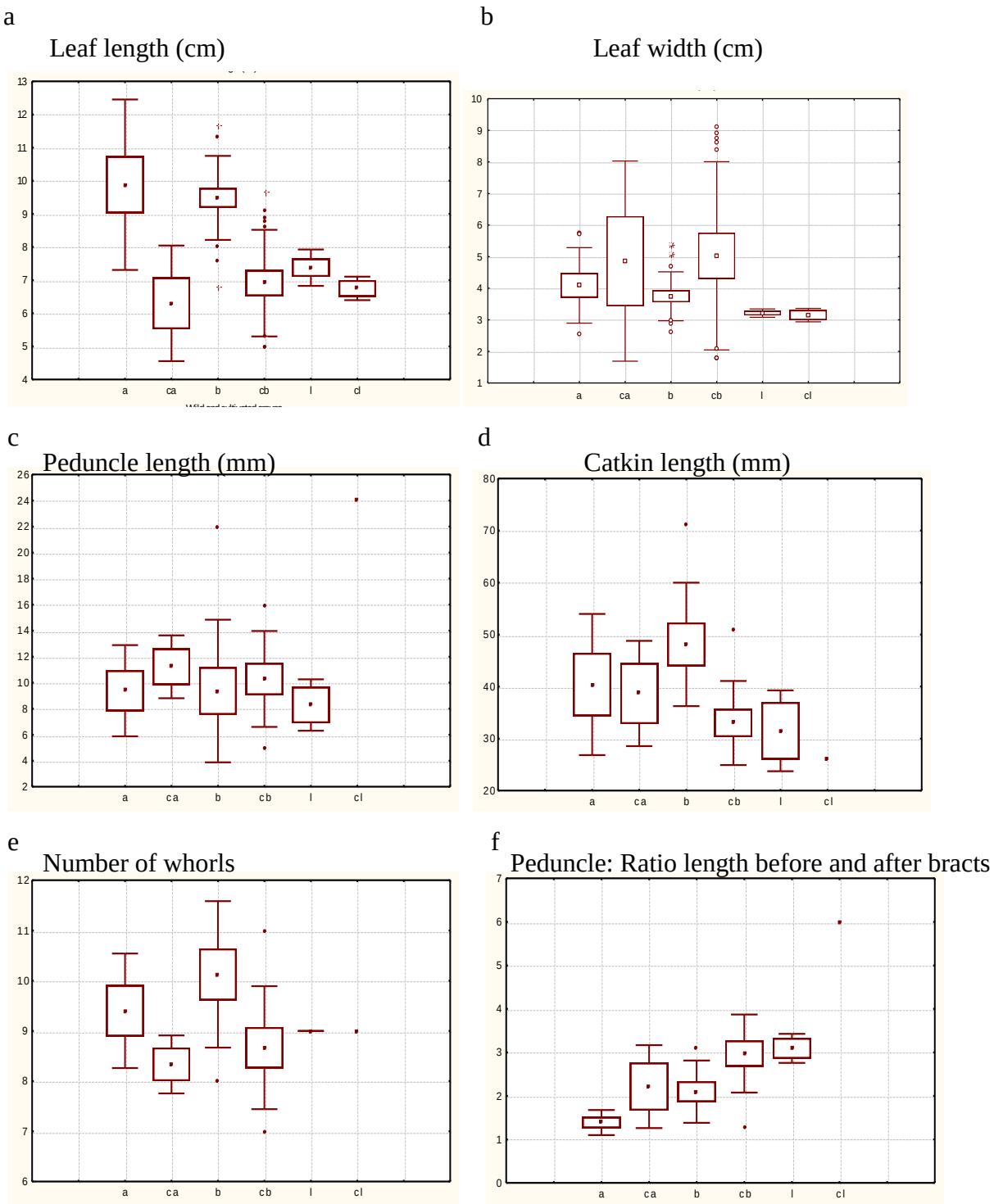


Figure 4.5 Comparison of variation in selected morphological characters of male plants of *G. africanum*, *G. buchholzianum*, and *G. latispicum*. Thirty specimens of both wild (a, b, l) and cultivated (ca, cb, cl) plants examined. Specimens identified by lettering as in Figure 4.2.

4.3.2 Phenetic analysis with female specimens of *Gnetum*

Cluster analysis

Sixty-two female specimens of *Gnetum* were clustered using UPGMA based on fifteen and fifty-seven characters for qualitative (Figure 4.6) and combined data (Figure 4.7) respectively. The analyses yielded phenograms with correlation coefficients r = 0.98 and 0.92 respectively indicating a good fit of the data to the tree for the qualitative and combined analyses. Distinct groupings of the various OTUs were evident according to the qualitative (Figure 4.6), and combined data matrices (Figure 4.7).

In the phenograms resulting from the qualitative analysis (Figure 4.6), all the cultivated specimens fitted the putative groups although in *G. latispicum*, the wild OTUs were nested in the cultivated. The cultivated specimens of *G. buchholzianum* followed the same pattern as the field collected specimens on the basis of the analysis in Chapter 2, i.e. they formed two distinct clusters that could be seen as two putative groupings. The various OTUs nevertheless, fitted in their respective presumed groupings.

In the combined qualitative and quantitative cluster analysis (Figure 4.7), the specimens of the cultivated plants, although they formed distinct groupings, clustered with their counterparts. This was true for the one cultivated specimen of *G. latispicum* (cl.C21), but the remaining OTUs of *G. latispicum* grouped with specimens of *G. buchholzianum*.

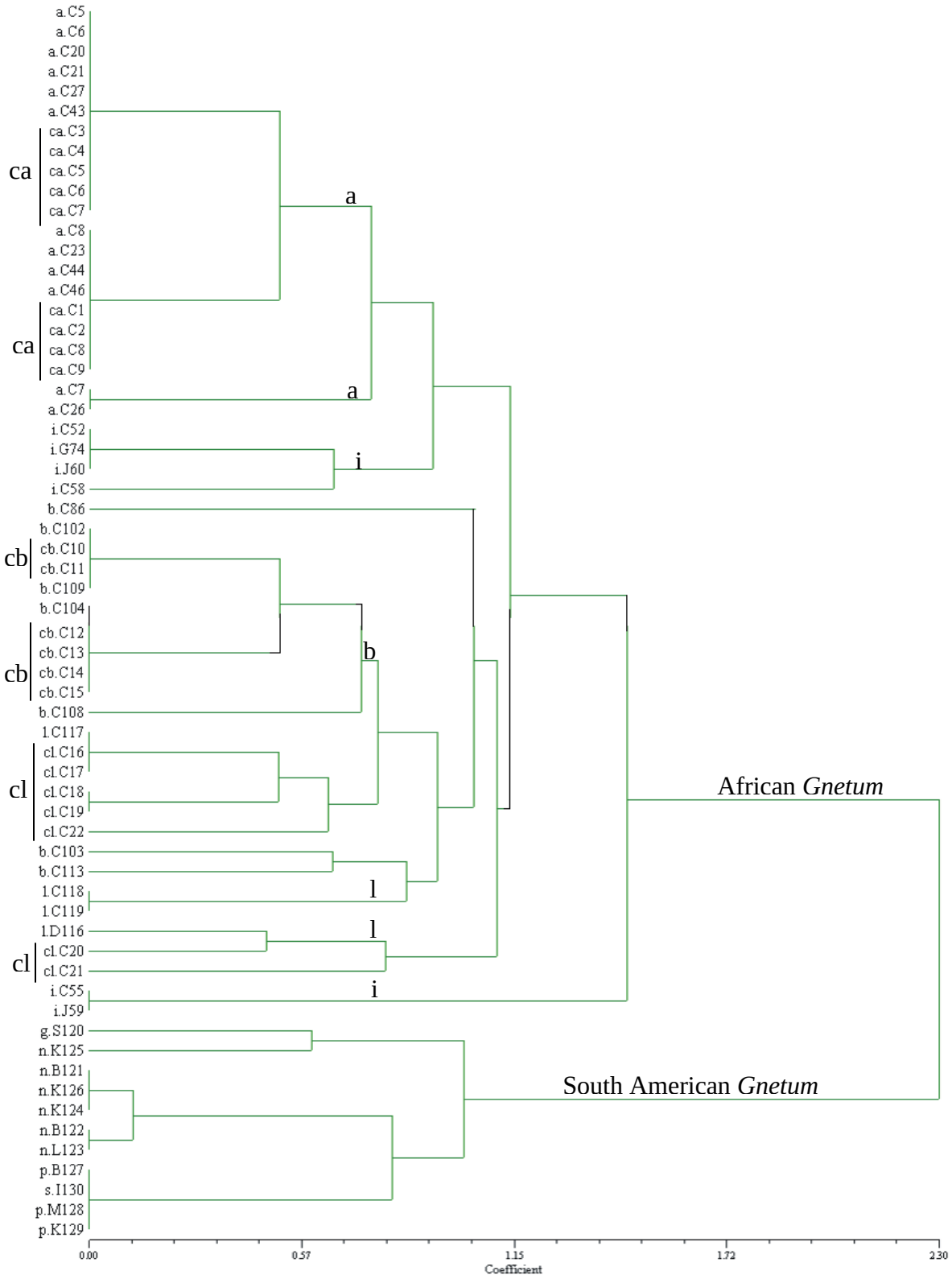


Figure 4.6 UPGMA phenograms using average taxonomic distance of the female specimens of *Gnetum* species in Africa based on qualitative data matrix. Abbreviations used to identify specimens as in Appendix 4.7.1.

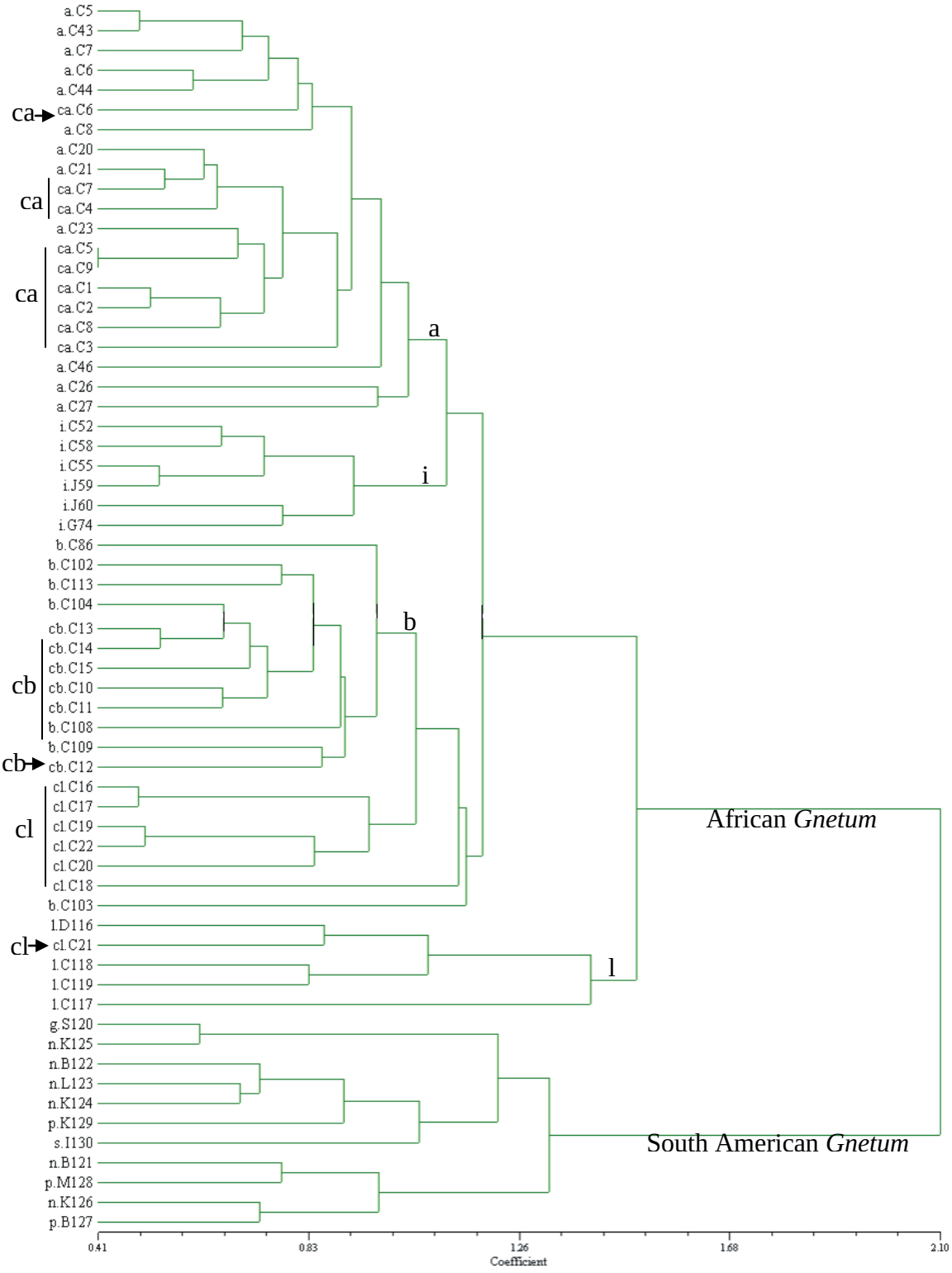


Figure 4.7 UPGMA phenograms using average taxonomic distance of the female specimens of *Gnetum* species in Africa based on combined quantitative and qualitative data matrix. Abbreviations used to identify specimens as in Appendix 4.7.1.

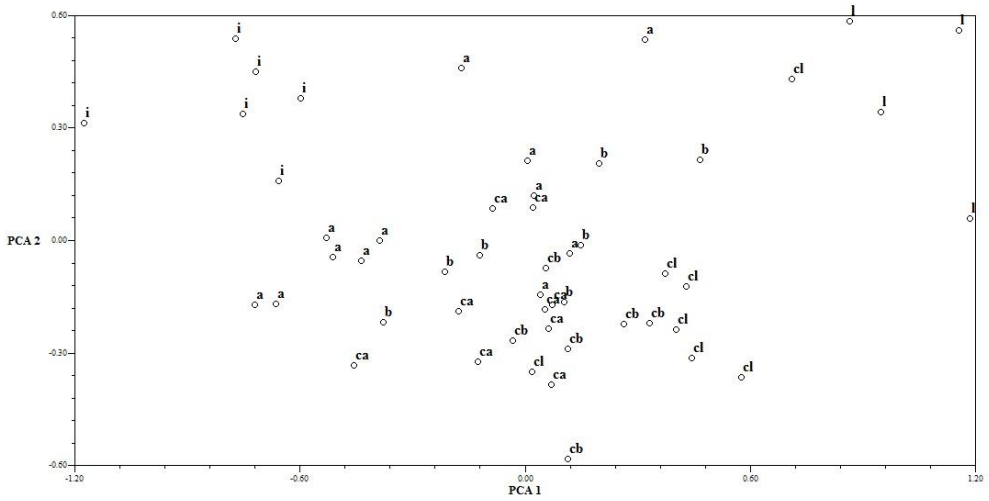
Ordination I

Principal Component Analysis (PCA)

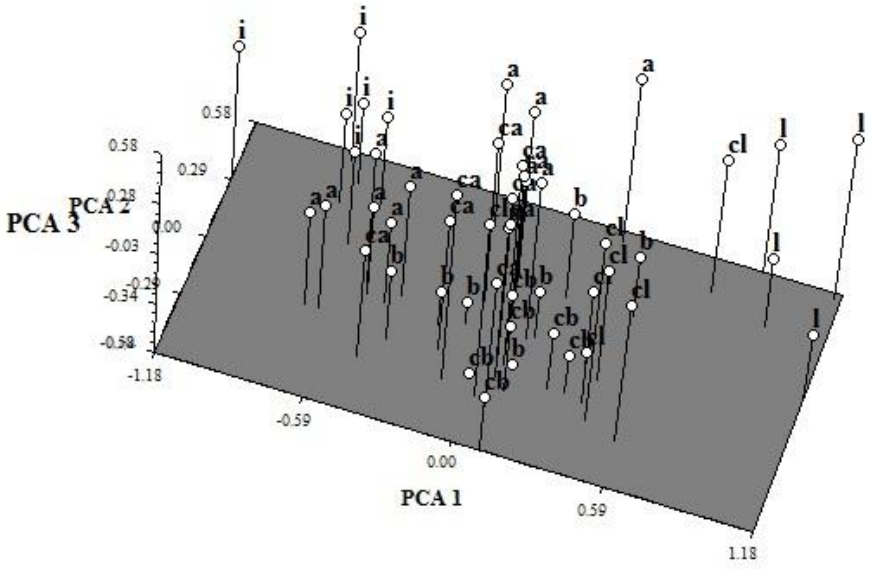
The 2D and 3D plots recovered from the PCA based on 42 quantitative characters resulted in the 51 female specimens across the plot(Figure 4.8). The first three axes accounted for 50.91% of the variation (Table 4.2) and the various groupings of OTUs remained separate from one another although *G. africanum* and *G. buchholzianum* were mixed on both plots. Most plants of *G. interruptum* and the wild-collected *G. latispicum* were distinctly grouped at the extreme left and right of the plot respectively. Plants of *G. africanum* and *G. buchholzianum* were placed at a more or less intermediate position along the second PCA axis although they somewhat overlapped. Generally the cultivated plants of each species grouped loosely with its wild counterpart, although these were more closely grouped together and not interspersed with the cultivated ones.

Table 4.3 Eigenvalues obtained from principal components analysis of the quantitative data matrix for the wild and cultivated male specimens of *Gnetum* species in Africa. Variation is concentrated in the first 13 axes (94.55%, with the first 3 axes accounting for 50.91%)

Axis	Eigenvalues	% Total variance contribution	Cumulative %
1	10.12050062	24.0964	24.0964
2	7.07511406	16.8455	40.9419
3	4.18511701	9.9646	50.9065
4	3.70575909	8.8232	59.7297
5	3.57981877	8.5234	68.2531
6	2.09159764	4.9800	73.2331
7	1.84912832	4.4027	77.6358
8	1.68954930	4.0227	81.6585
9	1.34552626	3.2036	84.8622
10	1.19306187	2.8406	87.7028
11	1.04849526	2.4964	90.1992
12	0.95405912	2.2716	92.4708
13	0.87483935	2.0830	94.5537
14	0.76877644	1.8304	96.3842
15	0.62961725	1.4991	97.8832
16	0.55306436	1.3168	99.2001
17	0.50454847	1.2013	> 100



(i)



(ii)

Figure 4.8 Scattergram (i) and 3D diagram (ii) generated by Principal Components Analysis based on quantitative data of female specimens of *Gnetum* species. The groupings of OTUs do not form distinct clusters in both 2-dimensional and 3-dimensional plots (not shown) but are separate from one another except for *G. africanum* and *G. buchholzianum* that are mixed. Wild and cultivated specimens identified by lettering as in Figure 4.2.

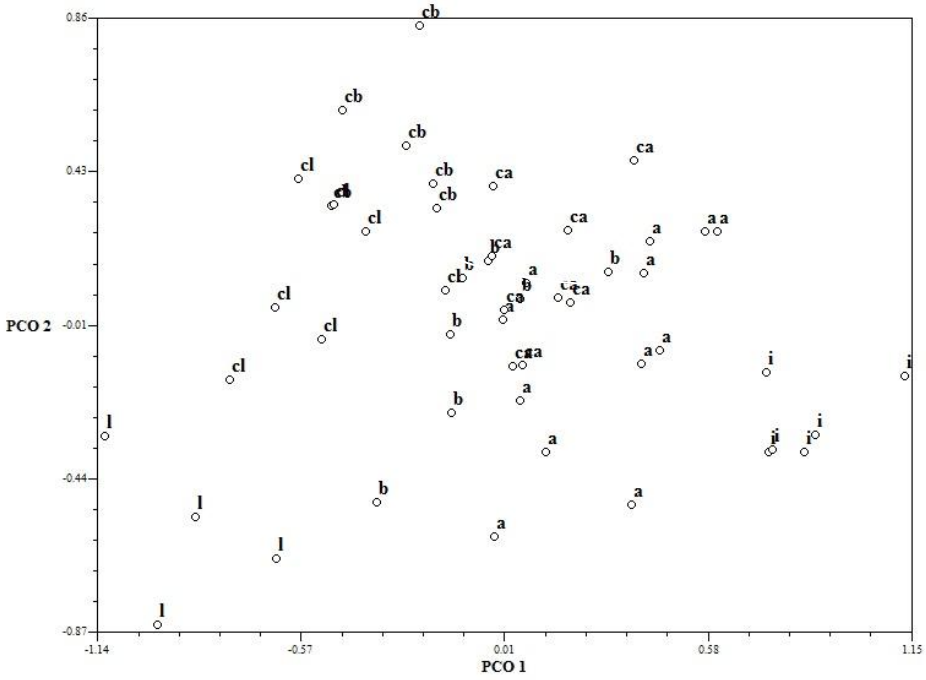
Principal Coordinates Analysis (PCO)

The scattergrams recovered from PCO analysis of 51 female specimens was based on the combined qualitative and quantitative data using 57 characters. The female specimens spread across the plot along both the first PCO and second axes (Figure 4.9). The cultivated plants were found close to the wild specimens for each putative group although they had a more or less intermediate position along the second PCO axis and extended towards the top of the plot. Both cultivated and wild plants of *G. africanum* and *G. buchholzianum* overlapped at the middle right side of the plot and those of *G. interruptum* and *G. latispicum* were placed respectively at the two extremes of the bulk of the African *Gnetum* (right and bottom left of the plot).

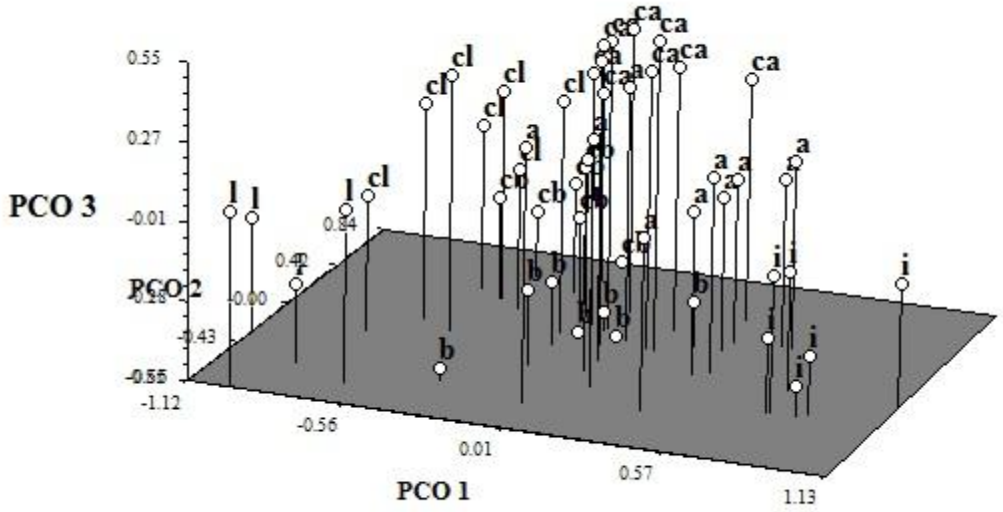
Table 4.4 Taxonomic values of quantitative and qualitative characters as loaded on the first three PCO axes

Characters generated	Characters	1st axis	3rd axis
Width of Leaf a	0.3467	0.2341	0.3885
Length/width ratio of a	-0.4655	-0.0078	**-0.8084**
Length of Petiole a	-0.0591	0.3375	0.1756
Length of Hl a	-0.3426	-0.0026	-0.3173
HL/Length ratio of a	-0.2325	-0.1368	0.0486
Width of Leaf b	-0.1077	0.3417	0.3663
Length/width ratio of b	-0.5989	0.2686	-0.1932
Length of Catkin unit	-0.1043	0.3258	0.2526
Length of stalk	-0.3707	0.2135	-0.1298
Diameter of stalk	-0.4053	0.2053	0.1965
Length of penduncle	0.1469	0.0153	0.3270
Diameter a penduncle	0.2885	0.0261	0.3649
Diameter b penduncle	0.1863	0.1888	0.4155
# of inflorescence	-0.4663	-0.0081	0.1224
Basal Inflorescence width a	0.4700	0.2937	-0.2062
Length of petiole b	0.2350	0.2239	0.0118
Length of apex b	0.0064	-0.3720	0.3240
Length HL b	**0.7243**	0.1999	0.0666
HL/Length ratio of a	0.5959	0.4725	0.1324
Lower inflorescence width b	0.2921	**-0.6969**	-0.3560
Lower inflorescence width a/b	**-0.7748**	-0.1761	-0.0425
Lower inflorescence length / width	**0.8044**	0.0815	-0.1142
Mid inflorescence width a	**0.7233**	0.3587	0.0492
Mid inflorescence width b	0.2881	**-0.7526**	-0.4365
Mid inflorescence width a/b	**-0.7449**	-0.0791	-0.0983
Mid inflorescence length/width	**0.7238**	0.3362	-0.0394
Upper inflorescence width a	0.3934	**-0.6545**	-0.1581

Upper inflorescence width b	**-0.6391**	0.0321	-0.2639
Upper inflorescence width a/b	**0.7327**	-0.0855	-0.0639
Upper inflorescence length/width	**0.6475**	0.1940	-0.0581
Length of staminal column	0.2683	**-0.7470**	-0.1385
Width of stamina column	-0.4799	0.1513	-0.3935
Length of bracts	-0.0674	-0.0496	0.2493
Basal inflorescence width b	**0.8261**	0.0215	-0.0526
Basal inflorescence width a/b	-0.1243	-0.0087	0.0820
Basal inflorescence length/width	0.2778	0.1453	-0.0523
Lower inflorescence width a	-0.1592	-0.3018	0.4537
Diameter of secondary stem	-0.4238	-0.1315	0.5738
Width of stalk	-0.4540	-0.2263	0.5668
Length/width ratio	-0.4617	-0.3690	0.4727
Length of stalk	0.2157	0.2369	**-0.7366**
Length of stalk/length of pedicel	**-0.6397**	0.3733	-0.3269
# of seeds	0.2115	-0.3063	0.2244
Leaf apex type	-0.4473	-0.2927	0.2421
# of inflorescence	-0.4465	-0.3221	-0.0704
Arrangement of spikes	-0.3856	-0.0846	-0.5236
Lamina ovate to oblong or elliptic	**-0.6103**	0.4627	-0.0475
Staminal column apex	**-0.7182**	0.5151	-0.1645
Space of seed insertion	-0.4725	-0.2281	0.0870
Pedicel:penducle length ratio	0.5616	0.0944	0.0630
Diameter of internode	0.5749	0.2305	-0.3275
Seed shape	**0.6607**	-0.5409	-0.1364



(i)



(ii)

Figure 4.9 Scattergram (i) and 3-D diagram (ii) generated by Principal Coordinates Analysis (PCO) based on the combined qualitative and quantitative data of the female specimens of *Gnetum* species. *G. africanum* (a) and *G. buchholzianum* (b) form a distinct cloud of OTUs, *Gnetum latispicum* (l) are separate in both 2-dimensional (a) and 3-dimensional (b) plots.

Phenotypic plasticity: The cultivated females possessed features that were almost equivalent in size to the wild samples for each species and the relative differences between the wild collected specimens and the cultivated material was much reduced compared to the males. Most of the characters overlapped between the naturally occurring and the cultivated specimens, as seen for instance in Figure 4.10a-e, but for the wild collected *G. latispicum* with outstanding features. In the ratio of lengths of peduncle below and above bracts, the difference between the plants collected in nature and cultivated materials was larger than that between taxa (Figure 4.10f) suggesting that this character is influenced by the environment and shows great phenotypic plasticity.

The difference between features to recognize species was minimized in cultivation compared to the naturally occurring plants, for most characters overlapped, such as the leaf width, peduncle length, the length of catkin, number of whorls, length and width of internodes of spikes and the length of bracts (Figure 4.5b-e). However, the shape and size of the internodes of spike, the ratio of their upper to lower widths are still good distinguishing characters for the three taxa involved in cultivation (Chapters 2 & 3).

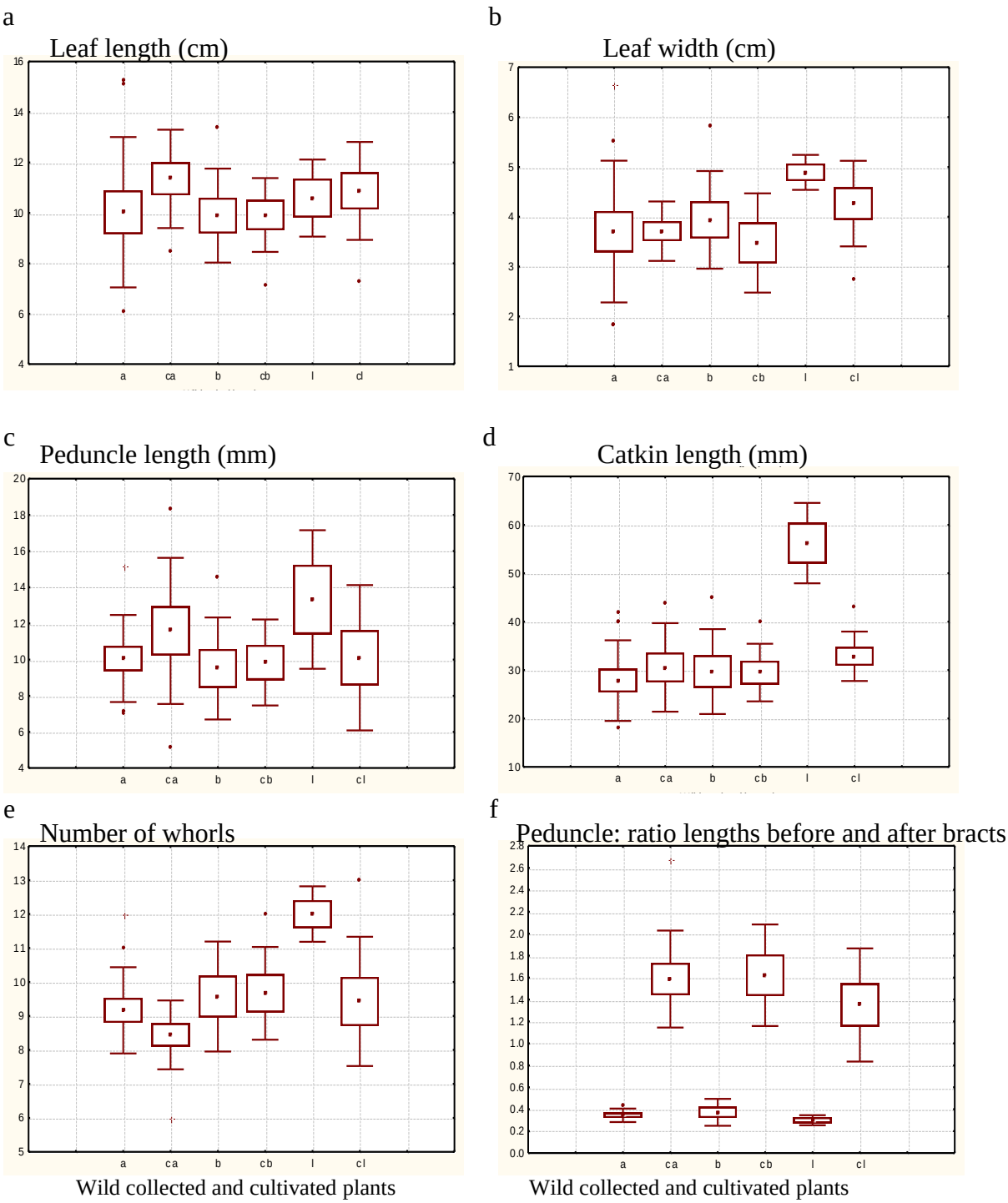


Figure 4.10 Comparison of variation in selected morphological characters of female plants of *G. africanum*, *G. buchholzianum*, and *G. latispicum*. Measurements were made on 45 specimens of both wild (a, b, l) and cultivated (cultivated: ca, cb, cl) plants. Specimens identified by lettering as in Figure 4.2.

4.4 Discussion

4.4.1 Taxonomic implication

Three species of *Gnetum* (*G. africanum*, *G. buchholzianum* and *G. latispicum*) have been found in cultivation in each of the three Institutions of Agriculture in Cameroon (Limbe Botanical Garden, South West Region; IRAD Barombi Kang, Kumba, South West Region; IRAD Nkolbisson, Yaounde, Central Region). The cultivated specimens used in the comparison were grown from cuttings of *Gnetum* plants from the wild and their selection was purposely intentional for a food source. Cultivated plant taxonomy is involved with the identification, description, naming of groupings of cultigens (Li, 1974; Jeffrey, 1968), although the nomenclature of cultivated plants is often complicated (Hetterscheid & Brandenburg, 1995). The identification of cultivated plants also presents challenges due to similarities among species based on their unclearly defined morphological character differences (Spooner *et al*., 2003). The three species of *Gnetum* in cultivation were identified according to the characters used to describe the natural wild plants.

Cluster and ordination analyses of morphological variation between wild and cultivated material of both male and female plants of *Gnetum* species in Africa has established that the cultivated forms are identical to three wild species recognized thus far (Chapters 2 & 3). Most cultivated forms were placed at the intermediate position and at a very short distance from their respective wild collected specimens in the 2D or 3D plots; this is an indication of their close similarity (Jeffrey, 1968). Points projected in ordination analysis indicate maximum variance (Rohlf, 2000) and characters that load heavily in this analysis are true indicators of taxonomic correlation.

The similarities observed between the wild and cultivated plants was due to some overlap in characters, especially among the female plants as organs such as cuttings successfully perpetuate desirable characters, especially in woody plants (Stearn, 1965; 1986; Giannattasio & Spooner, 1994). Plants of *Gnetum* propagated by cuttings have retained the distinguishing features observed in the naturally occurring specimens examined in the previous sections, although most characters were overlapping.

Characters often differ between wild and cultivated plants and as a result there is no major similarity observed between the plants. They might even appear as separate species (Ulukan,

2009). Some cultivated OTUs of *Gnetum* separated from their field counterparts forming distinct clusters. This might be due to the relatively shorter and broader leaves observed in the males and the characters pertaining to the peduncle in the females of cultivated plants when compared to the plants occurring in nature. These characters are influenced by environmental conditions (Ellison *et al*., 2004) causing differences in morphology, but the tendency is sometimes to recognize individuals as an additional putative group or cultigens or better still as cultivated varieties within the species. However, there is need for careful consideration for such groupings with ill-defined boundaries as species (Styles, 1986).

There are usually various variation patterns resulting from artificial selection within cultigens. These have often led to difficult taxonomic decisions and complicated definitions of species because of phenotypic plasticity in different environments (Spooner & Hijmans, 2001). Character variations occur in different directions and combinations, and such variations can thus be described when they are distinguishable but need not contribute to create any recognized grouping (Hawkes, 1986).

Cultivation is often coupled with a number of changes in phenotypic expression of characters (Schlichting & Levin, 1986). *Gnetum,* although still in cultivation trials, seems to exhibit some phenotypic plasticity among the wild and cultivated plants. The plasticity of cultivars has been noted to change very little during domestication but there could be a substantial phenotypic divergence from the wild (Schlichting & Levin, 1988; Ulukan, 2009). Despite the intermediate phenotypes that cause some specimens of various species of *Gnetum* to overlap, the three taxa are nonetheless morphologically well distinguished. The qualitative characters more clearly differentiated the three groupings (*G. africanum, G. buchholzianum* and *G. latispicum*) found in natural and cultivated conditions than the quantitative features, which were more plastic (most notably leaf length and width, as well as some of the reproductive characters). This implies that these important qualitative diagnostic characters were retained in the cultivated forms of the three species and that the distinguishing morphological differences have a genetic basis.

Based on the geographic origin of the naturally occurring taxa, it has been shown here (Chapter 2) that there is a continuous type of diversity which generally makes infraspecific identification or classification difficult (Hawkes, 1986; Styles, 1986; Sebola & Balkwill, 2009). Morphological variation due to geographic origin of the wild specimens was difficult to determine because not only was the source of the cultivated materials grown in Kumba

unknown but also the remaining OTUs, irrespective of their groupings, did not cluster based on the geographic origin to produce a pattern to recognize any variation.

4.5 Conclusion

The study has revealed that the cultivated plants of *Gnetum* have been selected from *G. africanum*, *G. buchholzianum* and *G. latispicum*, three species of wild collected *Gnetum* in Africa, mainly used in Cameroon. The cultivated materials were similar to and retained the qualitative distinguishing characters observed in the naturally occurring plants. It is known that variation is sometimes continuous at species level, thus most cultivated specimens fitted the respective putative groupings but some were mixed to an extent due to overlapping (mainly quantitative) characters.

In this analysis, the descriptive element was essentially the pattern of recognition and therefore ordination techniques applied indicated the closeness between cultivated and wild specimens. For future analysis of cultivated versus wild plants of *Gnetum*, a larger sampling strategy is needed; this was not possible because the cultivation of *Gnetum* is still not widespread. The human intention in cultivating these species of *Gnetum* is to increase their leaf biomass but so far it seems the plants are selected their success in cultivation rather than to improve their productivity as a source of food. Notwithstanding, molecular tools are deemed appropriate now to appraise and confirm the morphological correlation among and between wild individuals and their cultivated counterparts. Thus, the taxonomy of the genus *Gnetum* in Africa could be further explored based on their genetic patterns.

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APPENDIX

List of voucher specimens examined in the phenetic study arranged in order of identification number for male and then female (wild & cultivated) specimens.

The first letter of the code represents the species (a = *Gnetum africanum*; b = *G. buchholzianum*; i = *G. interruptum*; l = *G. latispicum*; n = *G. nodiflorum*; p = *G. paniculatum*; g = *G. gnemon*; s = *Gnetum* sp. from South America and the letter c indicates the cultivated materials); next letter = country of collection (A = Angola; B = Amazonia; C =Cameroon; E = Congo; F = Colombia; G =Democratic Republic of Congo; I = Ecuador; K = Guyana; L = Peru; M= Venezuela); followed by identification number.

Code	Collector, No. of specimens & herbarium	Collection Locality
Males		
a.A1	Welwitsch 1224 (PRE)	Golungo, Angola
a.G2	Wellens 449a (BR)	Mayombe, RDC
a.C3	Zenker s.n (BR)	Bipindi, South, Cameroon
a.A4	Gossweiler 5580 (LISC)	Faremla, Angola
a.E5	Devred 1009 (BR)	Mbwami, Congo
b.C7	Setten 346 (WAG)	Cameroon
b.C8	Bos 5242 (WAG, YA)	Cameroon
b.C9	Satabie 576 (P, YA)	Ndikinimeki, Centre, Cameroon
b.C17	Zenker 12 (BER)	Bipindi, South, Cameroon
b.C18	Thomas *et a*l. 5227 (P, YA)	Kumba, South West, Cameroon
b.C19	Letouzey 9754 (P, WAG, YA)	Ntui, Centre, Cameroon
b.C23	Biholong 258 (YA)	Yaounde, Centre, Cameroon
b.C24	Staudt 543 (BER)	Cameroon
b.C26	Zenker 522 (BER)	Cameroon
i.C34	Zenker 41 (P)	Bipindi, South, Cameroon
i.C35	Rudatis 42 (K)	Mamfe, South West, Cameroon

i.C36	Dinhlage 882 (BER)	Lokundje, South, Cameroon
i.C37	Manning 2002 (K, YA)	Libamba, Centre, Cameroon
i.C38	De Wilde 3835 (BR, K, WAG)	Eseka, Centre, Cameroon
i.C39	Bos 5465 (BR, WAG, YA)	Kribi, South, Cameroon
i.C54	Bos 5501 (LISC, PRE, WAG, YA)	Kribi, South, Cameroon
l.C55	Biye 603 (J)	Kumba, South West, Cameroon
l.C56	Biye 617 (J, YA)	Kribi, South, Cameroon
n.Col056	Rudas *et al*. 2536 (MO)	Leticia, Colombia
n.G057	Clarke 6954 (MO)	Takutu-U, Guyana
p.V058	Stergios et al. 9498 (MO)	Rio Siapa, Venezuela
p.G059	Hoffman et al. 2801 (MO)	Pomeroon-Supenaam, Guyana
p.Vs060	Holst et al. 2972 (MO)	Edo. Bolivar, Venezuela
Cultivated		
ca.C64	Biye 605 (J, YA)	Barombi Kang, S.W. Cameroon
ca.C65	Biye 609 (J, YA)	Barombi Kang, S.W. Cameroon
ca.C66	Biye 610 (J, YA)	Barombi Kang, S.W. Cameroon
cb.C67	Biye 604 (J, YA)	Barombi Kang, S.W. Cameroon
cb.C68	Biye 606 (J, YA)	Barombi Kang, S.W. Cameroon
cb.C69	Biye 607 (J, YA)	Barombi Kang, S.W. Cameroon
cb.C70	Biye 608 (J, YA)	Barombi Kang, S.W. Cameroon
cb.C71	Biye 611 (J, YA)	Barombi Kang, S.W. Cameroon
cb.C72	Biye 612 (J, YA)	Barombi Kang, S.W. Cameroon
cb.C73	Biye 614 (J, YA)	Barombi Kang, S.W. Cameroon
cb.C74	Biye 615 (J, YA)	Limbe BG, S.W. Cameroon
cb.C75	Biye 616 (J, YA)	Barombi Kang, S.W. Cameroon
cl.C79	Biye 618 (J, YA)	Barombi Kang, S.W. Cameroon
Females		
a.C5	Harris & Fay 1516 (PRE)	Sanaga, Centre, Cameroon
a.C6	Zenker 1061 (BER)	Cameroon
a.C7	Raynal 10247 (P, YA)	Ma'an, South, Cameroon
a.C8	Letouzey 11061 (P, YA)	Massangui, Centre, Cameroon
a.C20	Westphal 10075 (BR, WAG, YA)	Douala, Littoral, Cameroon
a.C21	Zenker 4380 (BR, P)	Bipindi, South, Cameroon
a.C23	Westphal 9832 (BR, K, WAG)	Yaounde, Centre, Cameroon

a.C26	Nkongmeneck 697 (YA)	Ndom, Cameroon
a.C27	Manning 1843 (K, YA)	Yaounde, Centre, Cameroon
a.C43	Bos 4144 (WAG)	Longii, South, Cameroon
a.C44	Wilde 3819 (WAG)	Eseka, Centre, Cameroon
a.C46	Surville 482 (YA)	Cameroon
b.C86	Zenker 2279 (WAG)	Bipindi, South, Cameroon
b.C102	Bos 5700 (WAG)	Kribi, South, Cameroon
b.C103	Bos 6434 (BR, WAG)	Kribi, South, Cameroon
b.C104	Bos 2993 (BR, WAG YA)	Kribi, South, Cameroon
b.C108	Leeuwenberg 9893 (BER, LICS, K, PRE, WAG, YA)	Nkam, Littoral, Cameroon
b.C109	Atangana 10 (YA)	Nkol Awana, Centre, Cameroon
b.C113	Biye 302 (J, YA)	Nkolo II, Centre, Cameroon
l.D116	Tisserant 1984 (BR, P)	Boukoko, Central African Rep.
l.C117	Bos 4267 (WAG)	Kribi, South, Cameroon
l.C 118	Letouzey 5249 (K, P, YA)	Mopwo, Cameroon
l.C119	Biye & SVO 301 (J, YA)	Sa'a – Lekie, Centre, Cameroon
i.C52	Zenker 4134 (BER, BR)	Bipindi, South, Cameroon
i.C55	Bos 3721 (WAG, YA)	Kribi, South, Cameroon
i.C58	Bos 4068 (K, WAG)	Kribi, South, Cameroon
i.J59	Wieringa 6460 (WAG)	Haut Ogouoe, Gabon
i.J60	Jongkind 5936 (WAG)	Cap Esterias, Gabon
i.G74	Lisowski 17686 (K)	Uele, RDC
g.D120	Botanical Garden (J)	Durban, South Africa
n.A121	Nascimento 396 (MO)	Rio Acara, Amazonia
n.A122	Encarnacion 1064 (MO)	Punto Negrococha, Amazonia
n.P123	Vasquez 19321 (MO)	Madre de Dios, Peru
n.G124	Hoffman 909 (MO)	Essequibo, Guyana
n.G125	Mutchnick 828 (MO)	Potaro-Sipatuni, Guyana
n.G126	Henkel 153 (MO)	Cuyuni-Mazaruni, Guyana
p.A127	Castill 4454 (MO)	Rio Orinoco, Amazonia
p.V128	Castillo 5740 (MO)	Rio Sipapo, Venezuela
p G129	Clarke 9203 (MO)	Potaro-Siparuni, Guyana
s.E130	Neil & Iguago 9046 (MO)	Canton Archidona, Ecuador
Cultivated		
ca.C1	Biye 414a (J)	Yokadouma, East Cameroon

ca.C2	Biye 414b (J)	Bertoua, East Cameroon
ca.C3	Biye 416 (J)	Limbe, South West, Cameroon
ca.C4	Biye 416a (J, YA)	South West, Cameroon
ca.C5	Biye 358 (J)	Yaounde, Centre, Cameroon
ca.C6	Biye 354 (J, YA)	Banga, South West, Cameroon
ca. C7	Biye 351 (J, YA)	Kumba, South West, Cameroon
ca.C8	Biye 422 (J, YA)	Yaounde, Centre, Cameroon
ca.C9	Biye 619 (J, YA)	Kumba, South West, Cameroon
cb.C10	Biye 620 (J, YA)	Kumba, South West, Cameroon
cb.C11	Biye 621 (J, YA)	Kumba, South West, Cameroon
cb.C12	Biye 355 (J, YA)	Kumba, South West, Cameroon
cb.C13	Biye 420 (J, YA)	Kribi, South, Cameroon
cb.C14	Biye 424 (J, YA)	South West, Cameroon
cb.C15	Biye 425 (J, YA)	South West, Cameroon
cl.C16	Biye 413a (J, YA)	Obala, Centre, Cameroon
cl.C17	Biye 413b (J, YA)	Sa'a, Centre, Cameroon
cl.C18	Biye 415 (J, YA)	Bertoua, East, Cameroon
cl.C19	Biye 426 (J, YA)	Soussa, Litoral, Cameroon
cl.C20	Biye 215 (J, YA)	Nkolmebenga, Centre, Cameroon
cl.C21	Biye 392 (J, YA)	Yokadouma, East Cameroon
cl.C22	Biye 423 (J, YA)	Yaounde, Centre, Cameroon

CHAPTER FIVE

THE ASSESSMENT OF GENETIC VARIATION IN *GNETUM* L. SPECIES IN AFRICA USING AFLP ANALYSIS

CONTENT

5.1 Introduction

- Background knowledge:

  Significance of molecular analysis to taxonomy

  AFLP compared to other molecular methods

- Problem statement:

  Scanty knowledge regarding genetic diversity and relationships among species of *Gnetum*

- Knowledge gap:

  Gnetum and molecular analysis

  Contribution of AFLP to the taxonomy of *Gnetum*

- Purpose of this research

  Aim and key questions

5.2 Materials and Methods

Plant material

DNA extraction

AFLP

5.3 Results

Band observation

Phenetic analysis

5.4 Discussion

5.5 Conclusion

REFERENCES

APPENDIX

Abstract

The variable vegetative morphology in the genus *Gnetum* had complicated species recognition but was helpful in revealing the correlation between the wild and their garden-grown counterparts. One of the factors influencing morphological variation in plants is their genetic composition. To improve the taxonomy of *Gnetum,* therefore, both morphological and molecular data are important to contribute to appropriate guidelines for conservation purposes. The AFLP technique is used here to appraise the genetic relationships among 45 accessions and the genetic similarities among wild and garden-grown individuals representing the four African species of *Gnetum*. The primer pair *Eco*RI/*Mse*I 2 was successful in detecting a high level of genetic polymorphism among the species. The polymorphism rates ranged from 40.14% to 85.71% with a mean of 64.63%. Species of *Gnetum* are genetically variable as revealed by the variously sized bands recovered. The number of different bands observed in *G. interruptum* was 59; 107 for *G. africanum*, 126 for *G. buchholzianum* and 88 bands in *G. latispicum*. Two major clusters were evident in the UPGMA dendrogram generated based on the Jaccard similarity coefficient. Cluster I comprised subgroupings of specimens of all four species of *Gnetum* recognized morphologically, while Cluster II included three of the four species. Several subgroups of species were thus evident in the dendrogram rather than distinct single clusters per species and this could be explained by *Gnetum* species often growing in isolated patches in the forest resulting in limited gene flow. The species of *Gnetum* show a relatively similar pattern in terms of genetic variation due to the high number of monomorphic fragments observed, although *G. interruptum* is more or less distinct; therefore they present equal importance for prioritization purposes. This first study of *Gnetum* species in Africa using AFLP analysis has provided some insight into the genetic similarities among these species, although there is need to improve the molecular analysis using other approaches such as DNA sequencing.

Key words: DNA Markers, Genetic Similarity, Gymnosperms, Taxonomy

5.1 Introduction

5.1.1 The limitations of vegetative morphology in African *Gnetum* species

Gnetum L. (Gnetaceae) is a gymnosperm that occurs in the tropical regions of the world, and when not flowering, could be mistaken for dicotyledonous plants (Chamberlain, 1966; Sporne, 1974). The genus comprises approximately 40 extremely variable species, mostly lianas and a few shrubs and trees; four have been reported to occur in Africa based on morphological features and these are all climbers (Hutchinson & Dalziel, 1954; Maheshwari & Vasil, 1964; Earle, 2001; Biye *et al.*, 2013).

The vegetative features of *Gnetum* are highly inconsistent and show within group variation, thus it is difficult to distinguish between the various species based on vegetative characters (Hutchinson & Dalziel, 1954; Chapter 2). Although the macro-morphological examination of *Gnetum* is not very helpful in species delimitation since most of the characters overlap, it has, however, contributed by showing the correlation between the wild collected and the cultivated specimens of this genus (Chapter 4). Diagnostic features distinguishing species of this genus are concerned with the spike which comprises an unbranched axis with internodes of various shapes and sizes correlating with different species (Chapter 3).

Morphological variation observed in plants is based on several interrelated factors. The environmental conditions in which the plant is found and its genetic composition, for example, can influence the growth and development of plant structures such as its vegetative features. The genetic constitution of a plant is constant and determines both its nature and its response to environmental changes (Bonner *et al*., 1959). Measurements of gross morphological structures (length, number and form of various plant parts) have been frequently used to assess genetic composition (Given, 1994; Chapters 2, 3 & 4), but examination of the DNA will provide direct genetic evaluation of population structure (Moran & Hopper, 1987). The use of molecular tools to detect the morphological variation observed in all forms of *Gnetum* (the wild and cultivated) is of interest here. These African species of *Gnetum* play a significant role in the regional nutrition, food security and economy, especially where they grow naturally (Besong *et al*., 2001; Sunderland & Clark, 2001; Clark *et al*., 2004), yet their taxonomy has needed clarification.

To improve the taxonomy of *Gnetum*, developing approaches that can accurately characterize genetic variation in its gene pool are required. It has been mentioned that wild and cultivated plants of *Gnetum* show a high level of morphological variation especially in their vegetative features (Chapters 3 & 4). Therefore molecular techniques that can detect variation within closely related genotypes are deemed necessary to provide additional information in order to assess taxonomic conclusions that were based on reproductive morphology and also to provide insight into the genetic relatedness among individuals (Herselman, 2003; Visser *et al*., 2008).

5.1.2 Significance of molecular analysis to systematics

The general belief that molecular information is more reliable and stable than morphological data because it is less influenced by environmental changes is one of the principal reasons behind most molecular analyses (Avise, 1994; Hillis *et al*., 1996). A number of molecular approaches to taxonomic study, such as DNA and RNA sequencing and molecular fragment analysis using restriction enzymes have been developed (Williams *et al*., 1990; Minelli, 1993; Ramirez & Haakonsen, 1999; Emadzade *et al*., 2010). Genetic markers are useful in molecular systematics because they often assist with understanding population processes and patterns of relationship among individuals (Patton & Smith, 1994; Hillis *et al*., 1996; Pilgrim *et al*., 2004).

Molecular markers are capable of identifying polymorphic variations (Vos *et al*., 1995; Palmer, 1992) and DNA marker technologies have become increasingly significant as resourceful tools in plant breeding systems. Variability has been found among wild diploid species of peanuts using DNA markers (Garcia *et al*., 1994; Stalker *et al.*, 1994; Herselman, 2003) for example. In a preliminary survey of the genetic relationship between *Erianthus rokii* and a wild relative of sugar cane '*Saccharum* complex', Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and microsatellites or Simple Sequence Repeats (SSR) analyses provided sufficient information to differentiate various genetic profiles of different forms of the same species (Cai *et al*., 2005). Various molecular techniques used to assess relatedness between organisms have been applied in numerous groups of plants (e.g. *Helicobacter* Godwin*; Withania* Pauquy; *Cineraria* L.; *Welwitschia* Reichb.; *Tolpis* Adans.) and have successfully determined limits within the various groups studied (Gibson *et al*., 1998; Negi *et al*., 2000; Cakir *et al*., 2002;

van de Wouw *et al*., 2003; Jacobson & Lester, 2003; Cron *et al*., 2008; Mort *et al*., 2010; Linz *et al*., 2010).

5.1.3 Efficacy of the AFLP technique

DNA fingerprinting techniques seem to be the most popularly used method for phylogenetic analysis (Avise, 1994), despite the regular use of sequencing nowadays. Although sequencing is mostly applicable at and above the genus level, it is often used at species level, but does not always show enough variation at and below species level and among very closely related genera (Koopman, 2005). Thus, restriction fragments, with sufficient phylogenetic signals, are considered suitable phylogenetic markers at the species level and among closely related organisms. For example, RFLP and SSR markers were used in high resolution mapping of loci conferring resistance to sugarcane mosaic virus occurring in maize (Xu et al., 1999).

Analysis of AFLPs is an established molecular marker technique that has often been used to study within and between species variation and genetic relationships (Mueller & Wolfenbarger, 1999; Cai *et al*., 2005), and has shown enough sensitivity for separating closely related cultivars (He & Prakash, 1997; Xu *et al*., 1999; Barbarosa *et al*., 2003; Chao *et al*., 2005). Unlike most other molecular techniques, the AFLP approach uses only two primers, produces repeatable results and no prior knowledge of the genomic sequence is required (Vos *et al*., 1995; Blears *et al*., 1998; Herselman, 2003; Koopman, 2005).

AFLPs have been used to investigate inherited agronomic traits in plant and animal breeding, pedigree and parentage analyses and as DNA markers to reveal patterns of gene flow (Blears *et al*., 1998). Moreover, they are able to scan through the whole genome to identify variation in forms, for it is a multilocus marker technique (Pejic *et al*., 1998). AFLPs are reported to identify a higher number of variations in a single assay compared to either RFLPs (Restriction Fragment Length Polymorphism), RAPDs or SSRs (Powell *et al*., 1996). Their ability to simultaneously investigate numerous fragments and not specific regions of each locus is referred to as their "assay efficiency index" (Pejic *et al*., 1998). AFLPs are inherited as Mendelian markers (Saghai-Maroof, 1984; Ajmone-Marsan *et al*., 1998) and are widely developed polymorphic markers that provide a potential source of phylogenetic information for systematic investigations (Mohan *et al*., 1997).

5.1.4 Problem statement

Previous delimitations of the African species of *Gnetum* have been based mostly on variations in morphology (Pearson, 1912; Hutchinson & Dalziel, 1954; Earle, 2001; Won & Renner, 2004; Biye *et al*. in press) and the recognition was largely based on reproductive features observed on herbarium specimens, as the vegetative characters overlap and cannot clearly distinguish between species (Biye *et al*. 2013; Chapters 3 & 4). This vegetative variability therefore, has in the past, led to difficulties in species' identification.

In order to appraise species diversity, accurate classification is important. Thus the identification of taxonomic units or rare species with a distinct genetic constitution from their more abundant relatives is essential especially when defining conservation strategies (Koopman *et al*., 2001). Variation observed in morphological characteristics needs to be interpreted genetically as these features are very plastic and cannot often be relied upon and may be related to molecular variation. Hence, the use of molecular markers in many plant studies is observed today. Both morphological and molecular data contribute towards determining appropriate management strategies for conservation. DNA finger-printing carried out in this study might facilitate the detection of variation within and between species in order to improve our understanding of the genus *Gnetum* in Africa.

5.1.5 Knowledge gap

Apart from phylogenetic investigations carried out by Won and Renner (2005a, b) mostly on non-African members of *Gnetum*, molecular work has focused on resolving ancestry relationships in the lineage rather than species delimitation. Even then, current information about these relationships is contradictory. Morphological characterization and molecular phylogenetics indicate that the three genera comprising the Gnetales are monophyletic (Price, 1996), but their phylogenetic position within the seed plants has been higly debated. Thus far, molecular studies on the Gnetales suggest that their angiosperm-like features are due to convergent evolution and not homology as a result of common ancestry with angiosperms (Doyle, 1998; Hansen *et al*., 1999; Won & Renner, 2003). Molecular studies suggest that their closest relatives are the conifers (possibly the Pinaceae) and they are therefore separate from angiosperms (Bowe *et al*., 2000). In addition, there has been horizontal gene transfer of the mitochondrial *nad1* intron 2 and adjacent exons b and c from angiosperms to *Gnetum* (Won & Renner, 2003). Conflict between phylogenies resulting from partitioned data sets

involving first and second codon positions (angiosperms and gymnosperms as sister clades and Gnetales as sister to Pinaceae) as opposed to third positions (Gnetales sister to all other seed plants) are seemingly due to heterogeneous evolutionary rates in these codon positions (Magallon & Sanderson, 2002). Thus, genetic markers are not always conclusive about the ancestral relationships of taxa, but provide complementary or sometimes contradictory evidence to morphological data.

Genetic markers have therefore previously been used for revealing evolutionary relationships at higher levels in the Gnetales (Hansen *et al.,* 1999; Jacobson & Lester, 2003; Won & Renner, 2003; 2005b; 2006; Yamada *et al*., 2008; Wang *et al.,* 2010) rather than to explore species boundaries in *Gnetum* for taxonomic purposes. To date, the use of genetic markers in the delimitation of African species of *Gnetum* is unknown. However, phylogenetic studies of *Gnetum* based on the chloroplast *trn*T-*trn*F region (Won & Renner, 2005a) and the ITS regions (Won & Renner, 2005b), including two specimens of *G. africanum* Welw. among most of the species from South America and Asia, placed the African species sister to the Asian ones with the South American species sister to the rest of *Gnetum*. Investigations revealed independent losses of a conserved motif in ITS 1 from the African species, *G. africanum* and some of the Asian species (Won & Renner, 2005b)**.**

As mentioned, AFLP analyses have been applied in investigations of various plant groups (Bachem *et al*., 1996; Herselman, 2003; Abdel-Satar *et al*., 2003; Schmidt *et al*., 2004; Cai *et al*., 2005; Meudt & Clarke, 2006; Visser *et al*., 2008). For example, AFLPs have been used to clearly differentiate two distinct gene pools of geographically isolated Sardinian and Sicilian populations of wild cardoon (*Cynara* L.) and most of the genetic variation was found within each population (Portis *et al*., 2005). However, the application of this analysis is uncommon to the study of the genetic diversity in gymnosperms in general and among African species of *Gnetum* in particular. The main objective here was to investigate the use of AFLP molecular data to confirm the African species distinguished based on morphological features, and their genetic similarities as well as the relationship between the cultivated and wild specimens. This might provide informative markers to complement morphological markers.

5.1.6 Purpose and key questions

In this study, AFLP markers are used to confirm species boundaries within the genus *Gnetum* in Africa, to investigate their genetic similarities and examine the genetic relationship among

African wild and cultivated members of the genus. Understanding the pattern of genetic variation between rare species is important for prioritizing species for protection, and for choosing seed sources for reintroduction and restoration.

The main questions this research attempts to answer are: (i) Can an AFLP generated genetic pattern assist in distinguishing *Gnetum* species in Africa? (ii) What is the distribution of genetic variability among the wild and cultivated plants within a species? (iii) To what extent are the wild varieties reflected genetically among cultivated plants of various species?

5.2 Materials and Methods

5.2.1 Plant materials

Forty-five accessions, representing the four African species of *Gnetum* (Fig. 5.1; Appendix 5.7.1) were collected either directly from the wild or from cultivated plants in the Limbe Botanical Garden, Cameroon, and dried in silica gel, or leaf material was obtained from selected specimens from the Yaoundé Herbarium (YA). Juvenile leaves were used for total genomic DNA extraction. Vouchers have been deposited at J and YA.

5.2.2 DNA extraction

The DNA was extracted using the Qiagen DNeasy Plant Mini kit and details of the modified extraction method are found in Appendix 5.7.2.

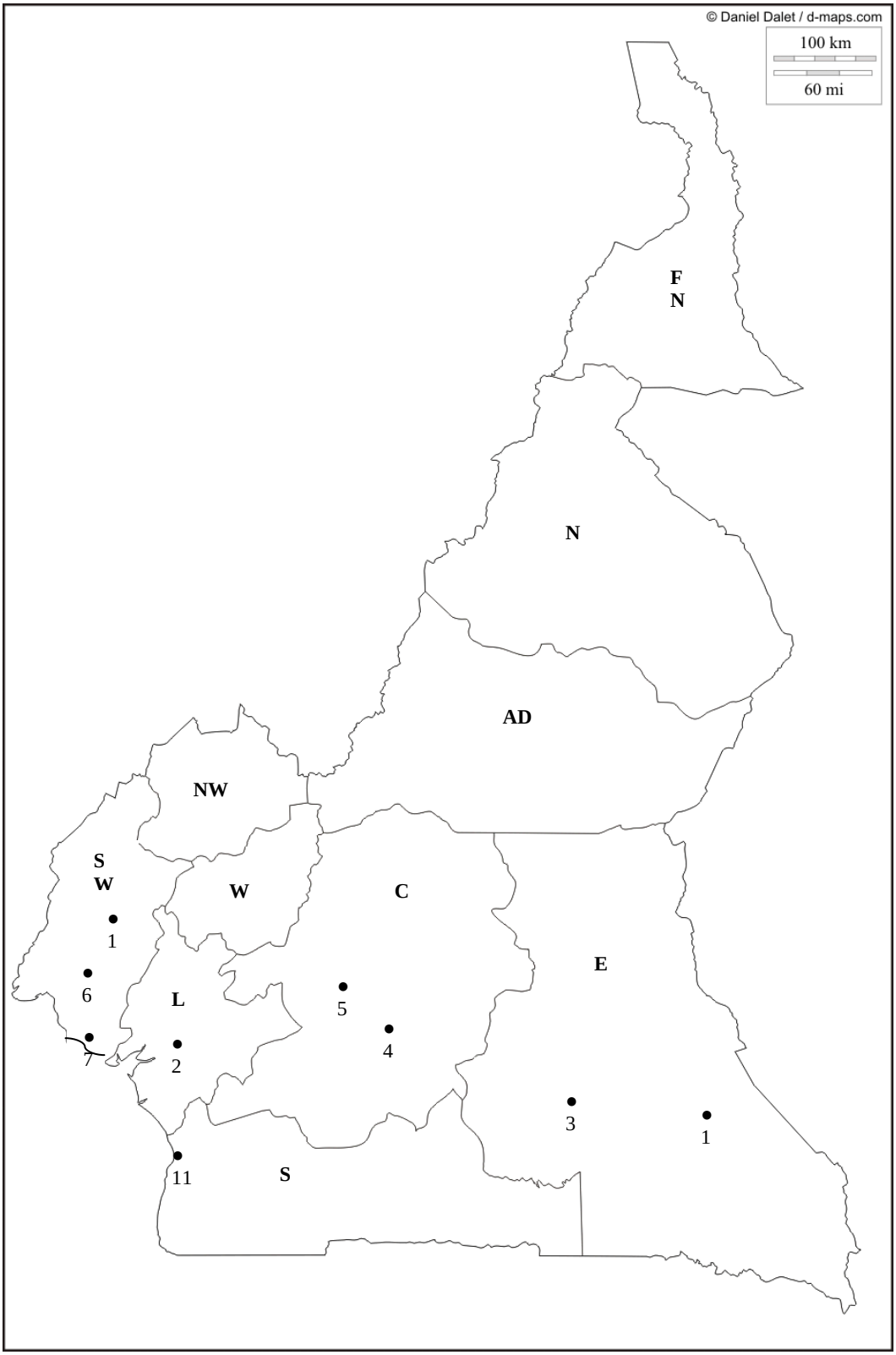


Figure 5.1 Location of collection sites of *Gnetum* species in Cameroon for use in the AFLP study/analysis. Numbers indicate the number of individuals sampled at that location. Provinces are: AD, Adamawa; C, Centre; E, East; FN, Far North; L, Littoral; N, North; NW, North West; S, South; SW, South West and W, West.

5.2.3 AFLP Analysis

Primers used for optimization of AFLPs in *Gnetum* were *Eco*RI and *Mse*I, and the selective nucleotides at the 3'-end are presented in Table 5.1 and oligonucleotides used for adapters were polyacrylamide gel electrophoresis (PAGE) purified. Adapters were prepared by adding equimolar amounts of both strands, heated for 10 minutes at 65 ºC in a water bath and then left to cool to room temperature. AFLP analysis was performed based on the methods of Vos *et al*. (1995), Angus (2005) and Reddy (2010) with some modification from Herselman (2003).

Table 5.1 Primer and adapter sequences used for AFLP analysis in *Gnetum*.

Primer/adapter	Type	Sequence(5'-3')
*Eco*RI	Adapter-F	CTCGTAGACTGCGTACC
	Adapter-R	AATTGGTACGCAGTCTAC
*Mse*I	Adapter-F	GACGATGAGTCCTGAG
	Adapter-R	TACTCAGGACTC**F** **N**
*Eco*RI + C	Primer + 1	GACTGCGTACCAATTCC
Apo I	Primer + 3	GACTGCGTACCAATTCCCA
*Mse*I	Primer + 0	GATGAGTCCTGAGTAA
	Primer + 2	GATGAGTCCTGAGTAACT

Restriction enzyme digestion and ligation reactions

The DNA was digested with two restriction enzymes and adapters with PCR primer sites ligated to the fragments and amplified using the polymerase chain reaction (PCR). Primers were used to further amplify the fragments from the first amplification and a few selective nucleotides were added to decrease the amplified fragments to facilitate fingerprinting with a clear number of bands. Restriction enzymes *Mse*l (a frequent cutter) and *Apo*l (a rare cutter) were used as described in Appendix 5.7.3. A thermal cycler (Bio-Rad M*y*Cycler) was used to carry out the digestion, ligation, pre-amplification and selective amplification reactions.

Preamplification reactions

Each 40 µl of pre-selective PCR reaction solution consisted of 8 µl of nuclease free water, 1 µl each of *Apo*I and *Mse*I primers and 20 µl of PCR Master Mix (Fermentas®) added to 10 µl of digested/ligated DNA solution. Reactions were performed using the following cycling programme: initial denaturation for 5 min at 72°C, 20 cycles of 30 s at 94°C, 30 s at 52°C,

followed by 60 s at 72°C and held at 18°C. The products were run on an agarose gel for 1 hr for visualization.

Selective amplification reactions

For selective amplification, pre-selective PCR products were diluted 10 fold in nuclease free water. 30 µl of selective PCR product was prepared by mixing: 10 µl of nuclease free water, 1 µl each of *ApoI* and *MseI* primers, 15 µl of 2x Fermentas PCR Master Mix to 3 µl of diluted pre-amplified products, pipetted into vials and run using the same PCR cycling protocol for selective amplification (Table 5.2). The 10 % denaturing polyacrylamide gels of length 50 cm used to separate fragments in the electrophoresis cell (Figure 5.2), was prepared beforehand as described by Angus (2005) and Reddy (2010) and summarized in Appendix 5.7.4.

Table 5.2 PCR profile for selective amplification.

Number of cycles	Temperature (ºC)	Time (seconds)
13	94.0 65.0 reduced by 0.7 /cycle 72.0	30 30 60
23	94.0 56.0 72.0	30 30 60

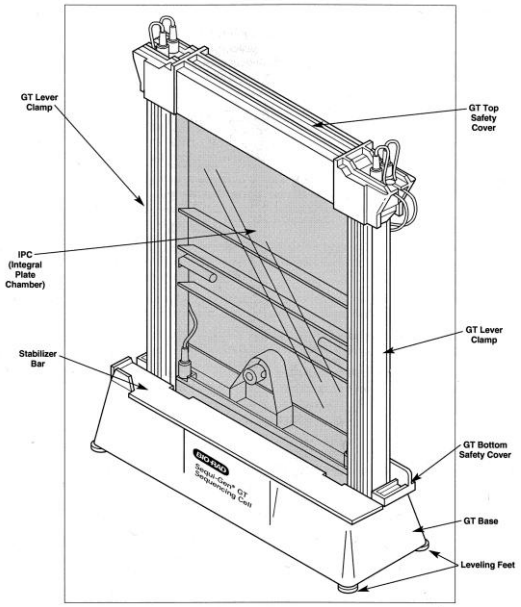


Figure 5.2 Sequi-Gen GT nucleic acid electrophoresis cell *(BioRad Instruction Manual)*

Preparation of samples

The tracking dye consisted of equal volumes of 10 mM EDTA (pH 8.0), 0.1% xylene cyanol and 1 mg/ml bromophenol blue placed in 98% formamide then heated at 90 °C for 3 minutes and quickly cooled on ice. A standard size molecular weight marker (Bio-Rad 100 bp Molecular Ruler) was run in the first and last lanes. 5 µl of each sample was loaded into the electrophoresis cell pre-heated to ± 45 – 50 ºC. The gel was run at 4 W for 15 min so as to stack the fragments and then run at 40 W for approximately 150 min until the dye reached 15 cm from the bottom of the gel. The long plate onto which the gel stuck, was separated from the short plate and the gel was silver stained.

Silver staining using a Promega kit

Visualisation was achieved using a Promega kit silver staining modified method as per instructions in the Promega Silver Sequence^{TM} DNA sequencing system manual. Three solutions (Fix/stop solution of acetic acid, staining solution of silver nitrate and developing solution of sodium carbonate) were prepared following the manual procedure and stored overnight at 4 ºC. The staining required the use of plastic trays which were rinsed with ultrapure water. Table 5.3 summarises the steps undertaken to visualize the bands. The stained gel plate was scanned on an Epson Expression 1640 XL scanner to enable the bands to be scored. Darker and clearer bands produced during visualization of the gels were also captured as a contact print on Kentmere Photographic Paper VC Select Glossy M.WT. 8388 HSE. The image was developed using Vario Grade paper developer (by Silvertone International Limited for black and white RC & fibre base paper) diluted to 1 part concentrate to 9 parts water for 1 min. The paper was then immersed in Rapid Fixer (Silvertone) for film and paper diluted to 1 part fixer to 9 parts water for 1 min with constant agitation before it was rinsed in tap water. Bands were scored using both the developed photographic paper and scanned images of the gel as seen in Figure 5.4.

Table 5.3 Silver staining protocol adapted from Promega manual

Steps	Solutions	Treatment time
Fix the gel using agitation	10% glacial acetic acid	30 minutes
Wash the gel using agitation (thrice)	Ultrapure water	1st time for 10 minutes, 2nd & 3rd times for 5 min each
Stain the gel using agitation	Silver nitrate and 3 ml of 37% Formaldehyde in 2 l distilled water	30 minutes
Prepare developing solutions	Sodium Carbonate in 2 l of distilled water + 3 ml of 37% Formaldehyde and 400 µl of Sodium Thiosulphate	Stir 30 minutes
Rinse the gel	Dip in ultrapure water	5 – 10 seconds
Develop the gel	Rocking of gel in developer till development of bands	Till bands appear
Stop development	Reuse 10% glacial acetic acid	2 – 3 minutes
Rinse gel twice	Ultrapure water	5 minutes
Dry gel	Leave at room temperature	

5.2.4 Data analysis

Bands were aligned by comparing the various samples to the molecular weight marker. The AFLP fragments were scored as dominant markers with individual profile as phenotypes. Only clearly defined bands were scored (‘1’ for presence and ‘0’) for absence for all the individuals and entered into a data matrix. Only reliable bands between 50 to 500 bp were considered. The binary matrix generated reflected AFLP fragments based on 45 individuals (Appendix 5.7.1). The amount (number and percentage) of loci that are polymorphic and specific fragments within and among species was calculated for every group using GenAlEx software (Peakall & Smouse, 2006). Monomorphic fragments were excluded from data analysis. Fragments that were separated by at least ± 5 bp were considered different.

Cluster analyses were performed using UPGMA (unweighted pair group method with arithmetic mean) in NTSYS-pc version 2.2 (Rohlf, 2000) A similarity matrix using the Jaccard correlation coefficient was based on the similarity data performed for the data matrix on NTSYS. This allowed me to compare the relationship between the genetic distance of the various species and the taxonomic distance. A dendrogram was generated using SAHN programme of NTSYS. To estimate the goodness of fit of the clustering to the data matrix, a

cophenetic correlation coefficient (r) was calculated by comparing the cophenetic matrix from the dendrograms with the similarity matrix i.e using COPH and MXCOMP options.

Principal coordinates analysis (PCO) was based on Jaccard similarity coefficients using the DCENTER and EIGEN programs of NTSYS-pc and 2D/3D plots were generated. Using the NTSYS-pc version 2.2 software (Rohlf, 2000), the Jaccard similarity coefficient (as suggested by Herselman, 2003) was calculated to perform the PCO.

5.3 Results

5.3.1 Band observation

Figure 5.3 shows eight of the 45 DNA extracts visualized on agarose gel. Most bands were of high quality and not degraded (lanes 2, 3, 4, 7, 8, 9). The DNA yield for specimens of *G. buchholzianum* was very low as indicated by lanes 5 and 6. This might be because the leaves in these samples were sticky during grinding and homogenization of the tissues was difficult. Of the four primer combinations (*Eco*RI/*Mse*I, *Eco*RI/*Mse*I + 2, *Apo*I/*Mse*I, *Apo*I/*Mse*I +2) screened to optimize AFLP analysis, the most informative primer combination was *Eco*RI/*Mse*I pair, which produced the highest number of bands. This primer combination, E + 1 with M + 2, amplified an average of 30 fragments per individual. Products from the digestion-ligation as well as the pre-amplification reactions were run on agarose gels and showed smears of genomic DNA indicating presence of product (Figure 5.4a & b). The final polyacrylamide gel and polymorphic bands were produced. Amplified fragment lengths varied from 500 bp and below. Faint bands presented difficulties when scoring.

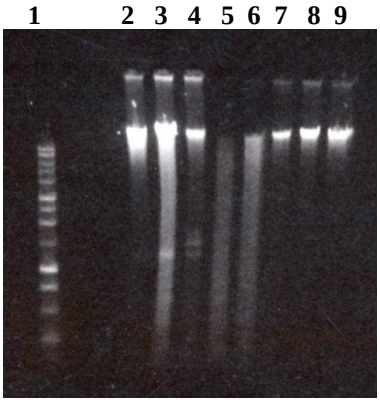


Figure 5.3 An agarose gel showing genomic DNA for selected *Gnetum* specimens (lanes 2 – 9). Lane 1: Molecular weight marker. Lanes 4 and 5 show samples with low DNA yield

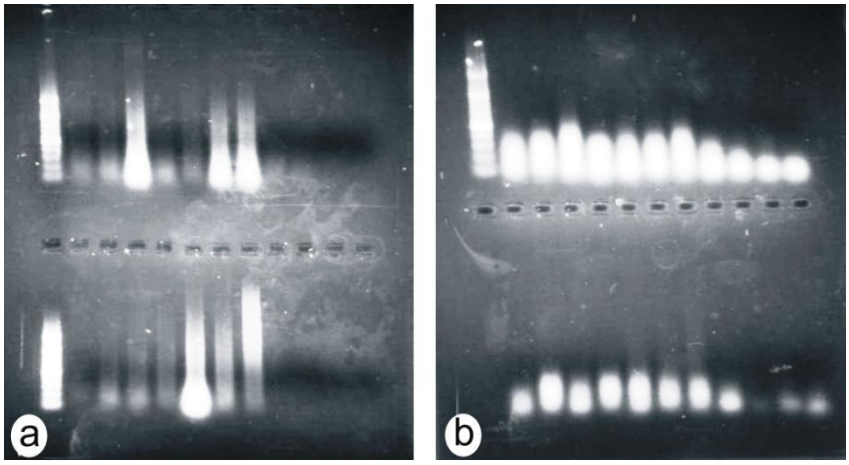


Figure 5.4 Agarose gels showing (a) products from the digestion-ligation reactions; and (b) reaction products from the pre-amplification reaction

5.3.2 Quantifying genetic diversity among *Gnetum* species

The scoring of AFLP gels generated from the one primer combination revealed the presence of 147 bands for individuals of the four species of *Gnetum*. The average percentage of all bands with different forms was 64.63 % ± 9.75 % (Table 5.4) and this represents an estimate of the total genetic diversity of all taxa. Of the total polymorphic bands observed, 24 were unique, i.e. applicable to all the plants of only a single species (Table 5.4). *Gnetum buchholzianum* had the highest percentage of polymorphic bands (~86%) and the highest number of bands (15) unique to the group (Figure 5.5 & Table 5.4). Five bands were unique to *G. africanum* and three unique bands were present in *G. latispicum* (Table 5.4; Figure 5.5). Although specimens of *G. interruptum* were fewer (n = 5), the polymorphic bands made up 40% of the total number of bands generated and only one unique band was recovered. The specimens of *G. interruptum* showed the lowest variance and had one unique marker, while

G. latispicum, *G. africanum* and *G. buchholzianum* each had 3, 5 and 15 bands respectively unique to the group (Figure 5.5).

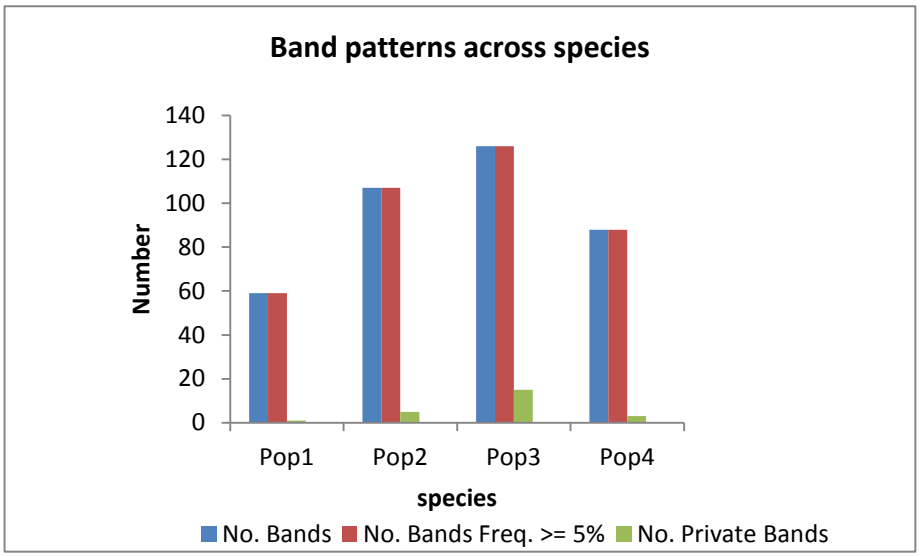


Figure 5.5 Band patterns across species of *Gnetum* using GenAIEx (Peakall & Smouse, 2005). Pop1 = *G. interruptum* (n = 5); Pop2 =*G. africanum* (n = 12); Pop3 =*G. buchholzianum* (n = 19); Pop4 = *G. latispicum* (n = 9). *[No. Bands = Number of different bands; No. bands freq. ≥5% = Number of different bands with a frequency ≥5%; No. private bands = Number of species-specific bands*

Table 5.4 Total band pattern for binary (diploid) data by *Gnetum* species in terms of number of bands, species-specific AFLP bands and percentage of polymorphic bands (%P) scored with the *Eco*RI/*Mse*I primer combination (GenAlEx software Version 6.4)

Species	n	Number of polymorphic bands	Number of species-pecific bands	% P
G. interruptum	5	59	1	40.14
G. africanum	12	107	5	72.79
G. buchholzianum	19	126	15	85.71
G. latispicum	9	88	3	59.86
				64.63 ± 9.75%

5.3.3 Cluster and ordination analyses

The dendrogram (Fig. 5.6), recovered from UPGMA cluster analysis indicates estimates of genetic similarity based on AFLP analysis with one primer combination. It shows the specimens of the various species forming subgroups of two (e.g. *Gnetum latispicum*) to eight (e.g. *G. africanum*) individuals of different species. Two major clusters I and II are evident at the 8% similarity level (Fig. 5.6). Cluster I comprises subgroupings of specimens of all four species of *Gnetum* recognized morphologically: *G. interruptum* (i), *G. buchholzianum* (b), *G. africanum* (a) and *G. latispicum* (l) and cluster II is made up of subgroupings of individuals of three of these species (*G. buchholzianum*, *G. africanum* and *G. latispicum*). All individuals of *G. interruptum* (i) group together in Cluster I at 31% similarity level distinguishing themselves from the other species, but including the specimen bC36. In addition, they were most similar to two individuals of *G. buchholzianum* (one wild, bMC23 and cultivated, cbE15).

The five cultivated individuals included in this analysis grouped with their wild collected counterparts where applicable, confirming their similarity to their respective species. The goodness-of-fit of the tree to the data set was confirmed by the high cophenetic correlation coefficient (r = 0.99). Some individuals within each species seemed to have grouped according to two main geographical localities (coastal and inland regions). For example, most of the specimens in the subgroup b_3, representing *G. buchholzianum*, grouped together based on similarity of the region of collection, i.e. inland or coastal. In contrast, the individuals of the subgroups corresponding to *G. africanum* grouped irrespective of geographical region as was the case with most individuals of *G. latispicum* in Cluster I. However, the subgroup l_2 of *G. latispicum* specimens in Cluster II comprised plants only from the coastal region.

The dendrogram (Fig. 5.6) thus presents a clustering pattern across the various species at similarity levels between 10% – 20% that do not reflect the morphological species boundaries recognized in this study. Subgroup I contains individuals of two species (*G. interruptum* and *G. buchholzianum*); Subgroup II is made up of specimens of three species (*G. africanum, G. buchholzianum* and *G. latispicum*); Subgroup III consists of specimens of two species (*G. africanum* and *G. buchholzianum*); Subgroup IV has elements from three species (*G. africanum,* and *G. buchholzianum*) and Subgroup V had two individuals of *G. latispicum*. The plants of *Gnetum* thus appear to share a large part of their genome as indicated by the number of monomorphic bands (67) observed. Nonetheless, some species-specific bands (24

in total) do reflect the currently recognized species limits and contributed towards the distinct subgroupings observed.

The results of the principal coordinates analysis based on the similarity matrix generated using Jaccard similarity coefficients (Fig. 5.7) provide a similar pattern to the cluster analysis shown above (Fig. 5.6). For example, four specimens of *G. africanum* are grouped in the top to middle left corner of the plot, two below the mid point on the second principal coordinate, while nine others are grouped at mid right of the plot. Most specimens of *G. buchholzianum* are spread out across the 3D space from below the mid point on the second coordinate (axis) towards the top right corner. Four specimens of *G. buchholzianum* (bSW38, bSW4, bWA13, bSW30 and bC36) were found on the top right of the 3D plot near to all the specimens of *G. interruptum*. The plants of *G. latispicum* are found near and below the midpoint on the first and second coordinates respectively.

There is thus a considerable degree of within-species genetic variability, especially among specimens of *G. buchholzianum*, and there is also variation between the species. The widespread yet unique distribution of individuals of each species with some overlap between them illustrates the high levels of genetic variation within the species sampled, and possibly, that insufficient genetic variation was used to reflect consistency among the l species as recognized morphologically. The individuals of each species did not group tightly together, however the first three principal coordinates accounted for only 31.4% of the total variation in the data set.

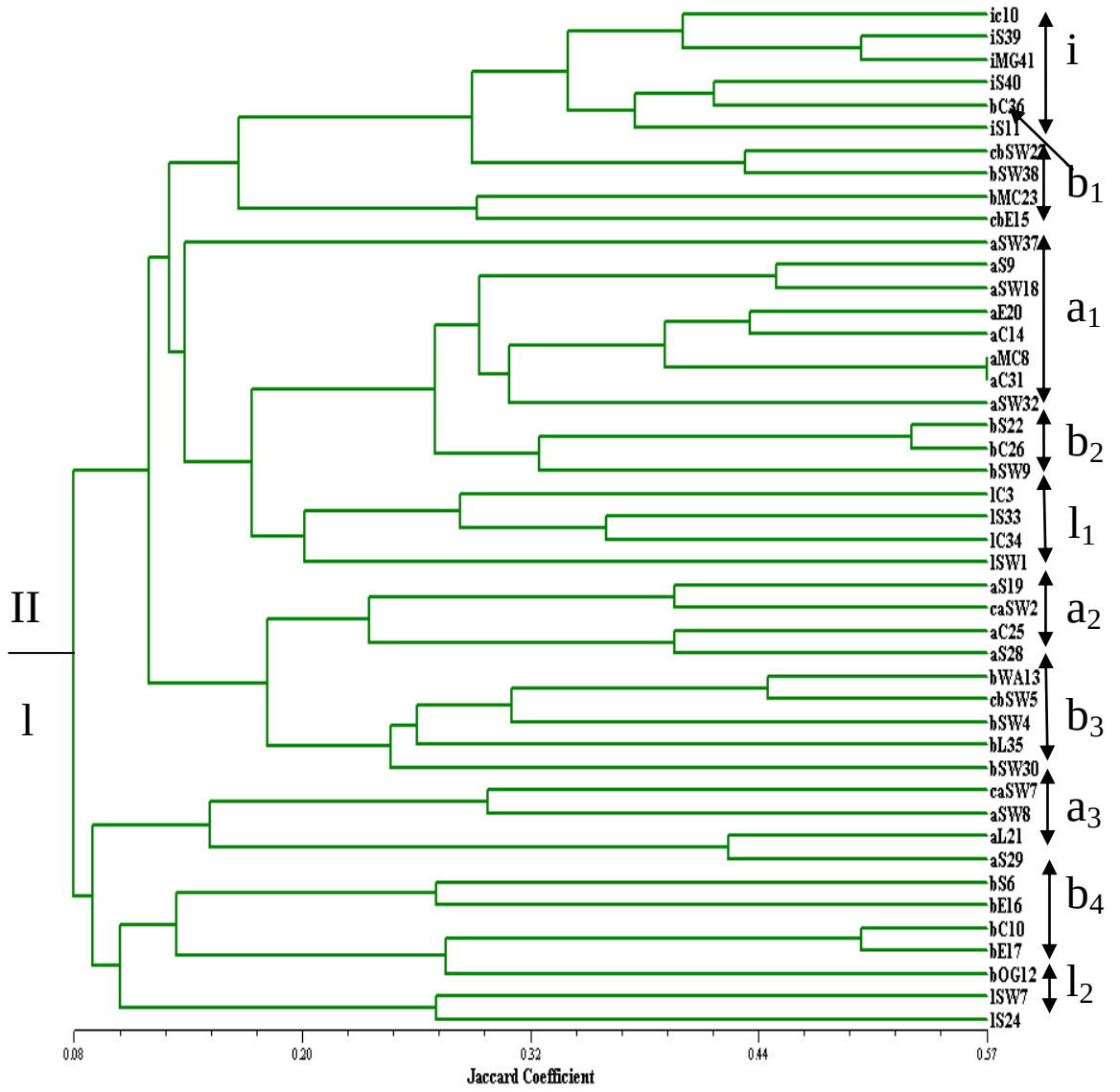


Figure 5.6 UPGMA Dendrogram using Jaccard's coefficient of similarity for the wild and cultivated samples of *Gnetum* species in Africa based on AFLP analysis with one primer combination. *G. africanum* (wild = a; cultivated = ca), *G. buchholzianum* (wild =b; cultivated = cb), *G. interruptum* (wild = i), *G. latispicum* (wild = l; cultivated = cl) + country or province of origin + identification number. a_1, a_2 and a_3; b_1, b_2, b_3, b_4 and l_1, l_2 represent groupings of *G. africanum* , *G. buchholazianum* and *G. latispicum* specimens respectively. Abbreviations used to identify specimens as in Appendix 5.7.1.

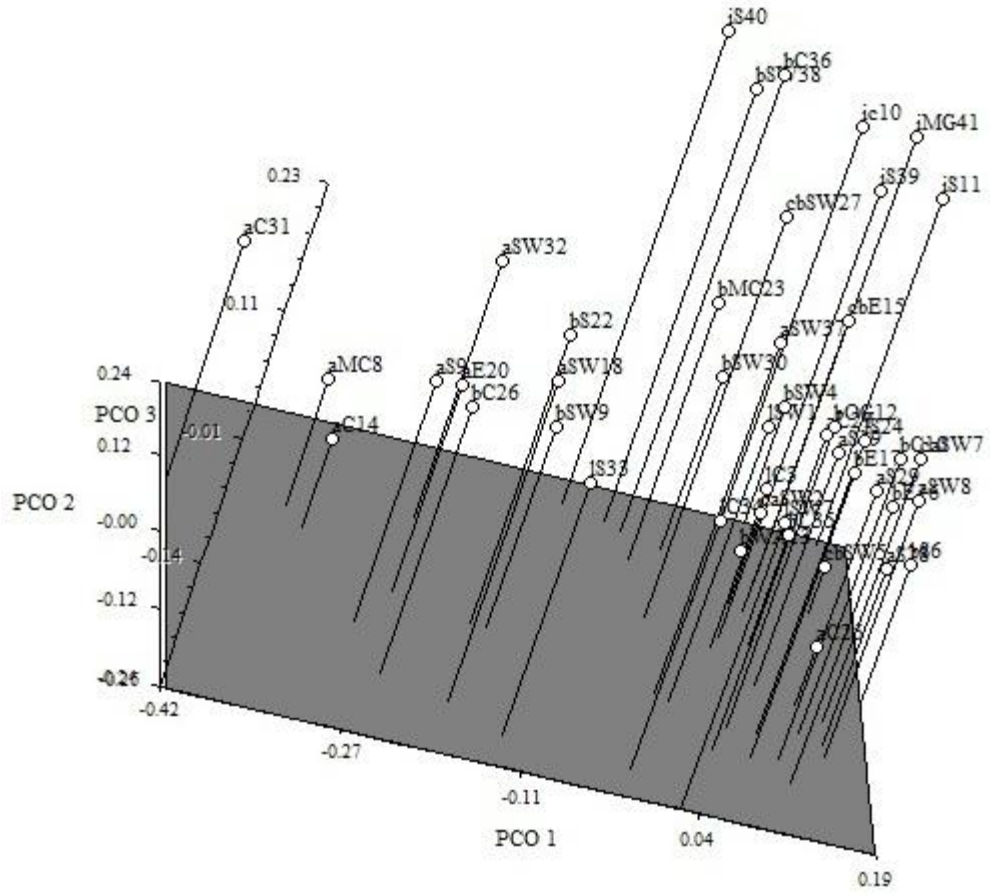


Figure 5.7 Three-dimensional diagram recovered from Principal Coordinates Analysis of individual specimens belonging to species of *Gnetum* based on AFLP markers. *G. africanum* (wild = a; cultivated = ca), *G. buchholzianum* (wild =b; cultivated = cb), *G. interruptum* (wild = i), *G. latispicum* (wild = l; cultivated = cl) + country or province of origin + identification number. Abbreviations used to identify specimens as in Appendix 5.7.1.

5.4 Discussion

This AFLP analysis has contributed towards assessing the genetic relationships among 45 accessions that represent four morphological species of *Gnetum*. One hundred and forty-seven bands were recovered from the AFLP analysis and the within species variance component accounts for 40% to 86% depending on the species. *G. buchholzianum* shows 85.71% within species variance while that for *G. interruptum* is least with 40%. A high level of variation is also known to occur in the vegetative morphology of *Gnetum* (Biye *et al.*, in press), and this is supported here by ± 65% of the total bands recovered being polymorphic indicating a high degree of genetic diversity among *Gnetum* genotypes. Similar results were obtained in *Albizia* species; their polymorphism is also high ranging from 96.15 % to 100%

with a mean of 98.06% for an average of 59.2 bands generated (Aparajita & Rout, 2010). These *Albizia* species also show remarkable morphological variability with regard to certain characters (Aparajita & Rout, 2010).

The average number of polymorphic fragments observed in AFLP analyses in general ranges from 18 to 97 per primer pair to effectively distinguish among species, not only because of the high polymorphism but also because many fragments are specific to a single species (Still *et al.,* 2005; Gaskin & Karmer, 2009; Russi *et al*., 2009). The number of polymorphic bands observed among *Gnetum* species falls within this range. Twenty-four species-specific fragments are evident in the data set for the four *Gnetum* species and are unique and distinguish the species (Wright, 1931; Larson *et al*. 1984; Russi *et al*., 2009), and are considered to represent true genetic differences in species (Slatkin & Maddison, 1990; Ramirez & Haakonsen, 1999; Nguyen *et al*., 2004). These molecular characters support the reproductive features that distinguish between these *Gnetum* species (Chapter 2), but were outweighed by the many shared bands. Therefore more primer combinations are required to target more specific and a larger number of fragments in order to fully investigate species limits in this genus.

According to the genetic factors, the plants of *Gnetum* show a high level of polymorphism and this indicates a reasonable evolutionary potential for other analyses like DNA sequences which is helpful to elucidate species relationships and to confirm the monophyly of genera. In reality, however, species of *Gnetum* are under threat due to increasing forest destruction and extensive harvesting losing genetic potential. The species of *Gnetum* are relatively similar in terms of genetic variation due to the high number of monomorphic fragments observed, although *G. interruptum* is more or less distinct; therefore they are equally important for prioritization purposes. Thus, the disappearance of one species will represent a loss in genetic diversity because each possesses bands that are unique to the group.

The bands produced in this analysis were of varying quality (size and intensity) suggesting that the band characteristics could represent real peaks but also possibly noise. The quality of the bands affects one's ability to score them clearly, although in this study this effect was ameliorated by discarding any bands that appeared to be noise at the number of band frequency less than 5 %. Bands may also overlap as a result of the many bands of high molecular weight produced due to partial digestion (Kapteyn *et al*., 2002). Weak bands are

associated with either contaminated DNA or incorrect thermal cycling and differing band intensities have been linked to primer matches and the amount of template interactions (Bachem *et al*., 1996).

No distinct exclusive group representing a single species of *Gnetum* (as recognized by morphological features) is recovered in the phenetic analyses performed here, although *G. interruptum* is fairly distinct in the 3D plot resulting from the PCO analysis and forms a single group in the cluster analysis (although including one specimen of *G. buchholzianum*). The other three species of *Gnetum* are not clearly evident in the groups resulting from these molecular-based analyses, although subgroupings of the species are seen. It seems that there is a high degree of genetic variation within these African species of *Gnetum*, and some degree of genetic differentiation among the species. This is similar to the minimal differentiation observed among these species based on vegetative features (Chapter 3).

Specimens of *G. buchholzianum* (both wild and cultivated) have shown a high variability in the morphological analyses, for example; two clusters were recovered within *G. buchholzianum* with no clear geographical pattern (Chapter 2). A large geographical distance between plants of the same species usually correlates with less genetic similarity (Bonnin *et al*., 1996; Helenurm, 2001; Gaskin & Kazmer, 2009), and in this molecular study individuals of *G. buchholzianum* from the coastal areas were grouped separate from those collected inland. A population study of *Welwitschia mirabilis* Hook. (another gymnosperm of the same order as *Gnetum*) has also shown a high genetic difference between populations and this was attributed to the geographic distance separating them (Jacobson & Lester, 2003). The high level of within-species genetic variation results in specimens of each species forming subgroups across the two main clusters of *Gnetum*.

The genetic differences found among individuals of a species could be attributed to either discontinuous distribution, or the small sample size per species, and/or limited numbers of primer pairs used (Hamrick & Godt, 1989; Helenurm, 2001; Kapteyn *et al*., 2002; Still *et al*., 2005; Russi *et al*., 2009). Due to cost and time limitations, only one primer combination was used. An important contribution to further this work would be to evaluate more markers produced by additional primer combinations. Although the use of a single primer pair combination could produce repeatable results (Vos et al., 1995), at least three primer combinations are ideal and are recommended by some authors (e.g. He & Prakash, 1997; Herselman, 2003; Reddy, 2010).

The pattern of genetic variation in plants is mainly influenced by their breeding system (Frisch & Rieselberg, 1992; Chauvel & Gasquez, 1994; Bonnin *et al.*, 1996; Helenurm, 2001). *Gnetum* is dioecious (male spikes have catkins of stamens; females catkins of ovules), thus the species are necessarily outcrossing in their mode of reproduction. Significantly, higher levels of genetic variation within populations and among populations occur more frequently in outcrossing species than in inbreeding ones (Helenurm, 2001). Moreover, populations occurring mostly in mainland habitats are known to experience more outcrossing than isolated inbreeding island populations (Baker, 1955; Slatkin & Barton, 1989; Broun *et al*., 1992; Helenurm, 2001). However, *Gnetum* species often grow in isolated patches of forest (habitat fragments) and this might result in limited gene flow which could be a possible explanation for some of the subgroups of species seen in the dendrogram, rather than distinct single clusters per species.

The subgroupings of *Gnetum* differentiating at the 35% similarity level (Fig. 5.6) are generally separated by a reasonable distance within their natural habitat as mentioned above. The African tropical forest in which *Gnetum* grows naturally covers an estimated surface area of over 29 million km² (Malhi, 2010). Species of this genus are distributed discontinuously across the forest from Nigeria through Cameroon, Central African Republic, Equatorial Guinea, Gabon, Congo, and Democratic Republic of Congo to Angola. Moreover; the subsequent physical disturbance (deforestation) also causes species to become progressively isolated with a high genetic variation. Phylogenetic analysis of data obtained from chloroplast and nuclear DNA sequences suggest that the genetic relationships within members of the genus *Ephedra* and among populations of *Welwitschia mirabilis* are more easily defined using the geographical region of origin rather than cone characters or other morphological features (Bowe *et al*., 2000; Jacobson & Lester, 2003; Won & Renner, 2006).

Very little is known about the pollination or seed dispersal of the African species of *Gnetum* and research is needed to elucidate these aspects of its life cycle. *Gnetum* seeds are brick red, a colour that suggests that the seed dispersal occurs mainly by birds and therefore the possibility of long distance seed dispersal exists, albeit within the 29 million km² of forest (Malhi, 2010). However, several species of forest interior birds remain mostly in the forest regions; they hardly cross roads or fly long distances because of barriers caused by the low quality of the secondary forest formed post-disturbance (Reijnen & Foppen, 1994; Arevalo & Newhard, 2011). Consequently, plants of *Gnetum* growing naturally in the forest will be

geographically isolated in the forest patches if these forest birds are effectively the only dispersal agents and are not flying long distances. It is known, for example, that the distribution of forest birds such as Hartlaub's duck, *Pteronetta hartlaubi*; Hawk, *Kaupifalco monogrammica*, is mostly based on the ecological conditions such as the nature of vegetation (Chapin, 1923).

The reproductive features of *Gnetum* provide the most discrete structures to clearly distinguish between the African species. The morphological species concept is thus applicable in the genus *Gnetum* in order to separate species easily because of the gap in morphology (Chapter 1; Mallet, 2007). The geographically isolation of some allopatric species may or may not result in their being reproductively isolated as predicted by the biological species concept.

Although very little is known about the pollination mechanisms that could result in gene exchange between the species, *Gnetum* is pollinated by insects (moths) which have commonly been observed on plants of this genus (Kubitzki, 1990), and insect pollination has been noted in *Gnetum cuspidatum* in the forest of Sarawak and in other Gnetales such as *Welwitschia mirabilis* (Kato *et al.,* 1995; Wetschnig & Depisch, 1999). Insect pollination has also been reported in other gymnosperms such as *Ginkgo biloba*, *Juniperus communis*, and *Cupressus sempervirens* (Labandeira *et al.,* 2007; Nepi *et al*., 2009.). The limited distance covered by most insects and especially moths could also result in the high within species genetic variation observed in *Gnetum*.

Further genetic studies on *Gnetum* should include more samples per species to provide more insight and clarification of the delimitation of and relationships among the species. Multi-primer combinations that might target not only specific but a large number of fragments could also be useful inputs to determine genetic relationships in *Gnetum*. This hypothesis needs to be tested. Because *Gnetum* also propagates vegetatively especially under cultivation, the degree of variation in characters of economic importance such as yield could be determined in future studies (Chapters 7 & 8).

5.5 Conclusion

Gnetum species are dioecious, allogamous plants with a high level of genetic polymorphism within species but a lack of clear genetic differentiation among the four species investigated here. In this first study of AFLP comparisons among *Gnetum* species in Africa, and despite considerable variation within groups, it has been revealed that molecular data do not confirm the morphological species boundaries. In addition, the data set gave only limited resolution of taxa due to substantial parts of the total variation being shared by the species being compared. However, the four species are differentiated from one another based on unique band for each species, but are strongly affected by the many-shared bands. Consequently, there is a lot of variation that does not distinguish between the species. This variation can be attributed to *Gnetum* species growing in isolation in forest fragments resulting in limited gene flow, which might explain the subgroups of species recovered in the dendrogram, rather than distinct single clusters per species. This preliminary study indicates the need to investigate the genetic relationship between the species of *Gnetum* using other approaches such as DNA sequencing in addition to using more primer combinations in further AFLP studies. The DNA sequence comparisons using rapidly changing genes or intergenic spacers in both the chloroplast and the nuclear genome would have the advantage of giving detailed phylogenetic information for the African taxa with the likely ease of comparison to outgroup taxa from Asia and the Amazon basin. In the future, AFLP or isozyme data also could be used to assess the level of variation within population (e.g. seed samples) among population and among species.

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APPENDICES

Appendix 5.7.1 List of voucher specimens used in the AFLP analysis arranged in order of identification number. The first letter of the code, sometimes with the prefix 'c' for cultivated specimen, represents the species epithet (a = *Gnetum africanum*; b = *G. buchholzianum*; i = *G. interruptum*; l = *G. latispicum*) followed by the locality and then the identification number.

Code	Collector, No. of specimens & herbarium	Collection Locality
lSW1	*Biye 403* (J; YA)	Kumba, SW, Cameroon
caSW2	*Biye & SVO 217* (J; YA)	Limbe, SW, Cameroon
lC3	*Biye & SVO 503* (J; YA)	Lekie, Centre, Cameroon
bSW4	*Biye 505* (J; YA)	Banga, SW, Cameroon
cbSW5	*Biye 506* (J; YA)	Kumba, SW, Cameroon
bS6	*Biye 357* (J; YA)	Kribi, South, Cameroon
caSW7	*Biye 401* (J; YA)	Limbe, SW, Cameroon
aSW8	*Biye 402* (J; YA)	Limbe, SW, Cameroon
aS9	*Biye 353* (J; YA)	Kribi, South, Cameroon
bC10	*Biholong 258* (YA)	Yaounde, Centre, Cameroon
iS11	*Bos 5465* (J; YA)	Kribi, South, Cameroon
bOG12	*Breteler 1553* (WAG, YA)	Ogoue, Gabon
cbWA13	*Setten 346* (WAG; YA)	Cameroon
aC14	*Biye & SVO 218b* (J; YA)	Lekie, Centre, Cameroon

cbE15	*Biye 414* (J; YA)	Bertoua, East, Cameroon
bE16	*Biye 220* (J; YA)	Bertoua, East, Cameroon
bE17	*Biye 405* (J; YA)	Batouri, East, Cameroon
aSW18	*Biye 402b (J; YA)*	Limbe, SW, Cameroon
aS19	*Biye 357* (J; YA)	Kribi, South, Cameroon
aE20	*Biye 403b* (J; YA)	Bertoua, East, Cameroon
aL21	*Westphal 100075* (YA)	Douala, Littoral, Cameroon
bS22	*Bos 2993* (YA)	Kribi, South, Cameroon
bMC23	*Bos 7192* (YA)	Mayombe, DRC
lS24	*Bos 4267* (YA)	Kribi, South, Cameroon
aC25	*Biye 413* (J; YA)	Yaounde, Centre, Cameroon
bC26	*Biye 401b* (J; YA)	Yaounde, Centre, Cameroon
cbSW27	*Biye 415* (J; YA)	Kumba, SW, Cameroon
aS28	*Bos 5242* (WAG, YA)	Cameroon
aS29	*Biye 358* (J; YA)	Kribi, South, Cameroon
bS30	*Biye 406* (J; YA)	Kumba, SW, Cameroon
aC31	*Biye 216* (J; YA)	Lekie, Centre, Cameroon
aSW32	*Biye 416* (J; YA)	Kumba, SW, Cameroon
lS33	*Biye 356* (J; YA)	Kribi, South, Cameroon
lC34	*Biye 217* (J; YA)	Lekie, Centre, Cameroon
lL35	*Biye 255* (J; YA)	Douala, Littoral, Cameroon
bC36	*Biye 219* (J; YA)	Lekie, Centre, Cameroon
aSW37	*Biye 400* (J; YA)	Limbe, SW, Cameroon
bSW38	*Biye 411* (J; YA)	Limbe, SW, Cameroon
iS39	*Bos 5465* (BR, WAG, YA)	Kribi, South, Cameroon
iS40	*Bos 4068* (YA)	Kribi, South, Cameroon
iMG41	*Los 1765* (YA)	Malibe, Gabon
lSW7	*Biye 603* (J; YA)	Kumba, SW, Cameroon
aMC8	*Wellens 449* (BR, YA)	Mayombe, RDC
bSW9	*Biye 416* (J; YA)	Limbe, SW, Cameroon
iC10	*Bos 3721* (YA)	Yaounde, Centre, Cameroon

Appendix 5.7.2 DNA extraction method

0.02 g of the dried young leaf tissue from each sample was ground in liquid nitrogen to obtain a fine powder which was transfered into cooled tubes. 800 µl Buffer AP1 and 8 µl RNase were added, centrifuged for 1 – 2 s and then incubated for 10 minutes at 65 ºC while inverting the tubes 2 or 3 times during incubation. 260 µl Buffer AP2 was added to the lysate, incubated for 5 min on ice and centrifuged for 5 min at 13000 rpm. Using a pipette, the lysate was collected and centrifuged for 2 min at 14000 rpm. The flow-through was transferred into new tube without disturbing the pellet and 1.5 volumes of buffer AP3/E was added to the lysate and mixed by pipetting immediately. 650 µl of the mixture was pipetted in a 2 ml collection tube and centrifuged for 1 min at 8000 rpm and the flow-through was discarded. The process was repeated with the remaining sample. The spin column was placed into a new 2 ml collection tube, 500 µl Buffer AW was added, and centrifuged for 1 min at 8000 rpm, the flow-through was discarded and the collection tube reused. 500 µl Buffer AW was added to the spin column and centrifuged for 2 min at 14,000 rpm. The spin column was transferred to a 1.5 ml or 2 ml tube and 100 µl nuclease-free water was pipetted directly onto the DNeasy membrane and incubated for 5 minutes at room temperature (15 – 25 ºC), and then centrifuged for 1 minute at 8000 rpm.

The DNA was thus eluted with 100 µl of nuclease-free water then with another 50 µl. Combining the two products provided a considerable amount of DNA. To investigate the extent of DNA present, 2 µl of extracted DNA was mixed to 1µl of 6X loading dye (80 % w/v sucrose, 10 mM tris-HCl, 10 mM EDTA, 0.3 % bromophenol blue) and run on 1 % agarose, 1X TAE (0.089 M tris, 0.089 M glacial acetic acid, 0.002 M EDTA, pH 7.8) gel in 1X TBE and observations carried out using ultraviolet light (U.V.I tec BTX 20 S) and photographed with a Polaroid Gelcam 0.85 electrophoresis hood.

Appendix 5.7.3 Digestion and ligation methods

To digest 50 µl *Apo*I for each sample, a mixture of 4 µl of 10x buffer, 25.64 µl of nuclease free water and 0.4 µl of *Apo*I enzyme (*Xap*I) was added to 10 µl extracted DNA and placed in an incubator at 37 °C for two hours. A mixture of 10µl volume of *Mse*l made up of 14 µl of 10x buffer, 8.64 µl of pure water and 0.4 µl *Mse*I enzyme (*TRU1*I) was then poured into the *Apo*I mixture and the resultant mixture further incubated at 65°C for another 2 hrs. This was

then confirmed by visualizing on an agarose gel and the image viewed using BIO-RAD Gel Doc XR+ and lab software.

The two adapters making the buffer added to 5 μM for *Apo*I adapter and to 50 μM for *Mse*l adapter were used to produce a double stranded adapter solution. This solution incubated at 95°C for 5 minutes and then allowed to cool before storage at -20°C. Ligation proceeded, for each reaction, with 1 μl of 10x ligase buffer to which was added 1 μl each of *Apo*I and *Mse*I adapters respectively, 6 μl of nuclease free water and T_4 DNA ligase mixed with 10 μl of digested DNA, then incubated at 37°C for 3 hours. These solutions were then preserved at -20°C.

Appendix 5.7.4 Gel preparation and Pre-electrophoresis

The polyacrylamide gel consisted of 10% acrylamide, 0.25% methylene bisacrylamide and the 7.5 M urea in 1X TBE made up of 0.089 M tris. 0.089 M boric acid, and 0.002 M EDTA, pH 7.8. The mixture was prepared by adding the urea in to the other substances at room temperature. To make up 50 ml of gel solution, 300 μl 10% ammonium persulphate solution and 50 μl TEMED were mixed, the gels injected using a syringe between plates of 20 cm x 50 cm x 0.4 mm apparatus and allowed to polymerise at room temperature overnight. The gel was cast following the instructions in the manual accompanying the BIO-RAD Sequi-Gen GT Nucleic Acid Electrophoresis Cell (figure 5.2). The gel was run at 40 W for 30 minutes to remove the polymerizing agents TEMED and ammonium persulphate so as to prevent any obstruction to DNA displacement.

CHAPTER SIX

UTILITY OF ANATOMICAL FEATURES IN DISTINGUISHING AFRICAN *GNETUM* L. SPECIES

CONTENT

6.1 Introduction

- Background knowledge:
  Importance of anatomical features to taxonomy

- Knowledge gap:

  Contribution of anatomical features to taxonomy of *Gnetum*

- Problem statement:
  Species recognition
  Matching of female specimens to corresponding males

- Purpose of this research

  Aim and key questions

6.2 Materials and Methods

Plant material
Leaf stomata preparation
Petiole preparation

6.3 Results

Characteristics of leaf epidermis
Characteristics of cross sections of petiole

6.4 Discussion

Anatomical features used in distinguishing species of *Gnetum* in Africa.

6.5 Conclusion

Evidence for intraspecific relationships in the genus

REFERENCES

APPENDIX

Abstract

The African species of the genus *Gnetum* are dioecious climbers distributed in the tropical forest regions. Field studies have shown that these species are morphologically variable and the male and female plants are difficult to distinguish in the vegetative state not only within groups, but also from dicotyledonous plants when not flowering. Although they are generally identified from their reproductive features, the leaf macromorphology is not helpful in species recognition in the genus *Gnetum* in Africa. Leaf and petiole anatomy are investigated here in four species of this genus in order to identify micro-morphological characters with taxonomic value to differentiate between the female plants to match with the respective male plant. Plant material was prepared using various techniques for conventional optical microscopy and was examined using a compound microscope.

Characters of taxonomic importance observed at the level of the petiole were size, shape and distance separating vascular bundles. These characters were similar for both male and female plants representing a species but varied between species. The leaves showed an uncommon type of sclereids, astrosclereids, and numerous anastomosing thicked-walled cellulosic fibres mostly in the adaxial subhypodermal layers in all the species. Both male and female elements of *G. africanum* presented stomata on both surfaces of the leaves at variable densities but mainly around the midrib of the adaxial surface. The walls of their epidermal cells were highly sinuous and irregular. Stomata were found on the adaxial leaf surface only for *G. buchholzianum*, *G. interruptum* and *G. latispicum* and the walls of their epidermal cells were less sinous to polygonal in shape. The anatomical characteristics of both petiole and leaf varied between taxa in both qualitative and quantitative values. The male and female plants of each of the four species of *Gnetum* resembled one another and presented similar anatomical features that can be used to establish intraspecific relationships among investigated groups. Species could be distinguished from each other on the basis of certain characters.

Key word: Anatomy, *Gnetum*, Leaf, Petiole

6.1 Introduction

Four African species are now recognized in the genus *Gnetum* L., of the phylum Gnetophyta and their taxonomic descriptions are provided (Chapters 2 & 3). These species are dioecious with the males producing catkins of stamens and the females catkins of ovules that are unprotected and the seeds are naked, as is common in gymnosperms. The reproductive features allow the male and female plants of a species to be matched easily (Biye *et al.*, in press). All other structures, for example, the vegetative features such as the leaves, vary not only between male and female plants but also among species. Thus, it is difficult to distinguish between species when specimens are sterile.

Anatomical features have been found to contribute to elucidating taxonomic relationships especially at the generic level (Carr *et al*., 1971; Radford, 1986; Poole *et al*., 1996; Lu *et al*., 2008). These structures have also proven useful in species identification problems in various genera such as *Pavetta* L., *Chionanthus* Gaertn., *Olea* L., *Combretum* L., and *Ephedra* L., and their application in interpreting species related problems is significant (Kiew & Che Su, 1982; Agbagwa & Ndukwu, 2002; Jauregui & Rojas, 2005; Mbagwu *et al*., 2009). Morphology (Chapters 2 & 3) and anatomy are both valuable in the study of plant structures, however, anatomical characters have been considered even more revealing than morphological features (Stace, 1980); and some anatomical features have become diagnostic and their use in species identification is now common (Cutter, 1971).

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6.1.1 Importance of anatomical features to taxonomy

Anatomy is concerned with the comparison of various internal plant structures and their organization at the cellular or tissue level. Comparative anatomy usually focuses on simple description of form and/or function of particular features of an organism. The anatomy of vegetative features is used more often, and usually offers new insights more frequently than the reproductive structures (Stuessy, 1990). Stem and especially leaf blade anatomy have been found to produce valuable evidence for systematic purposes (Cutter, 1971). Specifically, features such as sclereids, fibres, trichomes, stomatal types, layers of cells and tissues in leaf, petiole and stem sections, wood and bark anatomy have been useful in classification (Ananda & Bhupal, 1973; Weyers & Johansen, 1985; Mamay *et al*., 1988; Kubinova, 1994; Poole *et a*l., 1996; Carlquist, 1996; Lev-Yadun, 1997, 1999; Agbagwa & Ndukwu, 2002; Lu *et al*., 2008).

Anatomical features have been successfully used to identify *Ipomoea* species (Ogunwenmo and Ugborogho, 2003). Also, several diagnostic anatomical characters of the leaf and the petiole, such as the shape of the main vascular bundles, the number of radial rows of the vessel components in the main vascular bundles and the length of the petiole, have been found to be of taxonomic value in the African *Pavetta* species (Herman *et al*., 1986). The anatomy of trichomes, fibres and the presence of calcium oxalate crystals have provided very useful taxonomic information at all levels, from circumscription of family to separation of species and even varieties in some plant groups (Esau, 1960; Stace, 1965; 1973; Theobald *et al*., 1979; Lev-yadun, 1997; Inamdar *et al*., 1975; Mbagwu *et al*., 2009).

Sclereid diversity has been shown to be useful in taxonomy (Ananda & Bhupal, 1973; Khatun & Mondal, 2011); they are found in many different parts of plant organs and possibly serve for strengthening purposes (Horris, 1983). Thus, the tissues may become tough and less palatable to insects (Mauseth, 1988). Sclereids are classified in two main subdivisions, as either monomorphic or polymorphic based on the outline of the base form and whether or not they possess branches (Rao, 1968). There are seven main types of monomorphic and four polymorphic sclereids, namely ramiform, astrosclereids, polyramous sclereids and idiofibrosclereids (Ananda & Bhupal, 1973). The various types are found among different species and rarely in aquatic plants (Chiang & Huang, 1987; Kuo-huang, 1990). Astrosclereids are not a common type of sclereid and they are found in specialized tissues like the floating leaves of water lily (Chiang & Huang, 1987; Hartman *et al*., 2011).

The structure of stomata is also helpful in taxonomic work and the arrangement of subsidiary cells are most significant; thirty-one distinct patterns have been found in vascular plants, including a number known only in pteridophytes (Fryns-Claessens & Van Cotthem, 1973; Dilcher, 1974). The arrangement of subsidiary cells, when present, has proven useful in taxonomic studies (Radford, 1986); these cells flank the stomata and are involved in the osmotic regulatory movement of the guard cells (Cutler, 1978). Stomatal types are defined based on the arrangement of epidermal cells with respect to the guard cells. Thus, they are termed anomocytic, stephanocytic, paracytic and laterocytic and the side of stomata not surrounded by subsidiary cells often presents unspecialized epidermal cells (Fahn, 1969; Carpenter, 2005).

The epidermis of most leaves shows dorsiventral anatomy and plays a protective role at the interface of the plant with the environment. It also regulates the growth and expansion of internal organs (Becraft, 1999). The adult epidermal structure of the leaf and stem of *Ephedra foliata* Boiss., another member of the order Gnetales, showed either irregularly or transversely orientated stomata towards the leaf apex on both sides of the leaf, and this is very characteristic of this species (Pant & Mehra, 1964).

The petiole, which is a slender stalk that supports the leaf blade, can also provide useful characters in species identification and preparation of monographs (Essiett, 2010). Distinguishing characters of taxonomic value derived from the petiole anatomy include its shape, the variation in the number, arrangement and shapes of vascular bundles, and type and distribution of crystals (Essiett, 2010). The leaf epidermis and cross-sectional anatomy have received most attention in extensive taxonomic data collection for addressing taxonomic issues, although anatomical features are not always valuable for species identification purposes as the characters could be constant in all the species involved in a study (Herman *et al*. 1986; Olowokudejo, 1987).

6.1.2 Contribution of anatomical features to the taxonomy of *Gnetum*

The usefulness of anatomical evidence in species identification in *Gnetum* is no longer questionable. The ontogeny of stomata in two *Gnetum* species, *G. gnemon* L. and *G. ula* Brongn., and their radially oriented parenchyma cells in secondary phloem linked to companion cells have been described in order to distinguish between the two species (Maheshwari & Vasil, 1961). Leaf anatomical analysis carried out in Guianas showed that *Gnetum* species can be distinguished based on the presence or absence of subepidermal fibres found on the adaxial surface of the leaf (Stevenson & Zanoni, 1991), they are thus of taxonomic importance.

An influential study has identified essential morpho-anatomical characters to use in taxonomic delimitation of *Gnetum* in Venezuela (Jauregui *et al*., 2005). Six morphological features (the epidermis, length of internodes, size of leaves, seed colour, and the form and number of cotyledons) were used to distinguish the Venezuelan *Gnetum* species. Leaf anatomical characters such as presence of a hypodermis, crystals, layout and degree of thickening of cell walls and the number of vascular bundles in the midrib contributed to species delimitation of these South American *Gnetum* species (Jauregui *et al*., 2005).

Stomatal features were also used to distinguish between the Venezuelan species of *Gnetum*. Interestingly, there is a high similarity between the morphology and anatomy of the leaves of *Gnetum* with that of dicotyledonous angiosperms indicating the relative advancement of this genus compared with other Gnetaceae (Jauregui, 2005).

Very little has been published on the anatomy of the species of *Gnetum* in Africa. Duthie (1912), apparently the first to study the anatomy of *Gnetum* species in Africa and mostly dealing with the anatomy of the wood and bark of *G. africanum* Welw., indicated that the sclerenchymatous cells consisted of a network of very long and thick walls of both cells and fibres. The occurrence of tori has been revealed in the tracheary elements of *Gnetum* species in Africa and the direct or indirect origin of the lateral meristem was traced to cortical parenchyma (Carlquist & Robinson, 1995). *Gnetum africanum* and *G. buchholzianum* seemed to be distinguishable based on the circular bordered pits of vessel elements but were not regarded as taxonomically distinct (Carlquist, 1996), although *G. africanum* was considered to have distinguishing characters like narrower vessels with multiple perforations different from other *Gnetum* species plus helical thickenings observed at the vessel tips (Maheshwari & Vasil, 1961; Carlquist, 2012). Fortunately, to clarify this misinterpretation, the *Gnetum* species in Africa have been classified on the basis of both qualitative and quantitative characteristics using numerical taxonomic methods to distinguish species (Chapters 2 & 4); an identification key for the taxa and description of species are also provided (Biye *et al*., in press; Chapter 3).

Other than these few previous studies, there has been very little research on the comparative anatomical features of either the leaf or the petiole of the African species of *Gnetum*. Moreover, there is neither a specific investigation conducted on the anatomical features of the leaves of the four species now recognized in *Gnetum* in Africa (Chapter 2 & 3), nor is there any study that compares the male and female plants of each species. As a result, the significance of the anatomical characteristics of their leaves to clarify species delimitation in these African species of *Gnetum* is unknown. Therefore, the leaf and petiole anatomy of *Gnetum* species in Africa has been assessed in this chapter to reveal the many attributes of potential taxonomic importance they possess that are diagnostic at the species level.

6.1.3 Problem statement

Morphological descriptions of *Gnetum* species in Africa have been provided by Hutchinson and Dalziel (1954), Biye *et al.* (in press; Chapter 3), and the anatomy of the wood and bark of the two previously recognized African species of *Gnetum* (*G. africanum* and *G. buchholzianum*) has been described by Duthie (1912) and Carlquist and Robinson (1995). The investigation carried out by Jauregui *et al*. (2005) has shown the usefulness of anatomical features to distinguish *Gnetum* species in Venezuela, although knowledge of the use of anatomy to align the female individuals with their respective male plants was not emphasized.

This anatomical investigation was pursued for the African *Gnetum* species in order to differentiate between them and to match female plants with the respective male plants of each species. The aim of this study is to assess the value of anatomical features in matching the female plants to the respective male plants of each species and consequently to characterize the African species of *Gnetum.* The research is based on the hypothesis that male and female plants have recognizable matching anatomical features different from other groups and that these anatomical features can be used to distinguish between African species of *Gnetum* in general.

6.2 Materials and Methods

A total of fifteen male and female plants (four of each species of *Gnetum africanum*, *G. buchholzianum*, *G. interruptum* and three of *G. latispicum*) was used to conduct the anatomical investigation and specimens were deposited in YA were obtained from both field and herbarium specimens. The plant specimens (Appendix 6.7) were examined for valuable characters to be used in distinguishing species of *Gnetum.*

6.2.1 Leaf stomata preparation

The techniques of Shobe & Lersten (1967), O'Brien & von Teichman (1974) and Poriazis & Balkwill (2008) for clearing and staining plant leaves were applied. Intact leaves of similar size were placed in 80% ethanol in McCartney bottles and vacuum infiltrated until the leaves sank. The bottles were tightly capped and heated in a pressure cooker for 15 min at 1 atm. The tough leaves of *Gnetum* were heated further in 5% KOH at 1 atm for another 15 min.

The brownish leaves were placed in full-strength household bleach (JIK) till nearly colourless (5 – 20 min.). The specimens were then rinsed twice with water, stained with basic fuchsin and mounted in aqueous medium.

The stomatal size and frequency were determined using NIS-elements D 3.1 on an Olympus BH-2 compound microscope linked to a Nikon Digital Camera DXM 1200 and computer viewing screen. Stomatal size (area of closed guard cells) was averaged for 50 measurements per field of view per specimen. For each specimen, the number of stomata was obtained by counting in 20 microscopic fields of view under the 10X objective. Stomatal index was calculated for the field of view as the number of stomata divided by the sum of the number of stomata and epidermal cells multiplied by 100 (Pant & Mehra, 1964; Royer, 2001; Santos *et al*., 2008). Other leaf structures such as the size of fibres, type of sclereids and their distribution were also investigated and described.

6.2.2 Petiole preparation

The techniques used for petiole microscopy were provided by the Histology Unit of the University of the Witwatersrand (unpublished). The entire petiole (on average 0.5 mm long) of similar sized leaves per specimen was cut from each specimen of *Gnetum* and heated for 30 min in 1 – 2 cm^3 of "Glass Master" solution using a pressure cooker at 80 KPa and left overnight to cool at room temperature. The process of heating was repeated, because of the tough structures of *Gnetum*, for another 30 minutes after 18 to 24 hrs and then fixed in Formal Acetic Acid (FAA) for 48 hours to render the material hard enough for further handling.

For dehydration prior to embedding, the specimens were treated with a series of solutions containing increasing amounts of dehydrating agent and progressively less water. Tertiary butyl alcohol (TBA) was used as dehydrating agent. The specimens were then impregnated with wax and left in the embedding oven overnight. These were then arranged within the wax mould and the wax was allowed to solidify at room temperature. The wax blocks were trimmed to a suitable size and shape and stuck to a wooden block. Sections of 20 – 24 μm thick were cut using a microtome. The sections were dewaxed with pure xylene and rehydrated in an alcohol series (100%, 90%, and 70%) for 1 min each. Staining was achieved by dipping the slides in 1% alcian blue for about 5 min, washed with distilled water and

counter-stained with 1% safranin for 2 minutes. The stained section was again dehydrated through an alcohol series and mounted permanently in DPX or Canada Balsam.

This method was successful for only five of the specimens; therefore the freezing microtome method (Steedman, 1960) was used for the remaining 11 specimens. The microtome was equipped with a stage upon which tissue can be quickly frozen using liquid CO_2 that instantly freezes to solid and sections of about 10 – 20 μm thick were obtained. One or two drops of tissue-tek (O.C.T. 4583 compound) were placed on the block holder of the microtome; the specimen to be cut was placed on the top of the holder with the water beneath. CO_2 was then applied for a few seconds until the liquid was frozen. Using a sharp microtome knife, the specimens were cut into sections that floated off in water. These were stained with fasga stain and mounted in glycerine-jelly. The arrangement, number and the extent of the regions between vascular bundles were examined on sections midway in the petiole for utility to distinguish between species.

Photomicrographs were taken using an Olympus BH-2 compound microscope fitted with a Nikon Digital camera and the AxioVision Release 4.6.3. of the ZEISS Stereo Discovery V12 microscope. Measurements were carried out using the NIS-elements D 3.1 on Nikon DXM1200 session (1991 – 2009).

6.3 Results

6.3.1 Characteristics of leaf epidermis of four forms of *Gnetum* in Africa.

Epidermal cells

In three of the species examined, the epidermal cells of both abaxial and adaxial surfaces were sinuous but the cells on the adaxial surface seemed to be wider than those on the abaxial surface. The walls of the epidermal cells of *Gnetum africanum* (Figures 6.1a & b; 6.2a & b) were more sinuous and irregular than those of *G. buchholzianum* (Figures 6.1c & d; 6.2c & d) and *G. interruptum* (Figures 6.1e & f; 6.2e & f), which were less sinuous and appeared in rows. The pattern of distribution of these epidermal cells was more regular, forming ‘rows’, in *G. buchholzianum* and *G. interruptum* than in *G. africanum*, in which the cells were most irregularly arranged.

The lower epidermal cells of *G. latispicum* (Figures 6.1g & h; 6.2g & h) were the least sinuous compared to those of the other three species, while the upper epidermal cells were polygonal and more regular, but not undulate in shape. The upper epidermal cell walls were generally more thickened than the lower ones and the epidermal cells on this adaxial surface were relatively wider than those of the abaxial surface.The epidermal cells were similar in males and females of each species respectively (Figures 6.1 & 6.2). Nevertheless, the epidermal cells of the male plants were more sinuous than those of the corresponding female plants in all the species studied.

Stomata

The stomata were irregularly or transversely orientated, and evenly spaced on the abaxial surface (Figure 6.1) and varied with respect to the region of the surface of the leaf. In all the species studied, the stomata comprised guard cells surrounded by two subsidiary cells adjacent to and parallel to them and these were different from the ordinary epidermal cells. Moreover, no specialized cells covered the polar regions of the stomata. The side of the subsidiary cells towards the stomatal pore was generally thickened.

In *G. buchholzianum*, there were a few (2 – 4 mm^{-2}) stomata present on the adaxial surface but most (75 – 135 mm^{-2}) of the stomata were found on the abaxial surface. In *G. africanum*, a great number of these stomata was observed on both surfaces of the leaf (Table 6.1) and were more prevalent around the midrib on the adaxial surface, (125 – 160 mm^{-2} for males and 63 – 100 mm^{-2} for females). The stomatal index for the male plants of each species, was generally higher compared to that of females except in *G. africanum* where it was higher for the female than for the male plants (Table 6.1). Regarding the size, the stomata of *G. africanum* were smaller than those of the other three of *Gnetum* in Africa.

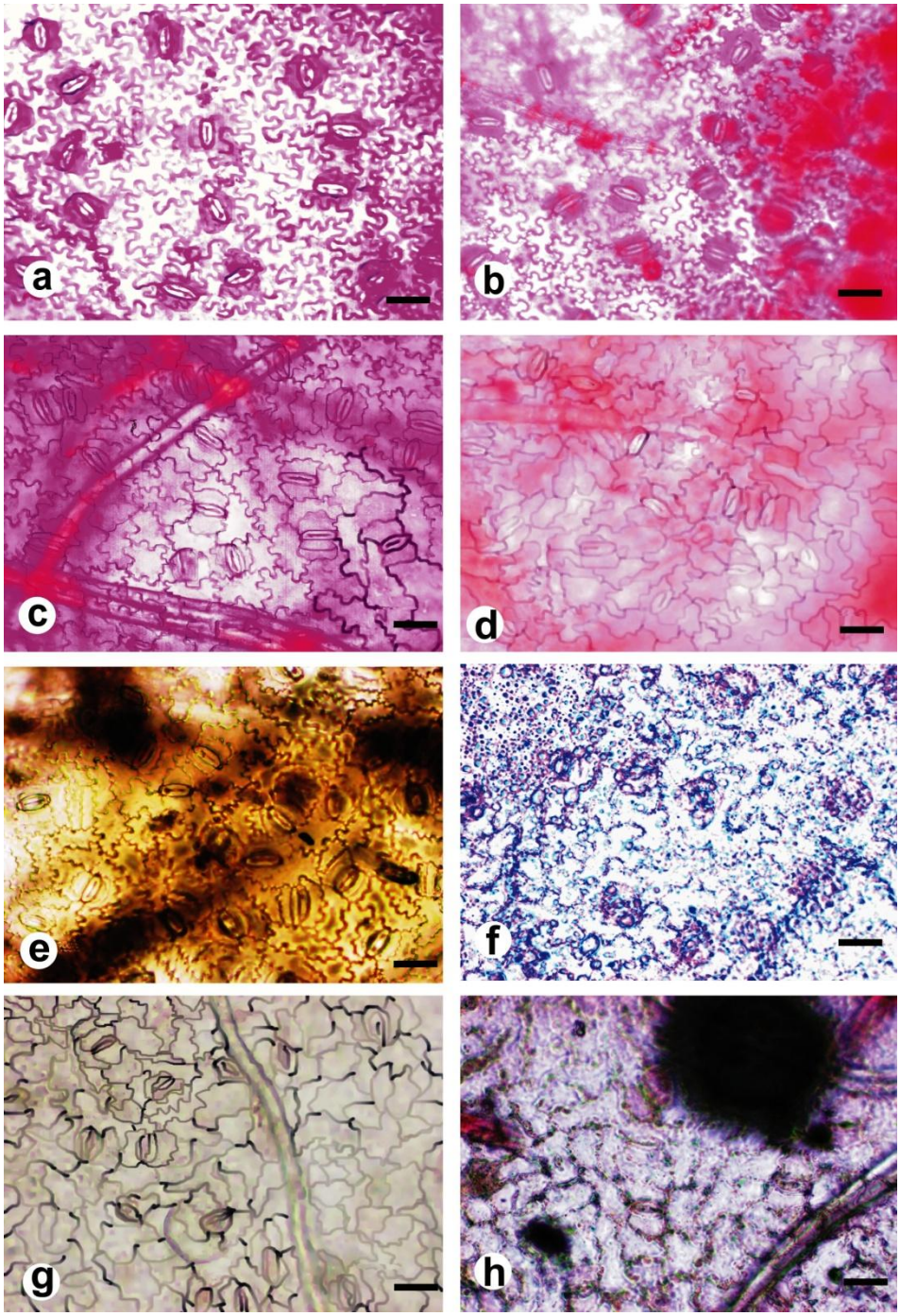


Figure 6.1 Light micrographs of the abaxial epidermis of *Gnetum* species in Africa. a. male & b. female *G. africanum*; c. male & d. female *G. buchholzianum*; e. male & f. female *G. interruptum*; g. male & h. female *G. latispicum*. Scale: 10 *μ*m.

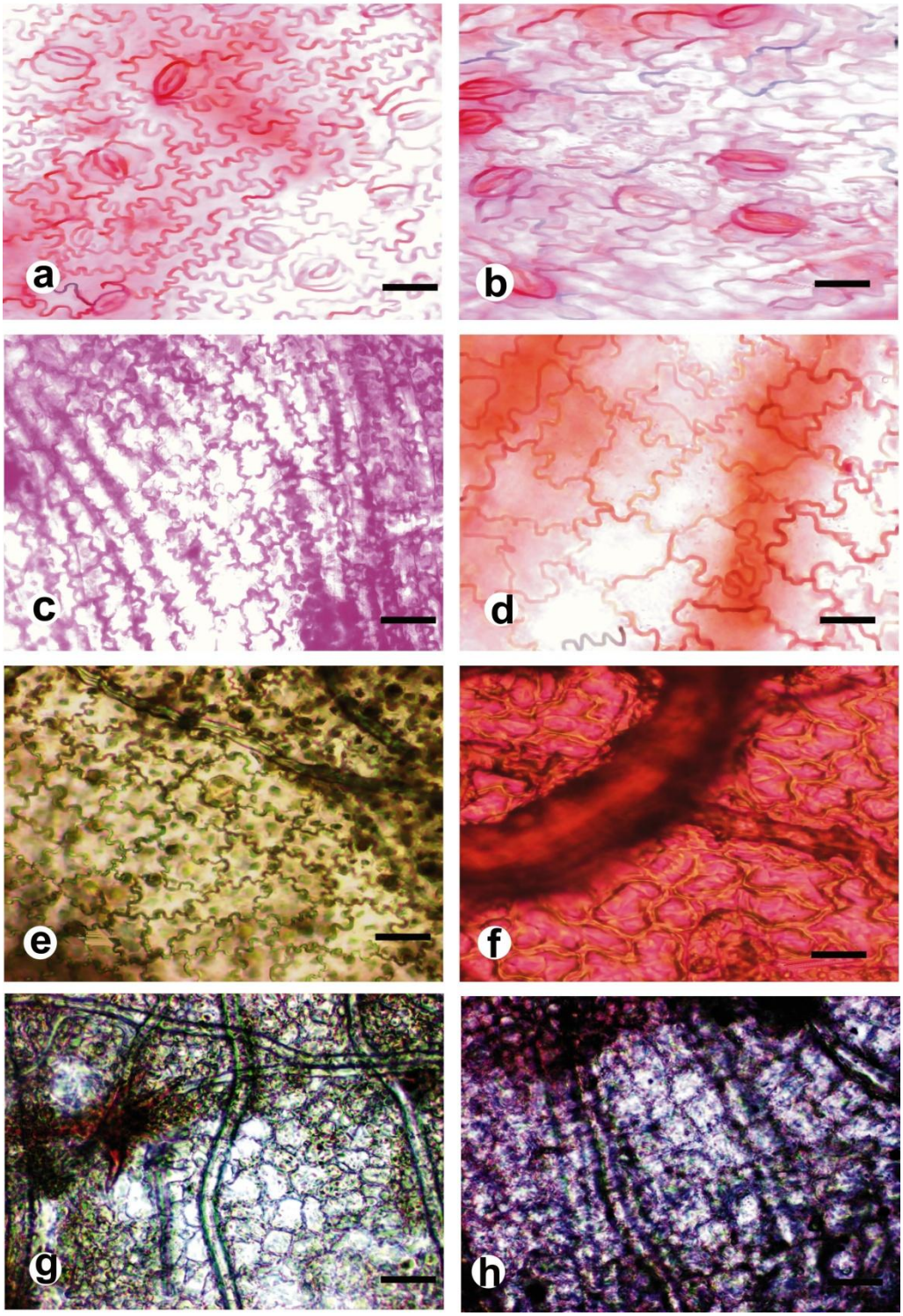


Figure 6.2 Light micrographs of the adaxial epidermis of *Gnetum* species in Africa. a. male & b. female *G. africanum*; c. male & d. female *G. buchholzianum*; e. male & f. female *G. interruptum*; g. male & h. female *G. latispicum*. Scale: 10 *μ*m.

Table 6.1 Stomatal characters examined in the leaves of the male and female specimens of four species of *Gnetum* in Africa.

Stomatal description → / Species ↓		Mean counts mm^{-2} n = 40	Mean stomatal area (μm^2) n = 100	Index n = 40	Location on leaf
G. africanum	Male	192.5 ± 45.1	457 ± 84	33.5	Abaxial Adaxial
	Female	131 ± 29	395 ± 79	39.3	Abaxial Adaxial
G. buchholzianum	Male	130 ± 7.1	514 ± 86	36.3	Mostly abaxial
	Female	95 ± 28.3	501 ± 109	14.1	Mostly abaxial
G. interruptum	Male	112.5 ± 17.7	524 ± 75	30	Abaxial only
	Female	120 ± 56.6	607 ± 96	28.6	Abaxial only
G. latispicum	Male	205.5 ± 78.5	509 ± 87	40.8	Abaxial only
	Female	158.5 ± 38.9	524 ± 92	25.6	Abaxial only

Fibres

Numerous fibres were present on both leaf surfaces of the male and female specimens of all the species studied (Figure 6.3). These were more abundant on the adaxial than on the abaxial surfaces. They appeared as filiform thick-walled sclereids, variable in size within and between species and formed a network of various proportions (Table 6.2). Fibres found in the males were wider than those found in the female specimens except in the case of *G. africanum* where those of the female were larger. The number of fibres in each surface varied and their path was irregular. The mature fibres were identified by the thick lignified wall. Because they appeared to double back on the themselves, plus their irregular course, fibre lengths were difficult to measure.

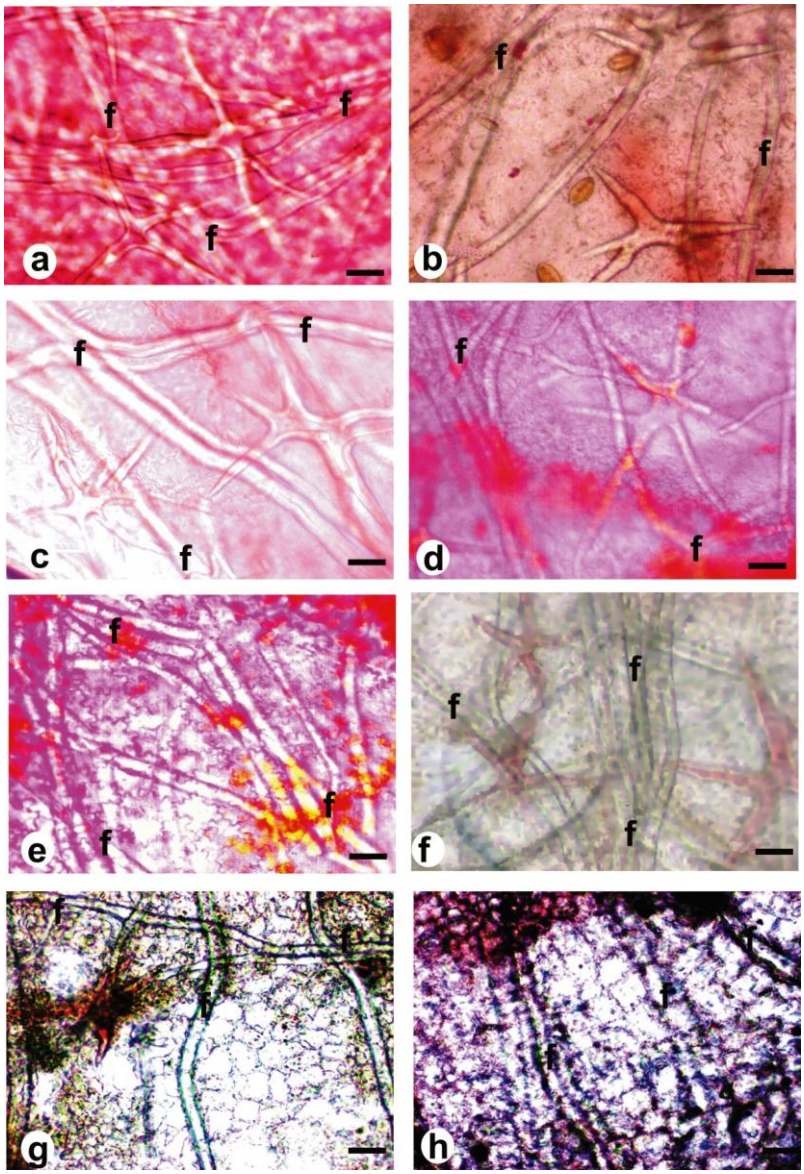


Figure 6.3 Fibre distribution in leaves of *Gnetum* species in Africa as seen under the light microscope. a. male & b. female *G. africanum*; c. male & d. female *G. buchholzianum*; e. male & f. female *G. interruptum*; g. male & h. female *G. latispicum*. Fibres indicated by f. Scale: 10 *μ*m.

Table 6.2 Mean diameter (± standard deviation SD) of fibres as seen on leaf surfaces of African species of *Gnetum* (n = 50 per specimen for each species).

Species → Character ▼	***G. africanum***		***G. buchholzianum***		***Gnetum interruptum***		***Gnetum latispicum***	
Fibre	Male	Female	Male	Female	Male	Female	Male	Female
Mean diameter ± SD (*μ*m)	14.0 ± 5.7	15 ± 7.1	19.5 ± 12	15 ± 8.5	19.25 ± 8.8	17.3 ± 6.1	16 ± 8.5	15 ± 7.1

Sclereid types

Most of the sclereids observed in the leaves of *Gnetum* species were astrosclereids (stellate forms) with at least four arms. They were present on both leaf surfaces in all four *Gnetum* species studied. The astrosclereids showed a great deal of diversity. They had a thickened central lumen, empty at maturity, with a number of radiating arms (> 4) of various sizes, branching irregularly and typically sessile (Figure 6.4). The arms of the sclereids in *G. africanum* were longer than those of the three other species and had pointed tips. The lumen of the sclereids in *G. buchholzianum* was wide and the tips of the arms were very acutely pointed. *G. latispicum* revealed astrosclereids with a thick broad lumen and the thickness extended to the arms, and appeared as osteosclereids in males. The tips were slightly blunt.

There was an almost equal density of astrosclereids on both surfaces in male individuals as in female plants. As many as 25 astrosclereids per 1 mm² area were counted in the leaves of the male specimens of *Gnetum interruptum.* It had the highest density of astrosclereids in its adaxial surface but they were not as dense on its abaxial surface. In contrast, the female specimens of *Gnetum interruptum* had relatively few astrosclereids on both surfaces (Table 6.3).

Astrosclereids were found to cluster in leaves of both male and female *G. africanum* and the clusters were unevenly distributed; more astrosclereids were present on the abaxial than on the adaxial surface. This pattern was not found in the other three species, viz. *G. buchholzianum, G. interruptum* and *G. latispicum.* Specimens of *G. buchholzianum* presented numerous astrosclereids almost evenly distributed throughout the surface, but these were fewer in the female than in the male specimens. *G. latispicum* exhibited astrosclereids dispersed on both surfaces of the leaf. The astrosclereids observed in all the species sometimes overlapped with fibres.

Table 6.3 Distribution and number of sclereids found in the leaves of *Gnetum* species in Africa (n = 50 per specimen for each species).

Species →	***Gnetum africanum***		***Gnetum buchholzianum***		***Gnetum interruptum***		***Gnetum latispicum***	
Character↓	Male	Female	Male	Female	Male	Female	Male	Female
Number /mm²	2 – 12	4 – 20	7 – 18	4 – 13	2 – 25	2 – 10	4 – 10	4 – 8
Distribution	clustered	clustered	diffuse	diffuse	diffuse	diffuse	diffuse	diffuse

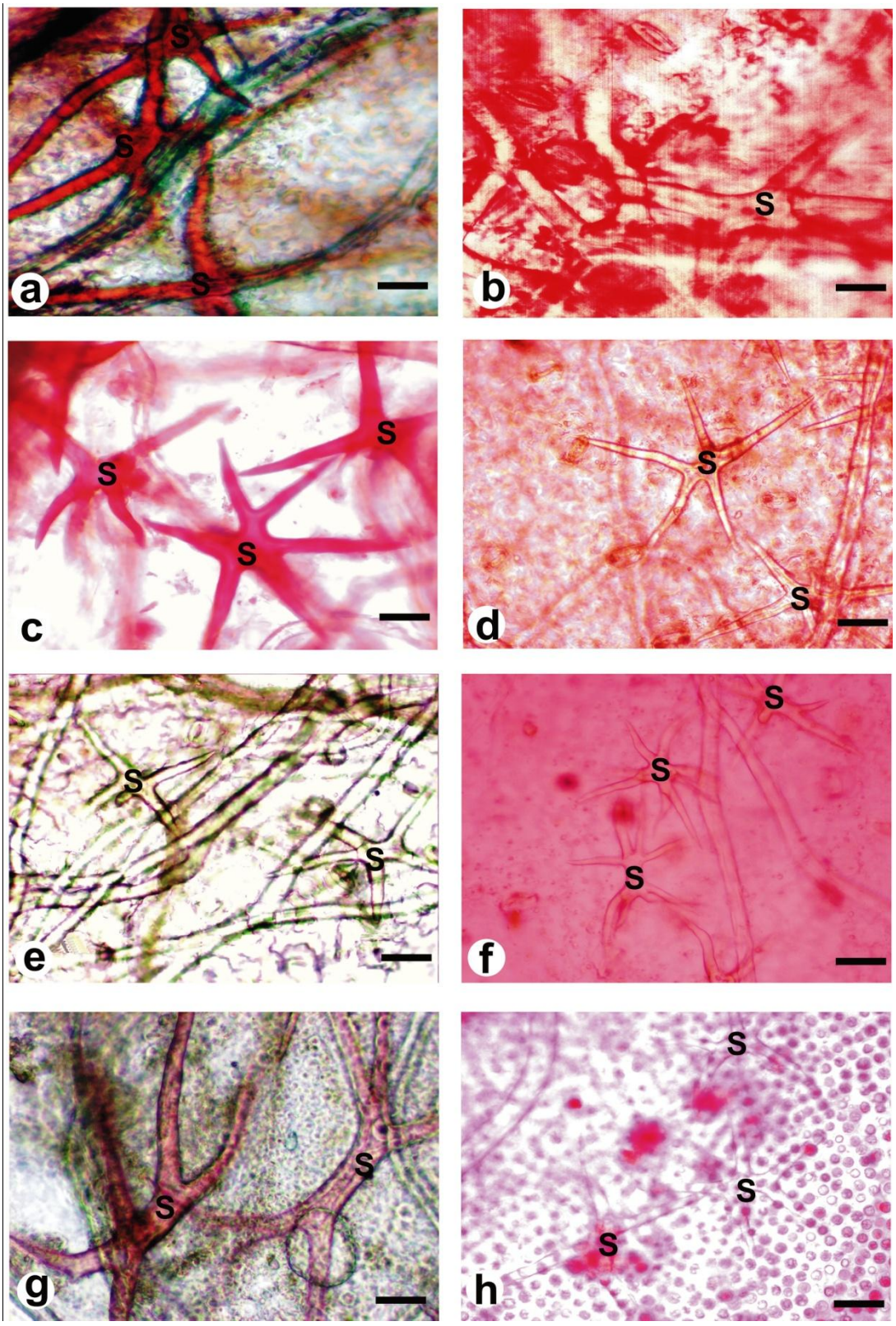


Figure 6.4 Light microscopy of adaxial surface of the leaf of *Gnetum* species showing astrosclereids (S). a. male & b. female *G. africanum*; c. male & d. female *G. buchholzianum*; e. male & f. female *G. interruptum*; g. male & h. female *G. latispicum*. Scale: 10 μm.

6.3.2 Characteristics of the petiole of *Gnetum* species in Africa

Transverse sections

The petioles vary from basically terete to D-shaped, i.e broader than thick, in transverse section (Figure 6.5). The petioles of the male and female of *G. africanum* and *G. latispicum* were D-shaped or subcircular with the abaxial surface convex and adaxial surface flat to slightly convex or shallowly concave. In both male and female specimens of *G. buchholzianum*, the petioles were basically terete but the male specimens had shallowly and broadly sulcate petioles while in the female specimens, they were shallowly and acutely sulcate. The petioles of *G. interruptum* were basically terete but esulcate in both male and female individuals.

The petiole epidermis consisted of a single layer of cells with thin cutinized outer walls. The epidermal cells of both surfaces were more or less spherical to irregular in shape with a few stomata present. No hairs or trichomes were found on the epidermises of the petioles in all the species studied. Beneath the epidermis was a well developed hypodermis with more or less cubical cells. The ground tissues comprised numerous irregularly shaped parenchyma cells that increased in size towards the cells adjacent to the vascular bundles.

In all *Gnetum* specimens examined, four adaxially located vascular bundles were present; these were single, collateral, closed, often symmetrical, and separated by parenchymatous cells (Figures 6.5 & 6.6). The xylem was adaxial and the phloem vessels abaxial in all bundles examined and surrounded by collenchyma cells. These vascular bundles were arranged in an arc and extended to the midrib and secondary veins of the leaf blade. The distance separating the two middle vascular bundles and that between paired vascular bundles on each side varied between species (Table 6.4).

The shape and sizes of the vascular bundles of the various species showed a degree of dissimilarity between species (Figure 6.6). In *G. africanum* (Figure 6.5a and b), the transverse section was more symmetrical with two vascular bundles closer to each other on either side of the midpoint, although they were more spaced out in the male than in the female specimens. The cross section of the petiole of *G. buchholzianum* (Fig. 6.6c and d) generally showed three large vascular bundles and one small and these were not uniformly spaced. In both male and female specimens of *G. interruptum* (Figure 6.6e and f), two of the four vascular bundles appeared equal in size although those of the male appeared slightly larger.

In *G. latispicum* (Figure 6.6g and h), there were three smaller vascular bundles and one large in the male specimens. The parenchyma regions appeared larger in *G. interruptum* and *G. latispicum* compared to *G. africanum* and *G. buchholzianum*.

Table 6.4 Size, shape and arrangement of vascular bundles (VBs) in the transverse sections of petioles of African *Gnetum* species.

Characters ⟶ / **Species** ↓		**Mean size of VBs (mm^2)**	**Mean distance between middle VBs (mm)**	**Mean distance between paired VBs on each side (mm)**	**Shape of VBs**
G. africanum	Male	0.01	0.25	0.155 ± 0.007	D-shaped; ½ as long as broad
n = 16	Female	0.01	0.235 ± 0.007	0.130 ± 0.014	
G. buchholzianum	Male	0.025 ± 0.020	0.195 ± 0.007	0.105 ± 0.035	Basically terete; almost as long as broad
n = 16	Female	0.020 ± 0.014	0.205 ± 0.007	0.105 ± 0.064	
G. interruptum	Male	0.02	0.135 ± 0.02	0.120 ± 0.057	Basically terete; 1 – 2 × as long as broad
n = 16	Female	0.02	0.195 ± 0.007	0.155 ± 0.007	
G. latispicum	Male	0.035 ± 0.021	0.27	0.13	D-shaped; ½ – 1 × as long as broad
n = 12	Female	0.030 ± 0.04	0.315 ± 0.035	0.110 ± 0.057	

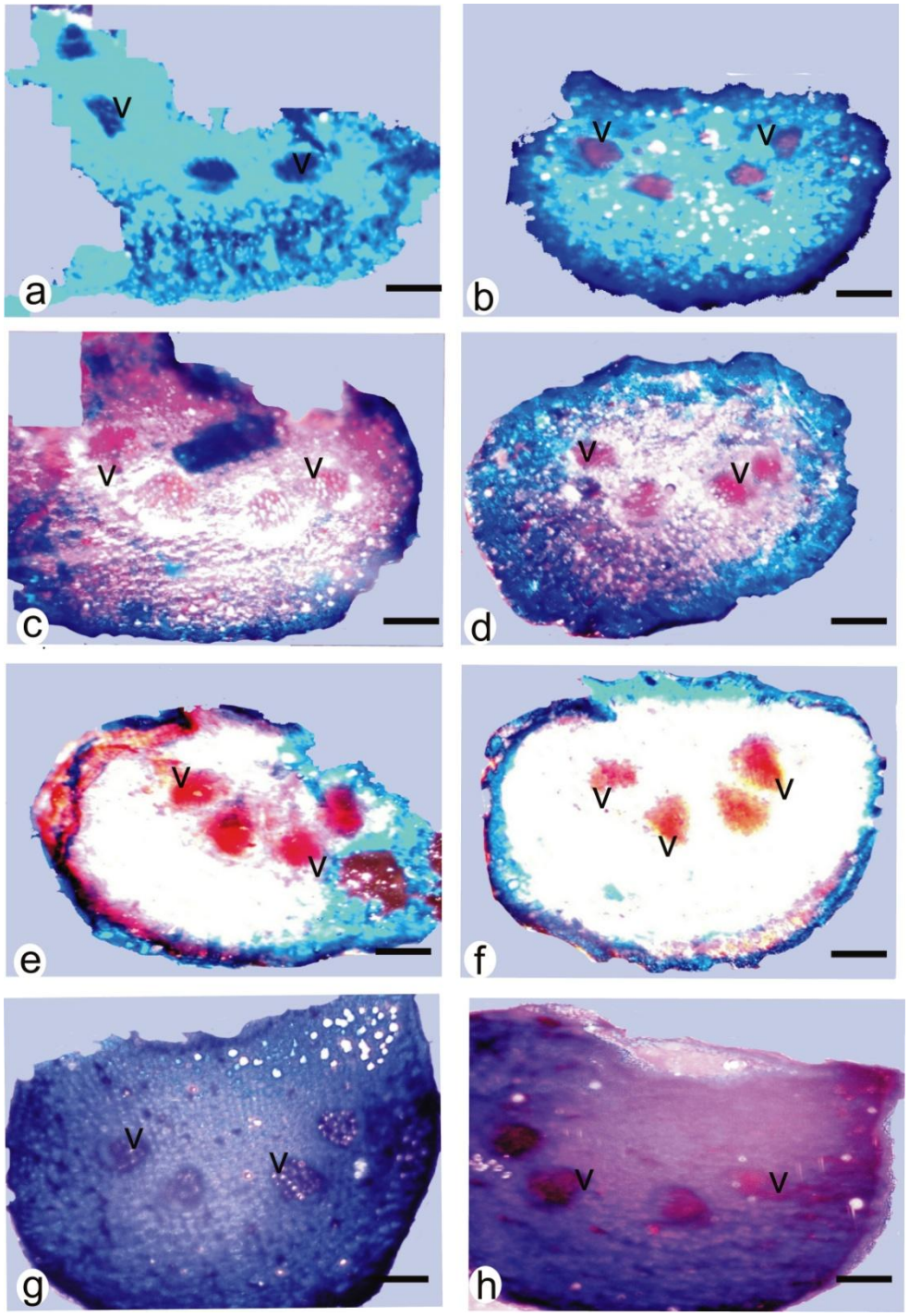


Figure 6.5 Transverse sections of the petioles of various species of *Gnetum* in Africa. a. male & b. female of *G. africanum*; c. male & d. female of *G. buchholzianum*; e. male & f. female of *G. interruptum*; g. male & h. female of *G. latispicum*. Two of the four vascular bundles in a cross section are indicated by V. Scale 10 *μ*m.

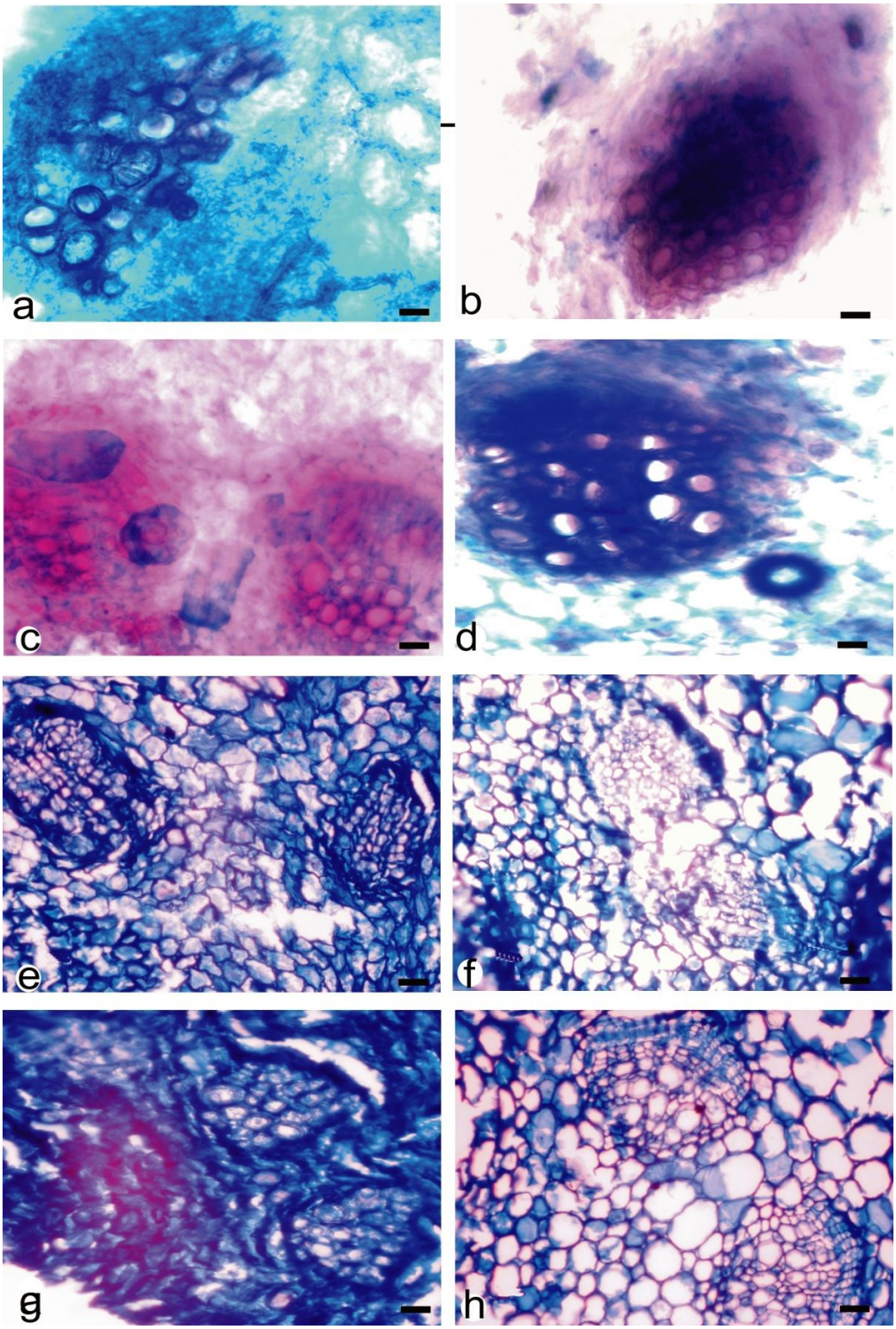


Figure 6.6 Vascular bundle structure of various species of *Gnetum* in Africa. a. male & b. female of *G. africanum*; c. male & d. female of *G. buchholzianum*; e. male & f. female of *G. interruptum*; g. male & h. female of *G. latispicum*. Scale 100 *µ*m.

Table 6.5 Summary of Anatomical Characteristics in the African species of *Gnetum*. Number of crosses indicates the degree of waviness of cell walls.

Species	***Gnetum africanum***		***Gnetum buchholzianum***		***Gnetum interruptum***		***Gnetum latispicum***	
Characters	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Leaf:								
Epidermal cell:								
Sinuous & irregular	+++	+++	++	++	+	+	Polygonal regular	Polygonal regular
Fibres	14	15	19.5	15	19.25	17.3	16	15
Sclereids								
Type	Astro-	Astro-	Astro-	Astro-	Astro-	Astro-	Astro-	Astro-
No. of arms	4 – 5	4 – 5	4 – 6	4 – 6	4 – 7	4 – 7	4 – 5	4 – 5
Distribution	Clusters	Clusters	Diffuse	Diffuse	Diffuse	Diffuse	Diffuse	Diffuse
Stomata:								
Surface located	Both	Both	Mostly abaxial	Mostly abaxial	Abaxial	Abaxial	Abaxial	Abaxial
Index	33.5	39.5	36.3	14.1	30	28.6	40.8	25.6
Petiole (t.s.):								
Outline	D-shape: broadly sulcate with obtuse margins	D-shape: flat adaxially with obtuse margins	Basically terete: shallowly and broadly sulcate	Basically terete: Shallowly and acutely sulcate	Basically terete: esulcate	Basically terete: esulcate	D-shape: Flat adaxially with erect magins	D-shape: Flat adaxially with acute margins
Vascular bundles								
Number	4	4	4	4	4	4	4	4
Type	Collateral	Collateral	Collateral	Collateral	Collateral	Collateral	Collateral	Collateral
Mean distance (mm)	0.25	0.235	0.195	0.205	0.135	0.195	0.27	0.315

6.4 Discussion

Different environmental conditions affect various structures in plants, for example, the anatomy of the leaves may reflect the environmental conditions of habitat where the plant grows (Herman *et al*., 1986). Because leaf features are influenced by environmental conditions, they often present adaptations to the conditions in which the plants are found (Esau, 1960). For instance, it was found that short and broad leaf epidermal cells were as a result of shading; but the number of layers of parenchymatous cells observed in the leaf could be linked with the duration of exposure to higher light radiation (Cutter, 1971, 1978). The African species of *Gnetum* grow naturally under dense shady conditions in the humid tropical forest. The epidermal cells on the adaxial surface of the leaf appeared broader compared to those on the abaxial surface in all the forms of *Gnetum* specimens studied. The broad adaxial epidermal cells observed among the male and female plants were considered to be an adaptation to forest conditions.

Although the macroscopic morphological features of leaf and petiole seldom distinguished the species of *Gnetum* in Africa (Hutchinson & Dalziel, 1954; Biye *et al*. in press; Chapters 2 & 3), the leaf blade and petiole of species of *Gnetum* provide some anatomical characters of taxonomic value. Leaf features, such as epidermal cells with either sinuous walls found in *G. africanum*, *G. buchholzianum* and *G. interruptum* or polygonal contours present in *G. latispicum*; the presence or absence of stomata on both surfaces of the leaves and the distribution pattern of sclereids largely contributed to distinguish between these species. Furthermore, the mean diameter of the fibres illustrated to a reasonable extent, the differences between species. The measurements of epidermal cells, expected to contribute towards distinguishing between closely related species, were limited by the variation observed among specimens of the same species and even among cells of leaves of the same plant.

Several authors (Stace, 1965; Herman *et al*., 1986; Lapp *et al*., 2004; Jauregui *et al*., 2005) have emphasized the usefulness of leaf epidermis in taxonomy and found that the types of epidermal cells enhanced species recognition in various

genera. The presence of epidermal cells with more or less polygonal contours and straight or slightly wavy walls found in *G. latispicum* is consistent with that observed in three Venezuelan species (Jauregui *et al*., 2005) and in *G. gnemon* from Indonesia, Ceylon and India (Maheshwari & Vasil, 1961). Epidermal cell contours can thus separate *Gnetum* species into two groups: the first group with cell walls more or less sinuous and irregular and the second with straight cell walls.

In all the species examined, the stomata were hypophyllus, except in *G. africanum* and some isolated cases in *G. buchholzianum* where they were found in the adaxial epidermis as well. Similarly, the presence of few or more stomata on the upper surface of the leaves was observed on specimens of *Gnetum ula* Brongn. and *Ephedra foliata* Boiss. (Pant & Mehra, 1964; Kausikan, 1974). In contrast, no stomata were found on the adaxial epidermis of all the leaves of *Gnetum* species studied in Venezuela (Jauregui, 2005). Based on stomatal location on the leaf surface, two groups of African *Gnetum* species can be recognized: the group with *G. africanum* and to some extent *G. buchholzianum* exhibiting stomata on both surfaces of the leaf, and the group of *G. interruptium* and *G. latispicum* with stomata on the abaxial surface only. The density of the stomata varied greatly with parts of the leaf surface in the African *Gnetum* species; this variation might mostly be influenced by environmental conditions known to affect plant structures (Cutler, 1978; Capellades *et al*., 1990; Casson & Gray, 2008). The size of the stomata also varied considerably within and between species; *G africanum* had the lowest mean area (i.e. less than 500 μm^2) compared to those of the other three species.

There was only one stomatal type observed in all *Gnetum* species in Africa, the paracytic type (Carpenter, 2005), and this is of little importance in distinguishing within and between species of *Gnetum* in Africa. The stomata observed in the Venezuelan *Gnetum* species were of two types, the paracytic where a subsidiary cell of similar size is attached on either side of the stoma and the duploparatetracytic with two cells attached on each side of the stoma. Stomatal 'twins' sharing subsidiary cells were frequent in the Venezuela specimens but

totally absent in the African species of *Gnetum* (Jauregui *et al*., 2005). However, the pattern of stomatal distribution appeared to be highly variable between species but might be regulated by mechanisms that maintain a minimum of one cell spacing between stomata (Casson & Gray, 2008).

Contrary to a study carried out in Guianas where *Gnetum* species have been separated based on the presence or absence of subepidermal fibres found on the adaxial surface of the leaf (Stevenson & Zanoni, 1991); fibres and filiform sclereids were found to be identical in all the species and at various stages of maturity in this study. They were well developed in the ground tissues and were present on both adaxial and abaxial surfaces. Therefore they could not distinguish between male and female individuals nor between species. The mean diameter of these fibres overlapped between species of *Gnetum* (Table 6.2), and generally the diameter ranged from 9 – 25 μm within and between species and could be influenced by the stage of maturity of the fibres. Actually, fibres vary in size and thickness with age (Tomlinson & Fisher, 2005), and therefore are not easily used taxonomically. Fibre initials, appearing after laticifer formation and extending by symplastic development but often intrusive apical growth, were less branched and irregular as they penetrate the mesophyll layers (Tomlinson & Fisher, 2005).

The distribution and the quantity of these fibres also varied within and between species. They were numerous and generally lengthy and had been described as a network of very long and thick walled structures in *G. africanum* (Duthie, 1912; Tomlinson & Fisher, 2005). The length of the fibres in the African species of *Gnetum* varied greatly and were presumed to be as long as 55 cm when examined after a special maceration (Aldaba, 1927). The fibres observed in this study were mostly a network of structures, although some occurred singly within a leaf. In *Cycas* leaflets, they were described as idioblasts as they were single but very often, fibres form bands or a network or an uninterrupted hollow cylinder in leaves of various plants (Fahn, 1969). The term idioblast was also applied to single sclereids causing tissues to be coarse and less tasty to insects (Khatun & Mondal, 2011).

The present examination also revealed a remarkable diversity in sclereid structures between male and female individuals of species of *Gnetum* in Africa. The stellate type of sclereid was present in all the African species of *Gnetum* studied, but varied in structure from four to seven armed. They were heterogenous, varying in number on both surfaces of the leaf and in number and shape within and between the species examined. They correspond to the uncommon complex type of sclereids, the astrosclereids with diverse shapes and sizes (Ananda & Bhupal, 1973; Chiang & Huang, 1984). The length, size and density unlike the type of these sclereids varied greatly and this might be in response to changing environmental conditions (Metcalfe & Chalk, 1950). Nevertheless, the sclereid characters (e.g. the acuteness or shape of the arms, the thickness of the lumen) could be valuable for interpreting and comparing not only the female and male plants but the African species of *Gnetum* as a whole.

With regard to the vascular system of petiole and especially, in terms of number of vascular bundles, the species of *Gnetum* in Africa were all similar. As a result, the number of vascular bundles was inadequate to distinguish between species. However, there was a degree of variation in the shape, nature and extent of the region between the two middle vascular bundles. The distance between the vascular bundles can help to distinguish between species in general. These distinguishing characters of taxonomic value were also observed in the petiole anatomy of *Eremomastax polysperma*, *Justicia insularis* and *Asystasia gangetica* (Essiett, 2010).

The vascular bundles in the African *Gnetum* elements appeared to have undergone a first dichotomy before separating from the central vein and then a second division towards the margin as they were arranged in pairs on each side of the cross section. There was thus a reasonable distance between them, especially between the two middle vascular bundles. Jauregui (2005) had identified this distance as an important distinguishing feature for the *Gnetum* species in Venezuela and described it as the extent of the regions between parenchyma bundle sheaths. This was considered as either narrow or broad. In all the species of *Gnetum* examined, the vascular structure consisted of four bundles grouped

towards the centre at the adaxial side forming a semi circle. The vascular system has been considered important in various studies of petiole anatomy (Essiett, 2010).

In all the African species of *Gnetum* studied, the parenchyma was thin as these plants are mostly found in shaded areas; a single layer of spongy parenchymatous cells was reported in the the mesophyll of *G. africanum* (Duthie, 1912). The difference in the number of layers of parenchyma cells observed in the various *Gnetum* species in Venezuela was taxonomically significant and the thick parenchyma region presented distinct characters for the delimitation of these species (Jauregui *et al*., 2005). Some other characters used elsewhere such as the presence of hypodermis, crystals, density, layout and number of vascular bundles (Olowokudejo, 1987; Kuo-huang, 1990; Kocsis, 2005; Akcin et al., 2011), when applied to the African species of *Gnetum* gave no clear distinction of the various species. Nevertheless, other major characters were found to be useful in the clarification of *Gnetum* species in Africa.

As mentioned, female plants presented almost as many similar anatomical features relevant in distinguishing species of African *Gnetum* as the corresponding male individuals. Most of the characters found in the male specimens were represented in the females, some at varying levels, and the female could possibly be matched to a corresponding male in the genus *Gnetum* in Africa. In sum, based on anatomical features of the leaf blades and petioles, male and female plants showed similar structures that facilitate within species matching. The relative homogeneity of the many anatomical characters in the group might reflect the close genetic relationships existing between male and female individuals and among the species as a whole. However, anatomical characters appear to be of greater value at the generic and higher taxonomic levels in determining clearer relationships than at the species and below species levels (Radford, 1986).

One of the widespread values of *Gnetum* is the use of its leaves as a major component in ‘eru’ meals. This anatomical study has revealed that the leaves of male and female plants are similar; hopefully, they might have similar taste or

chemical and genetic composition. This is an hypothesis that needs to be tested. However, perhaps one could anticipate that the leaves derived from either male or female plants from the same species have no influence on the taste of 'eru', as a result leaves from either male or female individuals or mixed leaves of both individuals of the same species should have the same taste when all other factors are constant. Any variation in taste among leaves of *Gnetum* species need not be due to the sex of the plant, but may be influenced by which species of *Gnetum* is collected. Investigating taste versus various forms of *Gnetum* could therefore be conducted regardless of the source of collection in terms of sex of the plant. This knowledge is thus essential when addressing some of the questions in the next stage, i.e. Chapter 7.

6.5 Conclusion

6.5.1 Evidence for interspecific relationships in the genus

The results based on anatomical features have indicated some usefulness in the clarification of the relationships within and between species of *Gnetum* in Africa in confirming the homologies of their morphological character states. There is a high degree of similarity between male and female individuals of species of *Gnetum* in Africa with respect to the leaf and petiole anatomy. Therefore, the male and female plants of the four forms of *Gnetum* respectively are similar but species can be distinguished from each other on the basis of certain characters.

Anatomical features of petiole which seems to be of taxonomic value are the size, shape and distance separating vascular bundles, while those of the leaf surface are size of stomata on both surfaces, the width of epidermal cells and the nature of the lower and upper epidermal walls, plus the distribution of astrosclereids. The anatomical study of *Gnetum* in Africa has strengthened the evidence to clarify the relationship between male and female specimens and the similarity among species. The anatomical characters are thus found useful here in addressing the specific identification issue concerning matching the female to the respective male plants.

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APPENDIX

Specimens of African *Gnetum* species used for the anatomical study

Species	Type of specimen	Locality	Collector and specimens number from YA
Gnetum africanum	female	Chailly, Congo	*Sita* 4079
G. africanum	female	Miang, Cameroon	*Letouzey* 14714
G. africanum	male	Cameroon	*Setten* 346
G. africanum	male	Faremla, Angola	*Gossweile*r 5580
G. buchholzianum	female	Kribi, Cameroon	*Bos* 2993
G. buchholzianum	female	Nkam, Cameroon	*Leeuwenberg* 9892
G. buchholzianum	male	Ntui, Cameroon	*Letouzey* 9754
G. buchholzianum	male	Sa'a, Cameroon	*Biye* 216
G. interruptum	female	Ma'an, Cameroon	*Raynal* 10247
G. interruptum	female	Kribi, Cameroon	*Bos* 4068
G. interruptum	male	Eala, Congo	*Corbivie*r 936
G. interruptum	male	Congo	*Louis* 12038
G. latispicum	female	Ovoabang, Cameroon	*Biye* 304
G. latispicum	female	Mopwo, Cameroon	*Letouzey* 5249
G. latispicum	male	Melen, Cameroon	*Biholong* 258

CHAPTER SEVEN

EVALUATION OF TASTE VARIATION BETWEEN VARIOUS SPECIES AND POPULATIONS OF *GNETUM* L. IN AFRICA: A CONTRIBUTION TO SELECTIVE CULTIVATION

CONTENT

7.1 **Introduction**

Background knowledge:

Significance of African nutritional resources

Conservation strategy

Evaluation of taste ability

Factors influencing plant taste

Problem statement:

The provenances of *Gnetum* population or species with good taste

Type of leaves of *Gnetum* to promote for sustainable utilization

Purpose of this research

Aim and key questions

7.2 Materials and methods

7.3 Results

Respondents' identification and questionnaire survey data

Gnetum groupings and taste analysis

The effect of provenance on *Gnetum* taste

Leaf developmental stage and taste of *Gnetum*

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7.4 Discussion

Correlation of taste with *Gnetum* forms

The influence of the *Gnetum* provenance on taste of 'eru'

The effect of the leaf age on the taste of 'eru'

Implication of the effect of tasting 'eru' to selective cultivation and conservation of *Gnetum*

7.5 Conclusion

REFERENCES

APPENDIX

Abstract

The leaves of *Gnetum* L. are important as a source of food and income to the people living around where it grows. To sustain these plants so that lives of those who depend on it are not affected in the near future, strategies are required to improve not only its productivity but also the method and extent of its harvesting. Thus this study investigates variation in taste of the leaves of *Gnetum* ('eru') meal with respect to species, provenance and age of leaf, so as to select the best local form(s) to propagate.

Twenty-five questionnaire surveys and 320 taste analyses of 'eru' meals were carried out to identify various elements related to species, provenance, and/or the age of the leaf likely to contribute to obtaining the best 'eru'. The leaves of *G. buchholzianum* provided the best tasting 'eru', closely followed by *G. africanum* and *G. latispicum*. *Gnetum* leaves obtained from most of the regions under study generally proved to be palatable for 'eru' meals, while young leaves tasted best among the three developmental stages investigated. These results permit the identification of genotypes suitable for propagation and improvement strategies that could be considered when sustaining species of *Gnetum*.

Keywords: Africa, Ecology, 'Eru', *Gnetum*, Physiology, Taste.

7.1 Introduction

7.1.1 Significance of African nutritional resources

Africa is a source of well known traditional food crops of worldwide, regional or local importance and the crops are also used as a source of income and/or employment. Moreover, forest species in particular are known to improve soil water-holding capacity, increase biological activities, suppress weed growth and decrease effects of diseases and insects (Onguene & Kuyper, 2001; Baligar *et al*., 2008). The genus *Gnetum* L., family Gnetaceae, is one of the most commonly consumed plants found growing naturally in the humid tropical rainforests of Africa. It rarely grows in gardens around villages, and then it serves mainly as a source of vegetable to sustain the family. The cultivation of *Gnetum* in farmland is not a common practice except in one or two places around Limbe and Kumba in the South West regions of Cameroon, where domestication trials have been conducted (Tekwe *et al*., 2003). All the African species of *Gnetum* are climbers and the leaves of this dioecious gymnosperm are opposite, entire, and net-veined, resembling those of dicotyledons. These leaves have a high economic value and are used for food at all levels of society not only in Africa, but also in some other parts of the world. It is quite common to find meals made of *Gnetum* leaves in several African restaurants and specialized African eating places in Europe and the USA (Tabuna, 1999; Tekwe *et al*., 2003).

Gnetum species play a crucial role in the lives of the people where they grow, particularly in local communities. Trade in *Gnetum* is one of the few income generating activities for women in marginalized rural areas and the species of this genus are reported to be the most exploited and commercialized vegetable in Cameroon (Besong *et al*., 2001). Depending on the ethnic groups using the *Gnetum* leaves, many local names are used across West and Central Africa, including ‘Eru’ or ‘Okok’ in Cameroon, ‘Mfumbwa’ or ‘Fumbwa’ in Kikongo in Congo, ‘M’fumbua’ or ‘Fumbua’ in Angola and ‘Koko, ‘Afang’, ‘Okazi’ or ‘Ukazi’ in Nigeria (Clark *et al*., 2004). *Gnetum* is commercially very important, for it is sold and exported. Nutritionally, *Gnetum* leaves are known to be rich in

minerals and protein (Sunderland & Clark, 2001), and to contain the eight essential amino acids in significant quantities (Mialoundama, 1993), and therefore can prevent malnutrition. Above all, economically, *Gnetum* contributes to poverty alleviation for the local women and children who collect and sell it all year round. Furthermore, *Gnetum* is shipped in considerable quantities from Cameroon to Nigeria to be used locally in the manufacture of a local gin (Burkill, 1994) and is also exported to Europe, USA and various African countries. A lot of *Gnetum* is gathered from the wild in a destructive way. There is therefore a rapid loss of *Gnetum* populations and its possible disappearance from the forest poses a great threat to human well-being.

7.1.2 Conservation strategy

Conservation is a major focus of environmental sciences and the main aims of a conservation strategy are not only to sustain vital ecological processes and the ecosystem to prevent habitat destruction but also to protect genetic diversity and to enable the sustainable utilization of species and their environment (Hambler, 2004). *Gnetum* species are valuable to people through a range of products and services and so ought to be preserved. They are therefore in need of a conservation strategy that may either prevent wild harvesting or lead to large-scale or household scale propagation of selected morphs of *Gnetum*. Measures undertaken for conservation purposes could be direct (e.g. domestication or creation of reserves etc.) or indirect such as applied in this study where taste testing is used in order to contribute towards sustainable use of *Gnetum* species in Africa.

The species that should be cultivated according to preferred taste and the parts of the plant, which should preferentially be used, should be identified prior to domestication. Shade leaves are recommended for the best tasty‘eru’ meal and these have been obtained with 50% net covering of plants under cultivation (Ndam *et al* ., 2001). To ensure the sustainability of *Gnetum*, the plants to provide leaves for ‘eru’ could be cultivated instead of harvesting the wild individuals.

Cultivation seems to offer an alternate source for harvesting for food, thus, the pressure on wild population is reduced.

7.1.3 Evaluation of taste ability

Plants not only secrete natural anti-feedants and other toxins as a means of protection, but also contain some bitter compounds that are medicinal (Drewnowski and Gomez-Carneros, 2000). Compounds that cause the plant to generally taste bitter, sweet, or sour rarely undergo any change in composition or structure during the developmental stages of the plant, although the concentrations might vary. Therefore, the plant retains its taste, but there are often fixed perceptions of taste based on the dislike of the bitterness of some plants even though they might be of medicinal value (Richerson and Boyd, 2004).

The ability to taste varies with individuals; some people have an enhanced ability to taste (hypergeusia) while others are either completely unable to taste (ageusia) or their ability to taste is greatly reduced (hypogeusia). Although there are five primary tastes: bitter, salty, sour, sweet, and savoury, and all other tastes result from a combination of these (Huang *et al*., 2006; Collings, 1974), a meal may be described as tasty, palatable, delicious, delectable or as tasteless, unpalatable, stale, unsavoury, etc. A particular taste can be judged "good" or "bad" or, when based on certain experiences, distinctions can be made between tasty and tasteless or unpalatable meals made of specific ingredients.

There is evidence that the ability to taste thiourea compounds indicates the capacity to taste a diet and the commonest of these compounds is phenylthiocarbamide (PTC) or phenylthiourea which is an organic compound (Wooding, 2006). PTC tastes either very bitter or practically tasteless depending on the genetic make-up of the taster. The ability to test PTC is a dominant gene and a good taster should taste PTC, although, the sense of taste might deteriorate with age in certain individuals (Koepnick, 2010). The PTC- blotting paper method has been described as unreliable and the use of Harris and Kalmus' (1949) eight-glass test for sensitivity to taste various substances was recommended (Swinson,

1973). Because PTC appears to have a strong genetic correlation that allows identification of individuals with the ability to taste, it has been used in this study.

The taste of a meal can be distinguished as either 'good and delicious' or 'poor and unpalatable' by the gustation method. Gustatory sensation tests have proven to be successful in the evaluation of taste in several studies, for example, Kataoka *et al*. (2005) investigated the taste and smell of commercial bottled drinks in Japan and classified 15 drinks into 4 groups based on palatability score. Nakamura *et al*. (2002) studied the mechanism of bitterness suppression of quinine, while Ishizaka *et al*. (2004) evaluated the bitterness in medicines used for children. These authors demonstrated the usefulness of taste sensor in predicting the bitterness in medicines. The taste of 'eru' meal is examined here based on human gustatory sensation test and artificial taste of the main component of 'eru', the leaves of *Gnetum*. Maintaining all other constituents constant and the only variable being *Gnetum* leaves, it might be possible to deduce that any change obtained in tasting 'eru' would absolutely be due to the effect of the *Gnetum* leaves. The most tasty genotypes to improve and promote for conservation of *Gnetum* could thus be identified.

7.1.4 Factors influencing plant taste

Several factors such as the genetic composition of the plant, its physiological condition and ecological interactions can influence its taste and these are interrelated. The genetic constitution of a plant for example, is constant and determines both its nature and its response to environmental changes (Bonner *et al*., 1959). Measurements of gross morphological structures (length, number and form of various plant parts) have been common in assessing genetic composition, but many other features such as chemical, ecological and physiological characters can be used as well (Given, 1994; Chapter 2, 3 & 5). Thus, careful analysis of macro characters and their variation allows for an indirect examination of the genetic composition (Ashton, 1987), although examination of the DNA will provide direct genetic evaluation of population structure (Moran & Hopper, 1987).

Among factors that influence the taste of plants is their physiological condition. Work was performed on the physiological state of *Gnetum* by assaying the proximate nutritional substances and fibre characters in relation to the leaf age in three varieties of *Gnetum* (Isong et al., 1999). A positive correlation was found between crude fibre length and age, whereas crude protein, in vitro digestibility and fibre width were negatively correlated with age. Therefore, it was assumed that the developmental age of leaves could affect the texture of the leaves of *Gnetum*, although the texture has no apparent direct influence on taste. More likely, the texture will affect ease of preparation, ease of chewing and digestibility. However, the texture of the leaf varied with its age and might subsequently influence the nature of the meal; this is a hypothesis that could be tested.

The taste based on the age of the leaf depends largely on the physiology of the leaf. Either metabolic products or the utilization of biosynthetic pathways as chemical proof (Radford, 1986) generally evidences physiological effects of organisms. It has also been demonstrated that physiological condition in natural populations of plants is related to a range of environmental factors (Crawford & Julian, 1976; Giannasi & Crawford, 1986). Ecological evidence usually deals with plant-environment interactions (Izco, 1980), which include the effects of the environment on plant features. The evidence is based on data from the soil (e.g. soil temperature and moisture), atmospheric temperature, humidity, light and rainfall (Searle, 1973); for example, differences in soil type usually have an effect on plant physiological conditions.

The ecological factors constituting part of the climate in which the plant occurs generally regulate its growth; organisms are sensitive to any fluctuation in climatic components (Bonner *et al*., 1959). Thus, plant growth and development are due to the interaction between their genetic constitution and environmental factors. Elsewhere, habitat types and associated species are also significant sources of ecological data and these could have the greatest impact at the species and infra-species levels (Stuessy, 1990). Taste might be influenced by environmental factors and could vary from one form of *Gnetum* to another. This

might thus provide a possible explanation for various plant tastes, although it is difficult to differentiate genetic from environmental effects on the basis of morphological variability often observed in plants.

7.1.5 Problem statement

Taste appreciation of *Gnetum* was carried out in Cameroon by Ndam *et al*. (2001) who arbitrarily sampled the opinion of twenty-three people after they had tasted cooked leaves harvested from three different light environments and scored the meals as very good, good, fair or poor. It was established that 80% shading was required to obtain good tasting *Gnetum* for 'eru' meals. Farmers involved in the domestication of *Gnetum* in the South West Region of Cameroon have indicated that its taste varies with the age of the leaves; they assumed that younger leaves were more bitter than the older ones (farmer *pers. com*.). Clearly, important questions need to be addressed concerning where or how the better tasting leaves for 'eru' can be obtained to minimize the massive destruction of the plants. It will also be of interest to determine whether the taste of *Gnetum* is affected by the age of the leaves or the environment.

Gnetum is threatened due to the extent and method of harvesting and the devastation of the forests in which it grows (Chapter 2). The application of conservation strategies is now more than ever necessary due to an increase in demand for *Gnetum* directly proportional to an obvious increase in human population. *Gnetum* is thus diminishing in the wild or its natural habitat for it is a constant source of food. Moreover, as the entire plant is uprooted during harvesting, it has therefore become rare in some provinces of Cameroon and might even be at risk of local extinction.

The focus of this study is to identify not only the species but also the provenance and leaf age of *Gnetum* that should be cultivated for sustainable utilization in Africa based on comparative data gathered by taste testing. Interpretation of results obtained from taste analysis of leaves at different stages of development of the various species and from different geographical regions could be used to

positively influence modes of harvesting of *Gnetum* plants by indicating the best leaves or species to be collected and/or the best areas for collection.

7.1.6 Purpose of this research

The purpose of this study was to investigate any variation in taste among *Gnetum* species and populations with respect to their geographical distribution and the leaf developmental age. This study aimed to recognize the species to promote for cultivation of *Gnetum* in Africa and was guided by the following key questions: Do different forms of 'Eru' taste differently? If yes, are differences in its taste related to the species, provenance, and/or the age of the leaf? What are the implications for sustainable utilization or harvesting of *Gnetum*?

7.2 Materials and Methods

Taste analyses were carried out on 320 meals of *Gnetum* leaf samples collected from 10 localities in the five regions of Cameroon in which *Gnetum* grows. Cooked meals of 'eru' (the leaves of *Gnetum*) were compared to determine the factors that most likely influence any differences in taste, i.e. provenance (origin), the age of the leaf or the species of *Gnetum* or a combination of these three variables.

Twenty-five volunteers participated in the questionnaire survey of which twenty were chosen for the tasting test. The participants were briefed about the study (Appendix 7.7.1) and a questionnaire (Appendix 7.7.2) was administered. Each participant was asked to respond to a structured sequence of questions (Appendix 7.7.2); in all, twenty-five questionnaires were distributed. A respondent was handed a questionnaire and asked to complete it while in the room in order to be assisted if necessary. The data were checked for accuracy and completeness in the presence of the participants and stored in a database structure (codebook) including the name, number, address and description of the respondent, as well as the method and date of the investigation in which they participated later. Data preparation and analysis was done using the method described by Trochim (2006).

The study was based on leaves of edible *Gnetum* bought at the markets in 10 towns of five regions of Cameroon, including all available morphological forms of *Gnetum* at each town. The vegetative structures of *Gnetum* species in Africa are strikingly similar and are thus unhelpful in the accurate recognition of species. Regrettably, most of the *Gnetum* samples found in the market were sterile specimens therefore, identification of forms/species was difficult. The harvesting of *Gnetum* for market use is based mostly on the size of the leaves to be shredded and not on reproductive features which are needed for its identification. Nevertheless, sufficient leaf samples with reproductive features enabled identification to species level and provided 20 participants with meals of three different species (*G. africanum*, *G. buchholzianum*, and *G. latispicum*) to determine the species with the best tasting leaves. *Gnetum interruptum* (Chapter 3) is not used in the cooking of 'eru'. The opinion of twenty volunteers was sampled after each had eaten three 'eru' meals. Difficulties in terms of essential logistics to preserve the leaves in a good state from the forest limited an extensive search for additional samples of the various species to use in the taste analysis.

Prior to the eru tasting test, a pretest was carried out to evaluate the sensitivity of all the potential tasters to phenylthiocarbamide (PTC)-blotting paper. Although this is not the best method as mentioned earlier, it is rather used here for its strong genetic correlation that could identify individuals with the ability to taste. Only those with the ability to taste PTC were chosen. It was ensured that all the tasters' ages varied between 15 and 30 years old, the age range of those with better tasting abilities, as the sense of taste deteriorates with age (Koepnick, 2010). Although, tasting ability was usually unstable, not only between individuals but within an individual at different periods, this was often associated with differences in physiological status of the taster and could be an index of unrecognized internal conditions at a given moment (Salmon and Blakeslee 1935). For this study, only healthy individuals were selected and they refrained from eating for at least 4 hrs before the testing time.

For the taste analysis based on provenance, ten localities were randomly selected in five regions (two per region) where *Gnetum* grows naturally in Cameroon (Figure 7.1). Prior, to cooking 'eru', just as it is traditionally prepared with mixed leaves, it was ensured that the three forms of *Gnetum* were in equal proportions. The nature of the vegetation, the climate and the soil of these localities were observed for any source of additional information that might extend beyond the data collection. The influences of developmental stage (i.e. age) of the leaves on the taste were also evaluated. These leaves were collected at three different intervals on fresh stems of *Gnetum* plants from two localities in the Central Province of Cameroon. The leaves used in the meal were collected from stems that were more or less equal in size and were designated as old (those found lower on the stem), medium (midway on the branch) and young (the first ten leaves from the stem or branch top), while assuming that all stems harvested were growing at the same rate.



Figure 7.1 Ten localities in five provinces of Cameroon where *Gnetum* was collected for gustatory sensation analysis. Centre: Yaoundé (C1), Oveng (C2); East: Bertoua (E1), Lele (E2); Littoral: Yabassi (L1), Douala (L2); South: Kribi (S1), Lobe (S2); South West: Kumba (SW1), Banga (SW2).

Meals from each of the five regions were first served (Table 7.1). Then, at a later date, volunteers were served *Gnetum* leaves of three different ages from Yaoundé (Central Region). The quantity and composition of each meal was the same: made up of finely chopped *Gnetum* leaves added to *Talinum triangulare* leaves of equal proportion then cooked in palm oil and pieces of meat added to the leaf mixture. The leaves of *T. triangulare* generally soften the tough fibrous chopped leaves of *Gnetum* producing a homogenous meal. Before and after tasting one sample, volunteers gargled with water and waited for at least 20 minutes before tasting the next sample. The volunteers were asked to score the sample as 'very good', 'good', 'fair', 'poor' or 'very poor' using the values 5, 4, 3, 2, 1 respectively.

Generally the randomized block design was used as a strategy for analysis; the tasters were grouped into various blocks according to their age. The blocking overrules factors, other than treatment group status like age and gender of tasters, that are likely to affect the end results. The meals were given within blocks of adjacent volunteers as laid out in Table 7.1. The number of blocks being the number of replicates and each meal was adjacent to any other, but each meal appeared once in each row and column within the block. ANOVA for randomized block design was performed. This was used to control any significant variation (http/www.statistics.com) in the different individuals likely to affect the outcome.

Table 7.1 Sample layout of tasters for 'eru' gustatory analysis for five meals at a time; C, (Centre); E, (East); L, (Littoral); S, (South); SW, (South West) represent the collection localities and I, II, III, IV, V represent various groups organized for the tasting.

Block no.	*Taster no.*	*1st meal*	*2nd meal*	*3rd meal*	*4th meal*	*5th meal*
I	1	C	E	L	S	SW
	2	E	L	S	SW	C
	3	L	S	SW	C	E
	4	S	SW	C	E	L
II	5	SW	C	E	L	S
	6	C	E	L	S	SW
	7	E	L	S	SW	C
	8	L	S	SW	C	E
III	9	S	SW	C	E	L
	10	SW	C	E	L	S
	11	C	E	L	S	SW
	12	E	L	S	SW	C
IV	13	L	S	SW	C	E
	14	S	SW	C	E	L
	15	SW	C	E	L	S
	16	C	E	L	S	SW
V	17	E	L	S	SW	C
	18	L	S	SW	C	E
	19	S	SW	C	E	L
	20	SW	C	E	L	S

7.3 Results

7.3.1 Respondent identification and questionnaire survey data

Thirteen themes emerged from interrogating the survey data; these are grouped into two main categories: the identity of the respondents i.e. gender, age and region of origin (Figure 7.2), and their response scores to various questions (Appendix 7.7.2) on *Gnetum* (Figures 7.3 & 7.4 and Table 7.2). There were almost equal numbers of respondents based on age and region of origin and according to gender, the females comprised 60% and the males 40% of the respondents (Figure 7.2).

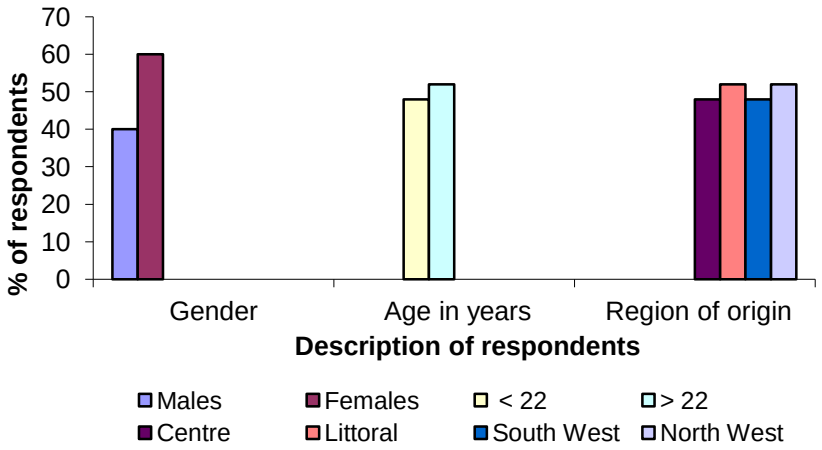


Figure 7.2 Comparison of various respondents' identification criteria used in the questionnaire focused on 'eru' taste.

Ten percent of males and 40% of females were able to recall when last they ate 'eru', although 100% of males indicated that there was a significant variation in 'eru' taste compared to only 75% of the female scores (Figures 7.3a & b). Based on taste versus provenance of *Gnetum*, there was an equal response from both males and females, 75% of either males or females had never wondered about the origin of the *Gnetum* in the meal. More than three-quarters of either males or females had never heard of 'eru' taste based on leaf age while the remaining males and females respectively left the question blank (Figure 7.3a & b). Over 50% of both male and female respondents eat 'eru' on a weekly basis and less than half of the respondents use a mixture of young, medium and mature or old leaves for 'eru' (Figures 7.3c & d).

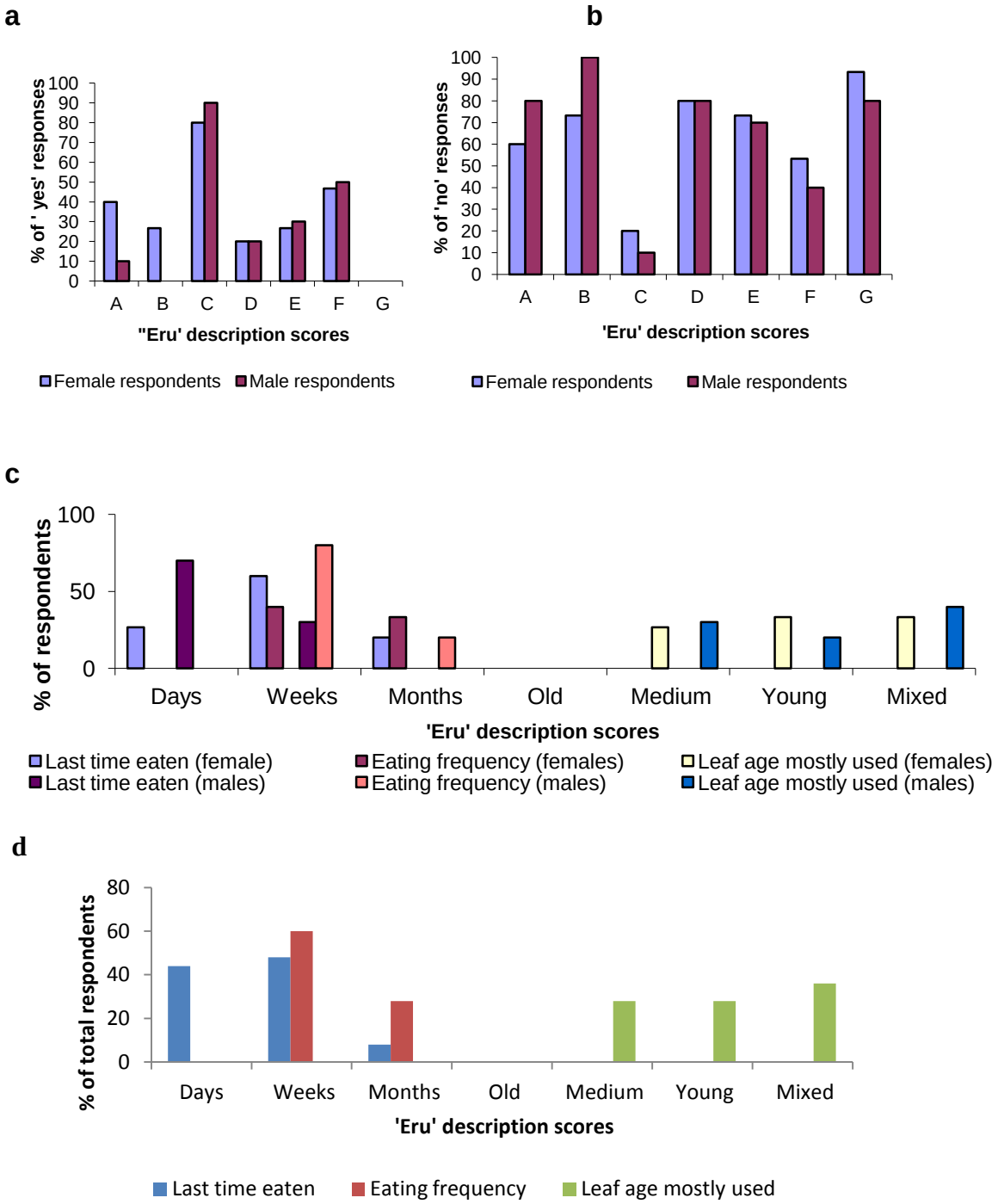
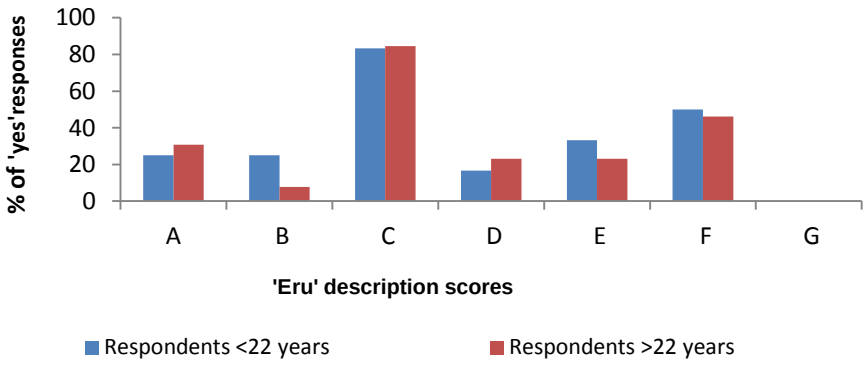


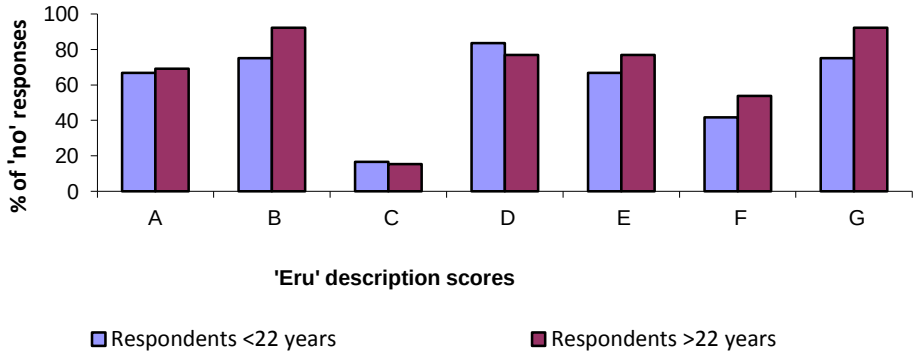
Figure 7.3 Comparison between gender of the respondents and their response scores to ten questions on 'eru', (*Gnetum*), total respondents males = 10 and females = 15. (a) male & (b) female percentage responses ('Yes' or 'No' respectively) to A, remembering the number of times the respondent had eaten 'eru'; B, taste always the same; C, awareness that taste differs; D, inquire about provenance; E, wonder about taste linked to provenance; F, heard of regional preference; G, heard of leaf age preference. Separate males and females % (c) & combined males + females (d) percentage responses related to the last time 'eru' was eaten, the frequency (how often it was eaten) and leaf age.

Figures 7.4a & c and Figures 7.4b & c show the percentage of respondents below and above twenty-two years old respectively and their responses to *Gnetum* description themes (above 21 years, one is considered mature in Cameroon). The pattern of distribution of various scores by respondents of both age groups was similar except that on the questions on regional preference of 'eru', fewer respondents younger than 22 attested to having heard of 'eru' preference based on collection localities compared to individuals above 22 years old. On the question as to when last 'eru' was eaten, over 62% of the participants younger than 22 years responded on 'weekly basis' while 58% of those above 22 said on 'daily basis' (Figure 7.4c). Although more individuals above 22 years preferred either medium aged or a mixture of leaves than those below 22 years, those who preferred the young leaves to either medium or mixed leaves, had an almost equal score when considering both groups of participants (Figure 7.4c).

a



b



c

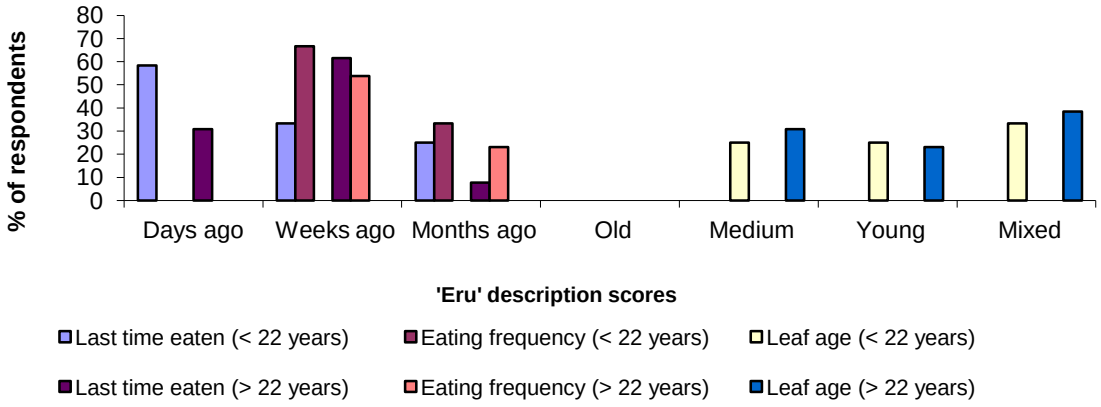


Figure 7.4 Comparison between respondents' age in years and their response scores to eight questions on 'eru' (*Gnetum*); total respondents <22 = 12 and >22 = 13. Percentage of respondents below (a) and above (b) twenty-two years and their responses ('Yes' & 'No') to A, remembering the number of times the respondent had eaten 'eru'; B, taste always the same; C, awareness that taste differs; D, inquire on provenance; E, wonder on taste linked to provenance; F, heard of regional preference; G, heard of leaf age preference; (c), the percentage of respondents of both ages, i.e. below and above twenty-two years, and their responses to the last time 'eru' was eaten, the frequency and leaf age use

The results based on the region of origin (Centre, Littoral, South West and North West in Fig. 7.1) of the respondents and their responses to question 12 (Appendix 7.7.2) regarding the age of leaves used in the cooking of 'eru' were inconsistent; the leaves used mostly varied between young, medium and mixed leaves (Figure 7.5). The responses from the Littoral participants did not vary (Table 7.2 & Figure 7.5), their choice of young leaves was total and 'eru' was frequently depending on individuals. Based on the leaf age mostly used (Figure 7.5), less than half of respondents from the Central region favoured either mixed, young or medium leaves. Most respondents from the South West region mostly use medium leaves and very few use young leaves; 75% of participants from the North West region preferred mixed leaves, followed by 12.5% for either medium or young leaves respectively. It was interesting to compare the responses of those from the North West region, a grassland region where *Gnetum* does not grow to the responses from other regions. The North West participants prefer 'eru' made from a mixture of leaves of various ages than that made from either young or medium leaves.

Table 7.2 Comparison between respondents' region of origin (number in bracket) and their response scores to eight questions on 'eru', (*Gnetum*). The percentage of respondents from the various regions of origin to respective *Gnetum* description themes were based on: A, remembering the number of times the respondent had eaten 'eru'; B, taste always the same; C, awareness that taste differs; D, inquire on provenance; E, wonder on taste linked to provenance; F, heard of regional preference; G, heard of leaf age preference. A dash indicates a blank for unanswered questions; 0 means the alternative had a total response

Respondent's origin (no. involved) → Description ↓	**Centre (7)**		**Littoral (3)**		**South West (8)**		**North West (7)**	
	% of		**% of**		**% of**		**% of**	
	Yes	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**
A	58	42	0	100	18	82	78	22
B	58	42	0	100	0	100	10	90
C	85	15	100	0	62	38	100	0
D	0	100	0	100	18	82	22	78
E	17	83	100	0	18	82	17	83
F	42	58	50	-	65	35	38	62
G	-	85	-	50	0	100	0	100

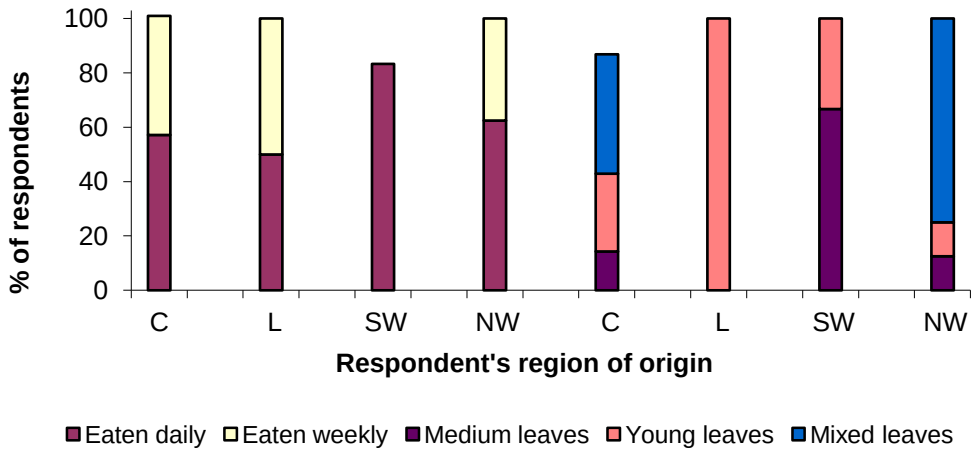


Figure 7.5 Comparison between respondents' region of origin and their response scores to the questions on consumption frequency and leaf age used for 'eru' (*Gnetum*). Regions: C, Central; L, Littoral; SW, South West; NW, North West

The various responses describing *Gnetum* could further be categorized as: consumption rate, taste diversity, 'eru' provenance and leaf age. On the basis of combined rating (Figures 7.3 & 7.4 and Table 7.2), more than 60% of the respondents had eaten 'eru' a hundred or more times and that it was not long since they had the meal; this was termed 'consumption frequency' (Figure 7.5). Over half of the respondents agreed that the taste of 'eru' varied but provided no reason for this variation. Twenty percent of the respondents suggested the amount of ingredients added or the method of cooking the 'eru' meal as a possible reason. Little attention was directed to the provenance of the *Gnetum* obtained from the market and their relation with taste. In terms of the last two questions (Appendix 7.7.2), the usage of young and the mixtures of leaves was established by 100% response.

Four of the fifteen variables were significantly different as shown in the analysis in Table 7.3 ($p<0.05$), viz the frequency at which 'eru' is eaten; the differences in 'eru' taste; the actual age of leaves used and the leaf age preference in terms of gender and age of the respondents respectively. In addition, in terms of the region of origin, there was a highly significant difference ($p<0.01$) based on the number of times, and the last time the respondent ate 'eru', the similarities and differences in the taste, taste depending on the provenance and the preferred provenance.

Table 7.3 Significant variation in the different respondents involved difference in survey questionnaire variables; tests are significant at * for $p \leq 0.05$ and ** for $p \leq 0.01$ (Statistics.com)

Subject → / Respondent	A		B		C		D		E		F		G		H		I		J	
	Z	p-value	Z	p-value	Z	p-value	Z	p-value	Z	p-value	Z	p-value	Z	p-value	Z	p-value	Z	p-value	Z	p-value
Gender	0.26	0.79	0.000	1.000	3.62	0.0003**	1.336	0.182	2.77	0.0056**	1.21	0.228	0.603	0.547	0.603	0.547	4.13	0.00003**	2.475	0.0133*
Age	0.87	0.37	0.32	0.75	3.61	0.0003**	2.21	0.027*	2.22	0.026*	1.66	0.096	1.21	0.228	0.0000	1.000	4.13	0.00004**	2.85	0.004**
Origin	1.94	0.052	1.92	0.055	0.87	0.382	2.12	0.033*	3.395	0.00069**	1.31	0.19	1.92	0.055	2.29	0.022*	0.29	0.79	1.565	0.117

A–Number of times ‘eru’ meal was eaten; B–Time when ‘eru’ meal was eaten last; C-How frequent ‘eru’ is eaten; D–Same ‘eru’ taste; E–Awareness of any taste difference; F–Inquire about ‘eru’ provenance; G–Does ‘Eru’ taste depend on provenance? H– Preference of ‘eru’ from a specific locality; I–Type of leaves used; J–Preference of a particular leaf age.

7.3.2 Taste analysis of 'eru' leaves

The three main components of this investigation are the taste of *Gnetum* according to species as identified in Chapters 2 & 3 and/or provenance, and the effect of leaf age on taste of *Gnetum.*

Gnetum species and their taste analysis

The three species of *Gnetum* used in the taste analysis were *Gnetum africanum, G. buchholzianum and G. latispicum.* The taste of all three species of *Gnetum* in all the provinces tested was appreciated with a mean score > 0.3 (Table 7.4). *Gnetum buchholzianum* was found to have the highest average scores implying that this form produced the best tasting 'eru', followed by *Gnetum latispicum* and *G. africanum* (Figure 7.6). A similar result was obtained from the combined scores (Figure 7.7), *G. buchholzianum* is identified as having the best taste score (94%), followed by *G. africanum* (78%) and then by *G. latispicum* (77%). The species of *Gnetum* with the best taste could be collected from the East and South West and then South regions (Table 7.4).

Table 7.4 Distribution of taste scores for various species of *Gnetum* with respect to region of collection. Central 1 (C1): Yaoundé, Central 2 (C2): Oveng, East 1 (E1): Bertoua, East 2 (E2): Lele, South 1 (S1): Kribi, South West 1 (SW1): Kumba. M = mean scores

Source of leaf →	***Gnetum africanum***			***Gnetum buchholzianum***			***Gnetum latispicum***		
Scores (points) ↓	C1	E1	M	E2	S1	M	C2	SW1	M
Very good = 2	2	6	4	16	10	13	6	10	8
Good = 1	9	7	8	5	8	6.5	9	9	9
Fair = 0	0	0	0	0	0	0	0	0	0
Poor = -1	-1	-6	-3.5	-1	0	-0.5	-3	-1	-2
Very poor = -2	-4	0	-2	-2	0	-1	-2	-8	-5
Average score	**0.3**	**0.35**	**0.33**	**0.9**	**0.9**	**0.9**	**0.5**	**0.5**	**0.5**

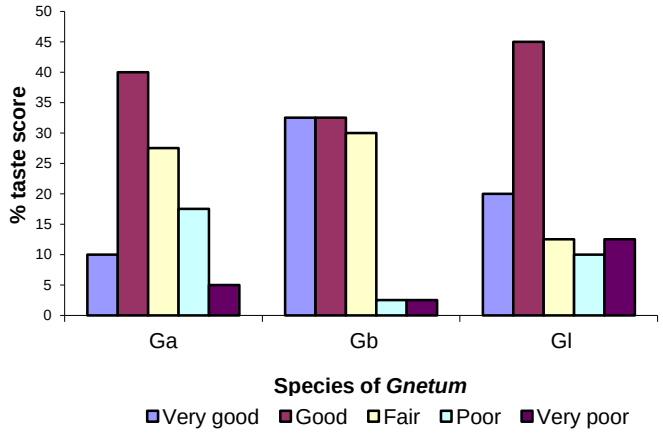


Figure 7.6 Distribution of taste score according to various species of *Gnetum* used in 'eru'. *Gnetum africanum* Ga; *Gnetum buchholzianum* Gb; and *Gnetum latispicum* Gl

The effect of provenance on *Gnetum* taste.

The average taste scores of *Gnetum* leaves with respect to provenance (Table 7.5; Figure 7.7) and the influence of geographical distribution on the taste based on combined rating as either good (very good + good) or fair or poor (poor + very poor) meal indicated that over 87% of tasters found the meal generally palatable and highly appreciated as compared to only about 12% who found the meal unsavoury.

Table 7.5 Taste scores for cooked *Gnetum* leaves in ten localities in Cameroon from 20 tasters. Centre 1 (C1): Yaoundé, Centre 2 (C2): Oveng, East 1 (E1): Bertoua, East 2 (E2): Lele, Littoral 1 (L1): Yabassi, Littoral 2 (L2): Douala, South 1 (S1): Kribi, South 2 (S2): Lobe, South West 1 (SW1): Kumba, South West 2 (SW2): Banga

Locality → **Scores (points) ↓**	**C1**	**C2**	**E1**	**E2**	**L1**	**L2**	**S1**	**S2**	**SW1**	**SW2**	**Total scores (points)**
very good = 2	2	6	6	16	0	2	10	10	10	2	**64**
Good = 1	9	9	7	5	1	3	8	8	9	3	**62**
Fair = 0	0	0	0	0	0	0	0	0	0	0	**0**
Poor = -1	-1	-3	-6	-1	-10	-3	0	-6	-1	-5	**-36**
very poor = -2	-4	-2	0	-2	-4	-6	0	-2	-8	-4	**-32**
Average score	**0.3**	**0.5**	**0.35**	**0.9**	**0.65**	**0.2**	**0.9**	**0.5**	**0.5**	**-0.2**	**2.9**

The ‘eru’ from Littoral Region (L1 and L2) was considered as not tasty while the one from Douala in the South (S1) was palatable to all the twenty volunteers (Table 7.5). Ranking based on combined ratings indicates that eru from Lobe in the South West Region (SW1) had the best taste as indicated by 70% of the participants, followed by eru from East E2, South S1 and S2 (65% of tasters) and the Central Region C2 (60% of tasters) while the most poorly rated tasting eru was obtained from the Littoral Province (Table 7.5; Figure 7.7). Although most volunteers rated the ‘eru’ from the regions East E2, South S1 and S2, the Central C1 and C2, South West SW1 as palatable, however, both very good and poor tasting meals originated from SW region. The ANOVA analysis (Statistica 6.0, 1984 - 2002) showed that there was a highly significant difference, p-value ($p<0.0001$) between ‘eru’ taste scores for the ten regions ($\chi^2= 41.06567$).

The forest where *Gnetum* mostly grows was observed to be dense with a closed canopy and an abundance of tree species in the East, South, Centre and part of the South West provinces. Around the Littoral Province and coastal areas of the South West, the forest was more secondary and abandoned farmland with less shading from sunlight. The soil was light, powdery, and either volcanic as in Banga in South West 2 or sandy as in Douala (Littoral 2) or clayey as in the Yabassi (Littoral 1), while the soil in the dense forest area where *Gnetum* with good tasting leaves readily grows was firm and ferralitic**.**

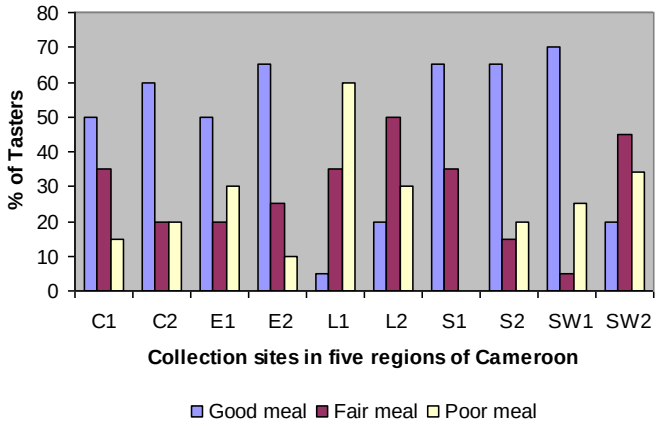


Figure 7.7 Taste correlation with provenance of *Gnetum* cooked leaves expressed as % of tasters who scored the meal on combined rating (very good + good), fair and (poor + very poor) against localities of collection. Central 1 (C1): Yaoundé, Central 2 (C2): Oveng, East 1 (E1): Bertoua, East 2 (E2): Lele, Littoral 1 (L1): Yabassi, Littoral 2 (L2): Douala, South 1 (S1): Kribi, South 2 (S2): Lobe, South West 1 (SW1): Kumba, South West 2 (SW2): Banga.

The leaf developmental stage and taste of *Gnetum*

Three categories of leaves were used and 20 volunteers tasted the 'eru' meals. The results indicate that 89% of the tasters scored the leaves used in 'eru' meal as palatable and the young leaves were very much preferred as indicated by 75% of the tasters; the average scores established in Table 7.6 for young, medium and old leaves are 1.25; 0.5 and 0.15 respectively. Over 30% of the tasters qualified the old leaves as producing a poor tasting 'eru' meal and some 60% of tasters recorded the young leaves as 'very good' for an 'eru' meal and 55% rated medium leaves as 'good' (Figure 7.8). The distribution of taste scores for the various meals was ranked based on combined ratings ("very good + good" and "fair + poor") as most preferred and least preferred meals. In this ranking young leaves are most suitable (75%) for 'eru' meals, followed by medium leaves (55%) and finally old leaves (30%). The colour of the old leaves after cooking was an unattractive dull brownish green compared to the shiny attractive greenish colour of the young leaves. ANOVA analysis of the scores revealed that there was a highly significant difference p-value ($p < 0.0069$) among tasters of eru cooked with different aged leaves ($\chi^2 = 9.9487$).

Table 7.6 Distribution of taste scores with respect to age of cooked *Gnetum* leaves collected from the Central province

Type of leaves→ **Scores ↓**	**Young**	**Medium**	**Mature**	**Total**
Very good = 2	24	2	6	**32**
Good = 1	3	11	3	**17**
Fair = 0	0	0	0	**0**
Poor = -1	-2	-3	-6	**-11**
Average score	**1.25**	**0.5**	**0.15**	**1.9**

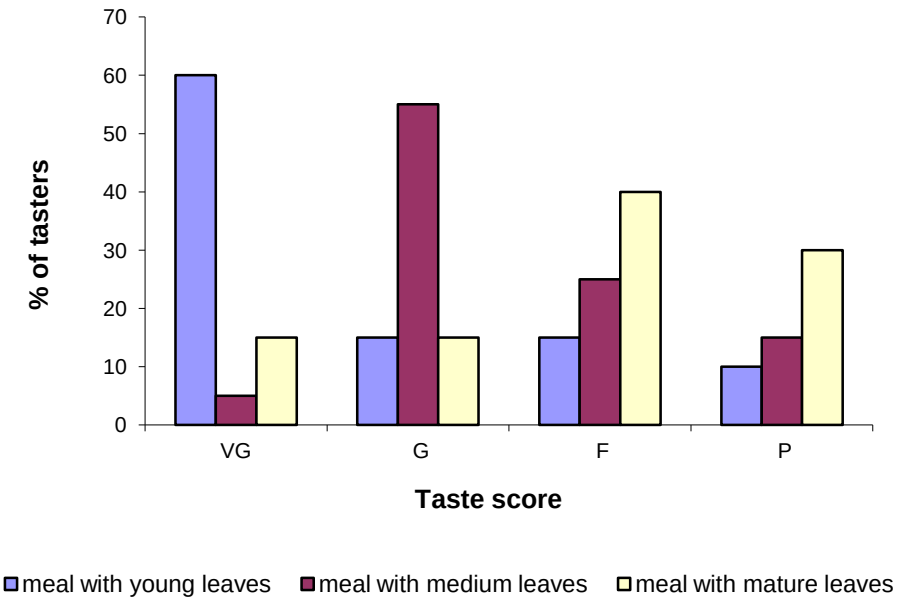


Figure 7.8 Taste appreciation of different ages of *Gnetum* leaves used in cooking 'eru'. (VG= very good, G = good, F = fair, P = poor)

7.4 Discussion

'Eru', irrespective of the province of origin of tasters, seems to be well appreciated and eaten by all age groups; male or female. There is evidence that the taste of the leaves varies according to geographical origin (Table 7.5 & Figure 7.7), age (Table 7.6; Figure 7.8) and species of *Gnetum* (Table 7.4; Figure 7.6). The variation in taste is usually negligible and depends on an individual's tasting ability, but these factors hardly influence the high rate of consumption of 'eru' worldwide.

7.4.1 Correlation of taste with genetic data

'Eru' resulting from the leaves of various species of *Gnetum* was generally delectable with an average score of over 3.5. The three different morphological forms of *Gnetum* identified prior to the taste analysis of 'eru' meal: *G africanum*, *G. buchholzianum* and *G. latispicum* tasted differently, as indicated by the various average taste scores 0.33, 0.9 and 0.5 respectively. Although the disparity in taste among the species of *Gnetum* could be due to genetic differences, or environmental conditions or the physiological state of

both plant and taster for example, the results of the taste scores were only slightly different reflecting similarity in taste between the species. Nevertheless, although the taste of all three species included in this study was satisfactory, *G. buchholzianum* was recognised as the most palatable *Gnetum* species in Africa. The slight differences in taste might reflect the genetic variation among the species (Chapter 5).

There were some differences in the leaf morphology of the three species as well as in their taste. For example, the leaves of *G. buchholzianum* with the best taste were mostly larger, softer and more easily shredded than those of either *G. africanum* or *G. latispicum*. *Gnetum africanum* had very tough leaves that were more difficult to shred. However, the leaves of the three species identified were normally large enough to be shredded easily, although there was a high within species variation (Chapter 2 & *per. obs*.), moreover, large leaves are also adaptations to capture maximum sunlight essential for growth (Chitwood *et al.*, 2012). *Gnetum buchholzianum* (average score 0.9) has been established as the best form for 'eru' meals as indicated by the results. Its average taste score was about thrice that of *G. africanum* and almost twice that of *G. latispicum*. *Gnetum buchholzianum* could therefore be the species to be promoted for cultivation, followed by *G. latispicum* and then *G. africanum*.

As mentioned, only three species of *Gnetum* are used in 'eru' meals and the fourth, *Gnetum interruptum* is not, as it is not eaten particularly in the East Province, but also where it grows in the country as a whole, therefore it has not been harvested for 'eru'. There is a general belief that this form of *Gnetum* is tasteless, besides, its leaves are often small and therefore difficult to shred (*pers. obs.).* Moreover, *G. interruptum* was unavailable at various markets in other regions, which further supports the conclusion that it is not used for 'eru' meals.

7.4.2 The influence of ecological interaction on the taste of 'eru'

With respect to provenance, there is variation in taste within and between provinces. *Gnetum* found in 80% of the areas of collection tastes 'good' in general, but the plants from Kumba (South West 1), Oveng (Central 2), Kribi (South 1), Lele (East 2) and Lobe

(South 2) were found to be particularly tasty and should thus be promoted for sustainable utilization. It has been shown that the taste of *Gnetum* leaves used in 'eru' varies with various light intensities in different environments (Ndam *et al*., 2001). These regions, where good *Gnetum* was collected, are typical dense forest zones where there is high humidity and light is available at the top of the canopy, but greatly reduces below the closed canopy (Prasad *et al*., 2009). The pattern of variation observed in a group of plants growing in an area can be due to environmental changes.

Both very good and poor tasting 'eru' are detected in the South West (SW) region, whereas the poorly rated 'eru' is found towards the coastal area. The variation in taste within this region appears to be linked to the genetics of the plants as well as to changes in environmental conditions e.g. the climate, the soil structure etc. *Gnetum* has been observed to produce different quantity and quality of leaf biomass as responses to various light environments and soil types (Ndam *et al*., 2001). *Gnetum* is generally found growing in the forest under optimal growing conditions, in a hot tropical climate with abundant rainfall throughout the year and no seasonal variation (Strahler & Strahler, 1984; Allaby, 1999; Palande, 2010). This environment is warm and humid and sunlight is a major limiting factor, hence the larger and darker green leaves in the denser forest plants (Chitwood *et al.*, 2012) compared to the much smaller and light green leaves of individuals along the coast. Specific leaf area was found to correlate negatively with species distributions along the insolation gradient, and this was noticeably lower in evergreen versus deciduous species (Ackerly *et al*. 2002) .

The results of this study reveal that *Gnetum* from the Littoral and South West regions produced 'poor' meals and was 'tasteless'. These regions are open canopy areas exposed to direct sunlight (*pers. obs*.). The taste of *Gnetum* has been reported previously to vary with light intensities (Mialoundama, 1993; Ndam *et al*., 2001), indicating that very good taste of 'eru' is possible when using leaves from 50 – 80 % shaded environment. However, plants require maximum light for their functioning and several authors have shown the correlation between the intensity of light with the growth of some tropical species (Ludlow *et al*., 1974; Eriksen & Whitney, 1981; Potter *et al*., 1999; Soontornchainaksaeng *et al*., 2001).

For *Gnetum* to grow properly, it requires between 20 – 50 % light intensity at most (Ndam *et al*., 2001); unfortunately, the Coastal (Littoral and South West) regions are at the equator, with sunlight at its fullest, and the canopy is open as the vegetation mostly consists of shrubs and herbaceous plants. An increase in light intensity in the environment of *Gnetum*, such as along the coast, consequently, decreases the quality of the leaves of *Gnetum* for 'eru' meals. Although several factors such as chlorophyll, leaf area and water content are known to influence plant growth, their correlation with the quality of plant taste has not previously been indicated, this hypothesis needs to be tested.

The variation in taste based on various collection localities might also be attributed to the differences in composition, temperature and moisture of the soil from the coastal areas inland. This is partly due to the lack of shading because of the considerable amount of open canopy in the secondary forests. Biomass production was tested in nine plants of *Gnetum* propagated in four different media at various light intensities; the volcanic soil with 50% light coverage produced the highest quantity of biomass (Ndam *et al*., 2001). The differences in soil therefore, not only influence the production of biomass but also usually affect the distributions of plants; consequently, a vertical stratification of three layers of trees is often observed in the primary forest (Sharma *et a*l. 1980; Rietkerk *et al*., 2002; Hewitt, 2004).

The soil around the coastal area hardly supports the growth of large trees like timber or cocoa trees found in the forest zone and trees are needed not only for shading *Gnetum* but also for mycorrhizal associations (Curtis, 1977; Onguene & Kuyper, 2001; Nwaga *et al*., 2003) and other factors that sustain growth in the forest. Therefore, the difference in the type of soils might mean a difference in vegetation between the various areas for it is known that soil types are also a major factor in determining the types of plants growing in a given area (Ewel, 1976). Subsequently, an open canopy type of vegetation might play a role in the taste of 'eru' for lack of shade produces *Gnetum* leaves less appreciated in 'eru'.

7.4.3 The effect of physiological condition on the taste of 'eru'

Leaf texture, maturity and bitterness are well known attributes that influence palatability and quality of food (Thapa *et al*., 1997; Ogundiwin & Oke, 1983). This study has established that the age of the leaves of *Gnetum* influences the taste of 'eru'. The young leaves were most suitable for 'eru' meals followed by medium leaves and the mature leaves were least preferred. The difference in taste between leaves of various ages might correspond to the ability of the leaves to actively export or receive sugars formed in the process of photosynthesis. Mature leaves are more photosynthetically active and export material, which is then translocated to younger leaves and other parts of the plants (Bonner & Galston, 1959; Noggle & Fritz, 1983). Almost the total photosynthate is exported to the young leaves, roots and stem. The photosynthate decreases with the age of the leaf such that the older the leaves, the more likely it is to find little or no photosynthate present in the form of starch or sugar (Quinlan & Weaver, 1970; Thorpe & Lang, 1983). Meanwhile, the young leaves tend to be fleshier and as a result were most appreciated in 'eru'.

Regarding the age of the leaves, the mature leaves could be considered as 'not delectable' when used in an 'eru' meal and their harvesting could be minimized. Plants of *Gnetum* are entirely uprooted during harvesting, therefore individuals are reduced. Harvesting young leaves that are soft and tasty for 'eru'and retaining the mature leaves on the plants will not only conserve the various species but also increase photosynthetic capacity, although the mature leaves will eventually senesce and therefore enough younger leaves are needed to continue photosynthesising. Besides, the uprooting of plants has been found to effect changes in certain nitrogenous constituents of plants as loss of protein and free amino acids in the roots appear to occur during uprooting (Lawrence & Muzik, 1962). *Gnetum* plants are known to be rich in protein and amino acids, so if these compounds are affected because of harvesting method, the taste might be poor due to their loss. This seems another disadvantage when *Gnetum* plants are uprooted.

Very little was previously known about the influence that leaf developmental stage could exert on the taste of 'eru'. Some farmers suggested that the young leaves of *Gnetum* had a

bitter taste. However, this study, based on both questionnaire survey responses and taste analysis, has shown conclusively that the best 'eru' meal was obtained when young leaves were used. Moreover, the survey indicated that 'eru' is not only a common and frequent source of food, but the variability in taste is apparently also influenced by the cooking methods as indicated in the responses to the question on the respondent's awareness to various possible taste of 'eru'.

The study has shown that young leaves are the best for 'eru' meals and a possible explanation for this outcome is that there are fewer fibres in the early developmental stages of *Gnetum* leaves (Tomlinson & Fisher, 2005). It was shown that laticifers appear before fibres as the leaf expands getting older, fibre initials are observed later (Tomlinson & Fisher, 2005). Therefore, young leaves are soft, easily chewable and are tasty with an attractive greenish colour. Because the old leaves contain a high amount of crude, tough fibres **(**Isong *et al.*, 1999), they are hard to shred or chew and are tasteless and are therefore generally avoided in the cooking of 'eru'. The mature cooked leaves normally appeared brownish green and unattractive, this could also be due to the presence of these crude fibres and/or less chlorophyll**.** It seems reasonable that the taste appreciation of *Gnetum* leaves in 'eru' could depend either on the texture and palatability of the leaves or on chemical compounds making up the plants.

7.4.4 Implication of the effect of tasting 'eru' to selective cultivation and conservation of *Gnetum*

'Eru' tastes differently depending on the species used, the provenance and age of the leaves. Although the leaves of the three species of *Gnetum* tasted good in 'eru' meals, those of *Gnetum buchholzianum* were most appreciated for their taste when used in 'eru', moreover, this species normally has broader leaves, an aspect valuable for marketing. Yet, the locals need to identify and harvest the right species with 'good' taste and right sized leaves. The *Gnetum* collected from most regions was acceptable in 'eru' meals, however those from five localities in the dense humid forest were most preferred.

For the cultivation of good tasting *Gnetum* leaves, the study has shown that the plants ought to be grown in shady areas with a limited amount of sunlight. Agroforestry should be practised whereby trees and shrubs are planted along side *Gnetum* to provide not only support but also 80% tree shade required for good tasting 'eru'. As an alternative to agroforestry, shade netting might serve the purpose although *Gnetum* requires other trees for support and the risk of the climbers being entangled with one another is reduced. Moreover, the trees are of various uses to humans. Nevertheless, 50 % of light with net cover produced leaves of *Gnetum* that were more palatable than the 50 % of light with tree cover (Ndam *et al*., 2001) but under large scale farming systems, the use of shade netting is almost impractical in the forest zone. It will mean massive destruction of the tall trees although this might be advantageous to *Gnetum* for competition for nutrients is decreased and a controlled environment created but several other environmental factors could be affected. However, 80% tree shade for growing *Gnetum* is just as good for large scale farming in the forests.

The *Gnetum* found naturally in every region is good enough to be promoted for cultivation, though regions like the South West or the East produce the best *Gnetum*. This is advantageous for domestication purposes. It therefore means that *Gnetum* can be collected locally within an area for cultivation thereby limiting costs such as transportation that might hinder its cultivation. Although it could be argued that only the mother stock can be transported and propagated from that, the stock will have to overcome and adapt to the new environmental conditions and its survival is not guaranteed. Awareness of the possibility of collecting tasty *Gnetum* locally throughout the areas where it grows needs to be promoted in rural areas. Nevertheless, this has the disadvantage that *Gnetum* might be gathered locally to extinction, as its harvesting will depend on the number of harvesters plus the extent of harvesting. Alternatively, in order to sustain *Gnetum* locally, the very best tasting genestock could then be selected and cultivated to make the cultivated material most desirable as the wild one becomes locally scarce. As a result, the price will improve and *in situ* conservation of genetic diversity of species promoted with gene banks established as back up.

The study also demonstrates that young leaves are best to be used for an 'eru' meal; it would therefore be beneficial for the plants if only young leaves are harvested rather than uprooting the whole plant (or stems cut down). This knowledge emphasising that the stem neither should be cut down nor uprooted needs to be publicized in rural areas. The harvesting of young leaves will also stimulate more foliage production for future 'eru' meals as more lateral as well as apical buds continue to develop. Young leaves have the tendency to hinder bud formation and prolonged inhibition leads to bud dormancy in *Gnetum africanum* (Mialoundama *et al*., 1984). Therefore, removal of the young leaves suppresses inhibition and prevents dormancy induction, thereby stimulating growth of the apical bud for more foliage. Usually the period of inhibition overlaps with the growth of these young leaves, which eventually stop growing, and dormancy is induced (Mialoundama *et al*., 1984). Thus if only leaves (especially young leaves) are harvested and the stem not destroyed, the plant rapidly resumes budding.

It is known that well-timed defoliation has been found to promote the production of not only new leaves, which causes an increase in ramification and leaf density on vigorous specimens (Diemer & Pfadenhauer, 1987), a hypothesis to test in *Gnetum* plants, but also the formation of a high percentage of roots and underground tubers (Van Noordwijk & Purnomosidhi, 1995; Tarragó *et al*., 2005). During unfavourable conditions, the underground tubers (reported edible) produced as food reserves remain viable many years after the vegetation and the *Gnetum* plants have been cleared and the soil laid bare (Shiembo, 1999). This means that when leaves are removed or the stem is destroyed, *Gnetum* plants have the possibility to regenerate from their underground tubers but how readily this occurs is not documented. In addition, neither the means of survival of these underground tubers nor their cultivation is known.

The uprooting of *Gnetum* plants during harvesting has resulted in a continuous reduction in the number of mature individuals. The plants are now endangered particularly in the Central and Eastern Provinces of Cameroon where no specific measures for protection are undertaken (Fondoun &Tiki, 2001) as compared to the South West where the Limbe Botanic Garden set up conservation trials for *Gnetum*. The plants of this genus are widely consumed as one of Africa's well known food resources from Nigeria, through

Cameroon, Central African Republic, Equatorial Guinea, Gabon, Congo, and Democratic Republic of Congo to Angola. The high rate of harvesting of these plants for food is one of the causes of its disappearance from its natural habitat. If guidelines on harvesting methods are provided, there will be a possibility of reducing the disappearance rate of *Gnetum*. Thus, conservation strategies such as those suggested here are required to sustain *Gnetum* species in Africa.

Over 3600 tons/year of *Gnetum* is harvested around the Central Region of Cameroon alone (Fondoun & Tiki, 2000). *Gnetum* has thus become the most exploited wild vegetable in most regions, not only because of its general good taste but also for its range of uses. The plants grow naturally and need no treatment, thus their production cost is low. As a result, *Gnetum* is found in all the markets around the country throughout the year and because it is harvested from the wild, it is affordable to all levels of society.

However, because of uncontrolled gathering of these plants, a lot of *Gnetum* is also lost due to bad marketing conditions and perishability, transportation difficulties or lack of accessibility to most areas, and the hard marketing processes whereby the local collector waits upon the arrival of an urban purchaser, for example. All these steps are undertaken because 'eru' is preferred when fresh leaves are used in cooking. Otherwise, some measures of preservation such as shredding, drying and packaging could solve perishability problems. Furthermore, either the exploration of other propagation methods like tissue culture or the production through seeds (Chapter 8) or large scale domestication through ex-situ cultivation are needed as strategies to accelerate *Gnetum* production in Africa to support the large demand in the local markets. Moreover, the cultivation of *Gnetum* could occur in the nearest forest to the main markets in the same area to limit excessive loss during its transportation and a possible approach to protect the forest and sustain *Gnetum* could be for harvesters to plant stem cuttings underneath trees (agroforesty system) during harvesting of *Gnetum* leaves. This could have low impact on the forest dynamics as well.

7.5 Conclusion

The results of this study are a contribution to the promotion of better tasting *Gnetum* for sustainable utilization in cultivation. Various 'eru' meals have been found to taste differently and the variation in taste is shown to be related to the species, the provenance and the developmental age of the leaves. This study, in which 360 meals of 'eru' were scored and the results analysed to determine the best taste scores, has established that the huge quantity of *Gnetum* found in the markets comprises a mixture of various morphological forms of *Gnetum* or species generally referred by the generic name '*Gnetum*' or 'eru', although only three of the four species are used. All three species are acceptable for 'eru', but *G. buchholzianum* produced the best tasting leaves for 'eru' meal followed by *G. latispicum* and *G. africanum*.

It has been revealed that there is some variability in taste for various populations of *Gnetum* within and between regions. *Gnetum* plants harvested in the dense inland forest regions tasted better compared to those harvested along the coast. The taste of *Gnetum* also correlated negatively with leaf age with older leaves being the least preferred. For cultivation, therefore, the three species of *Gnetum* studied collected from most regions give satisfactory results, and are suitable for cultivation, although *G. buchholzianum* is the most preferred, and should be promoted

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APPENDICES

Appendix 7.1 Subject Information Sheet

I am Elvire BIYE and am carrying out research on our traditional meal "Eru" made up of leaves from a plant scientifically called "*Gnetum*". This research is to fulfil one of the objectives of my study at the University of the Witwatersrand, Johannesburg. The meal "Eru" is based on the confusing plants of *Gnetum* with high economic value.

My intention is to determine where or how the better tasting "Eru" can be obtained, and how it is recognized in the field so that the cultivation of the best-tasting strain can be promoted.
You will therefore be invited for 30 minutes to 1 hour to my home to have a specific "Eru" meal. You will be requested to kindly complete the attached questionnaire. The last question has to be answered after the meal has been consumed. You have been selected among several volunteers because I believe I can trust your judgment. Your contribution to this taste appreciation test is thus highly valuable.

Nevertheless, your participation in this study is entirely voluntary and you are free to withdraw at anytime with no consequences whatever. If you do agree to participate, you are requested to answer the questions and hand the completed questionnaire to me after the meal(s). You should note that no name or address is required on the questionnaire but a code system will be used on the questionnaire to guarantee confidentiality and the information will be stored separately.

Thank you.

Signed

INFORMED CONSENT

I, …….. the undersigned, acknowledge receipt of your questionnaire which includes an invitation to voluntarily participate in tasting 'Eru" so as to fulfil one of the objective of your research in the University of Witwatersrand, Johannesburg.
I hereby inform you that, I will / I will not, take part in your study. *(Please underline your option).*

Yours sincerely,

Signature:

Date:

Contact: (Address and number)

For Official Use.
Code:

Appendix 7.2 Questionnaire

'Eru", *(Gnetum)* Taste Appreciation Test
Put an X in the box next to your answer.

1. GENDER: M ☐ F ☐
2. AGE:
3. AREA OF ORIGIN:
4. Can you remember the approximate number of times you have eaten "Eru"?

   Yes ☐ No ☐ If yes, how many times?

5. How long ago did you last have an "Eru" meal? Days ☐ Weeks ☐ Months ☐
6. How frequent do you eat "Eru"?

Once/day ☐ once / week ☐ Other ☐ Please specify:

7. Is the taste always the same? Yes ☐ No ☐

8. Are you aware that "Eru" may taste differently? Yes ☐ No ☐ If yes, what could be the possible reason?

9. Have you ever asked where the "Eru" you buy at the market comes from? Yes ☐ No ☐
10. Have you ever wondered if "Eru" taste will depend on where it comes from?

Yes ☐ No ☐
11. Has anyone ever mentioned his/her preference to "Eru" from a particular region?

Yes☐ No☐ If Yes, which region(s)?
12. Which plant leaves do you generally use?

Old leaves ☐ average ☐ young leaves ☐ mixture of leaves ☐
13. Have you heard of anyone preferring "Eru" made with leaves of a particular age?

Yes ☐ No ☐ any reason given?
14. After eating the "Eru" meal, will you please rate the taste as

Meal I very good ☐ good ☐ fair ☐ poor ☐ very poor ☐

Meal II very good ☐ good ☐ fair ☐ poor ☐ very poor ☐

Meal V very good ☐ good ☐ fair ☐ poor ☐ very poor ☐

THANK YOU.

Appendix 7.3.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (NON-MEDICAL)
R14/49/1 Biye

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER H080804**
PROJECT	The taxonomy and genetic variation of the genus Gnetum L in Africa and selection of suitable strains for cultivation
INVESTIGATORS	Ms EH Biye
DEPARTMENT	APES
DATE CONSIDERED	15.08.2008
DECISION OF THE COMMITTEE*	Approved Unconditionally

NOTE:

This ethical clearance is valid for 2 years and may be renewed upon application

DATE 22.09.2008 **CHAIRPERSON**: .....
(Professor R Thornton)

cc: Supervisor : Prof K Balkwill

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

.....
Signature

This ethical clearance is valid for two years from date of approval.
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

CHAPTER EIGHT

SEED GERMINATION TRIALS FOR SUSTAINABLE PROPAGATION OF *GNETUM* L.

CONTENTS

8.1 Introduction

- Background knowledge:
  Conservation through cultivation

- Problem statement:
  Sustainable utilization of *Gnetum* species

- Knowledge gap:

  Propagation of *Gnetum* using seeds

- Purpose of this research

  Aim and key questions

8.2 Materials and methods

Seed collection

Data collection

Seed viability test

Untreated seeds trials
Preconditioning seeds to stimulate germination

8.3 Result

Seed viability test

Germination rate

8.4 Discussion

The implication of seed viability as observed in two *Gnetum* species.

Factors affecting seed germination in the African species of *Gnetum*

Implications for conservation

8.5 Perspectives and Conclusion

REFERENCES

APPENDIX

Abstract

African species of *Gnetum* are in need of conservation as they are in high demand. Cultivation, either through seed germination or via vegetative organs, of wild threatened food plants is one of the viable measures often applied in species conservation. Propagation trials are underway using vegetative stem cuttings, but that impacts on the natural population as individual plants are over-harvested endangering the survival of *Gnetum* species. Cultivation through seeds seems to be one of the best methods for conserving plants when 20% of seeds per plant are collected as recommended. Individuals are retained after seed gathering, they are neither destroyed nor reduced in number. Unfortunately, seeds of *Gnetum* seldom germinate *ex situ*. Germination trials of this vulnerable gymnosperm were therefore set up to understand its seed germination dynamics in order to produce suitable guidelines addressing vital issues in *Gnetum* conservation, such as seed dormancy (e.g. environmental versus physiological) and other factors likely to influence seed germination.

Three pre-germination treatments were carried out (seed coat removal, scarification and acid treatment) on *Gnetum* seeds collected from Kumba, Barombi Kang in Cameroon. Although seeds remained viable for at least 12 months, a very low percentage germinated. Only the acid pretreated seeds germinated with subsequent seedling establishment. The acid-treated scarified seeds of *G. latispicum* germinated (15%) after 15 months in the substrate while the acid-treated, non-scarified (intact) seeds of *G. buchholzianum* sprouted (5%) after 18 months. The acid pretreatment deterred seed predation. No seedling emergence was observed before 12 months. However, as the seeds remained viable for 12 to 18 months, they are not recalcitrant, but orthodox, and appear to exhibit dormancy. Improved combinations and/or new pre-conditioned treatments are required to more rapidly break dormancy of *Gnetum* seeds.

Key words: Conservation, Dormancy, Gymnosperm, Orthodox Seeds, Viability

8.1 Introduction

8.1.1 Conservation of *Gnetum* species through cultivation

Biodiversity provides direct and indirect benefits to humans. Therefore it should be conserved to provide economically valuable resources, for ecosystem services (regulation and stability of the environmental processes), for ethical reasons (cultural values), and for pleasure e.g. by ornamental plants (Frankham *et al*., 2004). Conservation encompasses the protection, restoration, sustainable utilization and maintenance of the biodiversity and also the improvement of the natural environment (IUCN, 1980). It thus deals with persistence, extinction and replacement of one individual with another (Given, 1994) and some of its strategies include taxonomic practices and measurement of its effective action (Leadlay & Jury, 2006). Conservation thus embraces the management of rare and/or endangered populations in order to retain diversity. It is beneficial and uses theory coupled with techniques to reduce the threat of disappearance of vulnerable species (Given, 1994; Frankel *et al.*, 1995; Frankham *et al*., 2004).

For effective conservation, plants need to be identified. In addition, priorities are set for conservation addressing especially the disappearance or rarity of species; these are three issues that have engendered debates (Khanh *et al*., 2000; Christensen & Beal, 2001). There are various approaches to setting these priorities which depend on the abundance of the species and their level of endemism in a particular area, and the phylogenetic diversity they represent, thereby identifying and preserving genetic variation (Vane-Wright *et al.*, 1991; Bevill and Louda, 1999; Coyne & Orr, 2004). Restoration strategies that enhance the survival of rare and/or endangered endemic plant species are essential to maintain biodiversity (Vane-Wright *et al*., 1991), including strategies such as cultivation or domestication.

Cultivation reintroduces, restocks or restores wild plants either through seed germination (i.e. the resumption of active growth within the quiescent seed) or via propagation of vegetative organs and the subsequent reintroduction of plants to their natural or semi-natural habitat (Akeroyd, 2006). It is not only the act of producing and multiplying individuals, but much more importantly cultivation provides, as in this study, an alternate

source for harvesting for food, thus reducing the pressure on the wild populations. The number of plants is thus increased using limited organs such as stem cuttings and seed in order to perpetuate the species, although very often cultivated plants either lose their form (appearance, shape, structure) or revert to less desirable ones unless propagated under controlled conditions to preserve their useful characteristics (Hartmann & Kester, 1968). Propagation makes use of structures with temperature and light control (e.g. greenhouse) for seed germination or cuttings and also for the transplant and hardening of seedlings before exposure to the natural environment (Kains & McQuesten, 1948; Hartmann & Kester, 1968).

Plant propagation can be asexual or sexual through the use of vegetative organs such as stem cuttings or seeds respectively. Asexual propagation in higher plants is commonly via stems, rhizomes, bulbs, corms and tubers (Rao & Bapat, 1978). It presents some advantages. For example, only one parent is required thus special mechanisms such as pollination are not needed, however, only a few individuals can be propagated from each parent (except via tissue culture). The offspring resulting from a single parent are genetically identical and therefore advantageous traits can be preserved but the populations gradually lose their vigour because of lack of genetic variation (Chandler, 1959; Brydon, 1964). Asexual propagation is beneficial mostly to agriculturalists and modern techniques of tissue culture can be used for virus-free plants (Podwyszynska & Sochacki, 2010). The method is fast, often successful and the planting materials are available and low cost; however, a considerable amount of organ material is required. The method could be more destructive than sexual propagation and plants are reduced from their natural habitat.

Sexual propagation, via seeds, requires fertilization to occur (Meyer *et al*., 1973). This mode of propagation is advantageous as it promotes genetic variation within groups thus providing the populations with the ability to adapt to the environment over time; also, due to the fusing of gametes (one from each parent), the offspring usually display a mixture of characters (Offord *et al.*, 1999; Frankham *et al*., 2004; Butlin, *et al*., 2009). It is a less destructive, faster way to multiply plants and is very practical, easy to carry out and economical. Unfortunately, some plants are difficult to propagate from seeds and this is

true of *Gnetum* species in Africa. These plants have complex germination mechanisms influenced by several factors. Some seeds contain germination inhibitors in the juice of their pulp and ingestion by bird or their manual extraction from the pulp enhances germination (Yagihashi *et al.*, 1998; 2000).

Seeds are made up of three major parts, the seed coat, endosperm and embryonic plant (Hartmann *et al*., 2002). The rupture of the seed coat and the emergence of the new seedlings (i.e. germination) involve biochemical, physiological and morphological changes (Hartmann & Kester, 1968). Germination rate is the percentage of seeds that sprout and can be affected by seed viability, temperature, moisture, type of plant and quality of seed. Seeds must be viable and capable of germinating rapidly; viability can be affected during storage so conditions that slow respiration and other processes without damaging the embryo are applied. Some seeds require preconditioning in order to stimulate germination (Table 8.1). Seed propagation thus requires the understanding and management of germination conditions for individual kinds of seeds.

Twelve types of seeds have been distinguished based on embryo morphology, relative amount of endosperm, and position of embryo in relation to endosperm (Martin, 1946). These include broad, capitate, lateral, peripheral, rudimentary, dwarf, micro, linear, spatulate, investing, bent and folded seeds. All 12 types are found in dicotyledons, six in monocotyledons and only two (linear and spatulate types) are found in extant gymnosperms (Martin, 1946). The Gnetaceae is the only gymnosperm family with seeds having spatulate embryos (Martin, 1946; Kains & Mcquesten, 1948), i.e. the embryos are erect with cotyledons that are variable, thin to thick and slightly expanded to broad (Baskin & Baskin, 2001). These embryos, usually black, are large and, generally one quarter way towards the centre rather than peripheral with non-starchy endosperm. Spatulate embryos are ~60% fully developed (elongate) at seed maturity and are shown to have deep morpho-physiological dormancy (Hidayati *et al*., 2000) and therefore require treatment to break the dormancy. It has been suggested that further growth of spatulate embryos must precede germination as has been observed prior to radical emergence from seeds of *Sambucu*s (Hidayati *et al*., 2000).

Seeds that end their development abruptly may display viviparous, recalcitrant, or orthodox behaviour (Hartmann *et al*., 2002). This classification is according to seed longevity based on their sensitivity to drying and temperature. Viviparous and recalcitrant seeds will germinate before completing maturation, whereas orthodox seeds can be either dormant or non-dormant. Viviparous seeds are unable to reach the maturation stage of development and sometimes germinate before they are shed from the plant, while recalcitrant seeds can only tolerate a minimum of dryness. For example, mangroves produce massive seedlings from viviparous embryos germinating on the tree (Koller, 1959). The seedling eventually falls and is rooted in the surrounding mud. Also, wheat seeds often sprout prematurely in certain wet weather exhibiting vivipary, which is often inherited as a defective (in the agricultural sense) characteristic (Wellington & Durban, 1958).

Recalcitrant seeds do not survive freezing during ex-situ conservation (Corbineau *et al.*, 1986). They lose their viability when subjected to drying or when the critical values of their moisture content drops below 12 – 30% and at temperatures lower than 20°C; thus they cannot be stored for long periods like orthodox seeds (Hendry *et al*., 1992; Fernandez, 1993). Therefore, recalcitrant seeds, after extraction, must be planted immediately to ensure a high rate of germination. Although seeds of some temperate species can easily tolerate much lower temperatures which might stimulate germination, recalcitrant seeds are short-lived and remain viable for only a short period (from a few weeks to a few months) even if they are kept moist, and this depending on the species (Roberts, 1973). Recalcitrant species are mostly trees and shrubs of both moist tropics and temperate areas and some aquatic plants, including large-seeded tropical fruits, perennial crops and timber trees (Fernandez, 1993). Examples include avocado (*Persea americana*), black plum (*Syzygium cumini*), chestnut (*Castanea* spp.), cacao (*Theobroma cacao*), coconut (*Cocos nucifera*), mahogany (*Swietenia macrophylla*), mango (*Mangifera indica*), rubber (*Hevea brasiliensis*) and zapote (*Diospyros digyna*) (Fernandez, 1993).

Orthodox seeds are those that generally complete their maturation to the drying stage of development but may become either dormant or non-dormant after being shed from the

plant (Herrero, 1959; Pardo & Lazaro, 1983; Pinfield & Gwarazimba, 1992; Jensen & Eriksen, 2001; Hartmann *et al*., 2002). These seeds can continue to dry to about 10% moisture. They can tolerate drying as low as 5% moisture content under ordinary conditions and low storage temperatures, generally, although the propagation of orthodox seeds can be delayed by drying (Chin, 1992; Fernandez, 1993). Their life span in storage can be extended with low moisture and temperature. These seeds are relatively small and mostly produced by annual and biennial crops and agroforestry species.

Dormant seeds are usually viable but incapable of germinating in a specified period of time even when provided with conditions (enough moisture and oxygen, suitable temperature) considered adequate to stimulate germination (Justice, 1972; de Lima *et al.*, 1997; FAO, 2010). The lack of germination is often due to the seed rather than an environmental factor, although both generally influence germination. Organic dormancy occurs as a result of some seed characteristics and could either be endogenous or exogenous (Nikolaeva, 1969). In endogenous dormancy, physiological, morphological and morpho-physiological characteristics of the embryo prevent the seed from germinating, whereas exogenous dormancy involves physical, chemical and mechanical features (Table 8.1), such as endosperm and seed coats (Nikolaeva, 1977).

Five types of seed dormancy are currently recognised (Baskin & Baskin, 1998). This system has been divided into physiological dormancy (most common in gymnosperm and angiosperm seeds), morphological dormancy, morpho-physiological dormancy, physical dormancy and a combinational dormancy (physical and physiological dormancy). Physical and combinational dormancy are the only types of dormancy not previously found in gymnosperms (Baskin *et al.*, 2000), but physiological dormancy, for example, is known to occur in the gymnosperm, *Pseudotsuga menziesii* (Mirb.) Franco (Bianco *et al*., 1997).

Table 8.1 Categories of Seed Dormancy adapted after Hartmann *et al*. (2002)

Types of Dormancy	Causes of Dormancy	Conditions to Break Dormancy
1. Primary dormancy		
a. Exogenous dormancy	Imposed by factors outside the embryo	Scarification
Physical	Impermeable seed coat	Removal of seed covering (fruit)
Chemical	Inhibitors in seed coverings	Leaching seeds
b. Endogenous dormancy	Imposed by factors in the embryo	
Morphological	The embryo is not fully developed at the time seeds shed from the plant	Warm or cold stratification
Rudimentary	Small developing embryo	
Linear	Small differentiated embryo < ½ size of seed	Cold stratification and potassium nitrate
Physiological	Factors within embryo inhibit germination	Warm stratification and gibberellic acid
Nondeep	Positively photodormant (requires light)	Red light
	Negatively photodormant (inhibited by light)	Darkness
Intermediate	After-ripening	Short period of dry storage
	Embryo germinates if separated from the seed coat	Moderate periods (up to 8 weeks) of cold stratification
Deep	Often responds to gibberellic acid	
	Embryo does not germinate when removed from seed coat or will form physiological dwarf	Long periods (> 8 weeks) of cold stratification
c. Combinational dormancy	Combinations of different dormancy conditions that must be satisfied sequentially	
Morphophysiological	Combination of underdeveloped or rudimentary embryo and physiological dormancy	Cycles of warm and cold stratification
Epicotyl	Radicle begins growth when temperature and water permit, but epicotyls remain dormant	Warm followed by cold stratification
Epicotyl and radicle (double dormancy)	Radicle and epicotyls require chilling stratification, but radicle is released during first year and then epicotyls are released after a second chilling	Cold stratification followed by warm followed by a second cold stratification
Exo-endodormancy	Combinations of exogenous and endogenous dormancy conditions. Example: physical (hard seed coat) plus intermediate physiological dormancy	Sequential combinations of dormancy-releasing treatments. Example: scarification followed by cold stratification
2. Secondary dormancy		
a. Thermodormancy		Growth regulators or cold stratification
b. Conditional dormancy	After primary dormancy is relieved, high temperature induces dormancy	Chilling stratification
	Change in ability to germinate related to time of the year	

As noted, several factors have been shown to influence seed germination, including viability, water availability, free oxygen, heat, age and stage of maturity plus time of sowing (Kains & McQuesten, 1948; Justice, 1972; Krugman, 1974; Eriksen & Whitney, 1981; Tipton, 1992; Soontornchainaksaeng *et al.*, 2001). The seed coat is thought to influence seed germination, especially when hard and impermeable, by preventing water uptake, gaseous exchange or by restricting the growth process of the embryo (Watkins & Cantliffe, 1983). In such cases, a combination of treatments (e.g. acid scarification, hot water soaking and then moist-chilling) has been reported (Table 8.1) to improve germination rates (Hartmann & Kester, 1968; Justice, 1972; Eriksen & Whitney, 1981; Giri & Schillinger, 2003).

The endosperm of lettuce has been shown to control or prevent the emergence of the radicle (Watkins & Cantliffe, 1983); the endosperm cells at the micropylar end need to undergo structural changes before the endosperm can rupture and allow the radicle to emerge (Psaras *et al*., 1981). Two factors identified in the growing embryo of lettuce involved the mechanical force to push against the endosperm and the weakening of the endosperm by enzymes before the seed can germinate (Pavlista & Haber, 1970). Elsewhere, seedling emergence percentages for immature seeds of the endangered *Juniperus oxycedrus* in the south-west of Spain have been shown to be higher than mature seeds (Juan *et al*., 2006) so the stage of maturation of seeds seems also to be important for germination to occur in this species. The rupture of the seed coat and the emergence of the new seedling (i.e. germination) thus involve biochemical, physiological and morphological changes (Hartmann & Kester, 1968; Baskin & Baskin, 1998).

8.1.2 Problem statement: Sustainable utilization of *Gnetum* species

Gnetum L., endemic to tropical forest regions, is an important vegetable crop in Africa with an increasingly high national and international demand proportional to an obvious increase in indigenes. Presently, *Gnetum* is harvested directly from the wild and none is cultivated in plantations or village farms. Together, these factors mean that *Gnetum* is threatened by harvesting pressure and forest disappearance resulting in the regional scarcity of *Gnetum* in some areas, coupled with an increase in price of its products in the

market (Ndam *et al*., 2001). *Gnetum* is therefore at risk of either being harvested to extinction or disappearing with its habitat and to prevent this, interventions such as domestication, are required to increase the production of *Gnetum*.

Despite having been commonly found germinating in the wild, plus their dioecious state and outcrossing mode of reproduction resulting in seed availability, propagation trials were seen to rule out the multiplication of *Gnetum* by seeds as they mature slowly, take more than a year and a half to germinate and this seldom occurs under nursery conditions (Maheshwari and Vasil, 1961; Shiembo *et al*., 1996). There is therefore a need to develop mechanisms to break dormancy in the seeds of *Gnetum* in Africa and to enhance germination of viable seeds in nurseries. The advantages of this will be twofold: (i) increased availability of *Gnetum* leaves for the local populations and (ii) increased conservation of species diversity in the conservation areas and natural habitat. This study therefore investigates whether or not *Gnetum* seeds can readily germinate under artificial or nursery conditions, and the mechanisms required to promote seed germination of the African species of *Gnetum*.

8.1.3 Knowledge gap: Propagation of *Gnetum* using seeds

Although it is established that rare plants might disappear with their valuable genetic resources and knowledge of their various uses unidentified, in addition to the food security of locals affected (Tekwe *et al*., 2003), it is not yet clear whether the cultivation of rare plants will preserve genetic resources, increase production and also add to their variety (Hammett, 1973; Akeroyd, 2006; Iloh *et al*., 2009). In fact, a large variety of plants are being selected for cultivation basically for their usefulness as an additional food crop, for providing fibres, medicine, and for horticultural purposes, for example.

The entire *Gnetum* plant is generally uprooted during harvesting resulting in mass destruction of the plants. To sustain species of this genus in Cameroon, three research centres have successfully developed methods of asexual propagation of *Gnetum* plants (Ndam *et al*., 2001). Multiplication from vegetative parts of plants is possible because these plant parts (the stem cuttings) have a high capacity for regeneration (Kains &

McQuesten, 1948; Hartmann & Kester, 1968; Hartmann *et al*., 2002). *Gnetum* stem cuttings produce adventitious roots (Amissah *et al*., 2008) and are used for propagation. Any additional research contributing to optimize the domestication process is encouraged (FAO 2001) as the introduction of *Gnetum* into farmland systems is vital.

Methods to reduce the production period and to multiply *Gnetum* in greater quantities need to be identified for the realization of profit in a relatively short time (Ndam *et al*., 2001; Besong *et al*., 2001), and to ensure its conservation in the wild. Moreover, reproduction via seed promotes variation within groups and is essential for species adaptation to their environment.

8.1.4 Purpose of this research and key questions

This study investigates mechanisms required to break dormancy in seeds of *Gnetum* species in Africa and to improve and sustain the germination of viable seeds in order to promote the cultivation of more *Gnetum* and to enhance availability of leaves to local populations. In other words, germination trials have been carried out here in an attempt to maximize cultivation of *Gnetum* species in Africa for the 'eru' meals by potentially shortening the time of rupture of seed coats and the emergence of a new seedling plant (i.e. to break dormancy).

The key questions this research attempts to answer are:

(i) Are the seeds of *Gnetum* species in Africa viviparous, recalcitrant or orthodox?

(ii) Can seeds germinate readily under artificial or nursery conditions?

(iii) What mechanisms are involved in seed germination of the African species of *Gnetum*?

8.2 Materials and Methods

8.2.1 Seed collection

Seeds of *Gnetum latispicum* and *G. buchholzianum* were collected between 2009 and 2011 from at least twenty randomly selected individuals in the wild and in cultivation, numbers dependent on availability at the time and site of collection. For example, five hundred seeds, 200 wild collected from forests and 300 from cultivated plants, were obtained from the Research Institution, IRAD Kumba, Cameroon in March 2011. Only a small amount of seeds was collected per plant to avoid major damage to the populations; it is recommended that no more than 20% of the seeds are gathered (*www.rbgkew.org.uk*). The female plants of the dioecious species of *Gnetum* require about two years to develop mature, fleshy reddish cones from initially small and greenish ones. Cones at different stages of maturation were often seen on the same plant and occasionally on the same spikes. *Gnetum africanum* was rarely found with mature seeds at the times of collection and *G. interruptum* was absent at the sites of harvesting, so these species were not used in the germination trials.

8.2.2 Data collection

Three germination trials were undertaken on variously matured *Gnetum* seed batches brought from Cameroon in paper bags. For these trials, mature seeds (Fig. 8.1) of two species (*G. buchholzianum* and *G. latispicum*) harvested from both the protected and unprotected areas were used and only sinking seeds were selected as floating ones were generally empty. Prior to the third germination trial, a viability test was carried out on twenty seeds as described below.

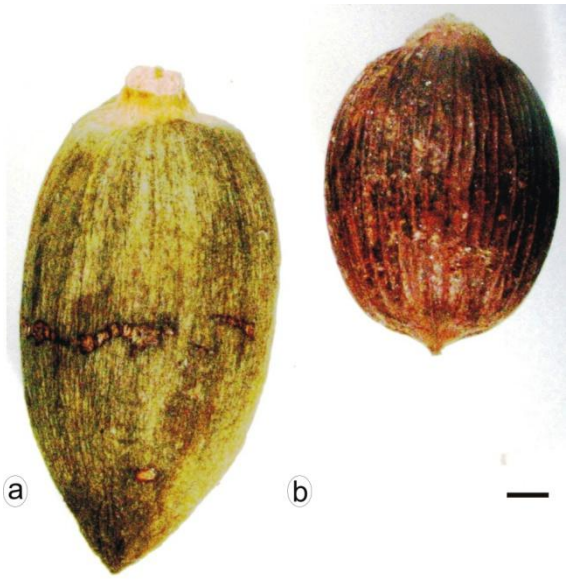


Figure 8.1 Mature seeds of *G. latispicum* (a) and *G. buchholzianum* (b) showing variation (difference in size, shape and colour; nature of seed coat; shape of apex and penducle) in intact seeds collected for the third germination trial. Scale 3 mm.

8.2.3 Seeds Viability test

To determine seed quality and its germination capacity, the seed viability was checked before and after starting germination trials using the tetrazolium oxidation test (Hartmann & Kester, 1968; ISTA, 1976; 1999). The first viability test was carried out in April 2011 when conducting the third germination trial. Due to the limited number of seeds available, only 20 randomly selected seeds were halved such that the embryo present within the seed was cut into two equal parts (Figure 8.2). These were immersed cut side down in 1% tetrazolium salt solution with pH 6 in petri dishes. They were placed in a dark cupboard for 30 – 60 min. and then examined to see if a pink colour developed indicating that respiration was occurring and thus a positive viability result.

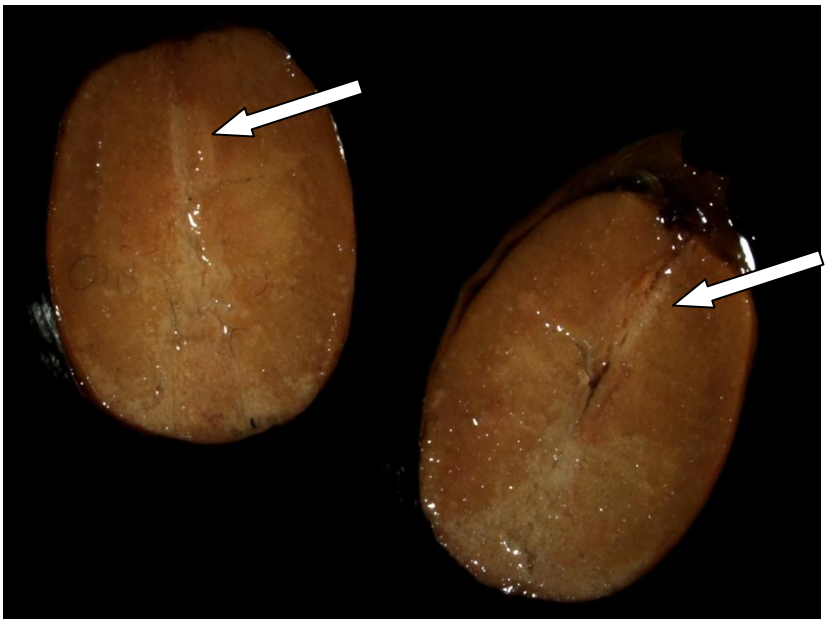


Figure 8.2 Longitudinal section of equal halves of a seed of *G. buchholzianum* used for the viability tests. Arrows point to the embryos surrounded by endosperm.

8.2.4 Trials of untreated seeds

A preliminary trial was conducted in April 2009 to investigate if *Gnetum* seeds could germinate with or without the seed coat removed. Ten seeds per species and geographic region were placed between two moistened Whatman No. 3 filter paper discs in a Petri dish for three weeks. Monitoring was done daily during which time the filter papers were kept wet.

A second trial was undertaken in May 2009 when 75 seeds (40 from the wild and 35 from cultivation) were put in similar pots containing a 50:50 ratio of sterile sand and vermiculite and placed in the greenhouse at the University of the Witwatersrand. Four to five seeds of each species were placed in each pot and maintained under greenhouse conditions: 25°C in daytime and 17 – 18°C at night. Half the seeds had their seed coats removed before planting and the other half still had their seed coats intact. The seeds were watered twice daily and 0.5 *l* of Nitrosol®, diluted 1:300, was added every fortnight. Nitrosol is a natural organic food readily absorbed by plants and is recommended for the cultivation of all in- and out- door foliage such as ferns, orchids,

palms etc. Because this product has been found to be effective in stimulating germination in some seeds, it was thought that it might trigger germination in *Gnetum*. Under the above conditions, i.e. in the presence of sufficient water, oxygen and light, plus nitrosol liquid, the seeds were expected to germinate. Observations took place every three days for one month and thereafter every two weeks for three months then monthly until March 2010.

8.2.5 Preconditioning seeds to stimulate germination

A third germination trial was carried out in April 2011 in the phytotron based on Watkins & Cantliffe's (1982) method, which involved coat removal of air dried seeds using a scalpel or blade, and subsequent scarification of the endosperm of some seeds (Hartman & Kester, 1968) as described below.

Mature seeds derived from the two species (*G. buchholzianum* and *G. latispicum*) and two types of sites (wild vs. cultivated). Twenty to twenty-five seeds were treated by (i) coat removal and/or (ii) scarification of the endosperm, and some seeds in each treatment were pretreated with acid, and then set out as shown in Table 8.2. All the treatments were replicated three to four times (20 – 25 seeds per replicate) and then placed in the phytotron at 25ºC in day time and 20°C at night; 12 h light and 12 h darkness. Seeds were examined for signs of radical emergence after every 72 hours and thereafter, each week for 9 months.

For the acid pretreatment (applicable for hard or impermeable seed envelops), the seeds were soaked in concentrated sulphuric acid at pH 0.2 for 10 – 15 min while gently stirring at intervals to prevent the formation of dark colour due to resinous material (Hartmann & Kester, 1968). The seeds were then flushed with a considerable amount of water and placed in Petri dishes while wet and allowed to dry prior to further treatment.

Decoated seed method: The seed coat was removed from air dried seeds by inserting a scalpel into the seed cavity and pulling off the seed coat before placing the seeds on moistened filter paper in a 5 cm Petri dish. These were also kept at constant temperatures of 25ºC during the day and 20ºC at night in the phytotron. Approximately a quarter of the

seeds were excised only around the area of radicle emergence. For this method, the following treatments were administered to seeds: Decoated seed not pretreated (DSNT); Decoated seed acid pretreated (DSAT); Raw (Intact) seed acid pretreated (RSAT); Raw seed not pretreated (RSNT) as control (Table 8.2).

Scarified endosperm test: Decoated and air dried seeds (with seed coats intact) were scarified by removing 0.5 mm of endosperm material directly in front of the radicle tip. Both the scarified and unscarified (control) seeds were allowed to germinate under the same conditions as described above. The seeds were therefore treated as follows: Scarified seed not pretreated (SSNT); Scarified seed acid pretreated (SSAT); Scarified + Seed with coat removal not pretreated (S+DSNT); Scarified + Seed with coat removal acid pretreated (S+DSAT) and the control. The layout for both methods described above (replication four times) is shown in Table 8.2.

After a period of nine months, the viability test was repeated with 20 of the seeds to determine if they were still capable of germinating and the percentage with germinating potential was calculated. The remaining seeds were transferred to the greenhouse (25°C during the day and 17 – 18°C at night) where they continued to be watered regularly for six to eight months (Figure 8.3). After 18 months, the viability test was conducted again to verify if the seeds still germinate. This was performed with 20 randomly selected seeds per species.

Table 8.2 Layout of variously treated seeds of *Gnetum* species (*G. buchholzianum* and *G. latispicum*) in the 3rd germination trial
Decoated seed not treated (DSNT); Decoated seed acid-treated (DSAT); Raw seed acid-treated (RSAT); Raw seed not treated (RSNT) as control; Scarified seed not treated (SSNT); Scarified seed acid-treated (SSAT); Scarified + Decoated seed not treated (S+DSNT); Scarified + Decoated seed acid-treated (S+DSAT) and the control.

DSNT	DSAT	RSAT	RSNT	SSNT	SSAT	S+DSNT	S+DSAT
S+DSAT	S+DSNT	SSAT	SSNT	RSNT	RSAT	DSAT	DSNT
RSAT	RSNT	S+DSAT	SSAT	S+DSNT	DSNT	SSNT	DSAT
SSNT	SSAT	S+DSNT	S+DSAT	DSNT	DSAT	RSNT	RSAT



Figure 8.3 Third germination trial transferred to the greenhouse after 9 months: Petri dish tops marked according to various treatments demarcate the differently treated *Gnetum* seeds buried in the substrate.

8.3 Results

8.3.1 Seed viability test

The results reported here were obtained from seeds used in the third trial. The seeds, selected for the viability test before and after the germination trial all contained an embryo (Figure 8.3), were viable but at different levels of viability (Table 8.3). In the presence of the tetrazolium salts, a colour change (colourless to pinkish or light red) was observed in the embryos of all the seeds examined and this was a positive test result for viability, indicating that respiration was occurring in the seeds. Out of the 20 seeds (per species) selected at random from the various treatments and tested if viable and capable of germination after 9 months of planting, 15 (75%) and 12 (60%) seeds from *G. buchholzianum* and *G. latispicum* respectively (Figure 8.5), showed a positive response to the tetrazolium test, thereby confirming their viability. Twenty seeds were randomly selected from the remaining seeds and were tested for viability. Six (30%) and five (25%) randomly selected seeds of *G. buchholzianum* and *G. latispicum* respectively, were still viable after 18 months. Most seeds not treated with acid had been eaten by the end of 18 months.

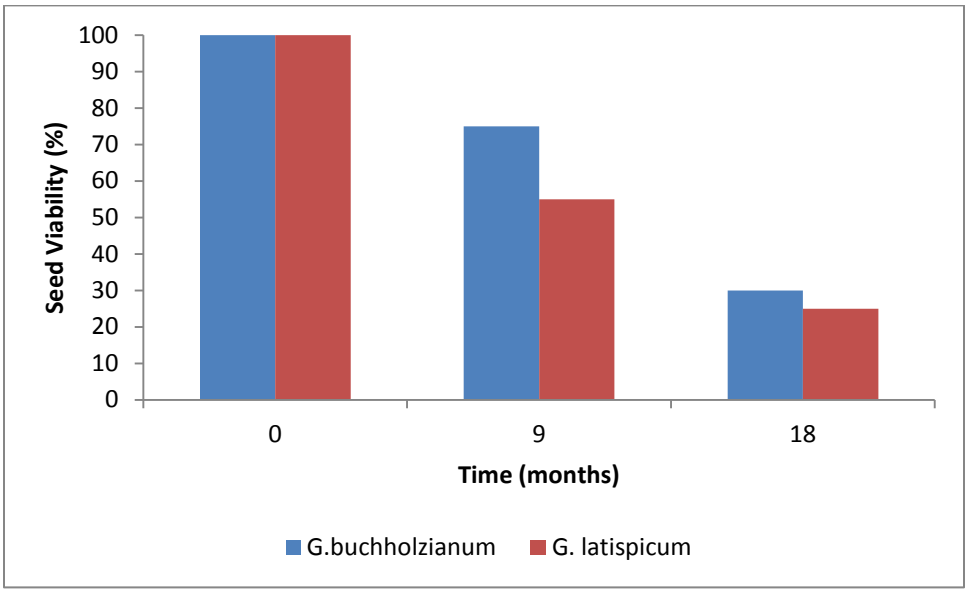


Figure 8.4 Viability percentages calculated for 20 seeds tested at three different periods for each *Gnetum* species sown in 2011 in a non-temperature controlled greenhouse. The last viability test after 18 months was calculated excluding those eaten by snails.

Table 8.3 Viability percentages observed for seeds of two species of *Gnetum* pretreated in various ways after 18 months in the substrate.

Pretreatments →		Acid-treated seeds				Non acid-treated seeds			
Species ↓	% of → Class of seeds ↓	Intact p<0.05	Scarified	Decoated	Decoated + scarified	Intact	Scarified	Decoated	Decoated + scarified
Gnetum buchholzianum	Viable	15	5	-	-	10	-	-	-
	Rotten	10	5	10	15	-	10	-	-
	Devoured	-	-	-	-	-	25	25	50
Gnetum latispicum	Viable	35	50	10	5	-	-	-	-
	Rotten	70	35	50	85	20	15	30	10
	Devoured	-	-	-	-	65	85	50	90

8.3.2 Germination trials

After three weeks into the first trial, there was neither change in seed size due to imbibition nor was any radical emergence observed. Thereafter, the seeds and filter paper in the Petri dish became mouldy.

The two treatments (removal of seed coats and seed coats intact + 0.5 *l* of Nitrosol® added) carried out on the seeds of *Gnetum* species in the second trial were expected to stimulate germination, but there was no seedling emergence. Although Nitrosol was added to the seeds every fortnight to promote germination in this trial, the seeds showed no seedling emergence in the duration of the trial (9 months).

Germination response of acid-treated seeds under greenhouse conditions

In contrast to the first two trials, the mature seeds of the two species of *Gnetum* used in the third trial produced a positive result with germination occurring, though at low percentages. The seedling emergence percentages for seeds of *G. latispicum* and *G. buchholzianum* were 15 % and 5 % respectively; the seeds germinated after 15 and 18 months respectively in the greenhouse (Figures 8.5a & b and 8.5c & d and Table 8.4). The t-test, single sample (Statistica) was significant for intact acid-treated seeds at $p \leq 0.05$ with t-value = 3.00.

Insects and snails ate more than 10 seeds per species, especially those without coat and/or scarified, and not treated with acid (Table 8.3) as the snails were found in the substrate. The seed coat of *Gnetum* seeds is a tough covering (Fig. 8.4b), hard enough for snails not to be able to break them open. This might be the reason why only the scarified seeds were easily consumed. The empty seed coats or shells were neither eaten nor broken but were abandoned intact (Figure 8.4a).

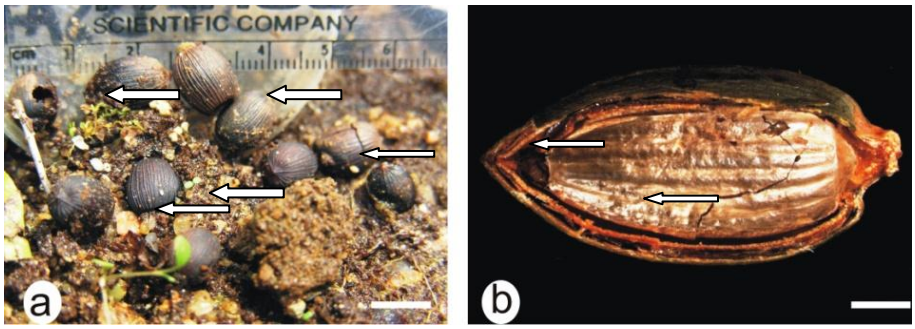


Figure 8.5 a, Seed coats (empty shells) of *G. buchholzianum* observed after the endosperm had been eaten by snails. Arrows point to openings of the empty shells; b, An intact seed of *G. latispicum* with partially removed seed coat to display the inner and middle coverings which including the coat are too tough for snails to break open. Arrows point to the coverings beneath the seed coats.

These germination percentages were very low (Table 8.4) and the germination requires a long period. The seeds that germinated in both species were acid-treated, but scarified in the case of *G. latispicum* (Figures 8.5a & b) versus intact in *G. buchholzianum* (Figures 8.5c & d). Seedling emergence percentages were calculated only for those treatments in which germination response was observed (Table 8.4). However, the overall germination rate for the 360 seeds planted was low approximately 2.5% and germination was delayed.

Table 8.4 Seedling emergence percentages observed in *Gnetum* species under greenhouse conditions

Germination period (months)	*G. latispicum* % of scarified acid-treated seeds that germinated (n=20)	*G. buchholzianum* % of intact, acid-treated seeds that germinated (n=20)
9 - 12	0	0
15 - 18	15	0
> 18	0	5



Figure 8.6 Young plants of two species of *Gnetum* that emerged from acid-treated seeds. The seeds used were also scarified in *Gnetum latispicum* and young plants a & b, were observed after 15 months; The seeds of *Gnetum buchholzianum* were non scarified and young plants c & d were observed after 18 months in the substrate

8.4 Discussion

8.4.1 The implication of seed viability as observed in two *Gnetum* species

The seeds of *G. latispicum* and *G. buchholzianum* exhibited 60% and 75% viability respectively after nine months. Although the cut seeds of *Gnetum* were submerged in tetrazolium salts for less than 1 h, the results of the viability tests on these two species' seeds were relatively high after the 9th months. However, it has been shown that the percentage of tetrazolium-stained seeds changes with the cut area, the temperature and infiltration time (Savonen, 1999; Juan *et al*., 2003). Thus, a longer submersion time could possibly have resulted in an even higher percentage of stained seeds in *Gnetum*. In

addition, the proportion of embryo and endosperm staining increases with more effective infiltration of the stained solution into the seed tissues (Savonen, 1999). Nonetheless, it has been shown that an incompletely stained embryo (indicating low viability) of a conifer seed was capable of developing into a seedling (Buszewicz & Holmes, 1957), but such levels of staining were not observed in *Gnetum* seeds.

Certain seeds become dormant as they mature; consequently fully matured seeds lose their germination capacity while immature seeds are able to germinate (Herrero, 1959; Pardo & Lazaro, 1983; Pinfield & Gwarazimba, 1992; Jensen & Eriksen, 2001). The high percentage of seed viability observed after more than 12 months for the two *Gnetum* species seems normal as it confirms previous observations that *Gnetum* seeds have been found germinating after two years in their natural habitat (Maheshwari & Vasil, 1961; Shiembo *et al.,* 1996; Onguene & Kuyper, 2001). Similarly, a high percentage of seed viability was also obtained with mature seeds of *Juniperus* species with low percentage germination (Juan *et al*., 2003).

Despite the high seed viability observed, germination of *Gnetum* seeds occurred only after 15 or 18 months in the greenhouse. This suggests that seeds of *Gnetum* species exhibit some type of dormancy, contrary to the well-known recalcitrant seeds of the forest such as *Persea americana*, *Lagerstroemia speciosa*, *Theobroma cacao*, *Cocos nucifera*, *Swietenia macrophylla*, *Mangifera indica*, *Hevea brasiliensis*, and *Diospyros digyna* (Fernandez, 1993). Lower levels of dormancy have been found in seeds derived from immature cones than those derived from mature ones of *Juniperus* species (Juan *et al*., 2006). This helps to explain the low germination rate found in species of some genera like *Juniperus* (Juan *et al*., 2006). Regrettably, all the seeds of *Gnetum* species used in this study were obtained from mature spikes, so it was not possible to test the influence of seed maturity on germination rate (and time) of *Gnetum* seeds.

As mentioned earlier, the seeds of *Gnetum* remained viable for a long period (at least for 12 months), but appeared to be incapable of rapid and vigorous germination during that period. This rules out the possibilities that *Gnetum* seeds are either viviparous (i.e. germinating before separating from the parents) or recalcitrant (i.e. tolerating a minimum

degree of dryness), but they are orthodox and clearly dormant. This implies that the seeds generally develop to the maturation stage prior to being shed, although parthenocarpy has been observed in a number of gymnosperms (Kozlowski, 1971; Lopez-Almansa & Gi, 2003). Parthenocarpy might not be applicable in *Gnetum* as there was an embryo in all the seeds sectioned for viability tests. Seeds of most gymnosperms are known to remain naturally dormant but alive for many years with the capacity for germination when exposed to suitable conditions that break their dormancy (FAO, 2010).

Dormancy protects the seed in its natural environment against harsh conditions and also increases the likelihood of its survival. The hard and impermeable seed coat has been found to present resistance to germination, i.e. it prevents the expansion of the embryo mechanically, obstructs the entrance of water, or inhibits gas exchange so that the embryo lacks oxygen on one hand (Graven *et al*., 1997). But it delivers nutrients (for example, early protein synthesis) to the embryo and provides adequate protection for some tropical species during the dry season on the other (Van Dongen *et al*., 2003; Essami & Mashair, 2005; Siddiqui & Khan, 2010). Dormancy is directly associated with embryo (imposed by factors outside the embryo), seed coat impermeability to water and/or the presence of germination inhibitors during difficulties in seed germination (Hajar, 1991; Cantos *et al*., 1998). Some gymnosperms exhibit a double dormancy characteristic, which involves both exogenous and endogenous factors (Prado & Lazaro, 1983), but seeds from conifers such as *Juniperus* showed only physiological dormancy with germination difficulties due to an inhibition mechanism (Baskin & Baskin, 2001).

There are various categories of seed dormancy due to multiple causes that require certain breaking conditions for germination to be effective. The cause of physiological dormancy in seeds, for example, is still not well understood although pretreatment techniques such as warm and/or cold stratification have been developed and have generally been successful (Krugman *et al*., 1974). To improve winter wheat production, seed priming was effective for germination in laboratory and greenhouse but not under field conditions (Giri & Schillinger, 2003). It has also been demonstrated that a combination of two or more treatments can overcome the effects of an impervious seed coat plus a dormant

embryo, a case of double dormancy, or promote germination in seeds with complex embryo dormancy (Hartmann *et al*., 2002).

It is essential to accurately pre-condition the seeds in order to stimulate germination. The seed coat of *Gnetum* seeds could be considered impermeable to water and necessitate further treatment as germination could be influenced by the texture of seed coat and surrounding envelopes (FAO, 2010). Hard and impermeable coats can inhibit water uptake or gaseous exchange for example thus preventing germination (Hartmann *et al*., 2002). Fleshy seeds of *Prunus* were found to rarely germinate while seeds within intact fruits did not germinate at all, suggesting that the juice of the pulp and the fruit pulp contained germination inhibitors (Yagihashi *et al*., 1998; 2000). An attempt to overcome the dormancy condition in *Gnetum* using acid treatment, coat removal or scarification of seeds was successful after a long period of more than a year; the acid treatment definitely made a difference irrespective of whether the seeds were intact or scarified. However, the germination characteristics of *Gnetum* seeds require greater understanding. This might be possible if various temperatures, ranges of acids and other special treatments such as stratification are administered to test for promotion of rapid seed germination. These treatments could be effective if they reflect the seed treatment in nature – e.g. if acids in the digestive system of animals assist in promoting germination and/or preventing predation of the seeds.

Maintaining conditions as close to the natural environment as possible is important for minimizing research bias (Mangarda *et al*., 2009). Fleshy *Gnetum* seeds are brick red, a colour that renders the seed likely to be bird-dispersed. It has been shown that most intact seeds of such bird-dispersed seeds like *Prunus* that take a long period to sprout under natural conditions cannot germinate without bird ingestion (Yagihashi *et al.*, 1998). Consequently, the *Gnetum* seeds are thought to go through a strong acidic environment in the bird's stomach (generally very low pH and increases depending on factors like, after feeding or before egestion) at the bird's body temperature before they can germinate.

The seeds of *Gnetum* remained viable for a long period, and, pre-germination treatments were unable to cause the seeds to germinate rapidly *ex situ*. It is recommended that in the

absence of specific knowledge on germination requirements, as in *Gnetum*, the natural environmental conditions associated with particular plants should be duplicated (Claussen, 1996). *Gnetum* seeds were therefore kept in the phytotron and later in the greenhouse under conditions (temperature and light intensity) that approximate those in tropical regions where they grow naturally. Despite the pre-treatments of scarification and/or acid-treatment adminstered in this trial, the seeds still took a long time to germinate.

8.4.2 Factors affecting seed germination in the African species of *Gnetum*

In non African Gnetum, it was noted that the embryo is still immature at the time of seed shedding and this would impose a delay in germination as the embryo matures (Maheshwari & Vasil, 1961). In gymnosperms, including *Gnetum*, it is now known that the food storage tissue in the seed, although commonly referred to as endosperm is formed by the growth of the female gametopgyte rather than as de novo endosperm tissue from the product of double fertilization. Gnetum species have a specialized form of embryo development within the seed in which the hypocotyls produces a vascularized lateral protuberance called a 'feeder'. The slow rate of germination might be linked to the state of the embryo, therefore the embryo type in the African Gnetum needs to be assessed directly from the dissected seeds.

Only fifteen percent of the acid-treated scarified seeds of *Gnetum latispicum* germinated after 15 months and 5% of the intact (non-scarified) acid-treated seeds of *G. buchholzianum* sprouted after 18 months. None of the seeds that were untreated (in acid) germinated during the first 12 months of the trial. That there was no seedling emergence from untreated seeds suggests that neither seed coat removal nor scarification of the endosperm enhanced rapid germination in *Gnetum* seeds, although most (>75%) of the seeds remained viable over 9 months. This implies that seedling emergence was lower than the viability levels so the seeds exhibit dormancy.

Although the seedling emergence percentages were relatively low, the germination trial is considered successful as *Gnetum* seeds were previously reported to only rarely germinate

under nursery conditions even though their seedlings are readily found in their natural environment (Maheshwari & Vasil, 1961; Shiembo *et al.,* 1996; Onguene & Kuyper, 2001). *Gnetum* seeds necessitate careful management in terms of their germination conditions and it appears that an acid pretreatment promotes germination in cultivation. This confirms the point that knowledge of an individual type of seed is essential for successful propagation (Hartmann & Kester, 1968; Wareing & Phillips, 1971; Hammett, 1973; Hartmann *et al*., 2002). The present work shows that the time taken for germination to occur is almost similar to that in their natural habitat. This suggests that the mechanism involved in *Gnetum* seed germination is complex and might not be determined by external factors only. Endogenous mechanisms of dormancy might also be operational in *Gnetum* seeds.

The seeds of *Gnetum* that germinated were all treated with concentrated sulphuric acid at pH 0.2 implying that the acid has an effect on the seeds, and could promote their germination. In short, better results might be obtained if different concentrations of acids (attempting to match with that found in a bird's stomach, from pH ~ 0.2, highly fluctuates) were used as only acid-treated *Gnetum* seeds germinated as ranges of acids have been found to enhance germination rate in several plants. It is known that the pH of a bird stomach varies e.g. continues to increase showing a regular pattern from after feeding to before egestion (Smith & Richmond, 1972). For example, germination was enhanced in seeds of tamarind (*Tamarindus indica* L.) and *Cassia auriculata* L. by increasing sulphuric acid concentrations (Muhammad & Amusa, 2003; Negi & Sharma, 2012), although poor responses were revealed when absolute sulphuric acid was used. Similar results were obtained with *Parkia biglobosa* (Jacq.) Don. (Awodola, 1994) and in *Pistacia mutica* (Caloggero & Parera, 2000). This implies that there are ranges of acid concentrations effective for seed germination.

Moreover, the results revealed that the insects and snails do not feed on the acid-treated *Gnetum* seeds. The acid treatment thus apparently protects the seeds from herbivores. The seeds then could survive the long period of dormancy until they germinate. It can also be argued that, seeds that were untreated (with acid) failed to germinate because they were devoured, thus their lifecycle was terminated abruptly. With this uncertainty, the acid

treatments need not be the active force in breaking dormancy in *Gnetum* seeds. However, the results suggest that *Gnetum* seeds can be triggered to germinate by acid treatments, moreover, not all the non-acid-treated seeds had been devoured during the trial period, but none of these had germinated after 18 months.

Gymnosperm seeds, especially those with embryos that are morphologically dormant, often undergo endogenous dormancy, they thus simply need time to germinate (Baskin & Baskin, 2001). Endogenous dormancy is either caused by a physiological inhibiting mechanism of germination, an underdeveloped embryo or both and this needs appropriate conditions for embryo germination, either warm and/or cold stratification to break dormancy (Nikolaeva, 1969) although it might not always be successful (Giri & Schillinger, 2003). Some acids are effective in breaking endogenous dormancy caused by small differentiated embryos that are less than half the size of the seed. *Araucaria angustifolia* (a gymnosperm in the Pinophyta) is a species with recalcitrant seeds. An increase in level of polyamines (PAs), indole-acetic acid (IAA) and abscisic acid (ABA) during seed germination was observed in the embryos (Fernanda *et al.*, 2011). Although the *Araucaria* seed had no connective tissue between the megagametophyte and the embryo, communication between the tissues was maintained, based on the likely transport of plant growth regulators (Fernanda *et al.*, 2011). The behaviour of the seeds under conditions with different levels of polyamine compounds and various acids is unknown in *Gnetum* as very little research on dormancy breaking mechanisms has been carried out, however, it is now known (from this study) that *Gnetum* seeds are orthodox and not recalcitrant.

8.4.3 Implications for Conservation

Gnetum is more easily propagated from cuttings than seeds. One of the successful strategies to conserve *Gnetum* will therefore be the domestication of the wild plants through vegetative propagation with the aim of rapidly increasing the production of *Gnetum* and thereby reduce impact on wild populations. This approach to propagating *Gnetum*, although still under trial, has produced results in a relatively short period, i.e. several plantlets in the nursery on weekly basis were produced from stem cuttings (*pers.*

obs). This is a much shorter time than the 15 or 18 months required for seed germination *ex situ*, as demonstrated here.

Vegetative propagation via stem cuttings of *Gnetum* as recommended by Shiembo *et al.* (1996) seems therefore for the moment to be the only rapid multiplication method applicable. The long period *Gnetum* takes to germinate might discourage farmers from cultivating these plants using seeds and they might revert to or maintain methods such as harvesting from the wild which impact on their biodiversity. Consequently, biodiversity projects currently involved in the propagation of *Gnetum* use simple and low-cost stem cuttings. Although, cultivation using stem cutiings as noted previously, also presents limited practical worth for *Gnetum* multiplication or its conservation in general. Perhaps, this is true only in terms of the number of individuals that are destroyed in order to provide enough stem cuttings for a large piece of land (e.g. one hectare of farmland).

Vegetative propagation has drawbacks in that it exerts pressure on the naturally occurring populations of *Gnetum* through the harvesting of stems, unlike if the seeds were collected and germinated. Although collecting seeds also puts pressure on wild populations, it could be minimized if only 20% of the available seeds per plant are gathered as recommended (www.rbgkew.org.uk). This will prevent major physical damage to the populations and fortunately, individuals that could produce many more seeds in future are not destroyed. Notwithstanding, collection of seeds also impacts on the natural populations because removing the seeds from the parental environment and planting them elsewhere can affect growth and development of the next generation. More importantly, it reduces genetic variation in the population and there may be fewer individuals to replace those that are damaged by harvesting. A good comparison of the levels of recruitment between natural and artificial conditions might be interesting but this is still untimely, as cultivation methods are not yet fully established.

Plants will respond differently to various fluctuations in environmental factors, thus knowledge of their responses is vital to determine the best method for restoration (Mangarda *et al*., 2009). Environmental conditions, including physical aspects, i.e light, water availability, the soil type, pH or organic matter and the biological environment, e.g.

physical arrangement of plants in time and in space, could therefore strongly influence the growth and development of plants (Orth *et al*., 2000). For instance, the parental environment influenced the primary root growth and gravitropism, the germination time of the young generation of *Arabidopsis*, their flowering and the weight of seeds they produced (Elwell *et al*., 2010). Therefore, to ensure cultivation of the suitable strains of *Gnetum*, for example, for sustainable use and diversity, parental environmental factors should be considered. Generally, domestication of wild and semi-wild food plants is thought to be a viable strategy for any threatened resource (Leakey *et al*., 1996), but the means of propagation with the least impact on the plant populations will be most beneficial for biodiversity conservation. For rapid production of leaves presently and in order to sustain *Gnetum* in Africa, it is hoped that low cost stem cutting propagation will be useful while investigations into mechanisms to stimulate rapid seed germination continue.

In order to promote the conservation of African *Gnetum* species, seed germination trials must be multiplied and the mechanisms to rapidly break dormancy identified. Because *Gnetum* seeds are persistent, take a long time to germinate (more than one year), endangering its survival, seed banks could be set up for long-term production of seedlings. Although the length of time for seeds in the seed bank can be crucial, seeds might lose their viability for example, as population stability might be affected by the availability of seed reserves through the next reproductive cycle, seed germination repeatedly over a long period could eventually assist in more seedling production in nurseries.

Unless other processes of propagating *Gnetum* are developed, it will continue to be scarce and its continued existence will be increasingly threatened. Domestication might be fully achieved if the price of *Gnetum* in the market reaches the point where it can be profitably grown, and people become interested in its propagation and cultivation. Regrettably, most propagation trials aiming to sustain *Gnetum* through cultivation initiated by the Cameroon National Institute of Agricultural Research for Development (IRAD) with some farmers by producing it in agroforestry systems (Shiembo 1999), have long been abandoned due to limited funds and lack of motivation. Moreover, the practice of

cultivating *Gnetum* is uncommon around the country and there is fear of the unknown plus the assumption that plants never go extinct. Unfortunately, there is no report of its cultivation in other countries, i.e. Central African Republic, Congo, Equatorial Guinea, Gabon, Nigeria and Angola where *Gnetum* also grows naturally in Africa.

8.5 Conclusion and Recommendation for Future Studies

This study demonstrates that sexual propagation is achievable from the seeds of *Gnetum* species in Africa, thereby maximizing genetic variability in domesticated plants. Also, *in situ* cultivation via seed germination is a possible strategy to undertake using various treatments especially various ranges of acids. Moreover, acid treatments seem to protect seeds from predation and might be of help in the production of least contaminated seedlings. The seed coat texture, imbibition time and temperature or endosperm strength are factors that influence germination in general and their investigation is needed to enlighten researchers on the mechanisms controlling seed dormancy in *Gnetum*. In other words, an investigation could be carried out to determine if the type of dormancy exhibited by the seeds of these species is endogenous, exogenous or combinational.

Because the acid-treated seeds showed some ability to germinate, other combinations of treatments and experimental conditions applied to *Gnetum* seeds could improve germination capacity e.g. ranges of acids, temperatures and time of imbibition. Both mature and immature seeds could be used in the trial to test whether immature seeds are less dormant than mature ones. It has been shown here that *Gnetum* seeds are dormant but the category of dormancy still needs to be identified in order to provide tools to enhance their germination.

The sexual approach to propagating *Gnetum* is needed to complement asexual propagation because what is currently carried out in research centres is not only insufficient to compensate for the degree of utilization of the species, but the method is not yet fully applicable in farmland production. Moreover, it is not sustainable because stem cuttings equally reduce the number of individual plants. These are further reasons why appropriate techniques to rapidly break seed dormancy need investigation and

cultivation via sexual reproduction should be promoted at once as seeds are, in my opinion, the best way to sustain *Gnetum* species in Africa.

In brief, for the propagation of *Gnetum* using seeds, methods to improve germination in a short time are still ideally required. Neither the acid treatments nor the seed coat removal nor scarification of the endosperm stimulated germination within a 12 month period, but the seeds of *Gnetum* remained viable. The acid pre-germination treatments allowed the seeds to germinate *ex situ* only after 15 months (*G. latispicum*) or 18 months (*G. buchholzianum*). The results suggest that these treatments were ineffective in rapidly breaking seed dormancy in *Gnetum* species in Africa. Since the germination rate of acid-treated seeds was also low in *Gnetum*, it appears that the low pH of the acid used failed to trigger early germination but was successful in protecting the seeds from predation. Scarification also did not stimulate seedling emergence, in other words, the treatment was unsuccessful in breaking seed dormancy, as in *Juniperus oxycedrus*. That seeds in the greenhouse took almost as long to germinate as in their natural habitat might be the confirmation that some type of dormancy control mechanism is involved in *Gnetum* seeds. However, it seems that the time *Gnetum* has taken to germinate is what its embryo requires to prepare before germination is established. Investigating the various aspects involved in dormancy might provide information to enable growers to trigger more rapid germination in *Gnetum* seeds.

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CHAPTER NINE

GENERAL DISCUSSION AND CONCLUSION

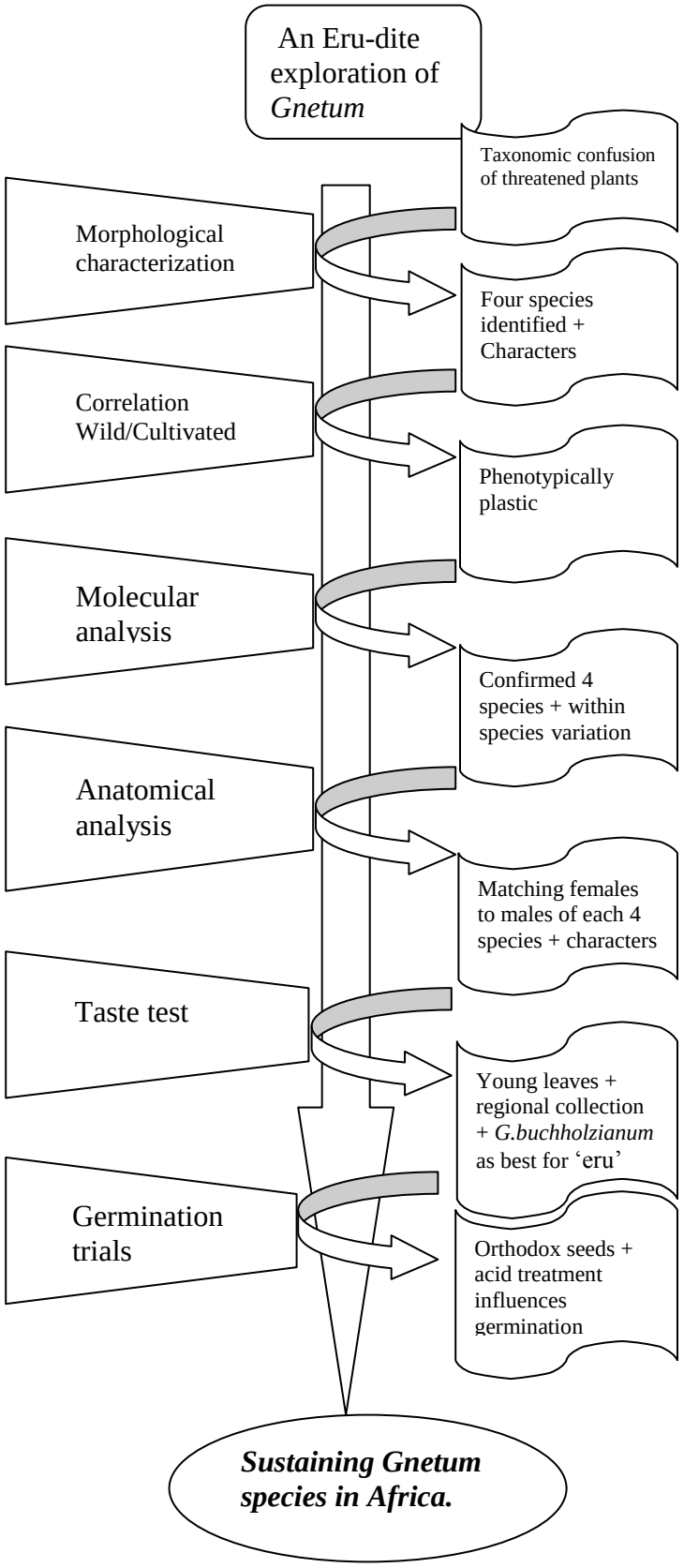


Figure 9.1 Synthesis of the eru-dite exploration of *Gnetum* Species in Africa.

9.1 General Discussion: An Eru-dite Exploration of *Gnetum*

'Eru' is a delicious common meal greatly appreciated by over 80% of the population of Cameroon. In various tropical African countries, the meal is widespread but its composition might differ from one region to the other. However, the major component of an 'eru' meal is the leaves of *Gnetum* species which are nutritionally and economically valuable to the local people. These leaves represent various forms within and between species of *Gnetum* and their variation have led to difficulty in identification of the African species of *Gnetum* (Hutchinson & Dalziel, 1954; Onguene & Kuyper, 2001; Won & Renner, 2003). The plants of this taxon are difficult to distinguish not only from one another based on vegetative features like the leaves, but also from dicotyledonous plants when sterile.

The leaves of *Gnetum* are gathered from various regions in huge quantities for 'eru'. This impacts greatly on the number of individuals as *Gnetum* climbers are often uprooted during harvesting (Mialoundama, 1993). The plants are therefore threatened due to over-exploitation and risk disappearing before they are sufficiently explored, most specifically those populations with the most popular taste. Both morphological and molecular data are essential input to determine the appropriate management strategies for conservation and improvement of *Gnetum* species in Africa. The present study thus provides the foundation for measures to be developed in order to reduce impact on natural populations. In this investigation, species limits have been clarified and the diversity exhibited by *Gnetum* species in Africa evaluated for agronomic and gustatory purposes using morphological, anatomical, molecular and biogeographical data (Figure. 9.1). The research has thus clarified the taxonomy of the African species of *Gnetum* by means of morphological features and genetic markers, and promotes sustainable utilization and conservation by identifying the best tasting species to propagate (Figure 9.1).

In the Introduction (Chapter 1), it was noted that previous classifications applied to the genus *Gnetum* in Africa had resulted in taxonomic confusion with one (Won & Renner, 2003), two (Hutchinson & Dalziel, 1954) or three (Onguene & Kuyper, 2001) species being recognized. This investigation, using morphological data together with objective

numerical approaches, anatomy and genetic markers for the first time, has clarified species circumscriptions; the use of reproductive characters has been key to this species clarification. The various thorough approaches applied in this study, have allowed species to be more accurately recognised, an essential step in setting up a species' conservation strategy. Thus, the taxonomic conclusions are enabling the development of conservation strategies for *Gnetum* species in Africa. Moreover, interesting conclusions about the use of numerical techniques and their importance in taxonomy as a whole have been reached.

9.1.1 The Taxonomy of *Gnetum* species in Africa

Four species of *Gnetum* are now recognized in Africa based on intensive micro- and macro-morphological investigations as well as multi-locus molecular markers (AFLPs). This study has provided a greatly improved classification and the confusion concerning the two previously described species: *G. africanum* Welw. and *G. buchholzianum* Engl. (Welwitsch, 1858; Engler, 1908; Hutchinson and Dalziel, 1954) has been clarified. Four African species of *Gnetum* have been revealed by phenetic analysis (Chapters 2 & 4), reproductive morphology (Chapter 3; Biye *et al.*, 2013), AFLP analysis to an extent (Chapter 5) and anatomy (Chapter 6). Two are newly described (Chapter 3, Biye *et al*., 2013), *G. interruptum* and *G. latispicum*; two were previously described, *G. africanum* and *G. buchholzianum* (Welwitsch, 1858; Engler, 1908; Hutchinson & Dalziel, 1954).

Various studies have shown that taxa are distinguished by different types of characters (e.g. Cron *et al*., 2007; Weigend *et al*., 2010; Strickland-Constable *et al*., 2010), and certain features, depending on the taxa, contribute more or less in the identification of species. As noted previously, reproductive features have been shown here to be extremely valuable in distinguishing among the African species of *Gnetum* (Chapters 2, 3 & 4). In the past, the vegetative features were used but posed problems as these structures are very unstable and are phenotypically plastic, varying with age and environment. The high degree of variability in the vegetative features within each species created doubts as to the number of species in the genus (Hutchinson & Dalziel, 1954; Carlquist, 1996; Isong, 1999; Earle, 2001; Onguene & Kuyper, 2001; Won & Renner, 2003). Thus, the species of this genus in Africa are not separated on a vegetative basis. In contrast, the non-African

species of this genus, especially the Venezuelan ones, are best distinguished by the size of the leaves and stem cortex surface (Jauregui *et al.* 2005).

Despite the variable vegetative features within African *Gnetum* species, the morphometric study coupled with phenetic analysis successfully differentiated between the four African species and reinforced the identification of distinguishing characters (Biye *et al.* 2013; Chapters 2, 3 & 4). The reproductive features that easily separate the four recognised African species are primarily the shape and size of the internodes of the jointed spikes, but also the number and arrangement of spikes, the shape of the staminal column tip and seed shape and size. These are all useful stable characters to recognise the various African species of *Gnetum*.

The genus *Gnetum* in Africa, on one hand, was judged to be made up of one species, if that was the case, *G. buchholzianum* and *G. africanum* become synonyms. There is no question of nomenclatural synonyms since different types, bearing different names (*G. buchholzianum* collected by Buchholz and *G. africanum* collected by Welwitsch), representing the two species of *Gnetum* do exist (Welwitsch, 1858; Engler, 1908). Their identity appears to be solely a taxonomic matter. On the other hand, it was supposed that *Gnetum* is made up of at least two species implying that there might be no gene flow between these two forms (*G. africanum* and *G. buchholzianum*), and so they could be considered as separate species. To address the problem of taxonomic synonyms thought to exist in the genus *Gnetum* in Africa, the type and the original description were used to identify and help in the attribution of the names to the different forms. Different names therefore might mean a break in gene flow. In taxonomic confusion of this nature, distinct groups need to be identified as belonging to definitions or concepts that allowed for species recognition in different studies (Mayr, 1970; Kornet & McAllister, 1993; Wheeler & Meier, 2000; Coyne & Orr, 2004; Mallet, 2007).

Two species concepts, the morphological species concept (Cronquist, 1968; Coyne & Orr, 2004) and the phenetic species concept (Sneath, 1988) have been used in this study to define species in the genus *Gnetum* in Africa. The interpretation of reliable morphological characters to define a species is the underlying principle of the

morphological species concept. The African species of *Gnetum* possess reproductive features that distinguish them from their closest relatives and these characters are thus taxonomically important. Actually, different views expressed on the total number of species was accounted for on the basis of morphological variations observed among and within populations. These variations might reflect the interaction between the environment and genotypes and could either be continuous or discontinuous with respect to gene flow.

Taxonomic clarification of *Gnetum* in Africa supported by phenetic species concepts to distinguish *Gnetum* species is based on recognizing phenetic clusters through multivariate analyses. Briefly, the phenetic species concept (describing the species as the lowest level at which distinct clusters are observed) provides necessary tools to examine the overall patterns of variation in order to identify discrete groups to classify as species. The criterion here is the phenotypic similarity in recognizing species. Besides the delimitation of taxa based on macro-morphological characterization of the genus e.g, using the leaf and spike features, anatomy and molecular methods such as AFLPs, were applied to further clarify and confirm whether the species boundaries are consistent with morphological data.

The AFLP analysis revealed the existence of two major genetically distinct groupings with a high genetic differentiation within species. *Gnetum interruptum* was recovered as a distinct cluster/group, but the others were scattered amongst the two main clusters reflecting their genetic variability. However, members of each species did cluster together to some extent and this reflects regional groups in some cases. Similarly, AFLP technology grouped six Indian species of *Cycas* into four clusters that did not reflect taxonomic groups and 78% polymorphism was recovered using two sets of primers (Radha & Singh, 2011). The high number of polymorphisms detected in *Cycas* is related to the genetic variation observed among species at the molecular level (Aparajita & Rout, 2010). These results were useful in the delimitation of *Cycas* in India as these highly endangered plants showed genetic dissimilarity among species. The African species of *Gnetum* are necessarily outcrossing and genetically variable with a mean polymorphism

slightly above 64%. A high level of this genetic polymorphism occurs within members of each species as detected using AFLP techniques as bands with various sizes and intensities were recovered (Chapter 5).

The AFLP analysis revealed considerable intraspecific genetic variation among the 45 specimens of *Gnetum*. This might suggest that insufficient genetic variation was resolved to find the consistency within the taxa as the molecular work was based on a limited number of samples representing both male and female, and wild and cultivated plants of *Gnetum* species in Africa, and not enough primer combinations were used as recommended for the technique. Therefore, if the sampling of each species was larger, it might have given results that may have been more convincing but this required additional financial input. Notwithstanding, the results obtained with a limited number of samples reflected the groupings of specimens according to the morphological study to some extent and the large number of unique bands supported their recognition as distinct species. To my knowledge, this is the first report of AFLP analysis applied to *Gnetum* specifically.

Just as leaf and stem anatomy provided distinct characters for the delimitation of *Gnetum* species in Venezuela (Jauregui *et al*., 2005), anatomical characters are shown here to be valuable in determining species limits among the African species (Chapter 6). The use of anatomy in taxonomy is now increasingly significant as more modern and sophisticated systems of data analysis are developed. The drawback in the use of anatomical characters is that they are often subject to more intraspecific variation than reproductive features (e.g. floral structures), but are reported to be more revealing than morphological characters, as in the Venezuelan *Gnetum* species (Stace, 1980; Jauregui *et al*., 2005). The anatomical variation is usually due to environmental factors in the plants' habitat and the amount of variation influences the phenotypes (Davis & Heywood, 1963).

The anatomical features of the leaves and petioles of the African *Gnetum* species have facilitated species' recognition, particularly by enabling the matching of female to corresponding male plants within the same species of similar morphological characters (Chapters 2 & 3). Taxonomically important anatomical characters that are applicable to the African species are the size, shape and distance separating vascular bundles of the

petiole, the type and size of stomata on both adaxial and abaxial leaf surfaces, the width of leaf epidermal cells and the nature of the lower and upper epidermal walls plus the distribution of astrosclereids on the leaf surface. In spite of difficulties of interpreting anatomical evidence in the light of intraspecific and environmental variation [as noted by Davis & Heywood (1963)], the established four African species of *Gnetum* could be identified based on the anatomy of leaf epidermis and petiole characters.

A number of conclusions may therefore be drawn from this taxonomic study. Firstly, there is no doubt as to the distinction between the four species of *Gnetum* in Africa, viz. *G. africanum*, *G. buchholzianum*, *G. interruptum* and *G. latispicum*, on the basis of reproductive morphological, anatomical and, to some extent, molecular characters. The success of this taxonomic study may be attributed to the multivariate approach to analysis. It has been suggested that characters be used from all parts of an organism when carrying out a numerical taxonomic study and this approach has proven to be the most satisfactory in that the combined effect of the characters is presented (Rohlf, 1963). It might be best to use all types of characters simultaneously and although this is possible with multivariate analysis, it was not applied in this study. Thus, although the multivariate analyses did not include anatomical characters, the overall relationships among taxa followed the same trends as in the morphology and molecular analysis and the four species are clearly distinct anatomically.

9.1.2 Implications of the taste tests and germination trials for conservation of African *Gnetum* species

Conservation strategies are needed for wild and semi-wild food plants, especially when they are threatened resources as in the case of *Gnetum* plants (Leakey *et al*., 1996). To improve the productivity and to guide also the method and extent of harvesting of *Gnetum* in Africa where it is commonly used as a high demand vegetable crop, this study adopted three approaches. The first was to assess the species and/or populations that taste the best and are therefore the best candidates for propagation. The second was to evaluate the age of the leaves that taste best in ‘eru’ in order to influence harvesting practices in

the wild and in cultivation. The third approach was to carry out germination trials with the seeds of two selected species of *Gnetum* in order to provide an additional means of propagation of this threatened resource.

Gnetum buchholzianum was found to taste the best of the three species scored in the taste trials (Chapter 7), followed by *G. africanum*, and then very closely by *G. latispicum*. Regional analyses showed that 'eru' meals from *Gnetum* grown in the littoral regions with sandy soils are the least preferred, whereas those from the dense forests and loamy soils are most favoured, especially from the South West Region. Young to medium leaves are clearly preferred over older leaves and all three species tasted are suitable and could be locally harvested and cultivated (Chapter 7). The taste of *Gnetum* depends on various factors (Mialoundama, 1993); this study has shown that taste is influenced by the species, provenance, and the age of the leaves (Chapter 7). These results thus indicate which special factors should be considered when promoting the multiplication and harvesting of *Gnetum*. Furthermore, for the best tasting 'eru', leaves should be obtained from *Gnetum* cultivated in shady areas, and thus its cultivation should be coupled with agroforestry whereby trees and shrubs are planted alongside *Gnetum* to provide not only support but also 80 % shade (Ndam *et al.* 2001).

Taste tests indicate that it is only worth harvesting medium to young leaves (Chapter 7), while the older, more fibrous (Isong *et al.*, 1999), less palatable, lower leaves and stems should be left. This would reduce the unnecessarily destructive practice of uprooting *Gnetum* plants, which, together with the uncontrolled deforestation in the tropics due to ever-increasing logging and farming practices, has resulted in a decrease of *Gnetum* species in their natural habitat. Moreover, the harvesting of the young leaves will stimulate continuous growth as their presence usually inhibits bud production for new shoots (Mialoundama *et al.*, 1984; Chapter 7).

In addition to reducing destructive harvesting of wild *Gnetum*, its multiplication via seed germination has become vital. Vegetative propagation trials have been conducted, but this is a less sustainable method of cultivation because it also impacts on the number of wild individuals. Therefore investigations into less destructive methods of cultivating

Gnetum in Africa are needed and this study is a first such attempt to carry out seed germination trials. Many species, especially tropical species, are known to have recalcitrant seeds (Chapter 8) which can only tolerate a minimum dryness (Koller, 1959). In contrast, *Gnetum* seeds in Africa are shown in this study to be orthodox exhibiting dormancy, but with further study and insight, the dormancy problem could be overcome. As shown here, *Gnetum* seeds can be germinated under artificial conditions, for example in the greenhouse, but methods to shorten the germination period are required.

Despite various treatments and suitable planting conditions applied in this study, the seeds of *Gnetum* only germinated after 15 months. As noted, they are not recalcitrant but are classified as orthodox seeds displaying dormancy (Chapter 8). Nonetheless, the seeds remained viable for at least 18 months (Chapter 8) and often retain this state for at least two years (Shiembo, 1996; Maheshwari & Vasil, 1961). Preconditioning treatments, such as warm and cold stratification, could be explored in addition to acid treatment and seed coat scarification to provoke rapid germination (Hartmann *et al.* 2002). These approaches would provide additional understanding of the type of seed dormancy exhibited by *Gnetum* plants.

As *Gnetum* seeds do not spontaneously germinate, their resistance to sprouting needs intense investigation to enhance rapid production of young plants so that farmers could be motivated to cultivate *Gnetum*. Unfortunately, the application of various combinations of treatment during this research did not stimulate rapid germination of seeds as anticipated, however, the acid treatment apparently prevented the predation of *Gnetum* seeds. The seeds could thus be facing either exogenous or endogenous or combinational dormancy (Hartmann *et al*., 2002). The stage of seed maturation is also an important factor that influences their germination. It was shown that seeds of *Juniperus* species obtained from immature cones germinated significantly more quickly than mature ones, suggesting that their dormancy level is lower than mature seeds in that gymnosperm (Juan *et al*., 2003).

One of the conservation strategies recommended to sustain the African species of *Gnetum* is their cultivation (Chapter 8) in order to increase their bulk production . The

domestication of the best tasting variants is specifically encouraged because if the ones that are cultivated do not taste good, then people will continue to harvest the wild ones to get preferred tasting material. Some research centres in Cameroon (e.g. The Institute of Agricultural Research and Development, IRAD; The Limbe Botanical Garden) have successfully cultivated *Gnetum* using stem cuttings obtained from the naturally occurring plants (Chapter 8). These plants collected from the wild had revealed some morphological diversity that engendered various conflicting identification and naming of the various specimens. The taxonomic confusion over the naming and description of the wild individuals in previous publications has most likely also been applied to the cultivated plants. Therefore their identity also needs to be clarified for accurate conservation of the various species via cultivation.

Plant cuttings used in the cultivation of *Gnetum* are those obtained from three species namely, *G. africanum, G. buchholzianum* and *G. latispicum* (Chapter 7), as their leaves are cooked as 'eru'. The study showed that the cultivated plants of these three species retain the main taxonomic distinguishing features observed in their wild counterparts (Engler, 1908; Welwitsch, 1858; Hutchinson and Dalziel, 1954; Chapters 4 & 5). There are no significant differences among cultivated plants collected from different regions of Cameroon and any morphological variation observed is due to species-specific characters (Chapters 4 & 5). Changes in phenotypic expression of characters have been observed in cultivation (Schlichting & Levin, 1986), however, the distinguishing morphological differences between various species of *Gnetum* are retained (Chapters 4 & 5) as they have a genetic basis.

Gnetum interruptum appears to be the rarest of the four species as revealed by this investigation, but is fortunately not harvested as a food source (Chapter 7). There were fewer specimens in herbarium collections and none has been collected post 1990. Fieldwork is needed to locate areas where *G. interruptum* could be found growing despite forest disappearance and then rapid propagation using stem cuttings promoted to ensure its persistence. Its conservation in the wild might be more complicated as there is uncontrolled felling of trees by the local populations for farming but its security could be guaranteed if it is found growing in existing protected areas.

Factors such as the number of herbarium records, habitat types and collection localities are indicators of the level of threat and /or rarity of a plant (Prance, 1984; Willis *et al*., 2003). The number of known collections and localities reflect the abundance of a species; this implies that if a species is widespread, it has more chance of being collected and of surviving local threat than a rare, localized and restricted one (MacDougall *et al*., 1998; Purvis *et al*., 2000; Schatz, 2002). However, regrettably, the collecting records of tropical African species are far less comprehensive than the European flora for example (Rhoads & Thompson, 1992), and these generally depend on factors such as research interest, availability of funding and botanists. Unfortunately, although *Gnetum* species are collected daily as a source of food, they are seldom used for research interest; consequently, there are very few herbarium collections of these plants (e.g. less than 15 specimens in YA).

9.2 Conclusions and Future Studies/Recommendations

The study has contributed to a taxonomic revision of *Gnetum* species in Africa using a phenetic approach and based on morphological and anatomical characterization. It differs from previous morphological studies carried out on this genus by the use of additional approaches like numerical techniques, molecular markers, and use of anatomical characters to match the female to male plants in the same species. It thus owes an interpretative debt to the phenetic concept.

The high variation in vegetative features, together with over 64% polymorphism reflecting within species genetic diversity has been the driving force behind the confusion observed in the past. However, the anatomical and reproductive features show various forms of unique structures that allow species recognition in this genus. Moreover, the difference in the number of unique species-specific bands recovered between species by molecular analysis supports the existence of four species in the genus *Gnetum* with a high intraspecific variation. The various species are also separated geographically and there is therefore a physical barrier to their inter-breeding and this further supports the idea of several species existing in the genus.

To sustain *Gnetum* plants in Africa, some clear conservation strategies are needed; for a start, it will be ensured that the results of this research are implemented by the government policy in order to constrain locals to apply the various findings and recommendations. The main strategy that could then be followed is the use of seeds in cultivation as most other methods are destructive to the individuals of this genus. Therefore, questions on how to break dormancy to facilitate rapid germination of *Gnetum* seeds in cultivation need to be addressed. I suggest that in future studies, germination of both mature and immature seeds in their natural habitat should be tested and a seed bank established as a backup to the in-situ conservation of genetic diversity of the species of *Gnetum*. Lack of sufficient knowledge on the dispersal and pollination mechanisms in *Gnetum* presents some gaps in various arguments. Observations need to be made on the various species' lifecycle, especially the seed dispersal and germination phases in their habitat in relation to seasonal changes in environmental conditions such as temperature and rainfall.

In the light of this study, questions such as: "What causes dormancy in *Gnetum* seeds and what conditions could break dormancy rapidly in order to motivate farmers to undertake *Gnetum* propagation using seeds?" need to be addressed. However, in order to ensure and enhance the sustainability of these species, more efforts of management and conservation are recommended. Educating the people harvesting plants for 'eru' could be promoted by introducing sensitization programs, seminars and communication within women social groups.

In brief, the leaves of *Gnetum* are used in 'eru' as one of its principal constituents and the plants of this genus are threatened because of the extent and method of harvesting. This research has contributed to improve the knowledge of the diversity of *Gnetum*. In addition to two previously known species *G. africanum* and *G. buchholzianum*, two new species, *G. interruptum* and *G. latispicum,* have been recognized and described and the distinguishing characters revealed for these taxa. Moreover, the study has established that young leaves of various forms of this genus and the most preferred *G. buchholzianum* could be collected for they taste best when used in the well appreciated meal 'eru'. As a

result, the extent of harvesting leaves could be limited as the old leaves will be retained. Likewise, the uprooting of the entire plant during harvesting of *Gnetum* leaves should be discouraged. Most importantly, the study reveals that *Gnetum* from any region where it grows naturally can be used for the delicious meal 'eru'. There is thus no need for long distance travel to other areas in search of *Gnetum* leaves with a possible good taste as these could be collected in the region of origin.

African species of *Gnetum* had previously been regarded as producing seeds that are incapable of germinating under artificial conditions. One of the most striking results of this research is the possibility of cultivating the African *Gnetum* species using seeds in nurseries so as to complement cultivation through stem cuttings as this impacts on plant individuals. Although stem cuttings may actually increase shoot production in some plants, removal of many stems for propagation purposes for example, can not only damage or weaken plants but can also reduce numbers of individual plants. *Gnetum* grows as high as its supports i.e. the neighbouring trees for example. Forest trees are tall trees therefore the *Gnetum* plant could go as tall as it supports, and young tasty leaves might be found high at the canopy level. The tendency might be to cut the climber at the ground level during harvesting of leaves for consumption. The plants are thus destroyed and the number reduced.

Government of Cameroon and several organizations comprise various structures in charge of promoting research with the aim to alleviate poverty and those that directly influence human life. The forestry department executes forestry policies and is concerned with conservation and managerial issues. These institutions are allocated an annual budget for various aspects such as forest management, especially non-timber forest products. Besides having funding to run these programs, the organizations are represented in very remote areas through local offices and could therefore be in contact and communicate with the local populations relatively easily. Consequently, the findings of this study will be made available to these institutions that are positioned to implement the major results of this investigation. In addition, various means of communication, including seminars, conferences, talks over the rural radio stations, etc. will be explored

to uphold and disseminate information on the sustainable utilization of the African species of *Gnetum.*

As mentioned, *Gnetum* species play an important role in meeting the nutritional and economic needs of rural households, and are reported as being particularly rich in protein, vitamin A, iron and calcium. They are among African indigenous vegetables identified for improvement and promotion through research. The various forms were difficult to differentiate from those with high nutritional value and good taste and to identify individuals with good agronomic traits for genetic enhancement. This study has thus contributed to identify, characterize, and delimit the genus *Gnetum* in Africa and has determined intraspecific variation. These aspects have clarified the taxonomic status of *Gnetum* species in Africa and therefore provided information to identify the forms with good agronomic traits. The role of systematics in the conservation of biodiversity is consequential; for not only does it provide a natural classification of organisms but also establishes principles of priority for their conservation. For the purpose of better management of *Gnetum* species in Africa for sustainable utilization, the number of species making up the genus especially the forms that taste best in 'eru' meals have been identified; where the best tasting species can be found and the type of leaves to value for specific uses e.g. in 'eru' in future was determined. Moreover, cultivation is suggested in this study as an alternate source for harvesting for food thus reducing pressure on the wild population, a measure to conserve and sustainably utilize *Gnetum* species in Africa.

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