ONE AFRICAN BAOBAB SPECIES OR TWO? USING MORPHOLOGY AND INFERRED PLOIDY LEVEL TO INVESTIGATE

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A research report submitted to the Faculty of Science, University of Witwatersrand, in partial fulfillment of the requirements for the degree of Master of Science of the School of Animal Plant and Environmental Sciences

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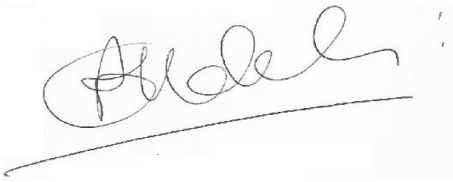
DECLARATION

I, Chukwudi Austin-willy Udeh, declare that this research report, apart from the contributions mentioned in the acknowledgements, is my own, unaided work. It is submitted for the Degree of Master of Science by coursework and research report to the University of the Witwatersrand. It has not been presented before for any degree or examination at any other University.



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ABSTRACT

Only one tetraploid (four sets of chromosomes) species of baobab (*Adansonia digitata*, L.) was recognized on mainland Africa. However, a study published in 2012 reported the existence of a new diploid baobab species known as *Adansonia kilima* which was said to co-exist on mainland Africa with *A. digitata*. This new species was identified on the basis of morphology (mainly floral and stomatal features), ploidy level and molecular phylogenetics. The two species of African baobabs were also differentiated according to their elevation preferences of between 650 m –1500 m above sea level (a.s.l.) and below 800 m a.s.l. for *A. kilima* and *A. digitata* respectively, which were said to rarely overlap. The report of this new species and the need to accurately determine the exact number of species of baobabs existing on mainland Africa have brought about a renewed interest in the study of the African baobabs and have necessitated this study. This research therefore compared *A. digitata* and *A. kilima* to assess the latter's authenticity as described by work. The objectives were to (i) examine floral and stomatal traits from samples across mainland African baobab populations to establish whether there are distinct differences that can be correlated with altitudinal differences in order to distinguish the species present in Africa, and (ii) use stomatal size and density to infer ploidy levels of baobabs that occur at low and high altitudes. Herbarium and fresh specimens of *A. digitata* and *A. kilima* from across Africa with both leaves and flowers were borrowed from various herbaria to represent the widespread distribution of baobab in Africa. Cluster analysis (CA), Principal Component Analysis (PCA) and Non-metric Multi-dimensional Scaling (NMDS) of seven floral traits of 124 African baobab specimens were used to analyze baobabs that occur at low or high altitude. These specimens did not form distinct clusters or separate groupings correlated with either low or high altitude. The inference of ploidy using stomatal size categories as per previous study showed no differences in ploidy level among the baobabs studied and the difference in morphological features between baobabs found at low and high altitudes also could not be linked to inferred differences in ploidy levels. Specimens were assigned diploids or tetraploids based on their stomatal length as defined by an earlier work and the altitude of each specimen was used to ascertain

whether the ploidy level correspond to the altitudinal category reported by a study published in 2012. Box and whisker plots were also used to compare the floral (pollen grain diameter, volume and density) and stomatal traits of baobabs that occurred at low and high altitudes. There was no statistical difference in the variation in floral features among African mainland baobabs found at low and high altitudes except in staminal tube length and style length. The stomatal traits results reveal that the mean stomatal length and density (per 1000 μm^2) between baobabs that occur at low and high altitudes were not statistically different except when mean stomatal length and density were grouped into the two putative ploidy categories as per previous study. Morphological features results suggest that the differences in floral size, and stomatal size and density of mainland African baobabs could be related to differences in climate, water availability and geographical variation. Based on the data analyzed for this work, it is concluded that *A. kilima* is not distinct enough from *A. digitata,* to be recognized as a new species. However, further research should investigate potential variation in genome size among African baobab that is correlated with altitude using flow cytometry analysis and/or chromosome counts.

Key words: altitudinal variation, mainland African baobab, multivariate analysis, ploidy level, pollen grains, species concept, stomatal density, sympatric speciation.

DEDICATION

This research report is dedicated to my wife (Judith Ngozi Udeh) and my daughter (Angel Amanda Udeh) for their support, patience, prayers and love throughout the whole period of this study.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

°C	degree Celsius
km	kilometer
m	meter
m.a.s.l.	meters above sea level
μm	micrometer
mm	millimeter
min	minute
%	percent
Π	Pi
μm^2	micrometer squared
mm^2	millimeter squared
s	second
s^2	second squared
QDS	quarter degree square
Id	specimen identity
χ^2	chi square

Glossary of Abbreviations

ANOVA	Analysis of variance
BBC	British Broadcasting Corporation
BSC	biological species concept
CA	cluster analysis
DRC	Democratic Republic of Congo
ESC	ecological species concept
ICUC	international centre for underutilized crops
NMDS	non-metric multi-dimensional scaling
PC	principal component
PCA	principal component analysis
PSC	phenetic species concept
OUT	operational taxonomic unit
SCUC	Southampton Centre for Underutilized Crops

CHAPTER ONE

1. LITERATURE REVIEW

1.1 IMPORTANCE OF AFRICAN BAOBAB

Native African fruit trees such as baobabs have many uses and have played vital roles in maintaining the food security, nutritional, health and economic wellbeing of the local communities wherever they occur (Gebauer *et al*., 2002; Sidibe and Williams, 2002; De Caluwe *et al*.,2009; Venter and Witkowski, 2013 a). They are useful for their social and aesthetic values (Akinnifesi *et al*., 2006). Some of the valuable local fruit trees include *Prunus africana* (Hook.f.) Kalkman (African plum), *Sclerocarya birrea* Hochst. (marula), *Adansonia digitata* L. (baobab), *Tamarindus indica* L.(tamarind), *Irvingia gabonensis* (Aubry-Lecomte ex O'Rorke) Baill. (wild mango), *Uapaca kirkiana* Müll.Arg. (wild loquat), *Strychnos spinosa* Lam. (monkey orange), and *Ziziphus mauritiana* Lam., (Shackleton *et al*., 2005; Akinnifesi *et al*., 2006; Jama *et al*., 2008; Wickens and Lowe, 2008). Various parts of native African fruit trees are used in a variety of ways and for various purposes by different African communities. For example, the trunks and leafy branches can provide shelter in homesteads and may be used as building and roofing materials and as stakes for yam barns and animal pens (Akinnifesi *et al*., 2006). The wood can also be used for carving, or as firewood for cooking and heating in homes. In addition, the leaves may be used as a vegetable or extracted for medical purposes. The bark, roots, and sap can be used for medicinal purposes and in the production of utensils, ropes and glues. Moreover, the fruit pulp may be used in making beverages, juice, jam, and wine, which add to the nutritional values of the local communities (FAO, 1996).

Reports have also shown that native African fruit trees are of great importance in the arid and the semi-arid African countries, where other fruit trees have difficulties surviving (Haq *et al*., 2008; Saied et *al*., 2008). These trees contribute to the food basket of the local population and are important to their nutritional, health and cash income needs, most especially for women and their children (Schreckenberg

et al., 2006; Gebauer *et al*., 2002) particularly when there is famine and at pre-harvest period (Wickens, 2004).

African baobab is highly valuable because of its dietary and therapeutic properties, which add to its socio-economic importance. Baobab fruit pulp has been used in the food industry to produce beverages, juice, and jam (FAO, 1996). The fruit pulp is also used to treat a number of diseases including asthma, fever, diarrhoea, malaria, small pox, and to curb inflammation related conditions, such as arthritis, allergies and type II diabetes (Erasmus, 2009; Wong 2012; Milner, 2013). Further, baobab products are frequently marketed as a source of antioxidants, which allegedly can help slow down the aging process and protect against ailments like heart disease and cancer. The baobab trees have been reportedly used as shelter. Hollow baobabs are used as water storage containers and to construct prisons, toilet facilities, bars, fencing for grave sites, animal pens, and storage rooms, watch towers as well as hives for honeybees (*Apis mellifera*, Hankey, 2004). Humans and animals chew the wood in times of drought to provide moisture in order to relieve thirst (Hankey, 2004). The roots of the very young trees are reportedly edible (Birnin-Yauri and Garba, 2011). The leaves may be eaten as a vegetable (Yazzie *et al*., 1994) whereas the fruit pulp is dissolved in water and used as a drink or eaten as porridge (Sidibe and Williams, 2002). It is a valuable source of vitamins C and B, phosphorus, calcium, magnesium, potassium, iron, carbohydrate, and dietary fibre (Osman2004; SCUC 2006; BBC 2008; Chadare *et al*., 2008). Moreover, the seeds are roasted, eaten raw or used as a thickener in soups (Sidibe and Williams, 2002). In addition, baobabs provide raw materials for carving, fishing and hunting as well as fibre for weaving and making rope. Almost all parts of the African baobab are useful to local communities. There has been increasing interest in the seed oil and dried fruit powder as consumer products since 2008 (Hills 2008). Oil extracted from African baobab seeds may be used as an industrial raw material in the production of food, facial and hair creams, paints, wood polish and medicines (SUCU, 2006). Consequently, the baobab has been acknowledged as the most important edible savanna tree with a recent approval of its fruit pulps and seed oil as export products to market in the European Union (2008/575/EC) and United States of America (GRAS Notice No. GRN 000273), thus giving the rural African populations

opportunity for income generation (Raebild *et al*., 2011). As of 2010, the potential international market income generation from baobab export was estimated at one billion US dollars per year (Lange, 2010). According to Gruenwald and Galizia (2005), the trade in baobab product has raised rural wealth in Zimbabwe by 250 %.

Baobabs are not useful as timber products, but as shown above and elsewhere, Buchmann *et al*. (2010) identified over 300 uses of African baobab in West Africa. All of these products obtained from baobab trees contribute to income generation and help to reduce poverty, enhance livelihood and allow participation of marginalized people in a growing cash economy (SCUC, 2006; Venter and Witkowski, 2013a). According to Jose (2009) and El Tahir *et al*. (2010), *A. digitata* offers many ecosystem services such as carbon sequestration, biodiversity conservation (habitat for other plants and animals), as well as soil, air and water quality enrichment.

The majority of previous studies on baobabs have focused on morphology, food value, socio-economic importance, ethnobotany and taxonomy (Sanchez, 2011; Sanchez *et al*., 2011b; Mpofu *et al*., (2012), whereas genetic studies have been reportedly rare (Sidibé and Williams, 2002; Kalinganire *et al*., 2008; Wickens and Lowe, 2008; Assogbadjo *et al*., 2009). This study was initiated following the description of a new species of baobab called *Adansonia kilima* Pettigrew, Bell, Bhagwandin, Grinan, Jillani, Meyer, Wabuyele, and Vickers (Pettigrew *et al*., 2012) in mainland Africa, and the need to know the exact number of species of baobabs in Africa and to test the hypothesis by Pettigrew *et al*. (2012) that two species of baobabs should be recognized on mainland Africa.

1.2 GEOGRAPHICAL DISTRIBUTION OF MAINLAND AFRICAN BAOBABS

Until the study by Pettigrew *et al*. (2012), there were eight species of *Adansonia* known in the world (Baum, 1995). Six are native to Madagascar; one is indigenous to mainland Africa, and one is endemic to Western Australia. In 2012, a ninth species, *Adansonia kilima*, was described from mainland Africa and noted to be diploid (Pettigrew *et al.*, 2012). The well-known tetraploid African baobab (*Adansonia digitata*) occurs across Africa in many countries (Fig.1.1). It is found in Benin, Togo, Zaire

(now Democratic Republic of Congo DRC) as well as the estuarine areas of Senegal and on the coastal plains of Ghana. In north eastern Africa, it grows in the Eritrean and Somalian lowlands as well as in the Nuba Mountains of south Sudan. In east Africa, baobabs are found throughout Kenya, southward to Mozambique, where the populations mainly occur on the coast and spread throughout the lowland bushy savannas, while in Tanzania they grow on the upland plateau. *Adansonia digitata* also occurs in Angola, Namibia, Botswana and Zimbabwe, and it grows as a savanna component in the Limpopo province of South Africa. African baobabs are associated with the savannah biome, especially the drier parts, and occur naturally in traditional agroforestry systems (Wickens, 1982).

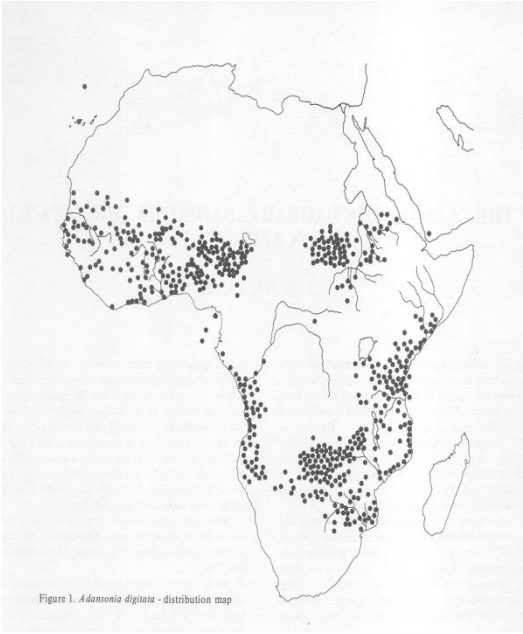

Figure1.1 Distribution map of *Adansonia digitata* in Africa (source: Wickens, 1982).

1.3 ALTITUDINAL DISTRIBUTION OF MAINLAND AFRICAN BAOBABS

The altitudinal range of baobabs is from sea level to about 1500 m a.s.l. (in Ethiopia and Tanzania; Baum, 1995), but baobabs occur most frequently between 450–500 m and 600–700 m a.s.l. (Baum, 1995). Surprisingly, Johansson (1999) recorded elevation range between 1600 and 1750 m a.s.l. for baobabs that occur on the Irangi Hills in the Kondoa District of Tanzania (an area dominated by

Brachystegia spiciformis–Julbernardia globiflora woodlands). However, Johansson (1999) suspected that the baobabs found on these hills were introduced (cultivated). This may suggest that though altitude does not restrict the range of African baobab, dispersal does. Even more surprising was a record of baobabs that occur at 2000 m a.s.l. on the Uluguru Mountains in Tanzania (Bruce, 1924). Dacrémont (1933) also recorded baobabs at an elevation of 1760 m a.s.l. in the Maladi Ducum District in Zaire, now the DRC. Although these baobab specimens occur at very high altitudes, they had no distinct morphological differences that could be linked to difference in altitude (Douie *et al.*, 2015). Interestingly, Pettigrew *et al.* (2012) differentiated the purported *A. kilima* from *A. digitata* due to their elevation differences (among other traits), suggesting that *A. kilima* is restricted to elevations of between 650 and 1500 m a.s.l., whereas *A. digitata* reportedly mainly occurred at elevations below 800 m.

Pettigrew *et al*. (2012) showed that the differences in stomatal size and density of the two purported species of African baobab were correlated with differences in altitude and that the differences in altitude could be linked to difference in ploidy. The number and density of stomata can also be influenced by the ploidy level of the plant (Ramsey and Schemske, 2002). Diploid plants often have leaves with greater stomatal densities that are smaller in size (aperture) than tetraploid plants (Judd *et al*., 2007). Pettigrew *et al*. (2012) reported that the purported diploid *A. kilima* leaves have smaller stomatal apertures (mean length of 26.1μm) and higher stomatal densities (5 per 100 μm^2) than the tetraploid *A. digitata. Adansonia digitata* leaves were said to have longer stomatal apertures (38.3 μm) and lower stomatal density (1.6 per 100 μm^2; Pettigrew *et al*., 2012). However, the stomatal density calculation reported by Pettigrew *et al.* (2012) does not seem realistic given that Douie *et al.* (2015) obtained similar stomatal density value (1.63) by calculating stomatal density as per 10,000 μm^2.

Based on a study of stomatal features, Douie *et al*. (2015) claimed that the purported *A. kilima* exists in Zimbabwe. Their study was conducted in low rainfall areas having annual rainfall of below 650 mm and the specimens were collected from baobabs that occur in natural forests with elevations ranging from 420 m to 1000 m. They examined the abaxial leaf surfaces of mature and healthy leaves from 63 baobab trees using clear nail polish peels to measure stomatal guard cells and calculated stomatal density.

The stomatal density was estimated by counting the number of stomata on five 10000 µm^2 squares on each leaf and taking the mean to obtain the stomatal density per 10000 µm^2. Douie *et al*. (2015) also reported that they tested for bimodality on stomatal length and density using Gaussian Mixture Modeling (GMM, Muratov and Gnedin, 2010).They reported that they established bimodality when plotting stomatal density versus length. However, they did not find any correlation in stomatal length and density with altitude amongst the baobabs they studied. Douie *et al*. (2015) interpreted the observed bimodality to infer two ploidy levels, one being the diploid *A. kilima* and the other the tetraploid *A. digitata* that co-existed at the same locations. They concluded that there were two distinct species of baobabs in Zimbabwe (viz. *A. kilima* and *A. digitata*), but their conclusion was derived from only stomatal size analysis with neither chromosome counts nor flow cytometry data to support it.

Stomatal length and density can be influenced by abiotic factors, such as altitude, temperature and soil moisture content (Wang *et al.*, 2014). Such influence may cause differences in the stomatal size of African baobabs and may have given rise to higher stomatal density and lower guard cell sizes in high temperature and low rainfall geographic locations in Benin (Sanchez, *et al*., 2010). Moreover, Chen *et al.* (2001) reported a correlation between stomatal density and leaf phenology, where younger leaves have higher stomatal densities. Therefore, the variation found in stomatal length and density may be linked to leaf maturity. Consequently, the differences in stomatal length and density reported in the works of Pettigrew *et al*. (2012) and Douie *et al.* (2015) might be as a result of growth changes or plasticity of the phenotype instead of difference in ploidy level.

1.4 MORPHOLOGICAL DIVERSITY IN MAINLAND AFRICAN BAOBABS

Adansonia digitata (Malvaceae) is a deciduous tree growing up to a height of 25 m and measuring about 5–10 m in diameter (Wickens, 1982; Baum, 1995). The crown is normally dense whereas the trunk tapers from top to the base or is bottle shaped or huge and tuberous or cylindrical and measures between 2–10 m in diameter (Sidibe and Williams, 2002). The branches are large, greyish and smooth.

Adansonia digitata has palmate compound leaves with caducous stipules as well as sessile leaflets that vary in size (5–15 × 3–7 cm; Wickens and Lowe, 2008).The flowers are simple and axillary with about five per branch. The buds are single and globose on pendulous flower stalks. The calyx lobes (3–5) are triangular and fused at the base and enclose the flower about 6 hours before anthesis (Wickens and Lowe, 2008). They are greenish and cream on the outside and inside respectively and are markedly reflexed. The petals are white and are also as long as they are broad; measuring between 4 and 8 cm, the androecium is white and is made up of a cylindrical staminal tube (3–6 cm long) with 720–1600 free filaments (of similar length) protruding from the distal end (Baum, 1995). The staminal corolla diameter is the distance between two stamens that are born directly opposite each other on a flower when they are spread out, especially in pressed flowers where the staminal corolla diameter is easy to observe and measure (Baum, 1995). The flowers of *A. digitata* have a superior ovary with hairs pointing upwards. The style is about 15 mm longer than the stamens and is reflexed or straight with a white stigma comprising 5–10 branches (Wickens and Lowe, 2008).

The fruits of *A. digitata* are quite variable, often spherical. However, some are oblate, ovate or cylindrical and are covered in soft golden or greenish hairs and measure about 7.5–54 cm in length and 7.5–20 cm in width (Sidibe and Williams, 2002; Wickens and Lowe, 2008; Zhigila *et al*., 2015). The white pulp is enclosed in a broad and woody pericarp. Seeds are deep brown or reddish black with a smooth testa that is wedged inside the pulp.

Adansonia digitata can be differentiated from other species of the genus *Adansonia* by its pendulous flowers, globose buds, and wide petals (Baum, 1995; Sidibe and Williams, 2002). The round tree apex and irregular branches are also other differentiating features. The flowering time varies significantly; in general, flowering can occur at any time of the year (except during the peak of the dry season), and whether leaves are present on the baobab tree or not (Baum *et al*., 1998).

Within the mainland African baobab population, there is proof for the existence of several morphotypes that can be differentiated by habit, vigour, size, quality of the fruits and vitamin content of the leaves (Gebauer *et al*., 2002; Sidibé and Williams, 2002). For example, a study from many

geographical locations of Benin has shown morphological variation based on climatic zones (Assogbadjo *et al*., 2005). Moreover, similar morphological variation has been reported in baobabs in Mali where farmers used individual differentiating features to identify four varieties of *A. digitata*, by looking at such characteristics as bark colour, height, and width of the tree or pulp and leaf taste (Sidibé and Williams, 2002). In the past, quantitative information relating to the genetic diversity of baobab was poorly documented (Wickens, 1982; Sidibé and Williams, 2002).

However, recent studies assessed the patterns of morphometric variability in the baobab populations across various geographical regions of the Republic of Benin and genetic differentiation as well as diversity of baobab in Malawi (Assogbadjo *et al*., 2006; Munthali *et al*., 2013). In addition, Wiehle *et al*. (2014) studied African baobab genetic resources in neglected populations of the Nuba Mountain in Sudan but further studies are needed to consider patterns of genetic diversity in relation to distribution and morphological variability found in the mainland African baobab. The differentiating morphological features between *A. kilima* and *A. digitata* as per Pettigrew *et al*. (2012) were based on the flower sizes, the staminal corolla diameter and the number of free staminal filaments among others.

1.5 POLYPLOIDY IN MALVACEAE

As noted above, apart from differences in morphological features *A. kilima* was differentiated from *A. digitata* on the basis of ploidy level. Polyploidy (whole genome replication) has been recognized as an important mechanism of species differentiation in plants (Ramsey and Schemske, 2002; Judd *et al*., 2007) and is linked to improved vigour, enhanced morphology, increased sterility, pest, and/or disease tolerance, and higher hybrid fertility (Ramsey and Schemske, 2002). In addition, polyploidy is linked to improved photosynthetic and respiration rates, higher tolerance to adverse temperature, drought, and flooding in plants (Chen, *et al*., 2001). Polyploidy has also been known to influence plant morphology and a major effect is an increase in cell size (Stebbins, 1971; Masterton, 1994; Baum *et al*., 1998). Pettigrew *et al*. (2012) reported that the two putative African baobab species have different ploidy levels, viz: diploid, *A. kilima* (2n = 88) and tetraploid, *A. digitata* (4n = 176). These ploidy levels were obtained

from chromosome counts on crushed and stained root tips and apical meristems of germinated baobab seeds using a field microscope. Specimens were also assigned to diploid or tetraploid based on stomatal length and density (Pettigrew *et al*., 2012). However, Sanchez *et al*. (2010) studied leaf morphology (e.g., leaf length and thickness, stomatal size and density on the leaf surfaces) of baobab trees in Benin from different agricultural and climatic zones and reported significant differences in leaf size and stomatal features (e.g., stomatal length and density) between these various zones. The authors concluded that the differences detected in leaf morphologies were due to the environmental and intrinsic drought tolerance of baobabs in that region. Another plausible explanation could be that there is a difference in ploidy level which led to the differences seen in the leaf morphologies. In this present study, ploidy level was inferred from stomatal length as per the differences noted between the diploid *A. kilima* and tetraploid *A. digitata* by Pettigrew *et al*. (2012).

Given the potential variation in ploidy level, or genome size, between the two presumed baobab species in mainland Africa, there may be two species of baobabs in Africa. The aims of this study were therefore to (1) examine morphological traits (mainly floral and stomatal features) from samples across mainland African baobab populations to establish whether there are distinct differences that can be correlated with the altitudinal differences in order to distinguish between *A. kilima* and *A. digitata* and (2) use stomatal size and density to infer ploidy levels of baobabs to test for correlations between ploidy and low and high altitudes.

1.6 SPECIES CONCEPTS

1.6.1 What is a species?

"The species are the fundamental units of evolution or the total genetic variability of nature organized in the form of discrete packages" (Mayr, 2005: 353), they are one of the basic units of comparison in all fields of biology, such as anatomy, ecology, evolution, genetics, molecular biology, paleontology, physiology and systematics. The importance of species in biology comes from their importance in systematics, which accounts for the taxonomic framework used in all fields of biology and

'species concept' has been the central concept in systematics (Mayr, 2005). Systematics is the study of biological diversification of living forms, both past and present and their correlations among living organisms through time. In systematics the correlations and dependence within, among, and between species are depicted as phenograms or phylogenetic trees. In order to delineate species, systematics uses taxonomy (a branch of systematics) as the primary tool to provide scientific names for organisms and describe them as well as preserve collections of the organisms and provide classifications for them. It also provides the key for their identification, data on their distributions and considers their environmental adaptations (Michener *et al*., 1970).

To further investigate whether *A. kilima* is the same species or different from *A. digitata*, the following species concepts were applied. Firstly, the Biological Species Concept (BSC) was useful, given that previous work by Pettigrew *et al*. (2012) indicated that the purported *A. kilima* and *A. digitata* have different ploidy levels which may prevent them from interbreeding (ploidy acts as a post- mating reproductive isolating barrier). This would then support their recognition as distinct species under the BSC. Pettigrew *et al*. (2012) reported that baobabs having stomatal length ranging from 20.40 μm to 31.80 μm were diploid whereas those having stomatal length ranging from 33.80μm to 42.30μm were tetraploid. Hence, ploidy levels were inferred from stomatal categories as per Pettigrew *et al*. (2012). Secondly, the Ecological Species Concept which is also informative and facilitated the determination of one or two baobab species on mainland Africa was employed. This was appropriate because the previous study by Pettigrew *et al*. (2012) indicated that difference in altitude was responsible for the morphological variation between *A. kilima* and *A. digitata*. Lastly, the Phenetic Species Concept was applied to determine if cluster and ordination analyses of equally weighted morphological trait measurements supported the hypothesis that there were two distinct species of baobabs in Africa.

1.6.2 Biological Species Concept

The Biological Species Concept (BSC) defines a species as "a reproductive community of populations that occupy a specific niche in nature or a set of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups" (Mayr, 2005: 66). Reproductive isolation is the central focus of the BSC. Reproductive isolating mechanisms prevent members of different species from producing offspring or ensure that offspring produced is sterile (Mayr, 2005). These barriers (reproductively isolating mechanisms) preserve the integrity of the species by reducing gene flow between related species (Mayr, 1996).

African mainland baobabs are said to occupy a specific niche and interbreed in nature. However, *A. digitata* does not interbreed with the Madagascan species where it has been introduced to Madagascar (Baum, 1995). The biological species concept is therefore, the result of genetic variation between species which produces distinct living forms that are separated from one another by barriers to reproduction (Mayr, 2005). These species are differentiated from one another by barriers to reproduction and the barrier to reproduction is the cause of disruption between sympatric organisms (Cain, 1953). If *A. kilima* and *A. digitata* have different ploidy levels as reported by Pettigrew *et al*. (2012), this may prevent them from interbreeding (ploidy acts as a post- mating reproductive isolating barrier: Futuyma, 1998), and were they to interbreed, the first generation hybrids might or might not be viable, whereas the subsequent progenies would be totally unviable or sterile (Mayr, 2005). The inability of the two populations with different ploidy levels to interbreed and produce viable offspring would then support their recognition as distinct species under the BSC.

It is important to note that the common features of a single species that are of vital importance in the BSC are those that provide the biological function of the species, that is the protection of compatible gene pools (gene combinations) known as isolating mechanisms (Mayr, 1996). The criterion of the biological species concept is interbreeding and this can only be tested by having two organisms next to each other (i.e. sympatric). Reproductive isolating mechanisms (barriers to reproduction) also play key roles in the BSC because they prevent reproduction, and thus gene flow between diverse sets of organisms, and sometimes guarantee genetic variation between groups (Mayr, 1996). There are two

forms: pre-mating or pre-zygotic mechanisms, which may be as a result of ecological or habitat isolation, seasonal or temporal isolation, sexual or behavioural isolation, mechanical isolation and gametic isolation. The second form is post-mating or post-zygotic isolating mechanisms that acts after mating has taken place to prevent fertilization or to prevent subsequent hybrids from transferring their genes. This may be as a result of hybrid non viability where zygotes are less viable thus, leading to hybrid sterility and hybrid breakdown where the first generation (F1) hybrids are viable and fertile but subsequent hybrid generations (F_2 and back crosses) are unviable or sterile. The BSC is important in this study as it is used to investigate whether the morphological features (aspects of flower size, pollen grain size and density) are correlated with differences in altitude and whether the differences in altitudes are linked to differences in ploidy level. The ploidy levels of African baobabs were inferred from stomatal categories as per Pettigrew *et al.* (2012) and included in a phenogram to see whether the specimens of different ploidies form distinct clusters and thereby indicate separate species.

1.6.3 Ecological Species Concept

The Ecological Species Concept (ESC) is defined as "a lineage (or intimately connected set of lineages) which occupies an adaptive zone minimally different from that of any other lineage in its range and which evolves separately from all lineages outside its range" (Van Valen, 1973: 49). A lineage is a clone or an ancestral descendant of a population, whereas a population is a group of individuals in which adjacent individuals occasionally exchange genes with each other reproductively, and in which adjacent individuals do so more frequently than with individuals outside the population (Van Valen, 1976). Lineages are closely related if they occupy the same adaptive zone as their latest ancestors. If their adaptive zones have changed since then, they are closely related if the new adaptations have been transferred among the lineages rather than originating separately in each. It is important that the following terms which are also relevant to the discussion of ESC in terms of establishing whether *A. kilima* and *A. digitata* are distinct or one species be defined here for clarity.

Species exist and interact with each other as well as with other organisms in nature for resources (such as nutrients, water, and sunlight) as a result of the adaptations or specializations acquired

by the species to survive in and exploit their environment. The interface at which these interactions occur is known as adaptive zone (Van Valen, 1971). It is a part of the ecosystem which is almost as distinct as the species that occupy it. The species in an adaptive zone exist independently from other species within the ecosystem. The "zone" implies that there are borders within the ecosystem. The boundaries of an adaptive zone may be permanent and may remain unchanged no matter the species in it. In some cases there may not be permanent borders but there is always an imposition of division by the species within the resource space. The Madagascan species (e.g. *A. madagascariensis* and *A. perrieri*) dominate the baobab forest in Madagascar and are generally low altitude species. They usually occur in dry deciduous forests (Baum, 1995). *Adansonia madagascariensis* usually prefers dry or moist soils particularly those that are made up of limestone, sandstone and gneiss soils (Sidibe and Williams, 2002). It occurs in the Antsiranana province in Madagascar close to the sea, whereas *A. perrieri* occurs in five known locations of predominantly evergreen forests on the banks of rivers or in dry deciduous forests in northern Madagascar. It is important to note that the resource spaces of the Madagascan baobabs are distinct and well defined (Baum and Oginuma, 1994). If the resource spaces of *A. kilima* and *A. digitata* hardly overlap (Pettigrew *et al*., 2012), this would mean that their separation would be quite distinct even spatially. ESC is therefore useful in placing my data into context to ascertain if the two presumed African baobabs exist in different ecological niches (i.e. at different altitudes) and if any observed difference in altitude is linked to differences in their morphology. This can be done with cluster analysis and principal component analysis to determine if the specimens under study will form distinct clusters (separate groupings) at low and high altitudes to indicate the two different species of baobab as described by Pettigrew *et al*. (2012). The ESC provides a useful conceptual framework for experimentation and observation at the boundary between systematics and ecology and has the potential to clarify variation in diversity and reproductive patterns. Besides, it also sheds light on the nature of variation patterns and might therefore help in taxonomic judgment where the BSC is conceptually inapplicable and the Evolutionary Species Concept fails to provide useful concepts, particularly in some taxonomically difficult cases (Andersson, 1990).

Since *A. kilima* and *A. digitata* reportedly occur at different altitudes, it is likely that if viable offspring are produced where the putative species co-occur, this would suggest there is only a single species. It has been shown by Douie *et al*. (2015) that the difference in stomatal features were not correlated with differences in altitude and that inferred ploidies coexisted at the same locality. It is likely that the morphological differences within the supposed *A. kilima* and *A. digitata* observed by Pettigrew *et al*. (2012) are not correlated with a difference in altitude. It remains to be seen whether they are linked to a difference in ploidy. The ESC is relevant to this study as it may be used to show whether the morphological differences observed between *A. kilima* and *A. digitata* by Pettigrew *et al*. (2012) are correlated with altitude and/or linked to differences in ploidy. The altitudinal differences observed in the few baobabs studied by Pettigrew *et al* (2012) might have occurred at different altitudes coincidentally and not be due to differences in ploidy level.

1.6.4 Phenetic Species Concept

To further examine whether the two reported baobab species in Africa are the same or different, their morphological characteristics were assessed with the Phenetic Species Concept. The Phenetic Species Concept recognizes species on the basis of as many morphological (or other) characteristics as possible and pheneticists may view species as groups of individuals with many similar diagnostic features. Therefore, the phenetic species concept has been defined as "a set of organisms that look similar to each other and distinct from other sets" (Sokal and Sneath, 1963:359). However, a more classical definition would indicate the extent of phenetic similarity, and the similarity would be measured by a phenetic distance statistics. In practice, the phenetic species concept would measure as many characteristics as possible in numerous specimens and identify phenetic clusters by multivariate statistics. These methods include, Cluster Analysis (CA), Principal Component Analysis (PCA) and Non-metric Multi-dimensional Scaling (NMDS, Sneath and Sokal, 1973). The clusters resulting from these analyses give an estimate of the level of difference that is enough for a set of organisms to be recognized as a species. The recognition and delimitation of species has often been random, personal choice as used in

classical taxonomy, however, the introduction of more objective quantitative methods makes it possible for one to measure the similarities, dissimilarities and distances between groups and make them clearer through the production of comprehensible phenograms (Sokal and Sneath, 1963).

To compare the morphological, reproductive and distributional properties of the species requires the use of phenetic analyses of as large a number of specimens as possible. There are several techniques for ascertaining distances between individuals and for the ordination of these specimens. They rely more on the method of ascertaining similarity matrices and clustering them to form hierarchic groups (Sneath and Sokal, 1973). According to Ridley (1989), it is the duty of the researcher to decide if taxonomic methods should use ordination or clustering techniques (that is, which is the more suitable technique to use). Clustering and ordination methods were applied in this study as they emphasize the different aspects of the relationships among the specimens sampled (referred to as 'operational taxonomic units' or OTUs by phenetists) based on the various morphological characteristics of African baobabs. Cluster analysis produces a better representation of higher distance relationship among specimens whereas ordination produces a better representation of the closest distance relationship among specimens (Sokal and Sneath, 1963). Other advantages of cluster analysis over ordination are that it makes few assumptions regarding the relationship in the data set and it does not impose a ranking structure on the data, such that it recognizes similar patterns (Faith and Norris, 1989). Cluster analysis on the other hand has the disadvantage of imposing a ranking structure on the dataset such that the analysis might exhibit distinct clusters even if variation is clinal (Thorpe, 1983).

Many studies used numerical taxonomy techniques to delineate species to determine whether multiple species should be recognized in a particular group. Examples of such studies include that of Cron *et al.* (2007) who studied morphological variation in *Cineraria lobata* in order to delineate the subspecies and to determine whether recognition at the intraspecific level is warranted. Goodman (1968 and Goodman and Paterniani 1969) also made similar studies on maize and Oka (1964) reported analogous studies on rice.

Other uses of geographic variation analysis include analysis of the geographic variation patterns in order to interpret these patterns as adaptations to variation in known environmental factors, for example climatic, topographic or edaphic variables, or variation in distributional, reproductive or ecological patterns within the species. Besides, geographic analysis can be used to allocate unidentified specimens to a given population or geographic locality with a stated probability of success (Gabriel and Sokal, 1969).

The Phenetic Species Concept is relevant to this study because it can assist me in determining whether there is any geographic pattern of variation in the morphological features of the African baobabs under study and to ascertain whether they are correlated with differences in altitude and if the differences in altitude are linked to differences in ploidy.

This study examines floral and stomatal traits of samples across mainland African baobab population to establish if there are distinct differences in order to identify the species in Africa. Ploidy levels were inferred from stomatal size and density as per Pettigrew *et al*. (2012) to test for correlation between baobabs that occur at low and high altitudes. This will help to know whether the presence of one or two baobab species should be recognized in Africa.

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APPENDIX 1

Baobab specimens used in this study

Bruce 210 (BM). Tanzania, Ulugurus, 1 Nov 1924, ca, 2000 m.

Dacrémont 366 (BR). DRC, 1 Dec 1933, 1760 m.

CHAPTER TWO

2.1. INTRODUCTION TO THE STUDY, METHODS, RESULTS, AND DISCUSSION

The potential foreign exchange earnings from baobab products are quite enormous (Lange, 2010). However, until the study by Pettigrew *et al*. (2012), there were eight known species of *Adansonia* L. in the world (Baum, 1995). Six are endemic to Madagascar; one is native to mainland Africa, and one is indigenous to Western Australia. In 2012, however, a ninth species, *Adansonia kilima* Pettigrew, Bell, Bhagwandin, Grinan, Jillani, Meyer, Wabuyele and Vickers (Pettigrew *et al*., 2012) was described from mainland Africa.

Morphological features, ploidy level, and molecular phylogenetic evidence were presented by Pettigrew *et al*. (2012) to distinguish the new species of baobab, *A. kilima* from *A. digitata*. The authors reported that *A. kilima* is a diploid (2n = 88), whereas *A. digitata* is a tetraploid (2n = 176). Morphological measurements were used in their study to distinguish the two species. Such measurements included: calyx length and breadth, petal length and breadth, free staminal filaments, diameter of staminal corolla, pollen spine density, pollen volume, as well as mean stomatal length and density (Appendix 2). *Adansonia kilima* was said to have smaller mean stomatal length and larger density per 100 μm^2 (26.1 ± 5.7 (S.E) and 5.0 ± 2.1) than *A. digitata* (38.3 ± 4.5 and 1.6 ± 0.7). The petals of *A. digitata* were also reported to be longer than the stamens and strongly reflexed when the flowers open but *A. kilima* has shorter stamens than the petals and the petals are not reflexed in the open flowers. According to Pettigrew *et al*. (2012), the size of the flowers of *A. kilima* was stated to be smaller (about half) than that of *A. digitata* with flowers usually numerous per tree contrary to only two or three open flowers produced by *A. digitata* per tree per day. Moreover *A. kilima* was said to have short petals when compared with the androecium in contrast, petals of the flowers of *A. digitata* were said to be longer than the androecium and were more reflexed in mature flowers. *Adansonia digitata* also showed noticeable kinesis (where the petals turn rapidly at night time to reveal the androecium that was originally concealed by the long petals), but flower kinesis was said to be absent in *A. kilima*. Further, it was shown that the large size and orientation of the

petals of *A. digitata* made it easily recognizable from a distance unlike the small petals of *A. kilima* which were partly enclosed by the calyx. In addition, flowers of *A. digitata* were said to have about twice the number of free staminal filaments (700–1600) than *A. kilima* (270–640), also the length of the style was said to be shorter in *A. kilima* whereas it was longer than the petals in *A. digitata*. Besides, the diameter of the staminal corolla (38–42 mm) in *A. kilima* was said to be about half that of *A. digitata* (95–106 mm). Furthermore, the pollen grains of *A. digitata* were said to be more easily distinguished from those of *A. kilima* using a field microscope because the former had twice the volume of pollen grain than the latter. *Adansonia digitata* was also said to have larger and stouter spines with lower spine density than *A. kilima*. The diameter and volume of the pollen grains were also smaller in *A. kilima* (43.7± 3.4 and 42.8–44.6μm^3 x 10^3) than *A. digitata* (63.4 ± 7.7 and 118.8–133.0 μm^3 x 10^3). *Adansonia kilima* reportedly had more numerous spines (142–158) per 1000 μm^2 than *A. digitata* (51–100). In addition, the small pollen grain diameter and volume were said to be in line with the pollen grains of other diploid *Adansonia* species, for example *A. madagascariensis* (Pettigrew *et al*., 2012).

Lastly, the phylogenetic evidence presented by Pettigrew *et al*. (2012) is inconclusive owing to the fact that the phylogeny based on ITS sequence data is not fully resolved within the *A. digitata/A. kilima* clade. In other words, the clade does not show two clear groupings, which means that the samples of *A. kilima* and *A. digitata* do not form distinct monophyletic clades.

However, the specimens used to collect measurement data of these features were not true representative samples of the baobab distribution in Africa. The data used in Pettigrew *et al*. (2012) were based on only six specimens of each species and only four samples of each were used in the counting of free staminal filaments. Given the small sample size and inconclusive phylogenetic evidence reported by Pettigrew *et al*. (2012), there is doubt about the presence of a second species of baobab in mainland Africa. Further, most of the samples used in the DNA and morphological studies had no vouchers, although Pettigrew *et al*. (2012) provided GPS co-ordinates for the trees in Tanzania. Moreover, standard techniques were not used for the measurement of the stomatal dimensions. Images of stomatal guard cells were measured on images captured with iphone camera instead of professional camera. In addition,

Pettigrew *et al*. (2012) examined the adaxial leaf surface of the leaflets, but these have been reported to have few or no stomata (Sanchez *et al*., 2010).The adaxial leaf surfaces of 16 herbarium and four fresh specimens were examined in this study and as I found no stomata on them, all stomatal measurements were done on the abaxial leaf surfaces. Given the above reasons, it is important to re-examine the characteristics measured by Pettigrew *et al*. (2012) and compare to a wide range of baobab specimens. This will provide evidence as to whether *A. kilima* and *A. digitata* are the same or different species. It is also important to accurately determine the identity of the various baobab populations in Africa, how many species are on the continent, and how they differ from each other to facilitate future identification of trees.

Therefore, this present study re-examines the hypothesis that two baobab species should be recognized in Africa as proposed by Pettigrew *et al*. (2012) by examining many of the same traits investigated in their study across a greater sampling of baobabs across the African continent.

In addition to traditional floral features, pollen traits, like spine density, can be used to ascertain differences between the two species and also indicate differences in ploidy. For example, *Adansonia digitata* reportedly has a lower spine density (51–100 per 1000 μm^2, Pettigrew *et al*., 2012) and the spines are also larger than the spines on the pollen grains of *A. kilima* (142–158 per 1000 μm^2, Pettigrew *et al*., 2012). It is worth noting that pollen grains from the diploid *A. madagascariensis* have a greater spine density than *A. kilima*, but pollen grains from the sister species of *A. digitata* (A. *gregorii* F. Muell.) have an even lower spine density than *A. kilima* (Pettigrew *et al*. 2012). According to Pettigrew *et al*. (2012), pollen grain diameter from tetraploid *A. digitata* (63.4 ± 7.7 μm) was one third larger than the pollen grain diameter of diploid *A. kilima* (43.7 ± 3.4 μm).

Besides the pollen size, the number and density of stomata on leaves of a plant can be a good indicator of ploidy (Sari *et al*., 1999). In general diploid plants tend to possess leaves with greater stomatal densities that are smaller in size (aperture) than tetraploid plants (Judd *et al*., 2007). *Adansonia kilima* was reported by Pettigrew *et al*. (2012) to have smaller stomatal length (26.1 ± 5.7 μm) and greater stomatal density (5.2 ± 2.1) per 100 μm^2 than the tetraploid *A. digitata* with both stomatal length and

density of 38.3 ± 4.5 µm and 1.6 ± 0.7 per 100 µm^2 respectively. Confirmation of diploidy and tetraploidy can be obtained by using flow cytometry analysis to compare the nuclear DNA content of the cells (Saltonstall *et al*., 2007) and inferred from the sizes of pollen grains (Rhodes and Zhang, 1999).

2.2. Objectives of the study

1 Examine morphological traits (floral and stomatal features) from samples across mainland African baobab populations to establish whether there are distinct differences that can be correlated with the altitudinal differences in order to identify the species present in Africa.

2 Use stomatal size and density to infer ploidy levels of baobabs that occur at low (< 800) and high (≥ 800) altitudes.

2.3. Questions

1. Are the morphological features of African baobabs (aspects of flower size, pollen grain size and density) correlated with differences in altitude and can they be linked to differences in ploidy?
2. Are the differences in stomatal size and density of the two species of African baobab correlated with differences in altitude and can they be linked to ploidy?
3. Do the findings of this work corroborate the differences between two species of baobabs that occur at low and high altitudes as reported by Pettigrew *et al*. (2012)?

2.4. MATERIALS AND METHODS

2.4.1. Study Samples

Baobab herbarium specimens, from across Africa with both leaves and flowers were loaned to represent the species' widespread distribution in Africa. Some baobab specimens from C. E. Moss Herbarium, University of the Witwatersrand, Johannesburg (J) were also used in this study. The rest of the specimens were loaned from the following herbaria: the British Natural History Museum (BM), Botanic Garden Meise Belgium (BR), Natural History Museum of Kenya (EA), Royal Botanical Garden Kew (K), Larry Leach Herbarium University of Limpopo (UNIN), Buffelskloof Herbarium (BNRH), Missouri Botanical Garden (MO), New York Botanical Garden (NY), Pretoria National Herbarium South Africa (PRE) and National Museum of Natural History Washington D.C. (NMNH). In addition, eight fresh specimens with leaves and flowers were collected from the Vhembe District of the Limpopo Province of South Africa and the Caprivi Strip in Namibia, including specimens from the same trees as the holotypes and the paratypes of *A. kilima* respectively.

2.4.2. Analyses of the floral features of African baobab

To differentiate between the two putative species, the following floral features were measured: calyx length, calyx breadth, petal length, petal breadth, staminal tube length, staminal corolla diameter, style length and stigma branch length. The measurements were taken from the floral parts of 124 baobab herbarium specimens using a 200 mm ruler. In addition, PCA, NMDS, and cluster analyses as well as, scatter plots, box and whisker plots were performed on the floral dataset to assess if there were differences among specimens and if there were any, to ascertain whether they were comparable to those reported in the previous study by Pettigrew *et al*. (2012).

2.4.3. PCA, NMDS, and Cluster Analyses

To test whether differences in floral features at different altitudes resulted in separate groupings representing the two reported species of baobabs in Africa, both cluster analysis and principle component

analysis (PCA) were conducted using NTSYS-pc version 2.2 (Rohlf, 2004). A ranking structure is imposed on data by cluster analysis, such that the analysis might exhibit distinct clusters even if variation is clinal as may be seen in ordination analyses (Thorpe, 1983). As a result, cluster and ordination analyses were both used in this study because the former produced a better representation of higher distance relationships among specimens whereas the latter produced a better representation of the closest distance relationships (Sneath, 1976). Non-metric multi-dimensional scaling (NMDS, Kruskal, 1964; Cox and Cox, 2000) was also carried out because it performs better than other ordination analyses when there are missing data (Kruskal, 1964; Cox and Cox, 2000).

In order to produce a phenogram using cluster analysis, comma delimited and standardized floral dataset was used. The characters were standardized by dividing the difference between the mean and the real measurement by the standard deviation using the standardization option in NTSYS-pc version 2.2 (Rohlf, 2004), then a dissimilarity matrix based on average taxonomic distance was calculated using the unweighted pair-group method and arithmetic averages algorithm (UPGMA) to hierarchically cluster the specimens. To test how well the tree fits the data, the cophenetic correlation coefficient (r) was plotted. A cophenetic correlation coefficient of 0.8 or 0.9, shows that the hierarchic classification obtained by the clustering method is a reasonably true representation of the original resemblance matrix [i.e. an indication of the goodness of fit of the tree to the dataset], (Sokal and Rohlf, 1962; Sneath and Sokal, 1973). Two phenograms were produced; the first phenogram investigated reported floral differences that correlated with altitude among African baobabs. Seven floral characters (Appendix 2) were examined for 124 specimens (Appendix 2) and each specimen was identified as occurring at either 'low' (< 800 m a.s.l.) or 'high' (≥ 800 m a.s.l.) altitude as per the definition of Pettigrew *et al*. (2012). It should be noted that altitude data were taken directly from collectors' notes on the herbarium specimens, if provided. Otherwise they were derived indirectly from the locality names using online resources to find out the altitude of the locality and using these as the altitude for the specimens. In addition, the altitudes of herbarium specimens were also derived indirectly from the quarter degree square (QDS) information

especially for the herbarium specimens collected from southern Africa if the QDS information was provided on the collectors' notes. This is verified using the altitude of the locality for accuracy. These altitude data were associated with the morphological evidence to determine if there was an ecological distinction between the two proposed groups.

Ten floral traits were initially measured and examined on 139 baobab specimens in this study. Fifteen specimens were missing four values of the ten examined characters and were discarded from the floral analysis. Three floral features (pollen grain diameter, pollen spine density, and stigma branch length) were measured on only 20%, 20% and 30% of the specimens respectively. Consequently, these characters were also excluded from the dataset. Ordination analyses were therefore performed on 124 specimens based on seven floral characters using NTSYS-pc version 2.2 (Rohlf, 2004). A dissimilarity matrix using average taxonomic distance was computed and the UPGMA clustering method was used to hierarchically cluster the specimens. The cophenetic correlation coefficient (r), which indicates the goodness of fit of the tree to the dataset, was computed for each resultant tree and its distance matrix (Sokal and Rohlf, 1962; Sneath and Sokal, 1973).

Principal component analysis was performed on the dataset. This was done by standardizing the matrix by variables, computing correlations among characters and using the eigenvectors to project the specimens in two dimensional and three dimensional spaces (Rohlf, 2004).

2.4.4. Box and Whisker plots for floral features

Box and whisker plots were generated using Statistica version 12.5 (Statsoft, 2014) to compare the variation in each of nine floral characters across the same 124 specimens from the multivariate analyses. Specimens in these analyses occur both below and above 800 m (a.s.l.). A t-test was performed to test if the specimens that occur at low and high altitudes differed significantly for each of the nine floral features of African baobabs. In addition, a t-test was used to establish whether the mean calyx length, petal breath, and staminal tube length of fresh specimens were significantly different from those of dry herbarium

specimens. Four fresh baobab specimens were compared to 120 herbarium specimens. The fresh specimens include three collected from the same trees where the holotypes and the paratypes were collected from the Vhembe District of the Limpopo Province of South Africa and the Caprivi Strip in Namibia respectively.

2.4.5. Pollen Analyses

Measurements of pollen grain diameters were carried out using a digital microscope (Axio imager M2, ZEISS M2 Professional, ZEISS International Company Germany). The pollen grains were collected from 42 herbarium specimens and eight fresh specimens. The grains were placed on a clean microscopic slide, with a drop of water for optimal viewing, covered with a cover slip and observed on a digital microscope at scale bar of 20 µm. Images of the pollen grains were captured using a digital camera (Axiocam 506 Colour, ZEISS M2 Professional). Pollen grain diameters were measured with the distance component of the ZEISS Axiovision software. Pollen grains from 20 baobab specimens were also examined using EFI Quanta 200 scanning electron microscope (SEM) to confirm the accuracy with the measurements of the pollen grain diameters made with the ZEISS Axiocam 506 compound microscope. The majority of the measurements of the pollen grain diameters were done with the compound microscope (Axio imager M2, ZEISS M2 Professional) because it is less expensive and assisted in a preliminary exploration into whether pollen grain size differs significantly among individuals. The mean pollen grain diameter for each specimen was calculated by calculating the average diameter of five pollen grains per field of view using digital images, while pollen spine density was calculated by counting the number of pollen spines on the visible surface of a pollen grain. Double-sided tape measuring 2 mm^2 was used to mount pollen grains on a silver disc for examination under the scanning electron microscope (FEI Quanta 200 Scanning Electron Microscope). The pollen grains from 20 of the 50 specimens were coated with gold palladium for optimal viewing. The pollen grains from each specimen were then viewed with a microscope controlled computer monitor linked to the SEM and images were captured in black and white (Fig. 2.1) using the linked infrared camera. The diameter of the pollen grains was calculated by

multiplying the value of the length of the distance across each pollen grain on the image by the actual scale bar on the computer and dividing the product by the measured length of the actual scale bar of the image. An example is shown below:

The length of pollen grain =106 mm, actual scale bar = 50 µm and measured length of scale bar = 113 mm.

$$\text{Pollen grain diameter} = \left(\frac{\text{length of pollen grain x actual scale bar of image}}{\text{measured length of scale bar}}\right)$$

$$= \left(\frac{106\text{ mm x }50\mu\text{m}}{113\text{ mm}}\right)$$

$$= 46.9\ \mu\text{m}$$

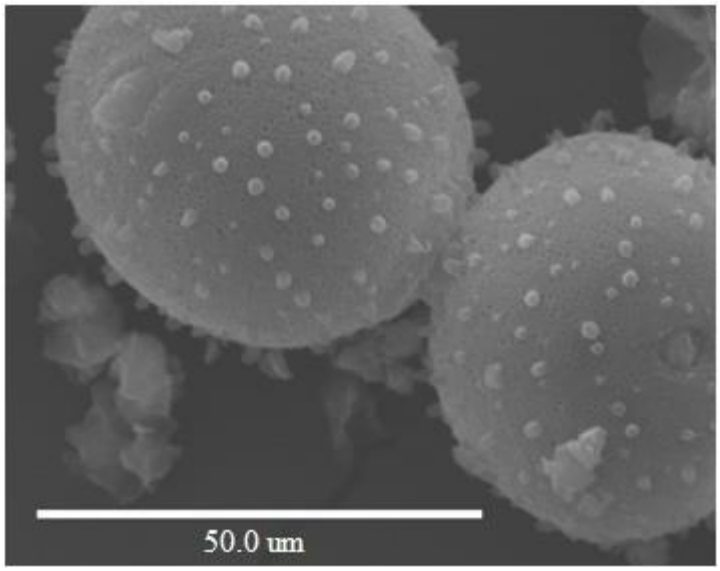


Figure 2.1. Digital image of African baobab pollen grains, showing pollen spines, Image was viewed with EFI Quanta 200SEM at 30 Kv and at 3060× magnification and photographed with internal TV camera (CCD). Specimen: *Greenway and Kanuri 14, 614* (MO). Scale bar = 50 µm. Photo: author.

The pollen spine density per 1000 μm^2 was calculated by dividing the number of spines (n) on the visible surface of a pollen grain (one hemisphere) by half the surface area (A) and multiplying the result by 1000: density = $\left(\frac{n}{0.5A}\right)\left(\frac{1000}{1}\right)$. The surface area ($4\pi r^2$ for a whole sphere) of one hemisphere of the pollen grain was calculated using the formula $2\pi r^2$ (in μm^2), i.e. the visible surface area.

The extent of variation and overlap in average pollen grain diameter between the specimens at low and high altitudes were investigated with box and whisker plot of the average diameter of five pollen grains per field of view. A t-test was performed on average pollen grain diameter to ascertain whether there was any significant difference between the specimens based on the two reported altitudinal ranges. Box and whisker plots were also used to verify whether the variation in pollen grain volume is linked to variation in the altitudes (high and low altitudes) of the African baobabs under study. A t-test was used to establish if there was any significant difference in pollen grain volume between the specimens occurring within the two altitudinal ranges.

2.4.6. Inferring ploidy level using stomatal features

2.4.7. Stomatal density

Clear nail polish (Revlon, USA) was lightly applied on the abaxial and adaxial leaf surfaces of selected leaves from pockets attached to 50 herbarium specimens from a range of localities across Africa. The nail polish was used to create an impression of the leaf epidermis on each surface as per Saltonstall *et al*. (2007). The nail polish was gently lifted from the surface of the leaf with a pair of forceps after it had dried to reveal an impression of the leaf epidermis. The peel was then mounted on a slide, with a drop of water added to soften and flatten it and then covered with a cover slip and observed on a compound microscope (ZEISS Axio Imager M2) at 400× magnification. Images of the stomata (Fig.2.2) were captured through a digital camera (ZEISS Axiocam 506 Colour digital camera) and the stomatal counts were carried out for three different fields of view on each peel using the digital images. Clear nail polish impression of the leaf epidermis from the midvein region according to Sanchez *et al.* (2010), from the adaxial surfaces of leaves of four fresh and 16 dry specimens of the 50 specimens used for stomatal analysis were also examined for the presence of stomata. This was necessary because Pettigrew *et al.* (2012) reported that their stomatal analysis was performed on measurements of stomata found on the adaxial surfaces of leaves of African baobabs. As no stomata were found on the adaxial surface of the leaves that were examined, stomatal measurements were restricted to the abaxial surfaces of the leaves

moving across each leaf epidermis from one end to the other in order to make sure that the measurements on each leaf epidermis were true representation of that particular leaf. The number and size of the stomata also varied near the midvein regions and measurements of stomata were done on mature leaves. The stomatal density (per 1000 μm^2) was calculated for each field of view by dividing the number of stomata per field of view by area of the field of view. It should be noted that the area of field of view was calculated on the image of leaf epidermis impression on the computer screen using Axiovision software which calculates the area of field of view by drawing a rectangle over the area of field of view on the computer screen using a graphical component called rectangle alignment. The formulae used are as shown below along with an example:

Stomatal density = number of stomata/(area of field of view) × 1000

Stomatal density = 48/(195991) ×1000/1(μm^2)

= 0.24 stomata per 1000 μm^2

This shows that 0.24 stomata would be found on every 1000 μm^2 of the abaxial leaf surface of that particular baobab leaf. A t-test was used to ascertain whether there was any significant difference between stomatal densities of baobabs found at low (< 800 m a.s.l.) or high (≥ 800 m a.s.l.) altitudes.

2.4.8. Measurement of stomatal guard cell

The same nail polish impressions of the leaf epidermis used for stomatal counts were used to measure the widest length and width of the stomatal guard cells (Fig.2.2) using the distance component of the ZEISS Axiovision software. A total of 15 stomata per specimen (five stomata per field of view and three fields of view per specimen) were measured for 50 leaf peels. A t-test was used to investigate whether there was any significant difference in the mean stomatal length between specimens that occurred at low or high altitude. The mean stomatal length was the average of fifteen stomata measured for the three fields of view that were observed for each leaf. The measurements of the three fields of view were used to provide within leaf replication and a t-test was used to ascertain if there was any significant difference in size between stomata within fields of view, among fields of view, and among specimens.

The area of the stomata was calculated using the area of the ellipse which best represented the shape of the guard cells: Area of stomata = $\pi r2$ or $\pi\left[\left(\frac{d1}{2}\right)\left(\frac{d2}{2}\right)\right]$ (where d_1 and d_2 are the diameters of the length and width of leaf stomata respectively)

Area of stomata = [(3.143) (28.139/ 2) (12.734/2)]

= 281.552 μm^2.

Box and whisker plots were used to compare the variation and overlap in average stomatal length of the stomatal guard cells between the baobabs found below and above 800 m a.s.l. These plots were also used to compare the variation and overlap in stomatal density (per 1000 μm^2) between specimens that occur within these two altitudinal ranges. The specimens were first grouped according to altitude, then according to the size range of the two ploidy categories as reported by Pettigrew *et al*. (2012) who showed that baobabs from low altitude (tetraploids) have stomatal lengths ranging from 33.80 to 42.80 µm (38.3± 4.5 µm), whereas those from high altitude (diploids) have stomatal length ranging from 20.4 to 31.80 µm (26.1 ± 5.7 µm), intermediates from the dataset between the two ranges were not included in these putative ploidy groups. In addition, a third group was created by dividing the dataset into two groups by drawing a midline in the gap between the reported ploidy categories, viz those specimens that had stomatal length of 31.90 µm and above were grouped together and those baobabs with stomatal length of 31.80 µm and below were grouped together (note that intermediates between the two ranges were included in this group). A t-test was used to determine if there was any significant difference between the two groups of baobabs under study according to altitude. Moreover, box and whisker plots were also used to compare the variation in the mean stomatal length and density of the strict ploidy groups. A t-test was used to ascertain if the mean stomatal length and density of the strict ploidy groups were statistically significantly different. A histogram of number of specimens versus mean stomatal length was plotted to confirm whether the data were bimodal or unimodal. In addition, t-tests were performed to establish if the mean stomatal length and density of fresh specimens were significantly different from the mean stomatal length and density of dry herbarium specimens. This will help to

confirm if data and information obtained from dry herbarium specimens are a true representation of the population and should be recommended for morphological studies.

Moreover, scatter plots of mean stomatal length versus width, number of stomata per field of view versus stomatal length and number of stomata per field of view versus stomatal width were generated in Excel (Micosoft Office 7, 2006, Redmond, WA, USA). The number of stomata versus stomatal length of the specimens were also conducted in Excel to determine whether there were two distinct groupings and thus, two species of baobabs in Africa as per Pettigrew *et al*. (2012) and to establish if stomatal length decreased with an increase in stomatal density.

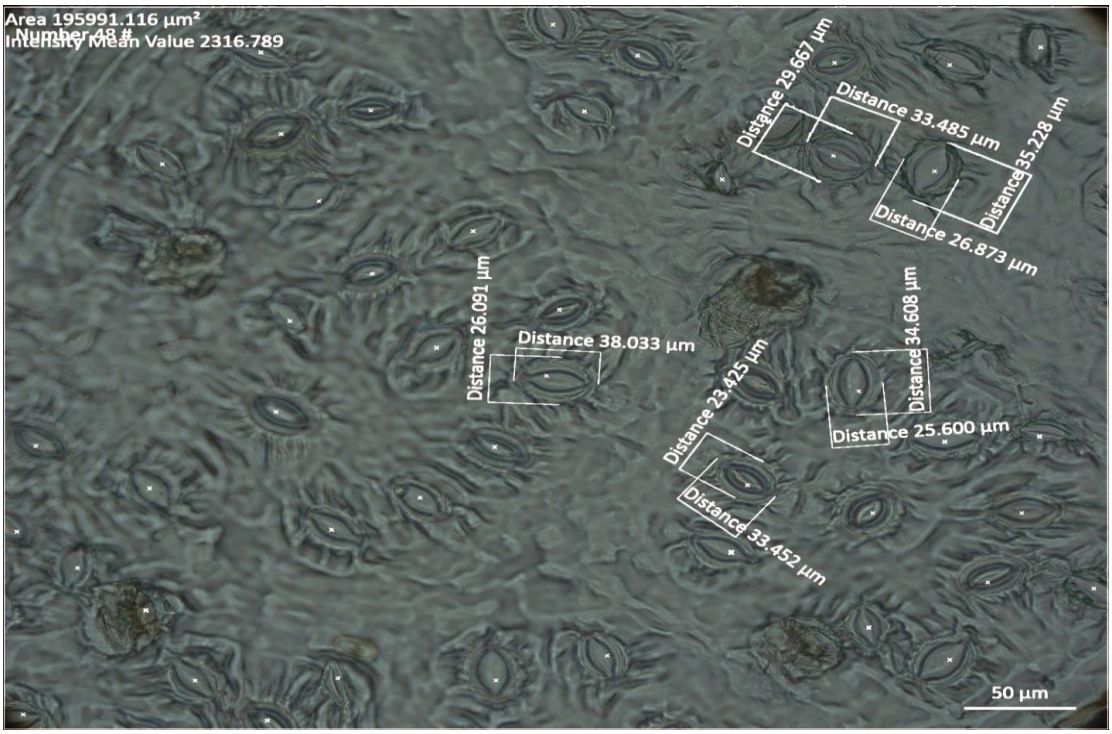


Fig.2.2. African baobab leaf with stomatal openings, showing the area of field of view from which number of stomata, guard cell sizes, stomatal length and width were measured. Image was viewed at 400× magnification, photographed with a ZEISS Axiocam 506 Colour digital camera (M2 Professional). Specimen: *Rodin 2637* (PRE). Scale bar = 50µm. Photo: author.

Species concepts are used in this study to put my data into context and delineate African baobabs using cluster analysis, principal component analysis, scatter plots as well as box and whisker plots to

ascertain whether baobabs found at low and high altitudes formed distinct clusters and groupings that are linked with ploidy. A t-test was applied to the morphological dataset to determine whether the baobabs under study were statistically significantly different between baobabs that occur at low and high altitudes. Therefore, elevation records from herbarium specimens were linked with the morphological evidence to determine if there was an ecological distinction between the two proposed groups.

2.5. RESULTS

2.5.1. Floral features

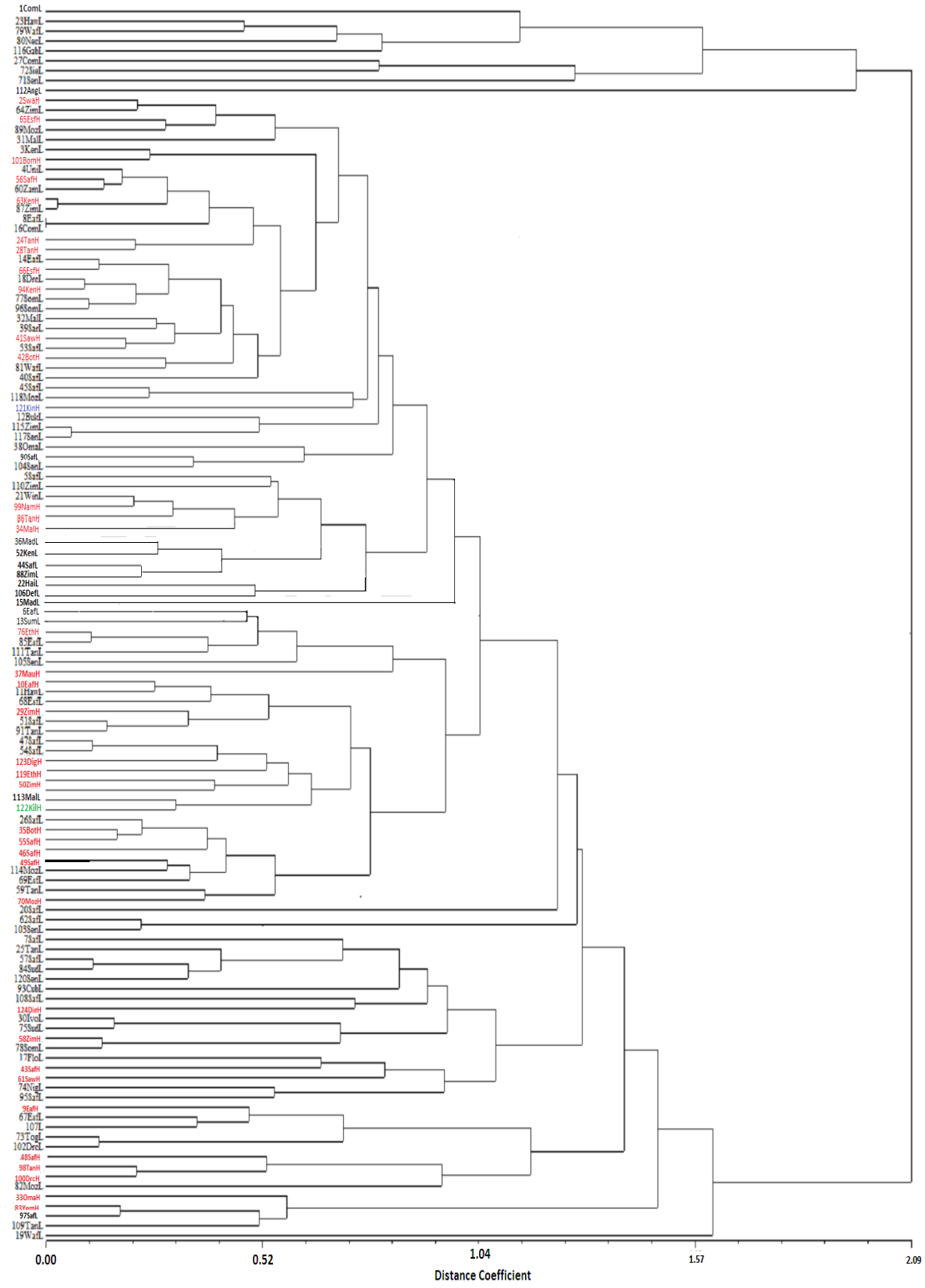
Cluster analysis of the floral dataset resulted in a phenogram, in which the specimens do not separate out into distinct groups or clusters. It should be noted that only 38 of the 124 specimens are found above 800 m a.s.l. The *A. kilima* holotype occurs at 825 m a.s.l. (#122) in the Vhembe District in the Limpopo Province of South Africa and was thought to be diploid (Pettigrew *et al.*, 2012). This individual is part of a cluster that includes a specimen that occurs in Malawi (762 m a.s.l., #113), two specimens found at low altitude in South Africa (518 m a.s.l., #47 and 274 m a.s.l. #54) and three specimens that occur at high altitude in Zimbabwe (1067 m a.s.l., #50), Ethiopia (1150 m a.s.l., #119) and the Vhembe District in the Limpopo Province of South Africa (818 m a.s.l., #123). On the other hand, the *A. kilima* paratype occurs at 932 m a.s.l. (#121) in the Caprivi Strip in Namibia and is thought to be diploid (Pettigrew *et al.*, 2012). This individual is part of a cluster that includes two specimens from the lowlands of Senegal (10 m a.s.l., #104 and 28 m a.s.l., #117), five specimens that occur in the low altitudes in Oman (150 m a.s.l., #38), Zimbabwe (610 m a.s.l., #115), Burkina Faso (410-578 m a.s.l., #12), Mozambique (0 m a.s.l. #118), South Africa (500-630 m a.s.l., #45) and one specimen that occurs at high altitude in Swaziland (1050 m a.s.l., #41: Fig.3.1a). Six specimens found at low altitude in Hawaii (122 m a.s.l., #23), West Africa (Nigeria, 7 m a.s.l., #79), Gabon (7 m a.s.l., #116), Sierra Leone (396 m a.s.l. #72), Senegal (0 m a.s.l., #71), Angola (140 m a.s.l., #112) and two specimens that occur in the lowlands of Comoros Island seem to have formed a cluster separate from the rest of the specimens at the base of the phenogram. The remaining 100 specimens also form mixed clusters with specimens found at both low and high altitudes. The correlation coefficient (r) of the phenogram is 0.70, which is a fair but not good fit of the data to the phenogram and the original distance matrix. In addition, the specimens I studied did not show any geographic pattern of variation.

The second phenogram shows the cluster of 35 specimens based on the same seven floral characters and the correlation with putative ploidy as inferred from stomatal length (Pettigrew *et al*.,

2012; Fig. 3.1B). These specimens also form mixed clusters at both the altitudinal levels and the ploidy categories (Fig. 3.1b). It is important to note that previous study reported that stomatal length ranging from 20.4–31.8 µm (26.1 ± 5.7 µm) were diploids whereas those ranging from 33.8–42.8 µm (38.3 ± 4.5 µm) were tetraploids. The *A. kilima* topotype occurs at 825 m a.s.l. in the Vhembe District of the Limpopo Province of South Africa according to earlier study. This individual was part of a cluster that includes one tetraploid specimen (1050 m a.s.l.) found in South Africa and one tetraploid specimen (500 m a.s.l.; Fig. 3.1b) planted in Florida. On the other hand, the *A. kilima* paratype occurs at 932 m a.s.l. in the Caprivi Strip in Namibia and is thought to be diploid as per previous study. This specimen is part of a cluster that includes two tetraploid individuals that occur in Tanzania (69 m a.s.l.) and Somalia (250 m a.s l.; Fig. 3. 1 b). The remaining 29 specimens also form mixed clusters at both the altitudinal levels and ploidy categories. The correlation coefficient (r) of the phenogram is 0.80. This is an indication that the hierarchic classification obtained by the clustering method is a fairly good representation of the original resemblance of the matrix.

Additionally, PCA and NMDS results also showed mixed groupings (Fig. 3.2 A, B; 3.3). The baobab specimens do not show clear separation according to altitude. However, some specimens that occur at the high altitudes (≥ 800 m a.s.l.) are placed more towards the mid to upper right side of the three dimensional space, whereas those that occurred at the low altitudes (< 800 m a.s.l.) are positioned more towards the mid to lower left side of the PC-3 axis (Fig.3.2 a, b). The specimens that occur at the low altitudes seem to have formed an envelope around the mixed grouping from the lower left towards the lower right and round to the upper left side of the three dimensional space through the upper right side. The topotype and paratype of *A. kilima* are placed at the upper left side and the upper mid side towards the right of the three dimensional space (Fig. 3.2B).

(3.1 a)



(3.1 b)

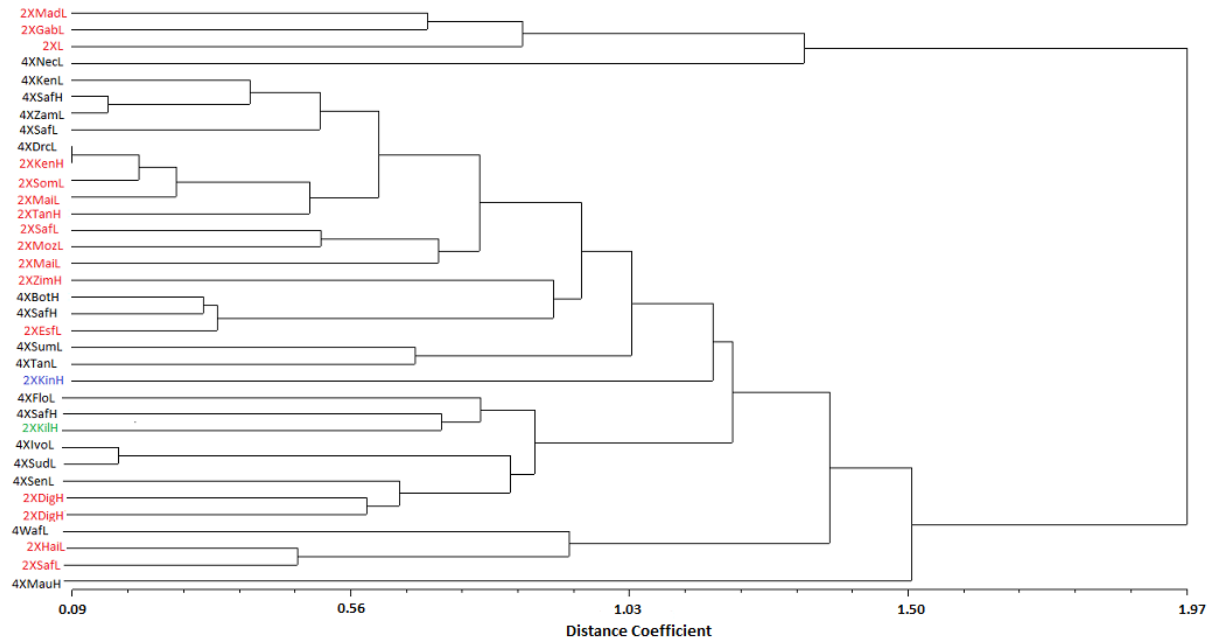
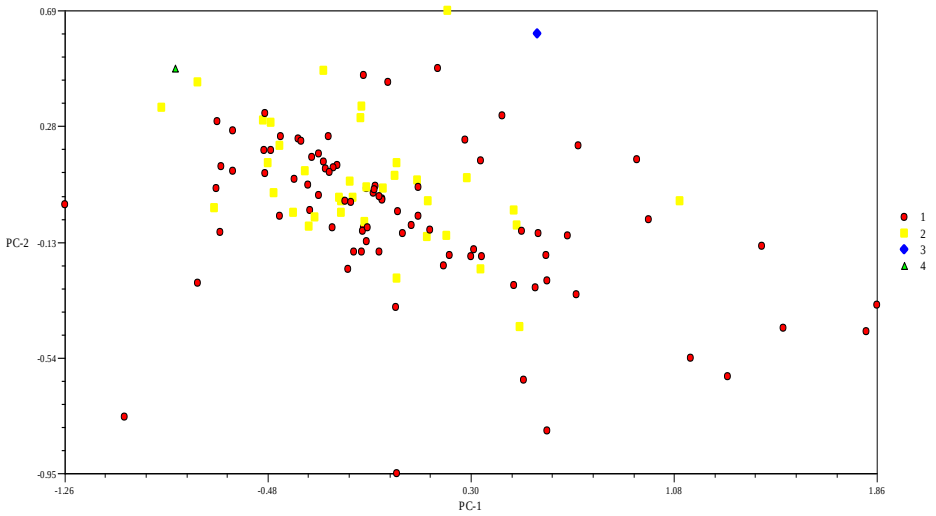


Fig.3.1Phenograms resulting from UPGMA cluster analysis of African baobab found at low (black) and high (red) altitudes, including specimens from the same trees as the topotypes (green) and paratypes (blue) of *A. kilima* and based on seven floral characters (a) for 124 specimens (r = 0.70) and (b) for 35 specimens where putative ploidy level is indicated according to the stomatal categories of Pettigrew *et al.* (2012), (r = 0.80). Pettigrew *et al*. (2012) reported that baobabs with stomatal length ranging from 20.4–33.8 mm were diploids whereas those with stomatal length ranging from 33.8–42.8 mm were tetraploids, tetraploid specimens (4x) and diploid specimen (2x).

Eigenvector values show that the staminal corolla diameter is the main determinant for distribution of specimens along the first and second PC axes, with specimens possessing large staminal corollas being distributed towards the right of the three dimensional space (Fig. 3.2 A and B; Table 1). Petal breadth is instrumental in influencing the distribution of specimens along the third PC axis (Fig. 3.2 A and B; Table 1). Specimens possessing broad and small petals are distributed towards the lower right and top left of the space along the PC-3 respectively (Fig. 3.2 A and B; Table 1). Surprisingly, petal length is not instrumental in influencing the distribution along the first three axes, but it is important to

note that 73.6% of the variation in the dataset is represented by the first three principal components (Table 1).

(A)



(B)

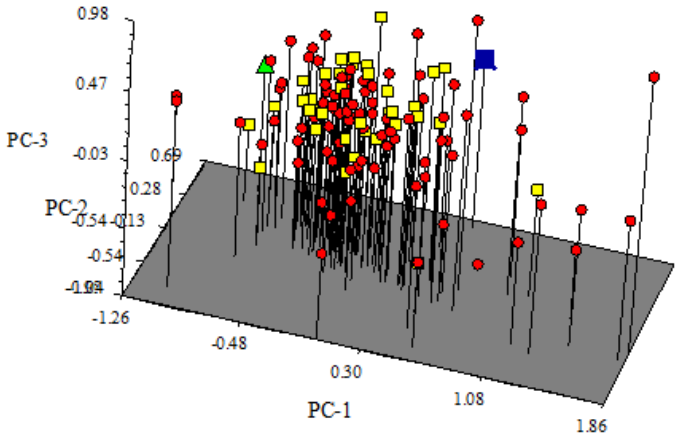


Fig. 3.2 (A) two dimensional and (B) three dimensional PCA plots of African baobabs based on seven floral characters and 124 herbarium specimens, including specimens from the same trees as the holotypes and paratypes of *A. kilima*. **Key**: (1) Red: Low Altitude (< 800 m a.s.l.), (2) Yellow: High altitude (≥ 800 m a.s.l.), (3) Dark blue: topotype of *A.kilima* (the Vhembe District). *Venter 2* (J); from the same tree

(holotype) described by Pettigrew *et al*. (2012), (4) Green: paratype of *A. kilima* from the Caprivi Strip Namibia); *Venter 1* (J).

(3.3)

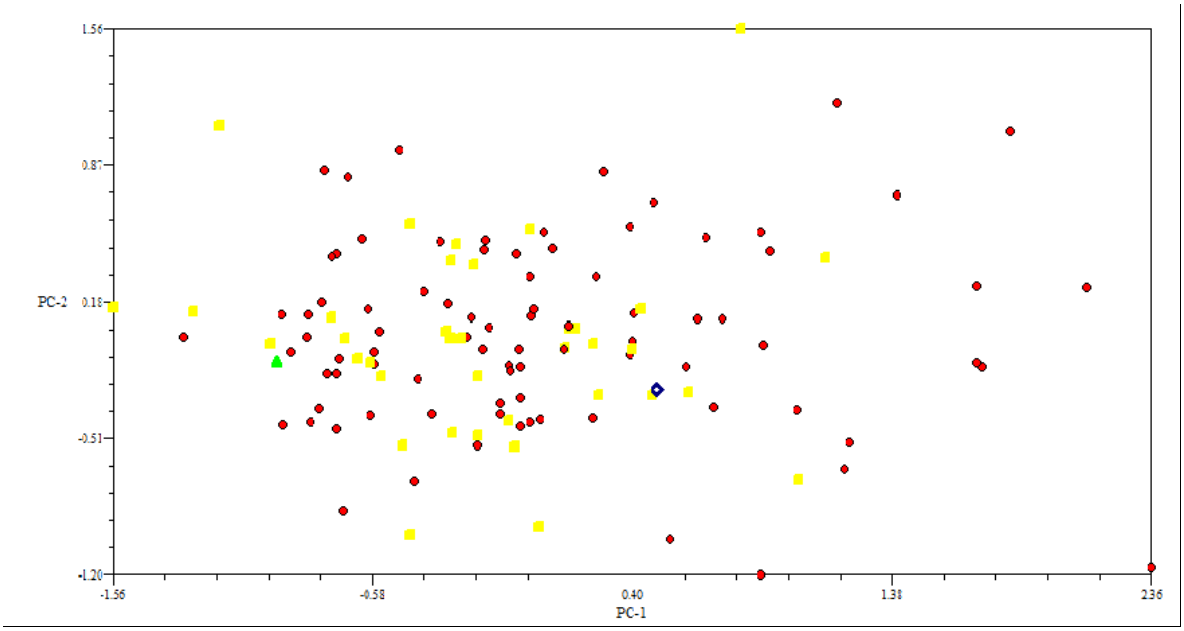


Fig. 3.3. A two dimensional NMDS of African baobabs based on seven characters and 124 herbarium specimens, including specimens from the same trees as the holotypes and paratypes of *A. kilima*. **Key**: (1) Red: Low Altitude (< 800 m a.s.l.), (2) Yellow: High altitude (≥ 800 m a.s.l.), (3) Dark blue: topotype of *A.kilima* (the Vhembe District in the Limpopo province of RSA). *Venter 2* (J); from the same tree (holotype) described by Pettigrew *et al*. (2012) (4) Green: paratype of *A. kilima* in Caprivi Strip Namibia); *Venter 1* (J).

NMDS results also produced mixed groupings of the specimens that occur at low and high altitudes (Fig. 3.3).

PCA, and NMDS of baobabs at low and high altitudes were performed excluding the cultivated specimens but (the results were similar to the PCA and NMDS that included the cultivated specimens) showed no separate groupings. The following 14 cultivated specimens were therefore, included in the analyses: Comoros Island– 1ComL, 16ComL, 23ComlL, 81ComL; Cuba– 82CubL; Florida– 15FloL;

Guyana– 97GuyL; Hawaiian Island– 19HawL; Haiti– 18HatL; New Caledonia– 71NecL; Oman– 33OmaH, 38OmaL; Yemen– 83YamH, and West Indies– 17WinL..

Table 1: Eigenvectors resulting from ordination of characters of specimens in 3D space along the PC1, PC2 and PC3) for the multivariate analysis of African baobabs (N=124). Percentage variation represented by each principal component is shown.

Character	**PC1 (49.3%)**	**PC2 (12.4%)**	**PC3 (11.9%)**
Calyx Length	0.7882	0.0553	0.0783
Calyx Breadth	0.5958	0.2752	-0.1965
Petal Length	0.6924	-0.1427	0.4246
Petal Breadth	0.5502	-0.1582	**0.7008**
Staminal tube Length	0.7711	-0.2699	-0.2648
Staminal corolla diameter	**0.8622**	**-0.2835**	-0.2911
Style length	0.8408	0.1368	-0.2216

2.5.2. Univariate analysis of floral features

Both calyx length and calyx breadth showed extensive overlap between specimens that occur at low and high altitudes (Fig. 3.4 A and B). There were no significant differences in calyx length ($t = 1.89$, $df = 76$, $P = 0.06$) and calyx breadth ($t = 0.83$, $df = 76$, $P = 0.41$) between specimens found at low and high altitudes (Table 4 A; Appendix 3). The petal length and petal breadth showed substantial amount of variation with altitude but neither was statistically significantly different between specimens that occur at low and high altitudes ($t = 1.95$, $df = 116$, $P = 0.05$; $t = -0.16$, $df = 116$, $P = 0.87$; Fig. 3.4 C, D; Table 4 A; Appendix 3). Similarly there was no significant difference in staminal corolla diameter between specimens found at low and high altitudes $t = 1.30$, $df = 108$, $P = 0.20$; Fig. 3.4 F; Table 4A; Appendix 3). Average pollen grain diameter, pollen grain volume, and pollen spine density per 1000 μm^2 all showed considerable amount of overlap, but none was significantly different between specimens that occur at low and high altitudes ($t = 2.60$ $df = 48$, $P = 0.13$; $t = 1.97$ $df = 48$, $P = 0.73$; $t = 0.64$, $df = 48$, $P = 0.52$; Fig. 3.4 H, I; J; Table 4 A). The pollen grain diameters recorded in this study for both *A. digitata* and *A. kilima* were 50.20 ± 8.59 µm and 59.85 ± 12.86 µm respectively. In addition, the pollen grain volume calculated in this study for *A. digitata* and *A. kilima* ranged from 253.6 – 2150.5 μm^3 (426.01 ± 172.45

μm^3) and 102.1 – 753. 2 μm^3 (428.62 ± 326.54 μm^3). Further, the pollen spine density per 1000 μm^2 recorded in this study for baobabs that occur at low and high altitudes ranged from 5.31– 32.81 (19.06 ± 13.75) per 1000 μm^2 and 9.36 – 24.28 (16.82 ±7.46) per 1000 μm^2. These volumes do not correspond to those (118.8 – 133.0 μm^3 and 42.8 – 44.6 μm^3) reported by previous study. Similarly, the pollen spine densities per 1000 μm^2 calculated for baobabs that occur at low and high altitudes differ from those (118.8 – 133.0 and 42.8 – 44.6) reported by previous work. However, staminal tube length and style length were statistically significantly different between baobabs that occur at low and high altitudes (t = 2.13, df = 110, P = 0.0; t = 2.10, df = 35, P = 0.04; Fig. 3.4 E, G; Table 4 A).

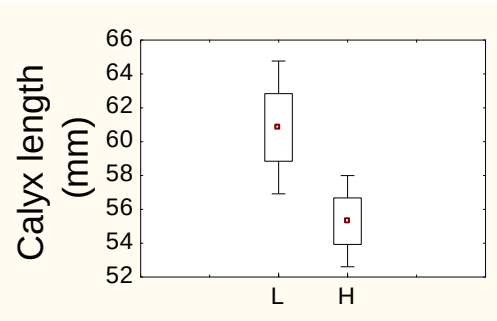
Moreover, there were no significant differences in petal breadth (t = 0.23, df = 116, P= 0.82), staminal corolla diameter (t = 0.84, df = 108, P =0.40), pollen grain diameter (t=0.73, df=48, P=0.47) and pollen grain volume (t=0.30, df = 46, P=0.97) between fresh and dry baobab specimens at significant level of $P < 0.05$ (Table 4 C; Appendix 3). Flower kinesis was mentioned as a differentiating feature between *A. digitata* and *A. kilima* in a previous study, however, there are no data in this study to confirm or refute the presence or absence of flower kinesis in either *A. digitata* or *A. kilima.*

2.5.3. Univariate analyses of Stomatal features

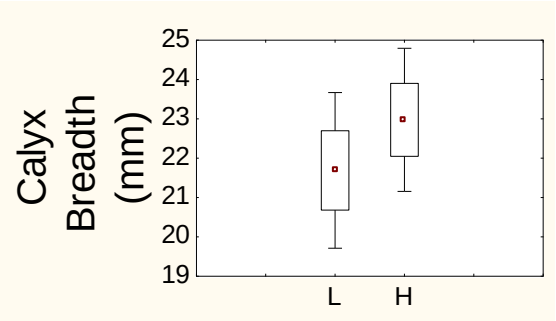
A box plot was used to compare stomatal size and density between specimens that occur at low and high altitudes (Fig. 3.5 a, b; Table 4 B). There were no significant differences between either stomatal length (t = 1.91, df = 44, P = 0.063) or stomatal density (per 1000 μm^2; t = -1.74, df = 44, P = 0.089) between specimens that occur at low and high altitudes respectively (Table 4 B; Appendix 3). There were however, significant differences in stomatal length (t = 9.40, df = 41, P = 0.00) between specimens when grouped according to stomatal length for the putative ploidy as per earlier study (Table 4 B). Also both stomatal length (t = -8.37, df = 48, P = 0.00) and stomatal density (t = -9.46, df = 48, P = 0.00) were significantly different between specimens when grouped according to the two putative ploidy groups with

a midline drawn in the gap between the two reported ploidy categories as per previous study (Table 4 B; Appendix 3).

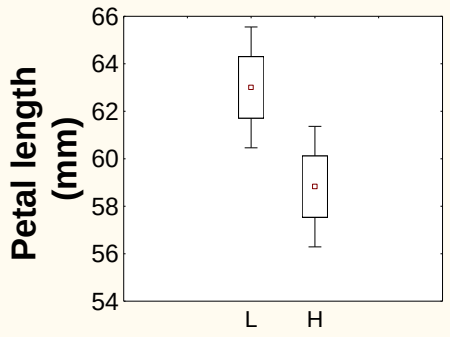
(A)



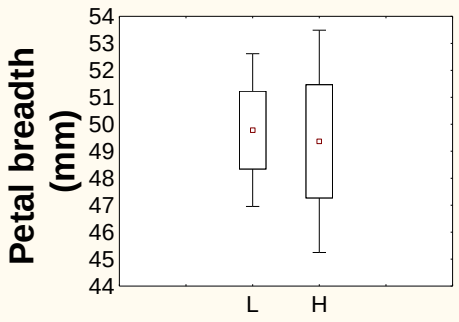
(B)



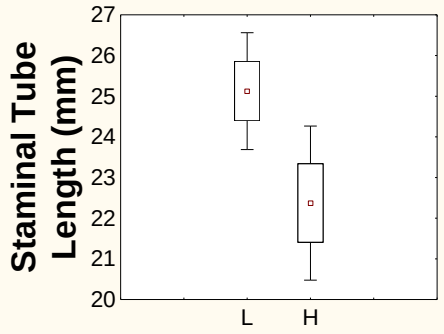
(C)



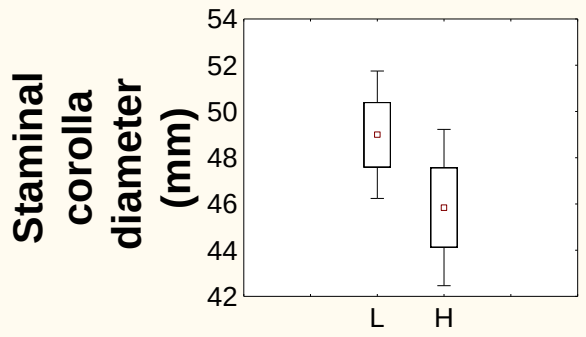
(D)



(E)



(F)



(G)

(H)

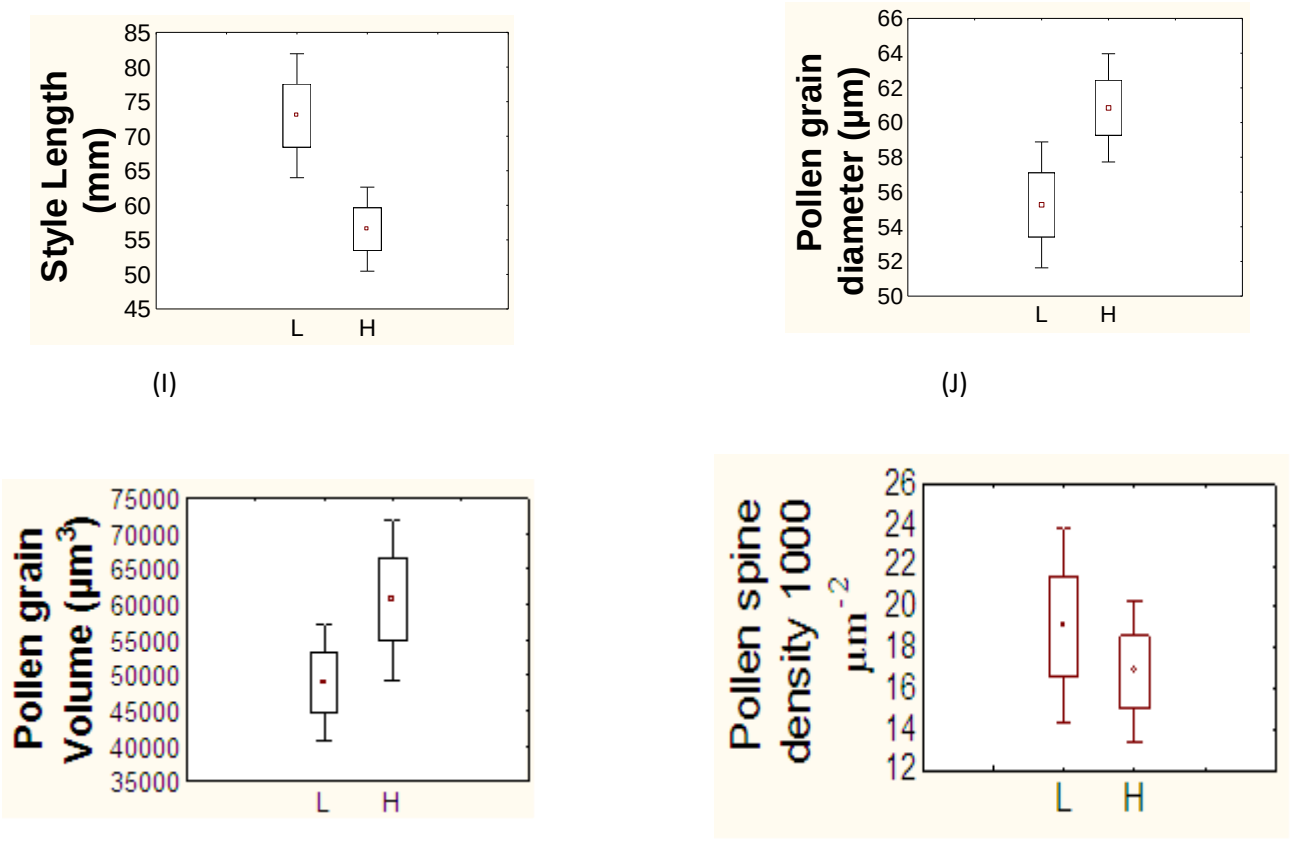


Fig. 3.4 Box and whisker plots showing variation in nine quantitative characters of African baobabs, *Adansonia* specimens (N=124) with altitude (A) calyx length (mm); (B) calyx breadth (mm); (C) petal length (µm); (D) petal breadth (mm); (E) staminal tube length (mm); (F) staminal corolla diameter (mm); (G) style length (mm) (H) pollen grain diameter (µm, N=50); (I) pollen grain volume (μm^3, N=41); (J) pollen spine density per 1000 μm^2. Keys: □ mean ± 2*SD; ⊥ Min-max; L low altitude (< 800 m a.s.l.); H high altitude (≥ 800 m a.s.l.).

Statistical comparisons of stomatal density values (per 1000 μm^2) between specimens could not be made when the specimens were grouped according to the reported putative ploidy categories as the density value of only one specimen fell within this category which violates the assumptions for t-test (Fig. 3.5 f). However, the histogram of number of specimens versus stomatal length represented a normal distribution curve suggesting that the stomatal length and density detected among African baobabs analyzed might be due to a single (unimodal) population (Fig. 3.6). In addition, the stomatal length and

stomatal density (per 1000 µm^2) of fresh and dry herbarium specimens were not significantly different in this study (t = -0.73, df = 48, P= 0.47; Table 4 D; Appendix 3).

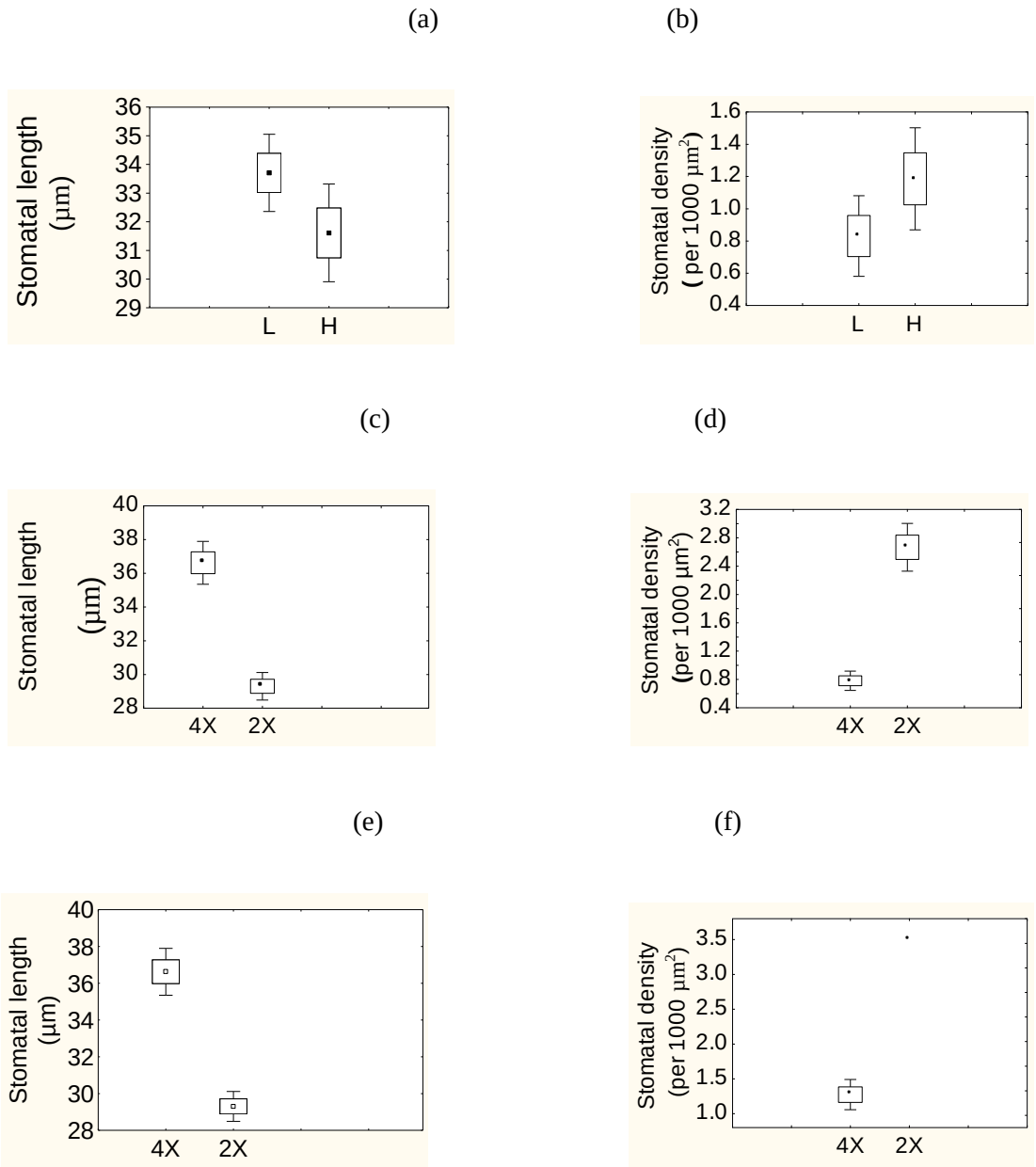


Fig. 3.5 Univariate analyses of stomatal features of African baobabs, *Adansonia digitata* specimens found at low (L, >800 m a.s.l.) and high (H, ≥ 800 m a.s.l.) altitudes between (a) putative ploidy groups (b) stomatal lengths (µm) are grouped according to strict putative ploidy categories; (c) stomatal length (µm) data are split with a midline drawn in the gap between reported ploidy categories; (d) stomatal density

(1000 µm^{-2}); (e) stomatal density (per 1000 µm^2) when data are grouped according to strict putative ploidy categories; (f) stomatal density (per 1000 µm^2) when stomatal density data are split with a midline drawn in the gap between reported ploidy categories. Keys: □ mean ± 2* SD, ⊥ Min-max. (A, N = 50; B, N = 46; C, N = 43; D, N = 16. Note: N is different for each plot because some values in the dataset fall outside the group range for the different groupings thus reducing the number of specimens used in each analysis)

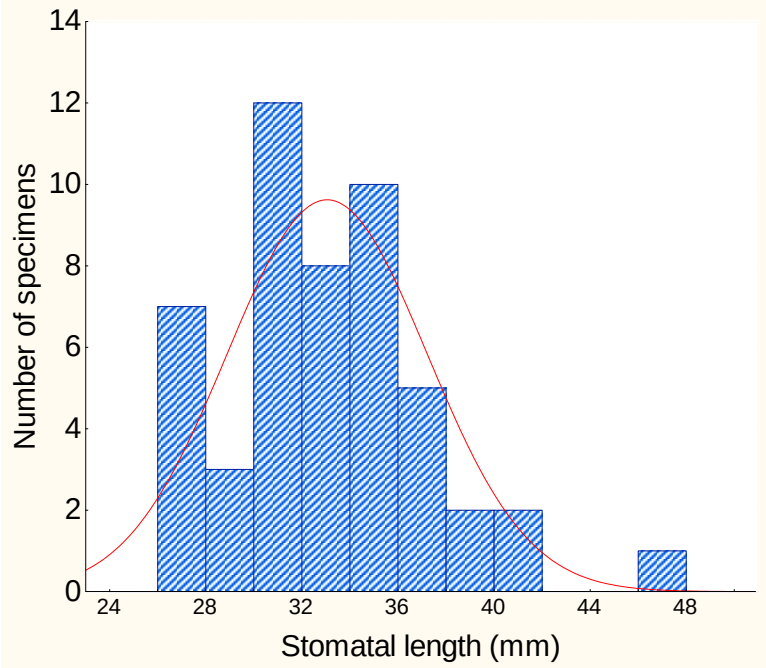
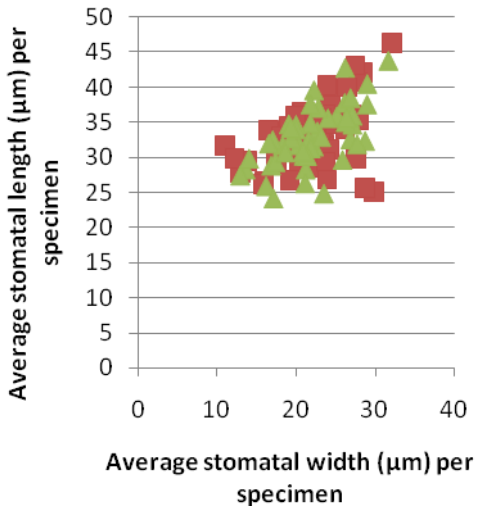


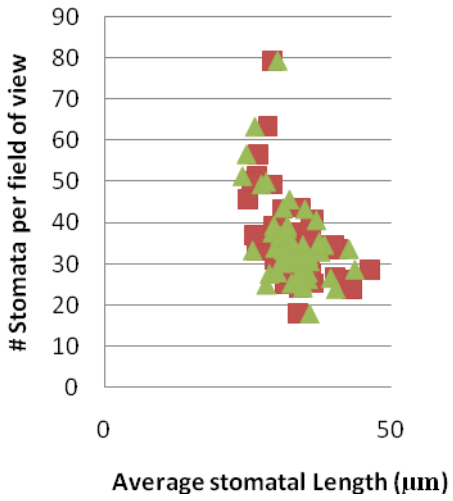
Fig. 3.6 Histogram of the number of specimens versus mean stomatal length (mm)

The scatter plots of average stomatal length per specimen versus average stomatal width per specimen, number of stomata per field of view versus average stomatal length per field of view, number of stomata per field of view versus average stomatal width per field of view and number of stomata versus average stomatal length all showed that specimens from low altitude are mixed with specimens from high altitude as they did not form two distinct groupings (Fig. 3.7 a, b, c and d).

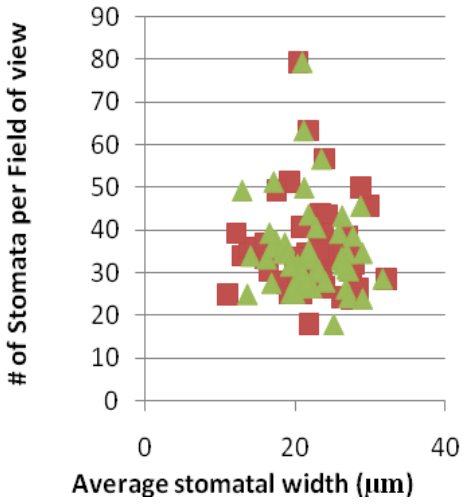
(3.7a)



(3.7b)



(3.7 c)



(3.7 d)

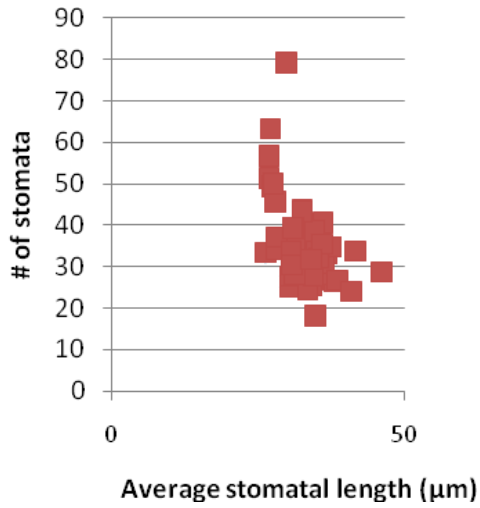


Fig. 3.7 Scatter plots of (a) stomatal length (µm, red) versus average stomatal width (µm, green) per specimen, (b) number of stomata (red) versus average stomatal length (µm, green) per field of view, (c) number of stomata (red) versus average stomatal width (µm, green) per field of view and (d) number of stomata (red) versus average stomatal length (µm, red)

2.6. DISCUSSION

2.6.1. Floral features

In this study, the results produced by both ordination and clustering methods show the formation of mixed clusters and groupings of baobab specimens that occur at low and high altitudes.The results show that flower size does not differ significantly between baobab specimens that occur at low and high altitudes, except in staminal tube length and style length. Both staminal tube length and style length did not show any pattern with increase in altitude. However, only the specimens found at low altitude could reach a length ranging from 37.5– 42 mm and 96 –126 mm for staminal tube length and style length respectively. The variation observed in flower size between specimens that occur at low and high altitudes may be due to the effects of environmental variables (e.g., temperature and rainfall; Assogbadjo *et al*., 2006). The effect of environment on biotic variables (e.g., flower and fruit sizes) has been reported in some edible trees in Africa (Sidibe and Williams, 2002). For example, a significant relationship between trait values (e.g., flower size, fruit size, shape, pulp sweetness and kernel content of the species) in the shea tree (*Vitellatria paradoxa*) and abiotic variables (e.g., temperature and rainfall) has been observed in the sub-Saharan Africa north of the equator (Maranz and Wiesman, 2003).

In addition, African baobabs have been reported to flower mostly in the rainy season (Pardy, 1953; Assogbadjo *et al*., 2005). Since the rainy season varies between different geographical locations, the flowering time also varies; the variation in season between different geographical locations creates an ecological isolation. The variation in seasons in different geographical zones that lead to variation in flowering time of a single species across its range may lead to potential reduction of gene flow. For instance, different climatic zones of Senegal was shown to have significant effects on the fruit pulp production of *Balanites aegyptiaca and Tamarindus indica* owing to variable flowering time (Soloviev *et al*., 2004).

Flower features such as size, shape and colour, are modified through selective forces influenced by pollinators (Silva-Montellano and Eguiarte, 2003). Variation amongst species in the expression of

flower characteristics might be due to natural selection and linked to their adaptive importance to surrounding conditions (e.g., climate; Schlenker and Lobell, (2010). Report has shown considerable variation in flower size, shape, colour and fecundity of *Agave lechuguilla* with changes in temperature from 20˚C in the South to 31.5˚C in the North of Chihuahuan desert in Mexico. Plants that occurred in the south of the desert have larger, longer, and tubular flowers, whereas plants found in the north had smaller, shorter, and more bowel-like flowers (Silva-Montellano and Eguiarte, 2003). The variation in flower size might have been due to selective force imposed by pollinators (Silva-Montellano and Eguiarte, 2003). Higher temperature may have resulted in more bowl-like and larger flower sizes that encourage day visitors, unlike the southern plants whose colour and size of flower encourage night pollinator visits. Increased or reduced temperature above or below an optimum temperature may affect flower size. This may have been responsible for the variation in baobab flower sizes. The variation in baobab flower size may be an adaptation resulting from selective pressure imposed by pollinators (Wickens and Lowe, 2008). This is because African baobabs are reportedly pollinated by bats and bush babies that often feed at night (Sidibe and Williams, 2002), and may selectively pollinate different sizes of flowers, as in the case of *Agave lechuguilla* (Silva-Montellano and Eguiarte, 2003).

Although there is some clustering in the phenogram, the specimens do not separate out into distinct groups that correspond with altitude or ploidy. This indicates that African mainland baobabs do not show distinct floral differences that can be correlated with altitudinal differences or linked to differences in ploidy level among the specimens I examined. In addition, it should be noted that both the topotype (#122KilH) and the paratype of *A. kilima* (#121KinH) that both occur at high altitudes form a mixed cluster with other baobab specimens from both low and high altitudes.

Only eight (6%) of the 124 specimens I investigated formed a distinct cluster at low altitude. These specimens include three that occur in coastal areas in West Africa (Gambia, Sierra Leone, and Senegal), two specimens that are planted outside Africa (Hawaii and New Caledonia) and one specimen that occurs in the Luanda District of Angola as well as two specimens found in the Comoros Island. These five specimens occur in different geographical regions but have one unique feature with the first three that

occur in West Africa. They also occur on the islands or coastal districts including Luanda District in Angola that is located on the country's coast with Atlantic Ocean.

A representation of the clustering of the specimens based on the same seven floral characters and correlation with ploidy level according to stomatal categories as defined by Pettigrew *et al*. (2012) also shows that specimens do not form distinct clusters according to inferred ploidy level. Both the topotype (#122KilH) and the paratype (#121KinH) of *A. kilima* that were reportedly diploid, occur in different mixed clusters with both diploid and tetraploid baobab specimens from low and high altitudes. This means that the morphological features (aspects of flower size, pollen grain size and density) are neither correlated with differences in altitude nor linked to differences in ploidy as reported by Pettigrew *et al*. (2012). However, some botanists, such as Masters (1868), Chevalier (1906) and Verdoorn (1973), reported variation in flower indumenta, peduncle length and flower size and their possible taxonomic importance in Malvaceae, particularly among the West African baobabs. Baum (1995a) also acknowledged some morphological variation among Africa baobabs whereas Baum and Oginuma (1994) reviewed their chromosome numbers but they could not find enough evidence in terms of morphology and ploidy level within *A. digitata* to warrant multiple species differentiation. In addition, morphological variation among baobabs that occurred in Benin and Mali has been attributed to variability in bio-climatic zones (Sidibe and Williams, 2002; Assogbadjo *et al*., 2005).

2.6.2. Stomatal size and density of African mainland baobabs

The analysis of stomatal size and density indicates a single population of *Adansonia digitata* and there are no distinct patterns in the stomatal length and density measurements that can be correlated with altitudinal differences. In other words, the differences in stomatal size and density of the two reported species of African baobab are not correlated with differences in altitude and are not linked to ploidy. My finding does not corroborate the hypothesis that there are two distinct African baobab species as reported by Pettigrew *et al*. (2012). This is because the stomatal size and density of specimens found at low and high altitudes do not correspond with those reported by Pettigrew *et al*. (2012). Moreover, the

mean stomatal length and density of baobab specimens that occur at low altitude are not significantly different from the mean stomatal lengths and density of baobab specimens that occur at high altitude. However, the mean stomatal length and density of African baobabs are significantly different when mean stomatal length and density are grouped according to strict putative ploidy categories. The mean stomatal length and density of the putative ploidy groups are also significantly different when the stomatal length and density data are split with a midline drawn in the gap between the reported ploidy groups. In addition, the mean and standard deviation of the greater stomatal lengths of 33.7± 3.6 µm and smaller stomatal length of 31.6 ± 3.8 µm obtained in this study for baobab specimens are not in agreement with the greater (38.3 ± 4.5 µm) and smaller (26.1 ± 5.7 µm) stomatal lengths published by Pettigrew *et al*. (2012) or to the larger (37.8 ± 2.2 µm) and smaller (26.6 ± 2.7 µm) stomatal lengths published for baobabs in Zimbabwe by Douie *et al.* (2015).

The stomatal length and density (floral and pollen traits) of baobabs found at low and high altitude did not differ statistically. It should be noted that no difference in ploidy level is inferred in this study between the two purported species of baobab in Africa because the CA and PCA of putative ploidy groups of Pettigrew *et al*. (2012), as well as the scatter plots produced did not form any distinct clusters or separate groupings for either the altitudinal or ploidy categories, indicating only one species of baobab in Africa. Besides, the histogram of number of stomata versus stomatal lengths in this study represented a normal curve. Thus, suggests a clear pattern of unimodal distribution. The histogram of number of specimens versus mean stomatal length (fig. 3.2) also represented a normal curve which illustrates that the data are unimodal. However, Douie *et al*. (2015) reported a bimodal distribution pattern in stomatal length and density (χ^2<0.001) for baobabs in Zimbabwe. They assumed that the bimodal distribution pattern implied differences in ploidy level which they assumed to be the two different species as reported by Pettigrew *et al*. (2012). Nevertheless, they did not confirm this assumption with chromosome counts. It is possible that the bimodality in their data might have resulted from limited sampling when compared to this work with a wider sampling. Similar to this study, Douie *et al*. (2015) concluded that variation in stomatal length and density was not correlated with altitude. My study confirms that there are no distinct

morphological features of African baobab that are correlated with altitude, contrary to what was reported by Pettigrew *et al*. (2012) for mainland African baobabs. Equally important to note, is that the mean stomatal lengths (33.71±3.57 µm and 31.61±3.79 µm) obtained here for *A. digitata* and *A. kilima* do not correspond to those (38.3 ± 4.5 µm and 26.1 ± 5.7 µm) published by Pettigrew *et al*. (2012) or those (37.8 ± 2.2 µm and 26.6 ± 2.7 µm) published for baobab specimens in Zimbabwe by Douie *et al*. (2015).

It is important to note that the unit of mean stomatal density of 1.6 ± 0.7 and 5.0 ± 2.1 per 100 μm^2 reported in previous study by Pettigrew *et al*. (2012) for baobabs that occur at low and high altitudes is unlikely to be accurate. This is because 100 μm^2 could not contain the numbers of stomata obtained in this work; as a result stomatal density was calculated per 1000 μm^2. Also in contrast to the previous study by Pettigrew *et al*. (2012), the mean stomatal density per 1000 μm^2 for baobabs that occur at low altitude does not differ significantly from those found at high altitude. Douie *et al*. (2015) obtained stomatal density values (1.63± 0.4 and 5.17 ± 0.77 per 10000 μm^2) for baobabs that occur at low and high altitudes in Zimbabwe by computing stomatal density per 10000 μm^2. Surprisingly, Douie *et al*. (2015) claimed that those set of density values they obtained were in agreement with those calculated by Pettigrew *et al*. (2012). Their density values may be more realistic, they are however, not in agreement with those calculated by Pettigrew *et al*. (2012) since Douie *et al*. (2015) calculated density figures per 10000 μm^2 and not per 100 μm^2.

Besides, the stomatal lengths of *A. kilima* and *A. digitata* were reported by Pettigrew *et al.* (2012) to be 26.1 ± 5.7 µm and 38.3 ± 4.5 µm respectively. It follows that their stomatal lengths range from 20.4– 31.8 µm and 33.8– 42.8 µm respectively. As such the stomatal areas (guard cells) of *A. kilima* and *A. digitata* in Pettigrew *et al.* (2012) would range from 323.65–794.23 μm^2 and 897.27–1438.72 μm^2 respectively. The range of areas above cannot be contained in 'the per' 100 μm^2 used by Pettigrew *et al.* (2012) for their density calculation. This is further evidence that 'the per' 100 μm^2 density calculation by Pettigrew *et al.* (2012) is impossible.

The stomatal size of the baobabs studied, though quite variable, suggests a single size class of stomatal length for *A. digitata* because of the non-significant difference in the stomatal length. This indicates that there is no quantitative difference in the baobabs studied to warrant multiple species differentiation among mainland African baobabs. The lack of significant quantitative difference in stomatal features is in conformity with Baum (1995a) who did not find any multiple species differentiation in the mainland African baobabs based on morphological features. It is also in agreement with Baum and Oginuma (1994) who found no variation in the ploidy level of mainland African baobabs to warrant the recognition of multiple species differentiation. Although stomatal length and density were not statistically significantly different between baobabs found at low and high altitudes, the stomatal sizes measured varied among the specimens studied. This variation may be due to soil drainage, moisture content, nutrient content, depth and climatic factors which influence plant growth rate and development (Mashapa *et al*., 2013). The poor availability of water resulting from a low water table at high temperature may potentially reduce the cell size of specimens at low temperature or high altitude areas (Sucker and Lopez-Gunn, 2014). This could be the case for the presence of higher stomatal density and smaller guard cell sizes in Benin with high temperature and low mean precipitation. In addition, Chen *et al*. (2001) established a correlation between stomatal density and leaf phenology. They concluded that higher stomatal densities were found in younger trees. So the difference in stomatal densities may be due to age of the leaves. Therefore, the variation in stomatal length and density published by Douie, *et al.* (2015) might be due to phenotypic plasticity or growth variation instead of differences in ploidy level.

The variation in stomatal size may also be explained by rainfall gradient and water availability because the growth rate of tropical trees is positively influenced by seasonal availability of water (Worbes, 1999). Therefore, the leaves of baobabs found in geographical locations with higher annual rainfall and well drained soils will be larger than those in specimens that occurred in arid areas (Assogbadjo *et al*., 2005). In addition, the age of tree has been highlighted as an important driving factor in morphological variation in the baobabs (Wiehle *et al*., 2014). Small size class distribution of baobabs

in ‘homesteads’ in the Nuba Mountains in Sudan were attributed to the young age of the baobabs (Wiehle *et al*., 2014), moreover, most young baobab trees produce simple leaves for several years in contrast to the palmate digitate leaves of the adult tree (Sidibe and Williams, 2002). In addition, stomatal density may be linked to leaf phenology such that younger leaves have greater stomatal densities (Chen *et al*., 2001).

2.6.3. Recognition of African baobab species or subspecies

Although several authors have reported that mainland African baobabs occupy a single niche wherever they occur (Baum, 1998; Sidibe and Williams, 2002), Pettigrew *et al*. (2012) has contrary view. Bimodal distribution patterns were found in stomatal length and density among African baobabs by Pettigrew *et al*. (2012) and in Zimbabwe by Douie *et al.* (2015). However, unlike Pettigrew *et al*. (2012), Douie *et al*. (2015) did not find any correlation in stomatal length or density with their altitudes. Moreover, in this present study, neither average stomatal length nor stomatal density per 1000 μm^2 is significantly different in baobab specimens that occur at low and high altitude. Additionally, the floral features I measured were not significantly different between baobabs at low and high altitudes with the exception of staminal tube length and style length. This suggests that there is no altitudinal pattern among African baobabs and *A. digitata* and *A. kilima* are not supported as distinct species because the variation found in their overall morphology did not seem to show a link with altitude.

Morphological similarities are recognized as the basis of phenetic species concept (Sokal and Sneath, 1963).The cluster and ordination analyses of equally weighted morphological character measurements do not identify two species. This is because neither CA, PCA, nor NMDS produced distinct clusters or groups for baobabs that occur at low or high altitude. Consequently, under the phenetic species concept, there appears to be only one species of mainland African baobabs based on the morphological data I collected.

The results of data analysis obtained in this study did not show any difference in ploidy level among African baobabs to support multiple speciation events under the BSC because the cluster analysis using inferred ploidy categories as per Pettigrew *et al*. (2012) based on stomatal length and density did not show distinct clusters. It also shows that morphological features (aspect of flower size, pollen grain size and density) are not correlated with differences in altitude and that differences in altitude of African baobab studied are not linked to differences in ploidy levels. This means that the baobabs under study can interbreed in nature to produce viable offspring. However, further studies such as chromosome count using root tip squash or flow cytometry analysis are recommended to confirm the presence of one or two ploidy levels in African baobabs.

2.6.4. Infraspecific ranks

The rank of subspecies is generally used when there is geographical or ecological difference between species and they are morphologically distinct (Hamilton and Richards, 1992), whereas the rank of variety is used when there is more overlap in these characteristics and no geographical distinctions. Assogbadjo *et al.* (2006) found patterns of genetic and morphological variability in baobabs across different climatic zones of Benin. This was also observed in Mali where farmers use different characteristics to identify four varieties of baobabs (Sidibe and Williams, 2002). Moreover, cluster analysis of quantitative and qualitative features of specimens collected from the savanna vegetations of Nigeria also identified four varieties of African baobabs based on morphological features and has proposed infraspecific ranks for *A. digitata* (Zhigila et al., 2015). Consequently, the baobab described by Pettigrew *et al*. (2012) may, at best, be described as a synonymy of *A. digitata*. However, more work would be necessary to test this hypothesis.

2.7. CONCLUSION

The results obtained in this study suggest that there are no distinct morphological character differences among baobab specimens that are correlated with altitudinal differences or linked to

difference in ploidy levels. The variation in floral and stomatal features of African baobabs might be due to edaphic or climatic factors. Moreover, ploidy was inferred from the stomatal categories defined by Pettigrew *et al*. (2012), but there were no differences in inferred ploidy level found among the specimens I studied that are correlated with altitude. However, further research should be conducted to determine whether there is potential variation in genome size among mainland African baobabs that are correlated with altitude using flow cytometry and / or chromosome counts.

The results obtained in this study suggest that there is no distinct altitudinal pattern among the mainland African baobabs studied and that *A. digitata* and *A. kilima* are not supported as distinct species. African baobabs investigated in this study were also not indicated as two species under the Phenetic Species Concept since the cluster and ordination analyses of equally weighted floral morphological characters did not identify two species. There was also not enough evidence based on my data set to support multiple speciations among African baobabs under the Biological Species Concept as the cluster analysis of the inferred ploidy groups based on stomatal length categories as per Pettigrew *et al*. (2012) did not produce distinct clusters. In other words, the data collected and analyzed in terms of the species concepts outlined above suggest that there remains only one species of baobab in mainland Africa. However, variation in morphological characteristics among African baobabs may suggest the existence of different varieties of baobab in Africa.

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APPENDIX 2

Table 2 Specimens of African baobabs, *Adansonia digitata* used in morphological study identifying specimen Id, collectors', numbers, date collected, locality information and herbarium information.

ID	*Collector*, Number & (Herbarium)	Locality	Date	Altitude (m) a.s.l.	Part of spcimens used
1 ComL	*Labat et al. 3204* (MO)	Nioumachoua, Moheli, Comoros Island.	23.11.1999	2	F,P & L
2 SwafH	*Rodin 9042* (NY)	20 km S of Oshikango, Namibia.	19.11.1947	1100	F
3 KenL	*Tanner 2833* (MO, NY)	Pembeya, Pangani Dist. Tanga Prov., Tanzania.	20.5.1956	0	F, P& L
4 UniL	*Venter 12248* (UNIN)	Nwanedi Game park Venda, RSA.	13.11.1986	720	F
5 SafL	*Maurin et al. om0747* (BNRH)	Kruger Nat.Park, RSA.	25.01.2006	286	L
6 SafL	*Momberg 141*(BNRH)	Messina Nat Res., Messina Dist., RSA.	27.11.1986	548.6	F
7 EafL	*Gerhardt & Steiner106* (PRE)	Gedi Forest, Kilifi District, Kenya.	12.05.1985	15	F
8 SafH	*Vahrmeyer 1523* (PRE)	Masequaspoort, Soutpansberg , RSA.	27.11.1966	1050	F& P
9 EafL	Koritschoner *1713* (EA, K, PRE)	Usanda, Shinanga, Tanzania.	11.1938	0	F
10 EafH	*Greenway & Kanuri 14614* (K)	T7, Ruaha Nat. Reserve, Near Nsembi Iringa Dist, Tanzania.	24.10.1970	823	F& P
11 EafH	*Napper.* (EA)	Kondoa, Tanzania.	01.1963	1219	F&P
12 BukL	*Garnier 15601* (B)	Tiana village on the road to Orodang and Sikasso, 37 km west of Bobo Dioulasso, Burkina Faso.	19.8.1973	410-578	F, &P
13 SumL	*Kilian & Lobin 2098 & 695* (BR)	Turn off to Brava at Mundun Brava District Lower Shabeelle, South Somalia.	01.08.1988	250	F & L
14 MadL	*Brunel 7694* (BR)	Rocherde, Rodokope, Madagascar.	06.1982	250	F&P
15 FloL	*Norman s.n. US 01223179 (MO)*	El Salvador (cultivated in Florida).	21.07.1954	500	F & L
16 DrcLF	*Aurich s.n B100366431* (B)	Idenga, Republic of Congo.	02.07.1977	319-321	F & L
17 winL	*Box 1550* (US)	Donovan's Antigua, West Indies.	26.09.1938	42	F& L
18 HaiL	*Cook 7* (NY)	Quartier Morin, Haiti.	17.08.1924	8	F, P & L
19 HawL	*Flynn 7124* (US)	Kauai, Koloa Dist. Hawai valley Hawaiian Island.	24.08.2004	122	F
20 BorH	*Mynharth 772* (NY)	Boroma Mithelap: Sangesi SE, Tanzania.	05.1938	1000	F&P
21 TanL	*Richards 26329* (NY)	Ruiha National Park Iringa District, Tanzania	26.10.1970	732	F&P
22 SafL	*Jooste & Sadie 30* (UNIN)	Venda, Nwanedi reserve at Luphephe Dam, RSA.	22.11.1978	720	F
23 ComL	*Rouhan, Pignal & Ibrahim 860* (MO)	Mwali Itsami, Comoros Island.	01.11.2008	16	F
24 TanH	*Mwangulango 1031* (MO)	Katavi Rukwa prot. Areas, Mpanda Dist., Tanzania.	02.10.2002	840	F, P & L

ID	*Collector*, Number & (Herbarium)	Locality	Date	Altitude (m) a.s.l.	Part of spcimens used
25 ZimH	*Grant 4534* (MO)	Victoria Fall, Zimbabwe.	22.01.1929	897	F & L
26 IvoL	*Amshoff 301* (MO)	45km East of Bouaké on the road toM'Bahiak.	17.06.1969	525	F, P & L
27 MalL	*Salubeni & Kwatta 4851* (MO)	Maluwa Village Chisi Island, Lake Chilwa Zouba Dist, Malawi.	10.11.1986	474	F
28 SenL	*Jacques* – Georges *8800* (MO)	Rufuique village; Senegal.	25.08.1934	0	F & L
29 MalL	*Pawek 12000* (MO)	Chikale beach Nkata bay, Nkhata Bay District, Malawi.	05.12.1976	471	F, P & L
30 EthiH	*Amshoff 4973* (MO)	Gondar Axun 265km from Gondar, Ethiopia.	05.11.1968	1150	F
31 SengL	*Jacques – Georges 1380* (MO)	M'bao.	08.06.1948	28	F & L
32 OmaH	Maconochie *3523* (K)	Wadi Ghazir, Oman.	02.07.1982	1200	F
33 MalH	*Salubeni 886* (K)	Chinseu village Ntakataka, Malawi.	13.11.1967	1400	F, P & L
34 ZamL	*Angus 1768* (K)	Great North Rd on the North side of Munal pass Nega Hill, Zambia.	13.10.1957	3	F, P & L
35 BotH	*A. Heat & R. Heat 1406* (K)	Selinda Reserve, Tswene, Botswana.	12.11.2007	951	F & L
36 MadL	*Baum 329* (MO)	Majunga (Mahajanga) Madagascar.	20.10.1991	2	F, P & L
37 MauH	*Lorence & Guého 1550* (K)	Mauritius Institute Port Louis, Mauritius.	17.12.1975	828	F & L
38 OmaL	*Miller 7039* (K)	West Hinna, East of Taqah, Oman.	28.05.1985	150	F&P
39 SarL	*Fosberg & Balakrishnan 53631* (MO)	Tirukketisvaran, Manner Dist., Sri Lanka.	11.12.1970	23	F
40 SwaH	*Pienaar & Vahrmeijer 178* (PRE)	East Caprivi Strip, Liambezi near, Katima Mulilo Dist., Namibia.	22.11.1973	950	F & P
41 SwaH	*de Winter 3638* (PRE)	Near Onakanyale mission, Ovamboland, Namibia.	14.11.1955	1100	F,P & L
42 BotH	*Allen 220* (PRE)	Orapa Baobab Dune, Botswana.	04.12.1974	945	F & P
43 SafH	*Obermeyer, Schweickerdt &Verdoorn 69* (PRE)	Farm, lower North slope of Soutpanberg, behind homestead, R.S.A.	11.1932	1050	F &L
44 SafH	*Dyer 1309* (PRE)	Dongola Reserve Soutpansburg, Limpopo, RSA.	02.12.1952	1052	F & L
45 SafL	*Reuter 4556* (PRE)	Haenertsburg, Soutpansburg, RSA.	12.1907	500-630	F & P
46 SafL	*Lang TRV31649* (PRE)	Kruger Nat. Park, Punda Maria, Soutpansberg East, RSA .	11.1932	508	F & P
47 SafH	*Van der Schijff 5961* (PRE)	Duiwels Kloof, Mpumalanga, RSA.	12.1961	816	F
48 SafH	*Van der Schijff5 929* (PRE)	Blyderivierspoort Nature reserve, Mpumalanga, RSA	07.12.1961	1354	F, P & L
49 ZimH	*Greenway and Karika 10953* (EA, PRE)	Lake Manyara, Msasa River, Mbulu District, Zimbabwe.	03.11.1934	1067	F & P
50 SafL	*Sachse 845* (PRE)	Farm Samaria, Mapungubwe Nat. Park, Alldays Dist., Limpopo, RSA.	04.12.2010	544	F & P
51 KenL	*Faden 74/297* (EA, PRE)	(1 Km north of Jadini hotel), Diani Beach, Moana, Kenya.	23.03.1974	5	F & P
52 SafL	*Evans 1* (PRE)	Tartarfontein Botanical Reserve, RSA.	18.12.1928	20	F
53 SafH	*Evans 47268* (PRE)	Dongola, Zoutpansberg, RSA.	20.11.1940	1050	F & P
54 SafL	*Straub153* (PRE)	Farm Breslau, along Limpopo River, Limpopo Province, RSA.	01.11.1982	518	F
55 ZimH	*Levy 111* (PRE)	Hwange Wankie, Zimbabwe.	12.1934	1080	F & P
56 TanL	*Greenway 6416* (PRE)	Ruaha Valley, Mtandik, Tanganyika territory, Tanzania.	06.11.1941	701	F & P

ID	*Collector*, Number & (Herbarium)	Locality	Date	Altitude (m) a.s.l.	Part of spcimens used
57 EasL	*Gillet &Hemming 910* (K, PRE)	West of juba river, South of 0° 30' W Tanzania	05.06.1983	550	F & L
58 ZimL	*Jacobsen 1909* (PRE)	Beit Bridge District, Zimbabwe.	21.11.1962	549	F & P
59 EasH	*Richards 13435* (K)	Wembere escapement, Nzega dist., Tanzania.	29.10.1960	1200	F
60 EasH	*Bjornstad AB1965* (K)	T7, Ruaha Nat. Reserve, at Great Ruaha river, 4Km Nem Nsembi iringa Dist, Tanzania.	28.11.1972	800	F
61 EasL	*Richards & Arasululu 26329* (K)	Track to Causeway, Ruaha National Park, Iringa Dist., Tanzania.	26.10.1970	732	F
62 EasL	*Wallace 514* (K)	Tanzania.	30.11.1932	0	F
63 EasL	*Faulkner 2735* (Mus. Bot. Berol)	Massazine , Zanzibar, Tanzania.	25.11.1960	0	F, & P
64 SenL	*Kesby 19* (K)	Near lake Tamna Kaya, Senegal.	17.07.1960	0	F
65 SieL	*Thomas 2626* (K)	Mmsaia, Sierra Leone.	02.10.1914	396	F
66 NigL	*Lowe 4701* (K)	Nursery Botany Dept. Uni. Ibadan, Nigeria.	12.07.1985	150	F & P
67 SudL	*Pfund 8177* (K)	Hessin, Sűd - Kordofan, Sudan.	16.07.1975	380	F & L
68 EriH	*Mooney 8583* (K)	Barentu, Western Eritria	25.05.1960	1000	F & P
69 SomL	*Gillet & Hemming190* (K)	Eastern foot of mountain, Bur Akada, Somalia.	20.06.1983	200	L
70 SomL	*O'Brien 122* (K)	Bur Heybe, South Somalia.	09.05.1985	210 - 220	F, P & L
71 NecL	*Mackee 196* (K)	Dumbea, New Caledonia.	30.01.1967	10	F & L
72 MauL	*Pignal & Bouffart 1849* (K)	Mascarene Island, Mayotte, Port Louis, Mauritius.	05.09.2000	219	F
73 EafL	*Hutchinson 2139* (PRE)	Dongola, Limpopo Province, RSA.	02.1868	236	F & L
74 YemH	*Wood Y/75/187* (BM)	Samsara Island, between Taiz & Turba, Yemen.	15.05.1975	1300	F
75 SudL	*BM001125915* (BM)	Tebeldi, Sudan.	1990		F
76 EasL	*Hildebrandt 1930* (BM)	Kriste Insepl, Mombassa, Zanzibar, Tanzania.	02/1876	0	F
77 TanH	*Bruce 210* (BM)	Uluguru Zoo, Tanzania.	Nov-24	2000	F
78 ZimL	*Swynnerton S.*(BM)	Melsetter Dist., Zimbabwe.	1958	344	F &P
79 ZimL	*Chase 3728* (BM)	Mutare (Umtali) District, Zimbabwe.	20.10.1948	762	F
80 MozL	*Balsinhas 22 (*BM)	Niassa area, Mozambique.	22.10.1953	508	F & L
81 ComL	*Humbolt 379* (BM)	Comoros Island.	1884	29	F
82 CubL	*Howard 4188* (BM)	Vicinity of Soledad, Santa Clara Province, Cuba.	08.1940	125	F, P & L
83 KenH	*Greenway 9505* (EA, K, PRE)	Ntito Andie to Kibwezi, Kenya.	12.11.1957	1189	F, P& L
84 KenH	*Verdcourt 1602* (PRE)	8 Km west of Kibwezi, Kenya.	4.11.1956	850	F
85 SafL	*Lebrun 2603* (EA, BR)	Buta, DRC.	26. 04.1931	431	F
86 SomL	*Gillet & Beckett 23217* (EA)	75 Km North east of Mogadishu, Somalia.	05.06.1981	20	L
87 TanL	*Harris 1203* (EA)	Near harbour, Dar es Salam, Tanzania.	16.11.1967	0	F
88 TanH	*Braum 6777* (EA)	Kiomoni by Tanga, Tanzania.	10.11.1909	1130	F
89 SafH	*Rodin 2637* (PRE, NY)	Bordering Angola near Oshikango, Ovamboland, Namibia.	19.11.1947	1100	F & L
90 DRCH	Baland *1507* (BR)	Eala DRC (Cult.).	07.06.1932	903	F
91 DRCH	*Panderyst 27201* (BR)	Boma, DRC (Zaire).	04.11.1930	903	F
92 DRCH	*Dacrémont 366* (BR)	Maladi Ducum, (Congo Beige), DRC.	12.1933	1760	F

ID	*Collector*, Number & (Herbarium)	Locality	Date	Altitude (m) a.s.l.	Part of spcimens used
93 DRCL	*Vermoesen 1300* (BR, MO)	Eun lualelia Congo Beige.	9.1919		F
94 SenL	*Van den Berghen 10519* (BR)	Abene Casamance, Senegal.	12.08.1997	10	F & P
95 SenL	*Vanden Berghen 5263* (BR)	Bouyouye Basse, Casamance, Senegal.	29.07.1982	8	F&P
96 L	*Jichefat 478* (BR, MO)	Sine Loc.		16	F
97 GuyL	*Granville 8341* (BR)	Bourgde Caynne, French Guyana.	2.11.1986	10	F
98 SafL	*Balakrishnan 605* (BR)	Tirukketiswaran, Manner District, Northern Province, Sri Lanka.	11.12.1970	23	F&P
99 TanH	*Bullock, 3462* (BR)	Zimba southward to the Mamba river, Milepa Ufipa District, Tanzania.	13.10.1950	1036	F
100 TogL	*Hakki, Leuenberg* & Shiers *124* (B)	Agoueve , NE Agouenyive, Sio-Flustal, Togo.	17.04.1978	69	F & L
101 TanL	*Schlieben 5406* (BM)	Bezirk, Lindi Lutamba 40 km west of Lindi Dist, Luanda- Viana, Tanzania.	28.09.1934	200-250	F
102 AngL	*Teixeira et al*., *10115* (BR)	Luanda District, Angola.	28.03.1996	140	F
103 MalL	*Chapman, Partel & Massige 5188* (BR)	Nambwale Village, foot of Tumitulu Hill lake Chilwa , Mulanje District, Malawi.	27.11.1980	762	F
104 MozL	*de Carvalho 868* (K)	Inhafenga, Buzi Chibabana, Mozambique.	28.10.1966	365	F
105 MozL	*Mendonca 3179* (K)	Mozambique.	30.11.1944	435	F
106 kinH	***S.Venter 1* (J)**	**Caprivi Strip, Namibia**	**14.11.2014**	**932**	**F, P & L**
107 KilH (topotype)	***S.Venter 2* (J)**	**S side of Route 523 to Sibasa between Tshkuwi & Tshirolwe, the Vhembe District , Limpopo Province,R.S.A.**	**18.11.2014**	**825**	**F, P & L**
108 DigH	*S.Venter 3* (J)	S side of Route 523 to Sibasa between Tshkuwi & Tshirolwe, the Vhembe District, Limpopo Province, R.S.A.	18.11.2014	818	F, P & L
109 DigH	*S. Venter 4* (J)	S side of Route 523 to Sibasa between Tshkuwi & Tshirolwe next to the taxi rank, the Vhembe District, Limpopo Province, RSA.	18.11.2014	811	F, P & L
110 SafL	*Baum & Mayne* 4 2014 (J)	Kruger National Park, RSA.	11.2014	206	F, P & L
111 SafL	*Baum & Mayne* 5 2014 (J)	Kruger National Park, RSA.	11.2014	228	F & P
112 SafL	*Baum &Mayne* 2 2014 (J)	Tshikuyu, RSA.	11.2014	372	P
113 SafL	*Baum & Mayne* 3 2014 **(J)**	Kruger National Park, RSA.	11.2014	221	F,P & L
114 SafL	*Straub* 755 (PRE)	Breslau farm, 2Km. Near Pontdrift.	08.2000	518	F,
115 EsfL	*Netshiungani* 624 (PRE)	Nzhelele (Dizanani), Nzhelele poort Venda, Limpopo Province, RSA.	07.11.1979	655	F
116ComL	*Leg 11802 (US)*	Near the Ocean, Comoros Island.	07.12.1967	0	F
117 SafH	*Smut & Gillett 3138 (PRE)*	Road to waterpoort, foot of mountain Zoutpansberg Dist. RSA.	NA	933	F
118 MozfL	*Timberlake et al 5737*	Below Nhica do Rovuma village along cut line 34, Way point JT514, Cabo Delgado, Palma Dist., Mozambique.		86	F
110WafL	*Huchinson & Dalziel 1821 (K)*	Senegal.	02. 1868	250	F
120 ZimL	Batsin *15721* (K)	Triangle Ranch, Forte Victoria Dist., Zimbabwe.	06.11. 1946	610	F& L
121 GabL	Leal et al 868 (MO)	On shell heap on the Island in estuary of Loango, Iguela.	12.11.2005	7	F, P & L

ID	*Collector*, Number & (Herbarium)	Locality	Date	Altitude (m) a.s.l.	Part of spcimens used
122Moz L	Hladik 6591	coastal vegetation,Tumble Zone, Mozambique.	11.04.2001	0	F
123 SafH	*Baum & Mayne* 1 2014 (J)	Beside route 523 to Sibasa between Tshkuwi and Tshirolwe 9 Km E of N1 highway in the Limpopo Province of RSA.	11.2014	803	L
124SenL	*Rottler 73 (BR)*	Senegal.	1908	15	F &L

Key: F- flower, L- leaf, P- pollen grain

Table 3: Data set for Principal Component Analysis ofseven characters, calyx length (mm), calyx breadth (mm), petal length (mm), petal breadth (mm), staminal tube length (mm), staminal corolla diameter(mm) and style length (mm, abbreviated in data set) of 124 African baobab specimens with 8.97% missing data.

ID	CalyxL	CalyxB	PetaL	PetaB	StamTL	StamC	StyleL	PolDiam
	---------- (mm) ----------							
1ComL	47.33	16.25	70.67	62.33	40	66	62	57.83
2SWaH	999	999	50.67	41	20	40	62	999
3KenL	999	999	61.67	48	26.25	40	999	58.02
4UniL	999	999	57.67	48.67	22	48	999	999
5SafL	50.75	13	60	26.5	999	999	999	999
6EafL	54.33	19	79.33	45.33	26	56	67	999
7SafL	56.5	22	60	41	34	46	999	50.18
8EafL	66	18.5	999	999	21	52	71	999
9EafH	61.75	20	65.5	67.75	24.5	56.59	76.5	56.9
10EafH	54.5	18	68.33	47.33	22	41	999	58.4
11HawL	999	999	66.67	52.83	23	38.5	999	999
12BukL	56	9	54	39	19.5	36	999	48.46
13SumL	45	19.5	76	40	21.67	52	999	999
14EafL	54	26.5	999	999	18	38	999	999
15MadL	84	27.5	56	41	22	46	99	53.87
16ComL	999	999	60	46.5	999	999	999	999

17FloL	74.67	25	57	64	30	62	999	999
18DrcL	999	999	58	45	20	39	999	999
19WafL	74.5	31	47.5	31.75	27	68	122.5	999
20SafL	44.5	18	55	57	17	30	41	32.81
21WinL	999	999	60	37	25.5	51.5	999	999
22HaiL	999	999	48.5	26	25	51.5	999	55.57
23HawL	75.67	19.67	77	68.33	42	73	85	999
24TanH	54	22	999	999	18	45	52	59.49
25TanL	59.33	21	66	39.5	28.5	59	999	34.71
26SafL	54	20.33	54	59.5	20	38	60	999
27ComL	66.67	17.67	89	63.33	23	41	999	999
28TanH	51	21	57	35.5	17	41	999	60.29
29ZimH	54	14	62.67	57.33	31	47	999	999
30IvoL	52	27	50	45	28	56	53	60.44
31MalL	38	20	40	49	22	41	999	999
32MalL	999	999	55	48	21	37	999	54.93
33OmaH	49	18.5	45	25	20	29	999	999
34MalH	48	20	53	41	27	52	999	69.66
35BotH	52	20	58	65	20	42	999	999
36MadL	61.5	18	55.67	28.33	21	40.5	59.5	59.18
37MauH	54	27.5	84.33	49.67	22	60	80	999
38OmaL	83	20	64.5	41.5	23	41	999	69.16
39SarL	999	999	60.5	48.5	22	37	999	999
40SafL	40	24	50	44	18	34	999	999
41SwaH	58	29	57	44.67	22	41	50	50.06
42BotH	999	999	57.33	48.67	17	41	50	54.92

43SafH	60.5	28	52	61	999	999	999	999
44SafL	999	999	50.5	34	23	45	999	999
45SafL	53	16.67	62.33	43.67	14.5	30	999	53.08
46SafH	56.67	21.67	65	64.33	19	41.33	999	54.95
47SafL	64	25	999	999	22	51	999	999
48SafH	63.5	23	70	68	15	41	999	999
49SafH	999	999	54	67	23	43	999	59.2
50ZimH	51	24	71	60.67	23.33	50	999	57.14
51SafL	999	999	66	53.33	27.5	54	999	56.92
52KenL	999	999	53	31	23	41	51	56.73
53SafL	999	999	53	46	22	41	999	999
54SafL	999	999	63.5	62.5	23	51	55	999
55SafH	999	999	57	62.33	20	39	999	54.13
56SafH	49.67	19.67	58.67	46	23	46.5	50	999
57SafL	999	999	65	47	30	999	999	999
58ZimH	999	999	56	43	37	74	999	56
59TanL	49.33	17	60	59.5	23	49	999	54.87
60ZamL	48.67	18.33	59.67	47.33	24.33	47.33	999	57.19
61SWaH	66	31.67	68.33	58.67	34	71	92	64.57
62SafL	999	999	65.5	40.5	13	34	45	999
63KenH	55	21	58.5	42	999	999	999	999
64ZimL	47	16.5	46.67	40.33	21	42	999	52.09
65EsfH	52	19.33	47.33	49	19.5	41.5	999	999
66EsfH	999	999	55.67	36.33	17	37	999	999
67EsfL	999	999	70.67	70	27.67	62.33	73	999
68EsfL	51.5	13.5	68	58	25	42	999	999

69EsfL	57	17	56	69	19	42	53	58.83
70MozH	49	19	52	54	22	51	61	999
71SenL	62	23	104	50	35	74	126	999
72SieL	79.5	22	90	50	999	999	999	999
73TogL	91	40.5	63	64	26	56.5	999	999
74NigL	999	999	66	51	38	72	999	47.56
75SudL	999	999	52.5	48	28	56	999	999
76EthH	56.5	22.5	69	42	25	50	999	79
77SomL	999	999	59.33	42.67	19	35	999	999
78SomL	56	21	54.33	43.67	999	999	999	57.42
79WafL	999	999	71.33	61.33	40.5	79	96	999
80NecL	88	31	83	68.5	40	80	98	999
81WafL	50.33	20.67	58	52	17	35	999	999
82MozL	83	24	76.67	83.33	24	44	999	999
83YemH	999	999	42.5	31.5	17	30	999	999
84SudL	61.33	18	66.33	45.67	999	999	54	999
85EsfL	999	999	70.67	42	24.5	51.5	999	999
86TanH	999	999	55.5	31.5	25	50	999	999
87ZimL	999	999	58.5	42.5	23.5	45.5	53	60.68
88ZimL	999	999	48	37	25	46	999	999
89MozL	46.33	16.67	47.5	52	18	40	999	999
90SafL	999	999	50	46	25	44	999	68.73
91TanL	53.5	16	66	56	999	999	60	999
92ComL	999	999	72.67	61.33	27	53	999	999
93CubL	68	32	71.5	38	31	63.5	999	52.25
94KenH	999	999	57.5	45	19	38	999	65.98

95SafL	82	35	58	61	37.5	75	107	999
96SomL	999	999	59.33	41	20	35	999	999
97TanL	37.5	14.5	43.5	34.5	999	999	999	57.77
98TanH	70.33	28	66	74.5	999	999	999	999
99NamH	999	999	61.67	33.33	24	50	999	999
100DrcH	67	29	999	999	16	39	64	999
101BomH	47.5	32	63.5	44	999	999	999	999
102DrcL	999	999	63.5	64	27.5	55.5	999	999
103SenL	65	42.5	66.5	44.5	999	999	999	999
104SenL	999	999	55	54	25	42	999	69.24
105SenL	999	999	75.5	45.5	29	46	999	67.73
106DefL	52	25	47.5	25	30.5	999	999	999
107L	999	999	72	76	30	60	84	999
108SafL	70	27	66.5	56.5	37	41	999	999
109TanL	46	12	47.5	30	12	33.5	50	51.02
110ZimL	58.5	15	67.5	32.5	26.5	56	66	999
111TanL	59	15.5	70	45	22	48	999	999
112NoaL	101	36.5	94	95	30	72	999	999
113MalL	44.5	22.5	65.67	58.33	20	46	999	999
114MozL	53.67	16	60	67.33	24	44	999	999
115ZimL	999	999	46	42	20	40	999	999
116GabL	999	999	80	74	38	62	999	71.76
117SenL	70	10	999	999	20	41	99	999
118MozL	999	999	59	47	16	28	999	999
119EthH	999	999	68	53	22	60	999	999
120SenL	999	999	70	50	32	63	999	999

121KinH	52	27.5	52.33	34.33	13	32	42	59.7
122KilH	60.67	27	67	61	27	49	82.5	76.34
123DigH	62.67	26.33	66.33	51.33	26	52	47	60.55
124DigH	53.33	23.67	65	55.67	33	52	58	67.21

TABLE 4: T-TEST RESULTS TO COMPARE BETWEEN ALTITUDES					
A.	Floral and pollen traits of African baobabs P< 0.05				
Variables	Low altitude (<800 m)	High altitude (≥800 m)	t-value	df	P
Calyx length (mm)	60.84±14.29	55.30±7.14	1.89	76	0.06^{ns}
Calyx breadth (mm)	21.69±7.20	22.98±4.82	0.83	76	0.41^{ns}
Petal length (mm)	60.01±11.77	58.83±11.43	1.95	116	0.06^{ns}
Petal breadth(mm)	49.79±13.01	49.37±12.61	0.16	116	0.87^{ns}
Staminal tube length (mm)	25.13±6.51	22.37±5.55	2.13	110	0.04*
Staminal corolla diameter (mm)	49.00±12.33	45.85±9.90	1.3	108	0.20^{ns}
Style length (mm)	72.96±23.75	56.55±9.81	2.1	35	0.04*
Pollen diameter (µm)	55.29±10.02	60.84±7.23	2.16	48	0.13^{ns}
Pollen volume ($\mu m^3 \times 10^3$)	48.29±22.70	61.45±24.29	1.97	48	0.73^{ns}
Pollen spine density 1000 μm^{-2}	19.06±13.75	16.82±7.46	0.64	48	0.52^{ns}
B.	Stomatal traits of African baobabs (P< 0.05)				
Stomatal length (mm)	33.71±3.57	31.61±3.79	1.91	44	0.06^{ns}
Stomatal length (mm) strict putative ploidy category	36.63±1.9	29.03±2.57	9.4	41	0.00***
Stomatal length (mm) putative ploidy split in the middle	35.72±3.11	29.30±1.9	-8.37	48	0.00***
Stomatal density per 1000 μm^2	0.83±0.66	1.19±0.7	-1.74	44	0.09^{ns}
Stomatal density 1000 per μm^2 (putative ploidy)	1.27±0.43	3.51±0.0	-5.05	14	0.00***
Stomatal density 1000 per μm^2 (putative ploidy split)	0.78±0.46	2.67±0.42	-9.46	48	0.00***
C. Floral and pollen traits of fresh and dry herbarium baobab specimens (P< 0.05)					
Variables	Dry specimens	Fresh specimens	t-value	df	P
Petal breadth(mm)	49.73±13.05	48.55±10.73	0.23	116	0.82^{ns}
Staminal Corolla diameter (mm)	48.33±11.77	44.17±11.18	0.84	108	0.40^{ns}
Pollen grain diameter (µm)	57.20±8.59	59.85±12.86	0.73	48	0.47^{ns}
Pollen grainVolume ($\mu m^3 \times 10^3$)	426.01±172.45	428.62±323.54	0.3	46	0.97^{ns}
D. Stomatal length and density of fresh and dry baobab specimens (P< 0.05)					
Stomatal length (mm)	33.79±5.59	30.35±1.60	1.22	46	0.23^{ns}
Stomatal density ($1000\mu m^{-2}$)	1.94±0.63	1.71±0.38	-0.73	48	0.47^{ns}

See text for details.ns – Not significant, * - P< 0.05, ***- P< 0.001 (significant)