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Assessing ploidy-level and gene flow between baobab (*Adansonia digitata*) fruit producers and poor producers in Limpopo

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A research report submitted to the Faculty of Science, University of the Witwatersrand, in partial fulfilment of the requirements for the Degree of Masters of Science by Coursework and Research Report.

Johannesburg

13 May 2014

Declaration

I, Ronie Tivakudze, 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 to any other University.



_____

(Signature of candidate)

13th day of *May* 2014

Abstract

The African baobab (*Adansonia digitata*) is a multi-purpose tree that is important among African villages as it provides food and a range of raw materials. Its fruits provide essential nutrients and are sold to generate income. As baobab fruits are important to the livelihoods of many people, it is important to understand the causes of differences in fruit production in order to maximise use and for conservation purposes. Many studies have examined fruit production to understand the causes of variation in fruit yields. In Venda, a region northern South Africa, differences in baobab fruit yield has been recorded for 8 years, thus classifying individual trees as either poor producers or producers (Venter and Witkowski, 2011). Poor producers are adult trees producing less than five fruits each year and some not producing at all. On the other hand, adult trees producing more than five fruits each year are referred as producers. Causes of this difference in fruit production have not been identified. Among other factors, the observed difference in fruit production could be related to differences in ploidy-level among baobab trees. Importantly, few or no studies to our knowledge have been carried out to confirm whether differences in fruit production among baobab trees are related to a difference in ploidy-level. The well-known and widespread mainland African baobab, *Adansonia digitata*, is known to be a tetraploid (four sets of chromosomes). Recently, a difference in ploidy-level has been revealed. A new diploid species, *Adansonia kilima*, has been identified in Africa (Pettigrew et al., 2012). Morphological characteristics (floral, pollen, and stomatal size and density), ploidy, and molecular phylogenetics suggest the presence of a new species. This new species has been reported to overlap the well-known and widespread tetraploid *A. digitata's* distribution in Venda. Consequently, the presence of a diploid species that reproduces with a tetraploid species could result in triploid progeny and contribute to the observed differences in fruit production in these baobab trees. The objectives of this study were (i) to assess if there is any difference in ploidy-level between the poor producer and producer baobab trees in Venda using flow cytometry, (ii) to assess if stomatal density and size correlate to differences in ploidy-level, and (iii) to use microsatellites to estimate levels of gene flow between these baobab trees. Morphological results showed that stomatal size and density were not significantly different between poor producer and producer trees and these features may not be true indicators of difference in ploidy-level for baobabs. Gene flow results showed that there was high mean genetic heterozygosity and low population differentiation expressed in all populations. This suggests that inbreeding was not responsible for the differences in fruit production between poor producer and producer

trees. Low population differentiation observed among the populations indicated that a large number of common alleles were shared among the populations. Therefore, the high gene flow observed among the populations suggests that poor producer and producer trees were sharing alleles, and what is causing the differences in fruit production remains unclear.

Keywords: African baobab, flow cytometry, fruit producers, gene flow, ploidy-level, poor producers, stomatal counts

Dedication

I dedicate this research report to my brother (F. R. Tivakudze) and his wife (L. T. Jabangwe) for their support throughout my studies. I also pay tribute to A. Magwali and O. M. Takura for accommodation they gave me for the entire period of my studies. Lastly, I dedicate this writing to my wife (R. R. Mugwechete) and my parents for their unconditional love and support they gave me.

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List of Symbols

^{o}C	degree Celsius
km	kilometre
m	metre
m.a.s.l.	metres above sea level
µl	microlitre
µm	micrometre
µM	micromolar
mg	milligram
mm	millimetre
min	minute
%	percent
π	PI
pg	picograms
μm^2	square micrometre
mm^2	square millimetre
s	second

Chapter 1.0

1.1 Literature Review

Indigenous fruit trees

Indigenous fruit trees have many uses and form an important part of the livelihoods of many African villages (Gouwakinnou et al., 2011; Shackleton, 2002). For instance, their importance is due to their nutritional value, medicinal uses, timber uses, social, and economic value (Akinnifesi et al., 2006). Some of the important fruit trees include the African plum (*Prunus africana* Hook.f; Kalkman), marula (*Sclerocarya birrea* (A. Rich; Hochst.), baobab (*Adansonia digitata* L.), tamarind (*Tamarindus indica* L.), wild mango (*Irvingia gabonensis* (Aubry-Lecomte ex O'Rorke; Baill.), wild loquat (*Uapaca kirkiana* Mull. Arg.), monkey orange (*Strychnos spinosa* Lam.) and ber (*Ziziphus mauritiana* Lam.) (Shackleton et al., 2000; Akinnifesi et al., 2006; Jama et al., 2007; Wickens and Lowe, 2008). Each part of many fruit trees can be used for a number of purposes. For instance, trunks and branches provide shade in homes and can be used to make wood carvings and firewood. Further, leaves may be used as relish or for extracts of some medicines. Bark and sap can be used to produce utensils, ropes, and glues. Fruit pulp is often used to make juices, wine, and jam, all of which contribute to the diet of African villages (FAO, 1996). Seeds from some fruits yield oil that is used in industry to make varnishes, paints and by pharmaceutical companies to produce facial creams (i.e., EcoProducts Baobab Oil; SCUC, 2006). Most importantly, fruits can be harvested and sold locally and internationally to generate income to meet livelihood needs (Leakey et al., 2005; Vedeld et al., 2007). Consequently, many villages value the fruit trees around them.

Fruit tree usage often depends on what products are most needed by people, and as a result, different villages prefer certain tree species to others (Poulton and Poole, 2001; Garrity, 2006; Wickens and Lowe, 2008). For example, if trees supply leaves used as relish, trees producing a lot of leaves may be preferred over those that do not produce many leaves. For trees harvested for use as fire wood, species that do not burn out quickly and do not produce too much smoke are preferable (Tietemam, 1991). In some trees where the leaves are harvested and cooked as relish, tree species that produce leaves regarded as good-tasting are often harvested (Dhillion and Gustad, 2004). On the other hand, if fruits are required for eating, trees that produce fruits with high nutritional value or are sweet may be preferable to those that do not produce sweet fruits (Babicz-Zielińska and Zagórska, 1998). Since fruit trees are harvested for a variety of purposes to meet the needs of villages, local

people play a central role in sustainably harvesting trees around them and conserving these natural resources (Agrawal and Gibson, 1999).

Despite having many uses, fruit trees play a major role in food supply among rural African communities. During periods of droughts and poor crop harvests, food becomes scarce and hunger becomes prominent (Akinnifesi et al., 2006). When such food shortages occur, fruit trees become vital in meeting the dietary requirements of people because they provide essential nutrients. Some fruits have been recorded to have high contents of vitamins, phosphorus, calcium, as well as other essential minerals, and can provide nutrition during food shortages (Akinnifesi et al., 2004). For example, baobab fruit pulp is known to contain more than 10 times as much vitamin C on a mass basis as orange (Sidibe and Williams, 2002). For these reasons, fruit trees are an important part of many rural villages.

Fruit production studies

Studies that have focused on fruit production have suggested several potential reasons behind differences in fruit production in a number of different tree species. Given the importance of fruit trees as a food source, fruit characteristics such as fruit yield (Shackleton, 2002), size, and taste have been well studied, often in order to maximize fruit production. Furthermore, these traits are also often useful criteria to determine which fruit tree species or individuals are preferable. Identifying causes behind difference in fruit production is necessary to build guidelines for sustainable harvesting and ensure trees will be available for future use by village dwellers. Consequently, much work has examined potential drivers behind difference in fruit yield in a number of fruit trees. Rainfall has been shown to affect fruit production in many tree species (Stephenson, 1981; Udovic, 1981). For example, rainfall received immediately after pollination has been shown to wash away pollen grains, thus resulting in low fruit set and ultimately low fruit production in both almond (Orteda et al., 2004) and loquat trees in Jordan (Freihat et al., 2008). Further, Shackleton (2002) found that rainfall differences could explain the difference in fruit production between two fruiting seasons in *Sclerocarya birrea* (marula) in South Africa.

In addition to rainfall, other environmental factors, such as soil type and land form, have been found to affect fruit production in marula trees in north-central Namibia (Botelle et al., 2002). Additionally, Botelle et al. (2002) noted that trees with larger trunk sizes yielded significantly more fruits than the trees with smaller trunks. In Mexican guava trees,

variation in fruit yield has been associated with soil conditions such as soil fertility and soil acidity, diseases, and other environmental conditions (Delgado et al., 2007). Alternatively, other factors may contribute to variation in fruit yield. For instance, differences in fruit yield may be due to the number of flowers and premature death of young developing fruits (Stephenson, 1981) or reduced pollinator activity (Freihat et al., 2008). It has also been suggested that fruit yield may be affected by damage on trees due to the harvesting of leaves and bark (Dhillion and Gustad, 2004). Clearly, difference in fruit yield is of considerable interest, yet a conclusion regarding potential reasons for observed differences has not been found.

Perhaps one of the best studied fruit trees is the iconic baobab tree (*Adansonia digitata*) due to its importance among African people and their communities. A better understanding of fruit production is necessary since baobab fruits are important in the livelihoods of many people, particularly those in the Venda region in the north-east of South Africa where the trees are economically important (Venter and Witkowski 2013a). In an effort to maximise use of fruit trees and baobabs in particular, local people often observe and note certain characteristics (Assogbadjo et al., 2009). Local people often look at characteristics of leaves, bark, and fruits, and often note differences among fruit trees. Through these observations, local people collect information about trees that is useful for both conservation and science. For example, very large differences in fruit yield have been observed in baobabs in both Benin and South Africa (Assogbadjo et al., 2008; Venter and Witkowski, 2011).

Similarly, observations of baobab fruit in Mali and Sudan noted differences in fruit yield, size, and nutritional value (De Smedt et al., 2011; Gebauer and Luedeling, 2013), as well as which trees produce tasty fruits. In these populations, fruit yield was negatively influenced by the degree to which people harvested fresh leaves for cooking, which in turn, resulted in the number of fruits per adult tree declining (Dhillion and Gustad 2004). Due to the importance of baobab fruits, locals observed that some baobab trees never produce any fruits, while others consistently produced fruits (Assogbadjo et al., 2008; Venter and Witkowski, 2011), thus identifying poor-fruiting trees as ‘male’ and fruiting trees as ‘female.’ However, the baobabs are bisexual (Sidibe and Williams, 2002; Assogbadjo et al., 2008) with both male and female parts in the same flower. Even though local people made these critical observations in distinguishing between these trees (Assogbadjo et al., 2008),

they had no scientific explanation as to what caused some baobabs to produce fruits and some to fail to produce any fruits despite producing flowers.

The observations made by local people have been corroborated by findings of Venter and Witkowski (2011). In that study, fruit production was found to differ markedly between baobab trees in Venda. Approximately 41% of adult trees consistently produced fewer than five fruits per year, and were then classified as 'poor producers'. Other trees in the same study area consistently produced more than five fruits (and usually many more than five) each year, and were thus classified as 'producers.' Interestingly, the 'poor producer' trees also produced many flowers; however, few of these flowers produce fruits (S. Venter, 2013, *pers. comm.*). Venter and Witkowski (2011) suggested that environmental conditions may not be causing differences in the observed differences in fruit yields because the poor producer and producer trees were often found growing next to each other and most likely to be sharing the same environmental conditions. Further, fruit production in these baobab trees also varied between years (Venter and Witkowski 2011). Venter and Witkowski (2011) also found that tree size and land-use type did not determine whether trees were poor producers or producers. Moreover, in the same study, rainfall received did not correspond to the fruit production in the same season. Therefore, the reasons behind some trees being poor producers and some producers need to be investigated.

Causes of differences in fruit yield

Although many ecological causes have been explored, relatively few genetic causes have been examined. One possibility is that inbreeding may result in reduced fruit production for some individuals. When deleterious alleles are passed in offspring, in such cases, inbreeding may lead to reduced fitness (inbreeding depression) for certain traits, such as germination rate, competitive ability, growth rate, pollen quantity, number of ovules, and amount of seed produced (Jain, 1976; Silvertown, 2001; Keller and Waller, 2002; Frankham et al., 2003). However, Baum (1995) conducted hand-pollination trials on Madagascan baobab trees (*Adansonia grandidieri, A. rubrostipa, A. madagascariensis* and *A. gregorii*) and found that there was no inhibition of pollen tube growth in the style, which suggests that these species may be self-compatible. Thus, if baobabs can self-pollinate, inbreeding depression could potentially cause the observed differences between poor producer and producer trees. In a similar hand-pollination trial, Baum (1995) further examined *A. gibbosa* and found about 98% delayed abortion of self-pollinated and non-

hand pollinated flowers approximately one month after pollination. In contrast, only 25% abortion was recorded for cross-pollinated flowers. As a result, the likelihood of baobab inbreeding and causing some baobab trees to be poor producers or producers is uncertain.

Other studies suggest that the mainland African baobab may be self-incompatible (unable to self-pollinate). For example, Rao (1954) noted that it is common to have sterile *A. digitata* trees, observing that fruits generally develop well with tender and juicy walls, but become hard after a while, resulting in the seeds failing to develop. These data suggest that *A. digitata* may be self-incompatible (Wickens and Lowe, 2008). Further, Assogbadjo et al. (2008) suggested that baobab trees in Benin that did not produce any fruits have been influenced by either inbreeding among particular baobab trees or some incompatibility within the reproduction system of baobab trees that did not produce fruits. In addition, *A. digitata* exhibits considerable morphological variation across its range. Assogbadjo et al. (2009) went on to study the genetic differentiation among eight different morphotypes observed within baobab populations in Benin. The different phenotypes were recognised through a morphological classification system which local farmers used for identifying trees with desired or undesired combinations of traits. Amplified fragment length polymorphism (AFLP) marker information was used, but found no genetic distinction among the morphotypes (Assogbadjo et al., 2009), which suggests that the eight different baobab phenotypes studied in Benin are genetically similar.

Another possible reason for the noted difference in fruit yield between poor producer and producer trees in Venda may be differences in ploidy-level in the genus *Adansonia*. *Adansonia digitata* is tetraploid (four sets of chromosomes) and is found only on mainland Africa, whereas *Adansonia* species found in either Madagascar or Australia are diploid (two sets of chromosomes, like most organisms; Wickens, 1982; Baum, 1995). Recently, work has suggested that there is a possibility that a diploid progenitor exists in mainland Africa (Pettigrew et al., 2012). This new diploid species, *Adansonia kilima* Pettigrew, Bell, Bhagwandin, Grinan, Jillani, Meyer, Wabuyele and Vickers, sp. nov, may have subtle morphological (floral and pollen characteristics, and stomatal length and density) and distribution differences (occurring at moderate elevations of about 650−1500 m) from the widespread *A. digitata*, though both species are said to overlap in northern South Africa in the Venda region (Pettigrew et al., 2012). Consequently, the presence of *A. kilima* may

represent a possible explanation for the observed difference in fruit production in baobab trees that occur in northern South Africa.

Polyploidy

Polyploidy (whole genome duplication) has long been reported in plants (Stebbins, 1971; Levin, 1983) and is associated with enhanced vigour, altered morphology, increased sterility, higher pest or disease tolerance, and restoration of hybrid fertility. In addition, it can influence reproductive compatibility and fertility (Stebbins, 1971). Ramsey and Schemske (2002) highlighted that infertility in polyploids is complex and may be due to meiotic aberrations, physiological effects of polyploidy, ecological factors, or genetic factors. Incidental effects of polyploidy may result in increased differences in the way information from genes is used in synthesis of functional genes, which reduces the number or viability of gametes produced and may also affect the growth and development of organisms (Ramsey and Schemske, 2002). Meiotic aberrations have been shown to be the most general factor affecting fertility in polyploids due to the high incidence of unpaired chromosomes and non-homologous chromosome pairing during meiosis (Stebbins, 1971; Ramsey and Schemske, 2002). Furthermore, reproduction between tetraploid (*A. digitata*) and diploid (*A. kilima*) baobab trees may have resulted in triploid offspring, which often result in infertility as suggested above. Therefore, infertility may be caused by a lack of homologous pairing due to the production of unbalanced, unviable, and semi-sterile gametes (Ramsey and Schemske, 2002) and, lead to differences in production between poor producer and producer trees in Venda.

Polyploidy often affects plant morphology, with the most direct and universal effect being an increase in cell size (Stebbins, 1971; Baum et al., 1998). Interestingly, within the baobab distribution, there is evidence indicating the existence of a number of forms differing in fruit size and shape, habit, vigour and leaf morphology (Pakenham, 2004; Pettigrew et al., 2012; Munthali et al., 2013). Many varieties have been described and may be a result of morphological and genetic diversity observed within the African baobab population (Pettigrew et al., 2012). For instance, Sanchez et al. (2010) studied leaf morphology (e.g., leaf length and thickness, and stomatal density and size on the leaf surfaces) of baobab trees in Benin from different agro-climatic zones and found significant differences in leaf size and stomatal characteristics. The authors linked the observed differences in leaf morphologies to the environment and inherent drought tolerance of baobabs. An alternative

explanation might be that there is a difference in ploidy-level that lead to the observed differences in leaf morphologies. The number and density of stomata can also be influenced by the ploidy-level of the plant. Diploid plants tend to possess leaves with greater stomatal densities and with stomata that are smaller in size (aperture) than in tetraploid plants (Stebbins, 1971). Interestingly, Pettigrew et al. (2012) found that *Adansonia kilima* (diploid) leaves have smaller stomatal apertures (mean length of 26.1 µm) and higher stomatal densities (5 per 100 μm^2) than the tetraploid *A. digitata. Adansonia digitata* leaves were found to have bigger stomatal apertures (38.1 µm) and lower stomatal density (1.6 per 100 μm^2). Given the potential variation in ploidy-level, or genome size, between the two presumed baobab species in mainland Africa, poor fruit production in baobabs may be related to infertility due to differences in ploidy-level. As a result, this study aimed to 1) determine if there are ploidy-level differences among the baobab trees sampled in northern Venda and on two islands off the coast of Mozambique and 2) determine if the observed differences in fruit production among the trees in Venda and Mozambique are linked to ploidy-level. The Mozambican trees sampled include trees that were also classed as 'poor producer' and 'producer.' Therefore, I included them in this study.

Use of molecular data

Prior to the advancement of molecular (DNA-based) data, genetic variation, kinship, and phylogenies were estimated using comparisons of phenotypic data from physiology, morphology, and behaviour observed in organisms (Avise, 2004; Conner and Hartl, 2004). Now, however, molecular approaches are widely used in population genetics to examine gene flow among individuals (Avise, 2004) and also to determine ploidy-level. Some of these molecular approaches include microsatellites, flow cytometry, and AFLP. Microsatellites are useful molecular markers to estimate gene flow from both parents due to their co-dominant nature. Moreover, microsatellites are typically characterized by high rates of mutation and hence a high level of polymorphism, and they are also fairly easy to develop and replicate, rendering them useful for fine-scale population structure, parentage and kinship analysis, and genome mapping (Tautz and Renz, 1984; Avise, 2000). Microsatellites or simple sequence repeats (SSRs) are stretches of short mono-, tri-, or tetra-repeats of DNA sequences of variable lengths and are distributed throughout the eukaryotic nuclear genome and are found in both coding and non-coding regions (Conner and Hartl, 2004; Moradi and Keyvanshokooh, 2013).

Genetic knowledge helps us better understand viability of species in the near future in view of environmental changes that may occur (Munthali et al., 2013). Use of molecular data can aid in better understanding of genetic variation in the poor producer and producer baobabs in Venda. Therefore, this project aimed to investigate whether the difference in fruit production between poor producer and producer baobab trees was linked to possible differences in ploidy-level among trees in the Venda region of the Limpopo Province, South Africa. In addition, I also aimed to estimate gene flow and test for potential inbreeding among the producer and poor producer trees.

Larsen et al. (2009) suggest that gene flow studies provide an insight into dispersal processes that shape the genetic structure, particularly of baobabs. The co-dominant nature of microsatellites and their wide dispersal across eukaryotic genomes (Koreth et al., 1996; Avise, 2000) makes them useful markers for the study of local gene flow and population structure by determining levels of genetic variation. Spatial genetic structuring in tree species has been shown to be influenced by many biological forces such as gene flow through seed and pollen dispersal, tree density, fragmentation, colonization history, isolation into small numbers, differential mortality, and micro-environmental selection (Kyndt et al., 2009). This same genetic structuring could be evident in the producer and poor producer baobab trees.

Molecular studies have been done in previous years on baobab trees from West Africa in order to assess genetic variation (Assogbadjo et al., 2009; Kyndt et al., 2009; Larsen et al., 2009), but few studies have used microsatellites. Most of these studies have been carried out in Benin, Ghana, Burkina Faso, and Senegal (Assogbadjo et al., 2009; Kyndt et al., 2009). These studies generally showed high levels of genetic variation and that genetic diversity varies between baobab populations in different climatic regions. The authors suggest that observed patterns of genetic variation are influenced by many factors such as seed and pollen dispersal, colonisation history, fragmentation, and micro-environmental selection (Heywood, 1991; Kyndt et al., 2009), which may affect the genetic structure in tree species (Kyndt et al., 2009). Recently, microsatellite primers developed by Larsen et al. (2009) have been used in Malawi to establish genetic differentiation and diversity in baobabs (Munthali et al., 2013). In my study, nine polymorphic microsatellite loci were used to assess gene flow between poor producer and producer baobab trees in Venda, South Africa, and poor producer and producer baobab trees from Mozambique.

Chapter 2.0

2.1 Introduction

The African baobab *(Adansonia digitata* L., Malvaceae) is an iconic tree (Venter and Witkowski, 2010) with multiple traditional uses across different African villages (Sidibe and Williams, 2002; Pakenham, 2004; Wickens and Lowe, 2008). For instance, it is a great source of food because it is a good source of vitamin C and phosphorus (SCUC, 2006). The pulp is mixed with water to make a refreshing drink and is also used as an ingredient in baking. The seeds of baobab fruits are roasted and ground to produce coffee (SCUC, 2006). Twigs, flowers, seeds, leaves and fruits are all used as common ingredients in traditional dishes for rural people (Sanchez et al., 2010). Furthermore, tender young baobab leaves in particular are used as vegetables; they can also be dried and cooked later as they are a good source of vitamin A and calcium (SCUC, 2006).

The economic value of the baobab is derived not only from its value as a food source, but also as an important raw material for a variety of uses. The seeds are crushed to extract oil that is used as an ingredient in the international cosmetic industry (Venter and Witkowski 2013a) and are burnt to ashes for use as soap. Empty seed pods are curved to make cups, fishing floats, and snuff boxes (Pakenham, 2004). Further, the pulp in the fruits contains sterols, saponins, and triterpenes that are used medicinally due to their pain killing (analgesic) and temperature reducing (antipyretic) effects (Pakenham, 2004; SCUC, 2006). The baobab bark is used for fibre to make ropes, fishing lines, nets, bark clothes, baskets and strong harnessing ropes (Pakenham, 2004). All of these products that are obtained from the baobab tree contribute to income and help to alleviate poverty, improve livelihoods and allows participation of marginalized people in a growing cash economy (SCUC, 2006; Venter and Witkowski, 2013a). In addition to industrial uses, huge, hollow African baobab trees have been used for other purposes, such as providing shelter, storage of water, as well as prisons or burial sites. Some are used as religious meeting places, stables, storage rooms, watchtowers, and as restaurants or pubs (Pakenham, 2004; SCUC, 2006; Pettigrew et al., 2012).

Given that baobabs are important for the livelihoods of African people (Sidibe and Williams, 2002; Venter and Witkowski, 2011), many studies have focused on this iconic tree. One particular area of interest is the dramatic difference in fruit production observed between individual trees. This difference has been observed by local people in Benin who

use baobab products, and as a result of this difference, they viewed trees that produce fruits in very low numbers as ‘male’ trees, and high fruit producing trees as ‘females’ (Assogbadjo et al., 2008). This pattern is also evident in South Africa in the Venda region, where poorly fruiting trees were named ‘poor producers’ and those producing many fruits ‘producers’ (Venter and Witkowski 2011). Despite a number of studies on variation in fruit production across many tree species, the causes behind these large differences observed among baobabs remain unresolved.

There are many factors that may cause variation in fruit production. Some of the factors suggested to be causing variation in fruit production include adverse conditions such as high or low temperature and low water availability, poor soil fertility, soil salinity and unfavourable soil pH (Stephenson, 1981; Botelle et al., 2002), predation and damage (Dhillion and Gustad, 2004; Venter and Witkowski, 2010). Additionally, variation in fruit production may be caused by limited activities of pollinator agents (Zimmerman and Aide, 1989).

In poor producer and producer baobab trees, causes of these clear differences in fruit yield remain unclear. Presumably, the observed huge difference in fruit production could be linked to the new species recently identified described by Pettigrew et al. (2012), viz., *Adansonia kilima*, using mainly morphological features (floral, pollen, and stomatal size and density) to describe this second mainland African baobab, *A. kilima*. This new species is noted to be diploid (having two sets of chromosomes) as compared to the widely spread tetraploid (four sets of chromosomes) *A. digitata*. Polyploidy is known to cause cell size increase (Stebbins, 1971) due to increased DNA content subsequently affecting morphology. Increased DNA content could be one of the reasons why there is a stark difference in fruit production between poor producer and producer baobabs. Further, mating between diploid *A. kilima* and tetraploid *A. digitata* could contribute to differences in fruit production among individuals. If mating occurs between diploid and tetraploid baobab trees, the offspring may be infertile triploids, due to unbalanced gametes (Ramsey and Schemske, 2002); this may be causing the differences in fruit production observed. This study aimed to investigate the causes of the large difference in fruit production between poor producer and producer trees, and specifically, to test if fruit production was linked to difference in ploidy-level. The study also aimed to examine and compare morphological features (stomatal density and size) of the poor producer and producer trees. Given that

Pettigrew et al. (2012) found differences in stomatal size and density between *A. digitata* and *A. kilima*, I tested whether stomatal size and density differed between producers and poor producers and whether this corresponds to a difference in ploidy. Another aim was to examine gene flow between the poor producer and producer trees using nine microsatellite loci from a sample of 30 individual trees across four populations in Venda, South Africa and one population from Mozambique.

2.1.2 Objectives of the study

1) To quantify stomatal density and measure stomatal size on the abaxial surface of baobab leaves and correlate these with any differences in ploidy.
2) To use flow cytometry to determine if there is variation in ploidy-level among the mainland African baobab trees in three populations in Venda, South Africa and one population from Mozambique and to correlate any differences with leaf morphology, notably stomatal features.
3) To examine gene flow between producers and poor producers using microsatellite loci.

2.1.3 Questions

1) Is stomatal density and size linked to a difference in ploidy-level? And does this match the differences reported by Pettigrew et al. (2012) between *A. digitata* and *A.kilima*?
2) Is there difference in ploidy-level between poor producer and producer trees in Venda, South Africa?
3) Is a difference in ploidy-level correlated with baobab fruit trees being poor producers or producers?
4) Is there gene flow between producer and poor producer baobab trees in the Venda region of South Africa?

2.2 Materials and methods

2.2.1 Study species

The genus *Adansonia* of subfamily Bombacoideae in the Malvaceae has eight species (Baum and Oginuma, 1994; Wickens and Lowe, 2008). All species are endemic to specific regions, *A. digitata*, is thought to be the only mainland African species that occupies the drier parts of the African continent, and *A. gregorii* F. Muell., is confined to western Australia. The other six species are endemic to Madagascar (Wickens and Lowe, 2008; Pettigrew et al., 2012; Gebauer and Luedeling, 2013). *Adansonia digitata* is the only species that is tetraploid, unlike the diploid species found in Madagascar and Australia (Wickens and Lowe, 2008; Pettigrew et al., 2012). Recent work by Pettigrew et al. (2012) suggests the presence of a new diploid species (*Adansonia kilima*), the type of which is in southern Africa – near Tshirolwe, in Venda, South Africa. Pettigrew et al. (2012 reported that *A. kilima* also grows in east Africa (e.g. on the eastern slopes of Mt. Kilimanjaro to southern Tanzania) as well as westwards to northern Namibia at altitudes between 650–1500 m.a.s.l., in contrast to the widespread *A. digitata* usually growing below 800 m.a.s.l. Surprisingly, this potentially new species went unnoticed despite many years of research on the genus *Adansonia* (Pettigrew et al., 2012).

In this project, I focused on the mainland African baobab tree (*Adansonia digitata*). The African baobab is a deciduous tree, shedding leaves mostly in the winter dry season and bearing leaves in summer (Wickens and Lowe, 2008). Baobab trees seldom exceed a height of 25 m. The cylindrical trunk gives rise to thick tapering branches resembling a root system, which is why it has often been referred to as the 'upside-down tree' (Gebauer and Luedeling, 2013). Baobab trees can be very long lived and previous age estimates suggest that the oldest baobab trees are over 2000 years old (Wickens, 1982). Interestingly, baobab seedling establishment in northern Venda has been episodic, possibly only occurring every 100−150 years (Venter and Witkowski, 2013b). Additionally, flowering of baobab trees is said to occur just before or at the start of the rainy season, and the age at which trees start producing fruits has been reported to vary across Africa (Wickens, 1982). In West Africa reports suggests that baobabs starts to flower and produce fruits at 8−10 years. Reports in South Africa suggests that trees cultivated at Messina started flowering when they were 16−17 years old, while in Zimbabwe first flowering of some baobabs has been reported to be 22−23 years (Wickens, 1982). This may be a reflection of different climatic regimes, and environmental factors affecting the baobabs.

In general baobab trees are restricted to hot, dry woodland on stony, deep well drained soils, in frost-free areas that receive low rainfall, and densities are very variable in the landscape. Baobab density in general is higher in cropland than in fallows or grazing land, as seedlings are more protected from fire and grazing in these areas (Dhillion and Gustad 2004; Venter and Witkowski, 2010). They are probably affected by a number of factors, such as competition for water (linked to the baobab's extensive root system), soil requirements, seed dispersal, predation, and human settlements (Wickens, 1982; Sidibé and Williams 2002; Wickens and Lowe 2008). In South Africa baobabs are found to be conspicuous constituents of the hot and dry Limpopo Province, mainly in the frost-free sandy areas to the north of the Zoutpansberg mountains and the Olifants River in the east. A few stragglers grow further south; some grow about 80 km to the south of the Olifants River, and a few in the Waterberg and in the Rustenberg district (Wickens, 1982; Wickens and Lowe, 2008).

2.2.2 Study Area

Young leaf samples were collected on 26 February 2013 from 26 individuals in the plains, rocky areas, fields, and villages of the Venda region in Limpopo province. Individuals sampled in this study are the same trees sampled in a study by Venter and Witkowski (2011). They were known as either 'producers' or 'poor producers' based on a study conducted by Venter and Witkowski (2011) that showed a large difference in fruit production in Venda with poor producer trees being adult trees producing less than five fruits per year or nothing at all, whereas producer trees consistently produced more than five fruits each year. Samples were also collected from three individuals from the Mozambican Islands, Quilalea and Senco, on 11 March 2013. Leaf samples were immediately placed in filter paper in resealable plastic bags with silica gel to rapidly dry the leaves and preserve the DNA. The sample collection was done based on the location these trees were found around the Venda villages, and this aided in naming these trees. Poor producer and producer trees were found growing mixed in the same area in all different locations. The locations were named A (most western locality near Muswodi village), B (most northern locality near Tshipise villages), C (most eastern locality near Tshikuyu village) and Q (Mozambican). The distance between villages A and B was approximately 25 km, and about 40 between villages B and C, with about 65 km between villages A and C. Included in the sampling was the type of *A. kilima* near Tshirolwe in Venda.

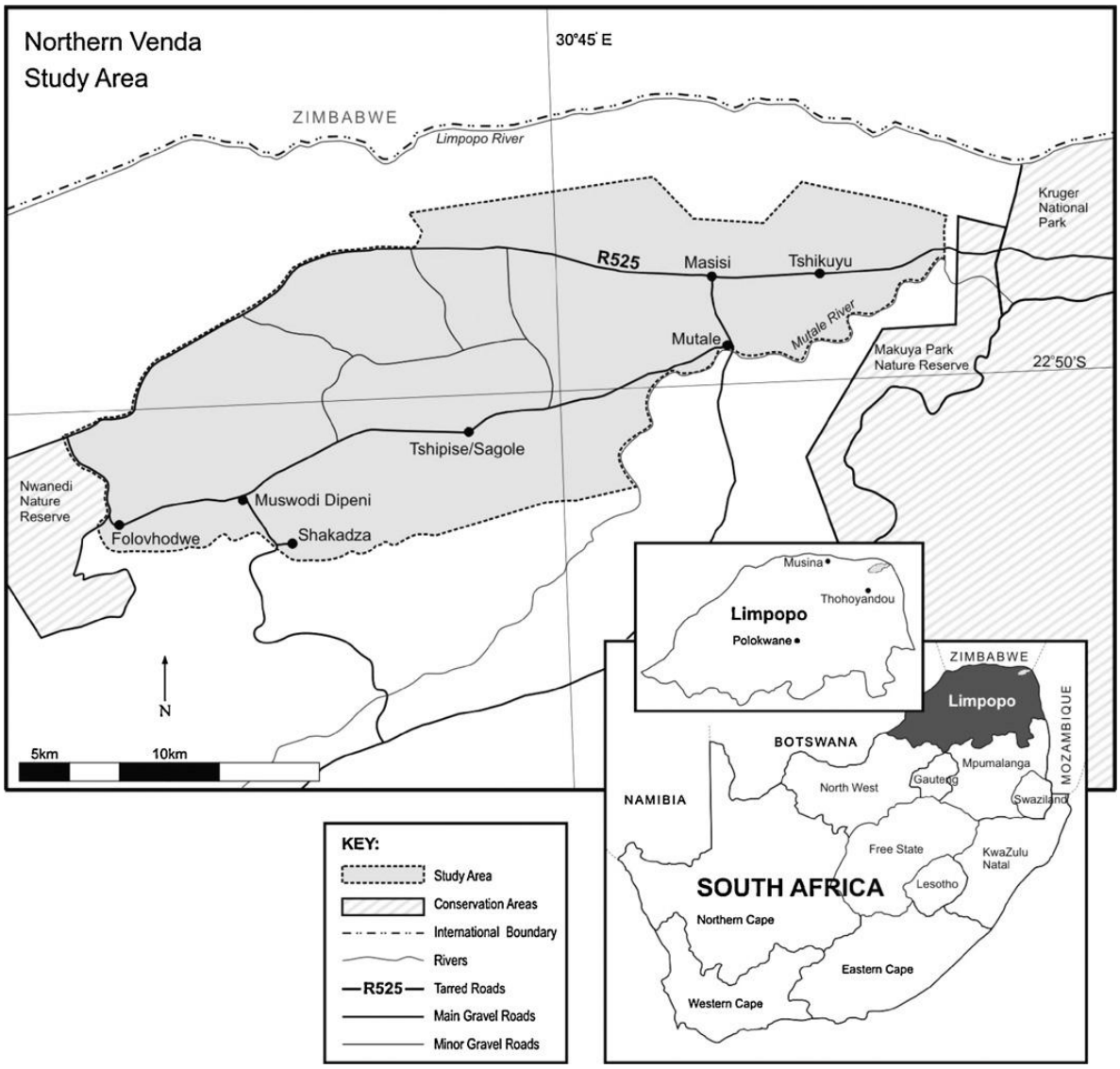


Figure 2.1. Map showing the study area in northern Venda, in South Africa (Map from Venter and Witkowski, 2010).

2.2.3 Stomatal analysis

To measure stomatal density and size, clear fingernail polish was used to create an impression of the abaxial surface of the leaf epidermis. The clear fingernail polish was applied on the abaxial epidermis of the selected leaf following methods outlined by Saltonstall et al. (2007). Once the clear nail polish had dried on the leaf surface, the dried layer was peeled off by firmly pressing sellotape at its edge, then carefully pulling it off. This peeled layer was then placed on a glass microscope slide, pressed flat using a cover slip, and observed using a light microscope (Olympus BH-2). Stomatal counts were recorded for three random fields of view per peel at 200X magnification. A systematic

approach to counting was done by observing a particular field of view by first counting from the top left side going down to the bottom, then taking a slight right turn, then counting going upwards, at the top end a right turn was taken again then counting proceeding going downwards. By so doing, all stomata in a single field of view were counted. A haemocytometer was then used in recount stomata to verify the initial counts. The grids on the haemocytometer allowed demarcation of a particular field of view.

The microscope field of view for a 200X magnification was found with the following formula:

$$\text{Field of View} = \pi r^2$$

$$= \frac{22\ (0.8\ \text{mm})^2}{7}$$

$$\text{Field of View area} = 2.01\ \text{mm}^2$$

Therefore, each field of view measured 2.01 mm^2, and counts were made for three separate fields of view within one leaf peel. An average was then calculated for the three fields of view to give an average number of stomata per 2.01 mm^2. The mean values of stomatal density were compared between producer and poor producers using the independent sample Welch t-test in statistical package R 2.12.1 version (R Development Core Team, 2010).

2.2.4 Guard cell size

The same peels of dried impressions used to count stomatal density were also used for guard cell size measurements. Measurements were done using the Nikon Imaging Software elements D3.1 (NIS-elements linked to a Zeiss compound microscope, Axio Imager M2). This software enables image capture, object measurement, and counting of objects on a screen from a microscope (Figure 1). First, calibration was done using a 2 mm micrometer that was placed under the microscope. A measurement of 0.1 mm was done on the micrometer using the NIS-elements and calibrated to measure in microns (1 mm = 1000 µm). The 0.1 mm was calibrated by equating it to 100 µm. After calibration, the dried peels were individually put under a microscope at 200X magnification. Thirty stomata were randomly selected to measure length (L) and width (W). The area of the stomata was calculated using the formula of an ellipse, which best represents the shape of the guard cells: Area = 0.5 π (L x W). The independent Welch t-test was also used to compare differences in the mean guard cell lengths between producers and poor producers. The

mean area of guard cells was also calculated and differences were compared between producers and poor producers using the Welch two sample t-test.

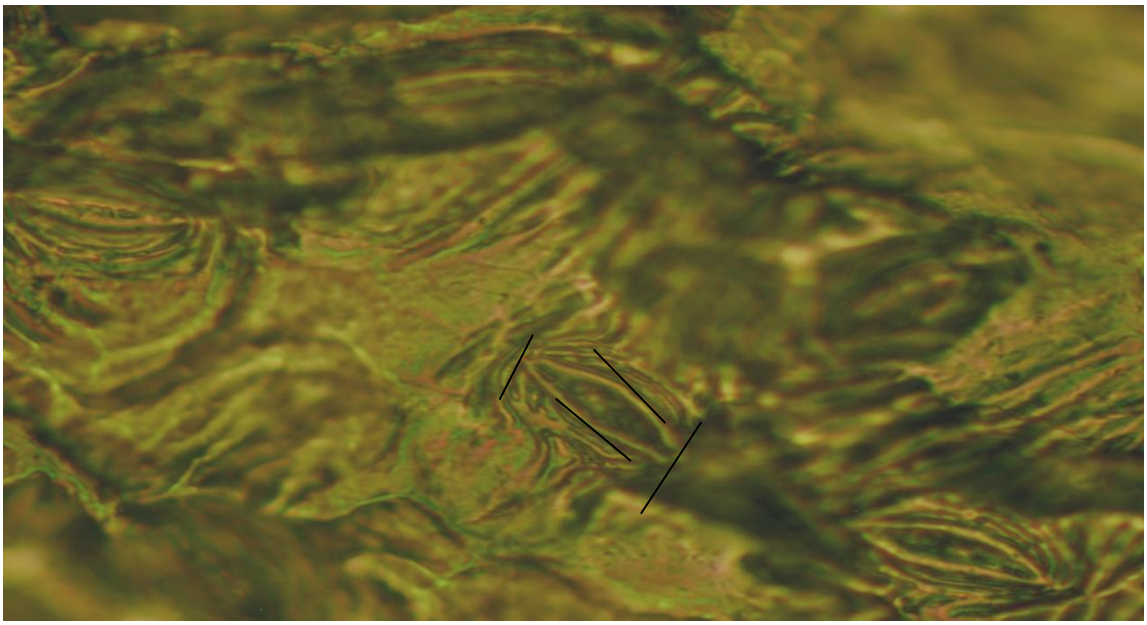


Figure 2.2. A baobab leaf stomatal opening, including guard cells surrounding it; photographed using the Nikon Imaging Software elements connected to an Olympus light microscope at 200X magnification (Photo: R. Tivakudze).

2.2.5 Gene flow analyses

DNA was extracted using a Qiagen DNEasy Plant Mini Kit following manufacturer's instructions with minor modifications; the volume of the buffers, AP1 and P3, was increased from 400 µl to 800 µl and from 130 µl to 260 µl, respectively. Previously published microsatellite primers for *Adansonia digitata* (Larsen et al., 2009) were used to amplify microsatellites to estimate gene flow between producers and poor producers. Optimum polymerase chain reaction (PCR) conditions were set for nine polymorphic markers (Table 2.1) to produce amplification products following Larsen et al. (2009). PCR reactions consisted of a 10 µl final volume; 1.5 µl of DNA template, 2 µl of nuclease free water, 0.5 µl of Bovine Serum Albumin (BSA), 0.5 µl each of 10 µM forward and reverse primers, and 5 µl Phusion Master Mix (Thermo Scientific; Inqaba Biotech, Pretoria, South Africa). The thermo cycler conditions followed instructions supplied with the Phusion Master Mix but annealing conditions followed Larsen et al. (2009). The PCR conditions were as follows: an initial denaturation step at 98 ^{o}C for 10 s, followed by 30 cycles of denaturation cycles for 10 s at 98 ^{o}C, annealing at 58 ^{o}C for 5 s and extension step at 72 ^{o}C

for 15 s, and final extension at 72 ^{o}C for 1 min, the reactions were held at 20 ^{o}C. The PCR products were then visualized on 1% agarose gels stained with SYBRSafe (BIO-RAD). After verification of the presence of a band within the correct size range, successful PCR products were multiplexed and sent to the Central Analytical Facility (CAF) at Stellenbosch University for analysis on an ABI 3130.

Table 2.1. Nine microsatellite loci used for *Adansonia digitata* with their base pair size ranges, and forward and reverse primers (Larsen et al., 2009).

			Primer sequences 5´−3´	
Locus name	**Size range**	**Motif**	**F**	**R**
Ad01	94−124	(AG)	CATTGCCAGGAATGCTTTTGC	GGATTGCCAGGTCTACTAC
Ad02	262−298	(TC)	TGCTGACTAGCAGTTTCCTATG	TCAGATGCCAAACATTCACACC
Ad04	176−224	(CT)	GTTGCTTGTGTGCTTACCC	CATCCCTCTCCCCATTCC
Ad08	265−301	(GAA)	TCTAAAGCCTGTAAGGAAAAATGGG	TTCTCCGTTCACTCTGTACTTCC
Ad09	181−211	(AAG)	TACCACTTCTCCAGATGCTAC	ACTGGCTAGAGATGCGTTG
Ad12	159−187	(AG)	GCTTGTCAAGCAATTCCCC	ACTTTGTCCCACCTGTTTCTC
Ad14	169−187	(AC)	CTTGATTGGAATACGGGAAATGGAG	CCAAACCAATTGGACTTTGACCTTC
Ad17	177−201	(AC)	GCGCCTTAGAAAGGACTTGTTAGAG	GCCAACAGCCTTAGTAGTCCAAG
Ad18	251−271	(TG)	ACCGCTTCCGTTCTCATTCC	ACCACCACTACACCGTCATTG

Microsatellites were visualized and recorded using PeakScanner v1 (Applied Biosystems, www.appliedbiosystems.com). PeakScanner was used to determine the size of the alleles found in each sample for the selected microsatellite locus. I calculated allele frequency, heterozygosity, inbreeding coefficients, and kinship coefficients between poor producers and producers using SpaGeDi (Hardy and Vekemans, 2002). Gene flow estimated between two subpopulations (producers vs. poor producers). In addition, individual trees were further divided into four groups based on the geographic location of the three different populations of the baobab trees in Venda and one population in Mozambique. All baobabs sampled were trees with 8 years of fruit production records (Venter and Witkowski, 2011), and these sampled trees were found growing among other baobabs not sampled for this study. The groups A, B, C comprised trees from Venda were grouped together and the Mozambican trees (Q) were separate. The grouping was done because the trees in each group were found in the same locality so grouping A, B, C, and Q together helped to analyse gene flow among trees. Nonetheless, gene flow between the three Venda populations may be possible as they are in relatively close proximity compared with the Mozambican population.

2.2.6 Flow cytometry for ploidy-level analyses

Flow cytometry was used to determine the relative DNA content for both producer and poor producer trees. A flow cytometer enables visualization and quantification of moving particles in a suspension (Johnston et al., 1999). The flow cytometer then converts the fluorescence signal obtained from the stained particles into a graph. All cells containing the same relative DNA content contribute to the same peak on the graph. Given the differences in fruit production and the presence of a potential new diploid species, I expected that poor producer and producer baobab trees would have different genome sizes. Fresh young baobab leaves were collected on 26 and 27 October 2013 in Venda for ploidy analysis (Figure 2.2). In the lab, these fresh leaves were weighed together with a standard, *Zea mays*, to obtain a combined mass of 0.05 mg. Both tissues were co-chopped using new razor blades in a petri dish and stained with 500 µl of DAPI One-step CyStain kit (Partec, Inc., USA) following the manufacturer's instruction to release nuclei. After chopping the sample for 45−50 s, 500 µl of DAPI stain was added and the chopped tissue was incubated for 2 min in the dark on ice to allow DNA staining to take place. After incubation, the mixture was filtered through a 30 µm mesh filter. Filtration was done to eliminate debris, such as the vacuole, cytoplasm and other soluble substances found in the plant cells,

obtained through the rough chopping of the plant tissues. The filtrate was then centrifuged at maximum speed for 30 s. The supernatant was discarded, and the DNA was resuspended using 1000 µl of DAPI stain. This solution was run through a Fortessa flow cytometer at the University of the Witwatersrand, Johannesburg Medical School.

These procedures were then repeated in November on a flow cytometer at Stellenbosch University Central Analytical Facility (CAF) to confirm relative DNA content. At Stellenbosch CAF a hybrid plum tree cultivar 'Marianne' (a hybrid of *Prunus munsonian* and *P. cerasifera*) was used as a standard. A Two-step CyStain kit (Partec, Inc., USA) was used, with an initial addition of 500 µl of lyse buffer followed by 80 µl of DAPI stain, and the other steps were similar to the single step CyStain kit described above. The DNA C-value (amount of DNA in picograms) of Marianne was not known and the values for the two parent species, *Prunus munsonian* and *Prunus cerasifera*, were obtained from Kew Royal Botanic Gardens DNA C-values data base (http://data.kew.org/cvalues). Although values were not available for the two parents, a literature search suggested that *P. cerasifera* was synonymous to *P. domestica* (2C DNA content = 0.66 pg; Loureiro et al., 2007) and that the other parent, *P. munsonian* was closely related to *P. angustifolia* (2C DNA content = 0.61 pg) and were found within a polytomy of the same clade (Baird et al., 1994; Shaw and Small, 2005). I therefore estimated the Marianne genome size by averaging the genome sizes of the close relatives (0.66 pg and 0.61 pg, respectively) of Marianne parents. The average estimated genome size (0.635 pg) was used as the standard in the equation below. Mean genome sizes (picograms) and standard errors for all samples were calculated using the following equation from Saltonstall et al. (2005):
Genome size = (Mean position of baobab peak/mean position of Marianne peak) X 0.63 pg

2.2.7 Statistical analyses

Mean stomatal density was compared between poor producer and producer trees using the independent sample Welch t-test in R version 2.12.1 (R Development Core Team, 2010). The same test was performed on the stomatal density obtained from the haemocytometer counts. Mean guard cell length and width were calculated for 30 randomly selected stomata per leaf sample. Guard cell area was also calculated for each stoma using the formula as mentioned above, obtaining area for the 30 randomly selected stomata. Mean guard cell area of the poor producer and producer trees were compared using the independent sample Welch t-test in R 2.12.1 version (R Development Core Team, 2010). The guard cell length

and width were also compared using the same t-tests to test if there were differences between poor producer and producer trees. A nested ANOVA was also conducted to determine if there were differences between individual trees and the two groups (poor producer and producer trees).

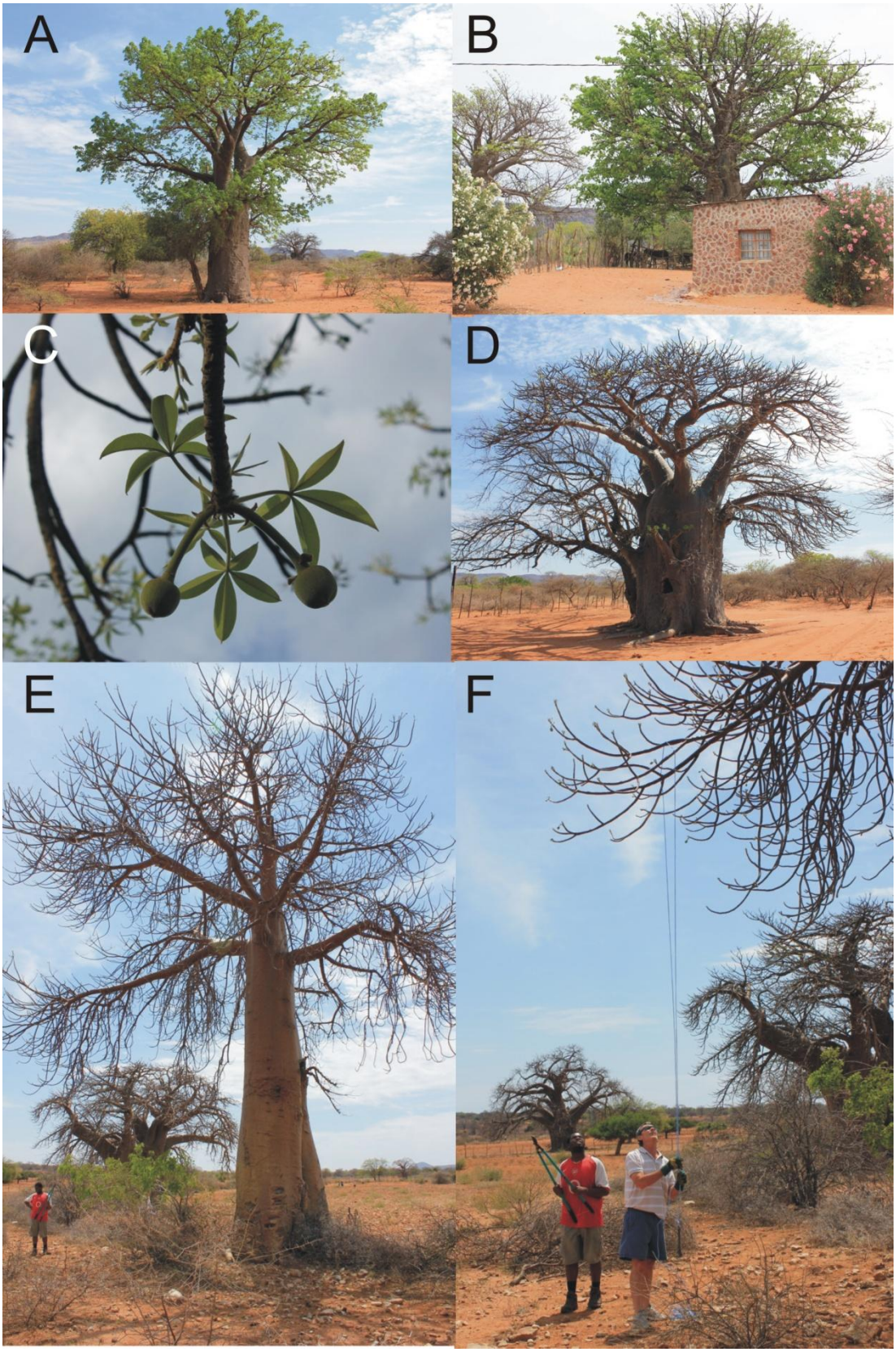


Figure 2.3. Baobab trees in Venda forming part of the sampled population; A, B, D, E, and F are trees in population A (in and around Muswodi village); C, Leaves and flower buds of the type of *A. kilima* (found near Tshirolwe village). Photographs: G. Goodman-Cron.

Chapter 3.0

3.1 Results

3.1.1 Stomatal density and size

The independent Welch t-test was used to compare the stomatal size and density between poor producer and producer trees. Stomatal density did not significantly differ between poor producer and producer trees (t = 1.4642, df = 24.66, *P* = 0.1558; Figure 3.1). Similarly, the length of the stomata was not significantly different between the poor producer and producer trees (t = −0.2713, df = 25.06, *P* = 0.7884; Figure 3.2). Finally, no significant difference was found in stomatal area between poor producer and producer trees (t = 1.2264, df = 25.214, *P* = 0.2314; Figure 3.3).

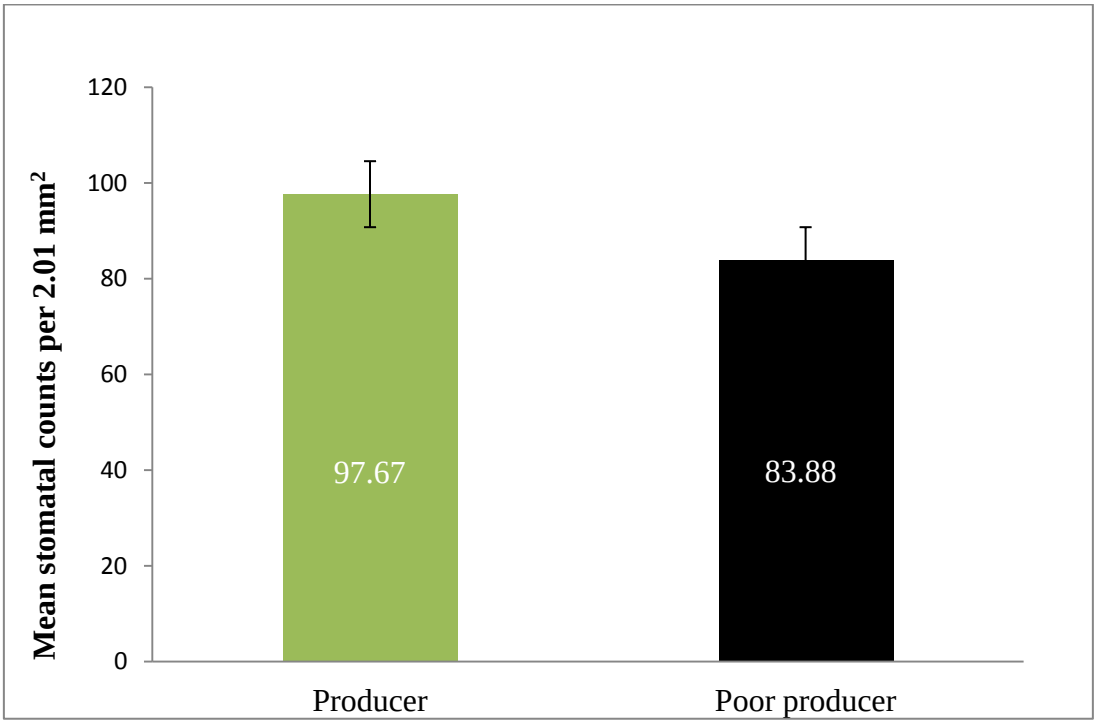


Figure 3.1. Comparison of mean (± S.D) stomatal counts between poor producer (N = 14) and producer (N = 14) baobab fruit trees showed no significant differences (*P* = 0.16, α ≤ 0.05).

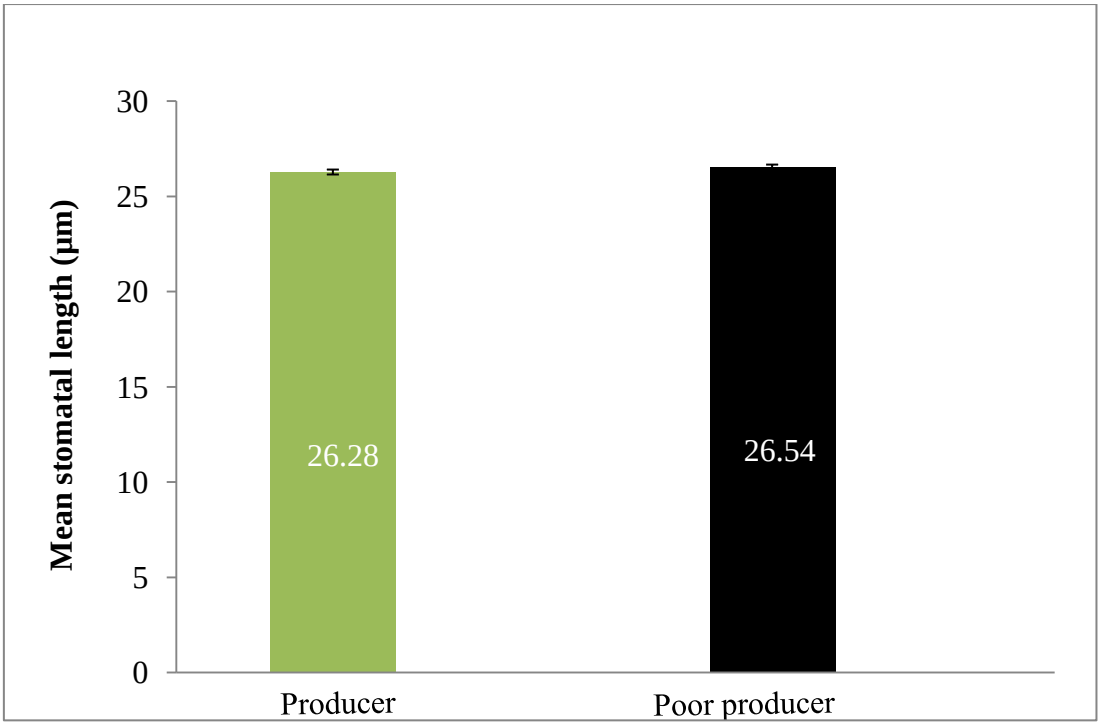


Figure 3.2. Comparison of mean (± S.D) stomatal length between poor producer (N = 14) and producer (N = 14) baobab fruit trees showed no significant difference ($P = 0.79$, $\alpha \leq 0.05$).

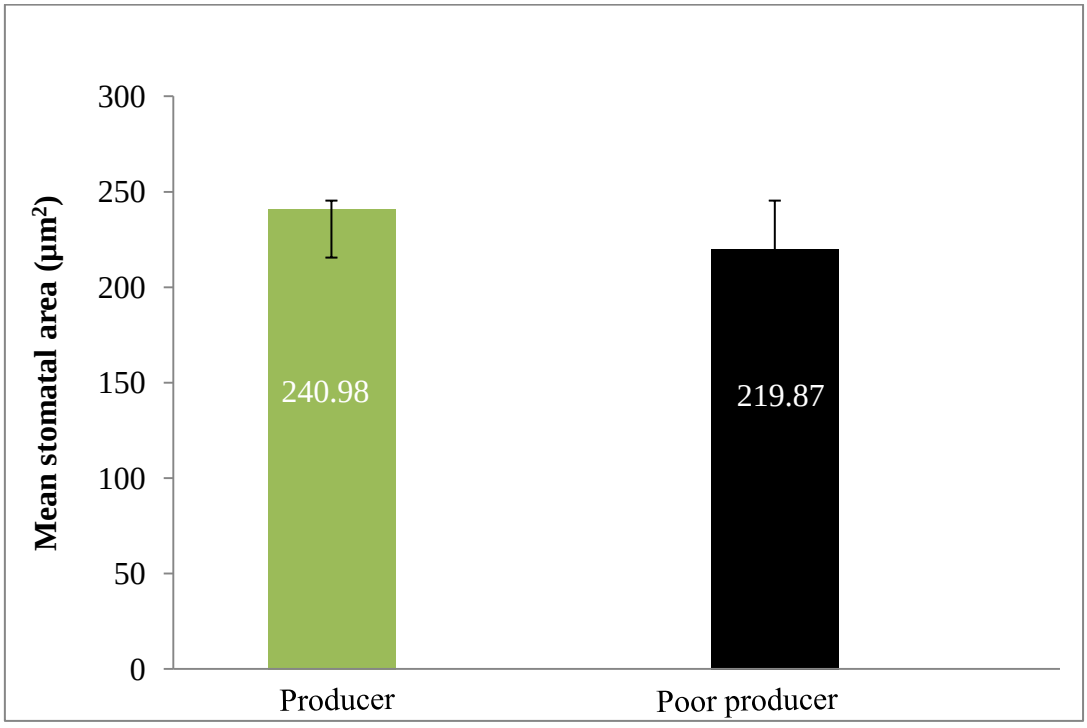


Figure 3.3. Comparison of mean (± S.D) stomatal area between poor producer (N = 14) and producer (N = 14) baobab fruit trees showed no significant differences ($P = 0.23$, $\alpha \leq 0.05$).

3.1.2 Nested ANOVA analyses

Results from the nested ANOVA did not suggest differences in stomatal density that were calculated for each individual sample nested within the producer group or the poor producer group. Stomatal density was not significantly different between the poor producer and producer trees (F = 2.14, $P = 0.55$; Table 3.12 in Appendix). However, stomatal density was significantly different among the individual samples (F = 21.48 $P < 0.01$; Table 3.12 in Appendix). Results from a nested ANOVA analysis for stomatal size showed no significant differences in stomatal length between poor producer and producer trees (F = 0.074, $P = 0.78$; Table 3.13 in Appendix), but stomatal length was significantly different among individual samples (F = 17.70, $P < 0.01$; Table 3.13). Stomatal area among poor producer and producer trees was not significantly different (F = 1.50, $P = 0.23$; Table 3.14 in Appendix), but stomatal area was significantly different among individual samples (F = 22.51, $P < 0.01$; Table 3.14 in Appendix).

3.1.3 Gene flow analyses

Total number of private alleles, and allele size (bp) varied among the populations across all nine loci (Table 3.5). The number of private alleles appeared to vary between poor producer and producer trees (average 10.33 alleles for poor producer trees vs. 11.67 alleles for producer trees; Table 3.5). The most and the fewest private alleles were scored at locus Ad04 (20) and locus Ad18 (8), respectively (Table 3.5). Allele sizes across loci ranged from 94 to 301 base pairs (bp).

Table 3.5. Summary of the private alleles found per locus between the producer and poor producer baobab trees.

	Number of private alleles		
Loci	**Producer**	**Poor producer**	**All**
All (average)	11.67	10.33	12.67
Ad01	15	11	16
Ad02	12	13	14
Ad04	17	17	20
Ad08	11	9	11
Ad09	9	9	9
Ad12	12	10	14
Ad14	8	7	9
Ad17	13	10	13
Ad18	8	7	8

The average number of private alleles across all four populations was 12.67 (Table 3.6). Generally, alleles were shared between trees from populations A, B, and C. Trees from population Q (Mozambique) did not share as many alleles with trees in locations A, B, and C. Trees in location Q (Mozambique) showed low gene flow between trees relative to those in locations A, B, and C. The average number of private alleles in trees from populations A, B, C, and Q was 8.78, 10.56, 8.0, and 4.78 respectively (Table 3.6).

Table 3.6. Summary of the private alleles per locus among the four populations, A, B, C, and Q (see text for details).

	Number of private alleles				
Loci	**A**	**B**	**C**	**Q**	**All**
All (average)	8.78	10.56	8.0	4.78	12.67
Ad01	9	14	8	5	17
Ad02	11	12	10	6	14
Ad04	12	15	9	6	19
Ad08	8	10	6	5	11
Ad09	7	8	7	3	9
Ad12	9	12	8	5	14
Ad14	7	6	7	3	9
Ad17	8	10	11	4	13
Ad18	8	8	6	6	8

To test if poor producers and producer baobab trees showed evidence of inbreeding, I calculated an inbreeding coefficient (F_I) for both poor producers and producers. Results among all the baobabs sampled suggest that the trees are outcrossing (mean $F_I = -0.154$; Table 3.7). Both the producer trees and poor producer trees are likely outcrossers (producer mean $F_I = -0.147$; poor producer mean $F_I = -0.167$; Table 3.7). The average heterozygosity (H_E; Nei, 1978) for all populations was high ($H_E = 0.856$; Table 3.7), indicating genetic diversity is high across the populations. Both the producer and poor producer groups showed high levels of heterozygosity ($H_E = 0.865$, $H_E = 0846$; Table 3.7).

Table 3.7. Summary of multilocus average expected heterozygosity (H_E), observed heterozygosity and inbreeding coefficient (F_I) for all samples and within the poor producer and producer baobab trees.

Multi locus average	H_E (expected heterozygosity, Nei, 1978)	Observed heterozygosity	F_I (individual inbreeding coefficient)
Producer	0.865	0.992	−0.147
Poor producer	0.846	0.987	−0.167
All populations	0.856	0.988	−0.154

The observed heterozygosity and inbreeding were also calculated for baobab individuals that were divided into the four populations recognised according to geography: A, B, C (Venda), and Q (Mozambique). Tree samples in all of the populations were found to be outcrossers (Table 3.8). The results showed that average heterozygosity (H_E; Nei 1978) for all populations was high (H_E = 0.857; Table 3.8).

Table 3.8. Summary of multilocus average expected heterozygosity (H_E), observed heterozygosity, and inbreeding coefficient (F_I) for all samples across the four geographical locations (see text for details).

Multi locus average	H_E (expected heterozygosity, Nei, 1978)	Observed heterozygosity	F_I (individual inbreeding coefficient)
A	0.849	0.989	−0.166
B	0.854	0.987	−0.156
C	0.868	0.999	−0.151
Q	0.794	1.009	−0.271
All populations	0.857	0.986	−0.15

In order to clearly understand the population differentiation among the poor producer and producer trees, Global F-statistics were used to incorporate three levels of population structure (within subpopulations (F_{IS}), among subpopulations (F_{ST}) and the individual differentiation within the population (F_{IT}). The average F_{ST} across all loci showed that there is little population differentiation between poor producer and producer trees (F_{ST} = 0.0018;

Table 3.9). The average F_{IS} for all loci demonstrated that the individual tree samples are out-crossing (F_{IS} = −0.1551), which corroborates inbreeding estimates (Table 3.9). Global F-statistics were also used to fully understand the population differentiation among the four geographic categories, A, B, C, and Q. The average F_{ST} across all loci is 0.0182, indicating that there is little population differentiation among the four locations. The average F_{IS} for all loci was −0.1652, demonstrating that the individual tree samples are out-breeding, which corroborates the F_I values (Table 3.10).

Table 3.9. Global F-statistics between poor producer and producer trees for individual loci and across all loci.

	Global F-statistics			
Locus	**F_{ST}**	**F_{IS}**	**F_{IT}**	**Pairwise D (Nei's 1978 standard distance)**
All (average)	0.0018	−0.1551	−0.153	−0.026
Ad01	0.0009	−0.1353	−0.1343	−0.0383
Ad02	0	−0.1334	−0.1334	−0.0408
Ad04	−0.0016	−0.0711	−0.0728	−0.0796
Ad08	−0.0028	−0.1264	−0.1295	−0.0561
Ad09	0.0224	−0.22	−0.1927	0.0625
Ad12	0.0048	−0.1541	−0.1486	−0.0073
Ad14	0.0027	−0.2696	−0.2661	−0.0248
Ad17	−0.0032	−0.1297	−0.1332	−0.0622
Ad18	−0.057	−0.1804	−0.1871	−0.0616

Table 3.10. Global F-statistics among the four geographic locations, A, B, C, and Q (see text for details).

	Global F-statistics			
Locus	**F_{ST}**	**F_{IS}**	**F_{IT}**	**Pairwise D (Nei's 1978 standard distance)**
All (average)	0.0182	−0.1652	−0.144	0.0908
Ad01	0.0234	−0.1528	−0.1258	0.1642
Ad02	0.018	−0.1439	−0.1233	0.0679
Ad04	0.0069	−0.0763	−0.0689	0.0337
Ad08	0.038	−0.1551	−0.1112	0.2909
Ad09	0.0272	−0.2244	−0.191	0.1602
Ad12	0.0143	−0.1671	−0.1504	0.0976
Ad14	0.0124	−0.2796	−0.2637	−0.0835
Ad17	0.0384	−0.1444	−0.1005	0.3351
Ad18	−0.0175	−0.165	−0.1854	−0.2538

3.1.4 Flow cytometry

Results obtained from the Fortessa flow cytometer at the University of the Witwatersrand Medical School were inconclusive. When a One-step CyStain kit was used, running the baobab stained DNA material alone, good output peaks were obtained (Figure 3.4). Similarly, when the standard was run alone in the Fortessa, it yielded good peaks with a defined position and size (Figure 3.5). However, when baobab DNA material was stained together with the standard (maize) the results showed unclear peaks, different from the ones obtained by baobab DNA alone, and maize DNA alone, making it difficult to distinguish the two peaks (Figure 3.6). Therefore, I was unable to calculate reliable estimates of DNA content using this approach. The samples analysed at Stellenbosch CAF provided clearer results using the *Prunus hybrid* cultivar Marianne as a standard and the Two-step CyStain kit. Although data obtained from the Stellenbosch CAF analyses suggest variation in genome size (DNA content) between poor producer and producer baobab trees, the genome size estimates obtained were also inconclusive. Estimation of relative DNA content of the unclear graphs obtained showed that the producer trees AP4, AP5 and AV1 may be diploids (0.35 pg, 0.44 pg and 0.47 pg respectively; Table 3.11), while some of the poor

producer (AP3 and BP4) and producer trees (CV5, AV3, AV4 and BF3; Table 3.11) may be tetraploid.

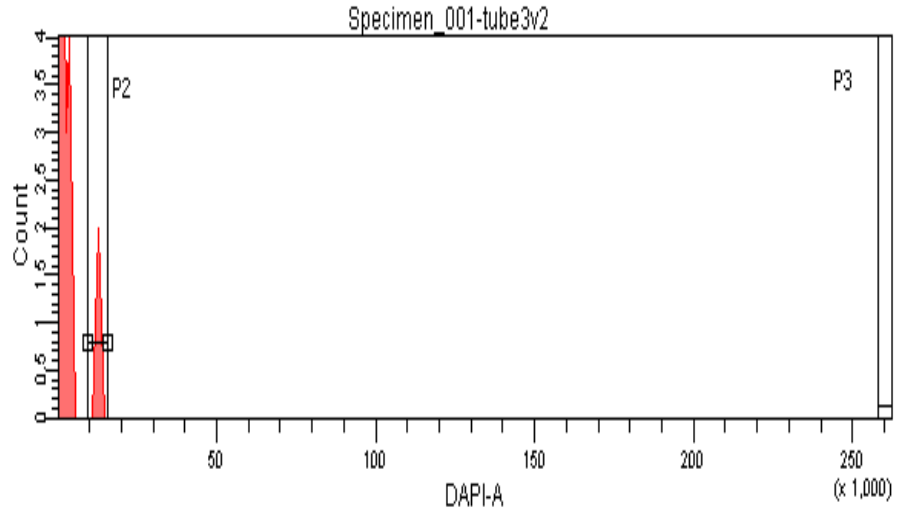


Figure 3.4. Flow cytometry analysis of relative fluorescence intensity of baobab nuclei alone.

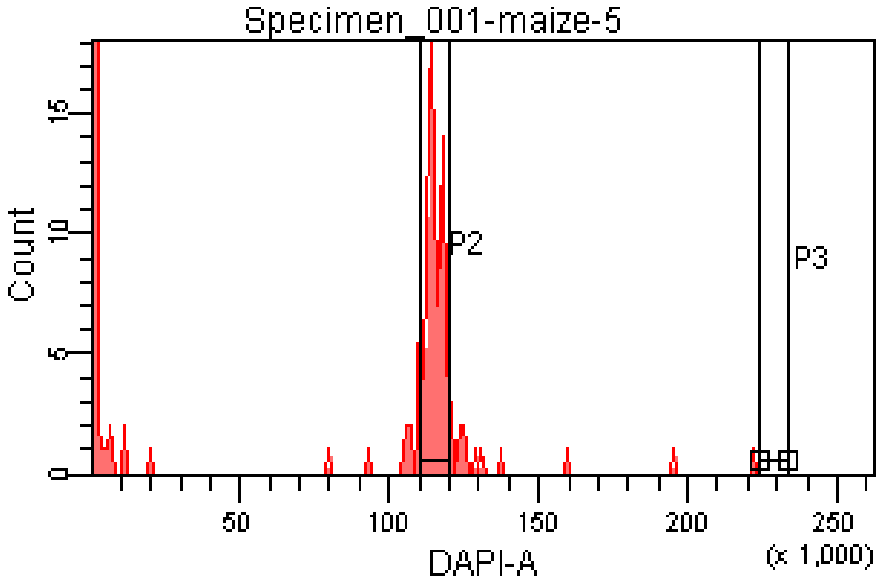


Figure 3.5. Flow cytometry analysis of relative fluorescence intensity of maize (standard) nuclei alone.

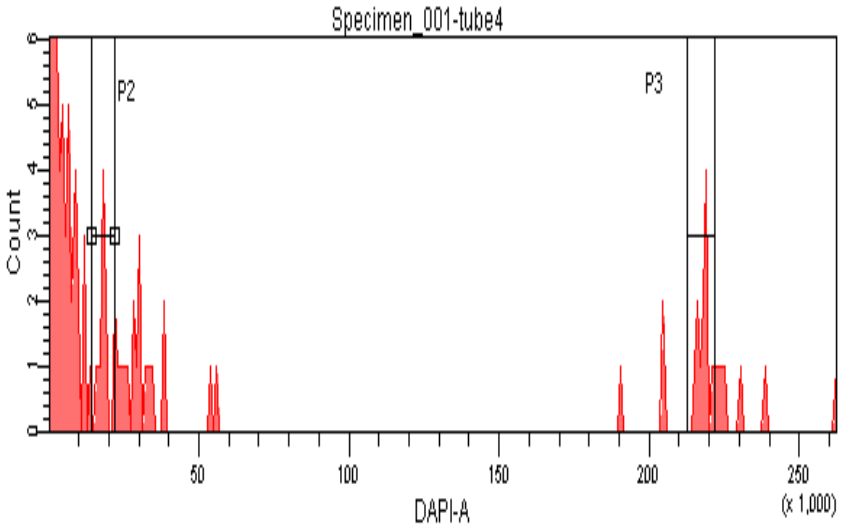


Figure 3.6. Flow cytometry analysis of relative fluorescence intensity of baobab (P2) and maize (P3) nuclei.

Table 3.11. Estimation of relative DNA content of baobab samples using flow cytometry. The *Prunus* hybrid cultivar 'Marianne' was used as the standard.

Fruiting history	Sample	Marianne Fluorescence Value	CV %	Baobab Fluorescence value	CV %	Putative Baobab ploidy estimates (pg)	
P	CV5	36.2	17	64.4	9.7	1.13	4x
PP	AP3	35.81	17.3	85.264	12.8	1.51	4x
P	AP4	61.022	9	34.09	12.7	0.35	2x
P	AP5	41.444	13	28.64	15.7	0.44	2x
P	AV1	41.145	13.1	30.741	15.2	0.47	2x
P	AV3	36.136	17.6	56.433	19.1	0.99	4x
P	AV4	42.465	12.7	69.063	22.5	1.03	4x
P	BF3	39.65	16.1	65.437	8.3	1.05	4x
PP	BP4	50.818	12.4	84.639	6.2	1.06	4x

P = producer, PP = poor producer, pg = pictograms, CV = coefficient of variation

Chapter 4.0

4.1 Discussion

4.1.1 Stomatal density and size

Polyploidy is known to influence the cell size of organisms and can affect reproductive function in plants. In addition, the number and density of stomata can change relative to ploidy-level. Diploid plants tend to possess leaves with greater stomatal densities and with stomatal apertures that are smaller in size than tetraploid plants (Stebbins, 1971). In baobab trees, Pettigrew et al. (2012) found that *A. kilima* (diploid) has significantly smaller stomatal apertures and higher stomatal densities than the tetraploid *A. digitata*. Tetraploid *A. digitata* individuals were found to have larger stomatal apertures and lower stomatal density. My study examined the stomatal density on the leaf surface, and the length and area of individual stomata to test for differences between poor producer and producer trees and to correlate the differences with ploidy-level. I also estimated the ploidy-level of the poor producer and producer trees using flow cytometry. I also examined gene flow between poor producer and producer trees, to test whether these trees are exchanging genes or if the differences in fruit production in these trees were due to inbreeding.

In this study, stomatal length and area were not significantly different between poor producer and producer trees (Figure 3.2; Figure 3.3). Poor producer and producer trees had mean stomatal lengths of 26.54 µm and 26.28 µm, respectively. Similarly, a mean stomatal length of 26.1 µm was obtained for the type of *A. kilima* (a diploid species; Pettigrew et al., 2012). Therefore, poor producer and producer baobab trees both have similar stomatal lengths to that of *A. kilima* as identified by Pettigrew et al. (2012), and poor producer and producer baobab trees in this study cannot be distinguished using stomatal length and size. Surprisingly, the results suggest that baobabs in Venda may all be diploid if only stomatal length is compared to findings of Pettigrew et al. (2012). However, it is more likely that stomatal density and stomatal size are not effective indicators of a difference in ploidy-level between poor producer and producer baobab trees. A similar study by Saltonstall et al. (2005) showed that stomatal density showed a significant relationship with subspecies and was useful in distinguishing between two subspecies of *Phragmites australis*, but DNA content was the same for both of the subspecies. They concluded that morphological features in *Phragmites australis* may not be accurate indicators of difference in ploidy-level. Therefore, the stomatal length and area in poor producer and producer baobabs may not be a true representative of whether the trees are diploid or tetraploid.

In addition, I found no significant difference in mean stomatal density between poor producer and producer trees. This suggests that poor producer and producer trees in Venda cannot be distinguished based on stomatal density. The similarity in mean stomatal density may be a result of these trees occurring in the same locality with similar environmental conditions. This is in accordance with Sanchez (2010) who, in a study on relationship between stomatal characteristics and drought adaptation in Benin and Malawi, found baobab leaves in Benin to have higher stomatal density but smaller guard cell length in high temperature and low rainfall areas. This relationship with environmental factors however was not consistently observed in baobabs in Malawi. Even though the mean stomatal density between poor producer and producer trees was not significantly different, it was difficult to compare the densities from this study and the one by Pettigrew et al. (2012). In this study, the abaxial surface of the leaflets was examined for stomatal length and density, whereas Pettigrew et al. (2012) reportedly studied the adaxial surface. No stomata were observed on the adaxial surface in this study when I examined the leaflets at 200X magnification. This is consistent with findings of Sidibe and Williams (2002), where stomata in baobabs were reported to occur only on the abaxial surface of the leaflets. However, Rao and Ramayya (1981) noted that stomata appear on both the abaxial and adaxial surfaces of the leaflets. Pettigrew et al. (2012) used a different microscope at 600X magnification, which may have aided observing stomata on the adaxial surface of the leaves. Sanchez (2010) observed (at 400 X magnification on a similar Olympus microscope) that stomata appear mainly on the abaxial surface but do occur on the adaxial surface of the medial leaflet where they are restricted to alongside the midvein. The stomata were noted to be absent from the adaxial lamina surface of the leaflets (Sanchez, 2010). It is possible that if the mid-vein area of leaflets of poor producer and producer trees had been viewed in this study at higher magnification, a few stomata might have been observed. However, leaflet impressions were easily peeled starting from the edge of the leaflets, whereas impressions from the mid-vein area of the leaflet were difficult to obtain for measurements in this study and were not viewed.

4.1.2 Gene flow

Inbreeding (mating of closely related organisms) may result in inbreeding depression if deleterious alleles are continuously passed in offspring. Inbreeding can also affect certain traits, such as germination rate, competitive ability, growth rate, pollen quantity, number of ovules, and amount of seed produced (Jain, 1976; Silvertown, 2001; Keller and Waller,

2002; Frankham et al., 2003). This study examined gene flow and inbreeding in poor producer and producer trees. I hypothesized that poor producer trees were more likely to be inbred relative to the producer trees. Further, I hypothesized that gene flow would be mainly occurring among producer trees and that poor producers would not contribute significantly to gene flow. However, results showed that both poor producer and producer trees have high heterozygosity (Table 3.7). This result suggests that both poor producer and producer trees outcross. Outcrossing is well-supported by the calculated inbreeding coefficients. Surprisingly, these results are not consistent with recent work on genetic differentiation and diversity carried out in Malawi. Munthali et al. (2013) found evidence for low genetic diversity among baobab populations in Malawi. However, genetic diversity, obtained from AFLPs of Benin baobab populations varies from high to low across the different climatic regions where the trees are found (Assogbadjo et al., 2009). In West Africa, spatial genetic structuring from AFLP data showed a high level of within-population genetic diversity (Kyndt et al., 2009). Collectively, these results may suggest that West African baobabs and Venda baobabs have different levels of gene flow and that population structuring may be more prevalent in East and West Africa relative to southern Africa.

Global F-statistics of population differentiation also suggested little population differentiation between producer and poor producer trees in Venda (average $F_{ST} = 0.0018$). The Global F-statistics values of less than 0.05 represent little population differentiation. Moderate population differentiation is shown when values are 0.05−0.15; values between 0.151−0.25 represent great differentiation and values above 0.25 represent very great differentiation (Conner and Hartl, 2004). These data also suggest that there is a high level of gene flow between poor producer and producer trees preventing differentiation. Little population differentiation suggests that there are many common alleles being shared between poor producer and producer trees, with few private alleles present. Again, southern African baobab populations differ from the genetic population in Malawi. For example, Malawian populations appear to be moderately genetically differentiated (Munthali et al., 2013). Compared to populations in Malawi, the poor producer and producer trees in Venda all show much higher levels of gene flow that is preventing population differentiation. Consequently, the baobabs of Venda appear to be a single, cohesive population, unlike baobab populations in other parts of Africa. This could be as a result of the Venda baobab

populations being more connected to each other, and the absence of major landscape features to really separate these populations.

The poor producer and producer baobab trees have shown high levels of gene flow and that they are more outbreeding with high heterozygosity, which is a healthy situation for this species. However, since it remains unclear what drives the difference in fruit production observed between poor producer and producer trees, further studies are required. Possible additional studies could include pollination studies, pollen viability, and self-incompatibility. Pollination studies, particularly controlled pollination trials, might help to test if the producer trees and poor producer trees contribute equally to gene flow and fruit production.

4.1.3 Flow cytometry and ploidy-level

Although estimates of DNA content suggest variation among the individual baobab trees tested, the estimates of DNA content were inconclusive. The peaks obtained were unable to distinguish between the baobab sample and the maize standard. However, the data do suggest that there is variation present, but it needs to be verified. The DNA estimates obtained showed that some producer trees may either be diploid or tetraploid, whereas the two poor producer trees are both tetraploid (Table 3.11). This seems to conflict with expectations that polyploidy results in increased vigour and increased productivity (Stebbins, 1971). In addition, these results appear to suggest that stomatal measurements do not always correlate with genome size estimates. Furthermore, fruit production does not appear to correlate with variation in DNA content either. However, earlier genomic size estimates of *A. digitata* using Feulgen microdensitometry (Fe) showed a DNA 2C-value of 7.7 pg (Bennet and Leitch, 1997). Relative DNA content of baobabs in the current study is very low with an average size of only 0.89 pg. The current study used flow cytometry, while Bennet and Leitch (1997) used Fe. This may be the reason why the genome sizes obtained are very different. On another note, difficulties faced in using flow cytometry to obtain good and reliable results can be attributed to secondary chemistry. Baobab leaves are known to contain toxicants such as hydrocyanic acid, oxalate, phytic acid, and tannins (Chadare et al., 2009). The possibility remains that these chemicals are reacting with the DAPI stain, thus preventing adequate staining of the nuclei for analysis. The staining protocol was altered several times in order to try and obtain clearer results. However, most alterations did not yield sufficient staining for subsequent analysis. Consequently, there is

need for an alternative method of DNA content determination or use of other staining protocols.

4.2 Conclusion

The results obtained in this study suggest that inbreeding and reduced gene flow did not cause the observed difference in fruit yield between poor producer and producer baobab trees in Venda. Furthermore, comparisons between stomatal measurements in this study and those of Pettigrew et al. (2012) suggest that either all baobabs in Venda are diploid, or that stomatal measurements are not a reliable measure of ploidy-level in baobabs. Future work should continue to assess ploidy-level using flow cytometry to better explore potential variation in genome size as a driver for the difference in fruit production. Due to the economic and nutritional value of baobab trees, producer trees remain the prime target for harvesting by local people.

To fully explore possible differences in ploidy-level, extensive sampling in southern Africa may be required, so that a much bigger area is covered. Moreover, seed germination followed by chromosome counts on root tips should be done to verify if there is any difference in ploidy-level among the baobab trees. In addition, more morphological features could be added to explore if they correlate with difference in ploidy-level, for example using features such as floral traits from the trees, or from voucher specimens collected; e.g. pollen grain diameter, stamen length and stalk diameter, maximum calyx diameter, and staminal corolla width could also be measured and the number of free staminal filaments could also be counted. Furthermore, hand pollinations could be carried out between poor fruit producers and producers to establish the viability of seeds from these crosses. Some studies can also be carried out to assess microsite environmental conditions including water availability in the soil as well as nutrient status of the soil. Therefore, it is clear that a number of factors, or even interactions exist that could cause the observed difference in fruit production, and further studies need to be carried out.

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Appendices

Table 3.12. Nested ANOVA of stomatal density comparing poor producer and producer baobab trees. No significant differences occurred between groups, but individual trees were significantly different.

Source of variation	D.F	SS	MS	F	P	Variance component in %
Between groups	1	3990.96	3990.96	2.144	0.155 ^{ns}	6.95
Among subgroups within groups	26	48402.40	1861,63	21.48	<0.01***	81.16
Within samples	56	4853.33	86.67			11.89
Total	83	57246.70				100.00

ns- not significant

*** Significant

Table 3.13. Nested ANOVA of stomatal length comparing poor producer and producer baobab trees. No significant differences occurred between groups, but individual trees were significantly different.

Source of variation	D.F	SS	MS	F	P	Variance component in %
Between groups	1	14.24	14.24	0.07	0.78 ^{ns}	0
Among subgroups within groups	26	5031.20	193.51	17.7	<0.01***	35.76
Within samples	812	8878.58	10.93			64.24
Total	839	13924.02				100

ns- not significant

*** Significant

Table 3.14. Nested ANOVA of stomatal area comparing poor producer and producer baobab trees. No significant differences occurred between groups, but individual trees were significantly different among the found.

Source of variation	**D.F**	**SS**	**MS**	**F**	**P**	**Variance component in %**
Between groups	1	93548.63	93548.63	1.50	0.231 ^{ns}	1.55
Among subgroups within groups	26	1617176.21	62199.08	22.51	<0.01***	41.11
Within samples	812	2243670.49	2763.14			57.34
Total	839	3954395.33				100.00

ns- not significant

*** Significant