Paternity, floral morphology and pollen viability analyses of African Baobab (*Adansonia digitata* L.) populations in South Africa

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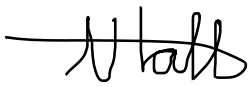
Declaration

I, Timothy Hall, declare that this dissertation, excluding the contributions mentioned in the acknowledgments, is my own, unaided work. This research is submitted to the Faculty of Science at the University of the Witwatersrand, Johannesburg in the fulfilment of a Master of Science and has not been previously submitted to any institution or degree for examination.



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Abstract

The African baobab (*Adansonia digitata* L.) is a valuable flagship, cultural and socio-economic species for communities across Africa. Not only is the baobab an important medicinal plant, but products such as seed oils and fruit pulp are sold to supplement rural community income. Baobab trees produce hermaphroditic flowers, but fruit numbers vary between individuals, with some trees producing many fruit (50 –200) every season, termed producers, and other trees producing little to no fruit every season (< 5), poor producers. This difference is not due to environmental conditions such as rainfall, soil conditions or land use type as tree types occur in the same environment, sometimes only meters apart. The aim of this study was to evaluate how floral reproductive traits and the movement of pollinators between *A. digitata* producer and poor producer trees may affect the paternity of seed sets from producer trees.

I measured seven floral traits of producers and poor producers to assess floral differences using one-way ANOVA between the tree types across three populations in the Vhembe region of South Africa. Floral traits included flower diameter, stamen ball diameter, filament length, anther length, stigma surface diameter, style length from stamen ball emergence and peduncle length. Producers had longer styles emerging from the stamen balls and longer peduncles, whereas poor producers had larger stamen balls, longer filaments, and larger anthers. There were no differences in flower diameter and stigma surface diameter between producer and poor producer trees. Similarly, canopy volume, width, and stem diameter revealed no difference between tree types. Additionally, I evaluated differences in pollen viability between producer and poor producer trees using Alexander's stain. Although both tree types possess viable pollen, poor producers had nearly 50% higher percentage viability compared to producers. There appears to be some level of functional dioecy across baobab individuals as we found that trees either allocate resources predominantly to either male or female floral structures. Assessing the movement of alleles as a measure of pollen movement between trees may aid in our understanding of how this functional dioecy is influencing allele movement within the population.

Eleven microsatellite loci were amplified to assess the paternity of 117 offspring from 13 'maternal' fruit-producing trees across three *A. digitata* subpopulations in Vhembe, South Africa.

The six nearest potentially paternal trees to each maternal tree were genotyped to test whether they were pollen donors. Parentage analyses conducted using POLYGENE revealed that offspring of maternal trees had alleles from multiple genotyped pollen donor trees outside of their six nearest trees with selected paternal trees contributing pollen to multiple maternal trees. Analyses suggested that pollen dispersed an average of 300 m within subpopulations, but also repeatedly dispersed distances over 40 km between subpopulations. The duration of pollen viability was determined through pollen tube germination trials and aids in our understanding of how long pollen remains viable for in the environment. Pollen grains that were 24, 48 and 72 hours old were deposited on receptive stigmas and allowed to germinate overnight. Scanning electron microscopy was used to confirm pollen tube growth for the three ages of pollen; it indicated that the pollen remains viable for at least 72 hours as it successfully germinated on stigmatic surfaces across these three treatments. This indicates that pollen present in the environment, especially on the surface of pollinators (e.g., moths, bats, or beetles) has the potential to pollinate flowers when up to 72 hours old, although pollen that is older than 72 hours may still be viable, which requires further investigation. The degree of functional dioecy in individual baobab trees, proportion of viable pollen, and extensive potential range of pollen dispersal, all contribute to our knowledge of *A. digitata* fruit production. Understanding baobab fruiting may aid in future conservation and regeneration strategies and would improve agroforestry security of the species.

Keywords: Agroforestry, Dispersal, Dioecy, Pollen movement, Tree morphology, *Malvaceae*, DBH, Pollination, Microsatellite, Hawkmoth, Parentage

Glossary: Terminology and abbreviations

AV/BR/BP/BF/BV/CV – Abbreviations denoting the land use types in subpopulations (see below) A, B and C; Rocky outcrop (BR), Plains/rangelands (BP), agricultural Fields (BF), and the Village (AV, BV, CV) subpopulations.

C.I. – Confidence Interval; primarily referring to the confidence levels from the paternity assignment.

CTAB – Cetyltrimethylammonium Bromide; Cationic detergent that is used to degrade polysaccharide membranes during DNA purification from plant tissue.

DBH – Diameter at Breast Height in meters (m).

Dioecy – The occurrence of staminate (male) flowers on one individual and pistillate (female) flowers on another individual of the same species.

Maternal individual – An individual *Adansonia digitata* tree that produces many fruit every season (producer; see below) from which seeds were collected for paternity analysis. All maternal trees are identified to be producer trees.

Paternal individual – A potential pollen donor to a maternal tree; with the six nearest trees to each maternal tree genotyped as potentially the likeliest pollen donors to maternal trees. Paternal donors may either be producers or poor producers (see below).

PCR – Polymerase Chain Reaction.

Poor producer – A tree that produces little to no fruits every fruiting season (less than five fruits annually) per Venter & Witkowski (2011, 2019).

Population – The grouping of subpopulations A, B or C as the sampled groups of baobab trees across the northern Vhembe region. These three subpopulations can interbreed and consist of collections of studied trees over multiple studies.

Producer – A tree that consistently produces many fruits every fruiting season (more than 5 fruits annually) per Venter & Witkowski (2011, 2019). A collection of producer trees in the Vhembe region are surrogate maternal trees for the paternity analysis.

Subpopulation – In this thesis, a subpopulation refers to three regions, A, B, and C, in the northern Vhembe region. Subpopulations A and C occur in villages whereas subpopulation B occurs over a variety of land use types (see above).

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1. Introduction

1.1 Rationale

The change in climate, rapid increase in human population, and land use change, have led to natural systems worldwide being subjected to increasing pressures such as overharvesting of plant and animal resources or the reduction of occupiable natural habitats through change in land surface and habitat fragmentation (Dale, 1997; Ehrlich & Holdren, 1971; Selwood et al., 2015). Southern Africa is expected to experience a decrease in mean annual rainfall and increase in mean annual temperature, which will likely strain natural environmental systems, including traditional indigenous agroforestry systems (Klopper et al., 2006; Neufeldt et al., 2013; Walker & Schulze, 2008). The African baobab, *Adansonia digitata* L. (Malvaceae; Baum, 1995a, Cron & Karimi et al., 2016), plays an important socio-economic role in traditional communities across Africa, particularly in the form of income generated from selling baobab products such as fruit, seed oil, tree fibres, etc. (Venter & Witkowski, 2011, 2013; Wickens, 2008). Long term data show fruit production discrepancies between *A. digitata* individuals within populations across Africa (Assogbadjo et al., 2005; Venter & Witkowski, 2011). Due to the extensive range of *A. digitata* across West, East and southern Africa, it occupies various climatic, environmental, and land-use conditions (Assogbadjo et al., 2005; Venter & Witkowski, 2011). Trees either produce many fruit seasonally (termed 'producers') or little to no fruit seasonally ('poor producers') and are locally referred to as 'female' and 'male' trees, respectively. Despite these differences in fruit production, producer and poor producer trees are visually similar and cannot be distinguished in the field during the non-fruiting season. Flowers are hermaphroditic and have functional 'male' and 'female' organs yet tree types seem to allocate resources differently to androecium and gynoecium structures (Chetty et al., 2021). *Adansonia digitata* is self-incompatible (Venter et al., 2017), meaning self-pollination does not result in ovum fertilization (Webb, 1994). The degree of self-incompatibility does not differ between producer and poor producer trees and the level of outcrossing and genetic diversity is similar between them, indicating that differences in fruit production are not due to selfing (Venter et al., 2017).

Chetty et al. (2021) suggested that a shift in sex allocation may be occurring within *A. digitata* populations, resulting in functional dioecy. Morphological differences between the flowers of producer trees and poor producer trees show functional sex differences that coincide with fruit production discrepancies. Producer trees generally tend to invest resources into the gynoecium (increased stigma receptivity and fruit production), whereas poor producer trees appear to invest resources into the androecium (high pollen count and viability). Considering the known self-incompatibility and possible functional dioecy in *A. digitata*, understanding the movement of pollen between individuals is essential to explore the observed functional reproductive differences among African baobab individuals and assess the impact of pollen movement and reproductive differences on future generations. Paternity analyses are useful to show the movement of male gamete (pollen) alleles and their potential recruitment into subsequent generations (Ramos et al., 2018). This type of analysis may confirm hypotheses that poor producers are acting primarily as functionally 'male' individuals in the population. Understanding floral discrepancies, as well as how movement of alleles occurs across tree types, may bolster our understanding of underlying mechanisms for the observed fruiting differences across tree types. Such understanding could inform management decisions for long-term sustainability of the traditional indigenous agroforestry of *A. digitata*, especially regarding fruit production.

1.2 Plant breeding systems

The evolution of intricate and complex mechanisms of plant reproduction has led to a diverse set of plant breeding systems. Amongst angiosperms, plants that have both male (androecium) and female (gynoecium) whorls, in particular, have evolved traits that enable them to effectively use the environment to give rise to reproductive opportunity and to spread both embryos (in seeds) and male gametes (in pollen) across environmental gradients, thereby colonizing species-specific niches (Richards, 1997). There are two distinct plant-breeding systems: 'sex systems' and 'mating systems' (Charlesworth, 2006). Sex systems refer to floral function within a population, such as, functional male and female flowers (hermaphrodites), separate male and female flowers on the same plant (monoecy), hermaphroditic plants interspersed with male plants (androdioecy), hermaphroditic plants interspersed with female plants (gynodioecy) or, male and female flower

morphs occurring at different flowering times (heterodichogamy) (Andrus, 1962; Charlesworth, 2006; Richards, 1997). Mating systems refer to how individuals in a population mate, inbreeding or outcrossing, which is often better identified in conjunction with assessing the sex system of a species.

Dioecy is a sex system that promotes heterogamy in a population and leads to the gain of male or female fitness (Andrus, 1962; Charlesworth, 2006; Richards, 1997), with individuals either allocating resources to male or female sexual expression and function. Dioecism provides several advantages that increase overall fitness in a population or species (Bawa, 1980; Käfer et al., 2017). For instance, genetic diversity is often increased as there is a spatial separation of sexes, which promotes outcrossing and reduces the chance of inbreeding (Bawa, 1980; Cronk, 2022). Having dedicated individuals that focus on investing in one sex results in better resource use and reproductive efficiency (Bawa, 1980; Spigler & Ashman, 2012). With increased genetic diversity and more efficient resource allocation, populations may be able to withstand greater disturbances and external pressures (Beach, 1981; Renner & Ricklefs, 1995). Despite these advantages, the spatial separation of sexes comes with a challenge in terms of pollination. Pollinator interactions are vital for dioecious plants as they require the movement of pollen between individuals (Beach, 1981).

1.3 Plant-pollinator systems

The contribution made by animals and their interactions with plants has facilitated great diversification in angiosperms (Benton et al., 2022; Crane et al., 1995). Often a striking feature of angiosperms is their sexual organ, the flower, which has regularly evolved mutualistic relationship with animals for pollination. Flowers can be considered ‘sensory billboards’ (Raguso, 2004); they attract an animal by use of visual, acoustic (e.g., bats), or olfactory signals (Simon et al., 2021). The animal then consumes pollen, nectar or other floral elements, and the flower may also provide shelter for the animal or an area to lay eggs (Walker, 2020). In turn, the plant usually benefits from pollination when male gametes, inside the pollen, are transported between flowers and placed onto the stigmas of conspecific flowers to enable fertilization to occur.

Pollinator behaviour has important consequences for the plant's mating system. For instance, the distance a pollinator flies influences the extent of pollen-mediated gene dispersal, which impacts the genetic structure of the populations (Mitchell et al., 2009). For self-incompatible species, the geitonogamous movement (transfer of pollen between flowers on the same plant) of pollen has strong negative implications for fruit and seed sets as self-pollen may clog stigmas and inhibit the growth of pollen tubes or attachment of pollen from outcrossed individuals (Barrett, 1996; Hessing, 1988; Snow et al., 1996). Additionally, pollen movement influences the genetic diversity of the species. However, in self-compatible species, the movement of pollen within and between individuals over short distances has minimal effect on seed set and fecundity, but increases selfing rates negatively influencing genetic structure due to inbreeding (Herrera & Pellmyr, 2009; Snow et al., 1996).

Mapping the movement of pollen (gametes) through plant-pollinator interactions, and subsequent genetic consequences, can be achieved through molecular methods, such as paternity analysis. Paternity analyses enable a comparison of putative parental and offspring genotypes. Comparing the genotype (allele composition) of a seedling with that of maternal and paternal genotypes, we can estimate the parentage of that seedling. In plants and animals, identifying the paternity of an individual gives us an understanding of the extent of gene flow and dispersal, given that the location of the paternal individual is known (Ramos et al., 2018). We can estimate the spatial movement of pollen by pollinators, which provides information on how effective pollinators are in pollinating a study species across its range and population (Ashley, 2010).

1.4 The African baobab

There are eight species of *Adansonia*, six species are endemic to Madagascar (*Adansonia grandidieri* Baill.*, Adansonia suarezensis* H.Perrier*, Adansonia rubrostipa* Jum. & H.Perrier*, Adansonia perrieri* Capuron*, Adansonia madagascariensis* Baill. and *Adansonia za* Baill.), the seventh species, *A. gregorii* F. Muell., occurs in north-western Australia (Baum, 1995a; Wickens, 2008) and the eighth species, the African baobab, *Adansonia digitata* L. (Malvaceae), being the only indigenous species spread throughout sub-Saharan Africa, and the most well-

known species world-wide (Baum, 1995a; Cron et al., 2016; Wickens, 2008). There are three distinct regional groups of *A. digitata*, two in West Africa and one in southern and eastern Africa (Tsy et al., 2009). *Adansonia digitata* is geographically widespread across the African continent, and occurs in a variety of habitat types, including both modified habitats such as rural villages and agricultural fields and unmodified, natural habitats such as rocky outcrops, rangelands/plains and river valleys (Venter & Witkowski, 2011; Wickens, 2008).

Adansonia digitata is a tetraploid pachycaul tree, relatively short (ca. 15 m in height) with a large (20+ m stem circumference), unevenly buttressed trunk with smooth grey bark. Its leaves are palmate with 5–7 tapering leaflets (<150 mm long) borne on a single petiole, except seedlings which have simple leaves. Velvety, egg-shaped fruits contain many kidney-shaped seeds, with each seed surrounded by an edible powdery white pulp. Primates are attracted by low hanging or fallen fruit and are seen splitting fruits to access the nutrient rich pulp. Ungulates and roaming livestock feed off the low hanging accessible leaves. The canopy of *A. digitata* provides refuge for insects, birds, and small mammals (Wickens, 2008).

1.4.2 **Economic importance and risk**

There are many economic uses of *A. digitata* ranging from traditional human and animal medicines, food and fodder production and natural resources such as wood and fibre (Romero et al., 2014; Wickens, 2008). Many of these products are sold by rural communities throughout southern, eastern and western Africa and provide significant income to these communities (Sidibe & Williams, 2002). There has been an increased demand for baobab products in recent years due to the fruit pulp's beneficial, high nutrient superfood properties, and moisturising properties of the seed oil extract (Darr et al., 2020; Meinhold & Darr, 2020). There is the risk that traditional indigenous agroforestry systems may not cope with this demand (Sanchez, 2011; Venter & Witkowski, 2011, 2013a, 2013b). Considering the lengthy timeframe to reproductive maturity of the baobab, in some cases 125 years (Mayne et al., 2022; Swanepoel, 1993), simply an increase in germination and growth programmes may be insufficient to meet near future demands (Venter & Witkowski, 2011). Additionally, Venter and Witkowski (2013b) found that low recruitment rates are dependent on microsite conditions, such as rainfall and seedling

herbivory from local animal husbandry. The combination of climatic changes and an increase in animal husbandry will drive impacts on natural recruitment and germination rates of *A. digitata*. Sustainable harvesting techniques, seedling management and germination programmes have been suggested as possible solutions for the increased demand for baobab products (Venter & Witkowski, 2013b).

1.4.3 **Floral biology and pollination**

Flowering in *A. digitata* commences after ca. 100 years of growth or up to 200 years in dry marginal climates and occurs annually during the early wet season (October – April) (Baum, 1995b; Mayne et al., 2022; Venter & Witkowski, 2019). *Adansonia digitata* can live up to ca. 3000 years and once reproductively mature, they continue to produce flowers, not entering a post-reproductive stage. Functionally hermaphroditic flowers undergo anthesis in the early evening with up to 50 flowers per tree opening per night. During anthesis, the calyx of a flower bud splits open and each lobe curls to the base of the flower until the petals and androecium are moderately expanded (Baum, 1995b). Anthesis occurs through the early evening with expanding filaments and a strong central axis turgor. In the early morning, turgor decreases and by dawn the corolla and androecium whorls have generally abscised leaving only the intact gynoecium. Baum (1995) suggests the gynoecium remains intact to ensure pollen tube growth in the style. Unfertilized flowers abscise within 24 hours and, although still receptive in the mornings, stigma receptivity decreases over time. (Baum, 1995b; Chetty et al., 2021). Flowers contain a copious amount (> 500 µl) of sucrose-rich nectar (sucrose: hexose ratio of 0.87) as an attractive reward for pollinators, particularly hawkmoths and bats (Baum, 1995b; Karimi et al., 2022). Post anthesis, flowers may emit a sour, musky scent which gradually weakens through the evening into the early morning.

Baobab populations exhibit unique patterns of fruit production; despite all individuals producing many flowers, fruit set has been found to be three orders of magnitude higher in some individuals compared to others (Venter & Witkowski, 2019). Populations comprise individuals that produce little to no fruit yearly (‘poor producers’), as well as individuals that produce many fruit annually (‘producers’), despite both tree types having similar flowering phenology (Venter

& Witkowski, 2019). This fruiting behaviour remains consistent among trees as they do not switch between producing or not producing fruit on a seasonal basis. When considering the economic importance and use of fruit and other baobab products from these trees by villagers, fruit production discrepancies pose a significant threat to poor producer trees as they are deemed economically useless to southern African local communities and such perceptions may lead to their removal. Venter and Witkowski (2019) suggested that *A. digitata* might be functionally dioecious, and a recent study by Chetty et al. (2021) supports this hypothesis. The authors found that poor producer trees prioritize androecium sexual fitness with a high viable pollen load, whereas producers prioritize gynoecium sexual fitness by increasing stigma receptivity and fruit set. With *A. digitata* being self-incompatible (Venter et al., 2017), functionally male, poor producer trees are acting as pollen donors whereas the producers serve as functional females, receiving pollen and producing fruit. For pollen transfer to occur between functionally dioecious self-incompatible individuals, pollinators play a critical part to baobab floral biology.

Extensive pollinator work has been done on eastern African *A. digitata* populations confirming bat pollination (Baum, 1995b; Swanepoel, 1993; Wickens, 2008). Evidence shows that fruit bats are the primary pollinators of east and west African *A. digitata*, with insects (hawkmoths, bees, butterflies) and small mammal (*Galago*; bushbaby) species considered nectar and pollen thieves (Baum, 1995b). Fruit bat species seen pollinating east and west African baobab populations include *Rousettus aegyptiacus* Geoffroy (1810), *Epomophorus gambianus* Ogilby (1835) and *Eidolon helvum* Kerr (1792) (Ayensu, 1974; Harris & Baker, 1959; Start, 1972), with all three genera also occurring in southern Africa (Monadjem et al., 2020). Yet, despite their presence in southern Africa, there has been no evidence of successful bat pollination leading to fruit yield in southern African *A. digitata* populations. Instead, preliminary studies indicate that hawkmoths may be the primary pollinators (Taylor et al., 2020). Significant fruit sets are produced by these southern African baobab populations which indicates that effective pollinators are not necessarily in short supply (Venter & Witkowski, 2010, 2011).

2. Aim and objectives

The aim of this study is to evaluate how floral reproductive traits and the movement of pollinators between *A. digitata* producer and poor producer trees may affect the movement of alleles between trees ultimately affecting seed set parentage from producer trees.

2.1 Objectives

For this study, *A. digitata* trees across three subpopulations were classified as either 'maternal' or 'paternal' individuals. Maternal trees were all producers, while paternal trees were selected based on proximity to individual maternal trees, not on their fruit production history. The six nearest baobab trees to a single maternal tree were considered as potential pollen donors to that maternal individual and were sampled as paternal trees. It is important to note that paternal trees could be producers (P), poor producers (PP), or have an unknown fruit production history.

The objectives of this study are as follows:

1) To identify and compare differences in floral morphology and pollen viability across paternal producers and poor producers and maternal producer *A. digitata* individuals. Quantifying functional morphological differences between trees will aid in understanding the fruiting discrepancies between tree types (producer & poor producer).

2) To assess the prevalence of alleles from paternal pollen donors in offspring of maternal (producer) trees. This may help identify if there are individual trees that contribute more pollen to the maternal trees and indicate how pollen is moving among trees in the study region.

3) To determine the length of time over which pollen stays viable by hand pollinating stigmas using pollen samples of different ages. This could improve our knowledge on how long pollen may stay viable on the surface of pollinators or in the latent environment.

3. Methods

3.1 Study site and sample collection

This study was conducted on a population of *Adansonia digitata* located in the northern Vhembe district (Figure 1; 22°19'S and 30°28'E), Limpopo province, South Africa. The region is classified as semi-arid savanna, dominated by several vegetation subtypes such as Musina Mopane Bushveld, Limpopo Ridge Bushveld, Soutpansberg Mountain Bushveld and Makuleke Sandy Bushveld (Mucina & Rutherford, 2006). Soils are characterized as deep sand with shallow sandy lithosols (Mucina & Rutherford, 2006). The region experiences hot summers (16°C – 40 °C) and mild winters (12°C – 22°C) with a mean annual rainfall of 334 – 423 mm and average altitude of 400 m above mean sea level (Mucina & Rutherford, 2006).

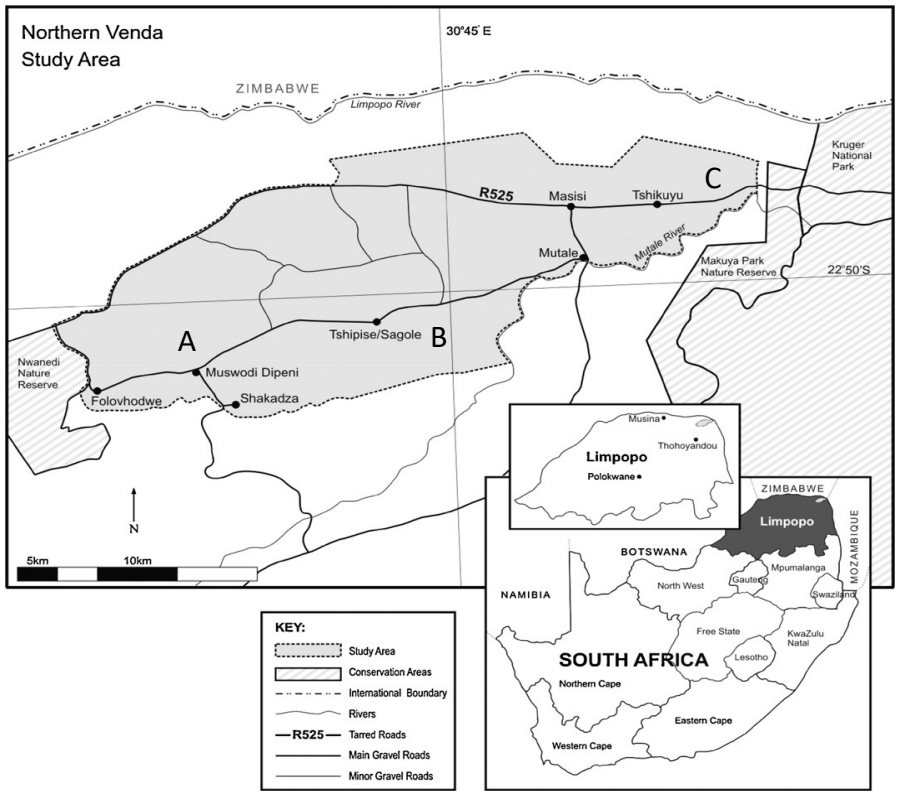


Figure 1. The study location of *Adansonia digitata* individuals located in the north-eastern province of South Africa, Limpopo. *Adansonia digitata* occurs throughout the Vhembe region, which was previously known as Venda. Three subpopulations (A, B and C), that have ongoing, long-term data collection, were used for this study. (Source: Venter & Witkowski, 2011).

The study area falls within the Vhembe Biosphere Reserve as inscribed by UNESCO World Network of Biosphere Reserves in 2009 (VBR, 2012). The bioregion is dominated by *Combretum apiculatum* Sond. (red bushwillow), *Terminalia sericea* Burch. ex DC. (silver terminalia) and *Colophospermum mopane* J.Kirk ex Benth. (mopane) vegetation. The current land-use activities include subsistence farming and animal husbandry within and outside local villages (Paumgarten et al., 2018; Venter & Witkowski, 2013b). Trees used in this study were distributed across multiple land use types, including villages, agricultural fields, undisturbed plains, and rocky outcrops ((Venter & Witkowski, 2010; Figure 2). Subpopulation A, the south-eastern most subpopulation occurs in a village (referred to as AV) and is ca. 23 km away from the B subpopulation. The B subpopulation comprises four land use types, viz., agricultural Fields ('BF'), undisturbed Plains (rangelands, 'BP'), Rocky outcrops ('BR') and a Village ('BV'). The B subpopulation marks the centre of the greater Vhembe *A. digitata* study population. It also includes the greatest number of genotyped individuals per subpopulation. Finally, the C subpopulation is the north-eastern most subpopulation and occurs in a Village (CV), ca. 23 km away from the B subpopulation and 46 km from the A subpopulation. Whilst the three populations were dispersed across a 46 km range, a significant portion of *A. digitata* trees in the regional area were not sampled.

Multiple studies have investigated selected *A. digitata* trees in the northern Vhembe region (Chetty et al., 2021; Venter & Witkowski, 2010, 2013b, 2019), and have established a long term fruit production history dataset. All maternal trees form part of this fruit production dataset however, many paternal trees have not been assessed for fruit production. Hence, during the fruiting season, when fruit could be seen on the trees prior to natural fruit abscission, paternal trees were putatively identified as producers or poor producers based on the number of fruit hanging on the tree.

During the flowering season in February 2021, young leaf material was collected from 13 known 'producer' maternal trees and placed into silica gel to dry tissue for DNA extraction. Leaf tissue was also collected from putative paternal trees (hypothesized pollen donors) that were identified as the six nearest trees surrounding each maternal tree. Generally, most paternal trees were within 100 m of maternal trees, or up to a maximum distance of 200 m. A total of 13 maternal

trees were selected with their corresponding six nearest paternal trees (13 maternal × six paternal = total of 84 trees); in some cases maternal trees had overlapping putative paternal trees. Whilst the paternal trees were dispersed across a 46 km range between the three populations, a significant portion of *A. digitata* trees in the regional area were not sampled. When fruits were ready to be harvested from maternal individuals, three accessible fruits were randomly collected from each of the 13 maternal trees, split open and three seeds per fruit were germinated (3 fruit per tree × 3 seeds per fruit = 9 seedlings per maternal tree). Seed coats were scarified using a soldering iron to break physical dormancy to aid in germination (Weiersbye & Witkowski, 2002). Germinated seedling leaf tissue was harvested and placed in silica gel to rapidly dry samples for DNA extraction.

Figure 2. Overview map of the genotyped pollen donors (green circles) and maternal trees (orange circles) from the A, B and C *Adansonia digitata* subpopulations in northern Vhembe, South Africa. The B subpopulation spatially occurs between the A subpopulation (ca. 23 km westwards) and C subpopulation (ca. 26 km eastwards). The B subpopulation is a collection of individuals on different land use types, including Plains, Fields, Rocky and Village areas. Note that the Rocky land use type is ca. 8.5 km northwards from the B Village, Fields, and Plains, but is still spatially included in the B subpopulation. Enlarged images from each land use type with its corresponding pollen donors are shown in subsequent Figures 15 to 19.

3.2 Floral morphology and tree structure

Morphological measurements were conducted across two flowering seasons (January 2021, November 2022) on both maternal and paternal individuals. Using tailors' tape, peduncle length and flower diameter (from petal tip to opposite petal tip) were measured on three flowers per tree following flower anthesis (after 19h00). Using a vernier calliper, stamen ball diameter, stigma diameter (from lobe tip to opposite lobe tip), style length from point of emergence from stamen ball, anther and filament lengths, were also measured on three flowers per tree following flower anthesis (after 19h00). Additionally, tree morphology measurements such as canopy width, canopy height, height to canopy from the ground, and stem diameter at breast height were also captured, where canopy width and height are the widest and tallest range of photosynthetically active material (i.e., leaves), respectively, and height to canopy is the height from the ground to the start of any photosynthetically active material. Canopy volume was estimated, as with a greater canopy volume we presumed there would be a proportional increase in floral capacity of that individual. Canopy volume was estimated for each maternal and paternal tree using a modified semi-ellipsoid formula (hemisphere shape),

$$2/3\pi \times r\text{^}2 \times \ (canopy\ thickness)$$

where canopy thickness is the height of the canopy (*total height – height to canopy = canopy thickness*) and *r* is the radius of the canopy (Huang et al., 2019; Thorne et al., 2002).
Floral morphology and tree structure measurements were explored using one-way ANOVA and Tukey posthoc comparison tests to assess differences between tree types. Additionally, linear regression analysis was conducted on relationships between tree measurements.

3.3 Pollen viability

During the November 2021 flowering season, 15 to 25 stamens per flower were removed from each sampled tree using forceps and placed into Eppendorf tubes containing 0.5 mL of Carnoy's fixative (6 ethanol:3 chloroform:1 acetic acid). Pollen viability was assessed for 99 flowers from

maternal producer (n = 19 flowers) and paternal poor producers (n = 66 flowers) and producers (n = 14 flowers) trees using an adapted Alexander's staining technique (Peterson et al. 2010). The Alexander's staining technique turns viable pollen grains a magenta-red colour and non-viable pollen a blue-green colour (Figure 3; Peterson et al., 2010). In the lab, tubes were vortexed for 30 seconds to evenly resuspend pollen within the fixative solution. For each sample, 30 uL of the fixative-pollen solution was placed onto a slide with one drop of Alexander stain solution (containing 95% alcohol, malachite green, distilled water, glycerol, acid fuchsin, orange G and glacial acetic acid). Slides were heated over a Bunsen burner within a fume hood until the stain solution was near boiling. Cover slips were placed onto slides after heating and left overnight at room temperature to ensure adequate stain diffusion into the pollen grains. Under a light microscope (Zeiss Discovery 2.0, Germany), viable (magenta-red) and non-viable (blue-green) pollen grains were counted across four fields of view for each sample at a 100× (10× ocular and 10× objective) magnification. To evaluate differences in pollen viability between maternal and paternal individuals and their fruiting strategies, a generalised liner model using a Gaussian distribution was created. Maternal producers, paternal producers, and paternal poor producer percentage of viable pollen was compared using one-way ANOVA and Tukey posthoc comparison tests.

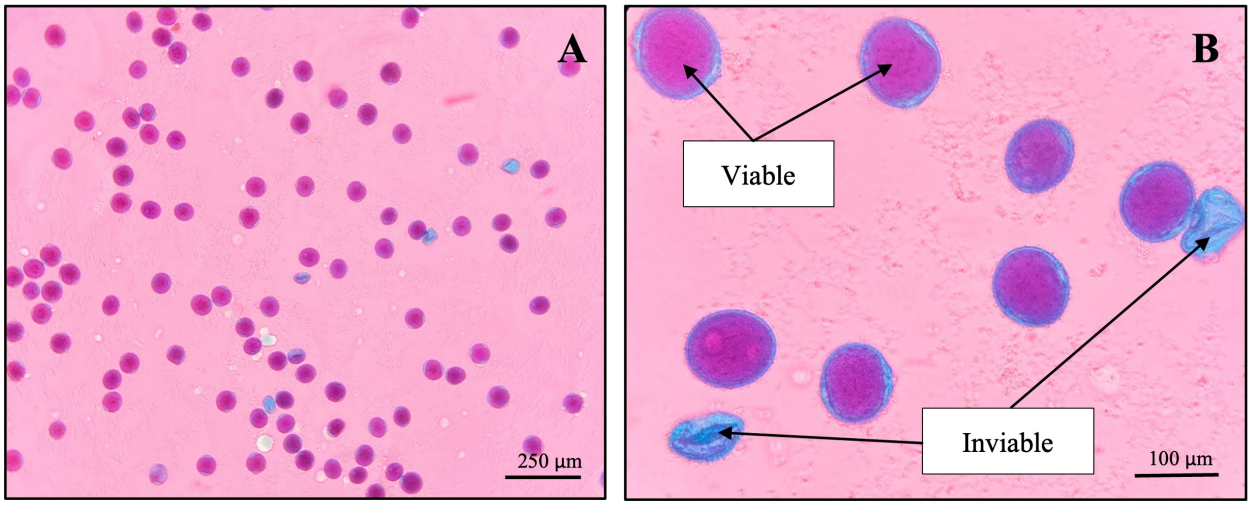


Figure 3. Images of Alexander-stained *Adansonia digitata* pollen grains. Pollen grains that are stained pink indicate viable pollen, while blue stain indicates non-viable pollen. A) Image of stained baobab pollen at 100×, B) Comparison of viable (pink) pollen and non-viable (blue) pollen grains (400×). Note the distorted shapes of the non-viable pollen grains.

3.4 Pollen structure and tube growth

To test viability of pollen of different ages, freshly released pollen was collected in folded filter paper pouches from one flower per tree, with five trees sampled at four time intervals, with 24-hour increments (0 hr, 24 hr, 48 hr and 72 hr; 5 pollen samples × 4 time intervals = 20 pollen samples). Prior to anthesis, at ca.16h00, 20 flowers on two known producer trees were bagged to prevent pollinator visitation upon anthesis. When styles had emerged from the flower bud (Figure 4A), flowers were emasculated, removing the petals, sepals, and the stamen ball (filaments and anthers; Figure 4B), flowers were then rebagged. At ca. 21h00, flowers were hand pollinated by dabbing filter paper with pollen directly onto the stigmatic surface using samples collected from flowers 24, 48 and 72 hours prior, and for the control treatment (time zero; 0 hr), pollen was collected from nearby *A. digitata* individuals' flowers. Following hand pollination, flowers were re-bagged. The following morning, at ca. 08h00, styles were cut above the ovary and stored in tubes containing 70% ethanol; 8 hours later, styles were transferred to tubes containing Carnoy's fixative (6 ethanol:3 chloroform:1 acetic acid) prior to pollen tube growth analyses.

Stigmas were removed from the fixative and allowed to dry on carbon stubs for scanning electron microscopy (Thermoscientific Phenom desktop SEM Generation 6). Once dried, stigmas were coated in carbon (C) and gold palladium (AuPd), to increase image resolution and quality of microstructure surface images (Fourie, 1982). Pollen on the stigmatic surface was then assessed for the presence of pollen tube growth as a sign of pollen germination and its ability to deliver the sperm to fertilise an ovum in the ovule. Additionally, the fresh pollen grains collected across the four time intervals were placed on carbon stubs for imaging to investigate any possible change in outer structure of pollen over time.

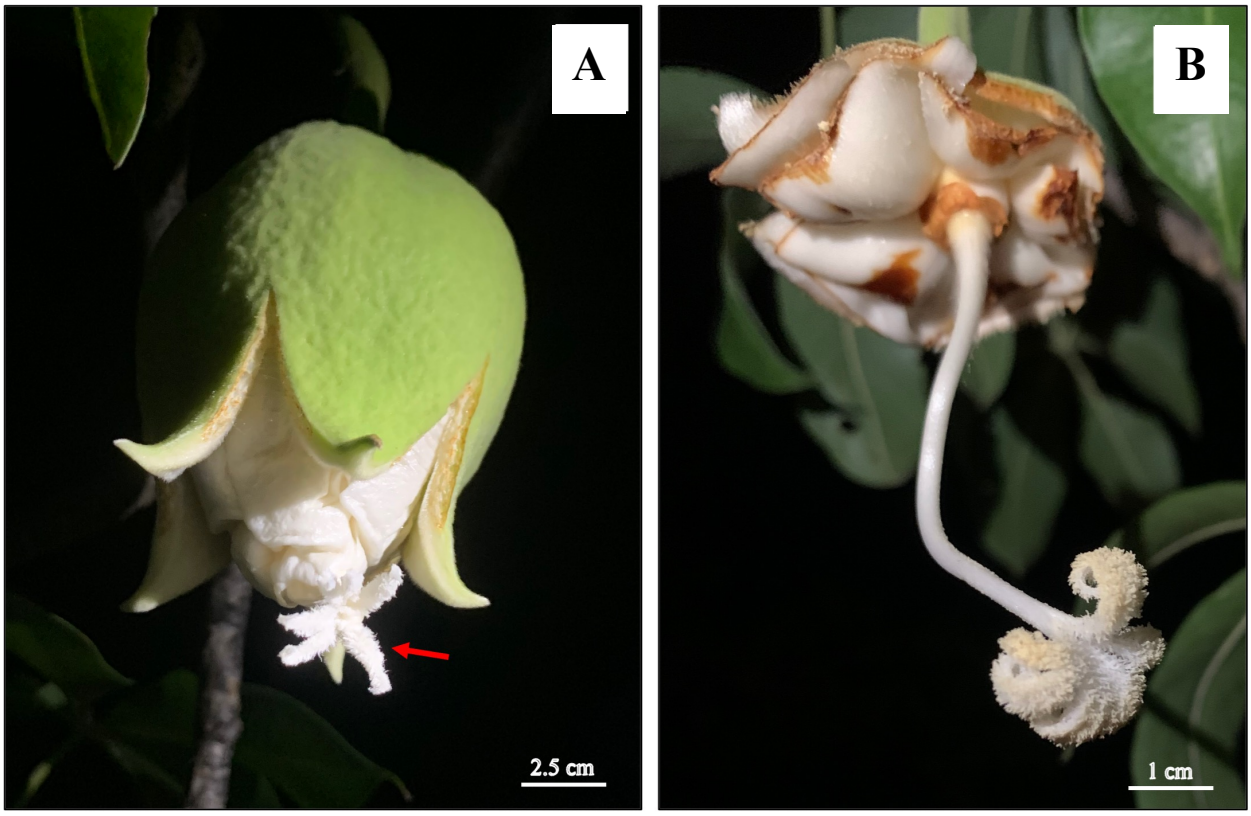


Figure 4. Flowers of *Adansonia digitata* showing a recently emerged stigma and a mature stigma. A) Stigma emergence (red arrow) from the flower bud in the early evening. B) An emasculated flower, with an intact stigma and style, note that the stigma has been manually pollinated. During emasculation, sepals, petals, and the stamen ball (filaments and anthers) are removed prior to flower maturation.

3.5 DNA extraction and genotyping with microsatellite markers.

DNA from maternal, paternal and seedling leaf tissue was extracted using the modified CTAB extraction protocol developed by Doyle & Doyle (1987) and Cullings (1992) with subsequent changes to waterbath and freezer times and solution volumes (see below). Silica-dried plant tissue (10-20 mg) was ground into fine powder and placed into Eppendorf tubes containing 700 µl of CTAB buffer (CTAB, PVP and B-mercaptoethanol). Following a 100 min incubation at 55 ^{o}C, 500 µl of 24:1 Chloroform:IsoAmyl Alcohol was added and vigorously mixed. After centrifugation for 5–10 min, the aqueous phase was pipetted into new Eppendorf tubes and volumes were estimated. To the aqueous tube, 0.54 volumes of cold Isopropanol were added,

mixed and allowed to cool in a −80 ºC freezer for 5 min and then for 15 min in a −20 ºC freezer. Thereafter, tubes were centrifuged for 5 min and the liquid from each tube was drained, being careful not to lose the DNA pellet. Cold 70% Ethanol (700 µl) was added to the tube, centrifuged for 5 min and liquid drained out to clean the DNA pellet. Pellets were dried and resuspended in 100 µl of TE buffer and refrigerated overnight. DNA was assessed using the microvolume UV-VIS spectrophotometer (Nanodrop, Thermo Fisher Scientific). DNA across all individuals was then diluted in molecular grade (DNA-RNA free) water to 100 ng/uL.

Twelve microsatellite loci (Ad01, Ad02, Ad04, Ad06, Ad07, Ad08, Ad09, Ad11, Ad12, Ad14, Ad17 and Ad18) developed for *A. digitata* by Larsen *et al*., (2009) were used to characterize 13 maternal trees, 72 paternal trees, and 117 seedling individuals. Microsatellites were amplified using the polymerase chain reaction (PCR) in a thermal cycler (BIO-RAD T100 Thermal Cycler; Hercules, CA, USA). Each ± 12.5 µL PCR reaction contained 0.5 µL of 50–100 ng genomic DNA, 0.2 µM of the forward primer and 0.2 µM of the reverse primer, and one unit of the One*Taq* 2× Master Mix DNA polymerase with standard buffer (6.25 µL). PCR amplifications started with initial denaturation at 94 ºC for 30 s followed by 30 cycles of 94 ºC for 20 s, 58 ºC for 45 s, 68 ºC for 60 s; and a final extension at 68 ºC for 5 min. PCR products were then pooled and sent to the Central Analytical Facility, Stellenbosch for fragment analysis and the resulting chromatographs were manually scored using the microsatellite plugin for Geneious Prime (Figure 5; version 2021.1.1, www.geneious.com; Biomatters Development Team, 2005) and a matrix of alleles was exported. One microsatellite locus (Ad02) did not consistently amplify and was excluded from analyses.

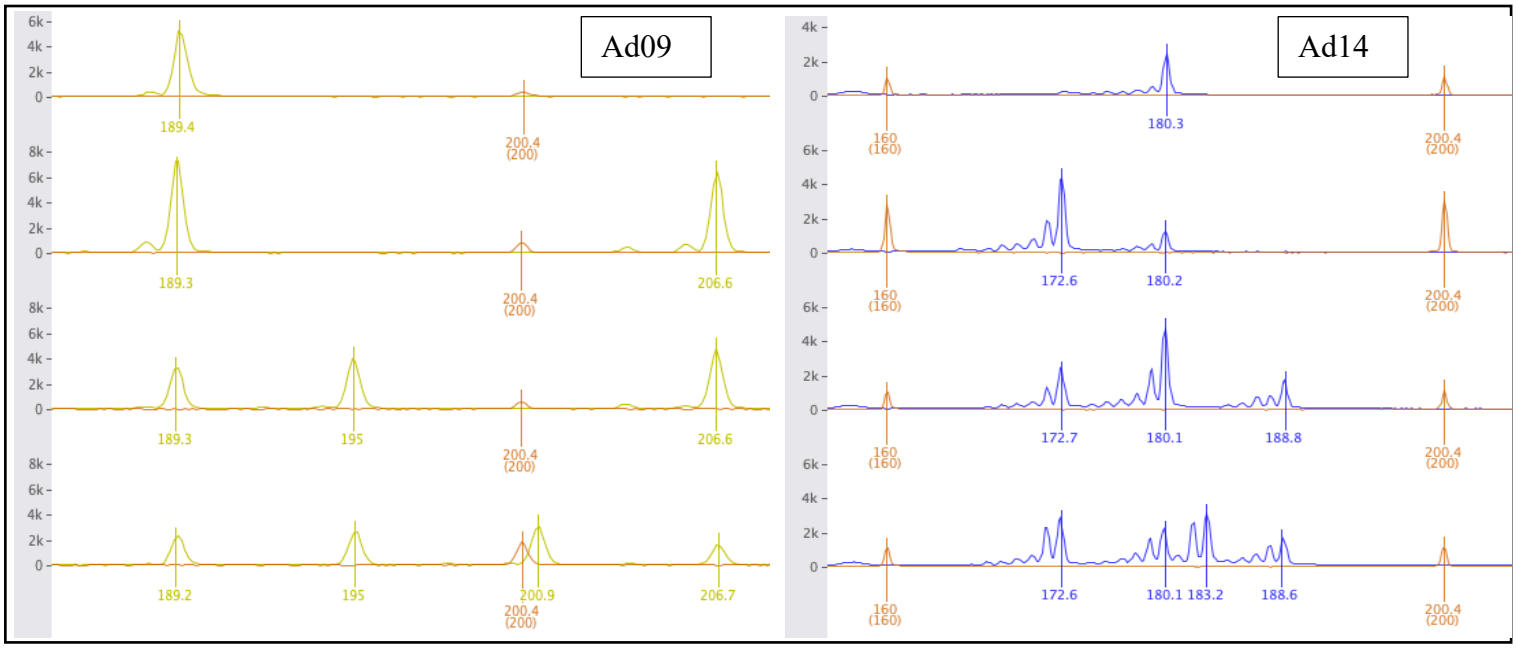


Figure 5. Electrophoretograms of two loci (left, Ad09 and right, Ad14) showing the microsatellite scoring of eight polyploid *Adansonia digitata* individuals. Each horizontal line is an individual. Single stutter peaks occasionally precede established alleles of Ad09, and multiple stutter peaks commonly precede alleles on Ad14.

3.6 Genetic diversity and paternity analyses

Adansonia digitata individuals are autotetraploids, which makes population genetic analyses challenging. Both paternity assignment and genetic diversity analyses were conducted using Polygene v 1.5 (Huang et al., 2020), which offers genomic tests that accommodate autotetraploid individuals and overcomes allelic dosage assignment. Genetic diversity indices were conducted on sampled offspring and adults (maternal, paternal, and seedlings) of *Adansonia digitata* individuals across the study population. Indices included the total number of individuals genotyped for each locus (N), the number of distinct alleles at each locus where the null allele is considered (K), the number of effective alleles at each locus (Ae), observed heterozygosity (Ho), expected heterozygosity (Hs), and the inbreeding coefficient (Fix).

Paternity assignment was conducted using the allelic phenotype method developed by Huang et al. (2021) implemented in Polygene v 1.5 (Huang et al., 2020). Multiple paternity programs exist

but are primarily tailored to computing diploid species parentage. Polygene not only performs well with autotetraploid species, such as *A. digitata*, but overcomes challenges such as dosage by converting genotypes of individuals to phenotypes in the case of unknown dosage. With this method, null alleles are integrated into the parentage model instead of assuming there are no null alleles with unknown allelic dosage. Paternity tests were conducted on 117 offspring using 85 candidate fathers, given that the mother for each offspring was known. Logarithm of odds (LOD) scores were produced for candidate fathers considering the known mother's genotype. Analyses were conducted under two assumptions, the first assumption was that subpopulations A, B and C are isolated from one another, this hypothesized that the pollen could not move between subpopulations, only within. The second assumption was that the three subpopulations are interconnected, and pollen could flow across the Vhembe region between the three subpopulations. When assessing parentage for each subpopulation, both paternal and maternal individuals were considered as potential fathers as all adult flowering trees could be considered pollen donors. Parameters were set at 50 000 simulations, 0.95 sample rate, 0.01 mistype rate; confidence intervals (C.I.) were set at 0.80 (80%) for relaxed and 0.95 (95%) as the strict C.I.. The top five fathers for each offspring based on trio LOD scores were assessed with the most likely father having the highest LOD score. Chord diagrams were constructed using identified potential pollen donors at all confidence intervals above 80%.

4. Results

4.1 Floral morphology

An analysis of floral morphology of the maternal producers and paternal poor producers and producers showed strong evidence for morphological differences between the two tree types (Figure 6 & Figure 7). Poor producers generally have a larger stamen ball ($F_{1,175} = 36.3$, $P < 0.001$), longer filaments ($F_{1,175} = 21.8$, $P < 0.001$), and larger anthers ($F_{1,175} = 54.5$, $P < 0.001$) than producers. In contrast, producers have longer peduncles ($F_{1,154} = 4.75$, $P = 0.03$) and a longer style emergence from the stamen ball ($F_{1,174} = 25.9$, $P < 0.001$). There were no significant differences in flower diameter ($F_{1,17} = 1.1$, $P = 0.13$) or stigma lobe diameters ($F_{1,174} = 2.3$, $P = 0.12$) among tree types.

When assessing the ratio of floral components, there were clear differences between the tree types (Table 1). There was a significant difference between producer and poor producer flower diameter:peduncle ratios with poor producers having higher ratios ($F_{1,154} = 7.56$, $P < 0.001$). Similarly, stamen ball:flower diameter ratios were significantly different between tree types showing larger ratios in poor producers($F_{1,175} = 20.7$, $P < 0.001$), while stigma lobe:flower diameter were not different between producers and poor producers ($F_{1,175} = 1.18$, $P = 0.28$). Interestingly, stigma lobe:stamen ball ratios were also statistically different between producers and poor producers with producers having a higher ratio ($F_{1,175} = 12.9$, $P < 0.001$).

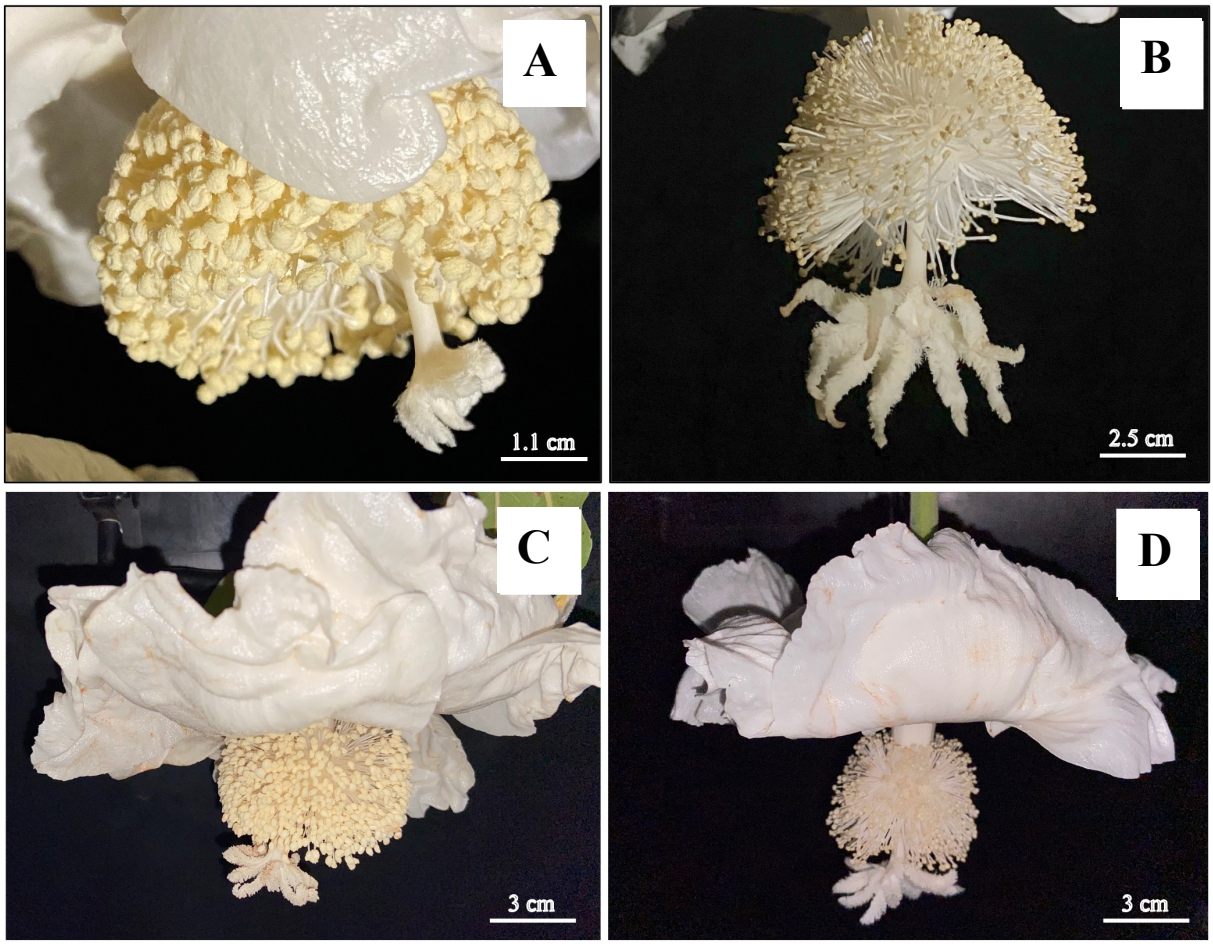


Figure 6. *Adansonia digitata* flower images (A–D) showing morphological dissimilarities among trees. Note differences in gynoecium and androecium morphology and colour with images (A) and (C) showing larger, swollen anthers with powdery, sticky pollen and smaller stigmatic surfaces; and images (B) and (D) showing smaller, beady anthers with larger stigmas. Pictures were taken ca. one hour after anthesis.

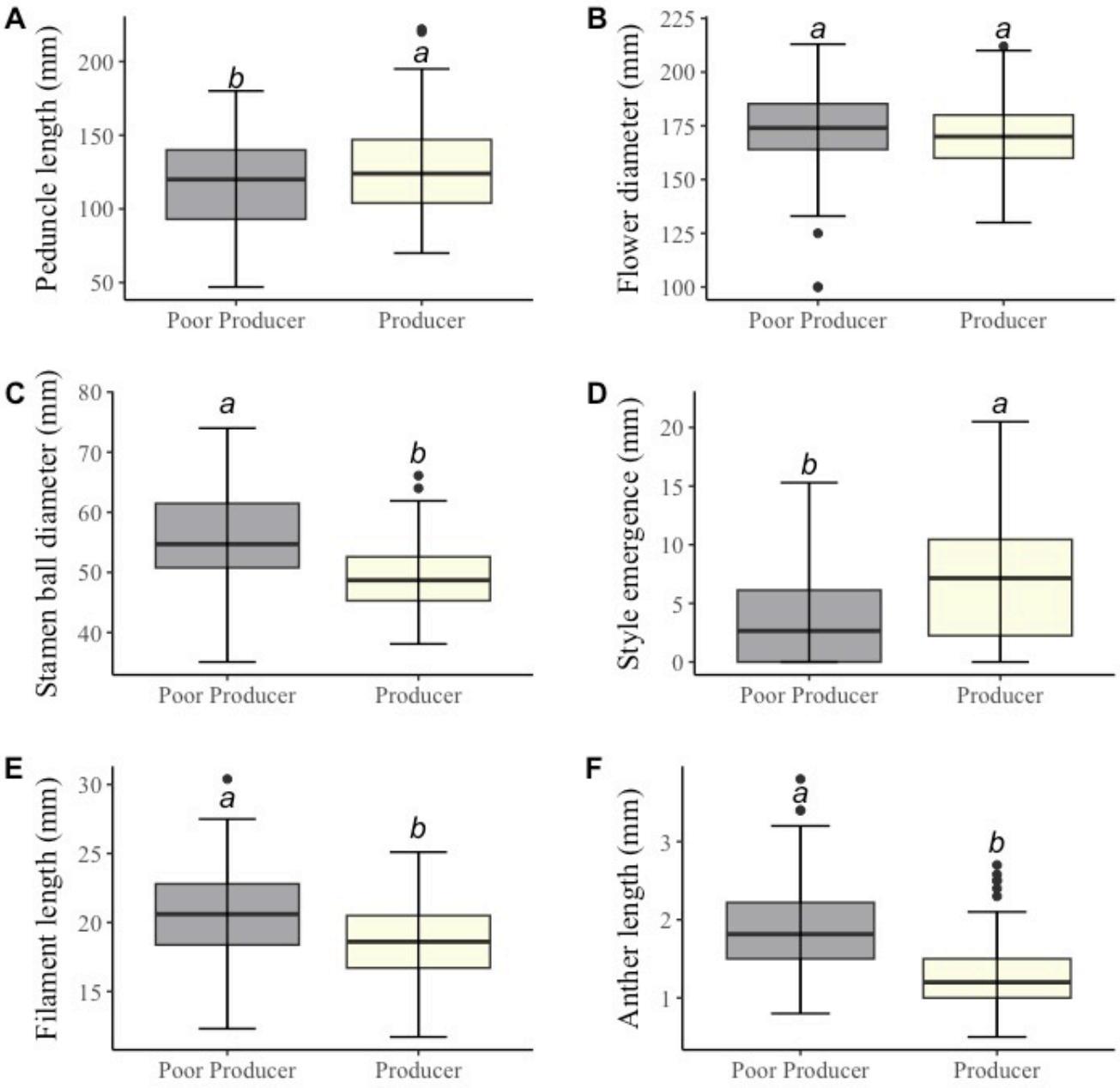


Figure 7. Reproductive trait differences between producer (n = 78 flowers) and poor producer (n = 77 flowers) trees across three subpopulations of *Adansonia digitata* trees in the northern Vhembe study region. The significance level is denoted by letters above the whiskers. The boxes indicate the 50% quantile of data points, with the upper and lower whiskers showing the highest and lowest data points in the 1.5 inter-quantile range, respectively. Points outside the whisker range are outliers.

Table 1. Means (± SE) of reproductive trait measurements of flowers between producer and poor producers across three subpopulations of *Adansonia digitata* trees in the northern Vhembe study region. †, indicates a significant difference (Tukey HSD, P < 0.01) between producer and poor producer trees.

Character	Poor Producer	Producer
Peduncle length (mm)	117 ± 3.64	128 ± 3.68
Flower diameter (mm)	174 ± 2.18	171 ± 1.96
Flower diameter: peduncle ratio	1.61 ± 0.06 †	1.42 ± 0.039
Stamen ball diameter (mm)	55.7 ± 0.843 †	49.2 ± 0.648
Stamen ball diameter:flower diameter ratio	0.324 ± 0.007 †	0.289 ± 0.004
Stigma lobe diameter (mm)	18.9 ± 0.61	20.7 ± 0.81
Stigma lobe diameter:flower diameter ratio	0.112 ± 0.004	0.119 ± 0.004
Stigma lobe diameter:stamen ball diameter ratio	0.346 ± 0.01 †	0.414 ± 0.02
Style emergence distance from stamen ball (mm)	3.43 ± 0.43 †	6.87 ± 0.57
Filament length (mm)	20.7 ± 0.36 †	18.4 ± 0.32
Anther length (mm)	1.92 ± 0.06 †	1.32 ± 0.05

4.2 Tree morphology

Tree morphology measurements such as tree canopy volume and canopy width did not differ significantly between producer and poor producer trees (Figure 8, Table 2; [Volume: $F_{1,67} = 0.714$, $P = 0.401$]; [Width: $F_{1,67} = 0.853$, $P = 0.359$]). Stem diameter at breast height also showed no differences between tree types (Figure 8, Table 2, $F_{1,67} = 1.267$, $P = 0.265$). Canopy height showed moderate statistical differences between producers and poor producers, with poor producers having taller canopies (Figure 8, Table 2, $F_{1,67} = 5.01$, $P = 0.029$). Poor producer trees in the A subpopulation showed higher canopy volumes compared to B Field poor producers (Figure 8; P < 0.05) and B Plains producers (P = 0.05). Based on the results of the multiple comparisons test (Tukey HSD, P<0.05), most of the canopy volumes did not differ between tree

types across the four land use types or across the six subpopulations (Figure 9; $F_{11,58} = 2.07$, P = 0.04).

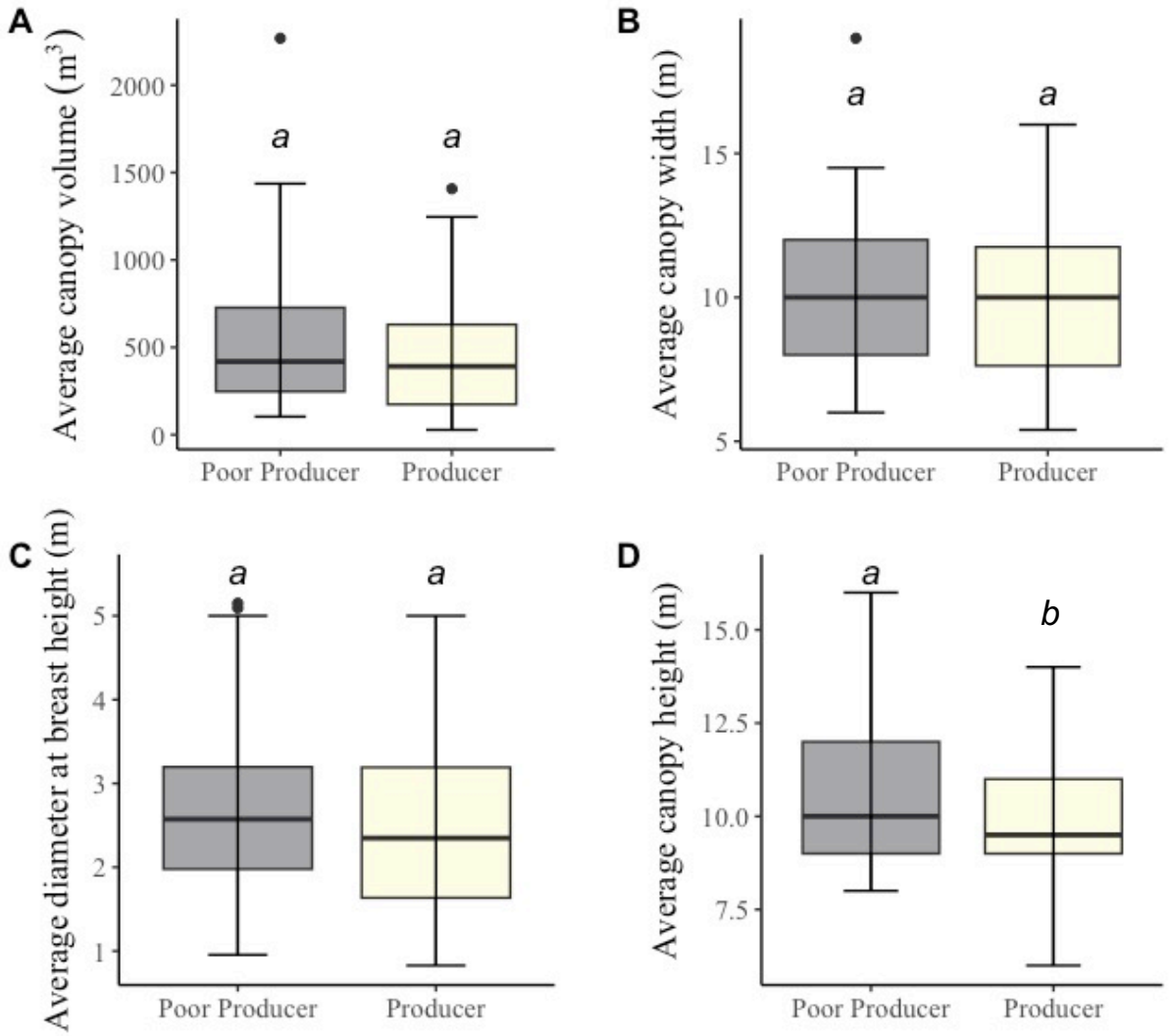


Figure 8. Average canopy volume (m^3), canopy width, height, and average diameter at breast height of poor producer and producer trees among *Adansonia digitata* subpopulations A, B and C across the northern Vhembe area. The significance level is denoted by letters above the whisker (Tukey HSD, P<0.05). The boxes indicate the 50% quantile of data points, with the upper and lower whiskers showing the highest and lowest data point in the 1.5 inter quantile range, respectively. Points outside the whisker range are outliers.

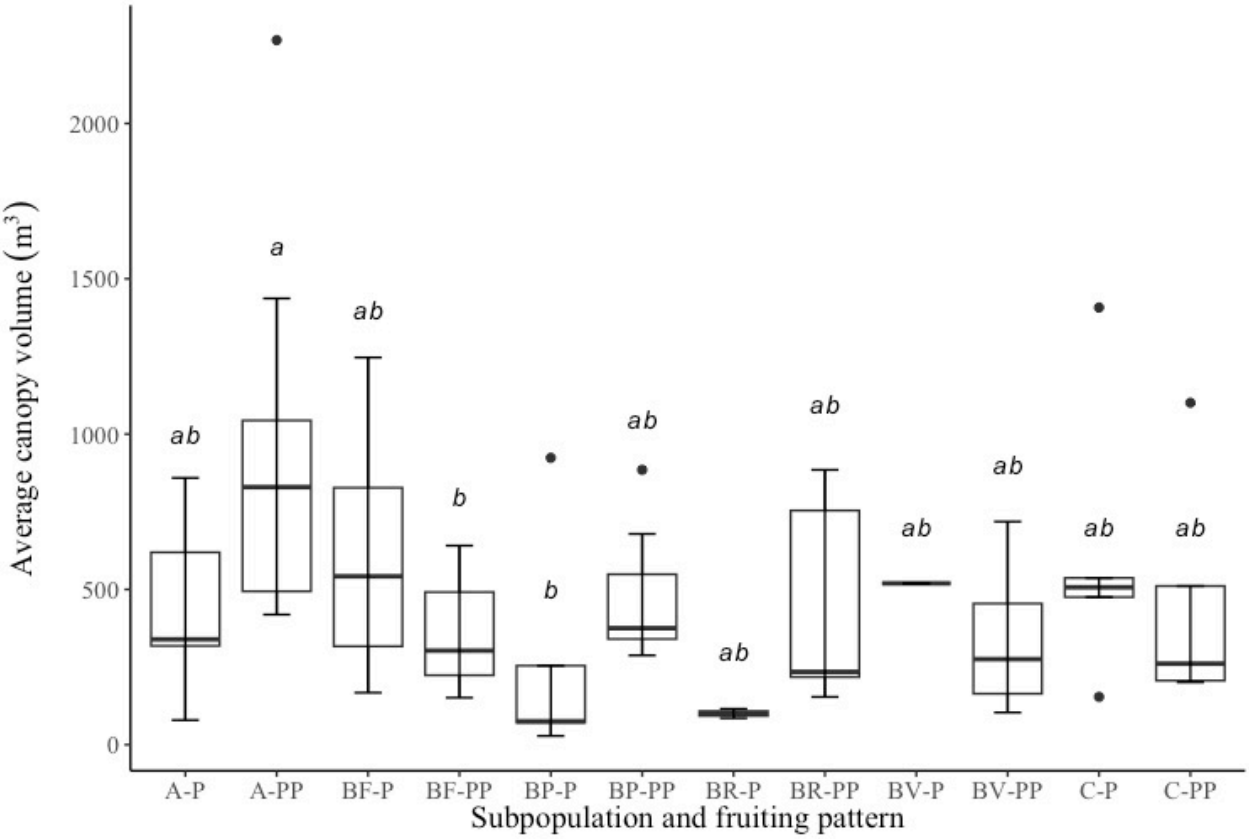


Figure 9. Average canopy volume (m^3) of poor producer (PP) and producer (P) trees among *Adansonia digitata* subpopulations A, BF (agricultural Fields), BP (rangelands/Plains), BR (Rocky outcrop), BV (Village) and C across the northern Vhembe area. The boxes indicate the 50% quantile of data points, with the upper and lower whiskers showing the highest and lowest data point in the 1.5 inter quantile range, respectively. Points outside the whisker range are outliers. The significance level is denoted by letters above the whisker (Tukey HSD, $P<0.05$).

Table 2: Comparisons (means ± SE) of tree canopy dimensions and stem diameter at breast height between producers and poor producers across three subpopulations of *Adansonia digitata* trees in the northern Vhembe study region. †, indicates a significant difference (Tukey, HSD, $P < 0.05$) between producer and poor producer trees.

Character	**Poor Producer**	**Producer**
Canopy volume (m^3)	575 ± 91.4	420 ± 62.9
Diameter at breast height (m)	2.72 ± 0.247	2.33 ± 0.206
Canopy height (m)	10.8 ± 0.366 †	9.65 ± 0.361
Canopy width (m)	10.7 ± 0.562	9.52 ± 0.569

Tree morphology regression analysis showed a strong linear relationship between DBH and canopy volume for producers and poor producers combined (Figure 9A; R^2= 0.4502, df = 68, P < 0.001). Additionally, there was a strong linear relationship between height and width for the tree types combined (Figure 9B; R^2 = 0.4917, df= 68, P < 0.001).

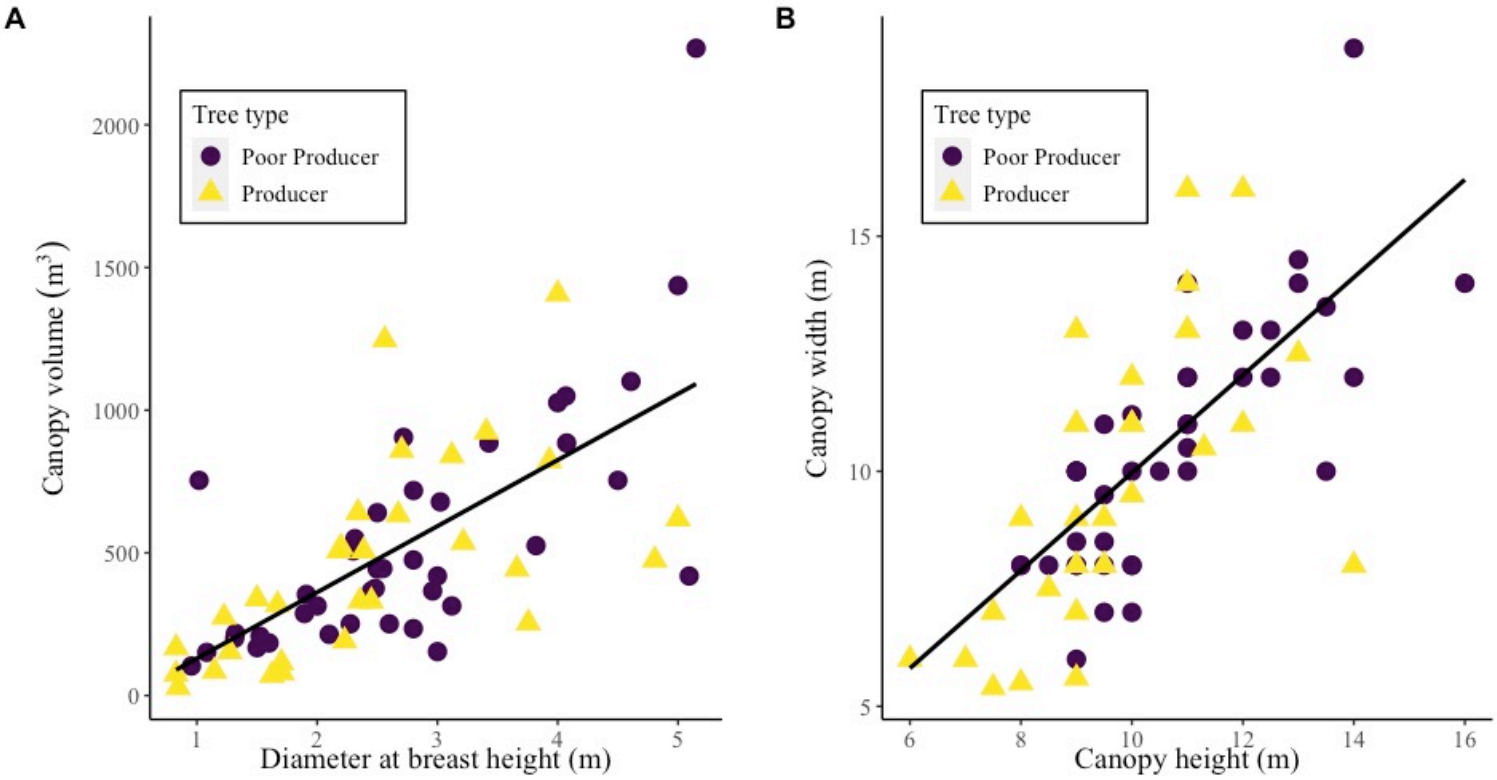


Figure 10. Tree morphology relationships of *Adansonia digitata* producer and poor producer trees with A) indicating the relationship between canopy volume (m^3) and stem diameter at breast height (m) and B) showing the relationship between canopy width and height (m). A linear regression line has been fitted to each graph.

4.3 Pollen viability

Pollen viability compared across 99 flowers showed maternal producers had a lower percentage of viable pollen (56.4 ± 9.92%) compared to paternal donors (Figure 11). Paternal poor producers had the highest percentage of viable pollen (94.3 ± 1.7%) compared to paternal producers (80 ± 9.1%; $F_{2,96} = 7.5$, $P < 0.01$).

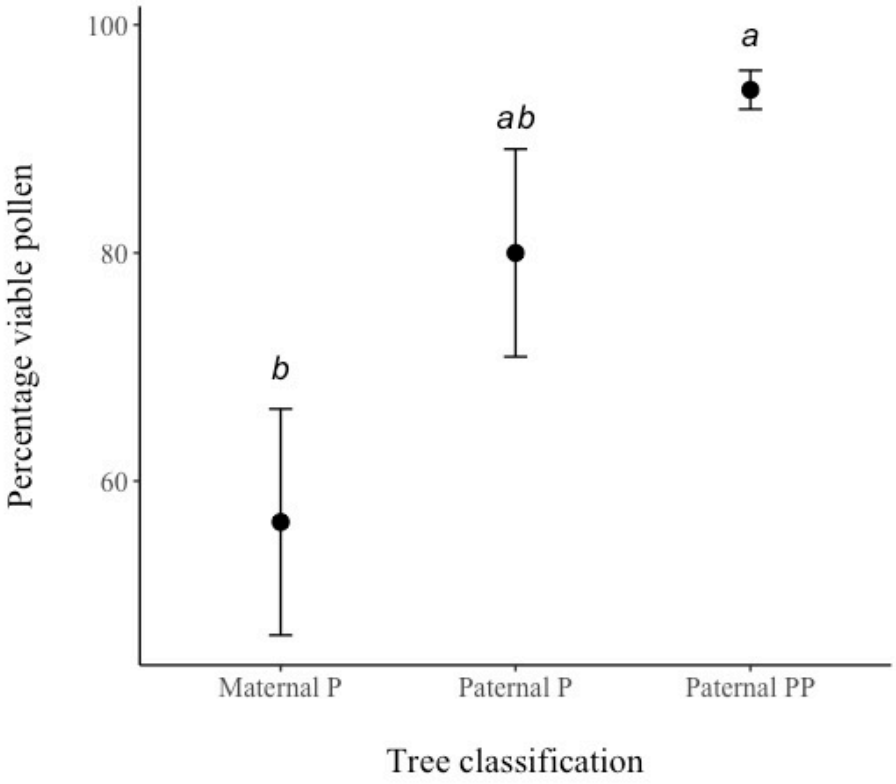


Figure 11. Comparison of the percentage of viable pollen between maternal producers (P; n=19 flowers), paternal producers (P; n=14 flowers) and paternal poor producers (PP; n=66 flowers) *Adansonia digitata* trees in Northern Vhembe, South Africa. The significance level is denoted by letters above the error bars (Tukey HSD, P<0.05).

4.4 Pollen structure and tube growth

Using scanning electron micrograph imaging, I found that pollen that was 24, 48, and 72 hours old, appeared to retain its spherical structure (Figure 12). Pollen tube growth trials showed all four treatments (0-, 24-, 48- and 72-hour old pollen) successfully germinated on stigmatic surfaces of *A. digitata* (Figure 13).

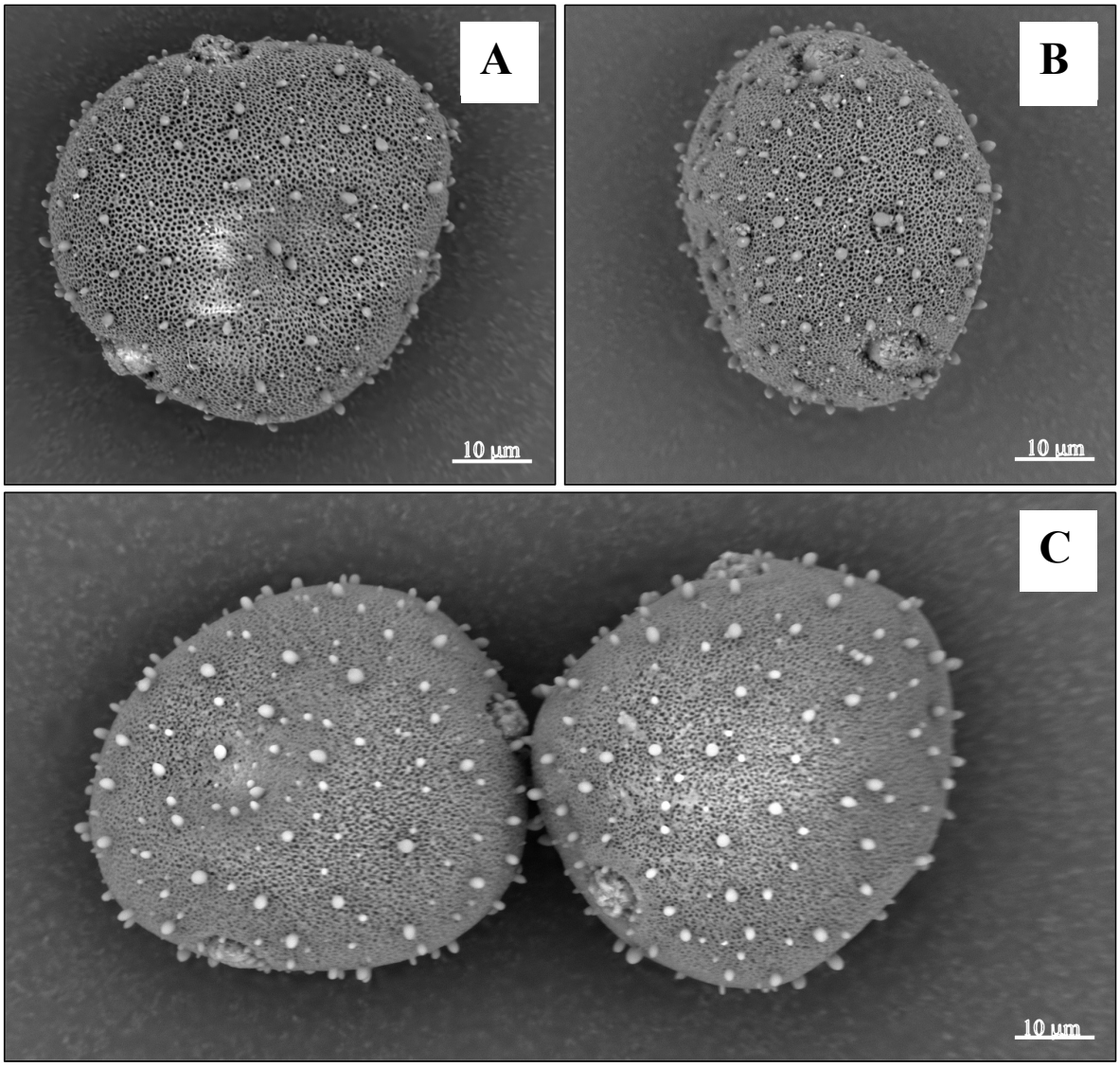


Figure 12. Scanning electron micrographs showing external structure of *Adansonia digitata* pollen. Pollen grains in panels A, B and C are 48, 72 and 96 hours old, respectively. The pollen in each panel was collected from different individuals.

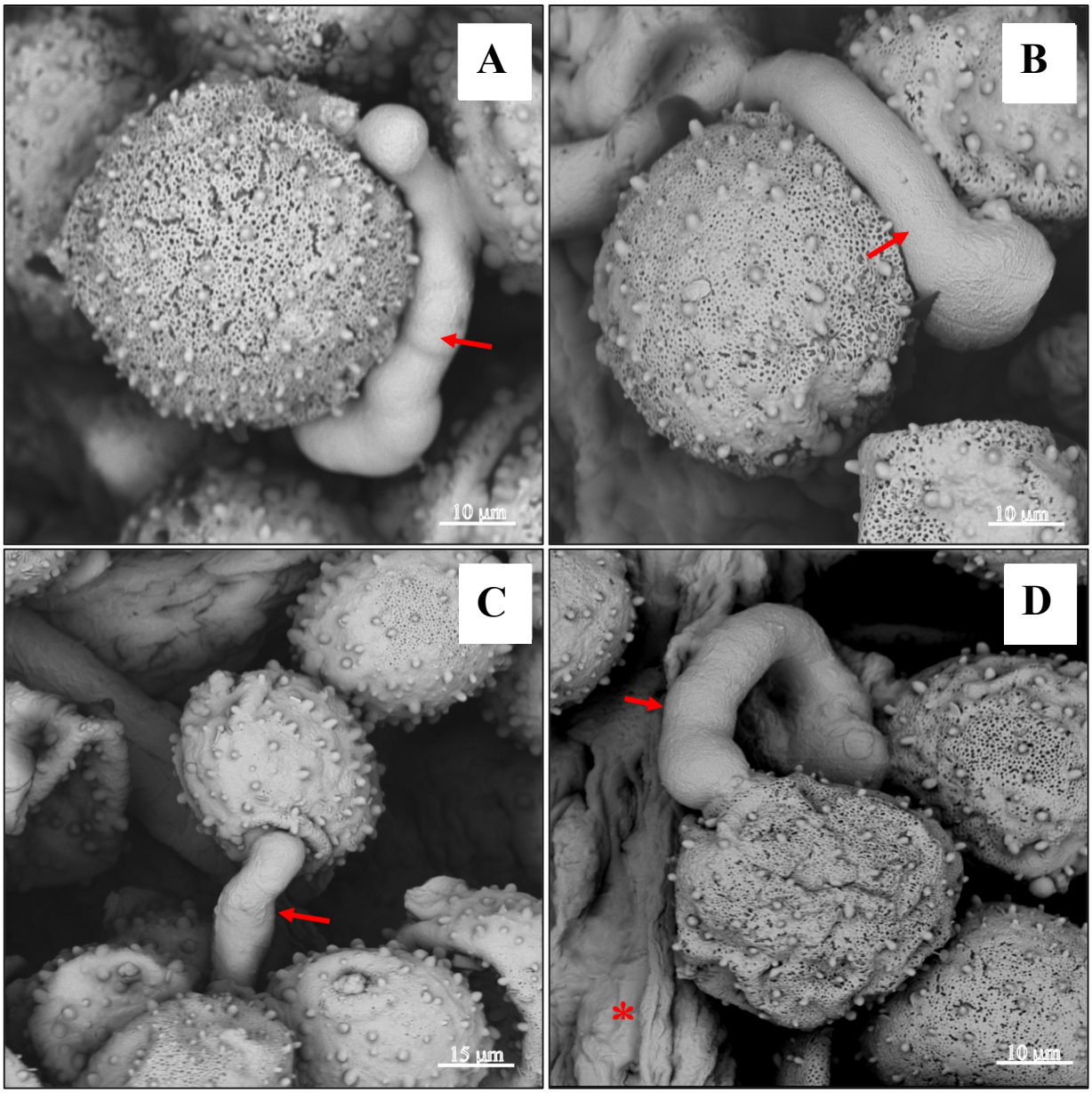


Figure 13. Scanning electron micrographs showing the growth of pollen tubes (red arrows), indicating pollen germination on stigmatic surfaces of *Adansonia digitata* flowers. Pollen in panel A is the control, which is from the same night as flower anthesis, pollen in panels B, C and D is 24, 48 and 72 hours old, respectively, all showing pollen tube growth. Note the stigmatic surface denoted by the asterisk (*) along the left side of panel D. Scale bars differ among the four images.

4.5 Genetic diversity

Overall allelic diversity was relatively high across the sampled individuals, with a total of 300 distinct adult alleles across 11 markers. Of this, there were ca. 42 effective adult alleles with an average of four informative alleles per locus. The number of effective alleles highlights the number of equally frequent alleles that it would take to achieve the same expected heterozygosity as in the study population. Overall, expected heterozygosity was higher (0.702) than the observed heterozygosity (0.593; Table 2). The sampled individuals showed low inbreeding coefficients, which indicates outcrossing (overall: 0.157; Table 2). Additionally, the additional Ritland's (1996) inbreeding coefficient calculated across all individuals yielded similar coefficients (0.182 ± 0.234).

Table 2. Genetic variation of the 11 microsatellite loci used to assess sampled offspring and adults (maternal, paternal, and seedlings) of *Adansonia digitata* individuals across the study population. Values shown are the total number of individuals genotyped for each locus (N), the number of distinct alleles at each locus where the null allele is considered (K), the number of effective alleles at each locus (Ae), observed heterozygosity (Ho), expected heterozygosity (Hs), and the inbreeding coefficient (Fix). Standard error values are in parentheses.

Locus	***N***	***K***	***Ae***	***Ho***	***He***	***Fix***
Ad01	162	31	2.713	0.493	0.631	0.220
Ad04	207	40	3.822	0.620	0.738	0.161
Ad06	271	30	8.793	0.826	0.886	0.067
Ad07	192	32	4.364	0.607	0.771	0.213
Ad08	182	27	4.126	0.591	0.758	0.220
Ad09	205	15	3.683	0.615	0.728	0.156
Ad11	236	22	2.437	0.542	0.590	0.081
Ad12	187	31	2.312	0.496	0.568	0.127
Ad14	205	21	3.065	0.583	0.674	0.135
Ad17	156	28	2.490	0.471	0.598	0.213
Ad18	222	23	4.564	0.675	0.781	0.135
Overall	202	27	3.852	0.593	0.702	0.157
S.E.	(±9.909)	(±2.036)	(±0.551)	(±0.030)	(±0.030)	(±0.016)
Total		300	42.370			

4.6 Paternity

Paternity analyses revealed an extensive allele sharing network (Figure 12), with interactions occurring between all three subpopulations of maternal and paternal individuals. Of the 117 seedlings, 56 potential pollen donors were detected in the paternity analyses, with 60 of the seedlings having producer sires, and 57 seedlings having poor producer sires. Nineteen paternal trees had alleles detected in one offspring, with the remaining 37 paternal donor's alleles detected in two or more seedlings. Two trees, from the BF and BP land use types contributed to 10 seedlings' alleles each. The maternal tree CV3 donated the most alleles among potential donors, with alleles detected in 13 seedlings amongst the three subpopulations. At an 80% confidence interval (C.I.), there was a 100% assignment rate of potential fathers to seedlings. Critical delta values are used to categorise potential fathers into confidence intervals. Critical delta values for 50000 simulations were 0.001 (80% C.I.), 0.878 (95% C.I.), 3.84 (99% C.I.) and 8.196 (99.9% C.I.). Across all candidate fathers, two individuals were identified at a 99.9% C.I. to two offspring and 15 fathers at a 99% C.I. to 13 offspring. Analyses showed high rates of outcrossing, with only one putative instance of selfing across subpopulations.

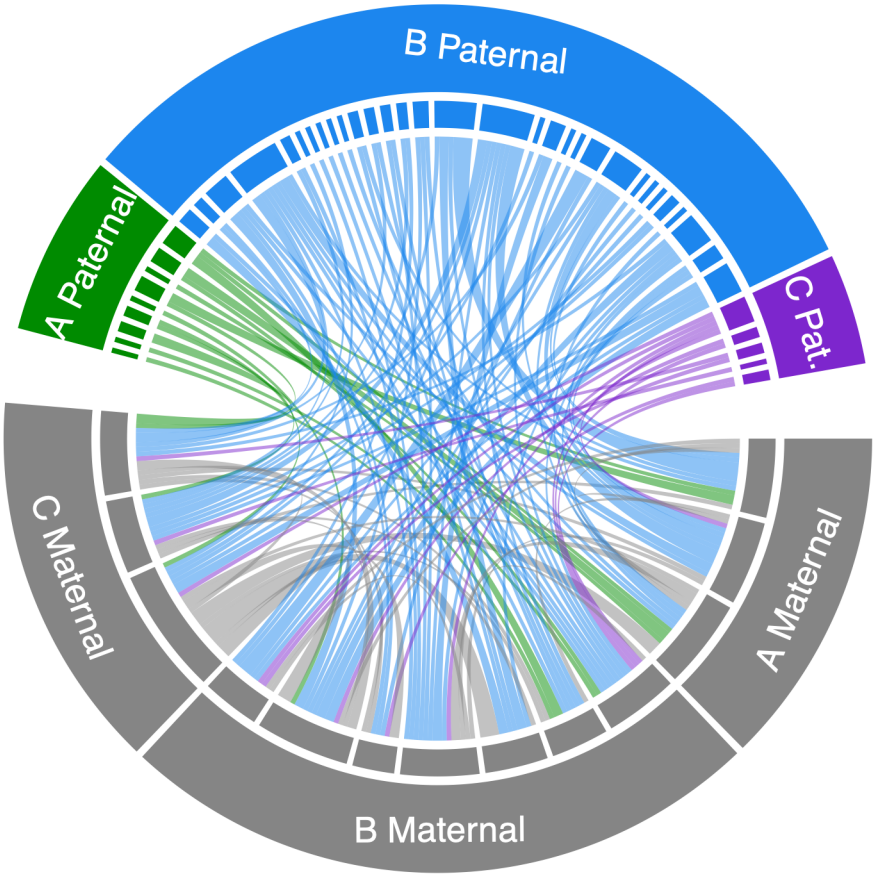


Figure 13. Network of pollen contribution among all sampled *Adansonia digitata* maternal and paternal individuals in the Vhembe region, South Africa. Direction of allele movement is from paternal pollen donors from subpopulations A (green), B (blue) and C (purple), toward the candidate A, B and C maternal producer trees (grey). Each segment in the inner circle is an individual; the width of the segment correlates with the proportion of seeds from a maternal tree sired by a given paternal donor, the larger the segment, the more seeds a paternal tree sired in maternal fruit.

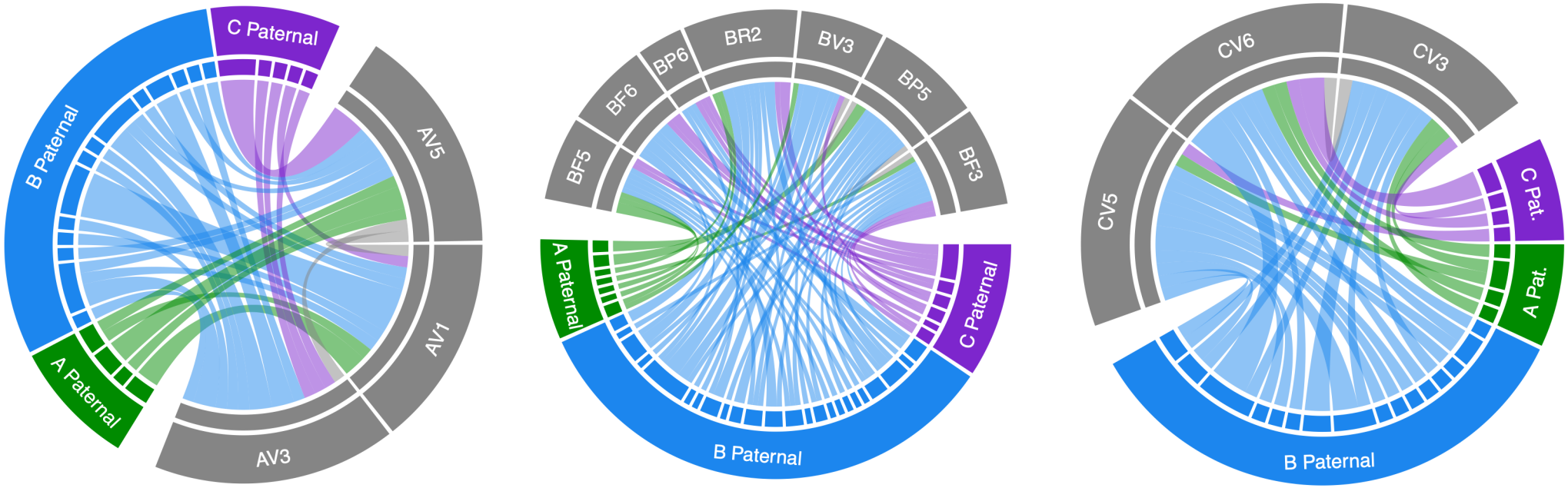


Figure 14. Network of allele sharing among *Adansonia digitata* maternal and paternal individuals from subpopulations A, B and C. Direction of allele movement is from paternal pollen donors from subpopulations A (green), B (blue) and C (purple), toward the candidate maternal individuals from each subpopulation (grey; A, B, C). Each segment in the inner circle is an individual; the width of the segment correlates with the proportion of seeds from a maternal tree sired by a given paternal donor, the larger the segment, the more seeds a paternal tree sired in maternal fruit.

Figures 15, 16, 17, 18 and 19 show analyses that assume the subpopulations are isolated from one another, where allele movement from one subpopulation to another was restricted. Across the three subpopulations, a total of 61 potential pollen donors were identified in the sample, and 38 trees donated pollen to two or more offspring. Maternal trees in subpopulation A showed a range of potential paternal candidates with 12 pollen donor's alleles found in the subpopulations' three maternal trees' fruit set (Figure 15). One pollen donor in subpopulation A was found at a 99.9% C.I. pollinating maternal tree AV3. Within subpopulation A's sampled trees, pollen appears to have dispersed a maximum range of 620 m from one side of the subpopulation to the other. Average pollen dispersal distance among sampled trees within the subpopulation was ca. 240 m.

The B subpopulation occurs at a greater spatial scale across multiple land use types compared to subpopulations A and C (Figure 2). The furthest pollen dispersal event in the B subpopulation was roughly 10 km from the rocky area to a maternal tree in Mbodi Tsha Fhasi village (Figure 16, Figure 17d), with an average of 1.2 km pollen dispersal distance within subpopulation B. All subpopulation B maternal trees received pollen from pollen donors outside of their six nearest trees, i.e., pollen did not exclusively come from trees within 200 m of each maternal tree, with many receiving pollen beyond their six nearest trees. Despite being relatively distant from the rest of the B subpopulation, the Rocky (BR) area still contributed pollen and received pollen from other trees in subpopulation B. Maternal Village tree, BV3, had only one potential pollen donor from within the village compared to 11 pollen donors from the Rocky, Plains and Fields B subpopulations (Figure 17e).

Maternal trees from the Plains land use type (BP5 and BP6) also showed high pollen acceptance from other pollen donors in the B area (Figure 17f & g). The Field area (BF) has the highest number of genotyped potential pollen donor trees. Despite the high number of potential pollen donors in the Fields, offspring still presented alleles from paternal trees from other areas in the B subpopulation, including the Rocky area (Figure 18). A wide canopy, producer tree donated pollen to both BF6 and BF3 shared a pollen donor tree to a 99.9% C.I.. Maternal Tree BF4 occurs on the outer edges of the Field area, with many pollen donors (8 of 13 potential pollen donors) coming from other areas in the B subpopulation (Figure 18j).

In subpopulation C, three maternal individuals with a total of 37 offspring were found to have alleles from 13 pollen donors (Figure 19). The furthest dispersal of alleles in subpopulation C was roughly 600 m, across the village from the only paternal tree found at 99.9% C.I. to maternal tree CV5 (Figure 19n). This donor tree is the largest genotyped tree, in terms of canopy volume, that occurs in subpopulation C and is a poor producer. Pollen dispersed an average of 300 m between individuals in the C subpopulation. The only selfing event across all subpopulations was recorded in tree CV3.

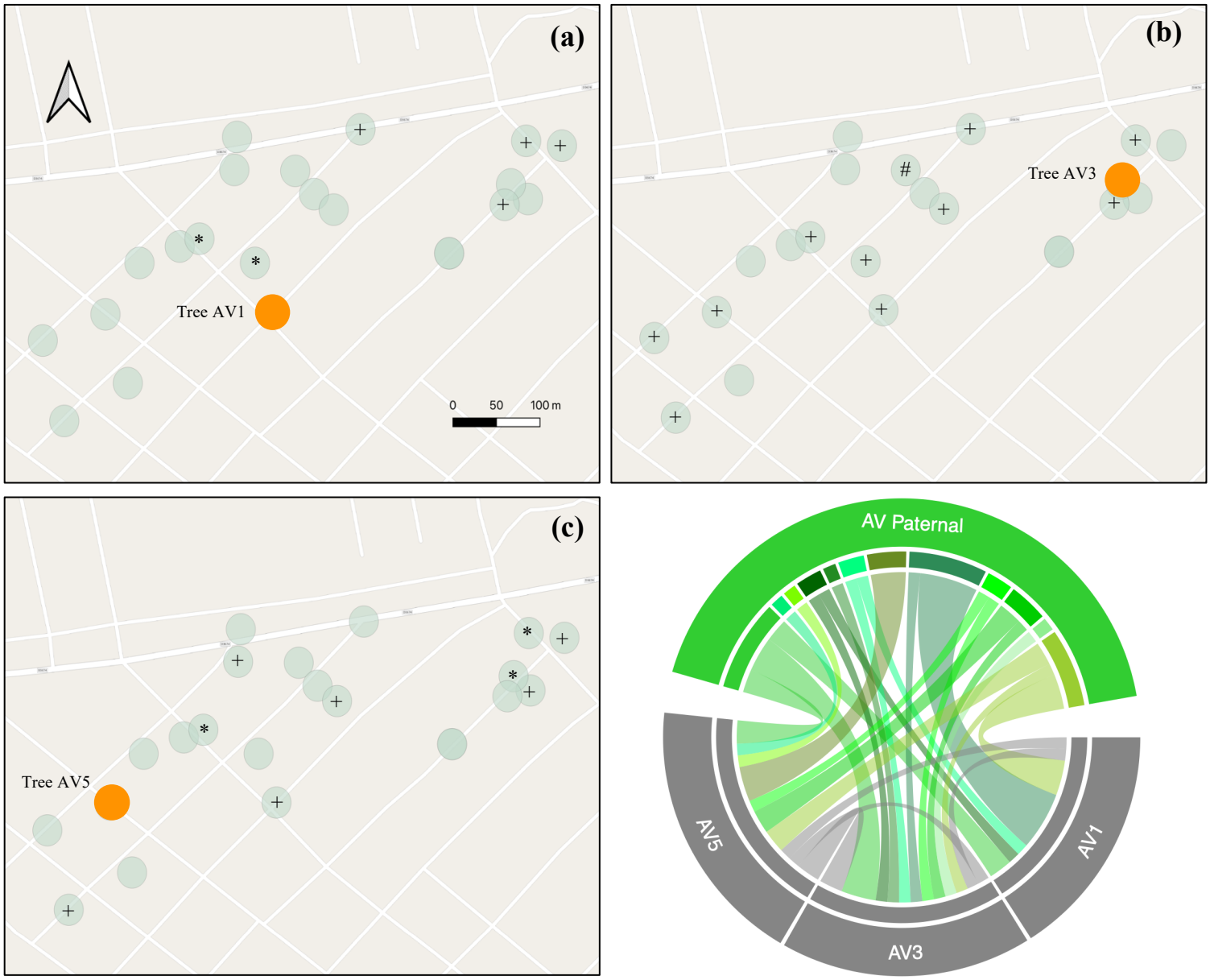


Figure 15: Distribution of genotyped pollen donors from the *Adansonia digitata* A subpopulation in Muswodi village, northern Vhembe, South Africa. Potential pollen donor trees are shown as transparent green circles, and selected maternal trees (AV1, AV3 and AV5) are shown as solid orange circles in panels (a), (b) and (c). The probability that a pollen donor sires offspring of maternal trees at a 95% C.I. is shown with a plus (+), at a 99% C.I. shown with an asterisk (*) and at a 99.9% C.I. shown as a hash (#). Trees without symbols were at a < 95% C.I.. Chord diagram (d) showing allele movement network of the A subpopulation where 'AV Paternal' (green) shows identified pollen donors contributing to offspring from three maternal (grey) trees. Each segment in the inner circle is an individual; the width of the segment correlates with the proportion of seeds from a maternal tree sired by a given paternal donor, the larger the segment, the more seeds a paternal tree sired in maternal fruit.

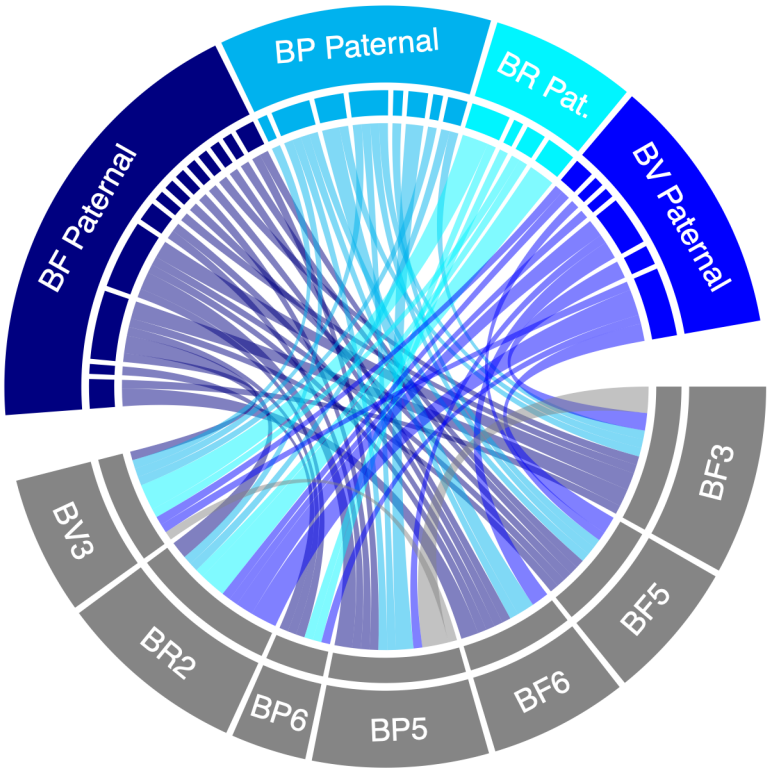


Figure 16. Chord diagram of the allele movement network where paternal subgroups are based on land use types: Field (BF), Plains (BP), Rocky (BR), and Village (BV) in different shades of blue. Each of the seven grey segments are maternal trees, to which alleles were dispersed to from adjacent coloured segments. Each segment in the inner circle is an individual; the width of the segment correlates with the proportion of seeds from a maternal tree sired by a given paternal donor, the larger the segment, the more seeds a paternal tree sired in maternal fruit.

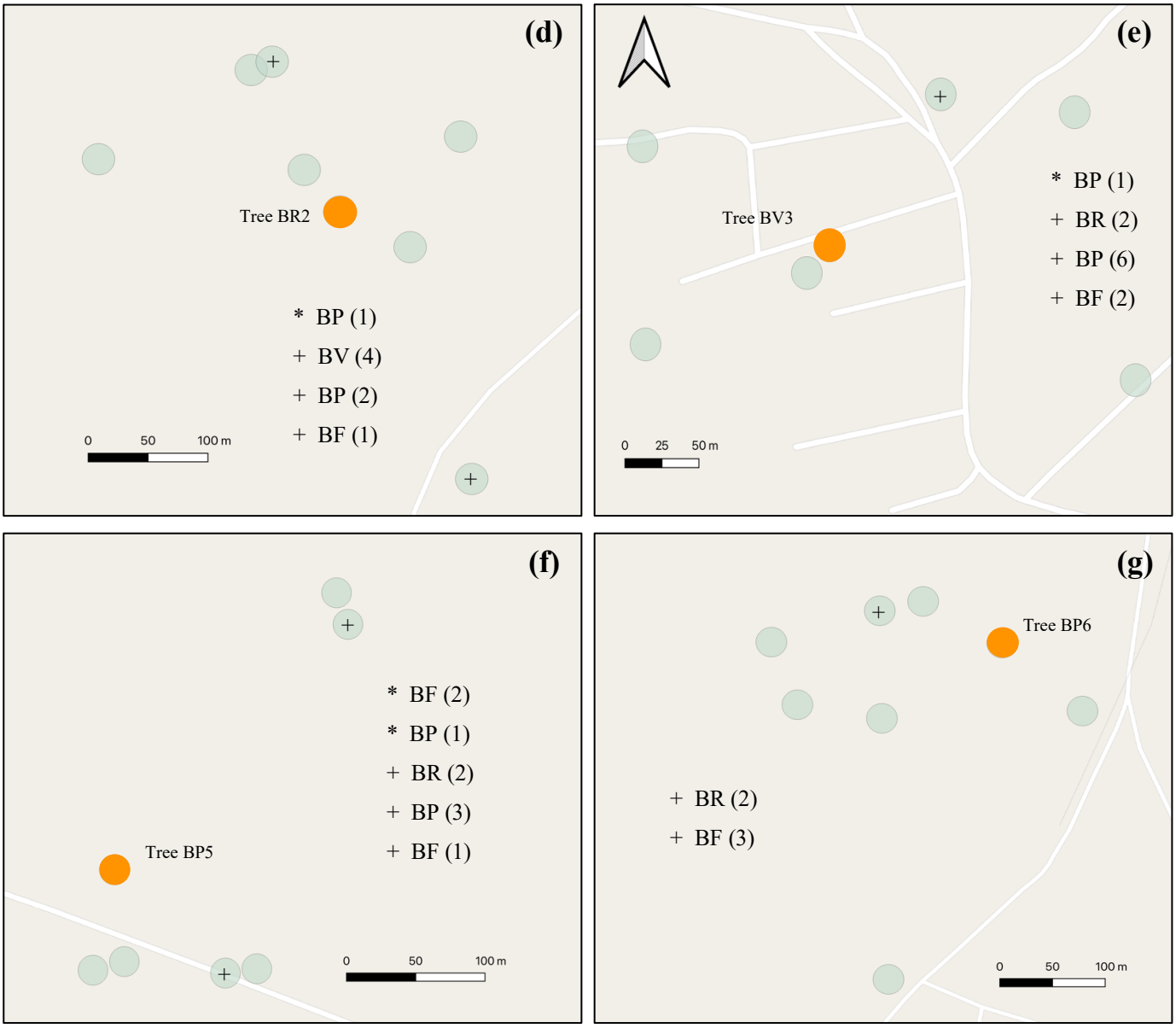


Figure 17. Distribution of genotyped pollen donors from the B subpopulation Rocky (BR), Village (BV) and Plains (BP) *Adansonia digitata* in northern Vhembe, South Africa. Potential pollen donor trees are shown as green transparent circles and selected maternal trees (BR2, BV3, BP5 and BP6) are shown as orange solid circles in panels (d), (e), (f), and (g). Each panel contains a list of additional pollen donors from other 'B' land use types with the number in parentheses indicating the number of potential fathers from each. The probability that a pollen donor sires offspring of maternal trees at a 95% C.I. is shown with a plus (+), at a 99% C.I. shown with an asterisk (*) and at a 99.9% C.I. shown as a hash (#). Trees without symbols were at a < 95% C.I..

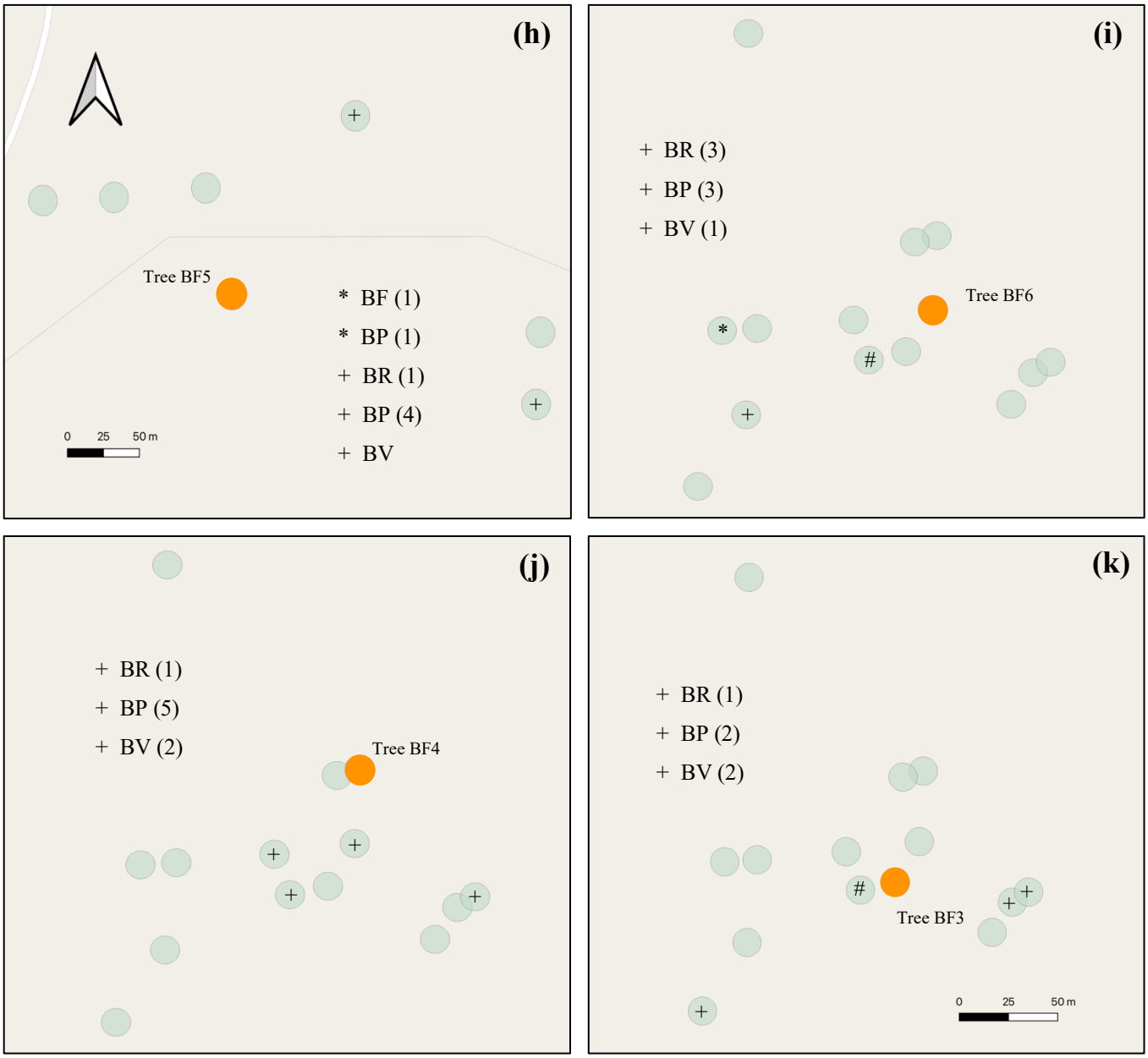


Figure 18. Distribution of genotyped *Adansonia digitata* pollen donors from the 'B' Field land use type occurring in northern Vhembe, South Africa. Potential pollen donor trees are shown as green transparent circles, and selected maternal trees (BF5, BF6, BF4 and BF3) are shown as orange solid circles in panels (h), (i), (j), and (k). Each panel contains a list of additional pollen donors from other 'B' land use types with the number in parentheses indicating the number of pollen donors from each. The probability that a pollen donor sires offspring of maternal trees at a 95% C.I. is shown with a plus (+), at a 99% C.I. shown with an asterisk (*) and at a 99.9% C.I. shown as a hash (#). Trees without symbols were at a < 95% C.I..

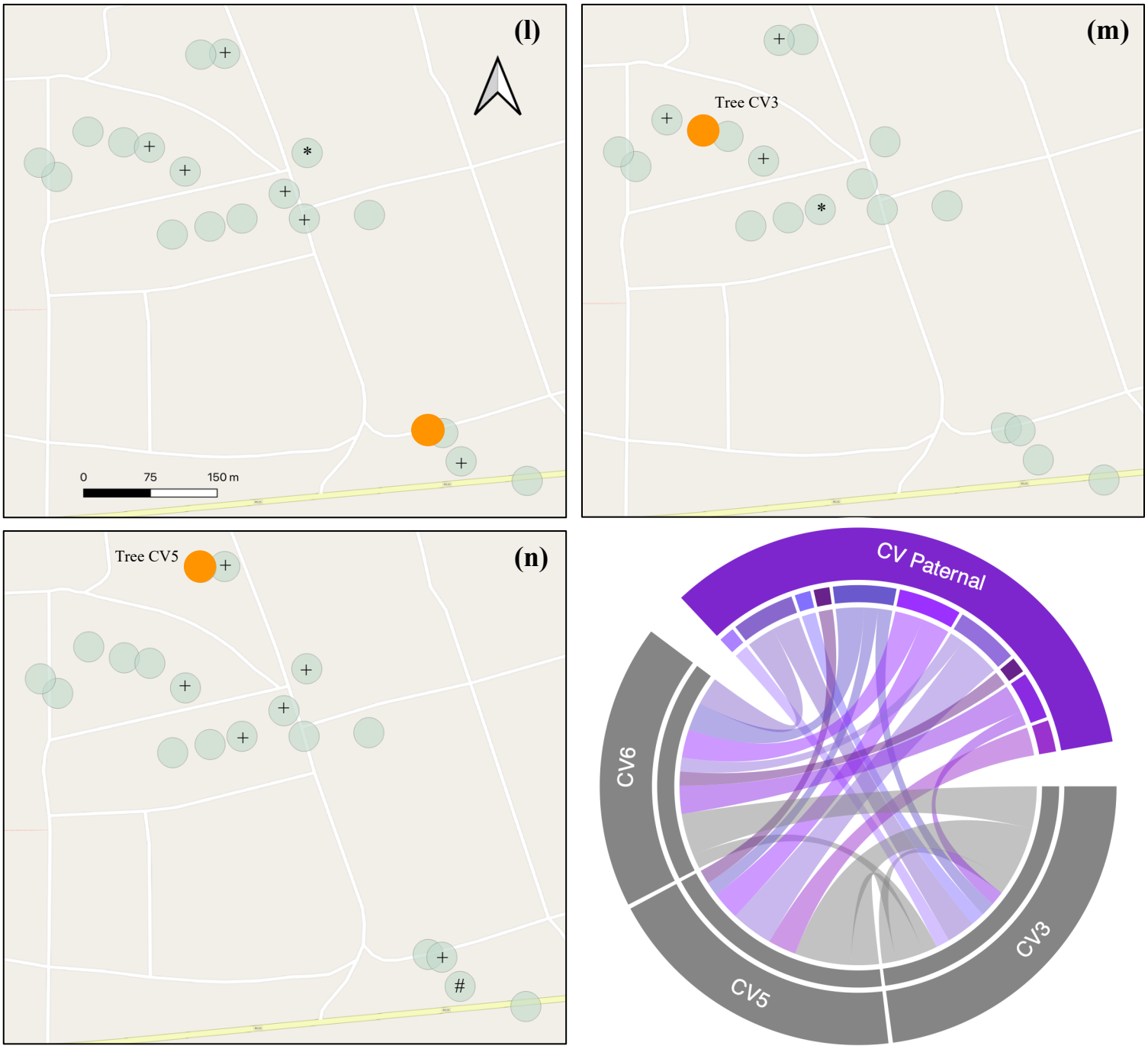


Figure 19. Distribution of genotyped pollen donors from the 'C' *Adansonia digitata* subpopulation occurring in Tshikuyu village, northern Vhembe, South Africa. Potential pollen donor trees are shown as green transparent circles, and selected maternal trees (CV6, CV3 and CV5) are shown as orange solid circles in panels (l), (m) and (n). The probability that a pollen donor sires offspring of maternal trees at a 95% C.I. is shown with a plus (+), at a 99% C.I. shown with an asterisk (*) and at a 99.9% C.I. shown as a hash (#). Trees without symbols were at a < 95% C.I.. Chord diagram (o) showing allele movement network of the C subpopulation

where ‘CV Paternal’ (purple) shows identified pollen donors contributing to offspring from three maternal (grey) trees. Each segment in the inner circle is an individual; the width of the segment correlates with the proportion of seeds from a maternal tree sired by a given paternal donor, the larger the segment, the more seeds a paternal tree sired in maternal fruit.

5. Discussion

Floral morphological differences exist between producer and poor producer baobab trees in the Vhembe region of South Africa. The data show that poor producers prioritise androecium features, with larger stamen balls, longer filaments, and larger anthers, compared to producers. In contrast, producer morphology appears to prioritise gynoecium features, having longer emergent styles. These data agree with Chetty et al.'s (2021) findings, i.e., fruit-producing trees generally had more functionally female flowers and poor fruit producing trees had more functionally male flowers, and they suggested a possible shift in sex allocation occurring in *A. digitata* (along a continuum). Species that exhibit dioecy, usually do so in response to various ecological factors, including resource limitation, sexual selection, or a difference in habitat (Bawa, 1980). Krishnamoorthy et al. (2022) found that Kenyan *A. digitata* fruiting is dependent on tree size and land use types. In southern African *A. digitata* populations, it appears environmental factors such as rainfall, soil conditions or land use types are unlikely to influence fruiting, as producers and poor producers often reside in the same land use type and do not differ in size. If environmental conditions were different, fruiting may be affected, for example, in the self-incompatible tree legume, *Prosopis glandulosa* var. *glandulosa* Torr., environmental pressures such as water stress negatively influenced fruit pod production (Lee & Felker, 1992). Additionally, this study found that fruiting patterns are unlikely to be correlated to canopy width, height, or stem diameter. There may be mechanisms acting in early development of the tree that dictate this fruit production discrepancy, but this would be challenging to study due to the long time it takes *A. digitata* to reach reproductive age, up to 200 years (S. Venter pers. comm.).

Flower recognition by nocturnal pollinators is dependent on the floral structure and the level of light scattering that occurs (van der Kooi & Kelber, 2022). In *A. digitata*, flower petal size is equally as large between tree types, which theoretically should be equally attractive to pollinators. Interestingly, peduncle length seems to be longer in producers compared to poor

producers in South African baobabs. Southern African *A. digitata* is primarily pollinated by hawkmoths (Karimi et al., 2022; Taylor et al., 2020), that hover in the air adjacent to the flower while accessing nectar. Longer peduncles allow the flowers to be held at further distances from the tree, possibly aiding in flower detection by pollinators, as there are fewer plant components to hinder visitation (Muchhala & Serrano, 2015; Schöner et al., 2016). Having an increase in visitation due to longer peduncles may increase the quantity, but also diversity, of imported pollen to producers from other trees. This would promote increased gene flow and seed set among producer trees. Another indirect strategy to increase visitation to trees is to have a larger canopy, which would have the potential to house more flowers and thus increase the chances of pollinator visitation. However, canopy size does not differ among producers and poor producers nor across the land use types.

Pollen appears to be viable for both tree types, but the proportion of viable pollen is greater in poor producers. Additionally, poor producers produced larger anthers, potentially increasing the pollen load of flowers and the quantity of pollen available to the environment. This combination of larger anthers and greater proportion of viable pollen should increase the proportion of alleles moving from poor producers to other *A. digitata* trees. The movement of pollen and its transfer onto stigmatic surfaces is dependent on pollinators, but understanding how long the pollen remains viable and its ability to germinate is equally important.

Pollen collected over the course of three days showed no external structural change, but importantly, remained viable and germinated successfully on stigmatic surfaces. In comparison, the economically important grass species (*Festuca arundinacea* Schreb., Poaceae), pollen only remains viable for up to two hours (Wang et al., 2004), whereas in orchid species of the genus *Phalaenopsis* Blume (1825) that house pollen in pollinia, pollen remains viable for up to four weeks in natural conditions (Yuan et al., 2018). Results from this study demonstrate that *Adansonia digitata* pollen may remain viable for longer than 72 hours and follow up studies should be conducted to estimate how much longer pollen remains viable in the environment. Pollen that can remain viable for long period of time is more likely to successfully pollinate following long distance dispersal (Liu et al., 2012). Flower stamen and petal sections abscise from the flower leaving only the gynoecium; viable pollen remaining on the stamen ball would

then become redundant as it has fallen to the ground, however pollen may have stuck to the bodies of pollinators prior to flower abscission. Observations of beetles, which usually visit *A. digitata* in the early morning, showed pollen scattered on their ventral sides, primarily on the thorax and densely accumulating around the coxa (proximal to the legs). Notably, pollen will remain viable for days on pollinators and, upon visitation to other flowers in subsequent nights, has the potential to successfully germinate after transfer to the stigmas. This finding explains why we see alleles shared between individuals, via pollen, that occur at distances of over 46 km apart in the paternity assessment.

Parentage analyses on *A. digitata* are limited by the longevity of individuals, their autotetraploid nature and their extensive population and range. Potential pollen donors outside of the sampled population may have contributed pollen to maternal individuals, but analyses are based on a best fit approach given individual genotypes and focused on surrounding focal trees of previously studied maternal trees. The paternity analysis suggested an extensive network of allele movement across a 46-km range between the three subpopulations' sampled trees at varying confidence intervals. Extensive pollen movement may be attributed to a variety of factors, such as pollinator range and pollination success, asynchronous flowering, the long flowering season of *A. digitata*, and the varying land use types in which these trees exist. Analyses showed that pollen is coming from both producers and poor producers at a roughly 50:50 ratio. Tree types are scattered across the landscape and often the nearest trees to each maternal tree were a mix of producers and poor producers. We know that, although the proportion of viable pollen is less in producers than in poor producers, producers still produce viable pollen. Together, this may explain why we see an even ratio of producer and poor producer alleles in the offspring of the maternal plants.

Baobabs have a long flowering season, during which the trees can produce hundreds of flowers every season (Venter & Witkowski, 2019). Additionally, asynchronous flowering occurs through the season where, for example, a tree can produce a flush of 25 flowers in one night, and only a few flowers the next (pers. obs.). Temporally, this may decrease the likelihood of pollinator visitation to that tree in one night, if only a few flowers have opened. Yet, across an entire flowering season, the likelihood of pollinator visitation to a particular tree increases significantly

as the number of flowers available to potential pollinators increases. Potential factors that may decrease visitation to *A. digitata* are distractors such as cultivated or natural/wild plant species flowering at the same time that also rely on similar pollinators (co-flowering competition; Krishnamoorthy et al., 2022), or deterrents such as human activity, for example, vehicle and human movement, light sources, or the creation of chemical signals such as smoke or brewing of sweet marula beer. These distractors and deterrents may explain why we see fewer alleles moving out from the Village land use type. However, we do see some alleles from Village trees in fruit from trees outside of the Village area. A model produced by Mesgaran et al. (2017) showed that for self-incompatible species, it may be beneficial to have co-flowering competition as other plants may attract pollinators into a region to mitigate pollination limitation; changing competition to facilitation. It is important to note that in cases where there is high co-flowering competition, it may limit pollination; however, this may be mitigated by the long asynchronous flowering exhibited by *A. digitata*.

Across the land use types, the B subpopulation consists of the most natural land use types and shows high proportions of alleles shared among trees within and from this area to genotyped *A. digitata* trees in subpopulations A and C. Undisturbed land use types, such as rocky outcrops and plains/rangelands, may promote visitation as there are fewer deterrents to pollinators. The Field land use type comprises large agricultural areas, yet limited agricultural crops were growing and most of the area was bare soil during this study. Had there been crops grown by communities, such as maize, they are unlikely to influence hawkmoth visitation of baobab trees in the land use type, as these crops are not pollinated by hawkmoths. This open landscape could then have facilitated pollinator visitation as there are little to no distractors in this area. However, alleles are still present from other areas in maternal fruit set, indicating that the lack of other vegetation in the Fields may be deterring hawkmoths possibly due to increased risk of predation or other factors.

During my limited pollinator observations in the field subpopulation, I saw hawkmoths swarming in groups and entering the tree canopy, often visiting multiple flowers within minutes. They did not typically spend a long time at one tree (~10 minutes or less) foraging and quickly moved to surrounding trees. One hawkmoth was caught roosting in the canopy in the BF

subpopulation and later identified as *Nephele comma* Hopffer (1857). This species was identified during citizen science pollinator observations that determined that southern African *A. digitata* populations were primarily pollinated by hawkmoth species (*N. comma* and *Sphingomorpha chlorea* Cramer, 1777; Taylor et al., 2020). These robust nocturnal insects have been shown to travel considerable distances across varying landscapes with remarkable navigation abilities (Lundmark, 2010; Menz et al., 2022). Depending on the species, hawkmoths can travel between 400 m to 32 kilometers in one day and can facilitate long distance pollen dispersal (Bawa, 1990; Linhart & Mendenhall, 1977; Skogen et al., 2019). It is beneficial to *A. digitata* to have a pollinator that travels long distances as this may facilitate allele dispersal if population decline or isolation were to occur. This long distance dispersal of alleles may be a contributing factor, along with the long life history of the species, why only one species of baobab occurs across continental Africa, as confirmed by Cron & Karimi et al. (2016).

In this study, the low inbreeding coefficient and genetic diversity, across all alleles, shows that there are many heterozygotes in the population overall. This is an indication of outcrossing, is common for autotetraploids, and can be expected from a species that is self-incompatible (Venter et al., 2017). Although expected heterozygosity was higher than observed heterozygosity, which is usually an indication of inbreeding, observed heterozygosity estimates are relatively high and comparable to multiple studies that indicate *A. digitata* is a genetically diverse species across Africa (Assogbadjo et al., 2009; Assogbadjoa et al., 2006; Chládová et al., 2019; Kyndt et al., 2009; Venter et al., 2017). This genetic diversity may be attributed to pollen mediated gene dispersal over long distances. In a scenario where there may be a reduction in individuals, or populations become isolated, genetic diversity is likely to remain high due to this extensive pollen movement in addition to the species' autotetraploidy.

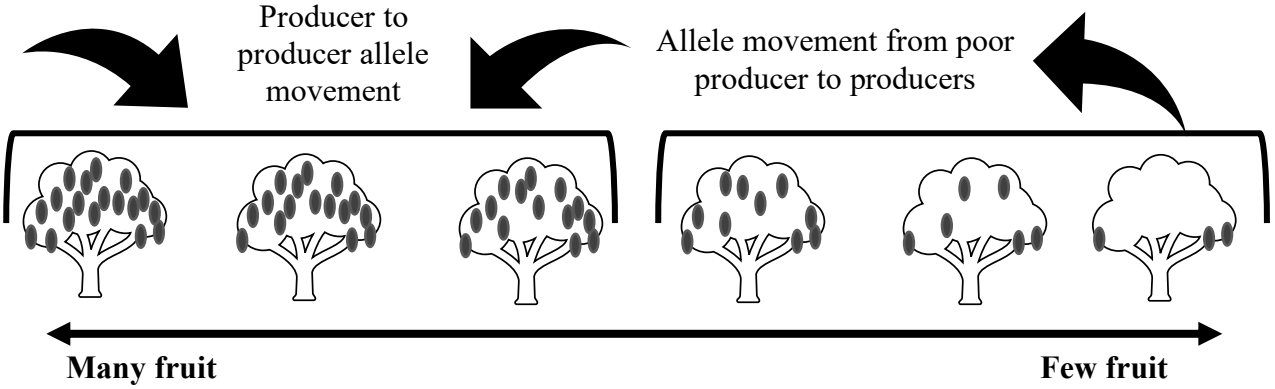
In conclusion, these data confirm that there is a shift in sex allocation between *A. digitata* trees, but there is little evidence supporting my hypotheses that poor producers may be acting as the only 'males' in the populations. Although paternal poor producers are clearly contributing pollen to producer fruit set, they are not acting alone as paternal producers are contributing an almost equal number of alleles to offspring. Nonetheless, the contribution of poor producer alleles is still essential in maintaining high genetic diversity within the baobab populations. The difference in

fruit set between trees may be attributed to the larger gynoecium structures, and longer peduncles on producer plants, making them more likely to be visited and pollinated by hawkmoths. Subsequent studies on gene expression of fruiting between individuals may shed light on molecular drivers of fruit differences among tree types, although this may be challenging due to the long time it takes for trees to begin fruiting. I show that the combination of the extensive pollinator range, pollen that remains viable for days, and the asynchronous and long flowering season, has led to extensive allele sharing among baobab trees.

Summary chart

1. Poor producer vs producer floral morphology? 2. Poor producer role in producer fruit set?

- Pollen movement detectable over 40 km region and remains viable for at least 72 hours.
- Maternal producer trees had poor producer and producer alleles present in fruit set, indicating producers are acting as both pollen donors and fruit producers in the population.



Producer

- Smaller anthers
- Longer style emergence
- Larger stigmas (Chetty et al. 2021)
- Smaller stamen balls
- Lower percentage viable pollen
- Longer peduncles

Poor Producer

- Larger anthers
- Shorter style emergence
- Smaller stigmas (Chetty et al. 2021)
- Larger stamen balls
- Higher percentage viable pollen
- Shorter peduncles

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