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Effects of ecology on *Ficus* population genetic structure in southern Africa

by

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Declaration

I, …….Junyin Deng……declare that the thesis/dissertation, which I hereby submit for the degree… PhD in Genetics ……at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: ..……..

DATE: …08/02/2020……

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Summary

South African forests have become fragmented due to both historical events and current human activities. Fragmentation may have increased population differentiation by reducing the gene flow between plants isolated in fragments. The genetic effects of fragmentation on species, especially plants, have received little attention in South Africa. Fig trees are keystone species in South African forests that provide food for many frugivores. Their population genetic structure has never been studied in South Africa. In my study, the population genetic structures of three forest fig trees (*Ficus bizanae, Ficus craterostoma* and *Ficus sur*) with different habitat specificity, geographical distribution, and level of endemicity were studied to explore the ecological factors (both non-biotic and biotic) that may contribute to their population differentiation.

First, I did a phylogeographic and population genetic study on the forest specialist *Ficus* species, *F. craterostoma*, from forests covering the species' range in South Africa. Chapter two reports this work. Three putative refugia were inferred, which played an important role in maintaining genetic diversity and facilitating recolonization by *F. craterostoma* after the LGM. In addition, comparing the genetic structures of nuclear and cytoplasmic DNA I found that nuclear DNA in *F. craterostoma* may be resistant to fragmentation while cytoplasmic elements are more vulnerable to fragmentation. This difference stems from the reduced effective population size of cytoplasmic DNA, but seed dispersal must also be limited whereas pollen dispersal, facilitated by a mutualistic pollinator fig wasp, is very extensive. In fact, a novel analysis that compared genetic variability with a combined forest size, suggested that forests within a 61 km radius of one another are genetically connected via the tree's fig wasps.

Differences in the biology of species can lead to differences in their responses to habitat fragmentation. Therefore, a comparison of genetic structure among species with different biologies may provide an understanding of the effect of fragmentation. I compared the

population genetic structures of three *Ficus* species (*F. bizanae*, *F. craterostoma* and *F. sur*) on both large and fine scales and report on these in my third and fourth chapters respectively. At a large scale, the two forest specialists have a stronger genetic structure compared to the habitat generalist (*F. sur*). Within the forest specialists, the species with a limited distribution (*F. bizanae*) showed stronger genetic structure, indicating local pollen and seed dispersal. At the fine scale, I found significant spatial genetic structure (SGS) in all three *Ficus* species, which may be due to limited seed dispersal between fragmented forests. Among them, *F. bizanae* had the highest positive kinship coefficient within a short distance as well as the highest *sp* statistic. This indicates that in addition to limited seed dispersal, there may also be limited pollen dispersal in *F. bizanae*. An increasing SGS of *F. craterostoma* was detected from saplings to adults at Ingeli forest (ING), which suggests that recent population fragmentation has stopped such long-distance immigration. Non-random mortality caused by microenvironmental selection may also contribute to the contrasting age-related fine scale SGS.

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Chapter 1. Literature review

1.1 Introduction

Population genetic structure reflects the distribution of genetic variation of a species within and between populations (Loveless and Hamrick, 1984). It indicates the genetic diversity of a species and also its potential ability to cope with environmental change (Loveless and Hamrick, 1984; Avise, 2000; 2009). Consequently, detecting genetic relationships between individuals within a population helps to estimate the sustainability of that population (Wagenius *et al.*, 2010), because inbred populations have a higher risk of local extinction (Dick *et al.*, 2008; Wagenius *et al.*, 2010; Baldauf *et al.*, 2014). The genetic structure within populations, and the factors and processes that promote population differentiation are also a fundamental part of evolutionary ecology, because of the effects of these factors on the survivability of species (Wright, 1977; Loveless and Hamrick, 1984; Bohonak, 1999).

Population genetic structure is affected by ecological factors including both abiotic (environmental changes such as geographic tectonics, climate oscillations) (Avise, 2000, 2009) and fragmentation (Young, 1996; Aguilar *et al.*, 2008) and biotic factors (including species' behavior, especially their dispersal ability) (Loveless and Hamrick, 1984; Hamrick and Godt, 1996; Galbusera *et al.*, 2004; Gaiotto *et al.*, 2003; Freeland *et al.*, 2012).

At large scale, plate tectonic processes have generated geographic barriers such as mountain ranges (Schwab *et al.*, 2012; Travers *et al.*, 2014; Nielsen *et al.*, 2018) and their associated big river systems that can restricted dispersal (Clancey, 1994; Turner *et al.*, 2016) and promote population differentiation. Quaternary climate oscillations caused periodic isolation and re-integration of populations, leading to population contractions and expansions. These oscillations may have resulted in the loss of overall

genetic diversity, but also may have promoted an increase in population genetic structure (Hewitt, 2000, 2004; Duminil *et al.*, 2015; Barnes and Daniels, 2019). Fragmentation caused by such historical paleogeographical events, or even as a result of more recent human activities (Axelrod and Raven, 1978; Berliner, 2009; Neumann *et al.*, 2010; Voelker *et al.*, 2010; Potts *et al.*, 2013) affect species' population sizes and connectivity between their populations. The resulting changes to the patterns of gene flow will affect population genetic structure (Young *et al.*, 1996; Fischer and Lindenmayer, 2007). However, species' responses to these environmental changes depend on their biological characteristics, especially their dispersal ability (Loveless and Hamrick, 1984; Hamrick and Godt, 1996; Freeland *et al.*, 2012). Thus, the interaction of biological differences between species with historical paleogeographical factors determines differences in how their genotypes are structured.

To investigate the influence of these historical paleogeographical and biological factors on plant population genetic structures we compared the population genetic structure of fig trees growing in highly fragmented forest environments. In this literature review I first summarize the factors that contribute to population genetic structure and how this population structure can be quantified. Then I briefly review the evolutionary history of forest expansion and fragmentation in South Africa; fig tree (*Ficus* spp., Moraceae) biology and population structure in general; and fig tree diversity and distribution in South Africa.

1.2 Population structure

1.2.1 Factors that affect population structure

The genetic structure of a population is determined by gene flow, drift, selection and mutation (Slatkin, 1987). Mutation is a random process that creates new genetic variation at a slow rate. Genetic drift is a process of allele frequency change due to

random sampling of gametes. It is more effective in smaller populations and ultimately results in the loss or fixation of new mutations. It is a slow process that takes in the order of 2N (effective population size) generations to fix a new mutation, i.e. the larger the effective population size, the longer it takes to become significant (Nielsen and Slatkin, 2013). Selection favours fitness-enhancing mutations and decreases frequencies of mutations that reduce fitness. As a result, selection leads to local adaptation. All three factors lead to differentiation among populations that exhibit restricted gene flow. Gene flow (the movement of gametes and/or individuals) has the effect of preventing differentiation (Slatkin, 1987; Bohonak, 1999). Therefore, the extent of genetic structure and variation across populations reflects the extent of gene flow and also effective population sizes.

Large mountain ranges or rivers that prevent dispersal can lead to population differentiation (Schwab *et al.*, 2012; Turner *et al.*, 2016). For instance, Ruiz Guajardo *et al.* (2010) found five genetic groups in *Senegalia mellifera* due to geographic barriers to gene flow created by the Kenyan Rift Valley. Geographic features are more effective barriers to dispersal for some species than others depending on their motility.

Past climate changes such as glacial cycles can affect effective population sizes by leading to population contraction and expansion (Hewitt, 2000). Any decrease in effective population size will strengthen the effect of genetic drift, and result in a greater loss of genetic diversity (Hewitt, 2000; 2004) as reflected by bottlenecks and founder effects (Yang, *et al.*, 2016). In addition, populations may retreat to different refugia during the coolest periods, leading to differentiation between isolated refugia (Hughes *et al.*, 2005; Bergh *et al.*, 2007; da Silva and Tolley *et al.*, 2017). For instance, isolation in two refugia resulted in two distinct lineages of *Juglans mandshurica* Maxim. that occur in two allopatric populations in northern China (Bai *et al.*, 2010).

Fragmentation is generated when continuous habitats become isolated into smaller units

(Fahrig, 2003). Fragmentation will reduce the effective sizes of populations and connectivity between populations (Young *et al.*, 1996; Aguilar *et al.*, 2008). Fragmentation can also change patterns of gene flow (Young *et al.*, 1996; Fischer and Lindenmayer, 2007). Theory predicts that fragmentation will lead to the loss of genetic diversity because of increased drift and inbreeding, and increases in genetic differentiation among populations because of more limited gene flow (Young *et al.*, 1996).

However, the results of these effects of fragmentation obtained from empirical studies are not always concordant with theoretical predictions, especially for plants (Hamrick, 2004; Kramer *et al.*, 2008; Vranckx *et al.*, 2012). These deviations can be mainly explained by differences in the life cycles, mating systems, rarity statuses and gene dispersal syndromes of different species. As a result species differ in their sensitivities and responses to habitat fragmentation (Kramer *et al.*, 2008).

Long-lived species are thought to be less affected by fragmentation than short-lived species (Young *et al.*, 1996; Hamrick, 2004; Kramer *et al.*, 2008). Exposure of relatively fewer generations to the effects of fragmentation results in lower negative genetic effects in long-lived species (Hamrick, 2004). In addition, due to the time lag between habitat fragmentation and loss of genetic variation (genetic extinction debt), recent fragmentation will be barely detectable in long-lived species (Vranckx *et al.*, 2012).

The relative vulnerability of self-incompatible species to fragmentation is debatable. Kramer *et al.* (2008) proposed that self-incompatible species have high genetic diversity within populations through outcrossing. They avoid close inbreeding and hence inbreeding depression through the avoidance of selfing; over longer time periods biparental inbreeding can nonetheless reduce fitness (Kramer *et al.*, 2008). However, Heinken *et al.* (2013) found self-incompatible species are more vulnerable to fragmentation than self-compatible species in terms of both genetic diversity and

reproductive fitness. Their findings suggested that the self-incompatibility that prevents selfing can also result in pollen limitation in small and isolated populations.

Rare species are usually characterized by narrow geographic ranges, restricted habitat specificities, or small local population sizes, which usually have low genetic variation (Ellstrand and Elam, 1993; Harnik *et al.*, 2012). Whether rare species are more vulnerable to fragmentation is also debatable. Leimu *et al.* (2006) found that genetic variation of rare plant species was reduced more in small populations than that of common species, but a meta-analysis by Honnay and Jacquemyn (2007) found the opposite. They emphasised that it is necessary to distinguish between species that became rare recently due to environmental or other changes and species that are naturally or historically rare. The former group is more sensitive to fragmentation and easily loses genetic diversity when their population size decreases dramatically. The latter group copes better with fragmentation due to their evolutionary history. Through generations of purging of deleterious mutations, they have become adapted to inbreeding, thus weakening inbreeding depression (Heinken *et al.*, 2013). In addition, they may also evolve long-distance dispersal that mitigates the negative genetic effects of small population size (Heinken *et al.*, 2013).

In fragmented habitats, plants maintain their genetic connectivity via gene flow that depends on vectors such as wind or animals (Kramer *et al.*, 2008). Pollen and seed dispersal vectors affect the spatial gene flow of plants, thus shaping the spatial genetic structure of the plant populations (Bacles and Jump, 2011). Fragmentation will reduce population sizes within populations and increase the geographical distance between populations (Young *et al.*, 1996, Ghazoul, 2005). This can indirectly change the behavior of pollen and seed dispersal vectors, and further affect a plant's spatial genetic structure (Young *et al.*, 1996; Ghazoul, 2005; Vranckx *et al.*, 2012).

The effects of fragmentation on pollination depend on how pollen vectors are affected

(Ghazoul, 2005; Vranckx *et al.*, 2012). For animal pollinated species, it may reduce the diversity of pollinators and may even completely disrupt their plant-pollinator interactions (Ghazoul, 2005; Vranckx *et al.*, 2012). This will reduce pollination efficiency and reduce outcrossing rates, leading to the loss of genetic diversity (Vranckx *et al.*, 2012). Furthermore, fragmentation can also change the pollinators' foraging behavior and increase more local pollination events, especially for weak flying vectors such as some insects and small birds. For example, short distance pollen flow in *Cabralea canjerana*, which is pollinated by a small moth, was increased in a fragmented Brazilian Atlantic forest because fragmentation restricted the pollinators' movements (de Oliveira Melo and Franceschinelli *et al.*, 2016). For wind pollinated species, fragmentation may reduce pollination efficiency by increasing the distances from pollen sources (Vranckx *et al.*, 2012; Heinken *et al.*, 2013). If most pollination takes place within populations, small population size will also reduce the number of pollen donors, increasing selfing and inbreeding and resulting in the loss of genetic variation (Albaladejo *et al.*, 2012; Breed *et al.*, 2012; Heinken *et al.*, 2013).

Like pollen dispersal, the effects of fragmentation on seed dispersal also varies from species to species, due to their different seed dispersal vectors (Henle *et al.*, 2004). In general, fragmentation can result in shortened seed dispersal by reducing movement of animal vectors (Sebbenn *et al.*, 2011) and it may be more serious in species where small arboreal, terrestrial or weak flying species are the vectors (Kramer *et al.*, 2008). Limited seed dispersal increases fine scale genetic structure and also reduces genetic diversity in newly recruited populations such as seedlings or saplings (Hardy *et al.*, 2006; Noreen *et al.*, 2016). This is especially true for low density populations (Ismail *et al.*, 2012).

1.2.2 Markers for detecting population structure

Both pollen and seed gene flow can affect the population genetic structure of plant species, therefore, markers that can track both pollen and seed dispersal should be

considered. In plants, markers from cytoplasmic and nuclear genomes are maternally and biparentally inherited respectively and are thus widely used to detect population genetic structure in angiosperms (Petit *et al.*, 2005; Avise, 2009).

Plants contain two circular cytoplasmic genomes, which are mitochondria (mtDNA) and chloroplasts (cpDNA) (Petit and Vendramin, 2007; Avise, 2009). Both mtDNA and cpDNA are highly variable in size, ranging from about 20- 2500 kb and 120-127 kb across species, respectively (Petit and Vendramin, 2007; Avise, 2009). They have a lower mutation rate than animal mtDNA. In plants, the mutation rate of cpDNA is about 3 to 4 times faster than mtDNA, which makes it useful and popular to estimate intraspecific phylogeographic relationships in plants (Petit and Vendramin, 2007; Avise, 2009). Some cpDNA regions, such as non-coding regions (intron trnL and inter spacer between 3' exon of trnL and trnF), have unusually high mutation rates (Taberlet *et al.*, 1991; Baraket *et al.*, 2010) and are thus frequently used for phylogenetic inference at the intraspecific level (Avise, 2009). Furthermore, the fact that it is non-recombining and normally maternally inherited and only transmitted through seed dispersal in angiosperms (Rajora and Dancik, 1992; Dumolin *et al.*, 1995; Gao *et al.*, 2019) make cpDNA valuable for tracking colonization routes in plants (Heuertz *et al.*, 2004; Bai *et al.*, 2010; Fu *et al.*, 2018; Urbaniak *et al.*, 2018; Pettenkofer *et al.*, 2019).

Nuclear markers can be divided into three classes depending on the technology employed: hybridization-based markers such as restriction fragment length polymorphisms (RFLP), PCR-based markers for example random amplification of polymorphic DNAs (RAPDs), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSR), and sequence-based markers, for instance, single nucleotide polymorphisms (SNP) (Varshney *et al.*, 2007). Among these, microsatellites (SSR) are widely used for estimating population structure (Varshney *et al.*, 2005; Selkoe and Toonen, 2006; Kalia *et al.*, 2011). Microsatellites are also known as simple sequence repeats (SSR), short tandem repeats (STR) and variable number tandem

repeats (VNTR) (Kalia *et al.*, 2011). They contain tandem repeated motifs of 1-6 nucleotides and are common in the nuclear genomes of eukaryotes (Buschiazzo and Gemmell, 2006; Selkoe and Toonen, 2006; Bhargava and Fuentes, 2010). Microsatellites are widely used for fingerprinting, parentage analyses and genetic mapping and they are also the most popular markers for population genetic studies due to their selective neutrality, co-dominance and high mutation rates that result in high polymorphism (Selkoe and Toonen, 2006; Bhargava and Fuentes, 2010; Guichoux *et al.*, 2011). They can sometimes be linked to genes so that they can be affected by selection, but normally they are not transcribed and are neutral (Putman and Carbone, 2014). The fact that they are co-dominant also means that both parents' genetic contributions can be seen. However, null-alleles can result in one allele not amplifying (Selkoe and Toonen, 2006; Putman and Carbone, 2014). Due to repeats, strand slippage can occur resulting in a mutation rate that is frequently orders of magnitude higher than point mutations (Selkoe and Toonen, 2006; Kalia *et al.*, 2011). Because of its high variation, the primer regions may be not conserved and fail to amplify across different taxonomic groups (Selkoe and Toonen, 2006). Therefore, SSRs markers usually are required to be designed denovo for each species (Selkoe and Toonen, 2006). However, microsatellite loci have now been isolated and developed in many species (Giraldo *et al.*, 2005; Caruso *et al.*, 2010; Ikten *et al.*, 2018). Some of these primers transfer to other species within the same genus (Giraldo *et al.*, 2005; Caruso *et al.*, 2010; Ikten *et al.*, 2018). As a result, a large microsatellite database is available to screen a novel species, and this simplifies their use in population genetic studies.

1.2.3 Methods for estimating population structure

Population genetic structure reflects the distribution of genetic variation among and within populations. Depending on the scale of the analysis, two approaches are common, regional and fine scale. Fine scale population structure is also referred to as spatial genetic structure (SGS). Regional scale studies focus on differentiation among

populations, while fine scale studies focus on the genetic relationships of individuals within a population.

Methods for detecting population genetic structure vary according to the scale of the study. Genetic structure among populations is detected with two main types of approach, descriptive statistics and clustering methods (Pritchard *et al.*, 2000; Putman and Carbone 2014). Fine scale analyses within populations rely on spatial autocorrelation (Sokal and Oden, 1978; Epperson and Li, 1996; Smouse and Peakall, 1999; Vekemans and Hardy, 2004). In this section, I provide a brief introduction to these approaches to the quantification of among and within population genetic variation.

1.2.3.1 Among population structure

Descriptive statistics

Wright proposed *F*-statistics to distinguish the effects of inbreeding and population subdivision on genotype frequencies. Three parameters, F_{ST}, F_{IT}, and F_{IS}, describe genotype frequencies and they are related to each other in the manner: $1-F_{IT} = (1-F_{IS})(1-F_{ST})$, where F_{IS} indicates the correlation between gametes within an individual relative to the population to which that individual belongs (Wright, 1949; 1965). F_{ST} and F_{IT} represent the correlation between gametes chosen randomly from within the subdivisions and within an individual respectively, relative to the total population. If populations are panmictic within each population $F_{ST} = F_{IT}$, since F_{IS} is equal to zero. If random mating occurs among the subdivisions, then $F_{ST} = 0$. It will increase as it departs from random mating (Wright, 1949; 1965). Therefore, F_{ST} is used to measure differentiation among subpopulations as it is not affected by inbreeding within populations. According to Wright (1978), when $F_{ST} < 0.05$, $0.05 < F_{ST} < 0.15$, $0.15 < F_{ST} < 0.25$, and $F_{ST} > 0.25$ this indicates weak, moderate, great and very great population

differentiation respectively. Wright (1965) expanded the F_{ST} definition, $F_{ST} = \frac{\sigma_p^2}{\bar{p}\,(1-\bar{p}\,)}$, where $\bar{p}$ and σ_p^2 are the mean and variance of allele frequencies in subpopulations. It can be interpreted as the proportion of genetic variation found between subpopulations at a locus, and allows the convenient estimation of F_{ST} from marker data in natural populations.

However, Wright's *F*-statistics are difficult to deal with when there are multiple alleles and unequal sample sizes, because it was derived for biallelic data and assumes infinite numbers of subpopulations. Therefore, lots of estimators of F_{ST} or *F*-statistics has been developed to address those limitations (Weir and Cockerham, 1984; Balding, 2003). Weir and Cockerham's θ is calculated using an analysis of variance (ANOVA) of allele frequencies and addresses unequal sample sizes during the variance estimation (Cockerham, 1973; Weir and Cockerham, 1984). It is a widely used F_{ST} estimator (Holsinger and Weir, 2009).

Nei (1973, 1977) defined another F_{ST} estimator, G_{ST}, (G_{ST} =(H_T-H_S)/ H_T), where, H_T and H_S represent the expected heterozygosity within subpopulations and the total population respectively. It can be interpreted as the proportional decrease in heterozygosity of subpopulations relative to that of the total population, estimated assuming Hardy-Weinberg equilibrium. Nei's G_{ST} can be applied for a large number of loci among a finite number of populations (Nei, 1973; 1977).

In order to identify hierarchical subdivision of haplotypic diversity, Excoffier *et al.*, (1992) defined ϕ_{ST} using analysis of molecular variance (AMOVA) to estimate variance components at different levels (Excoffier *et al.*, 1992; Meirmans and Hedrick, 2011; Wang, 2015).

Both G_{ST} and F_{ST} estimation rely on heterozygosity and will underestimate population differentiation when the mutation rate is high (such as in microsatellite loci) (Meirmans,

2006; Meirmans and Hedrick, 2011). Hedrick suggested standardizing G_{ST} (G'_{ST}) to decrease the within population diversity limits by dividing by the maximum value of G_{ST} (Hedrick, 2005). Similarly, Meirmans (2006) suggested a standardized genetic differentiation of F_{ST} (F'_{ST}) using the same method proposed by Hedrick (2005). An advantage is that the standardized parameters are suitable for comparing genetic differentiation between different organisms with different effective population sizes and among markers with different mutation rates (Meirmans and Hedrick, 2011).

F_{ST} and its related estimators such as θ, G_{ST} are estimated under infinite sites models, which may not be suitable for microsatellite markers. Therefore, Slatkin (1995) proposed R_{ST} to estimate population differentiation assuming markers following a stepwise mutation model, which is calculated from the variances of allele size and independent of within-population variation. However, many microsatellite loci do not follow the stepwise mutation model especially with regards to allele-size boundaries (Willems *et al.*, 2016), and as a result the estimation of R_{ST} is error prone (Balloux and Goudet, 2002).

Jost (2008) developed Jost's D to analyze population differentiation based on the effective numbers of alleles present. It reaches its maximum value if and only if the demes share no alleles. Unlike F_{ST}, which measures deviations from panmixia, D estimates deviations from total differentiation (Whitlock, 2011). His method tries to improve the population differentiation estimation for G_{ST} and F_{ST} estimators, which are very dependent on within-population gene diversity that leads to the underestimation of population differentiation for highly variable loci when migration is low (Hedrick, 1999; Balloux *et al.*, 2002; Jost, 2008; Ryman and Leimar, 2008). However, Whitlock (2011) argued that D is insensitive to any population genetic process when mutation rates are higher than migration rates. Furthermore, D cannot be used to infer population demography as it is specific to the locus being measured (Whitlock, 2011).

Clustering methods

Descriptive statistics can gauge how different populations are. In practice, knowing how many genetic groups there are, is also important to understand population genetic structure (Pritchard *et al.*, 2000; Guillot *et al.*, 2005; Jombart *et al.*, 2009; 2010; Putman and Carbone, 2014). In order to determine the number of genetic groups, clustering methods can be applied. They can be broadly divided into distance-based methods and model-based methods (Pritchard *et al.*, 2000).

In distance-based methods, a pairwise distance matrix between all pairs of individuals is calculated. Then it can be either represented using phylogenetic trees (unweighted pair group method with arithmetic mean (UPGMA) and neighbor joining (NJ)) or multidimensional scale plots (PCA, PCoA etc.). In such cases, clusters are identified by eye (Pritchard *et al.*, 2000; Jombart *et al.*, 2009). These methods depend on genetic distance measurement (Pritchard *et al.*, 2000). It is difficult to assess how confident conclusions should be as it is not possible to draw a confidence interval (CI) around each cluster (Pritchard *et al.*, 2000). Therefore, distance methods are more suitable for exploratory data analyses before model-based methods to form a prior impression of the genetic groupings in the data (Pritchard *et al.*, 2000; Putman and Carbone, 2014).

In model-based methods, each cluster is modeled by a set of parameters with X, Z and P respectively representing the genotypes of the sampled individuals, the unknown populations of origin of the individuals and the unknown allele frequencies in all populations. Then, a probability distribution of Pr (X| Z, P) is built and the parameters of interests, Z and P, is jointly inferred using statistical methods such as the Bayesian approach (Pritchard, 2000). Bayesian clustering algorithms are widely used for detecting population genetic groups and assigning individuals to each group, and are incorporated in many programs (Pritchard *et al.*, 2000; Corander *et al.*, 2003; François *et al.*, 2006; Chen *et al.*, 2007). Among them, STRUCTURE is one of the most widely

used (Chen *et al.*, 2007; Porras-Hurtado *et al.*, 2013; Putman and Carbone, 2014). It assumes Hardy-Weinberg equilibrium (HWE) within populations and linkage equilibrium between loci within populations. If these two conditions are met, the genotypes' probabilities will thus be maximized when Z and P are such that HWE and gametic linkage equilibrium are maximized (Pritchard *et al.*, 2000; Chen *et al.*, 2007; Porras-Hurtado *et al.*, 2013). The probability of the assignment of each individual genotype to its original population will be estimated (Pritchard *et al.*, 2000). STRUCTURE is a nonspatial Bayesian clustering program, and other spatial Bayesian clustering programs such as GENELAND (Guillot *et al.*, 2005) and TESS (Chen *et al.*, 2007) should be considered if the spatial distribution of each cluster needs to be incorporated.

1.2.3.2 Fine scale population structure

In Wright's isolation-by-distance model, individuals have random mating within a defined neighborhood, but dispersal limitation prevents mating outside that limit (Wright, 1943). Here, genetic similarity is expected to be higher among neighboring than among more distant individuals, resulting in spatial genetic structure (SGS) (Wright, 1943; Smouse and Peakall, 1999; Vekemans and Hardy, 2004). Spatial autocorrelation methods are also used to investigate such SGS within a population, which describe the correlation between the genetic similarity of two individuals and their spatial location within a population (Sokal and Wartenberg, 1983).

Genetic similarity can be estimated using a relatedness coefficient (Hardy, 2003). In general, there are two types of relatedness coefficient between individuals *i* and *j* (Hardy, 2003). The first one is 'kinship' or 'coancestry' coefficient F_{ij} representing the probability that a random gene from *i* is identical to a random gene from *j* and defined as $F_{ij} = (Q_{ij} - Q_r)/(1 - Q_r)$, where Q_{ij} represents the probability of identity in state of the two genes, while Q_r indicates the probability of identity in state of a

reference gene pair (Hardy, 2003; Vekemans and Hardy, 2004). The second one is a 'relationship' coefficient R_{ij} and defined as $R_{ij} = (Q_{ij} - Q_r)/(\frac{1+Q_0}{2} - Q_r)$, where Q_0 is the probability of identity in state between genes within individuals (Hardy and Vekemans, 1999; Wang, 2002, Hardy, 2003). All these parameters are related to each other. For example, in a k ploidy species, if the inbreeding index is F_I, then $R_{ij} = kF_{ij}/(1 + (k-1)F_I)$, (Cockerham, 1969; Hardy and Vekemans, 1999). Therefore, there is no need to use multiple indices to describe SGS.

Spatial autocorrelation can be visualized using spatial autocorrelogram (F(r)) and SGS by plotting the mean F_{ij} (or R_{ij}) estimates over pairs of individuals in a given distance interval r, against distance (Hardy and Vekemans, 1999). The regression slope (b) of F_{ij} on r_{ij} or $\ln(r_{ij})$ can be used for quantifying the extent of SGS, if F(r) tends to decrease linearly with r (one dimensional) or ln(r) (two dimensional) (Hardy and Vekemans, 1999; Vekemans and Hardy, 2004). In order to compare the extent of SGS among populations or species, Vekemans and Hardy (2004) proposed the '*Sp*' statistic ($Sp = -\frac{b}{1-F_{(1)}}$), where $F_{(1)}$ is the mean F_{ij} between pairs of individuals from the first distance class that does not sensitive to sampling scheme.

One of the major applications of spatial autocorrelation is indirectly inferring gene dispersal distances (Heuertz *et al.*, 2003; Hardy *et al.*, 2006; Wang *et al.*, 2009). When a population reaches migration-drift equilibrium, at a distance $\sigma \sim \frac{0.5\sigma}{(2\mu)^{1/2}}$, where σ is the average axial parent-offspring distance, the expected kinship coefficient between individuals decrease linearly with $\ln(r_{ij})$ at a rate of $b \cong -(1 - F_I)/(4\pi D\sigma^2)$, where D is the population density, F_I is the kinship coefficient between neighboring individuals (Heuertz *et al.*, 2003; Hardy *et al.*, 2006; Vekemans and Hardy, 2004).

An important approach is to see if differences in SGS are linked to a species' biology by comparing SGSs between two specific groups (De-Lucas *et al.*, 2009; Wang *et al.*,

2011; Nazareno *et al.*, 2013; Heer *et al.*, 2015). For example, in *Ficus* species, monoecious species are said to have longer pollen dispersal distances than dioecious species, and by comparing the SGSs of plants with these two breeding systems it can be seen if this is the case (Nazareno *et al.*, 2013).

1.3 Evolutionary history of forests in South Africa

While forests only cover approximately 0.41% of the land surface in South Africa, this habitat plays an important role by contributing disproportionally to the country's biodiversity (Berliner, 2009). Forests in South Africa have varied in area coverage and continuity through time and have become fragmented due to historical tectonic activities, climate oscillations and recent human activities (Axelrod and Raven, 1978; Berliner, 2009; Neumann *et al.*, 2010; Voelker *et al.*, 2010; Potts *et al.*, 2013). This is the case for the forests occurring along the whole eastern side of Africa that are constrained to the south or east of an escarpment that runs inland paralleling the southern and eastern coastlines.

Mucina and Geldenhuys (2006) identified 26 forest types in eight zones (southern and northern Afrotemperate forest, southern and northern Mistbelt forest, Scarp forest, southern and northern Coastal forest and Tropical dry forest group) and one azonal group based on the species composition of South African forests' floral communities. Lawes *et al.* (2007a) later simplified the classification of forests into three groups, namely Afrotemperate forests (Afrotemperate and mistbelt forests of Mucina and Geldenhuys (2006)), scarp forests (scarp and some of the northern coastal forests of Mucina and Geldenhuys (2006)), Indian Ocean coastal belt forests (most of the northern Coastal, Sand, Swamp and Mangrove forests of Mucina and Geldenhuys (2006)). In order to keep the classification manageable, I will hereafter use the three groups of Lawes *et al.* (2007a).

Ancient uplifting and warping facilitated formation of big internal mountain ranges and basins, and changed the geographic features of South Africa since Tertiary (Van Zinderen Bakker, 1983). This increase in presence of basins and mountain ranges facilitated expansion of arid areas, which split the previously continuous forests (Afrotemperate forests) (Sepulchre *et al.*, 2006). Quaternary climatic oscillation resulted in several glacial-interglacial cycles during a window period of at least two million years (Lawes, 1990). Alternating glacial-interglacial cycles led to forest contraction and expansion (Lawes, 1990; Lawes *et al.*, 2007a). During the Last Glacial Maximum (LGM) (18kyr BP), the Afrotemperate forests shrank dramatically and most fragments were eliminated (Lawes *et al.*, 2007a). Remnants were maintained in the original lowland Afrotemperate forests and fragments slowly migrated to Scarp forests close to the coast. Lawes (1990a) hypothesised that the forests that remained during the LGM became refugia for many plants and animals. After the LGM, about 14-12 kyr ago, the climate became warmer and humidity increased, favoring forest expansion (Geldenhuys, 1992; Eeley *et al.*, 1999). Indian Ocean coastal belt forests formed around the same time (8000 yr BP) through expansion from the east African coastal forests southward via the Mozambican coast to northern KwaZulu-Natal after the drop in sea level. These historical changes split species lineages, potentially promoted speciation and facilitated the formation of current forest biodiversity in South Africa, patterns which are reflected by many phylogeographic studies (Fjeldså and Bowie, 2008; Tolley *et al.*, 2011).

Recent human activities, including grazing, logging and agriculture have artificially increased forest fragmentation in South Africa (Hoffman, 1997; Lawes *et al.*, 2004; 2007b). Other disturbance such as drought and anthropogenic fires have compounded forest loss and degradation (Geldenhuys, 1993; 1994). The recent degradation of forests is reflected in the loss of small patches, size decrease of large patches, increase of edge effects, increased isolation among forest patches, a changed matrix surrounding forests,

and altered microclimate of the habitat (Berliner, 2009). These changes increase the risk of extinctions and may have decreased biodiversity in South Africa (Lawes, 2004; Lawes *et al.*, 2005; Wethered and Lawes, 2005; Fonseca, 2009).

Various studies have found either no (Berens *et al.*, 2014), or harmful (Lawes, 2004; Cooper *et al.*, 2017) ecological effects of fragmentation on species. Berens *et al.* (2014) found no significant effect of fragmentation on plant herbivorous communities (birds) in South Africa, which may be due to high fruit availability still remaining in fragments. In contrast, Lawes (2004) found that blue monkeys (*Cercopithecus mitis* Wolf) disappeared from smaller forest patches (<150ha) in South Africa. Similarly, a loss in overall bird diversity, especially of forest specialists, due to local extinction has been found in fragmented South African forests (Olivier *et al.*, 2013; Cooper *et al.*, 2017).

Like ecological effects, the genetic effects of fragmentation in South Africa are also varied. For example, Matolweni *et al.* (2000) found that historical isolation had limited gene flow and caused population differentiation of *Begonia dregei* and *Begonia homonyma* in South African coastal forests. Similarly, McManus *et al.* (2015) observed a subpopulation structure with low genetic diversity of a top carnivore (leopard) in a human-dominated landscape. For the species with extremely limited dispersal, forest fragmentation directly promotes speciation (Barnes and Daniels, 2019). However, Madisha *et al.* (2018) discovered no negative genetic effect in *Cercopithecus albogularis labiatus* (Samango monkey) in Hogsback, suggesting there was still high gene flow between populations.

Based on these studies, habitat fragmentation in South Africa can lead to different ecological and population genetic effects, due to species' different responses to fragmentation (Henle *et al.*, 2004; Kramer *et al.*, 2008). It is therefore impossible to make the general conclusion that forest fragmentation will lead to negative genetic effects. Therefore, more studies are needed to understand the genetic effects of

fragmentation in South Africa. Future studies should not only focus on rare endemics or species on the top of trophic pyramids, but also focus on common species with significant roles in ecosystems.

1.4 *Ficus* biology and population differentiation

There are around 800 described *Ficus* species worldwide (Compton *et al.*, 2010), which are distributed mainly in the tropical and subtropical regions. They have diverse life forms (trees, shrubs, stranglers) and occur in various habitats (forest understories and canopies, savannahs, riversides, even deserts) (Weiblen, 2002; Herre *et al.*, 2008). They have two breeding systems, monoecy and dioecy, with the former producing seed and pollinators within the same figs, the latter producing seeds in female plants and pollinators in male plants (Weiblen, 2002; Herre *et al.*, 2008). Monoecious *Ficus* species produce fruits year-round (although some exceptions may occur such as Windsor *et al.*, 1989) in order to maintain the cycling of each species' population of pollinator wasps. This phenology and their often large crop sizes make them keystone resources in tropical forest (Nason *et al.*, 1998; Harrison, 2003). Figs are therefore important food for tropical frugivores, especially because they have high levels of calcium (O'Brien *et al.*, 1998). However, some researchers have argued that the importance of figs is not universal in Africa, because of the low population density and limited fruit production of some species (Gautier-Hion and Michaloud, 1989). The same may apply in India (Borges, 1993). That said, Bleher *et al.* (2003) found that high numbers of animals were attracted by *Ficus thonningii* (= *Ficus burkei*) in South African coastal forests due to their high overall fruit biomass, suggesting that this species could be a keystone resource for the local frugivore community.

A fascinating part of fig tree biology is their relationship with pollinator fig wasps (Hymenoptera, Agaonidae) (Weiblen, 2002; Herre *et al.*, 2008). Fig trees rely solely on tiny, short-lived fig wasps to transfer their pollen and attract them by releasing volatile

components during a receptive period (van Noort *et al*, 1989; Weiblen, 2002; Herre *et al.*, 2008). Likewise, fig wasps need fig trees to complete their life cycle (Compton, 1993). The adult fig wasps are delicate and short-lived (surviving up to 1-3 days after emerging from their natal figs), and rely on air flow for long-distance dispersal (Ware and Compton, 1994a). Gene flow of the fig wasps and also the pollen flow of their host figs is likely to be influenced by their host's life-form, which influences where the fig wasps emerge and also the extent of the attractant cues generated by individual plants (Harrison and Rasplus, 2006; Chen *et al.*, 2011).

In forests, fig wasps that are released in the canopy are expected to travel further than those released in the understory, because wind speed is higher above the canopy than in the understory (Compton *et al.*, 2000; Harrison and Rasplus, 2006; Nazareno *et al.*, 2013). Most monoecious species are either big free standing or hemi-epiphytic trees, that produce large crops. In forests monoecious species usually release their pollinators above the canopy with some exceptions such as *F. sur* and *F. bizanae* that bear their figs on the main trunk, which is in contrast to dioecious species (Nason *et al.*, 1996; Harrison, 2003). Therefore, pollinators in monoecious *Ficus* species may often disperse further than dioecious species. Both field trap work (Harrison, 2003) and a comparison of genetic structure between monoecious and dioecious species (Nazareno *et al.*, 2013) have confirmed this. In monoecious *Ficus* species, fig wasps can travel very far, over 100 km (Ahmed *et al.*, 2009), and in a typical forested area of Panama pollination distances are routinely between 5.9 and 14.2 km (Nason *et al.*, 1996). Long distance pollinator dispersal of monoecious *Ficus* species can increase the breeding population size thus maintaining genetic connectivity and reducing population differentiation (Nason *et al.*, 1998). For example, limited population differentiation was found in *F. racemosa* within continental South-East Asia (Kobmoo *et al.*, 2010), which was pollinated by a single fig wasp species spanning well over 1500 km (Kobmoo *et al.*, 2010). In addition, another population genetic study of *F. racemosa* in urban vegetation

revealed no sign of inbreeding, suggesting long distance pollen dispersal mechanisms of such monoecious fig species allow them to maintain population genetic connectivity even at such scattered and fragmented areas as within a city (Krishnan and Borges, 2018).

Pollen-mediated gene flow is just one part of the story as seed-mediated gene flow also plays an important role in shaping population structure. *Ficus* species provide food for lots of frugivores, many of which are also seed dispersal vectors that help fig trees with range expansion and colonization (Thornton *et al.*, 1996; Shanahan *et al.*, 2003). Fig trees produce fruits with different size and color, which corresponds with different seed dispersal syndromes (Shanahan *et al.*, 2001; Lomáscolo *et al.*, 2008; 2010). In general, birds are attracted by small fruits of bright color, while mammals prefer large fruits that are dull in colour and have a distinctive smell (Lomáscolo *et al.*, 2008; 2010). The diverse frugivore communities of *Ficus* species indicate variation in seed dispersal distances in the genus. Among those frugivores, strong fliers such as large birds and bats are expected to transfer seed over long distances (Shanahan *et al.*, 2001). However, limited seed dispersal distances are reported in many *Ficus* species (Zhou and Chen, 2010; Yu *et al.*, 2010; Yu and Nason, 2013). This could only be explained if local seed dispersal is fairly common in these (often small) *Ficus* species. Local dispersal would thus increase fine scale genetic structure and increase the likelihood of genetic divergence of *Ficus* species in fragmented habitats, especially of maternally inherited DNA (Wang *et al.*, 2009; Heer *et al.*, 2015; Krishnan and Borges, 2018). For instance, spatial genetic structure was found in four monoecious species over a range of 1 km, despite pollen dispersal distance in excess of 1 km, suggesting local seed dispersal (Heer *et al.*, 2015). Similarly, even in *F. racemosa,* a species that shows long distance pollen dispersal strong spatial genetic structure was found within 1 km indicating clumped seed dispersal close to the natal tree (Krishnan and Borges, 2018).

1.5 *Ficus* in Africa

There are 113 indigenous species of *Ficus* in the Afrotropical region (Burrows and Burrows, 2003; Berg and Wiebes, 1992) of which 24 *Ficus* species occur in South Africa (Table 1.1). Their distribution and habitat preference varies, with one endemic species (Table 1.1). Among the non-endemics, 19 of them have distributions extending to central Africa and beyond, two arid-adapted species have distributions restricted to South-Western Africa (*F. cordata* and *F. ilicina*), and two of them have limited southern Africa distributions (*F. burtt-davyi* and *F. tettensis*) (Table 1.1). Within South Africa, the greatest concentration of species are in KwaZulu-Natal and north through Mpumalanga Province up to Limpopo Province. Only seven species are found further south down to the Eastern Cape (Table 1.1). Two species are forest specialists (*F. craterostoma* and *F. bizanae*), one of which, *F. bizanae*, is endemic to South Africa (Table 1.1). *Ficus* species play an important role in maintaining biodiversity in South Africa as was discussed above. The forest fragmentation changes due to historical climate oscillation and current human activities in South Africa have reduced forest species' population sizes and increased among-population distances, which may have affected *Ficus* species population genetic structure. However, population genetic structure of *Ficus* species in South Africa has never been studied. We selected three species (*F. bizanae*, *F. craterostoma* and *F. sur*) with different range sizes and habitat preferences to quantify and compare their population genetic structure.

Table 1.1 *Ficus* species in South Africa (Burrows and Burrows, 2003).

Species	African distribution	Within South Africa distribution	Habitat preference
F. capreifolia Del.	A	KwaZulu-Natal, Mpumalanga, Limpopo	Sandy riverbanks; thickets of river reed or along rivers in lowland forest
F. sycomorus L.	A	KwaZulu-Natal, Mpumalanga, Limpopo	Lowland riverine forest; savanna woodland
F. sur Forssk.	A	Eastern Cape, KwaZulu-Natal, Mpumalanga, Limpopo, Gauteng	Open grasslands, mixed woodlands, forest, rocky outcrops
F. verruculosa Warb.	A	Northern KwaZulu-Natal	Swamps, riverine vegetation, coastal marshes, spring seepage zones in deciduous woodlands, and along lake shores
F. salicifolia Vahl	B	Central KwaZulu-Natal, Mpumalanga, Limpopo, Gauteng	Rocky outcrops in dry, deciduous or semi-deciduous woodlands, rarely as a riverine constituent
F. cordata Thunb.	C	Western Cape Northern Cape	Steep rocky hillsides or cliff-faces
F. ingens Miq.	A	Eastern Cape, KwaZulu-Natal, Mpumalanga, Limpopo, Gauteng	Deciduous woodland, wooded grasslands, rocky hillsides, cliff-faces, riverine forest, coastal scrub and coral-rock outcrops
F. lutea Vahl	A	KwaZulu-Natal, Eastern Cape	Coastal forest, deciduous woodland, open wooded grassland and evergreen forest
F. glumosa Del.	A	KwaZulu-Natal, Mpumalanga, Limpopo, Gauteng	Rocks, cliff-faces
F. stuhlmannii Wrab.	B	KwaZulu-Natal, Mpumalanga, Limpopo	Mixed deciduous woodland, semi-deciduous coastal scrub-forest and riverine woodland
F. tettensis Hutch.	D	Limpopo	Rock cliff or hillsides
F. abutilifolia Miq.	A	KwaZulu-Natal, Mpumalanga, Limpopo, Gauteng, North-West	Near or on Rock outcrops
F. trichopoda Bak.	A	KwaZulu-Natal	Swamps or swamp forest
F. petersii Wrab.	A	Mpumalanga, Limpopo	Warm and relatively dry, semi-deciduous woodland
F. burkei Miq.	A	KwaZulu-Natal, Mpumalanga, Limpopo, Gauteng, Eastern Cape	Dry, semi-deciduous woodland
F. thonningii complex			
F. natalensis Hochst.	B	KwaZulu-Natal, Limpopo	Woodland
F. craterostoma Midlbr. and Burret	A	Eastern Cape, KwaZulu-Nata, Mpumalanga, Limpopo	Forest specialist
F. burtt-davyi Hutch.	D	Western and Eastern Cape, KwaZulu-Nata	Coastal scrub or thicket, coastal forest, rocky outcrops in inland areas
F. ilicina Miq.	C	Northern and Western Cape	Rocky faces
F. polita Vahl	A	KwaZulu-Natal	Coastal forest and woodland, scrub-forest in ravines
F. bizanae Hutch. and Burtt-Davy	E	Eastern Cape, KwaZulu-Natal,	Forest specialist
F. sansibarica Warb.	B	Mpumalanga, Limpopo	Mixed, semi-deciduous woodland, coastal scrub forest, and low-altitude riverine forest
F. tremula Warb.	B	Northern KwaZulu-Natal	Sand forest and sand dunes
F. bubu Warb.	A	Northern KwaZulu-Natal	Woodland, forest and rocky hills

A: Distribution extends to central Africa and beyond, B: Distribution reaches to central Africa through east Africa, C: South-Western Africa – arid-adapted species, D: Very limited distribution in southern Africa, E: South African endemic

1.6 Aims of the study

In this study, I quantified the population genetic structure of three *Ficus* species, *F. bizanae*, *F. craterostoma* and *F. sur,* in order to understand the ecological factors that have shaped and continue to shape their population structure. In the next three chapters I test the following hypotheses:

1) Based on the long-distance pollen dispersal of *Ficus* species, I expect that *Ficus* may show some genetic resilience to forest fragmentation. Furthermore, in contrast to pollen dispersal, seed dispersal is expected much more local. This will result in cytoplasmic DNA (that is only maternally inherited) showing substantially more genetic structure than nuclear SSRs that are dispersed via both pollen and seed. I therefore hypothesize that SSRs will be substantially less structured than cytoplasmic DNA. The genetic variation a population can sustain depends on its size. Since pollen can link forests if they are close enough to each other, I hypothesize that the genetic variation will be best explained, not by the size of forests from which the sample came, but by a summation of forest sizes that form a genetic unit, bound by wasp-assisted gene flow. I tested these hypotheses on the forest specialist species, *F. craterostoma*.

2) Different levels of genetic differentiation and ability to adapt to fragmentation would be expected among *Ficus* species that vary in ecological characteristics such as habitat preferences and fruiting biology. For example, trees that also occupy habitats located between forest patches should have less distinct forest populations. It has been shown that dioecious *Ficus*, have more genetically structured populations because they fruit asynchronously and frequently, resulting in wasps that tend to remain locally (Nazareno *et al.*, 2013), as opposed to monecious trees whose wasps often disperse very far (Compton *et al.*, 2000 and Harrison and Rasplus, 2006). However, we can expect any monoecious species with phenological similarities to dioecious trees to be more genetically structured. The wasps of trees that produce their fruit above the forest

canopy should be more likely to be dispersed by wind currents and their populations should be less distinct. Primates and other non-volant mammals are less likely to move between forests than birds (Davies *et al.*, 2000; Henle *et al.*, 2004) and plants that rely on primates for seed dispersal should have more genetically structured populations. By comparing three *Ficus* species, *F. bizanae*, *F. craterostoma* and *F. sur* among different sites we can address many of these ideas. We hypothesize that *F. sur* (a monoecious habitat generalist that relies on birds and mammals for seed dispersal) to show the least genetic differences between sample sites located in different forests. We hypothesize that *F. craterostoma* (which is a typical monoecious species with mainly bird dispersed fruit located above the forest canopy) to show little evidence of genetic structure. Finally, we hypothesize that *F. bizanae*, (a species that fruits asynchronously bears its fruit underneath the canopy and is likely to be mainly primate dispersed) should have the highest genetic differentiation between populations.

3) Any differences in the genetic structure of the three *Ficus* species may be linked to different levels of gene flow, which can be reflected in the fine scale population genetic structure. We hypothesized that the three *Ficus* species would show different degrees of fine scale SGS, with *F. bizanae* showing the strongest fine scale SGS. Finally, any changes in gene flow over time may also translate into differences of fine scale SGS between age classes. We expected that older age classes of the same species will have higher fine scale SGS, because of recent increases in the extent of forest fragmentation.

Chapter 2. Fig wasps help their host trees cope with forest fragmentation

This is a manuscript in preparation for submission to a peer-reviewed journal

Jun-Yin Deng, Simon van Noort, Stephen G. Compton, Yan Chen, Jaco M.Greeff

My contribution

Sample collection, all the lab work, data analyses and writing of the first draft and revisions.

2.1 Abstract

Population fragmentation is a frequent consequence of climate change. We used a phylogeographic approach to investigate the genetic consequences of fragmentation for a plant species, *Ficus craterostoma*. It is pollinated by an obligate mutualist fig wasp, *Alfonsiella pipithiensis,* and birds are its main seed dispersers. We collected samples throughout the range of *F. craterostoma* in South Africa, sequenced two chloroplast gene fragments (trnL, atpF-H; referred to as cpDNA) from a total of 150 *F. craterostoma* individuals and genotyped twelve polymorphic microsatellite loci from 443 individuals. We used genetic diversity and population structure, in combination with connectivity between forest patches to infer the processes that facilitated population differentiation. We detected significant between-population differentiation for both cpDNA and nuclear microsatellites, but chloroplast DNA had lower genetic diversity and higher differentiation than nuclear microsatellites. Keep in mind that cpDNA is maternally inherited and only dispersed via seed. Eight out of 15 sample sites were fixed for single haplotypes (four with H01, two with H02, one with H05 and one with H07), suggesting that either genetic drift occurred due to small population sizes or that founder events occurred. Mutations were frequently one mutational step apart, implying recent colonization and adjacent sample sites were often fixed for the same haplotype, indicating limited seed dispersal. Microsatellite data indicated two genetic

clusters. Some sample sites contained individuals that came from different genetic groups, suggesting long-distance pollen dispersal. This conclusion is further supported by a test for connectivity indicating that fig wasps travel sufficiently to cause random mating between trees from forest patches that are 76-242 km apart. While long range pollen dispersal by the obligate mutualist fig wasp, *A. pipithiensis* renders *F. craterostoma*'s nuclear DNA resistant to fragmentation, limited seed dispersal intensifies drift and makes its cytoplasmic elements more vulnerable to fragmentation. Population isolation caused by geographic barriers such as forest gaps and rivers are likely to have shaped the genetic structure of *F. craterostoma*. Two putative refugia close to the coast and one refugium situated inland may have played an important role in maintaining genetic diversity and facilitating recolonization of *F. craterostoma* after the LGM.

2.2 Introduction

The ice ages and more recent demographic processes such as dispersal and drift have left genetic imprints on populations that we can use to reconstruct their past. Several glacial-interglacial cycles during the last two million years (Tyson, 1986) greatly affected South African forests (Lawes, 1990). Forests require moderate rainfall (Lawes, 1990), but much of South Africa is now arid or semi-arid. Consequently, forests currently cover less than 0.41% of South Africa's land surface (Tyson and Dyer, 1978; Tyson, 1991; Fauchereau *et al.*, 2003; Berliner, 2009). The areas suitable for forests are naturally fragmented and occur mostly to the south-east of an escarpment that runs along the coastline (Figure 2.1).

The plants within South African forests have been classified into 26 distinct floral communities (Mucina and Geldenhuis, 2006), within three broader groupings (Lawes *et al.*, 2007a; Figure 2.1). These comprise 1) Afrotemperate forests (which includes Mucina and Geldenhuis's (2006) Afrotemperate and mistbelt forests), 2) Scarp forests

and 3) Indian Ocean coastal belt forest (called northern coastal forests by Mucina and Geldenhuis (2006)). The histories of these forests has been inferred from their community composition (Geldenhuys, 1989; Forbes, 2003; Lawes *et al.*, 2007a), climate change models (Eeley *et al.*, 1999; Potts *et al.*, 2013; Eberle *et al.*, 2017), fossil/palynological information (Scott *et al.*, 1997), and from phylogeographic and population genetic data (Hughes *et al.*, 2005; Willows-Munro and Matthee, 2011; Dalton *et al.*, 2015; da Silva *et al.*, 2017; Stone *et al.*, 2017).

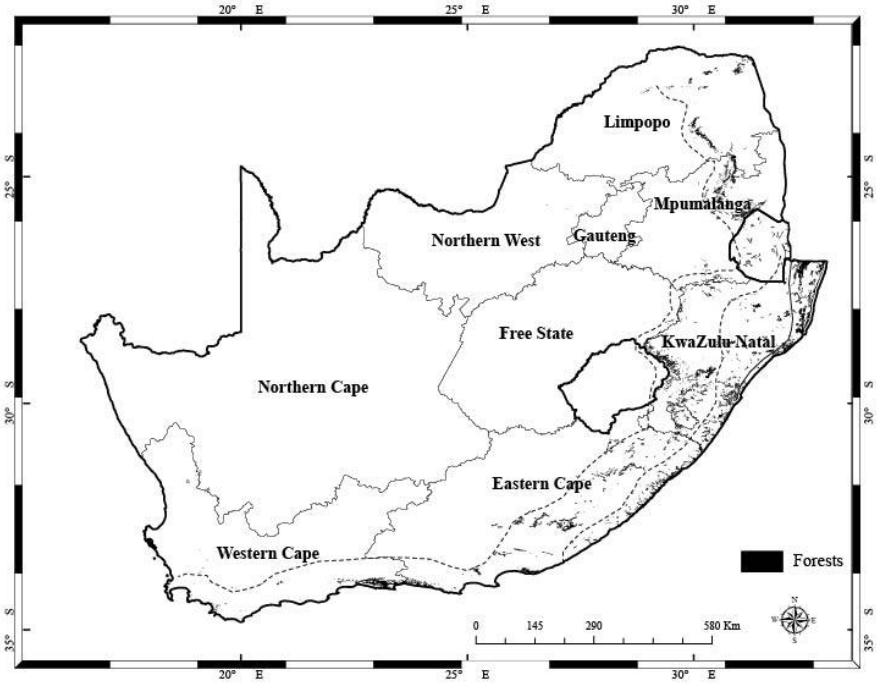

Figure 2.1 Forest locations indicated by black pixels (http://bgis.sanbi.org/vegmap 2018) throughout South Africa. Lawes *et al.* (2007a) grouped them into three broad kinds of forests - Afrotemperate forests are located between the two dashed dark grey lines (Afrotemperate and mistbelt forests according to Mucina and Geledhuis (2006)). Scarp forest occurs between the dashed dark grey line and dark grey solid line (or ocean). Indian ocean coastal belt forests are to the right of the dark grey solid line (northern coastal forest according to Mucina and Geledhuis (2006)). Bold and thin solid black lines represent state and provincial borders, respectively.

At the time of the Last Glacial Maximum (LGM; 18, 000 yr BP), Afrotemperate forests in South Africa shrank dramatically and most were eliminated (Lawes, 1990; Lawes *et al.*, 2007a). Remnants may have survived in lowland refugia close to the coast (Lawes *et al.*, 2007a) and inland Afrotemperate forest patches (Scott *et al.*, 1997). Climate

oscillations will also have affected the distribution of forest animals and that species that could not withstand these changes will have been filtered out from forest communities (Lawes *et al.*, 2007a). Remnant Afrotemperate faunas, like floras, may have persisted in local refugia or contracted to Scarp forest refugia located in KwaZulu-Natal and the Eastern Cape during the LGM (Lawes *et al.*, 2007a). This may explain the Afrotemperate faunas present in current Scarp forests (Lawes *et al.*, 2007a). Scarp forests are located between the Indian Ocean Coastal Belt and Afrotemperate forests and have elements of both, with current species diversity declining from northeast to southwest (Lawes, 1990; Lawes *et al.*, 2007a). They may have been the most important refugia for the Afrotemperate forests during LGM and formed a contact zone between Afrotemperate and Indian Ocean Coastal Belt forest during the Holocene (Lawes,1990a; Mucina *et al.*, 2006; Lawes *et al.*, 2007a).

When the climate became warmer and more humid after the LGM, forest expansion commenced from about 14-12,000 yr BP (Geldenhuys, 1992; Eeley *et al.*, 1999). The Indian Ocean coastal belt forests are thought to have formed around 8000 yr BP in association with a drop in sea levels and a southern expansion of the Mozambican coastal forest that had originated from tropical East African refugia (Lawes *et al.*, 1990a; Eeley *et al.*, 1999; Ramsay, 1996; Lawes *et al.*, 2007a). Periods of forest contraction and fragmentation, followed by expansion and fusion will have affected the genetic composition of the plant populations that survived. Fragmentation is expected to reduce effective population sizes and reduce connectivity between populations. The resultant reduction in gene flow allows populations to become genetically distinct and the increase in drift reduces genetic diversity within populations (Young *et al.*, 1996; Fischer and Lindenmayer, 2007).

Any genetic erosion of South African forests through fragmentation and drift is significant, because, despite the small size of the forests in South Africa, they contribute disproportionately to the country's biodiversity (Berliner, 2009). Continuing human

exploitation (Hoffman, 1997) and the expected further decline in rainfall across the region make these forests potentially very vulnerable (Tyson and Dyer, 1978; Tyson, 1991; Fauchereau *et al.*, 2003).

The threat of genetic erosion may be particularly true for plants that depend on specialist insect pollinators (Bond, 1994; Johnson and Steiner, 2000). *Ficus* spp. (Moraceae) are pollinated by host specific mutualist fig wasps (Agaonidae). This mutual dependency might be expected to increase the vulnerability of fig trees to fragmentation but some fig wasps are renowned for their long-distance dispersal (Ahmed *et al.*, 2009). Reflecting this, some fig tree populations show little or no nuclear genetic differentiation over distances extending to hundreds of kilometers (Zavodna *et al.*, 2005; Yu *et al.*, 2013; Bain *et al.*, 2016). In addition, fig wasps can find hosts that occur at low densities in forests (Mawdsley *et al.*, 1998) and this allows fig trees with widely-dispersing pollinators to reproduce rapidly following perturbations (Nason *et al.*, 1996; Harrison, 2003). The dispersal abilities of their pollinators suggest that many *Ficus* species may be naturally resistant to fragmentation (Chen *et al.*, 2008; Liu *et al.*, 2013).

In contrast to their frequently extensive pollen flow, seed dispersal is frequently spatially-restricted in *Ficus* species (Yu *et al.*, 2010; Yu and Nason, 2013; Krishnan and Borges, 2018). This is surprising given that the figs of many species attract a wide range of fruit-eating birds (Shanahan *et al.*, 2001) and bats (Shilton *et al.*, 1999; Zhou and Chen, 2010). Since only seed, and not pollen, transmits chloroplast DNA (cpDNA), *Ficus* cpDNA is expected to exhibit more spatial structure than nuclear DNA. In addition, cpDNA's effective population size is a half that of genomic DNA and it is thus more sensitive to genetic drift brought on by fragmentation. Hence, fragmentation of *Ficus* populations may have limited effect on nuclear genes while damaging cpDNA diversity.

Ficus craterostoma Mildbraed and Burret is a forest specialist fig tree in Africa (Burrows and Burrows, 2003) with a distribution in South Africa that will have been restricted to refugia during glacial periods. The structure of its genetic variation therefore allows us to test ideas about South African forest history. Here we quantify genetic variation in *F. craterostoma*. More specifically, we 1) use cytoplasmic genetic variation to show the footprint of habitat fragmentation on a naturally fragmented population over millions of years and identify potential forest refugia in South Africa; 2) contrast this variation with its nuclear genetic variation to examine the role that pollen dispersal plays in resisting genetic erosion caused by habitat fragmentation; and 3) estimate the distances over which fig wasp dispersal effectively binds fragmented *F. craterostoma* populations together.

2.3 Materials and methods

The study species

Ficus craterostoma is a monoecious species placed in subgenus *Urostigma*, subsection *Chlamydodorae* (Berg and Wiebes, 1992; Cruaud *et al.*, 2012). A hemi-epiphytic shrub or small tree growing up to 10m in height, it is found in lowland rainforest habitats of central and western Africa and at higher elevation in the afromontane forests of southern and eastern Africa (Berg and Wiebes, 1992; Burrows and Burrows, 2003). Trees in the two forest types are pollinated by two different fig wasps with largely allopatric distributions, - *Alfonsiella michaloudi* Wiebes (Berg and Wiebes, 1992) in western-central Africa and *Alfonsiella pipithiensis* Erasmus, van Noort, Jousselin and Greeff in southern Africa. (Erasmus *et al.*, 2007). Within South Africa, *Alfonsiella pipithiensis* is the only recorded pollinator. Birds, rodents and Samango monkeys are known to eat its figs and are the likely seed dispersers (Table S2.1, Gautier-Hion *et al.*, 1985; Lawes *et al.*, 1990; Basabose, 2002; Hart *et al.*, 2013).

Sampling

From 2008 to 2018, we collected leaves or bark of *F. craterostoma* from forests in the Limpopo (5), Mpumalanga (2), KwaZulu-Natal (5) and Eastern Cape (3) Provinces of South Africa (Table 2.1). The samples were stored and dried in plastic bags with silica gel or a NaCL-CTAB-azide buffer (Bhattacharjee *et al.*, 2009). We sampled from 11 to 110 individuals from 15 sample sites located in all three forest types (Afrotemperate, scarp and India Ocean coast belt forest) (Table 2.1).

Table 2.1 Sampled populations of *Ficus craterostoma* in South Africa and their genetic compositions. nSSR, ncpDNA, indicate the numbers of individuals used in microsatellite genotype scanning (SSR), and chloroplast gene sequencing, respectively. The chloroplast haplotypes present in each sample site are listed as H01-H10 with numbers in parentheses indicating the number of haplotype copies for each sample site. The classification of main and sub forest types was based on Laws *et al.* (2007) and Mucina and Geldenhuys (2006) respectively.

Code	Sample sites	Main forest type	Sub-forest type	Latitude (°S)	Longitude (°E)	n_{SSR}	n_{cpDNA}	Haplotypes
HAN	Hangklip	Afrotemperate	Northern misbelt	23.004	29.904	19	10	H01 (10)
ENA	Entambeni	Afrotemperate	Northern misbelt	23.006	30.242	14	10	H01 (10)
PIE	Piesanghoek	Afrotemperate	Northern misbelt	23.044	30.117	14	10	H01 (10)
THA	Thathe holy	Afrotemperate	Northern misbelt	23.252	30.345	19	10	H01 (10)
WOL	Wolkberg	Afrotemperate	Northern misbelt	24.037	30.058	23	9	H01 (8), H03 (1)
BUF	Buffelskloof	Afrotemperate	Northern misbelt	25.444	30.970	22	10	H02 (10)
SAD	Saddleback	Afrotemperate	Northern misbelt	25.798	31.142	11	10	H02 (10)
NGO	Ngome	Afrotemperate	Southern misbelt	27.826	31.419	23	10	H07 (8), H08 (2)
MAP	Maphalane	Indian Ocean	Northern Coastal	28.421	32.415	20	10	H05 (10)
ENT	Entumeni	Scarp	Scarp	28.887	31.371	13	9	H04 (8), H07 (1)
DLI	Dlinza	Scarp	Scarp	28.894	31.453	21	9	H04 (7), H06 (1), H07 (1)
ING	Ingeli	Afrotemperate	Southern misbelt	30.530	29.689	125	20	H07 (20)
MBO	Mboyti	Scarp	Scarp	31.435	29.688	21	9	H04 (1), H07 (8)
DWE	Dwesa	Scarp	Scarp	32.280	28.848	48	8	H07 (5), H09 (2), H10 (1)
MAN	Manubi	Scarp	Scarp	32.449	28.606	30	6	H04 (2), H07 (4)

DNA extraction, sequencing and genotyping

We extracted genomic DNA of *F. craterostoma* with the nucleoSpin, Plant II kit (Macherey-Nagel GmbHandCo.KG, Düren, Germany) using standard protocols. We sequenced two chloroplast DNA intergenic spacer regions trnL (Taberlet *et al.*, 1991; Baraket *et al.*, 2011) and atpF-H (Lahaye *et al.*, 2008; Li *et al.*, 2012) of 6 to 10 individuals per sample site. The 40 ul PCR reactions included: ddH_2O 27.6 ul, reaction Taq buffer 4 ul (10×), dNTP 4 ul (10 mM), primers 0.2 ul (10 uM), Taq polymerase (Takara) 0.4 ul (5U/ul) and DNA 4 ul (50 ng /ul). The thermal programs for *trn*L and *atp*F-H were respectively: 3 min of denaturation at 94 ℃, 35 cycles at 94 ℃ for 1 min, 55 ℃ for 1 min and 72 ℃ for 1 min and 10 min of elongation at 72 ℃; 5 min of denaturation at 94 ℃, 35 cycles at 94 ℃ for 45 s, 55 ℃ for 45 s and 72 ℃ for 1 min and 8 min of elongation at 72 ℃. We sequenced the PCR products in both directions on an ABI PRISM 3730. We checked the sequences manually and aligned them using MEGA7 (Kumar *et al.*, 2016). The accession numbers in GenBank for trnL and atpF-H are MN683996-MN684000 and MN684001-MN684006, respectively. The sequences of the two cpDNA regions from each individual were concatenated into a single sequence.

Twelve polymorphic microsatellite markers (Table S2.2) were selected and genotyped in all samples using an automated sequencer (ABI 3730) and scored loci using GeneMarker HID v.2.05 (Holland and Parson, 2011; further details are provided in Deng *et al.*, 2020).

Genetic diversity

We calculated genetic diversity parameters based on cpDNA sequences using DNASP 5.10 (Librado and Rozas, 2009). These included the number of haplotypes (*h*), average nucleotide divergence (*k*), haplotype diversity (*Hd*) and nucleotide diversity (π). For

microsatellite data, we used Micro-checker v.2.2.3 to check for any genotyping errors due to stuttering, large allele dropout and null alleles (Van Oosterhout *et al.*, 2004). Because null alleles were detected at most sample sites, subsequent analyses were conducted on all 12 loci, but also on the 9 loci least affected by null alleles (we deleted the loci that showed null alleles in more than 8 sample sites). We used FSTAT v.2.9.3 to confirm that no loci were in linkage disequilibrium with each other (Goudet, 1995). We calculated the deviation from Hardy-Weinberg equilibrium (HWE) in each sample site using the multi-locus exact test in GENEPOP 4.0 (Rousset, 2008). We specified a Markov chain method to estimate the *P* value of the test and calculated the number of alleles (N_a), observed (H_o) and unbiased expected heterozygosity (H_e) using FSTAT. We used HP-RARE to estimate allelic richness (A_r) after rarefaction to correct for different sample sizes between sample sites, and the average number of private alleles per locus (Kalinowski, 2005). We used 1000 permutations in FSTAT to estimate global F_{ST} and pairwise F_{ST}'s between sample sites. We also standardized these estimates to correct the maximum difference that could be obtained given observed allele numbers and frequencies using $F'_{ST} = F_{ST}/F_{ST\,max}$ as suggested by Meirmans and Hedrick (2011). We used RecodeData (Meirmans, 2006) to convert the data and imported them into FSTAT to estimate global F_{STmax} and pairwise population F_{STmax}.

Genetic differentiation and structure

We inferred networks between cpDNA haplotypes using TCS 1.21 with a connection limit of 95% (Clement *et al.*, 2000). Using ARLEQUIN 3.5 with 1000 permutations (Excoffier and Lischer, 2010) and (AMOVA) on cpDNA haplotypes we partitioned the genetic variance into within and among population (= sample site) components.

We used the Bayesian approach to detect genetic structure based on SSR data, implemented in STRUCTURE 2.3.4 (Pritchard *et al.*, 2000). We did 15 runs for each *K* from *K*=1 to *K*=16, with 10 000 burn-in and 100 000 MCMC repetitions for each run.

We determined the optimal *K* number with the *ΔK* method, using the website of STRUCTURE HARVESTER (Evanno *et al.*, 2005; Earl, 2012), and then assigned individuals into each group based on the *K* values, ignoring their geographic locations with 100 000 burn-in and 1 000 000 MCMC repetitions. In order to reveal relationships between sample sites we performed a principal coordinate analysis (PCoA) in GENALEX 6.5 using the cpDNA haplotypes and microsatellite data (Peakall and Smouse, 2006).

As with the microsatellite data, we used AMOVA as implemented in ARLEQUIN 3.5 (Excoffier and Lischer, 2010) to partition the variance of microsatellite data into within and among population components as well as within and among groups of populations basing our estimates on 1 000 permutations. We assessed global population differentiation and pairwise genetic differentiation between populations (= sample sites) using FSTAT with 1 000 permutations, applying sequential Bonferroni corrections.

Genetic barriers and isolation by distance

We explored genetic discontinuities between sample site pairs with Monmonier's maximum-difference algorithm based on the SSR components of each sample site using BARRIER 2.2 (Manni *et al.*, 2004), and estimated the robustness of the gene-flow barriers with 1 000 Nei's genetic distance matrices. We produced these distance matrices by bootstrapping over loci with MICROSATELLITE ANALYSER 4.05 (MSA; Dieringer and Schlötterer, 2003).

We estimated the role of geographic distance on SSR genetic differentiation by quantifying isolation by distance (IBD) between sample sites. Specifically, we regressed pairwise estimates of genetic distances F_{ST} / *(1-* F_{ST}*)* and F'_{ST} against the corresponding geographic distance (km) with a Mantel test using the R 3.3 package

'vegan' (http://www.r-project.org/), determining significance with 1 000 permutations. The tests were first performed on all sample sites, then 14 (excluding MAP) and 12 (excluding MAP, ENT and DLI) sample sites. We excluded these sample sites since they were unusually isolated and their higher genetic distances were clearly outliers compared to the remainder.

Forest patch connectivity analysis

We plot the histogram of pairwise geographic distance between forest patches (Figure S2.1), which suggest the size of a sampled forest may not reflect the true population genetic unit size of fig trees because of the potentially long-range dispersal of their pollinating fig wasps. We therefore calculated a distance (D-the connection radius) within which the forests can act as an interconnected unit. This concept is similar to Wright's neighborhood size (1943). Given a distance D, we calculated a corrected forest size as the sum of all fragment sizes within a radius D from the sampled fragment. To determine which values of D best explained the data, we utilized the well-supported proposition that larger populations can maintain more alleles. If this was true for *F. craterostoma,* the correlation between observed allele number and corrected forest size should be highest when a specified value of D is near the actual value of D. If the specified D is smaller, the corrected forest size will not include all the forests that actually make a genetic unit and when the specified connection radius is larger the corrected forest size would include forests that do not form part of the genetic unit. By systematically increasing D we were able to see which values of D gave the strongest correlation between corrected forest size and genetic variation. To achieve this we extracted the patch size and locations (geometric center) for each forest patch in Arcgis 10.2 based on the 2018 vegetation map (approximate scale 1: 250 000) from the SANBI website (http://bgis.sanbi.org). We reduced it to the four forest types that contain *F. craterostoma*: southern and northern mistbelt, northern coastal and scarp forests. Then we correlated allelic richness and corrected forest size (Pearson correlation) for a range

of D values (from 0 – 150 km in 1 km steps). For each value of D we added to the focal patch's size the size of any other patches whose geometric centre fell within the connection radius. By plotting the value of the correlation (r) against D it was possible to calculate the connection radius that best explained genetic the data and could be considered a genetic unit. To obtain 95 CI for the null hypothesis that the forests' location had no effect on the correlation, we repeated the following 1000 times: we randomly picked 15 forests and assigned the observed allelic richness values to them and calculated the correlations as D increased. All the correlations were calculated in R 3.3 (http://www.r-project.org/) using the geosphere package's distHaversine to calculate distances between localities.

2.4 Results

The combined cpDNA sequences were 1211 bp long. We obtained 150 unambiguous combined cp gene sequences from the 15 sample sites (Table 2.2). We distinguished 10 haplotypes based on 14 variable sites (9 substitutions and 5 indels; Table 2.2; Table S2.3), which provided sufficient variability to analyze population structure.

We scored 423 individuals at most of the 12 loci (Table 2.1, Table S2.1) and found no significant linkage disequilibria between loci, but 13 sample sites deviated from HWE (Table 2.2). True inbreeding or a Wahlund-like effect due to sampling family groups can contribute to deviation from HWE, but the most likely cause was the null alleles that MICRO-CHECKER inferred in all loci. The number of loci with null alleles and the frequency of inferred null alleles varied among sample sites (Table S2.4) and we therefore replicated all the subsequent analyses for the nine loci (excluding loci with null alleles in eight or more sample sites (Table S2.5-2.7, Figure S2.2-2.6).

Table 2.2 Genetic diversities of *F. craterostoma* based on chloroplast genes and microsatellites data. *S*, polymorphic sites excluding gaps; h, number of haplotypes; H_d, haplotype diversities; π, nucleotide diversities; *k*, average number of nucleotide differences;, number of alleles (N_a), allelic richness after rarefication (A_r), observed (H_o) and expected (H_e) heterozygosity with significance for deviation from HWE ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) after correction for multiple testing, inbreeding coefficient (F_{IS}) ($^{*}P < 0.05$), and private allelic richness (P_a). POP indicates the sample site. (12 loci)

POP	*Chloroplast gene*					Microsatellite data				
	S	*h*	H_d	π	*k*	N_a	A_r	H_o/ H_e	F_{IS}	P_a
HAN	0	1	0	0	0	7.33	5.96	0.56/0.64***	0.11	0.26
ENA	0	1	0	0	0	5.08	4.78	0.53/0.60**	0.12	0.17
PIE	0	1	0	0	0	5.08	4.69	0.60/0.61	0.01	0.17
THA	0	1	0	0	0	6.83	5.73	0.61/0.66***	0.07	0.10
WOL	1	2	0.222	0.0002	0.222	8.00	6.49	0.52/0.73***	0.29*	0.26
BUF	0	1	0	0	0	6.75	5.5	0.56/0.67***	0.18*	0.17
SAD	0	1	0	0	0	5.25	5.25	0.62/0.68***	0.09	0.14
NGO	0	1	0	0	0	5.75	4.60	0.67/0.63	-0.06	0.06
MAP	0	1	0	0	0	6.50	5.37	0.57/0.66***	0.14*	0.61
ENT	3	2	0.222	0.0006	0.667	7.92	7.39	0.72/0.77***	0.06	0.41
DLI	4	3	0.417	0.001	1.222	8.58	7.00	0.73/0.79***	0.08	0.52
ING	0	1	0	0	0	10.08	5.48	0.58/0.63***	0.09*	0.14
MBO	3	2	0.222	0.0006	0.667	7.50	6.05	0.48/0.66***	0.28*	0.19
DWE	2	2	0.536	0.0009	1.107	10.92	6.81	0.47/0.73***	0.36*	0.46
MAN	3	2	0.533	0.001	1.600	9.75	7.02	0.52/0.74***	0.30*	0.36

Genetic diversity of Ficus craterostoma

The cpDNA had low genetic diversity. Eight out of the 15 sample sites were fixed for one haplotype (four different haplotypes with nearby sample sites sharing the same one) (Table 2.1 and 2.2; Figure 2.2). This was the case for seven of the nine Afrotemperate forests and the single Indian Ocean coastal belt forest, but none of the five Scarp forests (Figure 2.2). Monomorphism in so many of the sample sites suggests a lack of gene flow through seed dispersal between most of the forests and also populations of limited size. The Scarp forest, especially sample sites DLI and DWE, had higher genetic diversity than the other forest types (Table 2.2, Figure 2.2) and raise the possibility that these areas are located close to Pleistocene refugia.

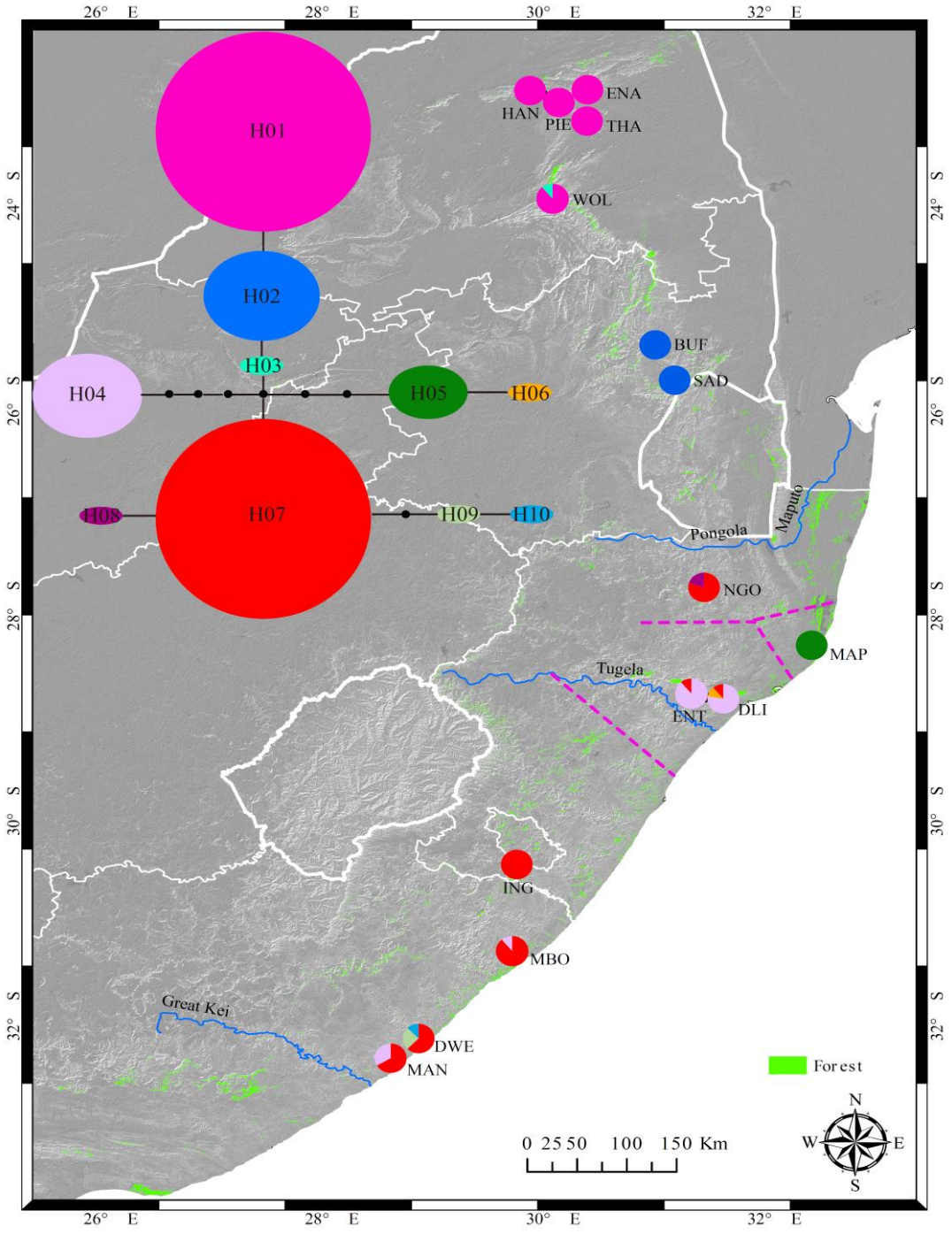

Figure 2.2 The northeastern part of South Africa where we sampled *Ficus craterostoma*. Green pixels indicate forest locations (http://bgis.sanbi.org/vegmap 2018). The chloroplast haplotype distribution is given in the pie charts, with colours corresponding to the haplotype network at the top-left corner (the black dots indicated mutations). Blue lines represent rivers, thin and bold solid white lines represent province and country boundaries and dashed purple lines indicate gene-flow barriers.

TCS linked all ten haplotypes into a network, based on the 95% statistical criterion (Figure 2.2). H01 was only found in the north, suggesting that there may have been a

northern refugium, but it must have been a small population considering the low genetic diversity present (Figure 2.2 and Table 2.2). It was notable that only haplotype H05 was found in MAP, despite it being close to DLI and ENT, where H04 was the dominant haplotype. Seven mutations separate H04 and H05 (Figure 2.2), suggesting a lack of seed-based gene flow between these areas and that the MAP sample site may have had a different origin.

All sample sites had SSR genetic diversity (Table 2.2, Table S2.5). The number of alleles per sample site ranged from 5.25 (SAD) to 10.92 (DWE), with a mean of 7.30. Allelic richness ranged from 4.60 (NGO) to 7.44 (ENT) with a mean of 5.82. The average observed heterozygosity of 0.59 was less than the expected heterozygosity of 0.68, suggesting some inbreeding. All 15 sample sites had indications of inbreeding (Table 2.2), but this is likely to be the result of null alleles and/or sampling family groups (the Wahlund effect). Private allelic richness ranged from 0.06 (NGO) to 0.61 (MAP). This again suggests that some of the sample sites may have unique origins or are located in refugial areas, in contrast to sample sites with lower values that are likely to reflect ore recent recolonizations.

Genetic differentiation and structure

Significant population differentiation was found in cpDNA sequences (φ_{ST} = 0.85, P < 0.001) and SSR alleles (F_{ST} = 0.08 (12 loci), F_{ST} = 0.07 (9 loci) P < 0.001; F'_{ST} = 0.25 (12 loci), F'_{ST} = 0.24 (9 loci)). The AMOVA revealed contrasting results for cpDNA and SSRs (Table 2.3). The major source of cpDNA variation occurred among populations (85.36%) whereas the major source of SSR variation was within populations (91.32%) (Table 2.3). These analyses suggest that pollen but not seeds is frequently transferred between forests. Even so, pairwise population differentiation tests based on microsatellites showed that most pairs of populations were genetically differentiated from each other (102 of 120 comparisons; Table S2.8). This suggests that

although there is gene flow between populations, it is not sufficient to homogenize them genetically.

STRUCTURE analyses of microsatellite data indicated that two genetic groups were optimal (Figure S2.7, designated as the red and yellow groups). When $K = 2$, sample sites from Afrotemperate forest were dominated by the red group, with some sample sites having a few individuals belonging to the yellow group (Figure 2.3, Figure 2.4a and Figure S2.3). Sample sites from Scarp and Indian Ocean coastal belt forest were dominated by the yellow group except for MBO (Figure 2.3 and Figure 2.4a). When $K = 3$, the Afrotemperate forests (red group) tended to stay the same, whereas the southern Scarp forests (blue group) (MAN, DWE, MBO) were separated from the other Scarp forests (yellow group) (indicated in blue in Figure 2.3 and Figure 2.4b). In addition, we also provided the genetic assignment result of each individual, when K values ranged from four to ten (Figure S2.8). Based on these results we can conclude that gene flow between forest types has been limited except between MBO and ING, which are situated close together (about 100 km). AMOVA of the sample sites when grouped into two and three genetic groups on the basis of the STRUCTURE results, confirmed the between-group distinctions (two groups: $F_{CT} = 0.05$, $P < 0.001$; three groups: $F_{CT} = 0.06$, $P < 0.001$).

Table 2.3 Molecular analysis of variance (AMOVA) results indicating the breakdown of chloroplast DNA (cpDNA) sequence and of nuclear microsatellite (nSSR) variability among and within populations of *Ficus craterostoma*. ($^{***}P < 0.001$)

	df	Sum of squares	Percentage of variation(%)
cpDNA			
Among populations	14	197.081	84.70***
Within populations	125	33.447	15.30
Total	139	230.529	
nSSR			
Among populations	14	319.874	8.17***
Within populations	831	3313.861	91.83
Total	845	3633.735	

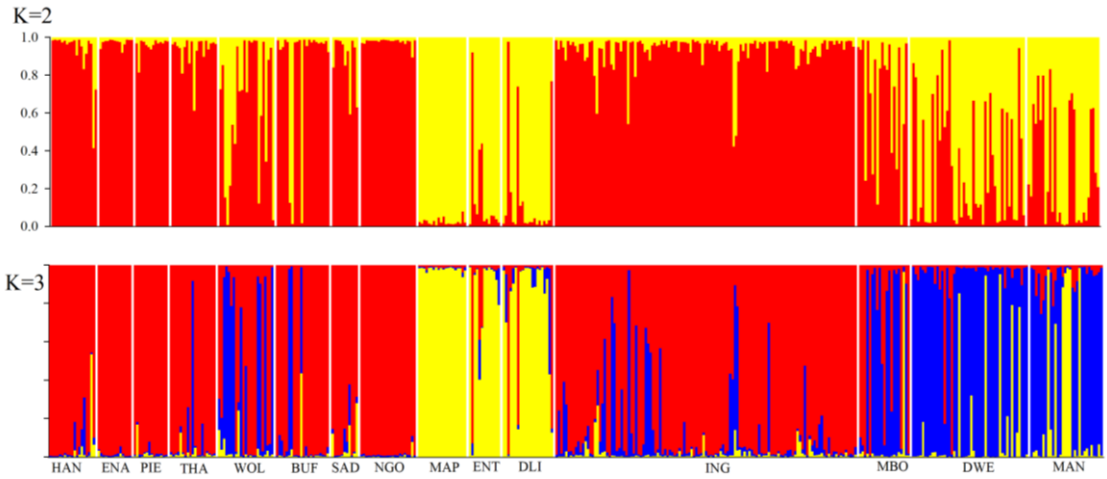

Figure 2.3 The microsatellite memberships of the *Ficus craterostoma* assigned by STRUCTURE for K = 2 and K = 3. Each vertical line represented one individual. The color indicates the group.

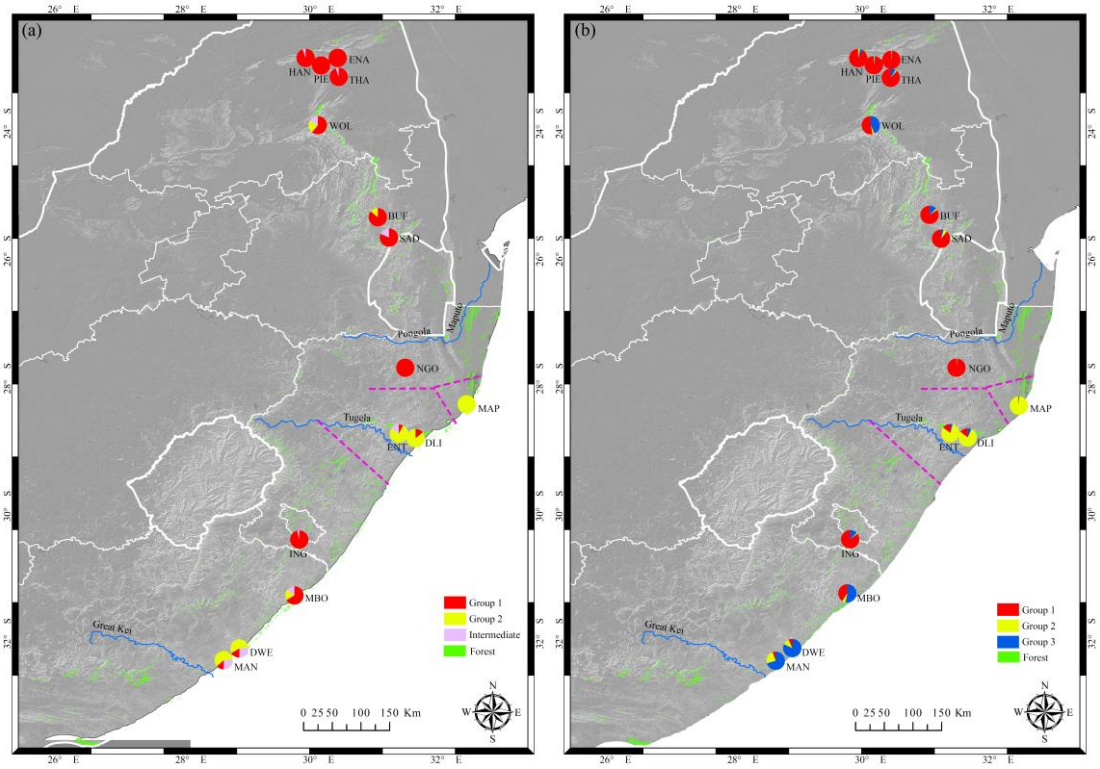

Figure 2.4 To illustrate the geographic structuring of the STRUCTURE groups in Figure 2, we indicate the groups on the maps for K = 2 (a) and for K = 3 (b). The group colours from Figure 2 were used to colour the pie-charts and pink was used for individuals that were ambiguous when K = 2.

The first axis in the principle coordinate analysis of cpDNA (Figure S2.9) explained 57.56% of the total variance and corresponded with the latitude of sample sites, and the

second axis roughly corresponded with the longitude of sample sites and explained a further 22.71%. Geographically close sample sites showed little or no separation. This analysis confirmed a spatial structuring of cpDNA that is consistent with low rates of seed dispersal between sample sites.

The first principle coordinate based on SSR data explained 44.99% of the variance and distinguished between sample sites located in the Afrotemperate forests and the Scarp and Indian Ocean coastal belt forests, except MBO. This axis is consistent with the two groups found by STRUCTURE when K = 2. The second axis corresponds to a longitudinal gradient and explained 19.09% of the variance (Figure S2.10). This analysis further emphasized the genetic distinction between forest types and showed that while spatial isolation with nuclear DNA was less clear, it was still present.

Gene flow barriers and isolation by distance

Based on the analyses of microsatellite data we found two genetic barriers that isolated the eastern sample sites from the others, with one isolating MAP from the rest and the other grouping ENT and DLI together (Figure 2.4). This indicates that the Indian Ocean coastal belt forest is isolated from the other sample sites and that the two nearby Scarp forests are also isolated from the more distant Scarp forests.

When all the sample sites were included, genetic isolation by distance was suggested, but not significant (r = 0.14, P = 0.078; Figure 2.5). It is clear from Figure 2.5 that genetic distances to MAP are outliers. When MAP was excluded, isolation by distance was highly significant (r = 0.34, P = 0.005; Figure 2.5). This trend was strengthened further when the other two geographically isolated sample sites (ENT and DLI) were also excluded (r = 0.69, P = 0.001) (Figure 2.5). Hence, while there are discrete gene flow barriers between some sample sites, distance also plays an important role in determining the extent of genetic separation.

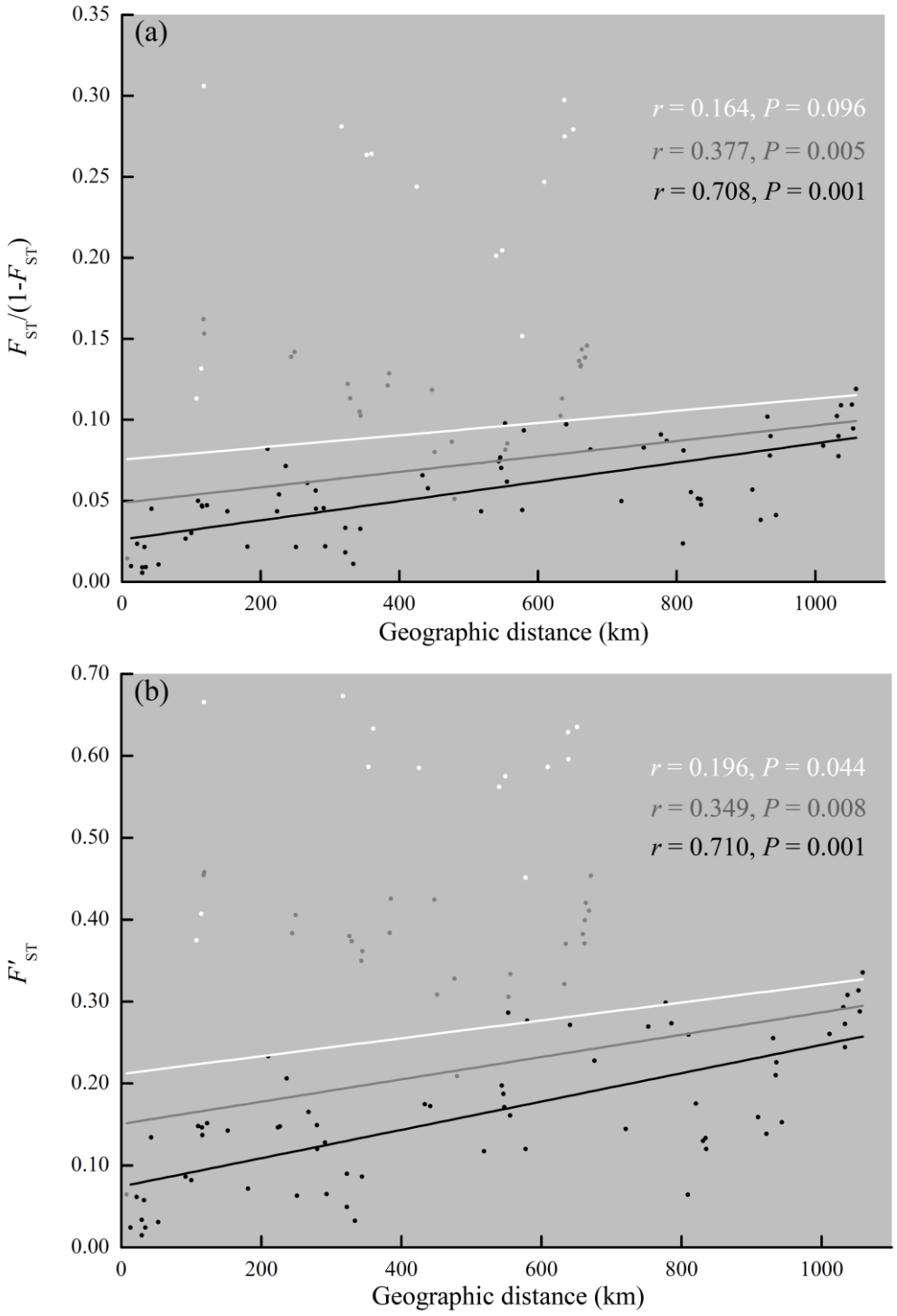

Figure 2.5 The relationships of genetic distances and geographic distances between sample site pairs in each region based on SSR genotypes. White, dark grey and black points indicate MAP comparisons, ENT and DLI comparisons and the remaining comparisons. (a) $F_{ST}/(1-F_{ST})$; (b) F'_{ST}

Habitat connectivity of F. craterostoma

In total, the northern and southern mistbelt forest, the scarp and northern coastal forests

consist of 10394 forest patches that varied in size from 1.32×10^{-5} to 96.46km^2, with a mean and median size of 0.30 km^2 and 0.03 km^2 respectively. Around 96% forests' are smaller than 1 km^2 in size. The correlations exceeded the upper 95% confidence limit for D values: 38<D<121 km (Figure 2.6), with a maximum correlation when D was equal to 61 km. Hence, although a connection radius of 61km gave the best correlation, there was a large range of significant connection radii, and wasp dispersal was binding together patches that were between 76 and 242 km apart.

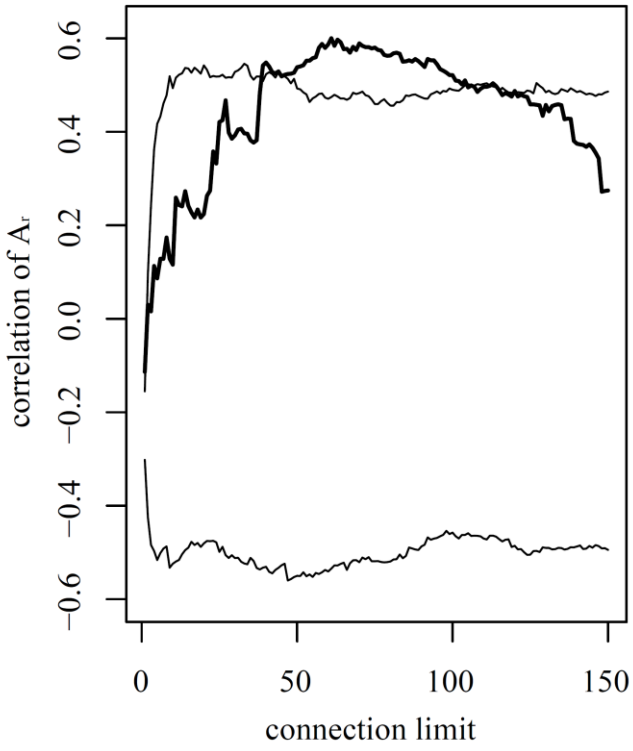

Figure 2.6 The correlation between allelic richness connection radius, the observation and 95% CI were represented by thin and thick dark line, respectively.

2.5 Discussion

The variation in cpDNA in the South African *F. craterostoma* supports there has been contraction and expansion of forests and suggests the locations of three putative glacial refugia. There was generally lower genetic differentiation between sample sites in *F. craterostoma* nuclear microsatellites compared to cpDNA, supporting the general

consensus that pollen flow in fig trees is more extensive than gene flow via seeds. Population connectivity analysis revealed that the fig wasps associated with *F. craterostoma* generate a pattern of random mating within their hosts across forests separated by between 76-242 km. Successful fertilization is more likely to occur between individuals from forests that are closer than 76 kms and less likely between individuals from forests separated by more than 242 kms. This means that pollen flow is extensive and occurs between forests of the same forest type, whereas seed-assisted gene flow is restricted to nearby forests. Geographic isolation has divided the forests into two genetic groups and isolated three of the sample sites (MAP, ENT and DLI) from the others. When these sample sites are excluded, genetic differentiation increases with the geographic distance separating sample sites and has generated a pattern of isolation by distance. Evidence of strong genetic drift was detected in cytoplasmic elements and this, together with its relatively restricted seed dispersal distances, is likely to make *F. craterostoma* cpDNA vulnerable to fragmentation, despite the genetic connectivity provided by long-range pollen dispersal that maintains within-population nuclear genetic diversity.

Forest fragmentation in South Africa and refugia of F. craterostoma

Glacial cycles have had major impacts on the distribution of forests in South Africa (Lawes, 1990; Lawes *et al.*, 2007a), with the harsh climatic conditions of glacial periods resulting in temporary contractions in range, the elimination of some forests and the retreat or migration of others to suitable refugia (Lawes, 1990; Lawes *et al.*, 2007a; Tolley *et al.*, 2018). Previous studies have pointed out that east coast Scarp forests (Hughes *et al.*, 2005; Dalton *et al.*,2015; Eberle *et al.*, 2017; Lawes *et al.*, 2007a) or some remnants of old Afrotemperate forests could have been the sites of refugia for Afrotemperate forest faunal communities during periods of range contraction (Lawes *et al.*, 2007a; Eberle *et al.*, 2017), while post-LGM colonization of Indian Ocean coastal belt forest faunas may have come from tropical East African refugia (Lawes *et al.*,

2007a). These refugia will have played an important role in preserving species and also facilitating species diversification, especially for species that only disperse locally (Tolley *et al.* 2008; da Silva and Tolley, 2017; Barnes and Daniels, 2019). Our genetic data suggest some potential refugia and we can also infer the recolonization route of *F. craterostoma* based on its haplotype diversity and distribution.

The pattern of genetic variation suggests that *F. craterostoma* in the northern Afrotemperate forests retreated to a refugium in Limpopo. Our results therefore provide further support for the existence of a northern Afrotemperate forests refugium in South Africa (Lawes *et al.*, 2007a; Coetzer, 2015; Coetzer *et al.*, 2019). This was possibly close to the WOL sample site, given that it has one polymorphic site and its sharing of the same haplotype as the other sample sites in Limpopo. The polymorphic sites in WOL (1) was nonetheless three times smaller than in the Scarp forest sample sites such as ENT (3) and MBO (3), suggesting that this refugium must have been small, with genetic drift leading to the loss of genetic diversity. BUF and SAD may have been recolonized from the north, with a single mutation step linking it to the potential refugium WOL haplotype. The northern and possibly southern recolonization of *F. craterostoma* from the putative refugium in Limpopo Province is likely to have resulted from seeds moved by birds (Coetzer, 2015; Coetzer *et al.*, 2019) and possibly monkeys (Lawes, 1990; Lawes *et al.*, 2007a; Dalton *et al.*, 2015).

Populations of *F. craterostoma* may also have persisted through the LGM in two putative Scarp forest refugia. One may have been located in northern KwaZulu Natal close to the ENT and DLI sample sites, the other in the north Eastern Cape close to the MAN and DWE sample sites. Refugial populations are characterized by their higher genetic diversity and private alleles (Provan and Bennett, 2008). The *F. craterostoma* populations close to the hypothesized refugia displayed these characteristics, with high nucleotide diversity in their cpDNA, high nuclear microsatellite heterozygosity and higher private allelic richness. The locations of these two putative glacial refugia

correspond with those identified for Samango monkeys (*Cercopithecus albogularis labiatus*) (Makahasi, 2013; Dalton *et al.*, 2015) and Cape Parrot (Coetzer, 2015; Coetzer *et al.*, 2019).

Indigenous forests in KwaZulu Natal were highly fragmented during the LGM and likely to have become restricted to areas that are now occupied by mistbelt and scarp forests (Eeley *et al.*, 1999). The plateau and highland region that runs parallel to the Tugela river occupied at present by Ngome forest and an archipelago of forests containing three forest fragments: Dlinza, Nkandhla and Qudeni (Eeley *et al.*, 1999), were important for preserving forest remnants during the LGM in KZN. Therefore, the NGO forest could either have derived from local refugia or could have been recolonized from northern KZN refugia. NGO shares its dominant haplotype (H07) with northern KZN forests, suggesting NGO was more likely repopulated from KZN refugia than derived from local refugia.

Only one haplotype was found in ING indicating that it stems from a recent recolonization. The most likely source may be located in the Eastern Cape, given its high frequency of the H07 haplotype and close proximity to the refugium postulated for the northern Eastern Cape (Figure 2.2). The northward recolonization of forest mammals (Lawes, 1990; Lawes *et al.*, 2007a; Makhasi, 2013) and birds (Forbes, 2003; Lawes *et al.*, 2007a; Coetzer 2015; Coetzer *et al.*, 2019) into Natal from the Eastern Cape refugium may have facilitated repopulation of *F. craterostoma* in southern KZN.

Although MAP had the highest number of private alleles, it is unlikely to have been close to a refugium because there has been fixation of only one haplotype and only moderate nSSR diversity. We interpret the high frequency of private alleles at MAP as the consequence of repopulation from a refugium outside of South Africa and being genetically isolated from other populations. MAP may have formed around 8000 yr BP with the expansion of Mozambique coast forest and forming of Indian Ocean coast belt

forests (Lawes, 1990; 2007a). It is fixed for one haplotype, which is 7 steps apart from the main haplotype of its nearby northern KZN refugial populations (DLI, ENT) (Figure 2.2), suggesting MAP may have been repopulated from another source. The alternative explanation, namely that mutations accumulated for a tree species within a relative short time, seems implausible. Afrotropical characteristics of birds' assemblages in Indian Ocean Coastal Belt forests (Forbes, 2003; Lawes *et al.*, 2007a) suggest the migration of birds from east Africa may facilitate the repopulation of *F. craterostoma* in Indian Ocean Coastal Belt forests. The second re-radiation of Samango monkeys after the LGM (*C. a. erythrarachus*) from east Africa via the Mozambican coastal forest south down to South Africa (Lawes, 1990; Dalton *et al.*, 2015) may also have helped with the recolonization of *F. craterostoma*. More samples from other coastal forests and East African populations are needed to test this idea.

Genetic differentiation of cpDNA and nuclear microsatellites

High genetic differentiation of cpDNA between populations has been attributed to limited seed dispersal in *Ficus* species such as *F. hirta* (Yu *et al.*, 2010) and *F. pumila* (Wang *et al.*, 2009; Liu *et al.*, 2015). This pattern is also present in *F. craterostoma*, where (85%) of the recorded cpDNA genetic variation was among sample sites. The potential seed dispersal agents of *F. craterostoma* include birds (Table S2.1) and arboreal mammals (Gautier-Hion *et al.*, 1985; Lawes *et al.*, 1990; Basabose, 2002). In South Africa the only mammal known to disperse *F. craterostoma* is the Samango monkey (*Cercopithecus albogularis*) (Dalton *et al.*, 2015). Movements of monkeys are constrained by geographic barriers such as rivers and mountain ranges (Turner *et al.*, 2016) and the genetic barrier detected close to the Tugela river may reflect this. Nonetheless, at least 39 bird species could be potential dispersers (Maclean, 1985). The distances that seeds are dispersed by birds varies greatly and some forest-living species are quite sedentary (Thornton *et al.*, 1996; Shanahan *et al.*, 2001; Moore *et al.*, 2008). Forest fragmentation leads to the loss of forest specialist bird species (Cooper *et al.*,

2017) and also Samango monkeys in small patches (Lawes *et al.*, 2004), so rates of seed dispersal may also have been depressed during periods of increased contraction.

One limitation of the use of microsatellites is null alleles, which increase the frequency of apparent homozygotes and lead to apparent deviations from HWE (Dakin and Avise, 2004). Here, a number of null alleles were detected across sample sites. Therefore, we conducted the analysis based on 12 loci and 9 loci and found the genetic structure were not affected by the existence of null alleles. We also found a lower genetic diversity after excluding some loci, with less sample sites showing significant inbreeding or deviating from HWE.

The nuclear microsatellites of *F. craterostoma* displayed a high level of genetic diversity, but with only moderate genetic differentiation between sample sites and less than 10% of total SSR variation came from among sample sites. As with the genetic structure of the sample sites, these provide evidence for long-distance pollen flow in *F. craterostoma.* Pollen flow has been occurring frequently between forest patches and even (at low rates) between different forest types. Long distance pollen flow is also shown by the connectivity analysis, which indicated that the fig wasps can facilitate mating within a range of 76-242 km. Some pollinators of *Ficus* species are known for their long-distance dispersal, which can extend to over 100 km and routinely more than 10 km in monoecious *Ficus* species such as *F. craterostoma* (Nason *et al.*, 1996; Ahmed *et al.*, 2009). This is achieved because although the fig wasps are small and weak fliers, they fly upwards from their natal figs, where they encounter faster-moving air which can carry them long distances down-wind (Ware and Compton, 1994a; 1994b; Compton *et al.*, 2000; Compton *et al.*, 2005; Harrison and Rasplus, 2006). Flight above the forest canopy by the pollinators of *F. craterostoma* may be facilitated by the tree's strangling habit because it normally bears its figs in the crowns of its host trees. This is around 10-20 meters above the ground, because the height of the canopy is relatively low in South African forests (Burrows and Burrows, 2003).

Both SSR variation and STRUCTURE of *F. craterostoma* indicate long distance pollen dispersal. In addition, the connection analysis suggest fig wasp-facilitated mating between forests that are 76-242 km apart. Beyond this distance forests should be more differentiated. Therefore, geographic distance contributes to the genetic differentiation of *F. craterostoma*.

Mantel tests suggest that even though their pollinators can disperse widely, most pollen dispersal events occur over relatively short distances. Within a given forest patch there are usually host trees with receptive fig crops available for pollination and oviposition and the immediate availability of attractant volatiles can draw in pollinators before they disperse further (McKey, 1989; Kjellberg and Valdeyron, 1990; Ware and Compton 1994a). Dispersal of fig wasps between forest patches may also be reduced by the less suitable environmental conditions they encounter away from the forests (Michaloud *et al.*, 1985). Fig wasps also fly closer to the ground in areas with lower and discontinuous canopies (Compton *et al.*, 2005), and wind speeds are typically slower close to the ground. *A. pipithiensis* is probably a nocturnal flier based on its colour and eye size. At night it may experience less wind than diurnal fig wasps would (Ware and Compton, 1994a) which would restrict the actual dispersal.

Genetic resilience of F. craterostoma

While long-range pollen dispersal may render *F. craterostoma* nuclear DNA resistant to fragmentation, its cytoplasmic DNA is experiencing stronger drift and will be more vulnerable in a genetic sense. We found a low genetic diversity and high genetic differentiation in its cpDNA, reflecting the impact of fragmentation. Chloroplast genes normally have uniparental inheritance, so their effective size is a half that of nuclear genes. This makes them more vulnerable to genetic drift, especially when population sizes are small (Petit and Vendramin, 2007). The fact that nine out of fifteen sample sites were fixed for one haplotype confirms this. Limited seed dispersal is also indicated

by nearby populations tending to be fixed for the same haplotype. The effectiveness of *F. craterostoma*'s fruit dispersal may have been strongly affected by the extinction of mammals in small forest patches (Lawes *et al.*, 2004), and the loss of many forest-dependent bird species in South Africa (Olivier *et al.*, 2013; Cooper *et al.*, 2017). Since these fauna are also more likely to be resident in local forests rather than travel between fragments (Bonnevie *et al.*, 2003), the observed strong genetic structure in cpDNA is not surprising.

Studies have often found that population fragmentation has a smaller effect on pollen migration than on seed migration (Bacles and Ennos, 2008; Wang *et al.*, 2011; Llorens *et al.*, 2012). This effect is magnified in most fig tree species by the long-distance dispersal that can be achieved by their pollinators. Our results confirm this pattern in *F. craterostoma*, because unlike cpDNA, most sample sites included individuals from more than one genetic group, and there was high nuclear microsatellite genetic diversity and low genetic differentiation between sample sites, all of which are indicative of long-distance pollen dispersal. In a typical forested area of Panama, fig wasps routinely disperse 5.9 to 14.2 km between trees (Nason *et al.*, 1996). Our result revealed that fig wasps can maintain the genetic connectivity of *F. craterostoma* in patches that are between 76 and 242 km apart. Long range pollen dispersal increases the effective population size of nuclear DNA, allowing selection to be more effective (Halliburton, 2004). It will simultaneously limit adaptation to local conditions because genes are frequently moved between environments (Kirkpatrick, 1996). On the other hand, long range pollen dispersal will not increase the effective population size of cytoplasmic elements and this may hamper the effectivity of selection on these DNA. Because cytoplasmic DNA is inherited without recombination, Muller's ratchet will systematically damage cytoplasmic genes (Gillespie, 2016). We can conclude that in *F. craterostoma*, long-distance pollen flow helps to maintain population genetic connectivity and diversity of nuclear DNA despite the fragmented habitats it occupies.

Because the nuclear DNA frequently admixes, chloroplast and nuclear genes should be co-adapted to each other. If active conservation measures were ever required for *F. craterostoma*, dispersal of the plant's seeds to augment existing populations would not run the risk of cyto-nuclear mis matches.

2.6 Supplementary materials

Table S2.1 The list of bird species that could potential dispersal *F.craterostoma*'s seed (Maclean 1985).

Common name	Scientific name
Rameron pigon	*columba arquatrix*
Delegorgues pigon	*columba delegorguei*
Green pigen	*Treron calva*
Cape parrot	*Poicephalus robustus*
Knysna lourie	*Tauraco corythaix*
Narina trogon	*Apaloderma narina*
Blackcollared barbet	*Lybius torquatus*
Goldenrumpod tinker barbet	*Pogoniulus bilineatus*
Black cuckooshrike	*Campephaga flava*
Blackheaded oriole	*Oriolus auratus*
Bush blackcap	*Lioptilus nigricapillus*
Blackeyed bulbul	*Pycnonotus barbatus*
Terrestrial bulbul	*Phyllastrephus terrestris*
Yellow streaked bulbul	*Phyllastrephus flavostriatus*
Sombre bulbul	*Andropadus importunus*
yellowbellied bulbul	*Chlorocichla flaviventris*
Olive thrush	*Turdus olivaceus*
Orange ground thrush	*Zoothera gurneyi*
Chorister robin	*Cossypha dichroa*
Natal robin	*Cossypha natalensis*
Cape robin	*Cossypha caffra*
Starred robin	*Pogonocichla stellata*
Garden warbler	*Sylvia borin*
Yellow breasted Apalis	*Apalis flavida*
Bluegrey flycatcher	*Muscicapa caerulescens*
Southern Black flycatcher	*Melaenornis pammelaina*
Southern Tchagra	*Tchagra tchagra*
Olive bush shrike	*Telophorus olivaceus*
Plumcoloured straling	*Cinnyricinclus leucogaster*
Glossy starling	*Lamprotornis nitens*
Blackbellied starling	*Lamprotornis corruscus*
Redwinged starling	*Onychognathus morio*
Cape white eye	*Zosterops pallidus*
Thickbilled weaver	*Amblyospiza albifrons*
Forest weaver	*Ploceus bicolor*
Spectacled Weaver	*ploceus ocularis*
Forest canary	*Serinus scotops*
Bully canary	*Serinus sulphuratus*
Protea Canary	*Serinus leucopterus*

Table S2.2 Twelve polymorphic nuclear loci used for exploring population genetic structure of *F. craterostoma* . bp = base pair, Na =Number of alleles.

Locus	Primer sequence (5'- 3')	Tm (°C)	Repeats	Na
N5-MFC1	F: ACTAGACTGAAAAAACATTGC	58	$(CT)_{13}$	13
	R: TGAGATTGAAAGGAAACGAG			
N11-Frac202	F: AGAGATATGATGTTCTAGTGCA	59	$(TG)_{13}$	28
	R: CTCGCTCCCACTTAAATACAAG			
N12-Frub29	F: CCACTTTGGAATGTCACTTGGA	59	$(AG)_{24}$	27
	R: TGAACACGCCAACTGAGAATG			
N14-Frub61	F: GTACACTCTCTTAGCTGCC	59	$(TC)_{24}$	23
	R: TACCTTTCTCTGGACATTC			
N17-Frub415	F: GCACGTAGTCGGTGTTAAGC	59	$(AC)_{10}$	15
	R: CTGTGCGGAATAAAAGCTAGC			
N24-FinsN1	F: AGGGCTGAGATAGGTTGATT	58	$(TA)_2(CA)_{10}(TA)_7$ CATA$(TG)_2$	26
	R: TAAGTTGGTGTGTGGCATC			
N29-FinsA1	F: AATCCCCGTACTTCACTTG	58	$(CT)_{12}$	21
	R: AGAACTTATTGCACGGACAG			
N33-FM3-64	F: GATGGTGTTGTTGTCGATGGTCAT	59	$(CAA)_8$	7
	R: GCGGCTCGGTGGAACTTGAG			
N34-FM4-70	F: CAGATGAGGTTGACGATGTTATTG	59	$(GAA)_{20-1}$	9
	R: TAAACCCTCTTCAAATTCACTCTC			
N40-LMFC20	F: ATGGAGGCTTAGATAGAAAT	54	$(AAG)_9(AG)_{18}$	17
	R: ACAACACAAAAAGAAATATCA			
N44-LMFC32	F: GAAAGAAAGTCGAATAATGTA	54	$(GA)_{23}$	22
	R: TATAAAGAGGGTGGTCTTAGT			
N49-Fsyc13	F: TGGAGATGTGAATATCATTGGACGTG	59	$(GAAA)_8$	17
	R: CGACGAACAGTTAGTATCGGTAACAGC			

Table S2.3 Variable sites in the aligned sequences of turL and atpF-H in the chloroplast gene fragments in 10 haplotypes of *Ficus craterostoma*.

	trn L										*atp* F-H			
Nucleotide Position:	30	102	164	262	276	748	750	851	903	904	1004	1019	1065	1147
H01	-	C	C	G	G	T	T	G	G	■	-	-	G	A
H02	-	C	C	G	G	T	T	G	G	■	-	-	G	T
H03	-	C	C	G	G	T	T	G	G	■	-	C	G	T
H04	-	C	C	C	A	T	T	T	-	-	-	C	G	T
H05	-	C	C	C	G	-	T	G	A	■	T	C	G	T
H06	-	T	C	C	G	-	T	G	A	■	T	C	G	T
H07	-	C	A	C	G	T	T	G	G	■	-	C	G	T
H08	-	C	A	C	G	T	T	G	G	■	-	-	G	T
H09	-	C	A	C	G	T	G	G	G	■	-	C	A	T
H10	▬	C	A	C	G	T	G	G	G	■	-	C	A	T

Black bar indicates insert of ATTGA, while black squares represent indels of GGTCTTGAATTAGGTATC

Table S2.4 Null allele frequencies for each locus at each sample site. (no represent no null allele)

	MFC 1	Frac 202	Frub 29	Frub 61	Frub 415	Fins N1	Fins A1	FM 3-64	FM 4-70	LMFC 20	LMFC 32	Fsyc 13
HAN	no	no	0.121	no	no	0.153	no	no	no	no	0.044	no
ENT	no	no	no	no	no	no	no	no	no	no	no	no
PIE	no	no	0.118	0.164	no	no	no	no	no	no	no	no
THA	no	no	0.101	no	no	no	no	no	0.122	no	no	no
WOL	0.178	0.114	no	0.12	0.218	0.292	0.1	no	no	0.092	0.112	0.131
BUF	0.123	no	0.082	no	no	no	no	no	0.116	no	0.162	no
SAD	no	no	no	0.233	no	0.254	no	no	no	no	no	no
NGO	no	no	no	no	no	no	no	no	no	no	no	no
MAP	no	no	no	no	0.116	0.173	no	no	no	no	no	no
ENT	no	no	no	no	no	no	no	no	no	no	0.153	no
DLI	no	no	no	0.127	no	0.136	no	no	no	no	0.176	no
ING	0.194	0.027	0.055	no	no	no	no	no	no	no	0.062	0.047
MBO	0.114	0.179	0.247	0.09	0.215	0.097	no	no	no	no	0.202	no
DWE	0.170	0.096	0.129	0.173	0.272	0.204	0.165	0.123	no	0.186	0.094	0.085
MAN	0.149	no	0.177	0.177	0.188	0.176	0.102	no	no	0.16	0.188	no

Table S2.5 Genetic diversities of *F. craterostoma* based microsatellites data (9 loci) in each sample site: number of alleles (N_a), allelic richness after rarefication (A_r), observed (H_o) and expected (H_e) heterozygosity with significance for deviation from HWE ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) after correction for multiple testing, inbreeding coefficient (F_{IS}) ($^{*}P < 0.05$), and private allelic richness (P_a). POP indicates the sample site. (9 loci)

POP	Microsatellite data (9 loci)				
	N_a	A_r	H_o / H_e	F_{IS}	P_a
HAN	7.222	6.05	0.649/0.68*	0.047	0.20
ENA	5.556	5.16	0.579/0.614	0.059	0.23
PIE	5.333	4.92	0.69/0.649	-0.067	0.13
THA	6.778	5.81	0.655/0.693**	0.057	0.05
WOL	7.333	6.22	0.544/0.728***	0.258*	0.16
BUF	6.111	5.22	0.603/0.693***	0.133	0.05
SAD	5.333	5.33	0.717/0.713	-0.006	0.19
NGO	6.000	4.71	0.733/0.672	-0.094	0.08
MAP	6.333	5.31	0.611/0.668***	0.088	0.57
ENT	7.778	7.24	0.752/0.748	-0.005	0.36
DLI	8.000	6.58	0.750/0.767	0.022	0.38
ING	9.222	5.52	0.609/0.654***	0.069*	0.08
MBO	7.333	5.93	0.521/0.658***	0.212*	0.19
DWE	9.889	6.41	0.477/0.735***	0.354*	0.41
MAN	8.889	6.58	0.554/0.734***	0.249*	0.25

Table S2.6 Molecular analysis of variance (AMOVA) results indicating the distribution of nuclear microsatellite (nSSR) (9 loci) variability among and within populations of *Ficus craterostoma*. ($^{***}P < 0.001$)

	df	Sum of squares	Percentage of variation (%)
Among populations	14	241.507	7.98***
Within populations	831	2576.037	92.02
Total	845	2817.544	

Table S2.7 Pairwise population differentiation (pairwise F_{ST}) of *Ficus craterostoma* (9 loci) in the bottom left triangle and geographic distances (km) between sample sites in the top right triangle. Only the sample site-pairs without significant differentiation were highlighted by NS ($P > 0.05$). The others were $P < 0.05$.

Pop.	HAN	ENA	PIE	HOL	WOL	BUF	SAD	NGO	MAP	ENT	DLI	ING	MBO	DWE	MAN
HAN		34.6	22.2	52.9	115.4	291.1	333.9	555.5	650.8	668.1	670.7	834.1	934.4	1033.1	1054.4
ENA	0.014 NS		13.5	29.2	115.7	279.9	322.4	546.9	638.2	661.2	663.5	835.4	935.6	1036.8	1058.8
PIE	0.028 NS	0.010 NS		32.8	110.1	279.5	322.2	545.7	638.7	659.3	661.7	830.5	930.7	1031.0	1052.8
THA	0.016 NS	0.003 NS	0.026 NS		91.7	250.9	293.3	518.1	609.0	632.7	634.8	809.0	909.1	1011.2	1033.5
WOL	0.045 NS	0.051	0.051	0.023		181.1	223.7	441.4	539.8	553.1	555.9	720.4	820.6	921.2	943.1
BUF	0.058	0.054	0.066	0.031	0.020 NS		42.9	267.7	359.7	383.6	385.3	577.5	675.7	785.4	809.9
SAD	0.018 NS	0.023 NS	0.033 NS	0.027 NS	0.050	0.055		226.4	316.9	343.0	344.4	543.4	640.6	752.3	777.3
NGO	0.072	0.077	0.089	0.051	0.059	0.072	0.061		118.0	117.6	118.4	343.6	433.7	552.4	579.6
MAP	0.183	0.201	0.164	0.158	0.145	0.185	0.188	0.196		114.3	107.7	352.8	425.3	548.2	577.2
ENT	0.105	0.098	0.086	0.067	0.070	0.098	0.075	0.118	0.124		8.0	244.3	325.7	447.3	475.6
DLI	0.120	0.121	0.093	0.080	0.077	0.107	0.080	0.116	0.095	0.011 NS		249.0	328.9	450.8	479.2
ING	0.058	0.050	0.062	0.028	0.046	0.053	0.077	0.037	0.175	0.099	0.107		100.3	209.9	236.4
MBO	0.081	0.102	0.103	0.055	0.059	0.091	0.104	0.064	0.180	0.103	0.104	0.025		122.9	152.0
DWE	0.081	0.108	0.096	0.072	0.034	0.081	0.079	0.085	0.135	0.092	0.065	0.073	0.050 NS		29.5
MAN	0.095	0.123	0.105	0.076	0.040	0.087	0.092	0.085	0.111	0.084	0.046	0.069	0.052	0.006 NS	

Table S2.8 Pairwise population differentiation (pairwise F_{ST}) of *Ficus craterostoma* (9 loci) in the bottom left triangle and geographic distances (km) between sample sites in the top right triangle. Only the sample site-pairs without significant differentiation were highlighted by NS ($P > 0.05$). The others were $P < 0.05$.

Pop.	HAN	ENA	PIE	HOL	WOL	BUF	SAD	NGO	MAP	ENT	DLI	ING	MBO	DWE	MAN
HAN			22.2	52.9	115.4	291.1	333.9	555.5	650.8	668.1	670.7	834.1	934.4	1033.1	1054.4
ENA	0.009^{NS}		13.5	29.2	115.7	279.9	322.4	546.9	638.2	661.2	663.5	835.4	935.6	1036.8	1058.8
PIE	0.023^{NS}	0.010^{NS}		32.8	110.1	279.5	322.2	545.7	638.7	659.3	661.7	830.5	930.7	1031.0	1052.8
THA	0.011^{NS}	0.005^{NS}	0.021^{NS}		91.7	250.9	293.3	518.1	609.0	632.7	634.8	809.0	909.1	1011.2	1033.5
WOL	0.045^{NS}	0.044	0.048	0.026^{NS}		181.1	223.7	441.4	539.8	553.1	555.9	720.4	820.6	921.2	943.1
BUF	0.043	0.043	0.053	0.021	0.021^{NS}		42.9	267.7	359.7	383.6	385.3	577.5	675.7	785.4	809.9
SAD	0.011^{NS}	0.018^{NS}	0.032^{NS}	0.021^{NS}	0.042	0.043		226.4	316.9	343.0	344.4	543.4	640.6	752.3	777.3
NGO	0.058	0.066	0.071	0.042	0.054	0.057	0.051		118.0	117.6	118.4	343.6	433.7	552.4	579.6
MAP	0.218	0.229	0.216	0.198	0.167	0.209	0.219	0.234		114.3	107.7	352.8	425.3	548.2	577.2
ENT	0.122	0.117	0.120	0.093	0.075	0.108	0.095	0.139	0.116		8.0	244.3	325.7	447.3	475.6
DLI	0.127	0.125	0.118	0.102	0.079	0.114	0.093	0.133	0.102	0.014^{NS}		249.0	328.9	450.8	479.2
ING	0.048	0.045	0.049	0.023	0.047	0.042	0.069	0.032	0.209	0.122	0.124		100.3	209.9	236.4
MBO	0.072	0.082	0.092	0.054	0.052	0.075	0.089	0.062	0.196	0.109	0.102	0.029		122.9	152.0
DWE	0.083	0.098	0.093	0.077	0.037	0.080	0.077	0.089	0.170	0.106	0.074	0.076	0.045		29.5
MAN	0.086	0.106	0.099	0.072	0.040	0.075	0.083	0.085	0.132	0.080	0.049	0.067	0.042^{NS}	0.009^{NS}	

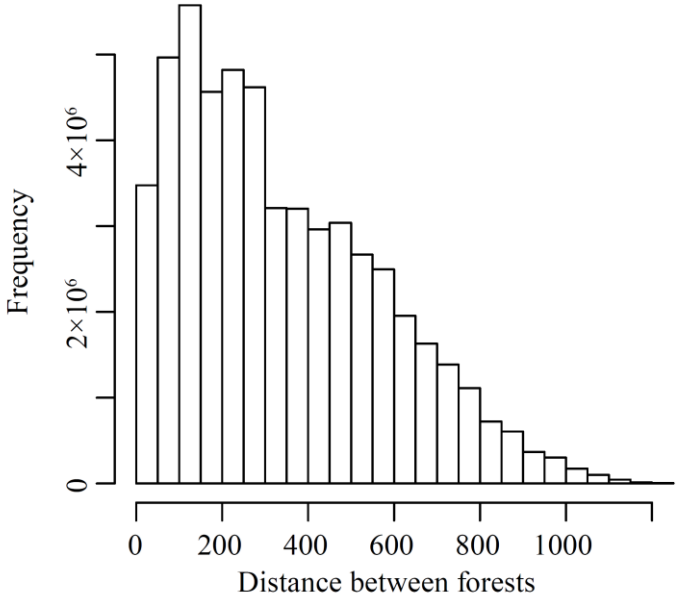

Figure S2.1 The histogram of pairwise geographic distance between forest patches

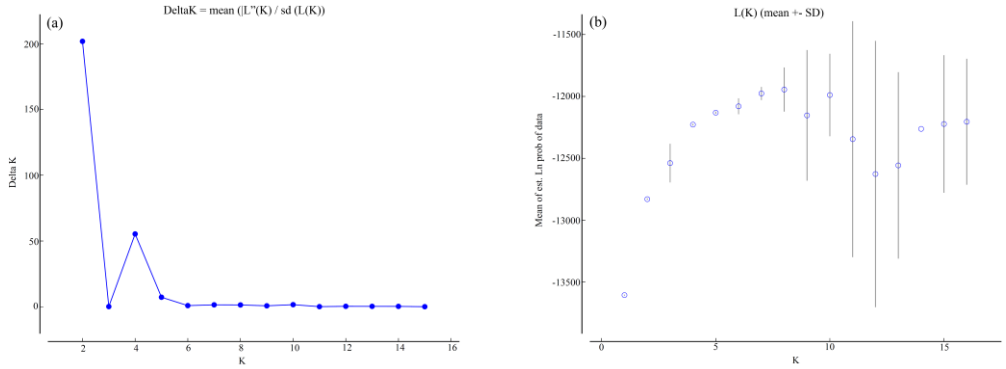

Figure S2.2 The magnitude of delta K (ΔK) simulated by STRUCTURE (a); the mean loglikelihood of the data for K (b) (9 loci).

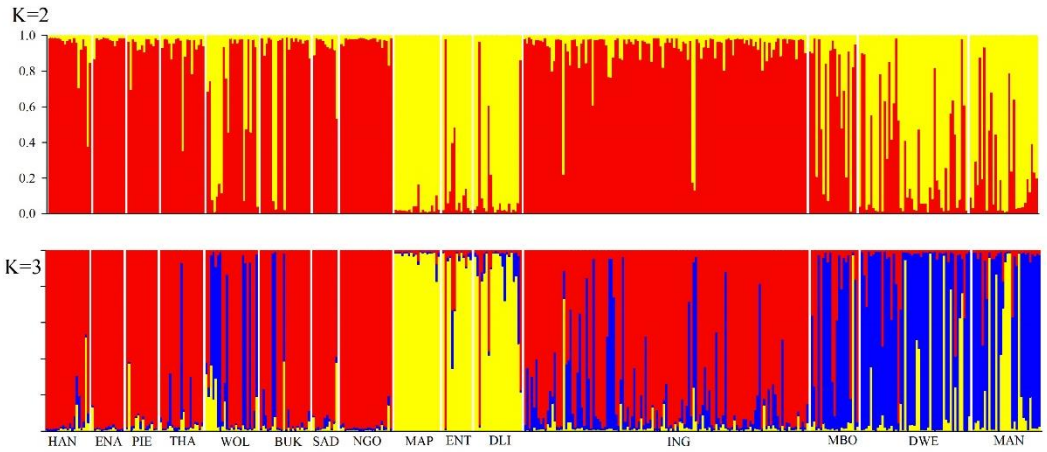

Figure S2.3 The microsatellite memberships of the *Ficus craterostoma* assigned by STRUCTURE for K = 2 and K = 3 for 9 loci. Each vertical line represented one individual. The color indicates the group.

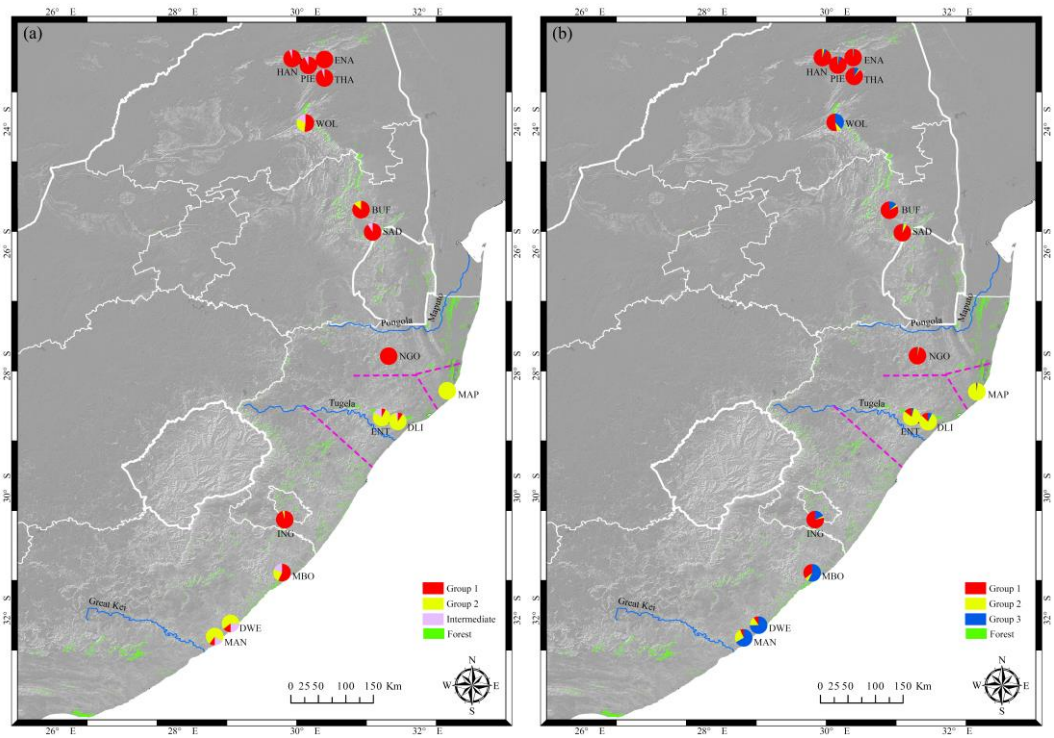

Figure S2.4 To illustrate the geographic structuring of the STRUCTURE groups in Figure S2.3, we indicate the groups on the maps for K = 2 (a) and for K = 3 (b). The group colours from Figure S5 were used to colour the pie-charts and pink was used for individuals that were ambiguous when K = 2.

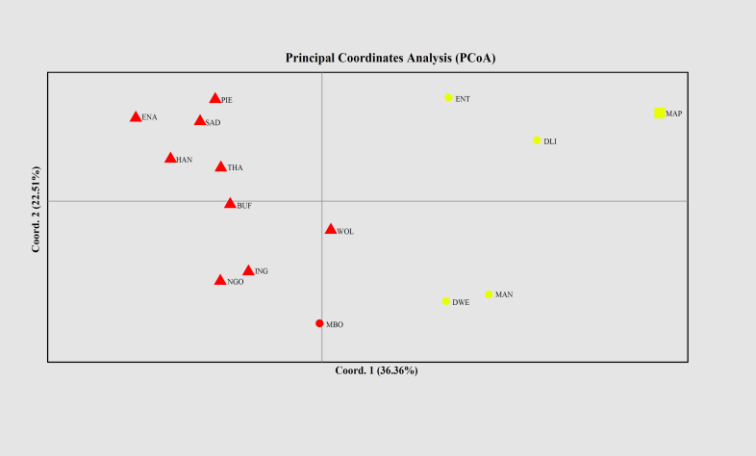

Figure S2.5 Principal coordinate analysis based on microsatellite data across all sample sites (9 loci)

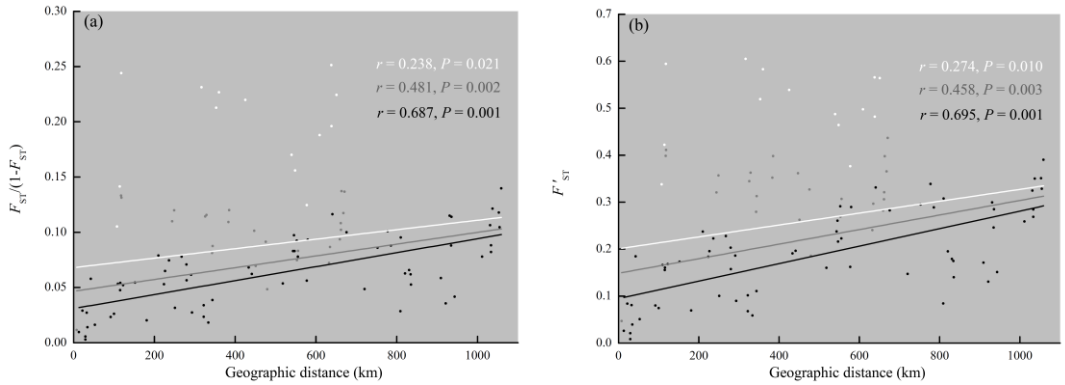

Figure S2.6 The relationships of genetic distances and geographic distances between sample site pairs in each region based on SSR genotypes at 9 loci. White, dark grey and black points indicate MAP comparisons, ENT and DLI comparisons and the remaining comparisons respectively. (a) $F_{ST}/(1-F_{ST})$; (b) F'_{ST}.

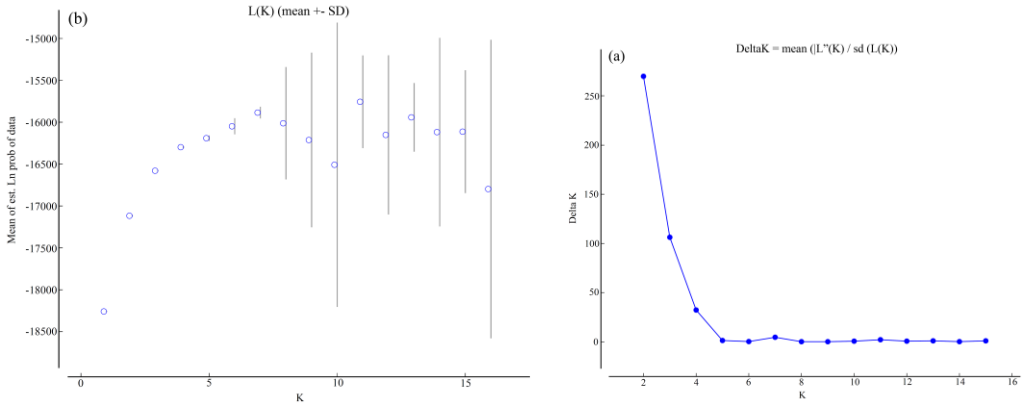

Figure S2.7 The magnitude of delta K (ΔK) simulated by STRUCTURE (a); the mean loglikelihood of the data for K (b).

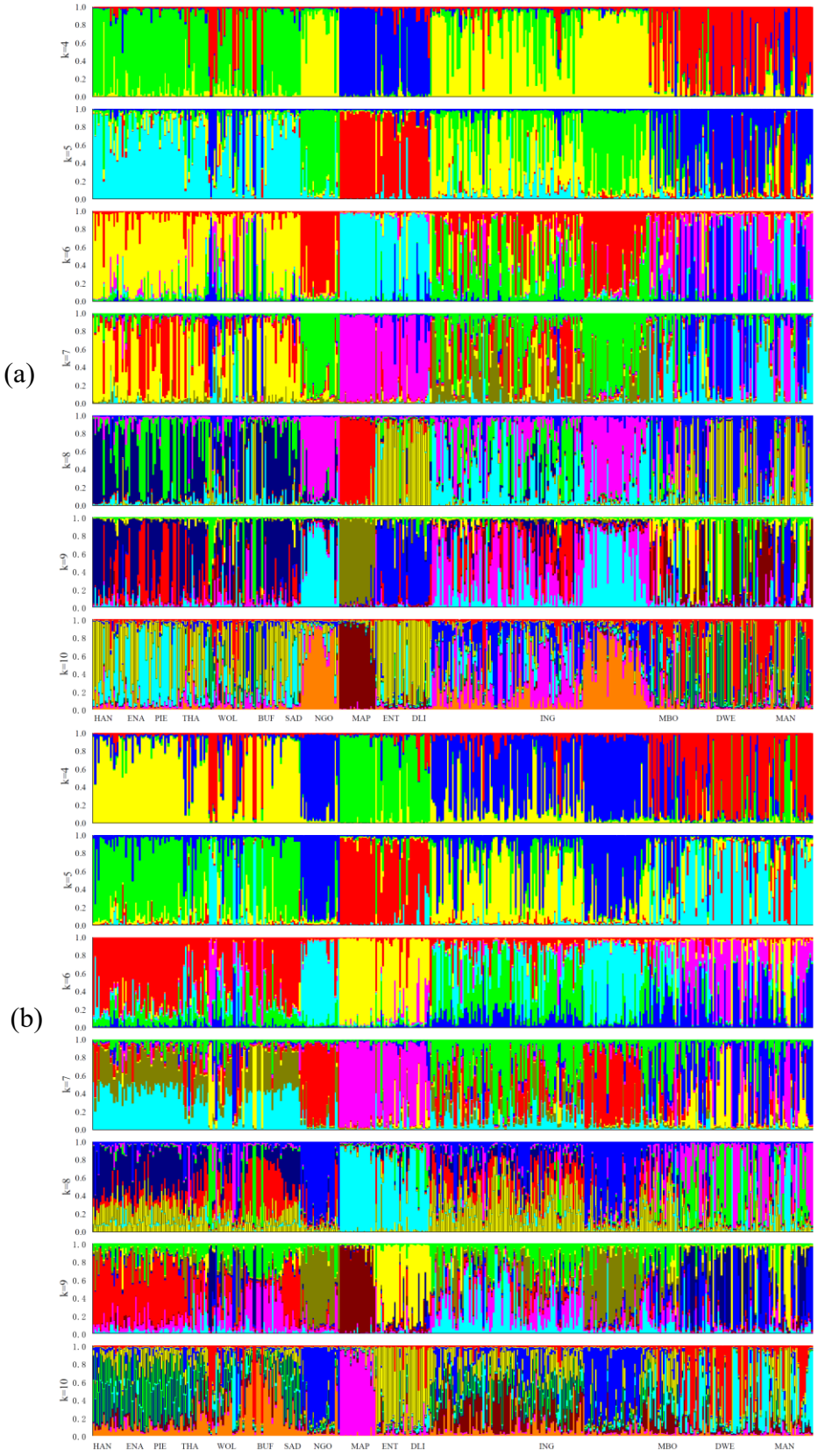

Figure S2.8 The microsatellite memberships of the *Ficus craterostoma* assigned by STRUCTURE (K from four to ten, a for 12 loci, b for 9 loci). Each vertical line represented one individual. The

color indicates the group.

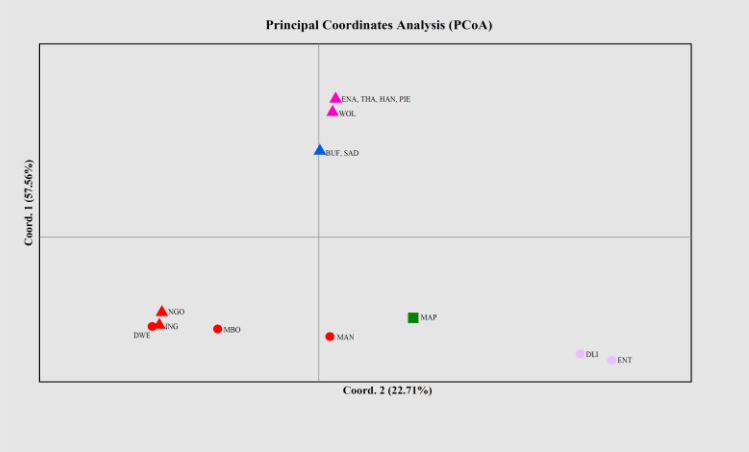

Figure S2.9 Principal coordinates analysis based on CP genes across all sample sites, triangle, circle and squares represent Afrotemperate, scarp and Indian Ocean forest, respectively. The colors from Figure 2.2 were used to color the shape.

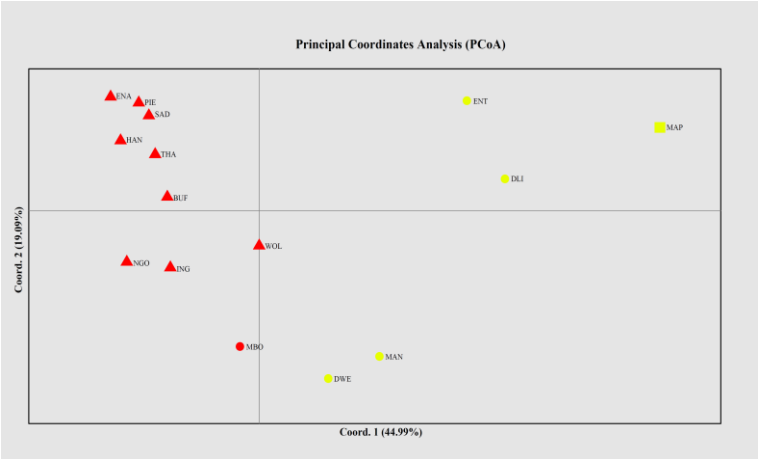

Figure S2.10 Principal coordinates analysis based on microsatellite data across all sample sites (12 loci). The clours represent different genetic group as same as in Figure 2.3.

Chapter 3. The genetic consequences of habitat specificity for fig trees in southern African fragmented forests

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My contribution

Sample collection, all the lab work, data analyses and writing of the first draft and revisions.

3.1 Abstract

Theory predicts that fragmentation will lead to reduced gene flow between populations, with losses of genetic diversity and increased population differentiation. However, these predictions may not always hold true, especially for long-lived woody plants and some fig trees (*Ficus* species) may not be affected by fragmentation because their fig wasps can transfer pollen for distances of over 100 km. Here we contrast the extent of genetic isolation caused by fragmentation among three southern African *Ficus* species with different habitat dependencies and distributional ranges. Two of the species are restricted to forest environments, which have been fragmented since at least the Pleistocene, and provide an indication of the long-term genetic effects of forest fragmentation. The third species is less forest-dependent, with a more general habitat association and more continuous populations. We found significant population differentiation in all three species. Populations of *F. bizanae*, a forest specialist with a highly restricted distribution, displayed the most genetic structure, followed by the second forest specialist, *F. craterostoma*. Populations of the habitat-generalist *F. sur* were the least genetically structured. Forest specialist *Ficus* species are clearly not

immune to habitat fragmentation, despite extensive pollen flow, and other southern African forest trees are likely to have experienced similar or greater effects of habitat fragmentation. The strong genetic structure of *F. bizanae* suggests a limited seed dispersal range and local dispersal by the fig wasp pollinator, a possible adaptation to the limited range of its host fig tree.

3.2 Introduction

Gene flow in most plants is achieved by a combination of pollen movement and seed dispersal. Gene flow acts to homogenize populations and maintains genetic diversity. Fragmentation of habitats can erode the genetic diversity of populations even if gene flow is extensive (Albaladejo *et al.*, 2012; Ismail *et al.*, 2012; Leonardi *et al.*, 2012; Morán-López *et al.*, 2016; Sampson *et al.*, 2014), but it can nonetheless buffer the effects of fragmentation brought about by factors such as climate change and other anthropogenic disturbances (Labra *et al.*, 2006; Byrne *et al.*, 2007; Llorens *et al.*, 2012; Liu *et al.*, 2013; Bai *et al.*, 2014; Colabella *et al.*, 2014).

The genetic erosion caused by fragmentation is typically reflected in the fixation of some alleles and the genetic isolation of populations (Young *et al.*, 1996). These effects eventually result in a loss of heterozygosity, which is really the last symptom of an ongoing process (Aguilar *et al.*, 2008; Vranckx *et al.*, 2012). Because the loss of alleles in response to population fragmentation is caused by drift, which is slow to act, the genetic effects of fragmentation are only manifested generations after fragmentation occurs (Young *et al.*, 1996; Kramer *et al.*, 2008; Bacles and Jump, 2011; Vranckx *et al.*, 2012). Furthermore, in long lived species such as trees we cannot hope to evaluate the genetic effects of fragmentation experimentally (Kramer *et al.*, 2008; Bacles and Jump, 2011). To understand the impact of fragmentation it is therefore important to study habitats that have been naturally fragmented for centuries or millennia.

South African forests are a prime example of a long-fragmented habitat. Across geological time scales, uplifting and warping facilitated formation of large internal mountain ranges and basins that dramatically changed the landscape of South Africa (van Zinderen Bakker, 1983). This facilitated expansion of arid areas, which split habitats that had previously comprised continuous forests (Sepulchre *et al.*, 2006). In addition, around 20 glacial-interglacial cycles have occurred during the last two million years (Lawes, 1990) and alternating colder and warmer cycles have led to periodic contraction and expansion of forest cover (Eeley *et al.*, 1999). During the Last Glacial Maximum (LGM) (18 000 years ago), the forests shrank dramatically and most were eliminated entirely (Lawes, 1990; Eeley *et al.*, 1999; Lawes *et al.*, 2007a). As with eastern African coastal forests, some remnants may have moved to lower elevations whereas inland forests retreated to higher elevations, contributing further to their disjunctive distributions (Axelrod and Raven, 1978). Most recently, human activities such as grazing, logging and agriculture have reduced the extent of forest cover and further aggravated forest fragmentation across the sub-continent (Hoffman, 1997; Lawes *et al.*, 2004; 2007b). Currently, South African forests cover an area of about 4981 km^2, which is only about 0.41% of the land area of the country. Furthermore, they are divided into an estimated 19470 tiny fragments with a median size of less than 0.02 km^2 and a mean of just over 0.25 km^2 (Mucina *et al.*, 2006; http://bgis.sanbi.org/vegmap).

The responses of species to forest fragmentation are highly variable (Henle *et al.*, 2004), and may result in no discernable effects (Berens *et al.*, 2014), a loss of genetic diversity and increased population differentiation (Matolweni *et al.*, 2000; McManus *et al.*, 2015) or even extinction of forest specialists (Lawes, 2004; Olivier *et al.*, 2013; Cooper *et al.*, 2017). The effects of fragmentation, although unlikely ever to be beneficial, are therefore unpredictable and remain poorly understood.

Fig trees (*Ficus* spp., Moraceae) are a diverse and ecologically significant component of the African flora (Berg and Wiebes, 1992; Shanahan *et al.*, 2001; Burrows and Burrows, 2003). Most species exhibit a monoecious breeding system and they typically have wide distributions across the continent (Berg and Wiebes, 1992). The *Ficus* species present in South African forests vary in the extent to which they are restricted to this habitat and in their overall area of distribution (Burrows and Burrows, 2003). They are pollinated by small (1-2 mm; Renoult *et al.*, 2009) host specific fig wasps (Agaonidae). The adults are short-lived (Warren *et al.*, 2010) and fly slowly, but can be carried passively by the wind over long distances (Ware and Compton, 1994; Ahmed *et al.*, 2009) and they are renowned for their dispersal ability (Kobmoo *et al.*, 2010; Liu *et al.*, 2013; Bain *et al.*, 2016). In a desert habitat one species, *Ceratosolen arabicus* was recorded to disperse the pollen of its host *Ficus* over a distance of 100 km (Ahmed *et al.*, 2009). As a result, fig tree populations often show little evidence of genetic isolation by distance (Liu *et al.*, 2013; Yu and Nason, 2013; Wang *et al.*, 2018; Yu *et al.*, 2019), although smaller species, with more aggregated populations, provide exceptions (Chen *et al.*, 2011).

Long-term fragmentation might therefore be expected to have had less effect on the population structure of fig trees than on other tree species in South African forests, because as few as four immigrants per generation can effectively homogenize populations (Hartl, 2000). It nonetheless remains unclear whether wasp-assisted pollen flow can be sufficient to connect forest specialist fig trees into a single large genetic unit across sub-continental scales, especially given that the dispersal of *Ficus* seeds often extends for only short-distances, despite the services of strong flyers such as frugivorous birds (Zhou and Chen, 2010). Seed dispersal distances in South Africa may have been reduced further by the loss of forest specialist birds and mammals after their own populations responded to fragmentation (Lawes, 2004; Olivier *et al.*, 2013; Cooper *et al.*, 2017).

Comparing allele numbers at microsatellite loci across species as a means of assessing genetic diversity is problematic because mutation rates differ between loci and even for the same locus, and ascertainment bias can also give misleading results (Jin *et al.*, 1996; Kalia *et al.*, 2011). An alternative approach is to estimate and compare genetic structure, because this is not prone to these shortcomings. We took this approach to describe the genetic effects of fragmentation on three related South African forest trees that differ in their habitat specificity and distributional ranges. Specifically, we asked whether two *Ficus* species that are restricted to forests (*F. bizanae* Hutch. and Burtt-Davy and *F. craterostoma* Midlbr. and Burret) exhibit a more developed genetic structure and less gene flow between populations than a habitat-generalist species (*F. sur* Forsk.) using microsatellite markers, and whether autecological characteristics may modify the impact of fragmentation on individual species.

3.3 Materials and methods

The study species

Ficus bizanae and *F. craterostoma* belong to section *Galoglychia* while *F. sur* belongs to section *Sycomorus* (Burrows and Burrows, 2003). These three *Ficus* species have different life forms, and they vary in their habitat preferences (Table 3.1, Figure 3.1). *F. bizanae* usually grows as a free-standing tree and can reach a height of 18 m. It is usually associated with rocky habitats within coastal forest environments, where it favours gently sloping rocky scree slopes (Pers. Obs.). The figs of *F. bizanae* develop on major stems, usually in clusters of 2-3 figs emanating from a single boss. The figs reach 25- 45 mm in size. Although they do not dramatically change colour on ripening, they develop a strong smell when they turn from bright green to a pale yellowish-green colour after the fig wasps have exited. These features suggest that both mammals and birds are likely to be attracted to the figs (Shanahan *et al.*, 2001). *Ficus bizanae* is a local endemic that only occurs in a few forests. Its entire range is shown in Figure 3.1.

Ficus craterostoma can reach heights of up to 20 m, and often grows as a strangler of other forest trees. Its figs are located in the leaf axils, where they reach 15-20 mm in diameter and turn yellow with red spots or entirely reddish when ripe. Consequently, birds are likely to be their main seed dispersal agents (Shanahan *et al.*, 2001). *Ficus craterostoma* has a wide distribution in Afromontane forests along the eastern side of Africa from just north of the equator to South Africa. It is also present in forests of central and West Africa. The third species, *Ficus sur,* is a large free-standing tree that grows up to 30 m in height. It is a habitat generalist, but is more common in forest and riparian habitats. The figs are produced in clusters on leafless branchlets arising from the trunk, from high in the canopy down to ground level. The figs reach 20-40 mm in diameter and turn red or dark orange with a pronounced sweet smell when ripe (Berg and Wiebes, 1992). The figs are likely to be dispersed by a wide variety of birds and mammals, including terrestrial non-volant species. *Ficus sur* has a wide distribution across Africa from the southern Cape coastal belt of South Africa northwards.

Table 3.1 The habitat preferences, life forms, South African pollinators and recorded seed dispersal vectors of three South African *Ficus* species.

Ficus (pollinator)	Habitats	Life form	Seed dispersal vector
F. bizanae *(Courtella sp.)*	Forest specialist[1]	Tree (up to 15-18 m), lithophytic or hemi-epiphytic[1,2]	No records, probably understory birds, bats and mammals such as monkeys
F. craterostoma *(Alfonsiella pipithiensis)*	Forest specialist[1]	Small tree (up to 5-10 m), shrub, lithophyticor hemi-epiphytic (occasionally reaching to 20 m) [1,2]	Birds, small rodents and Samango monkeys[3,4]
F. sur *(Ceratosolen capensis*)	Forest, savanna and woodland[1]	Medium to large size tree tree (up to 12 m in open woodland or to 30 m in forest) [1,2]	Birds, bats, rodents, primates (monkeys or baboons) [5,6]

[1] Burrows and Burrows (2003)

[2] Berg and Wiebes (1992)

[3] Gautier-Hion *et al.*, (1985)

[4] Basabose (2002)

[5] Blecher *et al.*, (2003)

[6] Linden *et al.*, (2015)

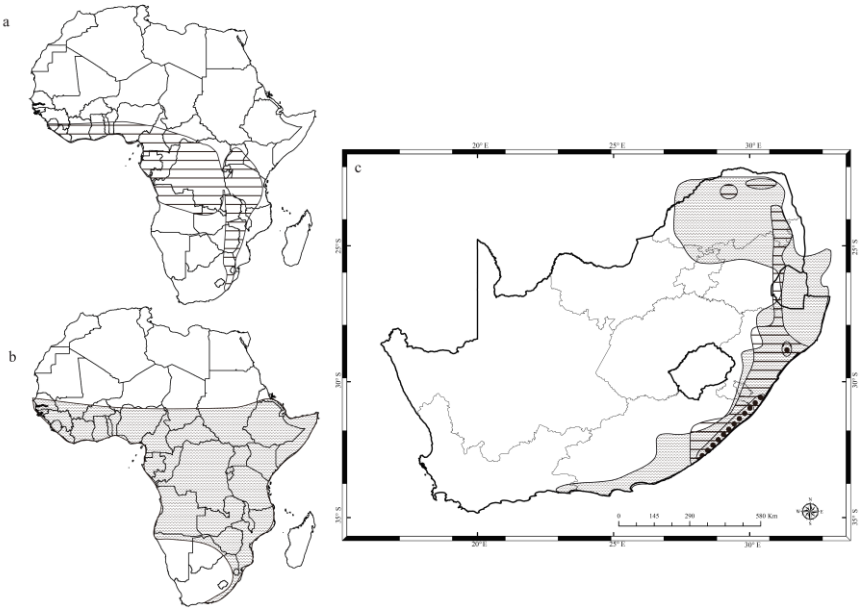

Figure 3.1 The distributions of *F. craterostoma* (horizontal lines, a) and *F. sur* in Africa (horizontal dashed lines, b). The distributions of *F. bizanae* (filled circles, its entire range), *F. craterostoma* (horizontal lines) and *F. sur* (horizontal dashed lines) in South Africa (c). Country borders in solid black bold lines and provincial borders in grey lines.

Sampling

We collected leaves or bark of the three Ficus species from six forests in the Eastern Cape and one forest in KwaZulu-Natal (Figure 3.2 and Table 3.2). We dried and stored most samples in bags with silica gel crystals. Some samples were stored in a NaCL-CTAB-azide buffer (Bhattacharjee *et al.*, 2009). Samples were collected from two types of forests as defined by Mucina *et al.* (2006): scarp and Southern mistbelt forests that occur as fragments along a southwest-northeast edge of southern Africa. Scarp forests are closer to the coast and mistbelt forests are more inland at higher elevations - usually above 1000 m. Montane mistbelt forests are botanically species-rich and contain subtropical floral elements. Scarp forests occur at lower elevations and are characterized by high rates of endemism.

In total, we sampled 108 *F. bizanae* individuals from three sample sites, 247 *F. craterostoma* individuals from five sample sites, and 186 *F. sur* individuals from six sample sites (Table 3.2, Figure 3.2).

Table 3.2 Sample sites and sample sizes in parentheses. The three letter code for each forest, the forest type and the latitude and longitude of each forest are given.

Code	Sample sites	Species	Forest types	Latitude(°S)	Longitude(°E)
NGO	Ngome	*F. craterostoma* (23), *F. sur* (13)	Southern mistbelt	27.826	31.419
ING	Ingeli	*F. craterostoma* (125)	Southern mistbelt	30.530	29.689
MKA	Mkambati	*F. bizanae* (54), *F. sur* (26)	Scarp	31.297	29.979
MBO	Mboyti	*F. bizanae* (32), *F. craterostoma* (21), *F. sur* (56)	Scarp	31.435	29.688
DWE	Dwesa	*F. bizanae* (22), *F. craterostoma* (48), *F. sur* (17)	Scarp	32.280	28.848
MAN	Manubi	*F. craterostoma* (30), *F. sur* (57)	Scarp	32.449	28.606
PIR	Pirie	*F. sur* (17)	Southern mistbelt	32.739	27.298

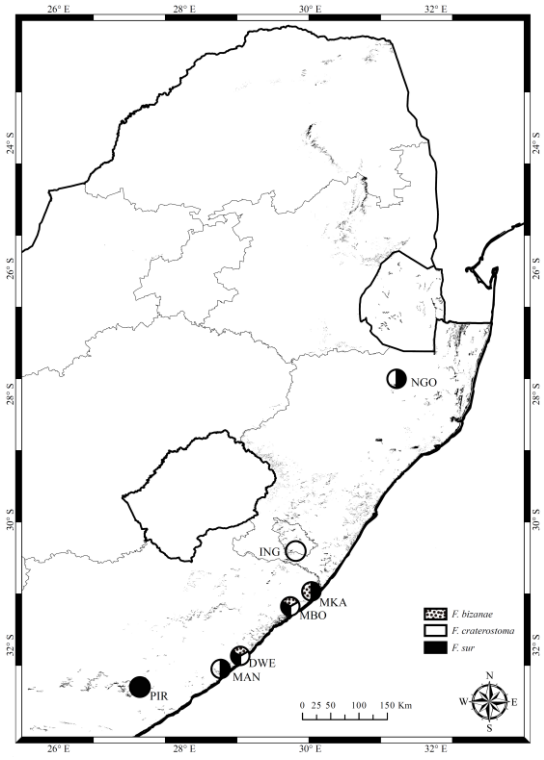

Figure 3.2 A more detailed depiction of the eastern part of Fig. 1 indicating sampled forests. Species sampled in each location are indicated (*F. bizanae* = dots filled, *F. craterostoma* = empty, *F. sur* = black filled) and forest distribution is given in black pixels.

DNA extraction and genotyping

Genomic DNA were extracted with the nucleoSpin, Plant II kit (Macherey-Nagel GmbHandCo.KG, Düren, Germany) using standard protocols. We selected 49 microsatellite (SSR) primer pairs that were developed for other *Ficus* species (Khadari

et al., 2001; Giraldo *et al.*, 2005; Zavodna *et al.*, 2005; Vignes *et al.*, 2006; Ahmed *et al.*, 2007; Crozier *et al.*, 2007; Heer *et al.*, 2012; Tan *et al.*, 2016). Amplifications for all primers of samples of each species were ran with 15 ul of polymerase chain reaction (PCR) mixture, including ddH2O 10.6 ul, reaction Taq buffer 1.5 ul (10x), dNTP 0.3ul (10 mM), primers 0.2 ul (10 uM), Taq polymerase (Takara) 0.2 ul (5U/ul) and DNA 2 ul (50 ng /ul). The published cycle programs were used for each primer and suitable annealing temperature were tested for those primers showing clear bands in amplifications. We then amplified DNA using all the primers again, but at their best annealing temperatures. The products were examined for polymorphism on an 8% polyacrylamide gel with a 50bp DNA ladder stained with silver nitrate. Twenty-three polymorphic SSR primer pairs were obtained and labelled with fluorescent markers giving 8 for *F. bizanae*, 12 for *F. craterostoma* and 10 for *F. sur* (Table S3.1). We genotyped the samples with these primers using an automated sequencer (ABI 3730) and scored loci using GeneMarker HID v.2.05 (Holland and Parson, 2011).

Confirming usability of loci

We examined the presence of any genotypic errors due to stuttering, large allele dropout and null alleles using Micro-checker v.2.2.3 (Van Oosterhout *et al.*, 2004). FSTAT v.2.9.3 were used to confirm that no loci were in linkage disequilibrium with each other (Goudet, 1995). The deviation from Hardy-Weinberg equilibrium (HWE) in each sample site was examined by the multi-locus exact test in GENEPOP 4.0 (Rousset, 2008). A Markov chain method was used to estimate the P-value of the test with default settings.

Genetic differentiation and structure

Global and pairwise population differentiation indexes (F_{ST}) for each species were estimated in FSTAT, jackknifing over all loci and using 1000 permutations to test significance and confidence intervals were calculated based on mean and standard error (CI = mean $\pm$ 1.96 × SE). We standardized these estimates to correct for the maximum difference that could be obtained given the variation using $F'_{ST} = F_{ST}/F_{STmax}$ as suggested by Meirmans and Hedrick (2011). RecodeData (Meirmans, 2006) was used to convert the data and imported them into FSTAT to estimate global F_{STmax} and pairwise population F_{STmax}.

We used an analysis of molecular variance (AMOVA) as implemented in ARLEQUIN 3.5 (Excoffier and Lischer, 2010) to partition the genetic variation of each species, into within and among population components with 1000 permutations to determine significance. In addition, for *F. craterostoma* and *F. sur*, we specified two groups of populations: montane (mistbelt forest) and coastal lowland forest (scarp). We used the AMOVA to partition the genetic variation into three components (within population, among populations and between groups). A 1000 permutations were used to estimate significance.

Isolation by distance

The effect of geographic distance on genetic differentiation between populations was measured using microsatellites. Specifically, we correlated pairwise estimates of genetic distances F'_{ST} against the corresponding geographic distance with a Mantel test using the R 3.3 package 'vegan' (http://www.r-project.org/). We determined significance with 1000 permutations.

3.4 Results

In total, we scored 541 individual trees: 8 loci in 108 *F. bizanae*, 12 loci in 247 *F. craterostoma* and 10 loci in 186 *F. sur* (Table 3.2, Table S3.1). We found no significant linkage disequilibrium. Several sample sites of each species deviated from HWE and some contained null alleles (Table S3.2). The excess of homozygotes in these sample sites may be due to inbreeding, a Wahlund effect, or the presence of null alleles (Hartl, 2000; Chapuis and Estoup, 2006).

All three species exhibited significant genetic structuring of their sample sites, with *F. bizanae* displaying the most structure and *F. sur* the least (Table 3.3). Similarly, among-population genetic variance was highest in *F. bizanae*, and lowest in *F. sur* (Table 3.3). Significant genetic difference (F_{CT} = 0.03, P = 0.02) was found between *F. craterostoma* in mistbelt (montane) and scarp (coastal lowland) forests, while no such difference was detected in *F. sur* (F_{CT} = 0, P = 0.60).

Table 3.3 Global F_{ST} with 95% confidence interval in parentheses, F'_{ST} and among population percentage of variation for each species.

Ficus species	*Global* F_{ST}	F'_{ST}	AMOVA: Percentage variation among populations (%)
F. bizanae	0.12 (0.07-0.17) ***	0.23	11.85
F. craterostoma	0.05 (0.01-0.09) ***	0.16	6.25
F. sur	0.04 (0.03-0.05) ***	0.11	3.89

***$P < 0.001$

All three species had indications of an increase in genetic distance as geographical distance increased, but isolation by distance was only significant in *F. craterostoma* (Figure 3.3). *Ficus bizanae* occurs in very few forest patches, which lowered the statistical power to detect a correlation, but its F'_{ST} was twice that of *F. sur* and nearly 1.5 times that of *F. craterostoma* (Figure 3.3; Table 3.3). Despite occurring in many forests, we were unable to detect any significant isolation by distance in *F. sur*. These findings support the impression that genetic structure is highest in *F. bizanae* and lowest

in *F. sur*.

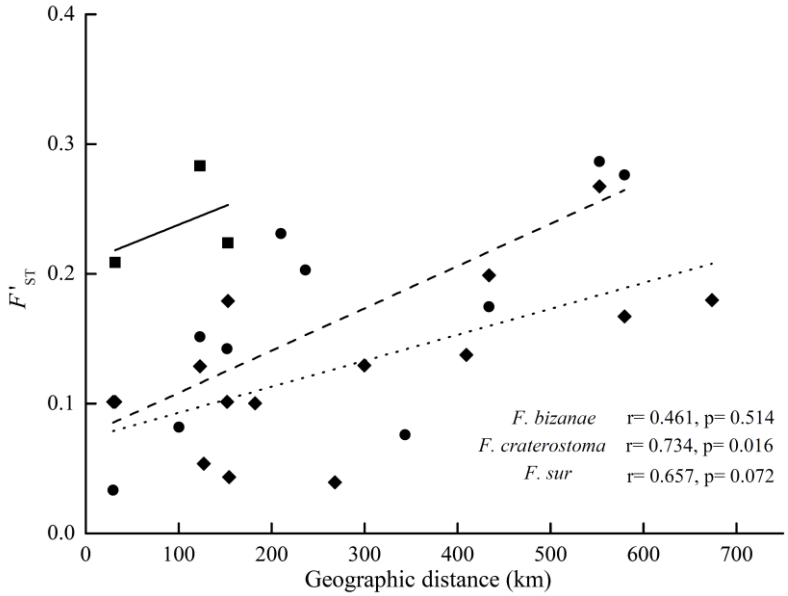

Figure 3.3 The relationships between genetic distances (pairwise F'_{ST}) and geographic distances between sample site pairs. *Ficus bizanae* = squares and solid line; *F. craterostoma* = circles and dashed line; *F. sur* = diamonds and dotted line.

3.5 Discussion

Despite the exceptional ability of some fig wasps to disperse pollen over long distances (Harrison and Rasplus 2006; Ahmed *et al.*, 2009; Kobmoo *et al.*, 2010; Nazareno *et al.*, 2013), we found that natural long-term fragmentation of South Africa's forests has resulted in stronger genetic isolation of populations of two habitat-specialist *Ficus* species than in the habitat-generalist *F. sur*. This is likely to be because forest populations of *F. sur* are linked genetically via individuals growing in other habitats between the fragmented forests. The extent of genetic isolation between populations was greater in the forest specialist species with a more limited distribution (*F. bizanae*) than in the more widely distributed *F. craterostoma*. This may be linked to differences in the distances that their seeds and pollen are dispersed.

Wright (1978) proposed that observed overall values of F'_{ST} can be characterized as belonging to one of three different qualitative categories, indicating very great

divergence ($F'_{ST} > 0.25$), great divergence ($0.25 > F'_{ST} > 0.15$) and moderate divergence ($0.15 > F'_{ST} > 0.05$). Based on these criteria, great divergence was found in both *F. bizanae* ($F'_{ST} = 0.23$) and *F. craterostoma* ($F'_{ST} = 0.16$), while moderate divergence was revealed in *F. sur* ($F'_{ST} = 0.11$).

The pairwise F'_{ST} 's of the three species nonetheless extend across Wright's categories, in response to the varying distances between sample sites (Table S3.3, Figure 3.3). More distant sample sites were more genetically dissimilar due to isolation by distance. This trend was only statistically significant for *F. craterostoma*, as a lack of statistical power limited our analysis of *F. bizanae*, despite it having greater genetic structure than *F. craterostoma*. For example, a comparison between the species shows that at a distance of 500 km the genetic distance between *F. craterostoma* sample sites is equivalent to that of *F. bizanae* sample sites located only 100km apart. The relationship between genetic and geographic distances between populations of the habitat generalist *F. sur* was not significant, suggesting that the gene flow is sufficient to homogenize samples that are over 600 km apart into one genetic unit (Figure 3.3). This result is similar to that recorded for some other fig trees in Asia, including species closely related to *F. sur* (Kobmoo *et al.*, 2010; Tian *et al.*, 2015; Wang *et al.*, 2018). The low gene flow of the two forest-specialists stems from the fact that these samples actually came from discrete populations, separated by 10's of kilometers rather than a semi-continuous distribution with individual trees more densely dispersed across the overall distributional range, as is the case for *F. sur* (Figure 3.1).

Whereas *F. bizanae* was only recorded in scarp forests, *F. craterostoma* and *F. sur* were recorded in both scarp and mistbelt forests. *Ficus bizanae* showed distinct genetic structure within scarp forest. In *F. craterostoma*, the AMOVA revealed that scarp and mistbelt forest sample sites were genetically distinct. Even so, pairwise F'_{ST} indicate some gene flow occurs between the two forest types when subpopulations are

geographically close (ING and MBO). In contrast, in *F. sur* no significant difference was found between scarp and mistbelt forests by AMOVA. Indeed, populations of this species from distantly located forest fragments, as well as those from different forest types can be genetically similar.

Working with animal rather than plant populations, Tolley *et al.* (2018) proposed an idiosyncratic process of vicariance in South African forests. They concluded that South African forests have undergone expansions, contractions and distribution shifts. Despite our current results being insufficient to elucidate the underlying evolutionary processes driving forest fragmentation in South Africa, we highlight dissimilar effects of the fragmentation process on different plant species with different ecological characters, as well as with some animal species.

The speciation process and evolutionary history of *F. bizanae* is currently unresolved. In particular, it is unclear whether the species has had a wider distribution in the past, or is a neo-endemic species that has always been restricted to the southern coastal areas of South Africa. *F. bizanae*'s overall range is much more restricted than that of *F. craterostoma* (Figure 3.1; Burrows and Burrows, 2003). The greater genetic isolation between its sample sites as compared to *F. craterostoma* suggests that the ecology of the two species differs in ways that influence their response to habitat fragmentation. Here we list a number of biological features of the two species and then relate these to their dispersal ability. *Ficus bizanae*'s figs are borne on the trunks (Burrows and Burrows, 2003) below the canopy of the forest. Phenology can be asynchronous within a tree allowing wasps to disperse from mature figs to immature figs on a single tree and thereby the plant can self-pollinate.

Ficus bizanae's crop sizes are far smaller than those of the other two species, and closely situated individual trees often simultaneously bear figs at different stages of development. These factors may favour more local dispersal of both pollen and seeds

and could explain the stronger genetic structure of *F. bizanae*. *F. bizanae*'s pollinators are produced by figs situated underneath the canopy and few or no pollinators may venture above the canopy to disperse passively over long distances using wind currents (Compton, *et al.*, 2000; Harrison and Rasplus, 2006). Intermittent asynchrony of *F. bizanae* crops will be conducive to pollinator cycling on the same tree, and could result in selfing. This was never the case in *F. craterostoma* where within-crop development is highly synchronised (Hossaert-McKey and Bronstein, 2001). Selfing should result in detectable inbreeding. However, if inbreeding offspring suffer from inbreeding depression it will reduce or even result in a negative inbreeding coefficient. In all the studied sample sites (bar one) of all three *Ficus* species, the inbreeding coefficient was significantly larger than 0. However, the excess homozygosity may be due to true inbreeding, null alleles, or Wahlund effect. Parentage analysis will be required to identify the cause of the observed excess homozygosity.

General island biogeographic effects may have selected for more localized movements of *F. bizanae* pollinators as seen in the reduced dispersal exhibited by animals on small islands (Macarthur and Wilson, 2001), and parasitoid wasps whose hosts are highly restricted to a particular habitat (van Noort *et al.*, 2014). *Ficus bizanae*'s restricted range means that long distance pollinator dispersal will always be unsuccessful and these pollinators may thus have evolved a reduced affinity to fly upwards towards strong wind currents, thereby avoiding the risk of ending up outside a forest (Smith and Bronstein, 1996; Gates and Nason, 2012; Fagan *et al.*, 2014). Such local wasp dispersal may also be responsible for restricting *F. bizanae*'s ability to found new populations because mature trees in newly established forest populations will never be pollinated.

Other factors may be restricting the ability of *F. bizanae* to colonise and establish new populations. *Ficus* seed dispersal is influenced by species-specific fruit traits that attract different animal species (Shanahan *et al.*, 2001; Lomáscolo *et al.*, 2008; 2010). Primates

may be the major seed dispersal vectors for *F. bizanae,* given the green colour and the sweet smell of its mature figs (Shanahan *et al.*, 2001; Lomáscolo *et al.*, 2008). Primates are usually not long-distance travelers and they frequently become extinct in small patches in fragmented habitats (Lawes, 2004) so low rates of seed dispersal, as well as short dispersal distances would not be surprising in *F. bizanae.*

In contrast, *F. craterostoma* trees, which bear large, dense and synchronous crops of small figs in the leaf axils, frequently protrude above the forest canopy Although *F. craterostoma* figs are also known to be eaten by monkeys (Basabose, 2002; Linden *et al.*, 2015), their small size and the fact that they ripen to a bright yellow and sometimes even to a reddish colour suggests that birds are important dispersers (Lomáscolo *et al.*, 2008), and figs in the canopy are more likely to attract birds that disperse over long distances (Shanahan and Compton, 2001). Continuous forest cover across parts of South Africa is believed to have started to fragment during the late Pleistocene (Lawes, 1990; Lawes *et al.*, 2007a; Eberle *et al.*, 2017). The extent to which the populations of the two forest-specialist fig tree species have diverged is consistent with such long-term fragmentation of their forest habitat and agrees with conclusions based on regional climatic conditions inferred from pollen studies (Scott *et al.*, 1997; Scott, 2002) and climatic reconstructions (Eeley *et al.*, 1999; Eberle *et al.*, 2017). Divergence has occurred despite the extensive pollen flow that characterizes monoecious *Ficus* species. Comparable genetic data is not available from other South African forest trees, but our results suggest that many other tree species that are restricted to South Africa's forests are likely to display similar or greater genetic effects from fragmentation, because their gene flow is likely to be less extensive than that of fig trees. Forest fragments form archipelagos in Afromontane areas across the whole eastern side of Africa, and fragmentation effects are likely to be widespread across a wide range of forest species (White, 1983).

Given the results of our study, it would be beneficial to both forest specialist *Ficus* species if nearby forest were linked genetically by transplanting seeds and seedlings. The artificial transfer of *F. bizanae* to other forest patches would also make populations of this rare endemic more resilient. The limited range of *F. bizanae* might nonetheless suggest that it has highly specific habitat requirements, but this remains to be confirmed.

In conclusion, we used a naturally fragmented population to study the effect of millennia of repeated forest contraction and expansion. It is clear that even for *Ficus*, with its high pollen dispersal ability, populations have become differentiated over time. Such differentiation is the result of a reduction of gene flow, which was more marked in forest specialists that are physically more isolated. The isolation was also more marked in the range-limited forest species where seed and pollen dispersal distances may both be reduced, which emphasizes the importance of individual species' ecology.

3.6 Supplementary materials

Table S3.1 Twenty three polymorphic nuclear loci used for exploring population genetic structure of three *Ficus* species (a,b and c represent *F. bizanae*, *F. craterostoma* and *F. sur* respectively)

Locus	Primer sequence (5'- 3')	Tm (°C)	Repeats	Species
MFC1[1]	F: ACTAGACTGAAAAAACATTGC R: TGAGATTGAAAGGAAACGAG	58	$(CT)_{13}$	a b
MFC8[1]	F: GTGGCGTCGTCTCTAATAAT R: TATTCTATGCTGTCTTATGTCA	58	$(CA)_9TA(CA)_{14}$ $(TA)_6$	c
Frac202[2]	F: AGAGATATGATGTTCTAGTGCA R: CTCGCTCCCACTTAAATACAAG	59	$(TG)_{13}$	b c
Frub29[2]	F: CCACTTTGGAATGTCACTTGGA R: TGAACACGCCAACTGAGAATG	59	$(AG)_{24}$	b c
Frub38[2]	F: ACACGTGCAGTGCTGCTGA R: ACAGCTGCCCAATTCCTTGA	59	$(AG)_8AAC$ $(GA)_{13}$	c
Frub61[2]	F: GTACACTCTCTTAGCTGCC R: TACCTTTCTCTGGACATTC	59	$(TC)_{24}$	a b c
Frub391[2]	F: AGATGTCAAATAAGGTCAGCT R: AGATGCAGTTCCATACAATTC	59	$(TG)_{19}$	a
Frub415[2]	F: GCACGTAGTCGGTGTTAAGC R: CTGTGCGGAATAAAAGCTAGC	59	$(AC)_{10}$	b
FinsI12[3]	F: GAACCTTCAACCTCAATCAA R: CTCCCCTTTCCTAGTCCTTA	58	$(TC)_5(CT)_{11}$	c
FinsJ10[3]	F: AGGTGGAATGAGGAGAGAGT R: AAACATCCTTTCTGGACTTG	58	$(TC)_{11}$	c
FinsM5[3]	F: ATGAATGGTGAAATCCTGAA R: CATGGCCTCAACTTAGAAAC	58	$(TC)_{13}$	c
FinsN1[3]	F: AGGGCTGAGATAGGTTGATT R: TAAGTTGGTGTGTGGCATC	58	$(TA)_2(CA)_{10}$ $(TA)_7$ CATA$(TG)_2$	b
FinsQ6[3]	F: TTCTCCAATTAAACCTCCAA R: CATGAAATCACCTTCCTCAT	58	$(TG)_7$ AG$(TG)_5$	a
FinsA1[3]	F: AATCCCCGTACTTCACTTG R: AGAACTTATTGCACGGACAG	58	$(CT)_{12}$	b c
FM3-64[4]	F: GATGGTGTTGTTGTCGATGGTCAT R: GCGGCTCGGTGGAACTTGAG	59	$(CAA)_8$	b
FM4-70[4]	F: CAGATGAGGTTGACGATGTTATTG R: TAAACCCTCTTCAAATTCACTCTC	59	$(GAA)_{20}$	b
FS4-11[4]	F: AAGGCAACGGGGATAAAGTATTCA R: CTCCGAGAGCAACTCCATCACG	59	$(CGA)_6$	c
LMFC20[5]	F: ATGGAGGCTTAGATAGAAAT R: ACAACACAAAAAGAAATATCA	54	$(AAG)_9$ $(AG)_{18}$	a b
LMFC32[5]	F: GAAAGAAAGTCGAATAATGTA R: TATAAAGAGGGTGGTCTTAGT	54	$(GA)_{23}$	b
Fsyc13[6]	F: TGGAGATGTGAATATCATTGGACGTG R: CGACGAACAGTTAGTATCGGTAACAGC	59	$(GAAA)_8$	b
FT02[7]	F: CAAAATCGGAATAATGACAGC R: AAAGACAACGAAACTAAGAGG	58	$(CT)_{13}$	a
FT07[7]	F: CCACAACTACCCAACCAAG R: GCTGGAGCCAAATCATCTA	58	$(TG)_7A(GA)_8$	a
FT14[7]	F: GAAGAGGCCCTGAGATAA R: GATCAAGCGATGACAACC	58	$(GA)_{12}$	a

[1] Khadari *et al.*, 2001; [2] Giraldo *et al.*, 2005; [3] Zavodna *et al.*, 2005; [4] Vignes *et al.*, 2006; [5] Ahmed *et al.*, 2007; [6] Crozier *et al.*, 2007; [7] Tan *et al.*, 2016

Table S3.2 Null allele frequencies for each locus at each sample site for each species. (no indicates no null allele)

Specis	Locus / Code	MFC1	Frub61	Frub391	FinsQ6	LMFC20	FT-02	FT-07	FT-14
Ficus bizane	MKA	no	no	no	no	0.142	no	no	no
	MBO	no	no	no	0.225	no	no	0.152	no
	DEW	no	no	no	0.151	0.290	no	no	0.211

Specis	Locus / Code	MFC1	Frac202	Frub29	Frub61	Frub415	FinsN1	FinsA1	FM3-64	FM4-70	LMFC20	LMFC32	Fsyc13
Ficus craterostoma	NGO	no	no	no	no	no	no	no	no	no	no	no	no
	ING	0.194	0.027	0.055	no	no	no	no	no	no	no	0.062	0.047
	MBO	0.114	0.179	0.247	0.09	0.215	0.097	no	no	no	no	0.202	no
	DWE	0.17	0.096	0.129	0.173	0.272	0.204	0.165	0.123	no	0.186	0.094	0.085
	MAN	0.149	no	0.177	0.177	0.188	0.176	0.102	no	no	0.16	0.188	no

Specis	Locus / Code	MFC8	Frac202	Frub29	Frub38	Frub61	FinsI12	FinsJ10	FinsM5	FinsA1	FM4-11
Ficus sur	NGO	no	no	0.163	no	no	no	no	no	no	no
	MKA	no	0.117	no	no	no	no	no	no	no	0.287
	MBO	0.080	0.180	no	no	no	0.076	no	no	0.116	0.111
	DWE	no	0.151	no	no	no	no	0.163	no	no	no
	MAN	no	0.130	no	no	no	no	0.113	0.080	0.027	0.064
	PIR	no	no	no	no	0.112	no	no	no	no	0.235

Table S3.3 Pairwise F'_{ST} for each species in the bottom left triangle and geographic distances (km) between sample sites in the top right triangle. (a *F. bizanae*, b *F. craterostoma*, c *F. sur*) ($^{*}P < 0.05$)

(a)

	MKA	MBO	DWE
MKA		31.632	152.813
MBO	0.209*		122.885
DWE	0.224*	0.283*	

(b)

	NGO	ING	MBO	DWE	MAN
NGO		343.634	433.674	552.393	579.648
ING	0.076*		100.348	209.89	236.395
MBO	0.175*	0.082*		122.885	152.025
DWE	0.286*	0.231*	0.152*		29.486
MAN	0.276*	0.203*	0.142*	0.033	

(c)

	NGO	MKA	MBO	DWE	MAN	PIR
NGO		409.238	433.674	552.393	579.648	673.459
MKA	0.138*		31.632	152.813	182.186	299.478
MBO	0.199*	0.101*		122.885	152.025	267.935
DWE	0.267*	0.179*	0.129*		29.486	154.221
MAN	0.167*	0.100*	0.101*	0.101*		126.909
PIR	0.180*	0.129*	0.039	0.043	0.054*	

Chapter 4. Gene flow and ecological processes that affect the fine scale population genetic structure of *Ficus* species in South African forests

This is a manuscript in preparation for submission to a peer-reviewed journal

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My contribution

Sample collection, all the lab work, data analyses and writing of the first draft and revisions.

4.1 Abstract

Gene flow, immigration, and non-random survival affect fine scale spatial genetic structure (SGS). Fragmentation of forests can reduce the effectiveness of pollen and seed dispersal within and between population fragments. Reduced and clumped immigration from distant fragments will reduce fine scale SGS, whereas reduced gene flow within fragments will increase fine scale SGS. In addition, if post-dispersal ecological processes result in non-random mortality of juveniles, the fine scale SGS of age-classes may differ. We studied the fine scale SGS of three *Ficus* species with different habitat preferences and distributional ranges in an archipelago of South African forest patches. Significant fine scale SGS in all three species suggests limited gene dispersal within forest fragments, probably due to limited seed dispersal. An endemic forest specialist, *F. bizanae*, has an unusually high fine scale SGS for a monoecious *Ficus*. This may be explained by this species having several features more typical of dioecious *Ficus* that reduce pollen dispersal distances. A significant negative kinship coefficient in one *F. bizanae* forest, and a significant fine scale SGS in adult *F. craterostoma* suggest that some clumped long-distance immigration may have occurred in the past. The lack of fine scale SGS in *F. craterostoma* saplings suggests that recent population fragmentation has stopped such long-distance immigration. Non-random mortality caused by microenvironmental selection may also contribute to the contrasting

age-related fine scale SGS.

4.2 Introduction

Wright (1943) proposed that even in a continuously distributed population, individuals are more likely to mate with close-by individuals than distant ones, due to limited dispersal. This results in fine scale spatial genetic structure (SGS) within populations (Wright, 1943; Sokal and Wartenberg, 1983; Epperson and Li, 1997; Smouse and Peakall, 1999; Rousset, 2000) that reflects the spatial impact on the gene flow taking place (Hardy and Vekemans, 1999; Hardy *et al*., 2006).

For sessile organisms such as plants, gene dispersal is limited to pollen and seed dispersal (Heuertz *et al*., 2003; Hardy *et al*., 2006; Dick *et al*., 2008). In general, long distance dispersal of seed and pollen will result in weak or no fine scale SGS, while short distance dispersal of seed and pollen will lead to a strong fine scale SGS (Dick *et al*., 2008). Extensive seed dispersal with limited pollen dispersal tends to result in weak fine scale SGS, while the opposite normally causes strong fine scale SGS, since pollen contains only a haploid set of DNA whereas seed is diploid (Dick *et al*., 2008). However, occasional long-distance clumped seed dispersal, especially from a distant population, will result in strong fine scale SGS (Pardini and Hamrick, 2008). This is the case because immigrants from a distinct gene pool will initially form a unique genetic cluster that will be homogenized through subsequent admixture. The pollen and seed dispersal of plants often relies on animals as vectors (Kramer *et al*., 2008). This means that by changing the number and behavior of pollen and seed dispersal vectors (Young *et al*., 1996; Ghazoul, 2005; Bacles and Jump, 2011), fragmentation can also change the extent of fine scale SGS (De-Lucas *et al*., 2009; Collevatti *et al*., 2014).

The non-random genotype distribution within populations measured by fine scale SGS can also be affected by post-dispersal ecological processes. These mainly comprise demographic thinning (Connell, 1971; Zhou and Chen, 2010; Ismail *et al*., 2012) and microenvironmental selection (Batista Leite *et al*., 2014; Berens *et al*., 2014; Helsen *et al*., 2015). If these result

in non-random mortality of individuals, especially juveniles figs, it will lead to fine scale SGS differences between age classes. Demographic thinning normally reduces fine scale SGS from juveniles to adults (Zhou and Chen, 2010; Ismail *et al*., 2012). This is because seedling mortality due to competition, predation and pathogens will be more severe on genotypes that resemble each other or the mother (Connell, 1971). On the other hand, microenvironmental selection should increase fine scale SGS from juveniles (Batista Leite *et al*., 2014; Berens *et al*., 2014) to adults due to the survival of similar genotypes in certain microenvironmental conditions (Troupin *et al*., 2006).

Since gene flow can change over time and survival is inherently temporal, fine scale SGS can differ between age groups in plants (Connell, 1971; Ismail *et al*., 2012; Berens *et al*., 2014; Helsen *et al*., 2015). For instance, demographic thinning due to high mortality of *Ficus cyrtophylla* Miq. seedlings near their mother tree reduces fine scale SGS from seedlings to saplings, to adults (Zhou and Chen, 2010). Temporal changes in gene flow can also change fine scale SGS of age groups since long-distance clumped immigration increases fine scale SGS. If long-distance gene flow is prevented for a period, fine scale SGS can then be reduced in younger age classes.

Ficus species are pollinated by tiny fig wasps, which are known for their long-distance dispersal, especially among species associated with monoecious *Ficus* species (Nason *et al*., 1996; Weiblen, 2002; Zavodna *et al*., 2005; Ahmed *et al*., 2009; Kobmoo *et al*., 2010; Heer*et al*., 2015). Ahmed *et al*. (2009) found that the pollinator of *F. sycomorous* can disperse pollen over 150 km. The pollinators of monoecious rainforest species in Central America routinely disperse pollen between 5.9 and 14.2 km far (Nason *et al*., 1996). Such long-distance gene dispersal of *Ficus* species may help them to homogenize the genetic structure within populations, resulting in no or weak fine scale SGS. However, *Ficus* species and their wasps vary in traits crucial to gene flow (Harrison and Shanahan, 2005). In forests, pollen flow in monoecious and dioecious fig trees tends to be very different. Monoecious trees bear infrequent, large synchronous crops accompanied by pollinators that make use of passive

wind dispersal by flying to above the forest canopy (Nason *et al*., 1996; Compton *et al*., 2000; Harrison, 2003; Harrison and Rasplus 2006). On the other hand, dioecious species have small, frequent and asynchronous crops and their pollinators rarely break through the canopy. Consequently, they rely more on short distance active flight for pollination (Compton *et al*., 2000; Harrison 2003; Harrison and Rasplus, 2006). As a result, dioecious *Ficus* typically have a higher fine scale SGS than monoecious *Ficus* (Dev *et al*., 2011; Nazareno *et al*., 2013).

Several *Ficus* studies reported fine scale SGS that may result from limited seed dispersal (Wang *et al*., 2009; Zhou and Chen, 2010; Dev *et al*., 2011; Heer *et al*., 2015; Krishnan and Borges, 2018). For example, SGS has been found within 1 km in four monoecious *Ficus* species despite pollen-dispersal distances that exceed 1 km. Similarly, in *F. racemosa*, which has pollen dispersed over long distances (Bain *et al*., 2016), strong spatial genetic structure was found within 1 km in an urban landscape indicating clumped seed dispersal close to natal trees (Krishnan and Borges, 2018).

Our inter-population analyses showed that species' autecology modulates the effects of fragmentation in Southern African fragmented forests (Deng *et al*., 2020). The inter-population comparisons of the genetic composition of three monoecious *Ficus* showed that, *F. sur*, which is a habitat generalist and occurs in between forests, had the least genetic structure (Deng *et al*., 2020). On the other hand, genetic structure was higher between populations of the two forest specialists (*Ficus craterostoma* and *Ficus bizanae*; Deng *et al*., 2020). An isolation by distance pattern showed that although the wasps are able to transport pollen between nearby forests, distant populations are more distinct genetically (Deng *et al*., 2020). These analyses also showed that populations of *F. bizanae*, a forest specialist that is more dioecious-like in its fruiting behavior, were more structured (Deng *et al*., 2020).

Intra-population SGS analyses can detect if gene flow is limited within forest populations and can test the hypothesis that *F. bizanae*'s pollinator displays local dispersal, rather than

flying up to above the canopy where it can be blown by the wind for long distances. Further, by looking at SGS in different age classes, temporal changes in the extent of inter-population dispersal can also be gauged. An avoidance of passive dispersal should translate into strong SGS patterns in older generation. Here we augment our previous inter-population findings with intra-population SGS analyses to answer the following questions 1) does the extent of fine scale SGS differ between three *Ficus* species found in Southern African forests? And 2) do different age classes of the same species vary in the extent of fine scale SGS?

4.3 Materials and methods

The study species

Three *Ficus* species with different life forms and habitat preference were chosen. *Ficus bizanae* and *F. craterostoma* belong to section *Galoglychia* (subgenus *Urostigma*) while *F. sur* belongs to section *Sycomorus* (subgenus *Sycomorus*; Burrows and Burrows, 2003). *Ficus craterostoma* is a strangler while *F. sur* and *F. bizanae* are usually freestanding trees. *Ficus bizanae* bear its fruit on the major stems and their mature figs are green with a strong smell (Berg and Wiebes, 1992), suggesting mammals may be its main target seed dispersal vectors (Shanahan *et al*., 2001). *Ficus craterostoma* figs develop in the leaf axils of branches that normally protrude above the forest canopy. Its figs turn yellow with red spots or entirely reddish when ripe, indicating their main seed dispersal agents are likely to be birds (Shanahan *et al*., 2001). The figs of *F. sur* turn red or dark orange with a sweet smell when ripe (Berg and Wiebes, 1992). They are clustered on leafless branchlets on the trunk, located from the canopy to below the ground. A wide variety of birds and mammals are likely to disperse its seed (Thomas, 1988).

Sampling and DNA extraction and genotyping

The study used the sample sites described in Deng *et al*. (2020) that contained 30 or more samples. The three *Ficus* species were collected from four forests in the Eastern Cape and

one forest in Kwazulu-Natal (Figure 4.1, Table 4.1). Each tree's location was recorded using GPS. We also recorded the age (saplings or adults) for the individuals of *F. craterostoma* in ING because this sample was large enough for a split analysis of two age classes. In total, we sampled 86 *F. bizanae* individuals from two sample sites, 188 *F. craterostoma* individuals from three sample sites, and 113 *F. sur* individuals from two sample sites (Figure 4.1, Table 4.1). DNA extraction and the selection of polymorphic markers were as reported in Deng *et al*. (2020). We genotyped *F. bizanae*, *F. craterostoma* and *F. sur* with 8, 12 and 10 polymorphic fluorescently-labelled SSR markers, respectively (Deng *et al*., 2020).

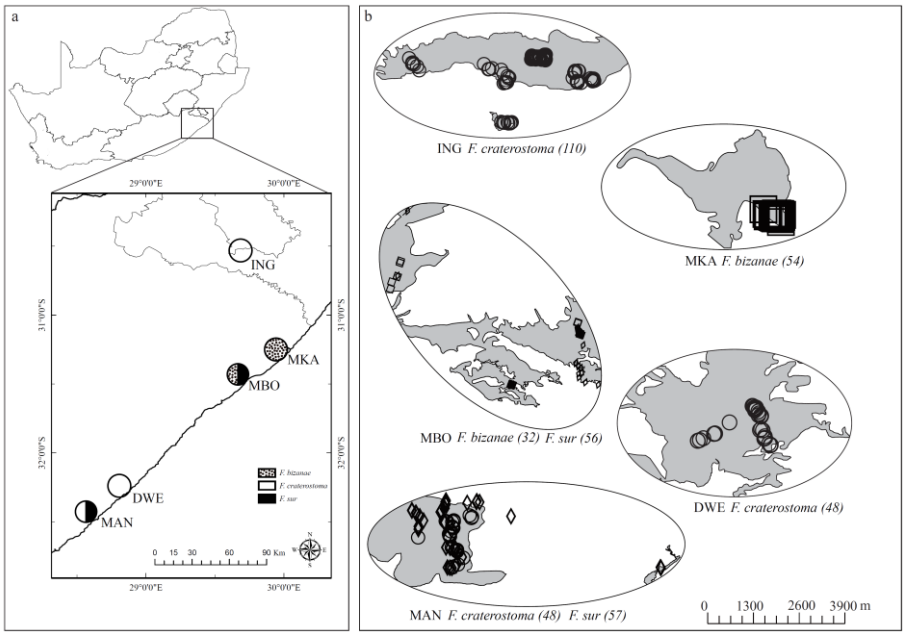

Figure 4.1 Species sampled in each sample site (a; *F. bizanae* = fill with dots, *F. craterostoma* = empty, *F. sur* = solid fill). (b) The locations of individual trees of each species sampled in each forest with sample sizes are given in parentheses (*F. bizanae* = squares, *F. craterostoma* = circles, *F. sur* = diamonds). The grey represents the forest patch of each sample site, which were downloaded from the website (http://bgis.sanbi.org/vegmap).

Spatial genetic structure

We analyzed the spatial genetic structure in each sample site of the three *Ficus* species using SPAGeDi version 1.5 (Hardy and Vekemans, 2002). For *F. craterostoma*, we collected 110 samples in ING, which allowed us to compare the spatial genetic structure of saplings and

adults. The kinship coefficient defined by Loiselle *et al*. (1995) was chosen, because it does not assume Hardy-Weinberg equilibrium (Hardy, 2003; Vekemans and Hardy, 2004) and has been widely used in other *Ficus* studies (Wang *et al*., 2009; Dev *et al*., 2011; Nazareno *et al*., 2013; Heer *et al*., 2015; Krishnan and Borges, 2018). The pairwise kinship coefficients (F_{ij}) (Loiselle *et al*., 1995) were estimated between individuals for each locus and then averaged over loci. We estimated the regression slope (b) by regressing the pairwise kinship coefficients (F_{ij}) against the natural logarithm of spatial distance ln(d_{ij}). Seven to fourteen distance intervals were defined (*F. bizanae*, MBO, MKA = 7; *F. craterostoma*, DWE = 14, MAN, ING (combined ages), ING (adults) = 12, ING (saplings) = 7; *F. sur*, MBO= 9, MAN= 8, Table S4.1). This followed the suggestion of Cavers *et al*. (2005) that at least 30 comparisons per class are needed to achieve statistical robustness (Legendre and Fortin, 1989). The fine scale SGS was further visualized in a spatial autocorrelogram by plotting the kinship coefficient against geographic distance (Vekemans and Hardy, 2004). A total of 10 000 permutations of locations and genes were performed to test the significance and determine the 95% CI for the index in each distance class. The standard errors for each comparison were estimated by jackknifing over loci for each comparison. To compare the intensity of fine scale SGS among different species as well as between different ages of *F. craterostoma*, the *Sp* statistic was calculated as $Sp = -b/(1-F_1)$, where F_1 is the mean F_{ij} in the first distance class (Vekemans and Hardy 2004). In order to compare to other *Ficus* species, we searched for published fine scale SGS studies using the web of science (http://www.webofknowledge.com) August of 2019.

Table 4.1 Sample sites with sample sizes in parentheses. The three-letter code for each forest and the latitude and longitude of each forest are also given.

Code	Sample sites	Species	Latitude (°S)	Longitude (°E)
ING	Ingeli	*F. craterostoma* (110)	30.530	29.689
MKA	Mkambati	*F. bizanae* (54))	31.297	29.979
MBO	Mboyti	*F. bizanae* (32), *F. sur* (56)	31.435	29.688
DWE	Dwesa	*F. craterostoma* (48)	32.280	28.848
MAN	Manubi	*F. craterostoma* (30), *F. sur* (57)	32.449	28.606

Estimation of effective ranges of gene dispersal

In Wright's isolation by distance model, the *Sp* statistic is expected to be equal to $1/4\pi D\sigma^2$ (1/Nb) in two-dimensional space when the population is at dispersal-drift equilibrium, where σ^2 is the square of half the average axial parent–offspring distance and Nb is Wright's neighborhood size (Heuertz *et al*., 2003; Vekemans and Hardy, 2004). We attempted to indirectly estimated Wright's neighborhood size (Nb) and gene dispersal distance (σ) through fine scale SGS analyses using an iterative procedure performed in SPAGeDi (Hardy and Vekemans, 2002). We can calculate the range of gene dispersal if σ converges. The calculation of Nb and σ relies on the effective population density (De). In natural populations, De is estimated through the census population density (D), which varied from D/10 to D/2, where D is the census population density (Hardy *et al*., 2006; Heuertz *et al*., 2003; Vekemans and Hardy, 2004). We did not search entire forests systematically and sampled only individuals where we could reach the figs. Not all trees will have been sampled and we therefore used D/2, D/4 and D/10 (D = number of samples per site/ forest patch size) for an effective population density to estimate gene dispersal distances.

4.4 Results

Significant fine scale SGS was found in all three *Ficus* species with kinship coefficient decreasing significantly as geographic distance increased (Figure 4.2, Table 4.2). All three species showed significant positive kinship coefficients at their first distance class, suggesting local gene dispersal in all three species.

The magnitude of fine scale SGS varied among species with the strongest fine scale SGS intensity in *F. bizanae* (average *Sp* = 0.025 $\pm$ 0.011), then *F. craterostoma* (*Sp* = 0.008 $\pm$ 0.005) and finally *F. sur* (*Sp* = 0.005 $\pm$ 0.003). This indicates that by comparison *F. bizanae* has much more local gene dispersal than the other two species. This difference also suggests that *F. bizanae* have restricted seed as well as pollen dispersal. The local gene dispersal was also supported by the kinship coefficient of *F. bizanae* (F_1= 0.106) at the first distance class,

which was almost five times larger than that of the other two species (*F. craterostoma*, F_1= 0.026; *F. sur*, F_1= 0.021).

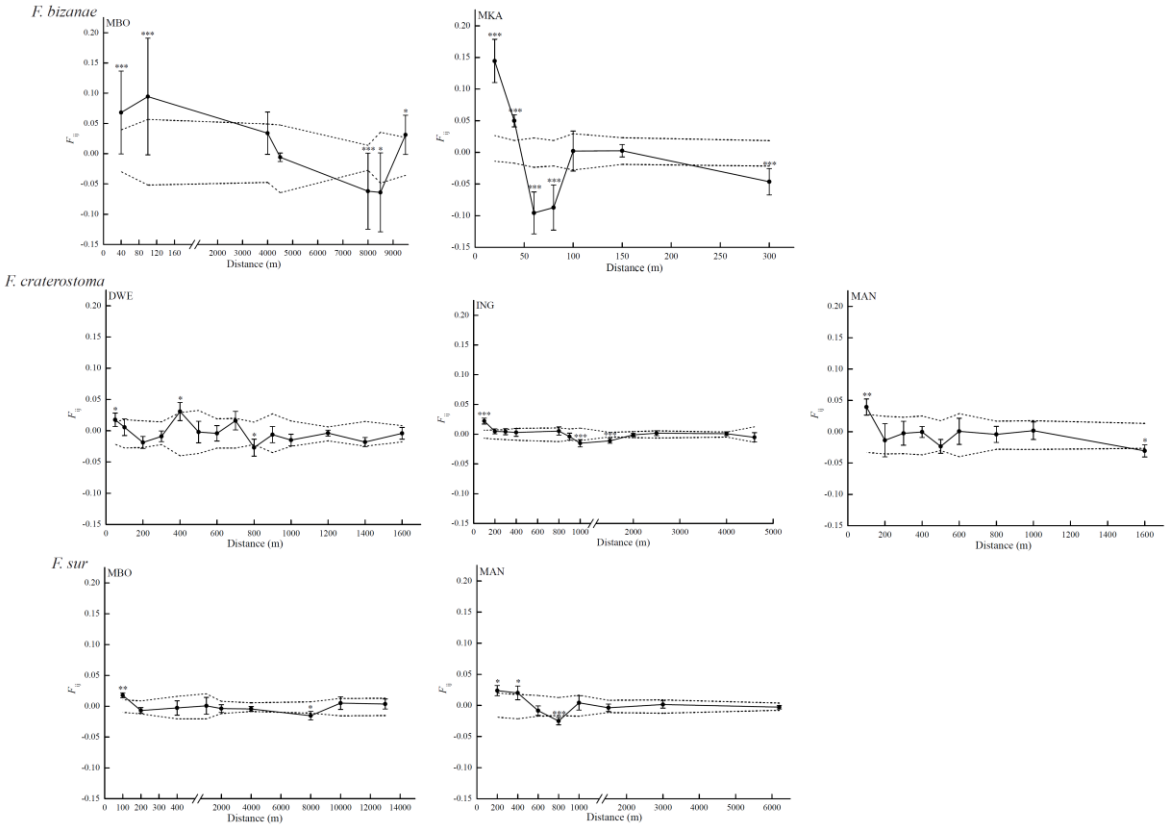

Figure 4.2 The kinship coefficient versus the geographic distance for each species in each sample site. The dotted lines are the 95% confidence intervals. The results of permutation tests are shown: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

The recorded fine scale SGS intensity (*Sp* statistics) of monoecious *Ficus* species ranges from 0.003 to 0.031 with an average of 0.012, while that of dioecious *Ficus* species is higher, ranging from 0.029 to 0.074 with an average of 0.042 (Table 4.3). The SGS intensity of *F. bizanae* is about two times higher than the average fine scale SGS intensity recorded for other monoecious species, which further supports the idea of local gene dispersal in *F. bizanae* (Table 4.3). On the other hand, the *Sp* statistics of *F. craterostoma* and *F. sur* are lower than the average for monoecious *Ficus* species (Table 4.3).

Table 4.2 Fine-scale spatial genetic structure parameters for each species. $b_{(log)}$ represented the regression slope of kinship coefficient F_{ij} on log spatial distance with standard errors given in parentheses. F_1 indicates the average kinship coefficient between individuals within the first distance class with standard errors given in parentheses. Significance is indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Species	Sample site	$b_{(log)}$	F_1	$Sp \pm$ 1.96SE	Forest size (km^2)
F. bizanae	MKA	-0.027*** (0.005)	0.144 (0.034)	0.031 ± 0.010	1.26
	MBO	-0.018** (0.006)	0.068 (0.026)	0.019 ± 0.012	13.89
	Average		0.106 (0.030)	0.025 ± 0.011	
F. craterostoma	DWE	-0.007** (0.003)	0.017 (0.010)	0.007 ± 0.006	21.46
	ING (all)	-0.005*** (0.001)	0.022 (0.008)	0.005 ± 0.002	7.18
	MAN	-0.011** (0.004)	0.039 (0.013)	0.011 ± 0.008	6.25
	Average		0.026 (0.010)	0.008 ± 0.005	
	ING (adult)	-0.006*** (0.001)	0.022 (0.007)	0.006 ± 0.004	7.18
	ING (young)	-0.002 (0.002)	0.010 (0.007)	0.001 ± 0.004	7.18
F. sur	MBO	-0.003*(0.001)	0.018 (0.004)	0.003 ± 0.004	13.89
	MAN	-0.006** (0.002)	0.024 (0.008)	0.006 ± 0.002	6.25
	Average		0.021 (0.006)	0.005 ± 0.003	

Table 4.3 Pubslished *Sp* statistics of spatial genetic structure in *Ficus* species.

Species	Sexual system	N	*Sp*	Continent
F. hispida	Dioecy	50	0.0350	Asia[3]
F. exasperata	Dioecy	45	0.0311	Asia[3]
F. pumila	Dioecy	129	0.0742	Asia[1]
F. cyrtophylla	Dioecy		0.0291	Asia[3]
Mean[7]			0.0423	
F. citrifolia	Monoecy	46	0.0086	South America[4]
		82	0.0075	South America[4]
		49	0.0042	South America[4]
F. citrifolia	Monoecy	52	0.0133	Central America[5]
F. eximia	Monoecy	48	0.0063	South America[4]
F. racemosa	Monoecy	47	0.015	Asia[6]
F. yoponensis	Monoecy	37	0.0083	Central America[5]
F. insipida	Monoecy	190	0.0034	Central America[5]
		155	0.0054	Central America[5]
F. obtusifolia	Monoecy	59	0.0311	Central America[5]
Mean[7]			0.0122	

[1] Wang *et al.*, 2009, [2] Zhou and Chen, 2010, [3] Dev *et al.*, 2011, [4] Nazareno *et al.*, 2013,[5] Heer *et al.*, 2015
[6] Krishnan and Borges, 2018,[7] Calculated as the average of the combined species

Due to a failure of σ to converge in all species, gene dispersal distances could not be estimated.

Within each species, the intensity of fine scale SGS also varied across sample sites (Table 4. 2). For *F. bizanae*, SGS was higher in MKA compared to MBO. The higher fine scale SGS in MKA was also indicated by the negative kinship coefficient of *F. bizanae* at the third (40-60 m) and fourth (60-80 m) distance classes (Figure 4.2). These observations suggest a recent clumped immigration by a number of seed that were related to each other, but unrelated to the rest of the surrounding population.

In *F. craterostoma* from ING, SGS was significant in adults ($Sp = 0.006 \pm 0.004$, $P < 0.001$), but not in saplings ($Sp = 0.001 \pm 0.004$, $P = 0.244$) (Figure 4.3). This could be the result of selective survival or a change in rates of immigration. If infrequent, clumped, long-distance immigration resulted in the fine scale SGS of older trees then this finding suggest that recent fragmentation stemmed such immigrations, while pollination within the fragment is sufficient to homogenize the population. Alternatively, the difference between age classes could indicate that effective gene flow that succeeds in generating saplings has then been reduced by local selection favouring specific genotypes in individuals by the time they reach maturity.

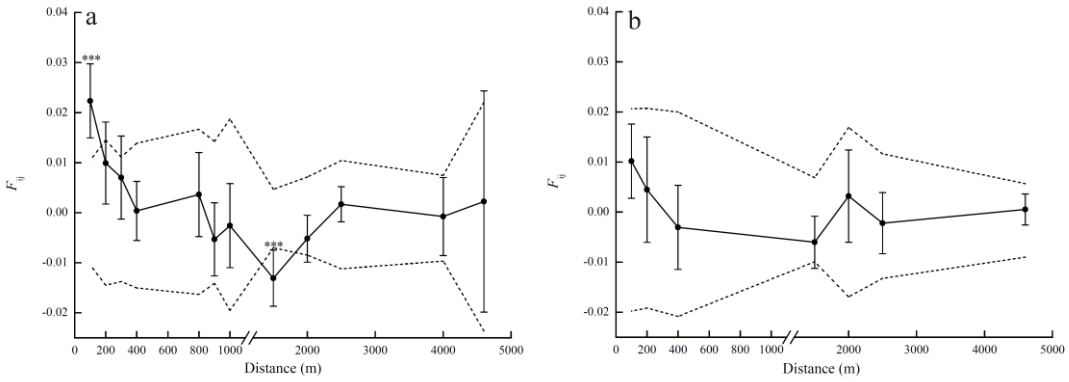

Figure 4.3 Correlograms of average kinship coefficients of *F. craterostoma* adults (a) and juveniles (b) at Ingeli forest (ING). The dotted lines are the 95% confidence intervals. The results of permutation tests are shown: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

4.5 Discussion

The forest fragments of all three *Ficus* species show some spatial substructure, suggesting short distance gene flow, presumably through limited seed dispersal. While two of these species were similar to other recorded monoecious *Ficus,* the substructure was substantially stronger in *F. bizanae*, confirming limited dispersal of its pollinator and seed. The extent of fine scale SGS within sample sites mirrored genetic structure between populations, suggesting that some determinants work at both scales of diversity (Heer *et al*., 2015). Variation in the degree of fine scale SGS within all three of the species suggested that either an equilibrium had not been reached or that the processes at work varied between populations. Gene dispersal distances did not converge, a result that could be expected if wasp mediated pollen dispersal distances vary tremendously between individuals, according to their fruiting phenology. The difference in fine scale SGS of age classes of *F. craterostoma* suggests that human induced fragmentation may have prevented recent long-distance clumped immigration into the populations.

All sample sites of the three species have significant fine scale SGS, suggesting frequent local gene dispersal. This is surprising for *F. sur* and *F. craterostoma* as typical monoecious *Ficus* pollinators are well known for their long-distance pollen dispersal (Nason *et al*., 1996; Zavodna *et al*., 2005; Harrison and Rasplus, 2006; Ahmed *et al*., 2009; Kobmoo *et al*., 2010; Bain *et al*., 2016). In fact, genetic variation of *F. craterostoma* suggests that forests as far as 242 km apart may be connected via pollen flow (Deng *et al*. in preparation). The genetic differentiation between *F. sur* populations is even less than that for *F. craterostoma* in forests that are equally distant (Deng *et al*., 2020). Therefore, the significant fine scale SGS of these two species are unlikely to be due to limited pollen dispersal, and suggest that seed dispersal in all three species must be predominantly local. Local seed dispersal is known to increase fine scale SGS in *Ficus* (Wang *et al*., 2009; Dev *et al*., 2011; Nazareno *et al*., 2013; Heer *et al*., 2015; Krishnan and Borges, 2018) and this was even inferred in a widespread South-East Asian *Ficus* (*F. racemosa*)that is pollinated by the widely-dispersing *Ceratosolen*

fusciceps (Kobmoo *et al*., 2010).

The intensity fine scale of SGS varied among the three species in South African forests, suggesting there may be variation in the extent of restriction to seed dispersal among the three *Ficus* species. Any differences may stem from different dispersal abilities of the predominant seed vectors of each species and different responses of the vectors to fragmentation.

Fruit characteristics tend to fall into discrete syndromes (Lomáscolo *et al*., 2008; Lomáscolo *et al*., 2010): birds are particularly associated with small and colored fruits such as *F. craterostoma*, while bats and other mammals prefer large fruit with a strong scent like *F. bizanae* (Lomáscolo *et al*., 2008; Lomáscolo *et al*., 2010). As for *F. sur*, a wide variety of birds, especially large ones and mammals, mainly bats (Shilton *et al*., 1999), disperse its large fruit that ripen to a red or dark orange color and with a sweet smell (Berg and Wiebes, 1992). *Ficus craterostoma* and *F. sur*'s lower SGS fits predictions based on these dispersal syndromes as birds and bats should disperse seed over greater distances than terrestrial mammals (Laurance, 1991; Davies *et al*., 2000; Henle *et al*., 2004). However, gut passage times also need to be considered (Guttal *et al*., 2011).

Habitat fragmentation also affects the migration of vectors between patches (Henle *et al*., 2004; Kramer *et al*., 2008). This is especially important in the context of South African forests that became fragmented millions of years ago (van Zinderen Bakker, 1983; Lawes, 1990a; Sepulchre *et al*., 2006; Lawes *et al*., 2007a). Mammals such as primates may be more vulnerable to fragmentation than birds and bats that can fly (Laurance, 1991; Davies *et al*., 2000; Henle *et al*., 2004). Therefore, very limited seed dispersal is expected in *F. bizanae* as reflected in it having the strongest SGS.

SGS may also be relate to germination patterns. For instance, if germination sites are diffuse, seedlings cannot germinate in close proximity to each other. This may explain why the hemi-epiphytic *F. craterostoma* has such a low SGS.

While pollen and seed dispersal distances are frequently estimated from such data (Heuertz *et al*., 2003; Vekemans and Hardy, 2004; Hardy *et al*., 2006), the fact that the average and variance of pollen flow failed to converge is hardly surprising given that *Ficus* pollen dispersal can vary dramatically depending on the trees' phenology (Bronstein *et al*., 1990; Hossaert-McKey and Bronstein, 2001). When a receptive tree is near to a tree releasing fig wasps, the pollen dispersal distance will be small, but when the wasp-producing tree is far away, pollen dispersal distance can be extensive (164km being the maximum distance recorded (Ahmed *et al*., 2009)).

The pollen dispersal distances of monoecious fig trees are expected to be further and more varied than dioecious species because they are typically a) more sparsely distributed (Harrison, 2003; Heer *et al*., 2015), b) grow higher in the forest structure (Harrison and Shanahan, 2005) c) bear infrequent, large and synchronous crops (Harrison and Shanahan, 2005), and d) their pollinators often travel above the canopy and over long distances (Compton *et al*., 2000; Harrison 2003; Harrison and Rasplus 2006). In contrast, it is expected that dioecious *Ficus* should have shorter and more uniform pollen dispersal distances because they frequently a) occur in dense stands (Harrison, 2003 b) are small trees or shrubs far beneath the canopy (Harrison and Shanahan, 2005), with c) frequent, small and asynchronous crops (Harrison and Shanahan, 2005) and d) have pollinator fig wasps that may often fly beneath the canopy (Compton *et al*., 2000; Harrison 2003; Harrison and Rasplus 2006).

Ficus sur and *F. craterostoma* fit this general expectation of monoecious *Ficus* well, but *F. bizanae* does not. *Ficus bizanae* has the highest *Sp* statistics and kinship coefficient at the first distance class, which indicate that in addition to limited seed dispersal, its pollen dispersal may also be local. Such limited pollen dispersal may stem from some similarities *F. bizanae* shares with dioecious *Ficus* - asynchronous crops and figs produced in the lower strata of the forest. These factors will only have an effect if the wasps have dispersal behavior that is different to that of species associated with other monoecious fig trees. We might

expect such a change since *F. bizanae* is a forest specialist with a very limited distribution (Burrows and Burrows, 2003). Therefore, like island species (Macarthur and Wilson, 2001), *F. bizanae* pollinators will be selected to avoid dispersal away from their native forest because this guarantees failure to reproduce (Gates and Nason, 2012). Unlike dioecious species, within-individual asynchronous flowering in *F. bizanae* (Pers. Obs.) offers a chance for selfing, thereby increasing the kinship coefficient even more (Hossaert-McKey and Bronstein, 2001).

One mechanism that can increase SGS is relatively recent immigration into a population by kin-groups unrelated to the previous individuals. This can result from occasional long distance seed dispersal. However, since older *F. craterostoma* trees in ING show a higher SGS than younger trees this suggests that some adults were the result of immigration which was not taking place more recently when saplings were generated. Increasing fragmentation during more recent decades may have been so severe as to sever immigration into ING, and possibly between the other sample sites, where our samples were too small to check.

Several other ecological factors that will result in the variation of SGS across different life stages have nonetheless been pointed out (Chung *et al*., 2003; Jones and Hubbell, 2006; Berens *et al*., 2014). For instance, better survival of heterozygous individuals (Ledig *et al*., 1983; Cohas *et al*., 2009).demographic thinning due to density-dependent predation, pathogen pressure, or higher mortality of juveniles germinating close to their mother will all reduce SGS by the time that trees become adults (Janzen, 1970; Connell, 1971; Ismail *et al*., 2012). However, we detected a significant SGS in *F. craterostoma* adults, but not in saplings. Higher SGS among older life stages has been found in many other plants(Jones and Hubbell, 2006; Batista Leite *et al*., 2014; Berens *et al*., 2014; Helsen *et al*., 2015), including other *Ficus* species (Heer *et al*., 2015). In addition to the cessation of clumped immigration, microenvironmental selection and overlapping generations may also explain this age-related SGS structure.

Almost all the fig tree species in Africa have a monoecious breeding system, and most have wide distributions across the continent. The limited distribution of *F. bizanae* appears to have selected for a different fruiting phenology and different dispersal behavior among its pollinators. Future work should explore this directly through doing paternity studies and germination trials of selfed seeds in order to examine further how geographical distribution influences the reproduction of fig trees and their pollinators.

4.6 Supplementary materials

Table S4.1 The defined distance intervals for every species at each sample site.

species	Sample site		Distance class												
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
F. bizanae	MKA	Max. distance(m)	20	40	60	80	100	150	300							
		No. pairs	245	278	220	217	111	189	171							
	MBO	Max. distance(m)	40	100	4000	4500	8000	8500	9500							
		No. pairs	82	37	45	32	178	42	80							
F. craterostoma	DWE	Max. distance(m)	50	100	200	300	400	500	600	700	800	900	1000	1200	1400	1600
		No. pairs	117	76	72	108	33	33	71	50	109	43	86	154	69	107
	ING (all)	Max. distance(m)	100	200	300	400	800	900	1000	1500	2000	2500	4000	4600		
		No. pairs	570	374	365	262	199	250	224	1421	793	639	739	159		
	MAN	Max. distance(m)	100	200	300	400	500	600	800	1000	1600					
		No. pairs	40	38	40	36	56	31	68	65	61					
	ING (adult)	Max. distance(m)	100	200	300	400	800	900	1000	1500	2000	2500	4000	4600		
		No. pairs	272	132	184	140	111	140	83	645	368	228	203	50		
	ING (young)	Max. distance(m)	100	200	400	1500	2000	2500	4600							
		No. pairs	55	49	49	210	70	105	165							
F. sur	MBO	Max. distance(m)	100	200	400	1000	2000	4000	8000	10000	13000					
		No. pairs	269	244	106	66	200	367	185	54	49					
	MAN	Max. distance(m)	200	400	600	800	1000	1500	3000	6200						
		No. pairs	122	131	156	206	164	386	269	162						

Chapter 5. General discussion

Forests in South Africa have been through repeated contractions and expansions, especially during the Quaternary climate oscillations (Lawes *et al.*, 2007a; Eberle *et al.*, 2017; Tolley *et al.*, 2018). However, our knowledge of ancient forest history in South Africa is based on extrapolations from pollen deposits at a few sites (Scott *et al.*, 1997), regional climatic conditions inferred from pollen studies (Scott, 2002), climatic reconstructions (Eeley *et al.*, 1999; Eberle *et al.*, 2017), comparisons of ecological communities (Lawes *et al.*, 2007a) and a few phylogeographic studies on animals (Eberle *et al.*, 2017; Tolley *et al.*, 2018). A phylogeographic study incorporating forest specialist plants can provide novel information about forest history in the region. In addition, South African forests have been fragmented for millions of years (Lawes *et al.*, 1990a; Eeley *et al.*, 1999; Eberle *et al.*, 2017). Habitat fragmentation results in population differentiation (Eberle *et al.*, 2017) and speciation (Barnes and Daniels, 2019). Our understanding of the effects of habitat fragmentation are taxonomically biased in South Africa with most studies based on animals (Matolweni *et al.*, 2000; McManus *et al.*, 2015; Madisha *et al.*, 2018; Barnes and Daniels, 2019). In this thesis, I used three *Ficus* species that are keystone resource species in South Africa (Bleher *et al.*, 2003) with varying dependence on forest habitats to fill this knowledge gap.

In the second chapter, I studied the phylogeography of a forest specialist *Ficus* species (*F. craterostoma*) using cpDNA. I found that forests in South Africa have been through contractions and expansions, as reflected by the haplotype network and their geographic distribution. Most of the forests were eliminated during the last glacial period. This was especially the case for Afrotemperate forests. Forest remnants survived in at least three putative refugia, which are located in the northern mistbelt forest (Limpopo province) and in two scarp forests (north KZN and EC province). The latter refugia have also been inferred from other studies (Lawes *et al.*, 2007a; Eberle *et al.*, 2017; Tolley *et al.*, 2018). Indian Ocean

coastal forests were formed quite recently and their components possibly originated from eastern Africa via the Mozambican coast down to South Africa. In the future, more samples from both Indian Ocean coastal and eastern African forests are required to thoroughly test this idea. In addition, by incorporating the population differentiation of *F. craterostoma* detected with nuclear microsatellite loci, and contrasting these to cytoplasmic DNA I discovered that nuclear DNA is more resilient to fragmentation than cytoplasmic DNA. Evidence of strong genetic drift was detected in cytoplasmic DNA, and this, together with its relatively restricted seed dispersal distances may make *F. craterostoma* cpDNA vulnerable to fragmentation. Long-range pollen dispersal, generated by its pollinating fig wasp, helps *F. craterostoma* to maintain genetic connectivity between forests and the analysis described here showed that forests as far as 242 km apart may be connected through pollen flow.

Ficus species, especially monoecious species, are well known for their long-distance pollen dispersal, (Harrison and Rasplus, 2006; Ahmed *et al.*, 2009; Kobmoo *et al.*, 2010; Nazareno *et al.*, 2013), which should make their nuclear DNA less vulnerable to fragmentation. In my third chapter I compared the population genetic structure of three *Ficus* species (*F. bizanae*, *F. craterostoma* and *F. sur*) with different habitat preferences and distributions, and found that stronger genetic isolation existed in the two habitat-specialist *Ficus* species, while less population structure was detected in the habitat-generalist species, *F. sur*.

Ficus sur's limited genetic differentiation is probably because forest are genetically linked via individuals growing in other habitats occurring between the fragmented forests. Within the two forest specialist species, higher population differentiation was detected in *F. bizanae*, which has a more limited distribution compared to *F. craterostoma*. Several factors may explain *F. bizanae*'s higher levels of genetic differentiation. The fruit characteristics of *F. bizanae* (Burrows and Burrows, 2003) suggest that mammals rather than vagile birds predominantly disperse its seed. Birds are more likely to move between forests than mammals other than bats (Shanahan *et al.*, 2001). This limited seed dispersal is echoed in

the species' very limited distribution (Burrows and Burrows, 2003). The limited distribution also means that pollinators that move outside one forest fragment are extremely unlikely to find another host and the pollinators, like island species (Macarthur and Wilson, 2001), would be selected to limit their long distance dispersal by wind. *Ficus bizanae* figs are located below the canopy, which reduces the chance of passive long distance pollinator dispersal. Finally, intra-individual asynchrony of *F. bizanae* means that a single tree can release and receive wasps, at the cost of self-pollination. This flowering phenology will also select for wasps to avoid long distance dispersal. All these factors contribute to genetic isolation of forest fragments.

The cycling of wasps on one tree should result in selfing, however, our data failed to find any indication of inbreeding through a positive inbreeding coefficient. However, inbreeding depression would reduce, or even make the inbreeding coefficient zero or negative. To answer this question future studies must conduct parentage analysis, potentially on the pollen carried by foundresses rather than seedlings that may already have undergone selection.

Ficus bizanae is the only endemic *Ficus* species in South Africa and has a very limited distribution (Burrows and Burrows, 2003). It would be interesting to see whether the species has had a wider distribution in the past, or is a neo-endemic species that has always been restricted to the southern coastal areas of South Africa. The combination of phylogeographic methods and species distribution models may help to answer this question.

Fine scale SGS can be affected by gene flow (Epperson, 2005; Hardy *et al.*, 2006) as well as post dispersal ecological processes such as demographic thinning (Zhou and Chen, 2010) and micro-environmental selection (Batista Leite *et al.*, 2013). SGS can thus indirectly reflect gene flow (Epperson and Li, 1997; Heuertz *et al.*, 2003; Epperson, 2005; Hardy *et al.*, 2006). In the fourth chapter, I studied the fine scale SGS of all three *Ficus* species. Surprisingly, they all had significant SGS in each sample site, which suggests limited gene dispersal in all three species. In two species pollen flow seems to be very effective - the

pollinators of *F. craterostoma* may facilitate gene flow between forests that are as far as 242 km apart and in *F. sur* gene flow is sufficient to prevent isolation by distance over distances of 600 km. Therefore, for these two species, their significant SGS are more likely to be due to local seed dispersal.

Among these species, the strongest SGS was found in the endemic forest specialist *Ficus* species (*F. bizanae*) suggesting extremely local gene dispersal that is likely to be due to both limited pollen and limited seed dispersal. This within-forest result echoes the conclusions reached by comparing gene frequencies among forests. Recent colonization of individuals from other population with different gene pools may result in strong SGS. However, given the limited gene dispersal of *F. bizanae*, it is unexpected to detect distinct genetic clusters within a population, suggesting recent colonization. Therefore, further studies should be conducted to test the colonization events perhaps through parentage analysis.

A single sample site of one species had a sample size that was sufficient to allow a comparison of SGS between adults and saplings. SGS was found in adults but not in saplings of *F. craterostoma* at ING. That suggests that recent population fragmentation has stopped such long-distance immigration. In addition, non-random mortality caused by microenvironmental selection may also contribute to the contrasting age-related fine scale SGS.

This could be related to post dispersal ecological processes such as overlapping generations and microenvironment selection.

Low nuclear DNA (microsatellites) and high chloroplast DNA differentiation have been reported in other *Ficus* studies (*F. insipida* Willd. and *F. adhatodifolia* Schott. of South America, *F. hirta* Vahl. and *F. pumila* L. of Asia) (Yu *et al.*, 2010; Yu and Nason, 2013; Honorio Coronado *et al.*, 2014; Heer *et al.*, 2015; Liu *et al.*, 2015; Costa *et al.*, 2017). In combination, they suggest extensive pollen and limited seed dispersal may be common in *Ficus* species. The extent of long distance pollen dispersal by different fig wasp species

varies from species to species, probably due to differences in host density and fig position (below or above the canopy) (Harrison, 2003; Harrison and Rasplus, 2006). For example, the pollinators of monoecious *Ficus* species usually transfer pollen further than dioecious ones because monoecious *Ficus* species usually are big free-standing trees or stranglers which release their pollinators above the canopy. Such difference result in different levels of genetic differentiation in *Ficus* species. For example, a lack of isolation by distance was detected in a monoecious *Ficus* species (*F. racemosa*) over South East Asia (Bain *et al.*, 2016), while significant population differentiation was found in an extremely dwarf dioecious *Ficus* species (*F. tikoua*) over distances as short as 31 km (Chen *et al.*, 2011).

Ficus species bear compound fruits (figs) with different morphological characters (sizes, colors and smell) that attract a wide, but varying range of frugivores (birds, bats, mammals etc.) to disperse their seeds (Shanahan *et al.*, 2001; Lomáscolo *et al.*, 2008; Lomáscolo *et al.*, 2010). Together with birds, bats are good fliers, which may facilitate the long distance transfer of *Ficus* seed (Shilton*et al.*, 1999). However, limited seed dispersal has been frequently reported in *Ficus* species (Yu *et al.*, 2010; Yu and Nason, 2013; Heer *et al.*, 2015; Liu *et al.*, 2015; Krishnan and Borges, 2018). The limited seed dispersal may restrict their range expansion especially after population contraction such as after the LGM (for example in *F. hirta* -Yu and Nason, 2013). It will also make cytoplasmic components more vulnerable to environment change such as fragmentation, resulting in loss of genetic diversity as explained in the second chapter. In addition, restricted seed dispersal is one of the major reasons for nonrandom distribution of genotypes within populations, forming SGS (Wang *et al.*, 2009; Dev *et al.*, 2011; Heer *et al.*, 2015; Krishnan and Borges, 2018). *Ficus racemosa* displays extremely long distance pollen dispersal, but significant SGS was detected within 1 km in an urban area, due to restricted seed dispersal (Krishnan and Borges, 2018).

Even though fig and fig wasps are tightly associated with each other, their gene flow may be quite different, because of their different effective population sizes, generation times and gene dispersal strategies (Liu *et al.*, 2015). Therefore, the trees and their pollinators may

respond independently to climate change and fragmentation, and this will lead to different population genetic structure (Liu *et al.*, 2013). The pollinators often have small effective population sizes and always have short generation times compared to fig trees, which make them more sensitive to climate change and fragmentation (Wang *et al.*, 2005). For instance, *Ficus pumila* pollinators, but not the trees experience reduced genetic variation in fragmented habitats (Chen *et al.*, 2008; Liu *et al.*, 2013). Population isolation together with their short generation times also make speciation in fig wasps more likely than in their hosts (Cook and Segar, 2010). More and more fig tree species have been found to be hosting cryptic pollinating fig wasps in different regions and even in sympatry (Molbo *et al.*, 2003; Chen *et al.*, 2012). Unfortunately, I was unable to detect different responses to fragmentation between pollinators and their hosts, as well as any cryptic speciation of pollinations, due to inadequate sample sizes.

Overall, my work inferred forest history in South Africa by studying the phylogeography of a forest specialist *Ficus* species (*F. craterostoma*). Furthermore, I found that even for *Ficus*, with its high pollen dispersal ability, populations are differentiated, most likely due to limited seed dispersal. More importantly, my work has emphasized that different species have different responses to fragmentation, due to their differing biological and ecological characteristics such as fruit position and habitat preference. The extent of genetic isolation was more marked in the range-limited forest species where seed and pollen dispersal distances may both be reduced. Comparable genetic data is not available from other South African forest trees, but our results suggest that many other tree species that are restricted to South Africa's forests are likely to display similar or greater genetic effects as a result of fragmentation, because their gene flow is likely to be less extensive than that of fig trees. Forest fragments form archipelagos in Afromontane areas across the eastern side of Africa. They were all similarly affected by tectonic activities and climate oscillations, so similar effects of fragmentation are likely to be seen across a wide range of forest species and forests of Africa.

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