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The population genetic structure of *Ficus craterostoma* in South Africa

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ABSTRACT

In the eastern part of its distribution, *Ficus craterostoma* occurs in Afromontane forests whereas it also occurs in low-lying scarp and Indian Ocean coastal belt forests in South Africa. *Ficus craterostoma* must have dispersed to these low-lying forests from the Afromontane forests, even though forests became highly fragmented during the Pleistocene. To understand how these ancient changes have impacted the distribution and population structure of *F. craterostoma* we quantified the genetic variation in its slow-evolving chloroplast DNA with limited dispersal ability via seeds, and its highly variable nuclear microsatellites that reflect exceptional pollen flow. The chloroplast variation was highly structured and frequently monomorphic in nearby forests while the nuclear variation showed little structure and isolation by distance. From these data we reach several conclusions. *Ficus craterostoma* may have become extinct from South Africa's northern Afromontane forests during the Pleistocene. These forests were possibly subsequently recolonized from southern forests that may have been scarp or Afromontane in nature. Additionally, there was one scarp and one Indian Ocean coastal belt forest refugium, both of which were very isolated and small. Nuclear gene flow caused by pollen flow is very effective along the western part of the South African population, knitting together Afromontane and scarp forest fragments, dispersed over 1000 km, into one genetic population. Conversely, the Indian Ocean coastal belt forest refugium appears to have been isolated in terms of gene flow, but more recent gene flow with two nearby inland forests may have started to homogenize their genetic variation. Due to the unusual pollination system of fig trees, other forest tree species may display very different dynamics.

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1. Introduction

In southern East Africa and eastern Southern Africa, *Ficus craterostoma* Warb. ex Mildbraed and Burret occurs in the Afromontane forests as a monoecious hemi-epiphytic tree (strangler) (Burrows and Burrows, 2003; van Noort and Rasplus, 2024; Fig. 1; supplementary text). While these populations are currently isolated "islands" they must have been continuous or near continuous at some stage (Kadu et al., 2011; Migliore et al., 2020; White, 1978). Views on when Afromontane forests expanded vary from during the cool and dry glaciations (Allen et al., 2021) to during wetter conditions (Ivory et al., 2018) and even during wetter and warmer conditions (Eeley et al., 1999). In South Africa, the climatic effects of increased altitude are replaced with increased latitude, which allows these forests to reach the coast (Mucina and Geldenhuys, 2006; White, 1978; Fig. 1). Consequently, in South Africa, *F. craterostoma* is not restricted to the southern extensions of the Afromontane forests, but also occurs in low-lying scarp and Indian Ocean coastal belt forests (Fig. 1). Given 1) the species' occurrence in three forest types with markedly different histories, 2) the fragmented distribution of these forests, 3) the proposed isolation and shrinkage of forests during the last glacial maximum (LGM), and 4) that species in the genus *Ficus* L. often have long distance pollen dispersal but relatively poor seed dispersal, *F. craterostoma*'s history and population genetic structure in the subregion is unclear.

The three major kinds of forests have very different histories in South Africa and currently only exist as fragments across the eastern and southern regions (Fig. 1; supplementary text; Geldenhuys, 1994; Lawes, 1990; Mucina and Geldenhuys, 2006) that total only 0.41 % of

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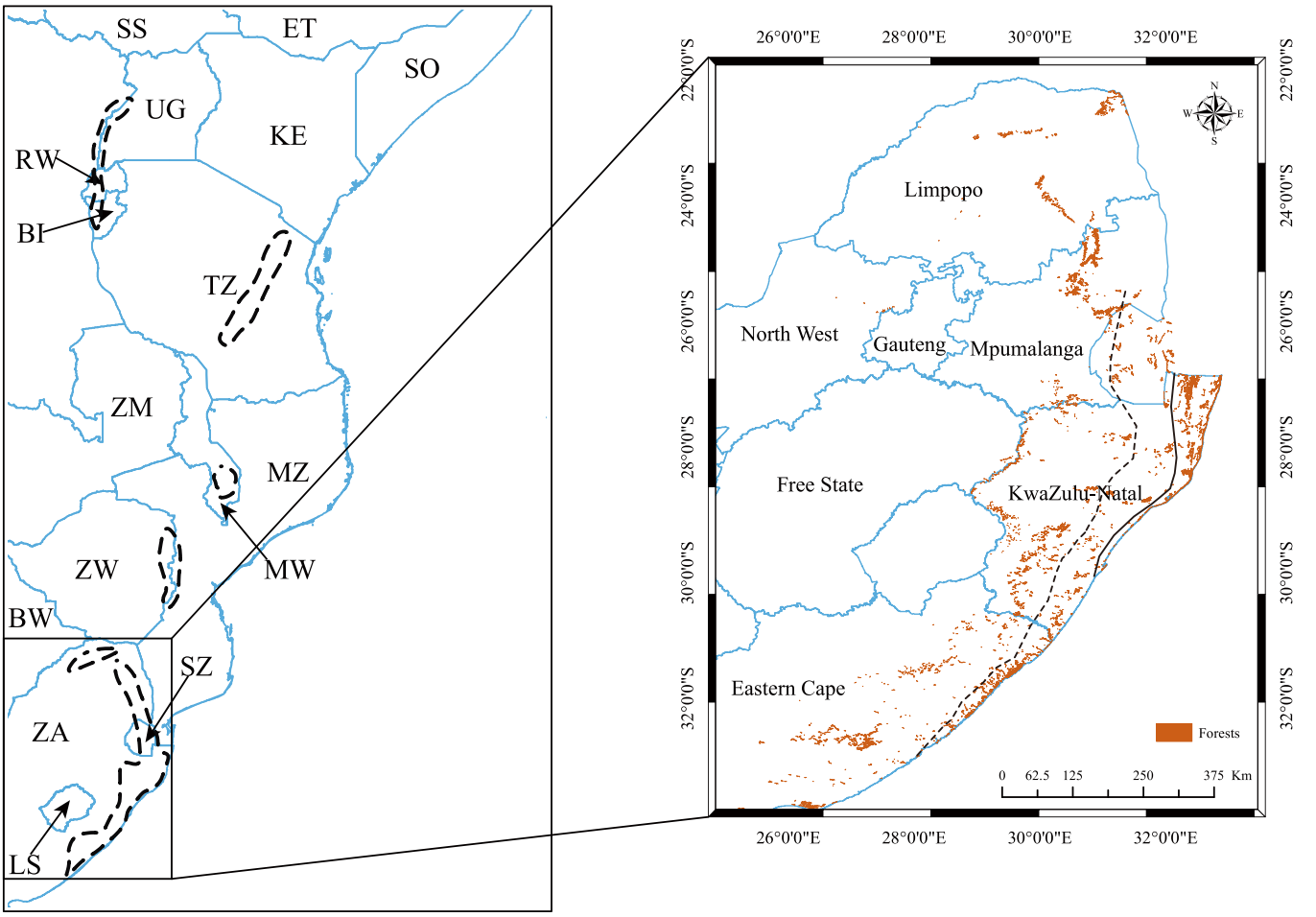

Fig. 1. The distribution of *Ficus craterostoma* in southern East Africa and eastern Southern Africa and forest locations in eastern South Africa. The former is based on records from gbif.org, Deng et al. (2020) and Burrows and Burrows (2003) and the latter in the inset from http://bgis.sanbi.org/vegmap 2018 (vermillion pixels). Note that in the left hand panel, areas demarcate where forests could be found rather than continuous populations and in the right-hand panel, pixel size is much larger than actual forest fragments in order for them to be visible (see supplementary text) and that we indicate all recorded forests, not just those containing *F. craterostoma*. Lawes et al. (2007a) grouped forests into three broad types - Afromontane forests are located to the west of the dashed black line and north of its end (include Mucina & Geldenhuys's (2006) Afrotemperate and mistbelt forests); scarp forest occurs between the dashed and solid black lines (or ocean); Indian Ocean coastal belt forests are to the east of the solid black line (northern coastal forest according to Mucina & Geldenhuys (2006)). Sky blue lines represent state and provincial borders. Countries are indicated by their ISO two-letter codes and provinces are given in the inset.

South Africa's land surface. This is less than one-tenth of the 7 % of the country that is considered suitable for forests based on rainfall alone (Forestwood, 2010). The difference is caused by other climatic, soil type and altitudinal requirements (Eeley et al., 1999), natural and human-induced fires (Geldenhuys, 1994; White, 1983), and agriculture and forestry (Eeley et al., 1999).

While the forests may have gone through brief periods of being more continuous in the past (Eeley et al., 1999; Ivory et al., 2018), for much of their past they may have been more fragmented than today (Eeley et al., 1999). In addition to this fragmentation, it is clear that Afromontane forest distribution must have changed substantially during the Pleistocene (Ivory et al., 2018). During the LGM (≈ 18,000 years ago), Afromontane forests in northern South African (Limpopo and Mpumalanga provinces) shrank and may have disappeared in some places (Lawes et al., 2007a; Scott et al., 1997). In central South Africa (KwaZulu-Natal province), Afromontane forests likely descended to lower elevations and were diminished in both size and number, with refugia likely persisting in low-lying areas now occupied by scarp forests (Eeley et al., 1999; Lawes et al., 2007a). After the LGM, much of the current Afromontane fauna repopulated westward to inland forests and northward via Eswatini to forests in Mpumalanga and Limpopo provinces (Fig. 1; Lawes et al., 2007a). The scarp forests may have been refugia for Afromontane forests and contact zones between Afromontane and the Indian Ocean coastal belt forests (Fig. 1; Lawes, 1990; Lawes et al., 2007a; Mucina and Geldenhuys, 2006). The Indian Ocean coastal belt forests likely formed around 7000 years ago (during the Holocene altithermal) with a drop in sea level and the southern expansion of the Mozambican coastal forest that had originated from tropical East African refugia (Zanzibar-Inhambane Regional Mosaic; Eeley et al., 1999; Lawes, 1990; Lawes et al., 2007a; Ramsay, 1996). However, because *F. craterostoma* is absent in these coastal forests of eastern tropical Africa (Burrows et al., 2019; van Noort et al., 2007), its populations in the Indian Ocean coastal belt likely stemmed from elsewhere.

Colonization by plants usually depends on seed dispersal. Seed dispersal in *Ficus* is often limited (Krishnan and Borges, 2018; Yu et al., 2010; Yu and Nason, 2013). Although Lawes et al. (2007b) estimated that *F. craterostoma* is capable of colonizing new fragments, its absence from several forest fragments (Deng et al., 2020a; Downs and Symes, 2004; Hart et al., 2013) suggests that it has limited colonization ability via seed dispersal often done by birds, rodents and samango monkeys that eat its figs (Basabose, 2002; Gautier-Hion et al., 1985; Hart et al., 2013; Lawes, 1990; Linden et al., 2015). Population genetics can assess seed movement using cytoplasmic DNA because this is only transmitted via seeds in angiosperms (Petit et al., 2005). Chloroplast DNA is an example of cytoplasmic DNA and due to its slow mutation rate (Wu et al., 2020) is useful for understanding the more ancient history of plants.

In contrast to the limited seed dispersal of *Ficus* species, fig pollen can be dispersed over long distances (up to 160 km; Ahmed et al., 2009). Indeed, some fig tree populations show little genetic differentiation across hundreds of kilometres (Bain et al., 2016; Yu and Nason, 2013; Zavodna et al., 2005). This extensive pollen flow is made possible through dispersal by host-specific mutualistic fig wasps (Agaonidae), which for Southern and East African populations of *F. craterostoma* are *Alfonsiella pipithiensis* Erasmus, van Noort, Jousselin & Greeff (Erasmus et al., 2007). In this study we quantify *F. craterostoma*'s population genetic structure and try to reconcile it with the history of the forests they inhabit.

2. Materials and methods

2.1. Sampling

Between 2008 and 2018, we collected leaves or bark of *F. craterostoma* from Afromontane, scarp, and Indian Ocean coastal belt forests across the species' South African range. The sampling included five forests in Limpopo province, two in Mpumalanga province, five in KwaZulu-Natal province, and three in the Eastern Cape province (Table 1; provinces indicated in Fig. 1). The samples were stored and dried in plastic bags with silica gel or a NaCL-CTAB-azide buffer (Bhattacharjee et al., 2009). Between 11 and 125 individuals were sampled from each of 15 forests. Two previous studies analysed a subset of these nuclear data, along with data from two other *Ficus* species, to investigate the role of habitat specificity in population genetic structure (Deng et al., 2020a) and to assess the conservation implications of fine-scale genetic structure (Deng et al., 2020b). The current study builds on this work by incorporating 10 more forests, where we sampled an extra 176 trees. In addition, we genotyped chloroplast DNA from 150 individuals across all 15 forests.

2.2. 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 for leaves and bark. We sequenced a chloroplast intron of trnL (Taberlet et al., 1991; primers c and d) and intergenic spacer of atpF-H (Lahaye et al., 2008) of 6 to 20 randomly selected individuals per forest. The 40 μl PCR reactions included: ddH2O 27.6 μl, reaction Taq buffer 4 μl (10 x), dNTP 4 μl (10 mM), primers 0.2 μl (10 μM), Taq™ polymerase (Takara) 0.4 μl (5U/μl) and DNA 4 μl (50 ng/μl). The thermal programs for trnL and atpF-H were respectively: 3 min of denaturation at 94 °C, 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and 10 min of elongation at 72 °C; 5 min of denaturation at 94 °C, 35 cycles at 94 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min and 8 min of elongation at 72 °C. We sequenced the PCR products in both directions on an ABI PRISM 3730. We checked the sequences manually and aligned them with clustal W using MEGA7 (Kumar et al., 2016). The accession numbers in GenBank for trnL and atpF-H are MN683996-MN684000 and MN684001-MN684006. The two cpDNA regions from each individual were concatenated into a single sequence because the entire chloroplast genome is in linkage disequilibrium and we did not fit an evolutionary model to it.

We selected twelve polymorphic microsatellite (SSR) markers developed in other *Ficus* species (Table S1), genotyped all samples using an automated sequencer (ABI 3730) and scored loci using GENEMARKER HID v.2.05 (Holland and Parson, 2011) following the method of Deng et al. (2020b). 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 over 67 % of the loci had inferred null alleles in three of the forests, with 11 inferred in DWE, eight in MAN and nine in WOL, we excluded these forests in the subsequent SSR analyses. In addition, we rescored loci with inferred null alleles in each population as missing data for that population in the STRUCTURE analyses. In order to avoid bias due to unbalanced sample sizes, we subsampled ING down from 125 to 25 trees by selecting the 20 individuals that were used for cpDNA and another 5 individuals randomly.

2.3. Genetic diversity

We calculated genetic diversity parameters based on cpDNA sequences using ARLEQUIN 3.5 (Excoffier and Lischer, 2010). These included the number of polymorphic sites and haplotypes, average nucleotide divergence, haplotype diversity and nucleotide diversity. We used FSTAT v.2.9.3 (Goudet, 1995) to confirm that no SSR loci were in linkage disequilibrium with each other. We calculated the deviation from Hardy-Weinberg equilibrium (HWE) in each forest using the multi-locus exact test in GENEPOP 4.0 (Rousset, 2008). We specified a Markov chain method to estimate the *P*-value and corrected for multiple tests using a sequential Bonferroni method (Rice, 1989). We calculated the number of alleles, observed and unbiased expected heterozygosity using FSTAT. We used HP-RARE 1.1 (Kalinowski, 2005) to estimate allelic richness after rarefaction to correct for different sample sizes between sample sites, and the average number of private alleles per locus.

2.4. Relatedness between individuals

For each population, we removed loci indicated to contain null alleles. To determine if the data contained first-degree relatives we used the remaining loci in COANCESTRY (Wang, 2010) to calculate relatedness between all pairs of individuals within each population. There were 9906 such comparisons. We calculated six relatedness estimators (excluding the dyadic maximum likelihood estimator) using the default settings and 100 bootstraps to estimate 95 % CI. We specified ten threads for each run and recorded the number of individuals that may be first-degree relatives as those ones with estimates greater than 0.45 for at least three of the estimates and where the lower 95 % confidence interval of the estimate was greater than 0.225.

2.5. Chloroplast genetic structure and gene flow

We calculated ΦST using ARLEQUIN 3.5 (Excoffier and Lischer, 2010). We partitioned the genetic variance of cpDNA haplotypes into within forests, among forests within forest types and among forest types with an analysis of molecular variance (AMOVA) using ARLEQUIN 3.5 (Excoffier and Lischer, 2010) with 1000 permutations. We inferred a haplotype network between cpDNA haplotypes using TCS 1.21 with a connection limit of 95 % (Clement et al., 2000).

2.6. Nuclear genetic structure and gene flow

Similar to chloroplast DNA, we used ARLEQUIN 3.5 (Excoffier and Lischer, 2010) to do an AMOVA on microsatellite genotypes, except that we included a level for variation within individuals.

We used 1000 permutations in FSTAT to estimate global F_{ST} and pairwise F_{ST}'s between forests. We also standardized these estimates to correct for the maximum difference that could be obtained given observed allele numbers and frequencies using $F'_{ST} = F_{ST}/F_{STmax}$ 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}.

We used a Bayesian approach to detect genetic structure based on SSR data, implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000). We ran the program for K from K = 1 to K = 13, with 200,000 burn-in and 900,000 MCMC repetitions for each run and 15 iterations for each value of K. The optimal K number was determined with the ΔK

method (Evanno et al., 2005) and Puechmaille's medMedK (median of medians), maxMedK (maximum of medians), medMeanK (median of means) and maxMeanK (maximum of means) estimators (Puechmaille, 2016) using STRUCTURE SELECTOR (Li and Liu, 2018). In addition, bar plots for the major modes were built for each K using the clumpak option in STRUCTURE SELECTOR.

We explored genetic discontinuities between sample site pairs with Monmonier's maximum-difference algorithm based on the SSR genotypes of each sample site using BARRIER 2.2 (Manni et al., 2004), and estimated the robustness of the gene-flow barriers with 1000 Nei's genetic distance matrices. We produced these distance matrices by bootstrapping over loci with MICROSATELLITE ANALYSER 4.05 (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 $F_{ST}/(1 - F_{ST})$ against the corresponding geographic distance with a Mantel test using the R 3.3 package 'vegan' (Oksanen et al., 2016), determining significance based on 1000 permutations. The tests were first performed on all forests, then on a subset of 9 forests, excluding three sample sites (MAP, ENT, and DLI) located near to each other. We excluded these three forests due to their isolation and the substantially higher genetic distances they exhibited, making them clear outliers compared to the other sites.

3. Results

3.1. Genetic diversity

The combined cpDNA sequences were 1211 bp long. We obtained 150 unambiguous combined cpDNA sequences from the 15 forests, from which we distinguished 10 haplotypes based on 14 variable nucleotide positions (Tables 1, 2 and S2).

We scored 423 individuals at most of the 12 loci (Tables 1 and 2; 1.1 % of genotypes failed, i.e. 55 of the total of 5076 = 423 individuals x 12 loci that were scored in total failed). All 15 forests displayed SSR genetic diversity (Table 2) but we excluded three of these forests, DWE, MAN and WOL, because of null alleles (Table S3). The number of alleles per forest ranged from 5.08 to 10.08. Allelic richness ranged from 4.60 to 7.39. Private allelic richness ranged from 0.06 to 0.61.

We found no significant linkage disequilibria between loci, and three forests deviated from HWE (Table 2). True inbreeding or a Wahlund-like effect due to sampling family groups can contribute to this deviation from HWE, but the most likely cause was the null alleles that MICRO—CHECKER inferred (Table S3).

3.2. Relatedness between individuals

As expected there were relatives in our samples. Of the 9906 within-population comparisons that can be made, only 132 pairs of individuals (1.3 %) were found to be first-degree relatives. Averaged over populations (rather than pairs), less than one percent of pairs are related to each other in the first degree (Table S4). The relatedness based on SSRs of the 779 possible pairs of samples with cpDNA data was lower than SSR samples, with only six pairs (0.79 %) being first-degree relatives. Therefore, a Wahlund-like effect is unlikely to explain deviations from HWE and the sampling of a few close relatives is unlikely to affect our conclusions.

3.3. Chloroplast genetic structure and gene flow

The cpDNA diversity was highly structured with significant population differentiation of cpDNA sequences between forests (Table 2; ΦST = 0.85, $P < 0.001$). The major sources of cpDNA variation occurred among forest types (53 %) and among forests within forest types (36 %) (Table 3). Eight out of the 15 forests were fixed for one haplotype. This was the case for seven of the nine Afromontane forests and the single Indian Ocean coastal belt forest (Tables 1 and 2; Fig. 2a). In contrast, none of the five scarp forests were fixed for a single haplotype (Tables 1 and 2, Fig. 2a). Nearby populations frequently have the same haplotype. TCS linked all ten haplotypes into a network that is easy to arrange so that it reflects the geographical distribution of the haplotypes (Fig. 2a). The network can shed light on the timing of events. There are seven mutational steps between haplotypes H04 and H05. Given the slow mutation rate of chloroplast DNA (Wu et al., 2020) this represents a timespan of almost 1 million years ($\approx 7/(2 \times 1211 \times 3 \times 10^{-9})$, if the per site per year rate is taken to be 3×10^{-9} (Shaw et al., 2005; Wolfe et al., 1987)), but note that the stochasticity in this processes is substantial. There are also seven mutational steps between H01 and H10. Given the number of mutational steps, it is clear that H04 and H05 must have been isolated from each other for a long time. Subsequently, H04 dispersed to the entire range of scarp forests. Except for H03 in forest WOL, the Afromontane forests' haplotypes reflect the network, with H07 in the south and H01 at the northern extreme of the South African range.

Table 1
Forests where we sampled *Ficus craterostoma* in South Africa and their genetic compositions. n_{SSR}, and n_{cpDNA} 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 (defined in Table S3) 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 Lawes et al. (2007) and Mucina and Geldenhuys (2006) respectively.

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

[1] This sample was subsampled to 25 trees.
[2] The lower part of NGO is classified as scarp forest, our samples are all from the southern mistbelt part.

Table 2
Genetic diversities of *Ficus craterostoma* based on chloroplast genes and twelve microsatellite loci. Polymorphic sites (S); number of haplotypes (h); H_d, haplotype diversity (H_d); nucleotide diversity (π); average number of nucleotide differences (k); number of alleles (N_a); allelic richness after rarefication (A_r); observed heterozygosity (H_o); expected heterozygosity (H_e) (heterozygosity with significant deviation from HWE ($^*P < 0.05$, $^{**}P < 0.001$) after correction for multiple testing); inbreeding coefficient (F_{IS}) ($^*P < 0.05$); and private allelic richness (P_a). Pop indicates the forest as given and arranged in Table 1.

	chloroplast diversity					microsatellite diversity				
pop	S	h	H_d	π	k	Na	Ar	Ho/He	F_{IS}	P_a
ING	0	1	0	0	0	10.08	5.48	0.60/0.63	0.05	0.14
NGO	1	2	0.355	0.0003	0.356	5.75	4.60	0.67/0.63	−0.06	0.06
BUF	0	1	0	0	0	6.75	5.5	0.56/0.67**	0.18*	0.17
ENA	0	1	0	0	0	5.08	4.78	0.53/0.60	0.12	0.17
HAN	0	1	0	0	0	7.33	5.96	0.56/0.64	0.11	0.26
PIE	0	1	0	0	0	5.08	4.69	0.60/0.61	0.01	0.17
SAD	0	1	0	0	0	5.25	5.25	0.62/0.68	0.09	0.14
THA	0	1	0	0	0	6.83	5.73	0.61/0.66	0.07	0.10
WOL	2	2	0.220	0.0004	0.444	–	–	–	–	–
MAP	0	1	0	0	0	6.50	5.37	0.57/0.66**	0.14*	0.61
DLI	8	3	0.308	0.002	2.472	8.58	7.00	0.73/0.79	0.08	0.52
DWE	3	3	0.607	0.001	1.321	–	–	–	–	–
ENT	3	2	0.220	0.0009	1.111	7.92	7.39	0.72/0.77	0.06	0.41
MAN	5	2	0.546	0.002	2.667	–	–	–	–	–
MBO	5	2	0.220	0.0006	1.111	7.50	6.05	0.48/0.66**	0.28*	0.19

3.4. Nuclear genetic structure and gene flow

Overall, populations were significantly differentiated for SSRs (F_{ST} = 0.09, $P < 0.001$; F'_{ST} = 0.28, $P < 0.001$). Pairwise population differentiation showed that most pairs of forests were genetically differentiated from each other (55 of 66 comparisons; Table S5). However, the microsatellite variation was not as structured as the cpDNA variation with only 12 % SSR variation stemming from the combined effects of differences among forest types and among forests within forest types and the majority was due to variation within individuals (77 %; Table 3). These analyses indicate that pollen is being transferred between forests.

STRUCTURE indicated that two or four genetic groups were optimal as determined by Evanno and Puechmaille's methods, respectively (Fig. S1 and S2). When K = 2, one group was composed mostly of Afromontane forests (coloured red in Figs. 2b and 2d), while the other group contained the Indian Ocean coastal belt forest and almost all the scarp forests (coloured yellow in Figs. 2b and 2d). Some Afromontane forests contained yellow or partially yellow individuals (Fig. 2d) while one scarp forest, MBO, contained mostly red group individuals (Fig. 2d). When K = 3, the Afromontane forest group remained the same (coloured red), whereas the former yellow group was split in two, one being the Indian Ocean coastal belt forest (coloured yellow) and the other being the northern scarp forests (blue group; ENT and DLI; Fig. 2d). When K = 4, a fourth group (coloured in light blue) is suggested that does not make sense because it consists of individuals and tiny parts of individuals from all forests spread over the entire South African range. It was most common in MBO and ING forests in the south as well as BUF and HAN forests in the north (Figs. 2c and 2d).

BARRIER corroborated the genetic barriers that isolated the eastern sample sites from the others, separating MAP from the rest and ENT and DLI from MAP and the others (Fig. 2a-c).

In the analysis where all the sample sites were included, genetic isolation by distance was not significant (r = 0.086, P = 0.234; Fig. 3). However, when the predominantly yellow group from K = 2 (MAP, ENT and DLI in Fig. 2b) was excluded, isolation by distance was significant (r = 0.70, P = 0.004; Fig. 3). This suggests that there is more pollen flow between nearby western populations, but that the eastern populations' very different genotypes disrupt the isolation by distance pattern.

Table 3
Percentage of variance explained by three levels of organization of chloroplast DNA sequences (cpDNA) and four levels of nuclear microsatellites (SSR) using Analysis of Molecular Variance (AMOVA). All estimates were significant ($P < 0.05$).

	% variance explained		
source of cpDNA variance	cpDNA	SSR	source of SSR variance
among forest types	52.68	7.60	among forest types
among forests within forest types	35.71	4.41	among forests within forest types
among individuals within forests	11.61	10.77	among individuals within forests
		77.22	within individuals

4. Discussion

Based on chloroplast variation, it appears that during the Pleistocene, *F. craterostoma* survived in isolated refugia, some of which would have been small, causing population bottlenecks. There were probably at least three refugia in the central and southern part of the species' South African distribution, which may have corresponded to the three forest types. When conditions allowed forest expansion, seed dispersal allowed recolonization of nearby forests, but given the number of mutations involved, such colonisations probably occurred over several glaciation events. On the other hand, nuclear genetic variation, which reflects pollen flow, indicates a far more connected population of forests along the western scarp and Afromontane forests that show genetic isolation by distance. Conversely, three forests at the extreme east of *F. craterostoma*'s distribution, but central in terms of its latitudinal range, are very isolated genetically from the other populations. By contrasting chloroplast and nuclear genetic variation it is clear that the tree's pollen disperses much more effectively than its seed and that it is this limited seed dispersal that makes it possible to reconstruct its history in the subregion.

4.1. Refugia and recolonization of *Ficus craterostoma* in South Africa

One of three putative refugia was potentially in the Eastern Cape montane area just south of Lesotho (Lawes et al., 2007a), with trees consisting mostly of H07. It was likely a large population because

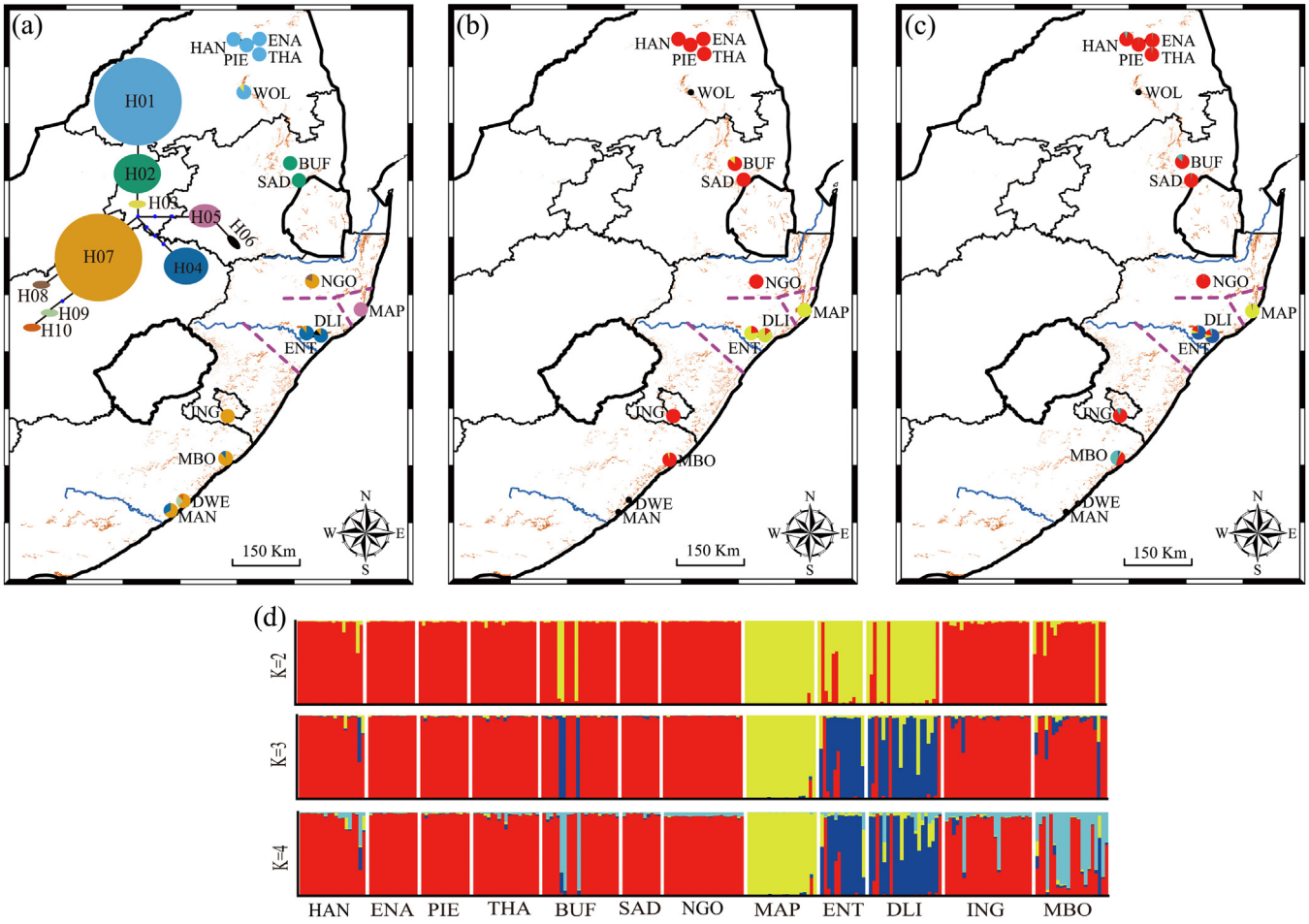

Fig. 2. Genetic variation in *Ficus craterostoma* in eastern South Africa. Vermillion pixels indicate forest locations (http://bgis.sanbi.org/vegmap 2018). (a) The chloroplast haplotype distribution is given in the pie charts, with colours corresponding to the haplotype network displayed on top of the map (the blue dots indicate inferred mutations). (b) The geographic structuring of the STRUCTURE groups of (d) for K = 2 and (c) for K = 4. The group colours from (d) were used to colour the pie-charts indicating the average assignment of individuals to each population. Blue lines represent rivers, thin and bold solid black lines represent province and country boundaries and dashed magenta lines indicate gene-flow barriers. (d) The microsatellite memberships of the *F. craterostoma* assigned by STRUCTURE for K = 2, K = 3 and K = 4. Each vertical line represented one individual. The colour indicates the group.

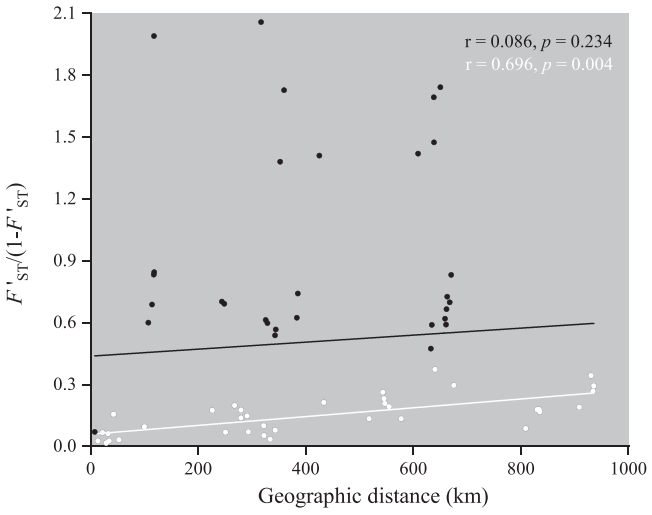

Fig. 3. Genetic isolation by distance. The relationships of genetic distances and geographic distances between sample site pairs based on SSR genotypes. Black points indicate distances involving MAP, ENT and DLI and white point the remaining distances. Black, and white lines respectively indicate regression of all comparisons (including white points) and comparisons excluding MAP, ENT and DLI (only white points).

H09 and H10 coexist even though it is in a scarp forest. We propose that from this core refugium, there started a stepwise recolonization of northern Afromontane forests, potentially over a time period of 550,000 years. This finding is in line with previous claims that east coast scarp forests (Dalton et al., 2015; Eberle et al., 2017; Hughes et al., 2005; Lawes et al., 2007a) or remnants of old Afromontane forests were refugia for Afromontane forest faunal communities during periods of range contraction (Eberle et al., 2017; Lawes et al., 2007a). The proposed northward recolonization of forest associated mammals (Lawes, 1990; Lawes et al., 2007a; Makhasi, 2013) and birds (Coetzer, 2015; Coetzer et al., 2019; Forbes, 2003; Lawes et al., 2007a) into KwaZulu-Natal from their Eastern Cape refugium would have facilitated *F. craterostoma*'s dispersal. As *F. craterostoma* recolonised towards the north, several small refugia along the northern extension of the great escarpment were potentially formed, as has been proposed for other species (Coetzer, 2015; Coetzer et al., 2019; Lawes et al., 2007a; Scott, 1987).

A second refugium seeded the scarp forests but was smaller because it only sustained a single haplotype that evolved into H04. It was most likely located in the central KwaZulu-Natal scarp area (Lawes et al., 2007a) and spread more recently to the southern extreme of the population, helped by potentially continuous stretches of this forest (Eeley et al., 1999). The presence of two refugia (scarp-like and Afromontane-like) fits with the two proposed refugia

suggested for samango monkeys (*Cercopithecus albogularis labiatus* Sykes) (Dalton et al., 2015; Makhasi, 2013) and Cape parrots (*Poicephalus robustus* Gmelin) (Coetzer, 2015; Coetzer et al., 2019), both of which live in forests.

The third putative refugium contained the population that is now found in the Indian Ocean coastal belt forest. Given the single haplotype, H05, it must also have been small. Since it could not have come from the Mozambican coast, and was strongly isolated from the scarp refugium, it may have been in the eastern part of the Lebombo mountain at the tripoint between South Africa, Mozambique and Eswatini (Lawes, 1990). The molecular data suggest that this refugium only came into contact with other forests recently because 1) MAP is monomorphic for the H05 cpDNA haplotype, 2) MAP has many private SSR alleles, indicating long isolation, 3) the inferred gene flow barrier may reflect an ancient divergence combined with recent admixture and 4) its genetic distances do not fit the isolation by distance pattern.

4.2. Seed dispersal and pollen flow between populations

Recolonization from refugia requires seed dispersal. The seeds of *F. craterostoma* are dispersed by birds and arboreal mammals (Basabose, 2002; Gautier-Hion et al., 1985; Lawes, 1990). The only mammal known to disperse *F. craterostoma* in South Africa is the samango monkey (Dalton et al., 2015). Since, 1) samango monkeys (Lawes, 2004) and forest specialist bird species (Cooper et al., 2017; Olivier et al., 2013) were lost from small patches; and 2) because these fauna are more likely to remain resident in local forests rather than travel between fragments (Bonnevie et al., 2003; Moore et al., 2008; Shanahan et al., 2001; Thornton et al., 1996) seed dispersal in this fragmented habitat would likely have been limited. Indeed, the concordance between the haplotype network and geographical position of haplotypes underscores such relatively poor seed dispersal. Two haplotypes that are more widespread, H05 and H07, probably benefitted from the recent near continuous distributions of scarp forests (Eeley et al., 1999). In addition, the frequent polymorphism in scarp populations is in part due to seed flow into the scarp forests from Afromontane and Indian Ocean coastal belt forests.

The lower nuclear microsatellite differentiation between forests compared to cpDNA supports the general consensus that in fig trees, as in plants in general (Petit et al., 2005), pollen flow is more effective than seed dispersal (Liu et al., 2015; Wang et al., 2009; Yu et al., 2010; Yu and Nason, 2013). In the case of figs, 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 (Compton, 2002; Compton et al., 2005, 2000; Harrison and Rasplus, 2006; Ware and Compton, 1994a, 1994b). One can expect nearby populations to have more gene flow and that is exactly what was found for the forest fragments scattered over 1000 km along the western scarp and Afromontane forests. This gene flow even occurs between different forest types (MBO and ING, and MAP and DLI and ENT). However, this gene flow is not enough to homogenize the populations genetically, because many F_{ST} values are significantly larger than 0.

At K = 3 and 4, MAP forms a cluster and DLI and ENT form another distinct cluster. This corroborates the BARRIER analysis that suggested gene flow barriers. At K = 2, however, MAP, DLI and ENT form a single cluster that is strongly isolated from the others. While these groupings can reflect an equilibrium reached over many years of characteristic gene flow, divergence models can also predict the situation. For instance, DLI, ENT and MAP were isolated from all the other forests long ago and more recently DLI and ENT split from MAP. However, a combination of these views is also possible, MAP was isolated from the others for a long time, building up allele frequency differences, and then recent gene flow between MAP and nearby DLI and ENT have started to homogenize these three forests so that MAP has similarities with DLI and ENT, while these scarp forests have similarities with MAP. This last interpretation seems the most parsimonious because only one population is required to be isolated in a species where pollinators can routinely disperse over tens of kilometres. Even the isolation of just one population is hard to imagine in the absence of mountains that can channel winds. If this is the correct interpretation, then the barrier results do not reflect current restrictions on gene flow, but differences built up in the past. The lack of recognizable current physical barriers to dispersal is another reason why divergence followed by recent admixture seems a more tenable explanation.

Alternatively, strong selection may prevent or retard gene flow between populations because alleles from another population result in seedlings failing to survive locally. If this is the case and MAP trees are adapted to their local conditions, then it would cause harm to move any plants between these coastal populations. In contrast, the movement of seedlings between adjacent western forests could be used to maintain or even start new *F. craterostoma* populations because local adaptation appears to be absent.

This study illustrates a now familiar pattern among *Ficus* species where very extensive pollen flow is combined with more spatially limited seed dispersal. Since seed dispersal is the only opportunity for cpDNA to be dispersed, we used it to infer the presence of a minimum of three ice age refugia for *F. craterostoma*. A large genetically diverse refugium is likely to have been located in the Eastern Cape, where it may have benefitted from the influx of genes from trees in other forest types. This refugium also seems to have been the source of the northward recolonization of Afromontane forests in South Africa. The other two refugia were possibly located in northern KwaZulu-Natal. They have preserved little or no cpDNA variation, implying they were much smaller than the Eastern Cape refugium. Their precise locations are uncertain, but one was probably in a scarp forest, and the other in Indian Ocean coastal belt forest. Our most Eastern sample, which is from an Indian Ocean coastal belt forest, is so genetically distinct that pollen flow with more inland forests has either only started recently, and/or, has been ineffective, possibly because of strong selection against admixture. In contrast, greater genetic similarity between western forests and a pattern of isolation by distance extended over a distance of >1000 km suggest pollen exchange has been frequent among nearby western forests.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Jun-Yin Deng: Writing – review & editing, Writing – original draft, Visualization, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Simon van Noort:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Stephen G. Compton:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Yan Chen:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Jaco M. Greeff:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Data availability statement

cpDNA sequences are available on GenBank and microsatellite genotypes and localities are available on Figshare at: 10.25403/UPresearchdata.19394153.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.sajb.2025.01.033.

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