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Spatial genetic structure of *Ficus superba* (Moraceae) in mainland and insular Singapore

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ABSTRACT. Monoecious *Ficus* L. (Moraceae) species have putatively effective long-distance pollen dispersal and low population densities. The lack of spatially defined populations and the assumption of high gene flow led to the dearth of work on the Spatial Genetic Structure (SGS) of monoecious species. Furthermore, as only forest or inland species were studied, the effects of landscape heterogeneity and discontinuous habitats have been overlooked. To address this, *Ficus superba* (Miq.) Miq., a monoecious spatially aggregated coastal and insular species from Singapore, was studied to ascertain if population homogenisation could result from pollen flow even when the assumptions of spatial and landscape homogeneity were violated. Individuals were genotyped using dominant Amplified Fragment Length Polymorphism (AFLP) markers. Bayesian clustering software and Principal Component Analysis (PCA) were used to test for population genetic structure. SGS analyses and Mantel's Tests were performed to test for the presence of significant fine-scale SGS and Isolation By Distance (IBD). Significant population genetic structure and SGS were found, with one mainland population more differentiated from the remaining insular and mainland populations. It is hypothesised that the spatial aggregation and landscape heterogeneity significantly distort gene flow through aggregated seed dispersal and greater impedance to pollen flow over land than sea. This calls into question the supposed dichotomy in gene flow characteristics of monoecious versus dioecious figs attributed to pollinator behaviour.

Keywords. AFLP, gene flow, landscape, pollinator dispersal, spatial aggregation

Introduction

The genus *Ficus* L. (Moraceae) consists of more than 700 species of figs worldwide (Berg & Corner, 2005), and is considered a keystone taxon in Southeast Asia and the Neotropics for its aseasonal fruiting that supports a wide range of vertebrate frugivores year-round (Leighton & Leighton, 1983; Terborgh, 1986; Lambert & Marshall, 1991). Each species is pollinated by one specific species of Agaonidae (Chalcidoidea) wasp, and the wasp is in turn dependent on the pollinated ovaries for raising its larvae (Galil & Eisikowitch, 1968; Wiebes, 1979).

Janzen (1979) realised that the low population densities of figs meant that the pollinators must traverse considerable distances. This was first demonstrated by

Nason et al. (1998) using genetic markers for pollinators of monoecious forest figs, a generalisation upheld by later work (Ahmed et al., 2009; Nazareno & Carvalho, 2009). The long-distance pollen dispersal of monoecious figs has been contrasted with limited dispersal of dioecious figs and explained by the differences in the pollinators' flight heights and dispersal efficiencies (Compton et al., 2000; Harrison & Rasplus, 2006). Furthermore, dioecious figs are typically found in locally dense clusters (Harrison, 2000; Wang et al., 2009), making long-distance dispersal unnecessary barring exceptional climatic events affecting reproduction of local fig populations (Harrison, 2000, 2003). Perhaps because of this preconception, work on population genetics (e.g., Valdeyron et al., 1985; Chen et al., 2008) and recent studies on spatial genetic structure (SGS) have focused on dioecious figs (e.g., Yu et al., 2010; Zhou & Chen, 2010; Dev et al., 2011). Even though monoecious species were overlooked, such studies would be valuable for discovering how aggregation across a heterogeneous landscape with patchy habitats modifies the population structure and SGS of a species despite unhindered pollinator dispersal. Only recently have there been a few studies in the Neotropics on monoecious figs, and these are limited to uniform landscapes and showed low but significant SGS (Nazareno et al., 2013; Heer et al., 2015). Thus, there is much to be learnt if the same applies to monoecious species in a different geographical region and over a varied landscape.

Hoping to address the gap, we chose to work on the monoecious, coastal, spatially aggregated and insular *Ficus superba* (Miq.) Miq. using Amplified Fragment Length Polymorphism (AFLP) marker system, which is applicable to many organisms without prior genetic knowledge or development cost of species-specific markers (Vos et al., 1995; Vos & Kuiper, 1997; Mueller & Wolfenbarger, 1999).

Materials and methods

Study species

The range of *Ficus superba* extends from Thailand to Java, the Lesser Sunda Islands, Borneo (Anambas and Natuna Islands), Celebes and the Moluccas (Ceram) (Berg & Corner, 2005). It is pollinated by *Platyscapa corneri* Wiebes (Wiebes, 1994). This deciduous hemiepiphytic fig, from *Ficus* subgenus *Spherosuke* Raf. (= *Urostigma* (Endl.) Miq.) section *Urostigma* (Endl.) Griseb., is found in coastal and monsoon forests, and often on rocks by the sea (Berg & Corner, 2005). Although it is a lithophyte in its natural habitat, it is commonly also found growing on planted trees in urban Singapore, eventually outgrowing the hosts (pers. obs.). The figs can become free standing reaching heights of up to 30 m with trunk diameter at breast height of up to 3.6 m (C.K. Yeo, pers. obs.). While the exact age of these large individuals is not known, they could probably grow indefinitely, with a propensity to sprout new shoots adventitiously in response to injury or incomplete attempts at removal.

Ficus superba is a predominantly bird-dispersed species: its fruits have been observed to be consumed by birds of various families, such as the house crow (*Corvus splendens* (Corvidae)), pink-neck pigeon (*Treron vernans* (Columbidae)), white-

vented myna (*Acridotheres javanicus* (Sturnidae)), glossy starling (*Aplonis panayensis* (Sturnidae)), and straw-headed bulbul (*Pycnonotus zeylanicus* (Pyconotidae) (C.K. Yeo, pers. obs.). Richard Corlett (pers. comm.) has also observed fruit bats, *Cynopterus brachyotis* (Pteropodidae), feeding on the ripe syconia.

A census was conducted from 23 July 2003 to 31 October 2004 in all parts of Singapore, based on previous records supplemented with visual surveys of locations with suitable habitats. In all, 359 individuals were found and the leaves were sampled for the population genetics study. As some plants were observed over an extended period of over a year in a phenological study (Yeo & Tan, 2009), a logistic regression model was developed (unpublished data), allowing all the individuals to be divided into cohorts based on them having reached sexually reproductive stage. This categorisation allowed the populations to be more finely genetically analysed by cohort.

Sample preparation

Each leaf sample was washed under running tap water, rinsed for five minutes in MilliQ water, five minutes in 30% Clorox (v/v) and three minutes in 10% ethanol (v/v) for surface sterilisation. It was then sealed in a resealable polythene bag and stored at -80°C until extracted.

A hexadecyltrimethylammonium bromide (CTAB)-based DNA extraction method modified from Lodhi et al. (1994) was used. About 0.05 g of each sample was pulverised in liquid nitrogen with mortar and pestle and placed in a 2 ml microtube (Axygen, MCT-200-C, USA) with 600 µl of DNA extraction buffer and incubated for 2 hrs at 60°C. The buffer consisted of 2% (w/v) CTAB, 20 mM ethylenediaminetetra-acetic acid (EDTA) (pH 8.0), 4% (w/v) polyvinylpyrrolidone (PVP), 1.4 M sodium chloride (NaCl), 100 mM Tris-HCl (pH 8.0), and 0.2% β-mercaptoethanol.

Upon cooling, 720 µl of chloroform:octanol (24:1) was added to each tube. The tubes were inverted 20 times and centrifuged at 7,200 rpm for 15 min (Eppendorf, 5415D, Germany). The aqueous layer was transferred to a new tube, and to each unit volume 0.5 volume of 5 M NaCl and 2.0 volumes of 95% ethanol chilled at -20°C were added and mixed by inversion. The DNA was precipitated overnight at -20°C. The tubes were centrifuged at 5,000 rpm for 5 min and the supernatant was decanted. The DNA pellet was washed with 300 µl of 76% ethanol (v/v) at 4°C, and centrifuged at 5,000 rpm for 5 min. The supernatant was decanted and the pellet was air-dried for 30 min then dissolved in 20 µl of Tris-EDTA (TE) buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and stored at -20°C.

AFLP

AFLP reactions followed Vos et al. (1995) with modifications. The oligonucleotide adapters and primers used are listed in Table 1. To each 1.75 ml microtube, 15 µl of 250 ng DNA and 10 µl of restriction digestion mix were added, followed by incubation at 37°C for 1 hr and cooling on ice for ligation. The restriction digestion mix consisted of 1× NEB Buffer 4 (New England Biolabs, USA), 1× Bovine Serum Albumin (BSA) (New England BioLabs, USA), 5 mM dithiothreitol (DTT) (Fermentas, Lithuania), and 2.5 u each of EcoRI (Pharmacia, UK) and MseI (New England Biolabs, USA).

Table 1. Sequences of adaptors and primers used in the AFLP reactions. All unlabelled oligonucleotides were supplied by 1st Base, Singapore. Fluorescently labelled primers for Eco 107, Eco 108, Eco 109, and Eco 110 were supplied by Applied Biosystems Inc.

Oligonucleotide type	Name	Oligonucleotide sequence
EcoRI Adaptor	EcoA 101	5'–CTC GTA GAC TGC GTA CC–3'
EcoRI Adaptor	EcoA 102	5'–AAT TGG TAC GCA GTC TAC–3'
MseI Adaptor	MseA 103	5'–GAC GAT GAG TCC TGA G–3'
MseI Adaptor	MseA 104	5'–TAC TCA GGA CTC AT–3'
Pre-selective PCR EcoRI primer	PEco 105	5'–GAC TGC GTA CCA ATT CA–3'
Pre-selective PCR MseI primer	PMse 106	5'–GAT GAG TCC TGA GTA AC–3'
Selective PCR EcoRI primer	Eco 107	5'–6FAM-GAC TGC GTA CCA ATT CAA C–3'
Selective PCR EcoRI primer	Eco 108	5'–VIC-GAC TGC GTA CCA ATT CAA G–3'
Selective PCR EcoRI primer	Eco 109	5'–NED-GAC TGC GTA CCA ATT CAC A–3'
Selective PCR EcoRI primer	Eco 110	5'–PET-GAC TGC GTA CCA ATT CAC T–3'
Selective PCR MseI primer	Mse 115	5'–GAT GAG TCC TGA GTA ACA A–3'
Selective PCR MseI primer	Mse 116	5'–GAT GAG TCC TGA GTA ACA C–3'
Selective PCR MseI primer	Mse 122	5'–GAT GAG TCC TGA GTA ACT T–3'

Two microtubes, with one containing EcoRI adaptors and the other with MseI adaptors, were prepared. For each sample, 1 µl of EcoRI adaptor mix and MseI adaptor mix were prepared in 10 mM Tris-HCl. The EcoRI adaptor mix contained 2.5 ρmol of each EcoA 101 and EcoA 102. The MseI adaptor mix contained 25 ρmol of each MseA 103 and 25 ρmol MseA 104. The two were placed into the thermocycler (GeneAmp PCR System 9600, Applied Biosystems, USA) and subjected to the following thermocycling conditions: 95°C for 10 min, 95°C to 4°C in 1 hr, 4°C incubation.

The ligation master mix consisted of 1× NEB Buffer 4, 1× BSA, 5 mM DTT, 1 mM adenosine 5'-triphosphate (ATP) (Fermentas, Lithuania), 1 µl each of EcoRI and MseI adaptors per reaction, and 0.5 µl of T4 DNA Ligase (New England BioLabs, USA) per reaction. To each tube of digested DNA, 5 µl of master mix was added, and then incubated at 15°C for 16–18 hours. The ligation product was diluted 10-fold for pre-selective polymerase chain reaction (PCR).

Subsequently, 5 µl of diluted ligation product was transferred to a 200 µl microtube on ice, and 15 µl of pre-selective PCR amplification master mix consisting of 1× PCR Buffer (Fermentas, Lithuania), 4 mM magnesium chloride ($MgCl_2$) (Fermentas, Lithuania), 0.2 mM of all four DNA nucleotides (dNTP) (Fermentas, Lithuania), 30 ng of each pre-selective PCR primers, PEco 105 and PMse 106, and 0.5 µl of *Taq* DNA Polymerase (recombinant) (Fermentas, Lithuania), was added and placed into a thermocycler and subjected to the following conditions: 25 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 1 min, then held at 4°C. The pre-selective PCR product was diluted 10-fold for selective amplification.

Subsequently, 5 µl of diluted pre-selective PCR product was transferred to a microtube on ice, and 15 µl of selective PCR amplification master mix consisting of 1× PCR Buffer, 4 mM $MgCl_2$, 0.2 mM dNTP, 30 ng of each component of a selective PCR MseI primer and fluorescently-labelled EcoRI pair, and 0.5 µl of *Taq* DNA Polymerase (recombinant), was added. The following combinations were used: Eco 107 + Mse 115, Eco 108 + Mse 116, Eco 109 + Mse 122, and Eco 110 + Mse 122. The tubes were transferred to the thermocycler and subjected to the following conditions: 94°C for 30 s, 65°C for the first cycle, dropping by 0.7°C for each of 13 cycles for 30 s, 72°C for 1 min; then 23 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, then held at 4°C. The marker products were analysed on ABI Prism 377 automated fluorescent sequencer with GeneScan 600 LIZ internal lane-size standards, and the electropherograms visualised using Genescan analysis software (Applied Biosystems Inc.). Fragments of size range of 50–500 bases were scored. In all, 91 markers, of which 82 were heteromorphic, were used to genotype 382 samples from 359 visually distinguishable plants. Large plants with multiple stems and crowns were sampled multiple times, in case ramets (genetic clones) or allofusions (grafting of genetically distinct individuals) were suspected. Eventually, 355 distinct genotypes were identified and divided into "reproductive" (sexually mature plants) and "juveniles" (non-sexually mature plants), using a logistic regression model based on 50 plants (unpublished data), observed over more than one year during a phenological study (Yeo & Tan, 2009).

Population delimitation

By visual inspection, the individuals of *Ficus superba* were divided into five geographic groups (Fig. 1). In Singapore, the larger mainland individuals were mostly found on the eastern side of the mainland (Singapore Island) in Bedok, likely remnants from past coastal population, which survived the land reclamation effort in the 1960s (Corlett, 1992). A smaller mainland geographic group was found centred on the Nanyang Technological University (NTU) campus in the west. Insular groups were found on islands around Pulau Sajahat in the northeast, Saint John's Island in the south and Pulau Salu in the southwest. The numbers of individuals recorded and genotyped from all the clusters are listed in Table 2.

Principal Component Analysis (PCA) was performed using MVSP 3.13p (Kovach Computing Services, Wales, 2007) with AFLP marker-derived Euclidean distances between the individuals. This was compared with the genetic clusters proposed by the Bayesian clustering software, STRUCTURE 2.3.3 (Pritchard et al.,

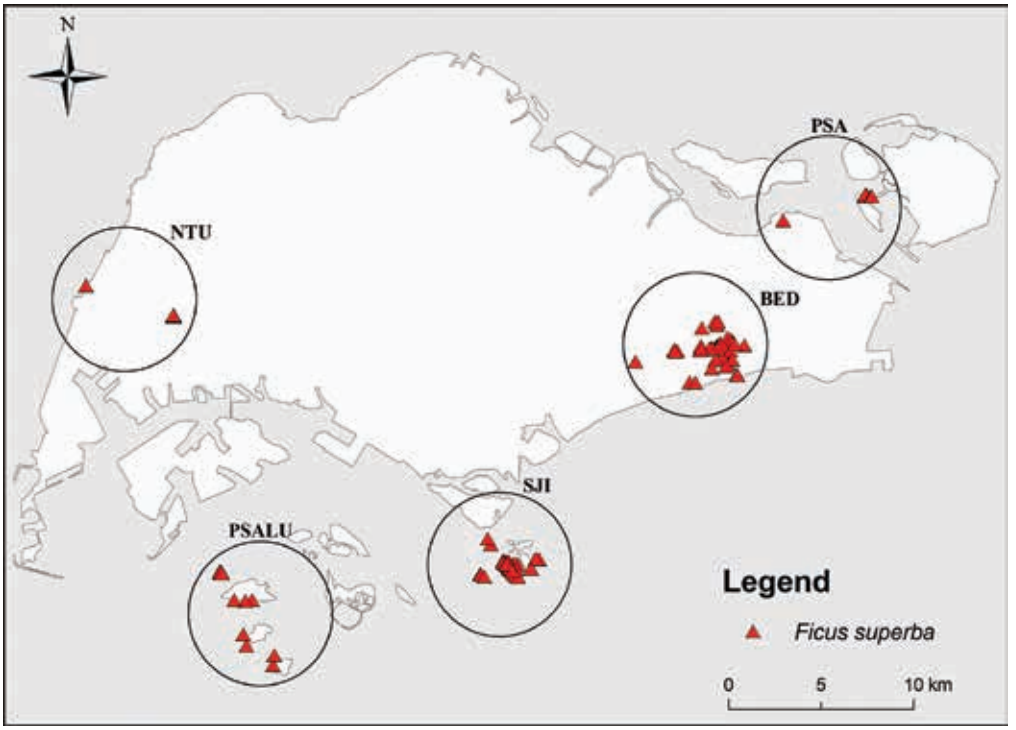

Fig. 1. Locations of the *Ficus superba* (Miq.) Miq. individuals sampled in Singapore. See Table 2 for the full form of the locality abbreviations.

Table 2. Number of *Ficus superba* individuals recorded and sampled from each of the five geographic groups in Singapore.

Location	Abbreviation	Number recorded	Number genotyped	Reproductives	Juveniles
Bedok	BED	74	73	12	61
Nanyang Technological University	NTU	17	17	1	16
Pulau Sajahat	PSA	131	131	15	116
St. John's Island	SJI	111	108	46	62
Pulau Salu	PSALU	26	26	16	10
Total		359	355	90	265

2000), which was used to estimate the number of genetically distinct clusters (K). The correlated allele frequency model for dominant alleles was used (Falush et al., 2003, 2007), with 10,000 burn-ins and 10,000 Markov chain Monte Carlo repetitions, setting K=1–20. For each K, the mean log-likelihood value was calculated and the ΔK statistics of Evanno et al. (2005) was derived to infer the number of populations from 15 independent runs to minimise spurious clusters (Frantz et al., 2009).

Secondly, Bayesian Analysis of Population Structure 5.3 (BAPS) was run with maximum K set to 10, 15, 20, and 25, with 10 replications for each under the spatial model (Corander et al., 2008). The non-spatial model was also run for comparison with STRUCTURE 2.3.3.

Population genetic structure

Genetic clusters suggested by the Bayesian clustering and PCA defined the populations analysed. AFLP-SURV 1.0 (Vekemans et al., 2002) and TFPGA 1.3 (Miller, 1997) calculated F_{ST} with allelic frequencies estimated according to Lynch & Milligan (1994), following the Bayesian approach assuming a non-uniform prior (Zhivotovsky, 1999). Inbreeding coefficient (F_{IS}) was assumed to be 0, and 999 permutations were used to test the significance of population differentiation (F_{ST}).

Genetic analysis in Excel (GenAlEx 6.41) of Peakall & Smouse (2006) was used to perform Analysis of Molecular Variance (AMOVA), and calculate the Φ_{PT}, an analogue of F_{ST}. Statistical significance of the estimates was then tested with 9999 permutations.

Fine-scale spatial genetic structure

Fine-scale SGS was analysed with GenAlEx 6.41 using the spatial autocorrelation methods of Smouse & Peakall (1999) and Peakall et al. (2003). The autocorrelation coefficient (*r*) was calculated for geographical distances at class intervals of 2 km. The null hypothesis of no SGS was tested with 999 permutations. Isolation By Distance (IBD) was tested using Mantel's Test. An alternative spatial autocorrelation test statistic, V, was used to detect departure of the average genetic distance of individuals in each distance class from random chance (Miller, 2005). Ten distance classes with 1000 permutations were used. Allelic Aggregation Analysis was also performed, using a modified aggregation index of Clark & Evans (1954) over all alleles (R_{ave}), to detect non-random spatial distribution of genetic variation.

SPAGeDi 1.3 of Hardy & Vekemans (2002) was used to calculate the *Sp* statistics based on the decrease of pair-wise kinship coefficient of Hardy (2003) with distance, as comparisons between different species could then be made, assuming IBD at drift-dispersal equilibrium (Vekemans & Hardy, 2004). Ten equal distance classes were used to obtain $\hat{F}_{(1)}$, the mean pair-wise kinship between individuals belonging to the first distance class, and $\hat{b}_F$, the regression slope of pair-wise kinship on ln(pair-wise geographic distance). *Sp* was calculated as $-\hat{b}_F/(1-\hat{F}_{(1)})$. The concavity or convexity of the pair-wise kinship coefficient relationship was used to gauge the relative contributions of seed versus pollen dispersal to the SGS following Heuertz et al. (2003).

Results

Population delimitation

The PCA accounted for 31.7% of the variations with the first three axes. The BED geographic group formed the most distinct cluster, while others were less resolved (Fig. 2). STRUCTURE 2.3.3 detected four genetic clusters, roughly corresponding to the geographic groups. Following majority membership detailed in Table 3 and Fig. 3, they corresponded to BED, PSA and SJI, while NTU was subsumed under PSALU (NTU-PSALU cluster). BAPS 5.3 recognised seven clusters using the non-spatial model, splitting each group into four to six clusters, while the spatial model detected four, supporting the four genetic clusters of STRUCTURE 2.3.3. This division into BED, PSA, SJI and NTU-PSALU was taken as the basis of subsequent population genetic analyses.

Population genetic structure

Nei's unbiased heterozygosity values were comparable between the populations and cohorts, except for the reproductives of BED and PSA having lower values than the respective juveniles and the same cohort from the other populations. As the number of individuals sampled in each genetic cluster was comparable, the older cohort of BED and PSA could be genetically less diverse rather than being an artefact of sampling (Table 4).

From the F_{ST} and Φ_{PT} values, the population genetic structure was significant and more differentiated for the reproductives than the juveniles (Table 5). Moreover, Nei's genetic distances (Table 6), pair-wise Φ_{PT} (Table 7) and pair-wise F_{ST} values (Table 8) all concurred that BED was the most distinct from other genetic clusters, and the reproductives were generally more different among the clusters than the juveniles. From the exact tests, the pair-wise F_{ST} values calculated by TFPGA for reproductives of NTU-PSALU:PSA and NTU-PSALU:SJI were non-significant. While the pair-wise F_{ST} values were significant for all individuals and the juvenile cohort, they tended to be lower between the insular genetic clusters. Therefore, NTU-PSALU was weakly isolated from other insular genetic clusters, while BED was the most reproductively isolated cluster.

AMOVA showed that most genetic variation was found within subgroups, followed by among groups, then least of all among subgroups (Table 9). The juveniles had more variation within the subgroups and less among the subgroups compared to the reproductives, while at the same time maintaining the same level of among-group variation, suggesting that there has been gene flow between the subgroups leading to more variations within subgroups. For both cohorts, similar levels of variation among groups were maintained suggesting much less gene flow between inland genetic cluster (BED) and offshore clusters.

Fine-scale spatial genetic structure

Spatial autocorrelation analysis for all individuals found significant coefficient *r*, alternating between positive and negative values over distance classes (Fig. 4A).

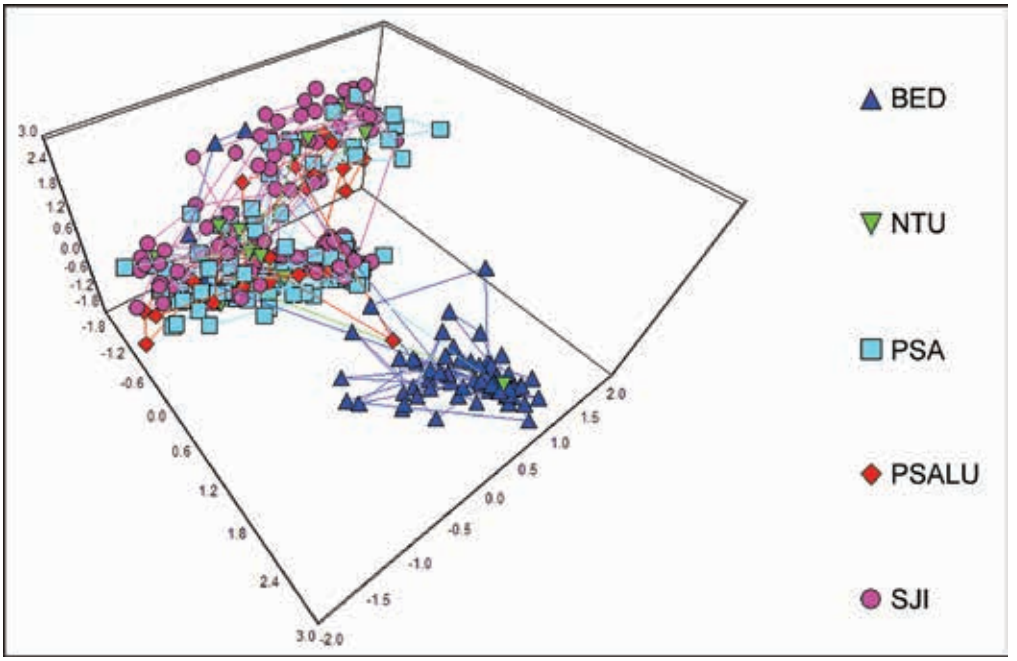

Fig. 2. Scatter plot of PCA done by MVSP 3.13p, based on the Euclidean distances between all individuals from the five geographic groups.

Table 3. A breakdown of geographic groups into component genetic clusters recognised by STRUCTURE 2.3.3.

Geographic groups	Component genetic cluster (%)			
	1	2	3	4
BED	0	0	100	0
PSA	49.2	12.3	1.5	36.9
NTU-PSALU	4.9	34.1	4.9	56.1
SJI	36.6	29.0	0	34.4

Separate analyses for the cohorts found more pronounced fluctuations for the juveniles than reproductives (Figs 4B, 4C).

The Alleles In Space (AIS) test statistics V was 0.03828 ($p < 0.00001$) for all individuals, thus showing that genetic distance was non-randomly spatially distributed. The corresponding results for the reproductives and juveniles were 0.03595 ($p < 0.00001$) and 0.03975 ($p < 0.00001$) respectively, showing that the spatial autocorrelation is stronger in the juveniles, though both are statistically significant.

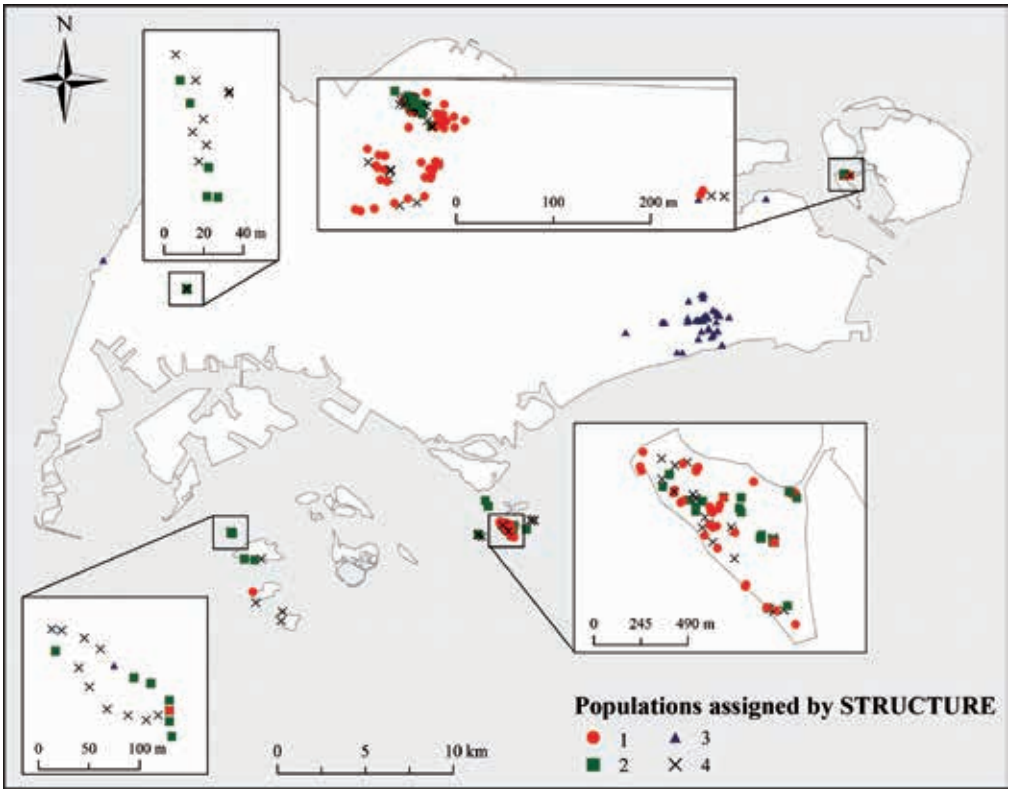

Fig. 3. Individuals plotted, with different symbols corresponding to genetic clusters proposed by STRUCTURE 2.3.3.

Table 4. Nei's unbiased heterozygosity calculated by TFPGA 1.3 using the genetic clusters assigned using STRUCTURE 2.3.3. The number of individuals sampled is given in brackets.

Genetic clusters	Overall	Cohorts	
		Reproductives	Juveniles
BED	0.1353 (73)	0.0846 (12)	0.1397 (61)
PSA	0.1389 (131)	0.0898 (15)	0.1384 (116)
NTU-PSALU	0.1501 (43)	0.1420 (17)	0.1454 (26)
SJI	0.1624 (108)	0.1694 (46)	0.1493 (62)

Table 5. Population differentiation estimates of F_{ST} and Φ_{PT} with p-values in brackets, based on the genetic clusters assigned using STRUCTURE 2.3.3.

	Φ_{PT} (GenAlEx 6.41)	**F_{ST} (AFLP-surv 1.0)**	**F_{ST} (TFPGA 1.3)**
Overall	0.421 (0.001)	0.175 (< 0.001)	0.2081 (< 0.0001)
Reproductives	0.474 (< 0.001)	0.197 (< 0.001)	0.2527 (< 0.0001)
Juveniles	0.417 (< 0.001)	0.170 (< 0.001)	0.2270 (< 0.0001)

Table 6. Nei's genetic distances calculated by AFLP-surv 1.0 in the lower triangle, and TFPGA 1.3 in the upper triangle, based on the genetic clusters assigned using STRUCTURE 2.3.3.

Overall				
	BED	PSA	NTU-PSALU	SJI
BED	0	0.0866	0.0744	0.1002
PSA	0.0740	0	0.0118	0.0105
NTU-PSALU	0.0644	0.0069	0	0.0132
SJI	0.0862	0.0082	0.0077	0
Reproductives				
	BED	PSA	NTU-PSALU	SJI
BED	0	0.1043	0.0870	0.1301
PSA	0.0665	0	0.0253	0.0365
NTU-PSALU	0.0678	0.0086	0	0.0225
SJI	0.1070	0.0257	0.0092	0
Juveniles				
	BED	PSA	NTU-PSALU	SJI
BED	0	0.0892	0.0832	0.0971
PSA	0.0744	0	0.0143	0.0091
NTU-PSALU	0.0672	0.0065	0	0.0163
SJI	0.0817	0.0059	0.0079	0

Table 7. Pair-wise Φ_{PT} values in the lower triangle with corresponding p-values in upper triangle, based on the genetic clusters assigned using STRUCTURE 2.3.3.

Overall				
	BED	PSA	NTU-PSALU	SJI
BED	0	0.001	0.001	0.001
PSA	0.441	0	0.001	0.001
NTU-PSALU	0.430	0.066	0	0.001
SJI	0.432	0.085	0.045	0
Reproductives				
	BED	PSA	NTU-PSALU	SJI
BED	0	< 0.001	< 0.001	< 0.001
PSA	0.558	0	< 0.001	< 0.001
NTU-PSALU	0.475	0.154	0	0.001
SJI	0.479	0.230	0.083	0
Juveniles				
	BED	PSA	NTU-PSALU	SJI
BED	0	< 0.001	< 0.001	< 0.001
PSA	0.431	0	< 0.001	< 0.001
NTU-PSALU	0.432	0.074	0	< 0.001
SJI	0.429	0.062	0.067	0

Table 8. Pair-wise F_{ST} values in the lower triangle calculated by AFLP-surv 1.0, and values calculated by TFPGA 1.3 in the upper triangle with p-values from exact tests within brackets, based on the genetic clusters assigned using STRUCTURE 2.3.3.

Overall				
	BED	PSA	NTU-PSALU	SJI
BED	0	0.3400 (< 0.0001)	0.3081 (< 0.0001)	0.3469 (< 0.0001)
PSA	0.2845	0	0.0593 (< 0.0001)	0.0510 (< 0.0001)
NTU-PSALU	0.2385	0.0349	0	0.0583 (0.0013)
SJI	0.2923	0.0410	0.0344	0
Reproductives				
	BED	PSA	NTU-PSALU	SJI
BED	0	0.5000 (< 0.0001)	0.3728 (< 0.0001)	0.4067 (< 0.0001)
PSA	0.2934	0	0.1382 (0.9916)	0.1621 (< 0.0001)
NTU-PSALU	0.2629	0.0442	0	0.0882 (0.3676)
SJI	0.3455	0.1139	0.0377	0
Juveniles				
	BED	PSA	NTU-PSALU	SJI
BED	0	0.3435 (< 0.0001)	0.3204 (< 0.0001)	0.3507 (< 0.0001)
PSA	0.2783	0	0.0704 (0.0005)	0.0463 (< 0.0001)
NTU-PSALU	0.2341	0.0321	0	0.0741 (0.0245)
SJI	0.2814	0.0301	0.0350	0

Table 9. AMOVA partitioning of genetic variation within subgroups, among subgroups and among groups, with one group consisting of BED and the other consisting of the PSA, NTU-PSALU and SJI subgroups, based on the genetic clusters assigned using STRUCTURE 2.3.3.

	Within subgroups (%)	**Among subgroups (%)**	**Among groups (%)**
Overall	58	5	37
Reproductives	52	11	37
Juveniles	58	4	38

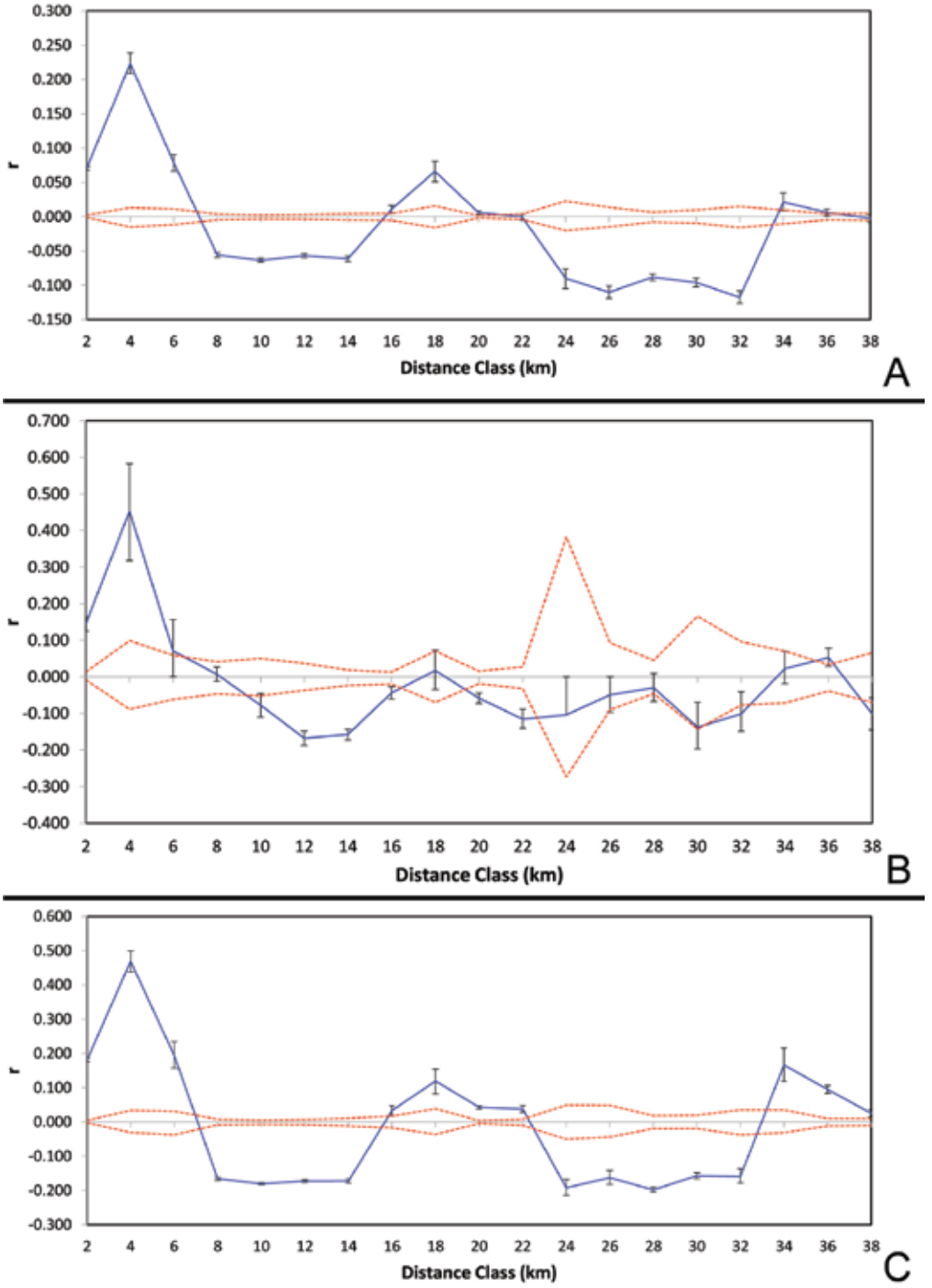

Fig. 4. Spatial autocorrelation analyses for (A) all individuals, (B) reproductives, and (C) juveniles. The error bars indicate the 95% confidence interval about the autocorrelation coefficient (r) as determined by bootstrap resampling, and confidence limits, bounded by the red dashed lines, indicate the 95% confidence interval about the null hypothesis of "no spatial structure".

However, the aggregation index for the Allelic Aggregation Analysis (R_{ave}) was 0.1636 (p = 0.064) for all, 0.3239 (p = 0.035) for the reproductives, and 0.1965 (p = 0.169) the juveniles. The $R_{ave} < 1$ values indicated aggregation, which was significant in the reproductive cohort but not in the juvenile.

The Mantel's Test correlation coefficient was 0.075 (p = 0.002) for all individuals. However, it was suspected that the mainland individuals may differ from the insular ones, thus it was repeated for the mainland and the minor offshore islands separately and found to be 0.735 (p = 0.001) for the former, and 0.0867 (p = 0.002) for the latter.

Sp statistics were calculated to be 0.00665, from mean kinship coefficient of the first distance class $\hat{F}_{(1)}$ of 0.0243 and regression slope $\hat{b}_F$ of -0.00649. The function was determined to be concave, i.e. above the regression line at short distances.

Discussion

Significant population genetic structure and spatial genetic structure were found for *Ficus superba*, which could not be simply attributed to geographical distances between the geographically based groups. Interestingly, one inland group was identified to be the most distinct from all others. An interpretation of the results is presented below, followed by a discussion on how they could have arisen owing to habitat destruction and landscape heterogeneity, despite long-distance pollen dispersal. With more recent research on monoecious and dioecious *Ficus* species, the possible contributions of reproductive characteristics and habitats could be explored. Notably, the work done on the neotropical monoecious species allows for comparison between distinct related monoecious lineages.

Population delimitation

AFLP was an informative marker system for studying the population structure of *Ficus superba* in Singapore. The mainland BED geographic group was distinctive in both PCA and Bayesian clustering, while the others were less-defined, showing an east–west gradient of membership composition. Discrepancies between the results of pieces of Bayesian clustering software were reported by Frantz et al. (2009) for a continuously distributed species, tending to over-estimate structure and not converge on the same solutions. By comparison, our study species was unproblematic. BED, PSA, SJI and NTU-PSALU geographic groups coincided with the genetic clusters accepted based on the results of STRUCTURE 2.3.3 and BAPS 5.3.

Population genetic structure

Based on the four populations defined by Bayesian clustering, Nei's unbiased heterozygosity, overall F_{ST} and Φ_{PT} values were calculated. The magnitude of the F_{ST} values indicated moderate differentiation between the populations (Wright, 1978). The lower Nei's heterozygosity values of BED (Bedok) and PSA (Pulau Sajahat) reproductives relative to the juveniles indicated that they might have experienced a bottleneck or been founded in the recent past. The first possibility is supported by

what is known of the land-use changes in Singapore. As reviewed by Corlett (1992), primary vegetation clearance started early with the founding of the British colony of Singapore in 1819 and rose quickly with increasing population that most of the original vegetation was lost by the 1900s. For the coastal *Ficus superba*, coconut cultivation along the sandy southeastern coast, and mangrove clearance, first for charcoal production and prawn-farming and later land reclamation for development, would have severely impacted any of its existing populations. While most offshore islands were likely less inhabited or affected by development as intensive reclamation only started in the 1960s (Glaser et al., 1991), Pulau Sajahat was different in being next to Pulau Tekong, which has been inhabited since 1857, and was frequented as a place of worship from the 19th century (Chen & Lee, 2012). The populations at Bedok and Pulau Sajahat probably recovered genetic diversity in recent years, as evident from the Nei's unbiased heterozygosity values of the juveniles approaching levels of other populations, with renewed recruitment as vegetation clearance and development had slowed down. In the case of Pulau Sajahat, this was owed to the eviction of the inhabitants when the island and Pulau Tekong were designated for military use in the 1970s (Chen & Lee, 2012).

Generally, the magnitude of the pair-wise Φ_{PT} and F_{ST} values between populations are statistically significant across cohorts, with the younger cohort generally slightly more similar compared to the older cohort. Though significant population structure is generally corroborated by the Nei's genetic distances, there is a small departure, where genetic distance, calculated by AFLP-surv 1.0, was higher for the BED:PSA juveniles than overall, which was in turn higher than genetic distance for the reproductives, suggesting that for BED:PSA the younger cohort have gotten more different than the older, but this was not replicated by TFPGA 1.3.

Taken as a whole, the magnitude of the genetic distances, pair-wise Φ_{PT} and F_{ST} values, overall and for all cohorts, between BED and any other population were higher than between other populations, supporting the more limited gene flow between the mainland and offshore island populations. This limited gene flow is corroborated by the high among group (BED:(PSA, SJI, NTU-PSALU)) genetic variation relative to among subgroup variation calculated by AMOVA. The BED genetic cluster is most genetically distinct from all other mainland and insular clusters, while all others could be considered panmictic. This distinction is well-supported by the STRUCTURE 2.3.3 assignment of all members of BED into genetic cluster 3.

Ficus superba is a species of open habitats, with the mainland population most differentiated, and the offshore island populations more similar. Thus, the sea is not a barrier for the species, unlike what Chen et al. (2008) and Wang et al. (2009) have reported for *Ficus pumila* L. Given the relatively flat topography of Singapore (Corlett, 1992) and the aseasonal phenology (Yeo & Tan, 2009), there are neither obvious physical nor phenological barriers to pollen flow, yet something is impeding gene flow and bringing about population differentiation. The possible causes of the limited gene flow over land relative to sea will be discussed in the following section on the SGS.

SGS and possible causes

The low *Sp* statistics of *Ficus superba* is typical of a "wind-pollinated tree" with an outcrossing breeding system and animal-dispersed seeds according to Vekemans & Hardy (2004). The concave kinship function would indicate that the seed dispersal is much more limited than pollen dispersal ($\sigma_s < \sigma_p$), thus the strengthening SGS could be caused by the former, while homogenisation of populations could be the result of the latter. Previous work on SGS has focused mainly on dioecious figs (e.g., Wang et al., 2009; Zhou & Chen, 2010; Dev et al., 2011), which reported similarly significant but higher SGS, and concluded that pollen flow greater than seed flow was responsible for the SGS. Given the more limited pollinator dispersal distances and more clumped distribution of dioecious versus monoecious *Ficus* species (Harrison, 2000, 2003; Harrison & Rasplus, 2006), the significant SGS was somewhat more unexpected for the monoecious *Ficus superba*.

Comparison of SGS with other monoecious and dioecious species

In recent years, more studies have been done on the neotropical monoecious *Ficus* subgenera *Pharmacosycea* (Miq.) Miq. and *Spherosuke* Raf. (= *Urostigma* (Endl.) Miq.). The latter are hemi-epiphytes like *Ficus superba*, from the same subgenus, so more direct comparisons can be made to understand if the SGS observed for *Ficus superba* is typical of monoecious species. Nazareno et al. (2013) compared the SGS of monoecious *Ficus citrifolia* Mill. and *Ficus eximia* Schott, both from subgenus *Spherosuke* (=*Urostigma*) section *Americanae* (Miq.) Miq. against dioecious figs, showing that the *Sp* statistics were six times as high in dioecious compared to monoecious species, and this was attributed to long-distance pollen dispersal and more limited seed dispersal and establishment, exacerbated by faunal loss. Though the low SGS values of *Ficus citrifolia* and *F. eximia* are just above and below what is seen in our study species, *F. superba* is from section *Urostigma*, sister to the rest of *Spherosuke* (see Gardner et al., 2023), thus it would be of interest to confirm with future work on other species of the section if low SGS values are common among the rest of the subgenus; interesting candidates include *F. religiosa* L. and *F. virens* Aiton. *Ficus religiosa*, though originally native to the Indian subcontinent and IndoChina, has naturalised far beyond Asia, with breeding populations in United States, Hong Kong, Israel, South Africa, Zambia and recently in Brazil as reviewed by Vianna-Filho et al. (2017). Therefore, it is an accessible model species for studying genetic structure in native habitats, newly invaded habitats and even novel ecosystems. *Ficus virens* is a species native to Singapore, and can be found in coastal, monsoon forest or savannah, on cliffs, and in secondary rainforest from Sri Lanka to southern China, across Southeast Asia to northern Australia (Berg & Corner, 2005). As it can be found in a wide range of habitats, it could be a good species to elucidate how habitat and landscape factors affect gene flow and population genetic structure.

Heer et al. (2015) similarly concluded that restricted seed dispersal could have resulted in significant but low SGS in *Ficus insipida* Willd. (subgenus *Pharmacosycea* section *Oreosycea* (Miq.) Corner) from Barro Colorado Nature Monument (BCNM) and along Pipeline Road in Soberanía National Park, despite its known long-distance

pollen dispersal. This was compared with the *Sp* statistics observed for three other monoecious species from BCNM. Interestingly, *Ficus yoponensis* Desv. (subgenus *Pharmacosycea* section *Pharmacosycea* (Miq.) Benth. & Hook.f.) showed the highest *Sp* statistics at about ten times as high as that of *F. insipida*, while *F. citrifolia* and *F. obtusifolia* Kunth, both from subgenus *Urostigma* (section *Americanae*), had intermediate values. The range of variation in the SGS was hypothesised to be caused by the differently aggregated distribution of the species and the different rates of recruitment from overlapping seed sources. However, as the two monoecious species from subgenus *Pharmacosycea* have pollinators from the subfamily Blastophagineae, which are closely related to pollinators of certain dioecious *Ficus* lineages (Cruaud et al., 2010), the pollinators of *F. yoponensis* could be similar to those associated with dioecious species with shorter dispersal distances, leading to the observed *Sp* statistics. It is unclear why it is not the case with *Ficus insipida*, suggesting that more work on species with related groups of pollinators could reveal if other factors are involved. Furthermore, while comparable F_{ST} values were also reported for populations of *Ficus insipida* (Heer et al., 2015) and *F. superba*, the pairwise F_{ST} and Φ_{PT} values, and AMOVA for *F. superba* clearly indicated greater similarity between the coastal and insular populations, with the inland BED genetic cluster most divergent. This contrasted with the work of Heer et al. (2015), which showed a simple relationship with distance. This is further support for the hypothesis that landscape heterogeneity may have a significant effect over physical distance on gene flow and therefore SGS.

Regarding the cohort study, only slightly stronger spatial genetic structure was detected in AIS in the younger juvenile cohort than the older reproductive cohort, while Allelic Aggregation Analysis found the juveniles to have non-significant aggregation compared to the reproductives. The latter agrees with genetic structure measures in population genetics showing juveniles to be less differentiated than reproductives.

The cohort study of dioecious *Ficus cyrtophylla* (Miq.) Miq. found significant SGS for the seeds and seedlings but not for the older cohorts (Zhou & Chen, 2010). Localised seed dispersal explained the SGS, while selection was invoked to account for the weakening SGS with age. In *Ficus superba*, the opposite was observed, with a weaker SGS in the younger cohort relative to the older from the Allelic Aggregation Analysis. Though limited seed dispersal is expected from what Laman (1996) demonstrated in his fig seed shadow study, and for a coastal and insular species like *Ficus superba*, seed dispersal and plant recruitment are probably even more clumped, as patchy habitable sites often end abruptly at the sea-land boundary, but a relatively low SGS was observed. Thus, long-distance pollen dispersal may have a greater effect than the short-distance seed dispersal in determining the cohort SGS. Furthermore, as the populations have been recovering from recent disturbance from vegetation clearance, with a preponderance of younger plants showing that the population dynamics is far from equilibrium, it would be interesting to monitor how the SGS of the species changes over time in the future.

There are theoretical grounds, however, for expecting that spatial aggregation alone could modify both pollen and seed dispersal. Morales & Carlo (2006) have shown that aggregation resulted in a more leptokurtic frugivore-aided seed dispersal, while Meagher & Vassiliadis (2003) have demonstrated that near-neighbour mating in

insect-pollinated plants is exacerbated by it. Robledo-Arnuncio & Austerlitz (2006) have also argued that aggregation has a non-negligible effect on pollen dispersal. Though aseasonal reproductive phenology of *Ficus superba* may reduce synchronised reproductive phases among individuals, it affects interpopulation and intrapopulation individuals alike. The predominance of intrapopulation mating is therefore also expected in *Ficus superba*, with the severity dependent on the scale of aggregation. This could be addressed with a future cohort study as the population dynamics move toward equilibrium, or more immediately with a seedling parentage study possibly at the crop-level.

It is more important to address the evidence against IBD, including the alternating sign of the spatial genetic autocorrelation coefficient, the disparity between the Mantel Test coefficients of the mainland and the offshore island individuals, and the genetically distinct mainland BED population. We could interpret the difference in magnitudes of the Mantel Test coefficients for the mainland individuals versus the insular individuals and the distinctiveness of the BED population, as indicating that pollinator dispersal aided by wind was less effective over land than over the sea. We would suggest that the difference in surface roughness is the cause for the observed difference in the effectiveness of the wind dispersal from what is known of wind-aided pollinator dispersal in *Ficus* (Compton et al., 2000; Harrison & Rasplus, 2006). More work on the movement of the pollinator over the heterogeneous landscape would clarify the causes of the departure from IBD, as most work on *Ficus* SGS to date have not considered the landscape matrix.

Conclusions

The present work has shown significant population differentiation and SGS in *Ficus superba* in Singapore. Furthermore, *Ficus superba*, a predominantly coastal and insular species, distributed over a heterogeneous landscape, has a highly aggregated distribution, unlike most well-studied, inland, monoecious species inhabiting a continuous, homogeneous landscape. We posit that the predominantly coastal and insular distribution and the heterogeneous landscape of Singapore has distorted gene flow (both seed dispersal and pollen flow), affecting the population structure and SGS, despite the putatively efficient long-distance pollen dispersal considered typical of the monoecious *Ficus* species.

Over relatively short historical timescales, land-use changes may alter the landscape matrix in which figs are distributed, even if the populations were left largely intact. What is the relevance of this in the conservation of fig species in a landscape affected by anthropogenic habitat fragmentation? It is conceivable that habitat fragmentation may impede some form of gene flow, e.g., via seed dispersal, while promoting others, e.g., via pollen dispersal depending on the pollinator's behaviour. Could this be amplified over evolutionary timescales, interacting with the speciation process caused by introgression between lineages (Gardner et al., 2023), and possibly accounting for why *Ficus* is especially species-rich in Malesia, with half of all its species (Berg & Corner, 2005)?

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