Wang T *et al.* (2021)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 49, Issue 3, Article number 12407
DOI:10.15835/nbha49312407
Research Article

Association between spatial genetic variation and potential distribution in tree fern *Alsophila gigantea* (Cyatheaceae) in Hainan Island, China

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Abstract

Spatial genetic variation involves spatial genetic structure (SGS) and genetic diversity is important genetic features of plants. We first evaluated spatial genetic structure (SGS) and genetic diversity among four populations of *Alsophila gigantea* from Hainan Island, China, using inter-simple sequence repeat (ISSR) markers. Significant but weak FSGS was found in *A. gigantea*. High genetic diversity was identified at the species level and the population level. AMOVA analysis revealed a low level of genetic differentiation among the four populations with high gene flow. Mantel test showed no significant correlation between genetic distance and geographic distance. It was found that association between annual mean temperature and annual precipitation with FSGS. Combined with these spatial genetic variation, abundant precipitation and suitable temperature create a stable environment for *A. gigantea* in Hainan Island, which allows the fern to expand rapidly during the LGM. These results further emphasized the role of outcrossing, and history and environmental factors in the evolution of *A. gigantea*. This study also provided new insights on in local adaptation of *A. gigantea* to environmental fluctuations, and available genetic data to enhance the conservation for relict tree ferns.

Keywords: *Alsophila gigantea*; environmental factors; genetic diversity; Hainan Island; ISSR; potential distribution; spatial genetic structure; tree ferns

Introduction

Spatial genetic variation includes spatial genetic structure (SGS) and genetic diversity, which are two important genetic features of plants. The former is referred to non-random distribution of individual genotypes in natural populations (Ramírez-Barahona and Eguiarte, 2015), while the latter is raw materials for against disease and adaptation to changing environment. The both genetic indices are affected by the similar factors including dispersal ability, mating system, life form, population density, human disturbance as well as historical events (Vekemans and Hardy, 2004; Chung and Chung, 2013; González-Díaz *et al.*, 2017). Among them, gene flow related to dispersal plays an important role (Troupin *et al.*, 2006). In addition, fine spatial genetic structure

Received: 20 Jun 2021. Received in revised form: 20 Aug 2021. Accepted: 13 Sep 2021. Published online: 23 Sep 2021.
From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

is also affected by environmental heterogeneity such as altitudinal, temperatures, and precipitation compared to genetic diversity (Troupin *et al.*, 2006; Torroba-Balmori *et al.*, 2017; Mosca *et al.*, 2018).

Gene flow is mainly from dispersal of pollen, seed, spore and propagule (Wang and Bradburd, 2014). For ferns, gene flow is closely related to a large number of tiny (0.029-0.031 mm) and wind-borne spores and unique mode of reproduction involving in independent haploid and diploid phase (Wang *et al.*, 2012; Bucharová and Münzbergová, 2012). Long distance spore dispersal can homogenize the differentiation among populations, reduce spatial genetic differentiation, and increase genetic diversity, which are further deepened in outcrossing rather than inbreeding ferns (Pryor *et al.*, 2001; Jiménez *et al.*, 2010; Wang *et al.*, 2012; Sessa *et al.*, 2016). However, it is unclear whether this hypothesis is acceptable for ferns in Hainan Island with several main mountains similar to "island within island", which greatly affects gene flow in the spatial scale (Saro *et al.*, 2019).

Hainan Island is the second largest island in China with abound ferns, which is isolated from mainland by Qiongzhou Strait with connection–disconnection events. It provides a very suitable island framework to address issues about pattern of spatial genetic structure (SGS) and genetic diversity. The island comprises diverse famous mountains far apart such as Jianfengling, Bawangling, Diaoluoshan, and Wuzhishan, which look like "independent islands" with spatio-temporal isolation within Hainan Island (Saro *et al.*, 2019). In general, island populations have low genetic variation due to colonization events, founder effects, finite population size, and adaptations to island environments (Frankham, 1997; Su *et al.*, 2010). The isolated spatial distribution of island populations further affects proportion of genetic variation among the populations (Nielsen, 2004). Due to high gene flow and complicated breeding systems, genetic features of ferns are generally contrary to these conclusions (Soltis *et al.*, 1991; Sessa *et al.*, 2016), with limited researches in *Pteris multifida*, *Dryopteris aemula*, *Cyrtomium falcatum*, and *Alsophila firma* (Murakami *et al.*, 1997; Jiménez *et al.*, 2010; Chung *et al.*, 2013; Ramírez-Barahona and Eguiarte, 2015). Ferns are key component of Hainan Island flora and play important roles in ecosystem (Dong, 2009). Especially, tree ferns represent a significant characteristic for rain forest of tropical and subtropical climate (Paul *et al.*, 2015). However, their survival crisis is attributed to the lack of effective gene flow between the fragmented small populations (Wang *et al.*, 2003). Hence, it is necessary to further investigate SGS and genetic diversity of tree ferns driven by gene flow in Hainan Island.

Suitable habitat is crucial for the survival and growth of ferns, especially in conservation biology of endangered ferns (Li *et al.*, 2019; Manel *et al.*, 2001). Climate has directly or indirectly great significance on distribution including suitable habitat, identification of which contribute to mitigate negative effects of climate fluctuation in global climate change. The heterogeneity of precipitation and temperature exerts great pressure to growth and reproduction of ferns, especially in island regions. The impact consequences and determinative factors can be determined by ecological niche modelling, which has been widely used in accurate distribution predictions of species (Zhang *et al.*, 2021). Combined with spatial genetic variation and biological characteristics of species, we can obtain distinct understanding to distribution of ferns in past, present, and future.

Alsophila gigantea is an important relict tree fern of family Cyatheaceae. As a giant fern, its erect stem is tall up to 2-5 meter with huge foliage. Fronds are bi or tripinnate with 2-3 m length. The stipe has dark brown and glossy scales (Wang *et al.*, 2019). The fern prefers to grow in moist open areas in dense forests at an altitude up to 1200 m in China including Hainan Island and other southeast Asian countries (Zhang and Nishida, 2013). As an ornamental and medicinal fern, it is regarded as a vulnerable species (Fu, 1992) and included in Appendix II of CITES (2017) due to illegal collection, human disturbance, and severe deforestation (Au, 2004; Ibars and Estrelles, 2012). Haploid spores are the main breeding way of *A. gigantea* with strict temperature requirement in storage, germination, development, and fertilization (Conant, 1990; Soltis *et al.*, 1990; 1991; Li *et al.*, 2010; Cao *et al.*, 2007; Du, 2009), which is a huge challenge for its conservation. In Hainan Island, *A. gigantea* mainly distributes in Jianfengling, Bawangling, Diaoluoshan, and Wuzhishan as isolated "island populations". It is suitable to investigate SGS mode and genetic variation, and related effect factors.

In this study, we adopted ISSR molecular markers and simultaneously combined multivariate statistics and spatial genetic autocorrelation analysis to survey SGS and genetic diversity of *A. gigantea* (Kalisz *et al.*, 2001; Vekemans and Hardy, 2004). Our goals are to present pattern of SGS and genetic diversity for *A. gigantea* in Hainan Island, and explore which environmental factors have exerted important effects on its SGS and genetic diversity.

Materials and Methods

Study region and species

We sampled 223 individuals from four populations of *A. gigantea* in Hainan Island (Figure 1), which were located in Diaoluoshan (DL, N=33; 425-470 m elevation), Wuzhishan (WZ, N=33; 705-785 m elevation), Jianfengling (JF, N=28; 806-926 m elevation) and Bawangling (BW, N=23; 919-1024 m elevation), respectively. Fresh and young leaves were collected and preserved in Ziplock plastic bags with silica gel immediately until extraction.

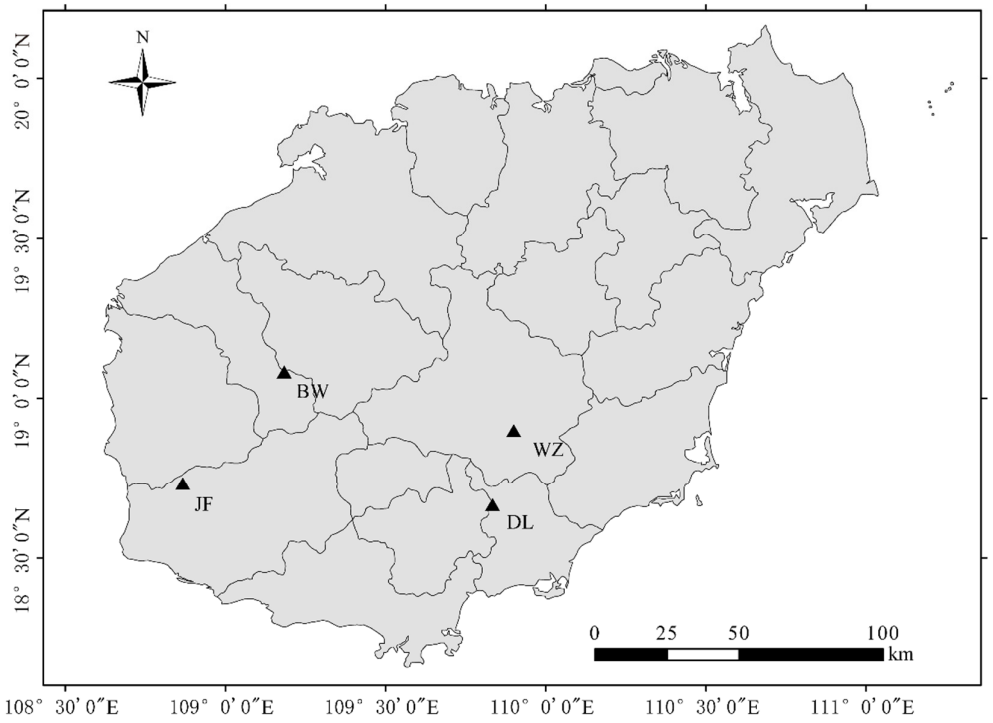

Figure 1. Geographic distribution of the four population of *A. gigantea* in Hainan, China
DL: Diaoluoshan; WZ: Wuzhishan; JF: Jianfengling; BW: Bawangling.

DNA extraction and ISSR amplification

A modified cetyltrimethyl ammonium bromide (CTAB) method was used to isolates genomic DNA, which was stored at -20°C until subsequent use (Su *et al.*, 1998). We screened out 23 primers from UBC ISSR Primers 801-900 (the Biotechnology Laboratory, University of British Columbia) (Table 1). PCR amplification reaction was performed in 20 μL volume, including 2.0 μL 10× buffer, 1.5 μL 20mM of $MgCl_2$, 0.4 μL 10mM of dNTP, 0.3 μL 0.011 mM of each primer, 1U of Taq polymerase and 50 ng DNA. Amplification procedures were as follows: an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 40s at 94 °C, 35s at 53 °C and 70s at 72 °C before final elongation at 72 °C for 5 min. The amplified products

were electrophored using 1.8% agarose gel with 100 bp DNA ladder, which was stained with Ethidium Bromide and visualized using 254 nm ultraviolet lamp.

Table 1. Primer sequences used in the study

Primer number	Primer sequences 5'- 3'	Primer number	Primer sequences 5'- 3'
UBC814	CTC TCT CTC TCT CTC TA	UBC815	CTC TCT CTC TCT CTC TG
UBC818	CAC ACA CAC ACA CAC AG	UBC825	ACA CAC ACA CAC ACA CT
UBC820	GTG TGT GTG TGT GTG TC	UBC827	ACA CAC ACA CAC ACA CG
UBC824	TCT CTC TCT CTC TCT CG	UBC841	GAGAGAGAGAGA GAG AYC
UBC844	CTC TCT CTC TCT CTC TRC	UBC845	CTC TCT CTC TCT CTC TRG
UBC846	CAC ACA CAC ACA CAC ART	UBC849	GTG TGT GTG TGT GTG TYA
UBC850	GTG TGT GTG TGT GTG TYC	UBC851	GTG TGT GTG TGT GTG TYG
UBC852	TCT CTC TCT CTC TCT CRA	UBC857	ACACACACA CAC ACA CYG
UBC854	TCT CTC TCT CTC TCT CRG	UBC859	TGT GTG TGT GTG TGT GRC
UBC860*	TGT GTG TGT GTG TGT GRA	UBC873*	GAC AGA CAG ACA GAC A
UBC864*	ATG ATG ATG ATG ATG ATG	UBC874*	CCC TCC CTC CCT CCC T
UBC880*	GGA GAG GAG AGG AGA		

R = (A, G), Y = (C, T), D = (A, G, T) (i.e. not C), H = (A, C, T) (i.e. not G), V = (A, C, G) (i.e. not T)

Data analyses

We adopted GENALEX v.6.41 software (Peakall and Smouse, 2006) to estimate genetic parameters, including the actual number of alleles (Na), the effective number of alleles (Ne), Shannon's information index (I), expected heterozygosity (He), unbiased expected heterozygosity (UHe) and percentage of polymorphic loci (PPL). Gene flow was calculated based on Nei's genetic identity (Inei) and genetic distance (D) in pairwise population using POPGENE v.1.31 (Yeh *et al.*, 1999). Isolation by distance (IBD) was further assessed using Mantel test (Miller, 1997) implemented in Arlequin v.3.5 (Excoffier and Lischer, 2010).

To investigate the population genetic structure, an individual-based Bayesian cluster analysis was conducted using STRUCTURE v.2.2 (Evanno *et al.*, 2005). We set the number of clusters as K= 1-7, and adopted other following parameters: admixture model, correlated allele frequencies, burn-in period of 10 000, Markov chain Monte Carlo (MCMC) repetitions of 100 000, and 10 iterations of K values. The optimal K number was determined by the maximum value of ΔK statistic based on LnP(D), which was performed in STRUCTURE HARVESTER (http://taylor0.biology.ucla.edu/structureHarvester/) (Earl and vonHoldt, 2012). For each optimal K model, the probabilities that each individual was assigned to the most likely clusters were averaged over replicate chains in CLUMPP Version 1.1.2 (Jakobsson and Rosenberg, 2007), and finally visualized in DISTRUCT (Rosenberg, 2010).

Based on the optimal K number, we used Arlequin v.3.5 to further evaluate genetic differentiation and variation among populations through analysis of molecular variance (AMOVA) with 10 000 permutations (Excoffier and Lischer, 2010). In addition, genetic differentiation was also estimated based on θII, a statistical value analogous to Fst, using HICKORY v.1.1 (Holsinger *et al.*, 2002). An optimizing model was determined by the smaller DIC from four models, which was obtained with the default parameters (burn in = 5000, sample = 100 000, thin = 20). If the difference of DIC among four models was less 5 or 6 units, the model with smaller value of the sum of Dbar (model fit) and pD (model complexity) had priority of adoption. HICKORY was run three times for each analysis to ensure consistency.

Based on squared allele frequency correlation coefficient (r^2) and statistical tests (p), linkage disequilibrium (LD) was assessed using TASSEL v.3.0.169 (Bradbury *et al.*, 2007), which was finally identified under the value of r^2 less than 0.1.

Spatial genetic structure analysis

Based on Shannon's information index, spatial autocorrelation was evaluated using SAM v.4.0 software (Rangel *et al.*, 2010). According to the maximum geographical distance among four populations, Moran's I statistics were calculated at four distance classes with intervals 1-30 km, 30-60 km, 60-90 km, and 90-120 km. The range of Moran's I is from -1 to 1, which indicates the complete discrete and correlation, respectively. Among them, 0 indicates the random distribution. Finally, 9999 random permutations were performed to obtain confidence interval, 95% of which determined significance of Moran's I with p-value.

Fine spatial genetic structure (FSGS) was further assessed using SPAGeDi v.1.3 (Hardy and Vekemans, 2002). To acquire more accurate results, we performed FSGS analysis among four population and four group, of which the latter was random combination of three of four populations (group1: DL+WZ+JF; group2: WZ+BW+JF; group3: DL+BW+JF; group4: DL+WZ+BW). Within six distance intervals, kinship coefficients between every pair of individuals i and j were estimated (Fij, Loiselle *et al.*, 1995). Based on the requirements of software for distance interval: % partic > 50%, and CV partic <= 1, six distance intervals with 200 m were set within the maximum distance of 1200 m among four populations. Similarly, for group1 and group 2, we set six distance intervals with 150 m and 170 m within the maximum distance of 900 m and 1020 m for group 4 and group 3, respectively. The kinship coefficients were further regressed on geographic distance between individuals and its natural logarithm (dij and ln(dij) to exhibit bd and bLd slopes, respectively. To obtain 95% confidence intervals, 10 000 permutations were performed. The statistic Sp was calculated to provide strength of FSGS using the formula Sp= -bLd /(1−F1), where F1 is the mean Fij of the first distance interval. The significance of FSGS was statistically determined by p-value and 95% confidence intervals.

Effects of environmental factors on genetic diversity and FSGS

In this study, environmental factors included three geographical variables (longitude, latitude, and altitude) and 19 climatic variables. The 19 factors were downloaded from WorldClim version 2 (*www.worldclim.org*) for the years 1950-2000 with a 2.5 minutes spatial resolution. We used ArcGis v.10.2 to extract climatic variables of four *A. gigantea* populations. Based on Pearson correlation coefficient, SPSS v.18.0.0 was used to evaluate correlation of 21 environmental factors and six genetic parameters (Na, Ne, I, He, UHe, and PPL).

We selected annual mean temperature (bio1) and annual precipitation (bio12) as target variables to analyze the effects of climatic factors on FSGS in four groups of *A. gigantea*. Linear regression model was used to investigate relationship between temperature/precipitation variables with Sp value of FSGS. The two climatic data were first converted into a standardized model using "vegan" package in R. Step by step regression by backward selection method was performed in R using the step () function in "mass" package (Venables and Ripley, 2002). The optimal model was determined based on the lowest Akaike Information Criterion (AIC) value.

Prediction of the potential distribution

Geographical distribution information of *A. gigantea* was collected from Global Biodiversity Information Facility (GBIF, https://www.gbif.org/) and China Virtue Herbarium (CVH, *http://www.cvh.ac.cn/*). All climatic data were obtained from WorldClim (*www.worldclim.org*). Except current and future climatic data, we also collected paleoclimatic data, including the Holocene (HOL), the Last Interglacial (LIG; 130-115 ka BP), and the Last Glacial Maximum (LGM, 25-17 ka BP). Two different global climate models were adopted: CCSM (Community Climate System Model) (Collins *et al.*, 2006) and MIROC (Model for Interdisciplinary Research on Climate) (Hasumi and Emori, 2004). MaxEnt v. 3.3.3k was used to predict the potential distribution (Phillips and Dudík, 2008) with ten crossvalidate replicated runs. Jackknife analyses were performed using default parameters (0.00001 convergence threshold, and 500 maximum iterations). Model performance was evaluated using the area under the Receiving Operator Curve (AUC), implying excellent if greater than 0.9.

Based on classification of habitat suitability (Qin *et al.*, 2017) and the IPCC Fifth Assessment Report (Mastrandrea *et al.*, 2010), four classes of potential habitats were estimated. Optimum growing area of *A. gigantea* was determined as suitable habitat (0.5-0.8) and highly suitable habitat (0.8-1).

Outlier detection

DFDIST and BAYESCAN v.2.0 were used to identify candidate loci under natural selection (Antao and Beaumont, 2011; Foll and Gaggiotti, 2008). In DFDIST, we detected outliers through comparing observed distribution with neutral expectations at a 99.5% confidence interval (CI) with 50 000 simulations. In BAYESCAN, a reversible-jump Markov chain Monte Carlo algorithm based on a Bayesian likelihood approach was used to estimate the ratio of posterior probabilities of selection over neutrality [the posterior odds (PO)]. Its parameters included 10 pilot runs of 5000 iterations followed by a burn-in of 50 000 iterations, and a sample size of 5000 with a thinning interval of 20. Only loci with log (PO) > 0.5 (Bayesian factor BF > 10) was considered to have substantial evidence for selection.

To ensure the accuracy of the results (Pérez-Figueroa *et al.*, 2010), we further used Samβada (Stucki *et al.*, 2017) to detect the correlation between outliers and 22 environment variables, including three geographical variables (longitude, latitude, and altitude) and 19 environmental variables (19 bioclimatic variables from WorldClim). Based on Wald and G, significant was acquired through comparing between models with and without environmental variables with an FDR cutoff of 0.01. In addition, linear regression with one variable model in SAM was also used to analyze association of outliers with geographical and environmental variables.

Results

Population genetic analyses

We detected 136 loci in four populations of *A. gigantea*, 116 of which were polymorphic. The percentage of polymorphic loci was 85.29% with variation from 73.53% in DL to 80.15% in JF (mean PPL=76.84%; Table 2). The effective number of alleles, Shannon's information index and expected heterozygosity were 1.627, 0.486 and 0.340, respectively (Table 2).

Two genetic groups were identified by Bayesian clustering analysis implemented in STRUCTURE. As shown in Figure 2, the best K was determined as 2 based on the highest ΔK value (33.95). WZ, JF and BW populations were clustered into one group, while DL population formed another.

Table 2. Locality information and population genetic parameters of the four population of *A. gigantea*

ID	Locality	Longitude	Latitude	Altitude (m)	N	Na	Ne	I	He	UHe	PPL
DL	Diaoluoshan, Hainan	E109°50'	N18°40'	425-470	33	1.735	1.615	0.469	0.329	0.334	73.53%
WZ	Wuzhishan, Hainan	E109°54'	N18°54'	705-785	33	1.779	1.621	0.488	0.340	0.345	77.94%
JF	Jianfengling, Hainan	E108°52'	N18°44'	806-926	28	1.801	1.662	0.511	0.358	0.364	80.15%
BW	Bawangling, Hainan	E109°11'	N19°05'	919-1024	23	1.757	1.611	0.476	0.332	0.340	75.74%
Total average					/	1.768	1.627	0.486	0.340	0.346	76.84%
Species level					117	1.853	1.718	0.548	0.385	0.387	85.29%

N: the sample size for each population; Na: the actual number of alleles; Ne: the effective number of alleles; I: the Shannon's Information Index; He: the expected heterozygosity; UHe: the unbiased expected heterozygosity; PPL: the percentage of polymorphic loci.

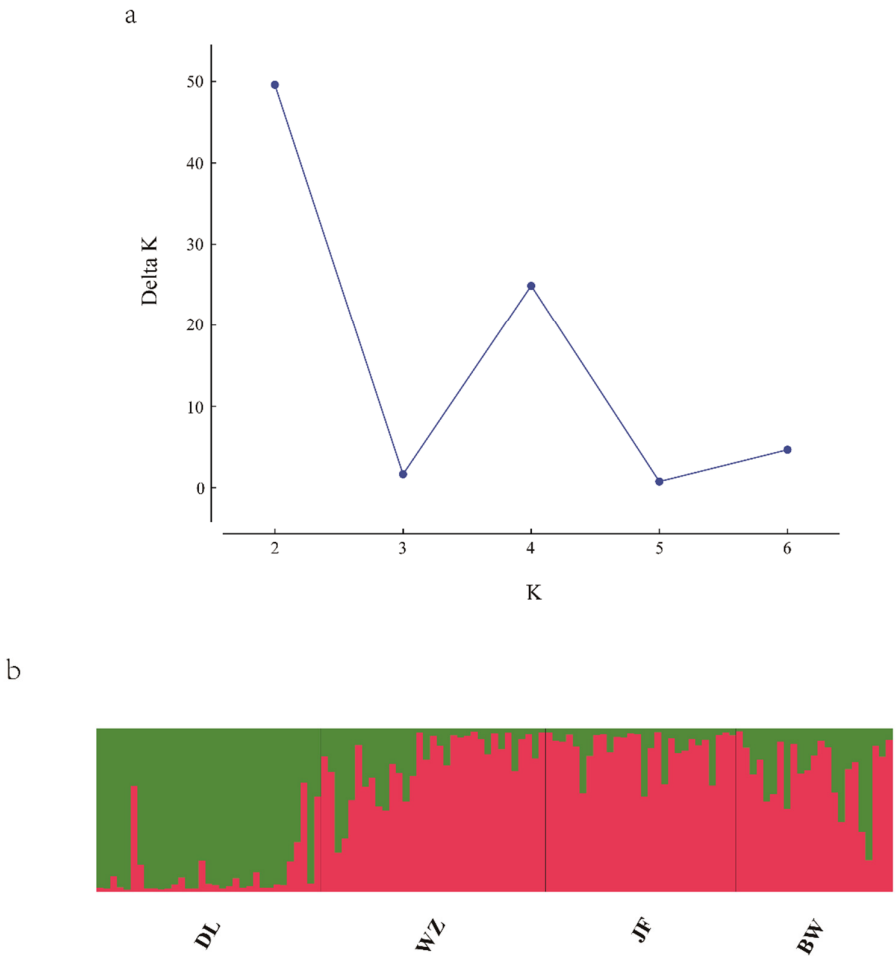

Figure 2. Genetic structure was detected using STRUCTURE based on Bayesian clustering
(a) Estimates of the posterior probability of the data for a given K. (b) Individual assignments for clustering scenarios of K = 2.

AMOVA analysis showed that the two STRUCTURE-based groups had a low genetic differentiation (Fst = 0.0801, P < 0.005; Table 3). Genetic variations mainly occurred within populations (91.99%, Table 3). Similar to AMOVA, HICKORY θII also indicated low genetic differentiation. Full model was chosen due to the smallest DIC value (θII = 0.0899175; CI: 0.0733515-0.108223). Gene flow among populations was estimated as 4.886. Nei's genetic identity ranged from 0.9210 to 0.9354, with an average of 0.9298 (Table 4). JF and BW populations had the largest Nei's genetic identity, whereas the smallest was between DL and JF. Mantel test showed that there was no significant correlation between genetic and geographical distance (r=0.454, p=0.17).

By using TASSEL, 136 linked-loci produced 11 of 9180 comparison pairs (0.12%). And linkage disequilibrium (LD) was detected with 22 of 136 loci (16.18%; r2 > 0.1, p<0.05; Table 5).

Table 3. Percentage of variation in the four population of *A. gigantea* based on AMOVA analysis

Source of variation	d.f.	Variance components	Percentage of variation	F statistics	p
Among groups	1	0.23958	1.02	Fct=0.01018	0.17129
Among populations within groups	2	1.64549	6.99	Fsc=0.07063	0.00000
Within populations	113	21.65230	91.99	Fst=0.08009	0.00000

Table 4. Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Pop	DL	WZ	JF	BW
DL	****	0.9324	0.9210	0.9229
WZ	0.0700	****	0.9323	0.9349
JF	0.0823	0.0701	****	0.9354
BW	0.0802	0.0673	0.0667	****

Table 5. Linkage disequilibrium analysis among 136 ISSR loci

Locus	r2	D'	p
56 and 19	0.104642	0.429268	0.00052394
61 and 29	0.126407	0.610433	0.000176258
72 and 60	0.129396	0.366359	0.000149455
74 and 48	0.101802	0.413194	0.000679577
77 and 76	0.250007	0.526611	0.000000087
88 and 67	0.102451	0.329688	0.001792001
100 and 27	0.108750	0.394679	0.000448761
108 and 107	0.111852	0.460829	0.001380078
111 and 101	0.117718	0.35	0.000439826
112 and 69	0.126999	0.385093	0.000376822
122 and 23	0.101011	0.468182	0.000813120

Spatial genetic autocorrelation analysis

No significant spatial autocorrelation was found at large scale among the four populations due to the lack of significance in mean Moran's I (Figure 3). By contrast, a significant positive Fij value was detected in the smallest distance interval (d≤0.2 km). Fij values were negative in other distance intervals with no significant correlation. The Sp statistics for *A. gigantea* was 0.0014 (Table 6; Figure 4).

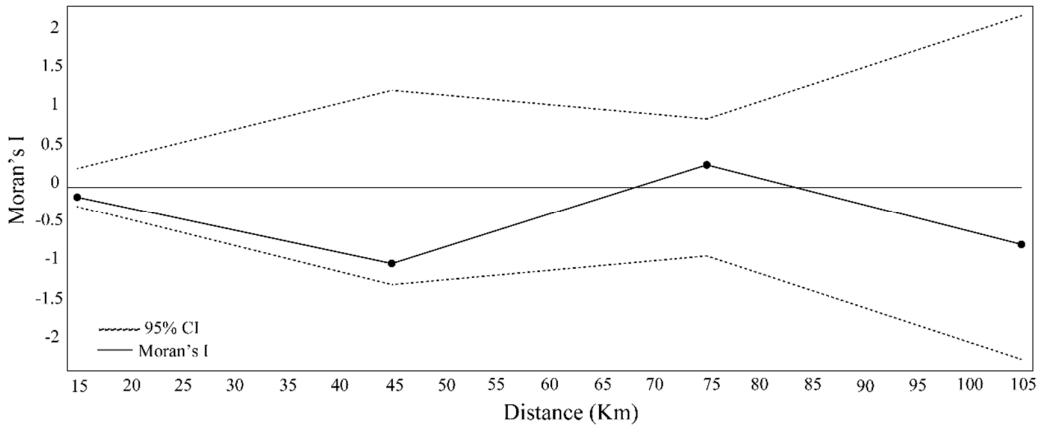

Figure 3. Spatial autocorrelation analysis in large-scale among four population of *A. gigantea* using SAM

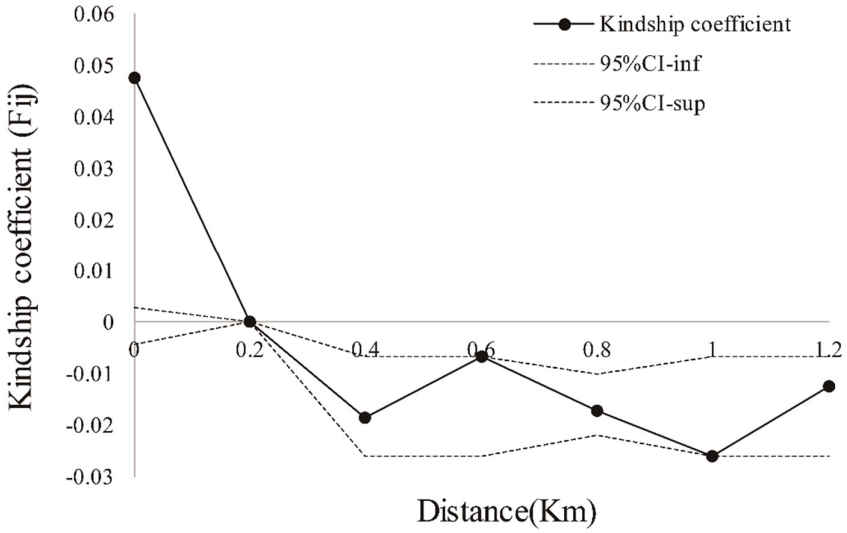

Figure 4. Fine-scale spatial autocorrelation analysis among four population of *A. gigantea* implemented in SPAGeDi

Table 6. Fine-scale spatial genetic structure (FSGS) among different population combinations

Group	Fis	F1	p	b-log	p	Sp	Sp-avg	Avg_T	Avg_P
Group1: DL+WZ+JF	0.95	0.0524	0	0.0019	0.8179	-0.0020	-0.0017	23.16	1549.67
	0	0.0339	0	0.0013	0.8168	-0.0013			
Group2: WZ+BW+JF	0.95	0.0461	0	-0.0098	0.4922	0.0103	0.0085	21.85	1565.67
	0	0.0292	0	-0.0066	0.4979	0.0068			
Group3: DL+BW+JF	0.95	0.0551	0	-0.0327	0.1696	0.0346	0.0288	21.94	1542
	0	0.0355	0	-0.0222	0.1608	0.0231			
Group4: DL+WZ+BW	0.95	0.0521	0	0.0055	0.4985	-0.0058	-0.0048	22.82	1582.67
	0	0.0334	0	0.0037	0.5036	-0.0038			
DL+WZ+JF+BW	0.95	0.0577	0	-0.0016	0.8863	0.0017	0.0014	22.44	1560
	0	0.0378	0	-0.0011	0.8796	0.0011			

b-log: slop of the regression of kinship with the logarithm of the distance; F1: mean Fij of the first distance interval; Sp-avg: average value of sp when fis is 0 and 0. 95; Avg_T: annual mean temperature of different population combinations; Avg_P: average annual precipitation of different population combinations

Effects of environmental factors on genetic diversity and FSGS

Based on Pearson correlation analysis, four genetic parameters (Ne, I, He, and UHe) exhibited a significant negative correlation with three environmental variables (bio2, bio3, and bio18; Table 8). The best linear model correlated FSGS with annual precipitation (Avg_P) and with group one (DL+WZ+JF) excluded (Adj.R2=0.9987, p=0.0161; Table 6; Table 7: Model D). Of note, Avg_P exerted a significant negative effect on FSGS. However, when both Avg_T and Avg_P or only Avg_T was considered, only a nonsignificant negative effect was detected.

Table 7. Best linear model correlating climatic factors and FSGS, as identified by AIC, after stepwise selection

Model	Variable estimates		Adj.R2	p
	Avg_T	Avg_P		
A	-0.0201	-0.0190	0.6434	0.3448
B	-0.0234	/	0.3754	0.2361
C	/	-0.0234	0.2013	0.3162
D	/	-0.0337*	0.9987	0.0161

A: complete model based on both variables; B: model without Avg_P; C: model without Avg_T; D: model without Avg_T and with outlier group1 (DL+WZ+JF) removed

Table 8. Correlation between genetic parameters and environmental variables based on Pearson correlation analysis

Genetic parameters	Environmental variables
Ne	Bio2 (r=-0.987; p=0.013)
I	Bio18 (r=-0.984; p=0.016)
He	Bio18 (r=-0.981; p=0.019)
UHe	Bio3 (r=-0.963; p=0.037); Bio18 (r=-0.996; p=0.004)

Predicting the potential distribution of A. gigantea

The Maxent model for *A. gigantea* performed well with AUC 0.962. Precipitation of driest month (bio14, 23.4%) contributed most to the model, followed by mean temperature of coldest quarter (bio11, 22.4%), precipitation of driest quarter (bio17, 16.4%), temperature annual range (bio5-bio6) (bio7, 9.7%) in temperature of coldest month (bio6, 8.2%) and isothermality (bio2/bio7) (* 100) (bio3, 7.4%). The cumulative contribution of these six factors was 87.5% (Table 9).

Table 9. Environmental variables used in the study and their percentage contribution

Code	Environmental variables	% Contribution
bio14	Precipitation of Driest Month	23.4
bio11	Mean Temperature of Coldest Quarter	22.4
bio17	Precipitation of Driest Quarter	16.4
bio7	Temperature Annual Range (BIO5-BIO6)	9.7
bio6	Min Temperature of Coldest Month	8.2
bio3	Isothermality (BIO2/BIO7) (* 100)	7.4
bio1	Annual Mean Temperature	4.1
bio2	Mean Diurnal Range (Mean of monthly (max temp - min temp))	3
bio15	Precipitation Seasonality (Coefficient of Variation)	1.8
bio4	Temperature Seasonality (standard deviation *100)	1.2
bio12	Annual Precipitation	0.6
bio13	Precipitation of Wettest Month	0.4
bio10	Mean Temperature of Warmest Quarter	0.4
bio18	Precipitation of Warmest Quarter	0.4
bio8	Mean Temperature of Wettest Quarter	0.2
bio9	Mean Temperature of Driest Quarter	0.1
bio16	Precipitation of Wettest Quarter	0.1
bio19	Precipitation of Coldest Quarter	0.1
bio5	Max Temperature of Warmest Month	0

We predicted the future, current, and historical distribution for *A. gigantea* (Figure 5). The current distribution prediction was basically consistent with the actual distribution, including southern Yunnan, southwestern Guangxi, southern Guangdong, Hainan, and Taiwan. Future habitats were predicted to concentrate in central Guangxi and a small region of western Guangdong and Hainan, with a tendency to expand into Tibet.

Based on MIROC model, the distribution of *A. gigantea* during HOL (69.51%) and LGM (96.08%) was similar to current distribution except slight contraction and more stable, respectively, whereas distribution in LGM was severely limited to a few parts of southeastern Yunnan and Tibet based on CCSM model (2.83%). In LIG (11.08%), suitable habitats of *A. gigantea* were found concentrated in Hainan. In comparison to LIG, models CCSM and MIROC produced very different distributions for LGM. The former predicted a significant narrow distribution (25.53%), whereas the latter projected a much wider distribution (86.75%) (Table 10). Overall, the suitable region of *A. gigantea* showed a trend of expansion from Paleoclimate to the present, but a trend of slight contraction in the future.

Table 10. Area of distribution range of *A. gigantea* under different climate scenarios

Class	Area (km^2)					
	Future	Current	HOL	CCSM	MIROC	LIG
0-0.2	8,260,608	8,194,304	8,334,208	8,749,280	8,273,856	8,788,848
0.2-0.5	445,696	456,000	380,800	109,056	386,576	40,352
0.5-0.8	134,912	207,440	141,712	6,000	197,056	22,928
0.8-1	11,200	4,784	5,808	0	6,848	576
0.5-1	146,112	212,224	147,520	6,000	203,904	23,504
*AOC	68.85%	/	69.51%	2.83%	96.08%	11.08%
*AOL	621.6%	902.9%	627.6%	25.53%	867.5%	/

*AOC: area of 0.5-1 class distribution range of each climate scenarios/ area of 0.5-1 class distribution range of current;
*AOL: area of 0.5-1 class distribution range of each climate scenarios/ area of 0.5-1 class distribution range of LIG

Loci under selection

DFDIST and BAYESCAN detected two (loci 18 and 62, Figure 6a) and one (loci 89, Figure 6b) outliers, respectively. However, no outlier was identified using Sambada. Linear regression with one variable model in SAM showed that loci 18 and 62 were positively and negatively correlated with six environmental variables (latitude, bio2, bio7, bio12, bio13, and bio16), respectively (Table 11). Loci 89 was positively correlated with nine environmental variables (longitude, bio1, bio3, bio5, bio6, bio8, bio9, bio10, and bio11) and negatively correlated with three environmental variables (altitude, bio4, and bio15) (Table 11).

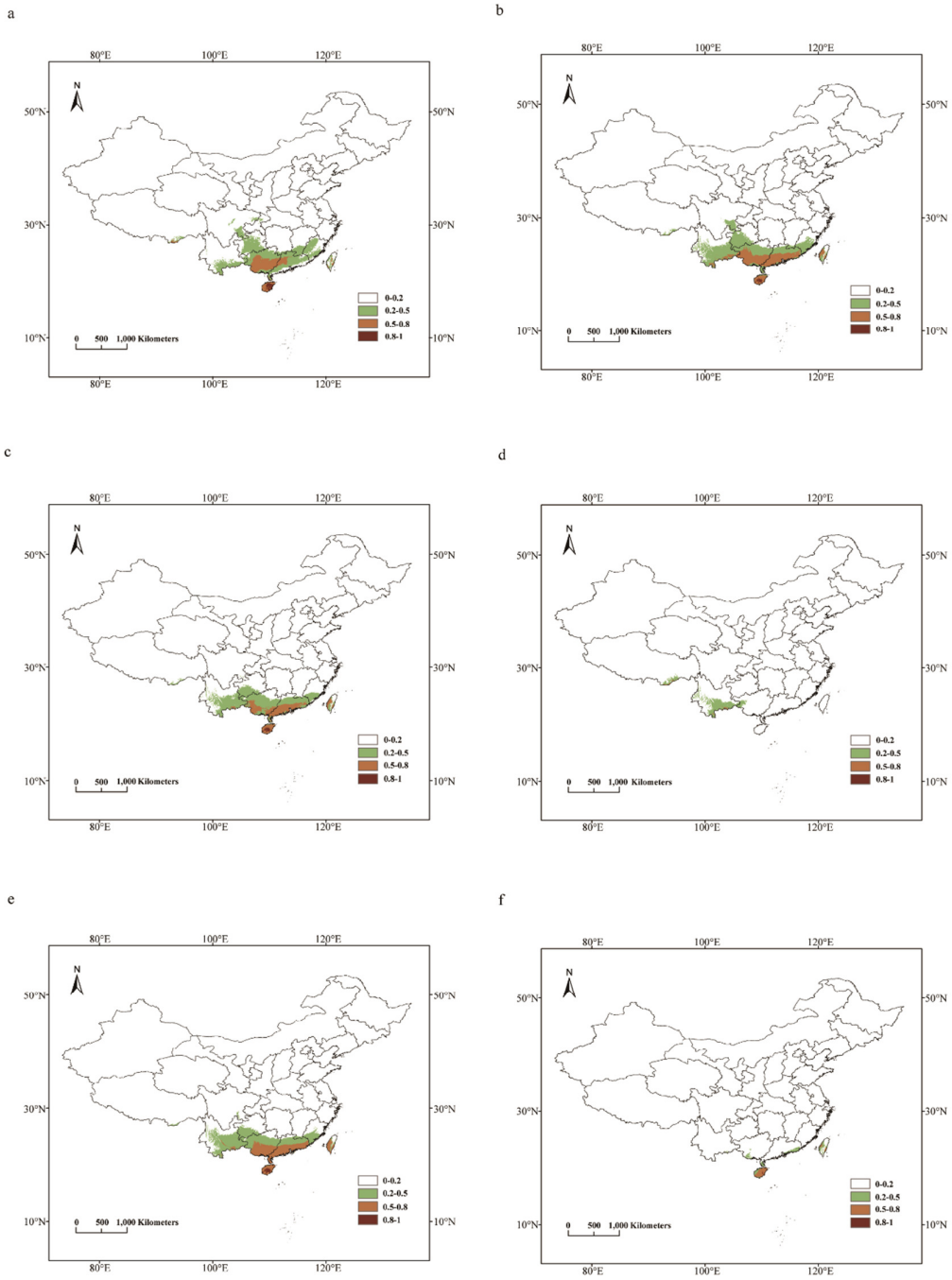

Figure 5. Predicted future, current and historical period habitat for *A. gigantea*
(a) Future suitable habitats (2070, average of predictions for 2061-2080); (b) Current suitable habitats; (c) Holocene (HOL); (d) Paleoclimate predictions from CCSM4 climatic model for the last glacial maximum (LGM, 25-17 ka BP); (e) Paleoclimate predictions from MIROC climatic model for the last glacial maximum (LGM); (f) Paleoclimate (LIG, the last interglacial period, 130-115 ka BP) predictions.

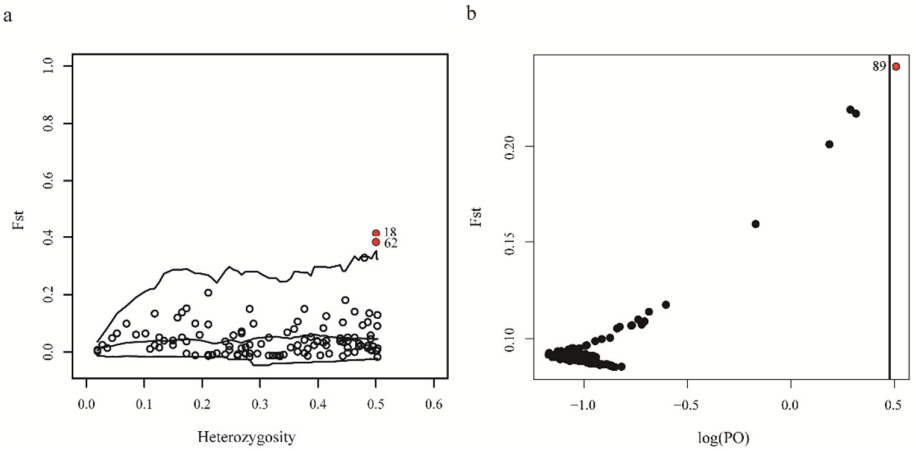

Figure 6. Outlier loci identified by DFDIST and BAYESCAN. (a) Outlier detection performed with DFDIST

The 0.5%, 50%, and 99.5% represented confidence intervals, respectively. Solid red dots above the 99.5% line were identified as outlier loci. (b) Plot of Fst values and log PO for 136 loci identified using BAYESCAN. Lines with log PO = 0.5 indicate "Substantial" evidence for selection corresponding to a posterior probability of 0.99. Solid red dots greater than log PO 0.5 represented outlier loci.

Table 11. Summary of outliers under selection identified based on genome scan methods (DFDIST and BAYESCAN), and their association with environmental variables based on the linear regression with one variable model

Method	Loci	Variable	Slope	r2
DFDIST	18	Latitude	1.774	0.789
		bio2	0.483	0.538
		bio7	0.431	0.82
		bio12	0.006	0.877
		bio13	0.101	0.94
		bio16	0.028	0.949
DFDIST	62	Latitude	-1.595	0.677
		bio2	-0.585	0.838
		bio7	-0.455	0.972
		bio12	-0.006	0.897
		bio13	-0.093	0.841
		bio16	-0.027	0.922
BAYESCAN	89	longitude	0.722	0.972
		altitude	-0.001	0.642
		bio1	0.18	0.898
		bio3	0.156	0.657
		bio4	-0.021	0.57
		bio5	0.185	0.978
		bio6	0.15	0.75
		bio8	0.245	0.983
		bio9	0.132	0.91
		bio10	0.19	0.912
		bio11	0.163	0.875
		bio15	-0.16	0.74

Discussion

In this study, we first estimated and reported in genetic diversity of four *A. gigantea* populations in Hainan Island. The result found that *A. gigantea* had high genetic diversity based on Shannon's information index (I=0.548), expected heterozygosity (He=0.385) and percentage of polymorphic loci (PPL=85.29%). Especially, expected heterozygosity and percentage of polymorphic loci were much higher than homosporous ferns (mean species-level estimates; PPL=36.1%, He =0.132; Li and Haufler, 1999). This result was similar to SSR analysis in *A. gigantea* (Ruan *et al.*, 2017). In addition, high genetic identity was found in pairwise populations of *A. gigantea*. The same phenomenon also occurs in four tropical Adiantum species based on ISSR marker (Korpelainen *et al.*, 2005).

Generally, island populations have lower genetic variation compared to their mainland counterparts due to founder effect (Frankham, 1997; Su *et al.*, 2010). Contrary to expectations, *A. gigantea* exhibited high genetic diversity in Hainan Island. On the one hand, we need to further survey and compare genetic variation of *A. gigantea* in mainland populations. On the other hand, *A. gigantea* still possibly maintains the previous high genetic variation as a long-lived relict tree fern. In addition, Neogene multiple connections between Hainan Island and Chinese mainland during the late Pleistocene–early Holocene period (Hope, 2005; Yan, 2006) have transformed more genetic variation from mainland population into island populations (Kolbe *et al.*, 2004).

The plant mating system is a key factor influencing the genetic diversity (Huang *et al.*, 2019). Homosporous fern with only one type of spore to form a bisexual gametophyte possesses three modes of sexual reproduction: intragametophytic selfing, intergametophytic selfing, and intergametophytic crossing (Wang *et al.*, 2012). Tree ferns are generally supposed to be outcrossing like *A. firma*, *Cyathea stipularis* and *Lophosoria quadripinnata* (Soltis *et al.*, 1991). Cyatheaceae species are especially intergametophytic mating (Chen, 1995; Chiou *et al.*, 2000, 2003), which are further confirmed based on ISSR investigations on *A. spinulosa*, showing that sexual recombination is the predominant source of genetic variation (Wang *et al.*, 2012). Although there is currently no report on the breeding mode of *A. gigantea*, we infer that it is outcrossing as a member of Cyatheaceae, which can promote population genetic variation. Except for sexual reproduction, some ferns also have vegetative propagation (Mcveigh, 1937; White, 1969; Johns and Edwards, 1991). As for *A. spinulosa*, most of natural populations are dominated by asexual reproduction, but there are some reproducing primarily by sexual reproduction (Wang *et al.*, 2012). Of note, somatic mutations may generate a considerable amount of genetic variation for clonal lineages (Wang *et al.*, 2012). Clonal growth is also indeed observed in other tree ferns such as *A. firma* (Mehltreter and García-Franco, 2008; Lehnert, 2011; Santiago and Luis, 2014). Hence, high genetic diversity of *A. gigantea* was possibly ascribed to sexual reproduction, and clonal growth with somatic mutations. Furthermore, the identified linkage disequilibrium can result in the fixation of different multilocus genotypes within populations to increase genetic variation (Jiménez *et al.*, 2010; Loveless and Hamrick, 1984). High genetic identity among four populations was possibly ascribed to the continuous presence of *A. gigantea* in Hainan islands in the past except high level of gene flow because island-like mountains originated from the upliftment of Hainan Island (Zhang and Liu, 1989) or the Quaternary transgression (Shi *et al.*, 2006).

The spatial genetic autocorrelation was also investigated in the four *A. gigantea* populations in Hainan Island. As expected, *A. gigantea* had a weak SGS in fine scale and lacked spatial autocorrelation in large scale among populations. The SGS was different among populations. SGS among DL, JF and BW (Spg3 = 0.0288) was stronger than that among WZ, JF and BW (Spg2= 0.0085) and among the four populations (Spall_pop =0.0014). This SGS result was contrary to the other tree fern like *A. firma* with strong SGS between two populations (Santiago and Luis, 2014). The reason might be attributed to factors other than life history and breeding system because all tree ferns have similar life form and outcrossing mode (Soltis *et al.*, 1991).

Compared to breeding system, spore dispersal ability has a deeper influence on the spatial genetic structure of fern population (Jiménez *et al.*, 2010; Pryor *et al.*, 2001). Ferns can produce a large amount of small and light spores (c. 0.02–0.13 mm; Knobloch, 1969; Makgomol, 2006), which plays an important role (Li *et al.*, 2010). The windborne spores usually have a high dispersal potential (500-800 km) and even up to 3,200 km (Tryon, 1970, 1972), which could span island-like habitat and complicated mountains system with deep and wide valley in Hainan Island leading to lack of spatial genetic structure. Although spores of *A. gigantea* lost viability quickly at room temperature (Li *et al.*, 2010), abundant rainfall in tropical habitats between May and October ensures their rapid germination and move requirement of spermatozoids for water, especially in June when spores mature (Peck *et al.*, 1990; Haufler *et al.*, 2000; Korpelainen *et al.*, 2005). Unique tropical humid climate in Hainan Island may promote the spread of fern spores and decreases population differentiation (Fst=0.0758) through monsoon and rainy season with storm and typhoon (Wang *et al.*, 2016). Lack of pattern of isolation by distance further justified the spatial genetic structure feature of *A. gigantea*. In this study, a negative correlation was detected between the annual precipitation and the strength of SGS quantified by the Sp value. Hence, the clonality, dispersal, and tropical climate may exert an overarching effect on SGS of *A. gigantea*. Compared to selfing plants, outcrossing plants generally tend to present a weaker SGS (Vekemans and Hardy, 2004). Moreover, the lack of genetic structure with high gene flow in *A. gigantea* populations implied that habitat was suitable for spore germination and gametophyte development and the effective mixing of different genotypes from spore dispersal (Jiménez *et al.*, 2010). The similar result was detected in *A. spinulosa* populations in Hainan Island (Su *et al.*, 2004). Of note, weaker SGS of *A. gigantea* occurred in the smallest distance interval (d≤0.2 km). This was possibly related to the formation of localized spore shadows around colonizing sporophytes (Chung and Chung, 2013) and some spores falling near the parent plants (Chung and Chung, 2013; Peck *et al.*, 1990; Dyer, 1994). Compared to co-dominant markers such as microsatellites, dominant makers may reveal FSGS with more accuracy by avoiding null alleles (Chung and Chung, 2013). It has been noted that AFLP-based Sp values are significantly higher than those from microsatellites (Gelmi-Candusso *et al.*, 2017; Jump *et al.*, 2012).

Natural selection can drive the formation of fine spatial genetic structure through local adaptation to environment (Lehnert *et al.*, 2019). In this study, we detected three outliers by DFDIST and BAYESCAN, which showed that *A. gigantea* had very strong adaptability to environment. Environmental factors, especially climatic conditions such as temperature and precipitation, have profound effect on the distribution of *A. gigantea*. Jacknife analysis indicated that temperature (47.7%) contributed most to *A. gigantea* distribution, followed by precipitation (39.8%). Annual precipitation and temperature also separately exerted on a significant negative effect on FSGS of *A. gigantea*. Hence, the two climatic factors might facilitate fine-scale genetic divergence between populations, where there were subtle differences in annual precipitation and temperature. Weak FSGS also showed that genotypes related to adaptation are limited to a small area, and not randomly distributed (Torres *et al.*, 2019).

Population density is also among the factors affecting spatial genetic structure (Zhang *et al.*, 2019). A reduced overlap of seed shadows can increase SGS in low density populations (Torroba-Balmori *et al.*, 2017; Vekemans and Hardy, 2004; Hamrick and Trapnell, 2011). Limited seed dispersal and the number and location of mature trees also affect the level and pattern of SGS (Hamrick and Trapnell, 2011). Generally, SGS in plants is the strongest at the least disturbed site with the lowest adult density (Gonzales *et al.*, 2010; Hamrick and Trapnell, 2011). In this study, four populations were from Hainan Island tropical mountain cloud forests (> 1200 m) including Wuzhishan, Bawangling, Jianfengling, and Diaoluoshan (Wang *et al.*, 2016), which was possibly the consequence of the island-like fragmentation of cloud forests from past connectivity (Ramírez-Barahona and Eguiarte, 2014). The similar high population density may make their spatial genetic structure similar, resulting in lack of spatial autocorrelation.

Potential distribution of *A. gigantea* was estimated using MAXENT with good AUC value. It was found to be minimal in the last interglacial period, expansive in the last glacial maximum (MIROC model), and further shrunken in the last interglacial period and in the future, respectively. Under two palaeoclimate models

(MIROC and CCSM), we obtained different predictions during the LGM. The same phenomenon was observed in another tree fern *A. firma* with a more stable distribution in the LGM period based on MIROC prediction rather than CCSM, which is probably closely associated with different precipitation level of the two models (Irez-Barahona and Eguiarte, 2014). It implies the importance of model selection and influence of model on prediction of past distributions of *A. gigantea*. During the LGM, climate and sea-level fluctuation during Quaternary glacial period imposed a huge impact on Hainan Island populations, although this area was not covered by ice sheets (Liu *et al.*, 2020). Hainan Island has a warmer and more humid climate from a stronger Asian summer monsoon (Liu *et al.*, 2020). Meanwhile, the level of precipitation became increasing with the formation of land-bridges between Hainan Island and mainland. As a result, stable habitats allowed *A. gigantea* to expand rapidly during the LGM (Liu *et al.*, 2013). In addition, the differences between the current and past distributions suggested that *A. gigantea* probably underwent distribution changes within a relatively short period of time (Ramírez-Barahona and Eguiarte, 2014).

The first x-intercept is considered as the genetic patch size when SGS is positive (Escudero *et al.*, 2003; Peakall *et al.*, 2003; Torres *et al.*, 2019), interpretation of which should be with caution because the sampling and the distance class also affect spatial autocorrelation (Vekemans and Hardy, 2004; Torres *et al.*, 2019). In this study, these limitations may not affect the analysis because our distance setting included all individuals and enabled to detect genetic structure.

Currently, ferns are facing serious threats due to habitat shrinkage caused by environmental changes, human disturbance, and overexploitation (Ibars and Estrelles, 2012). In this context, this study has provided valuable genetic information for *A. gigantea* conservation. With the development of tourism, human disturbance has been increasing in Hainan Island. The growth and propagation of *A. gigantea* suffer negative impacts (Dai and Zhou, 2000). Priority should be given for populations with high genetic diversity when protection measures are taken. In-situ conservation may also be considered in the natural reserve.

Conclusions

To our knowledge, it is the first to survey the spatial genetic structure and genetic diversity of *A. gigantea* populations in Hainan Island. All the four populations exhibit a high level of genetic diversity, a weak fine spatial genetic structure, and a lack of spatial autocorrelation at large scale. Factors such as long-distance spore dispersal, outcrossing system, and temperature and precipitation conditions are proposed to contribute to this phenomenon. Together with population genetic variation, the suitable precipitation and temperature may have created an adequate environment for *A. gigantea* in Hainan Island, allowing the fern to expand rapidly during the LGM. In addition, *A. gigantea* populations are found to maintain sufficient gene flows and a weak spatial genetic structure, which may be associated with its local adaptation to fluctuating environments. These findings are helpful to formulate the protection strategy of *A. gigantea* in Hainan Island.

Authors' Contributions

YS and TW proposed and designed this research. ZY and YS collected samples and conducted the experiments. XR, SL and ZW analyzed the data. YS, TW and SL wrote the manuscript, and YS, ZW and TW corrected the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by the National Natural Science Foundation of China [31670200, 31770587, 31872670, and 32071781], Guangdong Basic and Applied Basic Research Foundation [2021A1515010911], and Project of Department of Science and Technology of Shenzhen City, Guangdong, China [JCYJ20190813172001780].

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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