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Research Article

Genetic structure and phylogeography of a relict tree fern, *Sphaeropteris brunoniana* (Cyatheaceae) from China and Laos inferred from cpDNA sequence variations: Implications for conservation

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Abstract In this study, we analyzed the genetic structure and phylogeography of *Sphaeropteris brunoniana* from China and Laos. Combining cpDNA *trn*L-*trn*F and *atp*B-*rbc*L sequence variations, five haplotypes were identified from the 10 investigated populations. Moderate haplotype diversity ($h = 0.66580$) and low nucleotide diversity ($\pi = 0.23 \times 10^{-3}$) were detected. The *S. brunoniana* in Yunnan region had much higher genetic diversity ($h = 0.60195$, $\pi = 0.35 \times 10^{-3}$) than that of Hainan–Laos ($h = 0.00000$, $\pi = 0.00$). A high level of genetic differentiation (94.74%) between the two regions was revealed by AMOVA. Nested clade analysis identified two major clusters of the five haplotypes, one clade in the Yunnan region and the other in Hainan–Laos. The analysis indicated that restricted gene flow with isolation by distance and allopatric fragmentation were likely the major processes that shaped the spatial distribution of the haplotypes. The isolated distribution of clades implied the emergence of independent refugia of this species in each region during Quaternary glaciations. The Yunnan populations frequently contained an ancestral haplotype, and most of them harbored other descendent haplotypes. Based on the distribution pattern of haplotypes and the nested clade analysis results, the Yunnan region potentially had several refugia of this species during glacial periods, whereas the Hainan populations were probable new colonizations.

Key words *atp*B-*rbc*L, Hainan–Laos, phylogeography, *Sphaeropteris brunoniana*, *trn*L-*trn*F, Yunnan.

Population genetic structure of a species is not only strongly influenced by its life history and ecology characters, but also by geological historical events (Newton et al., 1999), especially climatic changes during the Quaternary period (Hewitt, 2000, 2004). Climatic oscillations during the last two million years of the Quaternary period probably played an important role in shaping the geographical distribution and diversity of many plants and animals (Hewitt, 1996). Phylogeography, the discipline that considers the historical events responsible for the present geographical distribution of a species, began approximately 20 years ago, and has proven effective in detecting historical events affecting species, such as fragmentation, restriction of gene flow by distance, long distance dispersal, and range expansion (Avise, 2000). Compared with seed plants, there are fewer published phylogeographical analyses about ferns. Trewick et al. (2002) summarized that this probably owed to the agreed fact that ferns have abundant, very small, and wind-dispersed spores and were expected to have high levels of gene flow, thus rendering them of little use for such studies. However, because spore vitality, germination, and gametophyte formation may be sensitive to environmental factors, effective migration is not as common as the fern life history suggests. In addition, mating systems and historical events could also induce genetic differentiation. As reviewed by Ranker & Geiger (2008) and others (e.g., Trewick et al., 2002; Su et al., 2005a; Shepherd et al., 2007; Chen et al., 2008), past studies have revealed interpopulation divergence and evidence of restricted gene flow in ferns.

Cyatheaceae Kaulfuss is a large pantropical family with approximately 500 extant species distributed globally (Tryon & Gastony, 1975). Although the approximately 120 species in the genus *Sphaeropteris* are distributed primarily in the Old World tropics (Korall et al., 2007), *Sphaeropteris brunoniana* (Hook.) R. M. Tryon is the only *Sphaeropteris* species in mainland China (Zhang, 2004). Cyatheaceae was listed in the Convention on International Trade in Endangered Species (CITES) in 1975 (Oldfield, 1995). Cyatheaceae was also listed in the secondary category of state-protected wild plants in China in 1999 (Yu, 1999). Studying population structure and phylogeography of a species can provide essential information to develop its effective

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conservation strategies (Arcand & Ranker, 2008). However, little is known about the genetic diversity and genetic structure of *S. brunoniana* populations.

Sphaeropteris brunoniana is a large tree fern with erect rhizome, which can grow to a height of over 20 m and can be found in tropical or subtropical lowland to submontane environments. The distribution of this species ranges from northeast India, through Bangladesh to Burma and into Vietnam, with its northern limit in China (Zhang, 2004). In China, its natural populations often occur in the margins or near to ravines of evergreen broad-leafed forests in Tibet, Yunnan, and Hainan provinces. This distribution provides the opportunity to analyze its population genetic structure and phylogeography. Such analyses would help us to understand its genetic variability and origin, and provide some constructive options for protecting this only *Sphaeropteris* species in mainland China.

Using cpDNA *trn*L-*trn*F and *atp*B-*rbc*L sequence variations, the phylogeography of *S. brunoniana* from China and Laos was analyzed in this study. We were particularly interested in addressing the following questions: (i) What are the levels of genetic diversity and genetic structure of *S. brunoniana*? (ii) What does this structure indicate about its postglacial history? and (iii) What are implications of the findings for developing its appropriate conservation strategies in China?

1 Material and methods

1.1 Plant materials

Plant materials were collected from nine populations in China and one population in Laos. Among the nine populations sampled in China, five were collected from roadsides, disturbed margins of secondary evergreen broad-leafed forests in Yunnan province; others were collected from natural reserve areas in Hainan province. A total of 85 individuals were sampled. Details of materials are given in Table 1. About 5 g of fresh leaf material per individual was collected and immediately dried using silica gel and stored at room temperature. Voucher specimens were deposited at the Herbarium of the Kunming Institute of Botany (KUN).

1.2 DNA extraction, amplification, and sequencing

Genomic DNA was extracted from dried leaves using the modified CTAB method (Doyle & Doyle, 1987). Polymerase chain reaction (PCR) amplification and DNA sequencing were carried out using universal primers c and f for *trn*L-*trn*F (Taberlet et al., 1991) and atpB-1 and rbcL-1 for *atp*B-*rbc*L (Chiang et al., 1998). Polymerase chain reaction was carried out in a volume of 25 μL containing 2.5 μL of 10× reaction buffer, 2.5 μL $MgCl_2$ (25 mmol/L), 0.5 μL dNTP mixture (10 mmol/L), 0.5 μL each primer (10 μmol/L), 1 μL bovine serum albumin (20 μg/μL), 1 U Taq polymerase, and 1 μL template DNA, and was run on an Eppendorf thermocycler with parameters set as: 94°C, 3 min; 35× (94°C, 40 s; 49°C, 50 s; 72°C, 90 s); 72°C, 10 min. As to samples collected from Yunnan, the *trn*L-*trn*F region was difficult to be amplified with universal primers, so substitute primers developed by Li et al. (2004) were used for amplification and sequencing. In addition, a two-step PCR protocol was carried out for those problematic DNA. The parameters were: 95°C, 3 min; 35× (95°C, 1 min; 66°C, 5 min); 66°C, 10 min. Sizes of PCR products were determined by agarose gel elecrophoresis. All PCR products were purified using a Multifunctional DNA Purification and Recycled Kit (Bioteke, Beijing, China), then sequenced in both directions for *trn*L-*trn*F and only one direction with primer rbcL-1 for *atp*B-*rbc*L using standard methods on an ABI 377 automated sequencer (Perkin Elmer) at the Shanghai Sangon Biological Engineering Technology & Service Co.

1.3 Data analysis

Sequences were aligned using the ClustalW program (Thompson et al., 1994), then adjusted manually to minimize the number of gaps. Because nucleotide repeats are prone to homoplasy (Ingvarsson et al., 2003), gaps caused by nucleotide repeats were excluded in all of the following analyses. Haplotypes were quantified based on both nucleotide substitutions and non-repeat indels. Haplotype diversity (h) and nucleotide diversity (π) were calculated at population, regional, and total levels with DnaSP 4.50 (Rozas et al., 2003). With this software, the neutral evolution of *trn*L-*trn*F and *atp*B-*rbc*L was also tested, including Tajima's criterion D (1989), Fu & Li's tests D* and F* (1993).

To analyze the population structure of *S. brunoniana*, an AMOVA was carried out and the significance was tested by 1000 permutations. Fixation indices were also quantified (Wright, 1978). To identify the isolation by distance model, a Mantel test (Mantel, 1967) was carried out between $F_{ST}/(1 - F_{ST})$ and the natural logarithm of geographical distance. Its significance was also tested using 1000 random permutations. Gene flow (Nm) was indirectly estimated from the expression $F_{ST} = 1/(1 + 2N_m)$ (Hudson et al., 1992). All analyses were carried out using Arlequin version 3.0 software (Excoffier et al., 2005).

To detect historical population expansion events, a mismatch distribution was carried out (Rogers & Harpending, 1992) with a total of 1000 replicates used to obtain an expected mismatch distribution under the sudden

Table 1 Populations of *Sphaeropteris brunoniana* sampled in this study

Population locality	Code	Coordinates	Altitude (m)	Sample size	Voucher
Yunnan region				48	
Yingjiang, Yunnan, China	B	97°35′ E/24°40′ N	684–1470	10	07031–07040 (KUN)
Luxi, Yunnan, China	D	98°29′ E/24°31′ N	1300	10	07061–07070 (KUN)
Hekou, Yunnan, China	M	103°57′ E/22°36′ N	321	10	08120–08129 (KUN)
Jinping, Yunnan, China	N	103°02′ E/22°38′ N	735	9	08135–08143 (KUN)
Xishuangbana,Yunnan, China	P	101°30′ E/21°31′ N	836	9	08180–08188 (KUN)
Hainan–Laos region				37	
Wuzhishan, Hainan, China	F	109°41′ E/18°54′ N	600–669	7	08031–08037 (KUN)
Diaoluoshan, Hainan, China	H	109°52′ E/18°44′ N	880–923	8	08061–08068 (KUN)
Jianfengling, Hainan, China	I	108°52′ E/18°44′ N	812–998	9	08076–08084 (KUN)
Changjiang, Hainan, China	J	109°11′ E/19°06′ N	1010–1020	8	08090–08097 (KUN)
Khamkeut, Laos	K	104°57′ E/18°12′ N	750	5	WS224–228 (KUN)

demographic expansion model. The sum of square deviations (SSD) between the observed and the expected mismatch were used as a statistic test, and the probability of a simulated SSD larger or equal to the observed SSD were quantified to determine whether or not to reject the expansion model. The raggedness index (R) was also calculated to detect the smoothness of the observed distribution (Harpending, 1994). Its significance was tested similarly to that of SSD. If the sudden expansion model was not rejected, the equation $Tau = 2ut = 2m_T\mu t$ was used to estimate the expansion age (t) (Rogers & Harpending, 1992), where m_T is the number of investigated nucleotides and μ is the mutation rate per nucleotide. The mutation rate of 1.3×10^{-9} substitution per site per year summarized by Richardson et al. (2001) was used to calculate the expansion time of *S. brunoniana*. Moreover, Fu's *Fs* test was carried out (Fu, 1997) with the significance tested using 1000 random samples under the hypothesis of selective neutrality and population equilibrium. The *P*-value was quantified as the proportion of random *Fs*-values less or equal to the observed ones. For the *Fs* test, $P = 0.02$ is considered to be significant at $\alpha = 0.05$ level (Fu, 1997). All of these analyses were also carried out using Arlequin version 3.0 (Excoffier et al., 2005).

To reveal the phylogenetic relationships of haplotypes, a parsimony haplotype network was constructed using ANeCAv1.2 (Clement et al., 2000; Posada et al., 2000; Panchal, 2007), with setting calculating connection limits as 95% and gaps as fifth state. Furthermore, a nested clade analysis (NCA) was carried out to infer the patterns of population history. Significance of each level of nested cladogram was estimated using 10 000 random permutation tests based on the statistics clade distance (D_c), nested distance (D_n), and the average interior distance minus the average tip distance (I-T). Results of historical processes responsible for the observed pattern of clade structure were auto-generated by the same software based on the methods of Templeton et al. (1995).

2 Results

2.1 Genetic diversity and genetic structure

For the *trn*L-*trn*F region (including *trn*L intron, exon and *trn*L-*trn*F intergenic spacer), the sequence sizes varied from 966 to 1001 bp with a consensus length of 1003 bp. This length polymorphism was caused by an imperfect TA repeat at sites 321–363 interrupted by one or two TATAGC inserts in Yunnan populations and a T mononucleotide repeat at sites 718–729. For the *atp*B-*rbc*L region, an A mononucleotide repeat was also identified at sites 462–471 and three nucleotide substitutions A↔C, A↔C, and T↔C were detected at sites 304, 438, and 530, respectively. The alignment of *atp*B-*rbc*L contained 726 sites. Excluding gaps caused by nucleotide repeats, the total alignment of *trn*L-*trn*F and *atp*B-*rbc*L sequences covered 1696 characters, of which nine were polymorphic sites and three were ascribed to substitutions. According to Tajima's criterion ($D = -0.63204$, $P > 0.10$) and the results of Fu & Li's tests ($D^* = -0.54798$, $P > 0.10$; $F^* = -0.67242$, $P > 0.10$), sequences of *trn*L-*trn*F and *atp*B-*rbc*L were neutral in terms of evolution. Based on both substitutions and non-repeat indels, five haplotypes (Hap1–Hap5) were identified from the combined data of those two sequences. The examined sequences were deposited in the GenBank database under the accession numbers HQ438266–HQ438271. Haplotype distribution, haplotype diversity (h), and nucleotide diversity (π) of each investigated population are listed in Table 2.

The haplotype diversity (h) and nucleotide diversity (π) at total level were 0.66580 and 0.00023, respectively. Across all populations, haplotype diversity ranged from 0.00000 to 0.77778, and nucleotide diversity varied from 0.00000 to 0.00066. The highest genetic diversity was found in population P, and the next were in populations D, M, and N. Sample sequences of four Hainan populations (F, H, I, J), one Laos population (K), and one Yunnan population (B) were homogeneously composed. As to *trn*L-*trn*F and *atp*B-*rbc*L, no

Table 2 Haplotypes, haplotype diversity (h) and nucleotide diversity (π) of *Sphaeropteris brunoniana* in each investigated population

Population	No. of haplotypes	Haplotypes (no. of individuals)	h	$\pi \times 10^3$
Yunnan region	4	Hap1 (1), Hap2 (2), Hap4 (16), Hap5 (29)	0.60195	0.35
B	1	Hap5 (10)	0.00000	0.00
D	2	Hap4 (5), Hap5 (5)	0.55556	0.33
M	2	Hap4 (5), Hap5 (5)	0.55556	0.33
N	2	Hap4 (4), Hap5 (5)	0.55556	0.33
P	4	Hap1 (1), Hap2 (2), Hap4 (2), Hap5 (4)	0.77778	0.66
Hainan–Laos region	1	Hap3 (37)	0.00000	0.00
F	1	Hap3 (7)	0.00000	0.00
H	1	Hap3 (8)	0.00000	0.00
I	1	Hap3 (9)	0.00000	0.00
J	1	Hap3 (8)	0.00000	0.00
K	1	Hap3 (5)	0.00000	0.00
Species level	5	Hap1 (1), Hap2 (2), Hap3 (37), Hap4 (16), Hap5 (29)	0.66580	0.23

genetic variation was detected in these six populations. When dividing the 10 populations into two groups, the Yunnan populations had much higher genetic diversity ($h = 0.60195$, $\pi = 0.00035$) than that of Hainan–Laos ($h = 0.00000$, $\pi = 0.00000$).

Analysis of molecular variance indicated that 94.74% of the total genetic variations occurred between regions, 0.44% among populations within regions, and 4.82% within populations (Table 3). The F-statistics (Wright, 1978) also revealed a high level of genetic differentiation between regions ($F_{CT} = 0.94738, P < 0.01$). Mantel's test indicated that the genetic differentiation of *S. brunoniana* among the investigated populations was directly related to physical distance ($r = 0.356$, $P = 0.000$). Calculated from the values of F_{ST}, the gene flow (Nm) among populations and between regions was 0.046 and 0.025, respectively.

2.2 Haplotype distribution and demographic history

Among these five haplotypes, Hap1, Hap2, Hap4, and Hap5 were distributed in Yunnan populations, and Hap3 was distributed in Hainan–Laos populations (Fig. 1 & Table 2). In the haplotype network (Fig. 2), Hap1 and Hap2 occurred only in population P placing in tip positions, Hap4 and Hap5 with high frequency located interior positions, whereas Hap3 in tip position through five undetected or extinct haplotypes connected to Hap5. Two groups were identified within the five extant haplotypes, one including Hap4 and Hap5 was geographically correlated to the Yunnan region, and the other composed of Hap3 was related to Hainan–Laos. In addition, a significant total cladogram was revealed in the haplotype geographic structure (Table 4). Through cladistic nested analysis, the evolutionary events, including restricted gene flow with isolation by distance at clade 1–3 and allopatric fragmentation in the total cladogram (Table 5), were inferred to be responsible for the geographic distribution of cpDNA haplotypes in *S. brunoniana*.

Mismatch distribution indicated that all of the four populations (D, M, N, and P) with intrapopulation divergence did not reject a sudden expansion model. Among them, populations D, M, and N had expansion ages at 197.1 thousand years ago, and population P at 287.9 thousand years ago (Table 6). Fu's *Fs* test showed that only population P had a negative *Fs* value, but its probability was insignificant; others had positive values and insignificant probabilities (Table 6). This result indicated that those populations did not experience demographic expansion.

3 Discussion

3.1 Low genetic diversity within species and high differentiation between regions

In this study, cpDNA *trn*L-*trn*F and *atp*B-*rbc*L were selected and combined to investigate the genetic variation and phylogeography of *S. brunoniana*. Through genetic analysis, a moderate level of haplotype diversity ($h = 0.66580$) and a low level of nucleotide

Table 3 Analysis of molecular variance for populations of *Sphaeropteris brunoniana* based on cpDNA *trn*L-*trn*F and *atp*B-*rbc*L sequences

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation indices	P-value
Among regions	1	128.031	3.05718	94.74	$F_{CT} = 0.94738$	0.0078
Among populations within regions	8	2.208	0.01423	0.44	$F_{SC} = 0.08382$	0.0753
Within populations	75	11.667	0.15556	4.82	$F_{ST} = 0.95180$	0.0000
Total	84	141.906	3.22697			

d.f., degrees of freedom.

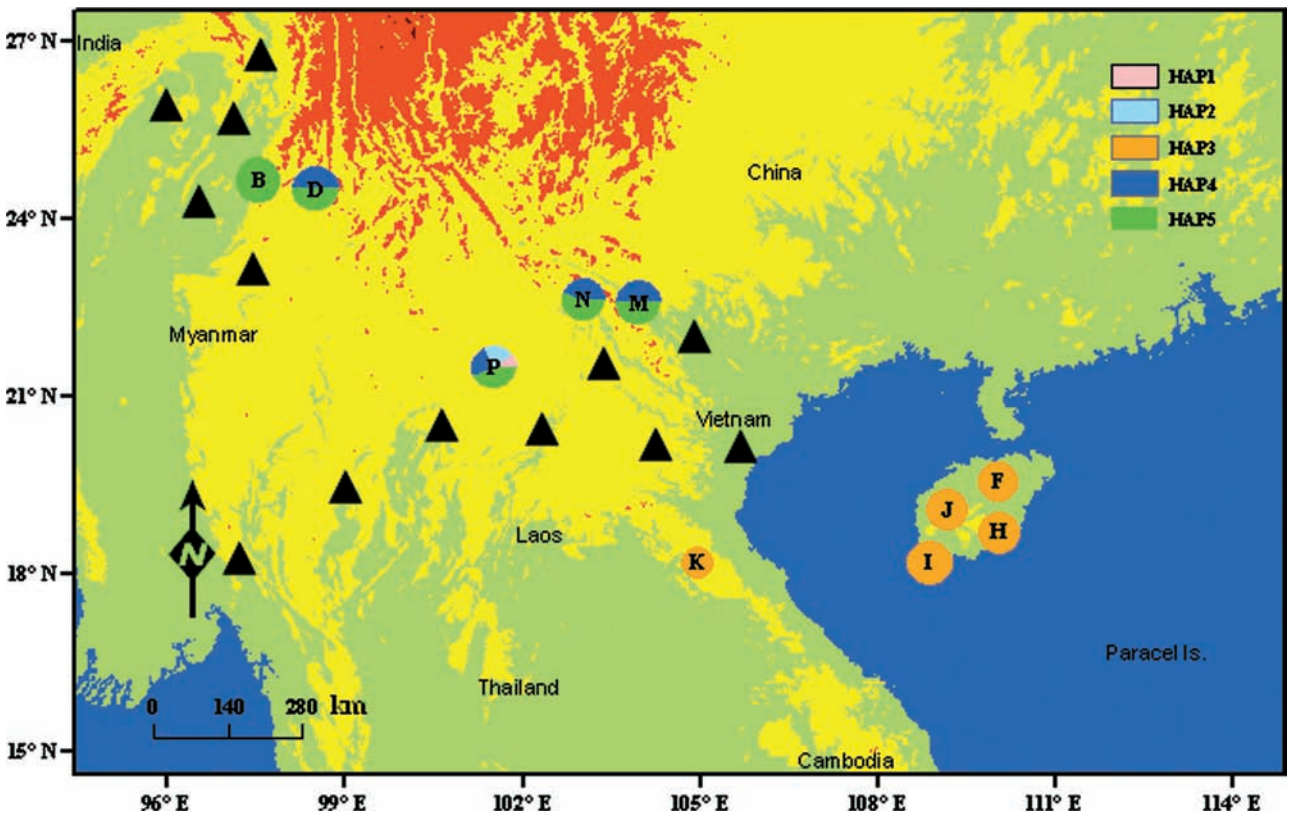

Fig. 1. Locations of investigated populations and geographical distribution of cpDNA haplotypes in *Sphaeropteris brunoniana*. Haplotypes are shown with different color pies. The letter in each pie represents the code of populations and the size of pies represents the numbers of sampled individuals in a certain population. Solid triangle, area where *S. brunoniana* was not sampled.

diversity ($\pi = 0.23 \times 10^{-3}$) were detected in the 10 investigated populations. The nucleotide diversity of this species was far less than that of other surveyed tree ferns, viz. *Alsophila spinulosa* ($\pi = 11.30 \times 10^{-3}$, *atp*B-*rbc*L; $\pi = 22.63 \times 10^{-3}$, *trn*L-*trn*F) and *A. podophylla* ($\pi = 2.08 \times 10^{-3}$, *atp*B-*rbc*L) (Su et al., 2004, 2005a, 2005b). At a regional level, the Yunnan populations had much higher genetic diversity than that of Hainan–Laos.

The results of AMOVA and NCA revealed that the genetic differentiation of this species between the two regions was high and their germ plasms were different from each other. Genetic differentiation of a species reflects the interactions of various evolutionary processes including the long-term evolutionary history, such as shifts in distribution, habitat fragmentation and population isolation, mutation, genetic drift, mating system, gene flow, and natural selection (Schaal et al., 1998). Estimated from F_{ST}, the gene flow between the two regions was only 0.025. Wright (1951) predicted that if $Nm < 1$, genetic drift would lead to genetic differentiation. In addition, the NCA result revealed habitat fragmentation of the two regions. Thus, in *S. brunoniana*, the high level of differentiation is probably caused by

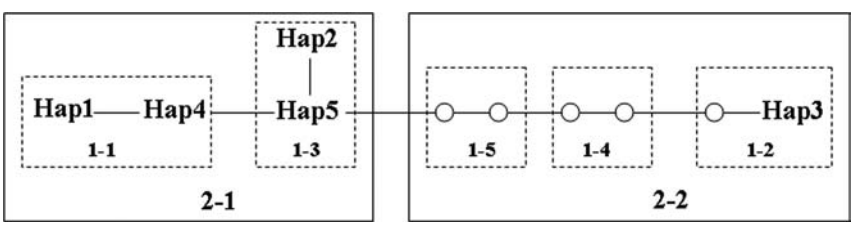

Fig. 2. Haplotype network and nested clades of the five haplotypes in *Sphaeropteris brunoniana*. Dashed line, a one-step clade; open circle, hypothetical node that was undetected or extinct in populations; solid line, a two-step clade.

Table 4 Results of nested clade analyses of geographical distance of cpDNA haplotypes in *Sphaeropteris brunoniana*

Nested clades and haplotypes	Type of geographical distance		Observed χ^2 statistic	Probability
	Within clade (D_c)	Nested clade (D_n)		
Clade 1–1			4.9583	0.1724
Hap1 (Tip)	0.0000	160.9948		
Hap4 (Interior)	239.9615	239.0668		
I-T	239.9615	78.072		
Clade 1–3			8.9080	0.0952
Hap2 (Tip)	0.0000	227.8310†		
Hap5 (Interior)	293.8528‡	294.3641‡		
I-T	293.8528‡	66.5331‡		
Clade 2–1			7.6812	0.1067
1–1 (Tip)	234.1873	262.8662		
1–3 (Interior)	289.7756	289.4382		
I-T	55.5883	26.5720		
Total cladogram			85.0000	0.0000
2–1 (Tip)	279.9904†	475.3991		
2–2 (Tip)	153.2610†	495.3948		

†D_c, D_n, or I-T (interior distance minus the tip distance) values that are significantly smaller than the expected at the 5% level based on 10 000 permutations. ‡D_c, D_n, or I-T values that are significantly larger than the expected at the 5% level based on 10 000 permutations.

habitat fragmentation, limited gene flow, and/or genetic drift.

3.2 Emergence of independent refugia in each region

Nested clade analysis indicated that allopatric fragmentation was responsible for the genetic distribution of this species between the two regions. This result suggested the emergence of independent refugia in each region during glacial periods.

Coalescent theory predicts that haplotypes found at interior positions coupled with a high frequency represent ancestral genotypes. Based on this theory, Hap5 is an ancestral haplotype of *S. brunoniana* in the Yunnan region. This ancestral haplotype occurred in all of the five Yunnan populations. Alternatively, NCA predicts that the distribution pattern of the observed haplotypes was caused by fragmentation and restricted gene flow by distance. These results imply that Hap5 was originally distributed in those populations rather than being widely dispersed from a single refuge. These results further imply that there were several refugia in Yunnan province during Quaternary glaciations.

Table 5 Inference chains based on results of geographical dispersion analysis

Clade	Chain of inference	Demographic event inferred
Clade 1–3	1–2–3–4-NO	Restricted gene flow with isolation by distance
Total cladogram	1–19-NO	Allopatric fragmentation

This table only includes clades in which the null hypothesis of no geographical association between haplotype variation and geographical distribution is rejected.

Hap3 was the only haplotype detected in Hainan populations and was in the tip position of the network. This result implies that the Hainan populations were established recently, which may explain why these populations showed less genetic variation than the presumably older ones in Yunnan. No haplotype of this species was shared between the two provinces of China. However, sample sequences of Hainan individuals were similar to those obtained from Laos. These results may imply that the genealogical origins of Yunnan populations and Hainan ones were different, but the origins of these populations require further studies to be well understood.

Restricted gene flow with isolation by distance was identified in clade 1–3 consisting of two haplotypes fixed in Yunnan. This finding seems to conflict with the result of AMOVA. A low level of genetic differentiation was quantified within regions (only 0.44%), implying unrestricted migration among intraregional populations. Mismatch distributions indicated that four of the Yunnan populations (D, M, N, and P) had experienced a sudden expansion 300–180 thousand years ago, corresponding closely to the great interglacial period. But Fu's *Fs* test did not detect such an expansion, all *P*-values were larger than 0.02 (Table 6) and did not reject the hypothesis of population equilibrium. Ramos-Onsins & Rozas (2002) showed that a mismatch distribution was a very conservative test to detect population growth and Fu's *Fs* test was more powerful. Rogers & Harpending (1992) showed that bottlenecks in population size generated similar mismatch distribution patterns to that produced by a sudden expansion. Thus, it is probable that populations of *S. brunoniana* resumed their sizes during interglacial periods after bottlenecks triggered by glaciation. Bottleneck and habitat fragmentation were interdependent. Although having expanded,

Table 6 Results of mismatch distribution analysis and Fu's *Fs* test for four populations of *Sphaeropteris brunoniana* from Yunnan, China

Population	SSD	SSD *P*-value	Raggedness index (R)	R *P*-value	Tau	Age†	*Fs*	*Fs P*-value
D	0.03716	0.168	0.32099	0.134	0.86914	197.1	1.09586	0.673
M	0.03716	0.170	0.32099	0.162	0.86914	197.1	1.09586	0.646
N	0.03716	0.225	0.32099	0.177	0.86914	197.1	1.01511	0.624
P	0.02563	0.427	0.18519	0.328	1.26953	287.9	−0.82233	0.154

†Age in thousand years. SSD, sum of square deviations.

those populations were no longer large and separated as isolation-by-distance units such that migration between those populations was not as effective as before.

In summary, fragmentation, restricted gene flow by distance, genetic drift, and different genealogical origins have resulted in the current genetic differentiation of *S. brunoniana* between the Yunnan and Hainan–Laos regions. The Yunnan region potentially had several refugia of this species during Quaternary glaciations, whereas the Hainan populations are likely to be established recently. Further studies, including samples from the entire range of this species westwards into northeast India, are required to fully understand the genetic make-up, the origin, and the migratory route of the relict tree fern.

3.3 Implications for conservation

This research provides some insights into the genetic diversity and genetic structure of *S. brunoniana*, which meet the basic requirements for conservation management. Considering the low genetic variation within populations and among populations within regions, and the significantly high genetic differentiation between regions, to retain existing diversity, whether *in situ* or *ex situ* conservation is used, the populations of Yunnan and Hainan should be equally considered. It is fortunate that Hainan populations of this species are generally distributed in natural reserve areas. In Yunnan province, however, *S. brunoniana* is usually found in disturbed habitats, such as population P with the highest genetic diversity but located along Xiaola highway. Thus, more conserved areas should be established in Yunnan province, and *ex situ* conservation should be adopted as soon as possible. Because of its long life cycle, spores and germplasm collections in botanical gardens or other institutions should be of practical value to conserve this species. It is also recommended that spores should be collected from the two different regions.

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