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Population genetic structure and phylogeographical pattern of a relict tree fern, *Alsophila spinulosa* (Cyatheaceae), inferred from cpDNA *atp*B–*rbc*L intergenic spacers

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Abstract Sequences of chloroplast DNA (cpDNA) *atp*B–*rbc*L intergenic spacers of individuals of a tree fern species, *Alsophila spinulosa*, collected from ten relict populations distributed in the Hainan and Guangdong provinces, and the Guangxi Zhuang region in southern China, were determined. Sequence length varied from 724 bp to 731 bp, showing length polymorphism, and base composition was with high A+T content between 63.17% and 63.95%. Sequences were neutral in terms of evolution (Tajima's criterion D=−1.01899, P>0.10 and Fu and Li's test D*=−1.39008, P>0.10; F*=−1.49775, P>0.10). A total of 19 haplotypes were identified based on nucleotide variation. High levels of haplotype diversity (h=0.744) and nucleotide diversity (D_{ij}=0.01130) were detected in *A. spinulosa*, probably associated with its long evolutionary history, which has allowed the accumulation of genetic variation within lineages. Both the minimum spanning network and neighbor-joining trees generated for haplotypes demonstrated that current populations of *A. spinulosa* existing in Hainan, Guangdong, and Guangxi were subdivided into two geographical groups. An analysis of molecular variance indicated that most of the genetic variation (93.49%, P<0.001) was partitioned among regions. Wright's isolation by distance model was not supported across extant populations. Reduced gene flow by the Qiongzhou Strait and inbreeding may result in the geographical subdivision between the Hainan and Guangdong + Guangxi populations (F_{ST}=0.95, Nm=0.03). Within each region, the star-like pattern of phylogeography of haplotypes implied a population expansion process during evolutionary history. Gene genealogies together with coalescent theory provided significant information for uncovering phylogeography of *A. spinulosa*.

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Introduction

Fossil records indicate that during evolutionary history, while many groups of plants became extinct entirely, plenty of others have persisted but with much diminished diversity. Ferns, however, attained remarkable richness in diversity from the Carboniferous Period to the Jurassic Period (Rothwell 1996; Collinson 1996; Skog 2001) and now still consist of more than 10,000 extant species, representing the second largest group of vascular plants (Smith 1972). Interestingly, recent research has suggested that extant ferns achieved their current diversity perhaps due to an ecological opportunistic response to the expansion of angiosperms, and hence had diversified much more recently (around 80 million years ago) than had been expected (Schneider et al. 2004). The new findings uncover crucial information about the evolutionary potential of ferns to shape their phylogeography.

Alsophila spinulosa (Wall. ex Hook) Tryon (Cyatheaceae) is a diploid tree fern with a single, erect, arborescent trunk up to 6 m tall. Historically, it was distributed worldwide during the Jurassic Period (180 million years ago) (Fu 1991). After being damaged by the Quaternary Period glaciers, the ancestral populations of *A. spinulosa* drastically dwindled (Tryon 1970). Its extant populations are mainly restricted to tropical and subtropical montane regions with relictual distributions and usually grow in acidic soil (pH 4.5–5.5) in warm, humid, and shady

habitats below an altitude of 1,000 m (Wang et al. 1996). It has been suggested that *A. spinulosa*'s poor adaptation to present environment and climate was relative to its biological characteristics, e.g., spore lifespan is short, and spore germination, gametophyte formation, and embryogenesis all require rigorously controlled temperature, humidity, and illumination conditions (Cheng et al. 1990; Tryon and Lugardon 1991). These explanations should be taken with caution, especially when it is known that possibly the whole idea of the evolutionary cul-de-sac of plants—at least for ferns—is basically flawed (Eriksson 2004; Schneider et al. 2004). In the past decade, within southern China, *A. spinulosa*'s habitats have been increasingly fragmented, and its populations have been greatly damaged because of local economic exploitation and human impact. Natural populations of *A. spinulosa* in China are extremely rare, and the species is regarded as National Protection Category 1-V in the Red List (Fu 1991).

Assessment of population structure and phylogeography will provide information essential for working out biologically sound conservation measures for threatened species (Zoller et al. 1999). Gene genealogies and coalescence theory develop powerful methodologies to investigate population-level phenomena such as gene flow, founder effects, and the history of lineages (Castelloe and Templeton 1994). Phylogeographical studies can also shed light on formation, distribution, and expansion of populations during geological processes (Hewitt 1996; Larena et al. 2002). Recently, cpDNA noncoding spacers have been frequently applied to survey population genetic variation and phylogeography of plants (Schaal et al. 1998; Huang et al. 2001; Chiang et al. 2001; Trewick et al. 2002; Lu et al. 2002; Hwang et al. 2003). Their uniparental inheritance and nearly neutral, fast evolution are well suited to reconstruct intraspecific phylogeographical patterns (Ferris et al. 1998). In addition, technically, DNA sequencing can avoid length homoplasies that usually occur when using restriction fragment length polymorphism and PCR-based fingerprinting methods (Chiang et al. 2001) and improves the level of resolution when sequence data are used to estimate population genetic structure and gene flow.

In this study, sequence variation of haplotypes of cpDNA *atp*B–*rbc*L noncoding spacers were utilized to examine the population genetic structure and phylogeographical pattern of ten relict *A. spinulosa* populations distributed across the Hainan Province, the Guangdong Province, and the Guangxi Zhuang autonomous region in southern China. The purpose of this investigation is (1) to determine whether geographical differentiation occurs among these populations at an interregion level, (2) to assess the level of genetic diversity and its hierarchical apportionment, and (3) to recognize the factors determining population genetic structure.

Materials and methods

Plant material

A. spinulosa is diploid ($2n$=138) as reported by Wang et al. (1997). Samples of *A. spinulosa* were collected from ten relict populations distributed in the Guangdong Province, the Guangxi Zhuang autonomous region, and the Hainan Province in the southern part of China (Table 1; Fig. 1). Plants grow on humid and sunny slopes in ravines or by streamsides in the montane forests at altitudes of 400–850 m. Young and healthy leaves were randomly sampled from individuals, with intervals of at least 5 m, and immediately preserved in silica gel. All samples were stored at −20°C until being processed. Vouchers from populations investigated have been deposited at the herbarium of Sun Yat-sen University, Guangzhou, China. All materials were identified by Prof. Bo-sun Wang at the Department of Biology, School of Life Sciences, Sun Yat-sen University.

Table 1 Material collected from populations of *Alsophila spinulosa* used for cpDNA sequencing

Populations	Localities	Coordinate	Sample size (n)	Haplotype diversity (h)	Nucleotide diversity (D_{ij})
A. spinulosa	–	–	75	0.744	0.01130±0.00197
Guangdong + Guangxi	–	–	61	0.619	0.00128±0.00031
SZTLS	Tanglangshan, Shenzhen, Guangdong	113°41′E, 22°37′N	9	0.694	0.00145±0.00048
YDDXS	Daxishan, Yingde, Guangdong	112°45′E, 23°50′N	10	0.679	0.00177±0.00073
FKHSD	Heishiding, Fengkai, Guangdong	111°53′E, 23°27′N	8	0.833	0.00137±0.00046
XXJC	Jicheng, Xinxing, Guangdong	111°57′E, 22°22′N	6	0.000	0.00000±0.00000
XXCF	Cangfo, Xinxing, Guangdong	112°31′E, 22°50′N	6	0.000	0.00000±0.00000
LPHNS	Huangniushi, Lianping, Guangdong	114°26′E, 24°07′N	6	0.000	0.00000±0.00000
ZQDHS	Dinghushan, Zhaoqing, Guangdong	112°35′E, 23°08′N	10	0.700	0.00110±0.00041
GXSJ	Sanjiang, Guangxi,	110°17′E, 24°40′N	6	1.000	0.00137±0.00068
Hainan	–	–	14	0.378	0.00138±0.00073
HNJFL	Jianfengling, Hainan	108°44′E, 18°23′N	8	0.464	0.00173±0.00084
HNBWL	Bawangling, Hainan	109°15′E, 18°25′N	6	0.000	0.00000±0.00000

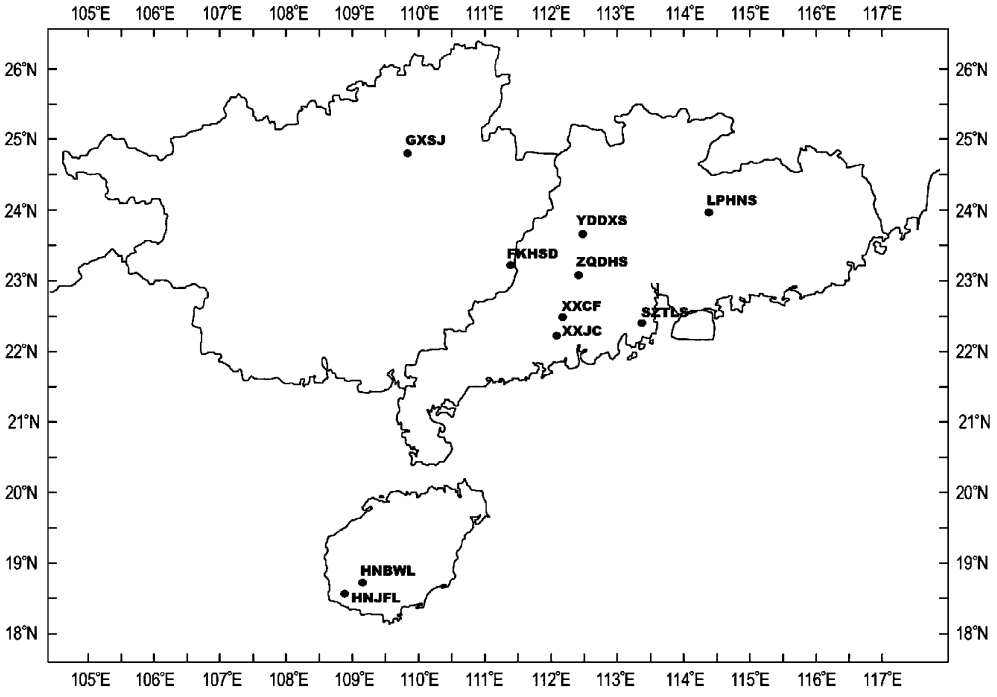

Fig. 1 *Alsophila spinulosa* sample locations in the Guangdong Province, the Guangxi Zhuang region, and the Hainan Province in southern China

DNA extraction and PCR amplification

Total genomic DNA was extracted from ground tissue, following the modified CTAB protocol (Su et al. 1998). DNA concentration and purity were determined by measuring UV absorption, using a Pharmacia 2000 UV/Visible spectrophotometer. DNA intactness was checked with 0.8% agarose gel electrophoresis. PCR was performed in a reaction volume of 100 μl, using 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM of each dNTP, 50 ng template DNA, 2 U *Taq* polymerase, and 40 pmol of each primer. Primers of Chiang et al. (1998) were used to amplify an *apt*B- *rbc*L noncoding spacer of cpDNA (Primer 1: 5′-AC ATCK-ARTACKGGACCAATAA-3′, primer 2: 5′-AACAC-CAGCTTTRAATCCAA-3′). Primers were synthesized by Shanghai Bioasia Biotech, China. The thermocycling profile consisted of 3 min at 94°C, 30 cycles of 40 s at 94°C, 50 s at 50°C, 80 s at 72°C, and an additional extension for 7 min at 72°C. The size of PCR products was determined by agarose electrophoresis.

DNA cloning and sequencing

The PCR products were purified by electrophoresis on a 1.0% low-melting point agarose gel. The desired DNA band was cut and recovered using the UNIQ-10 kit (Shanghai Bioengineering). Purified PCR product was ligated to a pMD18-T vector and then was used to transform competent *Escherichia coli* cells DH-5α. Positive clones was identified by blue/white selection and ascertained by PCR. For each plant sample, plasmid DNA prepared from three to five positive clones were independently sequenced in both directions by standard methods on an ABI 377 automated sequencer. Primers M13F and M13R, located on the pMD18-T vector, were utilized for sequence determination.

Data analysis

Sequences of the determined *atp*B–*rbc*L noncoding spacer of cpDNA were registered in GenBank with accession numbers of AY304397–AY304416. Sequences were aligned by the Clustal X program (Thompson et al. 1997), with the gap opening penalty set as 10.00, gap extension penalty as 0.20, delay divergent sequences as 30%, and DNA transition weight as 0.50. Length variation and nucleotide composition were calculated using BioEdit (Hall 1999). Haplotype diversity (h), nucleotide diversity (D_{ij}), tests of neutrality, and the determination of their associated significance were performed using DnaSP program (Rozas and Rozas 1999). A minimum spanning network (MSN) was constructed with the aid of the MINSPNET (Excoffier and Smouse 1994). Neighbor-joining (NJ) analysis by calculating Kimura 2-parameter distance was conducted using PHYLIP (Felsenstein 1995). Confidence of the clades reconstructed was tested by bootstrapping with 1,000 replicates. Studies on the molecular phylogeny of Cyatheaceae indicate that subgenus *Gymnosphaera*, to which *A. podophylla* and *A. denticulata* belong, is dichotomized as sister group of subgenus *Alsophila*, which contains *A. spinulosa* (Wang et al. 2003b); thus, both species *A. podophylla* and *A. denticulata* were utilized as outgroups for constructing an MSN and NJ tree. Gene flow within and among populations was approximated as *Nm*, the number of reproductively successful migrants per generation between populations. Because cpDNA employed in this study is

haploid and uniparentally inherited, *Nm* was estimated using the expression $F_{ST}=1/(1+2Nm)$, where *N* is the number of individuals in each population and *m* is the fraction of migrants in each population in each generation (Hudson et al. 1992; Slatkin 1993). Gene flow in ferns is dependent on the dispersion of spores between populations (van Zanten 1978; Schneller and Holderegger 1996; Vogel et al. 1999a, b). ARLEQUIN was used to deduce the molecular variance partition within and among populations (regions), based on square Euclidean distances (Schneider et al. 2000). The pattern of isolation by distance was assessed by plotting pairwise *Nm* values against geographical distance (Slatkin 1993). The significance of the association between *Nm* and distance was determined by a regression *F*-test using the SPSS program (version 11.0).

Results

Haplotype and nucleotide diversity

In this study, PCR-amplified *atp*B–*rbc*L intergenic spacer fragments of cpDNA were sequenced. Sequence length varied from 724 bp to 731 bp, with a consensus length of 739 bp; 68 sites (9.2%) were variable. Nucleotides A and T were shown to be common in the chloroplast sequence, with contents between 63.17% and 63.95%, which is consistent with the nucleotide composition of most noncoding regions and pseudogenes (Li 1997; Chiang et al. 2001; Lu et al. 2002). In total, 19 haplotypes of cpDNA *atp*B–*rbc*L spacers were identified in *A. spinulosa*. Most haplotypes were distributed among populations (Table 2). Sequence variation demonstrated non-significant deviation to expectations of neutrality, both by Tajima's criterion ($D=-1.01899$, $P>0.10$) and Fu and Li's test ($D^*=-1.39008$, $P>0.10$; $F^*=-1.49775$, $P>0.10$).

High levels of haplotype diversity ($h=0.744$) and nucleotide diversity ($D_{ij}=0.01130$) were detected within the whole species of *A. spinulosa* (Table 1). Across all studied populations, haplotype diversity and nucleotide diversity range between 0.464 and 0.833 and between 0.00110 and 0.00177, respectively, except for the homogeneous composition in the populations of LPHNS, XXJC, XXCF, and HNBWL. At the region level, haplotype diversity in Guangdong + Guangxi ($h=0.619$) is higher than that in Hainan ($h=0.378$), but its nucleotide diversity ($D_{ij}=0.00128$) is lower than that in Hainan ($D_{ij}=0.00138$).

Gene genealogies of cpDNA haplotypes

An MSN was reconstructed to recognize the phylogeographical pattern of *A. spinulosa* based on mutational changes between haplotypes of cpDNA *atp*B–*rbc*L intergenic spacer (Fig. 2). According to the network, haplotypes occurring uniquely in the two populations of Hainan separated first from those existing in the populations of Guangdong and Guangxi, indicating that *A. spinulosa* may be subdivided into two geographical groups: Hainan and Guangdong + Guangxi. In the populations of Hainan, three haplotypes were identified, which were HNJFL01 (=HNJFL06=HNJFL08 =HNBWL11), HNJFL02, and HNJFL03, and the genealogical relationships among them were simple; two of them, HNJFL02 and HNJFL03, rapidly coalesced to HNJFL01 with three mutations. By contrast, in the populations distributed in Guangdong and Guangxi, a total of 16 haplotypes were identified (Table 3), exhibiting a more complex pattern of genealogical relationships. Eight terminal haplotypes (ZQDHS14, ZQDHS82, FKHSD14, FKHSD10, GXSJ02, XXCF01, SZTLS37, and YDDXS13) coalesced to internal haplotype SZTLS23 (=GXSJ01=YDDXS14 =FKHSD06=SZTLS02) with one to three mutations; phylogenetically, this clade in the network has branched into a structure with a star-like pattern (Pages and Holmes 1998). For the rest of haplotypes found in Guangdong and Guangxi, terminal haplotypes YDDXS08 and FKHSD16, and the clade consisting of hapotypes LPHNS01 (=YDDXS16=YDDXS10=ZQDHS99=XXJC21), SZTLS55, XXCF02, and SZTLS30, coalesced to internal haplotype XXJC02 (=SZTLS51=ZQDHS66) rapidly, with merely one or two mutation changes. Interestingly, the genealogy of haplotypes was bifurcated with more branches near the

Table 2 Distribution of cpDNA haplotypes in each population of *A. spinulosa*

Population	Haplotype number	Haplotypes[a]
SZTLS	6	SZTLS02(3), SZTLS23(1), SZTLS30(1), SZTLS37(2), SZTLS51(1), SZTLS55(1)
YDDXS	5	YDDXS08(3), YDDXS10(2), YDDXS13(1), YDDXS14(2), YDDXS16(2)
FKHSD	4	FKHSD06(2), FKHSD10(2), FKHSD14(2), FKHSD16(2)
XXJC	2	XXJC02(3), XXJC21(3)
XXCF	2	XXCF01(2), XXCF02(4)
LPHNS	1	LPHNS01(6)
ZQDHS	4	ZQDHS14(2), ZQDHS66(2), ZQDHS82(2), ZQDHS99(4)
GXSJ	2	GXSJ01(3), GXSJ02(3)
HNJFL	5	HNJFL01(1), HNJFL02(1), HNJFL03(1), HNJFL06(1), HNJFL08(4)
HNBWL	1	HNBWL11(6)

[a]SZTLS02=SZTLS23=FKHSD06=YDDXS14=GXSJ01, YDDXS16=YDDXS10=XXJC21=ZQDHS99=LPHNS01, SZTLS51=XXJC02=ZQDHS66, HNJFL01= HNJFL06=HNJFL08=HNBWL11. Numbers in *parentheses* indicate replicate number of each haplotype

tips of network than near the root (*A. podophylla* and *A. denticulata*).

An NJ tree was also constructed based on the sequence variation of *atp*B- *rbc*L haplotypes of *A. spinulosa*, using *A. podophylla* and *A. denticulate* as outgroups (Fig. 3). Haplotypes of *A. spinulosa* formed two monophyletic clades, each supported with a bootstrap value of 100%. One clade consisted of populations from Hainan, and the other corresponded to populations from Guangdong + Guangxi. Thus, consistent with those recovered by MSN, the NJ tree also suggested a geographical subdivision between populations of *A. spinulosa* at interregional level.

Population structure and geographical differentiation

The population structure of *A. spinulosa* was assessed based on sequence variation of the cpDNA *atp*B–*rbc*L noncoding spacer. Significant differentiation between regions of Hainan and Guangdong + Guangxi was revealed by the estimates of F_{ST} (=0.95) and *Nm* (=0.03). At the intraregion level, F_{ST} of 0.00 and *Nm* of 8.75 were detected between HNBWL and HNJFL populations in Hainan, whereas F_{ST} ranging from 0.00 to 0.01 and *Nm* from 0.50 to 7.46 were estimated between populations in Guangdong + Guangxi (Table 3). Hierarchical analyses of sequence difference under analysis of molecular variance (AMOVA) indicated that 93.49% of molecular variance was attributed to difference among regions (P<0.001; Table 4). The isolation by distance model (Wright 1943) was not supported across populations (r^2=0.064, P=0.222). The *Nm* values observed between populations cannot be predicted by geographical distance.

With regard to DNA divergence, at the intraregional level, between populations in Hainan, the average number of nucleotide differences and the average number of nucleotide substitution per site are 0.625 and 0.00086, whereas between populations in Guangdong + Guangxi, the corresponding measures range from 0.000 to 1.306, and 0.000 to 0.00180, respectively (Table 5). At interregional level, the corresponding values between Hainan and Guangdong + Guangxi are 20.971 and 0.02913, respectively. Population DNA divergence between regions is apparently higher than that found within each region.

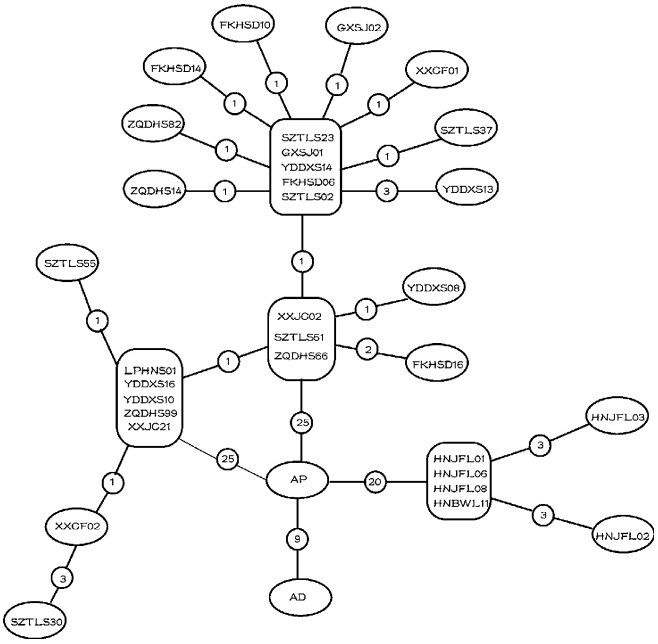

Fig. 2 The minimum spanning network, relating haplotypes of *atp*B–*rbc*L spacer of cpDNA found in populations of *A. spinulosa*. *A. podophylla*, and *A. denticulata* were used to root the tree. Major links between haplotypes are represented as *thick lines*. Other possible link is given as a *thin line*. *Numbers* superimposed on links between haplotypes indicate the number of mutational differences between haplotypes

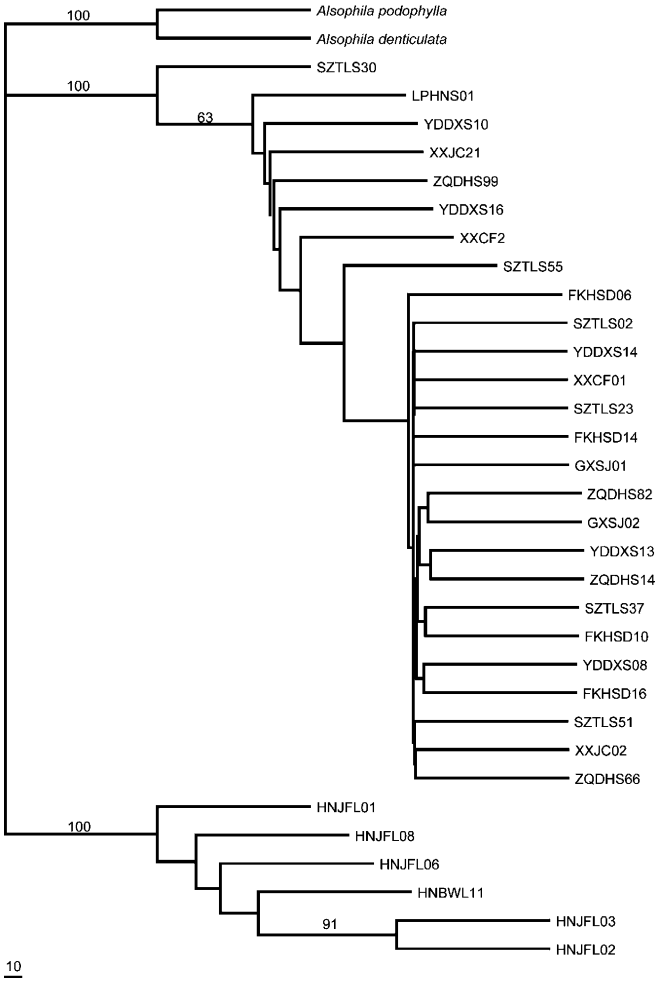

Fig. 3 The neighbor-joining tree of *A. spinulosa*, rooted using *A. podophylla* and *A. denticulata* as outgroups, based on sequences of haplotypes of the *atp*B–*rbc*L intergenic spacer of cpDNA. *Numbers* above branches indicate the bootstrap values of 1,000 replicates

Table 3 Pairwise comparisons of *Nm* (*above diagonal*) and F_{ST} (*below diagonal*) between populations of *A. spinulosa*, based on sequences of the *atp*B–*rbc*L intergenic spacer of cpDNA

	SZTLS	YDDXS	FKHSD	XXJC	XXCF	LPHNS	ZQDHS	GXSJ	HNJFL	HNBWL
SZTLS	–	1.88	2.44	7.46	7.46	7.46	2.72	2.14	0.03	0.03
YDDXS	0.10334	–	1.80	3.75	3.75	3.75	1.85	1.79	0.03	0.03
FKHSD	0.02632	0.08571	–	2.25	2.25	2.25	1.70	1.00	0.02	0.01
XXJC	0.05000	0.14286	0.00000	–	–	–	3.50	0.50	0.03	0.00
XXCF	0.05000	0.14286	0.00000	–		–	3.50	0.50	0.03	0.00
LPHNS	0.05000	0.14286	0.00000	–	–	–	3.50	0.50	0.03	0.00
ZQDHS	0.02907	0.09317	0.00000	0.00000	0.00000	0.00000	–	1.11	0.02	0.01
GXSJ	0.02632	0.08571	0.00000	0.00000	0.00000	0.00000	0.00000	–	0.03	0.01
HNJFL	0.94557	0.94334	0.95135	0.97110	0.97110	0.97110	0.95346	0.95135	–	8.75
HNBWL	0.97432	0.97044	0.97778	1.00000	1.0000	1.00000	0.98131	0.97778	0.00000	–

Discussion

Compared with seed plants, relatively few studies on patterns of genetic variation and geographical differentiation have been reported in ferns. Recently, Pryor et al. (2001) characterized microsatellites in the genome of the maidenhair fern (*Adiantum capillus-veneris*) and developed a marker system to dissect the genetic diversity and structure of its sporophyte populations within the UK and Ireland. Using microsatellite markers, high levels of genetic diversity were detected in *A. capillus-veneris*, with nearly all variation partitioned among rather than within populations. Trewick et al. (2002) explored origins of polyploidy and phylogeography of the rockfern *Asplenium ceterach* throughout Europe, based on cpDNA *trn*L–*trn*F noncoding sequence data. Apart from identifying at least six independent origins of polyploids, they found that tetraploid populations were widely distributed across Europe and beyond, whereas diploid populations were scarcer and predominated in the Pannonian–Balkan region (Trewick et al. 2002).

In seed plants, factors determining genetic structure of populations include mating system, gene flow, selection pressure, mutation, genetic drift, intraspecific phylogeny, evolution process, life history, and physical features of habitat (Loveless and Hamrick 1984). Whether these are also the determinants of populations of ferns still remain unclear. Influenced by the Quaternary glaciers, ancestral populations of *A. spinulosa* were forced to retreat into refugia and survived in the tropical and subtropical montane zone. Thus, they provide ideal materials for testing factors shaping fern population structures. In this study, we investigated the gene genealogies, apportionment of genetic diversity, and geographical differentiation of relict populations of *A. spinulosa*, based on sequence variation of the cpDNA *atp*B–*rbc*L intergenic spacer.

Effects of natural selection on the evolution of *atp*B–*rbc*L spacer of *A. spinulosa* were tested by Tajima's method, showing that the observed extent of sequence divergence was in agreement with the predictions of neutral theory. Thus, in terms of evolution, the spacer is neutral. This fact indicates that haplotype distribution of *atp*B–*rbc*L is influenced more by demographic events in population history than by selection, giving the spacer an advantage for reconstructing phylogeographical patterns (Beebee and Rowe 2004). *A. spinulosa* possessed a high level of haplotype diversity (h=0.744) and nucleotide diversity (D_{ij}=0.01130) as well. This is probably associated with its long evolutionary history, which accumulated genetic variation within lineages. Moreover, as a noncoding region, the *atp*B–*rbc*L spacer of cpDNA has relatively few functional constraints, mutations to some extent would have been retained within each lineage (Chiang and Schaal 1999). The same evolutionary feature of cpDNA spacers was previously reported in many tree species, such as oak, *Fagus*, and *Michelia formosana* (Demesure et al. 1996; Petit et al. 1997; Lu et al. 2002).

In the past, ferns have been expected to have very little population genetic structure because of their capability of producing abundant (several million each plant per year), very small (≈40 μm), wind-dispersed haploid spores and potential to found populations from single spores via intragametophytic fertilization (van Zanten 1978; Schneller and Holderegger 1996; Vogel et al. 1999a). However, in some fern species, interpopulation diversity and restricted gene flow have been revealed (Li and Haufler 1999; Ranker et al. 2000; Pryor et al. 2001). In this study, both the MSN and NJ tree constructed from *atp*B–*rbc*L haplotypes suggested the existence of two geographically differentiated groups, Hainan and Guangdong + Guangxi, in *A. spinulosa* (Figs. 2, 3). This population subdivision was also supported by high interregional observed F_{ST}

Table 4 Analysis of molecular variance for populations of *A. spinulosa*, based on sequences of the *atp*B–*rbc*L intergenic spacer of cpDNA

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	*P*-value
Among regions	1	318.796	13.89246	93.49	<0.001
Among populations within regions	8	19.665	0.23003	1.55	>0.05
Within populations	65	47.939	0.73752	4.96	>0.05
Total	74	386.400	14.86001		

Table 5 Average number of nucleotide differences (*below diagonal*) and nucleotide substitution per site (*above diagonal*) between populations

Populations	1 SZTLS	2 YDDXS	3 FKHSD	4 XXJC	5 XXCF	6 ZQDHS	7 LPHNS	8 GXSJ	9 HNJFL	10 HNBWL
1	–	0.00180	0.00145	0.00077	0.00077	0.00132	0.00145	0.00145	0.02942	0.02851
2	1.306	–	0.00172	0.00103	0.00103	0.00158	0.00103	0.00172	0.03099	0.03008
3	1.056	1.250	–	0.00069	0.00069	0.00124	0.00069	0.00137	0.03198	0.03108
4	0.556	0.750	0.500	–	0.00000	0.00055	0.00000	0.00069	0.02995	0.02905
5	0.556	0.750	0.500	0.000	–	0.00055	0.00000	0.00069	0.02999	0.02909
6	0.956	1.115	0.900	0.400	0.400	–	0.00055	0.00124	0.03051	0.02960
7	0.556	0.750	0.500	0.000	0.000	0.400	–	0.00069	0.02995	0.02905
8	1.056	1.250	1.000	0.500	0.500	0.900	0.500	–	0.03198	0.03108
9	21.181	22.375	23.125	21.625	21.625	22.025	21.625	23.125	–	0.00086
10	20.556	21.750	22.500	21.000	21.000	21.400	21.000	22.500	0.625	–

(=0.95) value and AMOVA, which indicated that 93.49% of variation was partitioned among regions ($P<0.001$, Table 4). Additionally, DNA divergence between pairwise populations from different regions was apparently higher than that within each region (Table 5). To search for factors resulting in this geographical division, initially we hypothesized that population genetic structure may be related to geographical distance, as some theoretical results showed that measures of genetic differentiation at neutral loci would increase with geographical distance (Kimura and Weiss 1964; Maruyama 1971; Nagylaki 1976). Thus, linear regression analysis was performed to assess the association between *Nm* and distance; however, no correlation was detected between them. The *Nm* value between Hainan and Guangdong + Guangxi was approximated as 0.03, far less than 1, indicating that ongoing gene flow was severely hindered according to Wright's theory (Wright 1931).

Distribution of *A. spinulosa* in China is restricted between 18.5°N and 30.5°N. In this area, during the late Tertiary and the early Quaternary Periods, Hainan was separated from the Chinese mainland by shifts in the location of regional landmasses due to plate tectonics and subsequent division by rising sea levels. During the late Pleistocene Epoch, global sea level dropping possibly linked Hainan to the mainland again, but with the advent of the following warm period in the Holocene Epoch, Hainan became isolated again (Xing et al. 1995). Since this time, migrations of individuals of *A. spinulosa* between Hainan and Guangdong + Guangxi were obstructed by the Qiongzhou Strait (with width of 20–40 km). Even though *A. spinulosa* produces very small, wind- or water-dispersed spores, spore dispersal across oceans was hardly achieved because of their weak vitality (e.g., loss of vitality around 8 days, Cheng et al. 1990). Changes in environmental factors, such as humidity, temperature, and illumination, were also lethal to germinating spores. Therefore, lack of effective gene flow due to vicariant events might be among the main reasons to facilitate interregional population differentiation of *A. spinulosa*. Previously, in other ferns, including *Polypodium* (Li and Haufler 1999), *Asplenium csikii* (Vogel et al. 1999a), *Odontosoria chinensis* (Ranker et al. 2000), and *A. capillus-veneris* (Pryor et al. 2001), restricted gene flow has been proposed as a major factor accounting for the interpopulation diversity observed.

The mating system of *A. spinulosa* may be another determinant for shaping its population genetic structure. In ferns, cross-fertilization requires two gametophytes to be close enough for the sperm of one individual to reach the egg of another (Tryon and Tryon 1982). Moreover, fertilization between prothallia of *A. spinulosa* highly depends on water in the niche, through which the sperm can swim from the antheridium to the archegonium (Cheng et al. 1990). These prerequisites hinder the chances of fusion of sperm and egg from different gametophytes. Observation on the reproduction of *Alsophila* species and hybrids noted that their female and male gametes mature at about the same time, indicating the potential for self-fertilization (Conant 1990). In seed plants, generally, inbreeding species partition most variability among populations, whereas outcrossing species maintain most genetic variability within rather than among populations (Brown 1979; Hamrick et al. 1979; Gottlieb 1981). This suggestion has been upheld in some pteridophytes, such as populations of *Blechnum spicant* and *Polystichum otomusui* (Soltis and Soltis 1988; Maki and Asada 1988). If this is also the case in *A. spinulosa*, either the fusion of sperm and egg resulting in a homozygote from the same gametophyte (intra-gametophic selfing) or the fusion of sperm and egg from different gametophytes derived from spores from the same parental sporophyte (inter-gametophic selfing) will enhance the apportionment of genetic variation among populations at interregional level.

At the intraregion level, frequent gene flow was maintained in populations of *A. spinulosa*, as demonstrated by an *Nm* of 8.75 within Hainan and an *Nm* ranging from 0.50 to 7.46 within Guangdong + Guangxi. However, here we would not propose efficient ongoing gene flow between extant populations of *A. spinulosa* within each region. Instead, we suggest that high *Nm* values are likely to represent historical migration events (Lu et al. 2001), considering its fragmentation of modern habitats,

the constraint of migratory capabilities of sporophytes, and gametophytes and the fragility of spore vitality. In this research, for both of the populations investigated in Hainan and six of the eight populations sampled in Guangdong + Guangxi, star-like phylogeographical patterns of *A. spinulosa* were revealed (Fig. 2). This relatively simple pattern features in most haplotypes linked to central haplotypes with short branch length and means that remnant populations preserved in refugia have experienced population expansion after glaciation. Since then, there has been insufficient time to form a more complicated population structure (Dynesius and Jansson 2000). Geological evidence has shown that during the early Pleistocene, a 20,000-year warm period followed ice ages that occurred at regular intervals of 100,000 years (Milankovich cycles, Bennett 1990). Accompanying the oscillation of climate, *A. spinulosa* survived in refugia, like some other ferns, via the production of abundant, long-range dispersed spores (van Zanten 1978), may have had to periodically expand populations into the low-light habitats within forest ecospaces created by the expansion of angiosperms (Schneider et al. 2004). Both the bottleneck effect and founder effect on the population structure of *A. spinulosa* have been inferred by using RAPD markers as well (Wang et al. 2003a).

This research investigated the genetic structure and geographical differentiation of relict populations of a tree fern species, *A. spinulosa*, based on sequence variation of the *atp*B–*rbc*L intergenic spacer of cpDNA. It provided some insights into the effects of factors, such as ongoing gene flow, historical migrations, and mating systems, on the population structure of ferns. Gene genealogies together with coalescent theory have been demonstrated as a useful tool for uncovering phylogeography of *A. spinulosa*. Further determination of ancestral haplotypes and their migratory routes will be helpful to understand the evolutionary history of the species.

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