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High genetic diversity and low genetic differentiation in the relict tree fern *Sphaeropteris brunoniana* (Cyatheaceae) revealed by amplified fragment length polymorphism (AFLP)

Zi-Juan WANG[1, 2] and Kai-Yun GUAN[1,*]

[1]*Key Laboratory of Economic Plants and Biotechnology, Kunming Institute of Botany, The Chinese Academy of Sciences, Kunming 650204, Yunnan, P.R. China*

[2]*Graduate University of Chinese Academy of Sciences, Beijing 100049, P.R. China*

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ABSTRACT. Amplified fragment length polymorphism (AFLP) was employed to analyze the population genetics of relict tree fern *Sphaeropteris brunoniana*. A total of 132 individuals from ten natural populations in China and Laos were collected for this study. Using two selective primers, 234 reliable bands were generated, of which 221 (94.4%) were polymorphic. Genetic analysis indicated an unexpectedly high level of genetic diversity in *S. brunoniana* (H_t= 0.333, H_{sp}= 0.499), but a low level of genetic differentiation among populations (G_{st}= 0.16, φ_{ST}= 0.12 and (H_{sp}-H_{pop})/H_{sp}= 0.16). This result may be due to its life traits, evolutionary history and gene flow. Two major genetic groups, one from Yunnan and the other from Hainan-Laos, were detected among the ten investigated populations. The Mantel test correlated this differentiation with geographic distance. However, the different population origins may also have an effect on this differentiation. Based on these findings, implications for conservation strategies of this species in China are discussed.

Keywords: AFLP; Conservation strategy; Genetic differentiation; Genetic diversity; *Sphaeropteris brunoniana*.

INTRODUCTION

Cyatheaceae is a relatively large pantropical family with about 500 extant species in the world (Tryon and Gastony, 1975). Because the tree-like rhizome of Cyatheaceae plants distinguishes them from other Filicales ferns, they are usually called tree ferns. It is said that some tree ferns are relics of a time when dinosaurs were common. Their trunks are used in the construction of garden troughs and as fiber pots. In order to protect these cherished plants from overexploitation and trade, the Convention on International Trade in Endangered Species (CITES) have recognized this family since 1975 (Oldfield, 1995). Cyatheaceae species were also listed in the secondary category of state-protected wild plants in China in 1999 (Yu, 1999). There are 14 species and 2 varieties distributed in China, while *Sphaeropteris brunoniana* (Hook.) R. M. Tryon is the only *Sphaeropteris* species found in mainland China (Zhang, 2004).

Sphaeropteris brunoniana is a large terrestrial tree fern endangered due to loss of ideal habitats in China. Its fronds are tripinnate and can reach 2~3 m in length and about 1.5 m in width. The fronds are not generally persistent and may leave distinct rounded scars on the trunk. The trunk is usually massive, bearing many fibrous roots, and can grow to a height of over 20 m. Unlike tree trunks, however, it lacks a secondary cambium and does not produce annual vascular rings, thus making age calculations difficult; nevertheless, Large and Braggins (2004) pointed out that some tree ferns can live over 100 years, some even in excess of 200 years. Mehltreter and García-Franco (2008) estimated that a 10 m tall *Alsophila firma* tree fern was probably 60 yr old based on the trunk growth rate. At present there is no information about the longevity of *S. brunoniana*, but based on the above sources/publications, it is probably a long-lived species. The distribution of *S. brunoniana* ranges from northeast India through Bangladesh to Burma and into Vietnam, with China as its northern limit (Zhang, 2004). It usually grows in the margins of evergreen broad-leaved forests and near ravines, where it is easily disturbed by humans. In southeast Yunnan province, many of its habitats have been occupied to grow rubber, tea or bananas, thus many natural populations have been severely damaged. Currently, many measures have been taken to protect this relict species in China. These include the es-

*Corresponding author: E-mail: guanky@mail.kib.ac.cn; Tel: +86-871-5223090; Fax: +86-871-5219933.

tablishment of nature reserves in Hainan province and the introduction of individual plants to Xishuangbana Botanical Garden and Kunming Botanical Garden. A few tissue culture studies have also been conducted by Kunming Botanical Garden and Harbin Normal University (Wang et al., 2007; Chen et al., 2008).

Genetic variation is usually believed to be a prerequisite for both the short-term and long-term survival of a species, and the importance of maintaining genetic diversity of both wild and domesticated species is widely acknowledged today (Shah et al., 2008). Genetic information about a species can provide useful insights when developing effective conservation strategies (e.g., Kingston et al., 2004; Chen et al., 2007; Kim et al., 2008). Studying genetic patterns within *S. brunoniana* is important to protect this single representative of the genus *Sphaeropteris* in mainland China. In the present study, we employed amplified fragment length polymorphism (AFLP) to conduct genetic analyses. AFLP, first described by Vos et al. (1995), has been widely used to investigate genetic diversity and population structure (e.g., Guthridge et al., 2001; Andrade et al., 2007; Tatikonda et al., 2009).

Using AFLP, a total of 132 individuals was analyzed. We were particularly interested in the following questions: (1) What is the level of genetic diversity in *S. brunoniana*? (2) How is the genetic diversity partitioned within and among populations? and (3) What do the results suggest for the conservation strategies of this species in China?

MATERIALS AND METHODS

Plant materials

Ten populations of *S. brunoniana*, nine from China and one from Laos, were used in this study. Among the nine Chinese populations, five were collected from the disturbed margins of secondary evergreen broad-leaved forests in Yunnan province, and the others were collected from natural reserve areas in Hainan province (Table 1). The number of individuals sampled per population ranged from 8 to 15. Young and healthy leaves from each individual were collected and immediately dried with silica gel and stored at room temperature. Voucher specimens were deposited at the Herbarium of the Kunming Institute of Botany (KUN).

DNA preparation and AFLP reactions

DNA was extracted from dried and frozen leaf tissues following the CTAB method (Doyle and Doyle, 1987). AFLP procedure was carried out based on the method of Vos et al. (1995) with some modifications. Genomic DNA was digested in a reaction mixture of 20μl by *Eco*RI and *Mse*I, respectively. The PCR program followed Saunders et al. (2001). The selective amplification was performed with two primer combinations: E-ACA/M-CAC and E-ACT/M-CAC, which were chosen from 16 primer pairs screened with 5 randomly selected samples. All reactions were run on an Eppendorf Master Cycler (Gradient

Table 1. Location, altitude, sample size, voucher, and genetic variability in the populations of *Sphaeropteris brunoniana* revealed by AFLP data.

Population code	Location	Altitude (m)	Sample size	Voucher	PPB (%)	H_e	I
Yunnan region			68		91.9	0.316 (0.162)	0.474 (0.213)
B	Yingjiang, 97°35′ E/24°40′ N	684-1470	15	07031-07045	85.0	0.297 (0.173)	0.445 (0.236)
D	Luxi, 98°29′ E/24°31′ N	1300	15	07061-07075	80.8	0.280 (0.187)	0.418 (0.257)
M	Hekou, 103°57′ E/22°36′ N	321	15	08120-08134	80.8	0.275 (0.183)	0.413 (0.253)
N	Jinping, 103°02′ E/22°38′ N	735	15	08135-08149	80.3	0.286 (0.185)	0.425 (0.256)
P	Xishuangbana, 101°30′ E/21°31′ N	836	8	08180-08187	76.5	0.277 (0.195)	0.411 (0.269)
Hainan-Laos region			64		94.4	0.307 (0.154)	0.466 (0.200)
F	Wuzhishan, 109°41′ E/18°54′ N	600-669	12	08031-08042	84.6	0.304 (0.178)	0.452 (0.243)
H	Diaoluoshan, 109°52′ E/18°44′ N	880-923	15	08061-08075	80.8	0.264 (0.184)	0.399 (0.252)
I	Jianfengling, 108°52′ E/18°44′ N	812-998	14	08076-08089	83.8	0.286 (0.180)	0.429 (0.246)
J	Changjiang, 109°11′ E/19°06′ N	1010-1020	14	08090-08103	79.5	0.275 (0.190)	0.411 (0.262)
K	Khamkeut, Laos, 104°57′ E/18°12′ N	750	9	WS224-232	73.9	0.262 (0.191)	0.391 (0.267)
Average within populations			13.2		80.6	0.281 (0.185)[1]	0.419 (0.254)[2]
Species level			132		94.4	0.333 (0.146)[3]	0.499 (0.189)[4]

PPB: percentage of polymorphic bands; He: Nei's gene diversity; I: Shannon's information index. Values in brackets are standard deviations.

[1]Average Nei's gene diversity within populations (H_s).

[2]Average Shannon's information index within populations (H_{pop}).

[3]Nei's gene diversity at species level (H_t).

[4]Shannon's information index at species level (H_{sp}).

5331). All enzymes were provided by Fermentas and adapters and primers were synthesized by TakaRa. AFLP fragments were separated and detected by the CEQ8000 Genetic Analysis System (Beckman Coulter) by loading 0.3 μl of selective amplification products with 20 μl of sample loading buffer and 0.2 μl of Beckman internal size standard-600.

Data analysis

AFLP bands of each individual were scored as absence (0) or presence (1). The resulting 0/1 data matrix was checked manually before being used for genetic analyses. The genetic diversity was measured at population and species levels using the percentage of polymorphic bands (*PPB*), Shannon's information index (*I*) (Lewontin, 1972) and Nei's (1973) gene diversity (H_e) (assuming Hardy-Weinberg equilibrium). To examine the genetic population structure, the coefficient of gene differentiation (G_{st}) was calculated among populations and between regions. The level of gene flow (*Nm*) was indirectly estimated from the formula: $Nm = (1- G_{st})/4G_{st}$ (Slatkin and Borton, 1989). The genetic differentiation between populations was also quantified using Nei's genetic distance (*D*) (Nei, 1978). To visualize their genetic relationships, based on this distance, a cluster analysis was performed using the unweighted pair group method of arithmetic averages (UPGMA) (Sneath and Sokal, 1973). These analyses were conducted with the software POPGENE 1.32 (Yeh et al., 1997). To test for a correlation between Nei's genetic distances and geographic distances (Km), a Mantel test (Mantel, 1967) was performed using TFPGA (Miller, 1997) with 1000 permutations.

Additionally, an analysis of molecular variance (AMOVA) was performed to estimate variance components for AFLP phenotypes, partitioning the variations between regions, and among populations and individuals using ARLEQUIN ver. 3.1 software (Excoffier et al., 2006). The φ-statistics (Excoffier et al., 1992) were also quantified. The significance of the variance components and φ-statistics were tested using 1000 permutations.

RESULTS

Genetic diversity

Across all samples of *S. brunoniana*, the two primer pairs generated a total of 234 reliable bands that ranged from 99 to 502 bp in size, of which 221 (94.4%) were polymorphic. The percentage of polymorphic bands (*PPB*) at population level ranged from 73.9% (K) to 85.0% (B), with 80.6% on average. Nei's gene diversity within populations (H_s) was 0.281 and at species level (H_t) was 0.333. Shannon's information index was 0.419 at average intra-population level (H_{pop}) and was 0.499 at species level (H_{sp}). Among the ten populations, population F had the highest genetic diversity, with H_e= 0.304 and I= 0.452, while population K exhibited the lowest diversity, with H_e= 0.262 and I= 0.391. According to their geographical distribution, when dividing the ten populations into two groups, the Yunnan group possessed 91.9% of *PPB*, while the Hainan-Laos group held 94.4% of *PPB*. Nei's gene diversity within the regions Yunnan and Hainan-Laos was 0.316 and 0.307, respectively; and Shannon's information index was 0.474 and 0.466, respectively. Based on the latter two parameters, *S. brunoniana* distributed in Yunnan had slightly more genetic diversity than that in Hainan-Laos. Details are shown in Table 1.

Genetic divergence

Nei's genetic distance (*D*) was highest (0.112) between populations B and J, while the lowest was 0.020, occurring between populations M and N (Table 2). This distance ranged from 0.020 to 0.066 between the Yunnan populations, from 0.023 to 0.060 between the Hainan-Laos populations, and from 0.076 to 0.112 between the populations of the two different regions.

The coefficient of gene differentiation (G_{st}) among populations was estimated as 0.16, indicating that most of the total genetic diversity occurred within populations (84.0%). The Shannon's information index also showed that the major variation was held within populations (H_{pop}/H_{sp} = 84.0%). These results were further confirmed

Table 2. Geographic distance (Km) (above diagonal) and Nei's (1978) genetic distance (*D*) (below diagonal) between populations of *Sphaeropteris brunoniana*.

Population	B	D	M	N	P	F	H	I	J	K
B	-	92	687	598	533	1403	1430	1340	1347	1047
D	0.027	-	596	508	456	1314	1342	1252	1258	970
M	0.046	0.053	-	94	279	725	753	670	670	500
N	0.056	0.066	0.020	-	201	807	835	748	751	532
P	0.056	0.054	0.033	0.034	-	901	927	829	844	514
F	0.086	0.077	0.089	0.092	0.087	-	28	88	57	505
H	0.101	0.085	0.087	0.087	0.091	0.024	-	105	84	523
I	0.091	0.086	0.083	0.087	0.080	0.028	0.028	-	53	418
J	0.112	0.099	0.091	0.098	0.105	0.027	0.027	0.023	-	457
K	0.102	0.103	0.091	0.089	0.080	0.060	0.060	0.050	0.057	-

Table 3. Analysis of molecular variance (AMOVA) of *Sphaeropteris brunoniana* based on AFLP data.

Source of variation	d.f.	SSD	Variance components		φ-statistics	*P*-value
			Absolute	%		
Total						
Among populations	9	917.336	5.037	12.4	φ_{ST}= 0.12	<0.01
Within populations	122	4355.982	35.705	87.6		
Yunnan vs. Hainan-Laos						
Among regions	1	433.508	5.641	13.0	φ_{CT}= 0.13	<0.05
Among populations within regions	8	483.828	1.894	4.4	φ_{SC}= 0.05	<0.01
Within populations	122	4355.982	35.705	82.6	φ_{ST}= 0.17	<0.01

d.f.: degree of freedom; SSD: sum of squared deviations.

by AMOVA analysis (Table 3), which revealed that 87.6% of the total genetic variance was attributed to intra-populations and only 12.4% was partitioned among populations. Calculated from the value of G_{st}, the level of gene flow (*Nm*) among populations was 1.31. The genetic differentiation of this species was low between the regions of Yunnan and Hainan-Laos, with G_{st}= 0.06 and *Nm*= 3.92. Shannon's information index and AMOVA analysis showed that only 5.8% and 13.0% of the total variation occurred between the two regions, respectively. Although the divergences at population and regional levels were low, they were significant (φ_{ST}= 0.12, *P*< 0.01; φ_{CT}= 0.13, *P*< 0.05) (Table 3). The Mantel test revealed that the genetic differentiation of *S. brunoniana* in the investigated populations was directly related to physical distance, with *r*= 0.837 and *P*= 0.001.

Two major clusters of populations that correlated to their geographical distribution were identified in the UPGMA dendrogram (Figure 1). One cluster was composed of populations from Yunnan region, while the other contained populations from Hainan-Laos. In the Yunnan cluster, populations B and D collected from the west of Yunnan province were further clustered, while the others collected from the southeast of Yunnan were grouped. In the Hainan-Laos cluster, all the populations sampled from Hainan province were clustered together. This result showed that the samples collected from areas that were geographically closer were grouped together and that the *S. brunoniana* distributed in the two regions were significantly different from each other.

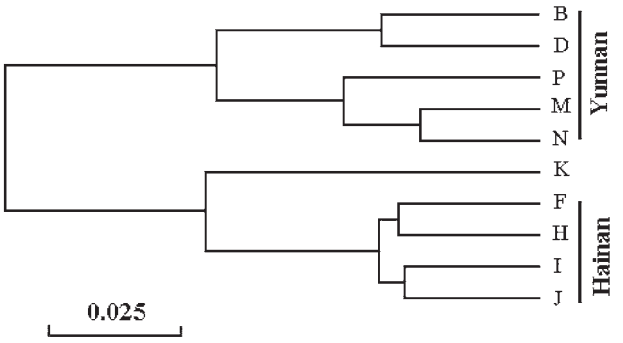

Figure 1. UPGMA dendrogram based on Nei's (1978) genetic distance showing relationships among *Sphaeropteris brunoniana* populations. Populations B, D, P, M and N located in Yunnan province. Populations F, H, I and J located in Hainan province and population K located in Laos.

DISCUSSION

High genetic diversity within the relict species

In this study, the genetic variation based on AFLP data was investigated for *S. brunoniana*. Despite its endangered status, unexpectedly high levels of genetic diversity were detected, with H_t= 0.333, H_{sp}= 0.499 and H_s= 0.281, H_{pop}= 0.419. These values were much higher than those for the other two Cyatheaceae species in China: *S. lepifera* (H_s= 0.057 and H_t= 0.064, Chen, 1995) and *A. spinulosa* (H_s= 0.141, Cheng et al., 2008) from Taiwan using allozyme markers, *A. spinulosa* from mainland China using RAPDs (H_s= 0.0132, H_{pop}=0.0136 and H_t= 0.0590, H_{sp}= 0.0560, Wang et al., 2004). The genetic parameters of *S. brunoniana* were also higher than the values reported for three other tree ferns from Costa Rica, based on allozyme data (Soltis et al., 1991). The different levels of genetic diversity for tree ferns may be explained, in part by the use of different molecular markers, and in part by the different sampling strategies, such as using the maximum geographic distance between sampled populations.

The genetic diversity within populations of *S. brunoniana* (H_s= 0.281) was also higher than the average value for plants (H_s= 0.23) compiled by Nybom (2004) based on AFLP literature, whereas it was close to the average values for long-lived (H_s= 0.25) and outcrossing species (H_s= 0.27) based on RAPD data statistics (Nybom, 2004). *S. brunoniana* must thus be a long-lived species. Moreover, Soltis et al. (1991) reported the outcrossing trait in three tree ferns, viz. *A. firma*, *Cyathea stipularis* and *Lophosoria quadripinnata*. Except *A. loheri*, the six other Cyatheaceae species native to Taiwan have been examined and have tended toward intergametophytic mating, especially intergametophytic crossing (Chen, 1995; Chiou et al., 2000, 2003). Although there is no information on the reproductive biology of *S. brunoniana* in the literature, the results

above suggest that outcrossing is the likely mating system. Generally, long-lived and outcrossing species tend to be more genetically diverse and have less genetic differentiation among their populations (Hamrick and Godt, 1996b; Nybom, 2004). In addition, geographically widespread species tend to possess higher genetic diversity than narrowly-distributed species (Hamrick and Godt, 1996a). The distribution range of *S. brunoniana* extends from northeast India, through Bangladesh and Burma, all the way to Vietnam (Zhang, 2004). Field observations showed that the vertical distribution of this species varied from about 300 m to 1,500 m (Table 1). To adapt to various environments, a species has to evolve and accumulate genetic variation. Hence, in *S. brunoniana*, the high level of genetic diversity may be due to its evolution and life history traits.

Low genetic differentiation among populations and between regions

Based on AFLP data, a low level of genetic differentiation was detected in the ten populations of *S. brunoniana*. The results of AMOVA, Shannon's information index and the coefficient of gene differentiation all revealed that the proportion of total variation partitioned among populations was small (12.4%, 16.0% and 16.0%, respectively). The value of G_{st} was lower than the average index (G_{st}= 0.21) compiled by Nybom (2004). The genetic differentiation of a species reflects the interactions of various evolutionary processes including 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). In *S. brunoniana*, the low level of differentiation may be a result of its outcrossing breeding system and frequent gene flow between populations. Outcrossing species tend to have less genetic differentiation among populations (Hamrick and Godt, 1996b). Ferns can generate enormous numbers of wind-dispersed spores; therefore, it is normal that a high level of gene flow was detected in this species (Nm= 1.31). Although the differentiation among populations was low, it was still significant (φ_{ST}= 0.12, P< 0.01). The Mantel test indicated that the genetic differentiation was correlated with the geographic distance among populations.

The genetic differentiation of *S. brunoniana* was also low but significant between the regions Yunnan and Hainan-Laos. The UPGMA dendrogram demonstrated that the investigated populations were subdivided into two geographical groups: Yunnan and Hainan-Laos. Similar population genetic structure was also detected in another Cyatheaceae species, *A. spinulosa*. Su et al. (2004, 2005) and Wang et al. (2004) found that the populations of *A. spinulosa* in China were subdivided into two genetic groups: Hainan and Guangdong-Guangxi, and they speculated such differentiation was related to the blocked gene flow by the Qiongzhou strait and an inbreeding system. Fern spores usually have a high dispersal capacity. Tryon (1970, 1972) stated that distances of 300~500 miles are only a slight barrier to spore dispersal, and that even distances of 2000 miles or more are possible for transport. Hence, it is surprising that the Qiongzhou strait, with a width of 20~40 Km, is able to block the migration of *A. spinulosa* between the two regions. Besides, intergametophytic mating, especially intergametophytic crossing was found in this species (Chiou et al., 2003). Unlike *A. spinulosa*, the interregional differentiation of *S. brunoniana* was related to geographic distance. Otherwise, this differentiation may also correlate with their different origins. According to specimen records and field investigation, we found that the provinces of Guangxi and Guangdong, adjacent to Yunnan and Hainan, had no distribution of this species. Additionally, the Hainan populations were grouped with the Laos population. It is thus probable that the Hainan populations were originally from Southeast Asia. Further studies, with samples from the entire range of this species, are required to fully understand the genetic differentiation of the two groups and the origin of this species.

Conservation implications

Knowledge of the level and distribution of genetic variation is a prerequisite for the establishment of effective and efficient conservation practices (Ge et al., 1998). A major goal of conservation for threatened and endangered species is the maintenance of genetic diversity (Avise and Hamrick, 1996), which is crucial to a species for adaptation to environmental changes, long-term survival and evolution. Based on genetic analyses of AFLP data, an unexpectedly high level of genetic diversity was detected in *S. brunoniana*. This result is encouraging, and suggests that management efforts can focus on other issues, rather than on increasing its genetic diversity. However, in order to maintain the existing diversity, effective actions must be taken on this relict species.

The genetic differentiation among populations of *S. brunoniana* was low. Therefore, *ex situ* species conservation should preferably be conducted on large populations. The Yunnan and Hainan-Laos groups are significantly different from each other. Therefore, whether *ex situ* or *in situ* conservation is used, the populations of the two regions have to be considered. In addition, because of its long life cycle, spore collection should be of practical value for the conservation of this species. It is also recommended that spores be collected from the two different regions.

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基於AFLP分子標記的白杪欐遺傳多樣性分析

王紫娟[1,2]　管開雲[1]

[1] 中國科學院 昆明植物研究所 資源植物與生物技術重點實驗室

[2] 中國科學院 研究生院

本文利用 AFLP 分子標記，對採自中國和老撾的 10 個白杪欐居群共 132 個個體進行了 PCR 擴增，2 對選擇性擴增引物共產生 234 條指紋清晰的可讀條帶，其中 221 條 (94.4%) 爲多態性條帶。分析結果表明，白杪欐具有較高的遺傳多樣性 (H_t= 0.333, H_{sp}= 0.499) 以及較低的居群間遺傳分化 (G_{st}= 0.16, φ_{ST}= 0.12 and (H_{sp}-H_{pop})/H_{sp}= 0.16)，這可能與它特殊的生活特性，進化歷史以及基因流有關。非加權平均法聚類分析 (UPGMA) 揭示這 10 個取樣居群可分爲雲南以及海南 - 老撾兩個不同的遺傳組，Mantel test 顯示這與它們的地理距離有關；此外，這也可能與它們不同的種質來源有關。另外，根據研究的結果我們討論了該物種的保護策略。

關鍵詞：AFLP；保護策略；遺傳分化；遺傳多樣性；白杪欐。