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Research paper

Allozyme Variations of a Widespread Tree Fern, *Alsophila spinulosa* (Hook.) Tryon (Cyatheaceae), in Taiwan

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【Summary】

Allozymes were used to detect the genetic diversity of *Alsophila spinulosa*, a widespread tree fern in Taiwan. Allozyme polymorphisms of 6 enzyme systems, including 9 loci and 12 alleles, were used to estimate the genetic diversity of *A. spinulosa* from 9 sites throughout Taiwan. The mean number of allele per locus (A) was 1.34, the percentage of polymorphic loci (P) was 34.5%, the observed heterozygosity (H_o) was 0.145, and the expected heterozygosity (H_e) was 0.141. The genetic diversity of *A. spinulosa* in Taiwan was higher than mean values of other diploid ferns and tree ferns. The large population size and an intergametophytic outcrossing mating system were thought to be important factors in *A. spinulosa* maintaining a higher level of genetic diversity. Inbreeding has also sometimes occurred, and this might have assisted the successful colonization by a single long-distance dispersal spore. At the population level, the Nanjenshan population (NJ), located in southern Taiwan, showed the highest expected heterozygosity (H_e). Additionally, lower levels of genetic differentiation among populations were detected and are thought to be a result of high gene flow due to spores.

Key words: allozyme variations, *Alsophila spinulosa*, Cyatheaeae, Taiwan.

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研究報告

台灣廣泛分佈之樹蕨類植物台灣桫欏的同功酶遺傳變異

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摘　要

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本研究利用同功酵素研究廣泛分佈於台灣的樹蕨類植物台灣桫欏(*Alsophila spinulosa*)的遺傳變異。測試6個酵素系統中，共獲得9個基因座(loci)和12個對偶基因(alleles)。分析各族群的遺傳歧異度，對偶基因平均數(A)為1.34，多型性基因座比率(P)為34.5%，異質結合度觀察值(H_o)為0.145，異質結合度期望值(H_e)為0.141。和其他蕨類或樹蕨類的遺傳歧異度平均值比較，台灣桫欏具有較高的遺傳歧異度。大的族群規模和配子體間的異交配育系統是造成台灣桫欏維持遺傳歧異度的重要原因。除此之外，部份的自交配育行為可能有助於單一孢子長距離傳播後的族群建立。以族群而言，南台灣的南仁山族群具有最高的異質結合度。經由孢子傳播進行的基因交流和廣泛分佈的特性，是台灣桫欏能維持族群間遺傳低度分化的重要因素。

關鍵詞：同功酶變異、台灣桫欏、桫欏科、台灣。

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INTRODUCTION

Genetic variation in a species indicates its evolutionary potential and the ability to adapt to various environments (Hamrick et al. 1992). Many factors have been considered to influence levels of genetic variation in plant species, including mating systems, gene flow, selection pressures, mutations, genetic drift, life history, and historical events such as glaciation (Loveless and Hamrick 1984, Hewitt 1996, Tomaru et al. 1997). These factors also influence genetic differentiations among populations and reflect levels of gene flow. For example, species with a continuous geographic distribution or seed dispersal by wind might be considered to have a higher efficiency of gene flow and to show lower levels of genetic differentiation (Hamrick et al. 1992). Numerous studies on the genetic diversity of seed plants have been published (Tomaru et al. 1997, Lin et al. 1998, Hiramatsu et al. 2001, Wu et al. 2001, Cheng et al. 2006); however, studies about ferns are relatively rare. In comparison with seed plants, ferns and their allies have different reproductive traits. Two independent generation phases, gametophytes and sporophtyes, are present in their life cycles. They also differ in the mechanisms of gene flow, which is primarily conducted by sexual reproduction by gametophyte and spore dispersal, including homospores and heterospores.

The breeding system is one of the major factors affecting levels of genetic variability both within and among populations (Loveless and Hamrick 1984, Hamrick and Godt 1989, Hamrick et al. 1992). Predominantly outcrossing species tend to have greater genetic diversity within populations and less genetic differentiation among populations than do predominant inbreeders (Hamrick et al. 1992). The breeding systems of ferns, including outcrossing and inbreeding, are primarily determined by independent gametophyte generation. There are 3 major types of mating systems in ferns and fern allies: intragametophytic selfing, intergametophytic selfing, and intergametophytic crossing (Klekowski 1969, 1979). The biology of fern gametophytes provides the opportunity for 2 forms of inbreeding. Intergametophytic selfing is similar to selfing in seed plants and occurs when 2 gametophytes are derived from the same sporophyte. Another type, intragametophytic selfing, is a more-extreme form of selfing which occurs when a single bisexual gametophyte produces a completely homozygous sporophyte. The latter mechanism allows a single dispersed spore to be an effective colonizer which can found a new population after long-distance dispersal. Intergametophytic outcrossing occurs when 2 gametophytes originating from spores of different sporophytes at the same site are sexual mature at the same time, and sperm must travel from 1 to the other gametophyte to unite with the egg.

Alsophila spinulosa (Hook.) Tryon, of the Cyatheaceae, is a diploid tree fern (n = 69, Tsai and Shieh 1983, 1984; 2n = 138, Wang et al. 1997) with a single, erect trunk. From fossil records, it is known that it was distributed worldwide during the Jurassic period (Fu 1991). Extant populations of *A. spinulosa* are mainly restricted to tropical and subtropical regions, including India, China, Japan, and Taiwan (Shieh 1994). This species is a homosporous tree fern, adapted to warm, humid, and shady habitats of lowland forests, and is widespread throughout Taiwan. Homosporous fern gametophytes may have the potential for self-fertilization because of their hermaphroditism. Several studies had proposed that intragametophytic selfing is the primary mode of reproduction for homosporous ferns (Klekowski 1979, Soltis and Soltis 1990a). However, Chiou et al. (2003) studied the mating systems of Cyathaceae native to Taiwan and found that *A. spinulosa* primarily produced sporophytes by intergametophytic outcrossing. Intergametophytic outcrossing might provide more opportunities to gain genetic material from other gametophytes, which would influence the genetic diversity and structure.

Allozyme analysis is a powerful tool for investigating levels of genetic variation within populations and the distribution of variations within and among populations (Loveless 1992, Lin et al. 2000, Cheng et al. 2006). Allozyme markers can also be used to estimate the actual incidence of inbreeding or outcrossing in natural populations (Korpelainen and Kolkkala 1996) and the evolutionary history of a species (Doyle et al. 2003, Crawford et al. 2006). In addition, allozyme data can be used to estimate levels of interpopulational gene flow (Ledig et al. 2006), which can also directly affect population diversity and genetic structure (Wright 1951).

In this study, allozyme loci were used to evaluate the genetic diversity of *A. spinulosa* in Taiwan. The objectives of this study were (1) to estimate the amount and distribution of genetic diversity within and among populations, (2) to compare our results with previous studies of other ferns, and (3) to estimate gene flow among populations. Additionally, factors influencing genetic diversity and structure are also discussed.

MATERIALS AND METHODS

Sampling

In this study, we collected samples of *A. spinulosa* from 9 natural populations in Taiwan; Chihnan (CN), Dasyueshan (DS), Fushan (FS), Hueisun (HS), Nanjenshan (NJ), Taimali (TM), Tengjhih (TJ), Yangmingshan (YM), and Zengwun (ZW) (Fig. 1). In total, 237 individuals were sampled, and the fresh fronds of 21~32 individuals of each population were collected from the field.

Enzyme electrophoresis

Fresh samples were ground up with liquid nitrogen and extracted with buffer according to the protocols of Feret (1971). The extracted enzymes were absorbed onto Whatman 3MM filters (4×12 mm), stored at -80℃ until needed for analysis. Allozyme variation was studied by means of horizontal starch gel electrophoresis using 12% starch gels. The following 6 enzyme systems were examined:

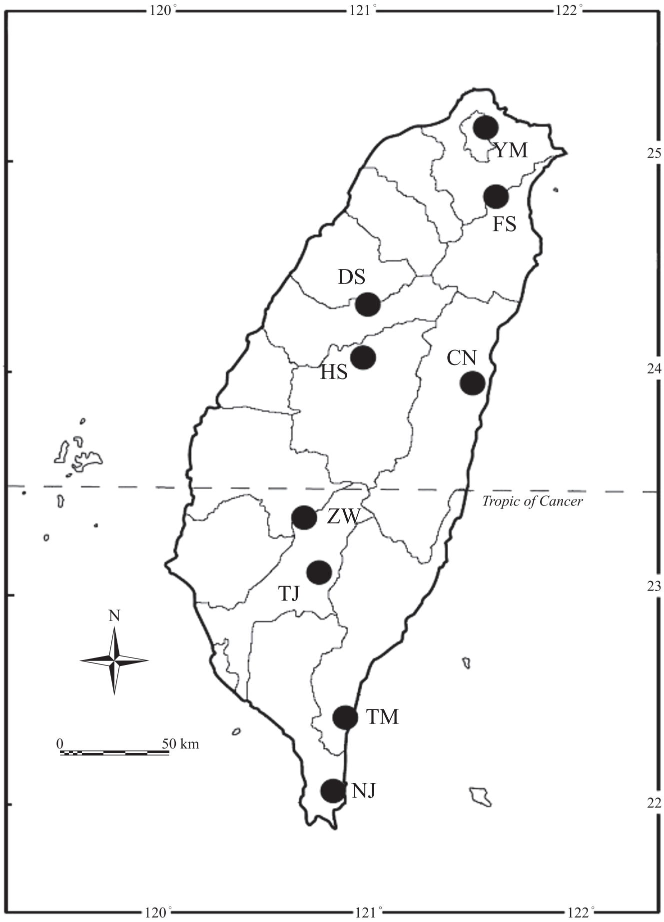

Fig. 1. Map of Taiwan and sampling sites of *Alsophila spinulosa* in this study. CN, Chihnan; DS, Dasyueshan; FS, Fushan; HS, Hueisun; NJ, Nanjenshan; TM, Taimali; TJ, Tengjhih; YM, Yangmingshan; ZW, Zengwun.

EST (esterase, E.C.3.1.1.1), IDH (isocitrate dehydrogenase, E.C.1.1.1.42.), MDH (malate dehydrogenase, E.C.1.1.1.37.), MR (meandione reductase, E.C.1.6.5.2.), 6PGD (6-phosophogluconate dehydrogenase, E.C.1.1.1.44.), and SkDH (shikimic acid dehydrogenase, E.C.1.1.1.25.). Electrophoresis and staining protocols followed the procedures described by Cheliak and Pitel (1984).

Data analysis

Allelic frequencies in each population of *A. spinulosa* were calculated from the loci examined in this study. The following estimated genetic parameters were used to quantify the level of genetic diversity within populations using the BIOSYS-1 program (Swofford and Selander 1981): the mean number of alleles per locus (A), the effective number of alleles per locus (A_e) (Crow and Kimura 1970), the proportion of polymorphic loci (P), the average observed heterozygosity (H_o), and the average expected heterozygosity or genetic diversity ($H_e = 1 - \Sigma p^2_i$, where p_i is the frequency of the *i*th allele at a locus) (Nei 1975).

Wright's (1969) *F*-statistics (F_{IS}, F_{IT}, and F_{ST}) were used to quantify the degree of differentiation among populations and to describe the genetic structure of this species. Values of F_{IS} and F_{IT} indicate the level of deviations from Hardy-Weinberg (H-W) expectations at the population and species levels, respectively. An F_{IS} value equal to or nearly zero indicates that the mating system is random; on the other hand, a significant negative value indicates an excess of heterozygotes, while a significant positive value indicates an excess of homozygotes. The Chi-squared test was used to evaluate whether F_{IS} and F_{IT} values for each locus significantly differed from 0 (Li and Horvitz 1953). Values of F_{ST} indicate genetic variations among populations or the levels of genetic differentiation among populations. Fixation indices (F), reflecting deviations from H-W equilibrium of each population, were calculated by the formula $F = (1 - H_o / H_e)$, and the outcrossing rate (t) was estimated using $t = (1 - F) / (1 + F)$ (Weir 1990). We also estimated the level of gene flow among populations using Wright's (1951) formula, $Nm = (1 - F_{ST}) / 4F_{ST}$.

Nei's (1978) unbiased genetic identity (I) and genetic distance (D) were calculated for all pairs of populations. A cluster analysis based on Nei's genetic distance (Nei 1978) via the unweighted pairwise group method (UPGMA) was used to present relationships among populations. A Mantel test (Mantel 1967) with 1000 random permutations was performed between the matrix of genetic differentiation and geographic distance to test whether isolation by distance was significant. Pairwise genetic differentiation between populations was expressed as $F_{ST} / (1 - F_{ST})$ (Rousset 1997).

RESULTS

Genetic diversity of *A. spinulosa*

The 6 enzyme systems resolved were interpreted as being encoded by 9 putative loci: *EST-2*, *MR-2*, *SkDH-1*, *IDH-1*, *IDH-2*, *MDH-1*, *MDH-3*, *MDH-4*, and *6PGD-1*. Among these 9 loci, *EST-2*, *MR-2*, *6PGD-1*, and *SkDH-1* were polymorphic for at least 1 of the population examined, while the others were monomorphic (Table 1). No significant correlations were found between allelic frequencies in relation to longitudinal or latitudinal gradients (data no shown). Only 1 rare allele, 6PGD-1b, was recorded in the southernmost population, Nanjenshan (NJ).

Measures of genetic diversity are presented in Table 2. The mean number of alleles per locus (A) was 1.34 (1.33~1.44); the effective number of alleles per locus (A_e) was 1.17, the average percentage of ploymorphic loci (P) was 34.5% with a range, from 33.3~44.4%; the observed heterozygosity (H_o) for each population varied from 0.122 to 0.199, with an average of 0.145; and the expected heterozygosity (H_e) varied from 0.120 to 0.172, with an average of 0.141.

At the population level, the southernmost population, Nanjenshan (NJ), exhibited the highest genetic diversity and maintained the highest values of A, P, and H_e, followed by the population at Tengjhih (TJ), southern Taiwan, and Yangmingshan (YM), northern Taiwan. Zengwun (ZW), a southwestern population, exhibited the lowest value of diversity.

Genetic structure and differentiation

Results of the genetic structure analyses of *A. spinulosa* carried out with Wright's *F*-statistics are given in Table 3. The mean F_{IS} value, representing the average deviation from H-W expectations within populations, for all polymorphic loci was 0.0161, and it showed no significant deviation from 0. That indicates that allelic frequencies within populations are close to random mating. Only 2 loci, *EST-2*

Table 1. Allelic frequencies of polymorphic loci in 9 populations of *Alsophila spinulosa* in Taiwan

Locus/Allele	Population								
	CN	DS	FS	HS	NJ	TM	TJ	YM	ZW
EST-2									
a	0.315	0.143	0.203	0.103	0.364	0.339	0.310	0.238	0.037
b	0.685	0.857	0.797	0.897	0.636	0.661	0.690	0.762	0.963
MR-2									
a	0.796	0.589	0.500	0.655	0.500	0.516	0.362	0.548	0.556
b	0.204	0.411	0.500	0.345	0.500	0.484	0.638	0.452	0.444
6PGD-1									
a	1.000	1.000	1.000	1.000	0.978	1.000	1.000	1.000	1.000
b	0.000	0.000	0.000	0.000	0.022	0.000	0.000	0.000	0.000
SkDH-1									
a	0.370	0.304	0.281	0.328	0.455	0.210	0.414	0.405	0.444
b	0.630	0.696	0.719	0.672	0.545	0.790	0.586	0.595	0.556

CN, Chihnan; DS, Dasyueshan; FS, Fushan; HS, Hueisun; NJ, Nanjenshan; TM, Taimali; TJ, Tengjhih; YM, Yangmingshan; ZW, Zengwun.

Table 2. Genetic diversity for each studied population of *Alsophila spinulosa* using allozyme analysis

Population (Code)	Elevation (m)	Longitude (E°)	Latitude (N°)	N	A	A_e	P[1]	H_o	H_e	*F*	*t*
1. Chihnan (CN)	400	121.50	23.92	27	1.33	1.16	33.3	0.123	0.138	0.109	0.803
2. Dasyueshan (DS)	1100	120.80	24.20	28	1.33	1.15	33.3	0.127	0.130	0.023	0.955
3. Fushan (FS)	700	121.58	24.75	32	1.33	1.16	33.3	0.170	0.139	-0.223	1.574
4. Hueisun (HS)	1000	121.00	24.08	29	1.33	1.14	33.3	0.199	0.122	-0.631	4.420
5. Nanjenshan (NJ)	300	120.83	22.08	23	1.44	1.21	44.4	0.130	0.172	0.241	0.612
6. Taimali (TM)	600	120.97	22.60	31	1.33	1.17	33.3	0.122	0.144	0.153	0.735
7. Tengjhih (TJ)	900	121.75	23.08	29	1.33	1.18	33.3	0.134	0.155	0.135	0.762
8. Yangmingshan (YM)	700	121.50	25.17	21	1.33	1.18	33.3	0.169	0.153	-0.104	1.232
9. Zengwun (ZW)	500	121.58	23.25	27	1.33	1.14	33.3	0.132	0.120	-0.100	1.222
Mean				26.1	1.34	1.17	34.5	0.145	0.141	-0.028	1.057

[1] A locus was considered polymorphic if more than 2 alleles were detected. *N*, number of individuals sampled; A, mean number of alleles per locus; A_e, effective number of alleles; P, percentage of polymorphic loci; H_o, observed heterozygosity; H_e, expected heterozygosity; *F*, fixation index, $F = (1 - H_o/H_e)$; *t*, outcrossing rate.

and *SkDH-1*, were found by the Chi-squared test to show significant deviations from H-W expectations. The mean F_{IT} value (0.0720) also showed no significant deviation at the species level. The F_{ST} value indicates the level of genetic differentiation or variation among populations. In this study, the F_{ST} value of each polymorphic locus ranged from 0.0197 to 0.0865, with an average of 0.0568; thus < 6% of the genetic variation was explained

Table 3. Contingency Chi-squared tests (with degrees of freedom, df) and estimates of Wright's (1951) *F*-statistics and gene flow for the polymorphic loci in *Alsophila spinulosa*

Locus	χ^2	df	F_{IS}	F_{IT}	F_{ST}	Nm
EST-2	127.334[1)]	1	0.7180	0.7424	0.0865	2.64
MR-2	2.257	1	0.0956	0.1505	0.0608	3.86
6PGD-1	0.122	1	-0.0222	-0.0120	0.0197	12.44
SkDH-1	85.980[1)]	1	-0.5900	-0.5437	0.0292	8.31
Mean	6.402		0.0161	0.0720	0.0568	4.15

[1)] Chi-squared values are significant at $p < 0.001$. F_{IS}, level of deviation from Hardy-Weinberg (H-W) expectations at the population level; F_{IT}, level of deviation from Hardy-Weinberg (H-W) expectations at the species level; F_{ST}, genetic differentiation among populations; Nm, level of gene flow among populations.

by differences among populations, which indicates a low level of genetic differentiation among populations (Wright 1978). Gene flow (Nm) (Wright 1951) estimated for each polymorphic locus ranged from 2.64 to 12.44 (average, 4.15), which indicated that high gene flow had occurred among *A. spinulosa* populations.

Mean fixation indices (*F*) for each population of *A. spinulosa* ranged from -0.631 to 0.241, with an average of -0.028 (Table 2). Four populations, FS, HS, YM, and ZW, exhibited negative values, indicating an excess of heterozygotes. The outcrossing rate (*t*) based on the fixation indices ranged from 0.612 to 4.420 (Table 2).

Genetic relationship among populations and test of isolation by distance

The unbiased genetic identity (*I*) and standard genetic distance (*D*) between populations (Nei 1978) are summarized in Table 4. The *D* values between populations ranged from 0.0003 to 0.0282 with a mean of 0.009. From the matrix of genetic distance, Fushan (FS) and Dashushan (DS) had the lowest genetic distance (0.0003). The highest genetic distance was between Hueisun (HS) and Nanjenshan (NJ) (0.0282). According to the theory of isolation by distance (Wright 1946), the genetic distance or genetic differentiation might be positively correlated with geographic distance. In this study, we used a

Table 4. Genetic distance (*D*) (below the diagonal) and genetic identity (*I*) (above the diagonal) for populations of *Alsophila spinlosa*

Population	1	2	3	4	5	6	7	8	9
1. Chihnan	****	0.9916	0.9874	0.9929	0.9754	0.9879	0.9768	0.9929	0.9835
2. Dasyueshan	0.0085	****	0.9997	0.9996	0.9782	0.9946	0.9897	0.9991	0.9972
3. Fushan	0.0127	0.0003	****	0.9966	0.9853	0.9982	0.9952	0.9993	0.9940
4. Hueisun	0.0071	0.0004	0.0034	****	0.9718	0.9900	0.9840	0.9972	0.9977
5. Nanjenshan	0.0249	0.0221	0.0148	0.0282	****	0.9877	0.9975	0.9906	0.9732
6. Taimali	0.0122	0.0054	0.0018	0.0100	0.0124	****	0.9928	0.9953	0.9825
7. Tengjhih	0.0235	0.0104	0.0048	0.0161	0.0025	0.0072	****	0.9966	0.9871
8. Yangmingshan	0.0071	0.0009	0.0007	0.0028	0.0095	0.0047	0.0034	****	0.9964
9. Zengwun	0.0166	0.0028	0.0060	0.0023	0.0272	0.0176	0.0130	0.0036	****

Mantel test to detect for a correlation between geographic distance and genetic differentiation measured as F_{ST} / (1 - F_{ST}). The result showed no significant correlation between these 2 matrices ($r = 0.047102$; $p = 0.384615$ with 1000 permutations), indicating that the genetic distance of each population pair had no correlation with its geographic distance.

The UPGMA dendrogram (Fig. 2) of 9 populations, produced using the pairwise genetic distances, roughly generated 2 major groups: 1 including NJ and TJ, located in southern Taiwan, and the other including YM, FS, HS, DS, CN, ZW, and TM located mostly in central and northern Taiwan.

DISCUSSION

Comparison of genetic diversities between *A. spinulosa* and other ferns

Alsophila spinulosa is a widespread and common tree fern in Taiwan. However, due to its rare and restricted populations in China, it is regarded as being in a National Protection Category on the Red List (Fu 1991). Sequence data of chloroplast DNA have been used to discuss genetic structure and phylogeographical patterns of *A. spinulosa* in China. Higher levels of haplotype and nucleotide diversities were detected and it was speculated that these were probably associated with a long evolutionary history (Su et al. 2004). However, a very low level of intraspecific genetic diversity was detected in China, as inferred from RAPD markers, which may have been due to both a bottleneck effect and founder effect associated with glacial events (Wang et al. 2004). In this study, the genetic diversity of *A. spinulosa* in Taiwan as estimated by the allozyme data was higher than values reported for other diploid ferns (Maki and Asada 1998) (Table 5). It was also higher than the average of other tree ferns examined by Soltis et al. (1991) and another tree fern

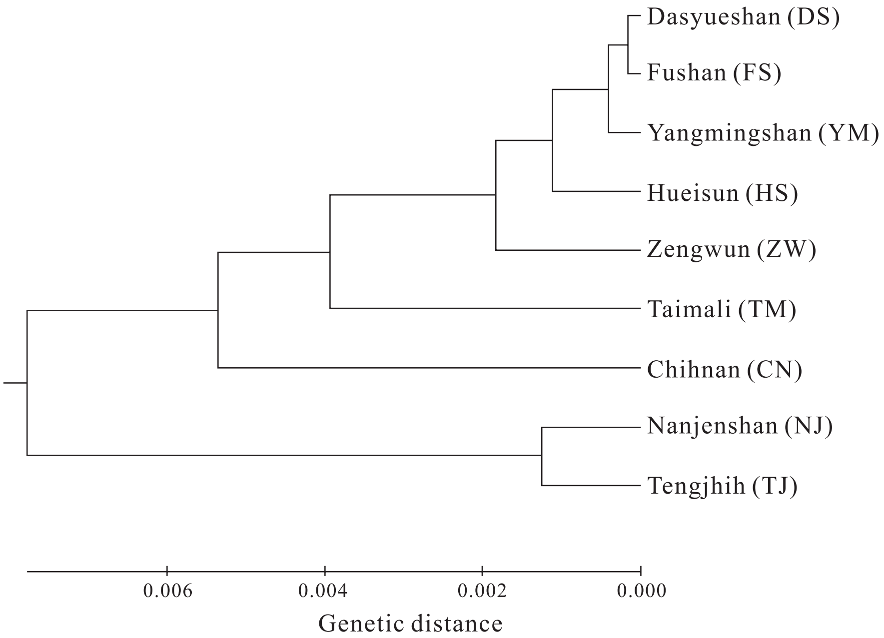

Fig. 2. Dendrogram of the cluster analysis for 9 populations of *Alsophila spinulosa* constructed using the UPGMA method based on Nei's (1978) genetic distance.

Table 5. Comparisons of genetic diversity in *Alsophila spinulosa*, other fern species, and tree species in Taiwan based on allozyme analysis

Species	A[1)]	P[2)]	H_e[3)]	Reference
Alsophila spinulosa	1.3	32.1	0.141	This study
Alsophila firma	1.24	20.0	0.078	Soltis et al. 1991
Cyathea stipularis	1.2	17.5	0.037	Soltis et al. 1991
Lophosoria quadripinnata	1.2	19.0	0.047	Soltis et al. 1991
Sphaeropteris lepifera	1.1	12.5	0.057	Chen 1995
Polystichum otomasui	1.93	61.9	0.177	Maki and Asada 1998
Diploid homosporous ferns	1.52	31.5	0.110	Maki and Asada 1998
Cinnamomum kanehirae	2.0	58.0	0.214	Lin et al. 1997
Mryica rubra	1.9	63.6	0.191	Cheng et al. 2000
Alnus formosana	1.8	59.1	0.175	Sue et al. 2000
Michelia formosana	2.0	75.6	0.241	Lin 2001
Castanopsis carlesii	2.5	69.0	0.270	Cheng et al. 2006
Widespread seed plants	1.72	43.0	0.159	Hamrick and Godt 1989

1) Mean number of alleles per locus.

2) Percentage of polymorphic loci.

3) Mean expected heterozygosity.

species with similar ecological characteristics, *Sphaeropteris lepifera*, in Taiwan (Chen 1995). When compared with other local tree species, the genetic variation of *A. spinulosa* was lower than some widespread tree species previously reported, such as *Alnus formosana* (Sue et al. 2000), *Castanopsis carlesii* (Cheng et al. 2006), *Cinnamomum kanehirae* (Lin et al. 1997), *Michelia formosana* (Lin 2001), and *Myrica rubra* (Cheng et al. 2000) (Table 5).

Factors influencing genetic variation

In a review by Hamrick et al. (1992), the range of distribution, breeding system, and life history were considered to be important factors influencing the level of genetic diversity and genetic differentiation of plant species. As in seed plants, geographically widespread species always have higher levels of genetic variation than their restricted congeners (Sherman-Broyles et al. 1992, Purdy and Bayer 1996). The large population size at the species level of *A. spinulosa* in Taiwan might provide a good opportunity to maintain higher genetic variation than seen for other tree fern species. Although no other congeneric species were studied, we think that *A. spinulosa* may have a higher level of genetic diversity than other species with restricted or isolated distributions in Taiwan.

The breeding system of a species is thought to be a major determinant of patterns of genetic variation in plants (Hamrick and Godt 1989). The outcrossing mating system is thought to predominate in terrestrial ferns (Soltis and Soltis 1987, 1990a, b). Although most diploid homosporous fern species exhibit high outcrossing (Soltis and Soltis 1992), a gametophyte of a homosporous fern may have the potential for self-fertilization because of hermaphroditism, an important mechanism to construct a population from a single spore after long-distance dispersal (Baker 1955, Klekowski 1979). Reported as an outcrossing fern species, *A. spinulosa* produces sporophytes primarily by intergametophytic mating, especially intergametophytic crossing (Chiou et al. 2003). The reasons that *A. spinulosa* maintains relatively higher genetic diversity than other ferns might be closely related to its mating system. The outcrossing habit of *A. spinulosa* may play a key role in the maintenance of its genetic diversity.

Genetic structure and population differentiation of *A. spinulosa*

The fixation index can be used to infer the relationship of a mating system in a population. From Wright's *F*-statistics, the estimated mean value of F_{IS} for the 9 populations showed no deviations from H-W equilibrium. The values of both F_{IS} and F_{IT} values being close to 0 indicates a random mating system. However, their slightly positive values indicate existing homozygosity, which originated from inbreeding within the population and at the species level. This phenomenon might be partially due to restricted dispersal of spores that fell near the mother tree and increased the opportunity for mating between congeners.

Inbreeding events were also indicated from the outcrossing rate (*t*). The *t* values of the populations sampled suggested that *A. spinulosa* is not a completely outcrossing species, but may permit some degree of inbreeding. If inbreeding is indeed significant, the observed heterozygosity should be lower than the expected heterozygosity, such as for the CN, DS, NJ, TM, and TJ populations. Homosporous ferns potentially have bisexual gametophytes that have been suggested to promote inbreeding in natural populations (Klekowski 1969, Haufler and Ranker 1985). Soltis and Soltis (1987) also discussed a variety of mating systems from inbreeding through mixed crossing to outcrossing for some homosporous ferns. The possibility of inbreeding in *A. spinulosa*, suggested by the values of the fixation indices and outcrossing rate conducted in this study, imparts an advantage of being a colonizer able to occupy distant habitats.

Genetic differentiation among populations is primarily a function of gene flow among populations via pollen and seed dispersal in seed plants (Loveless and Hamrick 1984). However, in ferns, gene flow is principally carried out by spore dispersal (Tryon 1986). In general, widespread species should have higher levels of gene flow than species with restricted or isolated populations. In homosporous ferns, the ability for spore dispersal is believed to be higher than the dispersal capability of most seed plants because of their aerodynamic properties (Tryon 1970, Soltis and Soltis 1990b). The low level of genetic differentiation among populations of *A. spinulosa* indicates extensive gene flow among them. The intergametophytic crossing system of *A. spinulosa* might provide many opportunities to increase the gene flow or gene exchange among individuals. Additionally, *A. spinulosa* produces light wind-dispersed spores. The taller trunk may also enhance the capability and opportunity for long-distance dispersal of spores by wind. Thus gene flow via spore dispersal is easy and frequent, which may reduce the genetic differentiation among populations. In conclusion, the higher level of gene flow in *A. spinulosa* is consistent with high spore production, high dispersability, and long spore viability in homosporous ferns (Tryon and Gastony 1975, Tryon 1986). On the other hand, an inbreeding capability enhances the possibility of successful production of a sporophyte from a single long-distance dispersal spore acting as a colonizer to establish a new population.

Correlations among populations

From the UPGMA of 9 populations (Fig.

2), 2 major groups were resolved. The isolation-by-distance analysis showed no significant pattern, suggesting that the distribution of genetic variation might not be explained by the geographic distances separating the populations. The genetic relationships among populations might be determined by gene flow among populations (Martinsen et al. 2001), selection pressures (Hamrick 1982), or historical events (Ge et al. 2005, Gonzalez-Astorga et al. 2005). No significant isolation by distance was also reported from an allozyme analysis of a widespread tree species, *Castanopsis carlesii*, in Taiwan (Cheng et al. 2006). The intermixing of different nuclear loci was thought to be a possible factor causing the erosion of the spatial genetic structure that led to no significance of the isolation-by-distance test for *C. carlesii*. That might also be a key factor for *A. spinulosa*. In addition, historical events have also been considered to be important factors affecting the regional geographic structure (Ge et al. 2005, Gonzalez-Astorga et al. 2005). No significant isolation by distance might also be influenced by postglacial recolonization. The colonization routes of *A. spinulosa* in Taiwan might have occurred in more than 1 direction from different glacial refugia. The multidirectional colonization effect would have promoted intermixing among populations of *A. spinulosa*. However, it is difficult to examine the effects of historical events on the distribution of *A. spinulosa* because the allozyme data cannot provide sufficient detail to reveal the migration history of this species. DNA markers, on the other hand, like chloroplast and mitochondrial DNA, are good at revealing the evolutionary history of plant species.

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