Bot. Bull. Acad. Sin. (2001) **42:** 173-179

RAPD variation in relation to population differentiation of *Chamaecyparis formosensis* and *Chamaecyparis taiwanensis*

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(Received August 25, 2000; Accepted January 19, 2001)

Abstract. The population differentiation of *Chamaecyparis formosensis* and *Chamaecyparis taiwanensis* based on random amplified polymorphic DNA (RAPD) variation is described. Two populations (Chilanshan and Alishan) of these two *Chamaecyparis* species are investigated for population differentiation. Shannon's phenotypic index was used to estimate *Ho* of these two *Chamaecyparis* species based on RAPD variation. RAPD analysis showed that the Chilanshan population had higher genetic diversity than the Alishan population in both *Chamaecyparis* species. These results correlated with the large population found in Chilanshan for both species. Based on RAPD analysis, there was 15.13% population differentiation between Chilanshan and Alishan of *C. formosensis* compared with 14.73% for *C. taiwanensis*. Higher levels of genetic variation and population differentiation indicated dynamic evolution in these two *Chamaecyparis* species in Taiwan as revealed by variation at RAPD loci.

Keywords: *Chamaecyparis formosensis*; *Chamaecyparis taiwanensis*; Population differentiation; RAPD.

Introduction

Information on the amount of genetic variation within a species, and its distribution within and between populations would aid in tree conservation planning. Allozyme has been employed to estimate the genetic variation and population divergence in many plant species (Hamrick et al., 1992). However, with the new types of molecular markers one may gain insight into DNA sequences other than nuclear coding loci for population genetic structure and the life history of long-living tree species.

Chamaecyparis formosensis and *C. taiwanensis* are endemic to Taiwan and are the most valuable timber produced from old-growth forest. According to Lin et al. (1994) there was only 4.6 and 3.9% genetic differentiation among populations of *C. formosensis* and *C. taiwanensis*, respectively, based on allozyme data. *Chamaecyparis formosensis* is found in the elevational range of 800 to 2, 500 m, but is most abundant from 1,500 to 2,100 m; *C. taiwanensis* grows at an elevation of 1,200 to 2,800 m (Liu, 1966). High gene flow would have occurred between populations in these two *Chamaecyparis* species with low population differentiation based on allozyme analysis. Polymorphisms in the chloroplast genome of *C. formosensis* and *C. taiwanensis* were investigated by a PCR-based RFLP (Hwang et al., 2000). No restriction fragment length differences were detected for *trn*V-*trn*M or *pet*G-*trn*P chloroplast intergenic spacers between Chilanshan and Alishan populations in both *Chamaecyparis* species.

RAPD, developed by Williams et al. (1990) and Welsh and McClelland (1990), uses random primers to reveal nucleotide sequence variation. RAPD markers are based on the amplification of unknown DNA sequences using single, short, random oligonucleotide primers. The RAPD system has been used in linkage map construction (Grattapaglia and Sedroff, 1994), insect resistance gene localization (Dweikat et al., 1997), hybrid origin identification (Friesen et al., 1997), and breeding utilization (Durham and Korban, 1994; Baril et al., 1997). RAPDs may also be useful for the design of collection strategies to maximize the sampling of genetic variation within the available gene pool (Dawson et al., 1993; Huff et al., 1993; Liu and Furnier, 1993; Nesbitt et al., 1995). Moreover, RAPD markers are capable of detecting variation in non-coding regions of the genome.

The objective of this study was to use RAPD markers to investigate the genetic variation and population differentiation in these two *Chamaecyparis* species between two populations located in two distant sites separated by high mountains and deep valleys.

Materials and Methods

Plant Materials

Two populations of young needle leaves were collected from natural stands for both *C. formosensis* and *C. taiwanensis* from Chilanshan (from 1,500 to 1,700 m in

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elevation) and Alishan (from 2,000 to 2,200 m in elevation) in Taiwan. Chilanshan is located in the north of the central mountain range and Alishan is located in the south of the central mountain range (Figure 1). Chilanshan is a large pure stand for both *Chamaecyparis* species, and Alishan is a much smaller population for these two species. In addition, the central mountain ridge that straddles from north to south separates the Chilanshan and Alishan populations. Thirty samples each for both species in Chilanshan and Alishan population were collected.

DNA Extraction and Quantification

Leaves (0.3 g) were ground with sea sand and liquid nitrogen, and the ground leaf powder was then placed in 5 ml of extraction buffer for genomic DNA extraction based on a modified CTAB procedure (Doyle and Doyle, 1990). DNA was precipitated with ethanol, and after washing with 70% ethanol, it was dissolved in 200 µl TE buffer, pH 8.0, and placed in 4°C. The DNA concentration was determined for each sample using the GeneQuant II RNA/DNA Calculator (Amersham Pharmacia Biotech).

RAPD Amplification

RAPD reactions were conducted on a DNA Programmable Thermal Cycler (PTC-100, MJ Research) with three steps: Step 1 was 3 min at 94°C. Step 2 was 45 cycles of 1 min at 94°C for denaturation, 1 min at 37°C for annealing, and 2 min at 72°C for polymerization. The last step was 10 min at 72°C for final polymerase reaction. Each reaction contained 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, 100 mM Tris-HCl (pH 8.3), 1 mM dNTPs, 2 µM

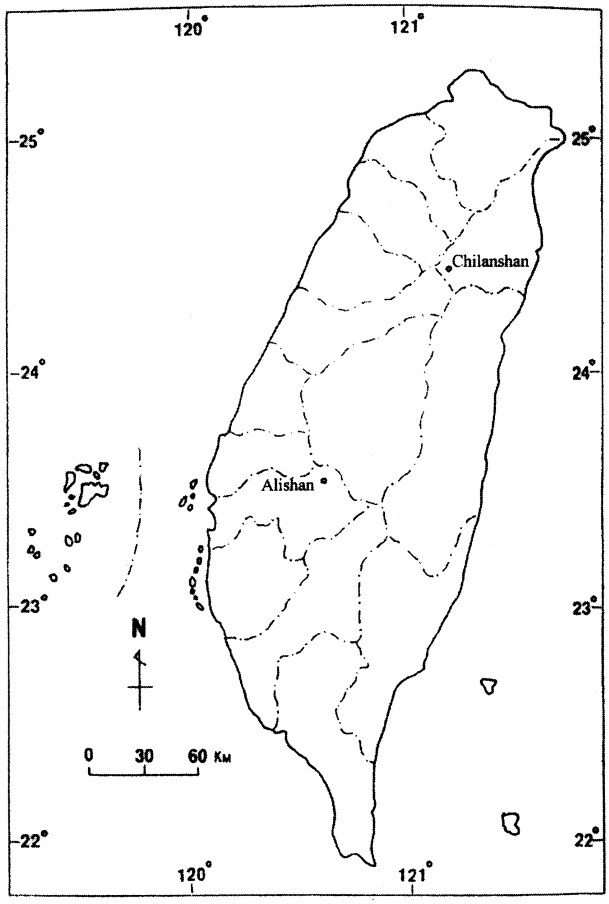

Figure 1. Collection localities of *Chamaecyparis formosensis* and *Chamaecyparis taiwanensis*.

Table 1. Attributes of oligonucleotide primers used for generating RAPD markers from 60 individuals of *Chamaecyparis formosensis* sampled from two populations in Taiwan.

Primer	Sequence (5'-3')	Total number of markers	PM and %P	
			Chilanshan	Alishan
P01	CCTGGGCTTC	7	3 (42.9)	4 (57.1)
P04	CCGGCCTTAC	10	3 (30.0)	3 (30.0)
P08	GCGCCCGAGG	12	5 (41.7)	4 (33.3)
P12	TAGCCCGCTT	10	6 (60.0)	5 (50.0)
P13	TAGCCGAGAC	12	10 (83.3)	7 (58.3)
P14	TGACCGAGAC	11	8 (72.7)	6 (54.5)
P15	TACGATGACG	13	11 (84.6)	9 (69.2)
P16	ATTGGGCGAT	9	8 (88.9)	3 (33.3)
P17	GTAGACGAGC	10	7 (70.0)	6 (60.0)
P18	ATCTGGCAGC	9	4 (44.4)	3 (33.3)
P19	GAAACAGCGT	11	7 (63.6)	5 (45.5)
P21	AAGCTGCGAG	11	11(100.0)	1 (9.1)
P27	TCCATGCCGT	11	9 (81.8)	6 (54.5)
P38	CAAGGGAGGT	10	4 (40.0)	4 (40.0)
P40	ATGACGACGG	10	9 (90.0)	7 (70.0)
P42	CAAACGGCAC	9	4 (44.4)	2 (22.2)
Pab3	CATCCCCCTG	8	4 (50.0)	3 (37.5)
Total		173	113 (65.3)	78 (45.1)

PM: no. of polymorphic markers; %P: percent of polymorphism.

Primer, 20 ng template DNA, 1 µg RNase, and 1.7 unit *Taq* polymerase (Amersham Pharmacia Biotech), to a final volume of 20 µl. Products amplified by PCR were resolved using 1.75% (w/v) Nusieve 3:1 agarose (FMC BioProducts) gel containing 0.1 g/ml ethidium bromide electrophoresed in 1X TBE under constant voltage (50V) for 2 h. A molecular size marker (ϕX 174/*Hae*III, Stratagene) was used to assign molecular weights for RAPD bands. Images of each gel were viewed by UV illumination, captured by "Grab It" software through a CCD camera, and stored as TIF files. Fifty primers designed by Mosseler et al. (1992) and another 20 primers (AB-0320-Kit 1) purchased from Advanced Biotechnologies (UK) were screened based on band resolution and band number for template DNA samples.

Data Collection and Analysis

All gel photographs were scored for the presence/absence of RAPD bands. The POPGENE computer package (Yeh and Boyle, 1996) was used to calculate Shannon's index of phenotypic diversity for RAPD diploid data according to $Ho = -\sum P_i \log_2 P_i$. P_i was the frequency of the presence or absence of the amplified fragment. A pairwise matrix of the genetic distances between individuals was obtained using an Euclidean distance measure (Huff et al., 1993), calculated from presence/absence data using RAPD istance (Armstrong et al., 1994). Components of variance partitioned into within and between populations were estimated from this matrix using AMOVA version 1.8 (Analysis of Molecular Variance, Excoffier et al., 1992). The number of permutations for significant testing was set at 1,000 for analysis. AMOVA variance components were used as estimates of the genetic diversity within and between populations.

Results

RAPD Polymorphism

To identify primers that detect polymorphism, 70 primers were screened using genomic DNA from needle leaves of both *C. formosensis* and *C. taiwanensis*. Seventeen and twenty primers produced polymorphic RAPD banding profiles for *C. formosensis* and *C. taiwanensis*, respectively (Tables 1 and 2). In total, 17 and 20 random primers yielded a total of 173 and 239 reproducible bands for *C. formosensis* and *C. taiwanensis*, respectively. On average 55.2% of 173 fragments scored in *C. formosensis* were polymorphic (65.3 and 45.1% for the Chilanshan and Alishan populations, respectively). For *C. taiwanensis*, on average 77.8% of 239 scored bands were polymorphic (81.6 and 78.1% for the Chilanshan and Alishan populations, respectively). The number of markers scored per primer ranged from 7 to 13 and from 8 to 17, respectively for *C. formosensis* and *C. taiwanensis* (Tables 1 and 2).

Shannon's Phenotypic Index Based on RAPD Variation

Table 3 presents the estimates of Shannon's phenotypic diversity for *C. formosensis* and *C. taiwanensis* for RAPD variation. The two *Chamaecyparis* species differed in the amount of *Ho*. The amount of *Ho* for Chilanshan and

Table 2. Attributes of oligonucleotide primers used for generating RAPD markers from 60 individuals of *Chamaecyparis taiwanensis* sampled from two populations in Taiwan.

Primer	Sequence (5'-3')	Total number of markers	PM and %P	
			Chilanshan	Alishan
P04	CCGGCCTTAC	12	11 (91.7)	11 (91.7)
P05	CCGGCCTTCC	16	14 (87.5)	14 (87.5)
P08	GCGCCCGAGG	12	11 (91.7)	10 (83.3)
P11	GGTGGGGACT	8	1 (12.5)	3 (37.5)
P13	TAGCCGAGAC	14	13 (92.9)	12 (85.7)
P14	TGACCGAGAC	13	11 (84.6)	9 (69.2)
P17	GTAGACGAGC	11	10 (90/9)	9 (81.8)
P19	GAAACAGCGT	12	10 (83.3)	8 (66.7)
P21	AAGCTGCGAG	12	9 (75.0)	9 (75.0)
P22	GGTCTCTCCC	10	7 (70.0)	6 (60.0)
P23	GTCGCATTTC	12	11 (91.7)	11 (91.6)
P27	TCCATGCCGT	12	11 (91.7)	10 (83.3)
P28	GCCTGGTTGC	9	6 (66.7)	3 (33.3)
P30	GAGCCCGTAC	11	7 (63.6)	4 (36.4)
P32	GAAGGCACTG	8	7 (87.5)	2 (25.0)
P34	ACGACGTAGG	10	9 (90.0)	10 (100.0)
P38	CAAGGGAGGT	13	10 (76.9)	10 (76.9)
P40	ATGACGACGG	16	16 (100.0)	15 (93.8)
P44	GCTGGACATC	11	6 (54.5)	5 (45.5)
Pab3	CATCCCCCTG	17	15 (88.2)	16 (94.1)
Total		239	195 (81.6)	177 (78.1)

PM: no. of polymorphic markers; %P: percent of polymorphism.

Table 3. Estimates of Shannon's phenotypic diversity index (*Ho*) for RAPDs for Chilanshan and Alishan populations of *Chamaecyparis formosensis* and *Chamaecyparis taiwanensis.*

Chamaecyparis formosensis			*Chamaecyparis taiwanensis*		
Primer	Shannon's index		Primer	Shannon's index	
	Chilanshan	Alishan		Chilanshan	Alishan
P01	0.31	0.358	P04	0.460	0.484
P04	0.187	0.130	P05	0.449	0.419
P08	0.205	0.145	P08	0.537	0.455
P12	0.341	0.333	P11	0.063	0.200
P13	0.536	0.361	P13	0.536	0.408
P14	0.417	0.336	P14	0.447	0.395
P15	0.523	0.402	P17	0.565	0.522
P16	0.481	0.219	P19	0.457	0.368
P17	0.410	0.373	P21	0.418	0.476
P18	0.256	0.187	P22	0.359	0.350
P19	0.383	0.287	P23	0.550	0.572
P21	0.632	0.060	P27	0.556	0.528
P27	0.559	0.319	P28	0.411	0.216
P38	0.229	0.247	P30	0.430	0.225
P40	0.538	0.373	P32	0.503	0.165
P42	0.283	0.154	P34	0.475	0.594
Pab3	0.337	0.257	P38	0.415	0.453
--			P40	0.603	0.557
--			P44	0.264	0.269
--			Pab3	0.469	0.518
Mean	0.390	0.267	Mean	0.448	0.409

Alishan also differed in the same species for RAPD markers. The mean RAPD *Ho* for *C. formosensis* was 0.329, with Chilanshan having the higher *Ho* (0.390) and Alishan the lower (0.267). The mean RAPD *Ho* for *C. taiwanensis* was 0.429 with Chilanshan having the higher (0.448) and Alishan the lower (0.409). RAPD *Ho* also varied among primers within population. For *C. formosensis,* primer P04 detected the lowest RAPD *Ho* (0.187) and P21 detected the highest RAPD *Ho* (0.632) in Chilanshan. Primer P04 also detected the lowest RAPD *Ho* (0.130), but primer P15 detected the highest RAPD *Ho* (0.402) in Alishan's *C. formosensis*. RAPD *Ho* in *C. taiwanensis* varied from 0.063 (primer P11) to 0.603 (primer 40) in Chilanshan and from 0.165 (primer P32) to 0.594 (primer P34) in Alishan.

Analysis of Molecular Variance

The results of the AMOVA partitioning of RAPD variance are shown in Table 4. There were highly significant (P<0.001) genetic differences between the Chilanshan and Alishan populations for both *Chamaecyparis* in Taiwan. Of the total genetic diversity, 84.9% was attributable to within population and only 15.1% to between populations in *C. formosensis*. For *C. taiwanensis*, 85.3% of the total genetic diversity was attributable to within population and 14.7% to between populations.

Discussion

A study of allozyme diversity showed that the average percentage of polymorphic loci per population was 20.6 and 22.5% for *C. formosensis* and *C. taiwanensis*, respectively (Lin et al., 1994). Moreover, no restriction length difference was observed for PCR amplified chloroplast DNA fragments among 60 individuals of both Taiwan *Chamaecyparis* species (Hwang et al., 2000). However, high levels of polymorphism based on RAPD were found

Table 4. Analysis of molecular variance for two populations of *Chamaecyparis formosensis* and *Chamaecyparis taiwanensis* based on RAPD variations.

Source of variation	df	MSD	Variance component	% Total	P value*
Chamaecyparis formosensis					
Between populations	1	103.47	2.91	15.13	<0.001
Within populations	58	16.30	16.30	84.87	<0.001
Chamaecyparis taiwanensis					
Between populations	1	200.45	5.60	14.73	<0.001
Within populations	58	32.43	32.43	85.27	<0.001

*Nonparametric randomization test (1000 permutation).

in this study (Tables 1 and 2) for both *Chamaecyparis* in Taiwan. The genetic parameters estimated from RAPD data indicated that there were substantial levels of genetic diversity in the natural stands of both Taiwan *Chamaecyparis* species. Based on RAPD variation, a higher percentage of polymorphic loci and higher Shannon's phenotypic diversity (*Ho*) were observed in Chilanshan compared with Alishan for both *Chamaecyparis* species. The results probably correlated with the large population size of Chilanshan, which contains more genetic variation.

The majority of RAPD variation in *C. formosensis* and *C. taiwanensis* was found within rather than between populations, which agrees with the data collected by allozyme analyses (Lin et al., 1994). That most genetic diversity existed within populations for the two *Chamaecyparis* species in Taiwan is consistent with the general trend in other outcrossing species (Huff et al., 1993; Nesbitt et al., 1995) based on RAPD variation. Plant species differ markedly in the way genetic diversity is partitioned between populations. The pattern of partitioning is correlated with the mating system and life-history parameters (Hamrick and Godt, 1989). Species that are primarily outcrossing and long-lived have most of their genetic diversity partitioned within populations. Our results are consistent with this pattern. It was reported that during the late Pleistocene, *C. formosensis* and *C. taiwanensis* along with other conifers dominated in the Tali glacial stage in Taiwan (Tsukada, 1967). Moreover, Liu (1966) reported that the distribution of the two wind-pollinated *Chamaecyparis* species is continuous in Taiwan. Therefore, most genetic variation apportioned within populations is not surprising and is possibly due to the high level of gene flow.

It was reported that in red pine (Mosseler et al., 1992) and *Amentotaxus formosana* Li (Wang et al., 1996), RAPD data concurred with the allozyme data in the level of population differentiation. Agreement between patterns of population structure revealed by isozyme and RAPD markers might suggest that neutral forces are more likely to be invoked than selection (Yeh et al., 1995). However, genetic differentiation between populations by RAPD analysis has been observed among races of Douglas fir with genetic differentiation revealed by RAPD exceeding by three times that for allozymes (Aagaard et al., 1995). Greater genetic divergence between populations revealed by RAPD markers in comparison with allozyme markers was also reported in bigtooth aspen and *Pinus leucodermis* (Liu and Furnier, 1993; Bucci et al., 1997b). In the present study, genetic differentiation between populations based on RAPD variation was observed to be about three times higher than that based on allozyme analysis (Lin et al., 1994).

Although it is not clear where in the genome RAPD bands are amplified, they are likely to be randomly distributed in the genome as suggested by moderate- and high-resolution RAPD genetic maps (Bradshaw et al., 1994; Grattapaglia and Sedroff, 1994; Gochmen et al., 1996; Bucci et al., 1997a). Most RAPD markers (53%) were mapped in the low-copy number region in *Eucalyptus grandis* and *E. urophylla* (Grattapaglia and Sedroff, 1994). Thus, it is likely that RAPDs provide an unbiased sample of DNA variation along the whole genome. The greater sensitivity of RAPDs to population divergence may be derived from rapid evolution of non-coding, repetitive DNA sequences detected by RAPDs (Plomion et al. 1995). Although variation detected by RAPD amplification may possibly have no phenotypic consequences, plant gene expression controlled by microsatellites has been reported (Ayers et al., 1997). Moreover, the maintenance of repetitive DNA sequences is important for plants to adapt to unfavorable changes in their environment (Rogers and Bendich, 1987). In conclusion, high levels of genetic polymorphism and genetic differentiation revealed by RAPD analysis might play a role in the dynamic evolution of *Chamaecyparis* species in Taiwan.

Acknowledgements. We thank Mrs. C.T. Wang and J.C. Yang for their assistance in the field. Financial support provided by the Council of Agriculture (Grant no. 87AST-1.2-FOD-04) to S.Y. Hwang is gratefully acknowledged.

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紅檜與台灣扁柏 RAPD 族群分化

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本文探討紅檜與台灣扁柏之族群分化問題。紅檜與台灣扁柏的棲蘭山與阿里山兩個族群以逢機擴增多型性 DNA 分子標記研究族群分化之問題。RAPD 變異以 Shannon's phenotypic index 來估算 *Ho*。無論是紅檜或台灣扁柏之棲蘭山族群都較阿里山族群有較高之遺傳變異。上述結果可能與棲蘭山為紅檜與台灣扁柏之大族群有關。基於 RAPD 之分析發現紅檜棲蘭山與阿里山族群間有 15.14% 之分化值，而台灣扁柏棲蘭山與阿里山族群間分化值為 14.73%。RAPD 基因座之高的遺傳變異與族群分化程度可能代表紅檜與台灣扁柏之動態演化有關。

關鍵詞：紅檜；台灣扁柏；族群分化；逢機擴增多型性 DNA。