Bot. Bull. Acad. Sin. (2002) **43:** 93-98

An assessment of DNA polymorphisms and genetic relationships of *Casuarina equisetifolia* using RAPD markers

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(Received August 3, 2001; Accepted October 29, 2001)

Abstract. One hundred forty-two individual samples belonging to 12 native accessions of *Casuarina equisetifolia* grown in an international Provenance trial garden in Taiwan were studied for RAPD variation. Twelve primers were used and 89 polymorphic bands were scored. An analysis of molecular variance (AMOVA) revealed that the percentages of variance attributable to variation among and within provenances were 39.28% and 60.72%, respectively. When accessions of north and south hemispheres were treated as two separate groups, the variance components between groups, among provenances within groups, and among individuals within provenances were 2.42%, 37.55%, and 60.03%, respectively. The difference between north and south latitude groups was not significant (p=0.1738). The average Nei's gene diversity for a location of origin was 0.1473. Cluster analysis and principal coordinates analysis revealed two major groupings. One group comprised solely the Cairns accession of Australia and identifies *C. equisetifolia* var. *incana* while the other group comprised the remaining accessions of *C. equisetifolia* var. *equisetifolia.* The RAPD data support the taxonomic treatment of two varieties in this species. Some regional relationships were observed within *C. equisetifolia* var. *equisetifolia*. Study results indicated a large genetic variation among the native accessions of *Casuarina equisetifolia*. Therefore, the results of provenance trials are expected to be useful in the selection of a suitable provenance for a particular coastal environment.

Keywords: *Casuarina equisetifolia*; Genetic variation; RAPD; Provenance trial.

Introduction

Casuarina equisetifolia Forst & Forst is the most widely cultivated species within the family *Casuarinaceae*. It is grown, especially in China and India, for landscaping, pulp, lumber, medicine, tannin, dye, and sand-shifting control in coastal areas (Pan et al., 1996). The species is distributed in Southeast Asia and Australia, which have large native populations, and is introduced into other subtropical and tropical areas (Doran and Hall, 1983; Wilson and Johnson, 1989). The plant was first introduced to Taiwan in 1897, employed as an important pioneer species in coast areas because of its salt and drought tolerance, fast growth, and suitability for sand-shifting control. Other *Casuarina* species have also been introduced in Taiwan. Introgressive hybridization among species may have occurred (Wang et al., 1984) because of the long cultivation history and the similar flowering periods of different species. Such hybridization can make species identification difficult (Hwang and Hsiao, 1985; Badran et al., 1976).

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In 1992, Taiwan Forestry Research Institute participated in an international cooperation program, which conducted a *Casuarina equisetifolia* provenance trial under the Forestry / Fuelwood Research and Development Project (F/FRED) of the WINROCK International Agriculture Development Research Institute and established a *Casuarina equisetifolia* provenance trial garden at the Suhu station on the western coast of Taiwan. Several other countries including Indonesia, Sri Lanka, Malaysia, and Thailand also participated in this program. The program was funded by the International Development Foundation in the United States and was overseen by the Commonwealth Scientific and Industrial Research Organization (CSIRO) of Australia. The seed sets were received from the program and were planted according to the standardized experiment design (Kamis, 1992). The purpose of this international project was to evaluate phenotypic responses to various habitats, to select a suitable provenance for a particular coastal environment, and to study the population genetic variation of the species. Twenty-eight provenances were included in this project. Among these 16 seed sets were collected from cultivated areas while 12 were collected from native populations. The latter were studied to evaluate the genetic variation of the native populations of the species.

Materials and Methods

Materials

Branchlet samples were collected from seven-year-old plants in the *Casuarina equisetifolia* international provenance trial garden at the Suhu station of the Taiwan Forestry Research Institute. Twelve plants for each native provenance were sampled except that ten plants were sampled for Panay (Philippines) provenance. A total of 142 plants were studied (Table 1). Fifteen branchlet were collected from each tree. The branchlet samples were kept in paper bags and dried with silica gel before DNA extraction.

DNA Extraction

Total DNA was extracted from 50 mg of dry branchlet material using a modification of the method of Doyle and Doyle (1987). The tissue was ground under liquid nitrogen and quartz sand, placed into a microfuge tube with 0.8 ml CTAB isolation buffer and incubated at 60°C for 15 min. The mixture was then centrifuged at 12,000 rpm in a microcentrifuge for 2 min. The supernatant was extracted twice with 0.5 ml chloroform and centrifuged at 12,000 rpm for 2 min before being collected and placed in a clean tube. Nucleic acids were precipitated by the addition of 0.8 ml ice-cold isopropanol and stored at -20°C for 6 h, pelleted at high-speed (13,000 rpm) for 2 min, washed in 0.8 ml 76% EtOH, and resuspented in 0.1 ml TE buffer.

RAPD Amplification

PCR reactions were performed in 25 µl volumes containing 1X reactions buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, gelatin 0.01% (w/v), Triton X-100 0.1% (w/v)), 100 µM dNTPs, 0.3 µM primer, 5 ng template DNA, and 0.4 units Taq DNA polymerase. RAPD amplification followed the method of Williams et al. (1990) with some minor modifications. The cycles were as follows: 94°C for 2 min; 45 cycles of 94°C for 30 s, 36°C for 30 s, 72°C for 2 min; a final step at 72°C for 5 min was followed by incubation at 15°C. The amplifications were carried out on a PTC-100 Thermal Cycler Controller (MJ Research Inc., USA). The DNA polymerase was purchased from HT Biotechnology (UK). Each amplification experiment was performed at least twice. Strong and reproducible polymorphic bands were used in the statistical analyses. The amplification products were separated by electrophoresis in a 1.5% agarose gel in 1× TBE (Tris-EDTA-borate) buffer and stained with ethidium bromide.

Data Analysis

Reproducible polymorphic bands from the RAPD analysis were screened qualitatively for presence (1) or absence (0) in each sample. Only intensely stained polymorphic bands were used in the following statistical analyses. A matrix of inter-sample distances was constructed using the formula of Excoffier et al. (1992), $D=N(1-(N_{11}/N)$, where N is the total number of polymorphic bands and N11 the number of bands shared by two samples. The matrix was analyzed in an analysis of molecular variance (AMOVA) using WINAMOVA 1.55 software (Excoffier et al., 1992). Genetic variation was partitioned within and among provenances and significant values assigned to variance components based on 9999 random permutations of individual samples assuming no genetic structure. Nei's gene diversity (H; Nei, 1973) for each provenance was derived using POPGENE 3.2 software (Yeh et al., 1999) assuming Hardy-Weinberg equilibrium. Band frequencies of each provenance were used to calculate genetic distances between provenances using Nei's formula (1972). The genetic distance matrix was used in a UPGMA cluster analysis and a principal coordinates analysis by employing the software NTSYS-pc (Rohlf, 1993).

Results

The primers of kits A, B, C, D, E, H, M, Q, and V of Operon Technology (USA) were screened. Out of the primers screened, twelve with better results were selected for this study and a total of 40 monomorphic bands and 89 polymorphic bands were scored from the amplifications using these primers (Table 2). The percentages of monomorphic and polymorphic bands were 31% and 69%, respectively. The high percentage of polymorphic bands

Table 1. The twelve native provenances studied, and Nei's gene diversity (H) within each provenance.

Provenance code	CSIRO seed No.	Locality	Latitude	Longitude	Altitude (m)	H
1	15958	Cairns, Australia	16º 41´ S	145º 34´ E	30	0.1210
2	18008	Darwin, Australia	12º 25´ S	130º 35´ E	20	0.1363
3	18121	Mariana Island, Guam	13º 40´ N	144º 04´ E	2	0.2700
4	18153	Papua New Guinea	9º 05´ S	147º 17´ E	10	0.1664
5	18117	Mindoro, Philippines	12º 25´ N	121º 03´ E	20	0.1162
6	18244	Sarawak, Malaysia	1º 44´ N	110º 30´ E	30	0.1566
7	18157	Pantai Moyog Sabah, Malaysia	5º 55´ N	116º 05´ E	2	0.1133
8	18158	Tanjung Aru Sabah, Malaysia	5º 55´ N	116º 05´ E	2	0.0815
9	18270	Viti Levu, Fiji	18º 11´ S	177º 35´ E	106	0.1402
10	18271	Vanua Levu, Fiji	16º 08´ S	178º 49´ E	30	0.1523
11	18154	Panay, Philippines	11º 55´ S	122º 30´ E	30	0.1583
12	18299	Trang, Thailand	7º 33´ S	100º 37´ E	2	0.1554

Table 2. Nucleotide sequence, GC content, number of fragments produced, and number and percentage of monomorphic and polymorphic fragments for each primer used.

Primer	Nucleotide sequence	GC content (%)	Number of fragments observed	Number of monomorphic fragments	Number of polymorphic fragments	Percentage of monomorphic fragments	Percentage of polymorphic fragments
OPA04	5′AATCGGGCTG3′	60	10	3	7	30%	70%
OPB01	5′GTTTCGCTCC3′	60	9	3	6	33%	67%
OPB20	5′GGACCCTTAC3′	60	10	2	8	20%	80%
OPC11	5′AAAGCTGCGG3′	60	14	6	8	43%	57%
OPC13	5′AAGCCTCGTC3′	60	9	3	6	33%	67%
OPC16	5′CACACTCCAG3′	60	9	2	7	22%	88%
OPD13	5′GGGGTGACGA3′	70	17	8	9	47%	53%
CPE16	5′GGTGACTGTG3′	60	8	1	7	13%	87%
OPH08	5′GAAACACCCC3′	60	14	6	8	43%	57%
OPQ09	5′GGCTAACCGA3′	60	8	1	7	13%	87%
OPV12	5′ACCCCCCACT3′	70	12	3	9	25%	75%
OPV15	5′CAGTGCCGGT3′	70	9	2	7	22%	88%
Total			129	40	89	31%	69%

is an indication of high genetic variation in this species. Theaverage number of polymorphic bands for a primer was 7.4.

Genetic Diversity

Nei's gene diversity for each provenance is shown in Table 1. The Mariana Island provenance of Guam had the highest diversity index (0.2700) while the Tanjung Aru Sabath provenance of Malaysia had the lowest index (0.0815). The average gene diversity was 0.1473.

Analysis of Molecular Variance

The results of AMOVA (Table 3) indicated that 39.28% of the total variation was attributable to the differences among provenances while 60.72% was due to the variation among individuals within provenances. The result of a random permutation test indicated that two variance components were both highly significant ($p<0.001$). When provenances of north and south hemispheres were treated as the two separate groups, the variance components between groups, among provenances within groups, andamong individuals within provenances were 2.42%, 37.55%, and 60.03%, respectively. The difference between north and south latitude groups was not significant ($p= 0.1738$).

Cluster Analysis and Principal Coordinates Analysis

The result of UPGMA cluster analysis based on the matrix of Nei's genetic distances among provenances (Table 4) is shown in Figure 1. The cophenetic correlation coefficient of this cluster analysis was 0.766. Two major groupings could be recognized in Figure 1. One group consists of Cairns provenance (Australia) only, belonging to *C. equisetifolia* var. *incana* Benth. Another group comprises the remaining provenances and belongs to *C. equisetifolia* var. *equisetifolia*. The RAPD data support the taxonomic treatment of two varieties in the species. Within the second group, some regional associations were observed. Pantai Moyog Sabah and Tanjung Aru Sabah provenances (Malaysia) showed the lowest genetic distance before they were joined by Sarawak provenance (Malaysia). Two Fiji provenances (Viti Levu and Vanua Levu) were also closely linked. The provenances of the second group were divided into three subgroups at the genetic distance of 0.48. The first subgroup was formed by Darwin (Australia), Viti Levu and Vanua Levu (Fiji), and Trang (Thailand) locations. The second subgroup included Mindora (Philippines), Sarawak, Pantai Moyog Sabah, and Tanjung Aru Sabah (Malaysia). The third subgroup included the Mariana Island (Guam), Papua New

Table 3. Hierarchical analysis of molecular variance (AMOVA).

Source of variation	df	SS	MS	Variance component	% Total variation	P value*
Twelve provenances						
Among provenances	11	839.78	76.34	5.7072	39.28	<0.0001
Within provenances	130	1146.84	8.82	8.8219	60.72	<0.0001
Two groups (North and South Hemispheres)						
Between groups	1	99.10	99.10	0.3554	2.42	0.1738
Among provenances	10	740.68	74.07	5.5179	37.55	<0.0001
Within provenances	130	1146.84	8.82	8.8219	60.03	<0.0001

*After 9999 random permutations.

Table 4. Nei's genetic distance between provenances based on RAPD data (Provenance codes as indicated in Table 1).

	1	2	3	4	5	6	7	8	9	10	11	12
1	0.0000											
2	0.7787	0.0000										
3	0.4186	0.4020	0.0000									
4	0.5978	0.6370	0.3435	0.0000								
5	0.7540	0.6925	0.4445	0.6331	0.0000							
6	0.6640	0.4784	0.3979	0.6110	0.4515	0.0000						
7	0.7469	0.6093	0.3482	0.6851	0.4072	0.1387	0.0000					
8	0.8021	0.7045	0.4758	0.7322	0.3751	0.1668	0.1348	0.0000				
9	0.7090	0.4672	0.3632	0.6370	0.4788	0.4446	0.4834	0.5208	0.0000			
10	0.6576	0.3142	0.2982	0.5715	0.2980	0.3482	0.3396	0.4025	0.2051	0.0000		
11	0.6934	0.5313	0.3450	0.5102	0.6303	0.5342	0.5589	0.7276	0.6212	0.4756	0.0000	
12	0.7548	0.3379	0.4056	0.6882	0.4466	0.3900	0.5381	0.5532	0.4289	0.2119	0.6561	0.0000

Guinea, and Panay (Philippine) locations. The result of principal coordinates analysis is shown in Figure 2. The first three coordinates accounted for 78.69% of the total variation. The relationships among provenances in this figure were supported by the result of cluster analysis. The Cairns of Australia was the most isolated provenance. The remaining provenances were divided into three clusters similar to the result of cluster analysis. The Cairns provenance (Australia) was close to the cluster of Mariana Island of Guam, Papua NewGuinea, and Panay of Philippine. Table 4 indicated that Cairns provenance of Australia was close related to Mariana Island of Guam (genetic distance 0.4186) and Papua New Guinea (genetic distance 0.5978). This relationship was not revealed in Figure 1 because Mariana Island and Papua New Guinea were members of the var. *equisetifolia*.

Discussion

Yasodha et al. (1999) developed an optimized protocol to generate RAPD markers in *Casuarina equisetifolia* and indicated the assay can be used to characterize closely related genotypes. However, they did not study the population variation of the species. The result of AMOVA of the present study can be compared with some other studies employing RAPD and AMOVA analyses (Huff et al., 1993; Vicario et al., 1995; Nolan et al., 1996; Sale et al., 1996; Black-Samuelsson and Andersson, 1997; Gabrielsen et al., 1997; Martin et al., 1997; Huff et al., 1998; Kolliker et al., 1998; Wen and Hsiao, 1999; Wen and Hsiao, 2001). The within-provenance component in *Casuarina equisetifolia* was at the low end while the differentiation among provenances was at the high end of the range. *Casuarina equisetifolia* is natively distributed in Malay Archipelago, Oceania, and the north and northeast coastal areas of Australia (Wilson and Johnson, 1989). Although the distribution areas of the species are around the equator and belong to the tropical climatic zone, its distribution is scattered through many islands, and therefore geographical isolation may occur. The species' wide distribution range and its geographic isolation may account for the observed large differences among provenances. When northern and southern latitudes were considered as two groups, the difference between latitudes accounted for only 2.42% of the total variation, and the difference was not significant (p= 0.1739). Therefore, the genetic variation is not associated with the difference in latitude and is probably related to habitat differences characterizing the regions.

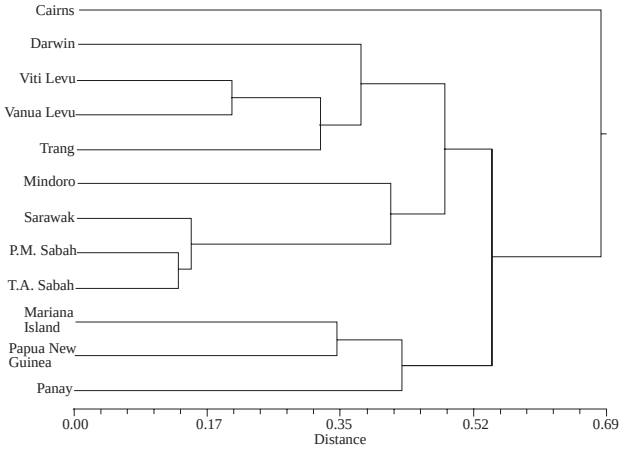

Figure 1. Dendrogram generated by UPGMA clustering of RAPD genetic distances.

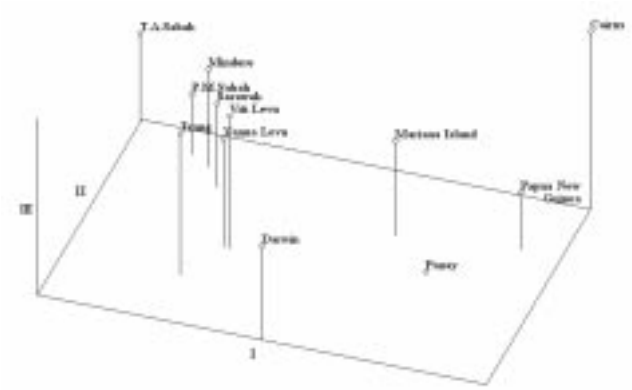

Figure 2. Principal coordinate diagram of provenances based on RAPD genetic distances.

The results of cluster analysis (Figure 1) and principal coordinates analysis (Figure 2) all indicated that the Cairns provenance of Australia was the most isolated provenance. *Casuarina equisetifolia* is taxonomically divided into two varieties. The variety *incana* is distributed in Australia from Darwin to the north coast of New South Wales while the variety *equisetifolia* is widely distributed form Malaysia to subtropical Australia, Melanesia, Micronesia, Philippine, and Polynesia (Wilson and Johnson, 1989). Cairns provenance was recognized in the present study as belonging to var. *incana* because the seeds from Cairns were collected in areas overlapping the distribution range of var. *incana*, besides, the trial in Taiwan revealed that the plants of this provenance grew slower compared to that of the remaining locations and has the botanical characteristics of var. *incana*. The result of genetic study based on RAPD data supported the taxonomic division of the species into two varieties. Within the var. *equisetifolia*, some regional associations were observed. Pantai Moyog Sabah, Tanjung Aru Sabah, and Sarawak (Malaysia) were closely linked in a single cluster, and all have their origin in Malaysia. Viti Levu and Vanua Levu (Fiji) also showed close relationship.

Yang et al. (1995) studied the variation of seed weight and seedling growth within the *Casuarina equisetifolia* species and concluded that the difference among provenances was highly significant and the variation was not continuous. The result of the present study indicates that a large genetic variation existed among the native provenances of *Casuarina equisetifolia*. Therefore, it is expected that the result of provenance trial can be useful in the selection of a suitable provenance for a particular coastal environment.

Acknowledgments. This study was supported in part by Grant 90AS-1.4.1-FI-G4 from the Taiwan Forestry Research Institute (TFRI contribution No. 181). The authors thank the institutions involved in the international cooperation program of *Casuarina* provenance trial for making the present study possible.

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應用 RAPD 研究木賊葉木麻黃之核酸多型性與親緣關係

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本試驗以 RAPD 研究木賊葉木麻黃 (*Casuarina equisetifolia*) 國際種源，在台灣之試驗的 12 個自生種源 142 個樣本，共使用 12 個引子，記錄了 89 個多型性條帶。AMOVA 分析結果顯示種源間及個體間的變方成分分別佔總變方成分的 39.28%及 60.72%；若將種源分成南北半球兩群，則其群間、種源間與個體間之變方成分分別佔總變方成分之 2.42%、37.55% 及 60.03%，而分成南北半球兩群是不顯著的 (p=0.1738)。平均的 Nei's 基因歧異度為 0.1473。歸群及主座標分析結果顯示了兩個主要的群團，一群為 *Casuarina equisetifolia* var. *incana* 的澳洲昆士蘭種源，另一群為 *C. equisetifolia* var. *equisetifolia* 的其他種源，故 RAPD 的結果支持本種有兩個變種的分類處理。從 *C. equisetifolia* var. *equisetifolia* 中可觀察到一些地區的關係，本研究結果顯示木賊葉木麻黃自生種源間存有較大的遺傳變異，所以，預期種源試驗的結果能選出適合的種源以適應特殊的海岸環境。

關鍵詞：木賊葉木麻黃；遺傳變異； RAPD；種源試驗。