Heredity **77** (1996) 388–395 *Received 14 November 1995*

Low genetic variation in *Amentotaxus formosana* Li revealed by isozyme analysis and random amplified polymorphic DNA markers

CHIEH-TING WANG†, WEI-YOUNG WANG†, CHIA-HUA CHIANG‡, YA-NAN WANG‡ & TSAN-PIAO LIN*†

†Silviculture Division, Taiwan Forestry Research Institute, 53 Nan-Hai Road, Taipei and ‡Department of Forestry, National Taiwan University, Taipei, Taiwan

The objective of this research was to use random amplified polymorphic DNA (RAPD) and isozyme analysis to investigate genetic variation in narrowly distributed populations of *Amentotaxus formosana* Li. A total of 20 loci from 10 enzyme systems were analysed in 50 individual trees from each of the two natural populations. No isozyme variation was observed in the Tsatsayalai population. Phosphoglucose isomerase (*Pgi-1*) was the only polymorphic enzyme in the Tawu population, giving 5 per cent polymorphic loci with 0.008 expected heterozygosity. No genetic distance was found between these two populations using isozymes. *Amentotaxus formosana* demonstrated a high proportion of monomorphic RAPD fragments, about 79 per cent, for 20 arbitrary oligonucleotide primers. High similarity (0.994) was found between the Tawu and Tsatsayalai populations. RAPD markers provided further confirmation of the low levels of genetic variation in *A. formosana* detected by isozyme analysis. The value of isozyme analysis was emphasized by the finding of the rare allele, *Pgi-1a*, which was present only in the Tawu population. Based on the analysis of 110 individuals, representing 16 per cent of a native population, it was found that the younger tree category had a higher frequency of *Pgi-1a* (0.125) than the older tree category (0.053), resulting in an expected heterozygosity of 0.250 and 0.105, respectively. It was inferred that the appearance of the *Pgi-1a* allele could be the result of a mutation in the Tawu population and that selection is acting directly upon trees carrying this allele.

Keywords: *Amentotaxus formosana*, genetic variation, isozyme, RAPD.

Introduction

Amentotaxus formosana Li is endemic to Taiwan, where it is probably the most narrowly distributed gymnosperm species. It is highly endangered and has received worldwide attention (Farjon *et al.*, 1993). It is a dioecious tree producing large and heavy seeds, but is poor in production. It grows in the broad-leaved forests of Taitung Forest District (Tawu) and the Pingtung Forest District (Tsatsayalai), at an elevation ranging from 900 to 1300 m. The Tawu population has about 700 trees greater than 1 cm in diameter and covers an area of about 86 ha. The Tsatsayalai population has about 800 trees and covers about 225 ha. In 1988 the Council of Agriculture, Taiwan, designated these two sites as natural reserves for *A. formosana*, because the populations were on the brink of annihilation. The climate and the components of the plant communities of these two habitats are very similar and belong to the warm temperate rain forest (Yang, 1994). The reversed J-type of the population structure, judged by the frequency distribution of breast height diameter (DBH) classes, indicated that *A. formosana* populations can grow continuously and stably under protection in these habitats (Yeh *et al.*, 1992; Yang, 1994).

The primary objective of this research was to investigate genetic variation within and between these two populations of *A. formosana*, using

*Correspondence.

©1996 The Genetical Society of Great Britain.

isozyme and random amplified polymorphic DNA (RAPD) markers (Williams *et al.*, 1990). Genetic studies based on isozyme data have major advantages over RAPD markers in that they are cheaper and easy to perform, but also give more information on genotypic relationships. Some evidence has suggested that allozyme variation may not be able to provide an accurate or complete measure of nucleotide variation in the genome (Wolff, 1991; Heun *et al.*, 1994; Meijer *et al.*, 1994). RAPD markers, on the other hand, may provide a less biased measure of genetic variability and a greater resolution of subtle genetic differences for inferring genetic structure. RAPD analysis has resulted in a more definitive grouping (Heun *et al.*, 1994; Maaß & Klaas, 1995), even though RAPD polymorphism is poorly understood and believed to be based upon either sequence variation or mismatches in the primer binding sites. However, in this report we present some unique information from the isozyme analysis that it is not possible to obtain using RAPD markers.

Materials and methods

Sampling

The locations of the two natural populations, Tawu and Tsatsayalai, are shown in Fig. 1. Fifty trees were sampled from each population for isozyme analysis, and 25 and 20 individuals from the Tawu and Tsatsayalai populations, respectively, for RAPD analysis. Fewer individuals were included in the RAPDs than the isozyme analysis because of practical constraints. Random sampling was applied to the trees; however, uniformity was not possible because some trees were growing on a steep slope. Young leaf tissue was collected in April 1994 and stored at −20°C until required and additional seeds were collected in January 1995 for gametophyte analysis. Young leaf tissue of an additional 60 individuals in the Tawu population was collected in April 1995, and a total of 110 individuals, which varied from <5 cm to 47 cm DBH, were used to compare the genotype distribution and allele frequencies of *Pgi-1*.

Isozyme electrophoresis methods

Horizontal starch gel electrophoresis was used to examine ten enzyme systems, namely: esterase (EST, EC 3.1.1.1); fluorescent esterase (F-EST, EC 3.1.1.1); L-aspartate aminotransferase (AAT, EC 2.6.1.1); isocitrate dehydrogenase (IDH, EC 1.1.1. 42); malate dehydrogenase (MDH, EC 1.1.1.37); 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.43); phosphoglucose isomerase (PGI, EC 5.3.1.9); phosphoglucomutase (PGM, EC 5.4.2.2); shikimate dehydrogenase (SKDH, EC 1.1.1.25); superoxide dismutase (SOD, EC 1.15.1.1). Young leaf tissue and megagametophytes were ground with extraction buffer (Feret, 1971). Electrophoresis and staining followed the procedures described by Cheliak & Pitel (1984).

Isozyme data analysis

Allele frequencies were calculated for each locus and population. The following four measures were used to quantify genetic variation within a population: (1) the expected heterozygosity (Nei, 1975) at each locus was calculated as

$$H_e = 1 - \sum_{i=1}^{k} P_i^2,$$

where P_i is the frequency of the ith allele, summed over k alleles; (2) the mean number of heterozygous loci per individual was calculated (Nei, 1973); (3) the mean number of alleles per locus was calculated by averaging over all polymorphic and monomorphic loci; and (4) the effective number of alleles per locus (A_e; Crow & Kimura, 1970), was defined as

$$A_e = 1 \Big/ \sum_{i=1}^{k} P_i^2 .$$

The number of alleles is maximized when the allele frequencies at any locus are equal. Both Wright's (1969) F-statistics and Nei's (1978) unbiased genetic identity (I_n) and genetic distance (D_n) were used to quantify the degree of differentiation among populations. The above calculations, with the exception of the effective number of alleles, were performed using BIOSYS-1 (Swofford & Selander, 1989).

DNA preparation

Total cellular DNA was prepared from 0.8 g of young leaf material using a modified mini-CTAB method (Murray & Thompson, 1980). Leaves were frozen in liquid nitrogen, ground to a fine powder and suspended in 15 mL extraction buffer (50 mM Tris–HCl, pH 8.0, 350 mM sorbitol, 5 mM sodium EDTA, 10 per cent polyethylene glycol 3350, 0.1 per cent bovine serum albumin, 0.1 per cent spermine, 0.1 per cent spermidine and 0.1 per cent 2-mercaptoethanol). The extraction was filtered through miracloth and centrifuged at 13 000 g for 15 min in a Kontron H-401 centrifuge. The pellet was resuspended in 350 μL resuspension buffer (50 mM Tris–

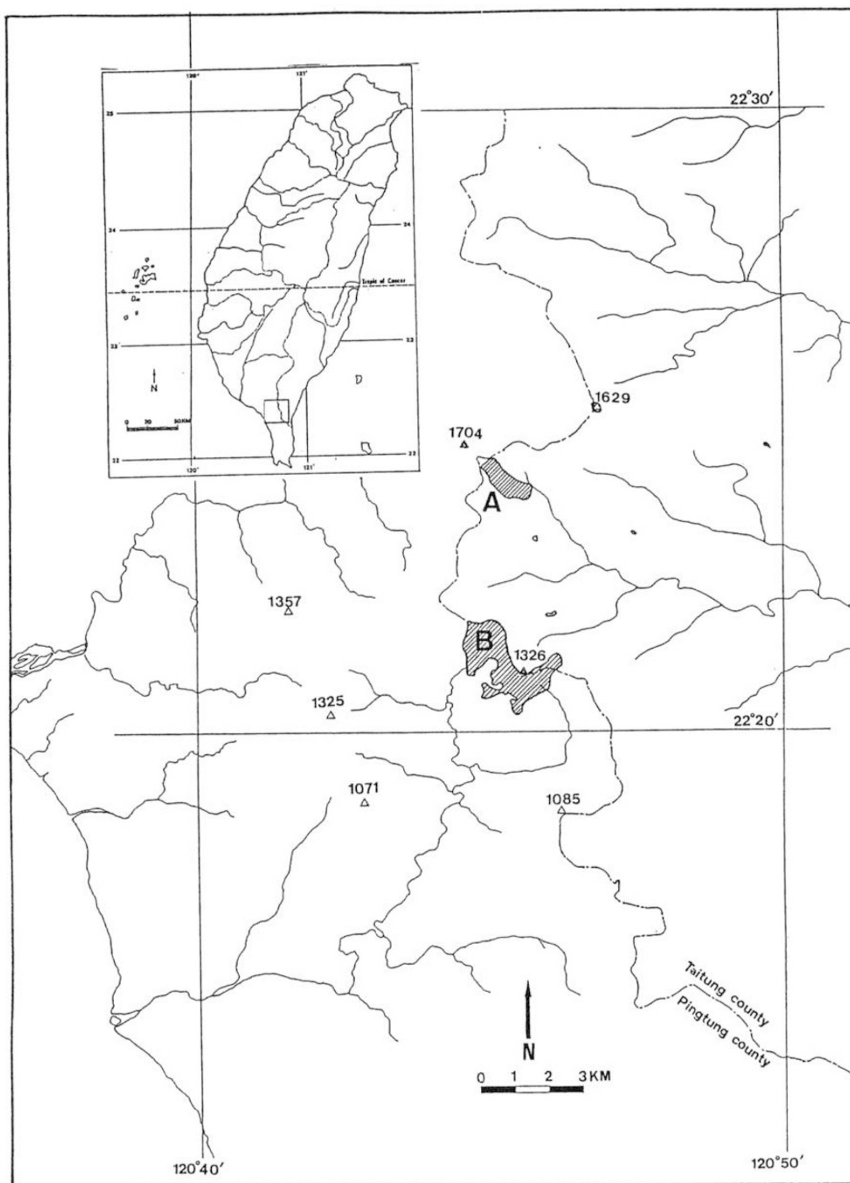

Fig. 1 The natural reserves for *Amentotaxus formosana*. A, Tawu; B, Tsatsayalai.

HCl, pH 8.0, 25 mM EDTA, 350 mM sorbitol and 0.1 per cent 2-mercaptoethanol) and the nuclei and organelles lysed by addition of 25 μL 20 per cent sarkosyl (N-lauryl sarcosinate) and incubating at room temperature for 15 min. After adding 70 μL 5 M NaCl and 55 μL 8.6 per cent CTAB (cetyltrimethylammonium bromide) and heating at 60°C for 10 min, the homogenate was extracted with 600 μL chloroform:isoamyl alcohol (24:1) and centrifuged in a Kubota KM-15200 microcentrifuge at 5000 ***g*** for 10 min. The nucleic acid was precipitated from the aqueous phase by adding 400 μL isopropanol and pelleted by centrifugation at 12 000 ***g*** for 10 min in the microcentrifuge, then washed with 70 per cent absolute ethanol. The pellet was dried and dissolved in 100 μL TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM disodium EDTA), containing 20 mg/mL RNase, and stored at -20°C. The DNA concentration was determined using a Hoeffer fluorometer and adjusted to 10 ng/μL for use in the polymerase chain reaction (PCR).

Polymerase chain reaction

PCR conditions for RAPD reaction with the Idaho Air Thermal Cycler are described as follows. Each sample, comprising 50 mM Tris–HCl buffer (pH 8.5) containing 20 mM KCl, 1.5 mM $MgCl_2$, 0.5 mg/mL

Table 1 Sequences and codes of random primers and the number of monomorphic and polymorphic fragments amplified

Primer	Sequence (5′→3′)	No. of monomorphic fragments	No. of polymorphic fragments	Total
OPE-2	GGTGCGGGAA	9	6	15
OPE-12	TTATCGCCCC	21	2	23
OPE-17	CTACTGCCGT	5	0	5
OPE-19	ACGGCGTATG	15	3	18
OPS-1	CTACTGCGCT	12	0	12
OPS-10	ACCGTTCCAG	10	7	17
OPS-13	GTCGTTCCTG	7	1	8
OPS-18	CTGGCGAACT	10	3	13
OPY-2	CATCGCCGCA	13	1	14
OPY-7	AGAGCCGTCA	7	0	7
OPY-9	AGCAGCGCAC	14	2	16
OPY-10	CAAACGTGGG	15	0	15
OPY-17	GACGTGGTGA	19	1	20
P-4	CGAAGCTTCG	13	1	14
P-6	CCGTCGACGA	5	8	13
P-10	ATTGCGTCCA	19	0	19
P-11	ATGTCCTCGA	10	0	10
P-13	TCAGCGTGCT	9	0	9
P-14	TACCGAACGT	8	19	27
P-25	GGTACCGTGC	8	6	14
Total		229 (79.2%)	60 (20.8%)	289

BSA, 200 μM each of dATP, dCTP, dGTP, dTTP, 0.4 μM 10-base primer, 60 ng of template DNA and 1.7 units of Taq DNA polymerase (Boeringer Mannheim Biochemica) at a final volume of 20 μL, was heat-sealed in a 25 μL glass capillary tube. Twenty random primers, 13 (OPE-2,..., OPY-17) supplied by Operon Technologies and 7 (P-4,..., P-25) synthesized by Oligos Etc., were included in the survey (Table 1). The amplification conditions included a total of 45 cycles with template denaturation at 94°C for 60 s, primer annealing at 37°C for 7 s, and primer extension at 72°C for 70 s during the first two cycles. The time for template denaturation was then reduced to 1 s for the remaining 43 cycles. Reactions were further incubated at 72°C for 4 min and the capillaries were stored at 4°C before the amplification products were analysed by gel electrophoresis.

Analysis of PCR products

PCR products were separated using 1.5 per cent NuSieve 3:1 agarose (FMC BioProducts) gels by electrophoresis in 1× TBE buffer, and detected by means of ethidium bromide staining, viewed under ultraviolet light. Specific amplification products were scored as present (1) or absent (0) in each DNA sample and similarity coefficients (*SC*) were estimated using Nei & Li's (1979) matching coefficient method

$$SC = 2N_{AB}/(N_A + N_B),$$

where N_A is the number of bands in individual A, N_B is the number of bands in individual B, and N_{AB} is the number of bands present in both A and B. Within-population similarity (S) was calculated as the mean of *SC* across all possible comparisons between individuals within a population. Between-population similarity, corrected for within-population similarity, was

$$S_{ij} = 1 + S'_{ij} - 0.5\,(S_i + S_j),$$

where S_i and S_j are the values of S for population i and j, respectively, and S'_{ij} is the average similarity between randomly paired individuals from populations i and j (Lynch, 1990).

Table 2 Allele frequencies and the expected (H_e) and observed (H_o) heterozygosities of the polymorphic locus in the two populations of *Amentotaxus formosana*

Locus and allele	Population		Avg.
	Tawu	Tsatsayalai	
Pgi-1			
a	0.090	0.000	0.045
b	0.910	1.000	0.955
H_o	0.180	0.000	0.090
H_e	0.164	0.000	0.086
Avg. H_o	0.009	0.000	0.0045
Avg. H_e	0.008	0.000	0.004

Expected heterozygosity for each population was calculated as the arithmetic mean at the 20 loci.

Table 3 The percentage of polymorphic loci, the percentage of heterozygous loci per individual, the mean number of alleles per locus, and the effective number of alleles per locus for each population of *Amentotaxus formosana*

	Tawu	Tsatsayalai
% Polymorphic loci*	5.0	0
%Heterozygous loci/individual†	0.009 (0.009)	0 (0.000)
Mean no. of alleles/locus†	1.05 (0.05)	1.00 (0.00)
Effective no. of alleles/locus	1.01	1.00

*The frequency of the common allele is <0.99.
†SE is shown in parentheses.

Table 4 Results of the χ^2 contingency test and F-statistics for *Pgi-1* in the two populations of *Amentotaxus formosana*

	χ^2	d.f.	F_{IS}	F_{IT}	F_{ST}
Pgi-1	0.804†	1	−0.099	−0.047	0.047
Average			−0.099	−0.047	0.047

†Not significant (5%).

Results

Isozyme analysis

Isozyme patterns from gametophyte and leaf tissue were compared to define the enzyme loci. With the exception of PGI, no differences in band number were found between the gametophyte and leaf tissue. The number of loci was determined according to Weeden & Wendel (1989).

Ten enzyme systems, with a total of 20 putative loci, were stained with consistently good resolution: two loci for PGM, PGI, IDH, AAT, 6PGD and SOD; three for MDH and EST; and one locus for SKDH and F-EST. All loci were monomorphic, with the exception of *Pgi-1*, which resolved two cathodally migrating alleles.

The observed allele frequencies, observed and expected heterozygosities at the polymorphic locus, and average heterozygosities at the population level are listed in Table 2. Allele *Pgi-1a* was found only in the Tawu population, whereas *Pgi-1b* was observed in both populations. Two genotypes, *ab* and *bb*, have been observed so far. The proportion of polymorphic loci, percentage of heterozygous loci per individual, the mean number of alleles per locus, and the effective number of alleles per locus were 5 per cent, 0.9 per cent, 1.05, and 1.01, respectively, for the Tawu population, whereas no variation was found for Tsatsayalai population (Table 3).

F-statistics are listed in Table 4. The χ^2-test was performed according to the formulae of Li & Horvitz (1953). The F_{IS} value for the *Pgi-1* locus was negative (−0.099), but the χ^2 analysis showed no significant deviation from zero at the 5 per cent level, indicating that the observed distribution of genotypes within a population was in Hardy–Weinberg equilibrium. Treating the entire species as a random mating unit, estimates of F_{IT} are closer to zero than F_{IS} for the locus surveyed. The extent of genetic differentiation among populations (F_{ST}) was 0.047. Thus, more than 95 per cent of the genetic variation resided within a population.

When the 110 individuals originating from the Tawu population were divided into four categories, based on their DBH, it was found that the young cohort with a DBH less than 5 cm had the highest heterozygosity ($H = 0.25$), whereas the older cohorts, with 15~25 cm and ≧25 cm DBH, gave lower values of $H = 0.105$ and $H = 0.111$, respectively (Table 5). The negative values of the fixation index indicated that the observed distribution of genotypes within a category had a slight excess of heterozygotes.

RAPD analysis

The 20 random primers used in this study generated a total of 289 DNA fragments (Table 1). Sixty of these fragments (20.8 per cent) were polymorphic, and 229 (79.2 per cent) monomorphic. The number

Table 5 Genotype distribution and allele frequency at *Pgi-1* in four DBH classes in the Tawu population of *Amentotaxus formosana*

	DBH (cm) No.	≦5 40	5~15 42	15~25 19	>25 9	Mean*
	aa	0	0	0	0	
Genotype	*ab*	10	9	2	1	
	bb	30	33	17	8	
Allele	*a*	0.125	0.107	0.053	0.056	0.100
	b	0.875	0.893	0.947	0.944	0.900
H_o		0.250	0.214	0.105	0.111	0.200
H_e		0.222	0.194	0.102	0.111	0.181
F†		−0.143	−0.120	−0.056	−0.059	

*Weighted by the number of individuals.
†Fixation index.

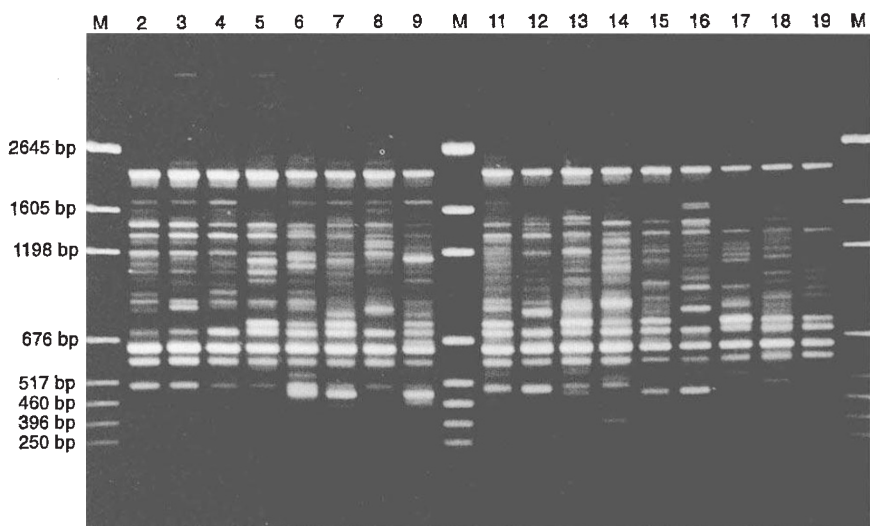

Fig. 2 RAPD polymorphism in *Amentotaxus formosana* using P-14. Lanes 2–9 represent eight individuals from the Tsatsayalai population; lanes 11–19 represent nine individuals from the Tawu population; M represents pGEM DNA size markers.

of scorable RAPD fragments generated per primer varied between five and 27, while the number of polymorphic bands per primer ranged between one and 19 (Fig. 2). The size of the DNA fragments ranged between 300–3000 bp. Seven of the primers (i.e. OPE-1; OPS-1; OPY-7; OPY-10; P-10; P-11; and P-13) detected no variation and the monomorphic profiles they amplified were shared by all individuals in both populations.

Observing the pairwise similarity coefficient (*SC*) across all possible comparisons, the maximum value of *SC* (0.992) was found within the Tawu population, and the minimum value (0.939) between the two populations. The average similarity coefficients within the Tawu and Tsatsayalai populations and between them were 0.974, 0.970 and 0.966, respectively (Table 6). The between-population similarity (S_{ij}), corrected for within-population similarity, was 0.994, and the corresponding genetic distance between the two populations was 0.006.

Discussion

The low genetic diversity detected in *A. formosana* during this study is most likely the result of the geological history of Taiwan. Strong tectonic activities (Penglai orogeny) were recorded in the middle of the Pleistocene (Teng, 1987). Several drastic vegetational changes were recorded in Taiwan during the Pleistocene and the last 60 000 years (Tsukada, 1967). The coldest climate prevailed in the Tali glacial age or early Würm glacial age, when a rapid expansion of the boreal elements took place (Tsukada, 1966). It is hypothesized that there was a drastic reduction in the number of trees in Taiwan during this geological age, forming a bottleneck that

Table 6 Average similarity coefficients (*SC*) within and between populations of *Amentotaxus formosana*

	Within population		Between populations	
	Tawu	Tsatsayalai	Not corrected	Corrected
Similarity*	0.974 (0.010)	0.970 (0.012)	0.966 (0.007)	0.994

*SE is shown in parentheses.

resulted in low genetic variation. Species such as *A. formosana* probably survived the extreme climate fluctuation by migrating to lower-elevation refugia during the Quaternary (Li, 1955). The geological reason for the genetic depauperation of *A. formosana* may be similar to that which caused the low genetic diversity in red pine; this low diversity resulted from passage through a genetic bottleneck during glacial episodes of the Holocene (Fowler & Morris, 1977; Simon *et al.*, 1986). The low genetic diversity could also be a result of the small populations of *A. formosana* confined to southern Taiwan. Because random genetic drift occurs particularly in small populations (Hartl, 1980), it results in fixation of alleles after many generations.

Genetic heterogeneity is often attributed to a local adaptation to environmental variations (Hamrick *et al.*, 1992). The F_{ST} value indicates that 4.7 per cent of the genetic diversity found in this study occurred between the two populations. This low interpopulational differentiation is consistent with data from many other conifers (Hamrick *et al.*, 1992).

The slight but not significant excess of heterozygotes in the Tawu population ($F_{IS} = -0.099$) is probably because no individuals with genotype *Pgi-1aa* were found. Indeed, *Pgi-1aa* is probably absent from the whole population as a total of 110 individuals, which comprises approx. 16 per cent of the Tawu population, was screened. However, as stated in 'Materials and methods', sampling was not absolutely random owing to inaccessibility. The chance of allele *Pgi-1a* not being picked up was always possible, given that it is a rare allele. Also the possibility exists that the apparent absence of the allele in the Tsatsayalai population is the result of sample size.

The increase in frequency of allele *Pgi-1a* in individuals with decreasing DBH (Table 5) may have several explanations. First, the allele *Pgi-1a* may be lost in the Tsatsayalai population but has remained unfixed in the Tawu population; this could have resulted from random genetic drift occurring in a small population. Alternatively, limiting ecological factors, including altitude, moisture, microclimate and their interactions found in natural habitats, suggest a strong selection pressure against *Pgi-1aa*. However, these two hypotheses do not account for the absence of the allele *Pgi-1a* in the Tsatsayalai population, which could be considered as a single panmictic unit with the Tawu population. *Amentotaxus formosana* was occasionally found between these two populations even if it is uncommon. As outcrossing wind-pollinated gymnosperms have the least variation among populations (Hamrick & Godt, 1990), the exchange of gametes between these two populations is always possible. The occurrence of the allele *Pgi-1a* may also be the result of a recent mutation in the Tawu population. The frequency of allele *Pgi-1a* increased from 0.056 in older trees to 0.125 in the youngest trees. This observation tends to support this hypothesis, as it may explain the absence of allele *Pgi-1a* in the Tsatsayalai population. The increase in frequency of allele *Pgi-1a* may have been caused by selection acting directly upon trees carrying this allele. The allele *Pgi-1a* may eventually be detected in the Tsatsayalai population, as no barrier has been found between them. However, dispersion of this allele may be slow because of the large and heavy seeds, which fall around the mother tree. The flowers may receive predominantly the pollen from nearby relatives, even though the pollen could also be transferred a long distance by wind.

The percentage of polymorphism detected using RAPDs was greater than that for isozyme markers. Unfortunately, no RAPD marker specific to either the Tawu or the Tsatsayalai population was found. However, the lack of variation between individuals within and between populations, as revealed by RAPD analysis, agrees with the low level found using isozymes. A similar observation, based on isozyme and RAPD data has been reported for red pine (Mosseler *et al.*, 1992).

Acknowledgements

We would like to thank Prof. Shong Huang (National Taiwan Normal University) for his helpful advice and for stimulating discussion. This research was supported by grant 84AST-2,3-FOD-04 from the Council of Agriculture, Taiwan.

References

CHELIAK, W. M. AND PITEL, J. A. 1984. Techniques for starch gel electrophoresis of enzymes from forest tree

© The Genetical Society of Great Britain, *Heredity*, **77**, 388–395.

species. *Can. Forestry Serv. Inf. Rep*. PI-X-42, pp. 19–45. Petawawa National Forestry Institute.

CROW, J. F. AND KIMURA, M. 1970. *An Introdution to Population Genetics Theory*. Harper and Row, New York.

FARJON, A., PAGE, C. N. AND SCHELLEVIS, N. 1993. A preliminary world list of threatened conifer taxa. *Biodiversity and Conservation*, **2**, 304–326.

FERET, P. P. 1971. Isozyme variation in *Picea glauca* (Moench) Voss seedlings. *Silvae Genet*., **20**, 46–50.

FOWLER, D. P. AND MORRIS, R. W. 1977. Genetic diversity in red pine: evidence for low heterozygosity. *Can. J. Forest Res*., **7**, 341–347.

HAMRICK, J. L. AND GODT, M. J. W. 1990. Allozyme diversity in plant species. In: Brown, A. H. D., Clegg, M. T., Kahler, A. L. and Weir, B. S. (eds) *Plant Population Genetics, Breeding, and Genetic Resources*, pp. 43–63. Sinauer Associates, Sunderland, MA.

HAMRICK, J. L., GODT, M. J. W. AND SHERMAN-BROYLE, S. L. 1992. Factors influencing levels of genetic diversity in woody plant species. *New Forests*, **6**, 95–124.

HARTL, D. L. 1980. *Principles of Population Genetics*. Sinauer Associates, Sunderland, MA.

HEUN, M., MURPH, J. P. AND PHILIPS, T. D. 1994. A comparison of RAPD and isozyme analyses for determining the genetic relationships among *Avena sterilis* L. accession. *Theor. Appl. Genet*., **87**, 689–696.

LI, C. C. AND HORVITZ, D. G. 1953. Some methods of estimating the inbreeding coefficient. *Am. J. Hum. Genet*., **5**, 107–117.

LI, H. L. 1955. The genetic affinities of the Formosan flora. *Proc. 8th Pacif. Sci. Congr*., **4**, 189–195.

LYNCH, M. C. 1990. The similarity index and DNA fingerprinting. *Mol. Biol. Evol*., **7**, 478–484.

MAAß, H. I. AND KLAAS, M. 1995. Infraspecific differentiation of garlic (*Allium sativum* L.) by isozyme and RAPD markers. *Theor. Appl. Genet*., **91**, 89–97.

MEIJER, G., MEGNEGNEAU, B. AND LINDERS, E. G. A. 1994. Variability for isozyme, vegetative compatibility and RAPD markers in natural populations of *Phomopsis subordinaria*. *Mycol. Res*., **98**, 267–276.

MOSSELER, A., EGGER, K. N. AND HUGHES, G. A. 1992. Low levels of genetic diversity in red pine confirmed by random amplified polymorphic DNA markes. *Can. J. Forest Res*., **22**, 1332–1337.

MURRAY, M. G. AND THOMPSON, W. F. 1980. Rapid isolation of high molecular weight plant DNA. *Nucl. Acids Res*., **8**, 4321–4325.

NEI, M. 1973. Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci. U.S.A*., **70**, 3321–3323.

NEI, M. 1975. *Molecular Population Genetics and Evolution*. American Elsevier, New York.

NEI, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590.

NEI, M. AND LI, W. H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. U.S.A*., **76**, 5269–5273.

SIMON, J. P., BERGERON, Y. AND GAGNON, D. 1986. Isozyme uniformity in populations of red pine (*Pinus resinosa*) in the Abitibi Region, Quebec. *Can. J. Forest Res*., **37**, 10–14.

SWOFFORD, D. L. AND SELANDER, R. B. 1989. BIOSYS-1. *A Computer Program for the Analysis of Allelic Variation in Population Genetics and Biochemical Systematics*. Release 1.7. University of Illinois, Urbana, IL.

TENG, L. S. 1987. Stratigraphic records of the Late Cenozoic Penglai orogeny of Taiwan. *Acta Geol. Taiwan*, **25**, 205–224.

TSUKADA, M. 1966. Late Pleistocene vegetation and climate in Taiwan (Formosa). *Proc. Natl. Acad. Sci. U.S.A*., **55**, 543–548.

TSUKADA, M. 1967. Vegetation in subtropical Formosa during the Pleistocene glaciations and the Holocene. *Palaeogeogr. Palaeoclim. Palaeoecol*., **3**, 49–64.

WEEDEN, N. F. AND WENDEL, J. F. 1989. Genetics of plant isozymes. In: Soltis, D. E. and Soltis, P. S. (eds) *Isozyme in Plant Biology*, pp. 46–72. Dioscorides Press, Portland, OR.

WILLIAMS, J. G. K., KUBELIK, A. R., LIVAK, K. J., RAFALSKI, J. A. AND TINGEY, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res*., **18**, 6531–6535.

WOLFF, K. 1991. Analysis of allozyme variability in three *Plantago* species and a comparison to morphological variability. *Theor. Appl. Genet*., **81**, 119–126.

WRIGHT, S. 1969. *Evolution and the Genetics of Populations*, vol. 2, *The Theory of Gene Frequencies*. University of Chicago Press, Chicago.

YANG, S. Z. Studies on the vegetation ecology of Tsatsayalai Shan nature reserve for *Amentotaxus formosana* in southern Taiwan. *Q. J. Chin. For*., **27**, 19–24 (in Chinese).

YEH, C. L., CHEN, C. C., CHUNG, Y. L. AND FANG, Q. T. 1992. Using GIS techniques to investigate changes of the *Amentotaxus formosana* population. *Remote Sensing*, **16**, 28–51 (in Chinese).