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Genetic diversity and biogeography of *Cunninghamia konishii* (Cupressaceae), an island species in Taiwan: a comparison with *Cunninghamia lanceolata*, a mainland species in China

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Abstract

Luanta-fir (*Cunninghamia konishii*), an endemic to Taiwan, is an outcrossing, long-lived conifer. Populations of *C. konishii* are generally fragmented due to a once high intensity of timber exploitation. *C. konishii* and *Cunninghamia lanceolata* are two sibling taxa constituting derivative-progenitor species relationship. The amount of genetic variations within and between 11 and 10 populations of *C. konishii* and *C. lanceolata*, respectively, were assessed using amplified fragment length polymorphism (AFLP) markers in this report. Three AFLP primer pairs generated a total of 357 and 226 markers for *C. konishii* and *C. lanceolata* samples, of which 56.1 and 65.3% are polymorphic, respectively. Analysis of molecular variance indicates a 4.78% variation between *C. konishii* and *C. lanceolata*. A relatively high value of genetic variation (24.60%) was apportioned between the populations of *C. konishii*. In contrast, a lower divergence value (12.21%) between populations was found for *C. lanceolata*. The population with the highest genetic diversity was found in Nantou County, which concurred with the results of many other tree species investigated in Taiwan. The estimates of the number of migrants between populations (*Nm*), obtained from population pair-wise Φ_{ST}, suggest that gene flow in *C. konishii* is efficient in some adjacent populations but is restricted in the rest. Individual UPGMA tree, generated based on AFLP markers, suggests six evolutionary lineages for *C. konishii*. All evolutionary lineages of *C. konishii* were derived from *C. lanceolata*. In conclusion, the migration patterns of *Cunninghamia* from mainland China may have been established following multiple sources, migrant-pools, long-distance dispersal events, and via different directions.

Keywords: AFLP; Biogeography; *Cunninghamia konishii*; Genetic diversity; Taiwan

1. Introduction

Population history plays a major role in shaping the spatial distribution of genetic diversity (Schaal et al., 1998; Taberlet et al., 1998). Higher level of allelic richness has been found in source populations than plants in colonized areas (Comps et al., 2001; Widmer and Lexer, 2001). Moreover, the cause for less genetic variability in the islands than in continental populations was attributed to bottleneck or founder effect occurred in island colonization (Barrettt and Shore, 1989; Nei, 1987). Loss of genetic variability is probably a general pattern of species with island-mainland distributions (Frankham, 1997).

The preservation of genetic diversity is a common emphasis of conservation programs. DNA type markers are able to detect the genetic variation beyond coding loci and to provide broader information on the amount of genetic variation and the genetic divergence among

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populations. Amplified fragment length polymorphism (AFLP) has been used successfully in the study of genetic diversity in many plant species (e.g., Keiper and McConchie, 2000; Larson et al., 2001; Muluvi et al., 1999; Palacios et al., 1999). The advantages of this technique include large number of loci assayed, high levels of polymorphisms, high reproducibility, no requirement of prior sequence knowledge, and genome-wide distribution of markers (Powell et al., 1996). Krauss (2000) showed that AFLP, as a dominant marker, is useful for the accurate estimation of genetic diversity. Moreover, AFLP is useful in the estimation of genetic diversity when compared to other types of neutral DNA markers (Powell et al., 1996).

The genus *Cunninghamia* R. Br. exRich. (Cupressaceae) consists of two extant members distributed in Taiwan and China. *Cunninghamia konishii* Hay. (=*Cunninghamia lanceolata* (Lamb.) Hook. var. *konishii* (Hay.) Fujita) is an endemic species in Taiwan and *C. lanceolata* (Lamb.) Hook. is a species that thrives only in mainland China. In the Tali glacial stage (50,000–10,000 years ago) during late Pleistocene, *C. konishii* dominated among other conifers when Taiwan was a part of mainland Asia (Tsukada, 1967). Presently, *C. konishii* is usually found scattered within forests of *Chamaecyparis*, *Pinus spp*., and *Pseudotsuga wilsoniana* at elevations of 1300–2800 m (Liu, 1966). Old growth of *C. konishii* is a valuable timber source, however, *C. konishii* is strongly threatened by anthropogenic disturbance of its habitats e.g., about 60 hectares of pure stand of *C. konishii* in Shianshanshan, Hsinchu County, Taiwan was completely deforested. Conservation of this species ex situ in seed orchards requires understanding of its genetic variation within and among natural populations as a first step in defining ex situ conservation programs.

Cunninghamia konishii has the highest genetic diversity ($He = 0.219$) found among the plant species revealed by allozyme assay from natural populations in Taiwan (Lin et al., 1998) which is higher than the average value reported for many other conifers (average $He = 0.151$, Hamrick et al., 1992). Moreover, the genetic diversity of *C. lanceolata* has also been assayed by allozyme and is significantly higher as compared to *C. konishii* in several studies ($He = 0.299$, Müller-Starck and Liu, 1989; $He = 0.394$, Yeh et al., 1994; $He = 0.343$, Lin et al., 1998). The high level of genetic diversity in *C. lanceolata* is probably related to the pooling of the *C. lanceolata* genetic materials from initially structured populations during the 2000 years of cultivation (Chen and Shi, 1987; Yeh et al., 1994).

Little genetic differentiation between *C. lanceolata* and *C. konishii* was found and progenitor-derivative species relationship with the latter species differentiated from the former was inferred (Hwang et al., 2003; Lin et al., 1998; Lu et al., 2001). The levels of genetic differentiation analyzed by allozyme variation among populations were 6 and 3% examined for *C. lanceolata* and *C. konishii*, respectively (Lin et al., 1998; Yeh et al., 1994). Low levels of population differentiation in both *C. lanceolata* and *C. konishii* were also found by chloroplast DNA (cpDNA) non-coding sequence data (Hwang et al., 2003). The levels of genetic differentiation among populations of these two *Cunninghamia* species are similar to those of other gymnosperms and concurred with species that are primarily outcrossing and long-lived (Hamrick et al., 1992). Low level of allozyme and cpDNA differentiation may be taken as evidence that *C. konishii* populations are genetically homogeneous across its geographic range and thus any population represents the diversity found in the species as a whole. Higher genetic differentiation among populations of forest trees based on neutral DNA markers has been found in *Pseudotsuga menziesii* (Aagaard et al., 1995), *Populus grandidentata* (Liu and Furnier, 1993), *Pinus leucodermis* (Bucci et al., 1997), *Chamaecyparis formosensis*, and *Chamaecyparis taiwanensis* (Hwang et al., 2001) in comparison with the allozyme data. Reassessment of population differentiation using neutral DNA type markers such as AFLP is crucial in conservation biology, which can provide information such as gene flow, and population history including the determination of evolutionary lineages (Ennos, 1996).

Genealogical analysis on *Cunninghamia* was first carried out by examining DNA sequences of one chloroplast intergenic spacer with limited sample size (Lu et al., 2001). Hwang et al. (2003) subsequently examined four chloroplast non-coding DNA sequences with a relatively larger sample size for both *Cunninghamia* species. Both studies revealed a close relationship between these two species. Furthermore, Lu et al. (2001) proposed a hypothesis of multiple sources and migrant-pool model for *Cunninghamia* invasion. This hypothesis was supported in part by Hwang et al. (2003) that most individuals from these two species shared the most common ancestral cpDNA haplotype. Sharing of the most common ancestral cpDNA haplotype indicated a possible migrant-pool introduction of *Cunninghamia*. However, many rare haplotypes (singletons) were derived independently following a glacial population bottleneck. It is therefore interesting to investigate further on the genetic relationship of these two species and to test whether multiple sources with different dispersal directions are likely to be the case in *Cunninghamia* introduction when more sampling across species ranges and multilocus molecular markers, i.e., AFLP are investigated.

The significance of this work is twofold. First, we investigated the molecular genetic structure in two extant members of *Cunninghamia*. Second, we assessed the level of gene flow, means of colonization, and the genetic relationship of these two species by individual AFLP haplotype analysis. We found that AFLP polymorphism provided insights that were not detected in

allozyme and cpDNA studies. We postulate that *Cunninghamia* invaded Taiwan from multiple sources of mainland China, and following migrant-pools and long-distance dispersal events.

2. Materials and methods

2.1. Plant materials

Eleven populations encompassing the whole distributional range and accounting for 146 individuals of *C. konishii* were collected from the seed orchard at Chuyunshan, a clonal garden at Lienhuachih Station, Taiwan Forestry Research Institute (TFRI), and from old growths of Shiyuan, Wuser, and Alishan. The sites of the 11 populations sampled in this study are shown in Fig. 1. The Chuyunshan seed orchard was established between 1968 and 1974 at an elevation of 700 m, and was composed of 25 grafted clones planted over 10 hectares. These 25 clones were originated from an old growth of *C. konishii* and grew at elevations of 1150–2350 m in central Taiwan. The Lienhuachih clonal garden, that consists of 139 clones originating from major old-growth forests of *C. konishii* and grew at elevations of 1500–2350 m, was established in June, 1978. Fifty-four individuals of *C. lanceolata* originating from 44 seed sources from China (10 provenances; expressed as populations) were also collected from the Lienhuachih Station (Table 1 and Fig. 1). Samples of *C. lanceolata* were used for the purpose of comparison with *C. konishii* in the estimation of genetic diversity and population differentiation, and for the examination of the individual genetic relationships.

2.2. DNA extraction and quantification

Total DNA was extracted from ground leaf powder according to a modified cetyltrimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle, 1987), which was described in detail by Hwang et al. (2001). DNA was precipitated with ethanol and after washing with 70% ethanol, it was dissolved in 200 μl TE buffer, pH 8.0, and placed at −20 °C. The DNA concentration was determined for each sample using GeneQuant II RNA/DNA Calculator (Amersham–Pharmacia Biotech).

2.3. AFLP amplification

AFLP analysis was performed essentially as described in Vos et al. (1995). Genomic DNA (300 ng) was first cut with 5 U of *Eco*RI restriction enzyme and then ligated with *Eco*RI adaptor. The *Eco*RI fragments were then subjected to PCR preamplification with the annealing of E00 pre-selective primers (Table 2). The preamplification was conducted in a reaction solution consisting of 0.15 μM E00 and M00 primers, 0.4 U

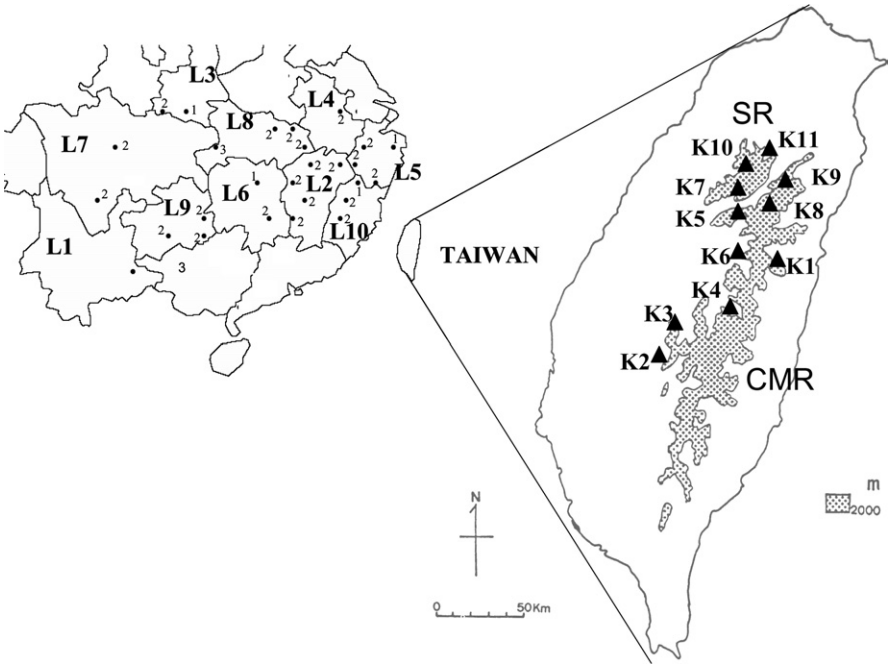

Fig. 1. Sample localities of *Cunninghamia konishii* and *Cunninghamia lanceolata*. The hatched area in Taiwan indicates the Shueshan Range (SR) and Central Mountain Ridge (CMR) in Taiwan. Populations K7, K10, and K11 of *C. konishii* are located in the SR. All other populations are located in the CMR. Population code labeled corresponded to that appeared in Table 1. The dots and the nearby numbers labeled for *C. lanceolata* in mainland China indicate the original site and number of plants collected.

Table 1
Site descriptions, specimens, expected heterozygosity (*He*), total number of amplified bands, percentage of polymorphism (%*P*, 95% criterion), and number of private fragments confined to only one population (U) of *Cunninghamia lanceolata* and *C. konishii*

Species and populations (code)	Sample size	Latitude (°N)	Longitude (°E)	*He*	Total bands	%*P*	*U*
C. konishii	146						
Yeinhai (K1)	6	24.23	121.24	0.2352	78	55.1	0
Alishan (K2)	13	23.31	120.46	0.1157	152	42.8	4
Chitou (K3)	6	23.42	120.47	0.3798	73	78.7	0
Denta (K4)	17	23.80	120.70	0.1628	142	59.2	0
Tajiann (K5)	16	24.10	121.00	0.1534	199	61.3	15
Wuser (K6)	12	24.00	121.00	0.1236	88	33.3	0
Tashueshan (K7)	8	24.30	121.10	0.2150	94	67.0	0
Shengkuang (K8)	9	24.20	121.20	0.2212	89	51.7	0
Shiyuan (K9)	11	24.26	121.20	0.1191	71	31.7	0
Kuanwu (K10)	29	24.75	121.10	0.1140	214	67.3	14
Shiouhluan (K11)	19	24.50	121.10	0.1815	186	68.8	6
Average			0.1838	113.3	56.1		
C. lanceolata	54						
Yunnan (L1)	3	23.00	104.21	0.3408	59	91.5	0
Jiangxi (L2)	10	26.50–28.30	114.10–114.25	0.2088	73	52.0	0
Sanxi (L3)	3	33.05–33.15	107.00	0.3619	86	95.3	0
Anhuei (L4)	2	32.50	118.20	0.3604	39	59.0	0
Zheijiang (L5)	9	28.06–29.50	118.35–120.90	0.2189	118	54.2	0
Hunan (L6)	3	26.52–25.80	109.41–113.00	0.3749	36	72.2	0
Xichuan (L7)	4	28.30–30.06	104.00–104.40	0.2962	61	96.7	0
Hubei (L8)	9	29.90–31.70	106.00–114.20	0.1786	112	41.1	1
Gueizhou (L9)	6	26.00–26.55	107.80–109.50	0.2731	47	40.4	0
Fuzeing (L10)	5	26.80–27.90	117.80–118.75	0.2490	73	50.7	
Average				0.2863	70.4	65.3	

Table 2
Adaptors and primers used for AFLP selective amplification

Adaptor site	Adaptor code	Adaptor sequence
*Eco*RI	Eco1	5′-CTCGTAGACTGCGTACC-3′ 3′-CTGACGCATGGTTAA-5′
*Mse*I	Mse1	5′-GACGATGAGTCCTGAG-3′ 3′-TACTCAGGACTCAT-5′
Pre-selective primers	E00	5′-GACTGCGTACCAATTC-3′
	M00	5′-GATGAGTCCTGAGTAA-3′
Selective primers	E00 + G	5′-GACTGCGTACCAATTCG-3′
	M00 + AG	5′-GATGAGTCCTGAGTAAAG-3′
	M00 + CT	5′-GATGAGTCCTGAGTAACT-3′
	M00 + GT	5′-GATGAGTCCTGAGTAAGT-3′

Taq polymerase, 0.5 μg/μl RNase, and 0.1 μM dNTPs in a reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0), to obtain a final reaction volume of 10 μl. The PCR program for preamplification was 20 cycles of 30 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C. The PCR preamplification was carried out using a Robocycler GRADIENT 96 temperature cycler (Stratagene). One microliter of this PCR product was then subjected to a second round of PCR amplification with Cy5 (Indodicarbocyanine phosphoramidite) labeled E00 + G and M00 + 2 primers (Table 2). The second round of PCR was conducted in a DNA Programmable Thermal Cycler (MJ Research). The fragments were amplified first at 94 °C for 3 min for 12 PCR cycles (30 s at 94 °C, 30 s at 65 °C with 0.5 °C touchdown per cycle, and 1 min at 72 °C), and then for 23 PCR cycles at 94 °C for 30 s, 30 s at 56 °C, and 1 min at 72 °C, and the last step was 5 min at 72 °C. The PCR products were separated by using 8% ReproGel High Resolution kit (Amersham–Pharmacia Biotech). The molecular size marker used was the Cy5 labeled 50–500 bp ladder. Electrophoresis was conducted on ALF Express II DNA Analysis System and the DNA fragments were recorded by ALFwin Fragment Analysis 1.01 software (Amersham–Pharmacia Biotech).

2.4. Data collection and analysis

All polymorphic bands were scored for the presence (1)/absence (0) across all loci. The computer program TFPGA (Tools for Population Genetic Analysis) was used to provide information on genetic diversity (*He*) for each population (Miller, 1997a). Average heterozygosity was calculated for each locus and averaged over loci according to the unbiased formula of Nei (1978). The AMOVAPREP program (Miller, 1997b) was used to prepare dominant marker data for AMOVA analysis. Components of variance partitioned within and between populations were estimated from a Euclidean distance matrix using WINAMOVA version 1.55 (analysis of molecular variance, Excoffier et al., 1992). The significance of AMOVA variance components was tested

using non-parametric permutation procedures. The AMOVA variance components were used as estimates of the genetic diversity within and between populations. By using Bartlett's test, the data were also tested for the assumption of homogeneity of molecular variance among populations and carried out simultaneously by WINAMOVA program. The UPGMA algorithm of Sneath and Sokal (1973) was used to generate a haplotype dendrogram. This algorithm was performed with SAHN (sequential, agglomerative, hierarchical, and nested clustering) routine using NTSYS-pc software version 2.0 (Exeter Software, Setauket, NY) based on a Jaccard's similarity matrix (Jaccard, 1908). This similarity algorithm was chosen because it ignores 0/0 matches, and is appropriate for molecular marker data producing only dominant bands.

3. Results

3.1. AFLP variation and genetic diversity

Initially, the AFLP procedure was conducted on a sample of 40 plants from 11 and 10 populations of *C. konishii* and *C. lanceolata*, respectively, to test the availability of 15 AFLP primer pairs. Only three AFLP primer pairs generated reproducible and unequivocally scorable fragments for further analysis. These three AFLP primer pairs employed generated a total of 357 and 226 markers for *C. konishii* and *C. lanceolata*, respectively. The molecular weights of the polymorphic fragments counted ranged in size from 50 to 350 bp. The *He*, total number of amplified bands, percent polymorphism, and private fragments for populations are shown in Table 1. The percent polymorphism that ranged 33.3–78.7% (mean = 56.1%) and 40.4–96.7% (mean = 65.3%) was detected across *C. konishii* and *C. lanceolata* populations, respectively. One hundred and thirty-five AFLP haplotypes were obtained for 146 *C. konishii* individuals and 54 were found for 54 *C. lanceolata*. The four populations in *C. konishii* that had population-specific AFLP fragments were Kuanwu 14, Alishan 4, Tajiann 15, and Shiouhluan 6, respectively. Hubei in *C. lanceolata* had one population-specific AFLP fragment.

The genetic diversity measures were estimated using TFPGA software (Miller, 1997a). Estimates of genetic diversity using AFLP data ranged from 0.1786 to 0.3749 with an average of 0.2863 in *C. lanceolata*. Hunan population had the highest *He* of 0.3749 and Hubei population had the lowest *He* of 0.1786 for *C. lanceolata*. Estimates of genetic diversity ranged from 0.1140 to 0.3798 with a mean of 0.1838 in *C. konishii*. Kuanwu population had the lowest *He* and Chitou had the highest *He* despite its small sample size examined. The population with the highest heterozygosity was found at a latitude of 23.42° corresponding to Chitou, which is located in Nantou County of central Taiwan. The Shengkuang, Shiouhluan, Yeinhai, and Tashueshan populations located between latitudes 24.20° and 24.50° also had high level of genetic variability.

3.2. Species divergence and population structure among populations

Genetic differentiation between *C. konishii* and *C. lanceolata* was low using AFLP markers ($\Phi_{CT} = 0.048$, $P = 0.016$) by AMOVA analysis (Table 3). The extent of genetic differentiation among populations was 24.6% ($\Phi_{ST} = 0.246$, $P < 0.001$ with 1000 non-parametric permutation, Table 3) and Bartlett's heterogeneity test was significant in *C. konishii* ($\chi^2 = 37.34$, df = 10, $P < 0.001$). The matrix of the pair-wise geographic distance (km) was not correlated with the corresponding matrix of pair-wise Φ_{ST} (Fig. 2; Mantel test: $r = 0.038$; $P = 0.0299$ with 1000 permutation). The effective number of migrants (*Nm*) for each population pair was calculated for the 11 populations of *C. konishii*, the most likely to exchange individuals (Table 4). Pair-wise Φ_{ST} values and the effective number of migrants indicated that the comparison of most population pairs differ significantly. Using AFLP high resolution markers, we found that the average number of migrants was 1.65 for populations of *C. konishii* and was moderate. Unexpected high levels of gene flow was found between

Table 3
Analysis of molecular variance based on AFLP amplification products

Source of variation	df	SSD	MSD	Variance component	% of total (*P*)
C. konishii and *C. lanceolata*	1	148.40	148.40	1.10	4.78 (=0.016)
C. konishii					
Among populations	10	895.66	89.57	5.74	24.60 (<0.001)
Within populations	135	2305.93	17.60	17.60	75.40
Total	145				
C. lanceolata					
Among populations	9	247.33	27.48	2.21	12.21 (<0.001)
Within populations	44	699.42	15.90	15.90	87.79
Total	53	946.75			

Levels of significance are based on 1000 random permutations.

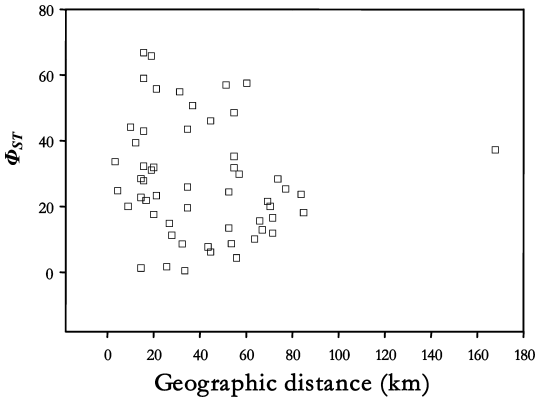

Fig. 2. Plot of the pair-wise Φ_{ST} values plotted against geographic distance (Mantel test) in the *Cunninghamia konishii* populations.

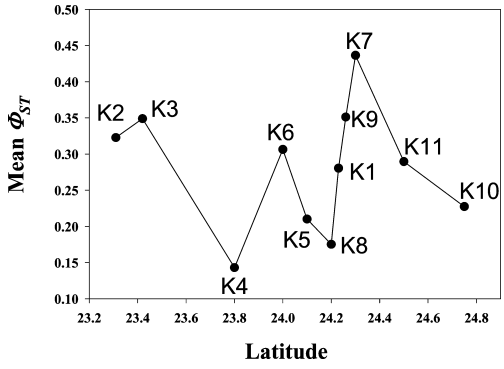

Fig. 3. Plot of the mean Φ_{ST} values of each population compared to every other population against the population latitude in *Cunninghamia konishii*. Population code labeled corresponded to that appeared in Table 1.

Yeinhai and Alishan ($Nm = 14.71$), between Alishan and Chitou ($Nm = 50.00$), and between Denta and Shiouhluan ($Nm = 18.66$). Only 12.2% ($\Phi_{ST} = 0.122$, $P < 0.001$ with 1000 non-parametric permutation) of the genetic differentiation was found among populations of *C. lanceolata*, and Bartlett's heterogeneity test was found to be insignificant ($\chi^2 = 1.62$, df = 9, $P = 0.059$, Table 3). Pair-wise Φ_{ST} values and the effective number of migrants were not estimated for *C. lanceolata* populations because of the pooling of genetic materials.

Interestingly, through the calculation of the average Φ_{ST} for individual *C. konishii* population in comparison with every other population, we found that Tashueshan (K7) was genetically the most distinct population followed by Shiyuan (K9) and Chitou (K3) (Fig. 3).

3.3. Genetic relationships of Cunninghamia individuals

Individual UPGMA tree revealed that *C. konishii* would originate from *C. lanceolata* (Fig. 4). Six clusters of *Cunninghamia* were classified in this dendrogram. The first major cluster (cluster A) represented individuals from populations K6, K9, and K10 of *C. konishii*. Cluster B contained only those individuals from K2, K3, K4, K8, and K9 of *C. konishii*. Cluster C was composed of individuals from K2, K5, and K10 of *C. konishii* and individuals of *C. lanceolata*. In cluster D, individuals from K1, K4, K5, K7, K8, and K11 of *C. konishii* were derived from individuals of Sanxi, Hubei, Gueizhou, and Fuzeing of *C. lanceolata*. Cluster E consisted of individuals from K2, K4, and K8 of *C. konishii* and two individuals of *C. lanceolata*. The F cluster consisted mostly of *C. lanceolata* and two individuals of *C. konishii*. All individuals of K1, K3, K6, K7, and K11 populations of *C. konishii* were grouped together in respective clusters. However, individuals of the *C. konishii* populations K2, K4, K5, K8, K9, and K10 were scattered in different clusters.

4. Discussion

4.1. Genetic diversity based on AFLP markers

The AFLP analysis was very efficient in detecting genetic variation in the genomes of both *C. konishii* and *C. lanceolata*. No population was characterized monomorphic using the AFLP polymorphisms. Higher number of

Table 4
Pairwise Φ_{ST} values (below diagonal) and the effective number of migrants (Nm) (above diagonal) illustrating population divergence in *Cunninghamia konishii* analyzed by AMOVA

	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10	K11
K1		14.7059	1.1416	1.9380	1.0979	0.6342	0.4241	1.2456	0.5429	1.0068	0.7869
K2	0.0170	***	50.0000	1.5423	0.8964	0.5743	0.3801	1.0221	0.4483	0.7835	0.4553
K3	0.2190	0.005	***	1.5060	0.8781	0.5663	0.3743	1.0540	0.4390	0.7750	0.4346
K4	0.1290	0.1621	0.1660	***	5.6818	0.9866	0.8381	2.2163	1.6779	2.4704	18.6567
K5	0.2277	0.2789	0.2847	0.0440	***	1.0711	0.7423	1.5985	0.9634	1.2463	3.2216
K6	0.3942	0.4353	0.4415	0.2534	0.2334	***	1.4188	1.3759	0.7096	0.8036	0.8806
K7	0.5895	0.6578	0.6679	0.2983	0.3368	0.1762	***	0.4931	0.4931	0.5823	0.5149
K8	0.2007	0.2446	0.2372	0.1128	0.1564	0.1817	0.2159	***	1.2749	2.0991	2.8902
K9	0.4605	0.5577	0.5695	0.149	0.2595	0.3523	0.5070	0.1961	***	2.8670	0.6713
K10	0.2483	0.3191	0.3226	0.1012	0.2006	0.3111	0.4293	0.1191	0.0872	***	1.8519
K11	0.3177	0.5491	0.5752	0.0134	0.0776	0.2839	0.4855	0.0865	0.3724	0.1350	***

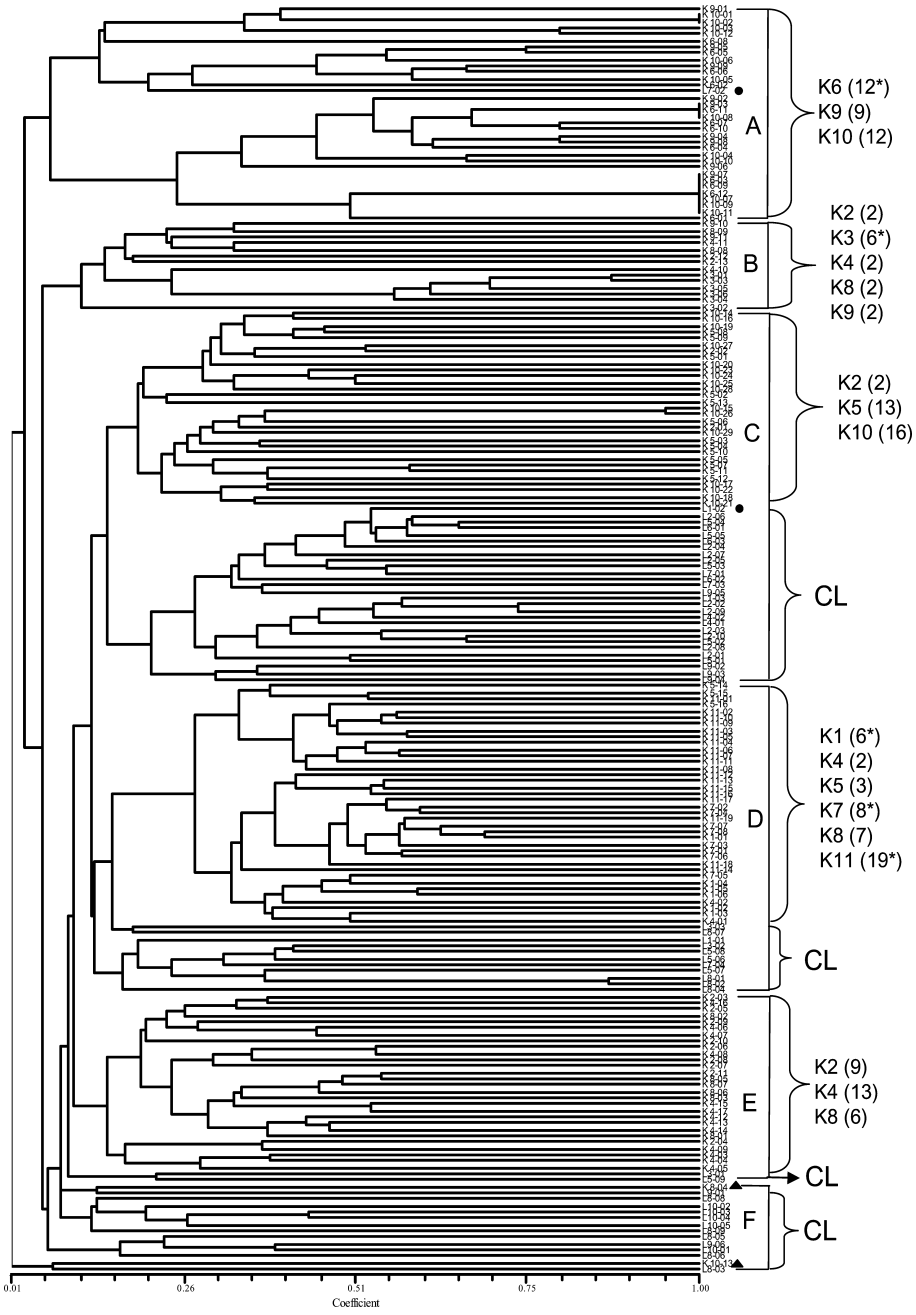

Fig. 4. Individual UPGMA tree. UPGMA algorithm of Sneath and Sokal (1973) was performed with SAHN routine using NTSYS-pc software version 2.0 (Exeter Software, Setauket, NY) based on a Jaccard's similarity matrix. Dots indicate misplacement of *Cunninghamia laneolata* with *C. konishii*. Triangle indicates *C. konishii* grouped with *C. lanceolata* in the base of the UPGMA tree. Six clusters were represented by A, B, C, D, E, and F. On the right of each cluster where population code labeled for *C. konishii* such as K3 (6*) indicates Chitou population of *C. konishii*, the number in the parenthesis indicates the number of individuals from this population grouped in that cluster; the star symbol represents all individuals from the population are grouped together in the same cluster.

resolved AFLP haplotypes indicated the existence of higher levels of genetic variations in the analyzed populations for both species. In general, AFLP generated a large number of polymorphic DNA fragments. The technical advantage of AFLP is that the accuracy of measurements of genetic distance increases with the number of loci examined (Travis et al., 1996). In this AFLP investigation, the average genetic diversity of *C. konishii* in Taiwan was considerable, but still greatly less in comparison with its presumed progenitor *C. lanceolata* both by allozyme (Lin et al., 1998) and AFLP (this study) assays. Population-specific AFLP fragments were more common in the populations of *C. konishii* than those of *C. lanceolata*. This could be resulted from the

genetic drift and population bottleneck occurred in the island species. Depauperate levels of genetic variation may be expected in a colonizing species if there are a low number of source populations and a high number of bottleneck events (Affre et al., 1997) resulting in reduced genetic variation since the introduction of *Cunninghamia* from mainland Asia to Taiwan.

Furthermore, the lower average genetic diversity found in *C. konishii* corresponded to the lower genetic variation observed in the marginal populations in comparison with more central populations located in mainland Asia. In many plants including gymnosperms and angiosperms, decrease in genetic variability towards species margins has been reported such as *Quercus spp*. (Dumolin-Lapègue et al., 1997), *Fagus sylvatica* (Demesure et al., 1996), *Pinus monticola* (Steinhoff et al., 1983), and *Picea mariana* (Yeh et al., 1986). This central-marginal decline in genetic variability is probably related to the hindrance of gene flow by effective barriers or the fragmentation and/or bottleneck of populations.

The geographical region in central Taiwan has been proposed as the major diversity center for several forest species based on allozyme variation (Lin, 2001). These species include *Myrica rubra* (Lour.) Sieb and Zucc. (Cheng et al., 2000), *Cinnamomum kanehirae* Hay. (Lin et al., 1997), *C. konishii* Hay. (Lin et al., 1998), and *Trochodendron aralioides* Sieb and Zucc (Wu et al., 2001). Moreover, island wide samplings of *T. aralioides* found a secondary diversity center between latitudes 24.7° and 25.0°. In *C. konishii*, the highest level of genetic diversity was found in Chitou population and was consistent with the results of allozyme data that the Nantou County in central Taiwan as the major diversity center harboring the greatest genetic variation for many plant species in Taiwan (Lin, 2001). Interestingly, populations around latitude 24.3° with relatively higher level of genetic variability assayed using AFLP polymorphism in this study concurred with the findings of allozyme data in several studies including *C. konishii* (Lin et al., 1998), *T. aralioides* (Wu et al., 2001), *Alnus formosana* (Sue et al., 2000), and *Taiwania cryptomerioides* (Lin et al., 1993). Similar results were also observed for cpDNA data from *T. aralioides* (Huang et al., 2004) and *Castanopsis carlesii* (Cheng et al., unpublished data).

4.2. Refugia inference for Cunninghamia konishii

The degree of average Φ_{ST} of each population in comparison with that of the remaining populations can be used to examine the consequences of historical and contemporary geographical population subdivision on evolutionary processes related to genetic variability (Johnson et al., 2000), and is important for reconstructing the phylogeographical history evolved during pre- and post-colonization events (Grant and Grant, 1997). A recent study shows that cpDNA variation in 22 widespread European trees and shrubs had genetically divergent populations in the Mediterranean regions that corresponded to the sites of glacial refugia (Petit et al., 2003). Interestingly, the region around Chitou and Alishan populations located in the Central Mountain Ridge (CMR), and the region around Tashueshan and Shiyuan populations located in Shueshan Range (SR) (Fig. 1), the two genetically divergent regions (Fig. 3), are probably the glacial refugia when the average temperature is 8–11 °C lower during glacial maximum in contrast to the present-day temperature (Tsukada, 1967). These two sites coincide with the region of major and secondary diversity centers mentioned above.

4.3. Species divergence and population differentiation

The 4.8% genetic divergence between *C. konishii* and *C. lanceolata* suggests their conspecific relationship. The lack of genetic divergence within the two species of *Cunninghamia* is consistent with the results analyzed by allozyme (Lin et al., 1998) and cpDNA variations (Hwang et al., 2003; Lu et al., 2001).

Higher population divergence (24%) found in *C. konishii* using AFLP markers in contrast to allozyme data (3%, Lin et al., 1998) is consistent with the results of other plant species when using dominant markers such as RAPD in contrast to codominant allozyme data (Aagaard et al., 1995; Bucci et al., 1997; Hwang et al., 2001; Liu and Furnier, 1993). The higher level of genetic differentiation revealed by AFLP was also reported for other plant species (e.g., Cardoso et al., 2000; Gaudeul et al., 2000; Palacios et al., 1999). Recently, higher value of genetic differentiation is observed based on RAPD markers for the threatened South American conifer *Pilgerodendron uviferum* (Cupressaceae) (Allnutt et al., 2003).

However, lower level of genetic differentiation among populations found in this study for *C. lanceolata* could be ascribed in part to the pooling of the *C. lanceolata* genetic materials (Yeh et al., 1994). Groupings according to ecotypes (Yu, 1996) failed to correlate well with either isozyme allele frequencies or heterozygosities in mature trees (Yang et al., 2000). Nine enzyme systems were used to investigate 16 populations of *C. lanceolata* in China, no correlation was found between the distribution of genetic diversity and both the geographic and climatic variables of the species, and this might have been caused by the lack of genetic isolation in the species (Yeh et al., 1994). Even though local populations of cultivated selections may contribute to the level of genetic diversity, interbred of different sources of *C. lanceolata* might have occurred and resulted in the lower level of genetic differentiation in contrast to *C. konishii*.

4.4. Gene flow and biogeography

Indirect estimates of gene flow measure the cumulative effect of migration acting over all temporal and spatial scales (Neigel, 1997). Gene flow in *C. konishii* would occur mainly due to the movement of pollens and seeds. The comparison of *Nm* values obtained from Φ_{ST} estimates suggests that high level of migration may have occurred for Yeinhai-Alishan, Alishan-Chitou, Denta-Tajiann, and Denta-Shiouhluan population pairs. Although high level of effective number of migrants might only reflect ancestral relationships, coupled factors of easily dispersed light seed and typhoon visiting during summer and autumn with high winds would have facilitated extensive gene flow among some adjacent populations, especially for Alishan and Chitou. The event of gene flow in *C. konishii* has not been limited to between nearest neighboring populations because individuals from those distant populations such as Shiyuan and Alishan are also grouped in the same cluster (cluster B) (Fig. 4). Effective gene flow, typical of most wind-pollinated conifers, would be hindered by local environmental differences that resulted in the failure of successful dispersal. Although a migration rate of 0.5 was considered sufficient to overcome the diversifying effects of random drift (Ellstrand and Elam, 1993), lower values of effective number of migrants observed in *C. konishii* might have resulted in a genetic drift in some populations.

Historical factors are also essential in the interpretation of the current patterns of genetic differentiation when using molecular markers in the analysis of the patterns of genetic variation (Newton et al., 1999). The present-day structure of genetic variation may be due to past decrease in population size rather than limited gene flow. Moreover, the observed genetic structure of *C. konishii* in this study might have derived from severe bottlenecks in some populations because low effective size of populations would have increased the genetic drift resulting in the loss of allelic diversity as that might have occurred in populations including Alishan, Denta, Tajiann, Wuser, Shiyuan, Shiouhluan, and Kuanwu. The large number of unique fragments found in Tajiann and Kuanwu further support the occurrence of bottleneck event during the past in this species.

Non-homogeneous genetic differentiation in *C. konishii* populations is probably associated with historical demographic changes among sampling sites. Despite an observed average moderate level of migration, the effective number of migrants between Tashueshan and Shiyuan, and between Shiyuan and Chitou were low. It is likely that Shiyuan with low genetic diversity and high divergence from the rest of populations suggests its small population size and limited recruitment by seed during postglacial colonization from glacial refugia. The overall pattern of genetic divergence among *C. konishii* populations reflects a history of separation and/or differences in the effective population size that is related to the bottleneck event (Hwang et al., 2003). An exception is the population from Denta, which displays a relatively higher level of migration event probably related to its geographic location that may play as a waypoint for migration.

Taiwan hosts more than 4000 species of vascular plants distributed from the sea level to 3900 m in elevation and is rich in endemism (Hsieh, 2002). Most of these plants are distributed within one of four floristic regions delimited distinctly in altitudes (Hsieh et al., 1994). It is unlikely that competitions of *Cunninghamia* with *Pinus spp.*, *Chamaecyparis*, and *P. wilsoniana* (Liu, 1966) as well as many other plant species for successful dispersal and survival in the local environments lead to successful introduction from only one single source. It is also quite impossible that from a single source of *Cunninghamia* introduction, the local spread occurred effectively despite niche competition and preemption (Silvertwon, 2004).

Multiple sources of *Cunninghamia* introduction are revealed by individual UPGMA tree (Fig. 4). This dendrogram reveals a pattern of individual groupings studded with an intermingling of different populations in *C. konishii*. One possible scenario ascribed to explain the intermingling of haplotypes is the multiple source introductions of *Cunninghamia* onto Taiwan via different directions in migrant pools and subsequently spread through local movements of these genetic variations after colonization. The multiple sources of *Cunninghamia* introduction from continental Asia via migrant-pool, long-distance seed dispersal had also been proposed (Lu et al., 2001). In this study, four migrant pools can be classified corresponding to the individuals of *C. lanceolata* in clusters C, D, E, and (A, B) (Fig. 4). The two individuals of *C. lanceolata* grouped with many individuals of *C. konishii* from various populations are possibly due to historical migration events instead of long-distance dispersal after glacial maximum. The same scenario could be applied to the two *C. konishii* individuals that were grouped together with individuals of *C. lanceolata* in cluster F.

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