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Postglacial population growth of *Cunninghamia konishii* (Cupressaceae) inferred from phylogeographical and mismatch analysis of chloroplast DNA variation

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Abstract

Phylogeographical and mismatch analysis of chloroplast DNA (cpDNA) variation were used to infer the temporal dynamics of distributional and demographic history of Taiwan fir (*Cunninghamia konishii*). We examined 64 and 52 trees from 17 populations of *C. konishii* and 14 provenances of *C. lanceolata*, respectively, by sequencing three intergenic spacers and one intron using cpDNA universal primers. Of the aligned 1888 base pairs (bp) sequence, 30 varied among 28 haplotypes, which consisted of three transitions, 14 transversions and 13 indels. One ancestral haplotype was found in 86 individuals across the surveyed range of both species, *C. konishii* and *C. lanceolata*, which was distributed in all populations and provenances. The 28 haplotypes also included 15 *C. konishii* specific and 12 *C. lanceolata*-specific haplotypes. Ancestral haplotype was found fixed in five populations of *C. konishii* and five provenances of *C. lanceolata*. Other haplotypes occurred mainly as singletons. The levels of population differentiation studied are relatively low in both *Cunninghamia* species. The nucleotide diversity (θ) of chloroplast DNA sequences within *C. konishii* was slightly higher than that of C. *lanceolata*. Excess in singletons as well as star-like phylogeny of haplotypes suggested no clearcut migration patterns of *C. konishii* after glacial maximum. One probable demographic history of *C. konishii* is the postglacial population growth of *C. konishii* after a glacial bottleneck event. This inference is supported by the combined results of fossil pollen record, low nucleotide diversity, significant Tajima's *D*-value, phylogeographical analysis and unimodal mismatch distribution. Similarities and discrepancies between our results and those of Lu *et al.* (2001) are discussed.

Keywords: cpDNA, *Cunninghamia konishii*, postglacial population growth, Taiwan

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Introduction

Temperature oscillations during ice ages of the Pleistocene forced species to move south or migrate to lower altitudes, where the environment may not be suitable for species to grow to a large population size, thus resulting in a bottleneck (Hewitt 1996). Species that survived in refugia in the south or in the lower elevations may recolonize habitats where plant species can grow for range expansion when climate becomes suitable in the north and at higher altitudes. Fossil pollen records as well as the present-day distribution of genetic variability can be used to trace backwards in time to know the demographic history of the species (Cruzan & Templeton 2000). Valuable information on the evolutionary history of forest trees can be obtained through sequence data that allow assessment of the relative roles of natural selection and genetic drift on genetic variation and population differentiation (Otto 2000). Chloroplast DNA variation is a useful tool in deciphering the spatio-temporal dynamics of the organism studied because of its

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small rate of structural and sequence evolution with rare or absent recombination and uniparental inheritance (Palmer 1987; Palmer *et al*. 1988). The cpDNA variation has been used for phylogeny reconstruction at the population level in many plant species, e.g. European oaks (Ferris *et al*. 1995; Dumolin-Lapègue *et al*. 1997; Matyas & Sperisen 2001), *Kandelia candel* (Chiang *et al*. 2001) and *Cyclobalanopsis glauca* (Huang *et al*. 2002).

Old growth of *Cunninghamia konishii* Hay. (= *C. lanceolata* (Lamb.) Hook. var. *konishii* (Hay.) Fujita) is usually found scattered within forests of *Chamaecyparis*, *Pinus* spp. and *Pseudotsuga wilsoniana* at elevations of 1300–2800 m (Liu 1966). *C. lanceolata* (Lamb.) Hook. is a species that occurs only in mainland China. Both *C. konishii* and *C. lanceolata* had high genetic diversity (Müller-Starck & Liu 1989; Yeh *et al*. 1994; Lin *et al*. 1998) revealed by allozyme assay. These two *Cunninghamia* species are related closely, with very little taxic differentiation according to allozyme data (Lin *et al*. 1998).

The lowland vegetation was dominated by cool–temperate forest composed of conifers mixed with deciduous hardwood species around 745.5 m altitude during the last glacial maximum according to fossil pollen evidence from Jih-Yueh Tan (23°49′N; 120°53′N) in central Taiwan (Tsukada 1967). The evidence of palaeontology indicated a once-downward migration of *C. konishii* during glacial maximum from higher altitude. Events of recolonization of higher altitude after glacial maximum are possible. Values of population differentiation for *C. lanceolata* and *C. konishii*, as estimated by Yeh *et al*. (1994) and Lin *et al*. (1998) using allozymes, are low and fall within the range observed typically for species with long generation times and high outcrossing rates, such as conifers (Hamrick *et al*. 1992). Another factor affecting the present-day distribution of genetic variation may be the rate at which *C. konishii* recolonized at higher altitudes.

In the present study, nucleotide diversity analysis of four chloroplast noncoding sequences including *trn*D-*trn*T, *trn*L-*trn*F, and *pet*G-*trn*P intergenic spacers and *trn*V intron together, with phylogeographical and mismatch analysis, were used to infer the temporal dynamics of distributional and demographic history of *C. konishii* throughout its native geographical range.

Materials and methods

Sixty-four individuals of *C. konishii* transplanted by grafting from 17 different natural populations were collected from seed orchards at Chuyunshan, Tongsyh and from a clonal garden established in 1978 at the Lienhuachih Station, Taiwan Forestry Research Institute (TFRI), as well as from old growth of Shiyuan, Wuser and Alishan. Fifty-two individuals of *C. lanceolata* originating from 44 seed sources from China (14 provenances) were also collected from Lienhuachih Station. The code names for populations and sample sizes for *C. konishii* and *C. lanceolata* are listed in Table 1 and Fig. 1.

Total DNA was extracted from ground leaf powder according to a modified cetyltrimethyl ammonium bromide (CTAB) procedure (Doyle & Doyle 1987) and described in detail by Hwang *et al*. (2001). Polymerase chain reaction (PCR) amplification was achieved for *trn*V intron (Huang *et al*. 2002), *trn*L-*trn*F (Taberlet *et al*. 1991), *trn*D-*trn*T (Demesure *et al*. 1995) and *pet*G-*trn*P (Hwang *et al*. 2000). PCR reaction conditions followed Hwang *et al*. (2001) with optimal annealing temperatures of 58 °C for *pet*G-*trn*P, 56 °C for *trn*L-*trn*F, 52 °C for *trn*D-*trn*T and 60 °C for *trn*V intron. The PCR mixture (50 μL) contained 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 10 mM Tris-HCl (pH 8.3), 100 μM of each dNTP, 0.2 μM primer, 20 ng template DNA, 1 μg RNase and 0.5 U *Taq* polymerase (Amersham Pharmacia Biotech). PCR products were purified and sequenced in both directions using *Taq* Dye Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and Model ABI373A automated sequencer (Applied Biosystems). All sequence polymorphisms were rechecked visually from chromatograms. Singletons were verified by resequencing.

Multiple alignments of the sequences were obtained using CLUSTAL W (Thompson *et al*. 1994) and subsequent manual adjustment. Haplotype diversity (h), nucleotide diversity (θ) (Nei 1987), Tajima's D (Tajima 1989) as well as Fu & Li's D^* test (Fu & Li 1993) for departure from neutrality on total number of segregating sites were calculated using DnaSP version 3.14 (Rozas & Rozas 1999). We used a pairwise mismatch distribution to test for population expansion (Rogers & Harpending 1992) by using the DnaSP program. Construction of the phylogenetic network was performed by TCS version 1.06, as described in Templeton *et al*. (1992). Two measures of population differentiation (G_{ST} and N_{ST}) were obtained according to Pons & Petit (1996).

Results

Sequence analyses, mismatch distribution and neutrality tests

Among 30 polymorphic sites, three transitions, 14 transversions and 13 indels were detected from the aligned 1888 base pairs (bp) sequences. The examined sequences consisted of *trn*V intron (472 bp, GenBank Accession nos AF549430–AF549440) and three intergenic spacers including *pet*G-*trn*P (441 bp, GenBank Accession nos AF549412–AF549415), *trn*D-*trn*T (664 bp, GenBank Accession nos AF549416–AAF549418) and *trn*L-*trn*F (311 bp, GenBank Accession nos AF549419–AF549429). In *C. konishii*, the mean nucleotide diversity is 0.00190 ± 0.00067, and the mean haplotype diversity is 0.463 ± 0.079 for 17 *C. konishii* populations. In contrast, for 14 *C. lanceolata* provenances, the mean

Table 1 Genetic diversity within and population differentiation between populations of *Cunninghamia konishii* and provenances of *Cunninghamia lanceolata*

Population (code/sample size)	Haplotype/ no. individuals	Haplotype diversity (h)	Nucleotide diversity (θ)	Tajima's D (P)	Fu & Li's D^* (P)	G_{ST}	N_{ST}
Cunninghamia konishii							
Total		0.463 ± 0.079	0.00190 ± 0.00067	−2.41047 ($P < 0.02$)	−3.74181 ($P < 0.001$)	0.073	0.119
Tashueshan (TH/4)	A/2, KTH1/1, KTH4/1	0.833 ± 0.222	0.00116 ± 0.00079				
Denta (DT/4)	A/4	0.000 ± 0.000	0.00000 ± 0.00000				
Kuanwu (KW/4)	A/3, KKW1/1	0.500 ± 0.265	0.00087 ± 0.00063				
Yenhai (YH/4)	A/3, KYH4/1	0.500 ± 0.265	0.00058 ± 0.00046				
Tayuanshan (TY/3)	A/2, KTY3/1	0.667 ± 0.314	0.00035 ± 0.00035				
Shengkuang (SK/4)	A/3, KSK4/1	0.500 ± 0.265	0.00029 ± 0.00029				
Chihlehhsi (CL/1)	A/1	0.000 ± 0.000	0.00000 ± 0.00000				
Shiyuan (SY/4)	A/2, KSY1/2	0.667 ± 0.204	0.00029 ± 0.00029				
Wuser (WS/4)	A/3, KWS3/1	0.500 ± 0.265	0.00029 ± 0.00029				
Alishan (AL/4)	A/3, KAL4/1	0.500 ± 0.265	0.00029 ± 0.00029				
Tajiann (TJ/4)	A/1, KTJ4/1, KTJ2/1, KTJ1/1	1.000 ± 0.177	0.00087 ± 0.00067				
Shiouhluan (SL/4)	A/4	0.000 ± 0.000	0.00000 ± 0.00000				
Chuyunshan (CY/4)	A/4	0.000 ± 0.000	0.00000 ± 0.00000				
Kuangmingchao (KM/4)	A/4	0.000 ± 0.000	0.00000 ± 0.00000				
Anmashan (AM/4)	A/3, KAM3/2	0.500 ± 0.265	0.00029 ± 0.00029				
Wulin (WL/4)	A/3, KWL2/1	0.500 ± 0.265	0.00058 ± 0.00046				
Chitou (CT/4)	A/2, KCT2/1, KTH4/1	0.833 ± 0.222	0.00087 ± 0.00063				
Cunninghamia lanceolata							
Total		0.440 ± 0.088	0.00176 ± 0.00065	−2.44968 ($P < 0.02$)	−4.46944 ($P < 0.02$)	0.017	0.014
Yunnan (YN/4)	A/4	0.000 ± 0.000	0.00000 ± 0.00000				
Jiangxi (JX/4)	A/3, LJX3/1	0.500 ± 0.265	0.00029 ± 0.00029				
Shanxi (SX/4)	A/2, LSX1/1, LSX3/1	0.833 ± 0.222	0.00087 ± 0.00063				
Anhui (AH/2)	A/2	0.000 ± 0.000	0.00000 ± 0.00000				
Zhejiang (ZJ/4)	A/3, LZJ4/1	0.500 ± 0.265	0.00058 ± 0.00046				
Hunan (HN/4)	A/3, LHN2/1	0.500 ± 0.265	0.00029 ± 0.00029				
Hubei (HB/4)	A/4	0.000 ± 0.000	0.00000 ± 0.00000				
Sichuan (SC/4)	A/3, LSC2/1	0.500 ± 0.265	0.00029 ± 0.00029				
Guizhou (GZ/4)	A/4	0.000 ± 0.000	0.00000 ± 0.00000				
Fujian (FJ/4)	A/3, LFJ4/1	0.500 ± 0.265	0.00029 ± 0.00029				
Guangdong (GD/4)	A/2, LGD2/1, LGD4/1	0.833 ± 0.222	0.00058 ± 0.00046				
Guangxi (GX/4)	A/2, LJS2/1, LGX3/1	0.833 ± 0.222	0.00087 ± 0.00063				
Jiangsu (JS/4)	A/2, LJS4/1, LJS2/1	0.833 ± 0.222	0.00087 ± 0.00063				
Henan (HE/2)	A/2	0.000 ± 0.000	0.00000 ± 0.00000				

nucleotide diversity is 0.00176 ± 0.00065 and the mean haplotype diversity is 0.440 ± 0.088. Populations of *C. konishii* from Tashueshan (θ = 0.00116), Tajiann and Chitou (θ = 0.00087) appeared to contain higher nucleotide diversity (Table 1). In *C. lanceolata*, provenances from Shanshi, Jiangshu and Guangxi (θ = 0.00087) had higher nucleotide diversity (Table 1).

We identified 28 unique haplotypes using the TCS program, in which one ancestral haplotype was identified in 86 individuals for both *C. konishii* and *C. lanceolata* along with 15 haplotypes specific to *C. konishii* and 12 haplotypes specific to *C. lanceolata*. Haplotype network depicted a 'star-like' phylogeny (Fig. 2a). Nine of 15 *C. konishii* specific haplotypes were found in Taichung County in central Taiwan, with four haplotypes found in population Tajiann. Haplotypes that belong to *C. konishii* are initialized with the letter K, while those that belong to *C. lanceolata* are initialized with the letter L in Figs 1 and 2a. The ancestral allele occurs in every population of *C. konishii* and every seed source of *C. lanceolata*. For *C. lanceolata*, haplotype LJS2 occurs in both Jiangsu and Guangxi provenances and other haplotypes are observed only in one single individual and characterized as autapomorphies. Provenances of Shanxi, Guandong, Guanxi and Jiangsu housed two haplotypes each. For *C. konishii*, haplotypes KSY1 and KAM3 were found in two individuals each of Shiyuan and Anmashan population, respectively. Most haplotypes occur as autapomorphies in *C. konishii*. Haplotype KTH4

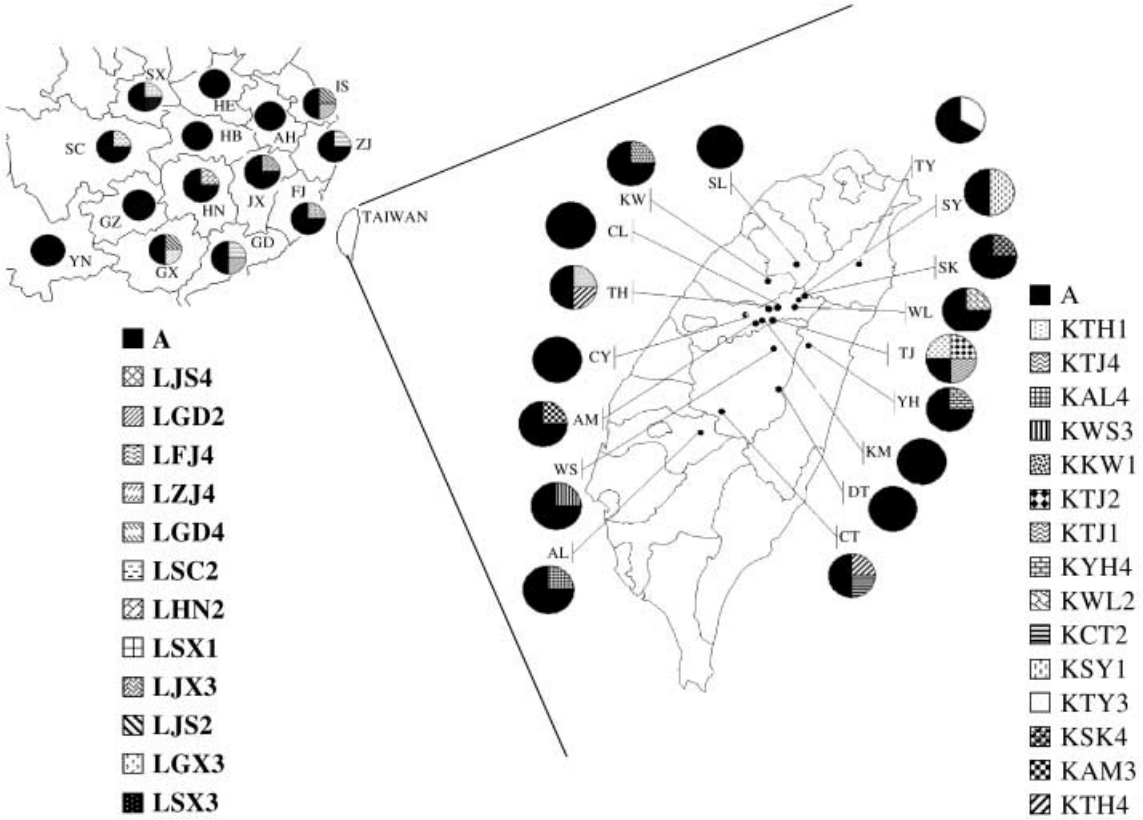

Fig. 1 Sample localities and geographical distribution of the cpDNA haplotypes of *Cunninghamia konishii* and *C. lanceolata* located in Taiwan and China.

was found in one individual each of Tashueshan and Chitou population. The significant skewness toward rare alleles in combined sequences of chloroplast *trn*V intron and three intergenic spacers indicating high level of singletons occur in *Cunninghamia*. Neutrality test by Tajima's D (–2.41047, $P < 0.02$) and Fu & Li's test statistic D^* (–3.74181, $P < 0.001$) showed the negative values significantly different from zero, indicating an excess of rare alleles over that expected for null neutral hypothesis in an equilibrium population.

To investigate the hypothesis of population expansion in *C. konishii*, we computed the distribution of pairwise differences from the segregating sites of cpDNA haplotypes in *C. konishii* using the DnaSP program. The mismatch distribution analysed showed no significant difference from Poisson expectation (Kolmogorov–Smirnoff test, $Z = 1.800$, $P = 0.4682$) and a unimodal mismatch distribution was observed based on the infinite-sites predictions and mutation rate homogeneity. The Harpending's raggedness index $r = 0.0432$ (Harpending 1994), indicating a smooth distribution, reflected a model of sudden expansion (Fig. 2b) in contrast to a constant population model. Because *C. lanceolata* has been cultivated for more than 2000 years (Chen & Shi 1983), exchange of seed sources might have confounded the explanation of demographic history; therefore, we will not attempt to make inference for the demographic history of *C. lanceolata*.

Analyses of population structure

Population differentiation according to frequencies and the degree of haplotype divergence of the 17 populations in *C. konishii* in Taiwan was 0.073 and 0.119 for G_{ST} and N_{ST}, respectively (Table 1). The G_{ST} and N_{ST} was 0.017 and 0.014 for 14 provenances in *C. lanceolata* (Table 1). The result indicated a low degree of genetic structure of cpDNA variations.

Discussion

Haplotype diversity and relationships

Fourteen of 15 haplotypes (93%) may be considered population-specific, as they are found uniquely in one stand only in *C. konishii*. The characteristic of population-specific haplotype is a general peculiarity in conifer species as described by Vendramin *et al.* (2000). One of the *C. lanceolata* haplotype, LGD2, gave rise to haplotypes LJS4, KKW1 and KTJ2. No reverse situation was found in which a *C. konishii* haplotype was ancestral to a *C. lanceolata* haplotype. This

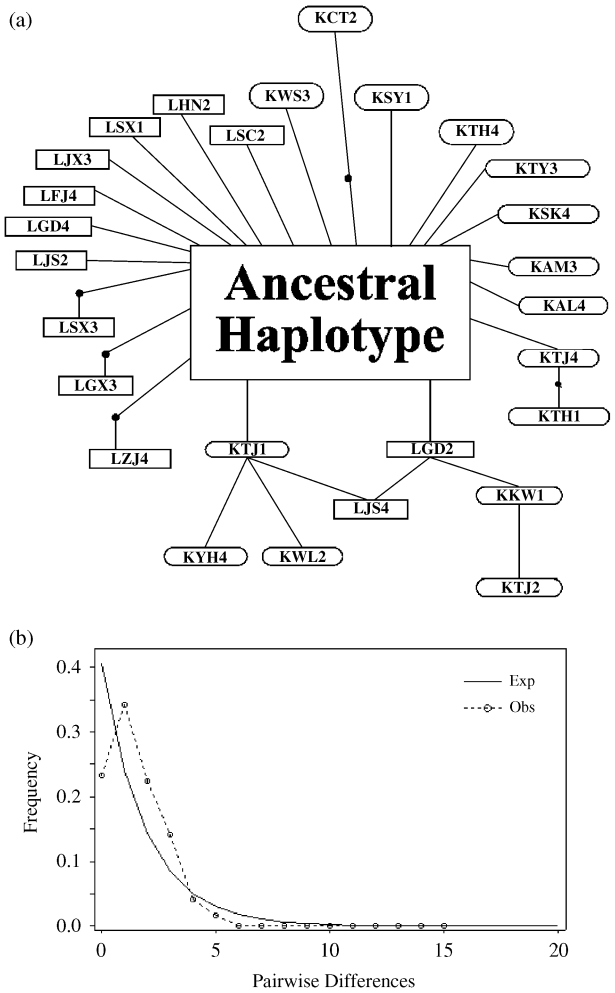

Fig. 2 (a) The star-like phylogenetic network depicting the haplotype relationships of *Cunninghamia konishii* and *C. lanceolata* based on *trn*V intron, *pet*G-*trn*P, *trn*L-*trn*F and *trn*D-*trn*T intergenic spacers of the chloroplast DNA. Each line between haplotypes represents a mutational step; the dotted line represents another mutational step between haplotypes. TCS software was used in this analysis. (b) Mismatch distribution established for *Cunninghamia konishii*; the thin line represents the expected mismatch distribution of a stationary population. The dotted line represents the observed mismatch distribution from segregating sites of the aligned sequences of *trn*V intron, *pet*G-*trn*P, *trn*L-*trn*F and *trn*D-*trn*T intergenic spacers of the chloroplast DNA in *Cunninghamia konishii*.

reveals that some *C. konishii* haplotypes are derived *C. lanceolata* haplotypes. We therefore hypothesize that Kuanwu, and especially the population Tajiann, was the site where *C. lanceolata* recolonized Taiwan from mainland China.

Population structure

In this study, low levels of population differentiation in both *Cunninghamia* species is consistent with the paternal inheritance in cpDNA of conifers (Vendramin *et al*. 1999; Bucci & Vendramin 2000; Vendramin *et al*. 2000; Ribeiro *et al*. 2002; Richardson *et al*. 2002). Lu *et al*. (2001) suggested maternal cpDNA inheriatnce for *C. konishii*, but this remains to be verified.

Occurrence of the same ancestral haplotype could be an indication of the same origin shared by populations because no sharing was found for those newly derived haplotypes in *C. konishii*. The following reasoning can be used to explain why there is no sharing of rare haplotypes found within *C. konishii*, and between *C. konishii* and *C. lanceolata*. First, the appearance of most *de novo* haplotypes was only very recent. Second, the newly developed alleles will have slow rate of dispersion because long regeneration time might limit the generation number to 20 since last glaciation. Because *C. konishii* is a long-life plant, 1–2 m in diameter is common in old-growth forest that was estimated to be more than 1000 years old. Moreover, high humidity growth environment limit the dispersion of pollen and seeds.

Inferences of demographic history of C. konishii

Taiwan would have been connected with Asian landmass by a landbridge when sea levels decreased during glacial stages (Liu 1988). This landbridge would have enhanced the gene flow of many species including *Cunninghamia*. The track of gene flow between two *Cunninghamia* species can be seen from the mingled genealogical relationships of two *C. lanceolata* haplotypes LGD2 and LJS4 to five *C. konishii* haplotypes, KKW1, KTJ1, KYH4, KWL2 and KTJ2, as depicted in the TCS haplotype network (Fig. 2a). However, gene flow of *Cunninghamia* from China to Taiwan during Holocene would be very rare or unlikely to occur, thus resulting in the independent evolution of *de novo* evolved rare cpDNA alleles.

The distributions of the haplotypes in *C. konishii* are uninformative in suggesting a clearcut postglacial migration pattern. This may indicate either that population and individual sampling was too limited to detect a postglacial migration pattern, or that *C. konishii* has spread from different cryptic glacial refugia within the Central Mountain Ridge, a lofty mountain range from north to south in the middle of island. On the other hand, altitudinal migration of species during glacial / interglacial extremes was suggested (Hewitt 1996). Although Taiwan had never been glaciated, the temperature was 8.0–11.0 °C cooler compared to the present-day temperature, as determined from one lake core in an altitude of 745.5 m in central Taiwan (Tsukada 1966). It is probable that during Pleistocene glaciation *C. konishii* migrated to lower altitude and recolonized the higher altitude during interglacial periods.

The 'star-like' phylogeny of haplotypes indicates positive selection or a population bottleneck in the historical demography of *C. konishii* (Nordborg & Innan 2002). Occurrence of a single, common, and often frequent ancestral

haplotype in all populations suggests population bottleneck event in red pine (Echt *et al*. 1998) and that may also be what has happened in *C. konishii*. The presence of excess rare alleles together with unimodal mismatch distribution, and significantly negative Tajima's D and Fu & Li's D^* indicated recent population expansion in *C. konishii* (Fig. 2; Table 1, Aris-Brosou & Excoffier 1996). The inference of population expansion may be supported further by the following reasoning. Growth of conifers may be strongly influenced by factors such as slope steepness, edaphic factors and humidity, as well as competition with other species. Consequently, decline in temperature in the lower elevation did not necessarily provide a suitable environment for the growth of *C. konishii*. A remarkably high value of herbs (Poaceae) indicates a dry environment according to fossil pollen records from Toushe (650 m high) and JihTan (750 m high) in central Taiwan (Liew & Chung 2001) during glaciation. In contrast, fluctuation in vegetational gain and loss during 10 000 and 47 000 BP in *C. konishii* was found and indicates recurrent population bottleneck and expansion in this species according to pollen analysis by Tsukada (1967). This dry climate prevailing is clearly a serious limiting factor in restraining *C. konishii* to several cryptic refugial habitats during glacial maximum. Because *C. konishii* survived only in cool temperatures with a high humidity climate, a dry environment would create a serious bottleneck effect with the result that only ancestral, the most common haplotype, survived after the glaciation.

The present study revealed some interesting similarities, but also discrepancies, with the study by Lu *et al*. (2001). The similarities are, first, that taxic difference is minimal between *C. konishii* and *C. lanceolata* based on chloroplast DNA variation; second, cpDNA nucleotide diversity tends to be higher within populations of *C. konishii* than within *C. lanceolata* populations; third, chloroplast DNA variation revealed no geographical structure. However, geographical structure was detected in some occasions (Lu *et al*. 2001). The total number of individuals and populations examined are higher in our study. The limited number of populations analysed in Lu *et al*. (2001) could lead to a biased inference of migration history of *Cunninghamia* from mainland China to Taiwan as cautioned by Templeton (1998). We found low nucleotide diversity in four chloroplast DNA fragments in contrast to the high nucleotide diversity of only one cpDNA fragment reported in Lu *et al*. (2001). Low nucleotide diversity was also found for several plant species in Taiwan, which grow in different altitudinal range, but may have experienced common geological history, including *Cyclobalanopsis glauca* (Huang *et al*. 2002), *Machilus thunbergii* (Hwang *et al*. unpubl. data), *Castanopsis carlesii* and *Trochodendron aralioides* (Lin *et al*. unpubl. data) from field-collected samples. A well-structured phylogenetic tree was found in Lu *et al*. (2001); however, our result indicated a star-like chloroplast DNA phylogeny. Our inference of the demographic history of *C. konishii* is supported by fossil pollen evidence (Tsukada 1967; Liew & Chung 2001). These differences suggest that further studies on the evolution and phylogeography of *Cunninghamia* in Taiwan are warranted.

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