Chloroplast DNA phylogeography of *Cunninghamia konishii* (Cupressaceae), an endemic conifer of Taiwan

Sheng-You Lu, Ching-I. Peng, Yu-Ping Cheng, Kuo-Hsiang Hong, and Tzen-Yuh Chiang

Abstract: In this study, we investigated the genetic structure and phylogeographic pattern of the genus *Cunninghamia*, a member of the Cupressaceae restricted to mainland China and Taiwan, based on sequences of the *trn*D–*trn*T noncoding spacer of the chloroplast DNA. Maternal inheritance of chloroplasts was determined experimentally. No paternal leakage was detected. Both parsimony and neighbor-joining analyses revealed the polyphyly of *Cunninghamia konishii*, populations of which were nested in clades of *C. lanceolata* from mainland China. The nucleotide diversity of chloroplast DNA sequences within *C. konishii* (0.0118) was higher than that between species (0.0104), which agrees with a previous allozyme investigation. Based on mutational differences between sequences, a minimum spanning network consisting of five clades was constructed. Significant genetic differentiation (Φ_{ST} = 0.130, $P < 0.001$) was detected between the clades based on AMOVA analyses. We infer several possible refugia in the Yunnan, Zhejiang, and Guangdong provinces of south China, all located in the minimum network as interior nodes. We also infer possible migration routes of *Cunninghamia* populations. The phylogeographic pattern shown in the reconstructed network suggests that the present-day *Cunninghamia* populations in Taiwan were derived from six different sources in continental Asia via long-distance seed dispersal. A migrant-pool model explains the heterogeneous composition of the organelle DNA in Taiwan's populations and the low differentiation between populations of Taiwan and China (Φ_{CT} = 0.012, P = 0.454). In contrast with the genetic heterogeneity within geographic populations, many local populations have attained coalescence at the *trn*D–*trn*T alleles, which has led to significant differentiation at the population level.

Key words: AMOVA, coalescence, cpDNA, *Cunninghamia konishii*, *Cunninghamia lanceolata*, minimum spanning network, phylogeography.

Résumé : Les auteurs ont examiné la structure génétique et la distribution phylogéographique du genre *Cunninghamia*, un genre de la famille des Cupressacées qu'on retrouve uniquement en Chine et à Taiwan. Ces études se sont appuyées sur la séquence de l'espaceur non-codant *trn*D–*trn*T de l'ADN chloroplastique. L'hérédité maternelle des chloroplastes a été déterminée expérimentalement. Aucune transmission paternelle n'a été détectée. Des analyses de parsimonie et « neighbor-joining » ont révélé la polyphylie du *Cunninghamia konishii* dont certaines populations s'inséraient au sein de clades du *C. lanceolata* provenant de la Chine. La diversité nucléotidique des séquences d'ADNcp au sein du *C. konishii* (0,0118) était plus élevée que celle détectée entre les espèces (0,0104), une observation qui concorde avec les résultats d'une étude antérieure réalisée avec des isoenzymes. Un réseau de couverture minimal comprenant cinq clades a été construit à partir des diverses mutations entre les séquences. Une différenciation génétique significative (Φ_{ST} = 0,130; $P < 0,001$) a été détectée entre les clades sur la base d'analyses AMOVA. Les auteurs en déduisent l'existence de plusieurs refuges possibles dans les provinces de Yunnan, Zhejiang et Guangdong dans le sud de la Chine, celles-ci formant des noeuds internes au sein du réseau minimal. Les auteurs déduisent également de possibles routes de migration pour les populations du *Cunninghamia*. La distribution phylogéographique illustrée dans le réseau ainsi établi suggère que les populations présentes du *Cunninghamia* à Taiwan sont dérivées de six sources différentes en provenance de l'Asie centrale grâce à la dispersion des graines sur de grandes distances. Un modèle reposant sur des groupes de migrants permet d'expliquer la composition hétérogène de l'ADN des organites au sein des populations taiwanaises et la faible différenciation entre les populations de Taiwan et de la Chine (Φ_{CT} = 0,012; P = 0,454). En dépit de l'hétérogénéité génétique observée au sein des populations géographiques, plusieurs populations locales ont atteint la coalescence des allèles *trn*D–*trn*T, ce qui a entraîné une différenciation significative au niveau des populations.

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S.-Y. Lu and Y.-P. Cheng. Division of Forest Biology, Taiwan Forestry Research Institute, Taipei 100, Taiwan.
C.-I. Peng. Institute of Botany, Academia Sinica, Taipei 115, Taiwan.
K.-H. Hong and T.-Y. Chiang.[1] Department of Biology, National Cheng-Kung University, Tainan 701, Taiwan.

[1]Corresponding author (e-mail: tychiang@mail.ncku.edu.tw).

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Mots clés : AMOVA, coalescence, ADNcp, *Cunninghamia konishii*, *Cunninghamia lanceolata*, réseau de couverture minimal, phylogéographie.

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Introduction

Molecular techniques have recently provided tools for unveiling the migratory footprints of plants and animals (Avise 2000) that evolved through glacial periods. Conifers with ancient histories are ideal materials in which to study the relationship between geological history and phylogenetic patterns. Many conifers, such as *Metasequoia*, were once widespread and became reduced in population number and size at times of glacial expansion. Fossil records provide fundamental evidence for reconstructing this aspect of evolutionary history. Nevertheless, fossil evidence is usually limited, owing to the fragile nature of most tissues. In addition, each fossil may provide evolutionary information covering only a short period of time. Complementing fossil evidence, a large amount of evolutionary information is stored in nuclear and organelle genomes, from which genetic information of lineages can be inferred.

Cunninghamia R. Br. ex Rich. (Cupressaceae) is one of the major coniferous timber genera in east Asia (Lin et al. 1998). Plants of the genus are restricted to mainland China (*Cunninghamia lanceolata* (Lamb.) Hook.) and Taiwan (*C. konishii* Hayata). Both species are limited in population number and size in the wild. The ancient evolutionary history of *Cunninghamia*, dating back to the early Cretaceous (cf. Ohsawa 1997), has attracted much attention from taxonomists and evolutionists. Nevertheless, the taxonomic status of *C. konishii* has been controversial (cf. Chiang and Peng 1998). Morphologically, *C. konishii* has smaller cones, seeds, and leaves and an earlier flowering season relative to *C. lanceolata*. An allozyme investigation revealed remarkably high genetic variation among populations of the genus *Cunninghamia*, but no differentiation between the two extant *Cunninghamia* species (Lin et al. 1998).

Taiwan is a continental island, 150 km east off the Chinese mainland. It is characterized by high levels of endemism and species diversity in floristic composition, which may, in part, be due to the maintenance of many relic species, such as *C. konishii*, that survived glacial periods and vicariance events. Geological evidence indicates that ice ages have occurred at regular intervals of approximately 100 000 years followed by warm periods of about 20 000 years (Milankovitch cycles) (Bennett 1990; King and Ferris 1998). During glacial expansion, many conifers and oaks previously dominant in the northern part of east Asia were forced to migrate southwards into scattered refugia in small patches across southern China and Taiwan (Chiang and Peng 1998). The current geographical distribution of *C. konishii* (medium-elevation mountains in Taiwan) may be the result of such a history.

Molecular markers of organelle DNAs that have a low frequency of genetic recombination have proved to be the most useful for resolving phylogeographic patterns and inferring the migratory routes of species evolving through glaciation events (Avise 1994). In plants, however, chloroplast DNA (cpDNA) is thought to evolve slowly, and has generally been used for studies at the level of higher taxonomic ranks. Recently, moderate to high levels of genetic variation have been detected between closely related species and between populations (Ohsako and Ohnishi 2000). Chloroplast DNA, especially the noncoding region, has thus become an appropriate marker for tracking migration routes in association with geological history (Ferris et al. 1995; Petit et al. 1997).

In phylogeographic studies, nested-clade analysis (Templeton 1998) is a useful tool for elucidating not only genetic structure or gene flow between populations, but also evolutionary genealogical information. Such analysis is complementary to conventional cladistics and can be used to examine intraspecific phylogenies and to resolve ambiguities in a cladogram and the rooting of the phylogeny (Crandall and Templeton 1993). Networks based on nested-clade analyses have been used to reveal possible historical migration routes, intergradation between species, origin of crops, and phylogeographic relationships (Avise 2000).

In this study, we examined nucleotide variation in the noncoding spacer between the *trn*D and *trn*T genes of the cpDNA in the genus *Cunninghamia*, to infer the phylogeographic pattern of *C. konishii*. Using a minimum spanning network, we traced the migration routes of *C. konishii* and identified possible glacial refugia. In contrast with the paternally inherited cpDNA in *Sequoia* (Neale et al. 1989) and *Taxu*s (Pennell and Bell 1988) of the Taxodiaceae, we demonstrated a maternal inheritance in *Cunninghamia*, based on the results of experimental pollination. Lin et al. (1998) suggested that wind-mediated pollen dispersal resulted in a low level of allozyme differentiation between populations. In contrast, the cpDNA markers, being maternally inherited, can only be carried via seeds. In *Cunninghamia*, seeds are dispersed by gravity or are carried by small mammals (such as squirrels; cf. Lin et al. 1998). The migration of seeds between populations is thus limited, owing to the constraints of seed weights and the migratory capacity of mammals. Therefore, an "isolation by distance" model, suggesting a high level of genetic differentiation among populations of *Cunninghamia*, is expected and tested in this study.

Materials and methods

Population sampling

Twenty-two individuals of *C. konishii* from five field populations (Showluan, Kuanwu, Tanta, Anmashan, and Tachien) in Taiwan were sampled (Table 1; Fig. 1). All the populations were found in mountains of medium elevation (altitude of ca. 1800–2500 m) in central Taiwan. Nineteen individuals of *C. lanceolata* planted in the Taiwan Provenance Test Plantation, which were originally collected from China (including Zhejiang, Guangdong, Hubei, Hunan, Yunnan, Xichuan, and Jiangxi provinces), were also sampled as references. Leaves from young shoots were collected and immediately preserved in silica gel.

Experimental pollination

To determine the inheritance of cpDNA in *Cunninghamia*, experimental pollinations were conducted. Prior to maturity, female cones of individuals K431, K533, and K550 (Table 1) were com-

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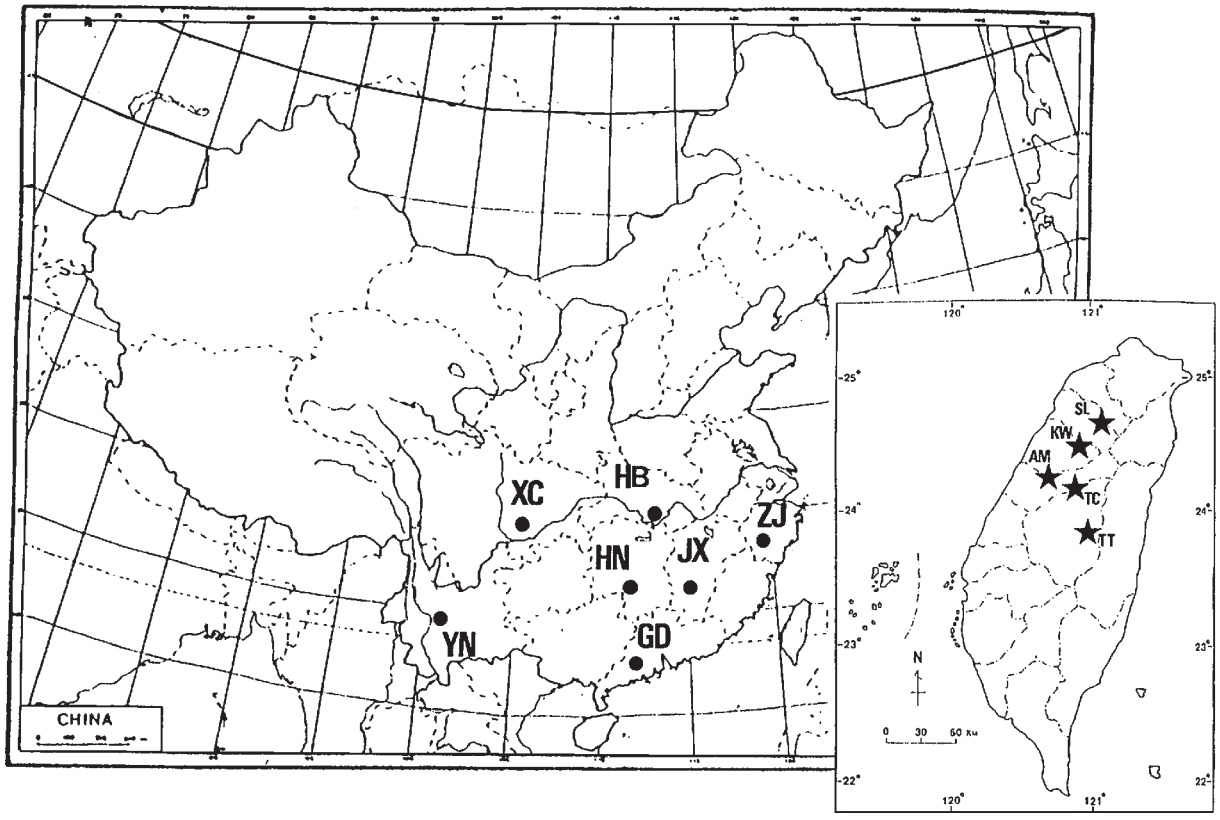
Table 1. Populations sampled for cpDNA sequencing.

Population	*n*	Haplotype diversity (*h*)	Haplotypes	Nucleotide diversity (D_{ij})
Cunninghamia	41	0.973		0.01018±0.00263
C. lanceolata	19	0.936		0.00741±0.00119
Xichuan	2	0	L157 (2)	
Yunnan	4	0.75	L100, L101, L102 (2)	
Hubei	2	0	L26 (2)	
Hunan	2	1	L113, L118	
Guangdong	5	0.8	L130 (2), L131, L136, L137	
Zhejiang	2	1	L15, L16	
Jiangxi	2	0	L106 (2)	
C. konishii	22	0.952		0.01181±0.00196
Tanta	5	0.6	K46 (3), K48 (2)	
Tachien	4	0.83	K431 (2), K550, K533	
Showluan	5	0.9	K528 (2), K452, K464, K441	
Anmashan	3	0	K154 (3)	
Kuanwu	5	0.8	K42 (2), K424 (2), K622	

Note: Haplotype diversity (*h*) is given for each population, each species, and the whole genus; nucleotide diversity (D_{ij} ± SE) is given for each species and the whole genus; *n* is the number of individuals sampled for cpDNA sequencing; and values in parentheses are the number of sequences for each haplotype, when there are more than one.

Fig. 1. Distribution of populations (filled circles) of *Cunninghamia lanceolata* in mainland China: HB, Hubei; HN, Hunan; GD, Guangdong; JX, Jiangxi; XC, Xichuan; YN, Yunnan; and ZJ, Zheijiang. Distribution of populations (filled stars) of *C. konishii* in Taiwan are shown in the inset: AM, Anmashan; KW, Kuanwu; SL, Showluan; TC, Tachien; and TT, Tanta.



pletely wrapped. Mature pollen was collected from male cones of plants K42, K424, and K622. After pollination, mature seeds, representing the F_1 progeny, were collected randomly and germinated. Genomic DNA was extracted from seedlings (see below) and the cpDNA *trn*D–*trn*T noncoding spacer was amplified and sequenced, using genomic DNA obtained from both parental plants and from seeds representing the F_1 progeny.

DNA extraction, PCR amplification, and sequencing

Leaf tissue was powdered in liquid nitrogen and stored at –70°C until use. Genomic DNA was isolated following the CTAB (cetyltrimethylammonium bromide) protocols of Murray and Thompson (1980) and gel-quantified. The noncoding spacer between the *trn*D and *trn*T genes of the cpDNA was amplified in 100-μL PCR reaction mixtures on an MJ Thermal Cycler (PTC

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Fig. 2. Variable sites of the aligned sequences of the *trn*D–*trn*T noncoding spacer of the cpDNA in *Cunninghamia konishii* and *C. lanceolata*. Nucleotide sequences were deposited in the EMBL database under the accession numbers AJ274382–AJ274408.

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            111 1111111111 1112222222 2222222233 3333333333 3444444444 5555555566 6666
     356899011 2345778888 8890001222 3556677802 2344556678 8002334788 0002247801 1456
    8442006445 7410021234 8982673016 5290714861 8205686921 9172788629 3484886780 5527
L-15 ATGGATTATT TTACTTGATG AATGAATCCT AGAAGGCGAA CTTTGACAGA TAAAAAAAAT TTTTTTTTTA AAAG
L-16 .......... .......... ........G. .......... .......... .......... .......... ....
L-26 .......... .......... .........C .......... .......... .......... .......... ....
K-42 .......... .......... .......TT. .......... .......... .....CC... .......... ....
K424 .......... .........- .......... .......... .......... .....C.... ..C....... ...T
K533 .....C.... .........- .......... .......... .......... .....C.... .......... ....
K464 .......... .........- .......... ...G...... .......... .....C...C .......... ....
K528 .......... .........- .......... .......... .......... ...GGC.... .......... ....
K452 .......... ..G......- .......... .......... .........G .....C.... .......A.. ....
L130 C......... .........- ....T..... .......... .......... .....C.... ....C..... ....
L131 C...T..... .........- ....T..... .......... .......... .....C.... ....C..... ....
L118 C...T..G.. .........- T.A....... .......... .......... .....C..-. A......... ....
L113 C......... .....G...- .......... ..G......G .......... .....C.... .......... ....
K154 .......... ...A.....- ......C... .......... .......... .....C.... C......... G...
K441 .C........ CA.......- .......... .T........ .......... .....C.... .......... ....
L106 ...T...... .........- .......... .......... .....T.... .G...C.... .......... ..G.
L157 .......... .........- .......... G......... ...CA..... C....C.... .........G ....
K431 .......... .........- .......... .......... .......... .....C.... .......... .T..
L101 .......... .........- .......... ........G. .......... .....G.... .......... ....
L102 .......... .........- .......... ........G. .......... .....-.... .......... ....
L100 .......... .........- .......... ........C. .......... .....C.... .......... ....
K622 .......... .........- ...A....T. ....C..... T.C...TC.. .....C..G. ...G.C.... ...T
K550 .......... .........- .....T.... ......T... .C........ .....C.... ......C.A. ....
L136 ......C... .........- .......... .......... .......... .....C.... .......... ....
L137 ......C... .........A .......... .......... .......... .....C.... .......... ....
K-46 ........AA ......TGAT .G........ .....A-T.. ........A. .....C.G.. .......... ....
K-48 ..A....... ....C..... .......... .....A-... .........-. ..G..C.... .C........ ....
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100) programmed for one cycle of 95°C for 4 min (denaturation); 30 cycles of 45 s at 92°C (denaturation), 1 min 15 s at 52°C (annealing), and 1 min 30 s at 72°C (extension); followed by 10 min at 72°C (extension). A pair of universal primers for amplifying the noncoding spacer between the *trn*D and *trn*T genes of the cpDNA (Demesure et al. 1995) were used. PCR fragments were eluted using the High Pure PCR Product Purification kit (BM, Mannheim, Germany) and ligated to a pT7blue T-vector (Novagen, Madison, Wis.). Plasmid DNA was purified using the Wizard Plus SV kit (Promega). Purified plasmid DNAs were sequenced in both directions by standard methods, using the Taq Dye Deoxy Terminator Cycle Sequencing kit (Perkin Elmer) on an Applied Biosystems Model 377A automated sequencer (Applied Biosystems).

Data analysis

Sequence alignment and phylogenetic analyses

Nucleotide sequences of the cpDNA were aligned with the program CLUSTAL V (Higgins et al. 1992). The cladistic analysis of sequence data was conducted using the program Phylogenetic Analysis Using Parsimony (PAUP, version 3.1.1.; Swofford 1993). Heuristic searches were undertaken with TBR branch-swapping option, stepwise addition of 10 random replicates, accelerated transformation (ACCTRAN), an unconstrained number of maximum trees, and retention of multiple most parsimonious trees (MULPARS). Neighbor-joining (NJ) analysis was also performed, using the program Data Analysis in Molecular Biology and Evolution (DAMBE, version 3.5.19; Xia 1999). Confidence limits for the cladistic analysis were tested by bootstrapping (Felsenstein 1985) with 1000 replicates, using unweighted characters.

The number of mutations between DNA genotypes in pairwise comparisons was used to construct a minimum spanning network with the aid of the program MINSPNET (Excoffier and Smouse 1994). Confidence of clades was tested using the bootstrap technique.

Population genetic analysis of cpDNA-sequence variation

Levels of inter- and intra-population genetic diversity were quantified by indices of haplotype diversity (*h*; Nei and Tajima 1983) and pairwise estimates of nucleotide divergence (D_{ij}; Jukes and Cantor 1969), using DnaSP (version 3.14; Rozas and Rozas 1999). We used AMOVA (version 1.55; Excoffier 1993) to estimate the significance of genetic differentiation between populations and between lineages, as well as among regions. The statistical molecular variants Φ_{CT} (among regions; i.e., between Chinese and Taiwanese populations), Φ_{ST} (among populations), and Φ_{SC} (among populations within regions) were estimated.

Patterns of geographical subdivision and gene flow were also estimated hierarchically with the aid of DnaSP. Gene flow within and among regions (populations) was approximated as *Nm*, the number of female migrants per generation between populations, which was estimated using the expression $F_{ST} = 1/(1 + 2\,Nm)$, where *N* is the female effective population size and *m* is the female migration rate. Geographical associations of haplotypes and clades within the minimum spanning network were tested using the program GeoDis (Posada et al. 2000). Two major statistics were calculated: the clade distance (D_c), a measure of the geographical spread of a clade, and the nested-clade distance (D_n), a measure of the geographical distribution of a clade relevant to other clades in the same higher-level nesting category. These measures of geographical distribution were used to infer historical processes following the methods of Templeton et al. (1995) (cf. Maskas and Cruzan 2000). The pattern of isolation by distance was assessed by plotting pairwise F_{ST} values against geographical distance. The significance of the association between F_{ST} and distance was determined by a regression *F* test using SPSS program version 6.0 (Norusis 1994).

Results

Maternal inheritance of chloroplasts

Genetic variation of the cpDNA spacer enabled us to dis-

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tinguish individual sequences and determine the mode of inheritance (Fig. 2). Complete maternal inheritance of chloroplasts in *Cunninghamia* was observed, based on an analysis of 60 F_1 progeny seeds from four crosses examined. Each F_1 offspring had the same sequence as the maternal plant. No paternal leakage was detected.

Haplotypes, nucleotide diversity, and phylogeny of *C. konishii* and *C. lanceolata*

Six hundred and sixty-seven base pairs of the noncoding spacer between the *trn*D and *trn*T genes of the cpDNA were sequenced and aligned. Seventy-four sites (11.1%) were variable (Fig. 2) and 20 were phylogenetically informative among sequences of both *Cunninghamia* species. In total, 13 haplotypes in *C. konishii* and 14 haplotypes in *C. lanceolata* were detected, yielding a haplotype diversity of 0.952 and 0.973, respectively (Table 1). Sequence variation was present both between and within populations. Populations of *C. konishii* from Showluan (h = 0.90) and Tachien (h = 0.833) appeared to contain higher haplotype diversity (h). All sequences in populations of *C. lanceolata* from Hunan and Zhejiang were unique (h = 1), but that may have been due to limited sampling.

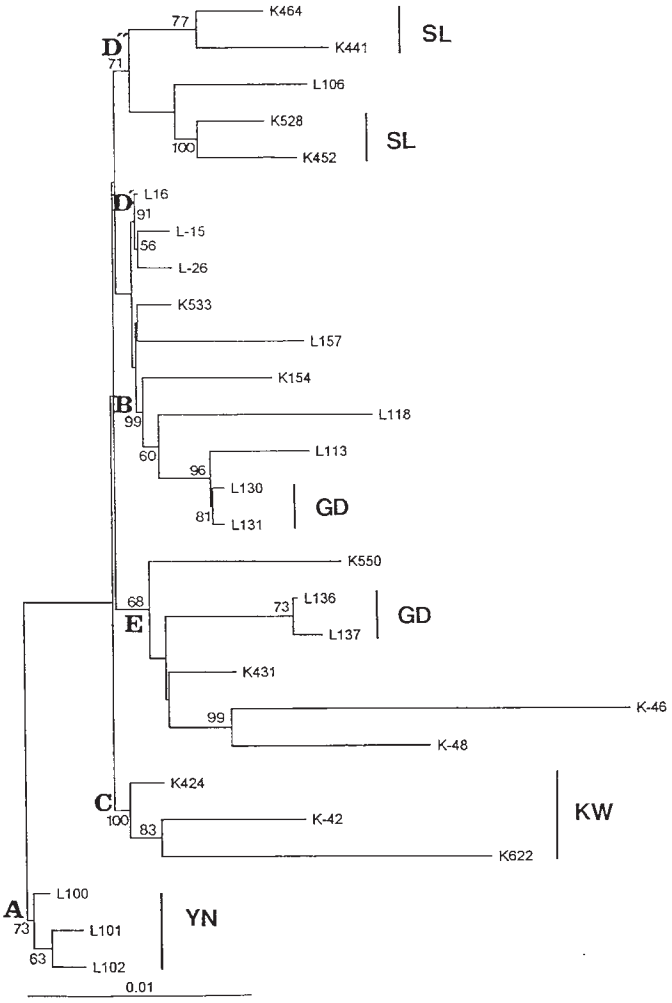
A high level of nucleotide diversity (cf. Jukes and Cantor 1969) was detected both within (D_{ij} = 0.00741 ± 0.00119 in *C. lanceolata* and 0.01181 ± 0.00196 in *C. konishii*) and between species (D_{ij} = 0.00991 ± 0.00175). When both species of *Cunninghamia* were considered as a whole, the nucleotide diversity was estimated to be 0.01018. Nucleotide diversity tended to be lower within populations of *C. lanceolata* than within populations of *C. konishii* (Table 1).

Parsimony analysis of haplotypes identified 16 equally parsimonious trees of 78 steps. The tree based on K2P genetic distance identified by DAMBE is consistent with the parsimony trees, except that the former had higher resolution (Fig. 3). According to both phylogenetic analyses, neither of the two species of *Cunninghamia* is monophyletic. Except for clade C, most of the samples of *C. konishii* were nested within the clades of *C. lanceolata*. At least five clades (A–E) were identified and their monophyly was mostly supported significantly with complete-and-partial bootstrap values. Two subgroups nested in clade E were also significantly supported (K46 plus K48 (99%) and L136 plus L137 (73%); Fig. 3).

Phylogeographic pattern of *Cunninghamia*

To improve the estimation of genealogical relationships between haplotypes in the genus *Cunninghamia*, a minimum spanning network was constructed by linking sequences in a hierarchical manner based on mutational changes between them (Fig. 4). Sequences were then grouped into higher clades, based on the results of the complete-and-partial bootstrap analysis. Closely related clades were linked further to each other to form a network. Five clades (A–E) were identified, three of which were significantly supported by bootstrap values. No higher-level clades grouping two or more of the above five clades were identified. As was found in the NJ tree, neither *C. lanceolata* nor *C. konishii* formed a monophyletic group.

Fig. 3. Neighbour-joining tree of haplotypes of the *trn*D–*trn*T noncoding spacer of the cpDNA of *Cunninghamia*. Numbers at the nodes indicate the bootstrap values obtained from the complete-and-partial bootstrap analysis. A, B, C, D′, D″ and E indicate reconstructed clades.



In the minimum spanning network, clades A (Yunnan and Sichuan) and D′ (Hubei and Zhejiang), both comprising *C. lanceolata*, were identified first as the interior nodes. Clades B (Hunan and Guangdong) and C were connected to clade A with four and three mutational steps, respectively. Likewise, clades D″ and E were connected to clade D′ with five and two mutations, respectively. An alternative linking between clades E and A with two mutations was also suggested by the program MINSPNET. Although the monophyly of *C. konishii* was rejected according to the network, sequences of the same populations were always grouped together. Clade C, an exclusive group of *C. konishii*, is composed of the sequences of the Kuanwu population. Sequences of the Tachien (haplotypes K431, K550, and K533) and Tanta (K46 and K48) populations, both in Taiwan, were linked to the sequences of the Guangdong population (L136 and L137), with two and six mutations, respectively. Two subgroups of the sequences of the Showluan population were identified (i.e., K528 and K452; K464 and K441), both of which were connected to the sequence of the Jiangxi popula-

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Fig. 4. Minimum spanning network based on the mutations between haplotypes. Numbers at the frames indicate the bootstrap values obtained from the complete- and partial-bootstrap analysis.

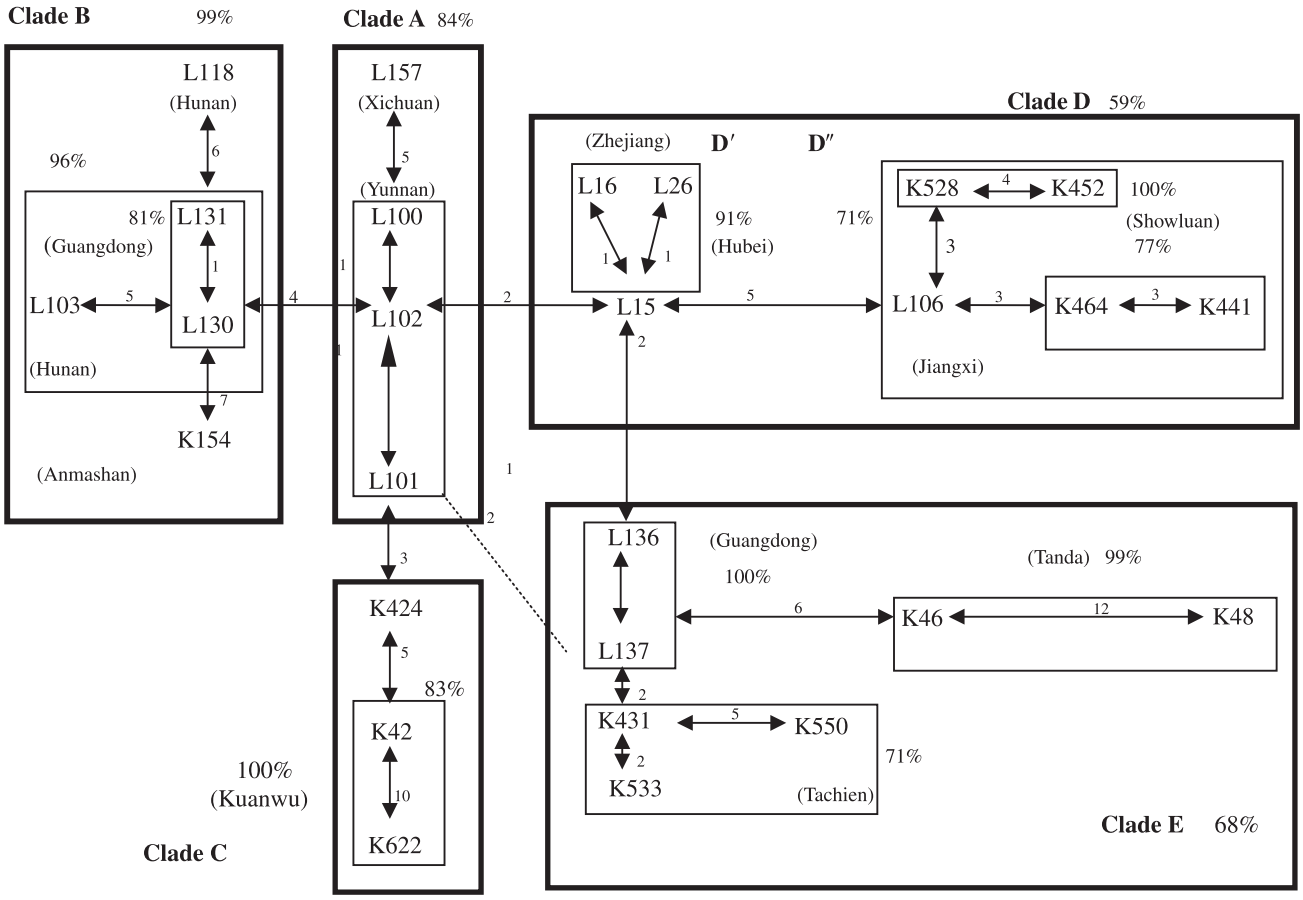

Table 2. Pairwise values of F_{ST} (above digonal) and *Nm* (below diagonal) between populations of *C. lanceolata*.

	Yunnan	Hunan	Guangdong	Hubei	Zhejiang	Jiangxi	Xichuan
Yunnan		0.33	0.51	1.00	0.80	1.00	1.00
Hunan	1.00		0.07	0.67	0.30	0.75	0.77
Guangdong	0.48	6.70		0.71	0.44	0.80	0.83
Hubei	0	0.24	0.03		0.78	1.00	1.00
Zhejiang	0.13	1.12	0.63	0.14		0.94	0.95
Jiangxi	0	0.17	0.02	0	0.03		1.00
Xichuan	0	0.15	0.02	0	0.03	0	

tion (L106) with three mutations. A single haplotype, K154, from Anmashan, Taiwan, nesting in clade B, was linked to haplotype L130 (Guangdong) with seven mutations.

Low genetic differentiation between *C. lanceolata* and *C. konishii* was indicated by the low F_{ST} value (0.0309) and high *Nm* value (15.68) obtained from DnaSP and was consistent with the cladistic analyses. Genetic differentiation between most populations of *C. lanceolata* was significant, with values of F_{ST} normally ranging from 0.30 (Hunan and Zheijiang) to 1.000 (Jiangxi and Yunnan) and of *Nm* ranging from 0.00 to 0.63. One notable exception involved the comparison between the Hunan and Guangdong populations, where low differentiation (F_{ST} = 0.07; *Nm* = 6.70; Table 2) indicated the validity of grouping populations together. Genetic differentiation among populations of *C. konishii* was also marked, with values of F_{ST} ranging normally from 0.30 (between Kuanwu and Tachien) to 0.63 (between Anmashan and Tachien) and of *Nm* ranging from 0.29 to 0.73. One exception involved the comparison between the populations of Showluan and Tanta (F_{ST} = 0.07; *Nm* = 5.77; Table 3).

When each lineage of the minimum spanning network was treated as a unit, genetic differentiation between lineages was nearly always significant, according to the Φ_{ST} statistics deduced from AMOVA, which ranged from 0.0780 to 0.2432 ($P < 0.01$). The only exception involved a comparison between lineages D and E (Φ_{ST} = 0.391, $P > 0.05$; Table 4). The overall Φ_{ST} of 0.130 ($P < 0.001$) also indicated significant differentiation among populations.

The isolation by distance model across overall populations was not supported (r^2 = 0.0225, P = 0.406; Fig. 5) by the

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Table 3. Pairwise values of F_{ST} (above diagonal) and *Nm* (below diagonal) between populations of *C. konishii*.

	Tanta	Tachien	Showluan	Kuanwu	Anmashan
Tanta		0.43	0.07	0.41	0.46
Tachien	0.65		0.40	0.30	0.63
Showluan	5.77	0.75		0.34	0.59
Kuanwu	0.73	1.12	0.99		0.50
Anmashan	0.60	0.29	0.34	0.50	

cpDNA markers, according to a regression *F* test between F_{ST} values and geographical distance. A test for geographical associations of haplotypes and clades within the minimum spanning network was conducted hierarchically using the program GeoDis. No geographical structure was suggested according to the entire minimum spanning network (χ^2 = 3.03, *P* = 0.270). Nevertheless, geographical structure was detected in nearly all clades, including clades A (χ^2 = 37.33, *P* = 0.013), B (χ^2 = 11.00, *P* = 0.011), D (χ^2 = 11.00, *P* = 0.011), and E (χ^2 = 10.00, *P* = 0.024) (Table 5).

Discussion

Genetic diversity and phylogeny of cpDNA in *C. konishii*

A conspecific relationship between *C. konishii* and *C. lanceolata* was suggested by the cpDNA tree based on nucleotide variation in the *trn*D–*trn*T noncoding spacer. Allozyme results (Lin et al. 1998) and cpDNA PCR-RFLP data (Lu et al. 1999) also support the conspecificity between *C. konishii* and *C. lanceolata*. However, Tsumura et al. (1995) detected one site change in the *rbc*L gene between the two taxa based on RFLP analysis. The lack of genetic differentiation detected by most of the above studies suggests that the recognition of *C. konishii* as a well-defined species merely on the basis of its smaller morph size and an earlier flowering season is inappropriate.

In contrast with the low level of genetic variation found in many relic species, for example, *Amentotaxus fomosanus* (Wang et al. 1996), *Archangiopteris itoi* (ferns; Hsu et al. 2000), and *Chamaecyparis* (Lin et al. 1994) of Taiwan, North American *Asclepias* (Broyles 1998), and alpine *Saxifraga* (Bauert et al. 1998), cpDNA variation was high in *Cunninghamia*. A total of 27 cpDNA haplotypes were detected among 41 individuals examined. The level of haplotype diversity in *Cunninghamia* is close to levels in *Beta vulgaris* ssp. *maritima* (13 cpDNA haplotypes; Desplanque et al. 2000), *Argania* (11 haplotypes; El Mousadik and Petit 1996), white oaks (23 haplotypes; Dumolin- Lapégue et al. 1997), and *Alnus* (13 haplotypes; King and Ferris 1998). The nucleotide diversity (D_{ij} = 0.01018) of the *trn*D–*trn*T spacer in *Cunninghamia* is close to values found for *Cycas taitungensis* (D_{ij} = 0.01268, for the cpDNA *atp*B–*rbc*L spacer) (Huang et al. 2001), *Michelia formosana* (D_{ij} = 0.01063, for the cpDNA *atp*B–*rbc*L spacer) (Chiang and Peng 1998), and *Kandelia candel* (D_{ij} = 0.02710, for the *trn*L–*trn*F spacer; Chen 2000), but higher than that of Californian pines (D_{ij} = 0.003; Hong et al. 1993) and *Begonia aptera* (D_{ij} = 0.003, for the *trn*D–*trn*T spacer; Liu 1999). High levels of polymorphism for cpDNA and allozymes (Muller-Starck and Liu 1989; Yeh et al. 1994; Lin et al. 1998; Wang and Lin 1998) may be associated with the long evolutionary history of *Cunninghamia* (cf. Ohsawa 1997), which has allowed mutations to accumulate within lineages. In addition, as a noncoding region, the *trn*D–*trn*T spacer of the cpDNA has relatively few functional constraints. With nearly neutral evolution, mutations, to some extent, would have been retained within each lineage.

Table 4. Pairwise values of Φ_{ST} (below diagonal) and of the probability that random distance (Φ_{ST}) is higher than observed distance (above diagonal) between clades A–E of *Cunninghamia*, estimated from the cpDNA sequence data; overall Φ_{ST} is 0.130 ($P < 0.0010$).

	A	B	C	D	E
A		0	0	0	0
B	0.2390		0	0	0
C	0.2432	0.2209		0	0
D	0.1079	0.1893	0.1838		0.5090
E	0.0780	0.1562	0.1432	0.0391	

Like many other plants, a very low level of recombination occurred in the chloroplast genome of *Cunninghamia* (cf. Desplanque et al. 2000). In an analysis using the software DnaSP, only one possible recombination (a DNA fragment between sites 80 and 206) was detected. The chloroplast genome is known to evolve mainly through point mutations or small indels (Clegg et al. 1994**)**. Clegg et al. (1994) found that indels tended to occur more frequently than point mutations. Although results of many studies (e.g., Desplanque et al. 2000) support their observations, the patterns of polymorphism in the *trn*D–*trn*T spacer presented here reveal a higher level of point mutations (Fig. 2).

Inevitably, the genetic composition of such species will have been affected by geological events, such as glacial cycles and vicariance events. According to geological evidence, Taiwan was linked to mainland Asia by a land bridge before the formation of the Taiwan Strait (Lin 1966), which dates back to the last glacial retreat some 18 000 – 20 000 years ago (cf. Bennet 1990). During the glacial maximum of about 100 000 – 20 000 years ago, ancestral populations of northern tree species were forced to migrate into refugia in the far south (cf. Chiang and Peng 1998; Hsu et al. 2000). Conifer species (e.g., *Chamaecyparis formosensis* Mats., *Juniperus tsukusiensis* Masam., *Picea morrisonensis* Hayata, and *Cephalotaxus wilsoniana* Hayata, all endemic to Taiwan) and oaks invaded Taiwan prior to its geographic isolation, survived, and subsequently speciated (cf. Chiang and Peng 1998). Plant populations in different refugia would have been geographically isolated from each other and likely to diverge genetically, owing to founder effects and genetic drift (cf. Ferris et al. 1995). Despite these possible effects, *C. lanceolata* has maintained genealogical cohesion with *C. konishii*, based on cpDNA (this study) and allozyme (Lin et al. 1998) evidence.

With limited seed dispersal, the cpDNA genetic structure of *Cunninghamia* populations would follow a one-dimensional stepping-stone model (Kimura and Weiss 1964). Small marginal populations would, as a rule, maintain lower genetic variation, owing to higher probabilities of genetic loss via stochastic processes. In *Cunninghamia*, however, a higher level of nucleotide diversity was detected

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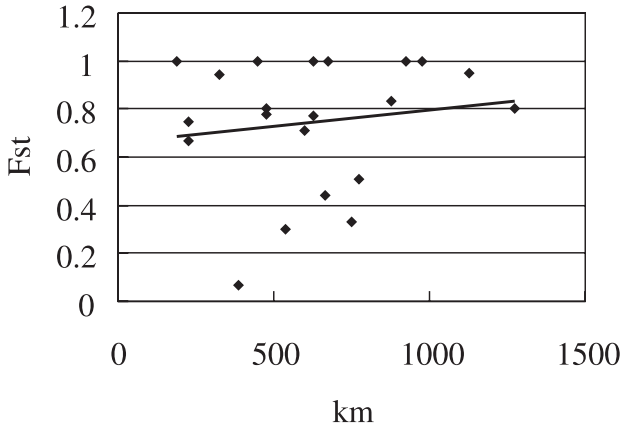
Fig. 5. Scatterplot of deduced F_{ST} values and geographic distance (km) between populations of *Cunninghamia*, based on cpDNA variation.



in populations from Taiwan relative to those of the mainland (0.01181 vs. 0.00741). Similar phenomena are also seen in European *Alnus* (King and Ferris 1998), *Fagus* (Demesure et al. 1996), and *Quercus* (Dumolin-Lapégue et al. 1997), as well as in the Japanese *Abies* (Tsumura and Suyama 1998), in which a higher level of cpDNA diversity was detected in southern than in northern populations. Such spatial distribution of genetic variation is thought to be associated with geological history.

cpDNA phylogeography of *Cunninghamia*

According to the experimental pollination, cpDNA is inherited maternally in *Cunninghamia*. Technically, to detect paternal leakage, large numbers of individuals from a single progeny are required (cf. Desplanque et al. 2000). Although the limited sampling of progeny in our study may not have been ideal for the purpose, no paternal leakage of cpDNA was detected based on the results of the experimental pollination. With maternal inheritance, and given no paternal leakage, the genealogical relationship between cpDNA haplotypes can be used to assess the phylogeographic pattern and to infer the migration routes via seed dispersal.

The minimum spanning network provides insights into the migration history and distribution of genetic variation in *Cunninghamia*. Golding (1987) pointed out that haplotypes of recent origin occur preferentially at the tips of the network. Crandall and Templeton (1993) argued that, according to their tests on empirical data, older alleles have a greater probability of producing mutational derivatives and, thereby, of becoming interior nodes, than do younger haplotypes. The association between the interior nodes of the network and possible glacial refugia was thereby demonstrated in various organisms (e.g., oaks; Dumolin Lapègue et al. 1997). In our study, the interior nodes of the *Cunninghamia* network inhabited a geographic range that coincided with hypothetical glacial refugia in southern China, that is, Yunnan, Guangdong, and Zhejiang provinces (cf. Chiang and Peng 1998; Hsu et al. 2000) and Kuanwu of Taiwan. Among these, two separate refugia in Guangdong Province, L136 and L130, linking to the Yunnan populations with two and four mutational steps, respectively, were identified (Fig. 4). These refugia may have provided colonizing centers for plants that survived the glacial maximum.

Apparently, the overall phylogeographic pattern of *Cunninghamia* did not match the model of isolation by distance (i.e., the correlation between degree of genetic differentiation and geographical distance) among populations (Fig. 5). A smaller genetic distance is frequently found between two geographically distant populations, such as the populations of Zhejiang and Yunnan provinces (with two mutations), than between sequences within the same population (e.g., 12 mutations between K46 and K48 and five mutations between K431 and K550 in Taiwan). Such patch-like distribution of closely related cpDNA haplotypes among populations has also been illustrated in many European tree species that survived glacial periods (Ferris et al. 1995; Petit et al. 1997; King and Ferris 1998). Long-distance seed dispersal during the glacial maximum was found to be one of the key mechanisms (cf. Petit et al. 1997) responsible for the high level of genetic heterogeneity within geographic populations, as it allowed individuals to invade newly opened habitats in the southern refugia. In *Cunninghamia*, an unexpectedly high value for *Nm* (15.68), deduced from cpDNA sequences between Chinese and Taiwanese populations and long links between haplotypes of geographically distant populations (e.g., six mutations between L137 and K46, seven mutations between L130 and K154, and three mutations between L106 and K528), suggested just such an unusual migratory history. It is almost impossible for the seeds of *Cunninghamia* to be dispersed across the Taiwan Strait in the modern environment, owing to the width of the strait and the constraint of the migratory capabilities of seed-carrying animals. Under such a migration model, the high *Nm* values for *Cunninghamia* deduced from F_{ST} values were invalid for estimating current population structure and ongoing gene flow. Instead, they are likely to represent historical migration events or the status of a lineage sorting of alleles (Chiang 2000).

Tests for geographical associations of haplotypes and clades within phylogenies provided further insights into these historical events (Table 5). Long-distance seed dispersal, which is characterized by significantly small values of D_c for tip clades and large values of D_c at some interior clades (cf. Templeton et al. 1995), was detected in clades B, D″, and E. No recent range expansion seems to have occurred, based on the inferences.

Accordingly, the reconstructed phylogeographic pattern of *Cunninghamia*, coupled with the apportionment of genetic variation among populations and the high deduced values of *Nm*, suggest a migrant-pool model (Wade and McCauley 1990), a model that describes a migratory pattern with colonists recruited from a random sample of all the other populations. Such unusual dispersal, as a historical event, would explain why the phylogeographic patterns of *Cunninghamia* and many other temperate species do not conform to an isolation by distance model at the scale of the entire species range. Nevertheless, in contrast with the lack of geographical structure in the entire cladogram, some significant geographical associations of haplotypes and clades were detected at the clade scale. The past-fragmentation model, which predicts restricted levels of D_c and a large D_n when ancestral populations were divided into two or more isolates (cf. Maskas and Cruzan 2000), could be used to explain the differentiation between clades D′ (the populations of Hubei

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Table 5. Nested contingency analysis of geographical associations and phylogeographic inferences made from a nested-haplotype analysis of the genus *Cunninghamia*.

Clade	Permutational χ^2	*P*	Clade key[a]	Inferences
A	37.33	0.013	1, 2, 3, 5, 15	Past fragmentation
B′	4.00	0.532	1, 2, 3, 5, 15	Past fragmentation
B	11.00	0.011	1, 2, 3, 5, 6, 7, 8	Restricted gene flow with some long-distance dispersal
D′	4.00	0.351	—	Inconclusive
D″	6.00	0.217	1, 2, 3, 5, 6, 7, 8	Restricted gene flow with some long-distance dispersal
D	11.00	0.011	1, 2, 3, 5, 15	Past fragmentation
E	10.00	0.024	1, 2, 3, 5, 6, 7, 8	Restricted gene flow with some long-distance dispersal
Entire cladogram	3.03	0.27	—	Inconclusive

[a]Numbers indicate the choice made in the dichotomous key given in the appendix of Templeton et al. (1995).

and Zhejiang) and D″ (the populations of Jiangxi and Showluan of Taiwan). Two other possible past-fragmentation events may also have occurred in the mainland populations of clades A and B′.

Such evolutionary history may account for the heterogeneous and polymorphic genetic composition in the marginal populations of Taiwan. Accordingly, at least six migration events from the Asian mainland to Taiwan are inferred. It is possible that multiple invasions from different source populations resulted in the heterogeneous composition and high genetic diversity found in Taiwan's *Cunninghamia*. According to the phylogram and minimum spanning network, all clades contained haplotypes of *C. konishii*, except clade A. This internal clade (A) was only three mutational steps from K424. In contrast, many exterior nodes of *C. konishii* in the network were distant from Chinese haplotypes by virtue of three to seven mutational steps. In addition, the unique and ancestral haplotypes of clade C possessed by the probable refugium population at Kuanwu suggest that the cpDNA genotypes became extinct in the Chinese populations, thereby increasing the genetic diversity in *C. konishii*. Altogether, the maintenance of both ancient and "newer" haplotypes is a result of a unique migration history in *Cunninghamia*—the migrant-pool model—that may have resulted in the unexpectedly high genetic variation in marginal island populations.

High level of cpDNA-allele differentiation among populations

In contrast with the low level of allozyme differentiation among populations (Lin et al. 1998), the *trn*D–*trn*T noncoding spacer of the cpDNA was highly differentiated in *Cunninghamia*. Our results agree with the theory of Wright (1977), which suggests that extinction and recolonization usually enhance genetic differentiation among populations because of founder events resulting from the colonization of vacant habitats by a small number of individuals. Such high levels of genetic differentiation among populations due to the postglacial recolonization occurred in Japanese *Abies* (Tsumura and Suyama 1998) and European common beech (Demesure et al. 1996). Nevertheless, the migrant-pool model of Wade and McCauley (1990) indicates that the level of genetic differentiation between populations through extinction–recolonization processes depends on the number of founders (colonists) and migrants (*Nm*). In our study, genetic differentiation between Taiwanese and Chinese populations of *Cunninghamia* was low at the level of the geographic region. As shown above, populations of *Cunninghamia* in Taiwan were established from different source populations on the mainland. Most polymorphic ancestral haplotypes in *Cunninghamia*, except for the ones in clade A, were transmitted to Taiwanese populations. Although ongoing gene flow (via seeds) is extremely limited, heterogeneous genetic composition, plus a short time span for isolation, apparently has maintained the paraphyly of most cpDNA *trn*D–*trn*T alleles within each region (China vs. Taiwan).

In contrast with low genetic differentiation at the level of the geographic region, populations within geographic regions were highly differentiated. With a small population (colonists) and a low level of gene flow, genetic drift would increase among-population heterogeneity and within-population homogeneity. In other words, many local populations have attained coalescence at the *trnD–trnT* alleles (cf. Hudson 1990), which has led to significant differentiation at the population level. Nevertheless, although geographic isolation is a hindrance for seed dispersal, wind pollination across a wide range could play an important role in homogenizing the differentiation of the nuclear genome between populations of *Cunninghamia*.

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References

Avise, J.C. 1994. Molecular markers, natural history and evolution. Chapman and Hall, New York.

Avise, J.C. 2000. Phylogeography: the history and formation of species. Harvard University Press, Cambridge.

Bauert, M.R., Kälin, M., Baltisberger, M., and Edwards, P.J. 1998. No genetic variation detected within isolated relic populations of *Saxifraga cernua* in the Alps using RAPD markers. Mol. Ecol. **7**: 1519–1528.

Bennett, K.D. 1990. Milankovitch cycles and their effects on species in ecological and evolutionary time. Paleobiology, **16**: 11–21.

Bryoles, S.B. 1998. Postglacial migration and the loss of allozyme variation in northern populations of *Asclepias exaltata* (Asclepiadaceae). Am. J. Bot. **85**: 1091–1097.

© 2001 NRC Canada

Chen, Y.J. 2000. Study on the genetic variation among populations of *Kandelia candel* (L.) Druce in East Asia. Master's thesis (M.A.), Department of Biological Sciences, National Taiwan Normal University, Taipei.

Chiang, T.Y. 2000. Lineage sorting accounting for the disassociation between chloroplast and mitochondrial lineages in oaks of southern France. Genome, **43**: 1090–1094.

Chiang, T.Y., and Peng, C.I. 1998. Phylogeography of the endemic plants in Taiwan. *In* Proceeding of the symposium on Conservation of Endemic Species, *Edited by* S.D. Yeng. Research Institute of Taiwan Endemic Species, Taipei, Taiwan. pp. 148–155.

Clegg, M.T., Gaut, B.S., Learn, G.H., and Morton, R.R. 1994. Rates and pattern of chloroplast DNA evolution. Proc. Natl. Acad. Sci. U.S.A. **91**: 6795–6801.

Crandall, K.A., and Templeton, A.R. 1993. Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstructure. Genetics, **134**: 959–969.

Demesure, B., Sodzi, N., and Petit, R.J. 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol. Ecol. **4**: 129–131.

Demesure, B., Comps, B., and Petit, R. 1996. Chloroplast DNA phylogeography of the common beech (*Fagus sylvatica* L.) in Europe. Evolution, **50**: 2515–2520.

Desplanque, B., Viard, F., Bernard, J., Forcioli, D., Saumitou-Laprade, P., Cuguen, J., and van Dijk, H. 2000. The linkage disequilibrium between chloroplast DNA and mitochondrial DNA haplotypes in *Beta vulgaris* ssp. *maritima* (L.): the usefulness of both genomes for population genetic studies. Mol. Ecol. **9**: 141–154.

Dumolin-Lapègue, S., Demesure, B., Fineschi, S., Le Corre, V., and Petit, R.J. 1997. Phylogeographic structure of the white oaks throughout the European continent. Genetics, **146**: 1475–1487.

El Mousadik, A., and Petit, R.J. 1996. Chloroplast DNA phylogeography of the argan tree of Morocco. Mol. Ecol. **5**: 547–555.

Excoffier, L. 1993. Analysis of molecular variance. Version 1.5.5. Genetics and biometry Laboratory, University of Geneva, Geneva, Switzerland.

Excoffier, L., and Smouse, P.E. 1994. Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: molecular variance parsimony. Genetics, **136**: 343–359.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution, **39**: 783–791.

Ferris, C., Oliver, R.P., Davy, A.J., and Hewitt, G.M. 1995. Using chloroplast DNA to trace postglacial migration routes of oaks into Britain. Mol. Ecol. **4**: 731–738.

Golding, G.B. 1987. The detection of deleterious selection using ancestors inferred from a phylogenetic history. Genet. Res. **49**: 71–82.

Higgins, D.G., Bleasby, A.J., and Fuchs, R. 1992. CLUSTAL V: improved software for multiple sequence alignment. Compu. Appl. Biosci. **8**: 189–191.

Hong, Y.P., Hipkins, V.D., and Strauss, S.H, 1993. Chloroplast DNA diversity among trees, populations and species in the California close-cone pines (*Pinus radiata*, *Pinus muricata*, and *Pinus attenuata*). Genetics, **135**: 1187–1196.

Hsu, J.W., Moore, S.J., and Chiang, T.Y. 2000. Genetic variability in relic *Archangiopteris itoii* based on RAPD fingerprints. Bot. Bull. Acad. Sin. (Taipei), **41**: 15–18.

Huang, S., Chiang, Y.C., Schaal, B.A., Chou, C.H., and Chiang, T.Y. 2001. Organelle DNAs phylogeography of *Cycas taitungensis*, a relict species in Taiwan. Mol. Ecol. In press.

Hudson, R.R. 1990. Gene genealogies and the coalescent process. *In* Oxford survey of evolutionary biology. *Edited by* D.J. Futuyma and J. Antonovics. Oxford University Press, New York. pp. 1–44.

Jukes, T.H., and Cantor, C.R. 1969. Evolution of protein molecules. *In* Mammalian protein metabolism. *Edited by* H.N. Munroled. Academic Press, New York. pp. 31–132.

Kimura, M., and Weiss, G.H. 1964. The stepping stone model of population structure and the decrease of genetic correlation with distance. Genetics, **49**: 561–576.

King, R.A., and Ferris, C. 1998. Chloroplast DNA phylogeography of *Alnus glutinosa* (L.) Gaertn. Mol. Ecol. **7**: 1151–1161.

Lin, C.C. 1966. An outline of Taiwan's Quaternary geohistory with a special discussion of the relation between natural history and cultural history in Taiwan. Bull. No. 23 of the Department of Archaeology and Anthropology, Institute of Sciences, Taiwan University, Taipei. pp. 7–44.

Lin, T.P., Lee, T.Y., Yang, L.F., Chung, Y.L., and Yang, J.C. 1994. Comparison of the allozyme diversity in several populations of *Chamaecyparis formosensis* and *Chamaecyparis taiwanensis*. Can. J. For. Res. **24**: 2128–2134.

Lin, T.P., Wang, C.T., and Yang, J.C. 1998. Comparison of genetic diversity between *Cunninghamia konishii* and *C. lanceolata*. J. Hered. **89**: 370–373.

Liu, S.L. 1999. Origins of *Begonia formosana* complex inferred from chloroplast DNA and nuclear DNA variation. Master's thesis (M.A.), Department of Biology, National Cheng- Kung University, Tainan, Taiwan.

Lu, S.Y., Chiang, T.Y., Hong, K.H., and Hu, T.W. 1999. Re-examination of the taxonomic status of *Cunninghamia konishii* and *C. lanceolata* based on the RFLPs of chloroplast *trn*D–*trn*T spacer. Taiwan J. For. Sci. **14**: 13–19.

Maskas, S.D., and Cruzan, M.B. 2000. Patterns of intraspecific diversification in the *Piriqueta caroliniana* complex in southeastern North America and Bahamas. Evolution, **54**: 815–827.

Muller-Starck, G., and Liu, Y.Q. 1989. Genetics of *Cunninghamia lanceolata* Hook. 2. Genetic variation within and between two provenance samples. Silvae Genet. **38**: 172–177.

Murray, M.G., and Thompson, W.F. 1980. Rapid isolation of high molecular weight DNA. Nucleic Acids Res. **8**: 4321–4325.

Neale, D.B., Marshall, K.A., and Sederoff, R.R. 1989. Chloroplast and mitochondrial DNA are paternally inherited in *Sequoia sempervirens*. Proc. Natl. Acad. Sci. U.S.A. **86**: 9347–9349.

Nei, M., and Tajima, F. 1983. Maximum likelihood estimation of the number of nucleotide substitutions from restriction sites data. Genetics, **105**: 207–217.

Norusis, M.J. 1994. SPSS for Windows. Version 6.0 Englewood Cliffs, N.J. Prentice Hall.

Ohsako, T., and Ohnishi, O. 2000. Intra- and interspecific phylogeny of wild *Fagopyrum* (Polygonaceae) species based on nucleotide sequences of noncoding region in chloroplast DNA. Am. J. Bot. **87**: 573–582.

Ohsawa, T.A. 1997. Phylogenetic reconstruction of some conifer families: role and significance of permineralized cone records. *In* Evolution and diversification of land plants. *Edited by* K. Iwatsuki and P.H. Raven. Springer, Tokyo. pp. 61–98.

Pennell, R.I. and Bell, P.R. 1988. Insemination of the archegonium and fertilization in *Taxus baccata* L. J. Cell Sci. **89**: 551–560.

Petit, R.J., Pineau, E., Demesure, B., Bacilieri, R., Ducousso, A., and Kremer, A. 1997. Chloroplast DNA footprints of postglacial recolonization by oaks. Proc. Natl. Acad. Sci. U.S.A. **94**: 9996 – 10 001.

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Posada, D., Crandall, K.A., and Templeton, A.R. 2000. GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. Mol. Ecol. **9**: 487–488.

Rozas, J., and Rozas, R. 1999. DnaSP: an integrated program for molecular population genetics and molecular evolution analysis. Version 3. Bioinformatics (Oxford), **15**: 174–175.

Swofford, D.L. 1993. PAUP: phylogenetic analysis using parsimony. Version 3.1.1. Available from the Illinois Natural History Survey, Champaign.

Templeton, A.R. 1998. Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. Mol. Ecol. **7**: 381–389.

Templeton, A.R., Routman, E., and Phillips, C A. 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. Genetics, **140**: 767–782.

Tsumura, T., and Suyama, Y. 1998. Differentiation of mitochondrial DNA polymorphism in populations of five Japanses *Abies*. Evolution, **52**: 1031–1042.

Tsumura, Y., Yoshimura, K., Tomaru, N., and Ohba, K.1995. Molecular phylogeny of conifers using RFLP analysis of PCR-amplified specific chloroplast genes. Theor. Appl. Genet. **91**: 1222–1236.

Wade, M.J., and McCauley, D.E. 1990. Extinction and recolonization: their effects on the genetic differentiation of local populations. Evolution, **42**: 995–1005.

Wang, C.T., and Lin, C.P. 1998. Inheritance and linkage relationships of allozymes, and estimation of outcrossing rates in a seed orchard of *Cunninghamia konishii* Hay. Silvae Genet. **47**: 33–37.

Wang, C.T., Wang, W.Y., Chiang, C.H., Wang, Y.N., and Lin, T.P. 1996. Low genetic variation in *Amentotaxus formosana* Li revealed by isozyme analysis and random amplified polymorphic DNA markers. Heredity, **77**: 388–395.

Wright, S. 1977. Evolution and the genetics of populations. Vol. 3. Experimental results and evolutionary deductions. Chicago University Press, Chicago.

Xia, X. 1999. Data analysis in molecular biology and evolution. Version 3. 5. 19. Department of Ecology and Biodiversity, University of Hong Kong, Hong Kong.

Yeh, F.C., Shl, J., Yang, R., Hong, J., and Ye, Z. 1994. Genetic diversity and multilocus associations in *Cunninghamia lanceolata* (Lamb.) Hook. from the People's Republic of China. Theor. Appl. Genet. **88**: 465–471.

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