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ORIGINAL ARTICLE

Contrasting phylogeographical patterns of two closely related species, *Machilus thunbergii* and *Machilus kusanoi* (Lauraceae), in Taiwan

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ABSTRACT

Aim The purpose of this paper was to study the patterns of genetic variation, demographic history, haplotype relationships and potential location of diversity centres of two closely related species, *Machilus thunbergii* and *Machilus kusanoi*.

Location The phylogeography of *M. thunbergii* and *M. kusanoi* was examined by sampling 110 and 106 individuals from 25 and 16 sampling sites, respectively, across their distributional range in Taiwan. *Machilus thunbergii* is distributed on the Asian mainland, South Korea, southern Japan, the Ryukyus, Taiwan and the Philippines, whereas *M. kusanoi* is endemic to Taiwan. These two species are closely related, and both are widely distributed in Taiwan but occupy different altitudinal zones and habitats.

Methods The range-wide variation of *M. thunbergii* and *M. kusanoi* in Taiwan was studied using chloroplast DNA (cpDNA) variations. A haplotype network was constructed with the computer program TCS. Nested clade analysis was conducted with the computer program GEODIS, and various parameters of genetic diversity were calculated and neutrality tested by the computer program DNASP. Population differentiation was estimated using the programs ARLEQUIN and HAPSTEP. The contribution of the populations to gene diversity and to allelic richness was calculated using the software CONTRIB. The level of divergence for each population from the remaining populations was calculated as the mean value of pairwise F_{ST} for each population against the rest of the populations.

Results Extremely low levels of genetic differentiation were found for both species. This result suggested that these two species probably survived in multiple relict refugia with different population sizes throughout the island during low-temperature periods of the Pleistocene. In addition, nested clade analysis (NCA) of cpDNA haplotypes indicated that restricted gene flow with isolation-by-distance characterized the recolonization after the Pleistocene by Tashueshan and Shiouhluan populations of *M. thunbergii* in the north-central area west of the Central Mountain Range (CMR). In contrast, NCA analysis indicated that a major diversity centre on the southern tip of the island (Kending population) and contiguous range expansion characterized the recolonization by *M. kusanoi* of northern areas along the east side of the CMR. The major diversity centres found for the two species examined were further supported by the results of the mean F_{ST} for individual populations in comparison with other populations, and of the contribution of the divergence component to the total diversity.

Main conclusions This research supports the multiple relict refugia hypothesis for both species investigated. Populations of *M. thunbergii* at Shiouhluan and Tashueshan in the north-central area west of the CMR represent a diversity centre currently expanding its size. A diversity centre at the southern-edge population of

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M. kusanoi, and a contiguous range expansion from Kending, were found. These results indicate that the *M. thunbergii* populations at Tashueshan and Shiouhluan and the *M. kusanoi* population at Kending, and even Soukar, are evolutionarily significant units for conservation programmes.

Keywords

Conservation, cpDNA, diversity centre, *Machilus kusanoi*, *Machilus thunbergii*, phylogeography, Taiwan.

INTRODUCTION

Interpretations on the historical framework of the geographical distribution of genetic variation in a species require an understanding of the spatial and temporal patterns that underlie population processes. Early studies used intuitive approaches to decipher phylogeographical data (Avise, 2000). Nested clade analysis (NCA) is a powerful tool for examining the geographical associations of haplotypes within a statistical framework. Knowles & Maddison (2002) raised concerns that NCA may lead to erroneous conclusions, and suggested that both intuitive and NCA approaches should be used in phylogeographical studies. Subsequently, NCA was examined on 150 actual data sets and performed well overall, but with some false positives (Templeton, 2004). Therefore the NCA inference key was modified to reduce the incidence of false positives. NCA allows for the analysis of more complex population processes, and provides a more objective assessment of the geographical partitioning of haplotypes.

Taiwan is an island supporting tropical to cool climate vegetation, due to its low latitude and high elevations approaching 4000 m. The Central Mountain Range (CMR) extends north to south following the axis of the island, and contains over 200 peaks > 3000 m in elevation. The CMR is generally divided into western and eastern parts, with the western part divided into the Shueshan Range in the north and the Yushan Massif in the south. The CMR forms the backbone of the eastern part. Although an individual peak may reach an elevation of more than 3000 m, the relief between two peaks is rarely more than 200 m. The remnants of glaciation at the top of some peaks indicate the former occurrence of glaciers (Böse, 2004). Ice coverage in the CMR is assumed to have been incomplete, allowing for forest patches to persist as refugia for temperate to subtropical species. This constitutes the multiple refugia hypothesis for taxa distributed today across latitudinal temperate to subtropical zones, as opposed to a single-refugium scenario. NCA analysis is probably suitable for revealing population processes for diverse groups of plants that are distributed in different altitudinal zones.

Recolonization patterns of many vascular plants after the Pleistocene can be examined by investigating representative tree species from different elevations. Moreover, historically unique populations or lineages can be identified for conservation efforts. Chloroplast DNA (cpDNA) is inherited maternally through seeds alone in most angiosperms, so phylogeographical analysis of cpDNA haplotypes can provide insights into recolonization and seed dispersal (Ennos, 1994). Phylogeographical studies based on cpDNA variations for widely distributed plants in Taiwan have been reported for *Cyclobalanopsis glauca* (Huang *et al.*, 2002); *Cunninghamia konishii* (Hwang *et al.*, 2003); and *Trochodendron aralioides* (Huang *et al.*, 2004). *Cyclobalanopsis glauca* is a subtropical-temperate species, whereas *C. konishii* and *T. aralioides* are both temperate species. Potential diversity centres located in the Shueshan Range, which is in the north-central area west of the CMR, were found consistently for these two temperate species. However, one diversity centre was inferred in the southern part of Taiwan for *C. glauca* (Huang *et al.*, 2002), which is different from the two temperate species *C. konishii* and *T. aralioides*. It is thus interesting to investigate whether another species distributed in both subtropical and temperate altitudes (*M. thunbergii*) shares a common diversity centre with *C. glauca*. Furthermore, the similarity and discrepancy in genetic diversity, phylogeographical structuring, and recolonization and dispersal patterns between the two closely related species *M. thunbergii* and *M. kusanoi* are worth investigating.

Machilus (Lauraceae) are evergreen trees or shrubs which consist of about 100 species distributed mainly in the tropical and subtropical areas of Asia (Liu *et al.*, 1994). *Machilus thunbergii* Sieb. & Zucc. is a large, evergreen tree distributed widely in the Asian continent, southern Korea, Japan, the Bonin islands, the Ryukyus, Taiwan and the Philippines. *Machilus kusanoi* (Hay.) [=*Machilus japonica* Sieb. & Zucc. var. *kusanoi* (Hay.) Liao] is endemic to Taiwan and is distributed from the lowlands to 1400 m, but occurs mainly within the *Ficus–Machilus* Zone (< 500 m, 23–26 °C) (Su, 1984). *Machilus thunbergii* is distributed at about 200–2000 m and occurs mainly in the *Machilus–Castanopsis* zone (500–1500 m, 17–23 °C) (Su, 1984).

We investigated whether *M. thunbergii* and *M. kusanoi*, two closely related species with different altitudinal distributions, differ in their patterns of genetic diversity and recolonization dispersal. We also tested whether the existence of a single refugium or multiple refugia most adequately explains the phylogeographical patterns of these two closely related species. To answer these questions, we

analysed variation in two chloroplast intergenic spacers within a statistical phylogeographical framework using coalescent mean F_{ST} for individual populations in comparison with the rest and divergence component contributions to the total diversity, and nested clade analysis to test hypotheses about population history.

MATERIALS AND METHODS

Plant materials and DNA purification

A total of 110 and 106 individuals were randomly sampled from 25 and 16 populations in Taiwan, encompassing the distributional range of *M. thunbergii* and *M. kusanoi*, respectively. Four individuals of *M. thunbergii* from Okinawa (the Ryukyus) were also collected. The population code, sample size, longitude and latitude for each population within Taiwan are shown in Table 1; collection sites are depicted in Fig. 1. Total DNA was extracted from ground leaf powder according to a modified cetyltrimethyl ammonium bromide (CTAB) procedure (Doyle & Doyle, 1987), described in detail by Hwang *et al.* (2001). DNA was precipitated with ethanol, and after washing with 70% ethanol was dissolved in 200 μL TE buffer (pH 8.0) and stored at −20 °C. The DNA concentration was determined for each sample using GeneQuant II RNA/DNA Calculator (Amersham Biosciences, Taipei, Taiwan).

Primers, PCR amplification and sequencing

Polymerase chain reactions (PCR) were performed with universal primers for *trn*V-*trn*M and *trn*L-*trn*F (Taberlet *et al.*, 1991; Demesure *et al.*, 1995). Amplifications were performed in a DNA programmable thermal cycler (PTC-100, MJ Research, Watertown, MA, USA): initial denaturation at 94 °C for 3 min followed by 42 cycles of 1 min at 94 °C, 1 min annealing at 52 and 50 °C, respectively for *trn*V-*trn*M and *trn*F-*trn*L, 90 s at 72 °C, and a subsequent 10-min final extension at 72 °C. The PCR mixture (25 μL) contained 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, 100 mM Tris–HCl (pH 8.3), 1 mM dNTPs, 2 μM Primer, 20 ng template DNA, 1 μg RNase and 0.5 U *Taq* polymerase (Amersham Biosciences). The PCR products were purified using a QiaGen purification kit and then sequenced in both directions using a *Taq* Dye Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and Model ABI373A automated sequencer (Applied Biosystems). For sequencing we used the same primers as those used for amplification. All sequence polymorphisms were visually rechecked from chromatograms. Repeated sequencing was performed for variation, which was identified as singletons. GenBank accession numbers for *trn*L-*trn*F and *trn*V-*trn*M were AY819940–AY819944 and AY819945–AY819950, respectively, in *M. thunbergii*. GenBank accession numbers for *trn*L-*trn*F and *trn*V-*trn*M were AY819927–AY819932 and AY819933–AY819939, respectively, in *M. kusanoi*.

Statistical analyses

Multiple alignments of the sequences were obtained using ClustalW (Thompson *et al.*, 1994) and subsequently adjusted manually. The number of haplotypes was measured within each sampling locality. Haplotype diversity (h), nucleotide diversity (π) (Nei, 1987), Tajima's D (Tajima, 1989), Fu and Li's D^* and Fu and Li's F^* test (Fu & Li, 1993) for departure from neutrality on the total number of segregating sites were calculated using DnaSP version 4.0 (Rozas *et al.*, 2003). Each indel was recoded as a transitional substitution for the DnaSP program, as indels are consistently and unambiguously alignable. Two measures of population differentiation G_{ST} and N_{ST} were analysed by the hapstep program (Pons & Petit, 1996). G_{ST} depends only on the frequencies of the haplotypes, but N_{ST} is influenced by both haplotype frequencies and the distances between haplotypes.

The network of chloroplastic haplotypes was reconstructed using the algorithm of statistical parsimony described by Templeton *et al.* (1992) and implemented in tcs version 1.06 (Clement *et al.*, 2000). We used nested clade analysis (NCA) to infer the population history of *M. thunbergii* and *M. kusanoi*. The NCA nesting design was constructed by hand on the tcs haplotype network following the rules given by Templeton *et al.* (1987) and Templeton & Sing (1993). The program GeoDis 2.2 (Posada *et al.*, 2000) was used to calculate the various NCA distance measures and their statistical significance. All statistical analyses in GeoDis were performed using 1000 permutations. Results obtained from GeoDis were then interpreted using the revised inference key of Templeton (2004). The statistics calculated for all clades were: (i) clade distance (D_C), which measures the average distance of all clade members from the geographical centre of distribution; (ii) nested clade distance (D_N), which measures how widespread a particular clade is relative to the distribution of other clades in the same nesting group; and (iii) interior-tip distances (I-TD_C and I-TD_N). This interior vs. tip contrast of clades corresponds to younger clades (tip clade) relative to their ancestors' clades (interior clades), common vs. rare under expectations from neutral coalescent theory (Crandall & Templeton, 1993). Testing for significantly small or large D_C or D_N distances in each nested clade is then used to reject the null distribution of no association between haplotype distributions and geography.

A Mantel test of the matrix of pairwise genetic distances against the matrix of pairwise geographical distances was performed by a simple Mantel test of zt: a software tool for simple and partial Mantel tests (Bonnet & Van de Peer, 2002). The contribution of the populations to gene diversity and to allelic richness was calculated following Petit *et al.* (1998) using the computer software contrib. The contribution of a population to the total diversity was calculated as the relative variation of the diversity on adding this population to the others. Similarly, the contribution to total diversity of a

Table 1 Population code, sample size, localities, chloroplast haplotype diversity, and nucleotide diversity for *Machilus thunbergii* and *Machilus kusanoi* in Taiwan

Population code	Sample size	Locality (°E/°N)	Haplotype (no. of individuals)	Haplotype diversity (h)	Nucleotide diversity (π) × 10^3
M. thunbergii					
Total	110			0.165 ± 0.049	0.31 ± 0.11
1 Qingrenhu	4	121.42/25.09	MTA (4)	0.000	0.00
2 Yangmingshan	6	121.51/25.18	MTA (6)	0.000	0.00
3 Pinglin	4	121.70/24.92	MTA (4)	0.000	0.00
4 Sanxia	7	121.42/24.56	MTA (7)	0.000	0.00
5 Zudong	4	121.05/24.44	MTA (3), MTH (1)	0.500 ± 0.265	0.97 ± 0.52
6 Shiouhluan	3	121.16/24.37	MTA (1), MTG (1), MTI (1)	1.000 ± 0.272	1.94 ± 0.68
7 Tashueshan	5	121.36/24.15	MTA (2), MTB (2), MTD (1)	0.800 ± 0.164	1.75 ± 0.69
8 Beidongyeinshan	3	121.21/24.49	MTA (3)	0.000	0.00
9 JiYuetan	5	120.54/23.52	MTA (4), MTF (1)	0.400 ± 0.237	0.39 ± 0.23
10 Tadongshan	3	120.41/23.30	MTA (3)	0.000	0.00
11 Duona	4	120.43/22.54	MTA (4)	0.000	0.00
12 Tahanshan	4	120.40/22.24	MTA (4)	0.000	0.00
13 Tawushan	4	120.54/22.21	MTA (4)	0.000	0.00
14 Taimali	4	121.01/22.36	MTA (4)	0.000	0.00
15 Jinping	4	121.10/23.07	MTA (3), MTE (1)	0.500 ± 0.265	0.97 ± 0.52
16 Yakou	4	120.58/23.15	MTA (4)	0.000	0.00
17 Zhongping	4	121.19/23.24	MTA (4)	0.000	0.00
18 Rueishuei	4	121.42/23.50	MTA (4)	0.000	0.00
19 Liyutan	4	121.30/23.56	MTA (3), MTC (1)	0.500 ± 0.265	1.94 ± 1.03
20 Herpin	4	121.42/24.18	MTA (4)	0.000	0.00
21 Wushibi	5	121.49/24.28	MTA (5)	0.000	0.00
22 Lupi	4	121.43/24.28	MTA (4)	0.000	0.00
23 Chilanshan	4	121.29/24.35	MTA (4)	0.000	0.00
24 Fushan	4	121.37/24.39	MTA (4)	0.000	0.00
25 Gueishan	4	121.57/24.51	MTA (4)	0.000	0.00
M. kusanoi					
Total	106			0.159 ± 0.048	0.51 ± 0.22
1 Wulai	10	121.34/24.51	mka (9), mkb (1)	0.200 ± 0.154	0.19 ± 0.15
2 Fushan	10	121.35/24.46	mka (10)	0.000	0.00
3 Zudong	4	121.04/24.44	mka (4)	0.000	0.00
4 Miaoli	8	120.45/24.22	mka (8)	0.000	0.00
5 Herpin	7	121.44/24.18	mka (6), mkj (1)	0.286 ± 0.196	2.48 ± 1.70
6 Lienhuachih	5	120.53/23.55	mka (5)	0.000	0.00
7 Liyutan	5	121.30/23.56	mka (4), mkd (1)	0.400 ± 0.237	0.39 ± 0.23
8 Fanlu	5	120.33/23.27	mka (5)	0.000	0.00
9 Zhongpin	6	121.18/23.23	mka (6)	0.000	0.00
10 Anton	6	121.19/23.17	mka (6)	0.000	0.00
11 Jiashein	8	120.35/23.04	mka (7), mkh (1)	0.250 ± 0.180	0.24 ± 0.17
12 Dongher	9	121.18/22.58	mka (8), mkg (1)	0.222 ± 0.166	0.21 ± 0.16
13 Honyeh	5	121.04/22.54	mka (5)	0.000	0.00
14 Laiyi	9	120.38/22.31	mka (9)	0.000	0.00
15 Soukar	5	120.50/22.14	mka (3), mkc (1), mki (1)	0.400 ± 0.237	1.15 ± 0.69
16 Kending	4	120.48/21.58	mka (2), mke (1), mkf (1)	0.833 ± 0.222	4.50 ± 1.23

population due to its own diversity and to its divergence was calculated. The contribution of a population to the total allelic richness was calculated by following the rarefaction method of Hurlbert (1971). The contribution of this population due to its own diversity and to its divergence was obtained from the partitioning of total allelic richness in similar ways to the contribution to total diversity.

Conventional F_{ST} (Wright, 1931), based on cpDNA sequences for population subdivision, was estimated using Analysis of Molecular Variance (AMOVA) implemented in the ARLEQUIN program (Schneider *et al.*, 2000). In addition, the level of divergence for each population from the remaining populations was calculated as the mean value of pairwise F_{ST} for each population against the rest of the populations.

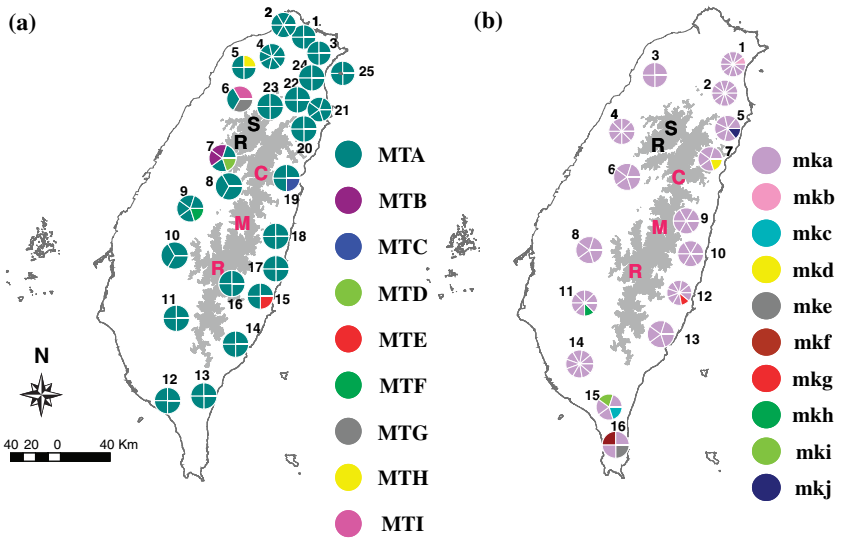

Figure 1 Map of Taiwan showing sampling sites and haplotype designations of (a) *Machilus thunbergii*; (b) *Machilus kusanoi*. The different number of pies in the haplotype circle for each population indicates the number of samples examined for each population. The shaded area indicates the Shueshan Range and Central Mountain Ridge in Taiwan.

RESULTS

cpDNA variation and haplotype relationships

Both *M. thunbergii* and *M. kusanoi* displayed very low levels of cpDNA nucleotide diversity (Table 1). Of the aligned 1031-bp sequences in *M. thunbergii*, 14 varied among nine haplotypes (indels at bases 109 and 110 were treated as one insertion–deletion event) (Table 2). In *M. kusanoi*, of the aligned 1041 bp, 20 polymorphic sites were observed among 10 haplotypes. Only one informative character was found at polymorphic site 162 in the *trn*V-*trn*M region in *M. thunbergii*. In contrast, three (polymorphic sites at 13, 57 and 69) in *trn*V-*trn*M and two (polymorphic sites at 730 and 758) in *trn*L-*trn*F were found in *M. kusanoi*. Haplotype MTA in *M. thunbergii* was fixed in 19 out of 25 populations investigated, with a haplotype frequency of 0.918 (Fig. 1). The haplotype frequency of rare alleles in *M. thunbergii* was either 0.009 or 0.018. In contrast, haplotype mka in *M. kusanoi* was fixed in nine out of the 16 populations examined, with a haplotype frequency of 0.915. All the rare haplotypes in *M. kusanoi* had a haplotype frequency of 0.009. Coalescent theory predicts that older (interior) haplotypes should be more common than derived (tip) haplotypes, and there is empirical evidence to support this idea (Crandall & Templeton, 1993). Following the rules of Crandall & Templeton (1993), tip haplotypes are defined as those connected to only one other haplotype, whereas interior haplotypes are connected to multiple haplotypes. The MTA haplotype in *M. thunbergii* is the most common and connected to multiple haplotypes (Figs 1 & 2). The most common haplotype is mka in *M. kusanoi*, which is connected to multiple haplotypes (Figs 1 & 2). Therefore haplotype MTA of *M. thunbergii* and mka of *M. kusanoi* are considered to be the ancestral haplotypes for *M. thunbergii* and *M. kusanoi*, respectively. Furthermore, only haplotype MTA was found in four individuals of *M. thunbergii* collected from Okinawa.

In *M. thunbergii*, the Shiouhluan and Liyutan populations had the highest level of nucleotide diversity, followed by Tashueshan. The highest cpDNA haplotype diversity was found in Shiouhluan, followed by Tashueshan. The *M. kusanoi* population in Kending had the highest level of nucleotide and haplotype diversity (Table 1). Moreover, the population group in the western CMR had higher haplotype and nucleotide diversities than the population group in the eastern CMR in *M. thunbergii* (Table 3). In contrast, the *M. kusanoi* population group in the eastern CMR had higher haplotype and nucleotide diversities than the population group in the western CMR.

Neutral evolution, haplotype structure and population history inferred from NCA

A neutrality test revealed a significant departure from equilibrium for all 25 *M. thunbergii* populations examined (Table 3). The neutrality test was also carried out on the population groups in the western and eastern CMR. The western group and the total populations had similar results, which showed a significant departure from equilibrium. A significant departure from equilibrium was also found for the total populations and for the eastern group of *M. kusanoi* populations examined (Table 3).

In *M. thunbergii* the N_{ST} estimate ($N_{ST} = 0.025$) was smaller than the G_{ST} estimate ($G_{ST} = 0.161$) in 25 populations, and the difference was significant (Table 3). This result indicated that haplotypes within populations had distant genetic relationships. A similar result was found for the populations west of the CMR. Mutation–drift equilibrium was found for the populations east of the CMR, with N_{ST}–G_{ST} approaching zero. In *M. kusanoi*, the N_{ST} estimate ($N_{ST} = 0.111$) was higher than the G_{ST} estimate ($G_{ST} = 0.044$) in 16 populations, and the difference was significant (Table 3). Mutation–drift equilibrium was found for the populations west of the CMR in this species. It is also clear that, in *M. kusanoi*, the eastern

Table 2 Polymorphic sites in the two intergenic spacers of cpDNA in *Machilus thunbergii* and *Machilus kusanoi*. Sequences are numbered from 5′ to 3′ for combined sequences from both cpDNA intergenic spacers

M. thunbergii

	trnV-trnM						*trnL-trnF*								
Haplotype	41	109	110	114	162	668	715	739	750	758	821	839	973	1014	1023
MTA	G	G	T	T	C	–	T	T	–	A	A	A	T	T	A
MTB	.	.	.	A	.	–	.	.	–	.	.	.	.	.	.
MTC	.	.	.	.	.	–	C	A	–	T	T	.	.	.	.
MTD	.	.	.	.	.	–	.	.	G	.	.	G	G	.	.
MTE	A	.	.	.	A	–	.	.	–	.	.	.	.	.	.
MTF	.	.	.	.	A	–	.	.	–	.	.	.	.	.	.
MTG	.	–	–	.	.	–	.	.	–	.	.	.	.	.	.
MTH	.	.	.	.	.	–	.	.	–	.	.	.	.	G	C
MTI	.	.	.	.	.	G	.	.	–	.	.	.	.	.	.

M. kusanoi

	trnV-trnM															*trnL-trnF*			
Haplotype	13	23	35	40	41	44	57	67	69	96	99	109	121	163	219	680	730	758	1009	1010
mka	G	C	T	G	G	–	G	C	C	G	G	A	A	C	A	–	C	C	–	C
mkb	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	–	.	.	T	.
mkc	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	G	.	.	–	.
mkd	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	–	.	.	–	A
mke	.	.	.	.	.	–	C	.	T	.	.	.	G	.	.	–	T	T	–	.
mkf	.	.	.	.	.	–	C	.	T	.	T	T	.	A	.	–	T	T	–	.
mkg	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	–	.	T	–	.
mkh	T	.	.	.	.	–	.	.	.	.	.	.	.	.	.	–	.	.	–	.
mki	T	.	.	A	.	A	.	.	.	.	.	.	.	.	.	–	.	.	–	.
mkj	T	T	G	.	A	–	C	G	G	A	.	.	.	.	C	–	.	.	–	.

The polymorphic site at 162 in *M. thunbergii* was phylogenetically informative.
The polymorphic sites at 13, 57, 69, 730 and 758 in *M. kusanoi* were phylogenetically informative.

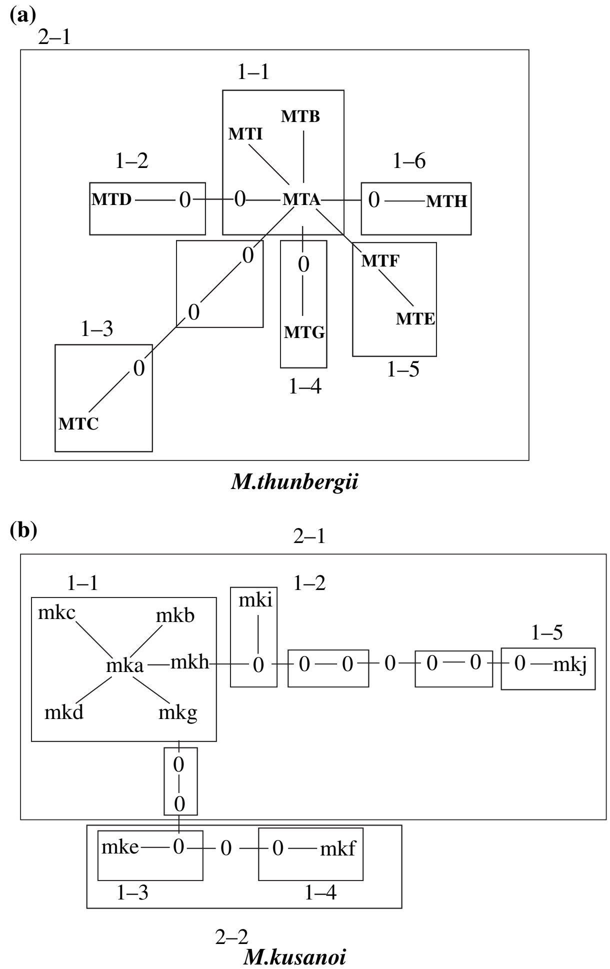

Figure 2 Nested cladogram of *Machilus thunbergii* (a) and *Machilus kusanoi* (b) haplotypes. Each line connecting one haplotype to another indicates one mutational step. Haplotype designations correspond to those in Table 1 and Fig. 1. Unsampled haplotypes are represented by 0. Hierarchical nesting design is specified by boxes and numbered clade designations.

group of populations showed significant non-random distribution of haplotypes when the ordered and unordered alleles are compared with estimated population differentiation that corresponds to the higher genetic similarity found within populations. Both these two closely related species had low levels of population differentiation.

Nested clade analysis showed a marginal relationship between genetic and geographical distribution in *M. thunbergii*. However, a significant relationship between genetic and geographical distribution was observed in *M. kusanoi* (Table 4). In *M. thunbergii*, restricted gene flow with isolation-by-distance was inferred between the populations on the Sheushan Range (clade 1–1), with an inconclusive result for clade 1–5 (Fig. 2a). Although no conclusive result was inferred for the haplotypes in clade 1–1 and one-step clade 2–1, contiguous range expansion was inferred from the NCA analysis of the two-step clade in *M. kusanoi* (Fig. 2b).

Table 3 Neutrality tests for 25 *Machilus thunbergii* and 16 *Machilus kusanoi* populations in Taiwan based on chloroplast intergenic spacers of *trn*V-*trn*M and *trn*L-*trn*F

Diversity parameter and test statistic	Total	West of CMR†	East of CMR‡
M. thunbergii			
h	0.165 ± 0.049	0.272 ± 0.084	0.070 ± 0.046
$\pi \times 10^3$	0.31 ± 0.11	2.19 ± 0.90	0.20 ± 0.14
$G_{ST(SE)}$	0.161 (NC)	0.164 (NC)	0.01 (NC)
$N_{ST(SE)}$	0.025 (NC)	0.037 (NC)	0.000
N_{ST}–G_{ST}	−0.136	−0.127	−0.01
Tajima's *D*	−2.440**	−2.297**	−2.079**
Fu and Li's *D**	−5.527**	−4.073**	−4.111**
Fu and Li's *F**	−5.245**	−4.114**	−4.065**
M. kusanoi			
h	0.159 ± 0.048	0.079 ± 0.052	0.19500 ± 0.069
$\pi \times 10^3$	0.51 ± 0.22	0.08 ± 0.05	0.84 ± 0.38
$G_{ST(SE)}$	0.044 (NC)	−0.009 (NC)	0.039 (NC)
$N_{ST(SE)}$	0.111 (NC)	0.000 (NC)	0.116 (NC)
N_{ST}–G_{ST}	0.067	0.009	0.077
Tajima's *D*	−2.467**	−1.464	−2.379**
Fu and Li's *D**	−4.615**	−2.533*	−3.390*
Fu and Li's *F**	−4.547**	−2.575*	−3.604**

†Includes populations 1, 2, 3, 4, 5, 6, 7, 8, 9,10 and 11 in *M. thunbergii*; populations 1, 3, 4, 6, 8, 11 and 14 in *M. kusanoi*.
‡Includes populations 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 and 25 in *M. thunbergii*; populations 2, 5, 7, 9, 10, 12, 13, 15 and 16 in *M. kusanoi*.
*$P < 0.05$; **$P < 0.02$.

Source populations of recolonization

A Mantel test of the matrix of pairwise genetic distance against the matrix of pairwise geographical distance resulted in a negative relationship between these two matrices ($r = -0.1775$, $P = 0.0035$, 10,000 permutations, one-tailed test) for *M. thunbergii*. Similarly, a Mantel test of the genetic distance matrix against the matrix of geographical distances also resulted in a negative relationship for *M. kusanoi* ($r = -0.1383$, $P = 0.0590$, 10,000 permutations, one-tailed test). However, when the eastern group of *M. kusanoi* populations was analysed, the genetic distances increased significantly with geographical distance ($r = 0.4108$, $P = 0.027$, 10,000 permutations, one-tailed test). Nevertheless, if recolonization was derived from a small number of source populations through stepping-stone colonization, we might expect a pattern in which the most important source populations showed a stronger contribution to the total diversity and allelic richness. Therefore the contributions of each population to the total diversity and allelic richness were analysed (Petit *et al.*, 1998). Results for *M. thunbergii* showed that the populations in Shiouhluan and Tashueshan contributed most to the divergence and to the diversity components of total diversity, due to their high haplotype diversity (Fig. 3a). The *M. kusanoi* populations in Soukar and Kending contributed most to the divergence and diversity components of total diversity (Fig. 3b). The two populations that contributed most to the divergence and diversity components of the total diversity in respective species also played an important role in the contribution of allelic richness. These results suggest that source populations do exist within local geographical areas.

Furthermore, the source population for recolonization from the centre of diversity can be further inferred from the mean F_{ST} for individual populations in comparison with the remaining populations. The mean F_{ST} results showed that the most genetically distinct populations were the area covering Shiouhluan and Tashueshan for *M. thunbergii*, and the area covering Kending for *M. kusanoi* (Fig. 4).

DISCUSSION

Genetic diversity and population differentiation

The low levels of cpDNA nucleotide diversity in *M. thunbergii* and *M. kusanoi* were consistent with other widespread tree species in Taiwan (Huang *et al.*, 2002; Hwang *et al.*, 2003;

Table 4 Inference of historical processes shaping the geographical distribution of *Machilus thunbergii* and *Machilus kusanoi* cpDNA haplotypes. Inferences are made from relative values of dispersion distance (D_C) and displacement distance (D_N) of tip and interior haplotype/clade (Fig. 2)

Nested clade	Permutational χ^2 statistic	*P*	D_C	D_N	Inference chain	Inferred pattern
M. thunbergii						
1-1	72.445	0.0490*	95.56^L	95.50^L	1-2-3-4-NO	Restricted gene flow with isolation-by-distance
1-5			37.50	77.69	1-19-20-2-11-17-NO	Inconclusive outcome
Total cladogram	136.880	0.0510	83.07	36.71^L	1-2-3-4-NO	Restricted gene flow with isolation-by-distance
M. kusanoi						
1-1	75.752	0.4380	91.31	91.29	1-2-11-17-NO	Inconclusive outcome
2-1	38.432	0.1230	92.10^S	92.09^S	1-2-11-17-NO	Inconclusive outcome
Total cladogram	51.481	0.0020*	92.74	-102.99^S	1-2-11-12-NO	Contiguous range expansion

S and L = D_C or D_N values that are significantly smaller (S)/larger (L) than expected at the 5% level based on 1000 permutations. *$P < 0.05$.

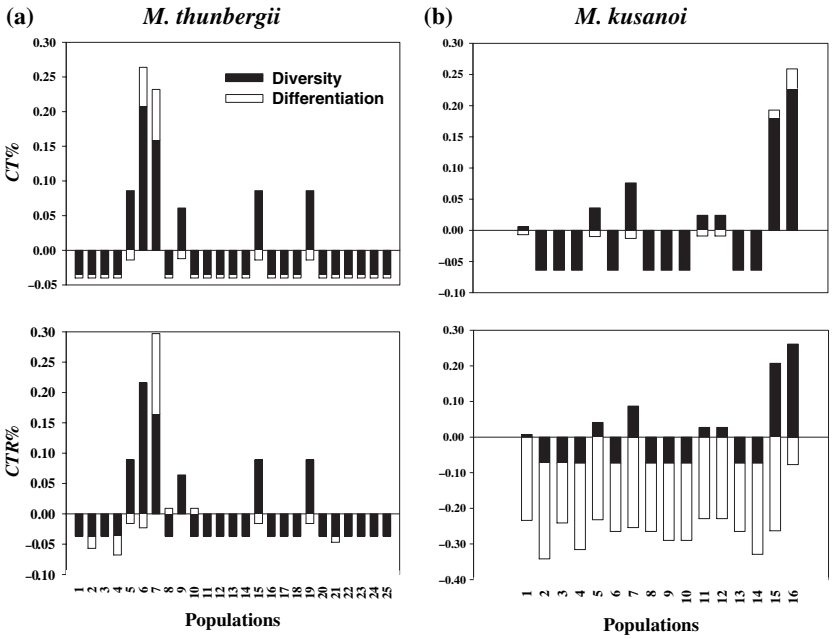

Figure 3 Contribution to total diversity (CT) and allelic richness (CTR) of each population of *Machilus thunbergii* (a) and *Machilus kusanoi* (b) using cpDNA haplotypes. Solid and open bars represent contributions of diversity and differentiation, respectively. Population numbers correspond to those in Table 1.

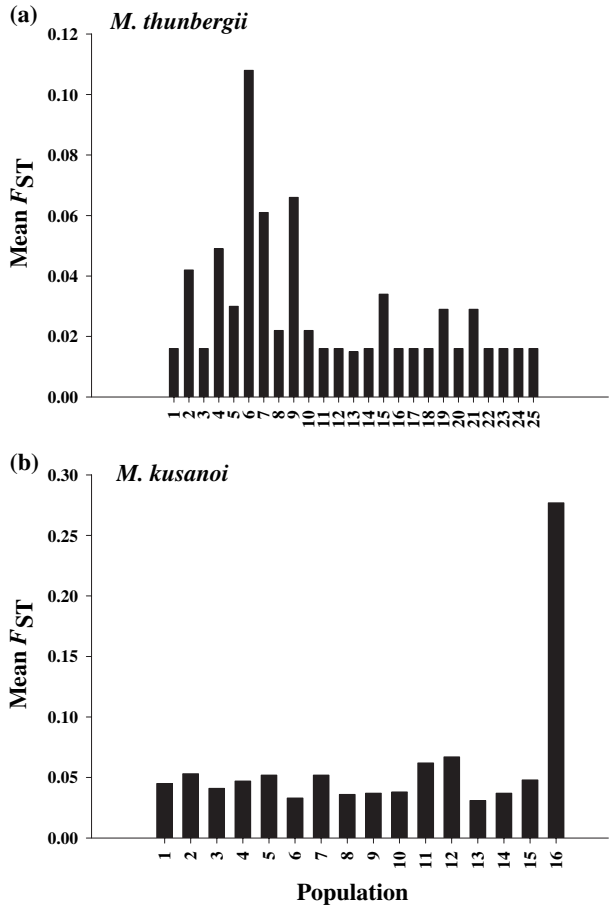

Figure 4 Degree of mean F_{ST} values of each population in comparison with those of the remaining populations. Population numbers correspond to those in Table 1.

Huang *et al.*, 2004). For the two closely related species investigated here, a contrasting level of cpDNA nucleotide diversity was found, as the same cpDNA intergenic spacer harboured different levels of nucleotide diversity in these two closely related species. It is also apparent that the island endemic *M. kusanoi* had a greater number of variable/informative molecular characters than its mainland counterpart *M. thunbergii*. This result is generally consistent with the more rapid evolutionary rate found in the island endemic species (Bromham & Woolfit, 2004). The higher level of cpDNA nucleotide diversity found in *M. kusanoi* compared with *M. thunbergii* is probably related to the faster rate of recolonization after cold periods in the Pleistocene.

Rates and patterns of dispersal and migration between populations will affect the genetic structure of species: the higher the dispersal ability, the lower the population structuring (Clegg *et al.*, 2002). Local adaptation and genetic drift may cause considerable phenotypic variation in widespread species (Joshi *et al.*, 2001). Populations along a colonization line following a glacial bottleneck can have a very low population differentiation ($F_{ST} = 0.005$), as found by maternal inherited mitochondrial DNA markers in *Pinus flexilis* (Latta & Mitton, 1997). This result was tested further, and suggested by the simulation of a one-dimensional colonization model for angiosperm tree species (Austerlitz *et al.*, 2000). The levels of genetic structure are unusually low in both species of *Machilus*; investigations of maternally inherited cpDNA in most angiosperms resulted in high levels of population differentiation (Petit *et al.*, 2005). We think that the low level of population differentiation found in *Machilus* may be related to the evolutionary history of this species, in which multiple relict refugia mainly hosted the ancestral haplotype. Little differentiation in the cpDNA of all populations investigated was equivocal with respect to the

multiple refugia hypothesis. Similar results have been documented by Soltis *et al.* (1997). Furthermore, if all haplotypes of *M. kusanoi* within a nesting level originated from a major source population, then relatively little genetic differentiation between haplotypes would be predicted.

Geographical patterns of genetic variation

It is typically reported that Pleistocene glaciation reduced species genetic variation in glaciated regions compared with regions that remained mostly ice-free. Although Taiwan was not glaciated, except for the high mountain peaks, the annual average temperature was 8–11 °C cooler (Tsukada, 1966), and a remarkably high proportion of grasses (Poaceae) indicates a dry environment in central Taiwan 20,000 years ago (Liew & Chung, 2001). This dry environment caused the deciduous broadleaved forests dominated by *Alnus* to replace the pre-existing evergreen broadleaved *Machilus–Castanopsis* forests in low, hilly areas (Liew & Chung, 2001). It is possible that most contemporary populations of *M. thunbergii* and *M. kusanoi* in Taiwan are descended from remnant populations that survived in some relict refugia during cold periods of the Pleistocene, and thus the ancestral haplotypes (MTA and mka) in both species are fixed in the majority of populations examined.

The significantly larger G_{ST} compared with N_{ST} indicated a random distribution of haplotypes in *M. thunbergii*. Multiple relict refugia existing during a period of cold climate, that hosted mainly the ancestral haplotype, would probably cause the shallow sequence divergence between haplotypes in *M. thunbergii*, which represents only an ancestral relationship. It is likely that the ancestral haplotype MTA became fixed by genetic drift in different relict refugial populations and therefore existed before the Holocene. It is interesting that cpDNA haplotypes showed a deeper sequence divergence in the haplotype network of *M. kusanoi*. Shen (1997) proposed that the flora of Taiwan could be divided into a southern Taiwan floristic superdistrict and a north-western Taiwan floristic superdistrict. The boundary between these two floristic superdistricts begins at Tahanshan (south Taiwan), proceeds northward along the eastern side of the CMR, and ends at Chingshuishan, close to the population at Herpin. The southern Taiwan floristic-superdistrict hypothesis, together with the non-random distribution of haplotypes (larger N_{ST} compared with G_{ST}) in *M. kusanoi*, allows at least two putative lineages to be inferred, although haplotypes in the lineages differ by several missing steps. The haplotypes mkh, mki and mkj formed one lineage; a second lineage included haplotypes mkg, mke and mkf (Fig. 2). Other rare haplotypes were probably derived randomly from ancestral haplotypes. Dominance of these two lineages in the eastern populations suggests the presence of a major source population at the southern tip of the island. It has been documented that interglacial periods became warmer and wetter compared with glacial periods, due to the intensification of summer monsoons (Huang *et al.*, 1997). One possible explanation for the longer branches exhibited by the southern haplotypes in *M. kusanoi* is that the trend of temperature rise after cold climate periods of the Pleistocene occurred from south to north, facilitating the growth and expansion of *M. kusanoi* at lower altitudes. In contrast, suitable habitats for *M. thunbergii* were at relatively higher altitudes, thereby constraining the recolonization process.

Phylogeographical patterns and local source populations of recolonization

Despite considerable interest in the effects of past climate change on plant migration, relatively little is known about the historical processes that have shaped present-day genetic diversity in regions such as Taiwan, which were mostly unglaciated. The geographical structuring of cpDNA haplotypes provided insights into the post-glacial history of two closely related species, *M. thunbergii* and *M. kusanoi*, within Taiwan.

Significant negative values obtained in a neutrality test for *M. thunbergii* may have been contributed mainly by the haplotypes collected from the area covering Tashueshan and Shiouhluan. However, this may not reflect a range-expansion event as a whole. Moreover, negative values of neutrality test statistics reflect an excess of rare haplotypes, which is consistent with either positive selection or an increase in population size. As the DNA fragments under investigation are not subjected to selective forces, it is possible that an increase in population size can be inferred. However, mismatch distribution analysis (data not shown) does not agree with the inference of an increase in population size. Nevertheless, the power is conservative in deciphering population history because mismatch distributions use little information accumulated in the data (Felsenstein, 1992; Ramos-Onsins & Rozas, 2002). Regional expansion in the area covering clade 1–1 in *M. thunbergii* did occur (Fig. 2a). This result can be explained by a short time to polymorphism accumulation recovered from an evolutionarily recent population bottleneck affecting *M. thunbergii* populations.

The haplotypes within clade 1–1 (haplotypes MTA, MTB and MTI; Fig. 2a) of *M. thunbergii* are limited primarily to the neighbouring populations at Tashueshan and Shiouhluan. This suggests a restricted gene flow with isolation-by-distance in this area in the north-central area west of the CMR. Indeed, NCA inferred a restricted gene flow event with isolation-by-distance in clade 1–1 (Table 4). However, the occurrence of ancestral haplotype MTA in all the populations investigated suggests that multiple refugia existed throughout the island, although most probably had small population sizes. Haplotypes MTF and MTE in clade 1–5 occur in two separate populations to the west and east of the CMR. This pattern allows no conclusion from NCA analysis, which further supports the multiple refugia hypothesis for *M. thunbergii*. Furthermore, populations in Shiouhluan and Tashueshan had the highest haplotype and nucleotide diversities, indicating that the area is probably the source of restricted gene flow, as revealed by the NCA analysis at a local scale. This is further supported by analysis of the contribution of total diversity by

divergence component (Fig. 3a). Previously, geographical regions in the north-central area, which is to the west of the CMR in Taiwan, have been proposed as the major diversity centre for several forest species based on allozyme variation (Lin, 2001). This north-central diversity centre in the western CMR was further revealed by amplified fragment length polymorphism (AFLP) analysis (Chung *et al.*, 2004). Although the contribution from the divergence component to the total diversity was not found for the eastern populations, Jinping and Liyutan contributed some allelic richness.

Although neutrality test statistics obtained were significantly negative, the 16 sampled populations of *M. kusanoi* show a mismatching distribution pattern that does not fit the range-expansion model (data not shown). However, as mentioned above, that mismatch distribution analysis has low power in detecting range expansion. In contrast, the Mantel test showed significant migration patterns for the eastern group of *M. kusanoi* populations, and the population history of contiguous range expansion was obtained from the NCA analysis (Table 4). NCA inference of contiguous range expansion or long-distance colonization is thought to be conservative, and not prone to false positives (Templeton, 2002). In *M. kusanoi*, the conclusion of a contiguous range expansion by NCA analysis is consistent with the analysis of isolation-by-distance (Mantel test) for the eastern group of populations, as the Mantel test found a significant correlation between geographical and genetic distances for the eastern group of populations. The haplotypes mka, mkb, mkd, mkg, mkh and mki are nested in clade 1–1, and are distributed along the eastern side of the CMR. Total diversity contribution analysis suggests that Kending contributed most to the divergence component, which supports the idea of migration along the eastern side of the CMR for *M. kusanoi*.

Comparison with *Cyclobalanopsis glauca*

Cyclobalanopsis glauca is also a widely distributed species in East Asia, and is the most commonly occurring plant among the 50 native species of the family Fagaceae in Taiwan (Huang *et al.*, 2002). *Cyclobalanopsis glauca* is found from sea level up to 1700 m, and is one of the dominant species in the *Machilus–Castanopsis* forests (Hsieh *et al.*, 1994). It is interesting that the distributional range of *C. glauca* covers the distributional range of *M. thunbergii* and *M. kusanoi*. Three populations of *C. glauca*, Yangmingshan (northern Taiwan), Wushe (central Taiwan close to the Tashueshan population of *M. thunbergii*), and Tahanshan (southern Taiwan close to the Kending population of *M. kusanoi*), showed higher haplotype diversity than other populations. Therefore the diversity centres of *C. glauca* corresponded to that of *M. thunbergii* in central Taiwan and to that of *M. kusanoi* in southern Taiwan, although they did not completely match geographically.

Conservation implications

Understanding patterns of genetic variation within tree species is of fundamental importance for successful management in tree-conservation programmes. Without knowledge of possible adaptive differences among areas, assessment of biodiversity within and among populations is central to revealing information on the population evolution of the closely related species *M. thunbergii* and *M. kusanoi*. The information can then be used in identifying and prioritizing areas with comparatively high genetic diversity for monitoring, management and protection. Knowledge of population structure is important for *ex situ* and *in situ* conservation of natural populations (Williams & Hamrick, 1996) by maintaining the total evolutionary potential and minimizing consanguinity. The present data on patterns of genetic diversity and phylogeographical structure suggest that the diversity centres, as well as some relict populations of both *Machilus* species, are worthy of conservation. The results of this investigation stress the importance of across-species range surveys to ensure a more representative sampling of the genetic diversity. Any *ex situ* conservation strategy should aim to include populations from both potential diversity centres, and from populations harbouring rare alleles for species that maintain mostly ancestral haplotypes.

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