

Phylogeography and genetic diversity of East Asian *Neolitsea sericea* (Lauraceae) based on variations in chloroplast DNA sequences

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Abstract *Neolitsea sericea* is an evergreen broad leaved tree in the warm-temperate regions of East Asia. This area is a hotspot for plant species richness and endemism caused by dynamic changes in land configuration during the Quaternary. However, the historical migration of such evergreen tree species is still poorly understood. In an attempt to reconstruct the phylogeographic history of *N. sericea* during the Quaternary, we identified the chloroplast DNA haplotypes of 287 individuals from 33 populations covering almost all of its geographic range. Analyses were based on sequence data from the *trnL-F*, *psbC-trnS*, and *rps16* regions. Nine haplotypes were identified. The majority included ancestral types in the southwestern part of the main islands of Japan, with other region-specific haplotypes being found in populations on the Korean Peninsula, Taiwan (Isl. Lanyu), and elsewhere in Japan. A statistical parsimony network revealed two lineages derived from Japanese main islands. One was represented on the Korean Peninsula, the other on Isl. Lanyu. The current distribution of *N. sericea* has been shaped by colonization via land bridges. During the glacial periods, two primary, but separate migration routes were followed—from the southwestern part of the Japanese main islands to either the Korean Peninsula or Taiwan. In addition, we believe the Zhoushan populations were shaped by post-glacial processes through an ECS land bridge (East

China Sea basin) from northern refugia that existed during the late Pleistocene.

Keywords Chloroplast DNA · East Asia · Evergreen broad leaved tree · Genetic diversity · Haplotype · *Neolitsea sericea* · Phylogeography

Introduction

Global climatic oscillations during the Quaternary have had a strong influence on species distribution and evolution. Thus, the present patterns of plant and animal species are the result of migration and extinction due to climatic changes (Hewitt 2000, 2003). In East Asia, fluctuations in sea levels that accompanied those oscillations produced dynamic changes in land configurations during the Quaternary (Kimura 1996, 2000; Kizaki and Oshiro 1977; Ota 1998). They have affected the fragmentation and rejoining of habitats for temperate plant species (Harrison et al. 2001; Qian and Ricklefs 2000). The modern genetic structure for temperate plant taxa distributed in that region would have been formed through expansion and contraction via land bridges (e.g., Bai et al. 2010; Li et al. 2008; Qiu et al. 2009a, b). Those warm-temperate climate zones of East China, southern Japan, and the southern tip of Korea are now hotspots for plant species richness and endemism (Qian and Ricklefs 2000; Xie 1997).

A simulated paleo-vegetation reconstruction by Harrison et al. (2001) has suggested that temperate forests are limited to a narrow belt at low elevations across the continental shelf that linked China, Korea, and Japan during the Last Glacial Maximum (LGM). In particular, warm-temperate evergreen forests that belong to temperate forest biomes are more extremely restricted than are temperate deciduous

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forests. However, this basic Quaternary expansion–contraction (EC) model of latitudinal range change is not paradigmatic due to differences in life cycles, habitat ecology, and a species' ability to disperse (e.g., Hewitt 1999; Qiu et al. 2011; Taberlet et al. 1998). Several studies in East Asia have led researchers to formulate various opinions about the historical migration of temperate taxa (Bai et al. 2010; Chen et al. 2008; Qiu et al. 2009b). However, such migration by warm-temperate evergreen tree species in this region is poorly understood. Few phylogeographical examinations have been made of East Asian species, and most population genetics studies have focused on very localized areas, such as China, Korea, Japan, or Taiwan (Aoki et al. 2006; Cheng et al. 2005; Chung et al. 2000; Wang et al. 2005).

Neolitsea sericea (Blume) Koidz. (Lauraceae) is an evergreen broad leaved tree growing in East Asian warm-temperate regions. It is commonly found in the evergreen forests of Korea and Japan, and shows a disjunctive and

restricted distribution on Lanyu Island of Taiwan and Zhoushan Archipelago of East China (Fig. 1) (Lee and Choi 2010). The Korean Peninsula and Japanese main islands represent the northwestern- and northeastern-most limits of its range (Ohashi et al. 2006; Lee and Choi 2010). This species is represented by two varieties: var. *sericea* and var. *aurata*. The latter is distinguished by its persistent golden-brown hairs on the lower surfaces of the leaves (Hatusima 1969). Its habitats are restricted to Lanyu and Ryukyu (Chen and Pan 1997; Hatusima 1969; Liao 1996). As such, it is a suitable model for obtaining historical information as it relates to climatic changes in the Quaternary.

However, Zhai et al. (2012) have suggested that *N. sericea* has two distinct lineages in areas north and south of the 'Tokara Gap', i.e., a sea strait between northern and central Ryukyu. Their findings serve to increase our understanding of allopatric speciation in East Asian plant endemics (Zhai et al. 2012). Nevertheless, we still lack a

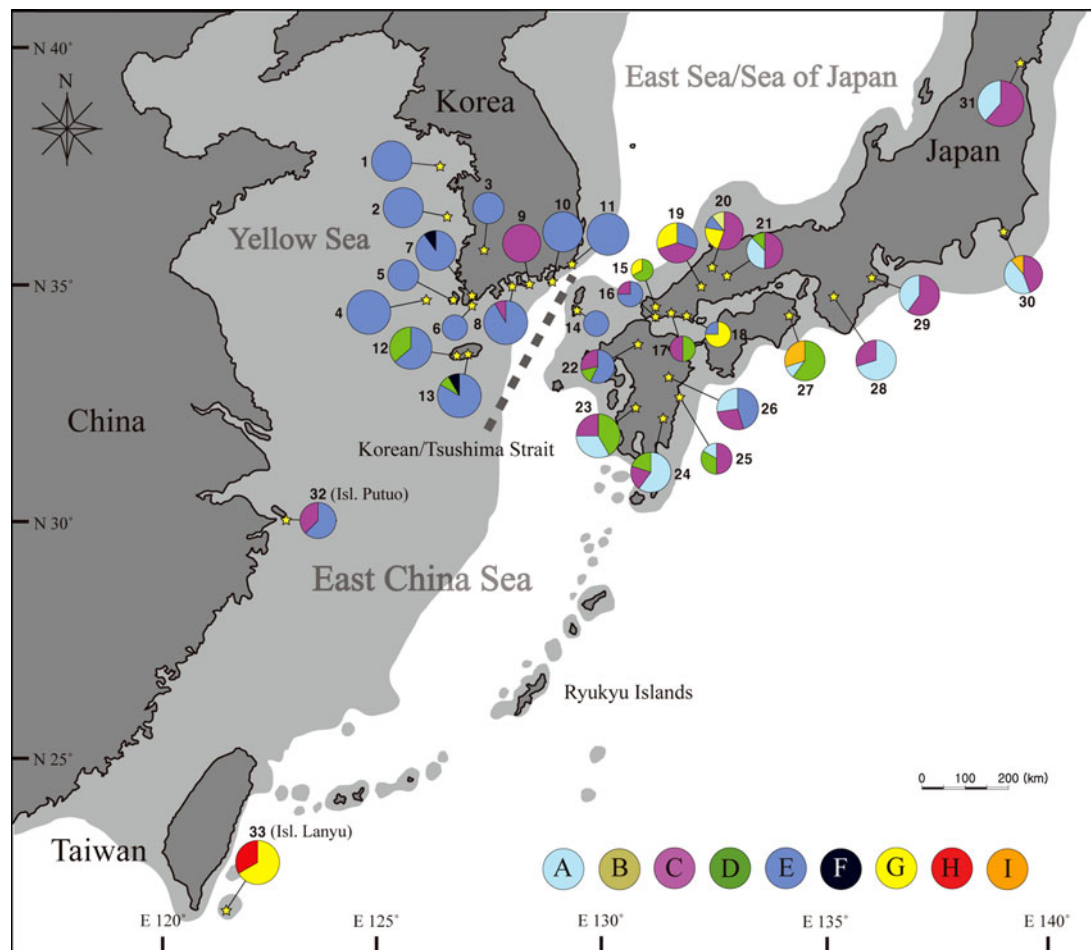


Fig. 1 Geographic distribution of cpDNA haplotypes detected in *Neolitsea sericea*. Dark shading indicates current mainland and island configurations; light shading shows exposed coastal areas and sea basins of East Asia during the time of glacial-induced alterations in

sea levels at late Pleistocene. Suppositions are based on a hypothesis by Kizaki and Oshiro (1977), then modified according to that of Hikida and Ota (1997)

clear picture of the phylogeographic structure for this species, especially within the Korean Peninsula. That region has played an important evolutionary role, and could have provided conditions suitable for generating new species via allopatric speciation, through the contemporary fragmentation of temperate forest combined with fluctuations in climate and sea levels (see Qiu et al. 2009b). Therefore, by analyzing that genetic structure on the Korean Peninsula, in essence covering its entire range, we can correctly acquire phylogeographical information for this species.

Our understanding of the processes that have influenced current distributions of populations and species has been greatly enhanced by the concept of phylogeography. This approach can be utilized to infer historical scenarios and account for spatial arrangements (Avice 2000). Chloroplast (cp) DNA is non-recombinant and maternally inherited (Corriveau and Coleman 1988), and it displays slow sequence evolution (Wolfe et al. 1987). Thus, cpDNA markers are useful tools for phylogeographic evaluations (Aoki et al. 2004, 2006; Heuertz et al. 2004; Petit et al. 2002; Rendell and Ennos 2003; Sugahara et al. 2011).

As has been observed with other temperate species in East Asia (Qiu et al. 2009b), we might expect that the distribution and genetic structure of *N. sericea* are deeply linked to paleogeography on the Korean Peninsula during the Quaternary (<2 million years ago). Therefore, to reconstruct its phylogeographic history, we investigated its variations in cpDNA sequences, paying particular attention to Korean populations. Our specific goals were to: (1) elucidate the genetic diversity within and among populations based on those sequences, (2) infer its historical migration routes in East Asia during the Quaternary, and (3) examine the taxonomic entity of *N. sericea* var. *aurata*.

Materials and methods

Sampling

Plants of *Neolitsea sericea* var. *sericea* were sampled from 32 populations in Korea, Japan, and China (Isl. Putuo of Zhou-shan Archipelago). One population of var. *aurata* was chosen from Taiwan (Isl. Lanyu). These populations represented almost the entire distributional range of this species except for the Islands of Ryukyu and Ogasawara. From each population, 3 to 13 plants were taken, for a total of 287 individuals (Table 1). Voucher specimens for these samples were deposited in the Herbarium at Inha University (IUI) (Table S1).

DNA extraction and PCR amplification

Genomic DNA was extracted from fresh leaves, using a G-spin™ Iip Kit for plants (iNtRON, Seongnam, Korea).

After preliminary screening of eight non-coding regions for cpDNA (*trnL-F*, *rps16*, *rpl16*, *psbA-trnH*, *psbC-trnS*, *rpoB-trnC*, *atpB-rbcL*, and *petD-rpoA*), we selected *trnL-F*, *psbC-trnS*, and *rps16* for the full survey because most of the variation was observed in those three regions (Lee et al. 2011). The regions were amplified by polymerase chain reaction (PCR). Primers c and f (Taberlet et al. 1991) were used to amplify the *trnL* intron and the *trnL-trnF* intergenic spacer (*trnL-F*). Primers *psbC-trnS/F*, *psbC-trnS/R*, *rps16/2F*, and *rps16/2R* (Nishizawa and Watano 2000) were used to amplify the non-coding regions of the *psbC-trnS* intergenic spacer and *rps16* intron.

PCR was conducted with a GeneAmp® PCR System 2700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Each reaction mixture contained 200 μM dNTPs (GeneCraft, Lüdinghausen, Germany), 1× PCR buffer with 1.5 mM MgCl₂, 1 U of Taq DNA polymerase (TaKaRa, Seoul, Korea), 10 ng of DNA, and an appropriate concentration of primers in a total volume of 50 μL. Conditions included an initial denaturation at 94 °C for 2 min; followed by 35 cycles at 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 1 min; with a final extension at 72 °C for 10 min. PCR products were visualized on 2 % agarose gels, purified with a high Pure PCR product Purification Kit (iNtRON), and sequenced with an ABI 3100 genetic Analyzer, using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). These sequences were deposited with their accession numbers in the GenBank database (Table S1).

Data analysis

All sequences were aligned manually with the program Clustal X version 1.83 (Thompson et al. 1997). Each indel was treated as a one-point mutation. The cpDNA haplotypes were determined based on those aligned sequences. The genealogical degree of relatedness among haplotypes was represented by a statistical parsimony network, generated by the program TCS version 1.21 (Clement et al. 2000). In addition, *N. aciculata* and *N. villosa* served as the outgroup to determine the ancestral haplotype.

Chloroplast DNA haplotype diversity (Nei 1987) was calculated for each population (h_S) and for the overall range (h_T), using DnaSP version 3.53 (Rozas and Rozas 1999). Two coefficients for gene differentiation— G_{ST} (all populations) and N_{ST} (differentiation influenced by both haplotype frequencies and genetic distances between haplotypes)—were estimated from cpDNA haplotypes following the methods of Pons and Petit (1996) and using the program PERMUT (available at <http://www.Pierroton.inra.fr/genetics/labo/Software/Permut/>). To test for the presence of a phylogeographical structure, we compared values for G_{ST} and N_{ST} with a permutation test that used

Table 1 Information about sampling populations of *Neolitsea sericea* in East Asia

Population code	Locality	Latitude/longitude	<i>n</i>	<i>h</i>	Haplotypes (number of individuals)
Korea					
1	Isl. Hagwangdae, Incheon	N37°02', E126°01'	10	0.000	E(10)
2	Isl. Oeyeon, Chungcheongnam-do	N36°13', E126°05'	10	0.000	E(10)
3	Mt. Bulgap, Jeollanam-do	N35°12', E126°32'	6	0.000	E(6)
4	Isl. Hongdo, Jeollanam-do	N34°41', E125°12'	12	0.000	E(12)
5	Mt. Cheomchal, Jeollanam-do	N34°28', E126°19'	6	0.000	E(6)
6	Isl. Wan, Jeollanam-do	N34°20', E126°41'	4	0.000	E(4)
7	Mt. Duryunsan, Jeollanam-do	N34°28', E126°37'	10	0.200	E(9), F(1)
8	Isl. Geumo, Jeollanam-do	N34°31', E127°45'	12	0.167	C(1), E(11)
9	Mijo-ri, Gyeongsangnam-do	N34°42', E128°02'	9	0.000	C(9)
10	Hakdong-ri, Gyeongsangnam-do	N34°45', E128°38'	10	0.000	E(10)
11	Taejongdae, Busan	N35°03', E129°04'	11	0.000	E(11)
12	Andeok Valley, Jeju-do	N33°15', E126°21'	11	0.509	E(7), D(4)
13	Mt. Sioreum, Jeju-do	N33°18', E126°30'	12	0.318	D(1), E(10), F(1)
	Regional mean			0.092	
Japan					
14	Mt. Daterayama, Isl. Tsushima	N34°14', E129°17'	4	0.000	E(4)
15	Mine-shi, Yamaguchi Pref.	N34°12', E131°17'	3	0.667	D(2), G(1)
16	Ube-shi, Yamaguchi Pref.	N34°02', E131°17'	4	0.500	C(1), E(3)
17	Komori Sano, Yamaguchi Pref.	N34°03', E131°31'	4	0.667	C(2), D(2)
18	Shunan-shi, Yamaguchi Pref.	N34°02', E131°56'	4	0.500	E(1), G(3)
19	Mt. Kanmuri, Hiroshima Pref.	N34°32', E132°23'	10	0.733	C(4), E(3), G(3)
20	Miyoshi-shi, Simane Pref.	N34°52', E132°41'	9	0.694	B (1), C(5), E(1), G(2)
21	Shobara-shi, Hiroshima Pref.	N34°47', E133°04'	8	0.679	A(3), C(4), D(1),
22	Mt. Koshoyama, Fukuoka Pref.	N33°28', E130°44'	7	0.667	C(2), D(1), E(4)
23	Mt. Shiroyayama, Kumamoto Pref.	N32°17', E130°42'	12	0.712	A(4), C(3), D(5)
24	Aya-cho, Miyazaki Pref.	N32°01', E131°10'	10	0.622	A(6), C(2), D(2)
25	Mt. Gongenyama, Miyazaki Pref.	N32°20', E131°37'	6	0.733	A(1), C(3), D(2)
26	Mt. Okueyama, Miyazaki Pref.	N32°44', E131°32'	11	0.710	A(3), C(3), E(5)
27	Mt. Bizan, Tokushima Pref.	N34°04', E134°30'	10	0.600	A(1), D(6), I(3)
28	Mt. Koya, Wakayama Pref.	N34°13', E135°32'	10	0.467	A(7), C(3)
29	Matsusaka-shi, Mie Pref.	N34°30', E136°27'	10	0.533	A(4), C(6)
30	Mt. Nokogiri, Chiba Pref.	N35°09', E139°49'	9	0.667	A(4), C(4), I(1)
31	Mt. Aobayama, Miyagi Pref.	N38°15', E140°51'	13	0.513	A(5), C(8)
	Regional mean			0.592	
China					
32	Isl. Putuo, Zhejiang Prov.	N30°00', E122°23'	8	0.536	C(3), E(5)
Taiwan					
33	Isl. Lanyu, Taitung	N22°00', E121°34'	12	0.485	G(8), H(4)
Total mean				0.390	

n number of collected individuals, *h* estimate of haplotype diversity

10,000 permutations. If $N_{ST} > G_{ST}$, then the closely related haplotypes occurred in the same populations, thereby indicating that such a structure existed (Pons and Petit 1996).

Population differentiation for between-region comparisons was assessed by non-hierarchical analysis of molecular

variance (AMOVA; Excoffier et al. 1992), using the program ARLEQUIN version 3.0 (Excoffier et al. 2005). We did not choose a suitable hierarchical model for calculating among-region differentiation via Φ_{CT} because this would have required multiple populations and only one population each exists in East China and Taiwan.

Mantel tests (Mantel 1967) were performed to test the correlation between the matrix of pair-wise Φ_{ST} values and the matrix of geographical distances between pairs of populations using ARLEQUIN version 3.0; AMOVA and Mantel tests were used to evaluate significance with 10,000 permutations.

The possibility of a geographical structure was also investigated by spatial analysis of molecular variance, using the program SAMOVA version 1.0 (Dupanloup et al. 2002). This method is based on a simulated annealing procedure that defines groups of populations that are geographically adjacent, geographically homogeneous, and maximally differentiated from each other. The most likely number of groups (K) was identified by repeatedly running the program for 10,000 iterations for $K \in \{2, \dots, 15\}$, using 100 random initial conditions, then retaining the largest Φ_{CT} values (i.e., the greatest proportion of total genetic variance due to differences between groups) as predictors of the best grouping of populations (Dupanloup et al. 2002).

Results

Variation and distribution of haplotypes

We sequenced three cpDNA regions from 287 individuals (33 populations) of *Neolitsea sericea*. Among these, the *trnL-F* (868–925 bp) region was the most variable, having three polymorphisms, i.e., a polynucleotide repeat-length and insertion-deletion (indel) that included a 52-bp mutation as well as a nucleotide substitution. The *rps16* (261–265 bp) region had two polymorphisms, including a nucleotide substitution and mononucleotide repeat-length, while the *psbC-trnS* (241 bp) region had only one polymorphism, a nucleotide substitution. When combined, their sequences were aligned for a consensus length of 1,431 bp. Based on these six polymorphisms, nine haplotypes were recognized for *N. sericea* (Table 2).

The distribution and frequencies of haplotypes A to I among the 33 sampled populations of var. *sericea* and var. *aurata* are shown in Fig. 1 and Table 1. No dominant haplotype was widespread over the entire distributional range of this species. Haplotype E occurred at high frequency in the populations from the Korean Peninsula and East China. Haplotypes A, C, and D were found at high frequency in Japan, with haplotype A occurring only in that country. Haplotype G was distributed in two disjunctive areas—the Chugoku region of Japan and Isl. Lanyu of Taiwan. In addition, three region-specific haplotypes (F, H, and I) were found in a few populations: F from populations 7 and 13 of the Korean Peninsula; H from population 33 of Isl. Lanyu; and I from populations 27 and 30 of Japan.

Table 2 Polymorphic sites and cpDNA haplotypes based on sequences of three non-coding regions from *Neolitsea sericea*

Haplotype	<i>trnL-F</i> aligned position			<i>psbC-trnS</i> aligned position		<i>rps16</i> aligned position	
	3	6	8	1		0	1
	4	2	1	8		4	4
	6	5	3	7		3	0
A	.	.	G	C		T	T ₁₅
B	T ₁₄
C	T ₁₂
D	.	.	.	T		.	T ₁₄
E	.	2
F	.	2	.	T		.	.
G	1
H	1	.	A	.		.	T ₁₆
I	1	.	.	.		G	.

1: Deletion = AAGGAAGAATCGAATATTCAGTGATCAAATCA TTCCTCCTCGGATAGATCT; 2: Duplication = TCTTT

Among the nine haplotypes identified in this species, all but two (F and H) were in Japan. Ten populations comprised just a single haplotype while the other 23 had two or more (Fig. 1).

Our SAMOVA results revealed that values for F_{CT} decreased progressively as the number of groups increased, and no geographical grouping of populations was apparent. In fact, each new group that was delimited was represented by a single population or else two geographically distant populations (data not shown).

Regional differences in haplotype diversity

Total genetic diversity ($h_T = 0.713$), based on cpDNA data across all populations, was much higher than the average within-population diversity ($h_s = 0.390$). A phylogeographic structure was found over the entire range and in Japan, but neither N_{ST} value was significantly higher than its corresponding G_{ST} value (range: $N_{ST} = 0.485 > G_{ST} = 0.458$; Japan: $N_{ST} = 0.263 > G_{ST} = 0.260$). By contrast, no such structure was found on the Korean Peninsula ($G_{ST} = 0.634 > N_{ST} = 0.616$). At the regional level, the highest average within-population diversity was detected in Japan ($h_s = 0.592$); East China (Isl. Putuo) and Taiwan (Isl. Lanyu) had values higher than the average whereas the Korean Peninsula had the lowest (below-average) value ($h_s = 0.092$; Table 1).

When compared among all regions, estimated divergences in terms of Φ_{ST} revealed that the Korean Peninsula, Isl. Putuo, and Isl. Lanyu were more genetically similar to Japan than to other regions. In each instance, highly significant genetic differentiation was found between Taiwan (Isl. Lanyu) and the Asiatic continent (Korean Peninsula:

Table 3 Divergence between regional groups of *Neolitsea sericea* populations based on cpDNA sequence data

Contrast regions	Φ_{ST}
Taiwan vs. Korean Peninsula	0.990
Taiwan vs. East China	0.960
Taiwan vs. Japan	0.844
East China vs. Korean Peninsula	0.558
Japan vs. Korean Peninsula	0.513
Japan vs. East China	0.214

$\Phi_{ST} = 0.990$, East China: $\Phi_{ST} = 0.960$; Table 3). The Mantel tests showed no significant correlation between population differentiation and geographical distance within either region (Korean Peninsula: $r = -0.044$, $P < 0.547$ vs. Japan: $r = 0.020$, $P < 0.356$). However, a significant positive correlation was noted over the entire range ($r = 0.428$, $P < 0.001$).

Phylogenetic relationships of haplotypes

Phylogenetic relationships among haplotypes were revealed in the statistical parsimony network (Fig. 2). All haplotypes were distinguished from each other by one or two mutational steps. Statistical parsimony analysis at the 95 % confidence limit initially resolved two separate networks—one including haplotypes G, H, and I, versus the remaining haplotypes. This was due to a 52-bp indel between the two groups, which could be joined only with a non-parsimonious connection (Fig. 2).

Haplotypes A and/or B were supported as basal types. The highest root probability was assigned by TCS as haplotype B ($P = 0.260$). In our outgroup comparison, the closely related *N. villosa* and *N. aciculata* had haplotypes A and B (data not shown). Furthermore, those two were located at the center of the network (Fig. 2). By contrast, haplotype C was distinguished from haplotypes A and B only by differences in their mononucleotide repeat-lengths. Thus, we assumed that haplotypes A, B, C, and D would be the basal group of *N. sericea*. In addition, two groups derived from the basal group were found in our statistical parsimony network—haplotypes G, H, and I, versus haplotypes E and F.

Discussion

Genetic diversity and population structure

Our cpDNA analysis of 33 East Asian populations of *Neolitsea sericea* revealed a high degree of total haplotype diversity ($h_T = 0.713$). This is generally thought to reflect

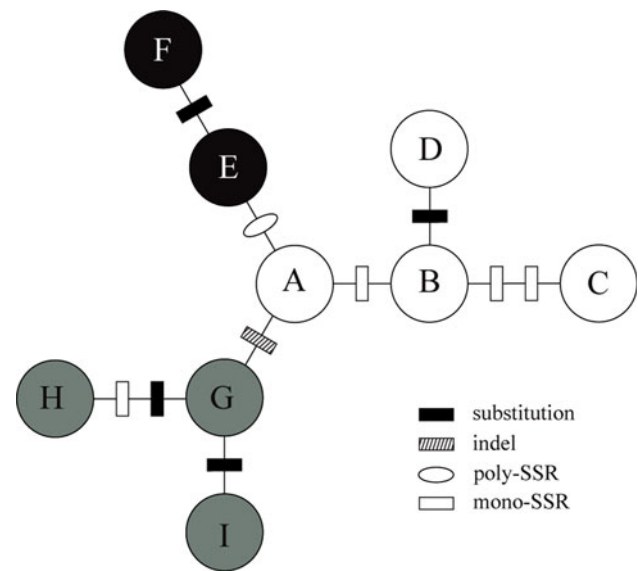


Fig. 2 Relatedness among chloroplast haplotypes (A–I) detected in *Neolitsea sericea*, represented in a statistical parsimony network. Identities of haplotypes are described in Table 2. Two phylogeographic lineages, derived from putative basal groups A, B, C, and D, are indicated by circles in black (Lineage I: E and F) and grey (Lineage II: G, H, and I)

a long evolutionary history (Huang et al. 2001). The average within-population diversity ($h_s = 0.392$) was considerably lower than the total haplotype diversity, probably because of the southwestern coastal populations (1–11) on the Korean Peninsula. Many of them (1–6, 9–11) occur in fragmented or small habitats that comprised a single haplotype, except for Isl. Putuo of East China. By contrast, populations found in continuous or large habitats had two or more haplotypes. This result is consistent with the theoretical expectation that larger populations have greater genetic diversity in the absence of selection (Kimura 1983).

Plants of *N. sericea* have an endozoochory type of seed dispersal that relies upon ingestion by vertebrate animals, such as monkeys and birds (Hong et al. 2007; Kominami et al. 2003). Species that produce ingestible seeds may exhibit weak population differentiation (e.g., Petit et al. 2003). However, the only life-history trait for maternally inherited markers that has been found to be significantly correlated with genetic differentiation among populations is the mode of seed dispersal (gravity vs. other means) (Duminil et al. 2007). We noted significant genetic divergence between the geographically adjacent Korean Peninsula and Japanese main islands ($\Phi_{ST} = 0.513$; Table 3). This also indicated that genetic exchange between regions through the action of birds was not frequent over long periods. In fleshy-fruited European *Frangula alnus* and Japanese *Photinia glabra*, high genetic differentiation and a clear phylogeographical structure have been detected

(Aoki et al. 2006; Hampe et al. 2003). The pattern of population differentiation identified here in *N. sericea* is not random, and genealogy is associated with the geographical distribution of cpDNA haplotypes.

Here, we identified seven of the nine haplotypes in the southwestern portion of those main islands (i.e., Kyushu, Shikoku, and Chugoku). Such haplotype richness provides a strong possibility for the existence of primary and persistent refugia in that area. During post-glacial re-colonization, the ancestral haplotypes were widespread, making it difficult to trace the sites for those glacial refugia, a process that often relies on derived or unique haplotypes for purposes of identification (Cheng et al. 2005). In fact, populations on the Korean Peninsula and in Japan and Taiwan (Isl. Lanyu) did have region-specific haplotypes (F, I, and H, respectively), which suggests that those locations were three separate refugia.

Our results indicated two geographically different haplotype lineages—I (E and F) and II (G, H, and I). The putative basal group (A, B, C, and D) showed a higher frequency in Japan than in other regions, and ancestral haplotypes A and B were distributed only in that country. By contrast, lineages I and II derived from the ancestral group were distributed in each of the other regions—I, on the Korean Peninsula, and II, in Taiwan (Isl. Lanyu). For comparison, East China (Isl. Putuo) did have haplotype C for the basal group and haplotype E within Lineage I.

Consequently, we suggest that population structuring of this species was shaped by colonization via land bridges. In particular, two main, but separate, migration routes were run during the glacial periods—from the southwestern part of the Japanese main islands to either Korean Peninsula or Taiwan.

Historical phylogeography and interpreting the genetic structure between Korea and Japan populations

During the glacial periods, the main islands of Japan (Kyushu, Shikoku, Honshu, and Hokkaido) were connected to the Asiatic continent (Korean Peninsula and East China) several times via paleogeographic land bridges (e.g., the Korean/Tsushima Strait, the East China Sea basin, and the Ryukyu Islands) (Kimura 1996, 2000; Kitamura and Kimoto 2006; Kizaki and Oshiro 1977; Ota 1998). In Korea and Japan, over the interval from 0 to 1.71 million years ago, the Korean/Tsushima Current Sea flowed into the East Sea (the Sea of Japan) during interglacial stages (Kitamura and Kimoto 2006). These periodical openings of the Korean/Tsushima Strait would have acted as a barrier to persistent contact of *Neolitsea sericea* through a land bridge between the Korean Peninsula and the Japanese main islands. In contrast, the periodic formation of a land bridge would have countervailed any genetic differentiation of *N. sericea*

populations between the Korean Peninsula and those Japanese islands. Thus, the population structure of these two regions would have been modulated by a balance between vicariance and immigration through historical processes that were linked to the Quaternary paleogeography. The phylogeographical structure of Korean populations is thought to have been derived from a small Korean glacial refugium, plus re-colonization from eastern Japanese refugia.

During the glacial periods, East China, like the Korean Peninsula, was repeatedly connected with the Japanese main islands by a land bridge over the ECS basin (Kimura 1996, 2000). However, in the early and middle Pleistocene, East China and the southern part of the Korean Peninsula would have been separated by the East China Sea (Kizaki and Oshiro 1977; Ota 1998; Fig. S1). This wide oceanic barrier would apparently have limited migration into East China from the Korean Peninsula and Japan. Moreover, wide stretches along the continental shelf of the East China Sea were exposed during the late Pleistocene (Kizaki and Oshiro 1977; Ota 1998). This might have provided ample opportunities for range expansion into East China. In addition, because we found no unique haplotypes at Isl. Putuo, we propose that, compared with the Korean populations, the disjunctive distribution of the Putuo population is relatively recent, possibly being post-glacial rather than evolving allopatrically due to vicariance during the early or middle Pleistocene. Our conclusion is consistent with that of Zhai et al. (2012), who suggested that species within the Zhoushan populations are remnants of a lineage that extended along the exposed ECS basin during the LGM, and that they colonized the area before the island became isolated (c. 9000 BP–7000 BP; Yiming et al. 1998).

By comparison, during the late Pleistocene, a connection is evident between the Asiatic continent and the Japanese main islands while a link between Japan and Taiwan is very weak (Kizaki and Oshiro 1977; Ota 1998; Fig. 1). Thus, our results show that much greater genetic divergence occurred between Japan and Taiwan than between Japan and the Asiatic continent, and that a completely different lineage of the haplotype network exists between the Asiatic continent and Taiwan. To explain this, we might assume that the Tokara Gap between central and northern Ryukyu acted as a strong barrier, causing the genetic lineages of *N. sericea* to be isolated during the late Pleistocene (Zhai et al. 2012). Therefore, we suggest that any intercontinental migration between Japan and Isl. Lanyu must have happened via the Ryukyu Islands bridge around early or middle Pleistocene when there was a strong connection between land masses (Kizaki and Oshiro 1977; Ota 1998; Fig. S1).

The paleo-vegetation reconstruction presented by Harrison et al. (2001) shows that the warm-temperate evergreen forests of East Asia were restricted to the

southern region of East China and the southern tip of Japan along the Pacific coastal areas during the LGM. Those forests rapidly expanded and recovered northward from refugia during the post-glacial era. Several phylogeographical studies in this region have suggested southward range contractions during the LGM, followed by rapid northward expansions after de-glaciation (Aoki et al. 2004; Huang et al. 2002; Ohi et al. 2003a, b). However, our results demonstrate that isolated populations of *N. sericea* in East China (Isl. Putuo) and Taiwan (Isl. Lanyu) can be more plausibly explained by southward expansion from previously existing populations that served as northern refugia during the LGM or glacial periods.

Taxonomic entity of *N. sericea* var. *aurata*

Neolitsea sericea var. *aurata* was originally described by Hayata (1911) at Isl. Lanyu of Taiwan as a separate species, *Litsea aurata*. Subsequently, Hatusima (1969) considered the species to be a variety of *N. sericea*. That variety is distinguished from var. *sericea* in having dense, golden-brown hairs on the lower surfaces of its leaves. This treatment has been maintained (Chen and Pan 1997; Liao 1996; Miyazaki et al. 2003).

Our results revealed that *N. sericea* var. *aurata* and *N. sericea* var. *sericea* cannot be completely separated by molecular data because they share haplotype G. Nonetheless, our non-hierarchical AMOVA presents greater genetic divergence in contrasts that include Isl. Lanyu. In particular, the populations of Taiwan (Isl. Lanyu) and the Asiatic continent (Korean Peninsula and East China) show completely different lineages in the haplotype network. Furthermore, the population of var. *aurata* at Isl. Lanyu has a private haplotype H. Based on these findings, both the morphological and molecular evidence suggest that these two varieties have undergone a speciation event by geographical fragmentation. However, we cannot confirm the taxonomic position of var. *aurata* because we lack materials from the Ryukyu Islands, where that variety is also distributed (Hatusima 1969).

In conclusion, most of the haplotypes, including ancestral types, are found in the southwestern part of the Japanese main islands. Our genealogical analysis of haplotypes indicated that the putative basal group leads to two lineages in each of the other regions. The current distribution of *N. sericea* in East Asia has resulted from separate historical migrations through land bridges that connected the Japanese main islands to either the Korean Peninsula or Taiwan during the glacial periods. The Zhoushan populations have been shaped by post-glacial processes from northern refugia that existed during the late Pleistocene. Further work using additional genetic markers and including broad areas such as the Ryukyu Islands and southern China might

provide a better understanding about the historical migration of evergreen tree species in East Asia.

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