Cytoplasmic DNA variation in and genetic delimitation of *Abies nephrolepis* and *Abies holophylla* in northeastern China

Zu Yao Jiang, Yan Ling Peng, Xiao Xiao Hu, Yong Feng Zhou, and Jian Quan Liu

Abstract: It has recently been hypothesized that genetic markers experiencing high rates of gene flow would be better suited to differentiate closely related species. We tested this hypothesis by examining genetic variation in the chloroplast (cp) DNA (*trn*S–*trn*G and *trn*L–*trn*F) and mitochondrial (mt) DNA (*nad*5 intron 4 and *nad*7 intron 1) of two fir species, *Abies nephrolepis* (Trautv.) Maxim. and *Abies holophylla* Maxim., with overlapping distributions in northeastern China. Two mitotypes were identified; one was common to both species, whereas the other occurred only in *A. holophylla*. However, four chlorotypes were identified, clustered into two species-specific groups exhibiting distinct mutations; species delimitation using these generated genetic variants was congruent with those obtained by morphological delimitation. Our findings supported the hypotheses that cpDNA, with its high rates of gene flow, is more useful than mtDNA for species delimitation in fir trees. In addition, the low intraspecific diversity observed for both species may result from their repeated range retractions and expansions in response to climatic oscillations in the history.

Résumé : Certains ont récemment émis l'hypothèse que les marqueurs génétiques caractérisés par un taux élevé de flux génique seraient plus aptes à différencier des espèces étroitement apparentées. Les auteurs ont testé cette hypothèse en étudiant la variation génétique de l'ADN chloroplastique (cp) (*trn*S–*trn*G et *trn*L–*trn*F) et de l'ADN mitochondrial (mt) (*nad*5 intron 4 et *nad*7 intron 1) de deux espèces de sapin, *Abies nephrolepis* (Trautv.) Maxim. et *Abies holophylla* Maxim., dont les aires de répartition se chevauchent dans le nord-est de la Chine. Deux mitotypes dont un était commun aux deux espèces et l'autre apparaissait uniquement chez *A. holophylla* ont été identifiés. Toutefois, quatre chlorotypes qui se regroupaient en deux groupes spécifiques aux espèces et démontraient des mutations distinctives ont été identifiés. La délimitation des espèces basée sur ces polymorphismes génétiques correspondait à celle obtenue à l'aide des caractères morphologiques. Nos résultats supportent l'hypothèse que l'ADNcp, avec son taux élevé de flux génique, est plus utile que l'ADNmt pour délimiter les espèces chez le sapin. Également, la faible diversité intraspécifique notée chez les deux espèces pourrait résulter de cycles répétés de contraction et d'expansion des aires naturelles de répartition en réponse aux oscillations climatiques passées.

[Traduit par la Rédaction]

Introduction

Genetic delineating of the diverse species is a key issue in biology, particularly in the conservation and utilization of natural resources (Comes and Abbott 2001; Van Dyke 2008). However, shared polymorphisms between species are common, especially in conifers (e.g., Perron et al. 2000; Jaramillo-Correa et al. 2008; Semerikova et al. 2011). This may result from incomplete sorting of ancestral polymorphisms and from genetic introgressions that occur during the second contact after an allopatric speciation event (Sites and Marshall 2003; De Queiroz 2007). In particular, the fixed genetic differences between species vary according to the genomes or genes studied (Petit and Excoffier 2009; Zhou et al. 2010). For example, in pines and spruces, the chloroplast (cp) DNA, which is primarily paternally inherited, is more effective in delimiting species than the mainly maternally inherited mitochondrial (mt) DNA (Petit et al. 2005; Du et al. 2009; Petit and Excoffier 2009). Two possible factors may account for these differences. First, cpDNA has a high rate of mutation in conifers (Wolfe et al. 1987; Wagner 1992) and as such may be more prone to developing species-specific variants (Sloan et al. 2008). Second, cpDNA has higher rates of gene flow than mtDNA, which may override introgression (Currat et al. 2008) and promote rapid lineage sorting of ancestral polymorphisms (Hoelzer 1997; Zhou et al. 2010).

It is very common for both mtDNA and cpDNA polymorphisms to be shared between fir species, although the geographical distributions of the recovered genetic variations were suggested to be highly correlated with historical range shifts of the studied species (e.g., Jaramillo-Correa et al. 2008; Semerikova et al. 2011; Wang et al. 2011). For example, genetic variants from these two genomes were shared between five Japanese fir species belonging to different sections

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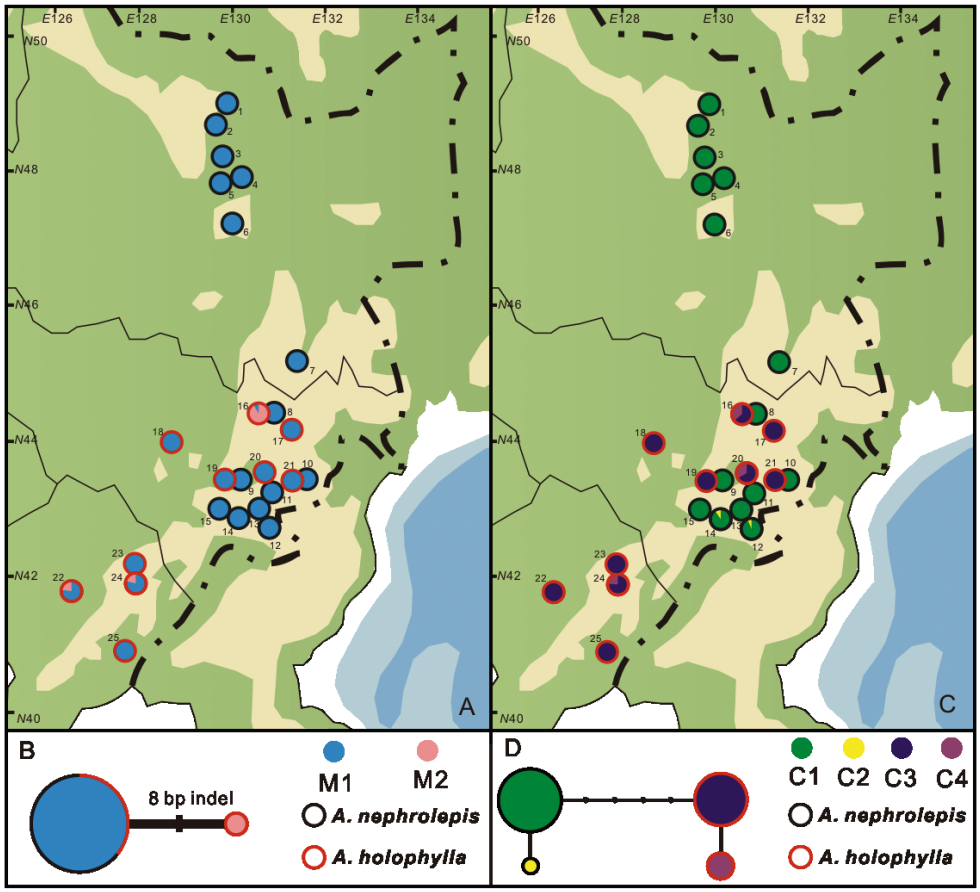
Z.Y. Jiang, Y.L. Peng, X.X. Hu, Y.F. Zhou, and J.Q. Liu. Molecular Ecology Group, Key Laboratory of Arid and Grassland Ecology, School of Life Science, Lanzhou University, Lanzhou 730000, China.

Corresponding author: Jian Quan Liu (e-mail: liujq@nwipb.ac.cn and ljqdxy@public.xn.qh.cn).

Fig. 1. (A and C) Distributions and (B and D) networks of mitotypes recorded in *Abies nephrolepis* (indicated by the circles with black boundaries) and *A. holophylla* (indicated by the circles with red boundaries). The black and red segments of circles in (B) and (D) indicate the relative proportions of each mitotype (B) or chlorotype (D) in *A. nephrolepis* and *A. holophylla*, respectively. Thick broken lines denote national borders; thin continuous lines denote provincial boundaries.



(Tsumura and Suyama 1998). Similarly, cpDNA variants are widespread among fir species occurring in Mediterranean and European regions, whereas mtDNA ones were more geographically correlated (Ziegenhagen et al. 2005; Liepelt et al. 2010). In Russia, South America, and central China, most mtDNA and some cpDNA variants are shared between species, but a few cpDNA ones are species-specific (Jaramillo-Correa et al. 2008; Semerikova et al. 2011; Wang et al. 2011). In fact, all of the results reported to date suggest that neither cpDNA nor mtDNA exhibits sufficient interspecies variability to allow for reliable species delimitation; this is largely due to incomplete lineage sorting and (or) frequent introgressions, especially between parapatric or sympatric species. In this study, we assessed the viability of species delimitation on the basis of variation in the cpDNA and mtDNA sequences of two fir species from Northeast China (where no other fir species occurs): *Abies nephrolepis* (Trautv.) Maxim. and *A. holophylla* Maxim. These two species are morphologically distinct and have overlapping distributional ranges in Northeast China (Fig. 1). The leaves of the former species are light green with apex emarginated, whereas those of the latter are dark green with apex acuminate or acute. These two species also differ slightly in the shape and color of their cones, seed scales, and bracts (Fu et al. 1999). As in other fir species, the pollen of these two species is wind-dispersed, while seeds are dispersed by animals (Fu et al. 1999). These two species were suggested to be closely related, but their phylogenetic relationship remains unclear (Liu 1971; Farjon and Rushforth 1989; Isoda et al. 2000), and a recent phylogenetic analysis of this genus did not include both species (Xiang et al. 2009). Therefore, both species together provide an ideal system for examining the hypothesis that the genetic variants from the genome with high rates of gene flow are more effective in delimiting species (Petit and Excoffier 2009). We collected 361 trees from 15 and 10 populations of *A. nephrolepis* and *A. holophylla*, respectively, with sympatric and allopatric distributions in Northeast China. We studied genetic variations of cpDNA and mtDNA, which have contrasted rates of gene flow and mutation in conifers (Wolfe et al. 1987; Wagner 1992). We aimed to address the following questions. (*i*) Are cpDNA variants more species-specific than mtDNA ones? If so, are there more variants shared between species in the sympatric distributions as suggested by the previous researchers? (*ii*) How are cpDNA and mtDNA polymorphisms geographically distributed within each species? Do their geographic distributions reflect range shifts of both species in response to historical climatic changes?

Table 1. Genetic diversity estimates for mtDNA and cpDNA.

Species	H_S	H_T	G_{ST}	N_{ST}
mtDNA variation				
A. nephrolepis	0.000	0.000	0.000	0.000
A. holophylla	0.063 (0.0394)	0.230 (0.1403)	0.725 (0.1679)	0.725 (0.1679)
Total	0.025 (0.0165)	0.095 (0.0684)	0.734 (0.1737)	0.734 (0.1737)
cpDNA variation				
A. nephrolepis	0.025 (0.0176)	0.026 (0.0181)	0.036 (NC)	0.036 (NC)
A. holophylla	0.135 (0.0694)	0.173 (0.0785)	0.219 (NC)	0.219 (NC)
Total	0.069 (0.0309)	0.535 (0.0522)	0.871 (0.0513)	0.968 (0.0125)**

Note: H_S, average gene diversity within populations; H_T, total gene diversity; G_{ST}, interpopulation differentiation; N_{ST}, the number of substitution types; NC, not computed. Standard deviations are given in parentheses.

Materials and methods

Population sampling

We sampled 15 and 10 populations of *A. nephrolepis* and *A. holophylla*, respectively. A total of 361 trees were collected throughout their current distribution range in northeastern China (Fig. 1; Supplementary Table S1).[1] At three localities (Dunhua, Fusong, and Helong), both species occurred together, whereas at the remaining localities, only one species was present. We sampled trees at least 100 m apart in each population and dried fresh needles with silica gel before further use.

DNA isolation, amplification, and sequencing

We used the hexadecetyltrimethylammonium bromide (CTAB) method to isolate the DNA (Doyle and Doyle 1987). Using primers described by Jaramillo-Correa et al. (2004, 2008), two mtDNA fragments were amplified and sequenced: intron 4 of subunit 5 of the NADH dehydrogenase gene (*nad*5 intron 4) and intron 1 of subunit 7 of the NADH dehydrogenase gene (*nad*7 intron 1). Similarly, two cpDNA fragments, *trn*L–*trn*F and *trn*S–*trn*G, were amplified and sequenced according to the method of Demesure et al. (1995). Polymerase chain reactions (PCRs) were carried out in a 25 μL volume, including 10–40 ng plant DNA, 50 mmol/L Tris–HCI, 1.5 mmol/L $MgCl_2$, 250 μg/mL BSA, 0.5 mmol/L dNTPs, 2 μmol/L of each primer, and 0.75 units of *Taq* polymerase. The products so obtained were purified using a TIANquick Midi Purification Kit (TIANGEN BIOTECH (Beijing) Co., Ltd., Beijing, China). Sequencing reactions were performed using an ABI PRISM BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, www.appliedbiosystems.com). DNA fragments were separated and identified using an Applied Biosystems 3730XL DNA Analyzer. All identified sequences were aligned using Clustal X (Thompson et al. 1997) and verified by visual inspection using MEGA version 4.0 (Tamura et al. 2007). A matrix of combined sequences was constructed for the 361 individuals examined. Multilocus mtDNA and cpDNA haplotypes (mitotypes and chlorotypes) were defined by assembling single-locus mtDNA and cpDNA genotypes, respectively. Two different mitotypes and four different chlorotypes were identified. These mitochondrial and chloroplast fragment sequences have been deposited in the EMBL GenBank under accession numbers HQ693528–HQ693536.

Data analysis

We constructed median-joining networks of mtDNA and cpDNA haplotypes with NETWORK version 4.2.0.1 (Bandelt et al. 1999), available at http://www.fluxus-engineering.com. We calculated the average gene diversity within populations (H_S), the total gene diversity (H_T), and two measures of population differentiation (G_{ST} (the coefficient of genetic variation over the whole population; Nei 1973) and N_{ST} (an analogous coefficient that accounts for sequence similarities between haplotypes)) for both mtDNA and cpDNA markers using PERMUT (available at http://www.pierroton.inra.fr/genetics/labo/Software/Permut/) (Pons and Petit 1996). A distinct phylogeographic structure is inferred if N_{ST} is significantly greater than G_{ST} (Pons and Petit 1996) because this suggests that closely related haplotypes are found more often in the same area than less closely related haplotypes. We used AMOVA to estimate the hierarchical partitioning of diversity between species, populations, and individuals using the ARLEQUIN version 3.0 software package (Excoffier et al. 1992, 2005), with significance tests based on 1000 permutations.

Results

mtDNA variation

The mtDNA analysis focused on intron 4 of the *nad*5 locus and intron 1 of the *nad*7 locus; polymorphism was only observed in the latter. Two mitotypes, M1 and M2, were identified, distinguished by an eight base pair indel in *nad*7 intron 1 at 320 bp. Mitotype M1 was widespread and observed in both species (Fig. 1A). All of the *A. nephrolepis* populations and most of the *A. holophylla* populations were fixed for mitotype M1. Only three *A. holophylla* populations in which mitotype M2 occurred were polymorphic. The total genetic diversity and average population genetic diversity were higher in *A. holophylla* ($H_T = 0.230$; $H_S = 0.063$) than in *A. nephrolepis* ($H_T = 0$; $H_S = 0$). The calculated values of G_{ST} and N_{ST} were similar (0.725 in *A. holophylla*; 0.734 between species), and there was no significant phylogeographic structure (Table 1) because mitotype M2 was concentrated in *A. holophylla*. Analysis of molecular variance (AMOVA) of

[1]Supplementary data are available with the article through the journal Web site (nrcresearchpress.com/cjfr).

Table 2. Analysis of molecular variance (AMOVA) of mtDNA and cpDNA variation.

Species	Source of variation	df	SS	VC	*V*%	*F* statistics
Mitotype						
All	Among species	1	8.971	0.02904	7.97	F_{CT} = 0.080
	Among populations within species	23	87.481	0.25832	70.93	F_{SC} = 0.771**
	Within population	337	25.890	0.07683	21.10	F_{ST} = 0.789**
	Total	361	122.343	0.36418		
A. nephrolepis	Among populations	14	0	0	0	
	Within populations	207	0	0	0	F_{ST} = 0.000**
A. holophylla	Among populations	9	87.481	0.68692	77.52	
	Within populations	130	25.890	0.19915	22.48	F_{ST} = 0.776**
Chlorotype						
All	Among species	1	435.627	2.53277	92.06	F_{CT} = 0.921**
	Among populations within species	23	15.937	0.03534	1.28	F_{SC} = 0.162**
	Within population	337	61.701	0.18309	6.65	F_{ST} = 0.933**
	Total	361	513.265	2.75120		
A. nephrolepis	Among populations	14	0.281	0.00048	3.59	
	Within populations	207	2.679	0.01294	96.41	F_{ST} = 0.036
A. holophylla	Among populations	9	15.656	0.09275	16.96	
	Within populations	130	59.023	0.45402	83.04	F_{ST} = 0.170**

Note: df, degrees of freedom; SS, sum of squares; VC, variance component; *V*%, percent of variation; ** indicates $P < 0.001$, 1000 permutations; F_{CT}, correlation of haplotypes within groups relative to total; F_{SC}, correlation within populations relative to groups; F_{ST}, correlation within populations relative to total.

Table 3. Variable nucleotide sites in two chloroplast DNA fragments that were used to identify four chlorotypes.

	Nucleotide variable positions										
	*trn*L–*trn*F							*trn*S–*trn*G			
Chlorotype	48	69	115	116	117	118	119	237	467	801	828
C1	G	G	—	—	—	—	—	C	T	A	C
C2	G	G	—	—	—	—	—	T	T	A	C
C3	T	T	—	—	—	—	—	C	C	C	T
C4	T	T	G	A	A	T	A	C	C	C	T

Note: Dashes (—) represent missing nucleotides.

two species showed that most of the variation occurred within species (92.03%), and that variation between species was negligible (7.97%). Among-population variation within *A. holophylla* was significant and accounted for 77.5% of the total variation (Table 2).

cpDNA variation

In cpDNA fragments *trn*S–*trn*G and *trn*L–*trn*F, six substitutions and one indel were detected, resolving four chlorotypes, two of which were highly frequent (C1 and C3) and two of which were less frequent (C2 and C4) (Table 3). Chlorotypes C1 and C2 were fixed in *A. nephrolepis*, whereas C3 and C4 were fixed in *A. holophylla*; no chlorotypes were common to both species. The minimum-spanning network of the chlorotypes indicated that the two frequent chlorotypes (C1 and C3) differ from each other by five site mutations, whereas C1 and C2 differ from each other by a single mutation step, and C4 contains an indel that is otherwise characteristic of C3 (Fig. 1C). Total genetic diversity and average within-population diversity were higher in *A. holophylla* (H_T = 0.173; H_S = 0.135) than in *A. nephrolepis* (H_T = 0.026; H_S = 0.025). Within each species, G_{ST} and N_{ST} showed the same value (0.036 in *A. nephrolepis*; 0.219 in *A. holophylla*), whereas G_{ST} and N_{ST} differed significantly between species ($N_{ST} > G_{ST}$; $P < 0.001$) (Table 1). AMOVA analysis indicated that 92.06% of the total variation was distributed between species. The among-population variation was significant and accounted for 17% of the total variation in *A. holophylla*, whereas the same component of variation was nonsignificant and accounted for only 4% of the variation in *A. nephrolepis* (Table 2).

Discussion

We compared genetic variants in the maternally inherited mtDNA and paternally inherited cpDNA between and within *A. nephrolepis* and *A. holophylla*, two fir species with overlapping geographical distributions in Northeast China. Our results indicate that cpDNA variants can always be used to distinguish between the two species, whether sympatrically or allopatrically distributed; mtDNA variants cannot. This finding obviously supports the hypothesis that genetic variants with high rate of gene flow are more effective in delim-

iting species (Petit and Excoffier 2009; Zhou et al. 2010). In addition, the low genetic diversity of each species in both the mtDNA and cpDNA sequence variations may mirror its large-scale range shifts in response to historical climatic oscillations.

Genetic delimitations between species

Our AMOVA analyses suggested that ~92% of the total cpDNA variation was partitioned between two species, whereas only ~8% of mtDNA variation was partitioned in this way (Table 2). mtDNA variants provided weaker solutions to delimitations of two species because mitotype M1 was widely distributed across both species (Fig. 1A). In contrast, all chlorotypes are species-specific, even in regions where they overlap (Fig. 1C). This is the first case in which it has been possible to reliably distinguish between fir species in a small region on the basis of cpDNA variation, although species-specific cpDNA variants have been identified in previous studies (Jaramillo-Correa et al. 2008; Semerikova et al. 2011; Wang et al. 2011).

It is well known that the polymorphisms that are common to different species may have arisen from introgressions during the second contact between the diverged species or from the maintenance of ancestral polymorphisms resulting from incomplete lineage sorting (i.e., De Queiroz 2007; Wiens 2007). The shared variants due to introgression between species would be expected to be geographically concentrated in parapatric populations (Palme et al. 2004; McGuire et al. 2007), whereas haplotypes resulting from maintenance of ancestral polymorphisms should be randomly distributed across the natural ranges of the derived species (Zhou et al. 2010). The high rate of gene flow may override introgression and lead to more species-specific cpDNA variants under some scenario, e.g., when an invading population is introgressed by the resident population (Petit and Excoffier 2009; Currat et al. 2008). On the other hand, if there are enough mutations, the high rate of gene flow may similarly promote rapid lineage sorting between species and fixing of the species-specific haplotypes (Zhou et al. 2010). For the two fir species examined in this work, the M1 mtDNA mitotype was fixed in most populations with no regard to distributional bias (no significant phylogeographic structure because the calculated values of G_{ST} and N_{ST} were similar; Table 1) because mitotype M2 was concentrated in *A. holophylla*, indicating that it may be a relic of ancestral polymorphisms rather than having arisen from introgressions between species. It is interesting that the species-specific chlorotype C4 cpDNA variant is also rare and appears in population 24 together with M2, indicating that these species-specific chlorotypes may also derive from ancestral polymorphisms. The near-complete absence of species-specific mtDNA polymorphisms may thus be primarily due to its low rate of gene flow rather than to the long mutation rate (De Queiroz 2007). However, the comparatively high rate of gene flow probably promoted cpDNA variants to become monophyletic between species with distinct interspecific phylogeographic structure ($N_{ST} > G_{ST}$; $P < 0.001$; Table 1) (Zhou et al. 2010). Our results thus support the hypothesis that in conifers, paternally inherited cpDNA, which has a higher rate of gene flow, is likely to be more useful for species delimitation than maternally inherited mtDNA (Du et al. 2009; Petit and Excoffier 2009; Zhou et al. 2010).

Genetic diversity and phylogeographic history

The observed mtDNA diversity was similar to that recorded for congeneric species in Europe, the Mediterranean region, and central China, where only one or two haplotypes were recorded for each species (Ziegenhagen et al. 2005; Liepelt et al. 2002; Wang et al. 2011). However, the observed cpDNA diversity was considerably lower than those reported in previous studies of different fir species; although these previous studies used different cpDNA markers (for example, microsatellite polymorphisms), most of them recovered more haplotypes and a higher level of genetic diversity for each fir species than was observed in the study reported in this paper (Ziegenhagen et al. 2005; Liepelt et al. 2010; Wang et al. 2011). Notably, in a study of fir species occurring in central China that focused on polymorphisms in the same two cpDNA regions as were examined in this work, more than 10 haplotypes were detected for each species (Wang et al. 2011). In addition, a study using the ISSR approach also indicated *A. nephrolepis* to have remarkably little diversity in its total genome (Woo et al. 2008). Other conifer species occurring in Northeast Asia at high latitudes were also found to have reduced diversity (Polezhaeva et al. 2010). The low diversity in this region may be a consequence of rapid range shifts caused by strong historical effects such as climatic oscillations (Hewitt 2000). During glacial periods, most high-altitude species had to retreat to glacial refugia or their distribution ranges shrank rapidly in situ. When the glacial periods ended, they expanded their ranges locally or recolonized their former distributions. The resulting founder and bottleneck effects undoubtedly resulted in reduced polymorphism within both populations and species (Hewitt 2000). This may account for the low diversity of the two fir species studied relative to their congeners in other regions such as central China (Wang et al. 2011).

It has been suggested that the forests in eastern Asia were replaced by herbs during the Last Glacial Maximum (LGM) and that the current forest distributions resulted from postglacial recolonizations from the refugia south of 30°N (Harrison et al. 2001). This suggestion was contradicted by the results of several phylogeographic studies of the forest species in northern China that suggested the existence of multiple refugia north of 30°N (Chen et al. 2008; Tian et al. 2009; Bai et al. 2010). However, all of these studies indicated that the recovered genetic haplotypes within Northeast China are highly uniform, which is consistent with postglacial recolonization from the southern regions. In fact, in this study, *A. nephrolepis* populations exhibiting polymorphism in both cpDNA and mtDNA were found only in the more southerly regions examined, which is consistent with the suggestion that this species might have recolonized the north postglacially. However, the other species examined in this work, *A. `holophylla*, had a more southerly distribution and two of its populations (16 and 24) were disjunctly fixed for mitotype M2 and chlorotype C4, indicating that this species existed in two glacial refugia during the LGM. Further phylogeographic studies examining a wider range of species will be needed to obtain deeper insights into forest retreats and recolonizations during the Quaternary oscillations in Northeast Asia.

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