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ScienceDirect
Molecular Phylogenetics and Evolution 41 (2006) 405–419
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Molecular phylogeny and biogeography of *Picea* (Pinaceae): Implications for phylogeographical studies using cytoplasmic haplotypes

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Received 22 January 2006; revised 17 April 2006; accepted 26 May 2006
Available online 6 June 2006

Abstract

The center of diversity is not necessarily the place of origin, as has been established by many plant molecular phylogenies. *Picea* is a complicated but very important genus in coniferous forests of the Northern Hemisphere, with a high species diversity in Asia. Its phylogeny and biogeography were investigated here using sequence analysis of the paternally inherited chloroplast *trn*C-*trn*D and *trn*T-*trn*F regions and the maternally inherited mitochondrial *nad*5 intron 1. We found that the North American *P. breweriana* and *P. sitchensis* were basal to the other spruces that were further divided into three clades in the cpDNA phylogeny, and that the New World species habored four of five mitotypes detected, including two ancestral ones and three endemics. These results, combined with biogeographic analyses using DIVA and MacClade and fossil evidence, suggest that *Picea* originated in North America, and that its present distribution could stem from two times of dispersal from North America to Asia by the Beringian land bridge, and then from Asia to Europe. Most of the northeastern Asian species and the European *P. abies* could arise from a recent radiation given the very low interspecific genetic differentiation and pure mitotype of them. Considering that the ancestral mtDNA polymorphism can be preserved in many descendant species, even distantly related ones, we suggest that more species, at least the closely related ones, should be sampled in the phylogeographical study using cytoplasmic haplotypes if possible. In addition, we also discussed the evolution and phylogenetic utility of morphological characters in *Picea*.

Keywords: *Picea*; Biogeography; Phylogeny; *trn*C-*trn*D; *trn*T-*trn*F; *nad*5; Himalayan–Hengduan mountains; Northeastern Asia

1. Introduction

Severe climatic oscillations associated with glacial cycles in the arctic during the late Tertiary and throughout the Quaternary resulted in great changes in species distributions and population structure (Böhle et al., 1996; Qian and Ricklefs, 2000; Liu et al., 2002; Petit et al., 2003; Hewitt, 2004). Meanwhile, descendent sea levels created land connections for intercontinental exchanges of flora and fauna, especially boreal species (Tiffney, 1985a,b; Wen, 1999; Xiang et al., 2005). With the advance and retreat of ice sheets, species went extinct over large parts of their range, and some populations dispersed to new locations or survived in refugia and then expanded again (Hewitt, 2000; Stewart and Lister, 2001). This repeated process would on the one hand stimulate adaptation and allopatric speciation (Hewitt, 2004), whereas, on the other, provide the opportunities for hybridization between recolonized populations, even reproductively unisolated species (Abbott and Brochmann, 2003). The reticulate evolution, and biological radiation resulted from climatic, ecological and geological changes bring many difficulties to the evolutionary and biogeographical studies of some taxa with long generation times, widespread distributions and low morphological divergence.

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doi:10.1016/j.ympev.2006.05.039

The genus *Picea* A. Dietrich (spruce) is a prominent component of the boreal, montane and sub-alpine forests in the Northern Hemisphere. It includes 28–56 species depending on different systems of classification used (Farjón, 1990; Ledig et al., 2004), and most of them are confined to Eastern Asia. Farjón (2001) recognized 34 spruce species in his conifer checklist, of which 24 natively occur in Asia, 8 in North America and 2 in Europe. Monophyly of *Picea* has never been debated (Wright, 1955; Prager et al., 1976; Frankis, 1988; Price, 1989; Sigurgeirsson and Szmidt, 1993), but infrageneric classification of the genus remains quite controversial (Liu, 1982; Schmidt, 1989; Farjón, 1990, 2001; Fu et al., 1999), owing to morphological convergence and parallelism (Wright, 1955), and high interspecific crossability (Ogilvie and von Rudloff, 1968; Manley, 1972; Gorden, 1976; Fowler, 1983, 1987; Perron et al., 2000). In addition, little is known about phylogenetic relationships of most species, especially the geographically restricted species growing in the montane regions of southwest China (LePage, 2001). Moreover, the origin and biogeography of *Picea* have drawn great interest from both geologists and biologists (Wright, 1955; Aldén, 1987; Page and Hollands, 1987; LePage, 2001, 2003), but they are still far from being resolved. For example, the two major hypotheses for the origin and evolution of North American spruces, both suggesting a dispersal from Asia (Wright, 1955; Nienstaedt and Teich, 1972), are in conflict with the finding of Sigurgeirsson and Szmidt (1993) that *Picea* might have an origin in North America. Therefore, a resolved phylogeny is very important for interpreting not only biogeographical patterns but also the morphological evolution in *Picea*.

Using the cpDNA-RFLP analysis, Sigurgeirsson and Szmidt (1993) constructed the first molecular phylogeny of spruces at the genus level, but relationships of many species were not resolved. In particular, the result of this study may be not very accurate due, as mentioned by the authors themselves, to limitations of RFLPs for detecting changes, such as the risk of non-homology of characters. A DNA-sequence based phylogeny of the whole genus *Picea* has not yet been obtained due possibly to the shortage of good markers. In recent years, the combined analysis of multiple genes from one or more genomes has been successfully used in robust reconstructions of complex phylogenies, and thus shed more light on biogeographical histories of many plant groups (e.g., Kusumi et al., 2002; Xiang et al., 2005). To resolve interspecific relationships, sequences of nuclear ribosomal DNA internal transcribed spacers (nrDNA ITS) and the chloroplast *trn*T-*trn*F region are most widely used (Wang et al., 1999; Wei and Wang, 2003; Shaw et al., 2005). However, the development of DNA markers in conifers has been hampered by: (1) a large nuclear genome with highly complex gene families (Kvarnheden et al., 1995; Kinlaw and Neale, 1997; Murray, 1998), which frequently give rise to the problem of gene paralogy; (2) a mitochondrial genome with the slow molecular evolution rate and high level of infraspecific polymorphism (Ahuja, 2001); and (3) a long nrDNA ITS region, which is too intragenomically variable in length to be used in investigating species phylogenies (Maggini et al., 1998; Wei et al., 2003; Campbell et al., 2005). So most previous molecular phylogenetic studies in conifers, especially at the genus level, were based on chloroplast gene markers (Sigurgeirsson and Szmidt, 1993; Wang et al., 1999, 2003; Kusumi et al., 2000; Wei and Wang, 2003).

In Pinaceae, the chloroplast, mitochondrial and nuclear genomes are paternally, maternally and biparentally inherited, respectively (Stine and Keathley, 1990; Sutton et al., 1991; Hipkins et al., 1994; Mogensen, 1996; Ahuja, 2001). Distinct phylogenies may be obtained from genes of the different genomes as a result of different inheritance pathways and responses to processes such as lineage sorting, gene duplication/deletion, and hybrid speciation (Doyle, 1997; Maddison, 1997; Wang et al., 2000). A good understanding of the inconsistency from distinct genomes will provide more valuable implications for the evolutionary process. Here, we reconstruct the molecular phylogeny of *Picea* using sequences of two cpDNA regions *trn*T-*trn*F and *trn*C-*trn*D. The later comprises the *trn*C-*pet*N intergenic spacer (IGS), *pet*N gene, *pet*N-*psb*M IGS, *psb*M gene and *psb*M-*trn*D IGS and has shown great potential in phylogenetic analysis at low taxonomic level (Lee and Wen, 2004; Shaw et al., 2005). Considering that the variation region in the first intron of *nad*5, a mitochondrial gene encoding subunit 5 of NADH dehydrogenase, used in the phylogeographic study of black spruce and red spruce is monomorphic in the other spruces and conifers surveyed by Jaramillo-Correa et al. (2003), we also sequence this region to obtain the genetic information of maternal lineages. Based on the joint cp- and mt-DNA analysis, we discuss the evolutionary history, biogeography and the evolution of morphological characters of this complicated genus.

2. Materials and methods

2.1. Plant materials

We sampled all of the 34 spruce species recognized in Farjón (1990, 2001) except *Picea aurantiaca*, an endangered species endemic to West Sichuan, China, which has been treated as a variety of *P. asperata* (Fu et al., 1999). Many species were represented by several individuals, and a total of 103 individuals were analyzed. *Cathaya argyrophylla* Chun et Kuang and two *Pinus* species, *P. strobus* L. and *P. thunbergii* Parl., were chosen as outgroups considering the close relationships among *Cathaya*, *Picea* and *Pinus* (Wang et al., 2000). Voucher specimens are deposited in PE. Sequences of the *trn*C-*trn*D and *trn*T-*trn*F regions of *P. thunbergii* (NC_001631) (Wakasugi et al., 1994) were retrieved from GenBank (www.ncbi.nlm.nih.gov/Genbank). The origins of the materials are shown in Table 1.

Table 1
Sources of materials

Taxa	Sources/individuals/vouchers	GenBank accession numbers		
		*trn*T-*trn*F	*trn*C-*trn*D	*nad*5
Sect. *Picea*				
Subsect. *Picea*				
Picea abies (L.) H. Karst.	Botanic Garden, Kunming Institute of Botany, Yunnan, China/1/Ran, J.-H. KM001	DQ358149	DQ358159	DQ358169
	Lushan Botanical Garden, Jiangxi, China/1/Ran, J.-H. LS001	Ditto	Ditto	Ditto
P. alcoquiana (Veitch ex Lindl.) Carrière	Jordan Botanic Garden, Geneva, Switzerland/1/Wang, X.-Q. 2144	DQ010598	DQ010553	DQ358170
P. asperata Mast.	Zhegu Mountains, Maerkang, Sichuan, China/1/Ran, J.-H. SCR044	DQ010599	DQ010554	DQ358171
	Botanic Garden, Institute of Botany, Beijing, China/3/Ran, J.-H. Ran012	Ditto	Ditto	Ditto
P. chihuahuana Martínez	Royal Botanic Garden, Kew, UK/1/Chase 18063	DQ358151	DQ358161	DQ358173
P. crassifolia Kom.	Qilian County, Qinghai, China/1/Liu, J.-Q. 716	DQ010602	DQ010557	DQ358174
P. glauca (Moench) Voss	Alberta, USA/2/Debreczy R018	DQ010605	DQ010559	DQ358177
P. glehnii (F. Schmidt) Mast.	Forestry and Forest Products Research Institute, Tsukuba, Japan/1/Liu, J.-Q. Gle	DQ010606	DQ010561	DQ358178
P. koraiensis Nakai	Changbai Mountains, Jilin, China/3/Wang, X.-Q. CBP001	DQ010608	DQ010563	DQ358180
	Botanic Garden, Institute of Botany, Beijing, China/6/Ran, J.-H., Ran013	DQ010610	DQ010565	Ditto
P. koyamae Shiras.	Royal Botanic Garden, Kew, UK/1/Chase 22940	DQ358153	DQ358163	DQ358205
P. mariana (Mill.) Britton and al.	White Lake, New Hampshire, USA/2/Debreczy 693234	DQ010621	DQ010576	DQ358183
P. maximowiczii Regel ex Mast.	Royal Botanic Garden, Kew, UK/1/Chase 18064	DQ358154	DQ358164	DQ358184
P. meyeri Rehder and E.H. Wilson	Botanic Garden, Institute of Botany, Beijing, China/2/Ran, J.-H. Ran006	DQ010622	DQ010577	DQ358186
	Botanic Garden, Institute of Botany, Beijing, China/2/Ran, J.-H. Ran002	DQ010623	DQ010578	Ditto
P. morrisonicola Hayata	Taiwan, China/5/CHC6888	DQ358155	DQ358165	DQ358185
P. neoveitchii Mast.	Baokang, Hubei, China/13/Jiang, M.-X. BK1	DQ358156	DQ358166	DQ358187
P. obovata Ledeb.	Altai Mountains, Xinjiang, China/6/Tan, D.-Y. P1	DQ010625	DQ010580	DQ358188
P. orientalis (L.) Peterm.	Jordan Botanic Garden, Geneva, Switzerland/1/Wang, X.-Q. 2161	DQ010626	DQ010581	DQ358190
	Samsun, Turkey/1/Wang, X.-Q. W02013	Ditto	Ditto	Ditto
P. retroflexa Mast.	Gongga Mountains, Sichuan, China/1/Ran, J.-H. SCR018	DQ010632	DQ010587	DQ358194
P. rubens Sarg.	White Lake, New Hampshire, USA/1/Debreczy 693235	DQ010633	DQ010588	DQ358195
P. schrenkiana Fisch. and C.A. Mey.	Tianshan Mountains, Xinjiang, China/1/Tan, D.-Y. Y001	DQ010634	DQ010589	DQ358196
	Tianshan Mountains, Xinjiang, China/1/Tan, D.-Y. Y002	DQ010635	DQ010590	DQ358197
P. smithiana (Wall.) Boiss.	Botanic Garden, Institute of Botany, Beijing, China/1/Ran, J.-H. Ran001	DQ010637	DQ010592	DQ358199
P. torano (Siebold ex K. Koch) Koehne	Botanic Garden, Institute of Botany, Beijing, China/1/Ran, J.-H. Ran008	DQ010627	DQ010582	DQ358200
P. wilsonii Mast.	Chengkou, Chongqing, China/1/Li, Z.-Y. CK01	DQ010638	DQ010593	DQ358201
	Shennongjia, Hubei, China/3/Yang, F.-S. Yang01	Ditto	Ditto	Ditto
	Botanic Garden, Institute of Botany, Beijing, China/1/Ran, J.-H. Ran005	Ditto	Ditto	DQ358202
Subsect. *Omorikae*				
P. brachytyla (Franch.) E. Pritz.	Gongga Mountains, Sichuan, China/3/Wang, X.-Q. 200304-2	DQ010600	DQ010555	DQ358172
	Gongga Mountains, Sichuan, China/2/Ran, J.-H. SC016	DQ010601	DQ010556	Ditto

(*continued on next page*)

Table 1 (*continued*)

Taxa	Sources/individuals/vouchers	GenBank accession numbers		
		*trn*T-*trn*F	*trn*C-*trn*D	*nad*5
P. breweriana S. Watson	Josephine Country, Oregon, USA/6	DQ358150	DQ358160	DQ358203
P. farreri C. N. Page and Rushforth	Drungzu, Cikai Labadi, Gongshan-Kongdong, Yunnan, China/2/Ran, J.-H. GS001	DQ358152	DQ358162	DQ358176
	Botanic Garden, Kunming Institute of Botany, Yunnan, China/1/GX01	Ditto	Ditto	Ditto
	Gongshan, Yunnan, China/2/Cun, Y.-Z. 01	Ditto	Ditto	Ditto
P. omorika (Pančić) Purk.	Royal Botanic Garden, Kew, UK/1/Chase 97.B	DQ358157	DQ358167	DQ358189
P. spinulosa (Griff.) A. Henry	Royal Botanic Garden, Kew, UK/1/Chase 22937	DQ358158	DQ358168	DQ358204
Sect. *Casicta*				
Subsect.*Sitchensis*				
P. jezoensis (Siebold and Zucc.) Carrière	Changbai Mountains, Jilin, China/3/Wang, X.-Q. CBP004	DQ010612	DQ010567	DQ358179
P. likiangensis (Franch.) E. Pritz.	Lijiang, Yunnan, China/4/Ding, K.-Y. DM455	DQ010618	DQ010573	DQ358182
	Linzhi, Xizang, China/1/Mao, J.-F. LZ062	DQ010619	DQ010574	Ditto
	Linzhi, Xizang, China/1/Mao, J.-F. LZ068	DQ010620	DQ010575	Ditto
	Jichou Mountains, Jiulong, Sichuan, China/2/Ran, J.-H. SC002	DQ010616	DQ010571	DQ358181
	Gongga Mountains, Sichuan, China/1/Ran, J.-H. SC035	DQ010617	DQ010572	Ditto
	Zheduo Mountains, Kangding, Sichuan, China/1/Ran, J.-H. SC006	DQ010615	DQ010570	Ditto
	Zhongdian, Yunnan, China/2/Liu, H.-M. YN305	Ditto	Ditto	Ditto
P. purpurea Mast.	Zhegu Mountains, Maerkang, Sichuan, China/1/Ran, J.-H. SC043	DQ010630	DQ010585	DQ358192
P. sitchensis (Bong.) Carriére	Yachts Oregon, USA/1/ Debreczy 69038	DQ010636	DQ010591	DQ358198
Subsect.*Pungentes*				
P. engelmannii Parry ex Engelm.	Jordan Botanic Garden, Geneva, Switzerland/1/Wang, X.-Q. 2162	DQ010603	DQ010558	DQ358175
P. pungens Engelm.	Botanic Garden, Institute of Botany, Beijing, China/1 Ran, J.-H. Ran019	DQ010628	DQ010583	DQ358191
Outgroups				
Pinus strobus L.	Botanic Garden, Institute of Botany, Beijing, China/1/Ran, J.-H. Ran027	DQ010640	DQ010595	
Pinus thunbergii Parl.	Wakasugi et al., 1994	NC_001631	NC_001631	
Cathaya argyrophylla Chun et Kuang	Dayao Mountains, Jinxiu, Guangxi, China/1/Wang, X.-Q. DY08	DQ010639	DQ010594	AF143415

2.2. DNA extraction, PCR amplification and sequencing

Genomic DNAs were extracted from needles of individual trees (single seeds were used for *Picea breweriana*) using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987; Rogers and Bendich, 1988). We amplified the *trn*C-*trn*D region with primers *trn*CN (5′-CCAGTTCGAATCCGGGTGTC-3′) and *trn*DN (5′-GGGATTGTAGCTCAATTGGT-3′) that were modified from *trn*C and *trn*D of Demesure et al. (1995), respectively. The *trn*T-*trn*F region and *nad*5 intron 1 were amplified using primer pairs a and f of Taberlet et al. (1991) and *nad*5-aF and *nad*5-bR of Wang et al. (2000), respectively. The PCR amplification was conducted in Tpersonal Thermocycle and T1 Thermocycle (Biometra, Goettingen, Germany) with a reaction volume of 25 μL containing 5–50 ng DNA template, 200 μmol/L of each dNTP, 6.25 pmol of each of the primer pair, 0.75 U of *Taq* DNA polymerase (TakaRa Biotech Co., Dalian, China). PCR cycles were as follows: one cycle of 4 min at 70 °C, 4 cycles of 2 min at 94 °C, 20 s at 55 °C (*trn*C-*trn*D) or 50 °C (*trn*T-*trn*F and *nad*5), and 2 min at 72 °C, followed by 36 cycles of 20 s at 94 °C, 20 s at 55 °C (*trn*C-*trn*D) or 50 °C (*trn*T-*trn*F and *nad*5), and 2 min at 72 °C, with a final extension step for 10 min at 72 °C.

The PCR products were purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, UK). Sequencing reactions were performed with the two PCR primers and several internal primers using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit or DYEnamic Energy Transfer (ET) Terminator Reagent Premix Kit (Amersham Biosciences). The internal primers included c (Wei and Wang, 2003) for the *trn*T-*trn*F region, *pet*N2G (5′-CTTGG GCTGCTTTAATGGTAG-3′), *psb*M2GF (5′-GTAGAG CAGCAATAAATGCAAG-3′), *psb*M2GR (5′-CTTGCA

TTTATTGCTGCTCTAC-3′) and *pet*N3G (5′-ATGGTA CGAGGTCCTTCATCC-3′) for the *trn*C-*trn*D region, and *nad*5-IF (5′-GGCTTTAGGGGGCCTTATG-3′) for the *nad*5 intron 1. *pet*N2G, *psb*M2GF and *psb*M2GR were modified from *pet*N2, *psb*M2 and *psb*M2R of Lee and Wen (2004), respectively. After precipitation in 95% EtOH and 3 M NaAc (pH 5.2), the sequencing products were separated on either an ABI 377 or MegaBACE 1000 automatic sequencer (Amersham Biosciences, Buckinghamshire, UK).

2.3. Data analysis

Sequence alignments were made with CLUSTAL X (Thompson et al., 1997) and refined manually. Nucleotide diversity (θ%) was estimated using DnaSP version 4.0 (Rozas et al., 2003). Three chloroplast datasets, *trn*C-*trn*D, *trn*T-*trn*F and combined *trn*C-*trn*D and *trn*T-*trn*F region sequences, were analyzed with the maximum parsimony method using *Cathaya* and two *Pinus* species as outgroups. Incongruence length difference (ILD) test (Farris et al., 1995) was conducted, using the partition homogeneity test in PAUP version 4.0b10 (Swofford, 2002), to examine the congruence between different datasets. Test settings were 100 random stepwise additions and 1000 replicates of heuristic search with TBR branch swapping using three outgroups.

The phylogenetic analyses were conducted using PAUP*4.0b10 with heuristic searches. The search options were 500 random addition sequence replicates, tree-bisection-reconnection (TBR) branch-swapping, MULTREES and a maximum of 1000 trees saved per round. Indels in the alignment induced by the ingroups were coded as 1/0 binary characters, and gaps of different lengths were all treated as single events. All character states were equally weighted. To evaluate relative robustness of the clades found in the most parsimonious trees, the bootstrap analysis (Felsenstein, 1985) was performed with 500 replicates using the same heuristic search settings except that a maximum of 100 trees were saved per round. We also constructed a phylogeny of the combined *trn*C-*trn*D and *trn*T-*trn*F region sequences with the maximum likelihood (ML) method. Modeltest 3.06 (Posada and Crandall, 1998) was used to determine the best model of sequence evolution for the combined two cpDNA regions, and the GTR+G model was suggested as the best using Akaike Information Criterion (AIC). Likelihood analysis was performed in PHYML version 2.4.3 (Guindon and Gascuel, 2003) using the GTR+G (Gamma distribution shape parameter = 0.5784) model. ML parameters were then optimized, with a BIONJ tree as a starting point (Gascuel, 1997). Support values for nodes on the ML tree were estimated with 500 bootstrap replicates (Felsenstein, 1985). Bayesian inference (BI) was conducted using MrBayes version 3.1.2 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). Indels were treated as same as in the MP analysis. We used the same model as in the ML analysis and a random starting tree. Four chains of the Markov Chain Monte Carto were run each for 1,000,000 generations and were sampled every 100 generations. The first 300 samples for each run were discarded as burn-in to ensure that the chains reached stationarity. Phylogenetic inferences were based on those trees sampled after generation 30,000.

Biogeographic analysis of the genus was performed using DIVA 1.1 (Ronquist, 1996, 1997; http://www.ebc.uu.se/systzoo/research/diva/diva.html) and MacClade 4.08 (http://macclade.org), respectively. DIVA assumes that geographic distributions can be the result of both dispersal and vicariance events. Ancestral states were reconstructed through minimizing the number of dispersal events. As a result, this type of analysis puts high emphasis on vicariance events. To simplify analysis we defined three geographic areas (North America, Asia and Europe) to cover distributions of all *Picea* species to reconstruct the continental biogeographic pathways. Clades in which component species had identical distributions were collapsed into a single terminal. MacClade 4.08 was also used to estimate the history of range change of the genus *Picea*. This method minimizes changes in distribution across the phylogeny, and favors dispersal over vicariance scenarios because they require fewer steps. The distribution settings in MacClade were as same as described above in DIVA. We did not include outgroups in the analyses due to the wide distribution of *Pinus* in the Northern Hemisphere and the relict distribution of *Cathaya*.

Since several intraspecific variants (mitotypes) of the *nad*5 intron 1 have been reported from some North American spruces by Jaramillo-Correa et al. (2003), we analyzed distribution patterns of the *nad*5 haplotypes in *Picea* referring to the chloroplast gene tree. To investigate the relationships among the mitotypes we found, a median-joining network (Bandelt et al., 1999) was constructed with the program NETWORK (http://www.fluxus-engineering.com).

3. Results

3.1. Sequence characterization

For the two cpDNA regions we analyzed, most *Picea* species do not have intraspecific variations based on the sampled individuals. Intraspecific variations were only detected from *P. brachytyla*, *P. koraiensis*, *P. likiangensis*, *P. meyeri* and *P. schrenkiana*, and nearly all of them are autapomorphies such as one substitution or indel (Fig. 1). Length of the *trn*C-*trn*D region is relatively conserved in the 33 *Picea* species sampled, ranging from 2324 (*P. sitchensis* and *P. smithiana*) to 2339 bp (*P. glauca*). However, it is much more variable in the three outgroups, being 2266, 2137 and 1955 bp in *C. argyrophylla*, *P. strobus* and *P. thunbergii*, respectively. The two genes *pet*N and *psb*M have no variation in *Picea*, and are 90 and 114 bp long, respectively.

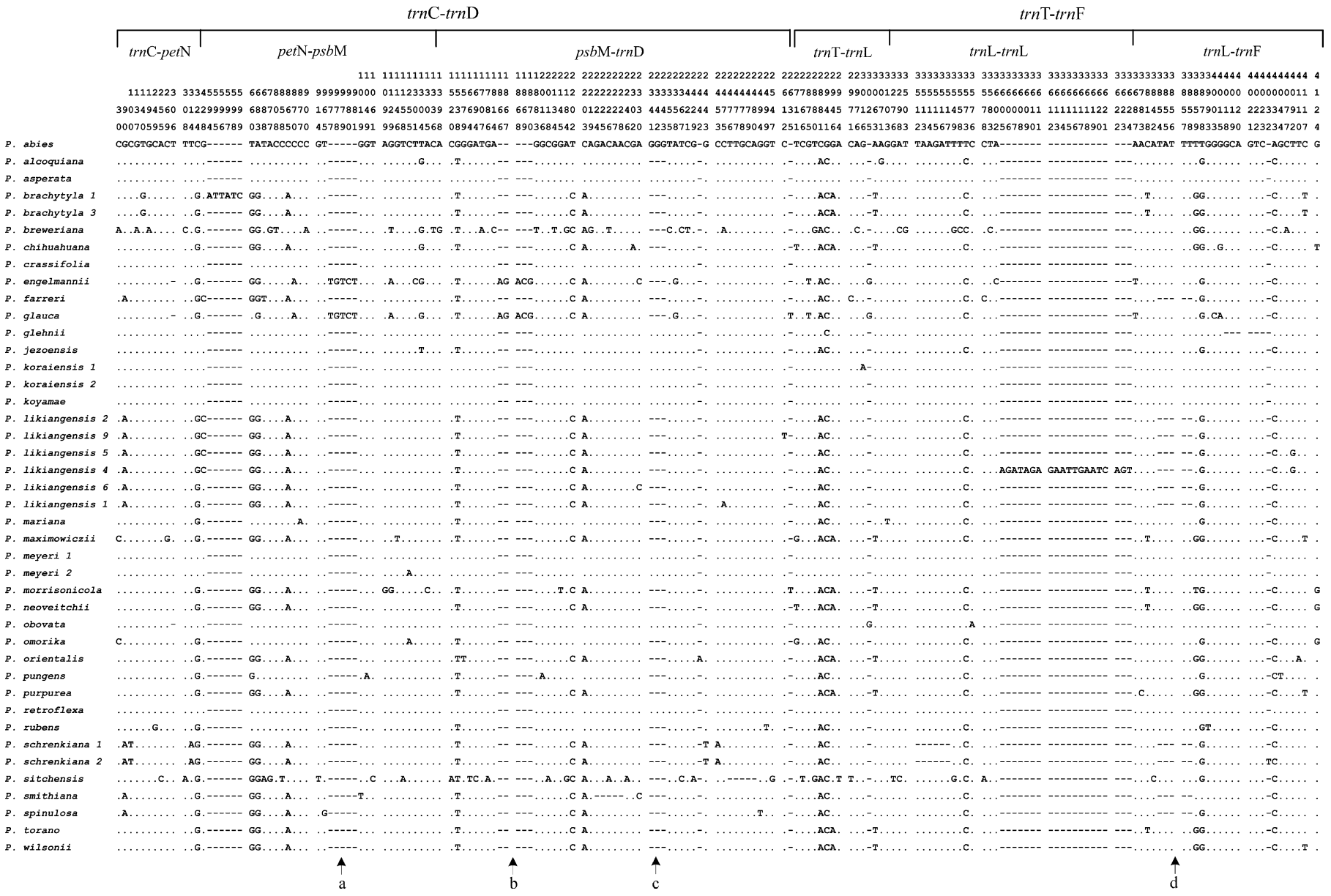

Fig. 1. Variable sites in the aligned *trn*C-*trn*D and *trn*T-*trn*F region sequences of *Picea*. Numbers on the rows denote the positions of nucleotides. Dots show identity to the first taxon sequence. Dashes indicate gaps, and a, b, c and d indicate the four indels at nts 977–981, 1866–1870, 2341–2343 and 3854–3858, respectively.

The alignment of the *trn*C-*trn*D region sequences has 2692 characters, of which 388 are variable and 131 are parsimony informative. Most of the indels were introduced by outgroups, especially in the *psb*M-*trn*D intergenic spacer. Nine gaps of 1–6 bp were found in the ingroups, and four of them are unique, including a 6-bp insertion in two of three individuals of *P. brachytyla*, a 1-bp insertion in *P. orientalis*, and two 5-bp deletions in *P. smithiana* and *P. sitchensis*, respectively. Two 5-bp insertions (a and b) are synapomorphies for *P. engelmannii* and *P. glauca*; one 3-bp insertion (c at nts 2341–2343) is present in *P. abies*, *P. asperata*, *P. crassifolia*, *P. glehnii*, *P. jezoensis*, *P. koraiensis*, *P. koyamae*, *P. meyeri*, *P. obovata* and *P. retroflexa*; one 1-bp deletion (T) is shared by *P. engelmannii*, *P. glauca* and *P. obovata*, and one 1-bp insertion occurs in *P. glauca* and *P. morrisonicola* (Fig. 1).

The *trn*T-*trn*F region of *Picea* species ranges in length from 1366 bp (*P. schrenkiana 1*) to 1392 bp (*P. likiangensis 4*) due to several insertion/deletion (indel) events in the 103 samples analyzed, and that of the outgroups varies from 1339 (*P. strobus*) to 1391 bp (*C. argyrophylla*) in size. The alignment of this cpDNA fragment is 1475 bp long, including 187 variable sites and 74 parsimony informative characters. Six indels occur in the ingroups, and four of them are unique to *P. schrenkiana* (nts 3512–3517), *P. likiangensis 4* (nts 3605–3624), *P. glehnii* (nts 4018–4023) and *P. schrenkiana 2* (nt 4032), respectively. One 1-bp (G at nt 3025) insertion is shared by *P. engelmannii*, *P. glauca* and *P. obovata*. The 5-bp deletion (d) at nts 3854–3858 is a synapomorphy of *P. farreri*, *P. likiangensis*, *P. schrenkiana*, *P. smithiana* and *P. spinulosa* (Fig. 1).

The ILD test indicated that the *trn*C-*trn*D and *trn*T-*trn*F datasets are significantly congruent ($P = 1$). When the two datasets were combined, the data matrix consisted of 4167 nucleotide sites, in which 575 were variable and 205 were parsimony informative. A total of 15 indels were coded, and they contributed additional 9 informative characters. Nucleotide diversity (θ%) of the two cpDNA regions for ingroups is shown in Table 2.

The primers used for the *nad*5 intron 1 allowed us to sequence 1167–1181 bp in *Picea* in this study. The alignment length was 1412 bp, in which nucleotides 595–609, corresponding to the highly variable region reported by Jaramillo-Correa et al. (2003), are variable among the *Picea* species we sampled. Based on the variations in this region, five mitotypes (A–E) were found (Table 3). Nucleotide diversity (θ%) of this intron was 0.151 for ingroups.

Table 3
The five *nad*5 intron 1 mitotypes detected from the genus *Picea*

Mitotypes	5 9 5	6 0 9
A	TG-	-------ACTTGAGT
B	TGACTTGCTTGACTTGAGT	
C	TG-----	CTTGACTTGAGT
D	TG-	-------------AGT
E	TT-	-------ACTTGAGT

All polymorphisms are due to the variations at nucleotides 595–609 in the alignment. Dashes indicate missing nucleotides.

3.2. Phylogenetic and biogeographic analyses and the distribution of mitotypes

Separate phylogenetic analyses of the *trn*C-*trn*D and *trn*T-*trn*F region sequences generated 5 and 180 most parsimonious trees (Table 3) and the two kinds of gene trees are very similar in topology, although many branches were not robustly supported by the bootstrap values (trees not shown). When the two cpDNA regions were combined, the heuristic search found 27 equally most parsimonious trees with a tree length = 683 (CI = 0.9327; RI = 0.9142). One of the trees with branch lengths is shown in Fig. 2. A monophyletic group comprising all sampled *Picea* species is strongly supported (100%) by bootstrap analysis. *P. breweriana*, a phenetically most distinct species, has a basal position, followed by *P. sitchensis*, a western North American species, and the remaining species were split into three clades corresponding generally to the distribution pattern of *Picea* (Fig. 2). Clade I was formed by *P. engelmannii* and *P. glauca* with a bootstrap value of 100% and supported by two 5-bp insertions (a and b in Fig. 1); Clade II included 14 species, i.e., eight species (*P. brachytyla*, *P. farreri*, *P. likiangensis*, *P. neoveitchii*, *P. purpurea*, *P. smithiana*, *P. spinulosa* and *P. wilsonii*) from the Himalayan–Hengduan Mountains and its vicinities, *P. schrenkiana* from the Tianshan Mountains (Middle Asia), *P. morrisonicola* from Taiwan, *P. orientalis* from West Asia, two Japanese species *P. maximowiczii* and *P. torano*, and the North American *P. chihuahuana*, but this clade only had 58% bootstrap support. Within the clade, five species (*P. farreri*, *P. likiangensis*, *P. schrenkiana*, *P. smithiana* and *P. spinulosa*) form a

Table 2
Summary of sequence variations in cpDNA *trn*C-*trn*D and *trn*T-*trn*F regions of the genus *Picea* and phylogenetic tree statistics

Region	Alignment length (bp)	Nucleotide diversity (θ%)	Substitution sites (informative)		Indels (informative)	Most parsimonious trees			
			Ingroups + outgroups	Ingroups		No.	Length	CI	RI
*trn*C-*trn*D	2692	0.335 ± 0.049	388 (131)	71 (24)	9 (6)	5	457	0.9387	0.9128
*trn*T-*trn*F	1475	0.390 ± 0.042	187 (74)	40 (17)	6 (3)	180	225	0.9244	0.9209
Combined	4167	0.356 ± 0.043	575 (205)	111 (41)	15 (9)	27	683	0.9327	0.9142

CI, consistency index; RI, retention index.

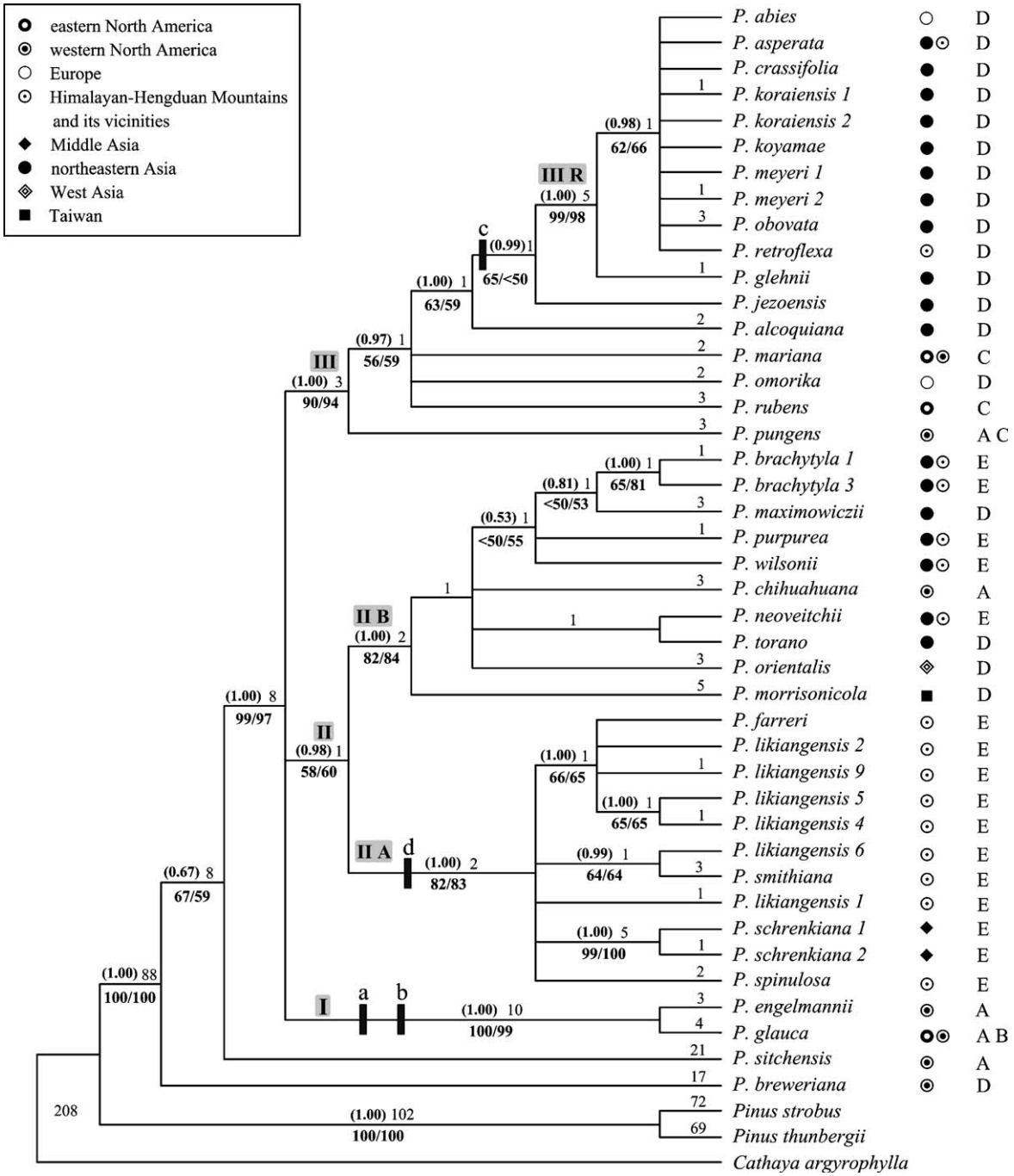

Fig. 2. One of the 27 equally most parsimonious trees constructed from sequence analysis of combined chloroplast *trn*C-*trn*D and *trn*T-*trn*F regions with *Cathaya argyrophylla* and two *Pinus* species as outgroups (length = 683; CI = 0.9327; RI = 0.9142). Numbers above the branches denote branch lengths Numbers below the branches are bootstrap values above 50% for the maximum parsimony analysis (left) and the maximum likelihood analysis (right), respectively. Bayesian posterior probabilities are shown in parenthesis. The symbols following species names show distributions of *Picea* species. Capital letters A, B, C, D and E on the right represent the mitotypes of *nad*5 intron 1 sequences. a, b, c and d indicate the indels shared by species in one clade, respectively.

sub-clade (II A, 82% bootstrap value) sister to the other sub-clade (II B, 82% bootstrap support) comprising the rest nine species, and the sub-clade II A was supported by one 5-bp insertion (d in Fig. 1). In Clade III, all northeastern Asian species sampled (except *P. maximowiczii* and *P. torano*), *P. retroflexa* and the European species *P. abies* clustered into a monophyletic group sister to two North American species *P. mariana* and *P. rubens*, and *P. omorika* from the Balkan Peninsula, with the North American *P. pungens* at the basal position. Nucleotide diversity values (θ%) of the combined two cpDNA regions of the three clades (I, II and III) are 0.161, 0.170 and 0.140, respectively. The topologies of trees generated by PHYML and MrBayes analyses (not shown) were nearly identical to the MP

tree (Fig. 2). Support values for nodes on the ML tree and Bayesian posterior probabilities were also shown in Fig. 2. Possible biogeographic pathways of the genus *Picea* suggested by DIVA and MacClade analyses were same, including an ancestral distribution in North America and five intercontinental dispersal events, i.e., two North America to Asia, one Asia to North America, one North America to Europe and one Asia to Europe (Fig. 3), which will be discussed later.

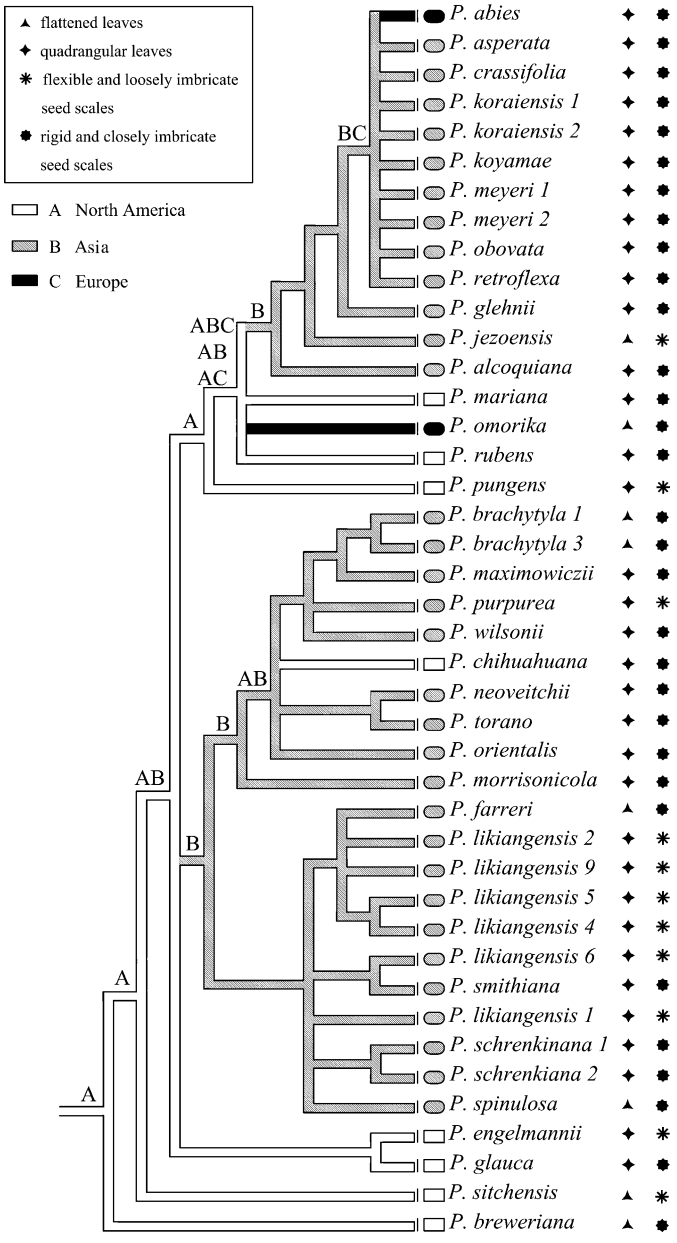

Fig. 3. A cladogram indicating the most parsimonious reconstruction of biogeographic pathways of *Picea* using MacClade. The results of DIVA reconstruction are also indicated above branches. Clades for which component species had identical distributions were collapsed into a single terminal in the DIVA analysis. The phylogeny used is the maximum parsimonious tree of the combined *trn*C-*trn*D and *trn*T-*trn*F regions, excluding outgroups. The symbols on the right indicate some morphological characters widely used in the classification of *Picea*.

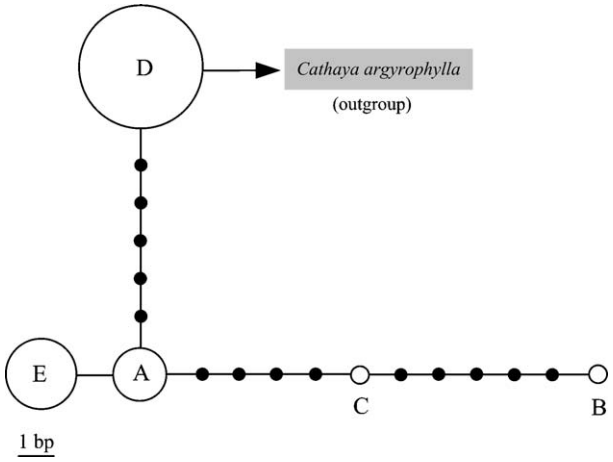

Fig. 4. Median-joining network of the five mitotypes of the *nad*5 intron 1 detected from the genus *Picea*. The network was rooted with *Cathaya argyrophylla*, a group with great divergence from *Picea*. The size of circle is proportional to the frequency of mitotype.

Mitotype A was shared by five North American species, i.e. *P. chihuahuana*, *P. engelmannii*, *P. glauca*, *P. pungens* and *P. sitchensis*; mitotype B occurred only in *P. glauca*; mitotype C was present in three North American species, *P. mariana*, *P. pungens* and *P. rubens*; mitotype D was harbored by all European, Northeastern and Western Asian species as well as *P. retroflexa* and *P. breweriana*; mitotype E was characteristic of *P. schrenkiana* and all species from the Himalayan–Hengduan Mountains and its vicinities except *P. retroflexa* (Fig. 2). The relationships among the five mitotypes are illustrated in a median-joining network (Fig. 4). Mitotype A has a center position, and is closely related to mitotype E (only 1-bp difference between them).

4. Discussion

4.1. Phylogeny and biogeography of the genus Picea: Implications for phylogeographical studies

Monophyly of *Picea* has long been commonly accepted (Wright, 1955; von Rudloff, 1967; Rushforth, 1987; Frankis, 1988; Sigurgeirsson and Szmidt, 1993), but the subdivision of the genus into subgenera, sections and series is greatly debated since most classification systems were formulated on few, easily scored characters from gross morphology (e.g., Colleau, 1968; Sudo, 1968; Bobrow, 1970; Schmidt-Vogt, 1977; Liu, 1982). Willkomm (1887) divided *Picea* into two sections, *Eupicea* and *Omorika*. Liu (1982) divided *Picea* into two subgenera, subgen. *Omorika* comprising sects. *Omorika* and *Morinda* and subgen. *Picea* including sects. *Picea* and *Casicta*. Schmidt (1989) also proposed a classification scheme of *Picea* with two subgenera and four sections, i.e., subgen. *Casicta* comprising sects. *Sitcha* and *Pungens* and subgen. *Picea* including sects. *Picea* and *Omorika*. Farjón (1990) largely followed Schmidt's system, but pointed out that the infrageneric differences are too small to warrant the distinction of subgenera. Therefore, he divided *Picea* into sect. *Picea*

and sect. *Casicta*, each with two subsections. None of the above classification schemes, either into subgenera or into sections and subsections, is supported by the present study. For example, sect. *Omorika* of Liu (1982) and Schmidt (1989) and subsect. *Omorika* of Farjón (1990) all included *P. brachytyla*, *P. breweriana*, *P. omorika* and *P. spinulosa*, but these species were placed in different clades of the cpDNA phylogeny (Fig. 2).

Previous phylogenetic inferences for *Picea* derived independently from morphology and chemical composition are rather discordant (Corrigan et al., 1978; Schmidt, 1989), and are very incongruent with patterns of crossability (Mikkola, 1969) and results of the present cpDNA analysis. Sigurgeirsson and Szmidt (1993) tried to resolve the phylogenetic relationships of *Picea* by the cpDNA-RFLP analysis. The UPGMA phenogram and the cladogram they derived are relatively congruent in topology, but differ greatly in basal clades and systematic positions of some species. In the cladogram, *P. breweriana* and *P. sitchensis* form a monophyletic group nested within Eurasian species (Sigurgeirsson and Szmidt, 1993, Fig. 2A). However, in the phenogram (Sigurgeirsson and Szmidt, 1993, Fig. 1), *P. breweriana* has a basal position, followed by a group comprising *P. engelmannii*, *P. glauca* and *P. mexicana* [*P. engelmannii* subsp. *mexicana* treated by Farjón (1990, 2001)] that are all from North America, then *P. sitchensis* (also a New World species) is sister to a clade comprising the remaining 26 species that were further divided into *P. brachytyla* alliance, *P. abies* alliance and a heterogeneous subgroup. In the present *trn*C-*trn*D and *trn*T-*trn*F phylogeny, *P. breweriana* and *P. sitchensis*, two western North American species, have the basal positions, and the other 31 species form three clades, one comprising two North American species that corresponds to the "*P. glauca* alliance" of Sigurgeirsson and Szmidt (1993), one comprising 13 Asian species (especially western China) and a North American species *P. chihuahuana*, and one including 11 species from northeastern Asia and Europe, two species from North America, and *P. retroflexa* from the Himalayan–Hengduan Mountains (Fig. 2). The recognition of "*P. brachytyla* alliance" and "*P. abies* alliance" by Sigurgeirsson and Szmidt (1993) is supported except the position of *P. alcoquiana*, and the systematic positions of some species placed in the heterogeneous subgroup of Sigurgeirsson and Szmidt (1993), comprising *P. jezoensis*, *P. likiangensis*, *P. mariana*, *P. omorika*, *P. pungens*, *P. rubens*, *P. schrenkiana*, *P. smithiana* and *P. spinulosa*, have been resolved in the present analysis (Fig. 2). *P. mariana*, *P. omorika* and *P. rubens* form a clade with 94% bootstrap support in Sigurgeirsson and Szmidt (1993), and artificial hybridizations between these species are highly successful (Mikkola, 1969; Ledig et al., 2004), but the three species do not show a very close relationship in the present study although they are all placed in clade III. This incongruence may result from the difference between the methods used. The RFLP analysis conducted by Sigurgeirsson and Szmidt (1993) screened more regions of the chloroplast genome than the present study, and thus could detect more synapomorphic characters for some species. At the same time, their RFLP analysis has limitations in detecting changes, such as the risk of non-homology of characters. Moreover, Ledig et al. (2004) acknowledged that relationship based on crossability may not be an accurate measure of total genetic similarity or difference.

Wright (1955) suggested two independent migrations of *Picea* to the America which gave rise to eastern and western *Picea* complexes, respectively. Nienstaedt and Teich (1972) hypothesized that an ancestral Asian *Picea* produced red, black and white spruce and that the other American *Picea* evolved from the white spruce lineage. Based on the cpDNA-RFLP analysis, Sigurgeirsson and Szmidt (1993) suggested that *Picea* originated in North America and that the colonization of Eurasia occurred through separate, intercontinental migrations. The North American origin hypothesis of *Picea* is supported by the present *trn*C-*trn*D and *trn*T-*trn*F phylogeny, in which *P. breweriana* and *P. sitchensis*, two western North American spruces, are basal to the other species (Fig. 2), and by the results of DIVA and MacClade analyses (Fig. 3). Moreover, all five mitotypes (A–E) of the *nad*5 intron 1 except E, a derivative from A (Fig. 4), were found in North American spruces, whereas only D and E were detected from the Eurasian species. This distribution pattern of mitotypes could be further evidence for the North American origin of *Picea*. The mitotypes A and D might represent ancestral types given their occurrence in several distinct lineages, and in particular in the two basal-most species, of *Picea* (Fig. 2), and a central position of A in the median-joining network (Fig. 4). This inference of an American origin of *Picea* is also congruent with paleontological evidence. The earliest fossil (pollen) of *Picea* occurred in Montana (USA) in the Paleocene (Wilson and Webster, 1946), and many spruce cone fossils have been reported from the Eocene sediments of North America (Axelrod, 1998; LePage, 2001). However, spruce fossil records in Asia are mostly from the Oligocene and then after, and those in Europe mostly from the Pliocene (cf. LePage, 2001).

Based on fossil records (Miller, 1989; LePage, 2001) and molecular clock estimation of the *mat*K gene (Wang et al., 2000), the origin of *Picea* could date back to the early Tertiary or late Cretaceous, when three land bridges, DeGeer Route, Thulian Route (McKenna, 1975) and Beringian Route (Wolfe, 1975; Tiffney, 1985a,b), were available to high-latitude floras and faunas for exchange between North America, Asia and Europe. However, the tropical to subtropical climate of Europe during the Paleocene and Eocene and the demise of the DeGeer and the Thulian routes afterwards would have prevented the eastward dispersal of northern temperate taxa. Therefore, it is unlikely that *Picea* has been dispersed through the Atlantic land bridge as suggested by Fowler and Roche (1976), and the dispersal of *Picea* directly from North America to Europe suggested by the DIVA and MacClade analyses is nearly impossible (Fig. 3). In contrast, the Beringian Route, an important exchange way for floras (Wen, 1999; Tiffney and Manchester, 2001), especially temperate and cold-tolerant

taxa (Xiang et al., 2005), existed throughout much of cretaceous and Tertiary time (Wolfe, 1975; Tiffney, 1985a,b), and most probably helped the migration of *Picea* from North America to East Asia during the early Tertiary. Considering that the spruce fossils are progressively younger moving westward from North America and southward from the Bering Straits, Ledig et al. (2004) inferred that *Picea* probably originated in boreal North America, then spread southward in North America and westward to Asia, and from Asia to Europe. This inference is corroborated by not only the present cpDNA phylogeny, but also the mtDNA data. In clade II, all species are from Asia, and have mitotype D or E (the close derivative of A), except that *P. chihuahuana* is endemic to Mexico, and habors mitotype A (Fig. 2). It seems to imply that the ancestral mitotypes A and D both could have been dispersed to Asia from North America, but the former might become extinct or evolve into new types afterwards. Although the position of *P. chihuahuana* in the subclade IIB seems to imply a dispersal from Asia to North America, which is also suggested by the DIVA and MacClade analyses, this species is more likely a progeny of an extinct American ancestor of Clade II considering its distribution in Mexico and mitotype A. In clade III, *P. pungens*, a western North American species has the basal position and mitotypes A and C, and another two North American species *P. mariana* and *P. rubens*, with mitotype C, are sister to the remaining species that are all from Eurasia, in particular northeastern Asia, and only harbor mitotype D. This indicates that the Beringian Route could also have bridged another recent dispersal of *Picea* from the New World to the Old World. Based on the above discussion, we infer that the present distribution pattern of *Picea* could stem from two times of dispersal from North America to Asia by the Beringian Route, and then from Asia to Europe. This inference is congruent with the results of the DIVA and MacClade analyses (Fig. 3).

Richardson et al. (2001) suggested that a high species diversity resulted from recent and rapid diversification (radiation) would be characterized by low genetic differentiation among taxa and a poorly resolved phylogeny. This hypothesis is also supported by many recent investigations such as in the gymnosperm *Ephedra* (Huang and Price, 2003) and the grass genus *Ehrharta* (Verboom et al., 2003). According to the present study, a monophyletic group (III R) in clade III comprises most northeastern Asian spruces, the European *P. abies*, and *P. retroflexa*, a species that has been merged into *P. asperata* distributed in northeastern Asia in Flora of China (Fu et al., 1999). This group, supported by five nucleotide substitutions and the indel c, could stem from a recent radiation through allopatric differentiation of populations in separate refugia, in consideration to the pure mitotype D (Fig. 2) and a very low interspecific genetic divergence ($\theta\% = 0.020$) of it, compared to that ($\theta\% = 0.356$) of the genus *Picea* (Table 2). Although most of the arctic and boreal region was covered by ice sheets in the Quaternary, increasing studies indicate the existence of plant refugia in some areas like Beringia, Northeast Russia and southern Europe (Abbott et al., 2000; Hewitt, 2000; Stewart and Lister, 2001). The relatively wide distribution of some northeastern Asian species such as *P. asperata*, *P. crassifolia*, *P. jezoensis*, *P. koraiensis*, *P. meyeri* and *P. obovata*, and the Norway spruce could be the result of the rapid postglacial re-colonization, whereas the limited present distribution of *P. omorika* in the Balkan Peninsula might be attributed to an unsuccessfully migrating back from the refugia after the Glacial Ages.

It may be argued that the chloroplast capture by hybridization could distort the present phylogenetic reconstruction. However, this should not have happened among the deep branches in that the species with different mitotypes are geographically isolated, and therefore do not have opportunities to contact, such as mitotype A endemic to North America, mitotype D mainly confined to the north of Eurasia, and mitotype E limited in the Himalayan–Hengduan Mountains and its vicinities except *P. schrenkiana*. One might still proposed that the maternal mtDNA could bear more evolutionary imprint, and thus has more phylogenetic utility than the paternal cpDNA. Obviously, this point does not fit for the present study. Intraspecific polymorphisms of the *nad*5 intron 1 region we used have been detected from some North American spruces such as *P. mariana* and *P. rubens* by Jaramillo-Correa et al. (2003), besides more than one mitotypes found in both *P. pungens* and *P. glauca* in our study. Lineage sorting would give rise to a misleading phylogeny, especially the topology of deep branches. Actually, we have tried several mtDNA fragments such as a *nad*1 intron, mh02 and mh27 (Jeandroz et al., 2002). Unfortunately, these markers either have no resolution, or are too polymorphic within species to be used at the genus level.

In recent years, the cytoplasmic haplotype analysis has been widely used in phylogeographical studies, especially in some boreal forest species (e.g., Jaramillo-Correa et al., 2004). However, most of these studies investigated a single species, such as Norway spruce (Gugerli et al., 2001), Scots pine (Sinclair et al., 1999) and ponderosa pine (Johansen and Latta, 2003). According to the present study, the ancestral mtDNA polymorphism can be preserved in many descendant species, even distantly related ones, such as mitotype D shared by the base-most species *P. breweriana* and species from sub-clade II B and clade III. This would increase the difficulty in retrieving the origin and evolutionary relationships of the haplotypes that are very essential to infer the population process, especially when interspecific gene flow and lineage sorting occur (Shaw, 2002; Xiang et al., 2005). We suggest that more species, at least the closely related ones, should be sampled in the phylogeographical study using cytoplasmic haplotypes if possible.

4.2. Evolutionary histories and systematic positions of some *Picea* species

As one of the plant diversity centers in the world (Meyers et al., 2000), the Himalayan–Hengduan Mountains, the southeastern part of the Qinghai-Xizang (Tibet)

Plateau, attracts the attentions of many botanists. Eleven out of 34 *Picea* species recognized in Farjón (2001) occur in this region and its vicinities, but only six of them, *P. aurantiaca*, *P. retroflexa*, *P. farreri*, *P. likiangensis*, *P. smithiana* and *P. spinulosa*, are endemics, and the first two species were merged into *P. asperata*, a species widely distributed in northern China, in Flora of China (Fu et al., 1999). The 11 species do not form a monophyletic group in the chloroplast *trn*C-*trn*D and *trn*T-*trn*F phylogeny, although most of them belong to clade II (Fig. 2), and therefore should not have a single origin. Some species could be dispersed from other areas. The climate of Eurasia was very warm in the early Tertiary, and the coniferous and broadleaf forests were distributed widely in the Arctic regions (Budantsev, 1994), whereas the Qinghai-Xizang Plateau was covered by tropical and subtropical forests (Wu et al., 1995). During that time, an arid zone from northwest to southeast in China prevented the exchange of flora between high latitude areas and the Qinghai-Xizang Plateau (cf. Sun, 2002). After the change in climate and environment of the Arctic regions resulted from the uplift of the Qinghai-Xizang Plateau and the disappearance of the arid zone, many arctic species migrated southward in Middle and Late Tertiary because of the colder habitats than before (Wu et al., 1995; Liu et al., 2002). On the other hand, the Qinghai-Xizang Plateau was not completely covered by the ice sheet during the Quaternary Ice Age (Shi et al., 1998; Yu et al., 2000), which would have been very helpful to the preservation of plant species diversity. Therefore, we can deduce that some spruces migrated from high latitude areas into the Himalayan–Hengduan Mountains when the world turned cold since the Miocene. This inference is also supported by the fact that the earliest *Picea* fossils in the Qinghai-Xizang Plateau appeared in the Miocene sediments (cf. Lü et al., 2004).

Many studies have been conducted on North American spruces (Sigurgeirsson and Szmidt, 1993; Weng and Jackson, 2000; Ledig et al., 2004; Jaramillo-Correa and Bousquet, 2005). The previous results showed that *P. engelmannii* is closely related to *P. glauca* and *P. mariana* is sister to *P. rubens*, whereas *P. breweriana* is phylogenetically isolated (Gorden, 1976; Schmidt-Vogt, 1977; Sigurgeirsson and Szmidt, 1993; Weng and Jackson, 2000; Ledig et al., 2004). These results are also supported by the present study. In addition, *P. sitchensis* is also an evolutionarily isolated species given its basal position in the cpDNA phylogeny (Fig. 2). *P. chihuahuana*, a Mexican endemic species that is morphologically distinct and reproductively isolated from other North-American species (Taylor and Patterson, 1980), was grouped with *P. glauca* in both Bobrow (1970) and Schmidt (1989), but was placed in a section with *P. engelmannii* by Aldén (1987). Our result is consistent with the finding of the cpDNA RFLP analysis by Sigurgeirsson and Szmidt (1993) that *P. chihuahuana* is closer to "*P. brachytyla* alliance" in Asia than to any other North American species.

There are six native *Picea* species in Japan, including *P. jezoensis* in subsect. *Sitchensis* and five other ones in subsect. *Picea*, i.e., *P. alcoquiana* (*P. bicolor*), *P. glehnii*, *P. koyamae*, *P. maximowiczii* and *P. torano* (*P. polita*) (Farjón, 1990, 2001). Except *P. glehnii* and *P. jezoensis*, the other four species are endemics. In the cpDNA-RFLP analysis of Sigurgeirsson and Szmidt (1993), all of the six species were sampled, and they, even the four species endemic to Japan, were clustered into several distantly related clades rather than a monophyletic group. Similar results were also found in the phylogenetic analysis of the sequences from the three non-coding regions between the *trn*T and *trn*F by Kobayashi et al. (2000). All Japanese spruces are nested within clade II or clade III in the present study (Fig. 2). Therefore, all of the DNA analyses suggest that the Japanese *Picea* should not have a single origin and that the species diversification could date back to at least the early Miocene, since the Japanese Archipelago was still a part of the Asian continent 17 Mya and began to separate thereafter (cf. Wang et al., 2003). Indeed, rich spruce fossils have been reported from the Miocene sediments of Japan (LePage, 2001).

Picea schrenkiana, a species distributed in the Middle Asia (the Tianshan Mountains, Xinjiang, China; Kazakhstan; Kirgizstan), forms a clade together with four species endemic to the Himalayan–Hengduan Mountains, i.e., *P. farreri*, *P. likiangensis*, *P. smithiana* and *P. spinulosa* (Fig. 2), which is supported by a base substitution at nt 90, a 5-bp deletion at nts 3854–3858 (Fig. 1), and the same mitotype. This species may be widely distributed in west China in the past, and its present limited distribution in the Middle Asia may result from the rapid uplift of the Qinghai-Xizang Plateau, which produced some very arid habitats in northwest China unsuitable for *Picea*.

4.3. Morphological evolution in Picea

The early classification schemes for *Picea* are exclusively or primarily based on the morphology of leaf [see the review by Liu (1982)]. Liu (1982) subdivided *Picea* into subgenera and sections according to the shape and texture of cone-scales, the shape and structure of leaves, the color and presence or absence of pubescences of shoots within characteristic buds, but he still paid more attention to the vegetative characters. Because reproductive organs are much less influenced by environmental factors than leaves, Schmidt (1989) relied primarily on cone morphology, with leave morphology as a secondary criterion. Weng and Jackson (2000) studied the needle morphology and anatomy of eight North American spruces, and concluded that the needle anatomy is under utilized in systematic studies of conifers. However, the study of LePage (2001) indicates that bract morphology is distinctive for each spruce and useful for species circumscription. Furthermore, Ledig et al. (2004) found that the DNA phylogeny of North American spruces contradicted intrageneric relationships constructed largely on cone morphology. The phylogenetic utility of

morphological characters such as cross-sectional shape of the leaves, arrangement of the stomata on the leaves, cone-scale morphology should be further evaluated for the difficult genus *Picea*.

In Fig. 3, *Picea* species do not form a clade corresponding to any of the four character states, i.e., flattened leaves, quadrangular leaves, loosely imbricate seed scales and closely imbricate seed scales. Therefore, these morphological characters should be used very carefully in the phylogenetic reconstruction and infrageneric classification of *Picea* since they would have evolved more than once given the topology obtained. It is interesting that the two basal-most species of *Picea*, i.e., *P. breweriana* and *P. sitchensis*, have flattened leaves. This kind of leaf, adapted to the relatively wet habitat, may represent a primitive state in *Picea*, considering its existence in nine of the eleven genera of Pinaceae, espeically in *Cathaya*, a close relative of *Picea* (Wang et al., 2000). Flattened leaves also occur in *P. brachytyla*, *P. farreri*, *P. jezoensis*, *P. omorika* and *P. spinulosa* that are distributed in the several main clades we found, respectively, and thus could have different origins in these descendant lineages or represent a retention of plesiomorphic condition. Although the molecular phylogeny of North American *Picea* constructed by Ledig et al. (2004) is in close agreement with the relationships based on needle anatomy (Weng and Jackson, 2000), the evolution of anatomical characteristics needs to be investigated in the whole genus in the future.

Acknowledgments

The authors thank the two anonymous reviewers for their insightful comments and suggestions on the manuscript. We also thank Drs. Mark Chase and Aljos Farjón of Royal Botanic Garden, Kew, UK for providing DNA samples of 5 *Picea* species; Dr. Zsolt Debreczy of the International Dendrological Research Institute, Wellesley, MA (USA) for his kind help in collecting leaf materials of some North American spruces; Dr. F. Thomas Ledig of USDA Forest Service, and Department of Plant Science, University of California, USA for providing some seeds of *Picea breweriana*; Drs. Zhi-Xiong Chen, Jian-Quan Liu, Dun-Yan Tan, Ming-Xi Jiang, Fu-Sheng Yang, Xue Yang, Ying-Jun Qiu and Hong-Mei Liu, Miss Yan Li, and Mr. Yu-Fei Li and Jian-Feng Mao for their help in the sample collections; Ms. Wan-Qing Jin, Cha-Cha Huang and Yin-Xue Sun for their assistance in the DNA sequencing. This study was supported by the National Natural Science Foundation of China (Grant Nos. 30425028, 30121003) and the Chinese Academy of Sciences (the special fund to Xiao-Quan Wang).

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