Molecular phylogeny and biogeography of *Pseudotsuga* (Pinaceae): Insights into the floristic relationship between Taiwan and its adjacent areas

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1. Introduction

The dramatic climatic cooling and major geological events since the late Tertiary have played important roles in driving species diversity and shaping the biogeographic distribution of extant organisms. Benefiting from the development of DNA technology and molecular analysis methods, studies of molecular phylogeny and biogeography have been particularly active in recent decades. Based on molecular dating, more and more phylogenetic studies have revealed striking chronological and geographical correspondence between evolutionary divergence and geological events (Mercer and Roth, 2003; Lemmon et al., 2007; Gamble et al., 2008). The disjunct distribution of morphologically similar plants between eastern Asia and eastern North America is one of the most remarkable biogeographic patterns in the northern hemisphere and has been extensively investigated (Wen and Zimmer, 1996; Xiang et al., 1998, 2000; Wen, 1999; Qian and Ricklefs, 2000; Milne, 2006; Nie et al., 2006; Havill et al., 2008). Over the last two decades, remarkable progress has been made in understanding the origin and development of this eastern Asian and eastern North American disjunction. A general pattern has been recognized that these disjunct distributions are relicts of the maximum development of temperate forests in the northern hemisphere during the Tertiary. Both the North Atlantic and the Bering land bridges were involved in the multiple origins of this pattern (Tiffney, 1985a,b; Wen, 1999). The traditional view of the floristic similarity between eastern Asia and eastern North America was also challenged. A closer biogeographic relationship between eastern North America and western North America than between eastern North America and eastern Asia was revealed (Wen et al., 1996, 1998; Xiang et al., 1998; Wen, 1999). By comparison, only a few studies were carried out on the plants with disjunct distributions in eastern Asia and western North America (Lee and Wen, 2002; Wei and Wang, 2003; Sun et al., 2004; Nie et al., 2005). Additional phylogenetic studies are needed of plants with wide distributions in the northern hemisphere or with disjunct distributions in eastern Asia and western North America to further test the origins of this biogeographic pattern. Furthermore, in eastern Asia, the floristic relationship among Japan, Taiwan, and mainland China is still an open question.

Taiwan is a continental island located off the southeast coast of mainland China and at the southern end of the Ryukyu Islands, which lie just south of the Japanese Archipelago. It is characterized...
by high floristic diversity and harbors many endemic plants (Lu et al., 2001; Cheng et al., 2005). Because of its botanical richness and biogeographic significance, the origin of the Taiwan flora has interested botanists for many years. In recent years, a number of molecular studies have been conducted to explore the evolutionary history of plants in Taiwan (Huang et al., 2001; Chung et al., 2004; Wang et al., 2004; Ge et al., 2005; Chiang et al., 2006; Huang and Lin, 2006). Wang et al. (2003) investigated the historical biogeography and phylogenetic relationships of the genus *Chamaecyparis* (Cupressaceae) based on cpDNA sequences and suggested a recent migration of *Chamaecyparis* to Taiwan from the Japanese Archipelago through Ryukyu islets that acted as stepping-stones for long distance dispersal. A similar distribution pattern was also found for species such as *Melichia compressa* and *Trochodendron aralioides* (Wang et al., 2004; Huang and Lin, 2006). In contrast, Lu et al. (2001) found a pattern of migration from mainland China eastwards to Taiwan for the species *Cunninghamia konishii*. Clearly, it is still a big challenge to make generalizations about the origins of the flora on this fascinating island. Further studies of more plant groups are obviously needed.

*Pseudotsuga* (Pinaceae) is an economically and ecologically important forest component in the northern hemisphere. It is distributed in western North America, Japan, Taiwan and mainland China and demonstrates a typical eastern Asia and western North America disjunct distribution pattern. *Pseudotsuga* was treated as eight species (*P. brevifolia*, *P. forrestii*, *P. gaussenii*, *P. japonica*, *P. sinensis*, *P. wilsoniana* and *P. macrocarpa*, *P. menziesii*) by Hermann (1982), but only four or five of them have been recognized in recent classifications (Farjon, 1990, 2001; Fu et al., 1999). Nevertheless, in all classifications, the existence of two North American (*P. macrocarpa* and *P. menziesii*) and one Japanese species (*P. japonica*) is well established. The circumscription of species in China is controversial, with a single species (*P. sinensis*) being recognized by Farjon (1990, 2001), while three species (*P. brevifolia*, *P. forrestii* and *P. sinensis*) are recognized by Fu et al. (1999). The species *P. wilsoniana* from Taiwan and *P. gaussenii* from eastern China recognized by Hermann (1982) were treated as synonyms of *P. sinensis* by Farjon (1990, 2001) and Fu et al. (1999).

Strauss et al. (1990) contributed the first molecular evidence (RFLP data of chloroplast, nuclear and mitochondrial DNA) to the evolutionary history of *Pseudotsuga* and hypothesized a stepping stone model, i.e., a North American lineage migrated across the Bering land bridge and gave rise to *P. japonica*, which then gave rise to *P. sinensis* and *P. wilsoniana*. But the RFLP data did not strongly support this hypothesis. Gernandt and Liston (1999) constructed a phylogeny of *Pseudotsuga* based on sequence analysis of the nrDNA ITS region, and found that the genus was divided into a North American clade and an Asian clade. However, interspecific relationships within the Asian clade were not well resolved. In addition, the two species *P. brevifolia* and *P. forrestii*, which have a distinct needle morphology and are endemic to south and southwest China, respectively, were not sampled in either study. It is obvious that further molecular studies are needed to clarify the evolutionary history of *Pseudotsuga*.

Phylogenetic reconstruction using multiple genes has been very successful for investigating the evolutionary relationships and historical biogeography of plants (e.g., Qiu et al., 1999; Solits et al., 1999; Xiang et al., 2005; Ran et al., 2006). Although sometimes the multiple-gene analysis may risk obtaining distinct phylogenies due to the different inheritance pathways and evolutionary mechanisms of the genes analyzed, the congruence of different gene trees most likely reflects the species tree. This is particularly true in the case of Pinaceae, since its chloroplast, mitochondrial and nuclear genomes are paternally, maternally and biparentally inherited, respectively (Hipkins et al., 1994; Mogensen, 1996). Moreover, low-copy nuclear genes take advantage of rapid evolutionary rates and biparental inheritance, which are particularly helpful in resolving close interspecific relationships and inferring the hybrid origins of some plants. For these reasons, they have been increasingly used in studies of plant phylogenetics and reticulate evolution (Sang et al., 1997; Wang et al., 2000; Kusumi et al., 2002; Sang, 2002; Small et al., 2004; Pend and Wang, 2008; Steele et al., 2008). Recently LEAFY, one of the key regulatory genes involved in the formation of flower meristem (Frohlich and Parker, 2000), has been used as a single-copy gene and efficiently reconstructed the phylogeny of some seed plants, such as *Amorophallus* (Groβ et al., 2004), Nellielaee (Oh and potter, 2005), *Gnetum* (Won and Renner, 2005), some conifers (*Pinus*, *Picea*, *Podocarpus* and *Taxus*) (Dornelas and Rodriguez, 2005; Vazquez-Lobo et al., 2007) and *Nothola* (Tu et al., 2008). Utilizing the LEAFY gene, as well as several cpDNA regions, nrDNA ITS and another low-copy nuclear gene, 4CL, Pend and Wang (2008) reconstructed the phylogeny of *Thuja* and explored its reticulate evolutionary history and biogeography.

In the present study, we used five chloroplast DNA regions (arpB–rbCL, trnL–trnF, trnC–trnD, petG–psaJ and trnM–trnS), two mitochondrial DNA fragments (cox1 and nad5 a/b intron) and the nuclear LEAFY gene to investigate phylogenetic relationships in the genus *Pseudotsuga* and to infer the biogeographical history of this eastern Asian and western North American disjunct genus. The floristic relationships among mainland China and its adjacent regions in eastern Asia, as well as the origin of flora of Taiwan, were also discussed.

2. Materials and methods

2.1. Plant materials

All species of *Pseudotsuga* were sampled based on the classification scheme of Hermann (1982), i.e., two species in North America (*P. macrocarpa*, *P. menziesii*), one species each in Japan (*P. japonica*) and Taiwan (*P. wilsoniana*), and four species in mainland China (*P. brevifolia*, *P. forrestii*, *P. gaussenii*, *P. sinensis*). More than one individual of each species was sequenced for cytoplasmic DNA markers. For the LEAFY gene, two individuals of each species were used for direct sequencing of PCR products, and one of these was further utilized in the cloning analysis. Two individuals (0203 and 0204) of *P. wilsoniana* were cloned due to the unexpected phylogenetic position of this species observed in the preliminary analysis. To investigate whether the cytoplasmic markers had intraspecific variation, six to ten individuals of *P. sinensis* were analyzed for each marker. *Larix griffithii* and *L. laricina* were chosen as outgroups because of the sister relationship between *Larix* and *Pseudotsuga* (Wang et al., 2000). The origins of the materials used are shown in Table 1. Voucher specimens were deposited in the herbarium of the Institute of Botany, Chinese Academy of Sciences (PE).

2.2. DNA extraction, PCR amplification and sequencing

Total DNA was extracted from silica gel dried needles using the CTAB method following the protocol of Rogers and Bendich (1988) and used as a template in the polymerase chain reaction. All the cytoplasmic DNA fragments (cox1, nad5 a/b intron, trnM–trnS, trnL–trnF, trnC–trnD and petG–psaJ) were amplified with the primers used in previous studies except atpB–rbCL (Taberlet et al., 1991; Wang et al., 2000; Duminn et al., 2002; Shaw et al., 2005; Huang and Lin, 2006; Ran et al., 2006). The primers for the amplification of the atpB–rbCL region were atpB-F (5′-TGAAGCCTTAGCAATRTTGTG) and rbCL-R (5′-ACATCCTAAACTCCCTACC). The LEAFY gene was amplified with the primers LPEY1F1 and LPEY1R1.

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and Wang (2008) and LFYE2R2 (5'-CCTTTGCAATATGTTGCACATC). The PCR reaction was carried out in a volume of 25 μL containing 5–50 ng of DNA template, 6.25 μmol of each primer, 0.2 mM of each dNTP, 1.5–2.0 mM MgCl2 and 0.75 U of Taq DNA polymerase. Amplification was conducted in a Tpersonal Thermocycle or T1 Thermocycle (Biometra, Goettingen, Germany). PCR cycles were as follows: 4 min at 70 °C, 4 cycles of 40 s at 94 °C, 20 s at 50–55 °C, and 1–2 min 30 s at 72 °C, followed by 36 cycles of 20 s at 94 °C, 20 s at 50–56 °C, and 1–2 min 30 s at 72 °C, with a final extension step of 10 min at 72 °C. PCR products were separated by 1.5% agarose gel electrophoresis and purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, UK). The purified PCR products were directly sequenced with the DYEnamic ET Terminator Kit (Amersham Pharmacia Biotech), using the PCR primers and several internal primers, including atpB–rbcL and rbcL–rbcL for the atpB–rbcL region (Chiang et al., 1998) and petN2G, psbM2GF and psbM2GR for the trnC–trnD region (Ran et al., 2006). Direct sequencing of PCR products of the LEAFY gene revealed that P. menziesii and P. wilsoniana have polymorphic nucleotide sites. Therefore, we cloned the LEAFY gene from all Pseudotsuga species with pGEM-T Easy Vector System II (Promega). Ten clones with the correct insertion (determined by digestion with EcoRI) were picked for sequencing with one PCR primer, and then all distinct clones were sequenced in both directions with the primers T7 and SP6 and internal primers LFYE1F (5’-TGTGATGGAaabccggagatgg) and LFYE2R (5’-AATTATAGCTATTCCTTGGAGG). After precipitation in 95% ethanol and 3 M NaCl (pH 5.2), the sequences were separated on either a MegaBACE 1000 (Amersham Biosciences, Buckinghamshire, UK) or an ABI PRISM 3730XL DNA analyzer. The sequences obtained in this study were deposited in GenBank under Accession Numbers (GU457439–GU457521).

### 2.3. Data analysis

Sequence alignments were made with CLUSTAL X (Thompson et al., 1997) and refined manually. MEGA version 4 (Tamura et al., 2007) was used for the molecular evolution analyses of the LEAFY gene, by calculating the distances of synonymous (ds) and nonsynonymous (dn) nucleotide substitutions (d). The dS and dN were estimated according to the Jukes–Cantor model in the Nei–Gojobori method (Nei and Gojobori, 1986), while the d value was calculated based on the Kimura two-parameter model (Kimura, 1980). Gaps/missing data were treated with pairwise deletion.

We first analyzed the five cpDNA data matrices separately, but then due to the low resolution of a single marker, we combined the cpDNA data for use in the final phylogenetic analysis. The incongruence length difference test (ILD) (Farris et al., 1994) was used to assess congruence between different cpDNA regions. For the LEAFY gene, all sequences of distinct clones were included in the phylogenetic analysis. Maximum likelihood (ML) and maximum parsimony (MP) analyses, as well as Bayesian inference (BI), were performed using PAUP version 4.0b10 (Swofford, 2002) and MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), respectively, with Larix laricina and L. griffithii as outgroups. The evolutionary models for the ML and BI phylogenetic analyses were determined by ModelTest 3.07 (Posada and Crandall, 1998) and MrModeltest 2.2 (Nylander, 2004), respectively. For the MP analysis, branch-and-bound searches were conducted with the MULTREES option. Gaps in the cpDNA data matrix were treated as single events, while all
character states in the LEAFY gene matrix were specified as unordered and equally weighted, with gaps treated as missing data. To evaluate the relative robustness of the clades found in the most parsimonious trees, bootstrap analysis (Felsenstein, 1985) was performed with 1000 replicates using the same search settings. The best-fit model obtained from ModelTest 3.07 was applied to each dataset (cpDNA and LEAFY gene) for the ML phylogenetic analysis. Optimal gene trees were found via heuristic searches with 1000 replicates of random sequence addition, and clade robustness was estimated by 1000 bootstrap replicates. For the Bayesian inference, priors for a number of the parameters in the DNA substitution models were applied to each partition. One cold and three incrementally heated Markov chain Monte Carlo (MCMC) chains were run for 1,000,000 cycles and repeated twice to avoid spurious results. One tree per 100 generations was saved. The first 300 samples for each run were discarded as burn-in to ensure that the chains had become stationary. The 50% majority rule consensus tree was obtained based on the trees sampled after generation 30,000. All variable nucleotide sites found in the two mitochondrial gene fragments were shown directly instead of by phylogenetic analysis due to the very low variation.

The r8s program (Sanderson, 2002) and Beast v1.5.3 (Drummond and Rambaut, 2007) were used for molecular dating based on the chloroplast and nuclear phylogenies. Considering that taxon number and collapsed branches can profoundly affect the nodes being calculated, a simplified LEAFY gene tree was used to estimate divergence times. In this tree, one clone was randomly selected from each species except for P. wilsoniana and P. menziesii, for which conspecific clones did not cluster together, and therefore more than one clone was selected. Rate constancy across lineages was examined for both datasets (cpDNA and simplified LEAFY gene analyses). Maximum parsimony analysis of the combined cpDNA data generated three most parsimonious trees, bootstrap analysis (Felsenstein, 1985) was performed with 1000 replicates using the same search settings. Confidence intervals of the divergence times were calculated by a nonparametric bootstrap procedure. Divergence time analyses were conducted on trees of fixed topology but variable branch lengths, which were obtained from 100 bootstrapped data sets generated by the SEQBOOT program in PHYLIP Version 3.67 package (Felsenstein, 2004). For Bayesian relaxed clock, GTR and HKY models were selected for cpDNA and the LEAFY gene, respectively. Priors for the MRCA of P. menziesii were fixed at 32 million years before present (mya). This was done by examining partial sequences of exon 1 (458 bp) and exon 2 (365 bp) and complete sequences of the first intron (1239–1268 bp). Two distinct clones were obtained from each species (individuals) except P. gaussei and P. sinensis. All conspecific clones from P. gaussei and P. sinensis had identical sequences and only three nucleotide sites in the intron region were different between the two species. As shown by the values of 0.002, 0.000, and d, as well as by the number of variable nucleotide sites in the Table 2, P. menziesii had the highest level of sequence divergence followed by P. brevifolia and P. wilsoniana, while P. gaussei, P. sinensis and P. macrocarpa had the lowest levels. In addition, a nucleotide substitution resulted in the replacement of a stop codon in one clone from P. japonica.

3.2. Phylogenetic analysis and molecular dating

The ILD test showed no incongruence (p = 1) between the five cpDNA fragments, so we combined all of them into a single dataset for phylogenetic analysis. The best-fitting models for the combined cpDNA data and the LEAFY gene were the K81uf+I and TIM models from the AIC test and the K81uf+G and HKY models (Hasegawa et al., 1985) from the hierarchical likelihood ratio test (LRT). The best-fitting BI models for the two datasets were the GTR+I and GTR models from the AIC test and the GTR+G and HKY models from the hierarchical LRT test. All of the phylogenetic methods (MP, ML and BI) with different models generated almost identical phylogenetic trees in both the cpDNA and LEAFY gene analyses. Maximum parsimony analysis of the combined cpDNA data generated three most parsimonious trees (tree length = 293 steps, consistency index = 0.956, retention index = 0.958). Also, the strict consensus tree was topologically identical to the ML and BI trees. For the LEAFY gene, a single most parsimonious tree was obtained (tree length = 208 steps, consistency index = 0.986, retention index = 0.986), and the tree was also topologically identical to the ML and BI trees. The MP trees of the combined cpDNA data and the LEAFY gene are shown with bootstrap support in Fig. 1a and b, respectively.

Both the cpDNA and LEAFY gene trees revealed two well-supported clades in Pseudotsuga: one was composed of the two North American species and T in all the eastern Asian species. The sizes of the two mitochondrial gene fragments, cox1 and the nad5 a/b intron, are 1171 bp and 1093–1098 bp, respectively. Only a single variable nucleotide site was found in cox1, with G in the two North American species and T in all the eastern Asian species. This nucleotide substitution also caused an amino acid replacement. Two variable nucleotide sites were observed in the nad5 a/b intron, but neither is phylogenetically informative. No intraspecific variation was detected in any of the cytoplasmic DNA markers.

The LEAFY gene ranged from 2062 to 2091 bp in length, including partial sequences of exon 1 (458 bp) and exon 2 (365 bp) and complete sequences of the first intron (1239–1268 bp). Two distinct clones were obtained from each species (individuals) except P. gaussei and P. sinensis. All conspecific clones from P. gaussei and P. sinensis had identical sequences and only three nucleotide sites in the intron region were different between the two species. As shown by the values of 0.002, 0.000, and d, as well as by the number of variable nucleotide sites in the Table 2, P. menziesii had the highest level of sequence divergence followed by P. brevifolia and P. wilsoniana, while P. gaussei, P. sinensis and P. macrocarpa had the lowest levels. In addition, a nucleotide substitution resulted in the replacement of a stop codon in one clone from P. japonica.

Table 2

<table>
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<th>Taxa (numbers of clones)</th>
<th>Exon variable sites</th>
<th>Intron variable sites</th>
</tr>
</thead>
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<td></td>
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<td>d5</td>
</tr>
<tr>
<td>P. brevifolia (2)</td>
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</tr>
<tr>
<td>P. forrestii (2)</td>
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</tr>
<tr>
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</tr>
<tr>
<td>P. sinensis (1)</td>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>P. japonica (2)</td>
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</tr>
<tr>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>P. menziesii (2)</td>
<td>7</td>
<td>0.009</td>
</tr>
</tbody>
</table>

3. Results

3.1. Sequence characterization

The lengths of the five cpDNA regions (atpB–rbcL, petG–psaJ, trnC–trnD, trnT–trnF and trnS–trnM) in Pseudotsuga are 1690 or 1694 bp, 846 bp, 2111–2153 bp, 1298–1319 bp and 850–905 bp, respectively. The combined cpDNA dataset is 7157 bp in length.
American species *P. menziesii* and *P. macrocarpa*, and the other was comprised of species from eastern Asia (Fig. 1a and b). However, within the eastern Asian clade, the interspecific relationships were inconsistent between the two phylogenies. Unlike the cpDNA tree, in which *P. japonica* was sister to the rest of the Asian species (*P. brevifolia*, *P. forrestii*, *P. gaussenii* and *P. sinensis* and *P. wilsoniana*), the LEAFY tree split the eastern Asian clade into two subclades and placed *P. brevifolia* and *P. forrestii* in subclade I and *P. japonica*, as well as *P. sinensis* and *P. wilsoniana*, in subclade II. Two divergent types of LEAFY sequences were found in *P. wilsoniana* (hereafter referred to as type I and type II). Type I and type II were clustered into subclade I and subclade II, respectively (Fig. 1b). Within the North American clade, instead of a monophyletic grouping of conspecific sequences, one sequence from *P. menziesii* showed a close relationship to *P. macrocarpa*. The variable nucleotide sites and indels in cox1 and the nad5 a/b intron are shown in Fig. 1a.

Based on the LEAFY gene data, the age of the split between subclade I (*P. forrestii–P. brevifolia–type I of *P. wilsoniana*) and subclade II (*P. japonica–P. gaussenii–P. sinensis–type II of *P. wilsoniana*), estimated using the PL method in the r8s program, was 20.26 ± 5.84 mya. The divergence time between type I and *P. forrestii* was 15.31 ± 4.99 mya and between type II and *P. japonica–P. gaussenii–P. sinensis* was 6.84 ± 2.54 mya. The two North American species *P. menziesii* and *P. macrocarpa* were separated 10.97 ± 4.22 mya (Fig. 2). A similar time span was also obtained using the NPRS method and the Beast program based on cpDNA data and the LEAFY gene, respectively. The results are shown in Table 3.

### 4. Discussion

#### 4.1. Phylogeny of Pseudotsuga: Implications for the floristic relationship among Japan, Taiwan and mainland China

The first molecular phylogeny of *Pseudotsuga* was constructed by Strauss et al. (1990) using restriction fragment length analysis of chloroplast, mitochondrial and nuclear DNA. In their study, two most parsimonious trees were obtained: one tree placed *P. macrocarpa* at the basal position, with *P. menziesii* as sister to the eastern Asian species, while the other suggested two reciprocally monophyletic groups that geographically corresponded to North America and eastern Asia. The sister relationship between North American and eastern Asian species of *Pseudotsuga* found in Strauss et al. (1990) is further supported by the nrDNA ITS phylogeny of Gernandt and Liston (1999) and corroborated by the present cpDNA and LEAFY gene data, as well as the single parsimony-informative nucleotide site detected in the mitochondrial gene cox1 (Fig. 1a). However, the sister relationship between *P. japonica* and the rest of the Asian species reported before is not supported by the present LEAFY phylogeny (Fig. 1b), although it is consistent.
with the cpDNA tree shown in Fig. 1a. It is interesting that two divergent types of LEAFY sequences occur in *P. wilsoniana*. Type I is closely related to *P. brevifolia* and *P. forrestii*, two morphologically distinct species native to south and southwest China, while type II groups with *P. japonica*, *P. sinensis* and *P. gaussenii* (Fig. 1b).

It may be argued that the inconsistent relationships among the eastern Asian species found in different studies could be attributed to the low resolution of the markers used and the absence of *P. brevifolia*, *P. forrestii* and *P. gaussenii* from the studies of Strauss *et al.* (1990) and Gernandt and Liston (1999). However, in the present study, we employed DNA sequences of more than 7 kb from five chloroplast markers and all species were sampled, and we were still unsuccessful in improving the resolution within the eastern Asian lineage. Therefore, an alternative explanation is that gene flow could be responsible for the phylogenetic relationships among the Asian species revealed by the LEAFY tree. Indeed, the Japanese Archipelago was connected to the Asian mainland during the late Pleistocene (Millien-Parra and Jaeger, 1999), and thereby might have served as a corridor for gene flow. This inference is basically consistent with the estimated age (4.64 ± 1.93 Myr) of the common ancestor of *P. japonica*, *P. gaussenii* and *P. sinensis* (Fig. 2). The high floristic similarity between east China and Japan has also been indicated by studies of phytogeography (Liu *et al.*, 1995; Hao *et al.*, 1996). Therefore, it is not unreasonable to hypothesize that *P. japonica* is much more closely related to *P. gaussenii* and *P. sinensis* than to the other species from mainland China.

The Taiwanese species *P. wilsoniana* is geographically isolated, but it is not surprising that the present phylogenies suggest this species has a close relationship with *P. gaussenii* and *P. sinensis*, two species distributed in southeast and central China, respectively, considering their morphological similarities and geographic closeness. Unexpectedly, the LEAFY gene tree also suggests a close relationship between *P. wilsoniana*, *P. brevifolia* and *P. forrestii* (Fig. 1b), particularly *P. brevifolia*, a species characterized by short linear leaves and restricted to the specific habitat of karst topography in Guangxi province, south China. The natural distribution of *P. forrestii* is narrow and only occurs in the Hengduan Mountains, southwest China. Although the
affinity between Taiwan island and southern or southwestern China/Himalayas has been proposed based on floristic comparisons (Wang, 1992; Zeng, 1993; Ying and Hsu, 2002), only a couple of molecular studies have investigated the floristic relationship between these regions (Lu et al., 2001; Ge et al., 2005; Chiang et al., 2006). Our study provides a good example for understanding the floristic relationships between Taiwan and mainland China, and suggests that the flora of Taiwan is not only closely related to the floras of eastern and southeastern China, but also to those of southern and southwestern China.

![Fig. 2. A simplified LEAFY gene tree for molecular clock dating. The node name and age estimated with standard errors (mya) are denoted above and below the branches, respectively.](image)

<table>
<thead>
<tr>
<th>Node</th>
<th>cpDNA</th>
<th>LEAFY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r8S</td>
<td>NPRS method</td>
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<tr>
<td></td>
<td>PL method</td>
<td>Beast</td>
</tr>
<tr>
<td>PwPj</td>
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</tr>
<tr>
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</tr>
<tr>
<td>PmPm2</td>
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<tr>
<td>PjPg</td>
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Table 3
Divergence times of Pseudotsuga estimated by the r8s and Beast programs based on cpDNA and LEAFY gene data. The nodes correspond to Fig. 2.
The oldest fossil record (~32 Myr) of a *Pseudotsuga macrocarpa*-like form found in Oregon (Schorn, 1994) could date the origin of *Pseudotsuga* back to the early Oligocene, which is consistent with the molecular clock estimation of the *matK* gene (Wang et al., 2000). Strauss et al. (1990) postulated a stepping stone model for the migration of *Pseudotsuga* after it originated in North America. That is, the ancestral form migrated north across the Bering land bridge and later reached Japan, and then, a Japanese lineage spread southwards and gave rise to the species in mainland China and Taiwan. The Beringian migration route of the genus is supported by the middle Tertiary fossils of *Pseudotsuga* from Alaska (Hermann, 1985). Actually, during most of the Tertiary, the Bering land bridge was an important high-latitude link between eastern Asia and North America for floras and faunas (Tiffney, 1985a; Novacek, 1985). During most of the Tertiary, the Bering land bridge was an important high-latitude link between eastern Asia and North America for floras and faunas (Tiffney, 1985a; Novacek, 1999). The present cpDNA and *LEAFY* gene phylogenies indicate that the North American and eastern Asian lineages evolved independently in the two continents after their separation. This hypothesis is also supported by the mitochondrial data. Molecular dating suggests that the Asian species last shared a common ancestor 20.26 ± 5.84 mya (Fig. 2) in the early Miocene, a period when a warming trend reduced the extent of the Antarctic ice-sheets that had been established from the earliest Oligocene to the latter part of the Oligocene (Zachos et al., 2001). This warm phase peaked during the late Mi–Miocene Climatic Optimum (MMCO, 15–15 Ma) and was followed by a gradual cooling and reestablishment of a major ice-sheet on Antarctica by 10 mya (Vincent et al., 1985; Flower and Kennett, 1995). These changes in the Antarctic ice-sheets indicate a globally relevant climatic oscillation which made the Bering land bridge available for exchange during a warm period (McKenna, 1983; Tiffney, 1985a,b). Based on the records of climatic changes, we could deduce that *Pseudotsuga* migrated into eastern Asia via the Bering land bridge following the climatic cooling and rapid expansion of the Antarctic ice-sheets in the late Oligocene and then developed in Asia during the late Oligocene to mid-Miocene warming period. This hypothesis is probably also supported by the fact that the extant *Pseudotsuga* species in eastern Asia are basically distributed in warm regions.

The *LEAFY* gene data provide interesting implications for the origin of *P. wilsoniana*. As mentioned before, *LEAFY* generally exists as a single-copy gene in the nuclear genome (Frohlich and Parker, 2000; Grob et al., 2004; Oh and potter, 2005; Won and Renner, 2005; Pend and Wang, 2008; Tu et al., 2008). In the present study, we obtained one or two distinct clones of the *LEAFY* gene from each species of *Pseudotsuga*. Conspecific clones are generally closely related, since they may represent different alleles. However, *P. wilsoniana* harbors two divergent types of *LEAFY* sequences (type I and type II) that are grouped into two sister subclades corresponding to geographic distribution, (Fig. 1b), one with species from south and southwest China and the other with species from East and central-southwest China and Japan. The two divergent types of *LEAFY* sequences may represent homologous genes that were derived from hybridization between *P. brevifolia* or its ancestor and the ancestor of the subclade II species. Molecular clock estimation suggests that type I diverged from *P. forrestii* at about 15.31 ± 4.89 mya while type II might have been inherited from the ancestor of the eastern China species 6.84 ± 2.54 mya (Fig. 1b), a key period in Cenozoic climatic evolution with several major geological events in eastern Asia. At that time, the uplift of the Tibetan plateau radically reshaped the topography of eastern Asia and led to remarkable environmental heterogeneity which significantly accelerated species diversification. Meanwhile, with the development of monsoons in East Asia (An et al., 2001; Spicer et al., 2003), southwest China became an important refugium when the global cooling forced many plants to migrate southward in the late Tertiary. It is well known that the Himalaya–Hengduan Mountains region is a hotspot of biodiversity and one of the plant diversity centers of the world (Myers et al., 2000). Therefore, the following biogeographic scenario for the origin of *P. wilsoniana* could be deduced. The ancestor of extant *P. wilsoniana* differentiated from the ancient populations of *P. forrestii* and *P. brevifolia* about 15.31 ± 4.89 mya. Then, with the further cooling and small-scale ice-sheet expansion on west-Antarctica (Kennett and Barker, 1990) and in the Arctic (Thiede and Vorren, 1994) that forced the assemblies of warm temperate to subtropical biotas southwards to refugial regions, it hybridized with the ancient population of the other lineage of *P. sinensis*–*P. gauensii*–*P. japonica* before it migrated into Taiwan, a continent island separated from mainland China 5–6 mya (Sibuet and Hsu, 1997, 2004). The extremely heterogeneous topography in southwest China could be a good explanation for the higher sequence divergence in *P. brevifolia* and *P. forrestii* than in *P. gauensii* and *P. sinensis*, two species distributed in the comparably homogenous environment of east China (Zhang et al., 2006).

Within the North American clade, two types of *LEAFY* sequences (type A and type B) were found in *P. menziesii* (Douglas-fir). Estimated divergence time showed that type A split from the ancestor 10.97 ± 4.22 mya while type B split 21.88 ± 6.00 mya, a time span similar to that of the eastern Asian species. It has been widely debated whether the Quaternary glacial and interglacial shifts, or the Tertiary or earlier global climatic changes, were responsible for the contemporary biological diversity (Hewitt, 2000; Richardson et al., 2001; Moritz et al., 2002; Rowe et al., 2004; Willis and Niklas, 2004; Lemmon et al., 2007; Gamble et al., 2008; Rull, 2008). The divergence of most *Pseudotsuga* species in the Tertiary, as suggested by molecular dating, may indicate that the glacial/interglacial alternations in the Quaternary had little influence on the species diversity of the genus. Nevertheless, the current geographical distribution of *Pseudotsuga* in North America implies that it has been inescapably affected by the Quaternary glaciations since most of North America was covered by ice-sheets during the Quaternary (Hewitt, 2000). Compared to the narrow distribution of *P. macrocarpa*, which is confined to the Coast Ranges of southern California within the California Floristic Province, an area that harbors more endemic plant and animal taxa than any other of comparable size in North America (Calsbeek et al., 2003), the range of *P. menziesii* extends from British Columbia to central Mexico with a wide span of climatic and topographical conditions and shows strong adaptation to different environments. More detailed phylogeographic studies are expected to shed some new light on how organisms responded to the geologic and climatic changes in this region.

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