Molecular Phylogenetics and Evolution 55 (2010) 776–785

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

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

Xiao-Xin Wei *, Zu-Yu Yang, Yan Li, Xiao-Quan Wang **

*State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China*

ARTICLE INFO

*Article history:*
Received 20 April 2009
Revised 25 February 2010
Accepted 3 March 2010
Available online 7 March 2010

*Keywords:*
Molecular phylogeny
Historical biogeography
Hybrid origin
Taiwan
*Pseudotsuga*
*LEAFY*

ABSTRACT

Climatic oscillations and geological events play major roles in shaping species diversity and the distribution of plants. The mechanisms underlying the high level of plant species diversity in eastern Asia are hotly debated. In this study, five cpDNA regions, two mtDNA fragments and one nuclear gene (*LEAFY*) were employed to investigate species diversification and the historical biogeography of *Pseudotsuga* (Pinaceae), a genus with a typical eastern Asia and western North America disjunct distribution. Both the nuclear *LEAFY* gene and cpDNA phylogenies strongly suggest that eastern Asian and North American species are monophyletic, respectively. Within the eastern Asia clade, the cpDNA tree placed *P. japonica* as sister to the rest of the Asian species, but the *LEAFY* gene tree showed a sister relationship between *P. japonica*–*P. sinensis*–*P. gaussenii* and *P. brevifolia*–*P. forrestii*. Molecular dating indicated that the Asian species last shared a common ancestor 20.26 ± 5.84 mya and the species diversification of *Pseudotsuga* was correlated with the Tertiary climatic and tectonic changes. These results, together with the fossil evidence, suggest that *Pseudotsuga* might have originated from North America and then migrated to eastern Asia by the Bering land bridge during the early Miocene. The Taiwanese species *P. wilsoniana* harbored two divergent types of *LEAFY* sequences, which implies that this species might have originated by hybridization between *P. brevifolia* or its ancestor and the ancestor of *P. japonica*–*P. sinensis*–*P. gaussenii*. Our study also suggests that Taiwan is closely related to both southwest and east China in flora.

© 2010 Elsevier Inc. All rights reserved.

## 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

* Corresponding author. Fax: +86 10 62590843.
** Corresponding author. Fax: +86 10 62590843.
*E-mail addresses:* weixx@ibcas.ac.cn (X.-X. Wei), xiaoq_wang@ibcas.ac.cn (X.-Q. Wang).

1055-7903/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2010.03.007

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 *Mechilia 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; Soltis 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 *Amorphophallus* (Grob et al., 2004), Neillieae (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 *Nolana* (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 (*atp*B–*rbc*L, *trn*T–*trn*F, *trn*C–*trn*D, *pet*G–*psa*J and *trn*fM–*trn*S), two mitochondrial DNA fragments (*cox*1 and *nad*5 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 ctyoplasmic 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 (*cox*1, *nad*5 a/b intron, *trn*fM–*trn*S, *trn*T–*trn*F, *trn*C–*trn*D and *pet*G–*psa*J) were amplified with the primers used in previous studies except *atp*B–*rbc*L (Taberlet et al., 1991; Wang et al., 2000; Duminil et al., 2002; Shaw et al., 2005; Huang and Lin, 2006; Ran et al., 2006). The primers for the amplification of the *atp*B–*rbc*L region were *atp*B-F (5′-TGAGCCTTAG-CAATRTTGTTG) and *rbc*L-R (5′-ACATTCGTAAACTGC TCTACC). The *LEAFY* gene was amplified with the primers *LFYE1*F3 from Pend

**Table 1**
Sources of materials used in this study.

| Taxa | Sources/individual/Vouchers/collectors | GenBank Accession Nos. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *atp*B–*rbc*L | *trn*C–*trn*D | *trn*fM–*trn*S | *pet*G–*psa*J | *trn*T–*trn*F | *cox*1 | *nad*5 | *LEAFY* |
| *P. brevifolia* Cheng et L.K.Fu | ChongzuoDaxin Natural Reserve, Guangxi, China/2/051001/Cun Y.Z | GU457439 | GU457496 | GU457506 | GU457486 | GU457516 | GU457449 | GU457477 | GU457459 GU457460 |
| *P. forrestii* Craib | Deqin, Yunnan, China/3/050903/Cun Y.Z | GU457440 | GU457497 | GU457507 | GU457487 | GU457517 | GU457450 | GU457478 | GU457461 GU457462 |
| *P. gaussenii* Flous | Huangshan, Anhui, China/3/PG007/Kan X.Z | GU457441 | GU457498 | GU457508 | GU457488 | GU457518 | GU457451 | GU457479 | GU457463 |
| *P. sinensis* Dode | Jinfoshan, Chongqing, China/4/PS01/ Wei X.X | GU457442 | GU457499 | GU457509 | GU457489 | AF440504* | GU457452 | GU457480 | GU457464 |
| *P. wilsoniana* Hayata | Forestry and Forest Products Research Institute, Tsukuba, Japan /1/0203/Liu J.Q | GU457443 | GU457500 | GU457510 | GU457490 | GU457519 | GU457453 | GU457481 | GU457465 GU457466 |
| | Taiwan/1/0204/Liu J.Q. | | | | | | | | GU457467 GU457468 |
| *P. japonica* (Shiras.) Beissn | Forestry and Forest Products Research Institute, Tsukuba, Japan/2/0202/Liu J.Q | GU457444 | GU457501 | GU457511 | GU457491 | GU457520 | GU457454 | GU457482 | GU457469 GU457470 |
| *P. macrocarpa* (Torrey) Mayr | California, USA/2/H05015/Chen W.L | GU457445 | GU457502 | GU457512 | GU457492 | GU457521 | GU457455 | GU457483 | GU457471 GU457472 |
| *P. menziesii* (Mirbel) Franco | Beijing Botanic Garden/1/965/Wang X.Q. | GU457446 | GU457503 | GU457513 | GU457493 | AF440503* | GU457456 | AF143416* | GU457473 |
| | California, USA/1/045/Chen W.L. | | | | | | | | GU457474 |
| *Outgroups* | | | | | | | | | |
| *L. griffithii* (J.D.Hooker) Parlatore | Linzhi, Xizang, China/2/LW9801/Wei X.X | GU457447 | GU457504 | GU457514 | GU457494 | AF440498* | GU457457 | GU457484 | GU457475 |
| *L. laricina* (Du Roi) K. Koch | Jordan Botanic Garden, Geneva, Switzerland/1/LW2166/Wang X.Q | GU457448 | GU457505 | GU457515 | GU457495 | AF440501* | GU457458 | GU457485 | GU457476 |

* From previous studies.

and Wang (2008) and *LFYE2*R2 (5′-CCTTTGCAATATGTTGCACATC). The PCR reaction was carried out in a volume of 25 μL containing 5–50 ng of DNA template, 6.25 umol of each primer, 0.2 mM of each dNTP, 1.5–2.0 mM $MgCl_2$ 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 *atp*B-1 and *rbc*L-1 for the *atp*B–*rbc*L region (Chiang et al., 1998) and *pet*N2G, *psb*M2GF and *psb*M2GR for the *trn*C–*trn*D 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 *LFYE1*F (5′-TGTTGATGGAAAACGCAAGATTG) and *LFYE2*R (5′-AATTATAGCATTATCTCCTTGAGG). After precipitation in 95% EtOH and 3 M NaAc (pH 5.2), the sequencing products 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 ($d_S$), nonsynonymous ($d_N$) and nucleotide substitutions ($d$). The $d_S$ and $d_N$ 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*) using a likelihood ratio test (LRT) (Felsenstein, 1988). When the data rejected the assumption of equal rates in sister groups, we used the nonparametric rate smoothing (NPRS) (Sanderson, 1997) and penalized-likelihood (PL) methods (Sanderson, 2002) in the r8s program, and the Bayesian approach in Beast v1.5.3. The age of the most recent common ancestor (MRCA) of *Pseudotsuga* was fixed at 32 million years before present (mya). This was based on the earliest known fossil records for *Pseudotsuga* which are for a *Pseudotsuga macrocarpa*-like form found in the early Oligocene lowland Willamette and Rujada paleofloras (Lakhanpal, 1958) of west central Oregon (Schorn, 1994). For PL methods, optimal values of smoothing were determined by a cross-validation procedure. 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 *Pseudotsuga* were set to 32 mya and root height to 45 mya (the oldest fossil record of *Larix*, Schorn, 1994) with a normal distribution. Markov chains in Beast were run for 10,000,000 generations, sampling every 1000th generation for a total of 10,000 trees, with a burn-in of 3000 trees. The node ages were displayed by FigTree.

## 3. Results

### 3.1. Sequence characterization

The lengths of the five cpDNA regions (*atp*B–*rbc*L, *pet*G–*psa*J, *trn*C–*trn*D, *trn*T–*trn*F and *trn*S–*trn*fM) 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. The sizes of the two mitochondrial gene fragements, *cox*1 and the *nad*5 a/b intron, are 1171 bp and 1093–1098 bp, respectively. Only a single variable nucleotide site was found in *cox*1, 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 *nad*5 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. gaussenii* and *P. sinensis*. All conspecific clones from *P. gaussenii* 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 $d_S$, $d_N$ and $d$, as well as by the number of variable nucleotide sites (Table 2), *P. menziesii* had the highest level of sequence divergence followed by *P. brevifolia* and *P. wilsoniana*, while *P. gaussenii*, *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

**Table 2**
Mean distance of synonymous ($d_S$) and nonsynonymous ($d_N$) substitutions calculated from the *LEAFY* gene exon regions and nucleotide substitutions ($d$) from the exon and intron regions of conspecific clones (except *P. gaussenii* and *P. sinensis*) of this gene.

| Taxa (numbers of clones) | Exon | | | | Intron | |
|---|---|---|---|---|---|---|
| | variable sites | $d$ | $d_S$ | $d_N$ | variable sites | $d$ |
| *P. brevifolia* (2) | 5 | 0.006 | 0.006 | 0.005 | 8 | 0.006 |
| *P. forrestii* (2) | 2 | 0.002 | 0.005 | 0.002 | 3 | 0.002 |
| *P. gaussenii* (1) *P. sinensis* (1) | 0 | 0.000 | 0.000 | 0.000 | 3 | 0.002 |
| *P. japonica* (2) | 2 | 0.002 | 0.000 | 0.002 | 2 | 0.002 |
| *P. wilsoniana* 0203 (2) | 2 | 0.002 | 0.000 | 0.002 | 15 | 0.012 |
| *P. wilsoniana* 0204 (2) | 2 | 0.002 | 0.005 | 0.000 | 12 | 0.010 |
| *P. macrocarpa* (2) | 1 | 0.001 | 0.000 | 0.002 | 2 | 0.002 |
| *P. menziesii* (2) | 7 | 0.009 | 0.022 | 0.005 | 16 | 0.013 |

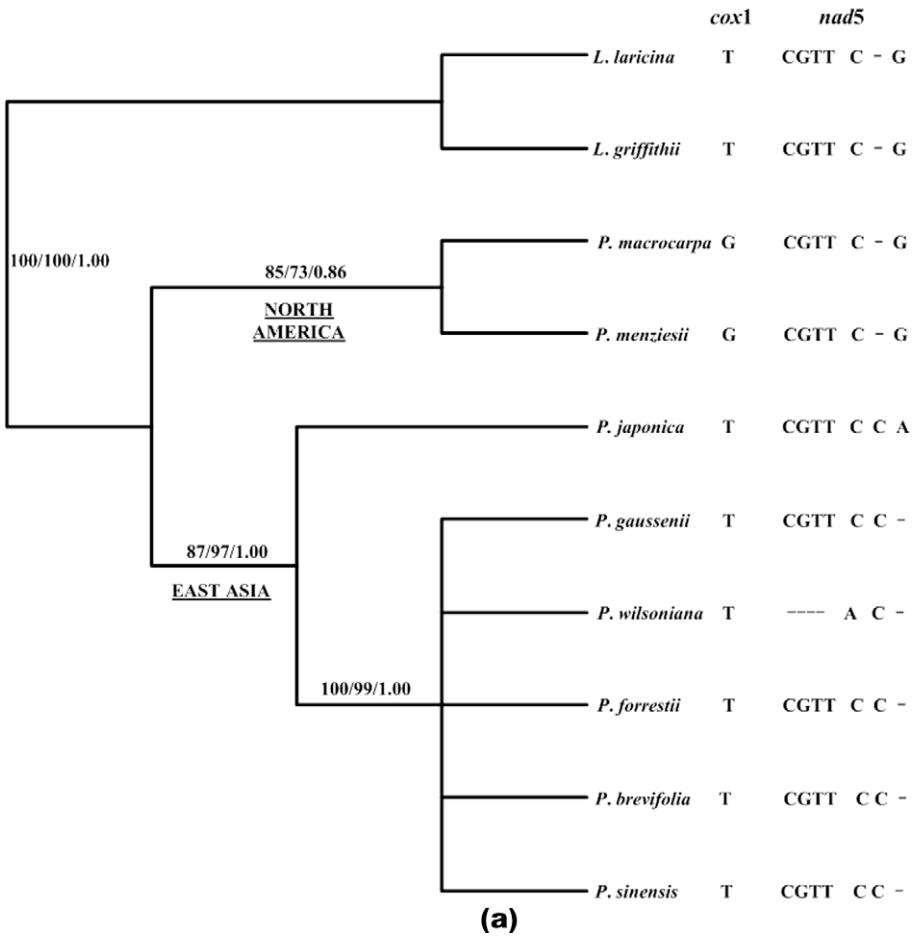


**Fig. 1.** The maximum parsimony trees of *Pseudotsuga* based on sequence analysis of the combined five chloroplast DNA regions (a) and the nuclear *LEAFY* gene (b) with *Larix* as the outgroup. Numbers on the branches indicate the bootstrap values of MP and ML, and the Bayesian posterior probabilities, respectively. Numbers following a species name represent clone numbers (except 0203 and 0204 as individual identifications). Variable sites in the two mitochondrial genes, *cox*1 and *nad*5, are shown to the right of (a).

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 *cox*1 and the *nad*5 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 ± 4.89 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 *cox*1 (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

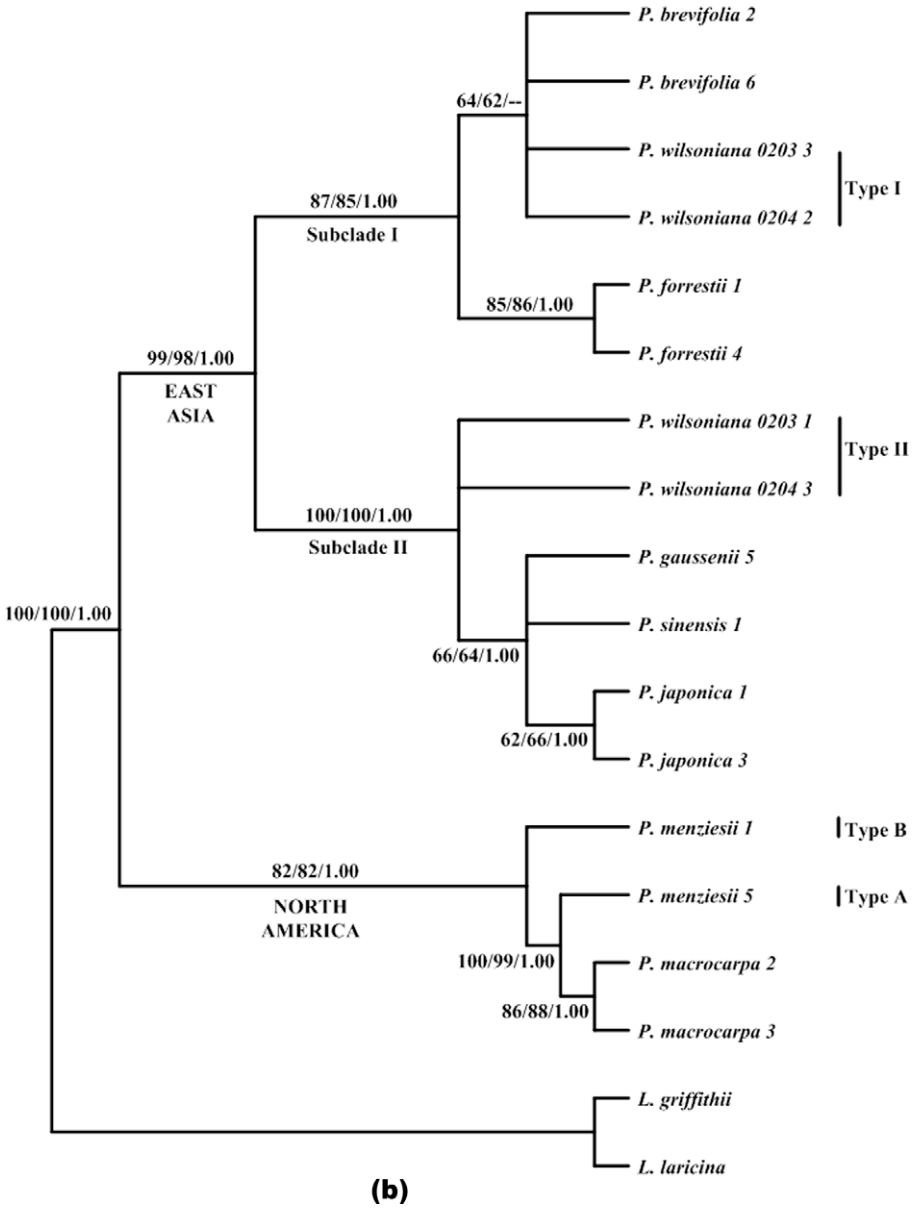


**Fig. 1** (*continued*)

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

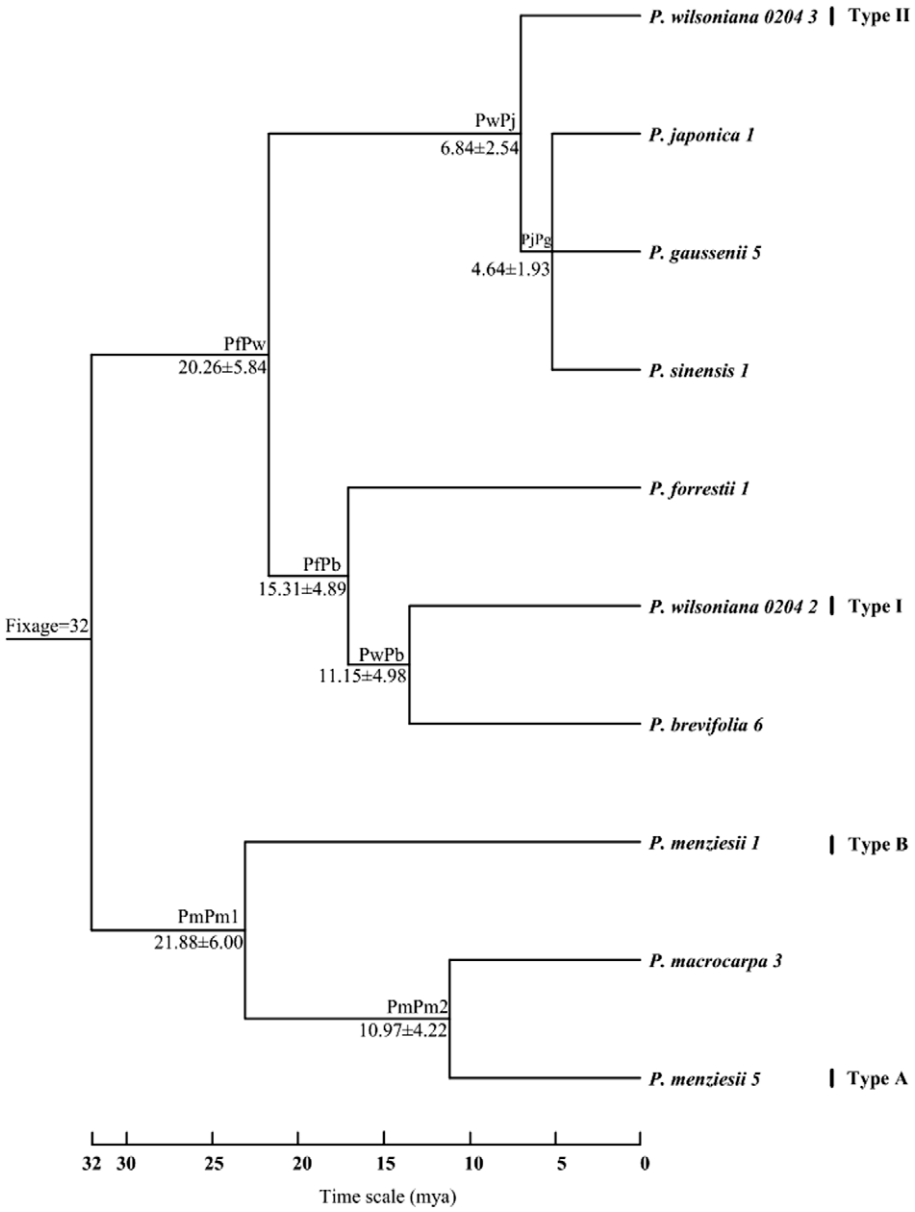


**Fig. 2.** A simplified *LEAFY* gene tree for molecular clock dating. The node name and age estimated with standard errors (mya) are denoted above and below the branches, respectively.

**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.

| Node | cpDNA | | | *LEAFY* | | |
|---|---|---|---|---|---|---|
| | r8S | | Beast | r8S | | Beast |
| | PL method | NPRS method | | PL method | NPRS method | |
| PfPw | 22.14 ± 7.25 | 22.45 ± 5.92 | 16.26 | 20.26 ± 5.84 | 20.95 ± 5.88 | 16.72 |
| PmPm1 | 18.37 ± 6.84 | 18.72 ± 5.46 | 12.24 | 21.88 ± 6.00 | 21.95 ± 5.95 | 16.44 |
| PmPm2 | — | — | — | 10.97 ± 4.22 | 10.98 ± 4.15 | 9.90 |
| PfPb | — | — | — | 15.31 ± 4.89 | 16.45 ± 4.81 | 5.33 |
| PwPb | — | — | — | 11.15 ± 4.98 | 12.94 ± 5.00 | 3.60 |
| PwPj | — | — | — | 6.84 ± 2.54 | 7.93 ± 2.65 | 2.30 |
| PjPg | — | — | — | 4.64 ± 1.93 | 5.08 ± 2.03 | 1.41 |

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.

### 4.2. Historical biogeography of Pseudotsuga and the hybrid origin of P. wilsoniana

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 *mat*K 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, 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 Mid-Miocene Climatic Optimum (MMCO, 17–15 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 early 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 assemblages 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 heterogenous topography in southwest China could be a good explanation for the higher sequence divergence in *P. brevifolia* and *P. forrestii* than in *P. gaussenii* 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.

## Acknowledgments

The authors thank the two anonymous reviewers for their valuable comments and suggestions on the manuscript, Profs. Deyuan Hong, Weilie Chen (Institute of Botany, Chinese Academy of Sciences) and Jianquan Liu (Lanzhou University), Drs. Yunfeng Hu (Guangxi Normal University), Xianzhao Kan (Anhui Normal University) and Yuzhi Cun (Institute of Botany, Chinese Academy of Sciences) for their kind help in the sample collection. Dr. Andrew Alverson (Indiana University, US) and Damien Hinsinger (Laval University, Canada) for their help in molecular clock dating and Dr. Mary Ann Feist (Illinois Natural History Survey, University of Illinois) for her assistance to go over the paper. We also thank Miss Wanqing Jin for her assistance in the laboratory. This study was supported by the National Basic Research Program of China (Grant No. 2007CB411600), the Chinese Academy of Sciences (Grant No. KZCX2-YW-415) and the National Natural Science Foundation of China (Grant Nos. 30500030, 30730010, 30425028).

## References


An, Z.S., Kutzbach, J.E., Prell, W.L., Porter, S.C., 2001. Evolution of Asian monsoons and phased uplift of the Himalaya–Tibetan plateau since Late Miocene times. Nature 411, 62–66.

Calsbeek, R., Thompson, J.N., Richardson, J., 2003. Patterns of molecular evolution and diversification in a biodiversity hotspot: the California Floristic Province. Mol. Ecol. 12, 1021–1029.

Cheng, Y.P., Hwang, S.Y., Lin, T.P., 2005. Potential refugia in Taiwan revealed by the phylogeographical study of *Castanopsis carlesii* Hayata (Fagaceae). Mol. Ecol. 14, 2075–2085.

Chiang, T.Y., Schaal, B.A., Peng, C.I., 1998. Universal primers for amplification and sequencing a noncoding spacer between the *atp*B and *rbc*L genes of chloroplast DNA. Bot. Bull. Acad. Sin. 39, 245–250.

Chiang, Y.C., Hung, K.H., Schaal, B.A., Ge, X.J., Hsu, T.W., Chiang, T.Y., 2006. Contrasting phylogeographical patterns between mainland and island taxa of the *Pinus luchuensis* complex. Mol. Ecol. 15, 765–779.

Chung, J.D., Lin, T.P., Tan, Y.C., Lin, M.Y., Hwang, S.Y., 2004. Genetic diversity and biogeography of *Cunninghamia konishii* (Cupressaceae), an island species in Taiwan: a comparison with *Cunninghamia lanceolata*, a mainland species in China. Mol. Phylogenet. Evol. 33, 791–801.

Dornelas, M.C., Rodriguez, A.P.M., 2005. A FLORICAULA/LEAFY gene homolog is preferentially expressed in developing female cones of the tropical pine *Pinus caribaea* var. caribaea. Genet. Mol. Biol. 28, 299–307.

Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214.

Duminil, J., Pemonge, M.H., Petit, R.J., 2002. A set of 35 consensus primer pairs amplifying genes and introns of plant mitochondrial DNA. Mol. Ecol. Notes 2, 428–430.

Farjon, A., 1990. Pinaceae: Drawings and Descriptions of the Genera *Abies*, *Cedrus*, *Pseudolarix*, *Keteleeria*, *Nothotsuga*, *Tsuga*, *Cathaya*, *Pseudotsuga*, *Larix* and *Picea*. Koeltz Scientific Books, Königstein, Germany.

Farjon, A., 2001. World Checklist and Bibliography of Conifers, second ed. Royal Botanic Gardens, Kew, England.

Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C., 1994. Testing significance of incongruence. Cladistics 10, 315–319.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Felsenstein, J., 1988. Phylogenies from molecular sequences: inference and reliability. Ann. Rev. Genet. 22, 521–565.

Felsenstein, J., 2004. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author Department of Genome Sciences, University of Washington, Seattle.

Flower, B.P., Kennett, J.P., 1995. Middle Miocene deepwater paleoceanography in the southwest Pacific: relations with east Antarctic ice sheet development. Paleoceanography 10, 1095–1112.

Frohlich, M.W., Parker, D.S., 2000. The mostly male theory of flower evolutionary origins: from genes to fossils. Syst. Bot. 25, 155–170.

Fu, L.K., Li, N., Mill, R.R., 1999. *Pseudotsuga*. In: Wu, Z.-Y., Raven, P.H. (Eds.), Flora of China, vol. 4. Science Press/Missouri Botanical Garden Press, Beijing/St. Louis, pp. 33–37.

Gamble, T., Simons, A.M., Colli, G.R., Vitt, L.J., 2008. Tertiary climate change and the diversification of the Amazonian gecko genus *Gonatodes* (Sphaerodactylidae, Squamata). Mol. Phylogenet. Evol. 46, 269–277.

Ge, X.J., Zhou, X.L., Li, Z.C., Hsu, T.W., Schaal, B.A., Chiang, T.Y., 2005. Low genetic diversity and significant population structuring in the relict *Amentotaxus argotaenia* complex (Taxaceae) based on ISSR fingerprinting. J. Plant Res. 118, 415–422.

Gernandt, D.S., Liston, A., 1999. Internal transcribed spacer region evolution in *Larix* and *Pseudotsuga* (Pinaceae). Am. J. Bot. 86, 711–723.

Grob, G.B.J., Gravendeel, B., Eurlings, M.C.M., 2004. Potential phylogenetic utility of the nuclear *FLORICAULA/LEAFY* second intron: comparison with three chloroplast DNA regions in *Amorphophallus* (Araceae). Mol. Phylogenet. Evol. 30, 13–23.

Hao, R.M., Liu, F.X., Yang, Z.B., Liu, S.L., Yao, G., 1996. The floristic similarity between east China and Japan. Acta Bot. Yunnan. 18, 269–276.

Hasegawa, M., Kishino, H., Yano, T., 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.

Havill, N.P., Campbell, C.S., Vining, T.F., LePage, B., Bayer, R.J., Donoghue, M.J., 2008. Phylogeny and biogeography of *Tsuga* (Pinaceae) inferred from nuclear ribosomal ITS and chloroplast DNA sequence data. Syst. Bot. 33, 478–489.

Hermann, R.K., 1982. The Genus *Pseudotsuga*: Historical Records and Nomenclature. Forest Research Laboratory Corvallis, Oregon.

Hermann, R.K., 1985. The Genus *Pseudotsuga*: Ancestral History and Past Distribution. Forest Research Laboratory Corvallis, Oregon.

Hewitt, G.W., 2000. The genetic legacy of the Quaternary ice ages. Nature 405, 907–913.

Hipkins, V.D., Krutovskii, K.V., Strauss, S.H., 1994. Organelle genome in conifers: structure, evolution, and diversity. For. Genet. 1, 179–189.

Huang, S.F., Lin, T.P., 2006. Migration of *Trochodendron aralioides* (Trochodendraceae) in Taiwan and its adjacent areas. Bot. Studies 47, 83–88.

Huang, S., Chiang, Y.C., Schaal, B.A., Chou, C.H., Chiang, T.Y., 2001. Organelle DNA phylogeography of *Cycas taitungensis*, a relict species in Taiwan. Mol. Ecol. 10, 2669–2681.

Kennett, J.P., Barker, P.F., 1990. Latest Cretaceous to Cenozoic climate and oceanographic developments in the Weddell Sea, Antarctica: an ocean drilling perspective, In: Proceedings of the Ocean Drilling Program, Scientific Results. Ocean Drilling Program, vol. 113. College Station, Texas, USA, pp. 937–960.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Kusumi, J., Tsumura, Y., Yoshimaru, H., Tachida, H., 2002. Molecular evolution of nuclear genes in Cupressaceae, a group of conifer trees. Mol. Biol. Evol. 19, 736–747.

Lakhanpal, R.N., 1958. The Rujada flora of west central Oregon. Univ. Calif. Publ. Geol. Sci. 35, 1–66.

Lee, C., Wen, J., 2002. Phylogeny and biogeography of the Asian clade of Araliaceae: insights from the nuclear ribosomal and chloroplast DNA data. Abstract, Botany 2002, Annual Meeting of Botanical Society of America, Madison, Wisconsin, USA. Available from: <http://www.botany2002.org/section12/abstracts/201.shtml>.

Lemmon, E.M., Lemmon, A.R., Cannatella, D.C., 2007. Geological and climatic forces driving speciation in the continentally distributed trilling chorus frogs (*Pseudacris*). Evolution 61, 2086–2103.

Liu, F.X., Liu, S.L., Yang, Z.B., Hao, R.M., Yao, G., Huang, Z.Y., Li, N., 1995. A floristic study on the seed plants from the region of east China. Acta Bot. Yunnan. (Suppl. VII), 93–110.

Lu, S.Y., Peng, C.I., Cheng, Y.P., Hong, K.H., Chiang, T.Y., 2001. Chloroplast DNA phylogeography of *Cunninghamia konishii* (Cupressaceae), and endemic conifer of Taiwan. Genome 44, 797–807.

McKenna, M.C., 1983. Holarctic landmass rearrangement, cosmic events, and Cenozoic terrestrial organisms. Ann. Mo. Bot. Gard. 70, 459–489.

Mercer, J.M., Roth, V.L., 2003. The effects of Cenozoic global change on Squirrel phylogeny. Science 299, 1568–1572.

Millien-Parra, V., Jaeger, J.J., 1999. Island biogeography of the Japanese terrestrial mammal assemblages: an example of a relict fauna. J. Biogeogr. 26, 959–972.

Milne, R.I., 2006. Northern hemisphere plant disjunctions: a window on tertiary land bridges and climate change? Ann. Bot. 98, 465–472.

Mogensen, H.L., 1996. The hows and ways of cytoplasmic inheritance in seed plants. Am. J. Bot. 83, 383–404.

Moritz, C., Patton, J.L., Schneider, C.J., Smith, T.B., 2002. Diversification of rainforest faunas: an integrated molecular approach. Annu. Rev. Ecol. Syst. 31, 533–563.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., Fonseca, G., Kent, J., 2000. Biodiversity hotspots for conversation priorities. Nature 403, 853–858.

Nei, M., Gojobori, T., 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. 3, 418–426.

Nie, Z.L., Wen, J., Sun, H., Bartholomew, B., 2005. Monophyly of *Kelloggia* Torrey ex Benth. (Rubiaceae) and evolution of its intercontinental disjunction between western North America and eastern Asia. Am. J. Bot. 92, 642–652.

Nie, Z.L., Sun, H., Beardsley, P.M., Olmstead, R.G., Wen, J., 2006. Evolution of biogeographic disjunction between eastern Asia and eastern North America in *Phryma* (Phrymaceae). Am. J. Bot. 93, 1343–1356.

Novacek, M.J., 1999. 100 million years of land vertebrate evolution: the Cretaceous-early Tertiary transition. Ann. Mo. Bot. Gard. 86, 230–258.

Nylander, J.A.A. 2004. MrModeltest v2. Program Distributed by the Author. Evolutionary Biology Centre, Uppsala Univ., Uppsala, Sweden.

Oh, S.-H., Potter, D., 2005. Molecular phylogenetic systematics and biogeography of tribe Neillieae (Rosaceae) using DNA sequences of cpDNA, rDNA, and *LEAFY*. Am. J. Bot. 92, 179–192.

Pend, D., Wang, X.Q., 2008. Reticulate evolution in *Thuja* inferred from multiple gene sequences: implications for the study of biogeographical disjunction between eastern Asia and North America. Mol. Phylogenet. Evol. 47, 1190–1202.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 917–918.

Qian, H., Ricklefs, R.E., 2000. Large-scale processes and the Asian bias in species diversity of temperate plants. Nature 407, 180–182.

Qiu, Y.L., Lee, J., Bernasconi-Quadroni, F., Soltis, D.E., Soltis, P.S., Zanis, M., Zimmer, E.A., Chen, Z., Savolainen, V., Chase, M.W., 1999. The earliest angiosperms: evidence from mitochondrial, plastid and nuclear genomes. Nature 402, 404–407.

Ran, J.H., Wei, X.X., Wang, X.Q., 2006. Molecular phylogeny and biogeography of *Picea* (Pinaceae): implications for phylogeographical studies using cytoplasmic haplotypes. Mol. Phylogenet. Evol. 41, 405–419.

Richardson, J., Pennington, R.T., Pennington, T.D., Hollingsworth, P.M., 2001. Rapid diversification of a species-rich genus of neotropical rain forest trees. Science 293, 2242–2245.

Rogers, S.O., Bendich, A.J., 1988. Extraction of DNA from plant tissues. Plant Mol. Biol. (Manual) A6, 1–10.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Rowe, K.C., Heske, E.J., Brown, P.W., Paige, K.N., 2004. Surviving the ice. Northern refugia and postglacial colonization. Proc. Natl. Acad. Sci. USA 101, 10355–10359.

Rull, V., 2008. Speciation timing and neotropical biodiversity: the Tertiary–Quaternary debate in the light of molecular phylogenetic evidence. Mol. Ecol. 17, 2722–2729.

Sanderson, M.J., 1997. A nonparametric approach to estimating divergence times in the absence of rate constancy. Mol. Biol. Evol. 14, 1218–1231.

Sanderson, M.J., 2002. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Mol. Biol. Evol. 19, 101–109.
Sang, T., 2002. Utility of low-copy nuclear gene sequences in plant phylogenetics. Crit. Rev. Biochem. Mol. Biol. 37, 121–147.
Sang, T., Donoghue, M.J., Zhang, D.M., 1997. Evolution of alcohol dehydrogenase genes in peonies (Paeonia): phylogenetic relationships of putative nonhybrid species. Mol. Biol. Evol. 14, 994–1007.
Schorn, H.E., 1994. A preliminary discussion of fossil larches (*Larix*, Pinaceae) from the Arctic. Quat. Int. 22 (23), 173–183.
Shaw, J., Lickey, E.B., Beck, J.T., Farmer, S.B., Liu, W., Miller, J., Siripun, K.C., Windel, C.T., Schilling, E.E., Small, R.L., 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am. J. Bot. 92, 142–166.
Sibuet, J.C., Hsu, S.K., 1997. Geodynamics of the Taiwan arc–arc collision. Tectonophysics 274, 221–251.
Sibuet, J.C., Hsu, S.K., 2004. How was Taiwan created? Tectonophysics 379, 159–181.
Small, R.L., Cronn, R.C., Wendel, J.F., 2004. Use of nuclear genes for phylogeny reconstruction in plants. Aust. Syst. Bot. 17, 145–170.
Soltis, P.S., Soltis, D.E., Chase, M.W., 1999. Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. Nature 402, 402–404.
Spicer, R.A., Nigel, B., Harris, W., Widdowson, M., Herman, A.B., Guo, S.X., Valdes, P.J., Wolfek, J.A., Kelley, S.P., 2003. Constant elevation of southern Tibet over the past 15 million years. Nature 421, 622–624.
Steele, P.R., Guisinger-Bellian, M., Linder, C.R., Jansen, R.K., 2008. Phylogenetic utility of 141 low-copy nuclear regions in taxa at different taxonomic levels in two distantly related families of rosids. Mol. Phylogenet. Evol. 48, 1013–1026.
Strauss, S.H., Doerksen, A.H., Byrne, J.R., 1990. Evolutionary relationships of Doughlas-fir and its relatives (genus *Pseudotsuga*) from DNA restriction fragment analysis. Can. J. Bot. 68, 1502–1510.
Sun, F.J., Downie, S.R., Hartman, R.L., 2004. An ITS-based phylogenetic analysis of the perennial, endemic Apiaceae subfamily Apioideae of western North America. Syst. Bot. 29, 419–431.
Swofford, D.L., 2002. PAUP*. Phylogenetic Analysis Using Parsimony (* and other methods). Version 4. Sinauer, Sunderland, MA.
Taberlet, P., Gielly, L., Pautou, G., Bouvet, J., 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol. Biol. 17, 1105–1109.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. doi: 10.1093/molbev/msm092.
Thiede, J., Vorren, T.O., 1994. The Arctic Ocean and its geologic record: research history and perspectives. Mar. Geol. 119, 179–184.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Tiffney, B.H., 1985a. The Eocene North Atlantic land bridge: its importance in Tertiary and modern phytogeography of the Northern Hemisphere. J. Arnold Arbor. 66, 243–273.
Tiffney, B.H., 1985b. Perspectives on the origin of the floristic similarity between Eastern Asia and eastern North America. J. Arnold Arbor. 66, 73–94.
Tu, T., Dillon, M.O., Sun, H., Wen, J., 2008. Phylogeny of *Nolana* (Solanaceae) of the Atacama and Peruvian deserts inferred from sequences of four plastid markers and the nuclear LEAFY second intron. Mol. Phylogenet. Evol. 49, 561–573.
Vazquez-Lobo, A., Carlsbecker, A., Vergara-Silva, F., Alvarez-Buylla, E.R., Pinero, D., Engstrom, P., 2007. Characterization of the expression patterns of LEAFY/FLORICAULA and NEEDLY orthologs in female and male cones of the conifer genera *Picea*, *Podocarpus*, and *Taxus*: implications for current evo–devo hypotheses for gymnosperms. Evol. Dev. 9, 446–459.
Vincent, E., Killingley, J.S., Berger, W.H., 1985. Oxygen and carbon isotope stratigraphy of the tropical Ocean. Geol. Soc. Am. Mem. 163, 103–130.
Wang, W.T., 1992. On some distribution patterns and some migration routes found in the Eastern Asiatic region. Acta Phytotax. Sin. 30, 1–24.
Wang, X.Q., Tank, D.C., Sang, T., 2000. Phylogeny and divergence times in Pinaceae: evidence from three genomes. Mol. Biol. Evol. 17, 773–781.
Wang, W.P., Hwang, C.Y., Lin, T.P., Hwang, S.Y., 2003. Historical biogeography and phylogenetic relationships of the genus *Chamaecyparis* (Cupressaceae) inferred from chloroplast DNA polymorphism. Plant Syst. Evol. 241, 13–28.
Wang, C.N., Moller, M., Cronk, Q.C.B., 2004. Population genetic structure of *Titanotrichum oldhamii* (Gesneriaceae), a subtropical bulbiliferous plant with mixed sexual and asexual reproduction. Ann. Bot. 93, 201–209.
Wei, X.X., Wang, X.Q., 2003. Phylogenetic split of *Larix*: evidence from paternally inherited cpDNA *trn*T–*trn*F region. Plant Syst. Evol. 239, 67–77.
Wen, J., 1999. Evolution of eastern Asian and eastern North American disjunct distributions in flowering plants. Annu. Rev. Ecol. Syst. 30, 421–455.
Wen, J., Zimmer, E.A., 1996. Phylogeny and biogeography of *Panax* L. (the ginseng genus, Araliaceae): inferences from ITS sequences of nuclear ribosomal DNA. Mol. Phylogenet. Evol. 6, 167–177.
Wen, J., Jansen, R.K., Zimmer, E.A., 1996. Phylogenetic relationships and DNA sequence divergence of eastern Asian and eastern North American disjunct plants. In: Nei, M., Takahata, N. (Eds.), Current Topics in Molecular Evolution. Hayama (Japan): publ. Jointly by Penn. State Univ., USA, and Grad. Sch. for Adv. Stud., Hayama, Japan, pp. 37–44.
Wen, J., Shi, S., Jansen, R.K., Zimmer, E.A., 1998. Phylogeny and biogeography of *Aralia* sect. *Aralia* (Araliaceae). Am. J. Bot. 85, 866–875.
Willis, K.J., Niklas, K.J., 2004. The role of Quaternary environmental change in plant macroevolution, the exception or the rule? Philos. Trans. R. Soc. B 292, 686–693.
Won, H., Renner, S.S., 2005. The internal transcribed spacer of nuclear ribosomal DNA in the gymnosperm *Gnetum*. Mol. Phylogenet. Evol. 36, 581–597.
Xiang, Q.Y., Soltis, D.E., Soltis, P.S., 1998. The eastern Asian and eastern and western North American floristic disjunction: congruent phylogenetic patterns in seven diverse genera. Mol. Phylogenet. Evol. 10, 178–190.
Xiang, Q.Y., Soltis, D.E., Soltis, P.S., Manchester, S.R., Crawford, D.J., 2000. Timing the eastern Asian-eastern North American floristic disjunction: molecular clock corroborates plaleontological estimates. Mol. Phylogenet. Evol. 15, 462–472.
Xiang, Q.Y., Manchester, S.R., Thomas, D.T., Zhang, W., Fan, C., 2005. Phylogeny, biogeography, and molecular dating of cornelian cherries (*Cornus*, Cornaceae): tracking Tertiary plant migration. Evolution 59, 1685–1700.
Ying, T.S., Hsu, K.S., 2002. An analysis of the flora of seed plants of Taiwan, China: its nature, characteristics, and relations with the flora of the mainland. Acta Phytotax. Sin. 40, 1–51.
Zachos, J., Pagani, M., Sloan, L., Thoma, E., Billups, K., 2001. Trends, rhythms and aberrations in global climate 65 Ma to present. Science 292, 686–693.
Zeng, W.B., 1993. The passageway of the flora migration on both sides of the Taiwan Strait in Pleistocene epoch. Acta Bot. Yunnan. 16, 107–110.
Zhang, Z.Y., Fan, L.M., Yang, J.B., Hao, X.J., Gu, Z.J., 2006. Alkaloid polymorphism and ITS sequence variation in the *Spiraea japonica* complex (Rosaceae) in China: traces of the biological effects of the Himalaya-Tibet Plateau uplift. Am. J. Bot. 93, 762–769.