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Phylogeny and Biogeography of *Tsuga* (Pinaceae) Inferred from Nuclear Ribosomal ITS and Chloroplast DNA Sequence Data

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Abstract—Hemlock, *Tsuga* (Pinaceae), has a disjunct distribution in North America and Asia. To examine the biogeographic history of *Tsuga*, phylogenetic relationships among multiple accessions of all nine species were inferred using chloroplast DNA sequences and multiple cloned sequences of the nuclear ribosomal ITS region. Analysis of chloroplast and ITS sequences resolve a clade that includes the two western North American species, *T. heterophylla* and *T. mertensiana,* and a clade of Asian species within which one of the eastern North American species, *T. caroliniana,* is nested. The other eastern North American species, *T. canadensis,* is sister to the Asian clade. *Tsuga chinensis* from Taiwan did not group with *T. chinensis* from mainland China, and *T. sieboldii* from Ullung Island did not group with *T. sieboldii* from Japan suggesting that the taxonomic status of these distinct populations should be reevaluated. The Himalayan species, *T. dumosa*, was in conflicting positions in the chloroplast and ITS trees, suggesting that it may be of hybrid origin. Likelihood-based biogeographic inference with divergence time estimates infers an Eocene basal crown group diversification and an initial widespread circumpolar distribution with subsequent vicariance and extinction events leading to the current disjunct distribution.

Keywords—ancestral area reconstruction, internal transcribed spacer, phylogenetic incongruence, *trnK–matK* intron, *trnL–F* region, *rpl16* intron.

Hemlock, *Tsuga* (Endlicher) Carrière (Pinaceae), is an important conifer of subalpine and lowland forest communities in North America and Asia (Wang 1961; Hirokawa 1972, 1976; Burns and Honkala 1990). Hemlocks are slow-growing, long-lived, shade-tolerant trees that cannot tolerate persistent drought. While certain species, especially *T. heterophylla* in western North America, are a good source of lumber and fiber, *Tsuga* is generally considered to be more valuable for its ecological functions and aesthetic value. Hemlocks provide important resources for wildlife, including deer (Mladenoff and Stearns 1993), birds (Benkman 1993), and salamanders (Brooks 2001). They are also horticulturally important with more than 250 named varieties and cultivars of *T. canadensis* alone (Swartley 1984; Cope 1992).

Up to 25 extant species of *Tsuga* have been described, with eight to 13 species accepted by various authors (Fitschen 1929; Flous 1936; Gaussen 1966; Cheng 1983; Silba 1986; Farjon 1990; Page 1990; Fu et al. 1999). We follow Farjon (1990) who recognized nine species, three of which occur in China and the Himalayas, two in Japan, two in western North America, and two in eastern North America (Fig. 1).

A comprehensive phylogenetic analysis of *Tsuga* has not been undertaken. Matsumoto et al. (1995) used eight morphological characters, and Taylor (1972) used phytochemical chromatography to infer *Tsuga* phylogeneny. Analyses of the nuclear ribosomal internal transcribed spacer (ITS) region agreed that the western North American species are sister to the remaining species, and that the two eastern North American species, *T. caroliniana* and *T. canadensis*, were not sister to each other, but that *T. caroliniana* is located in a clade that includes all of the Asian species (LePage and Bayer 1997; Vining 1999; LePage 2003a). The remaining relationships within *Tsuga* were not well resolved.

The objectives of this study were to reconstruct the phylogeny of *Tsuga* using nuclear internal transcribed spacer (ITS) and chloroplast DNA (cpDNA) sequence data, and to examine the biogeographic history of the genus. ITS is biparentally inherited, and cpDNA is paternally inherited in the Pinaceae (Mogenson 1996), which allows us to examine potential discordance in ITS and cpDNA phylogenies and to infer possible instances of hybridization. We used the *trnK–matK* intron, *rpl*16 intron, and *trnT–F* cpDNA regions, as these have been used successfully for phylogenetic studies of other groups of closely related plants (e.g. Gernandt et al. 2003; Bell and Donoghue 2005; Chaw et al. 2005; Ran et al. 2006; Liston et al. 2007). The ITS region is also commonly used for phylogenetic analyses of closely related plant taxa, including in the Pinaceae (e.g. Gernandt and Liston 1999; Liston et al. 1999; Gernandt et al. 2001; Wei and Wang 2004). In the Pinaceae, the ITS region is longer than in all other vascular plants, and the ITS1 region has distinctive subrepeats (Liston et al. 1996; Marrocco et al. 1996; Maggini et al. 1998; Gernandt and Liston 1999; Vining 1999; Gernandt et al. 2001; Wei et al. 2003; Campbell et al. 2005, Kan et al. 2007). In addition, ITS copies within a species do not always coalesce (Gernandt and Liston 1999; Wei and Wang 2004; Campbell et al. 2005). To accurately infer phylogenetic relationships among closely related taxa in the Pinaceae using ITS, it is helpful to examine and account for patterns of divergence among copies within and between species.

Materials and Methods

Sampling Design and Outgroup Selection—We obtained 2–10 samples of each *Tsuga* species and one sample of each outgroup (Appendix 1). Species of *Nothotsuga*, *Abies*, *Keteleeria*, and *Pseudolarix* were included for rooting purposes because these genera are thought to be most closely related to *Tsuga,* with *Nothotsuga* sister to *Tsuga* (Hart 1987; Price et al.

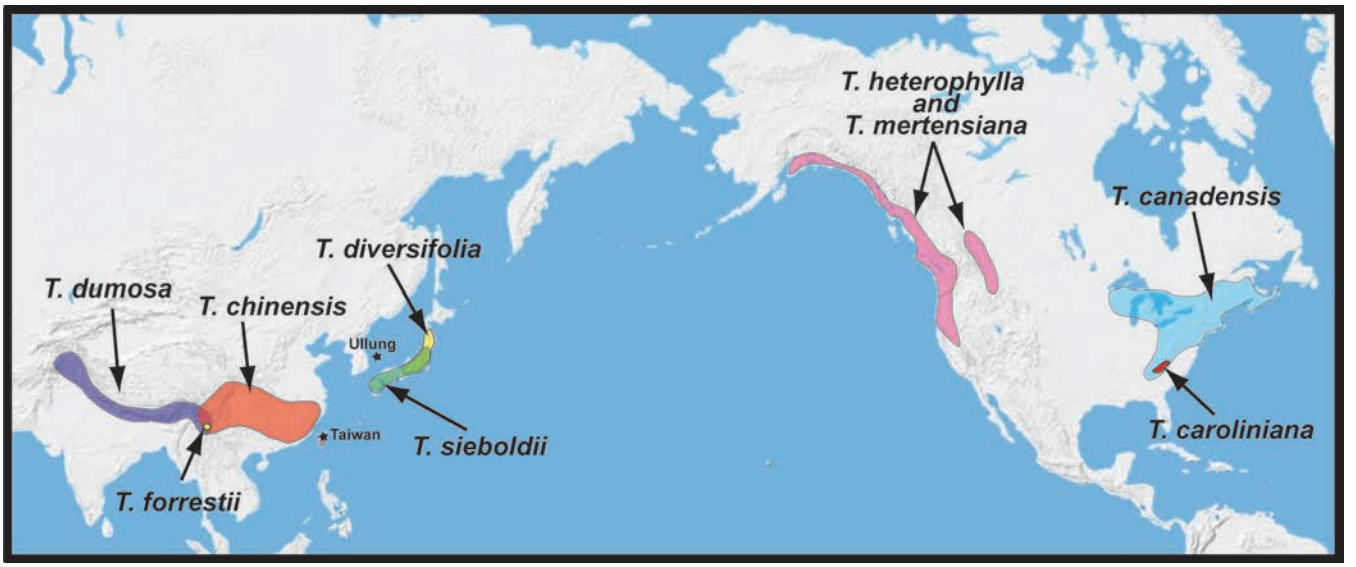

FIG. 1. Map showing the ranges of extant *Tsuga* species based on Little (1971), Hirokawa (1972; 1976), and Farjon (1990). The distributions of *T. mertensiana* and *T. heterophylla* are shown as sympatric for graphical simplicity although *T. mertensiana* generally occurs at higher elevations and in some regions such as the Sierra Nevada where *T. hererophylla* is not present. The islands of Ullung and Taiwan are indicated with stars.

1987; Farjon 1990; Vining 1999; Wang et al. 2000). The ITS region was rooted with only *Nothotsuga* because alignment of ITS1 from the other outgroup genera with *Tsuga* was problematic.

DNA Amplification and Sequencing—Total genomic DNA was extracted from fresh or dried leaves using the DNeasy plant mini extraction kit (Qiagen, La Jolla, California), except for *Keteleeria* and *Nothotsuga* for which DNA was kindly provided by X.-Q. Wang at the Chinese Academy of Sciences, Beijing.

The entire ITS region (ITS1–5.8S–ITS2; ca. 1730 bp) was amplified using the polymerase chain reaction (PCR) with the primer ITS-4 (White et al. 1990), and the new primer ITS-TsuFor1 (CGATGTCGTGAGAAGTTCACTG), which was designed using *Tsuga* sequences from Vining (1999). Thermocycling conditions were 5 min at 95°C, followed by 40 cycles at 95°C for 1 min, 54°C for 1 min, and 72°C for 1.5 min with a final extension at 72°C for 5 min. Amplification products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, California). For each sample, 27 colonies were screened for inserts by PCR using the amplification primers. Positive clones were sequenced using the amplification primers and the internal primers ITS-2d (5′-CGCAACGCCTTCGAGAGAAC-3′) and ITS-5c (5′-GTTGATCGGAAAGGGCTTGC-3′) (Vining 1999). Sequencing with ITS-2d and ITS-5c failed for *N. longibracteata* and *T. heterophylla*, respectively, so the additional primers ITS-NothoFor1 (5′-CTTGTGTGCCTCGGTCTTC-3′) and ITS-ThRev1 (5′-CATGGAGGACG GACTAGGG-3′) were designed using preliminary sequences.

Three cpDNA regions were amplified and sequenced in both directions. When necessary, new amplification primers were designed using sequences from other species in the Pinaceae from GenBank, and new internal sequencing primers were designed using preliminary *Tsuga* sequences. The cpDNA regions were (1) the *trnK* intron and a portion of the *matK* coding region (ca. 1240 bp) that were amplified using the primers *trnK*-11 (Young et al. 1999) and *matK*-TsuRev1 (5′-GAACCAAAATTTC-CGGATGA-3′), and sequenced with primers *matK*-TsuFor1 (5′-ATCCCATGAGTCAGGAGAGC-3′) and *matK*-TsuRev2 (5′-TCGTCATGAGGAAGAACGAA-3′); (2) the 3′ end of the *rpl16* intron (ca. 630 bp) that was amplified and sequenced using the primers *rpl16*-TsuFor (5′-CGTAAAGACTTTCTTCCACGAGA-3′) and *rpl16*-TsuRev (5′-CCAGAACCGGACATGAAAGT-3′); and (3) the *trnT–F* region (ca. 1460 bp) that was amplified and sequenced using combinations of the primers trnTa, trnLb, trnLc, trnLd, trnLe, and trnLf (Taberlet et al. 1991). Thermocycling conditions for *matK* and *rpl16* were 5 min at 95°C, followed by 40 cycles at 95°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min with a final extension at 72°C for 5 min. Conditions for the *trnT–F* reactions were the same except for an annealing temperature of 50°C.

Sequencing was performed using the BigDye Terminator kit (Applied Biosystems, Foster City, California) on an Applied Biosystems 3100 automated sequencer. Sequences were edited using Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, Michigan) and aligned using MUSCLE 3.6 (Edgar 2004). All sequences generated in this study have been deposited in GenBank (see Appendix 1) and all data matrices have been deposited in TreeBASE (study number S3450).

Phylogenetic Analyses—Evidence for ITS pseudogenes includes increased substitution rate and decreased GC content (Gernandt et al. 2001). To check for the presence of pseudogenes, the base composition of each ITS sequence was determined with PAUP* 4.10b10 (Swofford 2003).

Maximum parsimony (MP) analyses were conducted with PAUP* using heuristic searches with random sequence addition, tree-bisection-reconnection (TBR) branch swapping, collapsing zero-length branches, and equal weighting of all characters. Gaps were treated as missing data. Clade support was estimated using 1,000 bootstrap replicates with the same heuristic search conditions.

Bayesian analyses were conducted with MrBayes 3.1 (Ronquist and Huelsenbeck 2003) using default priors, four incrementally heated Markov chains, and two concurrent runs of 10,000,000 generations sampled every 50,000 generations for a total of 400 trees used to calculate posterior probabilities. The best fitting model for each cpDNA region and for ITS was determined using AIC as implemented by Modeltest 3.7 (Posada and Crandall 1998). Plots of log-likelihood scores versus generation time were used to assess stabilization of the Markov chains.

Shimodaira-Hasegawa (S-H) tests using the RELL bootstrap with 1,000 replicates as implemented in PAUP* were performed using the cpDNA and ITS data sets to test conflicting taxonomic hypotheses.

Divergence Time Estimation—The topology and branch lengths resulting from Bayesian analysis of the cpDNA data set were used to estimate divergence times. A likelihood ratio test was used to examine rate heterogeneity using likelihood scores with and without enforcement of a molecular clock. Because a constant rate among branch lengths was rejected, we used penalized likelihood (Sanderson 2002) with a rate smoothing parameter estimated using cross-validation using the software r8s 1.71 (Sanderson 2003). Means and standard deviations of divergence times were estimated by replicate analysis of the 400 sampled Bayesian trees.

Divergence times were calibrated using fossil evidence. The earliest known *Tsuga* megafossils are from Eocene deposits in Europe and western North America (LePage 2003b). However, assigning these fossils to phylogenetic positions within *Tsuga* is not always reliable because delineation of extant species requires a suite of leaf, cone, and shoot characters while the fossil deposits typically include only a subset of these (Szafer 1949; LePage 2003b).

Tsuga pollen is known from earlier times, making these records more informative because they can be used to constrain the age of the *Tsuga* stem. Pollen of *T. mertensiana* has two lateral sacs, as in most other Pinaceae, whereas pollen of the remaining *Tsuga* species is monosaccate with the lateral sacs reduced to a puffy equatorial fringe (van Campo-Duplan 1950; Erdtman 1957; Ho and Sziklai 1972; Owens and Blake 1983). *Nothotsuga longibracteata* pollen is bisaccate with small sacs that are variable in

size and shape (van Campo-Duplan 1950). Monosaccate *Tsuga* pollen differs among species in overall size, the size of the fringe, and certain aspects of surface structure (van Campo-Duplan 1950; Ueno 1957). Attempts have been made to associate fossil monosaccate pollen with extant *Tsuga* species, but whether these associations indicate phylogenetic relationships is unclear in the absence of a thorough phylogenetic analysis of fossil and extant *Tsuga* pollen. For example, Thomson and Pflug (1953) recognized two types of fossil *Tsuga* pollen from the Pliocene: a *diversifolia*-type with a fully developed fringe and a *canadensis*-type with a reduced fringe. However, other extant species such as *T. sieboldii* also have a reduced fringe (Ueno 1957). Sivak (1973) observed that it may be possible to associate fossil pollen with *T. canadensis* based on the diameter of the grain and the lack of spines on the surface, but that association of fossils with other extant monosaccate species was less convincing. The oldest record of *Tsuga* pollen is from late Cretaceous (ca. 90 mya) deposits in Poland (Macko 1963). These deposits contained bisaccate pollen, which he considered morphologically similar to *T. mertensiana*, and two monosaccate types, similar to *T. canadensis* and *T. diversifolia*. Based on this record, we calibrated the age estimates by fixing an age of 90 mya for the common ancestor of *Nothotsuga* and *Tsuga.*

***Historical Biogeography*—**Patterns of dispersal and vicariance were reconstructed with model-based likelihood inference using the software AReA 2.5 (note, AReA has since been superseded by the software package lagrange; Ree and Smith 2008). This method incorporates phylogeny, divergence times, fossil data, rates of lineage dispersal and extinction, and an independent model of the probability of connection among geographic areas over time (Ree et al. 2005; Moore et al. in press). Probabilities of ancestral movements among geographic areas are then estimated along each branch and for each node in a likelihood framework using Monte Carlo methods.

AReA analysis incorporated the tree topology and branch lengths derived from the cpDNA data set. Rates of 0.0003 for dispersal and 0.002 for extinction were estimated from the data under maximum likelihood using the genetic algorithm implemented in AReA 2.5. Macrofossils and pollen records indicate that *Tsuga* was present throughout Europe from the Cretaceous through the Pleistocene, but disappeared during the Quaternary due to cooler and drier climate and glaciations (Tiffney 1985; Liu and Basinger 2000; LePage 2003b). To incorporate the past distribution of *Tsuga* in Europe, fossils were placed along the branch leading to the most recent common ancestor of *T. mertensiana* and *T. canadensis*, and on the branch leading to the most recent common ancestor of *T. canadensis* and *T. caroliniana* based on multiple fossil records (summarized by LePage 2003b). The paleogeographic model of connection probabilities among Northern Hemisphere areas (Fig. 4, top) includes dispersal functions for the North Atlantic land bridges (between ENA and EUR), the Bering land bridge (between WNA and EAS), and the intracontinental connections (between ENA and WNA, and between EAS and EUR). The probabilities throughout the Cenozoic are based on paleoclimatic and paleogeologic factors synthesized by Tiffney and Manchester (2001) and implemented by Moore et al. (in press). Connection probabilities during the Mesozoic are based on paleogeographic history as summarized by Scotese (2001). Some features of this model include reduced dispersal probabilities between ENA and WNA in the late Cretaceous due to separation by the Western Interior Seaway, and between EAS and EUR from the Middle Jurassic to Oligocene due to the Turgai Strait. All probabilities are independent of the direction of migration.

Results

The aligned ITS data set of 116 cloned sequences was 1785 nucleotides long. Four clones of *N. longibracteata* and five to 15 clones across one or two individuals per *Tsuga* taxon were sequenced. The ITS1 region varied from 1342–1350 bp among *Nothotsuga* clones and from 1351–1364 bp among *Tsuga* clones, not including clone 23 of *T. chinensis* from Taiwan (hereinafter referred to as *T. chinensis* (Taiwan)), which was 1325 bp long due to a 37 bp deletion. Forty-four insertions or deletions (indels), all in ITS1, were required to align the data set.

While there appears to be a higher substitution rate in some clones of *T. chinensis, T. chinensis* (Taiwan), and *T. forrestii*, there is not a correspondingly low GC content. Mean GC content for all clones in our data set was 58.4 (s.d. = 0.004), and the narrow range of 57.1–59.1 indicates that there were no sequences with unusually low GC content. The 37 bp deletion in clone 04–85.23 from *T. chinensis* (Taiwan) suggests that this may not be a functional copy; however, there was otherwise no compelling reason to remove this sequence from the data set because its position in the tree was consistent with the other clones for this taxon and its GC content was 59.1%.

ITS1, 5.8S, and ITS2 contained 561 (39.6%), 27 (16.6%), and 64 (31.2%) variable sites, and 428 (30.2%), 9 (5.5%), and 38 (18.5%) parsimony informative sites, respectively. MP analysis of ITS resulted in 15,977 equally parsimonious trees with a length of 1191 steps, consistency index of 0.592 and retention index of 0.894. Bayesian analysis utilized a GTR + I + G model of nucleotide substitution. The Bayesian 75% majority rule consensus tree (Fig. 2) resolved the clones of *N. longibracteata, T. canadensis, T. caroliniana, T. dumosa, T. heterophylla,* and *T. mertensiana* as monophyletic for each species. Clones from *T. diversifolia* and *T. sieboldii* from Ullung Island (hereinafter referred to as *T. sieboldii* (Ullung)) formed a clade with the exception of four *T. diversifolia* clones that were part of a clade of *T. sieboldii* from Japan, *T. chinensis, T. chinensis* from Taiwan, and *T. forrestii.* When clones from more than one individual were included per taxon, sequences from an individual did not form a clade with the exception of *T. dumosa*. Taxa for which cloned sequences were monophyletic exhibited lower pairwise sequence divergence (range 0.007–0.013) than those that were not monophyletic (range 0.014–0.023), with the exception of *T. sieboldii* (Ullung) (0.007) (Table 1).

The aligned cpDNA data set of 37 sequences was 3456 nucleotides long (*matK* = 1246, *rpl16* = 680, *trnT–F* = 1530). Forty-eight indels were required to align the entire dataset (*matK* = 8, *rpl16* = 11, *trnT–F* = 29) including a poly-A and a poly-AT repeat in *rpl16*. Noteworthy indels, all in the *trnT–F* region, unique to one or two taxa included a 5 bp insertion and a 5 bp deletion in *T. diversifolia* and *T. sieboldii* (Ullung), a 15 bp insertion in *T. chinensis* and *T. forrestii*, a 17 bp insertion in *T. diversifolia*, a 69 bp deletion in *Nothotsuga*, two long deletions (68 and 83 bp) in *T. dumosa*, and a 112 bp deletion in *Keteleeria.* Indels were not coded as characters in phylogenetic analyses because the majority were found in single taxa and therefore not informative.

matK, *rpl16*, and *trnT–F* contained 113 (9.0%), 68 (10.0%), and 175 (11.4%) variable sites, and 61 (4.9%), 38 (5.5%), and 90 (5.9%) parsimony informative sites respectively. MP analysis resulted in four equally parsimonious trees with a length of 427 steps, a consistency index of 0.890, and a retention index of 0.914. The trees differed only by the placement of *T. caroliniana*. Bayesian analysis utilized a heterogeneous model of nucleotide substitution with separate unlinked GTR + I + G models for each of the three regions. The MP strict consensus tree was identical to the Bayesian 50% majority rule consensus tree (Fig. 3) except that in the MP tree *T. caroliniana* was not resolved as sister to *T. diversifolia* + *T. sieboldii* (Ullung), and the three *T. diversifolia* samples did not form a clade.

The cpDNA and ITS trees differed in the placement of *T. dumosa*. S-H tests with *T. dumosa* constrained to the different positions resulted in a marginally significant difference using the cpDNA data set (P = 0.07) and a significant difference for the ITS data set ($p = 0.03$).

Tsuga chinensis (Taiwan) did not group with *T. chinensis* from mainland China in the cpDNA tree, but instead was sister to *T. dumosa*. The S-H test was significantly different

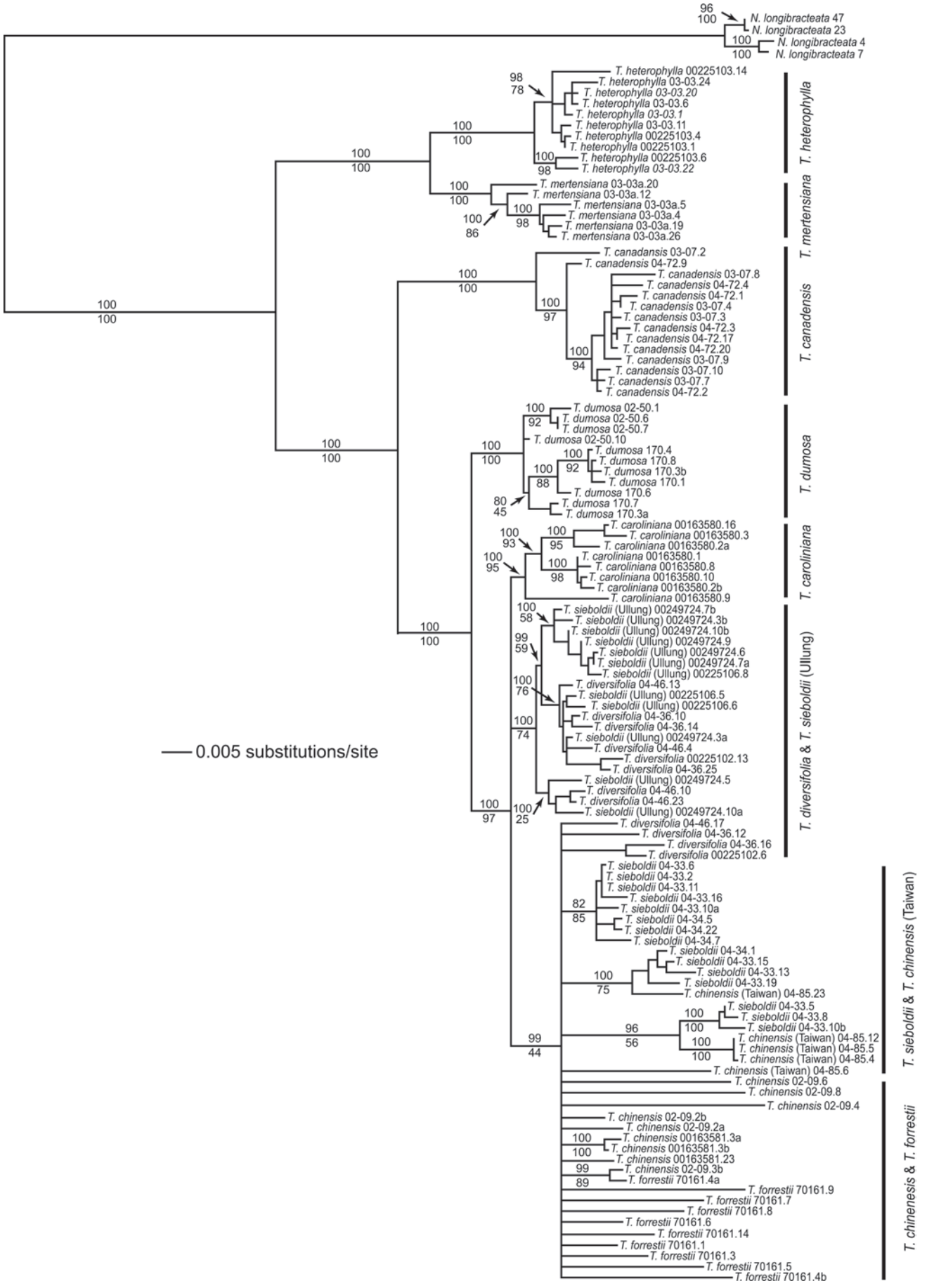

TABLE 1. Mean pairwise sequence divergence (Kimura two-parameter) with standard deviation for ITS1, 5.5S, and ITS2 clones within each taxon.

Taxon	No. individuals	No. clones per individual	ITS1	5.8S	ITS2	Combined
N. longibracteata	1	4	0.007 (0.005)	0.006 (0.007)	0.004 (0.003)	0.007 (0.004)
T. canadensis	2	7, 7	0.008 (0.006)	0.004 (0.005)	0.008 (0.005)	0.008 (0.005)
T. caroliniana	1	8	0.014 (0.008)	0.006 (0.005)	0.010 (0.008)	0.013 (0.007)
T. chinensis	2	3, 6	0.023 (0.011)	0.001 (0.002)	0.024 (0.015)	0.021 (0.010)
T. chinensis (Taiwan)	1	5	0.015 (0.010)	0.007 (0.006)	0.028 (0.022)	0.015 (0.011)
T. diversifolia	3	5, 5, 2	0.016 (0.006)	0.002 (0.003)	0.014 (0.007)	0.014 (0.006)
T. dumosa	2	4, 7	0.011 (0.005)	0.000 (0.000)	0.006 (0.005)	0.010 (0.005)
T. forrestii	1	10	0.025 (0.009)	0.010 (0.005)	0.021 (0.015)	0.023 (0.008)
T. heterophylla	2	4, 6	0.008 (0.004)	0.002 (0.003)	0.010 (0.007)	0.008 (0.004)
T. mertensiana	1	6	0.010 (0.004)	0.005 (0.004)	0.012 (0.011)	0.010 (0.004)
T. sieboldii	2	11, 4	0.019 (0.011)	0.004 (0.005)	0.014 (0.016)	0.017 (0.010)
T. sieboldii (Ullung)	2	9, 3	0.007 (0.003)	0.003 (0.004)	0.009 (0.006)	0.007 (0.003)

using the cpDNA data set ($p = 0.02$) between trees with *T. chinensis* (Taiwan) in the inferred position versus sister to *T. chinensis* from mainland China. Likewise, *T. sieboldii* (Ullung) did not group with *T. sieboldii* from Japan in either the ITS or cpDNA tree, but instead was sister to *T. diversifolia.* S-H tests found that the tree with *T. sieboldii* (Ullung) in the inferred position was significantly different from the tree with *T. sieboldii* (Ullung) sister to *T. sieboldii* from Japan using both the cpDNA ($p < 0.001$) and ITS data sets ($p < 0.001$).

S-H tests of the placement of the *Tsuga* root, with *T. mertensiana* and *T. heterophylla* as sister species versus with *T. mertensiana* sister to all remaining species, did not find a significant difference using the cpDNA data set ($p = 0.41$), but was significant using the ITS data set ($p < 0.001$).

Analysis with r8s using the sampled Bayesian cpDNA trees resulted in a mean rate of 0.0067 (± 0.0021 standard deviation) substitutions per site per million years. Estimates of divergence times (Table 2) and of biogeographic history (Fig. 4) reveal that the ancestral range of *Tsuga* comprised all four Northern Hemisphere areas until the late Eocene when a vicariance event separated the WNA lineage from the lineage in ENA, EUR, and EAS. The second divergence within *Tsuga,* dated to the Oligocene, resulted in a lineage in ENA leading to *T. canadensis* and a lineage with a widespread distribution in ENA, EUR, and EAS leading to the remaining species. This distribution was maintained until the divergence of *T. caroliniana* from *T. diversifolia* + *T. sieboldii* (Ullung). The *T. diversifolia* + *T. sieboldii* (Ullung) lineage in EAS split from the ancestor of *T. caroliniana* in ENA and EUR during the Miocene and extinction of the European trees left *T. caroliniana* in ENA.

DISCUSSION

ITS Variation—ITS clones of *Tusga chinensis*, *T. diversifolia*, *T. forrestii*, and *T. sieboldii* do not coalesce. A lack of coalescence of ITS clones has also been reported in closely related species in *Pinus* (Gernandt et al. 2001), *Larix* (Wei and Wang 2004), and *Picea* (Campbell et al. 2005). Lack of coalescence of some *Tsuga* ITS sequences may be due, in part, to incorrect taxonomic delimitation. This possibility is supported by strong evidence for non-monophyly of *T. chinensis* and *T. sieboldii* cpDNA sequences and only weak support for monophyly of *T. diversifolia* cpDNA sequences. The taxonomy of these species is discussed in more detail below.

ITS pseudogenes do not appear to be present in our data set, nor is there any obvious reason why PCR recombination (Cronn et al. 2002), if it has occurred, should be more prevalent in some species than others. Hybridization, which is more likely to occur between recently diverged *Tsuga* species (Taylor 1972; Wang et al. 1997; Bentz et al. 2002), could explain the high variation and lack of coalescence in *T. chinensis* and *T. forrestii*. *Tsuga sieboldii* (Ullung) is also recently diverged from *T. diversifolia*, but does not contain high sequence divergence, perhaps because of a bottleneck associated with island colonization and isolation from other *Tsuga* species.

Bouillé and Bousquet (2005) and Syring et al. (2007) indicated that retention of ancestral polymorphisms (incomplete lineage sorting) may be common for nuclear regions in the Pinaceae due to life history traits that lead to high effective population sizes. It also seems likely that concerted evolution, which is credited with homogenizing ITS regions in most plants, is retarded in the Pinaceae, possibly because of the separation of nuclear ribosomal DNA loci on numerous chromosomes (see Gernandt and Liston (1999) and Campbell et al. (2005) and references therein). Increased interspecific sampling and additional data from low-copy nuclear genes would be required to fully explore the influence of these factors.

While ITS has limited utility in resolving phylogenetic relationships among closely related taxa of *Tsuga*, it is also clear that ITS reliably infers deeper phylogentic relationships in the genus, as indicated by strong congruence between most of the deeper nodes in our ITS and cpDNA topologies.

Phylogeny and Taxonomy—*Nothotsuga longibracteata*, a native of southeastern China, was originally described as *T. longibracteata* (Cheng 1932). The latter designation is considered by some to be valid (e.g. Fu et al. 1999), but segregation into a monotypic genus is more widely accepted (e.g. Frankis 1989; Farjon 1990; Matsumoto et al. 1995). Vining (1999), Wang et al. (2000), and our cpDNA data (Fig. 3) show that *Tsuga* shares a more recent common ancestor with *Nothotsuga* than with the other outgroups. The percent sequence divergence between *N. longibracteata* and nearest *Tsuga* species is approximately twice the divergence between the two most distant *Tsuga* species (cpDNA: 2.6% versus 1.3%; ITS: 13.6 versus 7.4%) suggesting its use as an outgroup was well founded.

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FIG. 2. Bayesian 75% majority rule consensus tree of *Tsuga* ITS cloned sequences. Posterior probabilities are listed above and MP bootstrap values are below the branches. Herbarium voucher and clone numbers follow the species names (see Appendix 1).

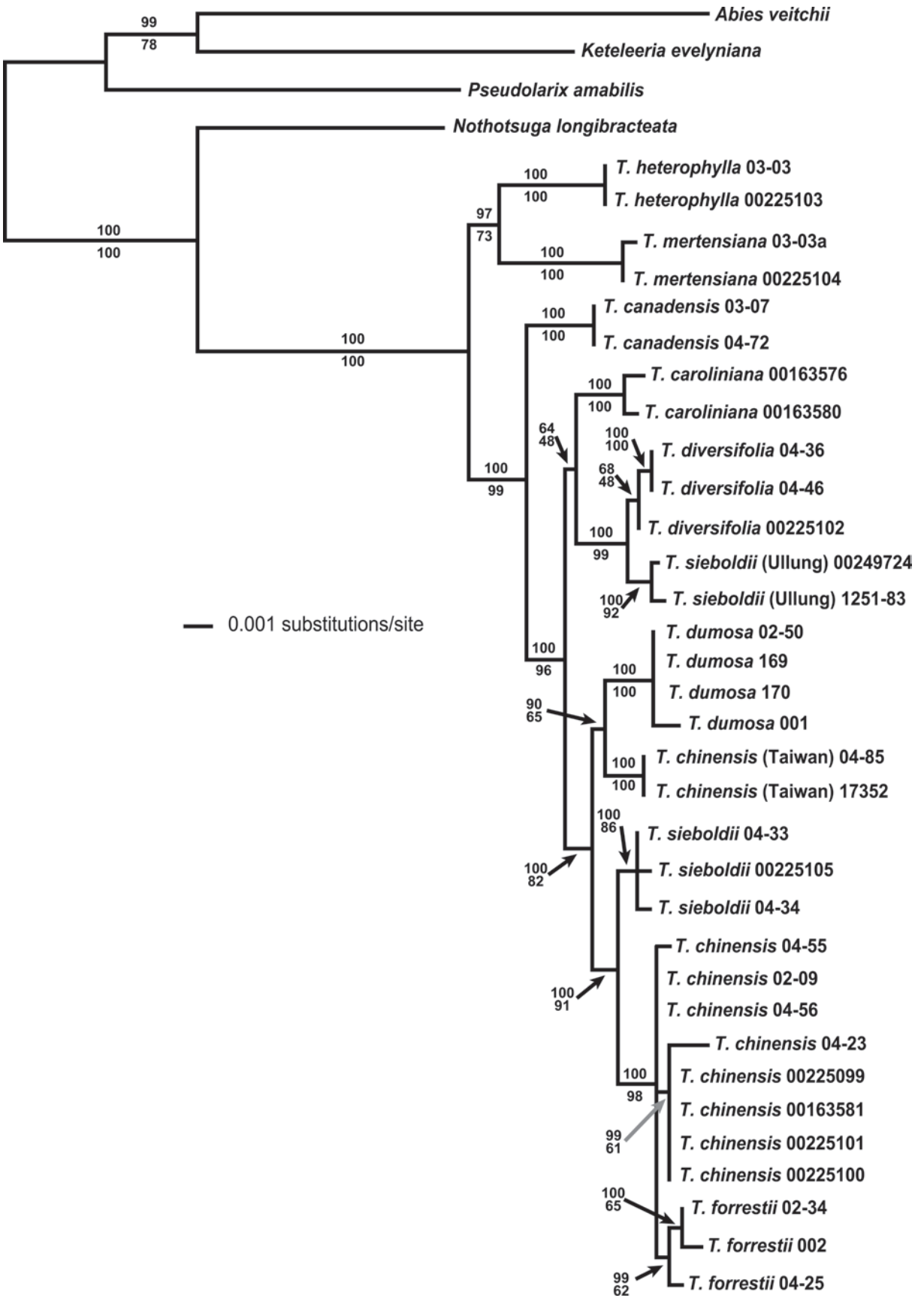

FIG. 3. Bayesian 50% majority rule consensus tree of *Tsuga* cpDNA. Posterior probabilities are listed above and MP bootstrap values are below the branches. Herbarium voucher numbers follow the species names (see Appendix 1).

TABLE 2. *Tsuga* divergence times (millions of years ± standard deviation) estimated by penalized likelihood using cpDNA data and calibrated using a 90 mya fixed age for the most recent common ancestor of *Nothotsuga* and *Tsuga* (Node B). Node designations are indicated in Fig. 4.

Node	Mean age ± standard deviation
A	171.26 ± 17.74
B	90
C	42.26 ± 8.02
D	34.62 ± 7.84
E	31.43 ± 8.46
F	24.73 ± 8.18
G	23.03 ± 8.08
H	8.76 ± 4.78
I	19.76 ± 7.63
J	16.79 ± 7.06
K	14.82 ± 6.84
L	4.84 ± 3.12

Tsuga mertensiana has been segregated to a monotypic section (Engelmann 1879; Dallimore and Jackson 1948; Farjon 1990) and the monotypic genus *Hesperopeuce* (Lemmon 1890). While use of the genus *Hesperopeuce* is not widely accepted (but see Ho and Sziklai (1972) and Page (1988)), the placement of *T. mertensiana* into its own section is generally recognized (Silba 1986; Farjon 1990; Vidakovic 1991; Flora of North America Editorial Committee 1993; Matsumoto et al. 1995).

We might have expected *T. mertensiana* to be sister to the remaining *Tsuga* species because it shares several apparently ancestral traits with other members of the Pinaceae, while these traits appear to be derived in the remaining *Tsuga* species. The inferred sister-group relationship of *T. mertensiana* and *T. heterophylla* implies either reacquisition of the ancestral traits in *T. mertensiana* or convergent loss of these traits in both *T. heterophylla* and the lineage leading to the remaining *Tsuga* species. For example, *T. mertensiana* has: (1) amphistomatic leaves, also found in *Nothotsuga, Abies*, *Cedrus*, and *Keteleeria,* versus hypostomatic leaves; (2) erect seed cones at pollination, also found in *Abies*, *Cedrus*, *Keteleeria,* and *Pseudolarix*, versus pendulous seed cones; (3) bisaccate pollen, found in all other Pinaceae except *Larix* and *Pseudotsuga,* versus monosaccate pollen. In addition, the pollination mechanism of *T. mertensiana* is more similar to *Abies* and *Cedrus* than to the remaining *Tsuga* species (Doyle 1945; Owens and Blake 1983; Owens et al. 1998; Fernando et al. 2005). To our knowledge, pollination mechanisms of *Nothotsuga, Pseudolarix,* and *Keteleeria* have not been described. In *T. mertensiana* the micropylar area is splayed out into a funnel where pollen lands on a pollination droplet. The micropyle faces down as a result of the cone being erect, and the buoyant sacs on the pollen cause it to float up into the ovule where it germinates. In contrast, *T. heterophylla* has no pollen drop, its ovules are not inverted because the cones are pendulous, and the surface of its pollen has minute spines that help the pollen adhere to the cone scales and bracts from which a long pollen tube grows to reach the ovule (Owens and Blake 1983; Owens et al. 1998). The pollination mechanism of *T. heterophylla* is considered to be typical of the other *Tsuga* species with monosaccate pollen (Owens et al. 1998), although, to our knowledge, the pollination mechanisms of the other species have not been examined in detail. While the sister relationship of *T. mertensiana* and *T. heterophylla* was supported by MP bootstrap and Bayesian posterior probabilities in all phylogenetic analyses (Figs. 2, 3), S-H tests that compare this relationship versus forcing *T. mertensiana* to be sister to all remaining *Tsuga* was significant for ITS, but not for cpDNA. The apparent discrepancies associated with this node could have resulted from historical hybridization or incorrect placement of the *Tsuga* root due to long-branch attraction. Data from additional nuclear regions may help to explain this result.

Tsuga caroliniana is not sister to *T. canadensis,* the other species from eastern North America. Rather it is nested in the Asian clade, a finding that is consistent with the capacity of *T. caroliniana,* but not *T. canadensis,* to interbreed with Asian *Tsuga* species (Bentz et al. 2002).

Tsuga from Taiwan was originally described as *T. formosana* (Hayata 1908) and later it was changed to *T. chinensis* var. *formosana* (Li and Keng 1954). The latter designation is commonly accepted (e.g. Li and Keng 1994; Fu et al. 1999), although Farjon (1990) did not favor treating it as a distinct variety because of a lack of diagnostic characters separating it from the mainland populations. Our results show *T. chinensis* (Taiwan) is not sister to or nested within *T. chinensis* from mainland China. This result, as well as the 15 bp insertion in the *trnT–F* region shared by *T. chinensis* and *T. forrestii*, but not by *T. chinensis* (Taiwan), supports its segregation as a separate species, *T. formosana*, rather than as a variety of *T. chinensis.*

Tsuga on Ullung Island has been consistently assigned to *T. sieboldii* (e.g. Wilson 1918; Kim 1988; Lee 1993; Lee 2001), but our results show it to be more closely related to *T. diversifolia*. Ullung is a small island in the Sea of Japan that formed from volcanic eruptions that began approximately 2.7 mya (Kim 1985). There are more than 30 endemic plant species on Ullung Island, which are thought to have diverged from sister species in Japan or mainland Korea (Sun and Stuessy 1998). Detailed analysis of inter and intra specific variation in *Tsuga* morphology is needed to further evaluate the taxonomic status of Ullung Island hemlock.

Several varieties of *T. chinensis* from mainland China (e.g. var. *tchekiangensis*, *robusta*, *oblongisquamata*, and *patens*), which are sometimes given species-level status (e.g. Cheng 1983; Fu et al. 1999), have been discriminated mostly by the size and shape of the needles and cones. It is not known whether these morphological variants correspond to evolutionarily or ecologically discrete units. We did not attempt to assign the samples of *T. chinensis* to varieties in our analysis. *Tsuga chinensis* individuals vary by only one to three substitutions in the cpDNA analysis, and there is no discernable pattern to differentiate the two individuals included in the ITS tree.

Tsuga forrestii has morphological characters that are intermediate between *T. dumosa* and *T. chinensis* (Farjon 1990), and it has been suggested that it may be a subspecies of either *T. dumosa* (Farjon 1990) or *T. chinensis* (Fu et al. 1999). Our cpDNA results suggest that *T. forrestii* may be nested within *T. chinensis,* and is not closely related to *T. dumosa.*

Tsuga dumosa appears in different positions in the cpDNA and ITS trees. Such discordance between cpDNA and nuclear DNA phylogenies has been presented as evidence for historical hybridization in other plant groups (e.g. Sang et al. 1997; Edwards et al. 2006). If the conflicting placement of *T. dumosa* in the cpDNA and ITS trees were the result of hybridization, then the paternal parent of *T. dumosa* would presumably have been Asian because its paternally inherited cpDNA is

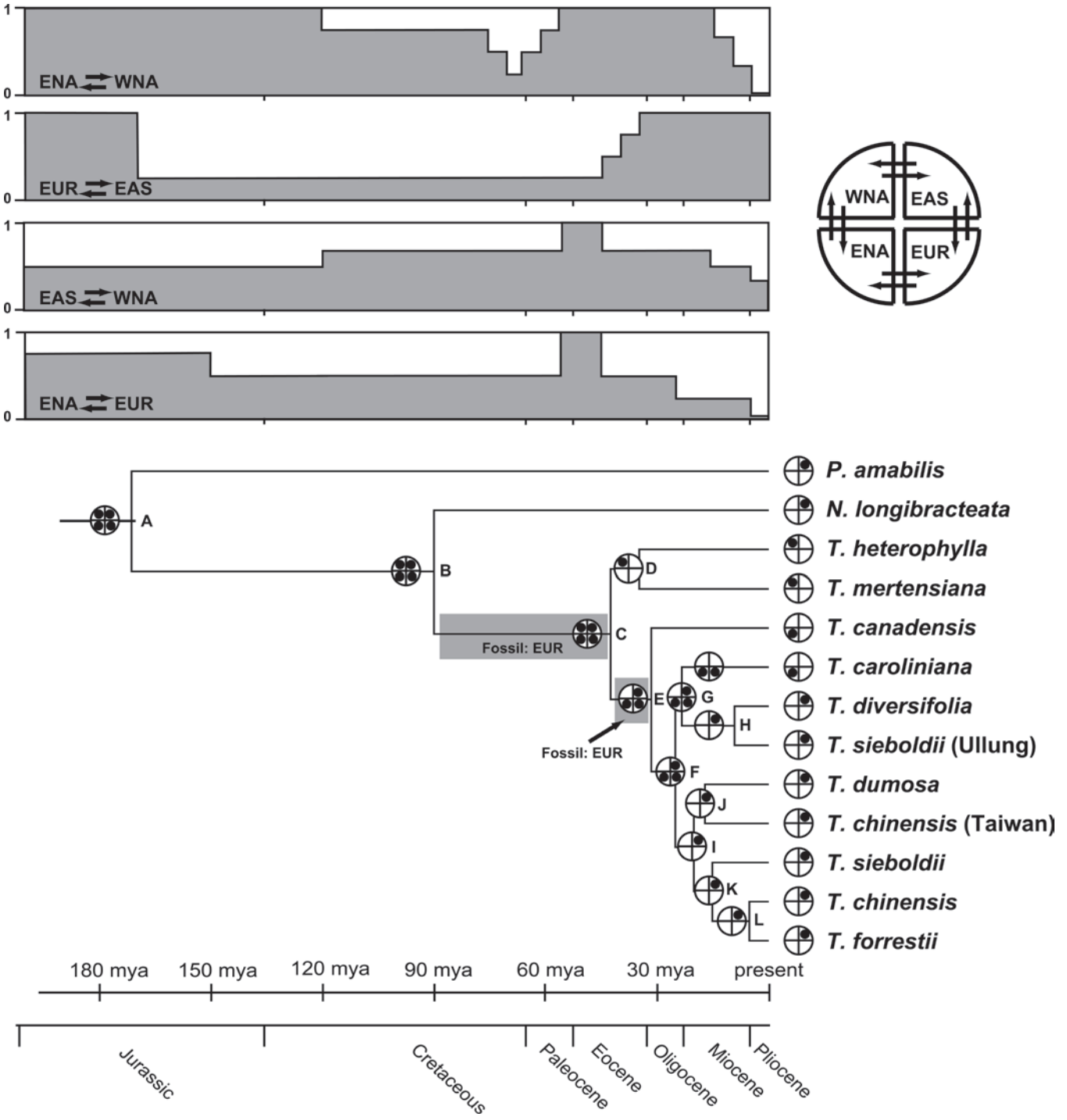

FIG. 4. Estimates of ancestral ranges for *Tsuga* from model-based likelihood inference using the software AReA. The graphs at the top show the paleogeographic model detailing the probability of connection among four geographic areas: eastern Asia (EAS), Europe (EUR), eastern North America (ENA), and western North America (WNA). Geographic distribution of extant species is shown on the tips of the tree. The tree topology and divergence times are derived from the cpDNA data set. Divergence times were calibrated with a fixed age of 90 mya for the split of *Tsuga* and *Nothotsuga*. Placement of European *Tsuga* fossils for AReA analysis are indicated on the tree, as are the resulting optimal estimates of ancestral range inheritance.

nested within an entirely Asian clade (Fig. 3). The geographic origin of its putative maternal parent is less clear. The ITS topology indicates that *T. dumosa* is sister to the clade containing the other Asian species and *T. caroliniana* from eastern North America. One possibility is that the maternal parent of *T. dumosa* was a member of a lineage that originated in Europe. This is consistent with the current distribution of *T. dumosa* as the western-most Asian species and closest to Europe. Although LePage (2003b) suggested that European populations of *Tsuga* were probably geographically isolated from east Asian populations during the late Oligocene and early Miocene, the lack of *Tsuga* fossils from the West Siberian Plain region does not preclude the possibility that European populations migrated east and that Asian and European populations of *Tsuga* successfully hybridized in eastern Asia. While assignment of *Tsuga* fossils to extant taxa should

be treated with caution, this scenario is consistent with the fossil record. Fossils said to resemble *T. dumosa* have been described from Oligocene through Pleistocene deposits in Europe (LePage 2003b), and from Pliocene deposits in Yunnan, China, that are within the current distribution of the species (Yi et al. 2005).

Historical Biogeography—Based on the fossil record, LePage (2003b) concluded that *Tsuga* was widespread during most of the Cenozoic with representatives in North America, Europe, and Asia and that their distributions became greatly reduced as global climate became cooler and drier during the late Tertiary.

Several of the closest extant relatives of *Tsuga* are exclusively found in eastern Asia, which might suggest that *Tsuga* had an origin in Asia. However, examination of the fossil record of these taxa show that they were all widely distributed in the past and therefore their modern ranges may have little to say about the area of origin for *Tsuga. Keteleeria* is currently found only in southern China, Laos, and Vietnam, but fossils are known from the Oligocene through Miocene in central Europe (e.g. Kirchheimer 1942; Kunzmann and Mai 2005) and western North America (e.g. Brown 1935; Lakhanpal 1958) as well as from the Miocene of China (Wang et al. 2006). *Pseudolarix*, which was found to be sister to the *Nothotsuga-Tsuga* clade by Wang et al. (2000), is currently found only in southeast China, but fossils are known in Asia, Europe, and western North American from the late Jurassic though to the Pliocene (LePage and Basinger 1995; LePage 2003a).

The fossil record of *Nothotsuga* is poor, with unequivocal reports only from the early Miocene of Germany (Kunzmann and Mai 2005), the Miocene (and possibly the Oligocene) of Russia (Karavaev 1958; Rayushkina 1979), and the Pliocene of Japan (Miki 1954, 1957). Based on these few reports we can only conclude that *Nothotsuga* was widely distributed during the Miocene and that it migrated west as far as Japan by the Pliocene.

Given the uncertainty in phylogenetic placement of *Tsuga* fossils, it is important to evaluate our molecular dating results in context with other related studies. Most studies place the divergence of extant Pinaceae genera between the late Jurassic and the early Tertiary (e.g. Miller 1976; Wang et al. 2000; LePage 2003a; Magallon and Sanderson 2005; Willyard et al. 2007). As discussed above, the inferred placement of the *Tsuga* root in our study implies reacquisition of bisaccate pollen in *T. mertensiana* or the convergent loss of sacs in both *T. heterophylla* and the lineage leading to the remaining *Tsuga* species. It is therefore not immediately clear whether the record of both bisaccate and monosaccate pollen from the late Cretaceous should constrain the *Tsuga* stem, crown, or other node.

If the *Tsuga* crown node is constrained to 90 mya, the most recent common ancestor of *Nothotsuga* and *Tsuga* is assigned an unreasonably early age of 310 mya using penalized likelihood (N. Havill, unpubl. data). Another calibration method that also results in unreasonably early ages relies on records of fossil pollen identified as *Cerebropollenites* (Couper) Nilsson. *Cerebropollenites* was first described as *Tsugaepollenites* by Couper (1958) and is similar in gross morphology and surface structure to monosaccate pollen of extant *Tsuga* (Batten and Dutta 1997; Shang and Zavada 2003). It has been suggested that *Cerebropollenites* is related to ancestral *Tsuga* (e.g. Batten and Dutta 1997; Shang and Zavada 2003), although megafossils have not been linked to it. *Cerebropollenites* first appears in the fossil record in the early Jurassic around the Hettengian-Sinemurian boundary (ca. 197 mya) (Pederson and Lund 1980; Batten and Koppelhus 1996), and its presence has been used to date other fossils, such as those of dinosaurs, to this time period (Martill et al. 2000). The presence of *Cerebropollenites* in the fossil record has also been used to delineate the *Cerebropollenites* Palynofloral Province, a distinct formation in the wet and cool circumpolar climatic zone dominated by gymnosperms and pteridophytes that was present in North America, Europe, and east Asia during the early Cretaceous (Srivastava 1994; Herngreen et al. 1996; Nichols 2003). If the *Tsuga* stem is constrained to 197 mya based on these records, the basal *Tsuga* crown divergence is assigned an age of approximately 62 mya and the most recent common ancestor of *Pseudolarix* and *Nothotsuga* is assigned the unlikely age of approximately 370 mya (N. Havill, unpubl. data). *Cerebropollenites* may not be closely related to *Tsuga* as previously suggested.

Constraining the *Tsuga* stem to 90 mya, as we report here, results in an age of 171 mya for the most recent common ancestor of *Pseudolarix* and *Nothotsuga* (Table 2; Fig. 4) which is consistent with the oldest record for *Pseudolarix* in the late Jurassic (LePage and Basinger 1995; LePage 2003a). This method also results in a basal *Tsuga* crown diversification during the Eocene that agrees with the proliferation of fossil pollen, seeds, and cones resembling *T. mertensiana*, *T. heterophylla*, and *T. canadensis* from this time (LePage 2003b).

Our results using likelihood-based biogeographic inference contrast with those using dispersal-vicariance analysis (Ronquist 1997) using the software DIVA (Ronquist 1996) which is commonly used to explore biogeographic history. Dispersal-vicariance analysis assumes that speciation occurs only by vicariance events and works by minimizing the number of dispersal and extinction events needed to explain the distribution pattern of extant species. In contrast, likelihood-based inference using AReA incorporates additional speciation scenarios that allow maintenance of widespread ranges through speciation events. AReA also allows direct incorporation of fossil data, which is only possible in DIVA by artificially considering them to be terminal taxa. This is valuable for inferring the history of *Tsuga* because the fossil evidence indicates *Tsuga* was extensive in Europe, but later went extinct. As such, Europe is an important area to include because it has been connected to east Asia and North America at various times in the past.

The results of DIVA reconstruction for *Tsuga* (N. Havill, unpubl. data) infer that the ancestor of the largely Asian clade that includes *T. caroliniana* was present only in eastern Asia. In this case, the presence of *T. caroliniana* in eastern North America must be explained by dispersal from Asia to North America, possibly through the Beringian Corridor. DIVA, by not allowing the retention of widespread ancestral ranges, may be more likely to yield an "out of Asia" pattern. Movements from Asia to North America have been inferred for other temperate plant clades, and this has been viewed as a common pattern (Donoghue and Smith 2004). Our results for *Tsuga* suggest that such inferences might depend heavily on the method of analysis.

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APPENDIX 1. Voucher specimens: locality; herbarium voucher; botanical garden accession (if applicable), and GenBank accession numbers ([1]*matK*, [2]*rpl16*, [3]*trnT–L*, [4]ITS). *Tsuga* nomenclature follows Farjon (1990). Botanical garden abbreviations: AA=Arnold Arboretum, RBGE=Royal Botanic Garden, Edinburgh, USNA = U.S. National Arboretum. Duplicates of Yale University Herbarium (YU) vouchers were deposited at the Institute of Botany, Chinese Academy of Sciences, Beijing (PE) or at the Osaka Museum of Natural History (OSA).

***Abies veitchii* Lindley**: Japan, Gifu; *Havill 04-40* (YU); [1]EF395569, [2]EF395379, [3]EF395416. ***Keteleeria evelyniana* Masters**: China; *Wang XQ2001-Ke* (PE); [1]EF395570, [2]EF395380, [3]EF395417. ***Pseudolarix amabilis* (Nelson) Rehder**: China, Zhejiang; *00225098* (A); AA 187-94-A; [1]EF395571, [2]EF395381, [3]EF395418. ***Nothotsuga longibracteata* Hu ex Page**: China, Hunan; *Luo ZC-002* (PE); [1]EF395572, [2]EF395382, [3]EF395419, [4]EF395453-EF395456. ***Tsuga canadensis* (L.) Carrière**: USA, Pennsylvania; *Havill 03-07* (YU); [1]EF395573, [2]EF395383, [3]EF395420, [4]EF395457-EF395463. USA, Connecticut; *Havill 04-72* (YU); [1]EF395574, [2]EF395384, [3]EF395421, [4]EF395464-EF395470. ***Tsuga caroliniana* Engelmann**: USA, North Carolina; *00163576* (A); AA 15803-J; [1]EF395575, [2]EF395385, [3]EF395422. Unknown wild source; *00163580* (A); AA 19447-O; [1]EF395576, [2]EF395386, [3]EF395423, [4]EF395471-EF395478. ***Tsuga chinensis* (Franchet) Pritzel in Diels**: China, Shaanxi; *Havill 02-09* (YU); [1]EF395577, [2]EF395387, [3]EF395424, [4]EF395479-EF395484. China, Hubei; *Havill 04-23* (YU); [1]EF395578, [2]EF395388, [3]EF395425. China, Sichuan; *Havill 04-55* (YU); [1]EF395579, [2]EF395389, [3]EF395426. China, Sichuan; *Havill 04-56* (YU); [1]EF395580, [2]EF395390, [3]EF395427. China National Tree Seed Co., unknown wild source; *00225099* (A); AA 100-94-CQ; [1]EF395581, [2]EF395391, [3]EF395428. China, Hubei; *00163581* (A); AA 17569-A; [1]EF395582, [2]EF395392, [3]EF395429, [4]EF395485-EF395487. China, Shaanxi; *00225100* (A); AA 243-2000-B; [1]EF395583, [2]EF395393, [3]EF395430. China, Shaanxi; *00225101* (A); AA 65-96-B; [1]EF395584, [2]EF395394, [3]EF395431. Taiwan, Taichung; *Havill 04-85* (YU); [1]EF395585, [2]EF395395, [3]EF395432, [4]EF395488-EF395492. Taiwan, Hualien; *Peng 17352* (HAST); [1]EF395586, [2]EF395396, [3]EF395433. ***Tsuga diversifolia* (Maximowicz) Masters**: Japan, Nagano; *Havill 04-36* (YU); [1]EF395587, [2]EF395397, [3]EF395434, [4]EF395493-EF395497. Japan, Yamanashi; *Havill 04-46* (YU); [1]EF395588, [2]EF395398, [3]EF395435, [4]EF395498-EF395502; Japan, Iwate; *00225102* (A); AA 1837-77-A; [1]EF395589, [2]EF395399, [3]EF395436, [4]EF395503-EF395504. ***Tsuga dumosa* (D. Don) Eichler**: China, Sichuan; *Havill 02-50* (YU), [1]EF395590, [2]EF395400, [3]EF395437, [4]EF395505-EF395508. Nepal, Singalia; *MacMillan 169* (E); RGBE 19790172; [1]EF395591, [2]EF395401, [3]EF395438. Bhutan, Wangchu; *MacMillan 170* (E); RBGE 19924259; [1]EF395592, [2]EF395402, [3]EF395439, [4]EF395509-EF395515. China, Sichuan; *S.E. Bentz 001* (NA); USNA 69969; [1]EF395593, [2]EF395403, [3]EF395440. ***Tsuga forrestii* Downie**: China, Yunnan; *Havill 02-34* (YU); [1]EF395594, [2]EF395404, [3]EF395441. China, Yunnan; *Havill 04-25* (YU); [1]EF395595, [2]EF395405, [3]EF395442. China, Sichuan; *S.E. Bentz 002* (NA); USNA 70161, [1]EF395596, [2]EF395406, [3]EF395443, [4]EF395516-EF395525. ***Tsuga heterophylla* (Rafinesque) Sargent**: USA, Washington; *Havill 03-03* (YU); [1]EF395597, [2]EF395407, [3]EF395444, [4]EF395526-EF395531. Canada, British Columbia; *00225103* (A); AA 22-94-C; [1]EF395598, [2]EF395408, [3]EF395445, [4]EF395532-EF395535. ***Tsuga mertensiana* (Bongard) Carrière**: USA, Washington; *Havill 03-03a* (YU); [1]EF395599, [2]EF395409, [3]EF395446, [4]EF395536-EF395541. Unknown wild source; *00225104* (A); A 693-77-A, [1]EF395600, [2]EF395410, [3]EF395447. ***Tsuga sieboldii* Carrière**: Japan, Osaka; *Havill 04-33* (YU); [1]EF395601, [2]EF395411, [3]EF395448, [4]EF395542-EF395552. Japan, Osaka; *Havill 04-34* (YU); [1]EF395602, [2]EF395412, [3]EF395449, [4]EF395553-EF395556. Japan, Tochigi; *00225105* (A); A 1007-80-B, [1]EF395603, [2]EF395413, [3]EF395450. Korea, Ullung Island; *00249724* (A); [1]EF395604, [2]EF395414, [3]EF395451, [4]EF395557-EF395565. Korea, Ullung Island; *00225106* (A); A 1251-83-A; [1]EF395605, [2]EF395415, [3]EF395452, [4]EF395566-EF395568.