PHYLOGENETIC RELATIONSHIPS OF *TORREYA* (TAXACEAE) INFERRED FROM SEQUENCES OF NUCLEAR RIBOSOMAL DNA ITS REGION

JIANHUA LI,[1] CHARLES C. DAVIS,[2] MICHAEL J. DONOGHUE,[3] SUSAN KELLEY,[1] AND PETER DEL TREDICI[1]

Abstract. *Torreya*, composed of five to seven species, is distributed disjunctly in eastern Asia and the eastern and western United States. In this study, interspecific relationships of *Torreya* were examined on the basis of sequences of the nrDNA ITS region. *Torreya taxifolia* and *T. californica* of the New World form a clade, as do the Old World species. This result suggests that the previous division of *Torreya* into two sections does not reflect phylogenetic relationships and that the diagnostic character for the two sections, namely, rumination of albumen, is homoplastic. It is possible that the present distribution of species of *Torreya* resulted from a single vicariance event separating the Old World and New World. However, the modern species are apparently young in age, and it will be necessary to integrate older fossils to ascertain the deeper biogeographic history of the genus.

Keywords: biogeography, phylogeny, Taxaceae, *Torreya*.

Five to seven species in *Torreya* Arn. are typically recognized, depending on the author (Cheng and Fu, 1978; Kang and Tang, 1995; Farjon, 1998).

Two *Torreya* species are present in North America, and the other three to five occur in Asia (Fig. 1; Li, 1952; Boufford and Spongberg, 1983; Wu, 1983; Axelrod et al., 1998; Wen, 1999). In the New World, populations of *T. taxifolia* Arn. are restricted to small areas along the Appalachicola River in Florida and Georgia, and this species has been listed as federally endangered (Burke, 1975; Stalter and Dial, 1984; Hils, 1996). *Torreya californica* Torr. is endemic to the coastal ranges and Sierra Nevada of California.

The eastern Asian species include *Torreya nucifera* (L.) Sieb. & Zucc., *T. grandis* Fort. ex Lindl., *T. jackii* Chun, *T. fargesii* Franch., and *T. yunnanensis* Cheng & Fu. *Torreya nucifera* is distributed in mountainous areas in central and southern Japan (Burke, 1975). The modern center of *Torreya* diversity is in the temperate and subtropical areas of China. *Torreya grandis* is widely distributed in eastern, central, and southwestern provinces, including Zhejiang, Fujian, Anhui, Jiangxi, Hunan, and Guizhou, whereas *T. jackii* occurs in a small area in Zhejiang. *Torreya fargesii* is found in several provinces, e.g., Anhui, Hubei, Henan, Shanxi, Gansu, and Sichuan, and *T. yunnanensis* occurs in Yunnan province, and probably in northern Burma.

Torreya is characterized by spiny leaf tips, a narrow stomatal band relative to the leaf midrib, and the production of two axillary female cones (Cheng and Fu, 1978). Cheng et al. (2000) have recently conducted a phylogenetic analysis of the Taxaceae using sequences of both nuclear and chloroplast DNA regions. Their results indicate that the two *Torreya* species sampled *(T. nucifera* and *T. californica)* form a clade, which is sister to *Amentotaxus* Pilger, a genus endemic to central, southern, and western parts of China, and northern Viet Nam. Similar results were obtained from sequences of chloroplast gene *mat*K by Wang and Shu (2000), who included *T. fargesii* and *T. grandis* in their phylogenetic analysis of Taxaceae and Cephalotaxaceae. Therefore, on the basis of both morphological and molecular evidence, *Torreya* appears to be monophyletic.

Hu (1927) studied Chinese *Torreya*s and recognized two sections on the basis of rumination

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[1]Arnold Arboretum of Harvard University, 125 Arborway, Jamaica Plain, Massachusetts 02130, U.S.A.

[2]Harvard University Herbaria, 22 Divinity Avenue, Cambridge, Massachusetts 02138, U.S.A.

[3]Department of Ecology and Evolutionary Biology, Yale University, P.O. Box 208106, New Haven, Connecticut 06520–8106, U.S.A.

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of the albumen. Sect. *Ruminatae*, including *Torreya jackii* and *T. fargesii,* is characterized by deeply ruminated albumen, whereas sect. *Nuciferae*, containing *T. nucifera* and *T. grandis,* is marked by only slightly ruminated albumen. Hu (1927) also recognized several forms and varieties of *T. grandis,* including f. *majus,* f. *non-apiculata,* var. *dielsii*, var. *sargentii*, var. *merrillii*, and var. *chingii*.

Torreya grandis has long been cultivated for its edible seeds in China, and many cultivars have been selected on the basis of the shape and size of the seed (Ching, 1927; Zen, 1935; Kang and Tang, 1995). These characters, however, vary greatly. Therefore, in their taxonomic treatment of Chinese *Torreya*s, Cheng and Fu (1978) combined all the varieties and forms, except for *T. grandis* cv. *Merrillii*.

The taxonomy of *Torreya fargesii* and *T. yunnanensis* has been controversial. Silba (1984) treated *T. fargesii* as a variety of *T. grandis,* and *T. yunnanensis* as a synonym of *T. grandis* var. *fargesii*. He later recognized *T. yunnanensis* as another variety of *T. grandis* (Silba, 1990). Kang and Tang (1995), on the other hand, recognized *T. fargesii,* and treated *T. yunnanensis* as a variety of *T. fargesii*.

In their worldwide review of *Torreya*, Kang and Tang (1995) recognized Hu's (1927) two sections, placing *T. californica* in sect. *Nuciferae* and *T. taxifolia* in sect. *Ruminatae*.

The objectives of this study were (1) to examine relationships of species of *Torreya* based on DNA sequence data, and (2) to consider taxonomic and biogeographic implications of the inferred phylogeny. We used sequences of the internal transcribed spacers of nuclear ribosomal DNA, as this region has been used successfully in estimating phylogenetic relationships of closely related species (e.g., Baldwin et al., 1995; Kim and Kim, 1999; Li et al., 1999, 2000; Liston et al., 1999; Gould and Donoghue, 2000).

Materials and Methods

Taxa

Fourteen individuals of *Torreya* were sampled in this analysis, representing the two sections and all species recognized by previous authors (Hu, 1927; Cheng and Fu, 1978; Silba, 1984, 1990; Kang and Tang, 1995). Table 1 lists collectors, sources of material, and accession numbers of the included species.

Molecular Techniques

DNAs were extracted from fresh leaves using the standard 2 X CTAB DNA extraction proto-

Table 1. Species used in this analysis.

Species	Source and AA Accession #	GenBank Accession #
Torreya taxifolia Arn.	Torreya State Park, Florida. 1441–85A	AF259271
	Florida. 71–97A	AF259272
	Flat Creek, Florida. 1051–89B	AF259273
T. californica Torr.	Mt. Tamalpais, California	AF259274, AF259275
T. grandis Fort.	Zhejiang, China. 1088–89A, 1088–89B	AF259276, AF259277
	China. 479–80C	AF259278
T. jackii Chun	Nanjing Bot. Gard., China. 780–94A	AF259282
T. nucifera S. & Z.	Japan. 490–97	AF259279
	Obara, Japan. 453–90A	AF259280
	Japan. 454–90A	AF259281
T. fargesii	Hubei, China. 14–97	AF259283
T. yunnanensis	Yunnan, China. 13–97	AF259284
Amentotaxus argotaenia (Hance) Pilger	China. 337–96	AF259285

Note: AA stands for the Arnold Arboretum collections. Vouchers are deposited in A, and all species except *T. californa* are cultivated at A.

col (Doyle and Doyle, 1987). Extracts were then purified using the Bio101 Geneclean Kit II (Bio101, Carlsbad, Calif.). Polymerase Chain Reaction (PCR) was conducted in a Perkin-Elmer thermocycler using the primers ITS4 and ITS5 of White et al. (1990), or ITS4 and ITS-Leu (see Baum et al., 1998). Each 25-μl reaction contained 2.5 μl of Taq polymerase reaction buffer, 4 μl of dNTP, 0.2 μl of Taq plolymerase (5U/μl), 0.8 μl of 50 mM $MgCl_2$, 1 μl of each of the two primers (10 μM), 50–100 ng of genomic DNA, 2 μl of DMSO, and an appropriate amount of sterilized distilled water. We sequenced the ITS region directly from PCR products for most of the sampled species; sequencing procedures are described elsewhere (Li and Donoghue, 1999). To check for multiple copies of ITS in *Torreya*, we cloned ITS PCR product for several species using the standard T-A cloning techniques. The pGEM®-T Easy Vector System (cat. #A1360, Promega, Madison, WI.) was used to ligate ITS PCR product into pGEM plasmids, which were then transformed into Epicurian Coli® XL1-Blue strain competent cells (cat. #200249, Stratagene, La Jolla, Calif.). Three white colonies for each selected accession were picked and cultured for 17 hours at 37°C, and their plamids were prepared using a Miniprep Kit (Qiagen, Santa Clarita, Calif.). Sequencing procedures for the plasmids were the same as for the PCR products.

Sequence Editing and Phylogenetic Analysis

For each accession, sequences generated using primers ITS4, ITS3B, ITS 5 or ITS-Leu (Baum et al., 1998), and 5.8Sgym (Liston et al., 1996) were edited using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, Mich.), and the ITS sequences were exported to PAUP* (version 4.0b3; Swofford, 2000). Sequences were readily aligned by sight. The limits of the ITS-1, 5.8S, and ITS-2 regions were determined by comparing published sequences in GenBank. We also sequenced the ITS region of *Amentotaxus argotaeda* (Hance) Pilger and included this species in parsimony analyses for rooting purposes. All characters were weighted equally, and their character states were treated as unordered; gaps were treated as missing data. Heuristic tree searches were carried out using PAUP* and search options included TBR branch swapping, simple sequence addition, MULPARS on, and steepest descent off. To evaluate branch support, we conducted 100 replicates of bootstrap analysis (Felsenstein, 1985) and obtained decay indices of up to five steps (Bremer, 1988; Donoghue et al., 1992).

Estimating Divergence Times

The maximum likelihood ratio (MLR) test was conducted using PAUP* to test whether the ITS region in *Torreya* evolved in a clocklike fashion (see Baum et al., 1998). One individual from each species except for *T. fargesii* (which is identical in ITS sequence to *T. yunnanensis*) was used in the MLR test, and several models were applied to the reduced data set, including F81, F84, and HKY (Baum et al., 1998). Rates of sequence evolution of the ITS region have been calibrated for Winteraceae (Suh et al., 1993) and *Robinsonia* (Sang et al., 1995). However, these rates differ by more than an order of magnitude, and there is no plausible reason to apply either one to *Torreya*. Fortunately, the rich fossil record of both *Torreya* and *Amentotaxus* (Florin, 1963; Cheng et al., 2000) enables us to independently calibrate the ITS base substitution rate in this group. Fossils of *Torreya* date to the middle Jurassic, and *Amentotaxus* has been reported from the late Cretaceous (Florin, 1963). On this basis it is likely that the two lineages diverged at least by the middle Jurassic (~165 mya).

RESULTS

In *Torreya* the ITS region ranged from 1158 to 1174 base pairs (bp) in length. The lengths of ITS-1 and ITS-2 were 762–791 and 235–237 bp, respectively, and the G+C content was between 60% and 62%. For *Amentotaxus argotaeda* the ITS region was 1040 bp long, and the lengths of ITS-1 and ITS-2 were 658 and 237 bp, respectively; the G+C content was 63%. The length of the 5.8S gene was 145 bp in all sampled species. The alignment of all sequences, including that of *Amentotaxus argotaeda*, required 84 indels, resulting in a data set of 1214 characters. Of the 225 variable characters, 40 were parsimony informative. All sequences have been submitted to GenBank (Table 1), and the data matrix and trees are available from the first author and in TreeBASE (http://www.harvard.edu/TreeBASE). Sequence divergences between *Torreya* and *Amentotaxus* ranged from 16.2% to 17.3%, with an average of 16.5%. Within *Torreya*, ITS sequences diverged from 0% between *T. fargesii, T. yunnanensis*, and two accessions of *T. nucifera*, to 3.6% between *T. taxifolia* and *T. grandis*.

Parsimony analyses generated three trees of 332 steps (CI = 0.95, RI = 0.91). A strict consensus tree is shown in Fig. 2. Two clades are strongly supported. The first contains the two North American species, *T. californica* and *T. taxifolia,* and the second includes all of the Asian species. Within the Asian clade, *T. jackii* and *T. grandis* form a clade, whereas *T. fargesii* and *T. yunnanensis* from China are allied with accessions of *T. nucifera* from Japan.

The MLR tests did not reject the hypothesis that the ITS region of *Torreya* and *Amentotaxus* evolved in a clockwise fashion (all $P > 0.35$). The substitution rate was calibrated as 5×10^{-10} bases per site per year, given a minimal divergence time between *Torreya* and *Amentotaxus* of 165 mya.

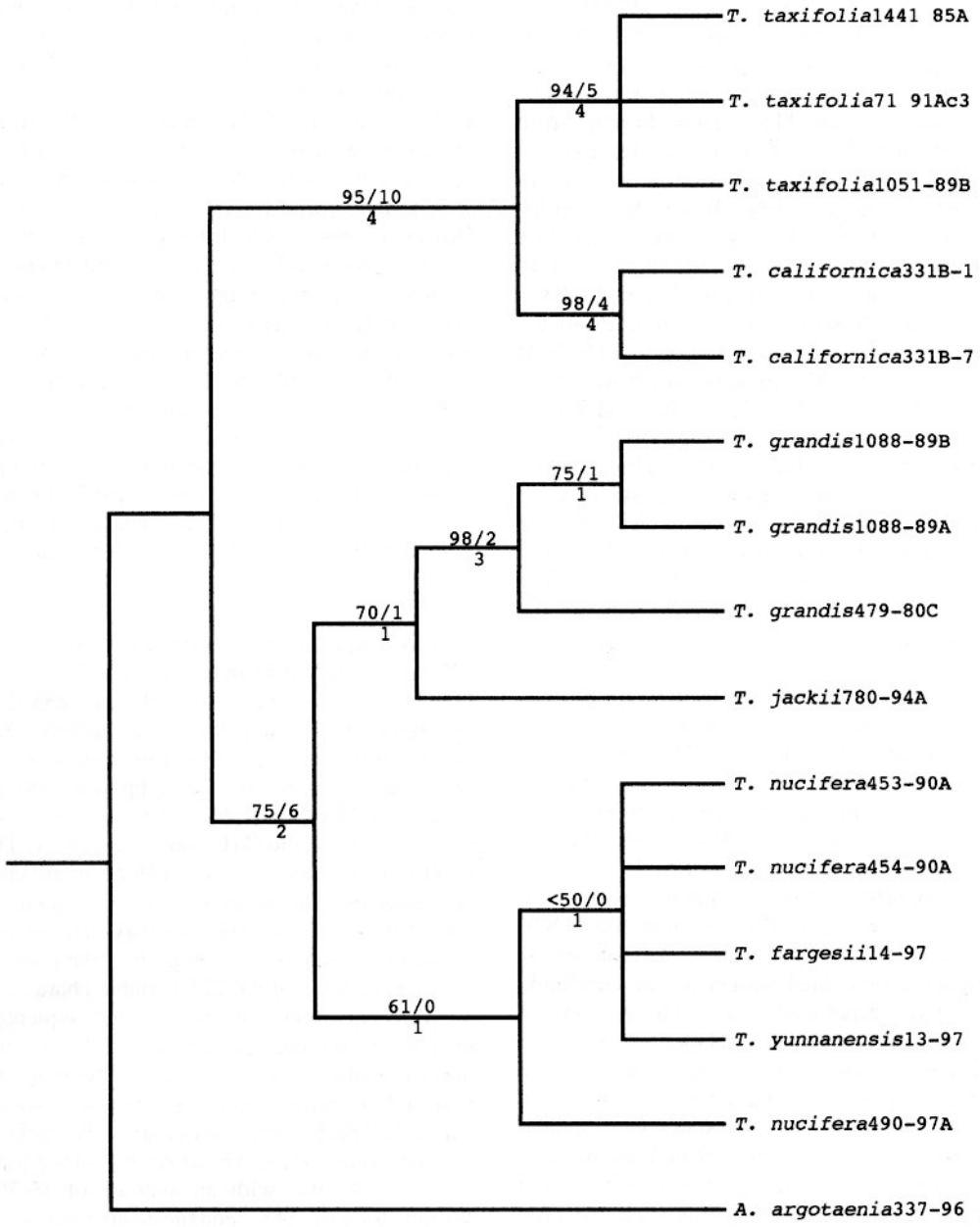

FIGURE 2. The strict consensus of three trees of 232 steps, based on sequences of nrDNA ITS (CI = 0.95, RI = 0.91). Numbers below and above the branches are decay values and bootstrap percentages/unambiguous base substitutions. Species names are followed by the accession numbers of the Arnold Arboretum.

Discussion

Sequence Characteristics

In conifers, the ITS region varies greatly in length in some groups, such as in *Pinus*, whereas in others there is little length variation, such as in *Cupressus* (Liston et al., 1996, 1999; Klein and Li, unpubl.). In *Torreya,* the ITS region is ~1170 bp in length, which agrees with the length estimate of 1200 bp based on RFLP data (Liston et al., 1996). The ITS-1 in *Torreya* is more than three times as long as the ITS-2, which corresponds to several other conifer groups (Gernandt and Liston, 1999).

Phylogenetic Relationships and Taxonomic Implications

The classification of *Torreya* has long been based on the rumination of the albumen (Hu, 1927; Cheng and Fu, 1978; Kang and Tang, 1995), and as a result two sections, *Ruminatae* and *Nuciferae* (= *Torreya*), have been recognized. The two North American species have been placed in separate sections because *T. californica* has ruminate albumen and *T. taxifolia* does not (Kang and Tang, 1995).

Our ITS trees do not support this traditional taxonomy of *Torreya*. In Fig. 2, the two North American species form a strongly supported clade (bootstrap = 95%; decay = 4). *Torreya fargesii,* which has ruminate albumen, is clustered with the Japanese species *T. nucifera,* which does not show distinct albumen rumination. Therefore, rumination of the albumen appears to be homoplastic in *Torreya*. As pointed out by Cheng and Fu (1978), albumen rumination is a variable character, and a thorough study is warranted to examine variation in this feature across all species of *Torreya*.

Cheng and Fu (1978) recognized both *T. fargesii* and *T. yunnanensis*. However, our specimens of these two species have identical ITS sequences, which supports the combination of the two taxa (Silba, 1984; Kang and Tang, 1995). Silba (1990) treated *T. fargesii* and *T. yunnanensis* as varieties of *T. grandis*. The ITS data, however, suggest that the former two species are more closely related to *T. nucifera* than to *T. grandis*. The virtual absence of ITS sequence divergence (0–0.8%) between *T. fargesii, T. yunnanensis,* and *T. nucifera* suggests that these species may best be treated as a single species.

Biogeographic Implications

Recognition of Hu's (1927) two sections of *Torreya* (Kang and Tang, 1995) implies that its modern distribution entailed at least two disjunctions between the Old World and New World. In contrast, our ITS phylogeny (Fig. 2) allows the possibility of just a single disjunction, perhaps the result of a single vicariance event separating the Old World and New World.

The oldest *Torreya* fossils have been recovered from the middle Jurassic deposits in southwestern Europe (Florin, 1963; Fig. 1), indicating that this clade had evolved by that time. In the middle

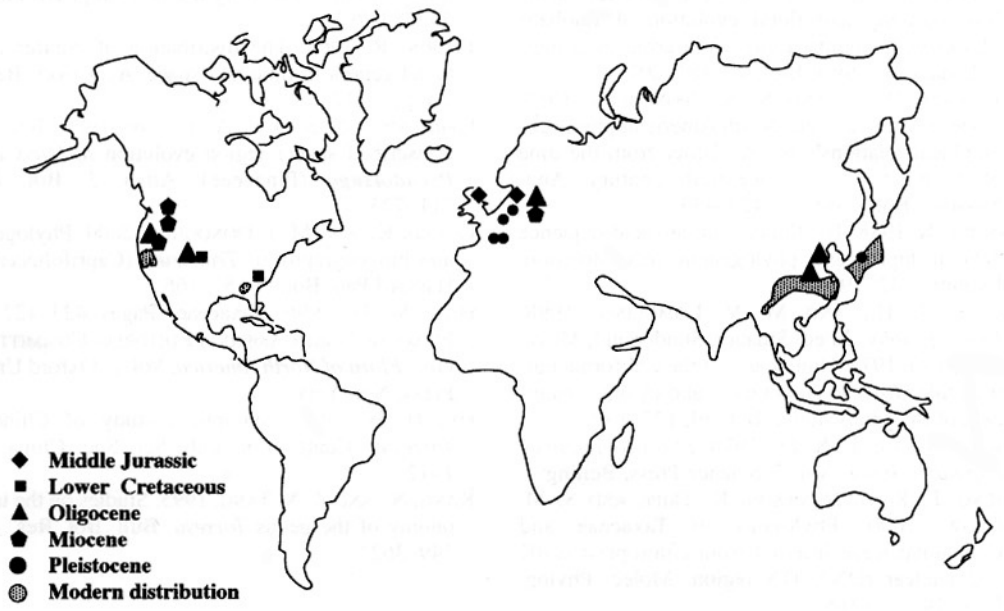

FIGURE 1. Fossil and modern distributions of *Torreya* (Florin, 1963; Li, 1995).

Jurassic the Turgai Sea was forming, separating Euramerica and Asia (see Brown and Lomolino, 1998). If disjunct populations of *Torreya* were created by the formation of the Turgai Sea, and the modern species were derived from these ancestral populations, we would expect high ITS sequence divergences between Asian and North American species. In contrast, the ITS sequences show rather little divergence, and the estimated time of divergence of the two species groups is only ca. 30 mya. On this basis it seems reasonable to reject the formation of the Turgai Sea as a relevant vicariance event.

Fossils of *Torreya* have been reported from the Lower Cretaceous to the Miocene deposits in the New World (Florin, 1963; Fig. 1). The separation of Europe from America started in the late Eocene, and the exchange of plant populations probably continued into the late Miocene via stepping stones (Tiffney, 1985). Our estimated time of divergence between Old World and New World *Torreya* species is ca. 30 mya (the early Oligocene). On the basis of the fossil record, *Torreya* does not appear in Asia until the Oligocene (Li, 1995); migration from Europe to Asia might have been facilitated by the land connection created by the disappearance of the Turgai Sea. In Europe, *Torreya* populations are known to have existed up to the end of Pleistocene (Florin, 1963). Climatic cooling and glaciation in the Quaternary eliminated many plant groups from Europe (Tiffney, 1985), apparently including *Torreya*. The modern species are apparently young in age, and it will be necessary to integrate older fossils to ascertain the deeper biogeographic history of this genus.

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