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Torreya jackii (Taxaceae): A Special Species that is Genetically Admixed, Morphologically Distinct, and Geographically Sympatric with Parent Species

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Abstract: *Torreya jackii* Chun is an endangered species (Taxaceae) confined to a few localities in China. However, the species status of *T. jackii* within *Torreya* Arn. has not been clearly elucidated under a phylogenetic context. In this study, phylogenetic analyses based on the nuclear internal transcribed spacer (ITS) and amplified fragment length polymorphism (AFLP) indicated that *T. jackii* is closely related with a sympatric species *T. grandis* Fort. ex Lindl. that is present due to cultivation. However, analysis based on the concatenated sequences of seven chloroplast loci resolved *T. jackii* as the first branch within the genus. Given their overlapping distribution and synchronous blooming, we suggest that the plastid-nuclear incongruence was derived from the dilution of the nuclear genome of *T. jackii* by *T. grandis* via pollen-mediated introgression hybridization when the two species met due to cultivation. Introgressive hybridization is fairly common in plants but few cases have been recognized as independent species. Our study highlights the complexity of protecting endangered species and the need for caution to prevent the unreasonable expansion of economic crops into the distribution ranges of their wild relatives.

Keywords: introgressive hybridization; phylogeny; *Torreya grandis*; *Torreya jackii*

1. Introduction

Torreya Arn. is a genus in Taxaceae, which is distinguished by its completely enclosed seed within an aril and the production of two axillary female cones [1–3]. Fossils of this genus are found widely across the northern hemisphere and the earliest has been dated to a Jurassic deposit in Europe [4]. However, only six highly endangered members are extant and they have disjunct distributions in North America (NA) and eastern Asia (EA) [2]. Two species, *T. taxifolia* Arn. and *T. californica* Torr., are endemic in NA, while four species are present in EA comprising *T. nucifera* (L.) Sieb. et Zucc., *T. fargesii* Franch., *T. jackii* Chun, and *T. grandis* Fort. ex Lindl. The first three species in EA are confined to a few localities in Japan or China, and the latter is cultivated due to its edible seed and oil in some southern provinces of China, where it overlaps the range with *T. jackii* (Figure 1) [1].

The morphological divisions within *Torreya* are not congruent with the geographical distribution of the species. This genus is divided into two sections in the only proposed infrageneric system [5,6]. Sect. *Ruminatae* Hu is defined by deeply ruminated albumen and it contains two species from EA and one species from NA, whereas sect. Nuciferac Hu has only slightly ruminated albumen and it contains the remaining three species. It is unusual that species from the same continent are not morphologically approximated. Furthermore, despite their sympatry, *T. jackii* and *T. grandis* are morphologically distinct

and ascribed to different sections due to differences in the albumen [5]. Moreover, their leaves differ in length, where those of *T. jackii* are at least 10 cm whereas those of *T. grandis* are less than 6 cm [1].

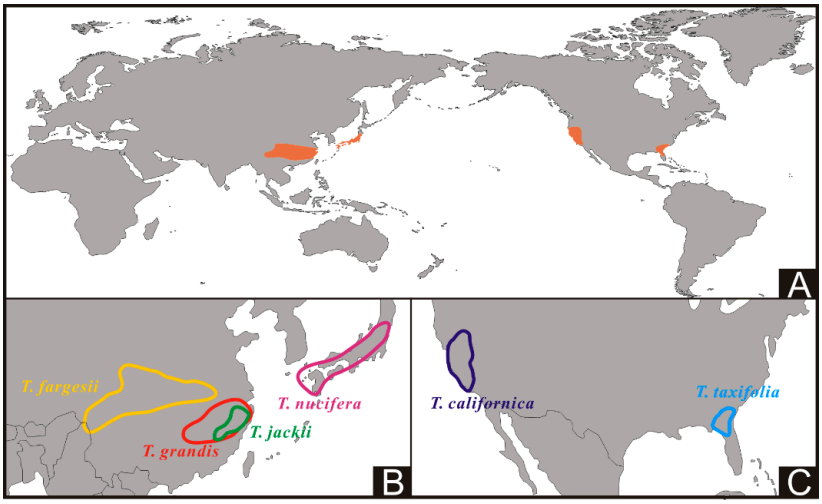

Figure 1. Geographical distribution of the extant *Torreya* species. (**A**), worldwide distribution (orange regions); (**B**), distribution in Eastern Asia; (**C**), distribution in North America.

The first comprehensive molecular phylogeny of *Torreya* reconstructed based on the nuclear internal transcribed spacer (ITS) contradicts the morphologically infrageneric division, but it is highly congruent with the geographical distribution [7] where the genera were resolved into two clades distributed in NA and EA. In addition, *T. jackii* and *T. grandis* were found to be closely related [7]. However, a subsequent study based on chloroplast (cp) DNA obtained different phylogenies by resolving *T. jackii* as the most basal clade [8]. Moreover, a second analysis based on ITS sequences that were mostly identical to those employed in the previous study found a different phylogeny [7,8]. These discrepancies in terms of the geographical distribution, morphological division, genetic information from different genomes, and analyses based on different methods or samples suggest that the genus *Torreya* seems to have a complex history, especially the conflict of phylogenetic position of *T. jackii* in the genus.

In the present study, compared with previous studies [7,8] we increased the sample size and analyzed more genetic information from both the chloroplast and nuclear genomes in order to explore the phylogenetic relationships within *Torreya*, mainly to elucidate the underling mechanism responsible for the incongruent phylogenetic context of *T. jackii* according to different lines of evidence.

2. Materials and Methods

2.1. Sample Collections

Leaf samples were collected from at least two individuals of all six species of *Torreya*. *T. jackii* and *T. grandis* were the main focus of the present study, and we collected six individuals from four localities and three individuals from three localities for these two species, respectively. *Amentotaxus argotaenia* (Hance) Pilger and *A. yunnanensis* Li were selected as outgroup based on previous studies [9]. Details of the samples, their voucher information, and GenBank accession numbers are listed in Table 1.

Table 1. Species, geographic origins, vouchers, and GenBank numbers used in this study.

Taxon	Geographic Origin/Voucher No.	GenBank No. (*mat*K, *rbc*L, *trn*L-*trn*F, *trn*D-*trn*T, *rpl*16, *psb*B_1-*psb*B_2, *trn*S-*trn*G, ITS)							
Torreya									
T. jackii -1	Xianju, Zhejiang/Liu and Noshiro 005	KJ588955	KJ589001	KJ589070	KJ589047	KJ589024	KJ588978	KJ589093	KJ588928
T. jackii -2	Shaowu, Fujian/Kou 0104	KJ588956	KJ589002	KJ589071	KJ589048	KJ589025	KJ588979	KJ589094	KJ588929
T. jackii -3	Jinyun, Zhejiang/Kou 1202	KJ588957	KJ589003	KJ589072	KJ589049	KJ589026	KJ588980	KJ589095	KJ588930-31
T. jackii -4	Xianju, Zhejiang/Kou 1506	KJ588958	KJ589004	KJ589073	KJ589050	KJ589027	KJ588981	KJ589096	KJ588932-32
T. jackii -5	Lishui, Zhejiang/Kou 1101	KJ588959	KJ589005	KJ589074	KJ589051	KJ589028	KJ588982	KJ589097	KJ588934-35
T. jackii -6	Lishui, Zhejiang/Kou 1102	KJ588960	KJ589006	KJ589075	KJ589052	KJ589029	KJ588983	KJ589098	KJ588936-37
T. grandis -1	Lin'an, Zhejiang/Liu and Noshiro 010	KJ588940	KJ588986	KJ589055	KJ589032	KJ589009	KJ588963	KJ589078	KJ588907
T. grandis -2	Zhuji, Zhejiang/Liu and Noshiro 013	KJ588941	KJ588987	KJ589056	KJ589033	KJ589010	KJ588964	KJ589079	KJ588908-09
T. grandis -3	Jinyun, Zhejiang/Kou 1208	KJ588942	KJ588988	KJ589057	KJ589034	KJ589011	KJ588965	KJ589080	KJ588910-11
*T. farges*ii -1	Jinfoshan, Chongqing/Liu and Noshiro 014	KJ588947	KJ588993	KJ589062	KJ589039	KJ589016	KJ588970	KJ589085	KJ588917-18
*T. farges*ii -2	Jinfoshan, Chongqing/Liu and Noshiro 016	KJ588948	KJ588994	KJ589063	KJ589040	KJ589017	KJ588971	KJ589086	KJ588919-20
*T. farges*ii -3	Shenlongjia, Hubei/Liu 200807-2	KJ588949	KJ588995	KJ589064	KJ589041	KJ589018	KJ588972	KJ589087	KJ588921-22
T. fargesii -4	Lijiang, Yunnan/Liu and Noshiro 018	KJ588945	KJ588991	KJ589060	KJ589037	KJ589014	KJ588968	KJ589083	KJ588914
T. fargesii -5	Weixi, Yunnan/Liu and Noshiro 019	KJ588946	KJ588992	KJ589061	KJ589038	KJ589015	KJ588969	KJ589084	KJ588915-16
T. nucifera -1	Forestry and Forest Products Research Institute, Japan/Nakais 200203	KJ588943	KJ588989	KJ589058	KJ589035	KJ589012	KJ588966	KJ589081	KJ588912
T. nucifera -2	Kunming Bot. Gard. (from Japan)/8	KJ588944	KJ588990	KJ589059	KJ589036	KJ589013	KJ588967	KJ589082	KJ588913
T. taxifolia -1	Kunming Bot. Gard./1062-89-c (Arnold Arborefum)	KJ588950	KJ588996	KJ589065	KJ589042	KJ589019	KJ588973	KJ589088	KJ588923
T. taxifolia -2	Kunming Bot. Gard./857-87*a	KJ588951	KJ588997	KJ589066	KJ589043	KJ589020	KJ588974	KJ589089	KJ588924
T. taxifolia -3	Kunming Bot. Gard./1054-89-I	KJ588952	KJ588998	KJ589067	KJ589044	KJ589021	KJ588975	KJ589090	KJ588925
T. californica -1	Philipps-Universität Marburg Bot. Gard./7	KJ588953	KJ588999	KJ589068	KJ589045	KJ589022	KJ588976	KJ589091	KJ588926
T. californica -2	San Francisco, California/Bruce Bartholomew, s.n. SFBG# XY-226	KJ588954	KJ589000	KJ589069	KJ589046	KJ589023	KJ588977	KJ589092	KJ588927
Amentotaxus									
A. argotaenia	Jinfoshan, Chongqing/3025	KJ588962	KJ589008	KJ589077	KJ589054	KJ589031	KJ588985	KJ589100	KJ588939
A. yunnanensis	Heilongtan, Kunming/3007	KJ588961	KJ589007	KJ589076	KJ589053	KJ589030	KJ588984	KJ589099	KJ588938

2.2. DNA Extraction, PCR Amplification and Sequencing

DNA was extracted from approximately 20 mg of leaves dried in silica gel using the cetyltrimethylammonium bromide (CTAB) method [10]. Seven cp gene loci, *rbc*L, *mat*K, *trn*L-*trn*F, *psb*B1-*psb*B2, *rpl*16, *trn*S-*trn*G, and *trn*D-*trn*T, and the nuclear internal transcribed spacer (ITS) were amplified with previously reported primers [11–14] according to the protocol described in our previous study [15]. PCR products from the ITS was purified using an Agarose Gel DNA Purification kit (TakaRa Inc., Dalian, China) and then cloned with the pMD19-T vector (TakaRa Inc., Dalian, China) according to the recommended protocol, before transformation into competent *Escherichia coli* JM109. The transformed bacteria were screened overnight on solid Luria–Bertani medium containing 100 mg/mL ampicillin at 37 °C. Five or more positive clones were amplified and sequenced using the universal primers M13-47 and RV-M. For all the cp loci, the primers used for amplification were employed for sequencing, which was conducted at the Beijing Genomics Institute. New sequences have been deposited in GenBank under the accession numbers listed in Table 1.

Amplified fragment length polymorphism (AFLP) analysis was conducted according to the protocol described in our previous study [16]. Selective amplification was performed using eight pairs of primer combinations, i.e., *Eco*RI-GTG/*Mse*I-CAA, *Eco*RI-GAG/*Mse*I-CTA, *Eco*RI-GTG/*Mse*I-CTA, *Eco*RI-GTG/*Mse*I-CTC, *Eco*RI-GAG/*Mse*I-CTG, *Eco*RI-GAT/*Mse*I-CTG, *Eco*RI-GTG/*Mse*I-CTG, and *Eco*RI-GTG/*Mse*I-CTT. Band data were collected using GeneScan 2.1 (Applied Biosystems, Foster City, CA, USA).

2.3. Data Analyses

Three datasets were constructed where one comprised the nuclear ITS sequences, the second contained the concatenated sequences of the seven cpDNA regions, and the third comprised the AFLP bands. Multiple alignments of sequence data were obtained using ClustalX 1.83 [17] and refined manually with MEGA 5.0 [18]. MEGA was also used to calculate the genetic distances according to the Kimura two-parameter model. The phylogenetic relationship was constructed using the maximum parsimony (MP) and maximum likelihood (ML) models implemented in PAUP 4.0 [19], and Bayesian inference (BI) in MrBayes 3.1.2 [20]. MP analyses were conducted by employing heuristic search to obtain the starting tree with stepwise, tree-bisection-reconnection (TBR) branch swapping, and the options of steepest descent, MulTrees, and Collapse. Bootstrap supports (BPs) were calculated by bootstrap analysis with 1000 replicates and the same settings as above. The optimal substitution models for the ML and BI analyses were found with ModelTest [21] based on Akaike's information criterion. The GTR+G+I model was selected for the seven combined cpDNA regions and the GTR+G model for the nuclear ITS gene. ML analyses were implemented via heuristic searches with 1000 replicates of random sequence addition, TBR branch swapping, and MulTrees. BPs were obtained using 1000 replicates by heuristic search with the same options. For BI, one cold and three hot Monte Carlo Markov chains were run twice for 2,000,000 generations, with sampling every 100 generations. Tracer v.1.5 (http://tree.bio.ed.ac.uk/software/tracer/) was used to choose a suitable burn-in period. PAUP program was used to calculate a consensus tree and posterior probabilities from the sampled trees after the burn-in period.

The multi-locus profiles of amplified fragment length polymorphism (AFLP) were scored for the presence (1) or absence (0) of fragments measuring between 50 bp and 300 bp. A neighbor-joining tree was constructed based on the Nei and Li distance [22] from the presence or absence matrix using the TREECON 1.3b program [23]. BPs obtained from 1000 pseudo-replicates were used as a measure of confidence in the reconstructed tree topology.

3. Results

The aligned lengths and variations in all the studied loci are listed in Table 2. The combined dataset of seven cp regions comprised 8241 bp, where 487 were parsimony-informative. The average

genetic distances between species are listed in Table 3 (upper right). The overall genetic distance was 0.28% while those within *T. grandis* and *T. jackii* were both 0. One to two different ITS sequences were found in each species, with a total of 33 sequences and a combined length of 1152 bp, where 212 were parsimony-informative. The average genetic distances between species are listed in Table 3 (lower left). The overall genetic distance was 1.17% while those within *T. grandis* and *T. jackii* were 0.06% and 0.32%, respectively. The AFLP dataset comprised 813 bands and 623 were variable, where 507 were parsimony-informative.

Table 2. Statistic for the sequences used in this study. PI: parsimony-informative sites; CI: consistency index; RI: retention index.

Loci	Length	Variable Sites	PI	CI	RI
ITS	1281	52	46	0.839	0.960
*mat*K	1434	14	13	1.000	1.000
*psb*B1-*psb*B2	1434	4	3	1.000	1.000
*rbc*L	1299	12	11	0.944	0.969
*rpl*16	962	13	11	1.000	1.000
*trn*D-*trn*T	1519	21	14	0.980	0.986
*trn*L-*trn*F	914	15	13	0.984	0.987
*trn*S-*trn*G	679	8	4	0.905	0.918

The MP strict consensus tree reconstructed with cpDNA datasets was largely consistent with those obtained by ML and BI in terms of the topology, as shown in Figure 2, where the support values from all three methods are indicated above the branches. The species *T. jackii* from EA branched off first with strong support, followed by the two NA species, *T. taxifolia* and *T. californica*, and the remaining *Torreya* species were resolved into two clades, which one contained *T. yunnanensis* and *T. fargesii* with strong support, and the other contained *T. grandis* and *T. nucifera* with moderate support.

The MP strict consensus tree (Figure 3) based on the nuclear ITS dataset recovered the same topology as the ML and BI trees. Compared with the tree obtained using the cp gene loci, the major difference was the position of *T. jackii*, which appeared in the base position according to the cp phylogeny, whereas it was closely related to *T. grandis* in the tree based on the ITS phylogeny. The AFLP data recovered an almost identical topology (Figure 4) to that using the ITS sequences, except *T. grandis* was resolved into polyphyly.

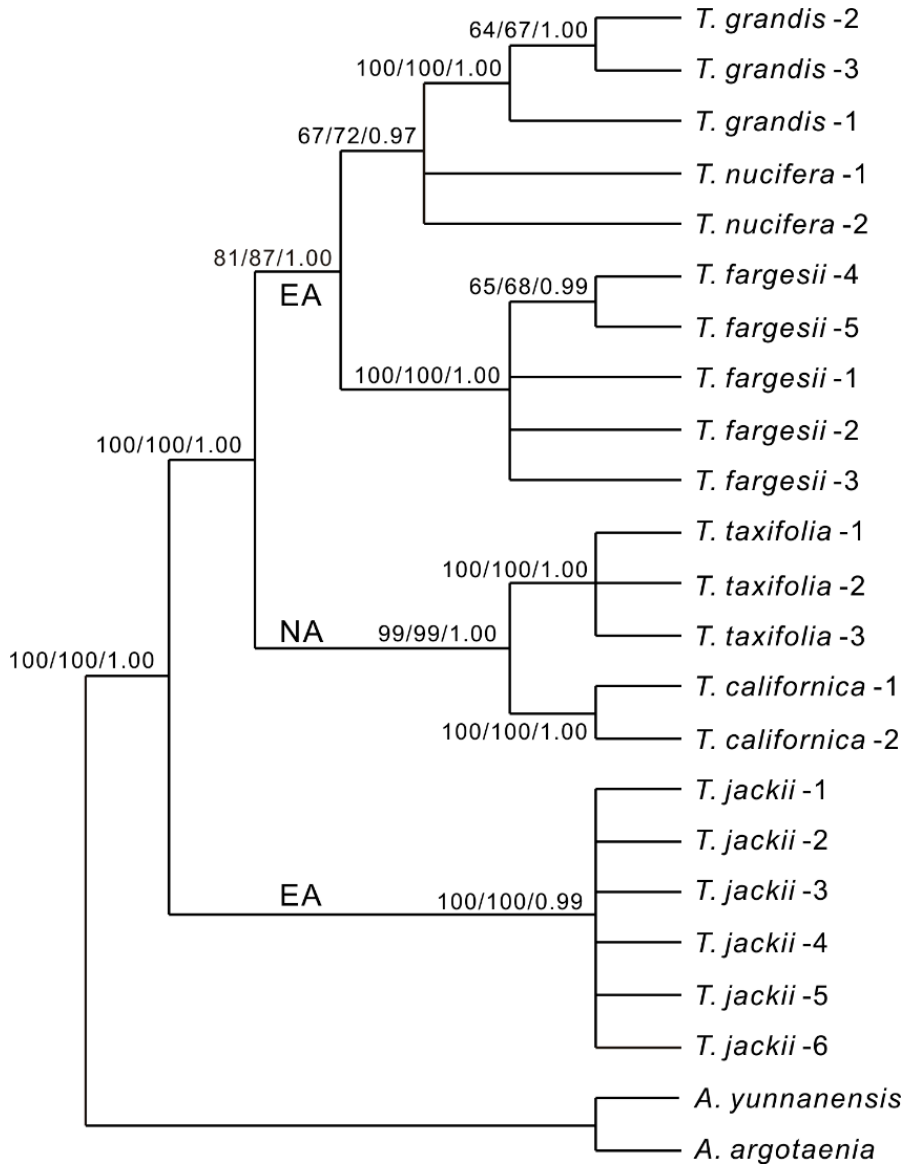

Figure 2. Strict consensus of the most parsimonious tree obtained for *Torreya* based on the seven combined chloroplast regions. Numbers above the branches denote the bootstrap values based on 1000 replicates from the maximum parsimony (MP) and maximum likelihood (ML) analyses, and the posterior probabilities from Bayesian analyses. EA, Eastern Asia; NA, North America.

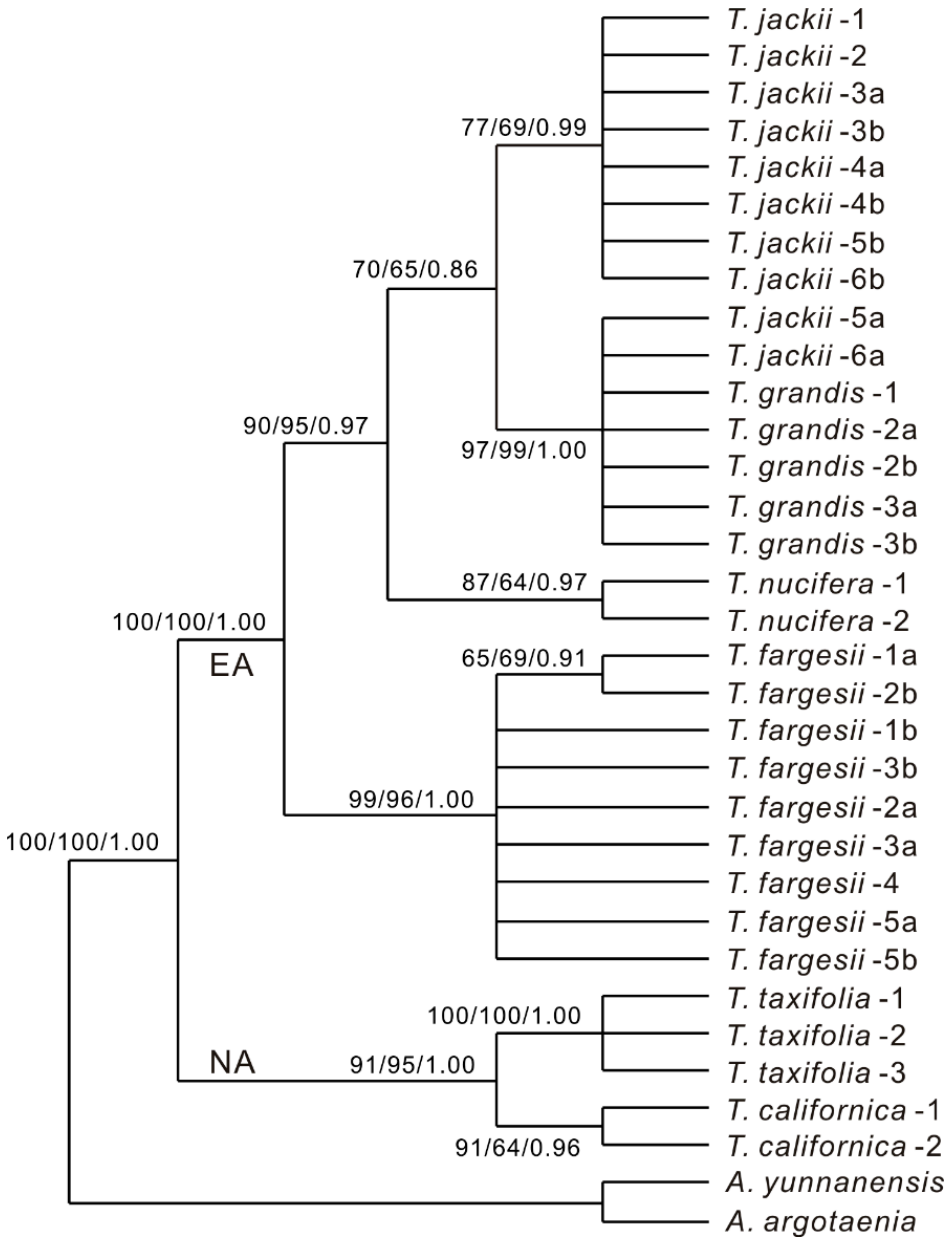

Figure 3. Strict consensus of the most parsimonious tree obtained for *Torreya* based on the nuclear internal transcribed spacer (ITS). The other details are the same as those in Figure 2.

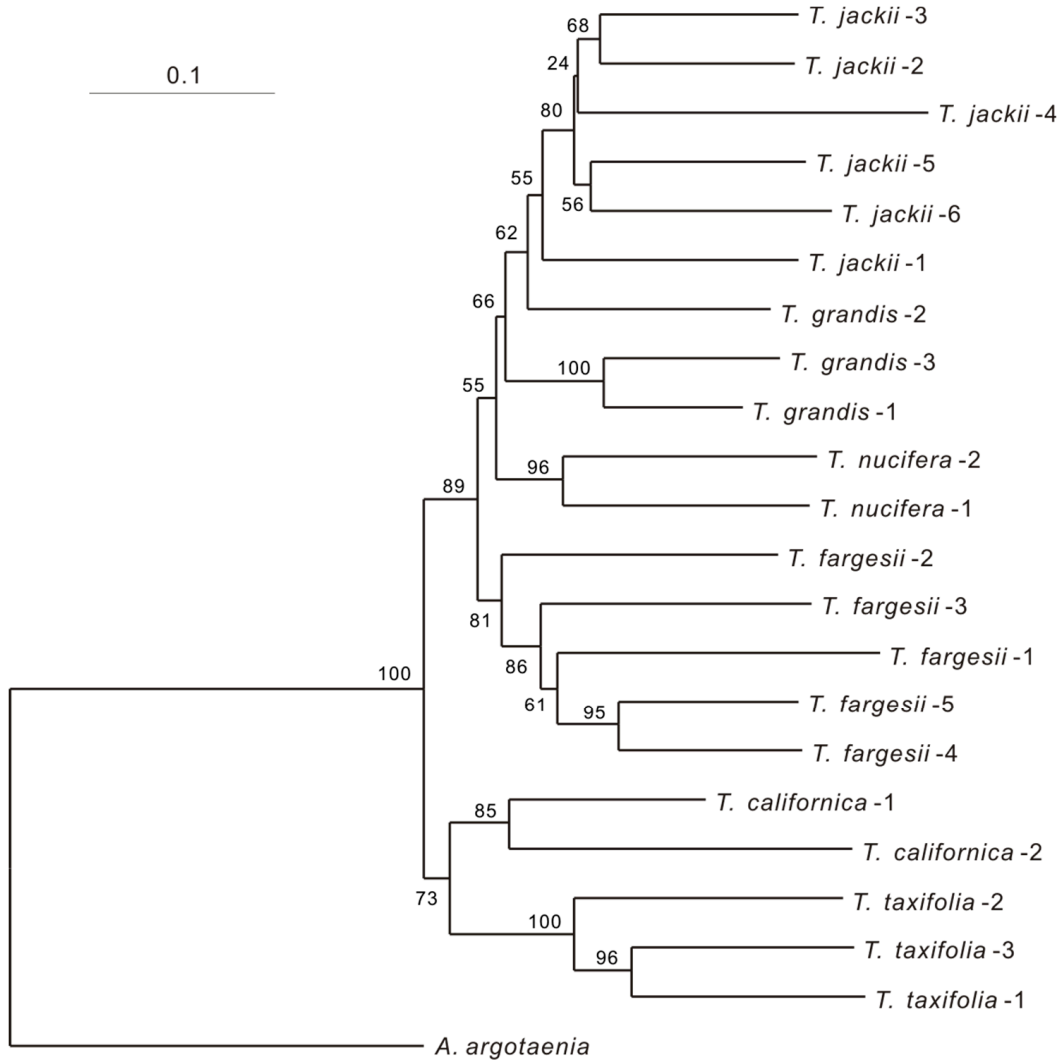

Figure 4. Neighbor-joining tree based on the amplified fragment length polymorphism (AFLP) data. Numbers above the branches denote the bootstrap values based on 1000 replicates from MP.

4. Discussion

Our phylogenetic analyses based on seven cp loci showed that *T. jackii* is the most basal branch of *Torreya*. The remaining species were resolved into two clades, where one comprised two species from NA and the other contained three Asian species, *T. grandis*, T. *fargesii*, and *T. nucifera* (Figure 2). Both ITS and AFLP, which are mostly representative of the nuclear genome, recovered a largely similar phylogeny (Figure 3), except that *T. jackii* was resolved as closely related to *T. grandis*. Among the six *T. jackii* sequences, two were identical to that in *T. grandis* whereas the other four differed by a distance of only 0.32%, which was much smaller than the overall mean distance (1.17%) for ITS sequences (Table 3). Incongruent phylogenies were also reported in a previous study based on different samples and analyses [7,8], thereby suggesting that technical issues such as insufficient taxon sampling, long branch attraction, or sequencing errors might not explain the incongruence [24].

Table 3. Pairwise distances (%) among ITS (lower left) and combined plastid (upper right) sequences from six *Torreya* species.

Plastid/ITS	*T. fargesii*	*T. nucifera*	*T. taxifolia*	*T. californica*	*T. grandis*	*T. jackii*
T. fargesii		0.20	0.42	0.40	0.27	0.33
T. nucifera	0.92		0.28	0.26	0.12	0.21
T. taxifolia	2.29	2.10		0.39	0.37	0.39
T. californica	1.90	1.72	0.68		0.35	0.36
T. grandis	1.35	0.74	2.54	2.16		0.33
T. jackii	1.20	0.60	2.39	2.01	0.57	

A number of biological factors could explain the incongruence between the phylogenies based on nuclear and cp DNA, including convergent evolution, lineage sorting, and introgressive hybridization [25]. Given our wide range of samples and the fact that we analyzed the cp and nuclear genomes, the first two explanations are unlikely to apply to *T. jackii*, but the last is difficult to reject [24]. Introgressive hybridization is a process where genes are transferred via the formation of an initial F_1 hybrid that subsequently crosses with individuals from one or both of the parental species [26,27]. The occurrence of this process requires that the two species meet some requirements including contact in space, overlapping blossoming periods, the same number of chromosomes, and the maternal inherence of cp DNA [26]. We found that *T. jackii* and *T. grandis* may satisfy all these requirements. First, *T. jackii* inhabits southern China where *T. grandis* is cultivated (Figure 1). In most localities, the two species are well within the distance of pollen dispersal, which is mediated by the wind in both species. Second, the male plants from both species blossom from late March until middle April, and the females from late March until early April [28–30]. Third, both species possess 11 chromosomes including one sex chromosome [31]. Finally, maternal inheritance is documented in *Torreya*, although the genome is usually inherited by the paternal donor in gymnosperms [32].

Both parents could be ancestors of the backcross, but a pattern that is often seen in instances of introgression is the asymmetric transfer of genetic material [33]. Therefore, one of the hybridizing lineages acts mainly as a donor and the other taxon as a recipient. It has been suggested that the quantity of pollen may be important and that pollen might be preferentially dispersed from a species with more pollen to one with less [34,35]. *T. grandis* produce a much higher quantity of pollen than *T. jackii* due to cultivation. Therefore, the female individuals of *T. jackii* (actually, its ancestor) might have been fertilized by the pollen from *T. grandis* to yield the F_1 hybrid, before multiple backcrosses with *T. grandis*. Finally, the genetic material from the mother donor was diluted over the generations to yield *T. jackii*.

Introgressive hybridization usually occurs between the ranges of two species and it leads either to speciation by adapting to a habitat different from those of the parents or extinction via genetic assimilation [26,27]. In each case, the offspring of hybridization are only a transient phase and they are seldom recognized as independent species. However, *T. jackii* is widely recognized as a species with little controversy, possibly due to its distinct morphology [1,2,36]. We suggest that this species is actually quite special and different from an ordinary species because it is not genetically or geographically isolated from one of the parents, and its cp donor or maternal ancestor might have become extinct, possibly due to the cultivation of *T. grandis* within the range of *T. jackii*. Therefore, we advise caution to prevent the unreasonable expansion of economic crops into the distribution ranges of their wild relatives, thereby highlighting the complex issues involved with the protection of endangered species.

5. Conclusions

In the present study, we explored the phylogenetic relationships of *Torreya* species based on the chloroplast and nuclear genome data, found the plastid-nuclear incongruent phylogenetic position of endangered species *T. jackii*, and elucidated the introgression hybridization responsible for the

incongruence due to the cultivation of sympatric species *T. grandis*. This finding implicates the complexity of protecting endangered species and the need for caution to prevent the unreasonable expansion of economic crops into the distribution ranges of their wild relatives.

Author Contributions: Y.-J.W. and Y.-X.K. designed the study, analyzed the data, and wrote the paper. K.X. and Y.-X.K. performed the experiments. All authors reviewed the manuscript.

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