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Original Research

Study of the genus *Torreya* (Taxaceae) based on chloroplast genomes

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Abstract

Background: Species of the genus *Torreya* are similar in morphology, and their morphological taxonomic characteristics are not stable because of environmentally induced changes. Therefore, morphology is insufficient for understanding their relationships. Chloroplast genome sequencing technology provides a powerful tool for molecular analysis to get more infomation for classification and identification of *Torreya* genus. **Methods**: A total of 4 chloroplast genome of *Torreya*, including *T. Parvifolia*, *T. nucifera*, *T. fargesii* var. *Yunnanensis* and *T. grandis* var. *jiulongshanensis*, were sequenced and annotated. Campartive genome and phylogenetic tree were provided for variation analysis. **Results**: The chloroplast genome size of the four samples is about 137 kb, the inverted repeat (IR) regions are identified in the genus *Torreya*. Genome comparison using mVISTA showed high sequence similarity among different species. Regions with divergence in exon regions include *accD*, *ndhB*, *ndhF*, *psbA*, *psbJ*, *rpl2*, *rps3*, *rps16*, *rps18*, *ycf1*, and *ycf2*. The phylogenetic tree based on 73 single-copy genes showed a clearer relationships among different species of *Torreya*. **Conclusions**: All genomes of the four *Torreya* species consist of two short IR regions, and results of the phylogenetic analysis concluded that *T. parvifolia* should be considered as *T. fargesii* var. *yunnanensis* or treated as a sister species. *T. grandis* var. *jiulongshanensis* should be treated as a variety of *T. grandis* according to molecular evidence, supporting the originally published proposal.

Keywords: *Torreya*; Chloroplast genome; Phylogeny; Single-copy orthologous genes; Molecular ecology

1. Introduction

The genus *Torreya* has a deep history, with the earliest fossils of the genus found in Europe being dated to approximately 170 million years ago, within the Jurassic period [2]. Because of the separation of continents, *Torreya* species were gradually distributed in North America and evolved distinct traits. Then, likely due to the drying up of the Turgai Sea, they migrated into Asia [2,3]. Because of climate change, human activity, and other factors, trees of this genus exist only in North America and East Asia, showing an obviously disjunct distribution.

The genus *Torreya* was originally proposed by Arnott in 1838, who published its type species *Torreya taxifolia* [4]. In 1846, the species of *Taxus nucifera* was designated by Siebold & Zucc, belonging to the genus *Torreya* [5]. *T. californica* was described in 1854 by John Torrey [6] and in 1857, *T. grandis* was published [7]. In 1899, Franchet published *T. fargesii* [8] whilst *T. jackii* was described in 1925 [9]. In 1975, *T. yunnanensis* was distinguished from *T. fargesii* in Flora Reipublicae Popularis Sinicae [10]. In 1995, Kang and Tang published *T. grandis* var. *jiulongshanensis* according to the morphological characteristics of the leaves. These authors also identified *T. fargesii* and *T. grandis* as two different species according to their morphological (especially endosperm) differences, geographic distributions and ecological characteristics. In addition, the authors considered Silba's change of *T. fargesii* to be a variety of *T. grandis* in 1984 as unreasonable and treated *T. yunnanensis* as a variety of *T. fargesii* [11]. In 2006, Yi *et al.* [12] published a new species of the Taxaceae, *T. parvifolia*. In 2010, Farjon placed *T. fargesii* as a species in the genus *Torreya* and treated *T. fargesii* var. *yunnanensis* as a variety of *T. fargesii*, which was consistent with the opinion of Kang and Tang (1995) and the classifications described in the Flora of China [13,14]. In 2017, based on a comparative analysis of leaf morphology in the genus, *Torreya*, *T. grandis* var. *jiulongshanensis* was treated as an independent species [15]. However, this variety was recently proven to be a natural hybrid species of *T. grandis* and *T. jackii* by analysis of gene fragments [16].

In summary, according to the latest classification data, there are currently 7 species and 2 varieties of the genus *Torreya* worldwide, which are distributed in East Asia and North America and have important economic and scientific

value [17–19]. *T. taxifolia* and *T. californica* are native to North America. *T. nucifera* is widely distributed in Japan, and arbors in the Korean Peninsula were introduced into China and planted as garden trees. There are 4 species and 2 varieties indigenous to China, namely, *T. parvifolia*, *T. fargesii* var. *yunnanensis*, *T. grandis* var. *jiulongshanensis, T. grandis*, *T. fargesii*, and *T. jackii*. *T. clarnensis* is an extinct species and was first described from a series of isolated fossil seeds in chert [20]. In this study, in order to determine the characteristics of *T. grandis* var. *jiulongshanensis*, chloroplast genomes of *T. nucifera* and *T. fargesii* var. *yunnanensis* were sequenced and compared by our team.

Phylogenetic relationships within the genus *Torreya* based on the nrDNA-ITS sequence, have strongly indicated that *T. fargesii* is closely related to *T. fargesii* var. *yunnanensis*, and there is no significant difference between *T. fargesii* and *T. nucifera*, suggesting that it is better to merge the two species into one [21]. Research using the *trnl-trnf* sequence supports combining *T. fargesii* var. *yunnanensis* into *T. fargesii* or treating it as a variety of *T. fargesii* [22]. Research using the *psbA-trnH* sequence combined with endosperm characteristics supports the treatment of *T. fargesii* var. *yunnanensis* as a variety of *T. fargesii* [23].

Chloroplasts play an important role in plant photosynthesis, moreover, they encode many key proteins important in other metabolic processes [24]. In general, the genome of chloroplasts is typically divided into four regions, including a large single-copy (LSC) region, a small single-copy (SSC) region and two inverted repeat (IRa and IRb) regions [25–27]. Chloroplast genomes are usually between 120 kb and 170 kb in length [28]. The chloroplast genome is the most valuable reference point for understanding plant evolution and phylogenetic relationships [23] and can be used in molecular phylogenetic and molecular ecological studies because of its highly conserved nature [29]. Phylogenomic analysis based on chloroplast genomes of the genus *Torreya* in Asia and North America has been performed, illustrating that *T. fargesii* var. *yunnanensis* is the sister species of *T. nucifera* + *T. fargesii* [1].

At present, the species relationship within the genus *Torreya* is still unclear. In this study, we aim to use the chloroplast genome to conduct a phylogenetic analysis of genus *Torreya* in China. We sequenced the chloroplast genomes of *T. parvifolia*, *T. nucifera*, *T. fargesii* var. *yunnanensis* and *T. grandis* var. *jiulongshanensis*. This study focused on the chloroplast genome structure, similarities, and phylogenetic relationships of members of the genus *Torreya* in East Asia by using maximum likelihood (ML) and Bayesian inference (BI) methods based on single-copy orthologous genes. Clarification of the phylogenetic statuses of these species could help understand their relationships.

2. Materials and methods

2.1 Sampling and DNA extraction

Four samples from China were sequenced in this study, and other genomes of the genus *Torreya* in East Asia were downloaded from GenBank database for comparison analysis (Table 1). Healthy young leaves were collected from each plant, wiped clean, numbered and placed in Ziplock bags. A large amount of color-changing silica gel was used for rapid drying.

The Hi-DNAsecure Plant Kit DP350 (Tiangen Biotech, Beijing, China) was used for DNA extraction following the manufacturer's instructions (include manufacturer).

2.2 Genome sequencing, assembling, and annotation

PE150 paired-end sequencing was conducted on an Illumina HiSeq X Ten genomic sequencer (Illumina Inc., San Diego, California, USA) at Majorbio Company (Shanghai, China). All sequencing depths were over 100×. The subsequent analysis was based on clean reads with high quality.

MITObim v1.8 [30] was used to assemble the chloroplast genome with default parameters, and the final circular structure was formed manually. Annotations of these chloroplast genomes were performed by using online software GeSeq [31] and BLAST+ 2.9.0 [32] with an E-value of 1e-5. The genome map was illustrated by using OGDRAW [33]. Annotated chloroplast genome sequences were submitted to GenBank and raw data were uploaded to Sequence Read Archive (SRA) of NCBI and will be released upon publication. GenBank accession numbers of genomes used in this study are listed in Table 1.

2.3 Genome comparison

Seven chloroplast genomes of the genus *Torreya*, including the four new genomes sequenced in this work, were compared by using mVISTA [34,35] based on the Shuffle-LAGAN method [36].

The seven chloroplast genomes of *Torreya* species were also compared with BLAST Ring Image Generator (BRIG) v0.95 [37], and BLAST+ v2.9.0 was used with an E-value of 1e-5.

2.4 Phylogenomic analysis

Single-copy orthologous genes were selected from the results analyzed by OrthoFinder v2.2.7 [38,39]. We used MAFFT v7.45 for multiple sequence alignment with the L-INS-i strategy for more accuracy [40,41]. Then, JModelTest v2.1.1.0 [42,43] was used to find the best-fitting models for single-copy orthologous genes according to the Akaike information criterion (AIC), where the lowest value showed the best fit [44].

A phylogenetic tree based on ML with single-copy orthologous genes was built by using RAxML v8.2.4 [45] with the best-fitting model and 1000 bootstrap replicates. A BI-based phylogenetic tree was constructed with single-

Table 1. Sampling information, GenBank and SRA accession number.

Family	Species	Location	GenBank accession number	SRA accession number
Taxaceae	*T. parvifolia*	Wandun mountain, Wuyi township, Butuo county, Liangshan Yi Autonomous Prefecture, Sichuan province, China	MN244711	SRR10769481
	T. nucifera	Nanjing University, Nanking, Jiangsu province (Jinling University introduced the species from Japan), China	MN244713	SRR10768423
	T. fargesii var. *yunnanensis*	Weixi County, Diqing Tibetan Autonomous Prefecture, Yunnan province, China	MN244712	SRR10758697
	T. grandis var. *jiulongshanensis*	Jiulong Mountain Nature Reserve, Suichang County, Lishui, Zhejiang province, China	MN244714	SRR10758782
	T. grandis	Non available	NC_034806	-
	T. fargesii	China	NC_029398	-
	T. jackii	Non available	KX902234	-
	T. jackii	Hangzhou Botanical Garden, China	MK249064	-
	T. nucifera	Lushan Botanical Garden, China	MK249060	-
	T. fargesii var. *yunnanensis*	Lijiang, Yunnan, China	MK249061	-
	T. californica	Royal Botanic Garden Edinburgh, UK	MK249062	-
	T. taxifolia	Atlanta Botanical Garden, USA	MK249063	-
	Taxus baccata	Non available	NC_035066	-
	Taxus canadensis	Non available	NC_041499	-
	Cephalotaxus oliver	Wuhan Botanical Garden, China	NC_021110	-
	Cephalotaxus sinensis	Taibai Mountain, Shaanxi, China	MF977938	-
	Amentotaxus argotaenia	Wuhan Botanical Garden, China	NC_027581	-
	Amentotaxus formosana	Academia Sinica and Taipei Botanical Garden, Taiwan, China	NC_024945	-
Podocarpaceae	*Podocarpus lambertii*	Lages, Santa Catarina, Brazil	NC_023805	-

SRA, sequence read archive.

copy orthologous genes in MrBayes v3.2.7 [46–48], running for 2×10^6 generations. The phylogenetic tree was illustrated by FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and TreeGraph v2.15.0-887 beta [49].

3. Results

3.1 Features of the chloroplast genome

Results for the four sequenced genomes, including genome structure and GC content, are shown in Table 2. The genome sizes of *T. parvifolia, T. nucifera*, *T. fargesii* var. *yunnanensis,* and *T. grandis* var. *jiulongshanensis* are 136781 bp, 136955 bp, 136807 bp, and 137320 bp, with an average genome GC content of 35.49%, 35.46%, 35.49%, and 35.41%, respectively. Their large single-copy (LSC) regions are 97386 bp, 97516 bp, 97421 bp, and 114484 bp, respectively. Their small single-copy (SSC) regions are 38799 bp, 38841 bp, 38790 bp, and 22148 bp, respectively. The inverted repeat (IR) regions are 298 bp, 299 bp, 298 bp,

Table 2. Statistics of chloroplast genomes.

Statistics	*T. parvifolia*	*T. nucifera*	*T. fargesii* var. *yunnanensis*	*T. grandis* var. *jiulongshanensis*
Genome size (bp)	136781	136955	136807	137320
LSC (bp)	97386	97516	97421	114484
SSC (bp)	38799	38841	38790	22148
IRa (bp)	298	299	298	344
IRb (bp)	298	299	298	344
Total genome GC (%)	35.49	35.46	35.49	35.41
LSC GC (%)	35.24	35.22	35.24	35.57
SSC GC (%)	36.15	36.1	36.15	34.45
IR GC (%)	33.67	33.56	33.33	41.11

LSC, large single-copy region; SSC, small single-copy region; IR, inverted repeat region.

and 344 bp, respectively. All of the genomes have similar small IR regions.

Chloroplast genome maps of *T. parvifolia, T. nucifera*, *T. fargesii* var. *yunnanensis,* and *T. grandis* var. *jiulongshanensis* are shown in Fig. 1. *T. parvifolia, T. nucifera*, and *T. fargesii* var. *yunnanensis* are very similar in gene number, order and names. However, *T. grandis* var. *jiulongshanensis* has a different gene number, order and name, especially gene order. In contrast to the other three taxa, *T. grandis* var. *jiulongshanensis* lacks the *rps11* gene but has the *clpP* gene as a unique gene. The locations of some genes in the chloroplast genome maps differ from others. For example, in *T. grandis* var. *jiulongshanensis*, the *atpA*, *atpF*, *atpH*, *atpI*, *rpoB*, *rpoC1*, *rpoC2*, *rps2*, *rps4*, *psaA*, *psaB*, *psbC* and *psbD* genes are located in the LSC region, and the *ndhF* and *ycf1* genes are located in the SSC region. However, in the other three species, the former group are located in the SSC region, and the latter group are located in the LSC region.

3.2 Comparative genomic analysis

Seven chloroplast genomes of species in the genus *Torreya*, compared by mVISTA with the Shuffle-LAGAN method and annotated according to *T. jackii* MK249064, are shown in Fig. 2. Overall, the sequence similarity is high among these species, especially in exon regions. Regions with such divergence in exon regions include *accD*, *ndhB*, *ndhF*, *psbA*, *psbJ*, *rpl2*, *rps3*, *rps16*, *rps18*, *ycf1*, and *ycf2*. tRNA or rRNA genes with high levels of divergence include *trnT-GGU*, *trnR-UCU*, *trnF-GAA*, *trnL-CAA*, *trnQ-UUG*, *trnN-GUU*, *trnM-CAU*, *rrn4.5* and *rrn5*. A comparison of the chloroplast genomes of seven species of the genus *Torreya* at the level of whole genome sequence was analyzed using BRIG, providing information for identifying the unique sequence of the chloroplast genome (Fig. 3). The sequence of *T. jackii* MK249064 was selected as a reference.

3.3 Phylogenetic analysis

Seventy-three single-copy orthologous genes were selected (**Supplementary Table 1**), and then a phylogenetic tree was built based on the ML and BI methods with these genes using the best-fitting model GTR + GAMMA + I. The ML and BI phylogenetic trees show the same relationships among the *Torreya* species (Fig. 4). *T. parvifolia* MN244711 is located closely to *T. fargesii* var. *yunnanensis* MN244712, and they are included in a clade with *T. fargesii* var. *yunnanensis* MK249061. *T. fargesii* NC_029398 and *T. nucifera* MK249060 form a single clade. *T. parvifolia* MN244711 and *T. fargesii* var. *yunnanensis* MN244712 are close to the clade containing *T. fargesii* NC_029398 and *T. nucifera* MK249060. *T. grandis* var. *jiulongshanensis* MN244714 is close to *T. californica* MK249062, and this clade is close to *T. grandis* NC_034806. The clade including *T. grandis* var. *jiulongshanensis* MN244714, *T. californica* MK249062 and *T. grandis* NC_034806 is close to our sample of *T. nucifera* MN244713. *T. jackii* KX902234 and *T. jackii* MK249064 are located in the most exterior part of the phylogenetic tree. The genus *Torreya* forms one clade in the phylogenetic tree. Based on phylogenetic trees, *T. nucifera* MK249060 is close to *T. fargesii* NC_029398, different from the results of Zhang *et al.* [1].

4. Discussion

4.1 Chloroplast sequence structure and divergence

Statistics of the genomes of four *Torreya* species showed that the IR regions were very short, with only 298–344 bp. Shrinkage or loss of the IR region is not rare in plants, and in a study of *Pinus thunbergii*, the IR region was found to have been reduced to 495 bp [50]. Loss of the IR region occurred in all cases of *Pisum sativum* L., *Vicia faba* L. [51], *Glyptostrobus pensilis* [52] and *Cryptomeria japonica* [53]. Pinaceae and non-Pinaceae conifers independently lost different copies of IRs, according to comparisons of the junctions near LSC regions and residual IR copies among gymnosperms [54], while the lack of an IR copy was considered a derived characteristic common to all

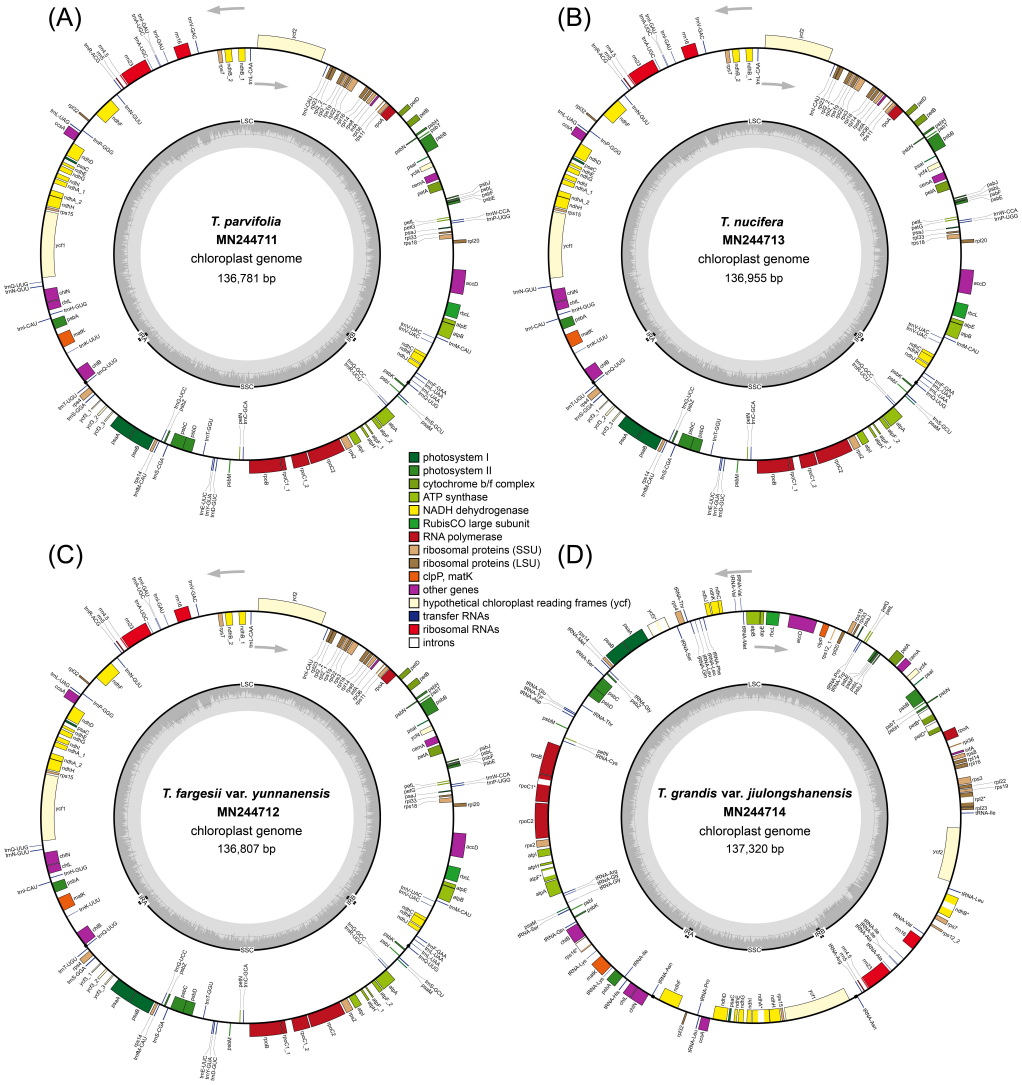

Fig. 1. Chloroplast genome maps of four species of the genus *Torreya* in East Asia. (A) The chloroplast genome map of *T. parvifolia*. Genes outside the circle transcribe counterclockwise, while those inside transcribe clockwise. Genes with different functions are marked with different colors. (B) The chloroplast genome map of *T. nucifera*. Genes outside the circle transcribe counterclockwise, while those inside transcribe clockwise. Genes with different functions are marked with different colors. (C) The chloroplast genome map of *T. fargesii* var. *yunnanensis*. Genes outside the circle transcribe counterclockwise, while those inside transcribe clockwise. Genes with different functions are marked with different colors. (D) The chloroplast genome map of *T. grandis* var. *jiulongshanensis*. Genes outside the circle transcribe counterclockwise, while those inside transcribe clockwise. Genes with different functions are marked with different colors.

conifers and "a single loss event defining the conifers as a monophyletic group" [55]. In addition, the four species shared similar GC contents (35.41%–35.49%). *T. parvifolia, T. nucifera*, and *T. fargesii* var. *yunnanensis* showed very similar chloroplast genome map structures, while *T. grandis* var. *jiulongshanensis* exhibited a very different order of genes. However, according to comparisons by both mVISTA and BRIG, there were no great differences among these species, especially in protein-coding regions. Therefore, this specificity could be due to genetic recombination.

4.2 Phylogenetic analysis

In a 2006 publication, *T. parvifolia* was treated as a new species [13]. However, based on the phylogenetic analysis reported here, *T. parvifolia* MN244711 and *T. fargesii* var. *yunnanensis* MN244712 have a close relationship, with a high correlation and form one clade with *T. fargesii* var. *yunnanensis* MK249061. Therefore, we believe that the species *T. parvifolia* should be considered as *T. fargesii* var. *yunnanensis* or treated as a sister species according to the molecular evidence. *T. nucifera* MK249060 was collected from Lushan Botanical Garden, China, and

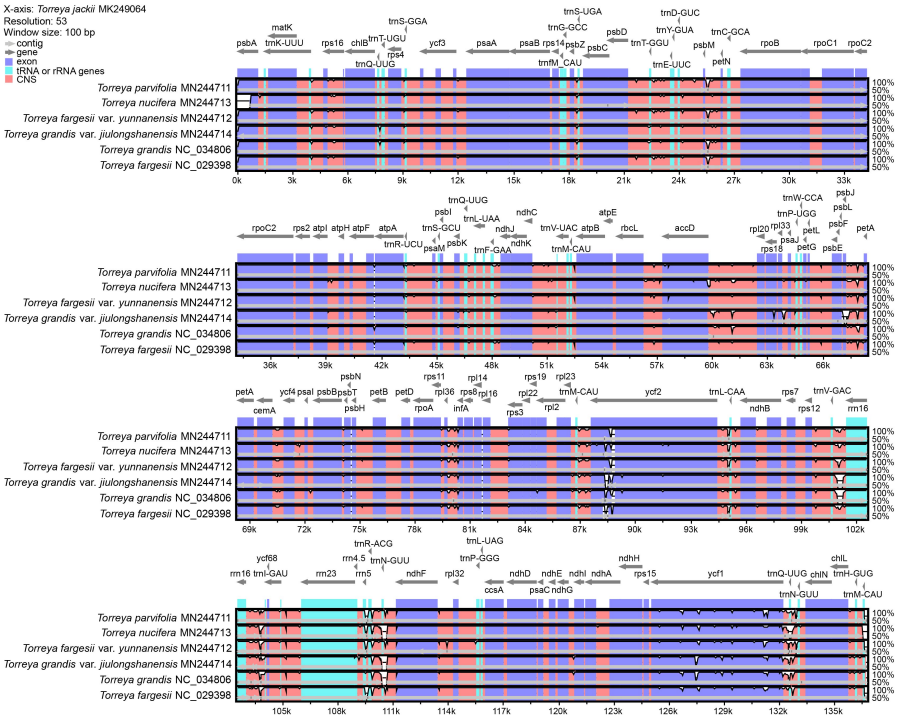

Fig. 2. Comparison of chloroplast genomes of seven species of the genus *Torreya* by using mVISTA based on Shuffle-LAGAN method. The sequence of *T. jackii* MK249064 is selected as reference. The thick gray arrow at the top of the array indicates gene orientations. The dark-blue regions, light-blue regions, and pink regions represent exon, tRNA or rRNA genes, and conserved non-coding sequences (CNS), respectively.

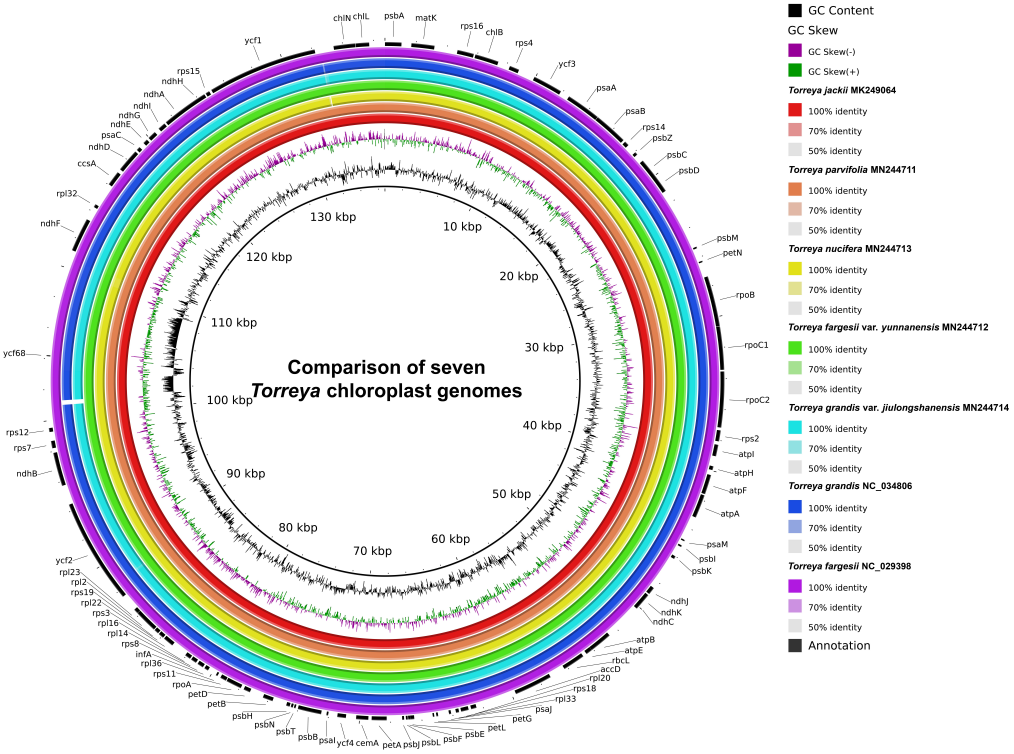

Fig. 3. Comparison of chloroplast genomes of seven species of the genus *Torreya* by using BRIG. The sequence of *T. jackii* MK249064 is selected as reference. Two of the innermost rings show GC content and GC skew from inside to outside, respectively.

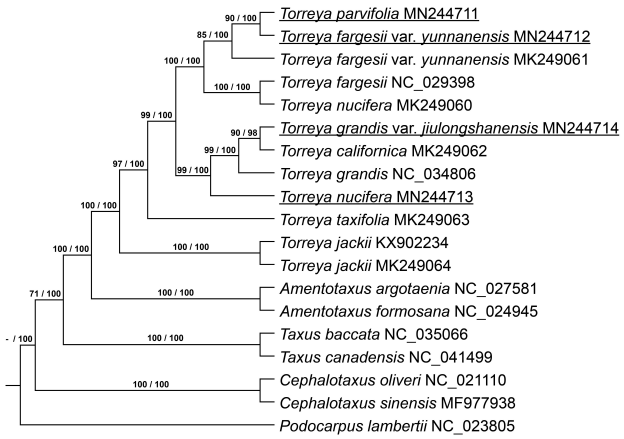

Fig. 4. Phylogenetic tree shows relationship and supported values. Both ML and BI tree show the same relationship. Supported values of ML and BI tree are showed on branch and divide by sign of division, respectively.

the material of *T. nucifera* MN244713 used in this study was collected from Nanjing University, Nanking, Jiangsu Province (Jinling University introduced the species from Japan), which has been verified by historical evidence. In the recent study [1], *T. nucifera* MK249060 and *T. fargesii* NC_029398 formed a single cluster with a very high association based on the ML method, and this cluster was closest to *T. fargesii* var. *yunnanensis* MK249061, followed by *T. grandis* NC_034806. However, in this study, *T. nucifera* MN244713 was closest to *T. grandis* NC_034806 and *T. grandis* var. *jiulongshanensis* MN244714 and then to *T. fargesii* NC_029398 and *T. fargesii* var. *yunnanensis* MK249061. Therefore, the molecular biological evidence in this study supports the treatment of *T. nucifera* MK249060 as *T. fargesii*.

In a recent study by Kou *et al.* [15], phylogenetic analysis of the nuclear internal transcribed spacer (ITS) and combined sequences of chloroplast *rbcL* and *rpl16* genes in the genus *Torreya* showed that *T. grandis* var. *jiulongshanensis* was close to *T. jackii* according to the combination sequences based on the ML method. However, it is insufficient evidence to suggest that *T. grandis* var. *jiulongshanensis* is a natural hybrid of *T. jackii* and *T. grandis,* basing on a few sequence fragments. In addition, the study comparing leaf variation among *T. grandis* var. *jiulongshanensis*, *T. grandis, T. fargesii* and *T. jackii* [16] proposed that *T. grandis* var. *jiulongshanensis* be treated as an independent species rather than a variety of *T. grandis* due to the morphological features of their leaves. The two studies above, examining gene fragments or just leaf variations, failed to offer hard evidence for related taxonomy. In our study, phylogenetic trees based on the whole genome-wide level single-copy orthologous genes showed consistent results by ML method and BI method, both illustrating that *T. grandis* var. *jiulongshanensis* MN244714 was close to *T. grandis* NC_034806. Therefore, according to our study, *T. grandis* var. *jiulongshanensis* should be treated as a variety of *T. grandis* rather than a natural hybrid between *T. jackii* and *T. grandis* or an independent species, supporting the original proposal [12].

5. Conclusions

In this study, we analyzed the complete cp genomes of four *Torreya* species. These genomes provided a basic genetic tool for species identification within the genus. All genomes sequenced by us, two short IR regions were identified in the work. We compared the cp genomes of different species of *Torreya*, and results showed that the gene size, content, and order were all similar. In contrast to the other three taxa, *T. grandis* var. *jiulongshanensis* lacks the *rps11* gene but has the *clpP* gene as a unique gene. Results of the phylogenetic analysis concluded that *T. parvifolia* should be considered as *T. fargesii* var. *yunnanensis* or treated as a sister species. *T. grandis* var. *jiulongshanensis* should be treated as a variety of *T. grandis* according to molecular evidence, supporting the originally published proposal.Generally, this study provides valuable genetic information of *Torreya* which can aid in further phylogenetic studies, species identification, and evolutionary relationships.

Abbreviations

SRA, Sequence read archive; BRIG, BLAST Ring Image Generator; AIC, Akaike Information Criterion; ML, maximum likelihood; BI, Bayesian inference; LSC, large single-copy; SSC, small single-copy; IR, Inverted repeat.

Author contributions

ZPM, XNN, LH and XH performed the experiments and data analysis. RBW and JHL collected samples. ZPM and XH wrote the manuscript. BBM and JHL revised the manuscript. ZPM, XNN, contributed equally to this work. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/FBL/27/1/10.52586/j.fbl2701009.

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