Research Article

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Identification and Genetic Relationship Analysis of *Torreya* Based on nrDNA ITS Sequence

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International Journal of Molecular Ecology and Conservation, 2021, Vol.11, No.2 doi: [10.5376/ijmec.2021.11.0002](https://doi.org/10.5376/ijmec.2021.11.0002)

Received: 11 Aug., 2021

Accepted: 19 Aug., 2021

Published: 28 Aug., 2021

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Preferred citation for this article:

Zhuo W., Liu Y., Bai J., Xiang R., Lu S.G., and Ren F.M., 2021, Identification and genetic relationship analysis of *Torreya* based on nrDNA ITS sequence, International Journal of Molecular Ecology and Conservation, 11(2): 1-8 (doi: [10.5376/ijmec.2021.11.0002](https://doi.org/10.5376/ijmec.2021.11.0002))

Abstract In order to evaluate the identification ability of nrDNA ITS sequences on *Torreya*, this study used ITS sequence and four different analysis methods (BLAST, K2P genetic distance, SNP analysis, NJ tree) to identify species of *Torreya*, and established phylogenetic trees to discuss their phylogenetic relationships. The results showed that the length of 48 ITS sequences was 1 095 bp~1 105 bp, the average intraspecific genetic distance was 0.001 6 and the average interspecific genetic distance was 0.015 0. At the species level, BLAST alignment and NJ tree had the highest efficiency in *Torreya*. The SNP loci analysis could effectively identify *T. grandis* cv. 'Merrillii' and *T. grandis*. The analysis of genetic relationship showed that all the other species of *Torreya* were single-line branches, but the quince tree of *T. yunnanensis* and *T. fargesii* were clustered into a cluster in the ML tree, indicating that the two were closely related. This provided evidence for the nuclear genome sequence proposed the incorporation of *T. yunnanensis* and *T. fargesii*. This study showed that ITS sequence could be used as a DNA barcode for the identification of *Torreya*, and providing reference for the identification and systematic relationship of *Torreya*.

Keywords DNA barcode; *Torreya*; ITS sequence; Identification

Torreya is the surviving relict plant of the Cenozoic Era, belonging to the Gymnospermae Taxaceae. According to the Chinese flora (FRPS), there are 7 species of *Torreya* of which 4 species from China (*Torreya fargesii*, *Torreya jackii*, *Torreya grandis* and *Torreya yunnanensis*), 2 species from North America (*Torreya taxifolia* and *Torreya californica*), 1 species from Japan (*Torreya nucifera*) (Zheng et al., 1978). Most of the *Torreya* is a rare species endemic to China with complex morphological characteristics. The main classification bases are leaf length, leaf apex length, and endosperm morphology (Shen, 2011; Teng et al., 2017). It is difficult to accurately classify *Torreya* because of different growth environment and morphological variation caused by extensive introduction and cultivation. In recent years, some scholars still proposed to classify *T. fargesii* and *T. yunnanensis* as one species, and the systematic relationship between them needs to be further clarified. In practice, the difficulty in identification of *Torreya* has a great impact on research and application. For example, The seeds of *T. grandis* "Xiangfeizi" are famous high-grade nuts that are known as "the king of dried fruits" (Li et al., 2014). *T. grandis* cv. 'Merrillii' is an excellent cultivated variety of wild-type *T. grandis*. In recent years, the *T. grandis* cv. 'Merrillii' cultivar has been vigorously developed in various places, and the supply of cedar tortoise seedlings is in short supply. However, due to the similar morphological characteristics of *Torreya*, other seedlings are often mixed with false *T. grandis* cv. 'Merrillii' seedlings in the market, which it is difficult to identify effectively. *T. grandis* has a slow growth and long fruition cycle, so the fake seedlings will cause serious economic losses to the growers. Therefore, accurate classification and identification of plants for *Torreya* not only helps to clarify phylogenetic relationship, but also has important significance for the reasonable utilization of resources of *Torreya*.

At present, domestic and foreign researches on *Torreya* mainly focus on germplasm resources investigation, community characteristics and biological protection (Yu et al., 2014; Bo et al., 2016; Gao et al., 2019), but there are few studies on classification and identification. The morphological characteristics of *Torreya* are not obvious,

especially in the long seedling stage before flowering and fruiting, which makes traditional morphological identification very difficult. DNA barcodes with the advantages of speed and accuracy have become an important tool for plant classification and identification which use a short and standard DNA fragment in the genome to identify species (Chen et al., 2010; Zhu et al., 2018; Li et al., 2020). ITS, ITS2, *matK*, and *psbA-trnH* and so on are commonly used DNA barcodes. China Plant BOL Group sampled 6 286 individuals representing 1 757 species in 141 genera of 75 families for data analysis. ITS showed the highest discriminatory power, and a combination of ITS and any plastid DNA marker was able to discriminate 69.9%~79.1% of species. In cases where it was difficult to amplify and directly sequence ITS in its entirety, just using ITS2 was a useful backup. China Plant BOL Group proposed ITS/ITS2 should be incorporated into the core barcode for seed plants (Li et al., 2011). Chen S.L research group evaluated the identification ability of ITS2 in a total of 6 600 medicinal plants in 753 genera, and found that the identification efficiency of ITS2 at the species level was 92.7%. Chen S.L research group recommended it as a standard barcode for medicinal plants (Miu et al., 2011). Tan et al. selected ITS sequence as the DNA barcode candidate sequence for *Celios cristata*, and successfully identified 6 common hybrids (Tan et al., 2020). Wang et al. selected ITS sequence to quickly identify succulents of *Sedum* and accurately identified 10 species of *Sedum* (Wang et al., 2020). Zhou et al. used the ITS2 sequence to successfully identify 25 species of “Xueteng”, and developed a medicinal material labeling system (Zhou et al. 2019).

In this study, nrDNA ITS sequences were used to identify and analyze the relationship between *Torreya*. Four different analysis methods (BLAST alignment, Kimura 2-parameter (K2P) genetic distance, SNP site analysis, and neighbor- Joining (NJ) tree) were used to identify *Torreya*. It is hoped to find an identification method for accurately identifying the *T.grandis* and *T.grandis* cv.'Merrillii'. At the same time, this study used the maximum likelihood (ML) to explore the genetic relationship of *Torreya*. The aim is to establish a rapid and accurate molecular identification method for *Torreya*, and provides a basis for the phylogenetic relationship of *Torreya*.

1 Results and Analysis

1.1 PCR amplification of ITS sequences

In this study, we used the DNA of the sample leaf as a template for PCR amplification, and the electrophoresis results showed that there were clear bands at the location of 1 000 ~ 2 000 bp (Figure 1). The PCR amplification effect was good which only a single and clear band was obtained. Then, the recovered and purified bands were sent to the sequencing company for sequencing.

Figure 1 PCR amplification of the sample ITS sequence

Note: M: DL 2000 bp Marker; No. 1~8 is *T. grandis* samples, No. 9~12 is *T.grandis* cv. 'Merrillii' samples; No. 13 is negative control

1.2 The ITS sequence features

In this study, the success rate of ITS sequence amplification and sequencing were 100%. After proofreading, the complete sequence was transmitted to NCBI database to obtain the ID. According to the preliminary analysis (Table 1), the length of ITS sequence in *Torreya* ranged from 1095 bp to 1105 bp, and the content of base C+G ranged from 59.9% to 61.5%, which was higher than A+T content. There were 51 variation sites and 1 051 conserved sites in ITS sequence.

We used BLAST comparison method to evaluate the identification ability of ITS sequence in *Torreya*. This method was able to effectively identify 6 species of *T. grandis*, *T. grandis* cv. 'Merrillii', *T. jackii*, *T. nucifera*, *T. californica* and *T. taxifolia*, but could not identify *T. yunnanensis* and *T. fargesii*.

Table 1 ITS sequence length and GC content of the sample

Genus name	Species name	Sequence length (bp)	C+G content (%)	Gene ID
<i>Torreya</i>	<i>T. grandis</i> cv. 'Merrillii'	1 103~1 104	61.2~61.3	MN888476-MN888483
	<i>T. grandis</i>	1 004~1 105	61.3~61.4	KJ588907, KJ588910, KJ588909, AF259278 MN880413-MN880416
	<i>T. jackii</i>	1 104~1 105	59.9~60.0	KJ588930, KJ588929, KU534171, KU534165
	<i>T. fargesii</i>	1 102	60.9	KJ588917, KJ588918, JF978979, JF978980
	<i>T. yunnanensis</i>	1 102	60.9	KJ588914, JF978983, JF978985, JF978981
	<i>T. nucifera</i>	1 103	61.2	KJ588912, AB024002, MK272739, AF259279
	<i>T. californica</i>	1 101~1 103	60.8~60.9	KJ588926, AB023997, AF259274, AF259275
	<i>T. taxifolia</i>	1 095~1 097	61.3~61.5	KJ588923, AF259271, AF259272, AF259273
<i>Taxus</i>	<i>T. wallichiana</i>	979	63.9~62.3	MH711827, HM590953, HM590952, HM590960
<i>Amentotaxus</i>	<i>A. argotaenia</i>	1 088~1 089	60.5~60.7	KJ588939, KU534173, KU534174, JF975879

1.3 The K2P genetic distance of ITS sequences

In this study, the genetic distance of *Torreya* was analyzed based on the K2P model, and the results showed that (Table 2): The average intraspecific genetic distance of *Torreya* was 0.006 (0~0.007 1), with the greatest genetic variation within *T. taxifolia*. The average interspecific genetic distance of *Torreya* was 0.015 0 (0~0.0302), among which the distance between *T. yunnanensis* and *T. fargesii* was the smallest, and the distance between *T. grandis* and *T. taxifolia* was the largest. By analyzing the K2P genetic distance of ITS sequence, we found that the minimum interspecific genetic distance was greater than the maximum intraspecific genetic in *Torreya*. which except *T. yunnanensis* and *T. fargesii*, *T. grandis* cv. 'Merrillii' and *T. nucifera*. K2P genetic distance could effectively identify 4 species of *T. grandis*, *T. jackii*, *T. californica* and *T. taxifolia*.

The barcoding gap showed (Figure 2) that there was no obvious barcoding gap on ITS sequence of *Torreya*, but the intraspecies and intraspecies genetic variation did not completely overlap. In the intraspecies genetic distance, 87.50% was less than 0.004 and 12.50% was between 0.004 and 0.008. The interspecific genetic distance was 75.00% greater than 0.008.

1.4 The SNPs site screening of ITS sequences

In this study, specific SNPs were screened by comparing ITS sequence variation sites in *Torreya*. The results showed that *T. grandis* cv. 'Merrillii' had no SNP sites; *T. grandis* had three SNPs sites; *T. nucifera* had a SNP sites; *T. jackii* had two SNPs sites; *T. yunnanensis* and *T. fargesii* had no SNP. Moreover, we found that the *T. californica* and *T. taxifolia* in North America had 16 loci which were clearly different from other species, the *T. californica* had 4 SNPs sites, and the *T. taxifolia* had 7 SNPs sites (Figure 3). Further analysis revealed that there were T-C mutations at the bases 302, 610, and 1 044 of ITS sequences of *T. grandis* cv. 'Merrillii' and *T. grandis*, which was the effective identification sites. SNP loci analysis could effectively identify 5 species of *T. grandis*, *T. nucifera*, *T. californica* and *T. taxifolia*.

Table 2 The genetic distance of ITS sequences in *Torreya*

Genus name	<i>T. grandis</i> cv. 'Merrillii'	<i>T. grandis</i>	<i>T. nucifera</i>	<i>T. jackii</i>	<i>T. fargesii</i>	<i>T. yunnanensis</i>	<i>T. californica</i>	<i>T. taxifolia</i>
<i>T. grandis</i>	***	0.006 4	0.002 4	0.006 7	0.011 1	0.011 3	0.019 4	0.022 1
<i>T. grandis</i>	0.004 3	***	0.006 1	0.006 1	0.012 6	0.013 0	0.023 3	0.030 2
<i>T. nucifera</i>	0.001 1	0.001 1	***	0.004 3	0.008 6	0.009 1	0.019 3	0.026 1
<i>T. jackii</i>	0.005 4	0.005 4	0.004 3	***	0.010 8	0.011 3	0.02 15	0.028 3
<i>T. fargesii</i>	0.009 7	0.011 9	0.008 6	0.010 8	***	0.000 0	0.020 4	0.027 2
<i>T. yunnanensis</i>	0.009 7	0.011 9	0.008 6	0.010 8	0.000 0	***	0.020 4	0.027 2
<i>T. californica</i>	0.018 4	0.020 6	0.017 3	0.019 5	0.018 4	0.018 4	***	0.013 6
<i>T. taxifolia</i>	0.026 2	0.027 3	0.024 0	0.026 2	0.025 1	0.025 1	0.008 6	***

Note: The minimum genetic distance between species is below the diagonal, and the average genetic distance is above the diagonal

Figure 2 The barcoding of ITS sequences in *Torreya*

Figure 3 Analysis of mutation sites in *Torreya*

Note: Red is the SNP site of *T. grandis*, purple is the SNP of *T. nucifera*, orange is the SNP of *T. jackii*, blue is the SNP of *T. californica*, and green is the SNP of *T. taxifolia*. The black frame is the difference site between *T. grandis* cv. 'Merrillii' and *T. grandis*

1.5 The Cluster analysis of ITS sequences

In this study, the NJ phylogenetic tree of *Torreya* was constructed based on ITS sequence, and the results showed that *Torreya* had good aggregation degrees of various species, and most of them was monophyletic (Figure 4A). Except *T. yunnanensis* and *T. fargesii*, other species of *Torreya* had obvious clustering without species crossing. Moreover, each species was clustered into a monophyletic group which had a high support rate. *T. wallichiana* and *A. argotaenia*, as two outgroups, were independent of *Torreya* and separately grouped into a single branch. NJ trees could effectively identify 6 species of *T. grandis*, *T. grandis* cv. 'Merrillii', *T. jackii*, *T. nucifera*, *T. californica* and *T. taxifolia*.

The ML tree was used to analyze the genetic relationship of *Torreya*, and the results showed that the clustering situation of ML trees was similar to that of NJ tree (Figure 4B). Except *T. yunnanensis* and *T. fargesii* croosed to form a single cluster, other species of *Torreya* had obvious clustering with a high support rate. This result indicated that *T. yunnanensis* and *T. fargesii* were closely genetic relationship. In addition, *T. grandis* cv. 'Merrillii' formed a parallel branch with *T. nucifera*, *T. jacki* formed a parallel branch with *T. grandis*, and *T. californica* formed a parallel branch with *T. taxifolia*, which showed a close genetic relationship between the two.

Figure 4 Constructing clustering tree based on ITS sequence
Note: A: Neighbor-Joining tree B: Maximum likelihood tree

2 Discussion

Traditional morphological identification mainly relies on the phenotypic characteristics of plants (Yang et al., 2017), while DNA barcode identification relies on the polymorphism and difference of standard gene fragments to achieve rapid and accurate identification of species (Gogoi et al., 2018; Mosa et al., 2018). Plant ITS sequences include ITS1 and ITS2 sequences. In the preliminary test, the ITS2 fragment was amplified, and it was found that the ITS2 sequence of the sample was highly similar and the identification efficiency was low. Gao et al. (2017) also chose ITS sequence as the core barcode and successfully identified peach kernel and almond due to the high similarity of ITS2 sequence. Therefore, The ITS sequences with 100% amplification rate and good sequencing quality were selected as the core identification sequence in this study. The results showed that all species of *Torreya* could be identified except *T. yunnanensis* and *T. fargesii*. If this single sequence could not fully identify the plant, the chloroplast gene could be used as an auxiliary barcode in combination for identification (Zhang et al., 2019; Chi et al., 2020; Intharuksa et al., 2020). The results showed that BLAST comparison, K2P genetic distance, SNP site screening and NJ trees could identify 6, 4, 5 and 6 species respectively, among which BLAST comparison and NJ trees had the highest identification efficiency. Yu et al. (2016) used RAPD method to identify *Torreya*, the method had poor stability and repeatability, and the reaction conditions needed to be optimized. But ITS sequence did not need to design multiple pairs of primers and optimized the reaction conditions. It has obvious advantages.

In this study, it was found that the *T. yunnanensis* and *T. fargesii* were very closely related. Not only was the K2P genetic distance between species small, but also the same evolutionary branch in the ML tree intersects with each other. In the FRPS, the *T. yunnanensis* and *T. fargesii* were divided into two different species. Silba et al. (1995) suggested that the two be merged under the *T. grandis* as a variant. However, Kang et al. (1995) suggested incorporating *T. yunnanensis* into *T. fargesii*. The results of this study supported the incorporation of *T. yunnanensis* into *T. fargesii* as variants, but not supported the incorporation of both as variants. Ma et al. (2014) and Zhou et al. (2015) analyzed the *trnL-trnF* and *psbA-trnH* genes of *T. fargesii* that reached the same conclusion. Later, in Flora of China (FOC), the *T. yunnanensis* was incorporated into the *T. fargesii* as a variant according to the same morphological characteristics such as the longitudinal groove of the leaf and the width of the midrib (Wu., 1999). The analysis results of the clustering tree in this study were basically consistent with the classification results of the traditional taxonomy in FOC, which could provide reference for the systematic classification of *Torreya*. At the same time, the study founded that the tree species located in North America had more mutation sites than Asian tree species. It not only was the mutation type more complicated, but the two were located in the two branches of the ML tree, which showed they were far from each other. It may be that different geographical growth environments gradually affect genetic characteristics. Geographic isolation had led to a reduction in gene exchange within the population, and genetic relationship had gradually become farther away (Guo et al., 2020). Similar geographical differentiation characteristics were also found in *Bletilla Striata* (Ren et al., 2019) and *Oryza rufipogon* Griff (Huang et al., 2019), the samples with closer geographical distribution had closer genetic relationships.

T.grandis cv.'Merrillii' is an excellent variety that has been cultivated and bred for a long time. In practice, *T.grandis* is often confused with *T.grandis* cv.'Merrillii' in the market, and it is difficult to rely only on morphological identification. This study found that the SNPs site and the NJ tree could effectively identify both. There were three bases serve as unique identification sites, and they were grouped into a monophyletic group without species crossover in the NJ tree. Therefore, in this study, nrDNA ITS fragments were used to successfully identify 6 species of *Torreya*, and the molecular identification between the *T.grandis* and *T.grandis* cv.'Merrillii' was carried out. It not only provides accurate identification of the plants of *Torreya*, but also helps to enrich the DNA barcode database of *Torreya*. At the same time, it can reduce the confusing use of *T.grandis* cv.'Merrillii' seedlings in the market, and rationally use *Torreya* resources to provide technical support.

3 Materials and Methods

3.1 Test materials and Reagents

The test samples were collected from Zhuji, Zhejiang (120°34 'E, 29°61' N), Shaoxing (120°44 'E, 30°07' N) and Yunyang County, Chongqing (108°67 'E, 30°95' N). They were identified as *T. grandis* cv.'Merrillii' and *T. grandis* by Liu Zhengyu, researcher of Chongqing Institute of Medicinal Plant Cultivation. Young and healthy plant leaves were taken, cleaned, quickly dried and preserved by silica gel. In this study, a total of 48 ITS sequences of plants for *Torreya* and outgroups were used for analysis, including 40 of *Torreya* (12 new ones) and 8 of *Taxus Wallichiana* and *Ametotaxus argotaenia*.

The plant genomic DNA Rapid Extraction Kit (centrifugal column) was purchased from Beijing Junord Biotechnology Co., LTD.I-5™ 2×High-Fidelity Master Mix was purchased from Beijing Qingke Biotechnology Co., LTD. DL2000 DNA Marker was purchased from Beijing Zhuang Meng International Biotechnology Co., LTD.ITS primer synthesis was completed by Beijing Qingke Biotechnology Co., LTD. Other experimental reagents were pure analytical reagents made in China.

3.2 Plant DNA extraction

The plant leaves were ground 1 800 times /min for 2 min with a ball mill, and 20 mg of sample fine powder was weighed. The sample DNA was extracted with the plant genomic DNA rapid extraction kit according to the instructions. The DNA purity and quality were detected by an ultraviolet-spectrophotometer and then placed at -20°C.

3.3 PCR amplification and sequencing

The reaction system included positive and negative primers (10 mol/L) 0.6μL, 2×*Taq* Mix 12.5μL, ddH₂O 9.8μL, template DNA 1μL. The amplification reaction procedure included predenaturation at 94°C for 5 min; then 40 cycles containing denaturation at 94°C for 30s, annealing at 53°C for 30s, and elongation at 72°C for 70 s. ITS primers (forward primers 5'-CCTTATCATTTAGAGGAAGG-3', reverse primers 5'- TCCTCCGCTT ATTGATATGC -3 '), PCR amplification products were detected by 1% agar-gel electrophoresis and sent to Beijing Qingke Biology Co., Ltd. for sequencing.

3.4 Data analysis

Sequencing results were edited and spliced by DNAMAN software. The software MEGA7.0.14 was used to analyze the sequence base variation sites, and Kimura 2-parameter model was used to analyze the genetic distance. The adjacent NJ trees were constructed for identification and analysis of *Torreya*, and the maximum likelihood ML trees were constructed for phylogenetic analysis. Then the Bootstrap value was set as 1000, and the support rate was more than 50%.

Authors' contributions

ZW was the experimental designer and executor of this research. LY and LSE completed the analysis of experimental results and the writing of the first draft of the manuscript; BJ and XR participated in data collection and experimental design; RFM was the initiator and responsible person of the project, guiding experimental design, data analysis, manuscript writing and modification. All authors read and approved the final manuscript.

Acknowledgements

This study was funded by the Basic Scientific Research Project of Chongqing (19KF10-2012).

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