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Evaluation of chloroplast and nuclear DNA barcodes for species identification in *Terminalia* L.

Stalin Nithaniyal, Madasamy Parani*

Department of Genetic Engineering, Center for DNA Barcoding, SRM University, Kattankulathur, 603203, India

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ABSTRACT

The genus *Terminalia* L. belongs to the Combretaceae family, which includes several medicinal and threatened species with high trade value. Species of *Terminalia* in India belong to four sections and species identification within the sections is considered to be complex due to the lack of sufficient taxonomical characters and the existence of morphotypes. Therefore, we tested the effectiveness of three chloroplast DNA barcodes (*rbcL*, *matK*, and *trnH-psbA*) and a nuclear DNA barcode (ITS2) for the discrimination of *Terminalia* species. A reference DNA barcode library consisting of 120 DNA barcodes from ten species of *Terminalia* was created. Intra-specific divergence was not observed among the accessions for any marker. Inter-specific divergence was highest in *trnH-psbA* (10.6%), followed by ITS2, *matK* and *rbcL* markers. The success of species differentiation by DNA barcodes was 100% with *trnH-psbA*, 80% with *matK* and ITS2, and 10% with *rbcL*. In the phylogenetic trees, the *rbcL* marker did not differentiate the species in any section. Two species from the section Catappa were not differentiated by *matK* and ITS2 markers. Only *trnH-psbA* resolved all the species and ranked the best among four markers for species identification. However, regarding species relationship studies, ITS2 was found to be better than other markers because it formed a separate clade for each section.

1. Introduction

Terminalia L. is the second largest genus in Combretaceae comprising 200 species with distribution in the tropical regions of the world (Mabberley, 2008). Characteristic features of this genus are nectar glands at the base of the lamina or petiole, flowers borne on spicate inflorescence, and flattened or winged fruits. There are about 12 reported species of *Terminalia* in India, belonging to four sections: *Catappa*, *Pentaptera*, *Chuncoa* and allied to *Catappa* (Clarke, 1879; Santapau and Henry, 1973; Vijayasankar et al., 2011). It is an economically important genus due to its medicinal and timber uses. Species of *Terminalia* are in high demand for the extraction of phytochemicals such as alkaloids, flavonoids, oleane type triterpenes, tannins, gums, and oils. The fruits and barks of *Terminalia arjuna*, *Terminalia bellerica*, *Terminalia cattapa*, *Terminalia chebula*, and *Terminalia elliptica* are traded in large volumes (from 2000 to 10,000 metric tons per year) due their medicinal value (Ved and Goraya, 2008). The large-scale sale of natural health products derived from *Terminalia* such as laxative, astringent, purgative and

Abbreviations: rbcL, ribulose-bisphosphate carboxylase; *matK*, maturase K; CTAB, cetyl trimethyl ammonium bromide; PCR, polymerase chain reaction; NCBI, national center for biotechnology information; BOLD, barcode of life data system.

* Corresponding author.
E-mail address: parani.m@ktr.srmuniv.ac.in (M. Parani).

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diuretic drugs, sold under such trade names as Ajuna, Haritaki, Baheda, and Triphala, makes it a valuable genus in herbal medicine (Nadkarni, 1976; API, 1999). Species such as *Terminalia pallida*, *T. arjuna* and *T. chebula* have been listed by I.U.C.N. in its threatened category at international, national or regional levels (www.iucnredlist.org; Ravikumar and Ved, 2000; Vijayasankar et al., 2008). Moreover, Nair (2004) considers these species as controversial drug plants prone to species adulteration in the trade markets. Therefore, species identification in *Terminalia* is essential to monitor trading of threatened species as well as authentication of raw drugs.

Systematic identification of *Terminalia* is complicated because inter-species differentiation is unclear and, at the same time, the species vary considerably in morphotypes, anatomy, and karyotypes (Parkinson, 1936; Excell and Stace, 1966; Srivastav et al., 1996). Morphological characters overlap among the species, which leads to difficulty in species identification (Maurin et al., 2010; Waman, 2015). Lack of sufficient taxonomical characters to discriminate the species of *Terminalia* necessitated exploring DNA barcoding for species identification. DNA barcoding is a rapid and cost-effective method that uses short DNA sequences for species identification (Hebert et al., 2003). DNA barcoding has been successfully used in various fields such as species identification (Gismondi et al., 2015; Angers-Loustau et al., 2016), discovery of new or cryptic species (Ragupathy et al., 2009), detection of plant adulteration in herbal drugs (Gismondi et al., 2013; Seberg and Petersen, 2009), and identification of fossil botanical species to reveal the origin of domesticated plant species (Gismondi et al., 2012, 2016). An ideal DNA barcode should be amenable to PCR amplification using universal primers, bidirectional sequencing using a single-primer pair, and should have higher inter-specific divergence than intra-specific divergence (Kress et al., 2005). A part of the CO1 (*cytochrome c oxidase* 1) gene is used as a universal DNA barcode for differentiating most animal species. However, CO1 is not a suitable gene for differentiating plant species due to low mutation rates and complex evolutionary processes (Rieseberg et al., 2006; Fazekas et al., 2009). Therefore, chloroplast and nuclear DNA barcodes have been suggested as appropriate for plants (Chase et al., 2007; Kress and Erickson, 2007; Hollingsworth et al., 2011). The CBOL Plant Working Group (2009) recommended the *rbcL* and *matK* as core barcodes, and *trnH-psbA* as a complementary marker for DNA barcoding of land plants (CBOL, 2009; Hollingsworth et al., 2011). However, recent studies show that ITS2 can also be used as a core marker for DNA barcoding in plants (Yao et al., 2010). A universal DNA barcode marker for plants is still elusive and it is often reported that one marker is more appropriate than others for a particular taxonomic group (Sun et al., 2012; Selvaraj et al., 2014; Cabelin et al., 2015; Vassou et al., 2015). The present study evaluated *rbcL*, *matK*, *trnH-psbA* and ITS2 for their ability to distinguish different species of *Terminalia*.

2. Materials and methods

2.1. Plant sampling

We collected 30 accessions representing ten species of *Terminalia* species from various locations in India (Table S1). Taxonomist carefully identified all voucher specimens and deposited at the SRM University Herbarium (SRMUH).

2.2. DNA isolation, PCR amplification, and DNA sequencing

Approximately 100 mg of fresh leaf sample was used for isolating genomic DNA following CTAB method (Saghai-Maroof et al., 1984) with minor modifications. Thoroughly ground samples were mixed with 500 μl of CTAB buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% beta-mercaptoethanol, and 2% polyvinylpyrrolidone), and transferred to 1.5 ml centrifuge tubes. The homogenized samples were incubated in water bath at 55 °C for 30 min. The samples were then extracted with an equal volume of chloroform, and centrifuged at 10,000 rpm for 10 min. The aqueous phase was transferred to 1.5 ml centrifuge tubes and precipitated by adding an equal volume of ice-cold isopropanol and centrifuged at 10,000 rpm for 10 min. The pellet was washed twice with 70% ethanol, air-dried at room temperature. The dried pellet was dissolved in 100 μl TE buffer, used as template for PCR amplification without RNase treatment.

PCR amplification of the DNA barcodes was achieved using universal primer pairs for *rbcL* (Levin et al., 2003; Fazekas et al., 2008), *matK* (Ki-Joong Kim, School of Life Sciences and Biotechnology, Korea University, Korea, unpublished), *trnH-psbA* (Kress et al., 2005) and ITS2 (Chen et al., 2010). PCR reaction mixture contained 1X buffer with 1.5 mM $MgCl_2$, 0.2 μM dNTPs, 5.0 pmol primers, 1 unit *Taq* DNA polymerase (GenetBio Inc., Korea) and 20–50 ng of genomic DNA. PCR amplification included initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, final extension at 72 °C for 5 min, and hold at 16 °C. The PCR amplified products were purified using EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc. Ontario, Canada). Sequencing of the PCR products was carried out with Big-dye terminator chemistry in 3130xl Genetic Analyzer (Life Technologies, California, USA) by following the standard manufacturer's protocol.

2.3. Data analyses

Sequence quality of each marker was checked visually using Sequence Scanner Software v1.0 (Applied Biosystems, CA, USA). Full-length sequences were assembled using a local alignment algorithm in CodonCode Aligner, version 4.2.4 (CodonCode Corporation). Database search was done using Basic Local Alignment Search Tool (BLAST) against non-redundant nucleotide database at NCBI (www.blast.ncbi.nlm.nih.gov/Blast.cgi) and BOLD database (www.boldsystems.org). Genetic