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Molecular analyses of genetic variation and phylogenetic relationship in Indian soap nut (*Sapindus* L.) and closely related taxa of the family Sapindaceae

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ABSTRACT

Molecular differentiation and phylogenetic relationships of Indian *Sapindus* and its closely allied taxa of the family Sapindaceae was studied using nuclear ribosomal DNA of internal transcribed spacer (ITS). Evolutionary divergence over sequence pairs between the tribes of the family Sapindaceae was estimated, and maximum divergence (0.20) was observed between the tribes, Paullinieae and Harpullieae, whereas the lowest (0.06) was found between Sapindeae and Lepisantheae. Phylogenetic tree based on maximum likelihood method revealed the separation of all three species of the *Sapindus* in different clusters. *S. delavayi* a Japanese species, showed a close affinity to the *S. mukorossi* with high bootstrap support (99%). There is a significant distinction between *S. emarginatus* and *S. trifoliatus* with high (99%) bootstrap support in the clade. It clearly indicates that both the species are distinct and no infra-specific categories existed among these taxa. ITS region has shown a reliable marker to differentiate different taxa considered in the present study. Furthermore, studies on comparative phylogenomics of different taxa might provide useful insight to understand the phylogeny of the family Sapindaceae in a more comprehensive way.

1. Introduction

The genus *Sapindus* L. (Sapindaceae) is represented by about 13 species, chiefly found in the warm regions of Asia, Australia, North and South America (Xia and Gadek, 2007). The genus *Sapindus* was studied by many scientists in different time intervals, and there has been a divergent opinion about the correct status and number of species of *Sapindus* in India (Table 1). Brandis (1874) described three species (*S. laurifolius* Vahl, *S. emarginatus* Vahl and *S. detergens* Roxb.) in Forest Flora of North West and Central India, whereas, Hiern (1875) reported 7 species (*S. attenuatus* Wall. ex Hiern, *S. bifoliolatus* Hiern, *S. danura* Voigt, *S. erectus* Hiern, *S. mukorossi* Gaertn., *S. thwaitesii* Hiern and *S. trifoliatus* L.), in the Flora of British India, and subsequently species like *S. erectus* Hiern, *S. thwaitesii* Hiern, *S. attenuatus* Wall. ex Hiern, *S. danura* Voigt and *S. bifoliolatus* Hiern were transferred to another genus *Lepisanthes* Blume due to changes in generic concept (Leenhouts, 1969). Later on the genus was treated variously by several contemporary botanists like Cooke, 1902; Duthie, 1903; Gamble, 1918 and Haines, 1920 in their respective Flora's. Gandhi (1976), Mukherjee (1980) and Chitra (1983) supported the taxonomic treatment given to the species of *Sapindus* by Gamble in his Flora. Prakash and Mehrotra (1990) and Pant (2000) also revisited the genus and their account differs from that of Hiern (1875), Cooke (1902), Duthie (1903) and Gamble (1918), not only in taxonomic treatment of the species but also on the number of species described from India. The identity and nomenclature of *S. trifoliatus* and its allied species, *S. emarginatus* are also very controversial in the Indian Floras. Hiern (1875) recognized *S. trifoliatus* and cited *S. laurifolia* and *S. emarginata* as synonyms of it, whereas Cooke (1902) treated *S. laurifolia* as a variety of it and cited *S. trifoliatus* of Hiern (not of L.) as synonym (in part) under both taxa. Duthie (1903) reported three species of the *Sapindus* in his Flora that was again not in conformity with the treatment accorded by other workers. Afterwards, Gamble (1918) recognized *S. laurifolius* and *S. emarginatus* as two distinct species with *S. trifoliatus* of Hiern (1875) as synonym (in part) under each. Subsequently, several workers like Santapau (1960), Gandhi (1976) and Chitra (1983) upheld Gamble's view and considered

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Table 1
Taxonomic treatment of *Sapindus* L. species as described by different workers.

Brandis (1874)	Hiern (1875)	Cooke (1902)	Duthie (1903)	Gamble (1918)	Prakash and Mehrotra (1990)	Pant (2000)
S. laurifolius *S. emarginatus* *S. detergens*	*S. attenuatus** *S. bifoliolatus** *S. danura** *S. erectus** *S. mukorossi* *S. thwaitesii** *S. trifoliatus*	*S. laurifolius* *S. laurifolius* var. *emarginatus*	*S. danura* *S. laurifolius* *S. mukorossi*	*S. emarginatus* *S. laurifolius*	*S. emarginatus* *S. mukorossi* *S. rarak* *S. trifoliatus*	*S. emarginatus* *S. mukorossi* *S. trifoliatus*

Species marked with asterisks (*) have been transferred to the genus *Lepisanthes* (Leenhouts, 1969).

that true *S. trifoliatus* does not occur in India and specimens assigned to this species by Hiern (1875) belong to *S. laurifolia* and *S. emarginata*, while Pant (2000) had considered *S. emarginatus* and *S. trifoliatus* as two distinct species, and *S. laurifolius* as synonym of *S. trifoliatus*. Obviously the taxonomic confusion still prevails because these studies were primarily based on phenotypic characters and therefore, genetic investigations are required to resolve the infra-specific as well as inter-specific impediments in *Sapindus* species and their closely related taxa of Sapindaceae.

There are several molecular markers available these days that are being used to unravel the molecular differentiation and the phylogeny in various plant groups. The internal transcribed spacer (ITS) region is one of the most widely used molecular markers for phylogenies of plants, animals, bacteria and fungi. The length of the ITS varies from 500–700 bp in angiosperms and 1500–3700 bp in some gymnosperms (Calonje et al., 2009). The ITS region comprises highly variable parts like ITS1 and ITS2, and the more conserved 5.8S gene in between. ITS is the most frequently sequenced region for plant phylogenetic studies because of its high species discrimination and technical ease of amplification (Alvarez and Wendel, 2003; Kress et al., 2005), therefore, there are large number of recent studies available, where ITS region has been successfully utilized to infer the phylogenetic relationships and identifications of various taxa (Ghada et al., 2013; Du et al., 2014; Hynniewta et al., 2014; Nazre, 2014; Shiran et al., 2014; Su et al., 2016, Kehie et al., 2016; Michel et al., 2016; Ndri et al., 2016).

A number of efforts have been made to elucidate the systematic and phylogenetic position of the family Sapindaceae primarily based on morphology and biogeography (Roadlkofer, 1890, 1933; Takhtajan, 1987; Cronquist, 1988; Dahlgren, 1989), pollen morphology (Muller and Leenhouts, 1976), molecular sequence data (Gadek et al., 1996; Savolainen et al., 2000; Angiosperm Phylogeny Group II, 2003; Harrington et al., 2005; Angiosperm Phylogeny Group III, 2009; Buerki et al., 2009, 2010, 2011a, 2011b) and phytochemical approaches (Umadevi and Daniel, 1991). However, phylogeny of the genus *Sapindus* and its relationships with closely related taxa are still not completely understood, and there is also a need to identify new synapomorphies in different groups of plants in the family Sapindaceae. Further, in the context of the various taxonomic treatments of the species of *Sapindus* in India and the fact that these Indian species have not been subjected to any rigorous tests of phylogeny, therefore, in the present study data obtained from the internal transcribed spacer (ITS) sequences of the nuclear ribosomal DNA (nrDNA) were used to assess phylogenetic relationships and differentiation of the three species of *Sapindus* in India. In order to carry out this study, relative to genera of different tribes under the family Sapindaceae, a wider representation of the family was included by (i) including ITS sequence data for other *Sapindus* taxa that already existed in the Genbank database and (ii) by generating the same for a few other taxa for which no prior data were available in the Genbank. To the best of our knowledge this is a maiden attempt to analyze the molecular differentiation and phylogeny in Indian representatives of *Sapindus* and their closely related taxa.

2. Materials and methods

2.1. Plant material

On the basis of the genetic diversity studies on the genus *Sapindus* (Mahar et al., 2011a, 2011b, 2013), 22 accessions belonging to three species of *Sapindus* (9 accessions of *S. emarginatus,* 6 accessions of *S. mukorossi* and 7 accessions of *S. trifoliatus*) representing different regions of India, and 13 accessions of other closely related taxa under the family Sapindaceae, were selected for the sequencing of ITS region of nrDNA. Voucher specimens have also been prepared for all the collected materials and have been deposited in the herbarium of CSIR-National Botanical Research Institute (LWG), Lucknow. A total of 72 accessions including 22 accessions of the genus *Sapindus* and 50 accessions of other related taxa belonging to 18 genera under the family Sapindaceae (APG III, 2009) were also considered for molecular phylogenetic studies, and taxonomic relationships of *Sapindus* species to its closely related genera of different tribes under the family Sapindaceae (Table 2).

2.2. Isolation of DNA and PCR amplification reaction

Total genomic DNA was extracted from fresh as well as silica-dried leaf tissues following CTAB method (Doyle and Doyle, 1990). Quantification of genomic DNA was carried out for its quality and quantity by gel electrophoresis on 0.8% agarose gel, staining with ethidium bromide, and comparison with a set of known DNA concentration standard, and by UV spectroscopy using a Nanodrop ND-1000 Spectrophotometer (Wilmington, DE 19810, USA). Universal ITS primers P4 (5′-TCCTCCGCTTATTGATATGC-3′) and P5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al., 1990; Baldwin, 1992) were custom synthesized from Merck Specialities Private Limited, India and used in the present study to amplify ITS region. The amplification of ITS region was carried out according to Allan and Porter (2000). Final concentrations or amounts of each reagent in 25 µl reaction volume were as follows: P4 and P5 primers (0.4 µM each), dNTPs (200 µM each in equimolar ratios), sterile water (13.0 µl), 10 × buffer A (100 mM Tris (pH 9.0), 500 mM KCL, 15 mM $MgCl_2$ and 0.1% Gelatin), 1.5 unit of *Taq* DNA polymerase (Merck Specialities Private Limited, Mumbai, India), and genomic DNA (50 ng). The amplification reactions were carried out using a Thermal Cycler (PTC 200, MJ Research, Inc., USA), which was programmed to include initial denaturation at 94 °C for 3 min, each cycle consisted of 1 min denaturation at 94 °C, 1 min of annealing at 50 °C, 1.5 min extension at 72 °C along with 7 min extension at 72 °C at the end of 35 cycles.

PCR products along with 100 bp DNA ladder (Merck Specialities Private Limited, India) was loaded in agarose gel as a standard were resolved on 1% agarose gel in 0.5 × TBE buffer at a constant voltage of 5 V/cm. After electrophoresis the gel was stained with ethidium bromide (10 mg/ml) and then visualized and archived using UV Tech Gel Documentation System (UK). Since the size of ITS region was known (approximately 600–700 bp in angiosperms), the bands of interest were identified with reference to the DNA ladder in the gels