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DNA barcoding to resolve phylogenetic relationship in *Myristica* spp.

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Abstract

Myristica is the largest and primitive genus of the taxonomically complex family Myristicaceae. DNA barcoding was used to study the evolutionary relationship between *Myristica* spp. and other genera of Myristicaceae. The barcoding loci namely, *rbcL, matK, psbA-trnH,* ITS and multilocus combinations were tested to assess their phylogenetic relationship. *psbA-trnH* locus revealed information regarding the relationship of species in *Myristica* genus. *M. fragrans* was found to be closely related to *M. beddomei, M. amygdalina, M. andamanica*1, whereas *M. fatua* was found to be distinct from *M. malabarica. Gymnocranthera* and *Knema* species were found to share sister relation with other *Myristica* spp.

Keywords: barcoding, evolution, *Myristica*, multilocus, nutmeg, phylogram

Introduction

Myristica is the largest and most primitive genus of Myristicaceae family (Sinclair 1958) that has around 18 genera and 300 species. New Guinea is considered as its centre of origin and distribution of *Myristica* genus (http://www.fao.org/docrep/x5047E/x5047E04.htm). They are pantropical and are seen in the rainforests of Asia, Africa, Madagascar, South America and Polynesia (Krishnamoorthy *et al.* 2007). Blume and Bentham divided the genus into four sections *viz*., *Knema, Irya, Pyrrhosa* and *Eumyristica* according to their androecium modifications (Hooker & Thomson 1855) while Hooker (1856) classified it into three sections *Knema, Myristica* and *Pyrrhosa*.

Presently four genera of Myristicaceae are found in India - *Myristica, Horsfieldia, Knema* and *Gymnocranthera,* mostly in Andaman and Nicobar Islands as well as in *Myristica* swamps found in valleys of Kulathupuzha, Shendurney, and forest ranges of Anchalin, South Western Ghats and the district Uttara Kannada of Central Western Ghats of India (Krishnamoorthy *et al.* 2007). There are about 120 species of *Myristica,* of which, 14 are found in India. *Myristica* genus is represented by species like *Myristica fragrans* Houtt., *M. malabarica* Lam., *M. andamanica*

Hook.f., *M. fatua* Houtt., *M. beddomei* King and *M. prainii* King (Krishnamoorthy *et al.* 2007), of which *M. fragrans* is the most important and commercially cultivated one (Bose *et al.* 2008). The seed and mace of *M. fragrans* (nutmeg) is widely used as a spice and in medicine, while mace of *M. malabarica* is exploited as a dye source. Other economically important species of *Myristica* genus are *M. andamanica* and *M. beddomei,* which are used in traditional medicine (Zachariah *et al.* 2008; Arunachalam & Subhashini 2011; Manjunatha *et al.* 2011). The wood of *M. beddomei* and *M. malabarica* is also used in making match boxes and splints (Sastri 1962; Bose *et al.* 2008). Inspite of the economic potential of the genus, the phylogeny and taxonomy of the genus are poorly understood.

Morphological characters are given dominance while studying phylogeny. But some of the inherent problems such as plasticity in phenotypic characters and variation in genotypic characters, interaction of the genotype with the environment, cryptic taxon and difficulty in finding reliable characters because of variation in expression or long maturity periods or incomplete penetrance reduce the reliability of morphological characters in phylogenetic studies (Abeysinghe *et al.* 2009).

Presently DNA barcoding, a technique based on the sequence variation in certain nucleotide sequences called barcodes has come up as a tool for molecular taxonomy (Hebert *et al.* 2003). In plants, the chloroplast genes like *rbcL* (coding for large subunit of RUBISCO), *matK* (coding for maturase), *psbA-trnH* and nuclear Internal Transcribed Spacer (ITS) regions serve as barcodes. The chloroplast DNA is considered as an informative phylogenetic marker for land plants (Bieniek *et al.* 2015). The predominant maternal inheritance and the conserved nature of chloroplast DNA have enhanced its application in evolutionary and phylogenetic studies (Schilling 1997; Mukherjee & Bhat 2013). Here an attempt is made to study the relationships within *Myristica* species and between *Myristica, Gymnocranthera* and *Knema* genera using chloroplast regions and nuclear ITS region.

Materials and methods

Collection of samples

Leaves were collected from 18 accessions representing six species of genus *Myristica,* one species each of the genus *Knema* and genus *Gymnocranthera* from different locations in India (Table 1).

DNA Isolation

Total genomic DNA was isolated from all the leaf samples collected from plant species given in Table 1 using the procedure for nutmeg leaf DNA isolation (Sheeja *et al.* 2008). The qualitative and quantitative estimation of the isolated DNA was done by measuring the absorbances at 260 nm and 280 nm in a Biophotometer (Eppendorf, Germany).

Amplification and sequencing of barcoding loci

Genomic DNA was amplified using the universal barcoding loci primers of *rbcL, matK, psbA-trnH* and Internal Transcribed Spacer (ITS) synthesized by Integrated DNA Technologies (IDT) (USA) (Table 2). The reaction mixture composition of all loci was optimized and was carried out in 50 µl reaction volume. The reaction mixture composition and the PCR reaction were performed as described by Swetha *et al.* (2017). The amplicons were purified using QIA quick PCR Purification kit (Qiagen, Germany) following the manufacturer's protocol. The purified products were sequenced bidirectionally at the DNA sequencing unit of SciGenome Labs Pvt Ltd (Cochin) Kerala.

Data analysis and submission of sequences to GenBank

The forward and reverse sequences obtained were assembled by the Cap 3 software (Huang & Madan 1999) to generate their consensus sequences and were checked against the

Table 1. Details of *Myristica* spp. used in the study

Species	No. of accession	Place of collection
M. fragrans Houtt.	3	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
	1	Nagercoil, Tamil Nadu, India.
	1	Andaman and Nicobar Islands, India.
M. malabarica Lam.	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
	1	Community Agro Biodiversity Center, Wayanad, Kerala, India.
	1	Nagercoil, Tamil Nadu, India.
	1	Ernakulam, Kerala, India.
	1	Kerala Forest Research Institute, Thrissur, Kerala, India.
M. andamanica Hook.f.	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
	1	Andaman and Nicobar Islands, India.
M. fatua Houtt. var. *magnifica* (Bedd.) Sinclair	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
	1	Kerala Forest Research Institute, Thrissur, Kerala, India.
M. amygdalina (Wall.) Warb.	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
M. beddomei King	1	IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala, India.
	1	Ernakulam, Kerala, India.
	1	Kerala Forest Research Institute, Thrissur, Kerala, India.
Knema attenuata (Hook. f. & Th.) Warb.	1	Kerala Forest Research Institute, Thrissur, Kerala, India.
Gymnocranthera farquhariana (Hook. f. & Th.) Warb.	1	Kerala Forest Research Institute, Thrissur, Kerala, India.

Table 2. Primers employed for amplification of barcoding loci

Primer name	Sequence (5′ - 3′)	Reference
*rbcL*af	5′ ATG TCA CCA CAA ACA GAG ACT AAA GC 3′	Kress & Erickson 2007
*rbcL*ar	5′ GTA AAA TCA AGT CCA CCG CG 3′	
matK 3F	5′ CGT ACA GTA CTT TTG TGT TTA CGA G 3′	Vijayan & Tsou 2010
matK 1R	5′ ACC CAG TCC ATC TGG AAA TCT TGG TTC 3′	
psbA f	5′GTT ATG CAT GAA CGT AAT GCTC 3′	Yang *et al.* 2011
trnH R	5′CGT AAC AAG GTT TCC GTA GGT GAA 3′	

GenBank database of NCBI to confirm the species identity using the BLAST tool (Altschul *et al.* 1997). *rbcL* and *matK* sequences obtained for the coding loci were translated by employing ExPASY translate tool (Gasteiger *et al.* 2003) and their identity was checked using Protein BLAST tool. The nucleotide sequences obtained were deposited in the GenBank nucleotide database (Table 3).

Phylogenetic analysis

In addition to the amplified *Myristica* sequences, sequences that showed an e-value of zero on BLAST analysis for each locus was also included for phylogenetic analysis in order to give a better picture regarding the phylogenetic power of the selected barcoding loci. *Magnolia acuminata* was selected as the outgroup for the phylogenetic analysis based on a previous study in *Myristica* species (Sheeja *et al.* 2014). Single locus (*rbcL, matK* and *psbA-trnH)* and multi locus barcode (*rbcL* + *matK*, *rbcL* + *trnH-psbA*, *matK* + *trnH-psbA* and *rbcL* + *matK* + *trnH-psbA*) approaches were tried for phylogenetic studies. The sequences were aligned using MUSCLE algorithm (Edger 2004) and manually trimmed using BioEdit software (Hall 1999). Multilocus barcodes were created for those loci that generated sequences for all loci by concatenating the sequence alignment files into one with *FAScon CATv1.0 tool (*Kück & Longo 2014*).* The evolutionary history was inferred using the Bayesian analysis, performed in MrBayes version 3.1 (Ronquist & Huelsenbeck 2003). Bootstrap analysis was performed with 10,000 replicates. All positions containing gaps and missing data were eliminated.

Table 3. GenBank accession numbers obtained for *Myristica* spp.

Species	Accession Number		
	rbcL	*psbA-trnH*	*matK*
M. fragrans 1	KT367808	KY966347	KT367809
M. fragrans 2	KT380141	KY966348	KT380142
M. fragrans 3	KT445277	KY966349	KT445278
M. fragrans 4	KY945257	KY966350	MF547523
M. fragrans 5	KY945258	KY966351	MF547524
M. malabarica 1	KY945259	KY966342	MF547530
M. malabarica 2	KY945260	KY966343	MF547531
M. malabarica 3	KY945261	KY966344	MF547532
M. malabarica 4	KY945262	KY966345	MF547533
M. malabarica 5	KY945263	KY966346	MF547534
M. andamanica 1	MF158638	MF086596	MF547528
M. andamanica 2	MF158639	MF086597	MF547529
M. fatua 1	MF186596	MF086592	MF547525
M. fatua 2	MF186597	MF086593	MF547526
M. amygdalina	MF417801	MF086598	MF547527
M. beddomei 1	MF186599	MF086594	MF547536
M. beddomei 2	MF186600	-	MF547537
M. beddomei 3	MF186601	MF086595	MF547538
K. attenuata	MF547520	MF086599	MF547535
G. farquhariana	MF547519	MF086600	MF547539

Results and discussion

Genomic DNA of high quality was obtained from the fresh leaves of *Myristica* species with yield in the range of 135 to 271 μg g^{-1} and purity ratio (A260/A280) in the range 1.8 to 1.9. A good amplification success was obtained for *rbcL, matK,* ITS and *psbA-trnH* loci with amplicons of size 600, 900, 750 and 450 bp (Fig. 1) respectively. High amplification success of these loci was previously reported (Kress *et al.* 2005; Li *et al.* 2012; Zhang *et al.* 2015; Kumar & Yusuf 2016).

Hundred per cent sequencing success was obtained for *rbcL*, *matK* and *psbA-trnH.* But for ITS, the forward and reverse reads could not be assembled in to contigs due to poor sequence quality. Sequencing failure of ITS has been reported previously in various works (Liu *et al.* 2012; Li *et al.* 2014; Bhagwat *et al.* 2015). Hollingsworth (2011) has reported that ITS may be present in numerous copies owing to incomplete concerted evolution and the multiple copies may be getting sequenced simultaneously, resulting in messy data that could not be processed further.

The sequence length range obtained for *rbcL, matK* and *psbA-trnH* was 510 bp - 579 bp, 695 bp - 782 bp and 299 bp - 413 bp, respectively. BLAST analysis of the contigs obtained for *matK, rbcL* and *psbA-trnH* exhibited maximum similarity to sequences of the respective locus of genus *Myristica* and other genera of family Myristicaceae. Protein BLAST done on the translated sequences of *matK* and *rbcL* exhibited maximum similiarity to maturase and RUBISCO sequences of Myristicaceae family. The generated sequences were deposited in the nucleotide database GenBank (Table 3).

Sequences of individual locus namely, *rbcL, matK, psbA-trnH* and concatenated sequences of multilocus combinations namely, *rbcL+matK, rbcL+psbA-trnH, matK+psbA-trnH* and *rbcL+matK+psbA-trnH* obtained were aligned and trimmed to uniform size. Sequences downloaded from GenBank database of NCBI were also included in the phylogenetic analysis of individual loci. *rbcL* sequences taken from GenBank are *M. fragrans* (AF206798), *M. fragrans* (AY298839), *M. fatua* (GQ248653), *Myristica cf. sepicana* (FJ976152), *Myristica globosa* (KF496610), *Staudtia kamerunensis* (KC628429), *S. kamerunensis* (KC628405), *Compsoneura mexicana* (EU090522) *Horsefieldia amygdalina* (KR529436) and *H. amygdalina* (KR529434). *matK* sequences taken from GenBank are *M. fragrans* (AJ966803), *M. fragrans* (EU669472), *M. fatua* (GQ248165), *Myristica maingayi* (DQ401374), *M. maingayi* (AY220452), *Myristica yunnanensis* (KR531264), *M. yunnanensis* (KR531265), *Myristica cinnamomea* (KJ709010), *M. cinnamomea* (KJ709009), *Compsoneura preussii* (AY743475), *C. preussii* (KC685082), *Knema globularia* (AB924720) and *K. globularia* (AB924868). *psbA-trnH* sequences taken from GenBank are *M. fragrans* (KT758178), *M. fatua* (GQ248350), *M. globosa* (GQ248351), *M. yunnanensis* (KR533281), *M. yunnanensis* (KR533282), *H. amygdalina* (KR533273), *H. amygdalina* (KR533274), *S. kamerunensis* (KC667945), *C. preussii* (KC688793) and *C. preussii* (KC688811).

Phylograms were constructed using single locus and multilocus combinations. The phylogenetic relationships between the *Myristica* species was studied using the individual (*rbcL, matK* and *psbA-trnH*) and concatenated (*rbcL+matK, matK+psbA-trnH, rbcL+psbA-trnH, rbcL+matK+psbA-trnH*) sequences with an outgroup using Bayesian inference (BI) methods. *rbcL & matK* phylograms were drawn using a final alignment of 474 bp and 688 bp, respectively. The phylograms obtained were rooted with *M. acuminata,* separated from the inner group. These phylograms were not able to provide any information regarding the phylogeny or discrimination of *Myristica* spp. The trees exhibited polytomy for the ingroup section. Difficulty of *rbcL & matK* loci in resolving closely related species was previously reported by Gielly & Taberlet (1994) and Tallei *et al.* (2015).

psbA-trnH phylogram was drawn using *psbA-trnH* sequences with a final alignment of 281 bp (Fig. 2). This locus was able to reveal the relationship between *Myristica* species. In *psbA-trnH* phylogram, *M. andamanica, K. attenuata* and *G. farquhariana* were found to be closely related. *M. fragrans* and *M. malabarica* appeared in separate clusters pointing to the distant relationship between them. *M. beddomei, M. amygdalina, M. fatua* and *M. andamanica*1 collected from the same habitat were found to have close association with each other. Sequences of other genera formed monophyletic groups and appeared to be sister groups of *Myristica* spp. The utility of *psbA-trnH* in discriminating plant species of different tree genera like *Alnus* (Ren *et al.* 2010) and *Quercus* (Simeone *et al.* 2013) have been previously reported.

The *matK+rbcL* phylogram was drawn using the concatenated *rbcL* and *matK* sequences. The data set contained 20 sequences trimmed to a final alignment of 1413 bp that was used to generate a phylogenetic tree (Fig. 3). The *rbcL+matK* phylogram was rooted with *M. acuminata* that branched separately from the inner group. High bootstrap support was obtained for the nodes but different accessions of the same species were seen in different clades. *rbcL+matK* phylogram showed close relation between *M. beddomei, M. fatua* and *M. andamanica*1. It showed the close association of *M. andamanica*2, *K. attenuata* and *G. farquhariana.* But this tree could not resolve

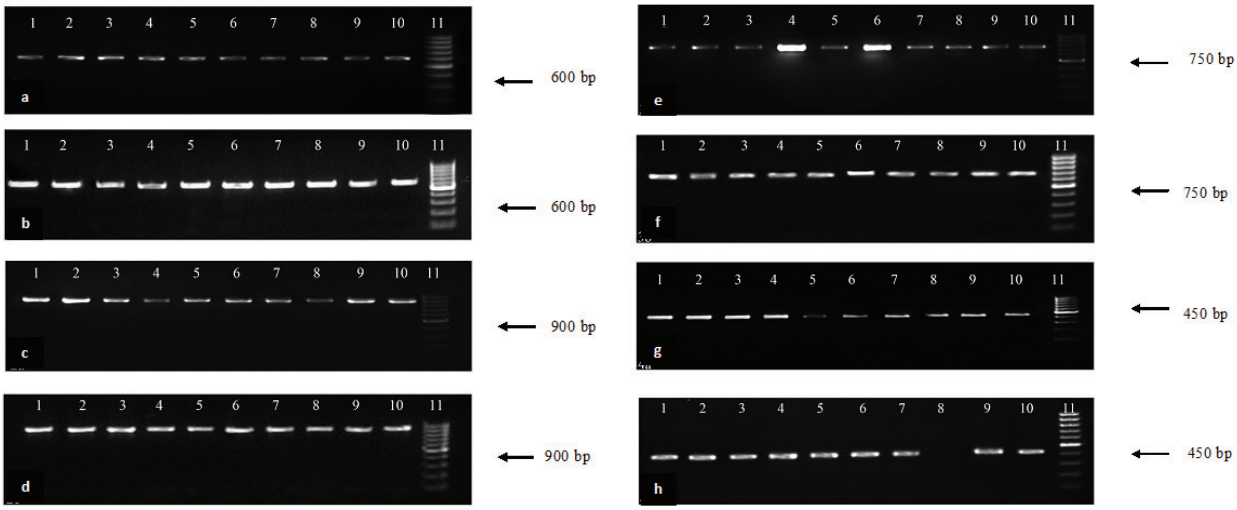

Fig. 1. Amplification of *rbcL* (a, b), *matK* (c, d), ITS (e, f) and *psbA-trnH* (g, h) loci in different *Myristica* accessions. Lane 1-5 and 6-10 in (a, c, e, g) was loaded with *M. fragrans* and *M. malabarica*, respectively. Lane 1-2; 3-4; 5; 6-8; 9 and 10 in (b, d, f, h) is loaded with *M. andamanica*, *M. fatua, M. amygdalina, M. beddomei, Knema attenuata* and *Gymnocranthera farquhariana*, respectively, lane 11 in both (a) and (b): 100 bp ladder (Fermentas)

M. fragrans, M. amygdalina and *M. malabarica.* Though *rbcL+matK* combination was proposed as ideal for plant species discrimination by Consortium for the Barcode of Life Plant Working Group, it failed to resolve *Myristica* spp. The failure of this combination in phylogenetic analysis has been previously reported by Li *et al.* (2014) in *Calligonum* species.

The topology of *rbcL+psbA-trnH* tree (Fig. 4) was similar *to psbA-trnH, matK+psbA-trnH* and *rbcL+matK+psbA-trnH* trees except for a single sample of *M. malabarica* positioned outside the *M. malabarica* cluster. That distinct specimen of *M. malabarica* was collected from Nagercoil, Tamil Nadu while rest of the samples were collected from Kerala. Geographical separation may be the reason for its positioning outside *M. malabarica* cluster. In these trees, *M. andamanica*1 collected from Andamans and *M. andamanica*2 collected from Kerala were also placed in different clades and showed close relation with different species which may also be due to geographical separation. Schneider *et al.* (2015) reported that genetic differences between species may be caused due to geographical separation. The accumulation of genetic differences by geographical isolation may have arisen in the process of adapting to different environmental conditions (Souframanien & Gopalakrishnan 2004). A close relation was observed between *M. beddomei, M. amygdalina, M. andamanica*1 and *M. fatua* in the present study. This is in agreement with the earlier study conducted by Sheeja *et al.* (2014) on Myristicaceae; the phylogenetic analysis done using 18S rDNA sequences that depicted the close relationship between *M. beddomei, M. andamanica, M. amygdalina* and their closeness was explained on the basis of the similarity in their seed and mace characters. *M. fragrans* was found to be closely related to *M. beddomei, M. amygdalina, M. andamanica*1 and *M. fatua* and distinct from *M. malabarica.* The close association between *M. fragrans* and the other species could be perhaps indicative of their common ancestry. *M. fragrans* and *M. malabarica* were clustered in two distinct clades due to the sequence divergence in *psbA-trnH* between these two species. *Gymnocranthera* and *Knema* species formed a sister clade within *Myristica* cluster. The sister relationship between *Knema* and *Myristica* was reported by Sauquet *et al.* (2003). *Gymnocranthera, Knema* and *Myristica* are Asian genera of Myristicaceae family. Clustering of *Gymnocranthera* and *Knema* along with *Myristica* may be due to the monophyly of the Asian genera of Myristicaceae (Sauquet *et al.* 2003).

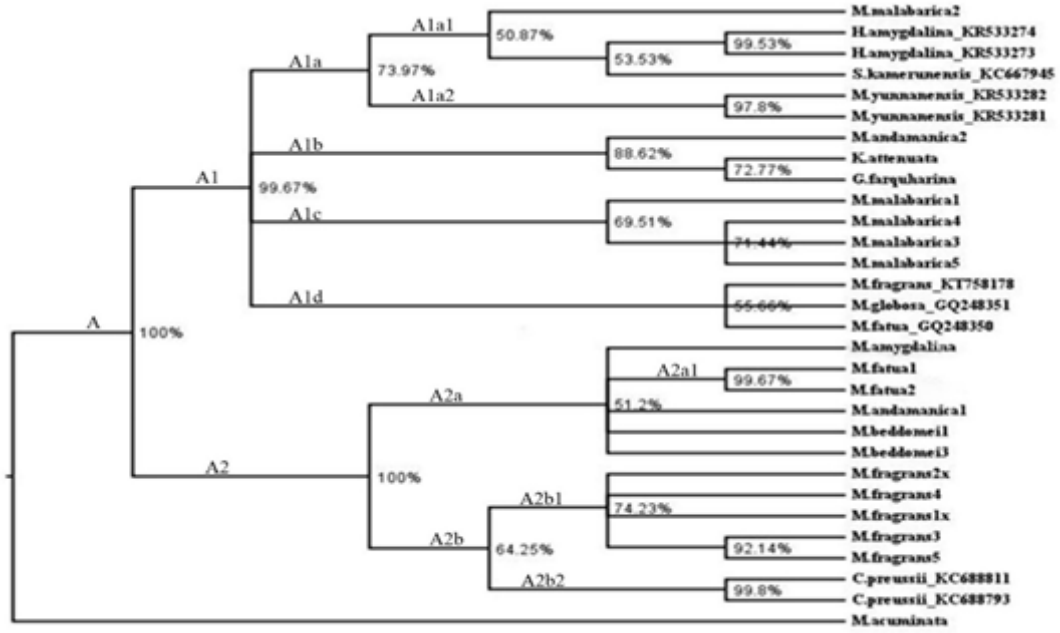

Fig. 2. Bayesian phylogram for *psbA-trnH* sequences

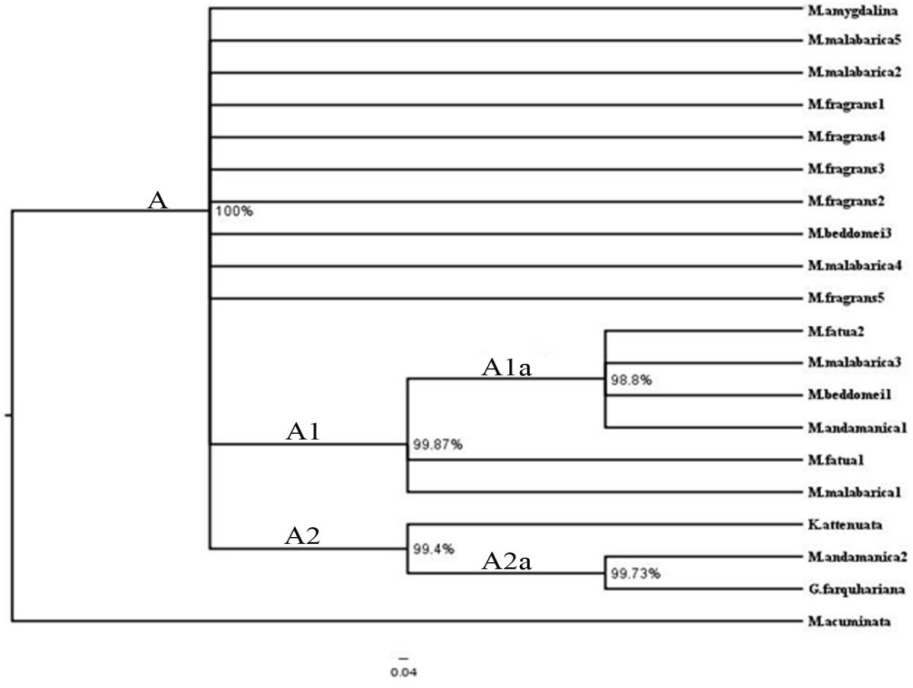

Fig. 3. Bayesian phylogram for *rbcL+ matK* sequences

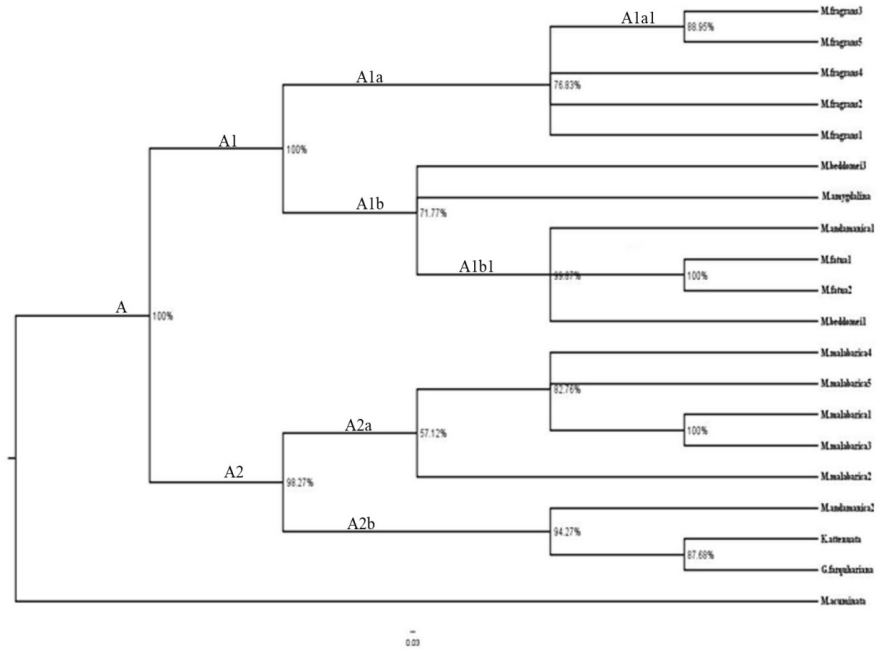

Fig. 4. Bayesian phylogram for *rbcL+ psbA-trnH* sequences

The results of the present study gave an insight on the genetic relationship among the members of *Myristica* genus. The locus *psbA-trnH* with its high sequence divergence provided information regarding the interspecific relation between species within the genus *Myristica*. Among the barcoding loci studied, only the locus *psbA-trnH* was informative to elucidate the relationships amongst the members of the family.

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