RAPD, SCAR and conserved 18S rDNA markers for a red-listed and endemic medicinal plant species, *Knema andamanica* (Myristicaceae)

T. E. Sheeja · P. R. Anju · R. S. Shalini · S. Siju ·
K. Dhanya · B. Krishnamoorthy

Published online: 24 February 2013
© Prof. H.S. Srivastava Foundation for Science and Society 2013

Abstract *Knema andamanica* is a red-listed endemic medicinal species of *Myristicaceae* restricted to Andaman and Nicobar (A&N) Islands, India. This species is used in tribal medicines and has immense bioprospective potential. With a view to generate suitable genomic markers for classification and identification, we have generated RAPD, SCAR and conserved 18S rDNA markers from *K. andamanica*. A unique 585 bp fragment, that distinguished it from seven other related species of *Myristicaceae* was first amplified using the random primer OPE 06 and converted to SCAR marker (GenBank accession # JN228256). The conserved sequences of 18S rDNA loci from *K. andamanica* were also amplified and sequenced (GenBank accession #JN228265). The sequence revealed deviations including 18 variable regions and 15 indels that were unique to *K. andamanica*. These markers can help in definite identification of *K. andamanica* even at the juvenile stages.

Keywords *Knema andamanica* · RAPD · SCAR · 18S rDNA and germplasm characterization · Genetic differentiation

Abbreviations

SCAR	Sequence Characterised Amplified Region
ISSR	Inter Simple Sequence Repeats
AFLP	Amplified Fragment Length Polymorphism
SSR	Simple Sequence Repeats
rDNA	ribosomal DNA
NCBI	National Center for Biotechnology Information
RAPD	Random Amplification of Polymorphic DNA
SNP	Single Nucleotide Polymorphism
BLAST	Basic Local Alignment Search Tool

Introduction

Myristicaceae is a primitive angiosperm family and is characterized by highly endemic species. Many of the *Myristicaceae* taxa are included in the IUCN threatened taxa or vulnerable taxa list (<http://www.iucnredlist.org/details/37093/0> accessed on 24.10.12). The family includes species that are rich sources of flavonoids (Valderrama and Martinez 2000) and *Knema andamanica* (Warb.) W.J.J.O.de Wilde, one of the most important members was chosen for this study. *K. andamanica* is a vulnerable and red-listed species, endemic to the tropical rain forests of A&N Islands. These islands are prone to frequent earthquakes, tsunami and anthropogenic intrusions (Roy and Krishnan 2005; Prasad et al. 2009). Hence conservation of this species in wild and protection from biopiracy are the major concerns. Referred to as ‘Oro’ or ‘Aurw’, by the Jarawa and Onge tribes of these islands, it possesses antimicrobial and therapeutic properties (Bhakuni et al. 1969; Sharief 2007). It is characterized with high amounts of alanine, threonine and total free amino acids and low phenylalanine (Maya et al. 2006), which indicates the bioprospecting potential of this species.

T. E. Sheeja (✉) · P. R. Anju · R. S. Shalini · S. Siju · K. Dhanya ·
B. Krishnamoorthy
Division of Crop Improvement and Biotechnology,
Indian Institute of Spices Research, Marikunnu PO,
Calicut 673012 Kerala, India
e-mail: teshee@rediffmail.com

T. E. Sheeja
e-mail: sheeja@spices.res.in

K. Dhanya
Weill Cornell Medical College in Qatar,
Qatar Foundation – Education City, P.O. Box: 24144, Doha, Qatar

Reliable identification techniques are an essential prerequisite for bioprospecting and conservation of this species. However, identification and distinction of *K. andamanica* from other related species is very difficult due to their close similarities. Also conventional systematic studies are limited and random (Rema et al. 2005). The main distinguishing feature in *K. andamanica* is the presence of a unique fused mace (aril) that does not help in discrimination at the juvenile stages. DNA markers like RAPD (Random Amplification of Polymorphic DNA) were found promising for fast and accurate identification in many endemic and endangered species (Sheeja et al. 2006; Castells et al. 2008; Sheeja et al. 2008; Yoo et al. 2011). Several species specific markers were detected from *K. andamanica* and related genera in an earlier study (Sheeja et al. 2013). However, considering the low reliability and reproducibility of RAPD markers (Rieseberg 1996; Bachmann 1997) we have successfully converted them to SCAR markers.

Many studies on herbal drugs emphasize the use of RAPD-SCAR in conjunction with 18S rDNA loci for classification and authentication (Sasaki et al. 2002; Lee et al. 2004; Zhang et al. 2007). The 18S loci are highly conserved and have great potential in identification of the genus or family of an unknown plant (Lee et al. 2010). Hence, cloning and sequencing of these loci was also of interest to the present investigation.

A limitation of traditional sources of systematic evidence and lack of genetic markers being a major impediment in *Myristicaceae*, our main objective was to develop DNA fingerprints and specific molecular markers from *K. andamanica* for its authentication, classification and protection.

Materials and methods

Plant materials

Fresh leaves of closely related members of family *Myristicaceae* viz., *M. fragrans* Houtt., *M. beddomei* King,

M. malabarica Lam., *M. prainii* King, *M. fatua* Houtt., *M. andamanica* Hook. f., *K. andamanica* (Warb.) W.J.J.O.de Wilde and *M. amygdalina* Wall. ex Hook. f. & Thomson were sampled from plants collected from different parts of the country and conserved in the IISR germplasm Repository, Experimental Farm, Peruvannamuzhi, Calicut, Kerala, India (Table 1). Three samples from each species were pooled for DNA isolation. Seven accessions of *K. andamanica* collected from different sites at Keechadnala (South Andaman), Monack Village (Nicobar) and Katchal Islands (Nicobar) were used for confirmation of the SCAR marker. DNA isolation was done as per the modified CTAB method (Sheeja et al. 2006). DNA concentration was assessed by taking absorbance at 260 nm. Working samples were prepared to a concentration of 20 ng/μL.

RAPD assay

All RAPD primers used in the study were random sequence, 10-base, oligonucleotide primers with G+C content ranging from 50 to 80 % (Sigma Chemical Company, USA). The RAPD analysis was done as per a standardized PCR protocol (Sheeja et al. 2006). The primers producing unique bands were identified from an earlier study (Sheeja et al. 2013) conducted by us on genetic diversity analysis of wild and related genera of *Myristica* using RAPD and ISSR markers.

Cloning of RAPD marker and development of SCAR

A prominent candidate band of 585 bp amplified by primer OPE 06 (AAGACCCCTC) present only in *K. andamanica* was chosen for development of SCAR marker. The gel region was excised and purified using Gel Extraction Kit (Sigma Gen Elute Gel extraction Kit) and subcloned into the PTZ 57R/T vector (Fermentas). The competent cell preparation and transformation were performed from an overnight

Table 1 Details of different species studied, their distribution, primer identifying the unique band and band size

Species	Place of collection (India)	Distribution	Primer identifying the unique band and band size (Primer _{bp}) ^a
<i>M. fragrans</i>	Kerala	Moluccas and Banda islands, cultivated in tropical regions	OPA-12 ₁₃₀₀ , OPA-19 ₅₄₃
<i>M. prainii</i>	Andaman & Nicobar Islands	Endemic to Andaman & Nicobar Islands	OPA-15 ₄₅₀
<i>M. andamanica</i>	Andaman & Nicobar Islands	Endemic to Andaman & Nicobar Islands	OPA-17 ₈₀₂ , OPA-17 ₁₇₆₇ , OPE-5 ₇₀₀ , OPE-6 ₅₀₀
<i>M. malabarica</i>	South Karnataka	Endemic to South India	OPE-17 ₇₀₀
<i>M. amygdalina</i>	Nagercoil	Myanmar, Bangladesh, India, Malaysia	OPE-17 ₄₅₀ , OPA-16 ₄₉₄
<i>K. andamanica</i>	Andaman & Nicobar Islands	Endemic to Andaman & Nicobar Islands	OPA-19 ₄₂₁ , OPA-20 ₂₇₅ , OPE-6 ₅₈₅ , OPE-5 ₃₀₀ , OPE-17 ₉₀₀
<i>M. beddomei</i>	Western Ghats	Endemic to South India	Nil
<i>M. fatua</i> var. <i>magnifica</i>	Western Ghats	Endemic to Western Ghats, Kerala, India	Nil

^a Data not shown except for OPE-6₅₈₅ (Fig. 1)

culture of DH5 α strain of *E. coli*. White colonies were confirmed by specific M₁₃F and M₁₃R primers. Plasmid isolation was done from six positive clones and custom sequenced at Bioserve Biotechnologies, Hyderabad, India.

Based on the characterized sequence, two specific primers (SCAR-01-FOR AAGACCCCTCCCTCCCCG and SCAR-01-REV AGACCCCTCGAGTATTACAAAGG) were designed confirming their thermodynamic efficiency with no potential dimers and hairpin formation (Using Fast PCR, primer biosoft, primer 3 programmes). Reactions were carried out in 25 μ L volumes containing 1X assay buffer, 1.5 mM MgCl₂, 10 pmole of each primer and 20 ng template using 1U Taq DNA polymerase. Amplification was performed with the following cycling conditions, 94 °C for 4 min, followed by 34 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min. The amplification products were resolved electrophoretically on 1.5 % TAE agarose. SCAR marker was screened in all *Myristica* species and also seven accessions of *K. andamanica* individually for confirmation.

Amplification of 18S rDNA gene and sequencing

The 18S rDNA loci of *K. andamanica* was amplified and sequenced for development of markers. Three sets of forward and reverse primers were designed using the sequence from National Center for Biotechnology Information (NCBI) (GenBank accession # AF206968) and Primer 3 software. The PCR product was generated as three overlapping fragments with three primer sets i) FOR(5'-ATTCGGACTGTGAACTGCG-3'); REV(5'-AGCTTGGGCTTGCTTTGAG-3'), ii) FOR(5'-CCATTGGAGGGCAAGTCTG-3'); REV(5'-GGTAAGTTTCCCCGTGTTGAG-3) and iii) FOR(5'-GTATGGTCGCAAGGCTGAAAC-3'); REV(5'-ACCTACGGAAACCTTGTTAC-3') with annealing temperatures in the manner, 53.5 °C 63.8 °C and 65.9 °C respectively, which was standardized by a gradient PCR.

The PCR programme involved an initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing for 45 s at temperature specific to each primer as mentioned in the previous paragraph, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. Thermocycling machine used was an MJ Research DNA Engine Tetrad. The amplified products were resolved and cloned into TOPO TA vector (Invitrogen Inc, USA) and transformed. Colony PCR and sequencing was done in the same manner as done for SCAR marker.

The partial sequences of 18S rDNA deposited by us (GenBank accession # JN228257-JN228265) and *M. fragrans* (GenBank accession #AF206968) available in NCBI were used for comparison with that of *K. andamanica*. Sequence alignment and trimming was done using BioEdit tool and analysis of

sequences was done using MEGA5.0 tool. The polymorphic sites, mutations and indels were identified using DNAsp.5 (Librado and Rozas 2009). Homology searches were performed within GenBank's non-redundant Viridiplantae database using BLAST 2.2.9 (Basic Local Alignment Search Tool; Altschul et al. 1990) algorithm at <http://www.ncbi.nlm.nih.gov/BLAST> of the NCBI, with the program BLASTN.

Results

Cloning and sequencing of putative RAPD marker

We had tested 25 random primers (Table 2) and shortlisted six primers that generated unique markers from the different species chosen for this study (Table 1.). Maximum number of unique bands were generated in *K. andamanica* with primers OPA- 19, OPA-20, OPE-5, OPE-6 and OPE-17 and a prominent band of 585 bp amplified by OPE-6 observed only in *K. andamanica* was selected for species-specific SCAR development. This band was eluted, cloned and sequenced. While doing transformation, we had chosen six white colonies for sequencing to ensure the retrieval of the most represented PCR product. The sequences from all the six clones were aligned using Clustal W software (<http://clustalw.genome.jp/>). It revealed a strong homology, confirming the presence of a unique simplified product in the RAPD marker band. The first 10 bp of the sequence was confirmed to correspond to the sequence of OPE-06 primer that identified the unique marker. Blast analysis revealed no sequence homology with any other sequences.

SCAR primer development and testing

While developing the SCAR primer, the RAPD primer sequences plus the next 9 to 10 bases in the RAPD marker sequence were used. The annealing temperature when tested through 60–68 °C, showed that the optimum annealing of the species specific primer was at 65 °C. SCAR primers generated a dominant single band of 585 bp in *K. andamanica* while no band could be observed in any of the other species of *Myristica* (Fig. 1). The SCAR primer pair was also tested with seven individual accessions of *K. andamanica* collected from different sites of A&N Islands. A bright, unique and easily identifiable band could be observed in all the accessions of *K. andamanica*. This proved that the SCAR primer designed in the study was species specific.

Classification and identification of markers from conserved 18S rDNA sequences

Amplification involving 18S rDNA primers yielded a product of 1,447 bp from *K. andamanica* after removal of vector

Table 2 Details of RAPD primers used for identifying unique bands from *K. andamanica* and other related species

No	Primer	Sequence 5'–3'	% Polymorphism	Band size (bp)
1.	OPA-5	AGGGGTCTTG	100	400–1,400
2.	OPA-7	GAAACGGGTG	100	450–1,900
3.	OPA-8	GTGACGTAGG	100	250–1,900
4.	OPA-9	GGGTAACGCC	100	275–1,600
5.	OPA-11	CAATCGCCGT	100	350–2,100
6.	OPA-12	TCGGCGATAG	95	200–1,500
7.	OPA-14	TCTGTGCTGG	100	250–1,400
8.	OPA-15	TTCCGAACCC	86	450–2,000
9.	OPA-16	AGCCAGCGAA	94	300–2,000
10.	OPA-17	GACCGCTTGT	92	500–1,500
11.	OPA-19	CAAACGTCGG	100	250–2,500
12.	OPA-20	GTTGCGATCC	100	300–1,800
13.	OPB-20	GGACCCTTAC	100	400–1,800
14.	OPC-2	GTGAGGCGTC	100	250–2,000
15.	OPC-6	GAACGGACTC	94	350–2,400
16.	OPE-1	CCCAAGGTCC	100	400–2,500
17.	OPE-2	GGTGCGGGAA	93	350–2,100
18.	OPE-3	CCAGATGCAC	100	400–1,500
19.	OPE-5	TCAGGGAGGT	100	275–2,000
20.	OPE-6	AAGACCCCTC	100	500–1,700
21.	OPE-12	TTATCGCCCC	100	300–2,200
22.	OPE-14	TGCGGCTGAG	100	250–2,000
23.	OPE-15	ACGCACAACC	100	350–1,500
24.	OPE-16	GGTGACTGTG	92	550–1,600
25.	OPE-17	CTACTGCCGT	100	250–2,000

sequences. For analysis 1,641, nucleotide sites were analysed including gaps in the multiple sequence alignment. Sequence analysis revealed a maximum dissimilarity of 5 % between *K. andamanica* and the rest of the species taken together. The sequence of *K. andamanica* was characterized with 18 variable regions, including 11 transitions and 5 transversions. There were 15 indels including 6 deletions and 9 insertions.

Fig. 1 SCAR marker showing amplification of 585 bp in *K. andamanica* accessions only. Lanes M- 100 bp marker. 1–8: *M. fragrans*, *M. beddomei*, *M. malabarica*, *M. prainii*, *M. fatua*, *M. andamanica*, *K. andamanica*, *M. amygdalina*, 9: Control, lanes 10–15: Accessions Nos. 8,9,15,16,18,19 of *K. andamanica*

Discussion

RAPD analysis revealed species specific markers in *K. andamanica*. Species specific markers for plant taxa need to be reliable, reproducible and amplifiable genomic sequences (Das et al. 2004). Many reports suggest better reliability of RAPD markers when converted to SCAR (Sun et al. 2011; Li and Park 2012). The SCAR marker identified in the present study discriminated *K. andamanica* from related species in a dominant manner. We observed only one marker band from the different accessions unlike the reports by Lee et al. (2004), where they observed several alleles in different cultivars. SCAR markers were found useful in differentiation of a number of endangered species (Ghosh et al. 2011; Lee et al. 2011). Very little amount of DNA is required and quality of DNA is not very stringent as in the case of RAPD analysis. The reaction is stable, reproducible and more specific due to the longer length and higher annealing temperature of the SCAR primers.

Analysis of 18S rDNA regions indicated a dissimilarity of 10 % between *K. andamanica* and the consensus sequence of rest of the *Myristica* species based on the transitions, transversions and indels identified. We observed that

K. andamanica maintained a unique identity and clustered separately from other *Myristica* species in RAPD and ISSR analysis (Sheeja et al. 2013). The 18S rDNA regions were observed to be conserved among eukaryotic organisms (Nei and Rooney 2005; Jo et al. 2009). These regions have been extensively used for phylogenetic analysis due to the features like conserved primary and secondary structure, mosaic pattern of variable and conserved regions and overall rate of homogeneity across lineages (Nickrent and Soltis 1995; Soltis et al. 1999). However, presence of sequence variations or polymorphism in rDNA coding regions was uncovered in many studies (Mentewab et al. 2011) and exploited for species identification and phylogeny either alone (Lee et al. 2010; Roy and Chattopadhyay 2011) or in conjunction with other markers like RAPD (Lee et al. 2004; Kim et al. 2005). In a similar manner, the variable gene regions identified in the 18S gene sequence of *K. andamanica* can be utilized for classifying markers by restriction digestion (Lee et al. 2004) or SNP based diagnostic PCR (Ding et al. 2012).

Accurate species identification and discrimination of closely related species is important for prioritization and chalking out appropriate strategies for conservation. Hence, molecular markers are nowadays considered as an integral component of biodiversity conservation programmes. In this study easy and fast molecular methods were devised based on RAPD-SCAR and 18S rDNA fingerprinting to distinguish between closely related species and also for generating species barcodes. We also intend to use this information for designing a core collection from the diversity of 484 accessions of *Myristica* and its related genera conserved in our germplasm repository.

Conclusion

Knema andamanica is a red-listed medicinal species restricted to Andaman and Nicobar islands, one of the oldest regions of human origin. Conservation of this species is of utmost importance considering the vulnerable nature of these islands, often faced with threats of anthropic elements and environmental calamities. Credible and convenient molecular marker methods were devised in the study based on RAPD-SCAR and 18S rDNA sequencing for authentication of this species and also for distinguishing from other closely related *Myristicaceae*. The dominant SCAR marker may be used as a DNA barcode for species authentication especially during the juvenile stages. The study also provides information on unique markers in seven other related, endangered members of *Myristicaceae*. These markers may be employed for germplasm patent disclosures or in legal issues regarding infringement. The information will be helpful in conservation, bioprospecting and augmenting traditional morphological systematics besides establishment of sovereign rights on endemic

species. However, it is recommended to involve larger populations that represent the available genetic diversity while undertaking such studies.

Acknowledgements The authors wish to thank the Director, IISR, Calicut for necessary facilities provided and DISC, IISR, Calicut for assistance in computation.

References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
- Bachmann K (1997) Nuclear DNA markers in plant biosystematic research. *Opera Bot* 132:137–148
- Bhakuni DS, Dhar ML, Dhar MM, Dhawan BN, Mehrotra BN (1969) Screening of Indian plants for biological activity, Part VIII. *Indian J Exp Biol* 7(4):250–262
- Castells CJ, Rodriguez MG, Rosas BM, Garca CN, Navarrete VB (2008) Population genetics of the endangered Canarian endemic *Atractylis arbuscula* (Asteraceae): implications for taxonomy and conservation. *Plant Syst Evol* 274:99–109
- Das M, Bhattacharya S, Pal A (2004) Generation and characterization of SCARs by cloning and sequencing of RAPD products: a strategy for species-specific marker development in bamboo. *Ann Bot* 95:835–841
- Ding G, Zhang D, Yu Y, Zhang B, Zhao L (2012) Genetic identification and relationship analysis of medicinal *Limonium* by rDNA ITS sequence, single nucleotide polymorphism (SNP) and amplification refractory mutation system (ARMS). *J Med Plants Res* 6:4535–4539
- Ghosh M, Chezian P, Ramasamy S, Ramasamy Y (2011) Development of SCAR marker in *Casuarina equisetifolia* for species authentication. *Trees* 25:465–472
- Jo SH, Koo DH, Kim JF, Hur CG, Lee S, Yang TJ, Kwon SY, Choi D (2009) Evolution of ribosomal DNA-derived satellite repeat in tomato genome. *BMC Plant Biol* 9:42
- Kim CS, Lee CH, Park KW, Kang SJ, Shin IS, Lee GP (2005) Phylogenetic relationships among *Pyrus pyrifolia* and *Pyrus communis* detected by randomly amplified polymorphic DNA (RAPD) and conserved rDNA sequences. *Sci Hortic* 106:491–501
- Lee GP, Lee CH, Kim CS (2004) Molecular markers derived from RAPD, SCAR and the conserved 18S rDNA sequences for classification and identification in *Pyrus pyrifolia* and *P. communis*. *Theor Appl Genet* 108:1487–1491
- Lee EJ, Hwang IK, Kim NY, Lee KL, Han MS, Lee YH, Kim MY, Yang MS (2010) An assessment of the utility of universal and specific genetic markers for opium poppy identification. *J Forensic Sci* 55:1202–1208
- Lee SY, Weber J, Mohamed R (2011) Genetic variation and molecular authentication of selected *Aquilaria* species from natural populations in Malaysia using RAPD and SCAR markers. *Asian J Plant Sci* 10:202–211
- Li G, Park YJ (2012) SCAR markers for discriminating species of two genera of medicinal plants, *Liriope* and *Ophiopogon*. *Genet Mol Res* 18:11
- Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25:1451–1452
- Maya KM, Zachariah JT, Krishnamurthy KS, Rema J, Krishnamoorthy B (2006) Fatty acids and leaf amino acids in *Myristica fragrans* Houtt. and related taxa. *Indian J Hortic* 63:316–318

- Mentewab AB, Jacobsen MJ, Flowers RA (2011) Incomplete homogenization of 18 S ribosomal DNA coding regions in *Arabidopsis thaliana*. BMC Res Notes 4:93
- Nei M, Rooney AP (2005) Concerted and birth and death evolution of multigene families. Annu Rev Genet 39:121–152
- Nickrent DL, Soltis DE (1995) A comparison of angiosperm phylogenies from nuclear 18S rDNA and rbcL sequences. Ann Mo Bot Gard 82:208–234
- Prasad PRC, Reddy CS, Lakshmi RKV, Kumari PV, Raza SH (2009) List of species. Angiosperms of North Andaman, Andaman and Nicobar islands, India. Check List 5:254–269
- Rema J, Krishnamoorthy B, Mathew PA (2005) Genetic diversity... Nutmeg (*Myristica fragrans* Houtt.). Indian Hortic 50:5–6
- Rieseberg LH (1996) Homology among RAPD fragments in interspecific comparisons. Mol Ecol 5:99–105
- Roy SC, Chattopadhyay A (2011) Evaluation of genetic diversity in mango germplasm resources using RAPD markers and characterization of cultivar Guti based on 18S rRNA gene sequence. Indian J Genet Plant Breed 71:254–261
- Roy S, Krishnan P (2005) Mangrove stands of Andamans vis-à-vis tsunami. Curr Sci 89:1800–1804
- Sasaki Y, Fushimi H, Cao H, Cai SQ, Komatsu K (2002) Sequence analysis of Chinese and Japanese *Curcuma* drugs on the 18S rRNA gene and *trnK* gene and the application of amplification-refractory mutation system analysis for their authentication. Biol Pharm Bull 25:1593–1599
- Sharief MU (2007) Plants folk medicine of Negrito tribes of Bay Islands. Indian J Tradit Knowl 6:468–476
- Sheeja TE, Rajesh Y, Krishnamoorthy B, Parthasarathy VA (2006) DNA polymorphism in clonal and seedling progenies of an elite nutmeg (*Myristica fragrans* Houtt.) by RAPD. J Plant Crop 34:558–561
- Sheeja TE, George KJ, Jerome J, Varma RS, Syamkumar S, Krishnamoorthy B, Parthasarathy VA (2008) Optimization of DNA isolation and PCR parameters in *Myristica* sp. and related genera for RAPD and ISSR analysis. J Spices Aromat Crops 17:91–97
- Sheeja TE, Sabeesh C, Shabna OV, Shalini RS, Krishnamoorthy B (2013) A comparative analysis of genetic diversity of wild and related genera of *Myristica* using RAPD and ISSR markers. J. Spices Aromat Crops 22 (in press)
- Soltis PS, Douglas ES, Paul GW, Daniel LN, Chaw SM, Russell LC (1999) The phylogeny of land plants inferred from 18S rDNA sequences: pushing the limits of rDNA signal? Mol Biol Evol 16:1774–1784
- Sun XQ, Wei YL, Zhou YF, Guo JL, Hang YY (2011) Development of species and region specific random amplification of polymorphic DNA- sequence characterized amplified region (RAPD-SCAR) markers for identification of the genuineness of *Spica prunellae* (Lamiaceae). J Med Plants Res 5:1677–1684
- Valderrama J, Martinez C (2000) Distribution of flavonoids in the *Myristicaceae*. Phytochemistry 55:505–511
- Yoo YK, Yuan T, Lee JS, Lee AK, Roh MS, Kurita S, Suh JK (2011) Species relationships of *Lycoris* endemic to Korea evaluated by RAPD and SNPs of nrDNA-ITS regions. Hortic Environ Biotechnol 52:145–151
- Zhang YB, Shaw PC, Sze CW, Wang ZT, Tong Y (2007) Molecular authentication of Chinese herbal materials. J Food Drug Anal 15:1–9