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Genetic diversity analysis of *Myristica* and related genera using RAPD and ISSR markers

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Abstract

Genetic diversity among seven species of *Myristica,* two of its related genera and an unidentified species was analyzed using 46 PCR markers (30 RAPD and 16 ISSR). This is the first study on molecular genetic diversity of the rare, endangered and endemic *Myristica* species and its related genera. RAPD and ISSR analyses yielded 497 and 262 bands with 98.1% and 97.3% polymorphism, respectively. By combining markers, a total of 759 bands were detected of which 743 (97.8%) were polymorphic with an average of 16.1 bands per primer. High level of existing genetic variability was evident from the high percentage of polymorphism. Combined analysis of RAPD and ISSR markers resulted in better distinction of species. The mean polymorphic information content (PIC) indicated that both the marker systems are effective in detecting polymorphism either individually or in combination. Similarity coefficient (Jaccards) varied from 0.22 to 0.62 when markers were combined and the pattern was similar to RAPD with a high Mantel matrix correlation (r=0.95). Principal Coordinates Analysis (PCA) conformed to cluster analyses. First three most informative PC components explained 51.1%, 49.3% and 46.5% of total variation. A maximum similarity of (63%) was observed between *Gymnocranthera canarica* and the unidentified species of *Myristica*. *Knema andamanica* and *Myristica prainii* were found to be the most distinct (17.7%). Similarities at molecular level were close to either the morphological traits (mace and fruit/seed characters) or the geographical location. Species specific bands could be identified from all the accessions under study, which has the potential for development into SCAR (Sequence Characterised Amplified Region) markers for genotype fingerprinting or development of specific DNA probes for identification and authentication.

Keywords: nutmeg, UPGMA, genetic variation, endemic, endangered, phylogeny

Introduction

Myristica is the largest genus with 120 species with Malayan region as its centre of origin. The family *Myristicaceae* has about 18 genera and 300 species. Among these four genera *viz., Horsfieldia, Gymnocranthera, Knema* and *Myristica* and about 15 species are found in India. Of these *Gymnocranthera canarica* and *M. fatua* are exclusive to the swamps. *M. malabarica* is occasional in the swamps and more frequent

in the evergreen forests as reported by Krishnamoorthy *et al.* (1997). *Gymnocranthera, M. fatua var. magnifica* and *M. malabarica* are endemic to the Western Ghats. *M. fragrans* is economically and medicinally popular and widely cultivated species. *M. prainii*, *Knema andamanica* and *M. andamanica* are endemic to the Andaman and Nicobar (A&N) Islands and are used in tribal medicine (Sharief 2007). Most of the species in the study are endangered, endemic/rare or threatened available only in the vulnerable hotspots and are used either in medicine or as spice. Despite great distances separating the members of this family, which are found in the humid tropics of different continents and oceanic islands, there is a striking similarity between them. Though distinguishable through morphological characters like shape of leaves and fruits/nuts, they require mature plants for identification and are unstable due to environmental influences. A broad based analysis of genetic diversity between and within wild species of *Myristica* is not yet done. In this context DNA markers like RAPD as suggested by Williams *et al.* (1990) and ISSRs as suggested by Zietkiewicz *et al.* (1994), are most appropriate for genetic diversity analysis, identification and conservation of the species. These methods are popular mainly because they are cheap, inexpensive, simple to perform and require no prior knowledge of DNA sequence and also require very little template DNA (Esselman *et al.* 1999). ISSR markers are highly polymorphic in plant populations with high consistency, reliability and co dominancy (Peng *et al*. 2006).

The main objective of the present study was to investigate phylogenetic relationships among the different species and related genera of *Myristica* and to identify species specific markers. The unique markers identified in the study can be used as species tags. This information may be utilized to identify duplicates in the large germplasm collection of *Myristica* and its wild and related genera at IISR (Indian Institute of Spices Research) for establishing a core collection.

Materials and methods

Seven species of *Myristica*, two of its related genera and an unidentified *Myristica* species collected from forests of Tamil Nadu, Kerala, Karnataka and Andaman and Nicobar Islands and conserved *ex situ* in the field gene bank of Indian Institute of Spices Research Farm, Peruvannamuzhi, Kozhikode, India (Krishnamoorthy *et al.* 1997) were used for the study (Table 1). Good quality DNA was isolated from fresh foliage samples from two to three individuals per species (Sheeja *et al.* 2008) and PCR amplification was performed as per Williams *et al.* (1990). Seventy five random primers (OPERON Technologies) were screened initially with two specimens to check

Table 1. Details of *Myristica* species and related genera used for study

Sl. No.	Names	Place of collection	Status
1	*Myristica fragrans* Houtt.	Kerala	Cultivated in tropical regions
2	*Myristica beddomi* King	Western Ghats, Kerala	Endemic
3	*Myristica malabarica* Lam.	South Karnataka	Endemic, rare threatened
4	*Myristica prainii* King	A & N Islands	Endemic
5	*M. fatua* Houtt. *var magnifica*	Western Ghats, Kerala	Endemic
6	*Myristica andamanica* Hook.f.	A & N Islands	Endemic, vulnerable
7	*Myristica amygdalina* Wall. ex Hook. f.& Thomson	Nagercoil	Native to South East Asia
8	*Myristica* sp.	Kerala	Not known
9	*Gymnocranthera canarica* (King) Warb.	Karnataka, Kerala	Endemic, threatened
10	*Knema andamanica* (Warb.)W.J.de Wilde	A & N Islands	Endemic, vulnerable

polymorphism and reproducibility. From these, 30 decamer primers (Table 2) showing clear banding pattern and polymorphism were used.

Preliminary screening was carried out with 42 ISSR primers (Sigma, USA), of which only 16 primers showed amplification (Table 3.). PCR reactions were performed as per Sheeja *et al*. (2006). The PCR program for ISSR was same as that of RAPD except the annealing temperature, which was optimized for each primer and number of cycle repeats was 32.

Data analysis

Only distinct and reproducible bands were scored as 'present' (1) or 'absent' (0). The discriminating power of primers was assessed by calculating percentage polymorphism, polymorphic information content (PIC) and the marker index (MI). PIC of individual primer was calculated to evaluate discriminatory power. The PIC value was calculated as per Roldan-Ruize *et al.* (2000): PICi = 2fi (1-fi), where fi is frequency of amplified allele (band present) and (1-fi) is frequency of null allele (band absent) of marker i. MI was determined as product of PIC and number of polymorphic bands per assay unit was determined as per Powell *et al.* (1996).

Marker index (MI) = PIC × No. of polymorphic bands

Table 2. Characteristics of polymorphic RAPD primers among *Myristica* and its related genera

Sl. No.	Primer	Sequence	Polymorphism (%)	PIC[a]	MI[a]
1	OPA-01	CAGGCCCTTC	94	0.39	6.24
2	OPA-05	AGGGGTCTTG	100	0.29	4.06
3	OPA-07	GAAACGGGTG	100	0.43	5.16
4	OPA-08	GTGACGTAGG	100	0.37	5.18
5	OPA-09	GGGTAACGCC	100	0.35	6.3
6	OPA-10	GTGATCGCAG	100	0.36	6.84
7	OPA-11	CAATCGCCGT	100	0.34	6.8
8	OPA-12	TCGGCGATAG	95	0.29	6.38
9	OPA-14	TCTGTGCTGG	100	0.40	4.8
10	OPA-15	TTCCGAACCC	86	0.29	3.77
11	OPA-16	AGCCAGCGAA	94	0.33	5.61
12	OPA-17	GACCGCTTGT	92	0.32	3.52
13	OPA-18	AGGTGACCGT	100	0.43	7.74
14	OPA-19	CAAACGTCGG	100	0.29	3.77
15	OPA-20	GTTGCGATCC	100	0.32	5.12
16	OPB-20	GGACCCTTAC	100	0.35	4.9
17	OPC-2	GTGAGGCGTC	100	0.37	6.66
18	OPC-6	GAACGGACTC	94	0.30	4.8
19	OPC-12	TGTCATCCCC	100	0.31	4.9
20	OPC-13	AAGCCTCGTC	100	0.39	8.97
21	OPE-1	CCCAAGGTCC	100	0.30	5.1
22	OPE-2	GGTGCGGGAA	93	0.29	4.06
23	OPE-3	CCAGATGCAC	100	0.34	3.74
24	OPE-5	TCAGGGAGGT	100	0.30	5.4
25	OPE-6	AAGACCCCTC	100	0.28	4.76
26	OPE-12	TTATCGCCCC	100	0.34	5.1
27	OPE-14	TGCGGCTGAG	100	0.31	5.58
28	OPE-15	ACGCACAACC	100	0.35	5.25
29	OPE-16	GGTGACTGTG	92	0.33	3.96
30	OPE-17	CTACTGCCGT	100	0.28	8.12

[sa] PIC, polymorphism information content; MI, marker index

Table 3. Characteristics of polymorphic ISSR primers among *Myristica* and its related genera

Sl. No.	Sequence	Polymorphism (%)	PIC[a]	MI[a]
1	$(CACG)_4$	100	0.39	5.07
2	$(AGC)_4GT$	100	0.48	4.8
3	$(CA)_8G$	91.0	0.31	3.1
4	$(CTC)_3GC$	100	0.39	5.46
5	$(GT)_8C$	93.3	0.35	4.9
6	$(AG)_8T$	85.7	0.19	1.14
7	$(CA)_8A$	92.8	0.33	4.24
8	$(CT)_8A$	100	0.42	6.3
9	$(TC)_7C$	94.1	0.33	1.98
10	$(TC)_7G$	100	0.32	6.72
11	$(AC)_7T$	100	0.39	8.58
12	$(AC)_7C$	94.7	0.31	5.58
13	$(AC)_7G$	95.8	0.34	7.82
14	$(CT)_7TG$	100	0.28	6.44
15	$(CT)_7AC$	100	0.36	5.04
16	$(GACA)_3GC$	100	0.36	8.28

[a] PIC, polymorphism information content; MI, marker index

The binary matrix prepared was used for calculating Jaccard's coefficient of genetic similarity using NTSYS-PC software version 2.0, Exeter Software, NY, USA by Rohlf (1998), where SIMQUAL program was used to calculate Jaccard's coefficients as:

$$N_{AB} / N_{AB} + N_A + N_B$$

Similarity coefficient values were used to construct a dendrogram using method of Unweighted Pair Group with Arithmetic Averages (UPGMA) and Principal Coordinates Analysis (PCA) was also carried out following NTSYS- pc version 2.02 (Rohlf 1998). The confidence limits for dendrogram groupings were performed by bootstrapping using Win Boot programme (http://irri.org/science/software/winboot.asp). Product-moment correlation (r) based on Mantel Z-value was computed to measure degree of relationship between similarity index matrices by any two markers as per Mantel (1967).

Results and discussion

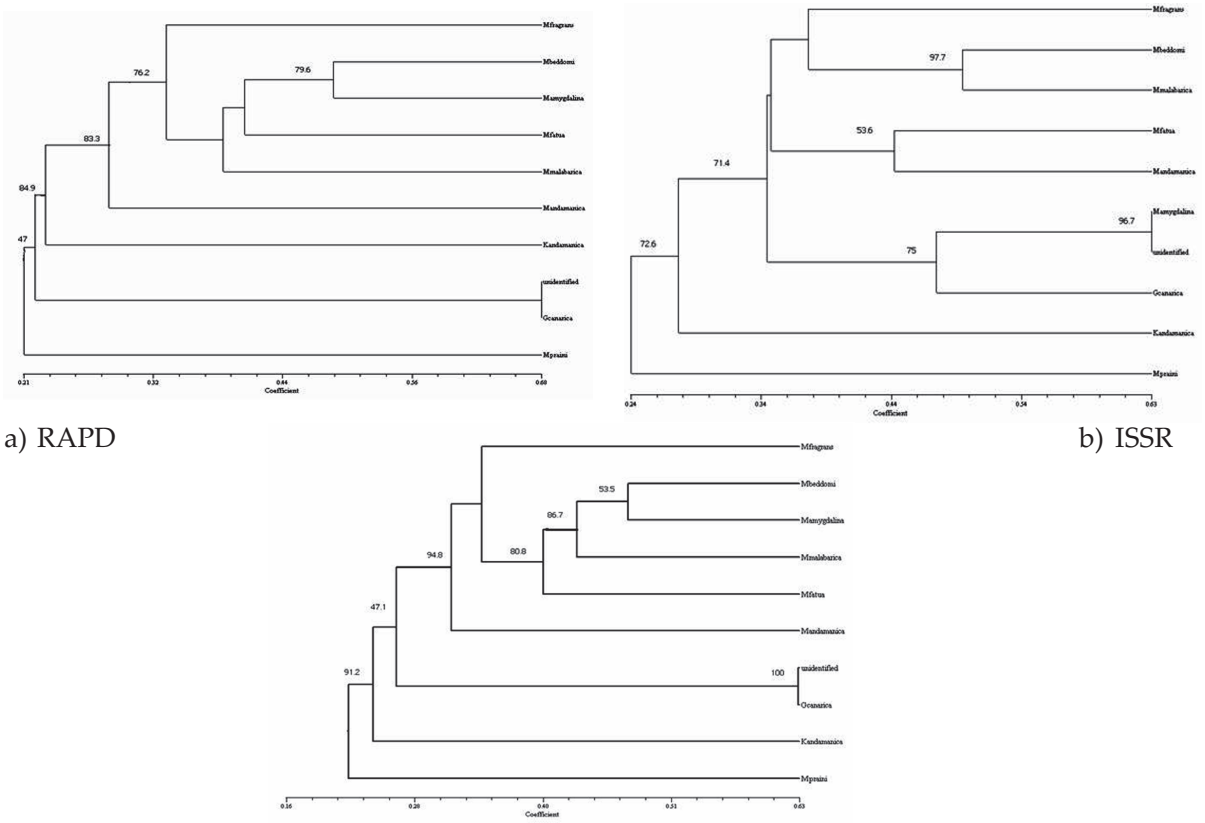
RAPD, ISSR and combined analyses

Total number of markers observed among genotypes based on RAPD (30 primers) and ISSR (16 primers) was 497 and 262, respectively. Number of scorable markers produced per primer ranged from 12 to 29 and 7 to 24, while size of amplified fragments ranged from 200 to 2500 bp in both. Total number of polymorphic markers and polymorphism were 488 and 98.1% respectively (Table 2 and 3).

PIC values, a reflection of allele diversity and frequency among genotypes, were not uniformly higher for all RAPD and ISSR loci tested. It ranged from 0.28 to 0.43 and 0.19 to 0.48 with a mean of 0.34 and 0.35 for RAPD and ISSR, respectively. The marker index (MI) ranged from 3.52 to 8.97 and 1.98 to 8.28 with an average of 5.4 and 5.3. The most informative primers were OPA-18, OPC-13 and OPE-17. The similarity coefficients based on 551 RAPD and 262 ISSR markers ranged from 0.23 to 0.67 and 0.21 to 0.63 and mean similarity indices were 0.26 and 0.32. In case of combined analysis it was 0.22 to 0.62 and 0.28, respectively.

Cluster analysis performed based on the Jaccard's similarity coefficient matrices calculated from both markers individually and combined generated dendrograms (Fig. 1) with species broadly grouped into two major clusters comprising of *M. praini* in first and all the rest in the second cluster. The combined analysis of RAPD and ISSR markers depicted the genetic relationships better than individual analysis. Earlier, Gupta *et al.* (2008) have also reported similar results in *Jatropha curcas*. Unidentified species showed 63% similarity with *G. canarica*.

The r and Z values denoted that correlation was not significant (0.68) for RAPD and ISSR, while for RAPD and integrated RAPD and ISSR and ISSR and integrated RAPD and ISSR it was significant at 0.95 and 0.86, respectively. PCA analyses were comparable to cluster analyses (Fig. 2). First three most informative PC components explained 51.1, 49.3 and 46.5% of total variation. *M. prainii* appears to be distinct from others in PCA. A number of unique species



a) RAPD b) ISSR

c) RAPD+ISSR

Fig. 1. (a-c). Dendrograms generated using UPGMA using RAPD, ISSR and combined marker data. Number of forks indicates confidence limits for grouping of those species in a branch occurred, based on 2000 cycles in bootstrap analysis, using Winboot program

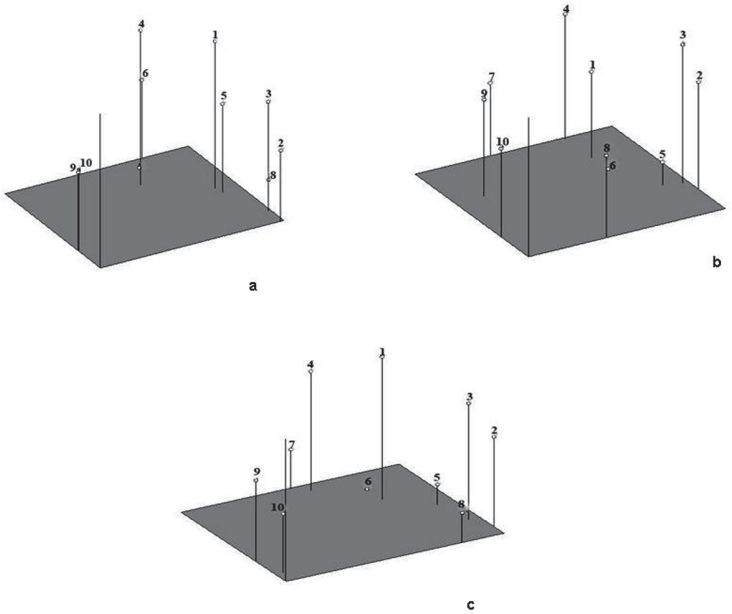


Fig. 2. Three-dimensional plot of PCA of *Myristica* and related genera using a) RAPD, b) ISSR, and c) combined analysis. The numbers plotted represents individual cultivars in Table 1.

specific RAPD bands detected from different species is given in Table 4.

Table 4. Selected RAPD bands having potential as SCAR markers for species identification

Sl. No.	Primer	Species	Unique band (bp)
1	OPA-12	*Myristica fragrans*	1300
2	OPA-15	*Myristica prainii*	450
3	OPA-16	*Myristica amygdalina*	494
4	OPA-17	*Myristica andamanica*	802
		Myristica andamanica	1767
5	OPA-19	*Myristica fragrans*	543
		Knema andamanica	421
6	OPA-20	*Knema andamanica*	275
7	OPE-5	*Myristica andamanica*	700
		Gymnocranthera canarica	350
		Knema andamanica	300
8	OPE-6	*Knema andamanica*	585
		Unidentified species of *Myristica*	550
		Myristica andamanica	500
9	OPE-17	*Knema andamanica*	900
		Myristica malabarica	700
		Myristica amygdalina	450

Most of the species like *M. malabarica*, *M. fatua*, *M. andamanica*, *M. prainii, K. andamanica* are endemic to India and hence very valuable. While *M. andamanica, M. magnifica var fatua, G. canarica* are rare/threatened and need to be conserved. *M. malabarica* is endangered as reported by Hammer & Khosbakht (2005). *M. fragrans* is the popular cultivated species known as a spice and medicine. Loss of this unique germplasm will be critical as far as biodiversity is concerned. The conservation and characterization of this germplasm demands that the genetic structure is well characterized and understood. Molecular information is also useful in unraveling the population and evolutionary genetics of these species. RAPD and ISSR markers are routinely employed for such purposes across a set of germplasm or cultivars. However, comparative studies in *Myristica* species involving molecular markers are very limited except for the reports by Sheeja *et al.* (2006 & 2008).

The discriminative power of primers for RAPD and ISSR marker systems revealed that polymorphism in RAPD (98.3%) is greater than ISSR (97.3%). Similar results were observed by Muthusamy *et al.* (2008) in *Vigna*. The ability to resolve genetic variation may be more directly related to the number of polymorphisms detected by the marker system as suggested by Sivaprakash *et al.* (2004). The results based on the two marker systems individually and combination, broadly grouped the genotypes into two major clusters. *M. prainii* was found to group separately in all the cases, showing its unique nature.

In the present investigation, the average number of fragments amplified by RAPD primers among the genotypes was 16.4 with a range of 12 to 29. Similar reports were made by Muthusamy *et al.* (2008) in rice bean. Such a high variation in the number of fragments produced by these arbitrary primers may be attributed to the differences in the binding sites throughout the genome of the accessions included. ISSR primers generated 90 to 121 markers with average of 103.4 per accession. The study showed that distribution of different microsatellite sequences in different plant genomes determines the possibility of using the method for DNA fingerprinting.

Comparison of PIC values for two marker systems indicated that the range of PIC values for RAPD and ISSR primers was 0.28 to 0.43. In case of ISSR only one primer possessed less than eight alleles, but in RAPD all the primers amplified more than ten alleles, indicating better resolving power of the RAPD markers. This is because of the polyallelic nature of RAPD markers as suggested by Muthusamy *et al.* (2008).

Correlation between RAPD and ISSR Jaccard's similarity coefficient values was low. A possible explanation for difference in resolution of RAPDs and ISSRs is that the two marker techniques target different portions of genome as suggested by Souframanien & Gopalakrishna (2004). Close correspondence between similarity matrices of RAPD and combined RAPD and ISSR and ISSR and

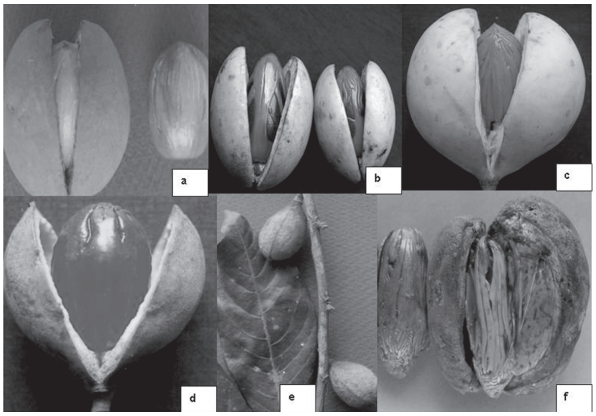


Fig. 3. Variation in fruit characters of some of the members of genus *Myristica* a) *M. beddomei* b) *M. andamanica* c) *M. fragrans*, d) *Knema andamanica* e) *M. prainii* f) *M. malabarica*

combined RAPD and ISSR was established by means of high matrix correlation value of 0.95 and 0.86, respectively. Hence, it can be concluded that both RAPD and ISSR either individually or combined can be effectively used in determination of genetic relationships. The mean marker index of RAPD was 5.4, while for ISSR it was 5.3.

Though pattern of clustering was similar in all three methods, individuals in each cluster showed mild difference in RAPD and ISSR. Earlier in rice bean (Muthusamy *et al.* 2008) and barley (Hou *et al.* 2005) a lack of congruence between RAPD and ISSR dendrograms was reported. Cluster combined marker system grouped genotypes differently with *M. prainii* and *M. beddomi* as most divergent. The consensus dendrogram is more similar to RAPD than ISSR. This revealed existence of sufficient amount of genetic variability among species. In general, similarity exhibited was low. Extensive genetic variability was reported by Singh *et al.* (1994), Hilu & Stalker (1995) in *Arachis*. The mean similarity index for ISSR was 0.32 and 0.26 for RAPD, which indicated a slightly higher genetic diversity detected by RAPD in conformity with results by Hou *et al.* (2005). The PCA analysis supported the UPGMA clustering in the present study.

Overall, molecular clustering showed close congruence to morphological characters of species in some cases and their geographical location in others. Maximum similarity was between *G. canarica* (63%) and unidentified species of *Myristica*. Among genus *Myristica*, *M beddomi* showed maximum similarity to *M. amygdalina* (47%) and *M. malabarica* (46%). All these three species were close to *M. fragrans*, possibly due to the similarity in their mace and fruit/seed characters. The former two are almost indistinguishable except for the yellow mace character of *M. amygdalina*. *M. fatua* (36%) and *M. amygdalina* (39%) were closest to *M. malabarica* due to their occurrence in marshy habitats. *M. fatua* possesses pheumophores for adaptation. The closeness among *M. fragrans, M. beddomi, M. amygdalina*, *M. fatua* and *M. malabarica* may be due to their same geographical origin (south India).

K. andamanica, *M. andamanica* and *M. prainii* with their common place of origin (A&N Islands), showed lesser similarities with rest of the species. *Myristica prainii* was the most distinct among all the members of *Myristica* species and grouped separately in all three cases. Geographically isolated population accumulates genetic differences as they adapt to different environment as suggested by Souframanien & Gopalakrishna (2004). RAPD markers were used earlier also to ascertain geographical origin among *Astragali radix* populations by Na *et al.* (2004).

Lowest similarities were shown in case of *K. andamanica* with *M. prainii* (17%) and *M. malabarica* (19%) since they belong to two different genera. The morphological characteristics of *M. prainii* are entirely different from rest due to compound fruits, panicles, entire mace, small fruit and seed size. However, *M. prainii* showed similarity in molecular profiles with *M. fragrans*, because they belong to the same genus.

Species specific bands identified (Table 4), have potential for conversion to SCARs for protection from biopiracy since the species under study are rare, threatened and endemic. Species-specific DNA markers are widely applied in the area of molecular taxonomy and molecular diagnosis of germplasm by Sucher & Carles (2008) Species-specific markers were

developed for six *Eucalyptus* members by Balasaravanan *et* al. (2006) and in *Dendrobium* species by Wang *et al.* (2009) using molecular markers.

It could be concluded from the study that RAPD and ISSR markers either singly or in combination can be used for detecting polymorphism in *Myristica* and its related genera. Both the marker systems could clearly distinguish accessions within the species and within the genera. Diagnostic markers can help in unequivocal identification and thus help from biopiracy of this valuable endemic/threatened flora. These can also be useful in germplasm and genebank management. This is the first study of its kind in *Myristica* and its related genera and is a stepping stone for further research on the population and evolutionary genetics of *Myristica*.

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