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Candidate molecular markers for monoecy in dioecious tree spice nutmeg (*Myristica fragrans* Houtt.) and analysis of genetic variability in a core collection

Nutmeg (*Myristica fragrans* Houtt.) is a major tree spice valued for its diverse uses in flavouring and pharmaceutical industry. Dioecious sex nature is the greatest bottleneck in its cultivation, and to avoid the male plants commercial orchards, propagation is necessitated through budding and grafting[1]. Previous attempts to develop molecular markers linked with the female sex form were differentially successful[2–4]. For higher yields, planting should be done using monoecious plants or at the sex ratio of one male plant for 10 female plants. Thus, the development of a marker will enable identification of monoecy at seedling phase itself and hence the selection of seedlings for planting.

The southern part of India has considerable genetic variability in this cross-pollinated crop, especially for growth, sex forms, yield and traits of fruits, mace and nuts[5–7]. A systematic genetic diversity analysis will enable determination of population structure[8] and to develop conservation strategies. The molecular marker-based analysis of genetic variability and population structure is an approved strategy and in plants with little genomic information, random primer-based systems are more reliable[9]. Hence, in the present study we used random amplified polymorphic DNA (RAPD) marker system[10]. The study performed using *Myristica fragrans* core collection from

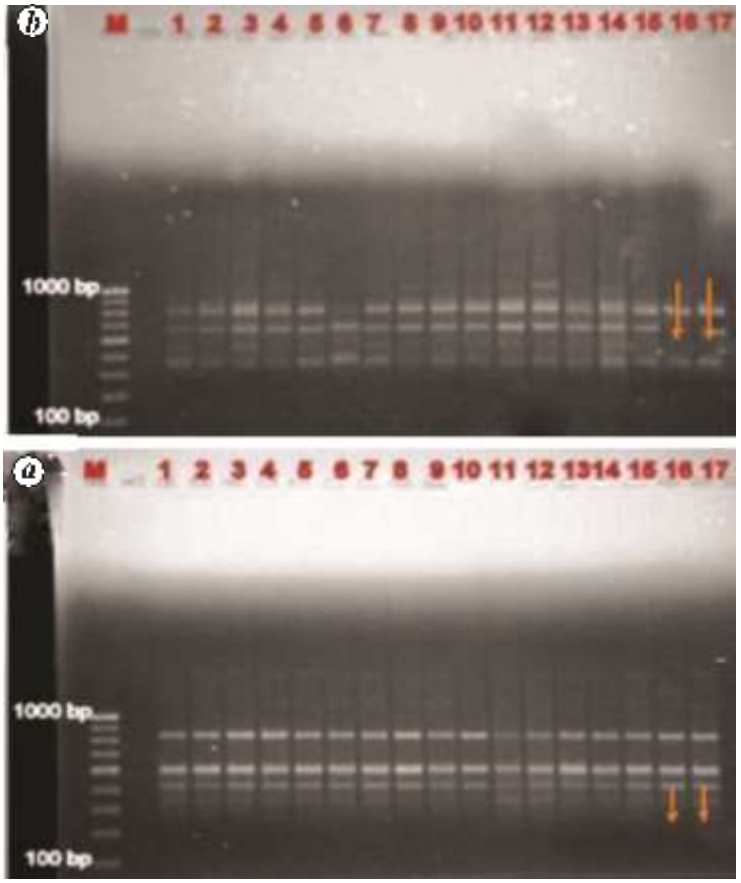

Figure 1. Amplification pattern in select nutmeg accessions with RAPD primers – ***a***, OPE15 and ***b***, OPE16. Lanes M, Ladder (*Eco*RI/*Hin*dIII, 1000 bp); lane 1, Acc.1; lane 2, Acc.5; lane 3, Acc.8; lane 4, Acc.9; lane 5, Acc.11; lane 6, Acc.14; lane 7, Acc.18; lane 8, Acc.21; lane 9, Acc.23; lane 10, Acc.24; lane 11, Acc.30; lane 12, Acc.35; lane 13, Acc.36; lane 14, Acc.37; lane 15, Acc.38; lane 16, Acc(H).1 and lane 17, Acc(H).4.

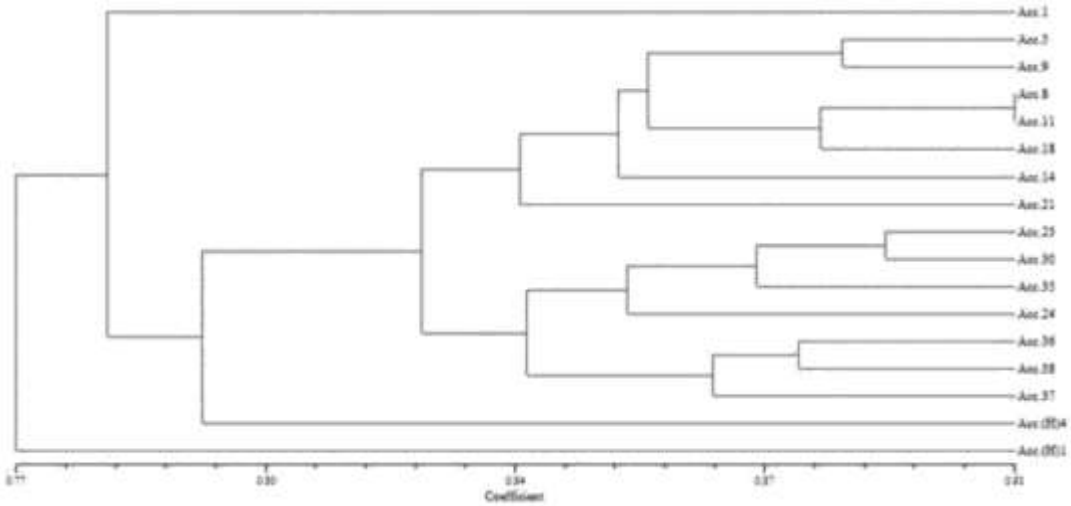

Figure 2. UPGMA dendrogram of RAPD profiling of select nutmeg accessions.

Kerala reveals the appreciable genetic diversity of this crop and valuable monoecy-linked markers.

Through a D^2 analysis on a core collection of 46 accessions (maintained in Chalakudy river basin of central Kerala, lat. 10.30°N, long. 76.33°E), 17 accessions – 15 female and 2 monoecious – with uniform age of 15 years were selected; care was taken to maintain adequate representation of all the distinct germplasm traits (Supplementary Table 1). Genomic DNA was extracted from the young tender leaves; third leaf from the shoot tip, using the protocol of Divyasree *et al.*[11], modified from the original cetyl trimethylammonium bromide (CTAB) method[12]. Initially, 43 primers were screened using bulk DNA and 21 primers generating distinct polymorphism were used for analysis. For thermal cycling and electrophoresis, the standard protocol was followed[11].

The analysis revealed 164 scorable bands, with the number of bands resolved per amplification varying from 5 to 10 and a mean polymorphism of 63.21% (Supplementary Table 2). Among the 21 primers used, 5, viz. OPA07, OPA02, OPB06, OPC05 and OPP13 yielded more than 80% polymorphism, with OPA07 showing the highest value of 90%. The amplification and polymorphism levels followed the existing reports on RAPD analysis in tree crops[13–15].

Primer OPE15 generated an additional band in both monoecious accessions at 500 bp; and this was absent in female accessions (Figure 1 *a*). Similarly, OPE16 yielded a characteristic band in monoecious accessions at 200 bp (Figure 1 *b*). The flowering and sex determination pathways in dioecious plants are highly complicated and poorly understood[16]. Presence of additional bands in monoecious accessions points to the possibility of their use as candidate markers for seedling selection in nutmeg. The uncertainty over the sex form of seed-germinated plants necessitates the propagation of nutmeg through budding or grafting. The identified markers will enable massive seedling screening at nursery phase itself and hence are commercially important.

Agglomerative hierarchical clustering was performed by UPGMA method using Jaccard's similarity coefficient matrix. Dendrogram was constructed on the basis of binary data generated by scoring the bands on 0 and 1 basis across the accessions. The pairwise similarity coefficient values varied between 0.77 and 0.91, suggesting high genetic variability across the core collection. Corresponding to a similarity coefficient of 0.86, all the 17 accessions were observed to group into 7 clusters (Figure 2).

The clustering pattern observed was found correlated with the morphological features of accessions. Cluster II hosted six accessions bearing the maximum number of fruits/tree and bold, thick mace. Four accessions in cluster IV were characterized by average fruit-bearing with bold nuts and mace, whereas three accessions in cluster V had medium-sized fruits with small nuts and mace. Rest of the clusters had one accession each, with unique qualitative and quantitative traits. These results suggest that molecular analysis successfully represent the morphological variation in the core collection. Even with its limitation of reproducibility, due to the use of random primers, RAPD analysis is highly useful in the generation of trait-linked markers, especially in plants with limited genomic information. Further, due to the capability to yield a large number of amplicons, it is widely employed in diversity studies as well[17].

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New record of *Chaetasbolisia erysiphoides* from cold arid soils of Zanskar (Kargil), India

Zanskar is a high altitude semi-desert valley in Ladakh (30°45′–35°50′N and 75°45′–80°31′E) lying on the northern flank of the Great Himalayan range. This mountain range acts as a climatic barrier protecting Zanskar valley from most of the monsoon, resulting in a warm and dry climate during the six months of summer, with a temperature range of 20–27°C, low humidity and scant precipitation. However, winter in this valley is freezing cold as the temperature goes below 0°C (–30°C) owing to heavy snowfall and some parts of it are considered as the coldest inhabited places of the world. The whole of Ladakh region usually remains land-locked and isolated from the rest of the country for about six months (November to May) because of continuous snowfall, snow blizzards and high velocity dust storms. Therefore, the soil usually remains covered by thick snow and its temperature is below zero.

The genus *Chaetasbolisia* Speg., typified by *C. erysiphoides*[1] embodies a rare group of microfungi characterized by superficial, unilocular, dark brown pycnidia with several setae scattered over its body; doliiform to ampulliform conidiogenous cells formed from the inner cells of the pycnidial wall and hyaline aseptate conidia[2]. The genus belongs to the family Didymellaceae in the Pleosporales, Dothideomycetes (ref: Index Fungorum). There are seven species, viz. *C. californiana*, *C. erysiphoides*, *C. falcata*, *C. falcata* var. *minuta*, *C. longiseta*, *C. microglobusa*, *C. raphiae* and *C. sapotae*, described in the genus, but so far no revision has been made[2]. Another species, *Chaetasbolisia indica*, was reported from India[3]. *Chaetasbolisia erysiphoides* initially described[4] as *Chaetophoma erysiphoides* was found to be hypophyllous on leaves of *Photinia loriformis* in China. It was later renamed as *Chaetasbolisia erysiphoides*[5]. The type species was redescribed[6] but not through an examination of the type collection.

In India, the genus was represented so far by only one species, *C. indica*, which was found to be lignicolous and showed differences from the type species *C. erysiphoides* in having much larger conidiomata, small blunt setae and slightly smaller eguttulate conidia[3]. During a mycological survey of cold arid soil of Zanskar valley (India), *Chaetasbolisia erysiphoides*, the type species of the genus was isolated and newly reported as

Table 1. Comparative account of two species of *Chaetasbolisia* reported from India

Species	Morphological characters			
	Conidiomata	Conidiogenous cells	Conidia	Setae
C. erysiphoides	Unilocular, brown, 92–184 μm in diam.	Ampulliform 7.4–9.4 × 1.4–2.5 μm	Oval, hyaline, minutely guttulate, 4.2–5.0 × 2.1 μm	Acute, irregularly verrucose, up to 122 μm long and 3.3–5.1 μm thick
C. indica	Unilocular, brown to blackish, 140–220 μm in diam.	Cylindrical to ampulliform, 4–9 × 2.5–4 μm	Oval, oblong-elliptical, eguttulate, 4–5 × 2 μm	Blunt, smooth, up to 20 μm long and 4–8 μm thick