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Identification of variety-specific ISSR markers in small cardamom (*Elettaria cardamomum* Maton.)

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Cardamom (*Elettaria cardamomum* Maton.) has recently become a major focus of Biotechnology research. It exhibits considerable variation under cultivation (Korikanthimath, 2003). There are three botanical varieties in this monotypic genus namely, Malabar, Mysore and Vazhukka with prostrate, erect and semi-erect panicle characteristics respectively, as the discriminative morphological markers. The identification of varieties is important during their breeding and registration process. All the varieties and races are interfertile and the observed variations are probably due to natural crossing (Korikanthimath, 2003).

Considering the difficulties involved in the traditional divergence studies, new methods based on studies at DNA level are being incorporated into breeding programmes because these techniques are reliable, unambiguous in nature and easy to adopt (Williams *et al*., 1990; Karp *et al*., 1997). They are widely used for the estimation of genetic diversity and are reported to be more precise than phenotypic markers (Gupta and Varshney, 2000). Inter simple sequence repeat markers (ISSR) use simple sequence repeats anchored at the 5' or 3' end by a short arbitrary sequence as PCR primers (Zietkiewicz *et al.*, 1994) and have been extensively used to characterize genetic diversity in several crop species (Arnau *et al*., 2003, Gomes *et al.*, 2009). They are of particular importance to study the variability, identification and germplasm conservation. ISSR markers differentiate closely related individuals at the inter-specific level because of high stability, good repeatability, speed and ease in handling (Wolfe and Liston, 1998).

Understanding the diversity of cardamom germplasm collections is important for effective exploitation of their genetic potential, selection as breeding lines, maintenance and for conservation. So, application of DNA marker technology in variety identification and diversity analysis in cardamom germplasm has very important practical value and theoretical significance in germplasm identification, genetic improvement, germplasm protection, core germplasm construction and importantly agronomic gene orientation. Molecular markers have been looked upon as tools for large number of applications ranging from characterization and evaluation of genetic diversity, localization of a gene to improvement of plant varieties by marker assisted selection *etc*. The exploration of rich biodiversity of cardamom has resulted in the release of a few high yielding clones/selections suited to different agro-ecological conditions of the cardamom tract (Kuruvilla *et al*., 2006). However, very limited work has been done on the characterization of cardamom germplasm with molecular markers (George *et al*., 2006). Moreover, there have been no reports on the development of variety specific markers in cardamom as well. The objectives of the present study were to assess the genetic diversity among the three botanical varieties and to develop variety specific markers.

Ten accessions each from the three different varieties of small cardamom (Table 1) were collected from the germplasm repository of Indian Cardamom Research Institute, Myladumpara. Genomic DNA was extracted from young leaves using a modified CTAB method (Mary *et al.*, 2013). The quantity and quality of DNA was checked with spectrophoto-

meter (Hittachi) and agarose gel (0.8%) electrophoresis. Absorbance ratio between 260 and 280 was computed and the quality of genomic DNA was confirmed. Final sample was stored at -20 ºC for downstream applications and the DNA concentration was adjusted to 25 ng μl^{-1} for PCR reactions.

ISSR primers (Sigma) were synthesized based on di- and tri-nucleotide repeats (GT, CT, AG, GAA and AGC) as a core sequence with a Tm value range of 47 to 56. Screening was carried out with 25 primers out of which 15 primers that gave clear banding patterns were used for confirmatory studies. Polymerase chain reactions were carried out in a reaction volume of 25 µl containing 25 ng of template DNA, 200 µM of dNTPs, 15 pM of primer, 0.5U of Taq DNA polymerase and 1X PCR reaction buffer with 15 mM $MgCl_2$. The optimum annealing temperature was determined for each primer. Amplification was carried out in a Biorad thermal cycler, with an initial denaturation at 94 ºC for 5 min followed by 39 successive cycles of denaturation at 94 ºC for 45 s, primer annealing at required temperature for 60 s, and extension of annealed primer at 72 ºC for 90 s, followed by a final extension at 72 ºC for 10 min. Amplicons were separated alongside a molecular weight marker (100 bp ladder, Bangalore Genei) by 2 per cent agarose gel Electrophoresis was done in 1X TAE (Tris acetate EDTA) buffer, stained with ethidium bromide and visualized in an UV trans-illuminator. Gel photographs were scanned through a Gel Doc System (Biorad, USA).

The ISSR marker was tested on 27 cardamom accessions that included 25 prostrate panicle varieties and 2 erect panicle accessions. The reaction mix (25 µl) consisted of 25 ng of DNA from each sample, 1X reaction buffer, 2.5 mM of Mg^{2+}, 200 µM each of dNTPs, 15 pM of each ISSR primer and 1 unit of Taq DNA polymerase. The annealing temperature and thermal cycler programming was the same. The products were resolved on 2 per cent agarose gel and the amplicons documented in a gel documentation system (Biorad).

Polymerase chain reactions were repeated twice to establish reproducibility of results under strict control of the reaction conditions. Distinct, reproducible, well resolved fragments were scored manually for the band presence (1) and absence (0) for each of the ISSR markers and different bands of different primers were used for constructing the fingerprinting of the selected cardamom accessions. The 0 or 1 data matrix was created and used to calculate the genetic distance and similarity using NTSYS-PC program (numerical taxonomy and multivariate analysis system program) (Rohlf,1993). The dendrogram was constructed by using a distance matrix using the unweighted pair group method with arithmetic mean (UPGMA) sub-program of NTSYS-PC.

Small cardamom accessions from three botanical varieties (Table 1, Fig. 1) were analyzed using ISSR primers. The analysis of 15 ISSR markers revealed that the PCR product size (bp) ranged from 250 to 1300. It resulted in the detection

Table 1. Ten accessions each of the three varieties of cardamom analyzed in the present study

Sl. No.	Malabar (Prostrate panicle)	Mysore (Erect panicle)	Vazhukka (Semi erect panicle)
1	ICRI 1 (14)	ICRI 2 (12)	MCC 4 (15)
2	ICRI 3 (11)	MCC 65 (10)	MCC 12 (14)
3	PV 1 (19)	KUTHU MYSORE (12)	MCC 8 (15)
4	MCC 34 (12)	MCC 6 (14)	MCC 334 (12)
5	MCC 44 (9)	MCC 67 (11)	MCC 55 (13)
6	MCC 58 (12)	MCC 260 (16)	MCC 64 (10)
7	MCC 60 (7)	MCC 39 (15)	MCC 66 (12)
8	CRS MUDIGERE 1 (12)	MCC 57 (8)	MCC 70 (11)
9	IISR VIJETHA (18)	MCC 59 (7)	MCC 71 (10)
10	IISR AVINASH (16)	MCC 61 (13)	MCC 72 (9)

Number in parentheses indicates the number of polymorphic bands for the ISSR primers (UBC841, UBC 866 and UBC 807) wherein maximum diversity is exhibited.

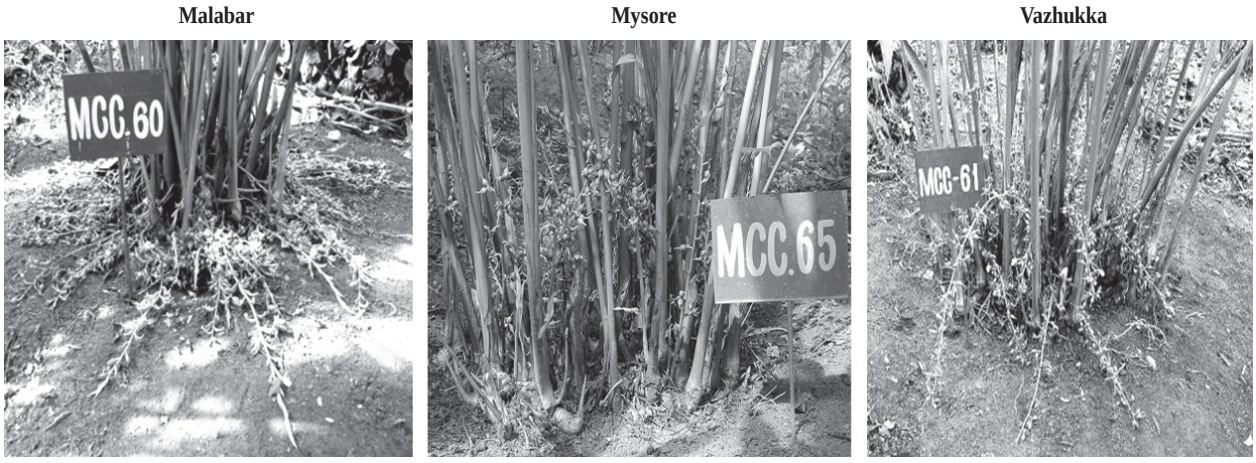

Fig.1. Botanical varieties of cardamom

of a total of 133 alleles, ranging from 14 alleles in UBC 866 to 5 alleles in UBC834, 873 and 867. Number of amplified fragments, number of polymorphic bands per primer and primer differentiation indices are given in Table 2. The levels of polymorphism were calculated based on the percentage of polymorphic bands. The highest number of amplified and polymorphic fragments was obtained using primer UBC 866. Some polymorphic fragments produced by ISSR primers were unique and could be used to discriminate specific varieties (Malabar, Mysore and Vazhukka) of cardamom.

Table 2. Genetic diversity parameters generated by 15 ISSR primers in ten selected cardamom germplasm of each botanical variety

Sl. No.	Primer sequence	Total no. of bands	No. of polymorphic bands	% of polymorphism
1	UBC 868	11	5	46
2	UBC 866	14	11	79
3	UBC 840	10	6	60
4	UBC 812	6	3	50
5	UBC 834	5	1	20
6	UBC 807	10	8	80
7	UBC 857	8	5	63
8	UBC 873	5	2	40
9	UBC 841	13	10	77
10	UBC 850	8	5	63
11	UBC 844	12	9	75
12	UBC 860	7	4	57
13	UBC 843	8	3	38
14	UBC 841B	11	6	55
15	UBC 867	5	2	40

The diagnostic potential of variety (prostrate panicle) specific marker was tested on another set of 26 cardamom accessions (Fig. 2) which included 25 different accessions of Malabar (prostrate panicle) and one accession of Mysore (ICRI 2) (erect panicle). The fragment (1250 bp) was present in 22 (88%) Malabar varieties and was absent in the tested Mysore variety. The specific fragment was absent in three Malabar varieties (MCC 103, MCC 104 and MCC 195) indicating the heterozygous nature of these accessions.

Cluster analysis of ISSR markers separated the cardamom genotypes into two major groups (Fig. 3). The first cluster comprised of all the genotypes except IISR Avinash, IISR Vijetha, ICRI 3, MCC 55 and MCC 59 which were in turn grouped in the second cluster. The first cluster further differentiated into several sub clusters. Some of the Malabar varieties IISR Avinash, ICRI Vijetha and ICRI 3 have come under one cluster. Similarly Malabar varieties ICRI 1, PV 1 and MCC 34 have clustered closely. Several of the Mysore varieties were grouped under the same sub cluster. Nevertheless, it could be seen that the cluster analysis was not in true agreement with the classification of the three varieties in cardamom as it could not clearly delineate all the tested genotypes to the three varieties. Similar results were observed in ginger (Wahyuni *et al.*, 2003) and *Musa* sp. (Wong *et al.*, 2001) using AFLP markers. Among the accessions, MCC55 and MCC59 showed very close similarity indicating duplication though these belonged to two different varieties. Among the

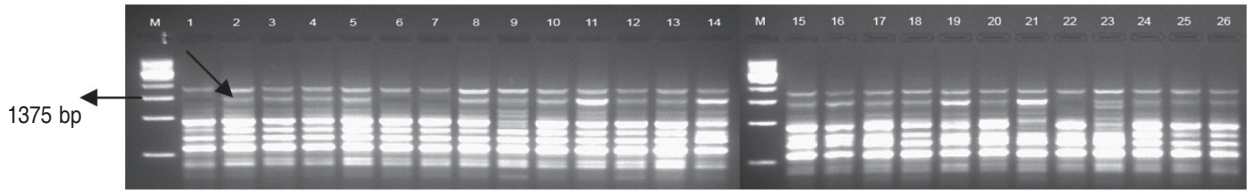

Fig. 2. Variety specific (prostrate panicle) marker confirmation in selected Mysore (lane-1) and Malabar varieties (lane 2-26)
M- Ecor1/HindIII, 1- ICRI 2 (Mysore variety), 2- Panikulangara 1, 3- Valley Green Gold, 4- Palakudy, 5- Panikulangara2, 6- MCC103, 7- MCC104, 8- MCC105, 9- MCC108, 10- MCC118, 11- MCC119, 12- MCC120, 13-MCC123, 14- MCC124, 15- MCC128, 16- MCC132, 17- MCC133, 18- MCC134, 19- MCC135, 20- MCC18, 21-MCC193, 22- MCC195, 23- MCC196, 24- MCC202, 25- ICRI4, 26- ICRI6

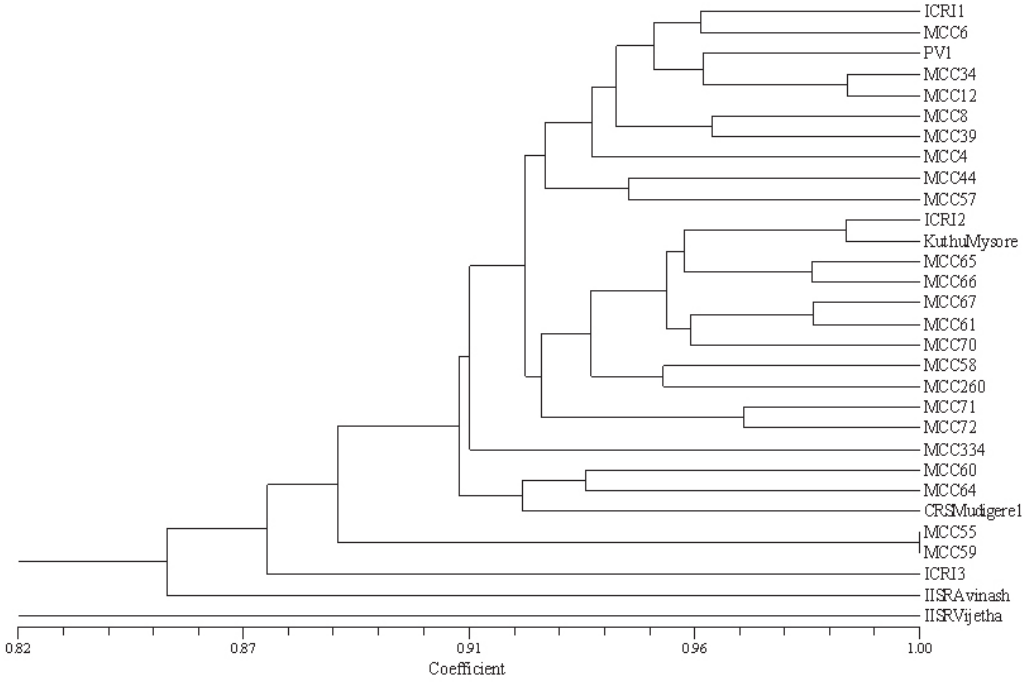

Fig. 3. Dendrogram generated by UPGMA cluster analysis illustrating genetic relationships among 30 small cardamom accessions

different accessions, MCC60 and MCC64 showed the least similarity at a co-efficient 0.93 and MCC34 and MCC12 showed maximum similarity (0.98). ICRI 2 a Mysore variety and Kuthu Mysore, typical Mysore appeared closely related with 0.97 similarity co-efficient. Further analyses using more ISSR markers have to be carried out to separate the accessions distinctly for assessing the variability among cardamom varieties. The advantage in the use of ISSR marker lies in their linkage to SSR loci which are likely to mark gene rich regions (Kojima *et al*., 1998). The variety specific markers using 10-mer arbitrary primers have been successfully used in distinguishing the rice varieties of distinctly different origins and with distinctly different characteristics (Ko *et al*., 1994).

In conclusion, ISSRs can be successfully employed to assess the level of polymorphism and diversity analysis in cardamom varieties. The results obtained in the present investigation could be useful for molecular identification, germplasm conservation and construction of core collections. The identified variety specific ISSR marker could be transformed to sequence characterized amplified regions (SCAR) after sequencing and designing primer pairs to develop markers specific for the variety.

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