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Diversity analysis of released varieties of Indian cardamom using ISSR markers reveal narrowing genetic base

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Elettaria cardamomum Maton the small cardamom of commerce is a monotypic genus in India under the family Zingiberaceae. Genetic diversity studies using inter simple sequence repeat (ISSR) markers were conducted on a total of 13 released varieties of cardamom including selections and hybrids from different research stations in South India along with a popular variety Njallani and a wild relative *Aframomum* sps as checks. ISSR markers however, revealed relatively high level of genetic redundancy among the tested varieties with the exception of a few. Nonetheless, unweighted pair group method of arithmetic average (UPGMA) based cluster analysis of the similarity matrix differentiated all the varieties tested with the exception of two and segregated the wild relative *Aframomum*. Considerable reduction of polymorphism percentage was observed on exclusion of *Aframomum* while studying ISSR polymorphism which could be an indicator of the narrowing genetic base in the released varieties. Morphological data were compiled based on International Plant Genetic Resources Institute (IPGRI) cardamom descriptor and revealed moderate variability among the varieties. The results highlighted the requirement of molecular characterization of unutilized germplasm accessions, related wild species, and exotic relatives using specific molecular markers to help refine breeding efforts and introgression of new alleles for further improvement and enhancement of genetic base of cultivated cardamom.

Keywords: *Elettaria cardamomum*, ISSR markers, varietal diversity, genetic base

Introduction

Elettaria cardamomum Maton or small cardamom 'the queen of spices' from Zingiberaceae is one of the most acclaimed of all spices for its unique flavor and pleasant aroma. A shade loving monocot with a rhizomatous herbaceous perennial habit, cultivated cardamom is monotypic in India[1-2]. Several documents endorse that cultivated cardamom is native to the evergreen forests of Western Ghats of South India within 8' and 30' N latitudes and 75' and 78'30' E longitudes, where it exhibits substantial variability[3-6]. Considering the abundance of genetic variability of the species apparently due to its cross pollinating nature[7-8] it is assumed that the Western Ghats must have been probably the major center of its genetic diversity.

Cardamom is a highly location specific crop with three domesticated natural cultivars namely Malabar, Mysore and Vazhukka[9]. Domestication of cardamom however started only in the 19th century and conservation of cardamom genetic resources started

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only in the early 1960s with six research organizations in the country[10]. The largest repository of cardamom collections with a total of 814 accessions is maintained at Indian Cardamom Research Institute (ICRI) at Kerala and its outstations. Morphological characterization and germplasm selection and less frequently hybridization have been carried out by several workers[11-17] that has led to varietal releases. Incidentally reports on molecular marker studies on cardamom that have been less frequent[18-21] remains to be assessed for its contribution to pre-breeding efforts.

The present study aimed for a perspective on the extent of genetic variability in a set of cardamom varieties released from India utilizing molecular marker data with a special focus on varieties released from ICRI *vis-a-vis* representative samples from other institutes.The study employed the multi-locus ISSR markers amplified using microsatellite primers, widely used for evaluating genetic diversity in crop plants[22]. It was also expected to ascertain uniqueness and distinctiveness of every released variety studied and be a pointer to subsequent genetic enhancement and crop improvement strategies in cardamom.

Materials and Methods

Twelve cardamom genotypes released from different cardamom research centers of Southern India and a farmer's popular selection (Njallani) were selected for the study. A different genus, *Aframomum melegueta* a wild relative was used as out group. All the ICRI released varieties *viz*. ICRI1, ICRI2, ICRI3, ICRI4, ICRI5, ICRI6, ICRI7, ICRI8 were selected from ICRI, Myladumpara, Kerala; Njallani farmer's selection from Idukki, Kerala and representative samples, IISR Avinash & IISR Vijetha from ICAR- Indian Institute of Spices Research (IISR), Kozhikode, Kerala, Mudigere 1 from UAHS, Mudigere, Karnataka, PV1 from Cardamom Research Centre, KAU, Kerala (Table 1). Morphological analysis of variability that forms a major part in the characterization of germplasm was done as per the

Table 1 — Released varieties of cardamom employed in the present study and their details

Sl No.	Variety	Centre developed	Year of release	Pedigree/ Parentage	Av. yield (kg/ha/)*	Dry recovery	Oil %	Salient features
1	ICRI 1	ICRI**	1992	CS Malabar	325	22.90	8.70	Early maturing, round extra bold dark green capsules, panicle medium sized
2	ICRI 2	-do-	1992	CS Mysore	375	22.50	6.67	Bold parrot green capsules, rot disease tolerant, medium long panicle
3	ICRI 3	-do-	1994	CS Malabar	440	22.00	6.60	Early maturing, rot tolerant, bold parrot green capsules suitable for Karnataka
4	ICRI 4	-do-	1997	CS Malabar	455	22.76	6.40	Early maturing, bold capsules, medium panicle, suitable for low rainfall areas, relatively tolerant to rot and capsule borer
5	ICRI 5	-do-	2006	Hybrid Vazhukka	1543	23.15	7.13	First hybrid, early maturing, high yielder moderately drought tolerant, capsule > 7 mm.
6	ICRI6	-do-	2006	CS Malabar	1200	19.00	7.33	High yielder, medium maturity, moderately drought tolerant, bold capsules > 7 mm
7	ICRI 7	-do-	2010	Hybrid Malabar	1400	22.00	8.84	Angular bold capsules, oleoresin 7.99%
8	ICRI 8	-do-	2014	CS Malabar	655	19.00	7.1	Oval, bold and pale green capsules
9	IISR Vijetha	IISR-ICAR *	2001	CS Malabar	643	22	7.9	Resistant to katte virus, bold capsules. Field tolerant to thrips and borer.
10	Mudigere	RRS-UAS***	1984	CS Malabar	300	20	8.0	Moderately tolerant to thrips, hairy caterpillar and white grubs, pale green, oval bold capsule
11	PV 1	CRS-ICAR*	1991	CS Malabar	260	19.9	6.8	Early maturing. short panicle, elongated ribbed light green, long and bold capsules
12	IISR Avinash	IISR ICAR*	2001	CS Malabar	847	20.8	6.7	Dark green capsules. Tolerant to rhizome rot, and shoot/panicle/capsule borer
13	Njallani	Farmer's selection	1990s	Selection Vazhukka	1600	25	9.01	High yielder non-pubescent, semi erect, globose extra bold and dark green capsules.

Courtesy: Cardamom cultivation practices Spices Board 2009, AICRPS- http://aicrps.res.in/sites/default/files/inline-files/Varieties.pdf
CS- Clonal selection, * Dry yield, ** Kerala state, *** Karnataka state.

descriptor based on IPGRI cardamom descriptor[23] and distinctness uniformity and stability (DUS) guidelines (Protection of Plant Varieties and Farmers' Rights Authority, Govt of India) in India[24]. Twenty five characters including eleven qualitative and fourteen quantitative characters were selected (Table 2) and data were compiled and documented for all the selected varieties.

Analysis of Morpho-Agronomic Data

Data on morphological parameters, yield and yield contributing characters of the 13 released varieties for 3 consecutive years were compiled simultaneously during the crop seasons of 2013-2016. Mean, standard deviation and correlation of variation of quantitative characters were calculated using standard procedures.

Genomic DNA Isolation

Isolation of total genomic DNA was done from young unopened leaves of the selected accessions during morning hours between 8 to 9 AM. Total genomic DNA was isolated employing a modification of cetyltrimethyl ammonium bromide (CTAB) method[25] incorporating 1% PVP, 0.04% v/v β- mercaptoethanol and 0.1% sodium metabisulphite for grinding the leaf tissue. All the other extraction procedures were carried out following standard procedures of CTAB protocol. The quality of DNA was checked on agarose gel and Nanodrop Spectrophotometer was used to check the quantity of the DNA. The total amount of DNA was quantified and normalized to 25 ng/µl for polymerase chain reaction (PCR) and genotyping.

ISSR Markers, ISSR-PCR and Agarose Gel Electrophoresis

For inter-simple sequence repeat (ISSR) assays forty primers designed by the University of British Columbia (UBC), Canada was selected and preliminary screening was done in 3 randomly selected cardamom accessions for selecting the most suitable primers for amplification of all the varieties. List of the primers, sequence and melting temperature (Tm) are provided in Table S1. The primers were selected based on their GC content and length. Optimal conditions of DNA amplifications were empirically determined by testing different concentrations of genomic DNA, $MgCl_2$ and primers.

The PCR amplifications were performed on Sigma SVI gradient thermo cycler. Twenty five µL reaction mixture containing 25 ng genomic DNA, 1X *Taq* buffer, 2.5 mM $MgCl_2$, 15 pM of each primer, 200 µM of each dNTP and 1 unit of *Taq* DNA polymerase was used for PCR reaction. The reactions were performed with the following temperature cycles. Denaturation at 95°C for 4 min, followed by 35 cycles of 94°C for 30 seconds, annealing temperature for 1 and 2 min extension at 72°C and a final extension at 72°C for 10 min.

Table 2 — Characters documented for morphological characterization

Sl. No	Characteristics	Mode of observations
	Morphological characters	
1	Panicle growth habit	Prostrate, semi erect or erect
2	Panicle height	Tall >3.5, medium 2.5-3.5 or short <2.5
3	Number of bearing tillers	Few <15, medium15-35 or high >35
4	Pseudostem pigmentation	Pale green, dark green or light purple
5	Pseudostem thickness	Thin <1, medium1-2 or thick >2
6	Leaf lamina shape	Lanceolate, oblong-lanceolate or ovate
7	Leaf colour	Light green, green or dark green
8	Leaf pubescence	Glabrous, puberulent or pubescent
9	Leaf anthocyanin pigmentation of midrib	Present or absent
10	Capsule shape	Globose, ovoid or ellipsoid
11	Mature capsule color	Yellow, pale green, parrot green or dark green
	Reproductive characters	
12	Panicles per tiller	Normal <3 or high ≥3
13	No. of panicles per plant	Less <30, medium 30-45 or high >45
14	Panicle length cm	Short <50, medium 50-75 or long >75
15	Panicle: simple/compound	Simple or compound
16	Panicle branching pattern	Branched or unbranched
17	Panicle internodal length cm	Short <1, medium 1-3 or long >3
18	Flower: Labellum variegation	Present or absent
	Yield characters	
19	No. of capsules per raceme	Low <2, medium 2-4 or high >4
20	No. of capsules per panicle	Low <30, medium 30-60, high 61-90 or very high >90
21	Capsule length mm	Short <10, medium 10-20 or long >20
22	Capsule width mm	Small <5, medium 5-10 or bold >10
23	No of seeds/capsule	Few <15, medium 15-25, high >25
24	Dry recovery %	Medium <18, high 18-24, Very high >24
25	Yield	Kg/hectare

The amplified products along with 100 bp or 250 bp ladder were analyzed by electrophoresis in 2% agarose gel containing ethidium bromide (0.5 µg/ml) and run in 1X TAE buffer (tris base, acetic acid, 0.5M EDTA) at a constant voltage for 3 hours. The gels were documented under gel documentation system.

Data Analysis

For the purpose of data analysis, existence and non-existence of bands were identified with numbers of 0 and 1 for all recognizable fragments, independent of their fluorescence intensity on the gel. Only the easily recognizable bands were recorded and the obscure bands excluded[26]. The binary matrix generated by scoring the bands were used to calculate the total number of scorable bands (NSB), number of polymorphic bands (NPB) and the percentage of polymorphic bands (PPB) for each ISSR marker. The PPB was determined as the percentage of polymorphic bands over the total number of scorable bands. To analyze the suitability of ISSR markers for evaluating genetic profiles of cardamom varieties, the performance of the markers was tested using four parameters: 1) polymorphism information content (PIC), 2) effective multiplex ratio (EMR), 3) marker index (MI) and 4) resolving power (Rp).

The PIC of each locus was calculated using the formula proposed by Roldan-Ruiz *et al*[27]. EMR was calculated as the product of fraction of polymorphic loci and the number of polymorphic loci for each primer according to Powell *et al*[28]. MI was calculated by the formula MI = PIC × EMR. The informativeness of each band Ib and resolving power Rp of each marker was calculated according to Prevost and Wilkinson[29]. Mean standard deviation and correlation of variation were calculated using standard procedures.

Genetic Distance and Diversity Analysis

The binary matrix obtained with ISSR markers were analyzed to examine the genetic relationship among the varieties selected. By making a pairwise comparison between all genotypes using the SIMQUAL module of NTSYS-pc software[30] version 2.01e, genetic distances based on the Jaccard's coefficient[31] were calculated. The similarity matrices were utilized to construct dendrogram using unweighted pair group method with arithmetic average (UPGMA) algorithm and sequential agglomerative hierarchical non-overlapping (SAHN) in NTSYS to represent genetic relationships.

Results and Discussion

The present study on morphological and molecular characterization of released varieties of small cardamom *Elettaria cardamomum* (Maton) was intended to make a comparative analysis of thirteen varieties of cardamom released by different organizations from the cardamom growing tracts of Kerala, Karnataka and Tamil Nadu states of India and a popular land race brought out by a farmer from ldukki District, Kerala. The varieties selected presented moderate variability in terms of morphological and agronomic characteristics and a few were with desirable traits like early maturity, tolerance to stresses. Based on the characters specified for cardamom characterization and DUS guidelines 11 qualitative and 14 quantitative characters (Table 3) were compiled and documented. Out of the eleven qualitative traits, variation among the genotypes was present for seven traits. Except for one selection with Mysore / erect panicle type (ICRI 2) and two for Vazhukka / semi-erect panicle type (Njallani & ICRI 5) all the varieties released belonged to Malabar / prostrate panicle type. Prostrate panicle type might have been the preference of breeders possibly due to several benefits it carried such as tolerance to drought[32], early maturing types[33], maximum flavonoid content[34] to name a few. No variation was observed in traits such as panicle branching pattern which invariably were simple and unbranched in all the released varieties. Even though collections with multiple branching and compound panicles were reported to have a higher yield potential[35] the varieties in this study revealed the narrow base from which selections were made at any rate for this character. Majority of the varieties studied had ovoid capsules in general with mild variations whereas mature capsule color varied from pale to parrot green to dark green and yellow. Earlier it was reported that the three natural varieties had distinctive features as plant stature being tall, medium or dwarf nature (Sudharsan *et al*, 1991). Conversely, in the present investigations, the least plant height of 1.66 m was exhibited by ICRI 6 and ICRI 8 and the tallest was at 3.55 m in IISR Avinash, all Malabar types with a coefficient of variation (CV) of 22.307 of mean 2.609 and standard deviation of 0.58 which appeared relatively homogenous. The qualitative characters did not have any close association to any of the three natural varieties in particular, revealing close affinities between the varieties. This could also mean that the general morphological features cannot be

Table 3 — Quantitative characters studied in small cardamom

Variety	Plant height (m)	No. of bearing tillers/ plant	Pseudo-stem thickness (cm)	Panicles/ tiller	No. of panicles/ plant	Panicle length (cm)	Inter-nodal length (cm)	No. of capsules/ raceme	No. of capsules /panicle	Capsule length (mm)	Capsule width (mm)	No. of seeds/ capsule	Dry recovery %	Average Yield Kg/ha
ICRI 1	2.99	32.45	2.62	2.2	71.39	55.12	2.02	10.63	313.4	15.6	13.2	17	24	650.33
ICRI 2	2.75	26.89	2.8	2	53.78	68.57	2.7	9.6	175	18.8	11.4	16.9	20.12	904.33
ICRI 3	2.4	28.45	2.5	2.9	82.505	87.7	4	2.9	53	16	10	22.3	24	523.8
ICRI 4	2.5	20.	4	2	40	46.88	2	5	55	7	5	18	17	548.9
ICRI 5	2.81	60	4.1	2.5	150	71.5	2.1	7.83	180	16.5	13.5	23	20.9	1216.33
ICRI 6	1.66	46	3.24	2.6	119.6	62.1	2.31	4.8	110.8	18.6	10.2	19	19	1052.25
ICRI 7	2.79	61	4.1	2.5	152.5	71.5	2.1	7.5	180	16.5	13.5	23	20.9	1452.49
ICRI 8	1.66	46	3.24	2.6	119.6	62.1	2.31	4.8	110.8	18.6	10.2	19	19	706.1
Njallani	3.39	42	2.3	2.72	114.24	93	3	3.38	51.6	7	13	18	25	1421.6
IISR A	3.55	9	2.9	2.5	22.5	46.2	3.5	4	139.4	14.3	11.1	13.8	25	847
Mudig1	1.95	7	2.4	1.9	13.3	25.4	2.6	2.9	45.24	14.4	10.4	19.3	23.3	275
IISR V	2.79	10	2.9	2	20	31.8	2	2	222	10.7	9.9	15.4	28.43	643
PV 1	2.68	34.2	2.4	1.76	60.192	50.22	1.8	7.93	130.36	18.3	11	12.95	19.9	545.6
Mean	2.609	32.54	3.038	2.322	78.43	59.39	2.495	5.636	135.9	14.79	10.95	18.28	22.04	829.7
SD	0.582	18.12	0.657	0.3608	46.86	19.73	0.656	2.768	78.73	4.122	2.261	3.205	3.193	363.6
CV%	22.307	55.685	21.626	15.538	62.188	33.221	26.292	49.113	57.932	27.87	20.648	17.532	14.487	43.823

linked to Malabar, Vazhukka or Mysore before panicle emergence which certainly offered possibilities for molecular level interventions.

Variability between the released varieties on the quantitative characters (Table 3) was indicated by the coefficient of variation which was highest in number of panicles per plant at 62.188% followed by number of capsules per panicle (57.932%). The lowest percent of CV was for dry recovery of capsules (14.487) followed by panicle per tiller (15.538) indicating that all the varieties were on par with respect to those traits.

Though the yield potential of several of the varieties studied were above 1000 kg/ha approximate yield realized varied. ICRI 7 and the popular land race outperformed the improved varieties with yields more or less the same. However, in general data on morphological/ phenotypic parameters revealed the narrow base of improved varieties in cardamom.

ISSR Profiling of Small Cardamom

Genomic DNA of thirteen released varieties of cardamom and one outgroup genotype were amplified using 40 ISSR markers that were previously checked for amplification and polymorphism. Twenty markers which produced moderate to high polymorphism were selected for the present study. ISSR locus diversity data comprising polymorphisms in small cardamom genotypes is summarized in Table S2. The 20 ISSR markers revealed 227 alleles of size ranging from 200 to 2000 bp (Table S2) with an average of 11.4 bands per marker out of which 197 (86.78%) were polymorphic (Fig. 1). Average number of polymorphic loci per primer was 9.85. Total number of amplified/ scorable bands (NSB), number of polymorphic bands (NPB) and percentage of polymorphic bands (PPB) of cardamom genotypes, Polymorphism information content (PIC), effective multiplex ratio (EMR), marker index (MI) and resolving power (Rp) calculated for each marker individually have been presented in Table 4.

Among the amplified fragments 197 bands (86.78%) were polymorphic. The frequency of polymorphic loci varied from marker to marker the highest with UBC 827 and lowest with UBC 809 at an average of 9.85 polymorphic bands per marker. The percentage of polymorphism across the released varieties of cardamom ranged from 62.5 to 100% with an average polymorphism of 86.78% per marker. Five markers *viz*., UBC 808, UBC 812, UBC 816, UBC 860 and UBC 880 generated 100% polymorphic loci. Least polymorphism was shown by UBC 809 with a percentage of 62.5%.

A comparative representation of ISSR marker polymorphism data including and excluding the wild genus *Aframomum* is given in Table 5. The amplified bands were 86.78% polymorphic including *Aframomum* and on eliminating the profiles generated for the wild species percent polymorphism was reduced considerably to 56.22%. The primers *viz*. UBC 808, UBC 812, UBC 816, UBC 860 and UBC 880 that generated 100% polymorphic patterns were also found to produce lesser polymorphism on excluding the wild genus. A net total percentage of 56.22% polymorphisms were uncovered by ISSR

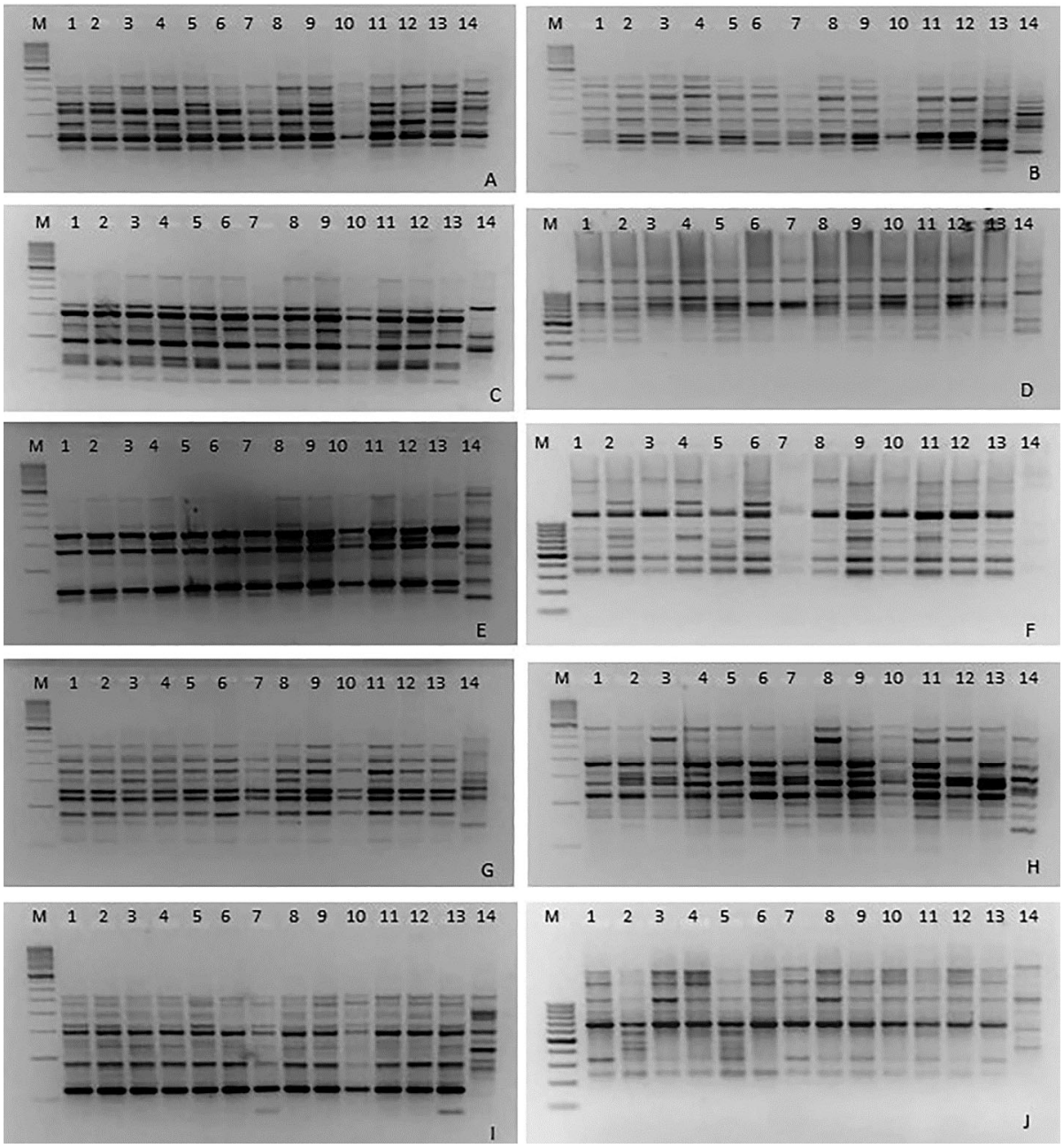

Fig. 1 — ISSR profiles of 13 small cardamom varieties and *Aframomum* sps. M-DNA ladder, 1-ICRI1, 2-ICRI2, 3-ICRI3, 4-ICRI4, 5-ICRI5, 6-ICRI6, 7-ICRI7, 8-ICRI8, 9-Njallani, 10-IISR Avinash,11-Mudigere1, 12-IISR Vijetha,13-PV1, 14-*Aframomum melegueta* A. UBC807 B.UBC 808 C. UBC 812 D.UBC 816 E.UBC 834 F.UBC 860 G. UBC 868 H.UBC 866 I.UBC 841 J.UBC 840

primers in the thirteen varieties of cardamom tested in the present study. The considerable reduction of polymorphism percentage on exclusion of *Aframomum* could be an indicator of the narrowing base in the released varieties. The present observations agree with those of Joshi & Dhawan[36] during ISSR profiling of medicinal plant *Swertia chirayita* where polymorphisms were considerably reduced from 98.7 to 42.5% when outliers were excluded. Nevertheless, the polymorphisms including those of *Aframomum* were taken into account for arriving at the genetic similarity indices of the different cardamom cultivars as a certain amount of ISSR amplifications were shared by cardamom and the wild genus and to observe how closely related are the two genera. The classification proposed by

Table 4 — Marker parameters studied using 20 primers in cardamom genotypes and the outgroup *Aframomum*

Sl. No	Primer	Primer sequence	Fragment size range	NSB	NPB	PPB	PIC	EMR	MI	Rp
1	UBC 807	AG_8TA	350-1250	11	8	72.73	0.16	5.81	0.93	2.43
2	UBC 808	AG_8C	250-1550	15	15	100	0.15	15	2.25	2.71
3	UBC 809	AG_8G	230-750	8	5	62.5	0.1	3.12	0.312	1
4	UBC 810	GA_8T	350-1750	12	10	83.33	0.13	8.33	1.08	1.86
5	UBC 812	GA_8A	230-950	11	11	100	0.19	11	2.09	3
6	UBC 816	CA_8T	300-1500	9	9	100	0.28	9	2.52	3.57
7	UBC 818	CA_8G	270-1750	9	6	66.66	0.12	4	0.48	1.29
8	UBC 827	AC_8YG	250-2000	19	18	94.74	0.29	17.05	4.9	8.57
9	UBC 828	TG_8A	350-1500	9	8	88.88	0.19	7.1	1.35	2.43
10	UBC 834	AG_8YT	300-1750	12	8	66.66	0.17	5.33	0.9	3
11	UBC 835	AG_8YC	380-1600	12	9	75	0.13	6.75	0.87	1.86
12	UBC 840	GA_8YT	250-1500	13	12	92.3	0.2	11.07	2.21	3.29
13	UBC 841	GA_8Y	300-1300	13	11	84.62	0.18	9.3	1.67	3
14	UBC 850	GT_8YC	300-1400	10	8	80	0.24	6.4	1.53	3.43
15	UBC 857	AC_8YG	200-1750	14	13	92.86	0.32	12.07	3.86	6.57
16	UBC 860	TG_8RA	350-1500	9	9	100	0.29	9	2.61	3.43
17	UBC 866	CTC_6G	300-1800	13	12	92.3	0.25	11.07	2.76	4.86
18	UBC 868	GAA_6	270-1500	13	12	92.3	0.16	11.07	1.77	2.86
19	UBC 873	$GACA_4A$	400-1300	9	7	77.78	0.19	5.44	1.03	2.57
20	UBC 880	$GGAGA_3$	250-1100	6	6	100	0.27	6	1.62	2.14
	Mean (μ)			11.35	9.85	86.133	0.200	8.695	1.837	3.193
	SD(σ)			2.8332	3.1348	12.125	0.0630	3.507	1.1000	1.7096
	CV%			25.61	32.65	14.44	32.26	41.38	61.43	54.92

NSB – Number of scorable bands, NPB – Number of polymorphic bands, PPB – Percentage of polymorphic bands, PIC – Polymorphism information content, EMR - Effective multiplex ratio, MI – Marker index, Rp – Resolving power.

Table 5 — Percent polymorphism detected in cardamom genotypes and the outgroup *Aframomum* – a comparison

Sl.	Primer	Polymorphism including wild genus			Polymorphism excluding wild genus		
No.		NSB	NPB	PPB	NSB	NPB	PPB
1	UBC 807	11	8	72.73	10	7	70
2	UBC 808	15	15	100	10	4	40
3	UBC 809	8	5	62.5	7	2	28.56
4	UBC 810	12	10	83.33	8	2	25
5	UBC 812	11	11	100	9	3	33.33
6	UBC 816	9	9	100	6	5	83.33
7	UBC 818	9	6	66.66	6	2	33.33
8	UBC 827	19	18	94.74	16	15	93.75
9	UBC 828	9	8	88.88	8	3	37.5
10	UBC 834	12	8	66.66	8	4	50
11	UBC 835	12	9	75	10	3	30
12	UBC 840	13	12	92.3	12	7	58.33
13	UBC 841	13	11	84.62	10	6	60
14	UBC 850	10	8	80	10	6	60
15	UBC 857	14	13	92.86	13	11	84.62
16	UBC 860	9	9	100	9	6	66.66
17	UBC 866	13	12	92.3	12	8	66.66
18	UBC 868	13	12	92.3	9	2	22.22
19	UBC 873	9	7	77.78	7	4	57.14
20	UBC 880	6	6	100	5	4	80
	Mean (μ)	11.34	9.85	86.133	9.25	5.2	54.021
	SD (σ)	2.833	3.134	12.125	2.5666	3.3023	21.382
	CV%	25.61	32.65	14.44	28.47	63.51	40.61

Kress *et al*[37] based on ITS sequences had grouped *Aframomum elettariopsis* closer to *Elettaria* and a few other genera in a single clade though cardamom appeared closer to *Amomum* and *Alpinia.*

Genetic Relationship and Cluster Analysis

The amplification profiles of the cardamom accessions generated using 20 ISSR markers were used to assess the discriminating power of markers. Four parameters were evaluated *viz*., PIC, EMR, MI and Rp (Table 4). Polymorphism information content value was used to indicate the ability of a primer combination to distinguish between genotypes[38]. The mean PIC value of the primers ranged from 0.1 UBC 809 to 0.32 UBC 857 with an average of 0.2 per primer and therefore UBC 857 had the highest discriminating power among the twenty primers tested. Similar PIC values as in current observations have been reported for other plant species such as mulberry, curry leaf, jaborandi as well[39-42] Reportedly the maximum PIC for dominant markers[42] is theoretically 0.50. Botstein *et al*[43] classified PIC v alue into three classes, slightly informative PIC < 0.25, reasonably informative PIC > 0.25 and highly informative PIC > 0.5. Based on this classification six ISSRs were found to be reasonably informative in the present study, suggesting their potential use for discriminating genotypes in diversity studies in cardamom. Likewise, UBC 827 had an EMR of 17.05 with a mean of 8.6 among the primers. The primers that showed higher polymorphism had higher EMR and MI values. The average MI was 1.837 the highest of 4.9 for UBC 827. Rp of the ISSR marker ranged from 1 (UBC 809) to 8.57 (UBC 827) with an average of 3.2 per primer. The markers that showed higher polymorphism had higher EMR and MI values. RP and MI being indexes of discrimination can also be used to infer marker efficiencies to differentiate between genotypes and have been widely used to measure the information content generated by molecular markers in various plant species[44-45]. The data generated show that indices such as PIC, EMR, MI and Rp using ISSR markers, were reasonably informative to discriminate the genotypes in the present study. Coefficient variation (CV) percentage was highest for MI followed by Rp of each marker.

Genetic similarity between all the varieties selected for the study was calculated using the binary matrix data generated by the ISSR primers. Jaccard's similarity matrix was estimated for all the varieties of small cardamom and *Aframomum* sp. using NTSYSpc software. The Jaccard's coefficient ranged from 0.11 to 1 (Table S3) demonstrating the existence of adequate variability among the 14 genotypes and confirming that ISSR markers were suitable for detecting genetic diversity within the released varieties of small cardamom though the markers did not expose the expected variability. This agrees with previous report that the genetic variation expressed by RAPD molecular markers was relatively low among cultivated cardamom though most of them were clear morphological variants which was expected from a monotypic genus like *Elettaria* (Nirmal Babu *et al* 2012). The lowest similarity matrix of 0.11 was generated between Njallani and *Aframomum* sp. followed by ICRI 1 and PVI with a similarity matrix of 0.20. ICRI 7 and ICRI 8 showed 100% similarity with a similarity coefficient of 1. Among the released varieties PV1 was the farthest from ICRI 1 indicating that the two genotypes were distinctly different from each other. Substantial variability existed in morphological and yield contributing characters between ICRI 1 and PV 1 as well.

The Jaccard's similarity matrix was used to construct a dendrogram using UPGMA method of cluster analysis and the dendrogram obtained is shown in Figure 2. The dendrogram consisted of two major clusters. Cluster I, consisted of all the cardamom genotypes which was further differentiated into sub-clusters. Cluster II, consisted of only the allied genus *Aframomum melegueta* a related genus, indicating the wide divergence from *Elettaria cardamomum*. The main cluster I, formed two large sub-clusters, and an outlier ICRI 1. In the first sub cluster ICRI 2 and ICRI 5 grouped closely together with IISR Avinash as has an outlier. ICRI 5, the first cardamom hybrid through breeding did not show the anticipated distinctive grouping with its parental lines *viz*. ICRI 1 and Njallani but it unexpectedly clustered

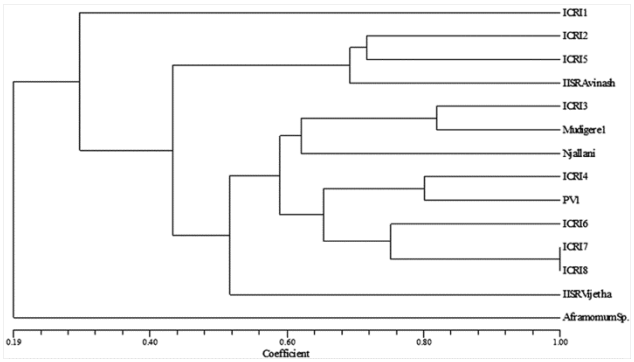

Fig. 2 — Dendrogram showing genetic relationship among the 13 small cardamom accessions and one outgroup genus on Jaccard's similarity coefficient generated by ISSR markers.

closer to ICRI 2. In the sub cluster II, there were nine genotypes evidently having a common origin but IISR Vijetha remained as an outlier. Among these ICRI 3 and Mudigeri 1 closely clustered indicating their clear divergence from the remaining genotypes. These were developed from the same region Karnataka indicating the narrow range of germplasm from which these selections could have been developed parallely by different groups. However, the popular variety Njallani green gold a farmer's selection from Idukki clustered to these as a distinct outlier. ICRI 4 & PV1 clustered together separately showing narrow divergence both being Malabar types that were not geographically much apart as regards regions of cultivation though the former is suited for lower altitudes. Furthermore, the varieties were more or less on par with respect to morphological characters and yield contributing characters except for variation among traits such as number of panicles per plant, number of capsules per panicle and capsule size (Table 3). The hybrid ICRI 7 also did not show parental linkage to one of its parents PV 1 even though both resembled in panicle morphology of prostrate trait. ICRI 6, 7 and 8 grouped closely though ICRI 6 formed a separate clade indicating that the new varieties released by ICRI could have a close agro-morphological association except for yield contributing characters (Table 3) such as number of panicles per plant and number of capsules per panicle. These varieties showed lesser variability in morphological parameters as well indicating that the varieties irrespective of their variability in quantitative characters were selected from a narrow genetic base.

In general, within the clusters all the varieties tested displayed a mixed pattern as regards their geographical locations. A few cardamom genotypes from the same location appeared in distinct clusters and conversely even with a geographical separation of several hundred kilometers. Some of the varieties clustered closely such as Njallani with ICRI 3 & Mudigere 1 and IISR Avinas has with ICRI 2 & ICRI 5. Therefore, substantial correlation between genetic variability and geographic origin or on even panicle morphology of the cardamom varieties as in ICRI 2 & ICRI 5 was not noticed with the ISSR markers. Earlier studies reported that clear divergence as observed in Kerala and Karnataka collections the two main centers of cardamom diversity does not support this observation (Nirmal Babu *et al,* 2012). Distinct grouping of hybrids and the resultant divergence was also not observed in the present study as was reported by them. Panicle morphology of most of the released varieties was prostrate coincidentally with the exception of ICRI 2 (erect), and ICRI 5 & Njallani both semi-erect which also evidently points out the narrow range of selection done. Prasath and Venugopal[46] reported that cluster analysis using morphological markers had demonstrated that there was no definite clustering of accessions for Malabar, Mysore and Vazhukka type of panicles as accessions of the three cultivar groups often grouped together in the same cluster suggesting some degree of ancestry between the three groups. Reportedly no reproductive barrier exists between wild and cultivated populations of cardamom with all the varieties and races being inter-fertile and the observed variations being probably due to natural crossing. The varieties ICRI 7 and ICRI 8 could not be discriminated in the present studies using the ISSR data analysis though the former is a hybrid and the latter a selection. This could be due to possible origin as segregating progeny from populations of narrow genetic diversity and collected possibly from same genetic stock.

Cluster analysis however has revealed that while generating new varieties through selection or breeding of cardamom a narrow germplasm base was depended upon as is apparent from the morphological and qualitative traits itself which do not show considerable variability. This might have occurred due to preference of traits contributing to high yield as one of the priorities during selection and potentially useful traits from wild accessions for hybridization was avoided inadvertently during breeding trials. Number of capsules per panicle, racemes per panicle, tillers per clump, panicles per clump and panicle length are the main yield contributing characters and have been used as selection criteria in the genetic improvement of cardamom. Except for ICRI 5 and ICRI 7 all the released varieties of cardamom in this study were generated through selection rather than hybridization. This could be probably due to the perennial nature of cardamom where breeding trials could be time consuming and cumbersome. However, the present selections would have been conducted based on utilization at particular locations as cardamom is a highly location specific crop and the three naturally occurring varieties are more or less suited to different geographic locations along the Western Ghats of South India. Another reason could be apparently due to the relatively late domestication and selection that started in cardamom in the 19th century. Mudigere 1, the first cardamom selection was released as a variety in the year 1984 (Table 1).

Domestication also resulted in large scale destruction of forest habitats and eventual decimation of the wild populations of cardamom. As recently documented natural populations of cardamom were replaced by 'selections' which narrowed down the genetic base and thus the evolutionary potential of cardamom. Cultivation of Njallani and a few other land races have spread to localities replacing the traditional novel cultivars leading to erosion of a number of local cultivars and landraces thus increasing the chances of narrowing of genetic base of cardamom. Though several efforts were made for discovering greater variability from the available conserved germplasm resources and have been potentially tested for genetic enhancement of the crop[47] it is limitedly reflected in the released varieties. Therefore, it can be assumed that the genetic base of cardamom in terms of released varieties might have become narrower due to selection consequential to domestication and more recently commercialization. Some of the recent studies also have concentrated more on yield performance of newer land races brought out by farmers as would be in any such crop. Bhandari *et al*[48] and Kuruvilla *et al*[49] reported that lopsided breeding practices focusing on improvement of only few traits like yield and its component traits could lead to depletion in natural variability and thereby genetic diversity. Only a few released varieties displayed potential characters such as that of biotic and abiotic stress tolerance in cardamom (Table 1). Phytochemical variations among four distinct varieties of Indian cardamom were reported recently[50].

Study of variability of genetic resources of a crop is the first step towards the understanding of genetic diversity of the genetic stock for use in crop improvement programmes. Since, genetic variations cannot be measured by the phenotypic evaluation alone diversity studies on the basis of morphological characters alone would not suffice in analyzing the extent of variability. Variability can be analyzed using molecular marker data which would enable categorization of varieties at genome level and could be used to complement conventional morphological characterization in identifying populations and for studying ongoing changes in the pattern of diversity. DNA markers provide clear insight into genetic variation at molecular level[51] and are unlimited in number and not affected by environmental factors and/or the developmental stage of the plant[52].

Given the fact that the number of accessions held in the *ex situ* gene banks of cardamom are fairly high and evidently only a limited contribution from that has been made in development of recent cultivars this points out the need for using molecular markers for evaluation of germplasm which can also help to minimize pre-breeding attempts for developing new varieties in cardamom. Molecular characterization would help in introgression of new alleles from unutilized germplasm accessions related wild species and exotic relatives which is vital for further improvement and enhancement of genetic base of cultivated cardamom. To conclude, the present study substantiates that more specific and polymorphic molecular markers should be used for estimating existing variability in germplasm for enhancing the genetic base of cardamom.

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