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RESEARCH ARTICLE

Isolation and characterization of genomic microsatellite markers for small cardamom (*Elettaria cardamomum* Maton) for utility in genetic diversity analysis

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Abstract Microsatellite markers in small cardamom (*Elettaria cardamomum* Maton) were developed using the selective hybridization enrichment method. A total of 140 microsatellite repeats were identified from 270 clones. Primers were designed for 58 microsatellites and 44 primer pairs amplified products of expected size in cardamom. These markers were used for studying the diversity of 20 important small cardamom genotypes, and six markers were found to be polymorphic. The number of alleles ranged from 2 to 7 with an average of 3.6 per locus. Polymorphic information content values ranged from 0.14 to 0.38 based on dominant scoring. The two markers ECM 47a and ECMG 28 generated specific banding patterns for the genotypes MCC7 (Pink tiller) and APG434 (MA18) respectively. Dendrogram illustrated the genetic similarity between different genotypes of Kerala and Karnataka regions. It differentiated the closely related genotypes and released varieties into separate groups. Principal coordinate analysis revealed PV1 and ICRI 1 as the most divergent genotypes. The study demonstrated that these markers are informative and can be further utilized for generating reliable molecular data for assisting the crop improvement of small cardamom. Cross generic transferability (71.4 %) of the developed primers proved that they are useful for phylogenetic studies in the family Zingiberaceae. This is the first report of de novo isolation, characterisation and utilization of microsatellite markers for the genetic diversity analysis of small cardamom.

Keywords *Elettaria cardamomum* · Small cardamom · Microsatellite markers · Cross generic transferability

Introduction

Cardamom (*Elettaria cardamomum* Maton) is one of the most important spice crop with the sobriquet "Queen of spices". It is a herbaceous rhizomatous monocot, belonging to the family Zingiberaceae. The crop has originated in the Western Ghats of India and is generally cultivated as an under crop in the hilly tracts of India at elevations of about 800–1300 m.a.s.l. It is also cultivated in many parts of the world such as Sri Lanka, El Salvador, Vietnam, Laos, Cambodia, Papua New Guinea, Tanzania and Guatemala. In Guatemala cardamom is grown on a commercial scale and is the largest producer of cardamom in world.

Cardamom is a perennial and which delays the conventional genetic characterization significantly. India has the largest variability in the germplasm of cardamom (Prasath and Venugopal 2004) and its conservation is realized by ex situ method as field gene banks. Based on the nature of panicles, there are three varieties of small cardamom (Sastri 1952). The variety Malabar is characterized by a prostrate panicle and Mysore variety possess erect panicle. The third type variety Vazhukka has semi erect or flexuous panicle and is considered as a natural hybrid between the other two varieties (Madhusoodanan

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et al. 2002). The basic chromosome number of small cardamom is X = 12 and 2n = 48 (Gregory 1936), which indicates a balanced tetraploid nature of the plant. Molecular characterization of cardamom will help not only in better understanding of interrelationships but also in developing breeding strategies for better recombination. In addition molecular markers could complement the conventional morphological studies for the discrimination of cardamom cultivars by providing a genetic background for the observed phenotypic variability. Molecular markers employed for characterization of small cardamom were limited to random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism PCR (RFLP PCR) and inter simple sequence repeat (ISSR) markers (Radhakrishnan and Mohanan 2005; Nirmal Babu et al. 2012).

Microsatellite markers have become one of the most widely used molecular markers for genetic analysis in recent years. They are present in both coding and non coding regions and are usually characterized by a high degree of length polymorphism. They have a major role in DNA sequence variation analysis and genetics studies because of their many desirable features like hypervariability, codominant inheritance, multiallelic nature, reproducibility, relative abundance and extensive genomic coverage (Wang et al. 2009). These markers have been utilized in the identification of novel marker alleles linked to genes involved in the expression of important traits, which will allow indirect selection of desirable traits in early segregating generations at the seedling stages (Gupta and Varshney 2000) and can be broadly employed during cultivar development in breeding programs (Garris et al. 2005).

In several crops, molecular markers which are closely linked to numerous traits of economic importance have been developed (Park et al. 2009). Newly developed genomic SSR markers have been reported in some of the economically important spice crops such as *Zingiber officinale* (Lee et al. 2007), *Vanilla planifolia* (Bory et al. 2008), *Piper nigrum* L. (Menezes et al. 2009; Joy et al. 2011), and *Curcuma longa* (Sigrist et al. 2009; Siju et al. 2010). Recently cross species transferability of EST and genomic microsatellites from *C. longa*, *Z.officinale* and *Amomum subulatum* to *E. cardamomum* was reported (Anu et al. 2015). The number of polymorphic markers from the study was very less and which indicates the need for development of microsatellite markers from small cardamom.

The present study was aimed to develop microsatellite markers from a small-insert genomic DNA library by selective hybridisation enrichment method and it is the first report about development of genomic SSR markers in cardamom. These markers were effectively utilized in the germplasm characterization of small cardamom.

Materials and methods

Plant material

Twenty varieties of small cardamom with diverse phenotypic characters (qualitative and quantitative) were collected from the germplasm maintained at ICAR-Indian Institute of Spices Research, Regional Station, Appangala, Madikkeri, Karnataka and Spices board research station Myladumpara, Kerala.(Table 1). These included released varieties of Indian Cardamom Research Institute (ICRI) and Indian Institute of Spices Research (IISR), farmer's selections and other varieties collected from different places of Kerala and Karnataka states in India.

For checking the cross generic transferability of SSR primers, plant material from five different genera (seven different species) under the family of Zingiberaceae were used with *Elettaria* viz. (1) *Amomum subulatum* Roxb., (2) *Amomum microstephanum* Baker., (3) *Amomum ghaticum K.G Bhat.*, (4) *Alpinia mutica* Roxb., (5) *Hedychium coronarium* J.Koenig., (6) *Elettaria cardamomum* Maton., (7) *Zingiber officinale* Rosc., (8) *Curcuma longa* Linn.

DNA extraction and quality analysis

Genomic DNA was extracted from the fresh young leaves of all the plants based on the protocol of Doyle and Doyle (1987) with slight modifications. Subsequently 2 µl of the DNA samples were run on a 1 % agarose gel to analyse the purity of the samples. Concentration of the samples were estimated using biophotometer (Eppendorf, Hamburg, Germany). The final DNA concentration was adjusted to 20 ng/µl for PCR analysis.

Construction of an enriched small insert genomic DNA library

A small insert genomic DNA library enriched for the microsatellite repeats $(AG)_{12}$, $(TG)_{12}$, $(ACT)_{12}$, $(AAAC)_6$, $(ACCT)_6$, $(ACTG)_6$ was constructed for small cardamom following the protocol of Glenn and Schable (2005). Approximately 1 µg of genomic DNA was completely digested with a frequent cutter restriction enzyme *Rsa*1 (NEB) or *Alu*1 (NEB). They were used in different reactions and were ligated into double stranded super SNX linkers (Super SNX 24 Forward-5′GTTTAAGGCCTAGC TAGCTAGCAGAATC-3′ and super SNX 24 + 4P Reverse-5′pGATTCTGCTAGCTAGGCCTTAAACAAAA-3′). Linker ligated DNA was denatured and hybridized to 3′ biotinylated oligonucleotide probes $(AG)_{12}$, $(TG)_{12}$, $(ACT)_{12}$, $(AAAC)_6$, $(ACCT)_6$, $(ACTG)_6$. Hybridized

Table 1 List of small cardamom accessions used for genetic diversity analysis

IC no.	Accession no.	Accession identity	Place of collection	Important character
584837	MCC 49	ICRI 1	Udumbanchola, Idukki, Kerala	High yielding, early maturing, profusely flowering variety, globose extra bold dark green capsule
584849	MCC 61	ICRI 2	Kodagu, Karnataka	High yielding, oblong bold parrot green capsules, tolerant to azhukal disease
349652	APG 360	ICRI 3	Saklespur, Karnataka	High yielding, oblong bold parrot green capsules, tolerant to rhizome rot disease
349383	APG 53	PV1	Manjerabad, Saklespur, Karnataka	High yielding, early maturing variety with slightly ribbed and narrowly ellipsoid to elongate light green capsules
349435	APG 113	Mudigere 1	Mudigere, Karnataka	High yielding, oval bold pale green capsules. Tolerant to thrips and shoot borer
349589	APG 296	IISR Suvasini/ CCS1/Appangala 1	Kodagu, Karnataka	High yielding, long panicle, oblong bold, parrot green capsules
349599	APG 306	IISR Vijetha/NKE 12	Hulagola, North Canara, Karnataka	Katte resistance
349591	APG 298	IISR Avinash/RR1	Kandanakolli, Kodagu, Karnataka	Rhizome rot resistance
349340	APG 10	MB3	Galebeedu, Kodagu, Karnataka	Multibranch/branched panicles
349550	APG 257	Green gold	Myladumpara, Idukki, Kerala	High yielding, globose extra bold, dark green capsules
349650	APG 358	Vander cardamom	Vander, Valliyatholva, Idukki, Kerala	Suitable for low elevation
584795	MCC 7	Pink tiller	Myladumpara, Idukki, Kerala	Pink colored tillers
547223	APG 454	Kalarickal white	Idukki, Kerala	White flower, high oil content
349447	APG 129	Narrow leaf	Idukki, Kerala	Narrow elongated leaf
547203	APG 434	MA18/CRC396	Kodagu, Karnataka	Foliar disease resistant
349634	APG 342	Coorg green	Kodagu, Karnataka	Typical coorg green
547218	APG 449	CRC 411	Kodagu, Karnataka	High yielding
584105	APG521	ASH	Appangala, Kodagu, Karnataka	Collected from Ashoka plantation; low thrips
349523	APG 217	Wayanad collection	Vythiri, Wynad, Kerala	Slender panicle
349530	APG 228	Wayanad collection (kothu)	Kalpetta, Wynad, Kerala	Very small capsule

DNA fragments were captured using streptavidin coated magnetic beads, (dyna beads, Dyna l Biotech, Oslo, Norway). After elution, the DNA fragments were amplified by PCR using Super SNX 24 forward primer and cloned to pCR® 2.1-TOPO®TA vector (Invitrogen, USA). The cloned vector fragments were used to transform One Shot® TOP10 chemically competent cells (Invitrogen, USA). Recombinant clones were identified as white colonies on LB/Kan agar plates. Positive clones were confirmed by colony PCR using M13 Forward (5′-GTAAAACGACGGCCAG-3′) and M13 Reverse (5′-CAGGAAACAGCTATGAC-3′) primers. Plasmid DNA was isolated from the selected 270 clones using QIA spin miniprep kit (Qiagen) (Qiagen Inc., USA) and sequenced at Chromus biotech, Bengaluru, Karnataka, India.

Analysis of nucleotide sequences and primer designing

All the nucleotide sequences were analysed for vector sequences using Vec Screen (NCBI) and the low quality end sequences were trimmed with BioEdit (version7.1.3.0) (Hall 1999). Clones with good nucleotide sequences were clustered into contigs and singlets using the software programme CAP3 (Huang and Madan 1999). The clustered sequences were analysed for microsatellite repeats using MISA (Thiel et al. 2003) and primers were designed from the flanking sequences using the software WEBSAT (Martins et al. 2009). The microsatellite repeats were defined based on the criteria: (unit size/minimum number of repeats): (1/10) (2/6) (3/4) (4/3) (5/3) (6/2), maximal

number of bases interrupting 2 SSRs in a compound microsatellite: 10. The SSR loci contain repeat units of 2–6 base pairs with ≥12 bp only were considered for primer designing. Mononucleotide repeats and most of the hexanucleotides repeating two times were avoided. The primers were custom synthesized at Sigma Genosys, Bengaluru, Karnataka, India.

Amplification and detection of microsatellite alleles in different genotypes of small cardamom

Amplification of microsatellite loci was carried out in a 25 µl PCR reaction mixture containing, 1 × PCR buffer, 2.0 mM $Mgcl_2$ (GeNeiTM, Bangalore), 0.1 mM dNTPs (Thermoscientific, USA), 5.0 pmol each of primers, 40–50 ng of genomic DNA and 0.5 U Taq DNA polymerase (GeNeiTM, Bangalore) in an Eppendorf thermal cycler (Master cycler EP Gradient S, Eppendorf, Germany) with the following amplification profile: initial denaturation at 94 °C for 30 s, annealing at 60–61 °C for 45 s, (depending up on primers used, see Table 2, Online Resource 1) extension at 72 °C for 1 min, final extension at 72 °C for 10 min and a hold at 4 °C. Amplified products were resolved in a 15 % denaturing polyacrylamide gel along with GeneRuler^{TM}100 bp and GeneRuler^{TM} ultra low range DNA ladder (Thermoscientific, USA). The gels were silver stained (Bassam and Gresshoff 2007) to view the bands and images were saved by scanning the gels.

Sequencing of microsatellite loci

In order to confirm the presence of SSR and the allelic variation of SSR loci, amplification products of the locus ECMG28 were isolated. PCR products were separated by 15 % polyacrylamide gel and targeted bands were excised and crushed in 10 µl nuclease free water. The samples were incubated for several hours at 37 °C. Polyacrylamide pieces were eliminated by centrifugation and DNA was recovered by ethanol precipitation. Eluted DNA was reamplified with the same primers and 4 µl was run on 2 % gel. The remaining PCR products were purified using QIA quick PCR purification kit (Qiagen) (Qiagen Inc., USA). The purified products were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed to JM109 *E. coli* cells. Positive clones were selected and isolated plasmid DNA samples were send for sequencing to Xcelris labs Ltd, Ahmedabad. The sequences were aligned using the software MEGA6.

Statistical analysis of data

For assessing genetic similarity among the small cardamom accessions, primers which are giving visible polymorphic bands were considered and only clear and prominent bands were scored. Others primers were excluded from the study. Allele sizes were calculated by comparing the bands to ultra low range DNA ladder (Thermoscientific, USA) in denaturing PAGE and 100 bp DNA ladder (Thermoscientifc, USA) in agarose gels. Since cardamom is a polyploid species, all the unique bands were considered as dominant markers. The presence (1) and absence (0) of bands were recorded for individual accessions and the Dice's similarity coefficients were calculated (Nei and Li 1979). Based on the similarity matrix a UPGMA based dendrogram was constructed using NTSYS-pc programme (Version 2.02i) (Rohlf 1998) and bootstrapping was carried out with 1000 random samplings to estimate the consistency of the dendrogram branches using Win Boot (Yap and Nelson 1996). The polymorphic information content for each microsatellite marker was computed using the formula: $PIC = 1 - \sum_{i=1}^{n} Pi^2$ (Weir 1990), where Pi is the frequency of the *i*th allele in the genotypes studied. For dominant markers, this formula can be simplified to PIC = 2PiQi (Roldan-Ruiz et al. 2000) where Pi is the frequency of presence and Qi is the frequency of absence of a particular band and the maximum value is 0.5. To calculate PIC value for a primer pair, the PIC values for all the polymorphic bands produced by the primer pair were averaged (Rana and Bhat 2004; Tehrani et al. 2008; Honig et al. 2010). Other variables of the diversity study could not be calculated due to the polyploid nature of cardamom and exact allelic dosages could not assign from the data.

Results

Identification of SSRs and primer designing

From the 270 clones sequenced, 198 good sequences were obtained after trimming and vector removal. Assembling of sequences using CAP3 (Huang and Madan 1999) resulted in 28 contigs and 137 singlets. Sequence analysis (MISA) (Thiel et al. 2003) of the 165 unigenes revealed the presence of one or more SSRs in 85 unigenes and 35 sequences containing more than one SSR. These sequences were deposited in NCBI. In total 140 microsatellite repeats which include di, tri, tetra, penta and hexa repeat motifs were identified out of which 12 sequences had compound SSRs and all others were perfect repeats (Table 1, Online Resource 1). Among the repeats 27 were class 1 (≥20 bp in size) and remaining were class 11 (≥ 12 bp ≤ 19 bp) (Mun et al. 2006). The size of the repeats varied from 10 to 54 bp. A total of 59 primer pairs were designed for the microsatellite motifs identified. Primers could not be designed for the remaining sequences due to inadequate number of base pairs to meet primer designing criteria and self complementarity of flanking sequences.

Table 2 Sequence characteristics of SSR primers amplified in small cardamom and related genera

Locus name	SSR motif	Forward primer (5′-3′)	Reverse primer (5′-3′)	Expected product size (bp)	Cross generic amplification	Gene bank accession no.
ECM2	(TTAGGA)2	CTTGATTTCTAGTGAATTGGGC	GACCTGAACCTAACCTTAACCC	261		KF537690
ECM10	(AT)6	TTGAGTGCGAGCCATAGTAAAT	TAGGTTGATTCTCCAATTCCAC	286		KC347542
ECM10a2	(GA)15	CTACATAAAGCTCTGCCTCTCT	GATCTACTCATTTCAACACATTCT	275		KC347542
ECM14	(CT)13	TAGCAGAATCACGTAAACATGG	TGAACATGGGTAGAGTAAAGGG	227		KF537691
ECM14a	(TCACAC)2	CTCTCTCTCTTCGTTCTCCCTT	TTTTCGGCTCCGCATATT	147		KF537691
ECM15	(CATACC)2	TAAGTATTCACCACCTCGATCC	GGACCAATGTCAACTCTTGC	201		KF545662
ECM18	(AAGTTT)2	CCCATAGGGAGAAAGGGAATAA	CATGTGTGGATGTGTGTGTTGT	158		KF648863
ECM21a	(AAAACA)2	TCACATCTCGTGTTCACATCAA	CCACTCAACGCTCTCTCTCTTT	382		KF648864
ECM21b	(AGC)4	GAGAGAGAGAGAGAGAGAGGGG	GAATAATAATGCGACTGCTGCT	276		KF648864
ECMC7	(CA)9	CCAAGACTCCCCTTCTGCTA	ATATTTCCCTCACATTTCTTTGG	294		KP982860
ECMC8	(AC)8	ACAGCATATACAAATCCTTGGC	GGGGTATCCAAAATCCATAATC	289		KP982861
ECMC11	(CA)27	TTTACCAACCATAGCACACCTA	AGGAAACGAATTAGAATATGCAG	164		KP982862
ECM47a	(CT)17TCAA(TC)9	CTCCCTCTTCCTCTTTTCTTTT	CCATATCACAGACATAGCAAGG	**137**		KP982864
ECM53	(TG)8	CCATTCCCAGAACATCACTAAC	GCAGAATCCTCAGAAAATGACT	276		KP982866
ECM64	(TG)10	TACTCATGTTGTCCGATGAAAG	TAAGTAAGATGGAAGAGCAGCC	186		KP982867
ECM70	(TTC)4	CAGTGTTTGTTTTCCTTGTCCT	ATAATAGGTGTTGCTGCGTCTT	287		KP982868
ECMG1	(GA)15	AATTCGCCCTTGTTTAAGGC	ACACTAGGATGATGATGTCCCC	189	*	KJ938508
ECMG2	(AC)7	TGGATGCAATCAGGGACT	GGCGTATATCAACTGCAAAAC	242	*	KJ938509
ECMG3	(AG)10	TTCTCCGAACCATTCTTCTTTC	CCCTCAAGAGCCATACAACAA	162	*	KJ938510
ECMG5	(TC)7	TTTTCCCTCTCCATCCCTCTA	GCCGTCAAGTAAAACACAAACA	116	*	KJ938512
ECMG6	(GA)16	ATTTCCCCAATTACCCTCAATC	GTCTGCACAAACTCCATGTACC	212	*	KJ938513
ECMG7	(CT)10	TGCAGGAAGTGTGGATCTTTTA	ATTCTATTCCGTGGCATGAGTT	176	*	KJ938514
ECMG9	(ATTTT)3	ATCGCTCCTTCCTTCACTTGT	GAATCCTTGCTTTTGTTGATGG	133		KJ93851
ECMG10	(CT)8	ATCTCTTGATTGCCCTCGAA	AATTCGCCCTTGTTTAAGGC	190		KJ938516
ECMG11	(GT)9	TGGACTCTCAGCATACAGACTT	TGTCTACCACCATCATTTCTTG	161	*	KJ938517
ECMG13	(AAAC)4	AATCCAAGTTTATGTTTACAAGACC	TGATTCTTTAGTTGCAGTGACAT	181	*	KJ938519
ECMG14	(CAAA)5	TAGCCAAAGCATCAGAACAAAA	CTAGCAGAATCCTCTCTGTCGG	176	*	KJ938520
ECMG15	(CTT)10(TC)8	GATCCCACTTGATAGCATCTTGA	CTAGCAGAATCCTCCCTTTGGT	241		KJ938521
ECMG16	(CT)8	TCCGTGTACCAATTCGACTAAA	TAGCAATCCATCGTCTCATTTG	152		KJ938522
ECMG17	(CAA)4	AGTGAGCAGAAGATACCCCAAT	CCGACCCCATCTAGTGAATAAG	123	*	KJ938523
ECMG20	(ATTTT)3	CGCTATTGAACTCTATTCCTTACTC	ATACGTGTGTATGTGTGCGTTT	226		KJ938525
ECMG21	(TATT)4	GTCGCCTAAAATTGTTGCTTTC	CTAGCAGAATCCTTTCCACCAT	233	*	KJ938526
ECMG22	(AG)10	CAGCGTTATCAAATTCTCCACA	CACATCAGGTAAAGTGCCAACA	180	*	KJ938527
ECMG23	(TTTG)4	TCTAAAGGAGGGAACATGGATA	GGTTAAGATGAAGGCAAAAGAG	236	*	KJ938528
ECMG24	(CAAA)4	TCTCATGTGGGAACATTTTGAC	AAACGCTGAAACAATCTGTCG	207		KJ938529

Table 2 continued

Locus name	SSR motif	Forward primer (5′-3′)	Reverse primer (5′-3′)	Expected product size (bp)	Cross generic amplification	Gene bank accession no.
ECMG25	(GA)8	GAAAAGGACAGGAAGGTTCAAG	CAGGACTGTGTTCCAGAAGATG	209		KJ938530
ECMG26	(CA)11	CAATTACTCAGCGAAACCTGTG	GAGCTTCTAAACTGGTGCGAAT	206	*	KJ938531
ECMG27	(CCTA)6	GGAATGGGTTTAGTGAGGTCAT	GCTGGAGTATTTATCTGGTGGG	234		KJ938532
ECMG28	(TATC)5TA (TTTG)4 TC(TATT)3	TGTTCAGAGGAGTCAGCAGGTA	GCCTCAAACTTCTTGTCCATCT	148	*	KJ938533
ECMG30	(CCGT)3	ATCGGACTTATAGGGGATTCGT	GTTACAAAGCGGCCCAAA	102	*	KJ938534
ECMG32	(TG)7	AATTAACCGCCCCATAATACCT	TTAAATTGGTGAGTGCATCCTG	113	*	KJ938536
ECMG33	(AG)6	GATCACAAAAGCACTCTTCACG	GATCTCCGCTATCCATAAGGC	100	*	KJ938537
ECMG34	(TTTG)3	GATGAACGGATATGTTGGGAA	GTGTTGGCTTCTTGGATCAAC	162		KJ938538
ECMG35	(AC)6	TTTGTAATTTGGAGTGACGGTG	AGAGTTCTTGTGCATTCTTCCC	155	*	KJ938539

* Primers amplified in related genera

Table 3 Characteristics of polymorphic SSR primers developed from small cardamom

Locus name	Ta[a] (°c)	Observed product size (bp)	N_a[b]	PIC[c]
ECM14	61	221–227	2	0.36
ECMC11	61	140–160	4	0.14
ECM47a	61	110–137	7	0.26
ECMG15	63	235–241	2	0.26
ECMG16	63	148–152	2	0.38
ECMG28	63	144–160	4	0.30

[a] Ta Optimized annealing temperature of the primer

[b] N_a Number of alleles

[c] PIC Polymorphic information content

Characterisation of microsatellite primers and polymorphism

Out of the 58 primer pairs designed 44 (Table 2) were amplified to give expected product size ranging from 100 to 382 bp. Among them six (Table 3) showed polymorphism in the selected 20 cardamom genotypes. A total of 22 different alleles were detected on these accessions. The number of allelic variants ranged from 2 to 7 alleles with an average of 3.6 alleles per locus. A maximum of seven alleles were observed for the locus ECM47a and MCC7 (Pink tiller) showed a genotype specific banding pattern for the same locus. It indicates that this marker may be linked to pink colouration of the stem. The genotype APG 434 (MA18) showed specific banding pattern for the locus ECMG28. The PIC values ranged from 0.14 to 0.38 based on the dominant scoring of bands.

Sequence comparison of alleles

For understanding of the SSR polymorphism, four different alleles of the locus ECMG28 were sequenced. The sequences were aligned to the original locus, SCGG125 from which the SSR marker ECMG 28 was designed. Multiple sequence alignment of the sequences showed that flanking sequences of the SSR were highly conserved and the allelic variation is due to the length polymorphism of repeats (Fig. 1, Online resource 2).

Genetic diversity analysis

Clustering of the 20 accessions using dominant scoring based on UPGMA has separated them into two major clusters at 60 % similarity (Fig. 1). The cluster one consisted of two groups with ICRI1 alone in a separate group diverged at 66 % similarity and PV1, APG342 (Coorg green) and MCC7 (pink tiller) in another group. In group two, MCC7 diverged at 67 % similarity. Cluster two

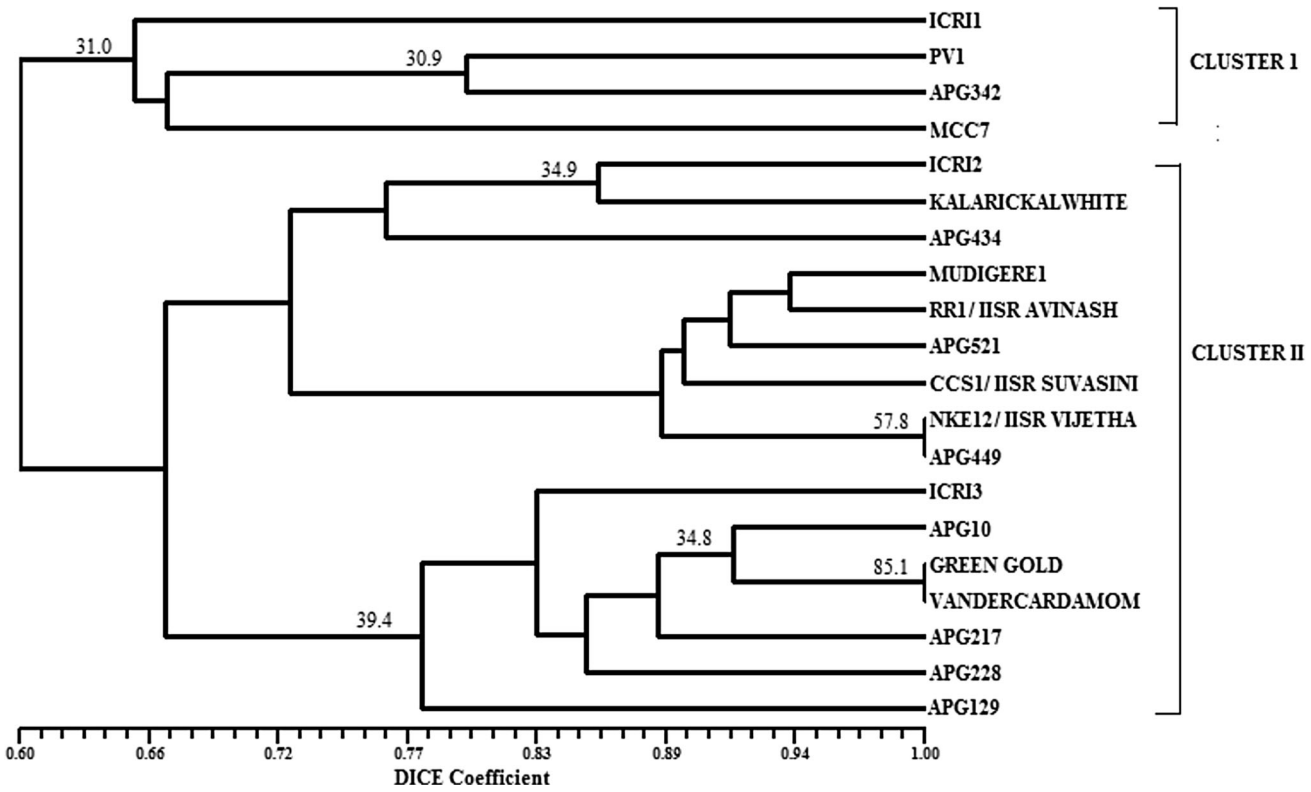

Fig. 1 Dendrogram showing the genetic divergence of 20 small cardamom accessions based on the Dice similarity coefficient using six genomic microsatellite markers. The genotypes were discriminated into two major clusters at 60 % similarity

consisted of mainly two groups: group three and four. Group three includes Karnataka varieties and farmer's selection Kalarickal white while group four consisted of remaining Kerala varieties with APG10 (MB3) and ICRI3. Among the genotypes used for the study, the three IISR varieties and the unique varieties like Njallani Green Gold, Kalarickal white, pink tiller were clearly separated into different groups. The two closely related Wynad collections APG217 and APG228 were differentiated from each other using the primer ECM14. PCoA plot (Fig. 2) gave a better depiction of the grouping of different genotypes. The grouping was similar to that of the dendrogram and showed PV1 and ICRI1 as the most divergent ones.

Transferability to other genera in Zingiberaceae

In total 44 of 59 primers were amplified in cardamom. From that a set of 28 (ECMG) primers (Table 2) were selected for amplification in the five related genera. Out of which 20 primers amplified a band in one or more related genera. Over all cross generic transferability rate was 71.4 %. Among the 20 primers three primers (ECMG1, ECMG3, and ECMG11) were amplified in all the seven genera. ECMG1 gave a similar banding pattern in all the seven species and *Elettaria* which shows the genetic similarity between these genera at this locus. All other markers revealed the genetic variability among different genera. 17 (60.7 %) primers were amplified in at least one among the three different species of *Amomum*. Out of them *Amomum subulatum* showed more resemblance to *Elettaria* and *Amomum ghaticum* the least. *Zingiber officinale* amplified 13 (46.4 %) microsatellite loci. *Alpinia mutica* amplified 10 loci. *H. coronarium* and *C. longa* amplified 10 (35.7 %) and nine (32.1 %) loci respectively.

Discussion

Isolation of microsatellites and enrichment efficiency

In the present study, a robust set of 44 microsatellite primers were developed for use in small cardamom. A microsatellite enrichment efficiency of 52 % (out of 165 unigenes, 85 sequences with SSR motifs) was observed using the protocol of Glenn and Schable (2005). Comparing this with the studies in other crop in Zingiberaceae the enrichment efficiency is lower than *Curcuma* (84 %) (Siju et al. 2010), and higher than *Zingiber* (41 %) (Lee et al. 2007). A total of 85,452 bp sequences were analyzed for microsatellites and 140 SSR motifs were identified. As the

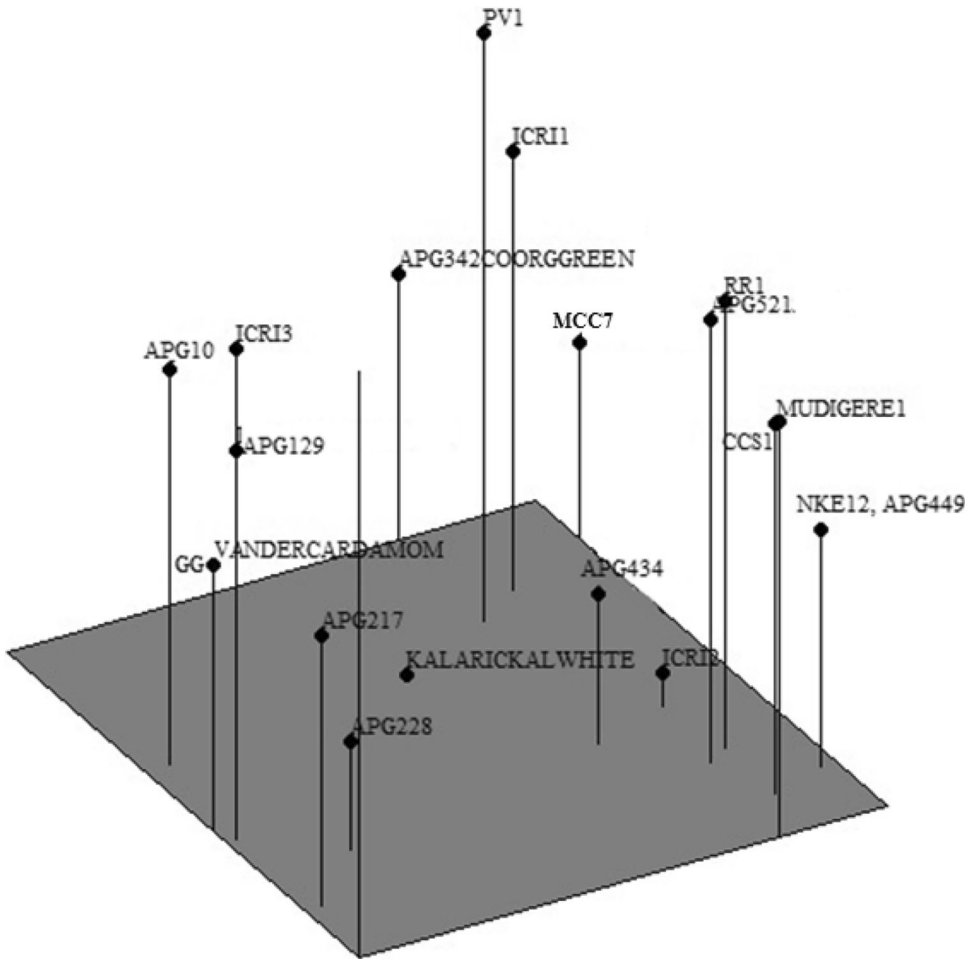

Fig. 2 Three dimensional PCoA plot based on the genomic microsatellite analysis data generated for the 20 small cardamom genotypes

dataset was limited, low stringent conditions were applied to identify SSRs from sequences. The identified SSRs include both class I (≥20 bp size) and class II (≥ 12 bp ≤ 19 bp size) (Mun et al. 2006) repeat motifs. The observed frequency of microsatellites in genomic DNA was 1.63 SSR/kbp (including hexa nucleotides with 12 bp size). The frequency of class I microsatellites is 0.32/kbp and class II is 1.32/kbp. Even though the values were lower, it is comparable with the frequencies in monocot plants like rice (class I 0.6/kbp and class II 14.7/kbp) and in dicot plants like *Medicago truncatula* (class I 0.6/kbp and class II 12.1/kbp) (Mun et al. 2006). Mun et al. (2006) reported that the frequency of SSRs was 1.3–2.8 fold higher in genomic sequences than in EST sequences. The differences in frequency of microsatellite motifs in plants depend on the size of data set, search criteria and different bioinformatics tools used (Varshney et al. 2005). The present data indicate the chances of small cardamom to be a microsatellite less abundant crop.

Most of the SSR motifs identified were class II microsatellites and majority of which includes hexanucleotides. This indicates the abundance of class II microsatellites in the genome than class I microsatellites. However, most of the hexanucleotide motifs repeating two times (12 bp) were not considered for primer designing. The similar studies were reported earlier in *Medicago truncatula* (Mun et al. 2006) where class II microsatellites include most of the hexa and hepta nucleotides. The class I microsatellites comprised of mostly dinucleotides with one or two SSR motifs from other repeat types. The results of the present study indicate the abundance and adequate distribution of dinucleotides than trinucleotides (Table 1, online resource 1). Among dinucleotides the most common motif was (AG/CT)n (62 %) then (AC)n (36 %) (Fig. 3), which is in agreement with other studies in different crop species (Gupta and Varshney 2000). Trinucleotides represent comparatively a small portion of the SSR motifs isolated (Table 1, online resource 1). Out of which (AAC/GTT) and (AAG/CTT) repeats were predominant (Fig. 3). In contrast (GGC)n was observed in rice (monocot) and in legumes (ATT)n repeats were over represented in genomic sequences (Mun et al. 2006). But the presence of AT or ATT repeats were very less in the

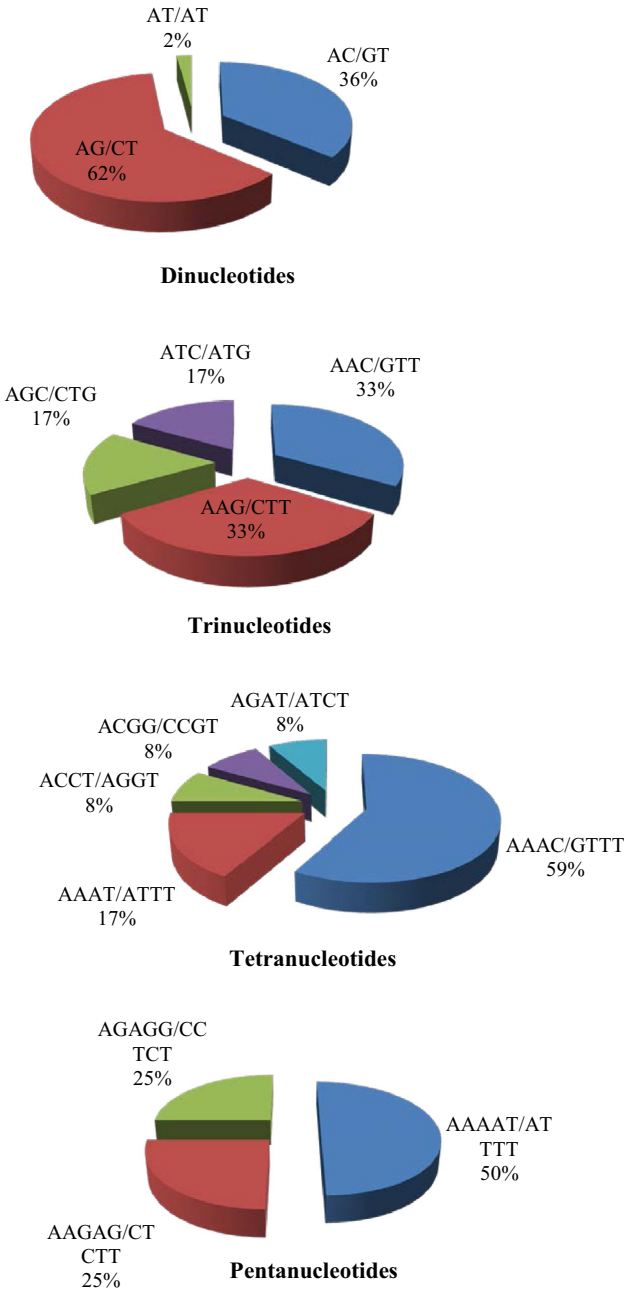

Fig. 3 Distribution of different motifs of dinucleotide, trinucleotide, tetranucleotide and pentanucleotide repeats obtained from the genomic DNA library of small cardamom

present study. The possible reason for the difference in microsatellite repeat may be the different biotinylated oligos used for microsatellite isolation. In addition taxon specific accumulation of microsatellites is reported in many species by different authors (Toth et al. 2000; Katti et al. 2001). Varshney et al. (2000) reported that library enriched by hybridization may not detect (AT)n motifs due to secondary configuration resulting from intra-strand pairing between A and T nucleotides.

Evaluation of SSR markers for genetic diversity study

Primer pairs were designed successfully for 58 SSR motifs. Out of which 44 (76 %) were amplified fragments of expected size in cardamom. Among the amplified loci six (13.6 %) markers showed consistent polymorphism in 20 genotypes. In this study the microsatellites with more number of repeats were given polymorphism. Out of them four were dinucleotides and three motifs were compound microsatellites. Apart from that more variation was observed in the microsatellite motif (AG/CT)n. The locus ECM47a with the compound microsatellite motif $(CT)_{17}TCAA(TC)_9$ showed an allelic variation of more than 25 bp. Similar reports are there in jute (Das et al. 2012) and rice (Blair et al. 1999) where more polymorphism was detected in dinucleotide repeats especially (AG/CT)n Among the amplified primers 86.4 % was monomorphic and 13.6 % was polymorphic. The total number of alleles observed for polymorphic markers was 22 with an average of 3.6 alleles per locus. When compared to the number of markers, the number of alleles detected was high. The PIC value ranged from 0.14 to 0.38 based on the dominant scoring of bands. This implied that these markers contained high genetic information for cardamom.

The polymorphic markers discriminated 16 out of 20 genotypes into different groups as shown in the dendrogram (Fig. 1). Even though most of the microsatellites isolated were in conserved regions, the six polymorphic markers were able to differentiate the major cultivars and released varieties from each other and also showed genotype specific banding pattern. The loci ECM14 differentiated the two genotypes collected from Wynad, APG217 and APG228 from each other. The three IISR released varieties IISR Suvasini, IISR Vijetha and IISR Avinash were differentiated from each other at the same locus ECM14. The released varieties ICRI1, ICRI2 and ICRI3 were distinguished from each other with the two markers ECM47a and ECMG28. Among the 20 cardamom varieties, the genotype APG10 (MB3) amplified both rare and abundant allele for most of the loci employed in this study.

The percentage of polymorphism detected was low in cardamom as these markers could not detect minor variations in cardamom and which is expected in cardamom because diversity at species level is absent in Indian population. The low level of polymorphism was also reported in different crops. In saffron (Mir et al. 2015) the molecular markers did not reveal significant polymorphism at molecular level. Totally 209 markers including RAPD, ISSR and SSR were tested with 31 morphologically distinct saffron selections and 34 markers were polymorphic. In cotton (Rungis et al. 2005) 5 % polymorphic SSR markers were reported between cultivars. The 20 genotypes were selected based on a criterion that it can represent the wide

variability in the germplasm. However these 20 genotypes may not be enough to represent the large germplasm of cardamom with more than 500 accessions and huge number of morphological variations (Prasath and Venugopal 2004). So the monomorphic primers may show polymorphism when tested in different genotypes in the germplasm with special characteristics and with sophisticated techniques which can resolve the allelic variations at single base pair changes. More over sequence analysis at microsatellite regions may help to detect the morphological variations at molecular level.

Microsatellite markers has become one of the most widely used molecular markers for genetic analysis in recent years. Accurate knowledge of genetic variability among small cardamom accessions is important for establishing core collections of germplasm and in aiding breeding work. The present study identified divergent genotypes of small cardamom accessions and unnamed landraces; and revealed the genetic similarity between hybrids and selections with popular known cultivars (Fig. 3). The results indicate the divergence between Kerala and Karnataka collections and less divergence within the population as reported by Nirmal Babu et al. (2012). It is because most of the collections originated from open pollinated seedlings. However the dendrogram point towards the possible chances of hybridisation between different distant groups.

Cross generic transferability of developed cardamom microsatellite markers to other Zingiberaceae members showed that these markers are informative and can be used for phylogenetic studies. Over all transferability rate was 71.4 % which is comparable with markers developed in other crops. From pear to Rosaceae over all rate of transferability was 61.2 % (Fan et al. 2013). The transferability rate of small cardamom SSR markers was lower than that achieved for apple EST SSR to Rosaceae (75 %) (Gasic et al. 2009) and higher than which obtained for ginger EST SSR (36.4 %) and genomic SSR (12.5 %) to small cardamom (Anu et al. 2015). Also we have observed a high transferability (71.4 %) rate of *Amomum* primers to *Elettaria* (Anu et al. 2015). The present study also reports a high transferability rate (60.7 %) of *Elettaria* primers to *Amomum*. The results indicate that *Elettaria* is closely related to *Amomum* than other genera. It is in agreement with other studies (Fischer 1956; Kress et al. 2002, 2005; Nirmal Babu et al. 2012) which state the relationship of *Elettaria* to *Amomum*.

In conclusion, the novel SSR markers were able to discriminate the divergent genotypes in cardamom germplasm, which shows that these markers are informative and reliable. Two markers produced genotype specific banding patterns and can be used as variety specific marker. Genome of cardamom is mostly conserved as it is a monotypic species in India. Among the markers identified earlier, only ISSR markers gave high polymorphism (Nirmal Babu et al. 2012) followed by these SSR markers. Hence SSR coupled with earlier reported markers RAPD, RFLP-PCR, ISSR (Nirmal Babu et al. 2012) can be used for differentiating the cultivars and mapping population and for developing linkage maps. Construction of integrated genetic linkage map using RAPD, ISSR and SSR have been reported in different crops (Venkateswarlu et al. 2006; Priyamedha et al. 2012). This report is an initiative towards molecular profiling of more unexploited accessions of small cardamom that can provide insights into patterns of genetic diversity. Being a first report, fingerprinting study discerned by SSR markers is informative to study genetic architecture and interrelationship in the genus *Eletteria*, however, generation of more number of polymorphic SSR markers using next generation sequencing and high resolution electrophoretic techniques viz. MultiNA can provide a better understanding of the genome which will aid in the utilization of markers in varietal identification, conservation, and development of core collection of small cardamom genotypes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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