Transferability of ginger, turmeric and large cardamom SSR primers to small cardamom (*Elettaria cardamomum* Maton)

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Abstract
Small cardamom (*Elettaria cardamomum* Maton) of the family Zingiberaceae is an important spice crop and research is in progress to develop molecular markers to study genetic diversity and taxonomic inter-relationships in this crop. In an effort to develop a repository of molecular markers for genetic analysis of small cardamom, a set of 79 SSR (simple sequence repeat) markers reported from the related genera (*Amomum subulatum*, *Zingiber officinale* and *Curcuma longa)* were screened for cross generic amplification in cardamom. The study has demonstrated the transferability of 26 SSR markers to small cardamom. When tested in a group of 20 genotypes of small cardamom, the SSR profiles were often monomorphic except for three unique polymorphic SSRs. The ginger SSR marker ZOC100 produced a variety specific banding pattern for the genotype APG10 (MB3). The genotypes were heterozygous with most of the loci tested. The total number of alleles obtained was 50 with a range of one to seven alleles per SSR locus. Multiple sequence alignment of different alleles of the marker ASM1 confirmed the presence of microsatellite motifs in the alleles and the allelic variations were due to length polymorphism of microsatellite repeats. The results indicated that cardamom genome, as it is a monotypic genus, is highly conserved with little detectable molecular variations at microsatellite regions. Furthermore, the study underlined the earlier reports which state the genetic similarity and closeness of *Amomum* to *Elettaria.*

Key words: *Elettaria cardamomum*, SSR transferability, *Amomum subulatum*, *Zingiber officinale*, *Curcuma longa*.

Introduction

Cardamom, the Queen of Spices is one of the major spices in the world. It is the small cardamom (*Elettaria cardamomum* Maton) which belongs to the family Zingiberaceae and genus *Elettaria.* The genus consists of about six (Mabberly, 1987) or seven (Sakai and Nagamasu, 2000) species distributed in India, Sri Lanka, Malaysia and Indonesia. Out of them *E. cardamomum* Maton is the only economically important species and India has the largest variability in this commercially important crop (Prasath and Venugopal, 2004). The morphological diversity is fairly high in cardamom and may be because of the cross pollinated nature of the crop (Prasath et al., 2009). The chromosome number of small cardamom is 2n=48 and X=12 (Gregory, 1936). Even though it is one of the costly plants among the members of Zingiberaceae family, the efforts to unravel morphological diversity in terms of molecular diversity are very limited. Conservation of genetic material has utmost importance in plant breeding programmes (Madhusoodanan et al., 2002) and conversely, it is related to the removal of duplicates from the germplasm materials. The earlier studies in cardamom using RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeats) (Radhakrishnan and Mohanan 2005; Nirmal Babu et al., 2012) markers were not able to distinguish the high level of morphological variations at molecular level. Ashitha et al. (2013)

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also reported that significant variation could not be observed and more molecular markers are needed to assess the allelic variation.

Molecular markers are important tools for dissection and analysis of genomes for better understanding of genome architecture. This helps breeders to incorporate important agronomical traits in breeding programmes by marker assisted selection (Christiansen et al., 2002). Among the molecular markers, microsatellite (Simple Sequence Repeat) markers are widely accepted for genetic diversity studies because of their reproducibility and co-dominant nature (Zane et al., 2002). They show allelic variation and can be used for germplasm studies, linkage mapping and breeding studies. However, development of SSR markers for each species is very difficult and time consuming. Hence the markers which are transferable to other species or genera (Agrawal et al., 1999) will be more preferred. Moreover this will assist in comparative genomics, QTL analysis and evolutionary studies of different plant genomes. There are several studies which demonstrate the presence of conserved flanking regions of SSR motifs between species/ genera (Agrawal et al., 1999). In recent years the utility of cross species/ generic transferability of SSR markers, especially for minor crops has been reported increasingly (Kuleung et al., 2004; Barbará et al., 2007; Park et al., 2009; Yu et al., 2011).

The members of the family Zingiberaceae are underutilized crops and molecular markers are not available for most of the species. The exceptions are ginger (*Zingiber officinale* Rosc.) and turmeric (*Curcuma longa* L.) in which a robust set of newly developed SSR markers were reported (Lee et al., 2007; Siju et al., 2010a; Siju et al., 2010b). Furthermore, Jatoi et al. (2006) reported the usefulness of rice (*Oryza sativa* L.) microsatellites for diversity studies in ginger and Basudeba et al. (2013) employed rice microsatellites in different species of Zingiberaceae family. Since there was no information on the genome of cardamom, cross generic studies offered more potential to develop a repository of molecular markers for utility in this crop. In the present study a set of 79 SSR markers from the related genera were tested to demonstrate their utility in cardamom.

Materials and Methods

Plant Material

The plant materials used for the study were collected from the germplasm maintained at ICAR-Indian Institute of Spices Research, Regional Station, Appangala, Madikkeri, Karnataka and Indian Cardamom Research Institute, Myladumpara, Kerala. Twenty diverse genotypes were shortlisted *viz*., ICRI 1, ICRI 2, ICRI 3, PV1, Mudigere 1, IISR Suvasini, IISR Vijetha, IISR Avinash, APG10 (MB3), Green Gold, Vander Cardamom, MCC7 (Pink tiller), Kalarickal White, APG129 (Narrow Leaf), APG434 (MA18), APG342 (Coorg Green), APG449 (CRC411), APG521 (ASH) and two Wynad collections APG 217 and APG228 based on phenotypical differences.

DNA Extraction

Total genomic DNA was extracted from the fresh leaves following the protocol of Doyle and Doyle (1987) with slight modifications. The quantity and quality of the isolated DNA was estimated using biophotometer (Eppendorf, Germany) and 2 µl of the samples were run on a one per cent agarose gel. The final DNA concentration was adjusted to 20 ng μl^{-1} for PCR analysis.

SSR Markers Used for the Study

A total of 79 SSR primers from different genera under the family Zingiberaceae were utilized for the study *viz.* ginger: 22 EST SSR primers selected based on the functional annotation (unpublished personal data) and 8 ginger genomic SSR primers (Lee et al., 2007); turmeric: 17 EST SSR primers (Siju et al., 2010a) and 18 genomic SSR Primers (Siju et al., 2010b) and 14 large cardamom (*Amomum subulatum* Roxb.) SSR primers (unpublished personal data).

Amplification of DNA, Electrophoresis and Sequencing

All the amplification reactions were carried out in 25 µl reaction volume containing 20 ng template DNA, 1X PCR buffer, 2 mM $MgCl_2$, (GeNei™, Bangalore),0.1 mM each dNTPs mix (Thermo Scientific, USA), 0.5 picomol forward and reverse primers and 0.5U Taq DNA polymerase (GeNei™, Bangalore). The amplification was performed in a gradient thermocycler, (Eppendorf, Germany) using the following programme: 95°C - 3 min.; then, 35 cycles of 95°C - 45 sec., 52°C - 65°C (varied for different primers) - 45 sec., 72°C for 1 min; then 72°C for 10 min. and hold at 4°C. Amplified products were resolved in a 15 per cent denaturing polyacrylamide gel along with GeneRuler™100bp and GeneRuler™ ultra low range DNA ladders (Thermo Scientific, USA). The gels were silver stained (Bassam and Gresshoff, 2007) to view the bands, and images were acquired by scanning the gels. Genotypes which showed allelic variations were selected based on the size of DNA fragments and the PCR products were purified and sequenced at SciGenom Labs Private Ltd, Cochin, India. The sequences were aligned to the original sequences using the softwares MEGA6.05 (Tamura et al., 2013) and BioEdit (Hall, 1999).

Data Analysis

Allele sizes were calculated by comparing the bands to GeneRuler™ ultra low range DNA ladder (Thermo Scientific, USA) in denaturing PAGE and GeneRuler™ 100bp DNA ladder (Thermo Scientifc, USA) in agarose gels. Because of the polyploid nature of the cardamom species, the banding pattern observed at each locus were taken as "allele phenotypes" and all the unique bands were treated as dominant markers (Honig et al., 2010). The data were scored based on the presence (1) or absence (0) of a band for each genotype. The polymorphic information content (PIC) of each SSR marker was calculated 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 where Pi is the frequency of presence and Qi is the frequency of absence of a particular band. 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).

Results and Discussion

Amplification of SSR markers

Thirty ginger SSR primers including 22 ginger EST SSR primers and eight ginger genomic SSR primers were tested in cardamom, out of which eight EST SSR primers (36.4 per cent) and one (12.5 per cent) genomic SSR primer were successful (Table 1). Among the 35 turmeric SSR primers, six EST SSR primers (35.3 per cent), and one genomic SSR primer (5.5 per cent) were amplified in cardamom. From the genera *Amomum* 10 primers (71.4 per cent) produced discrete bands in small cardamom (Table 1). A total of 50 alleles were generated by the markers with an average of 1.9 alleles per locus which reflects that the genotypes were heterozygous at most of the loci tested.

Compared to *Zingibe*r SSR markers, the percentage of *Curcuma* SSR markers amplified in cardamom (30 per cent and 20 per cent respectively) were less (Table 2). This could be due to the failure of primers selected from turmeric to amplify regions in cardamom. It also indicates that *Curcuma* is more distantly related to *Elettaria* than *Zingiber*, which is supported by the earlier phylogenetic studies in the family Zingiberaceae (Kress et al., 2002). The success rate of transferability of SSR markers is dependent on the phylogenetic distance between the species and conservation of sequence in the tested species (Wang et al., 2009). Barbará et al. (2007) reported that transfer of polymorphic markers in monocot plants is successful within genera (40 per cent in the reviewed monocots) and difficult across genera within the family. On an average, 35 per cent of the EST SSR primers and 9 per cent of genomic SSR primers from ginger and turmeric were

Table 1. Primer sequences and characteristics of 26 SSR primers which were successful in cross generic amplification of microsatellite loci in small cardamom

Locus name	Repeat Motif	Forward Sequence(5'-3') Reverse Sequence(3'-5')	Ta[a] S	Exp. Product ize (bp)	Obs. product Size(bp)	No. of loci.	N_a[b]	PIC[c]	Species
ZOC11	(CTG)9	GCTGCTGGTACTTGGCTTTC CTCCTTCCTTTGCCTATCAAGA	60	233	233-235	1	2		*Zingiber officinale*
ZOC22	(TTC)8	GGAGATTGGGTTCATCGTCTAC AGCAAAGAAATAGGACAAGGCA	60	270	270	1	1		
ZOC28	(GCCTTC)4	GCCTTCTTCGGAGTGTCCTT AACCAAAGCCTAATCCAAAACC	60	268	268	1	1		-do-
ZOC91	(GA)14	GCAGATCCAGAACTCCACCTAA ATTTCCTCTGTTCCATCTCAGC	60	238	270	1	1		
ZOC92	(CGG)9	GTAGTCCCCAAACAGAAACTCG AGATCGAGGTGGTCAGCAAT	60	206	185	1	1		
ZOC98	(CT)14	TGCCACTCAATAACATGAACC CGATACATAACAAGCAAGCAAC	58	275	290	1	1		
ZOC100	(CGA)9	CATCCCACTGAAGCGTACAAAC AGGTCGGAGGTGAAGTCTCTG	61	142	130-170	2	7	0.28	
ZOC102	(GAAAA)5	TCTCGGGCACCAAAATAAATAC TCATCGTGTTGTAGAAAATGGC	60	179	150	1	1		
GB-ZOM-140	$(GGA)_4$	AGGGGGCAGTGGAGAG ACGTTCCTGCACTTGACG	55	100-280	280	1	1		
CLEST SSR-04	(AAAC)6	ACACAACATTCAGTTTAGCAC TCCCTATTCTTTCCTCTCG	62	184	180-184	1	2		*Curcuma longa*
CLEST SSR-07	$(AT)_8(AC)_{14}$	AGACAGAAGAAGAGGCAGAAG AAATGATGACCACGGACTAC	62	152	180-186	1	2		
CLEST SSR-08	(AGAT)8	GATGCACACATTGCCCGTG GGGTGCAATTCTTGGTCCG	60	171	152-180	1	4		-do-
CLEST SSR-13	(AAT)7	TGTACAAGCTCCAAATAAGTCAAG CAGGAGTGTTCTAATGTTGCCC	65	154	156-160	1	2		
CLEST SSR-15	(CCT)7	GCCAAAGAAAGAACTGACATCC TTACAACCCTCCTCCCATTAGA	65	172	162	1	1		
CLEST SSR-16	(AGG)5 (AAG)7	AAGCAGTCCGTGGGAGAAG CTTCCTCAATCGAATGGCCG	65	173	154-210	1	2		
CuMiSat-10	$(AG)_9$	CACCCTATGAGTGCTAACTGAAG ACCTGCACCACGATCAAC	60	186	180	1	1		
ASM1	(TTTG)5	AAGGATCAAAATTGCCTCACAC GCTTGCTCTCAAAGTCATCAAA	60	234	220-224	1	6	0.4	*Amomum subulatun*
ASM2	(CT)6	GCAATTCCCGATATGTTTGT GGCCCTTATTTTACGATACACT	57	113	108-114	1	2		
ASM3	(CTT)4	AAGATACCTGAAGAGGATGGGG AGAAGGCTAAGAAGGCAAAACC	60	145	145	1	1		-do-
ASM5	(AC)8	GATGATGTTGGGCATTGATTT CATGGCTCTTTTCTATAATCCAAGT	59	193	192	1	1		
ASM6	(AC)17	AAATGACTCTATGTCACGCCCT TCGGTATGCACTATGCTGAGTT	60	226	196	1	1		
ASM7	(TG)7	CATCCTCCTTCGAGGTAAAGAA TAAATGAGTTGTCCTGCCTTCC	60	141	130	1	1		
ASM8	(AAG)5	AGGGGTTAGGTTCATTTTACCG CAAAGGGGTGTTCGAGAGAA	60	249	380-400	1	3		
ASM11	(CT)6T(CT)9	AATTTCTGTTGTGGAAACGGAG GACGGTGCGTGTGTGTGT	60	165	132-140	1	2		
ASM12	(TGTTT)2	AAAGAGATTGCACAAAGACGTG CAACAACAACAACAACAACAAAA	59	126	170-174	1	2	0.49	
ASM14	(GCATGA)2	GAGGGGATCATAGGGACTTCTT GCGAGTTTATCATGCAACCATA	59	270	268	1	1		

[a]Ta- annealing temperature, [b] N_a –Number of alleles, [c]PIC- Polymorphism information content , the polymorphic primers were given in italics.

transferable to cardamom. It shows the conservation of expressed portion of DNA sequences in related genera under the same family Zingiberaceae as evidenced in different studies. In Rosaceae family 75 per cent of apple EST SSRs were transferred to other Rosaceae genera (Gasic et al., 2009) while 61.2 per cent genomic SSRs were transferred from pear to other Rosaceae genera (Fan et al., 2013) and genomic SSRs expressed more polymorphism.

Among the large cardamom SSR primers tested, 71.4 per cent were transferable to cardamom which indicates that the flanking sequences of the genomic microsatellite primers were conserved between the two genera. High level of transferability of large cardamom genomic microsatellite markers to small cardamom was ascribed to the fact that both genera belong to the same tribe Alpinieae under the family Zingiberaceae. According to earlier studies the genera *Elettaria* is closely related to the genera *Amomum* (Holttum, 1950; Fischer, 1956; Kress et al., 2002; Kress et al., 2005; Nirmal Babu et al., 2012). Our findings are in agreement with the earlier reports which state the similarity and closeness of *Elettaria* to *Amomum*.

Polymorphism of SSR Markers

Twenty six SSR primers from different related genera were used for cross amplification studies in 20 different genotypes of small cardamom. Out of them three markers were polymorphic among different genotypes of small cardamom, one ginger EST SSR primer (11.1 per cent) and two large cardamom primers (20 per cent) (Table 2). These three polymorphic markers could well distinguish the selected accessions of cardamom with the PIC value ranges from 0.28-0.49 (Table 1) based on dominant scoring. The average value was 0.43 for the markers when maximum PIC value for dominant markers is 0.5 (De Riek et al., 2001; Bolaric et al., 2005) which implied that they are significant for genetic research. A total of 15 alleles were detected by the three polymorphic markers. The average number of alleles was five with a range of two to seven alleles per SSR marker. The greatest variation was detected for the SSR marker ZOC 100 with seven alleles in 20 different genotypes of small cardamom and the marker ASM1 detected four alleles. The difficulty in amplifying ASM 12 primer in all the genotypes indicates the presence of null alleles in cardamom genome.

The experiment displayed the utility of SSR markers at varietal level identification. The marker ZOC 100 distinguished the variety APG10 (MB3) specifically from others with a different banding pattern. Amplification of the marker ZOC100 at multiple loci suggests the presence of duplicated regions in the genome. Similar studies were earlier reported in bread wheat (Roder et al., 1998) and Egyptian hexaploid wheat (Salem et al., 2015).

Sequence Analysis

Sequencing of the selected alleles obtained from small cardamom confirmed the presence of microsatellite motifs in the amplicons. Alignment of the sequences from different alleles of ASM1 marker to the original sequence of large cardamom disclosed the presence of insertions and deletions at the microsatellite region in cardamom genome and the flanking regions were conserved (Figure 1). The allelic variations were due to the length polymorphism of microsatellite repeats. The flanking regions were diverged for marker ASM12

Table 2. Amplification frequency of cross generic SSR primers in *Elettaria cardamomum*

Species in which the SSR primer is reported	Successful amplification of SSR primers in small cardamom			
	Number of primers amplified	Percentage of successful primers	Number of polymorphic primers	Percentage of polymorphic primers
Zingiber officinale	9	30	1	11.1
Curcumalonga	7	20	-	-
Amomum subulatum	10	71.4	2	20

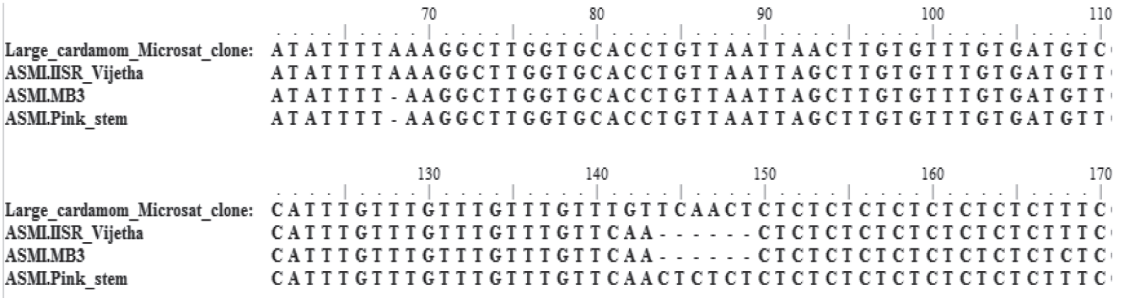

Figure 1. Multiple sequence alignment of the sequences obtained from different alleles of marker ASM1 with their original sequence. Sequences confirmed the presence of (TTTG)n and (CT)n motif with variable number of repeats and conserved flanking sequences.

and marker ZOC100. Targeted microsatellite motifs could not be observed in the sequences obtained for the marker ZOC100 and indels were present at a new microsatellite motif present in the flanking region. Allelic variations were owing to the length polymorphism of new microsatellite repeat in cardamom genome.

Cardamom is a monotypic species originated in the Western Ghats of India and wide variability is observed in the crop for morphological characters. Investigations on the crop were limited to yield and hybridization studies and genetic diversity studies based on morphological variations and RAPD, RFLP-PCR and ISSR markers. There is only little information available on the molecular characterization of genome. Previous studies reported the requirement of more molecular markers (Nirmal Babu et al., 2012; Ashitha et al., 2013) for characterization of the unexploited large germplasm of small cardamom with more than 500 accessions.

The present study reports the transferability of 26 SSR primers *viz*. nine (30 per cent) SSR primers from *Zingiber*, seven SSR (20 per cent) primers from *Curcuma* and 10 SSR primers from *Amomum* to *Elettaria*. Compared to primers from the other two genera, large cardamom SSR primers gave a better cross generic transferability (71.4 per cent) to cardamom which in turn proved the possibilities of high rate of cross generic transferability of SSR markers between closely related genera of monocots. High rate of intergeneric transferability of SSR markers were reported in many other species: from tall fescue to several grasses (~ 57 per cent) (Saha et al., 2006), *Setaria italica* to six grass species (~ 74 per cent) (Gupta et al., 2012) and guava to eucalyptus, bottlebrush and clove (71.2 per cent) (Rai et al., 2013). A comparatively higher rate of transferability was reported from *Litchi chinensis* to *Euphoria longan* (~ 91 per cent) (Viruel and Hormaza, 2004) and a lower rate was reported (<50 per cent of SSR primers) between *Fragaria* and *Rubus* and also from other genera under Rosoideae (Lewers et al., 2005). All these studies demonstrated sharing of similar primer regions in different genera of the same family.

In conclusion, the study points out that though genomic microsatellite markers from other genera could not be effectively utilized in cardamom, microsatellite markers from *Amomum* were effective. Since the genotypes had a narrow genetic base, sufficient numbers of markers with techniques which can detect single base pair changes in the genome were required to characterize the germplasm for development of core collection. However, the SSR based study confirmed the presence of significant diversity in cardamom germplasm. The sequencing results proved that these markers are reliable for phylogenetic studies. The present markers can be used in the germplasm for a various number of applications including molecular breeding, QTL mapping, comparative

mapping, genetic diversity studies, marker assisted selection, and cultivar identification.

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