Assessment of ISSR Markers for Tagging Genetic Variability for Yield Components in Small Cardamom (*Elettaria cardamomum* Maton)

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**ABSTRACT**

Genetic diversity among ten small cardamom accessions having variability in yield traits was investigated using inter simple sequence repeat markers (ISSR). A better understanding of the variability in yield is essential for the proper utilization of genotypes in breeding programmes. Of the 10 primers analysed, 5 reported polymorphisms and generated a total of 47 scorable loci, of which 32 were polymorphic revealing 68% polymorphism. The molecular weight of the fragments ranged from 200-1100 bp. Specific banding pattern was observed in high yielding group using ISSR7 (800bp) and with ISSR 3 (800bp) for low yielding group. Genetic diversity analysis within and among the two groups showed maximum diversity in high yielding varieties based on the values of Nei’s genetic diversity (h) and Shannon’s informative index (I). Dendrogram based on Jaccard’s similarity coefficients were generated based on an average linkage algorithm (UPGMA) using marker data. Clear grouping of the ten accessions of small cardamom into two clusters was observed. These results suggested that ISSR markers could efficiently differentiate the small cardamom genotypes based on yield and can be useful for future cardamom improvement programmes.

**Key words:** Cluster analysis, *Elettaria cardamomum*, Genetic variability, ISSR, Yield trait.

**INTRODUCTION**

Small cardamom (*Elettaria cardamomum* Maton), popularly known as ‘Queen of Spices’ is a rhizomatous perennial herb belonging to *Zingiberaceae* family. It is one amongst the most ancient and economically important spice crop that has been used as a spice, flavouring agent, cosmetics and also in native herbal medicines (Korikanthimath et al., 2001). Small cardamom registered maximum export growth during April-December 2017 for a total of 4180 tonnes valued for Rs 456.01 crore compared to the 2,910 tonnes for Rs 297.80 crore in 2016 (Anbuchelvi, 2019). The natural habitat of this crop serves to be the moist evergreen forest of Western Ghats of Southern India, that lies within 8°30’ and 14°30’N latitude and 75-70°E longitudes with Kerala accounts for 60% of the cultivation and production followed by Karnataka 30% and Tamil Nadu 10% (Ravindran, 2002). It is cultivated either as a monocrop under the forest canopy or as an intercrop. The optimum yield of small cardamom is obtained on warm (10 to 35°C), humid (with >1500 mm of well-distributed rainfall) and mountain slopes at 600-1500m above sea level (Reyes et al., 2006).

Genetic identification is the first key step in breeding programs and molecular markers are valuable tools for identifying and characterizing diverse genotypes (Ramakrishnan et al., 2019). Molecular markers based on polymerase chain reaction (PCR) are widely used since they require a relatively small amount of DNA and are technically simple and not affected by environmental, pleiotropic and epistatic effects (Mondini et al., 2009). Among the variable molecular markers, ISSRs have been widely used to study plant genetic variations such as gene tagging (Gopalakrishna and Joshi-Saha, 2007), genetic diversity (Abirami et al., 2018), fingerprinting (Bhagyawant and Srivastava, 2008) and marker-assisted selection (Vijayan et al., 2006). The principle of ISSR is similar to RAPD in which random primers are used. However, the ISSR primer sequences consist of a di- or tri-nucleotide repeated motifs targeting the regions between inversely oriented repeats (ISSRs) makes them more reproducible than RAPD markers (Semagn et al. 2006). There have been several genetic diversity studies reported in small cardamom. Babu et al. (2012) evaluated the genetic relationship between *Elettaria* and related genera using ISSR, RAPD and RFLP markers. The usefulness of ISSR markers in assessing genetic diversity among small cardamom accessions was reported by Jose et al. (2014) and Anjali et al. (2016). All these procedures were performed to assess small cardamom diversity, in order to differentiate elite varieties as well as duplicate genotypes in the germplasm repositories.
However, no studies were reported in detail that could associate yield trait of small cardamom at the molecular level. So, there arose a need to develop a screening method by which high yielding varieties could be distinguished from other cardamom genotypes. According to (Prasath et al., 2009), enough variability for economic traits exists in natural populations of small cardamom and this might be due to the cross-pollinating nature of the species. The identification of markers closely linked to important agronomic traits will allow their utilization in marker-assisted selection and thus increase the efficiency of selection in breeding programs.

Keeping the usefulness of ISSR markers for diversity analysis and need to characterize the small cardamom germplasm with respect to yield trait, the present study is aimed to assess the genetic diversity among and within ten selected small cardamom genotypes having variability with respect to yield trait and also to identify yield specific markers.

MATERIALS AND METHODS
Sample collection
Small cardamom samples were procured from germplasm repository of Indian Cardamom Research Institute, Idukki, Kerala, India. Collections included five high yielding varieties according to the yield parameters described in (Hrideek et al., 2008 and Jose, 2016) and five randomly selected low yielding accessions (Table 1). Accessions were selected with respect to the IPGRI descriptor (IPGRI, 1994). The samples were individually placed in sealable bags, transported to laboratory and stored in deep freezer until DNA extraction. The experiment was carried out in 2015 for a period of six months at the Department of Biotechnology, Mar Athanasius College, Kothamangalam.

DNA isolation and quantification
DNA extraction was carried out from fresh tender leaves of small cardamom using Nucleospin R plant II isolation kit (Macherey-Nagel, Germany) following manufacturer’s instruction. The concentration of DNA was estimated spectrophotometrically at 260 nm and the purity was measured by the ratio of the absorbance at 260 nm and 280 nm. The samples were then diluted to 25ng/µl and stored at -20°C until use.

ISSR assay
A total of ten ISSR primers (Table 2) representing di-repeats with specific modifications at 3’ end was selected for this study. The optimised amplification reaction mixture (25µL) contained 25ng of DNA template, 2X Orion Taq mix, 10 pmol of each ISSR primer and PCR grade dH₂O. The PCR amplification were carried out in a Mastercycler personal (Eppendorf) using the following programme, initial denaturation at 94°C for 2 min; 40 cycles of denaturation at 94°C for 30 second, primer annealing at 44°C for 45 seconds, extension at 72°C for 2 min and final extension at 72°C for 10 min. The amplification products were separated by electrophoresis in 1.5% agarose gel treated with 0.5 mg/ml of ethidium bromide and visualised in UV transilluminator. The electrophoretic profiles of the bands were photographed using the E-Gel imager (Life Technologies) photo documentation system.

Data analysis
Each band was considered as an individual locus and were scored visually as (1) for present and (0) for absent, unclear bands were not counted. The reactions were done twice in order to confirm reproducibility. POPGENE 1.32 software mentioned by Yeh et al. (1999) was used to measure the following parameters: Shannon’s information index (I) which measures gene diversity; Nei’s gene diversity index (h); total genetic diversity (HT); genetic diversity within populations (HS); observed number of alleles (na); effective number of alleles (ne) which is the number of alleles that can be present in a population; Percentage of polymorphic loci (PPB); genetic similarity index. Dendrogram was also created using the POPGENE 1.32 software.

RESULTS AND DISCUSSION
ISSR profiling
In the present study, ten small cardamom accessions possessing important agronomic variability with respect to yield was analysed using molecular markers with the goal to differentiate high yielding clones (Table 2). ISSR markers were employed for this study because of the simple, fast, cost effective and highly discriminative nature of the marker (Nayak et al., 2011). Ten primers were initially used to analyse the ten small cardamom accessions and five ISSR primers produced reproducible, scorable, polymorphic banding pattern were evaluated further (Table 3). Five primers produced 47 amplified fragments of which 32 (68%) were polymorphic (Table 3). The total number of

Table 1: Details of small cardamom accessions analysed in the present study.

| Sl. No | High-yielding varieties | Sl. No | Low-yielding varieties |
|-------|--------------------------|-------|------------------------|
| 1     | MCC 260                  | 6     | MCC 255                |
| 2     | ICRI 1                   | 7     | MCC 253                |
| 3     | ICRI 2                   | 8     | MCC 249                |
| 4     | Valley greengold         | 9     | MCC 251                |
| 5     | Panikulangara            | 10    | MCC 252                |

Table 2: ISSR primers analysed in ten small cardamom accessions.

| Primer | Motif | Sequence          |
|--------|-------|-------------------|
| ISSR 1 | (CA)6RY | CACACACACACARY     |
| ISSR 2 | (CA)6RG | CACACACACARG      |
| ISSR 3 | (AG)7YC | AGAGAGAGAGAGAGYC   |
| ISSR 4 | (GT)6YR | GTGTGTGTGTGYR     |
| ISSR 5 | (GT)6AY | GTGTGTGTGTGTAY     |
| ISSR 6 | (CT)8RG | CTCTCTCTCTCTCTCTCT |
| ISSR 7 | (GA)8T  | GAGAGAGAGAGAGAT    |
| ISSR 8 | (AG)8YT | AGAGAGAGAGAGAGAYT  |
| ISSR 9 | (AG)8YA | AGAGAGAGAGAGAGYA   |
| ISSR 10| (TC)8RG | TCTCTCTCTCTCTCTC    |

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fragments amplified per primer ranged from 7 (ISSR 1) to 12 (ISSR 6) with an average of 9.4 bands per primer with sizes varying from 200 to 1100bp. Primers ISSR 7 and ISSR 6 produced the highest number of scorable loci, 12 and 10 respectively. Polymorphic fragments ranged from 3 (ISSR 1) to 11 (ISSR 6) with a mean of 6.4. Also, the highest polymorphic percentage 91.7% was seen with the primer ISSR 6, followed by 88.9% with the primer ISSR 8. The average percentage of polymorphic bands amplified by all five ISSR primers was 65.81%.

ISSR markers have demonstrated their efficiency in genetic variability studies in small cardamom germplasm. Jose et al. (2016) analysed intra-specific variation of small cardamom using ISSR markers and reported high genetic variation (87.63% polymorphism) among the small cardamom accessions. Jose et al. (2014) analysed the efficiency of ISSR markers for analysing genetic diversity in small cardamom and differentiated small cardamom accessions.

**Genetic diversity and cluster analysis using ISSR markers**

The Shannon’s information index (I) and gene diversity (He) analysis were conducted to further understand the genetic diversity of the studied accessions. As per the results showed in (Table 4), maximum diversity was observed within the high yielding group. The gene diversity (h) for high yielding group was 0.2038. A similar pattern was observed for the Shannon’s information index (I), with the highest value of 0.3004 observed in high yielding group. These results agreed with finding of others, that highest genetic diversity was observed within the population (Narendrula and Nkongolo, 2012) than among the population. This was explained by (Nybom, 2004) that long-lived out crossing species with wide and continuous range retain most of their genetic variation within populations.

Unweighted pair-group method with arithmetic (UPGMA) cluster analysis based on genetic similarity values among the 10 small cardamom accessions yielded the dendrogram shown in (Fig 1). The dendrogram derived from UPGMA analysis clustered into two groups. The first cluster consisted of all five samples considered as high yielding group and the second cluster consisted of low yielding accessions. As seen in the dendrogram, the most genetically similar accessions were observed from low yielding group (0.9362); ACC 252 and ACC 251 followed by ACC 249 and ACC 253(0.9149) (Fig 2). Babu et al. (2012) studied genetic diversity between small cardamom cultivars and related genera using 50 RAPD, 6 ISSR and 2 PCR-RFLP primers and indicated that *Elettaria* is closer to *Ammonum* and *Alpinia*.

**Identification of yield specific markers**

If genetic markers could be associated with yield, then they will be quite useful in marker-assisted selection to identify desired traits at an early stage and also increase the possibilities of selection of parental lines for future breeding applications.

Attempts have been made earlier to identify specific ISSR markers associated trait in small cardamom and other species. Jose et al. (2013) identified an ISSR marker for tagging Malabar varieties (prostrate panicle) of small cardamom. ISSR marker associated with leaf yield in mulberry was identified by (Vijayan and Chatterjee, 2003). ISSR markers have also been used for linkage map construction in several plant species (Hashizume et al., 2003; Galvez et al., 2003). All these studies clearly indicate that ISSR markers could be associated with agronomic traits and they can be used as an initial screening step. A similar approach was attempted in this study and was successful as unique bands of molecular weight 800 bp were observed with the primer ISSR 3 and ISSR 7. ISSR 3 (Fig 3A) produced specific bands for low yielding accessions and ISSR 7 produced bands specific for high yielding accessions (Fig 3B). The present investigation was fortunate on identifying specific markers for the studied groups, however, use of

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**Table 3:** Primers employed to evaluate genetic variability among ten small cardamom accessions.

| Primer name | Repeat type and length | Range of amplicon (bp) | No. of clear scorable loci | Monomorphic loci | Polymorphic loci (PL) | Percentage of polymorphic bands (PPL) |
|-------------|------------------------|------------------------|---------------------------|------------------|----------------------|------------------------------------|
| ISSR 1      | (AG)8YT                | 200-900                | 7                         | 4                | 3                    | 42.85                              |
| ISSR 3      | (CA)6RY                | 250-1010               | 9                         | 4                | 5                    | 55.6                               |
| ISSR 6      | (CT)8RG                | 400-1100               | 12                        | 1                | 11                   | 91.7                               |
| ISSR 7      | (AG)6RG                | 350-1100               | 10                        | 5                | 5                    | 50                                 |
| ISSR 8      | (GA)8T                 | 400-1100               | 9                         | 1                | 8                    | 88.9                               |
|             |                        |                        |                           |                  |                      |                                    |
| Total       |                        |                        |                           |                  |                      |                                    |
| Average     |                        |                        |                           |                  |                      | 65.81                              |

**Table 4:** Genetic diversity analysis of two populations obtained with ISSR primers.

| Population     | No. of Samples | Observed no. of alleles (na*) | Effective no. of alleles (ne*) | Nei’s gene diversity index (h*) | Shannon’s Information Index (I*) |
|----------------|----------------|------------------------------|-------------------------------|---------------------------------|---------------------------------|
| High yielding   | 5              | 1.5319                       | 1.3566                        | 0.2038                          | 0.3004                          |
| Low yielding    | 5              | 1.2553                       | 1.1605                        | 0.0922                          | 0.1373                          |
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**Fig 1:** Dendrogram showing distinct clustering between high yielding and low yielding accessions of small cardamom.

| pop ID | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1      | 0.7660| 0.6809| 0.7021| 0.7660| 0.7660| 0.6596| 0.6170| 0.6383| 0.6170|       |
| 2      | 0.2666| 0.7021| 0.6809| 0.7447| 0.7021| 0.5957| 0.5957| 0.5532|       |       |
| 3      | 0.3844| 0.3536| 0.7660| 0.7021| 0.7021| 0.6383| 0.5957| 0.6596| 0.6170| 0.6383|
| 4      | 0.3536| 0.3844| 0.2666| 0.7234| 0.5957| 0.5957| 0.6170| 0.6383| 0.6170|       |
| 5      | 0.2666| 0.2948| 0.3536| 0.3238| 0.7021| 0.5532| 0.5532| 0.6170| 0.5957|       |
| 6      | 0.2666| 0.3536| 0.3536| 0.5179| 0.3536|       | 0.8085| 0.8085| 0.8723| 0.8511|
| 7      | 0.4162| 0.5179| 0.5179| 0.5921| 0.2126| 0.8085|       | 0.9149| 0.8511| 0.8723|
| 8      | 0.4829| 0.5179| 0.5179| 0.4829| 0.5921| 0.2126| 0.0889|       | 0.8936| 0.9574|
| 9      | 0.4490| 0.4829| 0.4162| 0.5179| 0.4829| 0.1366| 0.1613| 0.1125|       | 0.9362|
| 10     | 0.4829| 0.5921| 0.4490| 0.4829| 0.5179| 0.1613| 0.1366| 0.0435| 0.0660| 0.9362|

* Represents Nei’s genetic identity (above diagonal) and genetic distance (below diagonal).

**Fig 2:** Correlation of genetic identity and genetic distance between the varieties.

**Fig 3:** ISSR-PCR amplification profile of the 10 accessions of small cardamom using ISSR 3 and ISSR 7: Lane A - E, high yielding varieties; M 100 bp ladder; Lane F - J, low yielding varieties.

more markers and germplasm lines are required for successful validation.

**CONCLUSION**

This preliminary study has revealed valuable information to associate ISSR markers and small cardmom accessions based on yield. The specific bands at 800 bp produced by the two primers ISSR 7 and ISSR 3 can be further sequenced and validated so that they can be used to detect promising genotypes at the seedling stage. Through this study we clearly proved the potential of ISSR markers to evaluate genetic diversity in small cardamom and we were also successful to identify genetic markers with respect to yield trait.

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