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# Cross genera amplification of ginger EST-SSRs in large cardamom using genomic DNA isolated from standardized simplified protocol

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Large cardamom (*Amomum subulatum* Roxb.) is one of the most important cash crops of Sikkim. The issues crippling its production have been largely addressed through improved agronomic practices but efforts for genetic improvement have not been made. Being an orphan crop with regard to its genomic resources, the present study was carried out to standardize DNA isolation protocol for large cardamom using minimal resources and cross amplification of ginger expressed sequence tag (EST) based simple sequence repeat (SSR) markers in large cardamom. The DNA isolation protocol was standardized through various modifications in the general cetyltrimethyl ammonium bromide (CTAB) procedure. The DNA isolated through standardized protocol was of high quality confirmed through both electrophoretic (clear and intact bands) and spectrophotometric studies ($A_{260}/A_{280}$ ratio 1.68 to 1.97). The isolated DNA of all the six large cardamom samples was employed for PCR studies with 73 EST-SSR primers of ginger, out of which 18 showed cross amplification. Out of 18 primers, only 5 exhibited polymorphism showing maximum of 2 alleles per locus. In total, the PIC ranged from 0 to 0.63. A total of 23 alleles were amplified with average of 1.3 alleles per marker. A null allele marker was also observed. The results indicated low cross amplification rate (24.6%). DNA isolation protocol standardized in the study can be used across labs for extraction of quality DNA with minimal resources and primers showed cross-amplification may be further used for various molecular studies in large cardamoms.

**Keywords:** Large cardamom, ginger EST-SSR primers, DNA isolation, standardization protocol

## Introduction

The North Eastern (NE) region of India is known for its diversity in spice crops which are in huge demand both at national as well as global levels. Spices are high value cash crops which play pivotal role in boosting farmers' economy and has tremendous production potential in NE region due to congenial climatic conditions for its cultivation. Large cardamom (*Amomum subulatum* Roxb.) is one of the most important cash crop of the eastern Himalayan region being majorly grown in Sikkim and Darjeeling areas contributing around 80% of the India's large cardamom production[1]. Along with cultivated species, the state is home to various wild and related species of large cardamom where it flourishes luxuriantly in the wild. However, the declining production during last decade due to various biotic and abiotic factors has raised serious concerns for its sustained production. Attempts to improve its production and quality using various agronomic and post harvest means have been reported[2-4] but efforts for its genetic improvement is still lacking. Being a crop of regional importance, genetic improvement program is needed to evolve superior genotypes/clones having tolerance to stresses along with high capsule yield. Biotechnological approaches play an important role in crop improvement programs and have made significant impact in improvement of major food grain crops. However, the full potential of these genomic and molecular approaches have not been harnessed in spice crops. Along with conventional approaches, genomic assisted crop improvement utilizing genomic tools like genome wide genetic diversity studies, gene mapping, marker assisted selection and genomic selection[5] in neglected spice crops like large cardamom is necessary to unravel the hidden genetic information. DNA is the most important macromolecule for carrying out genetic studies and its

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success in molecular studies depends on its quality which depends on the method of DNA extraction, type of species and tissue, storage conditions during storage and handling during PCR reactions. Species of Zingiberaceae are rich in secondary metabolites and polyphenols which usually interfere in isolating quality genomic DNA needed for further PCR reactions and for which specific protocols and precautions have to be followed during extraction process[6]. The polyphenols in its oxidized form covalently bind to DNA giving it brownish yellow colour and reduce the storage time making it useless for molecular studies[7]. One of the major problems faced in carrying out well planned studies in remote locations is unavailability/untimely supply of necessary items like liquid nitrogen, lab chemicals, costly kits, delay in sample freezing after collection from source. Also hazardous chemical like phenol are toxic, hazardous, expensive and require special facilities for storage. Most of the currently available DNA extraction protocols need liquid nitrogen, tissue grinders, -80$^{o}$C refrigeration, commercial kits and other cumbersome equipments. Although various protocols for isolating DNA from species rich in secondary metabolites including species of Zingiberaceae have been reported[8-12] but as per as our knowledge only one protocol for DNA extraction specifically for large cardamom have been reported[13].

Unlike other spice crops, scanty literature is available on genomic resources of large cardamom. Information on cross genera/species transferability of molecular markers has been reported in various spice crops[14-18] and a few reports of transferability of SSR markers of members of its family have been reported[19-21]. 'Genetic variability bottleneck' is generally associated with clonally propagated crops like large cardamom and the limited available information is generally based on phenotypic characters which are often associated with restricted genetic resolution and usually requires multi-environment evaluation. Ginger is a well explored crop worldwide and plenty of genomic information is available. Large cardamom is a relative crop to the same family Zigiberaceae, therefore transferability of molecular markers could provide initial information on the conserved and variable locus/sequences and markers thus identified could be utilized in large cardamom. SSR markers are best choice for it due to their reproducibility, multiallelic nature, codominant inheritance and relative abundance and good genome coverage[22]. With the availability of expressed sequence tags (ESTs) of genic microsatellites a wealth of DNA sequence information has been generated and is available in online database. Genic SSRs have some advantages over genomic SSR markers due to their presence in expressed regions of the genome and expected transferability as the primers are designed from the conserved coding regions of the genome[23]. The most significant application of EST-SSR is the comparative mapping, diversity studies and marker assisted selection. Its rapid and inexpensive development and cross taxon portability makes it better choice than genomic SSR. With this background, the study was carried out with twin objectives to (i) standardize a simplified protocol for quality genomic DNA isolation from large cardamom and ginger & (ii) generate genomic information on large cardamom by cross amplification of ginger EST-SSR markers.

## Materials and Methods

### Collection and Preparation of Plant Samples for DNA Isolation

The tip of young, tender unfolded leaf from six local large cardamom cultivars of Sikkim namely *Sawney, Seremna, Varlangey, Ramsey, Ramla* and *Dzongu Golsai* (Table 1) maintained at research farm of Indian Cardamom Research Institute (Spices Board) at Pangthang, Gangtok, East Sikkim and ginger leaf from local cultivar '*Bhainse*' were collected as samples for the study during 2013-14. Before clipping, the top

Table 1 — Sample names, their general features and quantitative estimates of DNA concentration using UV spectrophotometer.

| Sample name | Species | Sample weight (mg) | DNA concentration ng/µl | Absorbance ratio λ 260/280 |
|---|---|---|---|---|
| Sawney | *A. subulatum* | 300 | 557.9 | 1.78 |
| Seremna | *A. subulatum* | 300 | 383.2 | 1.77 |
| Ramsey | *A.subulatum* | 300 | 159.6 | 1.79 |
| Varlangey | *A.subulatum* | 300 | 340.2 | 1.89 |
| Dzongu Golsey | *A.subulatum* | 300 | 470.0 | 1.74 |
| Ramla | *A. subulatum* | 300 | 111.8 | 1.68 |
| Bhaise | *Zingiber officinale* | 300 | 650 | 1.97 |

tender emerging leaves were wiped with alcohol to remove impurities, wrapped in aluminum foils, kept in thermocol containers containing ice packs during collection and transportation and stored at -20°C for safe storage to retain the freshness of the plant samples until further use.

**Extraction Method**

The cetyltrimethyl ammonium bromide (CTAB) DNA extraction method described earlier[24-25] was employed as reference protocol for extracting DNA from the study samples. Initially we followed the above mentioned protocol but the results were not satisfactory either due to failure to isolate DNA or contamination in the DNA finally extracted. Hence the proposed method was optimized in our laboratory for quality genomic DNA isolation with several modifications to the reference protocol:

**Standardized DNA Extraction Protocol**

i The extraction buffer (CTAB buffer) was pre-heated without addition of β-merceptoethanol for about half an hour in water bath at 65°C.

ii The leaf samples were first washed with distilled water, dried on filter paper and then cut into small pieces using sterilized blade or scissors to facilitate grinding. About 300 mg of these leaf samples of each cultivar of large cardamom and ginger were ground in pre-chilled mortar and pestle (-20°C) using the pre-heated extraction buffer. The samples were grinded for about 3 - 4 minutes until thick paste was formed. (*This method can be used for DNA isolation to avoid usage of liquid nitrogen*)

iii About 750 µl of ground tissue was transferred to 2 ml sterilized microcentrifuge tubes to which same quantity of the hot extraction buffer was added followed by addition of 2 µl each of 0.2% β-merceptoethanol and 3% polyvinyl pyrrolidone (PVP). The tubes were gently shaken by inverting for about 1 - 2 minutes.

iv The tubes were incubated in water bath maintained at 65°C for one and half hour (90 mins) with intermittent slow vortexing at regular interval of 10 mins to facilitate uniform mixing of buffer with plant tissue and avoiding lump formation.

v After incubation, the samples were kept at room temperature for 2 mins and about equal volume of chloroform : isoamyl alcohol (24:1) was added and vortexed slowly for 2 - 3 mins until the sample was well mixed.

vi The sample tubes were centrifuged at 10,000 rpm for 15 mins at 21°C and the top aqueous phase was transferred into a new 1.5 ml sterilized microcentrifuge tubes. Wide bore sterilized tips were used for transferring the aqueous phase to avoid mechanical damage to DNA and also to prevent mixing with the precipitate.

vii Equal volume of chilled absolute isopropanol (-20°C) was added and incubated at -20°C for 12 hrs to allow proper precipitation of DNA. (*Longer duration of chilled incubation helps in better precipitation of DNA*)

viii The DNA was pelleted by centrifugation at 6000 rpm for 8 mins at 4°C.

ix The DNA pellet was washed by adding absolute chilled ethanol, the pellet was spooled carefully and centrifuged again at 6000 rpm for 5 mins at 4°C (*This process cleared the impurities present in the DNA*).

x The supernatant was discarded and the pellet was air dried by inverting the tubes at room temperature. It was ensured that no ethanol was left out in the tubes and over drying was also avoided to avoid problem in resuspension of the DNA pellet.

xi The dried pellet was completely dissolved in 100 µl tris EDTA buffer followed by addition of 2 µl of RNAse enzyme to each sample tube and incubated at 37°C for 30 mins for complete removal of RNA contamination.

xii The DNA samples were labeled and stored at -20°C until further use.

**Quantitative and Qualitative Analysis of Extracted DNA**

The DNA yield and quality were assessed by electrophoresis of all DNA samples (5 µl) in 0.8% agarose gel, stained with ethidium bromide and bands were observed in gel documentation system. The DNA purity was determined by calculating absorbance ratio at $A_{260/280}$ using spectrophotometer (Thermo Scientific Nanodrop 2000c).

**PCR Reaction and Cross Amplification of Markers**

The DNA isolated from the standardized protocol was employed for PCR reaction using ginger SSR marker (DY 345089) which showed amplification during primer screening. A total of 73 EST-SSR markers of ginger were employed for cross amplification in large cardamom. The expressed sequence tag (EST) sequences of *Zingiber officinale* were downloaded from the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov). EST-SSRs were mined using microsatellite identification tool

(MISA, http://pgrc.ipk-gatersleben.de/misa/). Primers used for EST-SSR study were designed using PRIMER3 software (http://primer3.ut.ee/). PCR reaction was performed in 25 µl reaction volume containing PCR master mix (2X) having 0.05 unit/µl *Taq* DNA polymerase (Promega), reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP, 1 µl of each forward and reverse primer and 25 ng of sample DNA. Amplification was performed using a thermal cycler (Applied Biosystem) with an initial denaturation at 94°C for 5 min, followed by 35 cycles each of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final extension at 72°C for 30 min. The amplicons were then visualized on 3% MetaPhor™ Agarose (Lonza, USA) under gel documentation system.

## Results and Discussion

Spice crops are generally rich in secondary metabolites such as polyphenols and phenolic acids like tannic, gallic, caffeic, cinnamic, chlorogenic, ferulic and vanillic acids[26] and particularly crops from Zingiberaceae are abundant in monoterpenes like limonene, sabeinene, terpinenes and pinenes[27] which are known to hinder effective isolation as well as quality of DNA. In addition to phenolic compounds, the presence of pectin like polysaccharides also interfere with biological enzymes, such as polymerases, restriction endonucleases and ligases by forming tight complexes with nucleic acids resulting in unsuccessful amplification during PCR reactions[28]. Hence, isolation of high quality genomic DNA a prerequisite step for any molecular studies[29] has been a challenging task as these compounds get accumulated and interfere with isolation and purification[30]. In such cases, the DNA isolation methods need to be optimized for each plant species and even to each plant tissue due to presence of these metabolites[31].

We came across some difficulties in isolating quality DNA from initial stages of cell lysis to DNA separation while following the reference protocol. Issues generally encountered were highly viscous and sticky suspension during cell lysis, presence of brown pellets due to contamination, low yield and quality of DNA in case of double washing with ethanol making it unfit for utilization for molecular studies. Hence, in the present study we made certain modifications in the DNA extraction protocol to obtain good quality and yield of DNA using leaf tissues of large cardamom and ginger. The unfolded young leaves (tips) of large cardamom and ginger plants were selected as sample tissues for extraction of DNA as the DNA yield and quality was low in mature/older tissues due to formation of sticky suspension during extraction process. The use of pre-chilled pestle & mortar and -20°C stored leaf sample successfully substituted the use of costly liquid nitrogen which is largely inaccessible for the remote locations like Sikkim. The leaf surfaces of large cardamom and ginger are waxy in nature thus require more grinding time in hot buffer to break the cell wall. Increasing the duration of sample grinding (minimum 3 mins) along with incubation at 65°C for minimum of one and half hour resulted in improving both quality and quantity of DNA. We recommend 4 - 5 mins grinding of tissues with hot extraction buffer for better results. The ground tissue was also viscous in nature so the tubes were vortexed at regular interval to facilitate the uniform mixing of buffer and enable cell wall lysis. This also avoided lump formation during incubation which is not desirable. The addition of PVP and β merceptoethanol in CTAB protocol is highly essential due to their nature as strong reducing agent[30] that inhibits oxidation processes and eventually direct or indirect damage to DNA. Appearance of brownish colour in DNA pellets is undesirable and could not be removed when we used merceptoethanol only. To counter the effect of polyphenol oxidation, PVP has been used frequently in CTAB extraction protocols[32]. In the present study we used both PVP (3%) and merceptoethanol (0.2%) for removal of phenolic compounds and polysaccharides which otherwise interfere with DNA amplification during PCR reactions. The DNA thus obtained was devoid of brown colour. For pellet formation and further removal of impurities the tubes were centrifuged at 6000 rpm. The centrifugation was kept lower to avoid shearing of DNA. Unlike in previous study on optimization of genomic DNA isolation from large cardamom the present study standardized DNA extraction without using liquid nitrogen which is substituted with chilled pestle and mortar, increase in grinding time of tissues with hot extraction buffer and double centrifugation of DNA pellet with chilled ethanol for extracting pure DNA devoid of impurities.

Electrophoresis of the DNA thus obtained showed bright bands with no sign of impurities or RNA contamination (Fig. 1a & 1 b). The purity of extracted DNA was very good as evident by $A_{260}/A_{280}$ absorbance ratio that ranged from 1.68 to 1.97 in the nanodrop spectrophotometer (Table 2) suggesting absence of proteins and polyphenolics/polysaccharide compounds. There was no DNA fragmentation due to

shearing in any of the samples which substantiates the high purity of DNA. It has been reported that the shearing of DNA in extraction process directly or indirectly interfere with enzyme reactions during molecular studies[33]. The DNA thus obtained was employed for PCR studies. Suitability of the isolated DNA for PCR reaction was confirmed by amplification of DNA of all the six large cardamom samples using SSR primer (F: TCGGTGCACACTAC GACTTC R:GGTACATGAAAGTGCGCCTG). Clear and bright amplicons were observed in all the six large cardamom samples indicating its suitability for downstream reactions (Fig. 2a & 2b). Out of 73 ginger EST-SSR markers assayed, 18 showed amplified fragments in DNA samples of all the six large cardamom samples with cross amplification frequency of 24.6%. List of the EST-SSRs primers amplified in large cardamom has been shown in Table 2. Of the

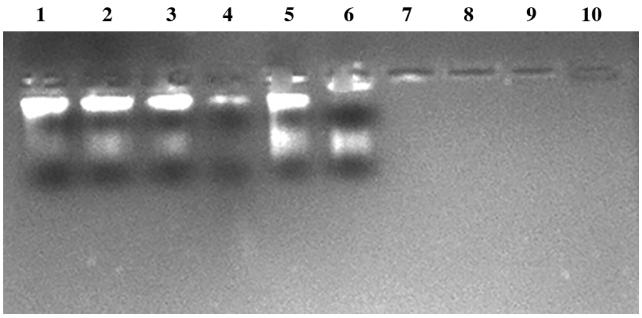


Fig. 1 — (a) Electrophoresis of genomic DNA of *Amomum subulatum* (lane 1-6) and 1 (b) *Zingiber officinale* (lane 7-10) on 0.8% agarose gel.

Table 2 — Properties of EST-SSRs of ginger cross amplified in large cardamom.

| S. No | Locus | Sequence of primers (5'-3') | Annealing temperature ($^oC$) | Motifs | No. of alleles | Size (bp) | PIC |
|---|---|---|---|---|---|---|---|
| 1 | DY344705 | F TTGTCGACCGGTACATGAAA<br>R TCGGTGCACACTACGACTTC | 56 | $(AT)_6$ | 1 | 200 | 0 |
| 2 | DY344866 | F GCATCCCGTACGATCTTGTT<br>R GCGCTCCGTGGAAATAAGTA | 56 | $(AG)_8$<br>$(GCA)_7$ | 1 | 149 | 0 |
| 3 | DY345189 | F TCGGTGCACACTACGACTTC<br>R GGTACATGAAAGTGCGCCTG | 57 | $(TA)_6$ | 2 | 191 | 0.63 |
| 4 | DY345190 | F GGTACATGAAAGTGCGCCTG<br>R TCGGTGCACACTACGACTTC | 56 | $(AT)_6$ | 2 | 191 | 0.63 |
| 5 | DY345991 | F TCCTTGTCGACGGTACATGA<br>R TCGGTGCACACTACGACTTC | 56 | $(AGA)_4$ | 2 | 202 | 0.63 |
| 6 | DY346011 | F TGTCGACAGGCACATGAAAG<br>R TCGGTGCACACTACGACTTC | 56 | $(AT)_6$ | 2 | 199 | 0.63 |
| 7 | EL645422 | F GATGACACATCCGGTCACTG<br>R TCCCCATCACCTAGTTCTGC | 56 | $(CAG)_6$ | 1 | 193 | 0 |
| 8 | DY344733 | F GCGAGTGAAGGCAGAGAGAC<br>R GAGTGGATGGACGACTTCCT | 56 | $(GGC)_6$ | 1 | 160 | 0 |
| 9 | DY344866 | F CCTGCAGACGCTGTATCTCA<br>R GTCTTCATGGATCTGCCCAC | 56 | $(AG)_8$ | 1 | 229 | 0 |
| 10 | DY345180 | F AGAGCCTGAAGAAGAAGGGC<br>R CCGTGGCTCAGTTTTCACTT | 56 | $(CGG)_7$ | 1 | 245 | 0 |
| 11 | DY345181 | F ATCCTCGCCAGCAGTAAAGA<br>R GAAACCCAGGACGAGAACAG | 55 | $(GCC)_7$ | 1 | 265 | 0 |
| 12 | DY345207 | F GACATATATGCACCCTGGGC<br>R GCAGGATGCCTTTGTTTAGC | 56 | $(ATT)_5$ | 2 | 202 | 0.44 |
| 13 | DY345245 | F ACGTACTGCTTGATCACCCC<br>R CGTCCTCCTTCCTTTTAGGG | 56 | $(CTC)_7$ | 1 | 222 | 0 |
| 14 | DY345331 | F AGGCTGAGGCTGAACACCTA<br>R TGCTAATGGCATGACAGCTC | 56 | $(GGT)_6$ | 1 | 250 | 0 |
| 15 | DY345335 | F CGTGGGTCCCTTATCTTCAA<br>R AGGGTGTCAGGATATGCAGG | 56 | $(AAT)_5$ | 1 | 246 | 0 |
| 16 | DY345865 | F CACAGGAGAAACAACAGCCA<br>R ACCAATGATTCCAGCAAAGG | 56 | $(GCA)_5$ | 1 | 193 | 0 |
| 17 | DY345966 | F GCATCAATCGCCTTATAGCC<br>R AGACCATCATGGCATCCTTC | 56 | $(GCA)_5$ | 1 | 278 | 0 |
| 18 | DYDY346649 | F ACGCTTCGAGCTTCTTTCTG<br>R GCTCCCCAATAACATAGGCA | 56 | $(CTT)_5$ | 1 | 273 | 0 |

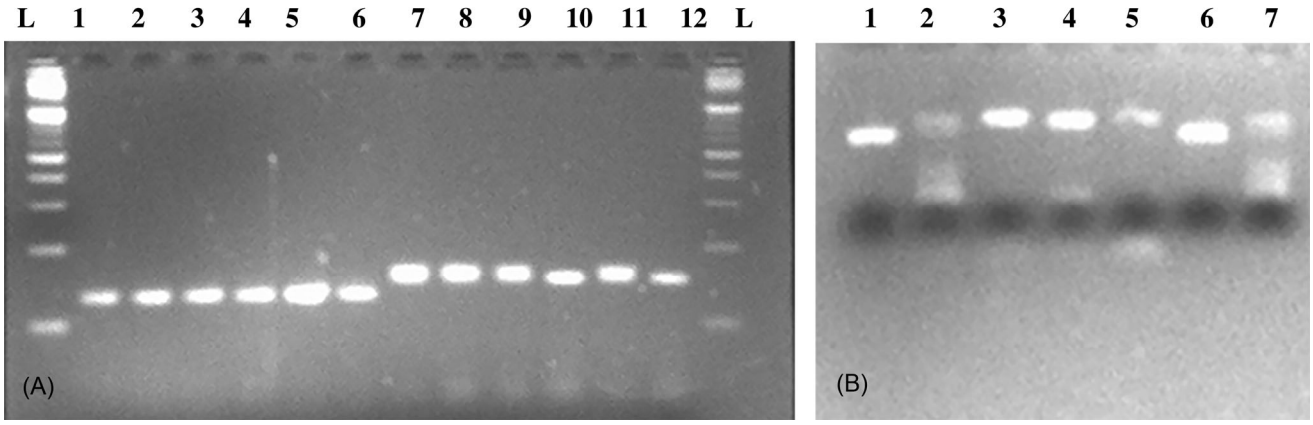


Fig. 2 — (a) Amplification of large cardamom DNA samples using ginger SSR primer DY 345089 (lane 1-6) and DY 345190 (lane 7-12) (b) large cardamom and ginger sample using SSR primer DY 344733 with null allele in sample no. 2 (sample 1-6 of large cardamom and 7 of ginger) using 3% metaphor agarose. L: 100 bp DNA ladder.

18 markers which showed cross amplification only five were polymorphic and remaining were monomorphic. Out of these five markers one primer (DY 344733) showed null alleles in two of the samples after repeated PCR reactions. Null alleles have also been reported in other important crops like kiwi fruit, rice, spruce and wheat. Null allele with one the primer could possibly be due to the deletion of microsatellites at a specified locus and mutation in the primer binding site. The amplicon size ranged from 149 to 278 bp. A total of 23 alleles were amplified by 18 microsatellite markers and we detected maximum of two alleles per microsatellite locus with an average of 1.3 alleles per marker with polymorphism information content (PIC) ranged from 0 to 0.63. Transferability of markers from ginger to large cardamom indicates that the regions flanking these microsatellites are conserved to allow locus amplification. SSR transferability depends on various factors like number of markers tested, amplification conditions and plant species used in the study. Cross amplification with primers from other species/genera depends on the evolutionary distance and also on the rate of evolution of the genomic sequences[34]. The utility of cross species/generic transferability of SSR markers, especially for minor crops has been reported increasingly in past few years[35-38]. Cross amplification of turmeric, ginger and large cardamoms SSRs have been observed to small cardamom with amplification rate of 71.4%. DNA markers have been utilized in members of the Zingiberaceae family in turmeric[39-40], large cardamom[41] and ginger[42] for which robust set of SSR markers have been developed and successfully employed in cross genera amplification. The study revealed low rate of cross-genera amplification of ginger ESTs to large cardamom possibly as these both belongs to different tribes (Zingiberaceae and Alpiniae resp.). EST-SSRs have been reported to be less polymorphic compared to genomic SSRs because of higher DNA sequence conservation in transcribed region. Relatively, low polymorphism has been reported by various workers with EST-SSRs as compared to genomic SSRs in grapes, spruce, rice, durum wheat and barley[43-50]. Large cardamom is majorly a clonally propagated crop and inherently associated with 'diversity bottleneck' like in other asexually propagated crops. The cultivated *A. subulatum* species evolved from the wild species over a period of time and the variation observed in cultivated ones are mostly in plant morphology and capsule size and shape. Considering species with very scarce genomic resources the information generated in the present study can be utilized for large cardamom studies. Polymorphic markers identified in the study can be used for assessing diversity in cultivated and wild genetic resources of large cardamom. The DNA extraction protocol standardized in the present study is a simplified one which demands less resources and can be followed in labs with minimal facilities. Apart from its application in PCR reactions the DNA isolated from the simplified protocol can be employed for more downstream applications like restriction enzyme digestion, analysis using modifying enzymes, cycle sequencing, amplification based single nucleotide polymorphism (SNP) genotyping and array based genotyping. Genic markers specifically for large cardamom can be successfully extracted through *de novo* sequencing, cDNA cloning and shot gun approach. The information generated through transferable SSRs from ginger can be useful genomic resource for comparative genome analysis and will add to the scarce pool of markers in large cardamom for gene mapping, marker assisted selection and further diversity analysis.

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