RESEARCH ARTICLE

Molecular characterization of large cardamom cultivars using *mat*K and *rbc*L genes

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Large cardamom is an important high value cash crop of Himalayan region of India, Nepal, and Bhutan. It is widely used in food industry as spice and as medicine in herbal practices. This rhizomatous perennial plant is one of the main revenue earners of these regions and the livelihood of many people is associated with it. However, due to the various factors such as climate change, emergence of highly virulent diseases, and absence of resistant varieties, the production of large cardamom has declined considerably in the last two decades. The genetic diversity study amongst the cultivar forms the basis of crop improvement to address these problems. Cultivars of large cardamom have only been characterized morphologically till date. The molecular characterization using barcode loci recommended by Consortium for Barcode of Life (CBOL) is not only needed for resolving the different large cardamom cultivars but also to give insight into the understanding of the genetic distance between themselves, which will be helpful in identifying the distantly related cultivars for crop improvement such as in plant breeding. The two barcode loci maturase K (*mat*K) and ribulose-bisphosphate carboxylase (*rbc*L) genes of chloroplast DNA were found to be good candidates for barcoding of large cardamom cultivars. The mutations in these two loci at cultivars level were low but distinctive enough to differentiate and diverse themselves to form the same tree topology by different phylogeny methods, thereby successfully resolving the different cultivars. It was found that the cultivars which share common ancestors in phylogeny also share one or two distinct common genetic sequence mutation patterns in both loci indicative of common origin. The phylogenetic tree construction using two barcode loci i.e., *mat*K, *rbc*L, and combined genes (*mat*K+*rbc*L) by three different method viz. unweighted pair group method with arithmetic mean (UPGMA), Neighbor-joining, and Maximum-likelihood based on Kimura 2-parameters amongst the five large cardamom cultivars successfully resolved the cultivars of large cardamom as they formed same tree topology except by Neighbor-joining method of *rbc*L gene. Two distinct monophyletic groups were formed by all nine trees constructed by three different methods indicating resolvability of the cultivars. The phylogenetic study shows "*Varlangey*" and "*Seremna*" are closely related cultivars and share common ancestor with "*Sawney*" cultivar. Similarly, "*Green Golsey*" and "*Dzongu*" are closely related cultivars and formed distinct cluster. The *mat*K, *rbc*L, and combined gene loci exhibited highest genetic distance between "*Dzongu*" and "*Seremna*"' cultivars. The present study paved the way for barcoding of number of other large cardamom cultivars, which will be helpful not only for inferring evolutionary relationship but also for selection of genetically distant cultivars having desired characteristics to produce hybrid with maximum hybrid vigor.

Keywords: Large cardamom; barcoding; cultivars; genetic diversity; phylogenetic tree.

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Introduction

Amomum subulatum Roxb., commonly known as large cardamom or black cardamom is an important cash crop of Sikkim Himalayas and is used largely as flavoring agent in confectioneries and food preparations. It is also used in ayurvedic system of medicine. The species belongs to family Zingiberaceae under the order Scitamineae. It is a perennial rhizomatous herb which grows under the shade trees in a humid area preferably near the perennial source of water. The capsules encapsulating seeds are aromatic and contain volatile oil, chiefly, 1,8-cineole, α-terpineol, α and β-pinene, and *allo*-aromadendrine [1]. The dried seeds or sometimes whole capsule are used as spice, flavoring agent in confectioneries, making perfumes, and other medicines [2]. The crop is now cultivated in other parts of country including north eastern states, Uttarkhand, Himachal Pradesh, and other countries like Bhutan and Nepal mainly imported from Sikkim. There are number of cultivars under cultivation evolved naturally over a period of time. However, only five cultivars viz. "*Dzongu*", "*Green Golsey*", "*Sawney*", "*Seremna*", and "*Varlangey*" are taxonomically authenticated based on critical morphological studies [3]. Traditionally, the identification and characterization of cultivars and species are based on morphological and physiological properties. However, this identification is not effective and reliable [4]. The chloroplast *rbc*L and *mat*K genes have been recently recognized as the DNA barcoding regions for plants by the Consortium for the barcoding of Life (CBOL) [5]. On evaluation and analyzing nine plastid and two nuclear barcode loci in family Zingiberaceae 60 accessions of 20 species belonging to seven genera from India, *mat*K and *rbc*L consistently resolved 15 species (75%) into monophyletic groups and five species into two paraphyletic groups. The results support using *mat*K and *rbc*L loci for barcoding Zingiberaceae members and highlight the poor utility of Internal Transcript Spacer (ITS) and also caution against proposing ITS loci for barcoding taxa based on limited sampling [6]. The highly conserved nature of Maturase K gene located within the intron of *trn*K in chloroplast is recognized as one of the barcode loci for species and cultivar identification at molecular level. The 1,500 bp gene which codes a protein is involved in Group II intron splicing. *Mat*K gene is also found to be a good candidate for DNA barcoding of plant family Zingiberaceae [7]. Cultivar differentiation of large cardamom based on genetic studies has not been carried out. Two barcode regions, maturase K (*mat*K) and ribulose-bisphosphate carboxylase (*rbc*L) loci, located in the circular DNA of chloroplast offer authentic regions for species and cultivar differentiation at the molecular level.

The study on the genetic mutation at the barcode region and phylogeny of the large cardamom cultivars is an interesting area which helps us to understand the evolutionary history of different cultivars. Further, it helps us to identify the distantly related cultivars which, if with desired character, can be used to produce hybrid having hybrid vigor. The isolation of quality DNA from the sample and polymerase chain reaction (PCR) amplification of gene of interest is a pre-requisite for any downstream applications such as sequencing and gene studies. The detail protocol for DNA isolation and PCR from large cardamom has not been described. The protocols for isolation of quality DNA from large cardamom leaves and PCR targeting two barcode loci, *mat*K and *rbc*L, are standardized and described in this paper. The sequences of two barcode loci of five popular large cardamom cultivars were submitted to National Center for Biotechnology Information (NCBI) database (Bethesda, MD, USA). It will pave the way for the amplification and sequencing of more cultivars, which may be helpful in understanding the phylogenetic relationship among them. Genetic diversity study on large cardamom using randomly amplified polymorphic DNA (RAPD) markers revealed the highest genetic distance between "*Ramla*" cultivar and "*Churumpha*" cultivar and lowest genetic distance was exhibited by "*Chivesay*" and an unidentified cultivar [8].

Various scientists have tested different barcode regions for amplification as well as reliability to use as DNA barcode for identification of various species and cultivars. The highly variable *mat*K region has lower PCR amplification success than the more conserved *rbc*L gene [9]. The *rbc*L gene was found to be more reliable to be used as DNA barcode for identification of *C. variegatum* than *mat*K gene [10]. Analysis of chloroplast genome sequences such as *rbc*L and *pbsA-trn*H can be valuable tool in establishing the phylogenetic analysis and variability in taro cultivars grown in Brazil [11]. The phylogenetic trees constructed on the basis of *mat*K and *rpo*Cl suggested that the *mat*K alone or in combination with *rpo*Cl can be used for determining the levels of genetic variations and for barcoding [12]. Determination of the individual region of *mat*K that best represents the entire gene and designing the primers in adjacent and more conserved regions of the area is an important step to test the universality of primer that best represent wider taxon [13]. A set of universal primers that can be multiplexed in one PCR to amplify *mat*K successfully in angiosperms was reported [14]. However, it was also reported that production of a single universal primer pair for *mat*K across the whole of the plant kingdom is unlikely because of the extensive variation exhibited within the regions [9]. Out of the previously published primers, matk, 390F, and 1326R have the highest percentage match for monocots [5]. The genetic diversity study of five tomato varieties using *atp*B gene promoter sequence has shown variations and genetic diversity [15]. The *rbc*L gene located on the chloroplast DNA (cpDNA) encodes the large subunit of ribulose 1,5-bisphosphate carboxylase/ oxygenase, an enzyme that catalyze carbon fixation in photosynthesis. Compared to most genes encoded in the cpDNA, the *rbc*L gene has a relatively slow nucleotide substitution rate. Since *mat*K has a relatively fast mutation rate, it evolves faster than the *rbc*L gene [16]. The cpDNA has several characters that are stable structure, a small genome with high conservative region, and low substitution of nucleotide. The *rbc*L has a low level of mutation compared with other barcode regions. Therefore, in-depth study of intraspecies genetic and phylogenetic variations can be done using this gene [17]. Various barcoding of species and cultivars were done by previous studies. Phylogenetic analysis of eight Malaysian pineapple cultivars using *rbc*L gene formed several groups or subclades due to similar genetic patterns. The study confirmed that the *rbc*L gene is a good indicator to determine the phylogenetic relationship of the Malaysian pineapple cultivars [18]. The DNA barcode study in Fig cultivars revealed the ITS sequence presented highest variation rates, while the phylogeny constructed with the *mat*K sequence obtained the highest percentage of solved monophyletic groups [19]. The molecular techniques based on the sequence of evolutive markers (nrITS, *rbc*L, and *mat*K) revealed more detailed information on the phylogenetic relationships between rose cultivars and rose species [20]. The genetic diversity study of rice indicated that simple sequence repeats (SSR) marker is neutral and co-dominant, which could be a powerful tool to assess the genetic variability of cultivars [21].

The present study aims to understand the genetic diversity amongst large cardamom cultivars, which enables not only to know the genetic distance between themselves but also to identify the distantly related cultivars and is useful for new variety development.

Material and methods

Sample collection

The leaf samples of large cardamom were collected from the germplasm maintained at Biotechnology Research and Application Centre, Sikkim State Council of Science and Technology, Sajong, Rumtek, E. Sikkim and brought to the Molecular Biology Laboratory, Vigyan Bhawan, Gangtok, E. Sikkim. The fully expanded young leaves of about 20-25 days old, which were recently emerged from new shoots of the existing mother plant, were collected and immediately placed in little amount of water just enough to dip the lower region. The leaves were

transferred to the laboratory under the same condition. The DNA extraction was carried out either immediately or within 1-2 days. This new procedure of sample collection yielded good quality DNA from large cardamom as compared to conventional methods.

DNA extraction

The extraction of DNA from leaf sample was carried out using cetyltrimethylammonium bromide (CTAB) method with few modifications. Briefly, 1% polyvinylpyrrolidone (PVP) (100 mg/10 mL) and 0.3% β-Mercaptoethanol (30 uL/10 mL) were added in CTAB buffer before heating at 65°C in hot water bath. The optical density (OD) value and concentration were checked by using Spectrophotometer (DeNovix, Wilmington, DE, USA) at 260/280 nm. The sample having OD value around 1.8 was taken for PCR. The quality of DNA was also checked by using 1.2% agarose gel electrophoresis.

PCR reaction

The total 25 µL PCR reaction volume was prepared by sequentially mixing 12.5 µL of 2X Taq PCR Master Mix (Qiagen, Germantown, MD, USA), 2 µL of each reverse and forward primers (10 ng/µL), 1 µL of DNA template (50 ng/µL), and 7.5 µL of nuclease free water. The mixing procedure was done in minicooler. The PCR primers for *mat*K gene amplification were matK-390F (5'-CGATCTATTCATTCAATATTTC-3') and matK-1326R (5'-TCTAGCACACACGAAAGTCGA AGT-3'). The PCR conditions were initial denaturation at 94°C for 5 minutes followed by 38 cycle of denaturation at 94°C for 40 sec, primer annealing at 48°C for 40 sec, extension at 72°C for 40 sec. Final extension was carried out at 72°C for 7 minutes, and then, reaction was kept on infinite hold at 4°C till further application.

The same PCR reaction mixture was used for amplification of *rbc*L gene with the specific *rbc*L gene amplification primers of gtrbcL.1 F (5'-TCTGTTACTAACATGTTTACTTC-3') and gtrbcL.1 R (5'-TCCCTCATTACGAGCTTGTACACA-3'). The PCR conditions for amplification of *rbc*L gene consisted of initial denaturation at 95°C for 5 minutes followed by 38 cycles of denaturation at 95°C for 30 sec, primer annealing at 55°C for 40 sec, and extension at 72°C for 1 minute. Final extension was carried out at 72°C for 7 minutes, and then, the reaction was kept at 4°C infinite hold.

The PCR product was visualized in 1.2% agarose gel aligned with 100 bp DNA ladder. The gel loading mixture contained 3 µL of PCR product, 1 µL of 6X loading dye, and 2 µL of distilled water. The amplified DNA based on gel observation was sent for sequencing.

Bioinformatical analysis

The DNA sequencing was done by Centyle Biotech Private limited (Rudrapur, Uttarakhand, India) using Sanger dideoxy sequencing method. After sequencing, two sequences were received from each sample generated one from forward and another from reverse primer. The consensus sequence from these two sequences was generated by using BioEdit, a free software for sequence analysis and sequence alignment editor. BLASTn search tool of NCBI was used to compare the consensus sequence with the nucleotide sequence from GenBank.

The phylogenetic tree was constructed using MEGA-X software which was freely downloaded from internet. The *mat*K and *rbc*L gene sequences of five cultivars were subjected to three different methods of phylogenetic tree construction including Neighbor joining method, Maximum Likelihood, and unweighted pair group method with arithmetic mean (UPGMA) method to resolve the individual cultivars. Kimura 2 parameters were used in all tree construction with 1,000 bootstrap replicates. The phylogenetic tree using combined gene (*mat*K and *rbc*L) sequences of two barcode loci was also constructed using the same three methods to see the robustness of the tree. A distantly related species of same family, Zingiberaceae, was used as out-group during tree construction to have a greater resolvability. Ten sequences of two gene (*mat*K and *rbc*L) loci from five large cardamom cultivars were submitted to NCBI GenBank.

Table 1. The gene sequences with accession number submitted to NCBI GenBank.

	*mat*K		*rbc*L	
Cultivars	**SeqID**	**Accession No.**	**SeqID**	**Accession No.**
A. subulatum cultivar Dzongu	Seq1DZ_M	MW086614	Seq1DZ_R	MW086619
A. subulatum cultivar Green Golsey	Seq2GG_M	MW086615	Seq2GG_R	MW086620
A. subulatum cultivar Sawney	Seq3SW_M	MW086616	Seq3SW_R	MW086621
A. subulatum cultivar Seremna	Seq4SR_M	MW086617	Seq4SR_R	MW086622
A. subulatum cultivar Varlangey	Seq5VAR_M	MW086618	Seq5VAR_R	MW086623

Results and discussion

The CTAB method of DNA extraction with some modifications in protocol yielded a good quality DNA from the large cardamom leaves having OD value ranging from 1.75 to 1.89 with number of accurate purity level of 1.80 at 260/280 nm. The isolation of quality DNA is pre-requisite for all successful downstream application such as PCR and sequencing. The proper collection of sample, simple storage method, and immediate use as soon as possible were found to secrete less phenolics during DNA isolation. The collection of young, fully expanded, undamaged leaves and immediate dipping the lower region of leaves in small amount of water provided less time to phenolic response against the plant damage. The addition of 0.3% β-Mercaptoethanol and 1% PVP in CTAB buffer was found indispensable during the DNA isolation process especially in phenolic rich sample.

The PCR conditions proposed by Dunning *et al*. [5] for amplification of *mat*K gene have successfully amplified with standard PCR mix. The *rbc*L gene was successfully amplified by the PCR conditions proposed by Vinitha *et al*. [6]. The minimum of 38 PCR cycles was found appropriate to generate sufficient PCR product. The gene sequences of five large cardamom cultivars with both *mat*K and *rbc*L genes were deposited in GenBank. The accession numbers are presented in table 1.

BLASTn results showed the 100% of query cover, 0.0 of E value, and percentage identity of 99.57-100%. Phylogenetic tree was constructed with *mat*K, *rbc*L, and combined (*mat*K + *rbc*L) gene sequences of five large cardamom cultivars using three different methods including Neighbor-joining, UPGMA, and Maximum-likelihood method based on Kimura 2-parameters with the bootstrapping of 1,000 replicates to evaluate the recovery of cultivars. A distantly related species belonging to the same family was used as outgroup. All the nine phylogenetic trees including combined gene trees formed same tree topology except Neighbor-joining method of *rbc*L gene, in which the common ancestor of "*Sawney*" cultivar was shared by all four remaining cultivars (Figure 1). Two distinct clusters were formed by all three methods. One cluster was formed by "*Varlangey*" and "*Seremna*" cultivars and another by "*Green Golsey*" and "*Dzongu*" cultivars. "*Sawney*" cultivar separated and formed different branch and shared common ancestor with "*Green Golsey*" and "*Dzongu*" cultivars. It can be assumed that, if the different methods yield the same tree, then a robust estimation will become possible [22]. Some of the bootstrap values showed high confidence level up to 100%. It was found that the "*Dzongu*" and "*Green Golsey*" cultivars forming a monophyletic group sharing the common ancestor as the same case with "*Varlangey*" and "*Seremna*" cultivars. The tree topology indicated that the "*Sawney*" cultivar shared the common ancestor with "*Varlangey*" and "*Seremna*" cultivars. These also indicated that the "*Varlangey*" and "*Seremna*" cultivars were closely related cultivars. Similarly, "*Green Golsey*" and "*Dzongu*" cultivars were found to be closely related cultivars. The sequence alignment and comparison indicated very few polymorphic sites in these two barcode loci. However, one or two distinct mutation points in the same

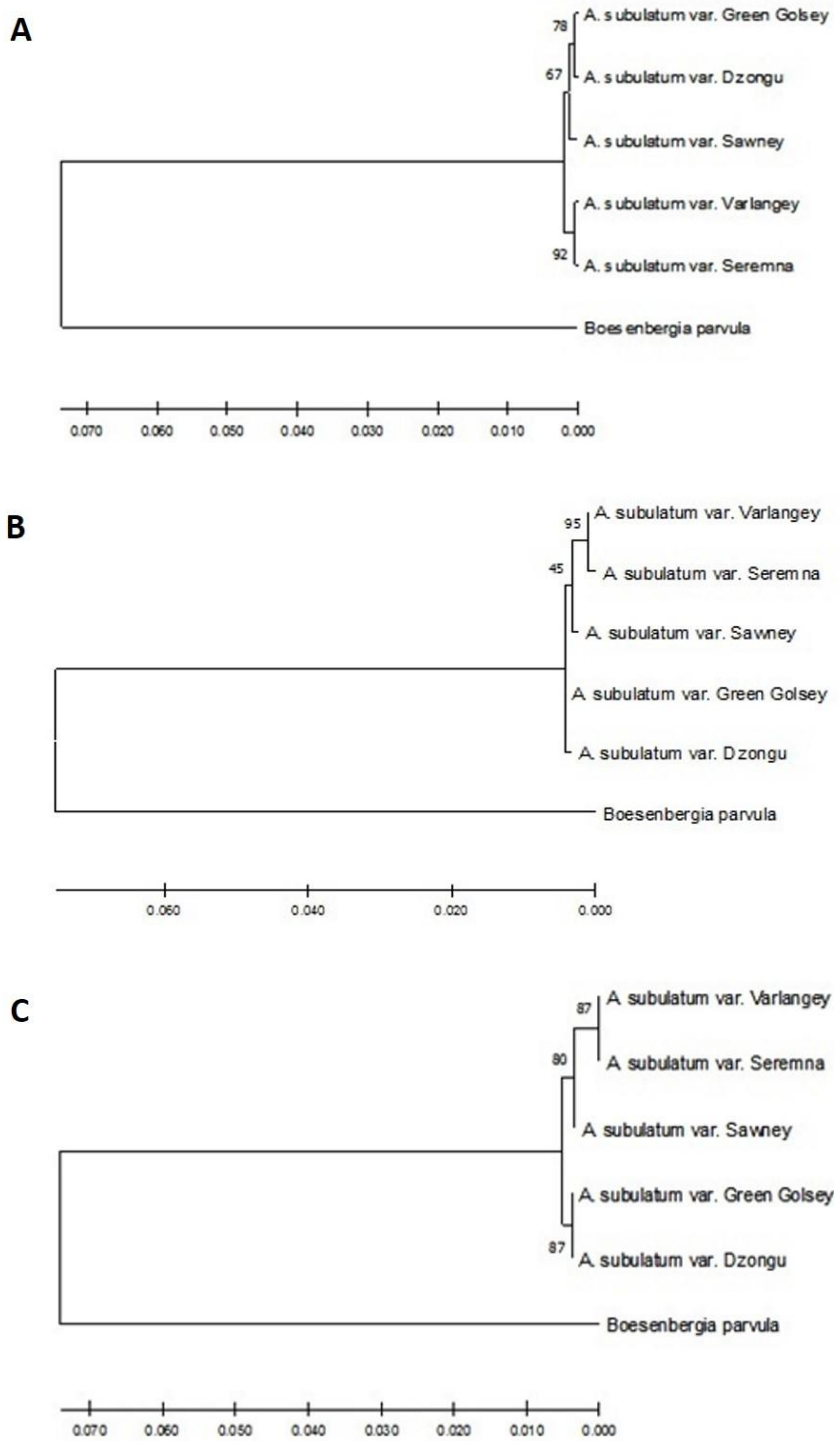

Figure 1. Phylogenetic trees based on Kimura 2-parameter of the combined barcode loci (*mat*K + *rbc*L). Bootstrapping method with 1,000 replicates adopted. **A:** unweighted pair group method with arithmetic mean (UPGMA) method. **B:** Maximum-likelihood method. **C:** Neighbor-joining method.

sequence location occurred amongst the cultivars that shared the common ancestor. In case of intra and interspecific variations, the sequence variation must be lower enough to distinguish the variations [23].

Both barcode loci provided better cultivar recovery in large cardamom cultivars. The pairwise genetic distance ranges from 0.00107 to 0.00538 with overall average distance of 0.00322 in case of *mat*K gene sequence of five cultivars. The highest genetic distance is between "*Dzongu*" and "*Seremna*" cultivars. Similarly, in case of *rbc*L gene, the average pairwise genetic distance ranges from 0.00109 to 0.00547 with an overall average distance of 0.00328. This gene locus also showed the highest genetic distance between "*Dzongu*" and "*Seremna*". Further, the combined gene loci of *rbc*L and *mat*K showed the pairwise genetic distance ranging from 0.00108 to 0.00542 with an overall average distance of 0.00325. The combined gene (*rbc*L + *mat*K) also exhibited highest genetic distance between "*Dzongu*" and "*Seremna*" cultivar.

The study also revealed an important aspect, useful for breeding program to get hybrid vigor breed. The production of large cardamom has declined considerably, directly affecting farmers, due to number of factors including disease infestation, climate change, soil fertility, loss of moisture, and lack of disease resistant and high yielding hybrid. The present study paved the way for selection of genetically distant cultivars having desired characteristics to produce hybrid with maximum hybrid vigor. The genetic diversity study of large cardamom not only provides insight into the understanding of phylogenetic relationship amongst different cultivars but also offers possibility of producing hybrid with hybrid vigor.

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