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RESEARCH ARTICLE

GENETIC DIVERSITY IN LARGE CARDAMOM (*Amomum subulatum* Roxb.) DISSECTED USING RAPD MARKERS.

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Abstract

Large cardamom is major cash generating spice having high commercial impact on farmers for its high economical values, beside spice it is used for medicinal purposes. Present study was done to explore genetic variation present in Nepal cardamom (*Amomum subulatum* Roxb.). So collections of large cardamom were done from three districts of Eastern hills of Nepal during 2012. Presence of wild cardamom in forests of Nepal made possibility of high genetic diversity of large cardamoms found in Nepal. All together 16 samples were analyzed using 25 random primers that generated 169 different loci and were found highly polymorphic (90.90%). The Nei's genetic diversity among all collected accessions was found to be 0.295 similarly; Shannon Information Index value of 0.448 also suggested high genetic diversity for collected large cardamom accessions. Dendrogram based on Dice coefficient separates all the cultivars into 4 major clusters at 0.68 level. Wild relatives of large cardamom formed separate cluster similarly cultivar Varlange also formed separate cluster depicts large variation in these cultivars in respects to cultivated cultivars viz. Ramsay, Golsay, Ramla, Chivesay and Jirmale. This result shows the potentiality of RAPD markers in accessing genetic diversity in clonally propagated crop such as large cardamom and also to distinguish difference among large cardamom accessions.

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Introduction:-

The genus *Amomum* is the second largest genus of the family Zingiberaceae with c. 150 species (Thomas et al., 2009). This species is indigenous to moist deciduous and semi-evergreen forests of sub-Himalayan tracts at an altitude ranging from 500- 2000 m. (Sharma, 2009). Nepal is top most producer of large cardamom in the world (Shrestha et al., 2008). The annual production is 6026 Mt from areas of 11665 hectares (MOAD, 2012). Large Cardamom is mostly grown in Nepal, India and Bhutan. Regeneration through seed is difficult in large cardamom due to dormancy of seed (Bisht et al., 2010) hence; clonal propagation is widely adopted among farmers. Large cardamom has high value because of its medicinal properties such as for stomachic, carminative and stimulant (Singh, 1982) and has high demand in world market. Its cultivation has drastically increased in Nepal due to its high economic impact and also social and environmental advantages (Niroula, 1998).

Random Amplified Polymorphic DNA markers are widely employed in genetic research due to its simplicity and speed (Williams et al., 1990; Welsh and McClelland, 1990). RAPD are effective in producing species specific fingerprints therefore used in several crops for identification, characterization and estimation of genetic relatedness (Ganesh Ram et al., 2008 and Yeotkar et al., 2011) so these markers also can be used for assessment of genetic variation that are important key tools for crop improvement and germplasms conservation (KalyanBabu et al., 2007). Molecular markers which detect variation at the DNA level overcome most of the limitations of morphological and biochemical markers hence potential application of RAPD markers has been demonstrated in many crops since long time (Bowditch et al., 1993 and Zhang et al., 2011). Due to polymorphism shown by these markers it is quite useful for genetic diversity analysis in most of the crops (Pandiyani, 2010, Bardakci, 2001). The

aim of this study was to compare RAPD profiles of sixteen large cardamom cultivars and to estimate phylogenetic relationships between the investigated cultivars on the basis of the obtained RAPD profiles. Large cardamom nomenclature is interesting in Nepal as the farmer named large cardamom cultivars according to the adaptability of individual accessions to their climatic situation so researcher are confused on the diversity situation of large cardamom so this paper also aimed to differentiate the large cardamom accessions popular in different geographical regions of Eastern part of Nepal.

Materials and methods:-

The sixteen plant material (*Amomum subulatum*) used in this study were collected from Taplejung, Panchthar and Ilam districts of Eastern Nepal (Figure 1) during 2012. Immature leaf samples were collected from various eco-geographical locations of different altitude irrespective of wild as well as cultivated genotype. The samples were transported to the laboratory as soon as possible and stored at -40°C until DNA extractions. Geographical situations of collected samples were recorded with the help of GPS machine (GPS Garmin). Each sample were reduced to fine powder through mortar and pestle with addition of liquid N_2 and transferred to a 2.0 ml eppendorf tube. DNA extraction was performed using a modified CTAB method (Doyle and Doyle, 1990), where modification was done for CTAB buffer reaction time at 65°C for 1 hour. DNA concentration was estimated by Q5000 UV vis Spectrophotometer (Quawell).

RAPD amplification was performed with random decamers (Kit from Operon Technologies, Alameda, CA, USA). On preliminary experimentation sixty primers were tested but only twenty five arbitrary RAPD primers (Table 1) showed reproducible banding pattern and were chosen for the analysis based on highly readable and polymorphic bands as described by Pandiyan et al., 2010.

DNA amplifications were performed in 15 μl reaction volume containing 50ng template DNA, 2X Green GoTaq® Reaction Buffer (pH 8.5), 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP and 3mM MgCl_2 . (Promega) and 0.6picomole of primer. The mixture was gently mixed and centrifuged in shaker for 60 seconds prior to adding two drops of mineral oil. After addition of mineral oil it was transformed to thermocycler, All the amplifications were performed in the same thermocycler (MULTIGENE OPTIMAX, Labnet International, Inc.) programmed for 1 cycle of 95°C for 5 min followed by 40 cycles of 95°C for 60 sec, 36°C for 1 min 30 sec, 72°C for 2 min, and final 1 cycle of 72°C for 10 min. Samples were kept at 2°C until further analysis. 12 μl aliquots of amplification products were loaded in a 1% (w/v) agarose gel (Bioneer) for PCR product separation in 1X TAE buffer (Bioneer) in an electrical field (120 V) for 90 minutes. Gels was stained with Ethidium Bromide (0.5 $\mu\text{g}/\text{ml}$ for 30 min) and visualized under exposure of UV light under gel doc system (UVDI, Major Science). For scoring band and to confirm consistent amplifications during the whole experiment standard 1 kb Ladder (Bioneer) was used.

RAPD profiles of 16 different cultivars (Figure 1) were scored on binary system as 1 for presence of band and 0 for absence of band. Only the bands that are clear and reproducible were scored. Polymorphic Information Content (PIC) for each primer were calculated as described by Roldan Ruiz et al., (2000) as $\text{PIC} = 2P_i(1-P_i)$, Where P_i is the frequency of i^{th} null allele. Scored bands were subjected to study the Dice similarity coefficient with the help of NTSYS pc version 2.01 and based on genetic distance UPGMA genetic map were created. To confirm the clustering pattern further principle coordinate analysis was performed. GeneAlex 6.1 was used to estimate Nei's genetic diversity and Shannon's Information Index.

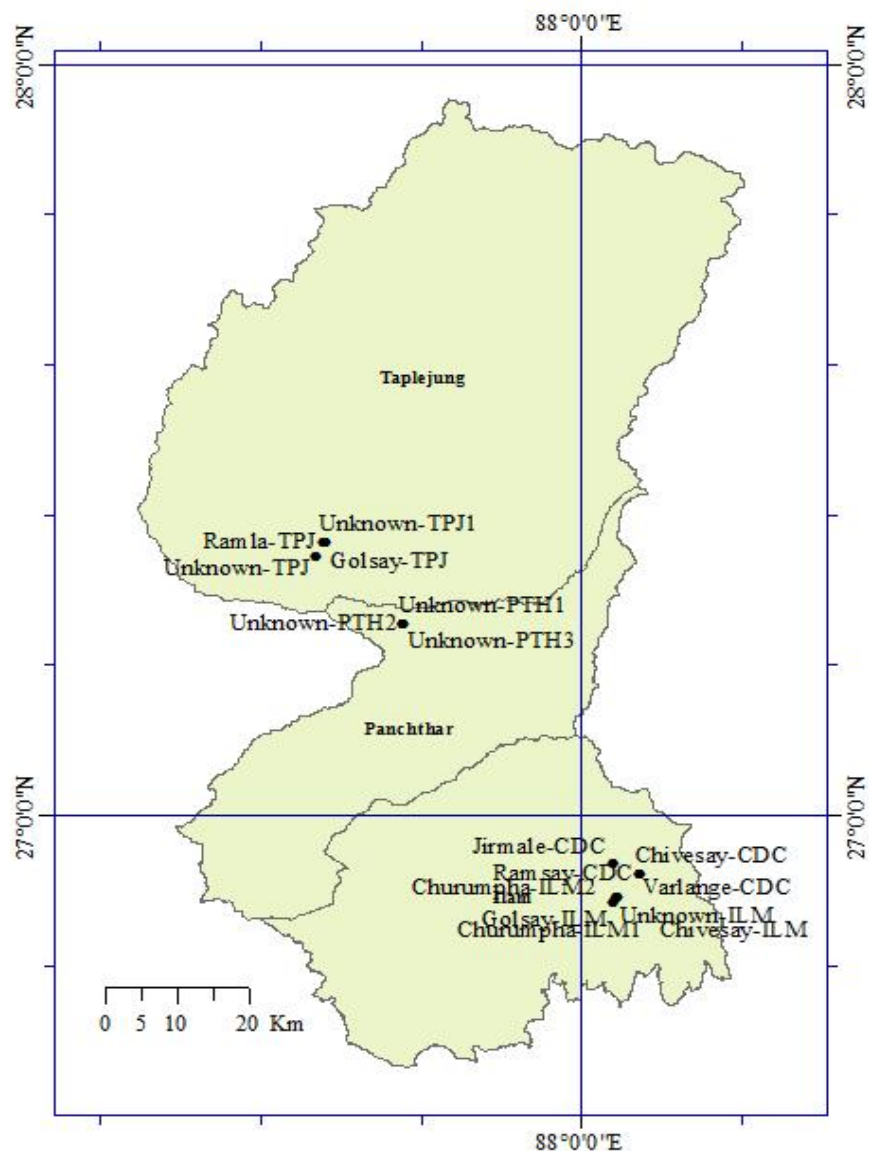


Figure 1: Large cardamom sample collection places in Mechi zone of Nepal

Results and discussion:-

A total of 169 loci were generated by 25 random decamers for overall 16 cultivars with average of 6.76 loci per primers. Nine different primers produce 7 loci each; however, the polymorphic information content varies among primers suggest that there is high level of gene diversity among collected accessions. Minimum number of loci generated is 4 by primer OPB-11 and maximum of 10 by primers OPA-03, OPB-01 and OPB-02. Polymorphic information content varies from 0.15 by primer OPAH-09 to 0.47 by OPB-16 with average of 0.34 ± 0.15 .

Highest genetic distance (0.694) was found between Ramla cultivar of Taplejung district with Churumpha cultivar collected from Ilam district. Similarly, lowest genetic distance (0.111) is found between Chivesay cultivar of Ilam district with unidentified cultivars of Panchthar district.

Nei's heterozygosity for all the loci ranges from 0 to 0.5 with mean 0.295 ± 0.013 , similarly unbiased heterozygosity was 0.304 ± 0.013 . Shannon's information index was found to be 0.448.

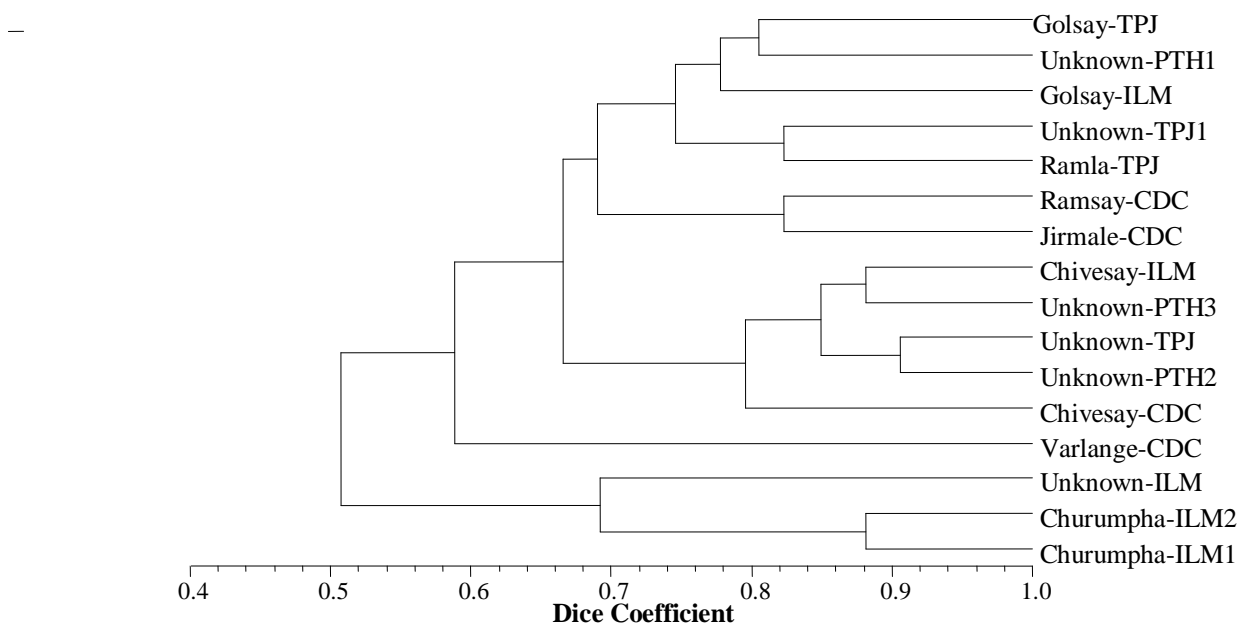
This suggests broad genetic base for selected cultivars of large cardamom. Selection of wild relatives of large cardamom in this study is major reason for broad genetic base among collected large cardamom accessions. The present molecular diversity study revealed a high genetic variability as reported earlier in zingibers by Jaleel and Sasikumar, 2010.

For phylogenetic study UPGMA clustering was performed as it is simplest method for constructing tree or phenogram. UPGMA assumes a constant rate of evolution and it was originally used to represent the extent of phenotypic similarity for a group of species in numerical taxonomy. Four different clusters (Figure 2) were formed after Dice similarity coefficient at 0.68; wild relative of large cardamom (Churumpha) formed separate cluster with other two cultivars from Ilam district. Varlange cultivar collected from Cardamom Development Center, Ilam was found in another separate cluster and is entirely different from other accessions. This may be due to abundance of this cultivar only in high altitude with greenish color of the stem as well as inflorescence. This cultivar produces relatively large size capsule than other cultivars (Thomas et al., 2009). Chivesay cultivars collected from Ilam district along with other three unidentified cultivars form separate cluster, this type of cultivars are different from other in terms of capsule size (relatively small). Though Golsay cultivars form separate cluster it is closely related with Ramla, Ramsay and Jirmale cultivar; production of confectionery type of capsule are best described to these cultivars.

Principle coordinate analysis (PCoA) is realistic in representation of cultivars based on mean values. PCoA analysis was found informative in deciding nature of cluster and the variances shown by the respective coordinates are highly used in agricultural researches (Maji and Shaibu, 2012). The cluster pattern generated through UPGMA clustering is well supported by the principle coordinate analysis that also separates all cultivars into four major groups (Figure 3). Upon PCoA analysis variation in X axis was found 35.87 % in Y axis 23.51% and in Z axis it is 13.69% (Figure 3). These cluster patterns showed that wild relatives of large cardamom were distinct from cultivated cultivars and there was high genetic dissimilarity between wild genotypes to the locally cultivated genotypes. Use of RAPD marker in such genetic variability analysis was previously described by Belaj et al., 2003, Mignouna et al., 1998, Raghunathachari et al., 2000 and Salazar et al., 2006 in different crops and plants. Gupta et al. (2006) described the variation in large cardamom based on certain morphological traits. Similarly, our results based on molecular analysis also reveal the highly diverse nature of large cardamom exist in nature. Cross pollination nature (Sinu and Shivanna, 2007) of this crop may also be the reason for forcing this crop as heterogeneous in nature.

Table 1:-List of primers, sequence, number of band amplified, range of amplified bands and polymorphic information content.

S.N.	Primer code	Primer sequences (5' to 3')	Number of band amplified	Approx. Range of amplified bands (kb)	Polymorphic Information Content
1	OPA-03	AGTCAGCCAC	10	0.3-1.8	0.38±0.06
2	OPA-10	GTGATCGCAG	7	0.3-1.6	0.36±0.18
3	OPA-11	CAATCGCCGT	7	0.6-1.6	0.33±0.20
4	OPA-13	CAGCACCCAC	8	0.5-1.5	0.31±0.15
5	OPA-17	GACCGCTTGT	6	0.3-1.6	0.34±0.14
6	OPA-18	AGGTGACCGT	5	0.3-1.5	0.37±0.10
7	OPA-19	CAAACGTCGG	6	0.3-2.2	0.33±0.08
8	OPAH-05	TTGCAGGCAG	8	0.4-1.5	0.38±0.18
9	OPAH-08	TTCCCGTGCC	7	0.4-1.5	0.32±0.23
10	OPAH-09	AGAACCGAGG	7	0.8-1.6	0.15±0.17
11	OPV-19	GGGTGTGCAG	6	0.7-1.25	0.25±0.19
12	OPT-01	GGGCCACTCA	7	0.5-1.5	0.35±0.13
13	OPT-07	GGCAGGCTGT	7	0.5-1.61	0.37±0.12
14	OPB-01	GTTTCGCTCC	10	0.4-2.0	0.38±0.13
15	OPB-02	TGATCCCTGG	10	0.2-2.1	0.39±0.12
16	OPB-09	TGGGGGACTC	4	0.3-1.0	0.36±0.16
17	OPB-10	CTGCTGGGAC	7	0.3-2.0	0.29±0.21
18	OPB-11	GTAGACCCGT	4	0.3-1.8	0.22±0.16
19	OPB-12	CCTTGACGCA	9	0.2-2.2	0.39±0.10
20	OPB-13	TTCCCCCGCT	7	0.3-2.9	0.38±0.18
21	OPB-14	TCCGCTCTGG	7	0.2-1.6	0.36±0.13
22	OPB-15	GGAGGGTGTT	5	0.4-1.6	0.38±0.15
23	OPB-16	TTTGCCCGGA	5	0.2-1.2	0.47±0.05
24	OPB-18	CCACAGCAGT	5	0.3-2.0	0.25±0.16
25	OPB-19	ACCCCCGAAG	5	0.2-2.2	0.35±0.15
Mean			6.76 (169)		0.34±0.15

**Figure 2:-** UPGMA clustering of 16 accessions based on 169 loci generated by 25 random primers.

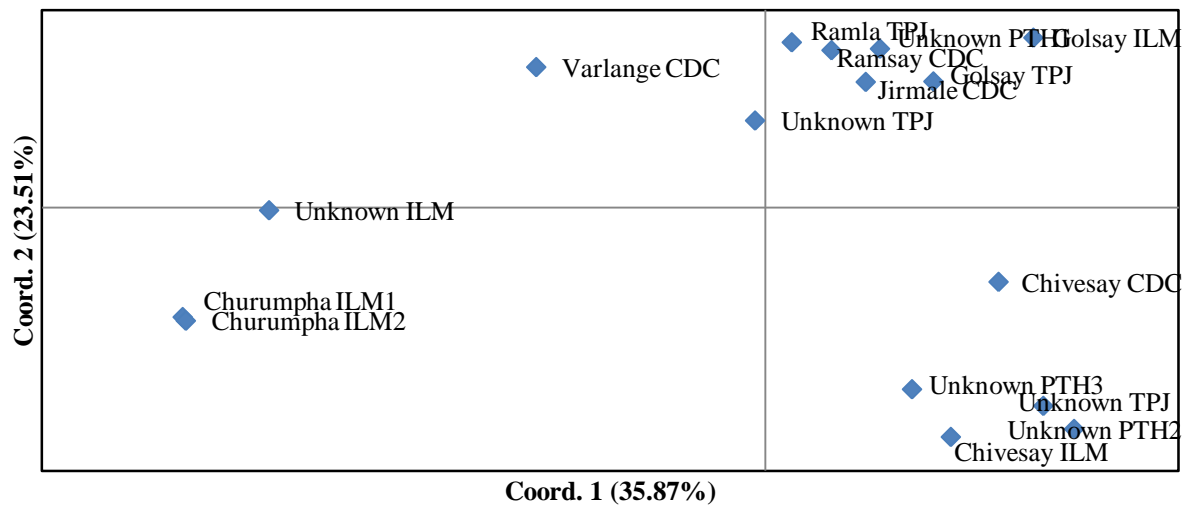


Table 2:-Genetic distances among 16 large cardamom accessions based on 169 loci generated by 25 random primers.

	Golsay-TPJ	Unknown-PTH1	Unknown-ILM	Chivesay-ILM	Unknown-TPJ	Unknown-PTH2	Unknown-PTH3	Unknown-TPJ1	Ramla-TPJ	Golsay-ILM	Churumph a-ILM1	Churumph a-ILM2	Chivesay-CDC	Ramsay-CDC	Jirmale-CDC	Varlange-CDC
Golsay-TPJ	0.000															
Unknown-PTH1	0.221	0.000														
Unknown-ILM	0.592	0.591	0.000													
Chivesay-ILM	0.325	0.381	0.568	0.000												
Unknown-TPJ	0.273	0.333	0.549	0.143	0.000											
Unknown-PTH2	0.299	0.349	0.580	0.111	0.082	0.000										
Unknown-PTH3	0.329	0.429	0.571	0.127	0.184	0.140	0.000									
Unknown-TPJ1	0.333	0.455	0.649	0.424	0.419	0.438	0.371	0.000								
Ramla-TPJ	0.306	0.294	0.625	0.477	0.425	0.444	0.443	0.313	0.000							
Golsay-ILM	0.230	0.244	0.664	0.353	0.301	0.304	0.396	0.353	0.313	0.000						
Churumpha-ILM1	0.542	0.581	0.362	0.497	0.497	0.538	0.523	0.616	0.675	0.638	0.000					
Churumpha-ILM2	0.603	0.632	0.411	0.524	0.546	0.565	0.525	0.628	0.694	0.689	0.143	0.000				
Chivesay-CDC	0.224	0.333	0.506	0.205	0.156	0.179	0.226	0.380	0.400	0.288	0.515	0.580	0.000			
Ramsay-CDC	0.233	0.325	0.537	0.406	0.348	0.341	0.350	0.466	0.395	0.289	0.519	0.562	0.269	0.000		
Jirmale-CDC	0.278	0.341	0.516	0.371	0.298	0.323	0.341	0.461	0.453	0.261	0.512	0.577	0.265	0.172	0.000	
Varlange-CDC	0.441	0.493	0.466	0.503	0.515	0.534	0.475	0.513	0.540	0.520	0.535	0.585	0.459	0.392	0.380	0.000

Conclusion:-

This study demonstrates that genetic base for collected large cardamom is very high and there is potentiality of RAPD markers in differentiating cultivars distributed in Eastern hills of Nepal. The feasibility of the RAPD marker technique for quantifying genetic distance among large cardamom accessions is found useful and additional use of markers will help to verify the pattern of variation of large cardamom exist in nature.

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