GENETIC DIVERSITY IN NEPALESE LARGE CARDAMOM GENE POOL BASED ON RAPD MARKERS

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ABSTRACT

Large Cardamom (Amomum subulatum Roxb.) is a major cash generating spice crop of Nepal, mainly confined to the eastern hills of Nepal. Fourteen large cardamom samples collected from Taplejung, Panchthar and Ilam Districts of Nepal during 2012 were used to evaluate genetic diversity using RAPD markers. Total of fifty one decamer primers (Operon series) were initially used for the experiment but only eighteen primers were selected based on nature of polymorphic and reproducibility for the analysis. Out of 133 loci generated 97.74 % were found to be polymorphic with number of alleles 1.977±0.013 and number of effective alleles1.409±0.024. Considering a single population Nei's genetic diversity over all loci generated was found to be 0.263±0.012 with Shannon's information index 0.416. On UPGMA clustering, two main clusters were observed that clearly separate wild cultivars (Churumpha) from commercially grown cultivars. The RAPD markers showed sufficient polymorphism for genotype discrimination and experimental reproducibility. Incorporation of molecular approaches over conventional method of genetic diversity assessment is very much effective in pinpointing the potential genetic diversity for cardamom improvement.

Key words: Large cardamom (*Amomum subulatum*), *Churumpha*, RAPD, decamer, genetic diversity

INTRODUCTION

The genus *Amomum* Roxb. is the second largest genus of the family Zingiberaceae with *c.* 150 species (Thomas et.al., 2009; Tripathi & Prakash, 1999). This species is indigenous to moist deciduous and semi-evergreen forests of sub-Himalayan tracts at an altitude ranging from 500- 2000 m. in the world (Shrestha et al., 2001). Nepal is top most producer of large cardamom. The annual production is 5232 Mt from areas of 14001 hectares (MOAC 2010). More than 70,000 families around Nepal are directly and indirectly involved in Large Cardamom farming (Sector study on Large Cardamom, 2007). It is said to be one of the oldest species indigenous to the eastern Himalayan region, probably in Nepal and hence known as Nepal Cardamom (AEC/FNCCI, 2006). It is mostly grown in Nepal, India and Bhutan. It is a clonally propagated crop having high value for its medicinal properties. Its cultivation has drastically increased in Nepal due to its high value economic, 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 markers are effective in producing species specific fingerprints and, therefore, used in several crops for identification, characterization and estimation of genetic relatedness (Ganesh Ram et al., 2008; Rahunathanchari et al. & Yeotkar et al.,2011). The aim of this studty was to compare RAPD profiles of fourteen large cardamom cultivars and to test the usefulness of the amplified fragments as taxon-specific markers. Furthermore, the purpose of this study was to estimate phylogenetic relationships between the investigated cultivars on the basis of the obtained RAPD profiles.

MATERIALS AND METHODS

Sample Collection

The plant material (*Amomum subulatum*) used in this study were collected from three districts Taplejung, Panchthar and Ilam of eastern Nepal. For the purpose of DNA extraction, immature leaf samples were collected from various eco-geographical locations (Table 1). Samples were collected from different altitude irrespective of wild as well as cultivated genotypes. The samples were transported to the laboratory as soon as possible and stored at -40^{0}C until DNA extractions. Geographical situation of collected samples were recorded with the help of GPS machine (GPS Garmin).

Table 1 : Collection sites, Cultivars, Latitude, Longitude and Elevation of Samples.

S.N.	Collection site	Cultivar	Latitude	Longitude	Elevation m. a.m.s.l.
1	Phuling-7 ,Taplejung	Golsahi	N 27.36^0	E 087.68^0	1672
2	Phuling-7 ,Taplejung	Golsahi	N 27.33^0	E 087.68^0	1672
3	Nagi,Pachthar	Unknown	N 27.25^0	E 087.77^0	1377
4	Panchkanya-6,Ilam	Unknown	N 26.89^0	E 088.044^0	1709
5	Panchkanya-6,Ilam	Chivesay	N 26.89^0	E 088.044^0	1709
6	Phuling-7 ,Taplejung	Golsahi	N 27.36^0	E 087.68^0	1672
7	Nagi,Pachthar	Unknown	N 27.25^0	E 087.77^0	1377
8	Nagi,Pachthar	Unknown	N 27.25^0	E 087.77^0	1377
9	Hangdeva-7,Taplejung	Churumpha			
10	Phuling-7,Taplejung	Golsahi	N 27.36^0	E 087.68^0	1672
11	Phuling-7,Taplejung	Ramla	N 27.36^0	E 087.67^0	1687
12	Panchkanya-6,Ilam	Golsahi	N 26.88^0	E 088.04^0	1680
13	Panchkanya-6,Ilam	Churumpha	N 26.89^0	E 088.04^0	1709
14	Panchkanya-6,Ilam	Churumpha	N 26.89^0	E 088.04^0	1709

Isolation of Genomic DNA

Each sample were reduced to fine powder through mortar and pestle with liquid N_2 addition 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^{0}C for 1 hour. DNA concentration was estimated by spectro-photometery technique with absorbance at 260 nm and 280 nm with dilution factor of 50.

RAPD Primers

Rapid amplification was performed with random decamer primer obtained from Operon Technologies (Alameda CA, USA). On preliminary experimentation fifty one primers were tested, but only eighteen arbitrary RAPD primers (Table 2) showed reproducible banding pattern and were chosen for the analysis based on highly readable and polymorphic (Pandiyan et al., 2010) bands.

Table 2 : List of Arbitrary Decamer Primers of Operon Series

S.N	Primer code	Primer sequences 5' to 3'	Nucleotide length	Annealing temperature	Number of cycles
1	OPA-03	AGTCAGCCAC	10-mers	27^0 C	45
2	OPA-10	GTGATCGCAG	10-mers	37^0 C	45
3	OPA-11	CAATCGCCGT	10-mers	37^0 C	45
4	OPA-13	CAGCACCCAC	10-mers	37^0 C	40
5	OPAH-05	TTGCAGGCAG	10-mers	37^0 C	40
6	OPAH-08	TTCCCGTGCC	10-mers	37^0 C	40
7	OPAH-09	AGAACCGAGG	10-mers	37^0 C	40
8	OPB-07	GGTGACGCAG	10-mers	33^0 C	40
9	OPC-02	GTGAGGCGTC	10-mers	27^0 C	40
10	OPC-09	CTCACCGTCC	10-mers	37^0 C	45
11	OPC-10	TGTCTGGGTG	10-mers	37^0 C	45
12	OPD-08	GTGTGCCCCA	10-mers	27^0 C	40
13	OPG-13	CTCTCCGCCA	10-mers	37^0 C	40
14	OPT-01	GGGCCACTCA	10-mers	37^0 C	40
15	OPT-07	GGCAGGCTGT	10-mers	37^0 C	40
16	OPT-08	AACGGCGACA	10-mers	37^0 C	40
17	OPU-15	ACGGGCCAGT	10-mers	37^0 C	40
18	OPV-19	GGGTGTGCAG	10-mers	37^0 C	40

RAPD Amplification

DNA amplifications were performed in 10 µl reaction volume containing 50 ng template DNA, 2X Green GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl2. (Promega) and 0.4 picomole of primer. The mixture was gently mixed and centrifuged in shaker for 60 seconds prior to adding 2 drops of mineral oil. After addition of mineral oil it was transformed to thermocycler. All the amplifications were performed in the same thermocycler (PTC-100, MJ Research, Inc., Watertown, Massachusetts, USA) programmed for 1 cycle of 95° C for 5 min followed by 40 to 45 cycles of 95° C for 60 sec, 27°C to 37°C for 1 min 30 sec, 72° C for 2 min, and finally 1 cycle of 72° C for 10 min (Table 2). Samples were kept at 2^0 C until further analysis. 8 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). Gels was stained with ethidium bromide (0.5µg/ml for 30 min) and visualized under exposure of UV light. For scoring band and to confirm consistent amplifications during the whole experiment standard 1 kb Ladder (Bioneer) was used.

Statistical Analysis of RAPD fingerprints

RAPD profiles of 14 different cultivars 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 was calculated as described by Roldan Ruiz et al. (2000) as PIC= 2Pi (1-Pi), where Pi is the frequency of i^{th} null allele. Scored bands were subjected to study the Jaccard's 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 component analysis was performed. Genealex 6.1 was used to estimate Effective number of alleles, Nei's genetic diversity and Shannon's Information Index.

Result and discussion

Out of 51 primers studied on *Amomum subulatum*, only 18 primers showed banding patterns (Table 2) that generated 133 different bands with average of 7.38 bands per primer with 97.74d which were polymorphic in nature. Primer OPC-09 produced the highest number of polymorphic bands. The lowest numbers of bands were produced through the primers OPAH-08 and OPC-10.

Table 3 : Name of Primers with Amplified Bands, Size of Bands and Polymorphic Information Content

S.N.	Random decamer	Size of Band (kb)	No. of bands amplified	PIC*
1	OPA-03	0.3-1.8	9	0.41±0.07
2	OPA-10	0.3-1.6	7	0.36±0.18
3	OPA-11	0.6-1.6	5	0.41±0.10
4	OPA-13	0.5-1.5	11	0.39±0.09
5	OPAH-05	0.4-1.5	8	0.33±0.09
6	OPAH-08	1.4-1.5	2	0.48±0.00
7	OPAH-09	0.8-1.6	6	0.19±0.08
8	OPB-07	1.0-1.3	4	0.24±0.15
9	OPC-02	0.2-1.3	9	0.27±0.14
10	OPC-09	0.3-2.0	12	0.31±0.15
11	OPC-10	0.4-1.5	3	0.43±0.04
12	OPD-08	0.75-1.7	5	0.41±0.07
13	OPG-13	0.6-1.2	5	0.39±0.08
14	OPT-01	0.5-1.5	11	0.40±0.09
15	OPT-07	0.5-1.61	8	0.34±0.14
16	OPT-08	0.9-1.1	11	0.33±0.15
17	OPU-15	0.2-1.0	11	0.33±0.18
18	OPV-19	0.7-1.25	6	0.27±0.15
	Mean		7.38(133)	0.35±0.10

*PIC: Polymorphic Information Contents

The polymorphic information contents ranged from 0.19±0.08 in primer OPAH-09 to 0.48±0.00 in OPAH-08 (Table 3); mean 0.35±0.10 showed high gene diversity for selected primers. OPA-13 and OPT-08 had same number of amplified band though there was difference in PIC suggested gene diversity in large cardamom cultivars, similarly in OPA-03 and OPC-02 have same number of amplified band but OPA-03 showed high PIC hence high gene diversity as suggested by Anderson et. al., 1993.

Nei's heterozygosity for all the loci ranged from 0 to 0.498 with mean 0.263±0.012; similarly unbiased heterozygosity was 0.273±0.013. This may be due to high genetic material transfer (suckers) between these districts as a source of propagation. Shannon's information index was found to be 0.416 where number of alleles was 1.977±0.013 with effective number of alleles 1.409±0.024 (Table 4).

Table 4 : Genetic Diversity of Eighteen Primers for 14 Large Cardamom Samples

				N	Na	Ne	I	He	uHe
Mean				14.000	1.977	1.409	0.416	0.263	0.273
SE				0.000	0.013	0.024	0.016	0.012	0.013

Where, Na = no. of different Alleles
Ne = No. of Effective Alleles = 1 / (p^2 + q^2)
I = Shannon's Information Index = -1* (p * Ln (p) + q * Ln(q))
He = Expected Heterozygosity = 2 * p * q
uHe = Unbiased Expected Heterozygosity = (2N / (2N-1)) * He

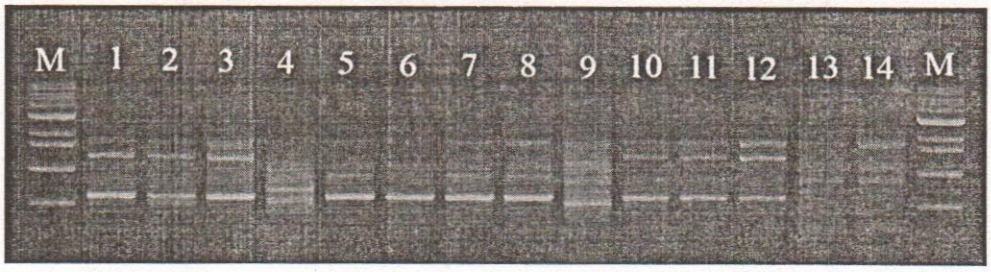

Figure 1: Banding pattern shown by primer OPA-10, M: Standard 1 kb size ladder (Bioneer); 1: Golsahi TPJ ; 2: Golsahi TPJ; 3: Unknown PTH; 4 : Unknown ILM; 5: Chivesay ILM; 6: Golsahi TPJ; 7: Unknown PTH; 8: Unknown PTH; 9: Churumpha TPJ; 10: Golsahi TPJ; 11: Ramla TPJ ; 12: Golsahi ILM;13: Golsahi ILM; 14: Churumpha ILM

Table 5 : Nei's genetic distance matrix of 14 Large Cardamom cultivars based on RAPD analysis

	Golsahi TPJ	Golsahi TPJ	Unknown PTH	Unknown ILM	Chivesay ILM	Golsahi TPJ	Unknown PTH	Unknown PTH	Churumpha TPJ	Golsahi TPJ	Ramla TPJ	Golsahi ILM	Churumpha ILM	Churumpha ILM
Golsahi TPJ	0.000													
Golsahi TPJ	0.204	0.000												
Unknown PTH	0.326	0.357	0.000											
Unknown ILM	0.733	0.766	0.729	0.000										
Chivesay ILM	0.413	0.531	0.300	0.636	0.000									
Golsahi TPJ	0.509	0.535	0.406	0.658	0.200	0.000								
Unknown PTH	0.400	0.431	0.441	0.686	0.232	0.238	0.000							
Unknown PTH	0.467	0.367	0.531	0.780	0.495	0.458	0.302	0.000						
Churumpha TPJ	0.733	0.781	0.848	0.684	0.775	0.802	0.736	0.738	0.000					
Golsahi TPJ	0.481	0.364	0.559	0.872	0.707	0.687	0.527	0.365	0.750	0.000				
Ramla ILM	0.573	0.469	0.424	0.842	0.550	0.605	0.626	0.443	0.783	0.292	0.000			
Golsahi ILM	0.320	0.435	0.277	0.769	0.315	0.486	0.395	0.461	0.730	0.447	0.378	0.000		
Churumpha ILM	0.654	0.656	0.684	0.448	0.596	0.600	0.583	0.667	0.627	0.740	0.733	0.650	0.000	
Churumpha ILM	0.654	0.699	0.705	0.390	0.596	0.618	0.600	0.711	0.573	0.792	0.787	0.689	0.096	0.000

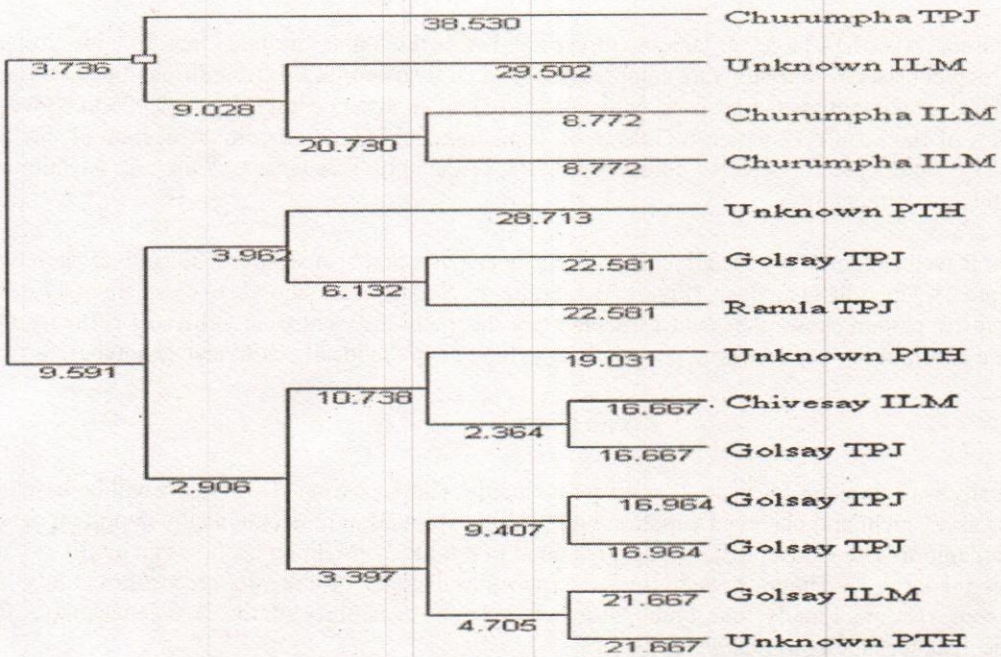

Figure 2: Dendrogram showing 14 Large Cardamom accessions based on Nei's genetic distance using UPGMA methods with bootstrap of 1000 replications.

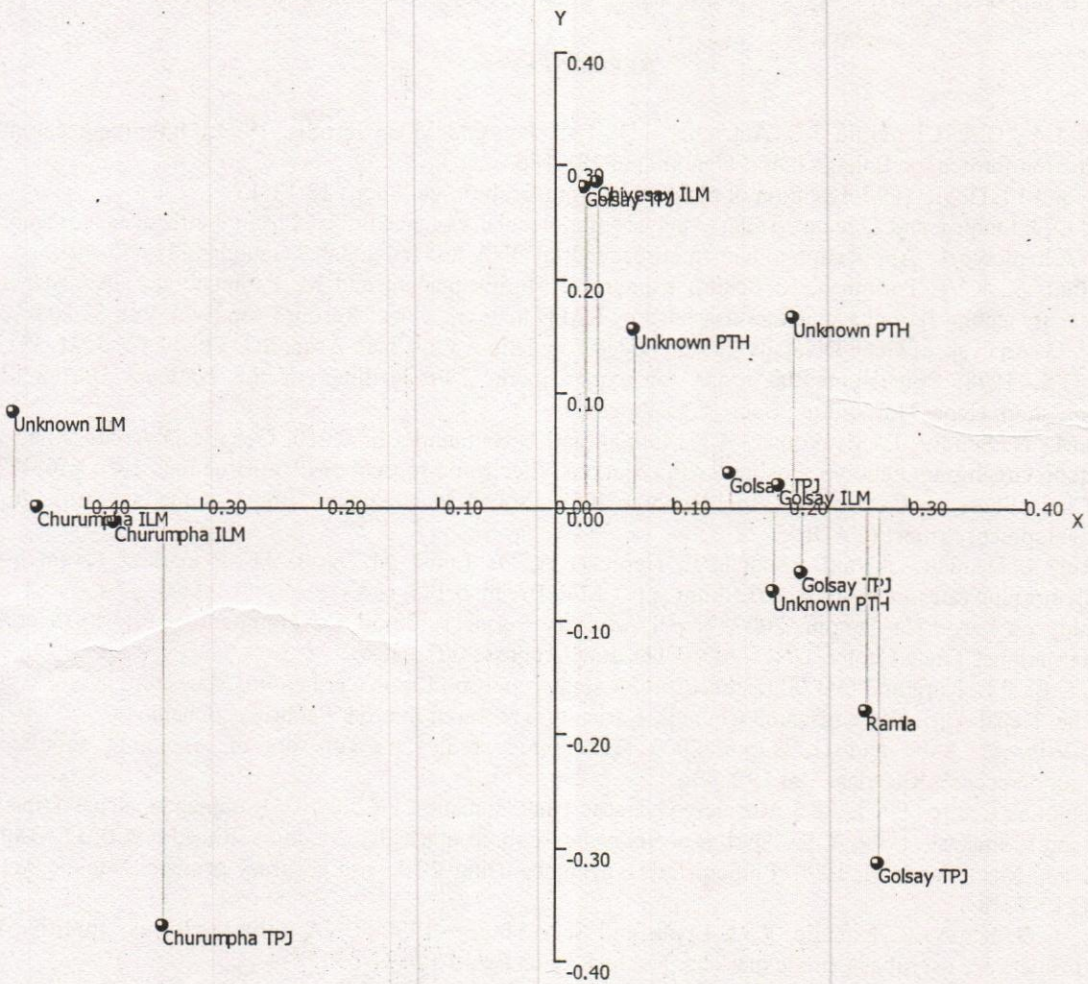

Figure 3: Genetic relationship of 14 large cardamom cultivars based on Principle Coordinate analysis

Hierarchical cluster analysis of 14 large cardamom cultivars shows accession are grouped into 2 major clusters (Figure 2). First group consists of four cultivars comprises of wild cultivars collected from different districts and another cluster comprises ten cultivars apart from wild cultivars. Genetic distance ranges from 0.096 in between Churumpha samples of Ilam to 0.872 between Golsahi of Taplejung and Unknown cultivar of Ilam (Table 5). This suggests broad genetic base for selected cultivars of large cardamom. This occurs as there is selection of wild cultivars for this experimentation.

This cluster pattern is well supported by the principle coordinate analysis that also separates all cultivars into two major groups (Figure 3). Upon PCO analysis (Figure 3) variation in X axis was found 48.16 % where as 22.27% in Y axis. These cluster pattern shows that wild cardamom are distinctly different from cultivated cultivars and there is high genetic dissimilarity between wild genotypes as compared to the locally cultivated genotypes.

CONCLUSION

High genetic diversity was obtained for over all cultivars including wild accession. This results will be baseline for future diversity assessment and observed variation will help in exploitation of economically important genes present in large cardamom. The genetic distance data obtained in this study might be useful as an auxiliary tool in future strategies for large cardamom breeding purpose and the diversity of cultivars creates possibility for genetic resource conservation. Finally, our findings demonstrate the feasibility of the RAPD technique for quantifying genetic distance among *Amomum* accessions.

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