Assessment of Genetic diversity by using RAPD markers of three *Curcuma* species from Melghat forests of Amravati, (MS), India

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Abstract
Genus Curcuma L. (Zingiberaceae) comprising of 120 species is distributed throughout South and South-East Asia with few species extending to China, Australia and South Pacific.; 40 species being recorded from India. Four species of Curcuma are reported from Melghat. Of these C. longa L. is cultivated while C. inodora Blatt., C. pseudomontana J. Graham and C. decipiens Dalzell are wild. C. decipiens being rare could not be collected. Curcuma inodora Blatt. known as 'Jangali Halad' is a common herb of Melghat at higher elevations. In Melghat area, populations of C. inodora are found to show many distinct variations in aerial as well as underground characters. Twelve distinct variants of C. inodora and one accession each of C. pseudomontana and C. longa were collected.

Molecular genetic fingerprints of three Curcuma species and twelve variants of one species were developed using RAPD marker to elucidate the genetic diversity relatedness among the species and within the species. Five RAPD primers produced 125 bands of which 124 were polymorphic. A total of 125 bands range from 200 to 4850 basepairs and some were amplified. Dendrogram was constructed based on UPGMA. Cluster analysis of data placed two species and twelve variants of one species in to four groups. C. pseudomontana and C. longa stand distinct from C. indora variants.

Keywords: *Curcuma inodora* Blatt., *Curcuma pseudomontana* J.Graham, *Curcuma longa* L., Melghat Forest, RAPD, Cluster analysis, Genetic Diversity.

Introduction

Genus *Curcuma* L. (Zingiberaceae) comprising of 120 species is the largest genus in the primarily tropical Zingiberaceae widely used in spices, medicines, dyes as well as ornamental plants[13]. The genus is easily recognized by its inflorescence, a spike with prominent spiral bracts which laterally fuse to form pouches, each subtending circinnus flowers and cluster of often coloured sterile, terminal bracts forming 'comma'.

The identification of *Curcuma* has traditionally been achieved using morphological data. However, *Curcuma* species exhibit large morphological variations both intra and inter species, but in some cases, especially early flowering groups show a very similar pattern of morphology between them leading to confusion in their identification[1]. Conventional taxonomic techniques in conjunction with molecular biology may go a long way in resolving taxonomic confusion prevailing in the genus. Estimation of genetic variations is increasingly being based upon information at the DNA level by various molecular techniques such as RAPD (Randomly Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), ISSR (Inter Simple Sequence Repeat) etc.

RAPD marker generated by PCR is widely used since 1990's to assess infraspecific genetic variations at nuclear level[15]. RAPD is PCR based technique for identifying genetic variation. Several DNA marker systems are now commonly used in genetic diversity analysis of plants. RAPD technique is popularly used in genetic studies[16]. RAPD technique was employed for determination of the phylogenetic relationship among 15 *Curcuma* species from Thailand by Orawan et al.[10] Sixteen *Curcuma* species and 96 individuals from Bangladesh were investigated to understand the genetic relationship and distance by Mohamad Islam[7].

Present study was undertaken to understand the closeness and genetic distance between the three species and 12 variants found in Melghat.

Material and Methods

Curcuma pseudomontana J.Graham , *Curcuma longa* L. and twelve variants of *Curcuma inodora* Blatt. were selected for genetic variability analysis. These samples were collected from various locations in Melghat Forests. Identification of *Curcuma* species was done by using standard floras[2,3,5,17]. Leaves were washed with distilled water. Fresh material was used for molecular studies; whenever required, the material was cryopreserved. Genetic diversity analysis was done by using RAPD molecular marker.

DNA Isolation: The Genomic DNA Was isolated by CTAB method[8]. PCR mix was prepared for the DNA samples. Total 5 PCR reactions were performed using five primers for each sample. Final volume of each reaction was 25.0 µl. The reaction mix was prepared for all samples and added into 200 µl PCR tubes. Genomic DNA was added later to each tube as per table 1. After addition of genomic DNA to PCR tubes, tubes were subjected to thermal cycling programme shown in table 2.

Table 1
Addition of genomic DNA to PCR Tubes

Materials	Volume (µl)	Final Concentration
Genomic DNA (1: 10 and 1:5 diluted)	5.00	100-200 ng
10X PCR buffer	2.50	1X
50mM $MgCl_2$	1.00	2.5mM
0.5mM dNTP Mix	1.00	0.1 mM each dNTP
10 pmole primer solution	2.00	0.2 mM
Taq DNA polymerase (5.0 units/µl)	0.50	2.5 unit/reaction
Nuclease Free water	13.00	

Table 2
Thermal Cycling

Stage	Temperature (^{0}C)	Time (min:sec)	Cycles
Initial denaturation	95	5:00	35 cycles
Denaturation	95	1:00	
Annealing	37	1:30	
Extension	72	2:00	
Final extension	72	10:00	
Hold	4	Until use	

RAPD PCR: Unlike normal PCR which uses two, RAPD uses only one primer with an arbitrary sequence. Therefore, amplification in the RAPD process occurs anywhere along a genome that contains two complementary sequences to the primer which are within the length limits of PCR (~3 kb). PCR reactions were performed using five 10 mer primers for each sample following Sharma et al.[12] Agarose gel electrophoresis of the RAPD products was performed using 1.0% (w/v) agarose gel and standard 0.5 x TBE gel electrophoresis buffer.

RAPD data analysis: The scoring was done in samples where bands were clearly visible and amplified product was reproducible over two repeated amplifications. Amplified fragments were manually scored for presence 1 and absence 0 and binary matrics were subjected to statistical analysis using MEGA software. The similarity matrix were computed and corresponding dendrogram of genetic relatedness was constructed by applying UPGMA clustering algorithm.

Dendrogram was drawn using MEGA software. Briefly all the RAPD bands were scored in binary format. For RAPD dendrogram collectively all primers data was used for construction of Neighbor-Joining (NJ) tree[6]. Similarity matrix of RAPD was calculated as per the method prescribed by Souframanien and Gopalakrishna[14].

Results and Discussion

All the 14 accessions were studied for genetic diversity. For the agarose gel electrophoresis, genetic DNA was isolated from 14 samples.

RAPD Profile: The RAPD analysis was performed for fourteen samples using five different random primers as in table 3. In all five cases, the profiles of DNA fragments found for all the samples were not similar. With these primers, DNA fragment length polymorphism was observed, the resultant also includes some similar sized fragments. For each primer, the array of DNA fragments consists of bands of high or low intensities of ethidium bromide staining. The higher-intensity bands may be due to the amplification of repetitive DNA sequences, the influence of neighboring sequences on hybridization to the target sequence, or fewer mismatches to the target sequence.

In contrast, the low-intensity bands may have been generated inefficiently because of a higher degree of mismatch between the primer and the target sequence[11].

Agarose Gel electrophoresis of the RAPD PCR: Among the tested primers, a total of 5 primers showed reliable banding pattern with high reproducibility and clear band resolution were used for further analysis. These 5 primers produced a total of 125 distinct amplification products ranging from 200 bp to 2500 bp. Agarose Gel product obtained with primers is enlisted in table 3 and figures 2 to 6.

The number of scored bands per primer ranged from 15 (OPX 06) to 36 (OPM05) with mean number of 25 per primer as in table 3. These two primers are also representative of the lowest (15) and the highest (36) number of polymorphic product respectively. Among these amplified products, a total of 124 bands were polymorphic. The average number of polymorphic markers across the primers was 98.94 % and ranged from 94.73 produced by OPMO6 to 100% obtained by 4 primers OPMO5, OPX06, OPA07 and OPX01.

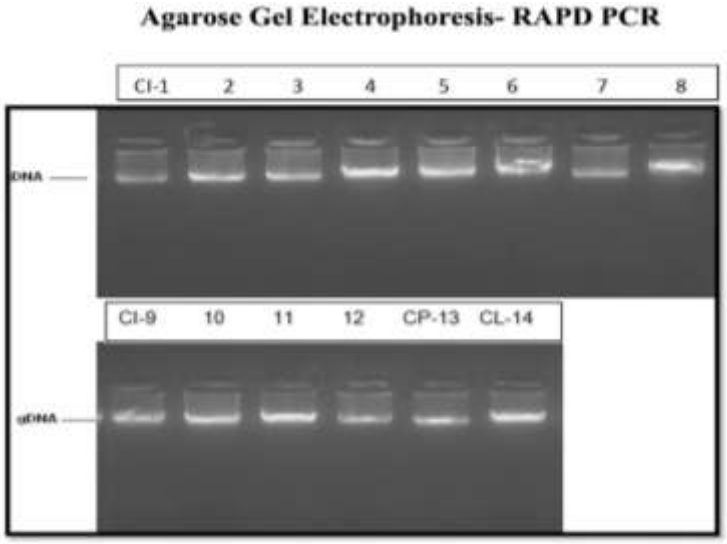

Figure 1: DNA isolated from CI-1 to 12, CP-13 and CL-14 samples. 5uL of the sample was loaded in each well.

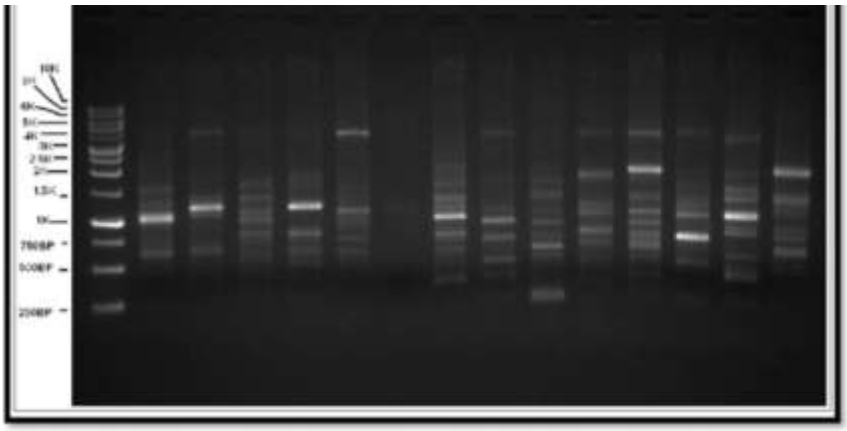

Figure 2: Agarose (2% w/v) Gel Electrophores is of RAPD PCR products for fourteen samples.
Lane M: DNA size standard- Lane 1- CI-1; Lane 2- CI -2; Lane 3- CI -3; Lane 4- CI -4; Lane 5- CI -5; Lane 6- CI-6; Lane 7- CI -7; Lane 8- CI-8; Lane 9- CI-9; Lane 10- CI-10; Lane 11- CI-11; Lane 12- CI-12; Lane 13- CP-13; Lane 14- CL-14 PCR products generated using primers OPM05.

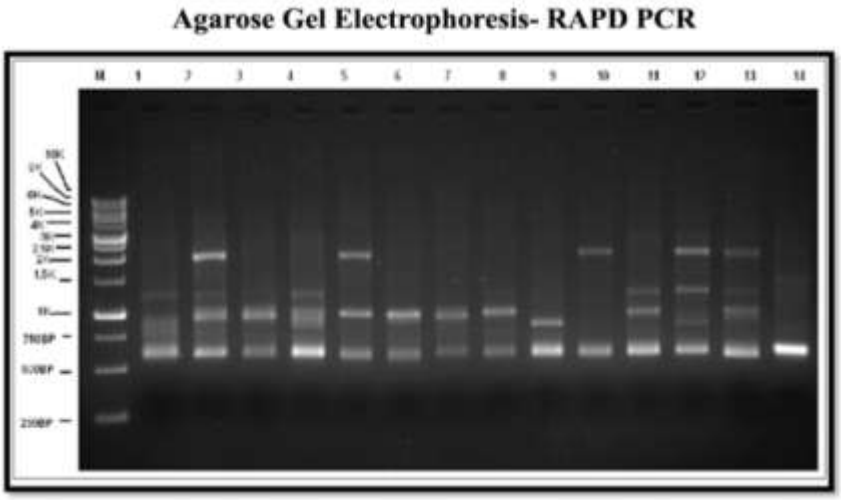

Figure 3: Agarose (2% w/v) Gel Electrophores is of RAPD PCR products for fourteen samples.
Lane M: DNA size standard- Lane 1- CI-1; Lane 2- CI-2; Lane 3- CI-3; Lane 4- CI-4; Lane 5- CI-5; Lane 6- CI-6; Lane 7- CI-7; Lane 8- CI-8; Lane 9- CI-9; Lane 10- CI-10; Lane 11- CI-11; Lane 12- CI-12; Lane 13- CP-13; Lane 14- CL-14 PCR products generated using primers OPX06.

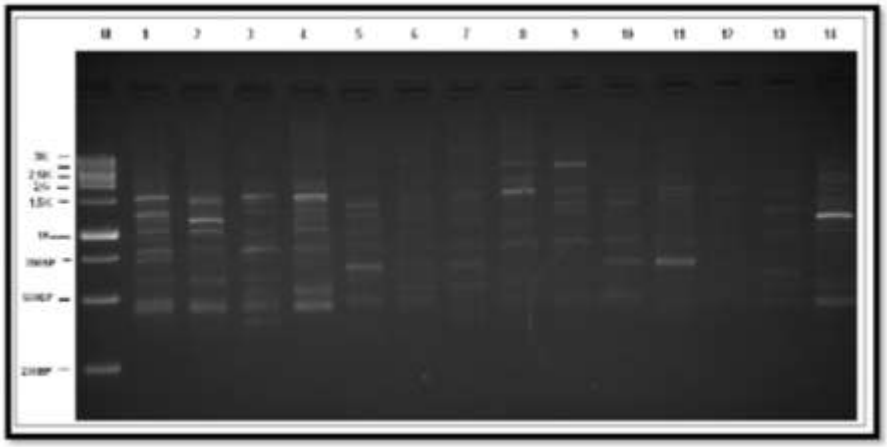

Figure 4: Agarose (2% w/v) Gel Electrophores is of RAPD PCR products for fourteen samples.
Lane M: DNA size standard- Lane 1- CI-1; Lane 2- CI-2; Lane 3- CI-3; Lane 4- Cl-4; Lane 5- CI-5; Lane 6- CI-6; Lane 7- CI-7; Lane 8- CI-8; Lane 9- CI-9; Lane 10- CI-10; Lane 11- CI-11; Lane 12- CI-12; Lane 13- CP-13; Lane 14- CL-14 PCR products generated using primers OPA07.

Agarose Gel Electrophoresis- RAPD PCR

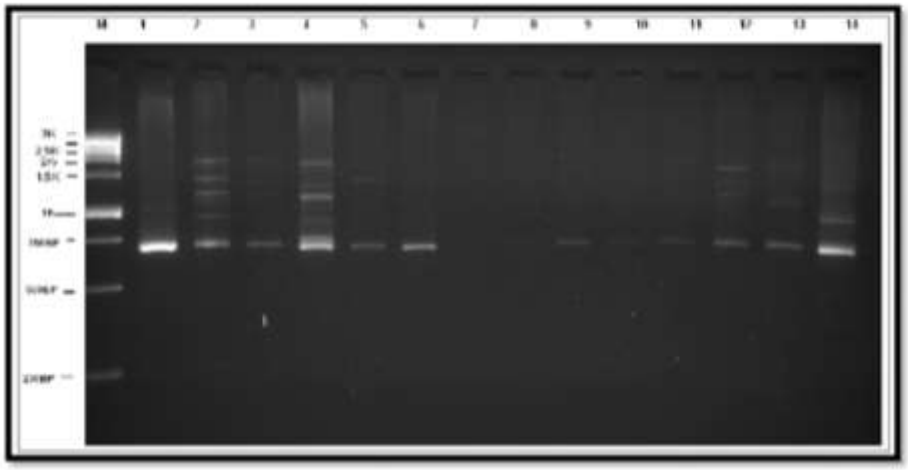

Figure 5: Agarose (2% w/v) Gel Electrophores is of RAPD PCR products for fourteen samples.
Lane M: DNA size standard- Lane 1- CI-1; Lane 2- CI-2; Lane 3- CI-3; Lane 4- Cl-4; Lane 5- CI-5; Lane 6- CI-6; Lane 7- CI-7; Lane 8- CI-8; Lane 9- CI-9; Lane 10- CI-10; Lane 11- CI-11; Lane 12- CI-12; Lane 13-CP-13; Lane 14- CL-14 PCR products generated using primers OPM06.

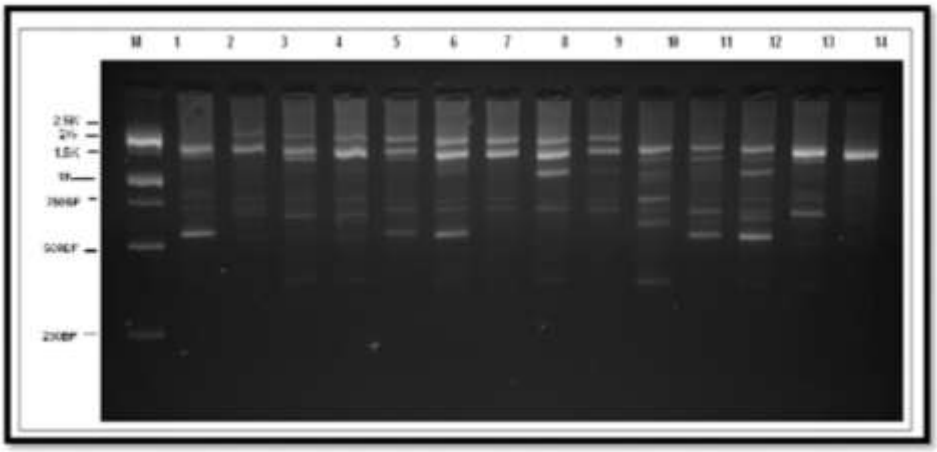

Figure 6: Agarose (2% w/v) Gel Electrophores is of RAPD PCR products for fourteen samples.
Lane M: DNA size standard- Lane 1- CI-1; Lane 2- CI-2; Lane 3- CI-3; Lane 4- Cl-4; Lane 5- CI-5; Lane 6- CI-6; Lane 7- CI-7; Lane 8- CI-8; Lane 9- CI-9; Lane 10- CI-10; Lane 11- CI-11; Lane 12- CI-12; Lane 13- CP-13; Lane 14- CL-14 PCR products generated using primers OPX01.

The level of polymorphism is different with different primers among different samples. Only 13 fragments were amplified from sample 6 genomic DNA with 5 primers as compared to the total of 322. Highest number of fragments 32 was found to be in sample CP-13. Fragments from the rest of the varieties ranged from 19 to 29. The number of amplified fragments also varied with different primers. A minimum of 39 fragments were amplified with primer OPM-06 and a maximum of 80 bands with primers OPM-05. The size of amplified fragments also varied with different primers. A 4850 bp fragment was amplified from sample 3 (CI-3) with primer OPM-05 while the smallest-size (300bp) fragment was amplified by primer OPM-05.

Similarity Matrix: A similarity matrix based on the proportion of shared RAPD fragments was used to establish the level of genetic relatedness among the 12 variants and 2 species genotypes studied during the present investigation. (Table 4) Pair-wise estimate of similarity for 14 samples belonging to the 3 species ranged from 92.31 to 100. Variants CI-8 and CI- 5 show highest similarity index of 95.30 and CP-14 and CI-3 show lowest similarity indices.

Table 3
Polymorphism generated by samples using different primers:

S.N.	Primer	Nucleotide sequence (5'-3')	Total bands	Polymorphic bands	Monomorphic bands	Percent polymorphism
1	OPM05	ACGCCAGAGG	36	36	0	100
2	OPX06	GGGAACGTGT	15	15	0	100
3	OPA07	CTGGGCAACT	31	31	0	100
4	OPM06	GAAACGGGTG	19	18	1	94.73
5	OPX01	CTGGGCACGA	34	34	0	100
		Total	**125**	**124**	**01**	
		Mean	**25**	**-**	**Average**	**98.94**

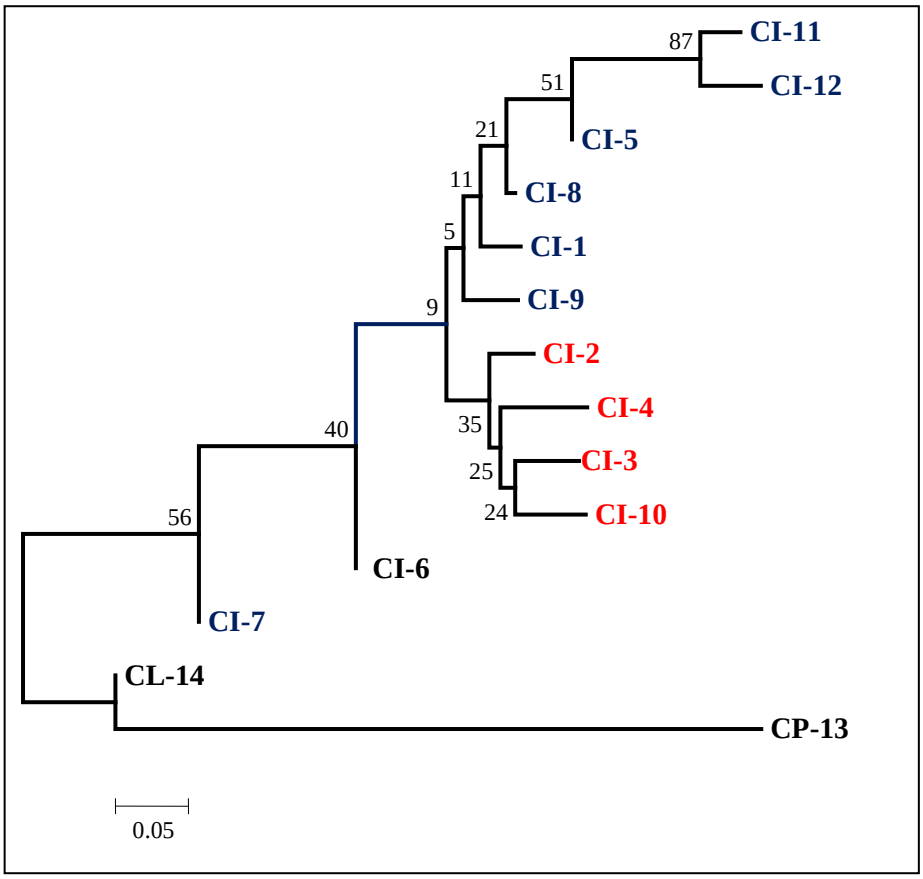

Figure 7: Dig: – Dendrogram (RAPD data)

Dendrogram Clusters:
Cluster 1 – CP-13, CL-14
Cluster 2- CI-7
Cluster 3- CI-6
Cluster -4 Subcluster 4.A- CI-2, CI-4, CI-3, CI-10
Subcluster 4-B – CI-9, CI-1, CI-8, CI-V, CI-11, CI-12.

Table 4
Similarity Matrix: (RAPD data)

0	CI-01	CI-02	CI-03	CI-04	CI-05	CI-06	CI-07	CI-08	CI-09	CI-10	CI-11	CI-12	CP-13	CL-14
CI-01	100													
CI-02	94.614	100												
CI-03	93.442	93.366	100											
CI-04	94.522	93.755	93.755	100										
CI-05	94.522	93.917	94.432	94	100									
CI-06	95.417	94.522	94	94.803	95	100								
CI-07	94.343	94.614	93.917	94.343	93.835	94.803	100							
CI-08	93.835	93.755	94.803	93.835	95.309	94.614	94.169	100						
CI-09	94.522	94.255	93.917	94.522	94.901	94.803	94.522	94.343	100					
CI-10	94.169	93.755	93.755	93.835	94	94.255	94	94.522	93.835	100				
CI-11	93.675	93.291	93.144	93.519	94	94.255	93.519	94.169	93.675	94.169	100			
CI-12	94.343	93.755	93.442	94.169	93.835	94.255	93.835	94.522	94	94.708	93.835	100		
CP13	94.169	93.291	92.318	93.835	93.366	93.917	93.835	93.519	93.217	94.343	93.519	93.835	100	
CL14	94.614	93.675	94.343	94.083	94.803	94.708	94.083	94.432	94.255	94.255	93.755	93.917	93.442	100

Cluster Analysis: Genetic diversity acquired from RAPD data was used to make cluster diagram (Dendrogram Dig. no. figure 7). Dendogram was drawn using MEGA software. Briefly all the RAPD bands were scored in binary format. Collectively all primers data was used for construction of Neighbor-Joining (NJ) tree[6].

C. pseudomontana and *C. longa* cluster together but interestingly genetic distance between two is maximum. Population variations exhibited by *C. inodora* are continuous variations. Clusters of *C.inodora* variants show that genetically CI-6 and CI-7 stand distinct from rest of the variants. In course of evolution, there is possibility that variant CI-6 and CI-7 in due course of time can evolve as distinct varieties **.** Rest of the variants are genetically more closer to each other.

In present study the RAPD technique was found to be suitable in revealing infraspecific genetic variation in *C. inodora*. Not a single primer has shown same pattern of bands, also not a single primer has shown clear polymorphism amongst all the accessions. The primers used produced almost 100 % polymorphism indicating great genetic variability. The level of polymorphism is different with different primers among different samples. According to Nayak et al[9] also the main cause of the high level of polymorphism could be intraspecific variation.

High genetic variability in *Curcuma* populations studied can be easily understood because of the fact that Melghat region from where the populations are collected, has a long history of forest management and reservation. Since 1860, Melghat forests are well managed and today it enjoys the status of special forest i.e. a tiger reserve. Most of the *Curcuma* species reproduce asexually. *C. inodora* reproduces sexually and produces viable seeds which allows gene exchange.

Conclusion

Primers used in RAPD produced almost 100% polymorphism indicating the great genetic variability. Genetic polymorphism is the central issue of the genetic conservation since it is the primary source for morphological and physiological variation of plants. This leads to evolutionary changes within and among species and to genetic diversity. Different geographical and ecological niches are expected to produce variations.

However, the populations studied here grow in the same geographical and ecological niche and still show great variations not only at morphological[4] but also at genetic level also. This indicates the adaptive and evolutionary potential of *C. inodora* populations. It is therefore suggested that these populations growing in Melghat should be conserved with at most care to save the future evolutionary lines.

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