BIOLOGIA PLANTARUM 52 (4): 627-633, 2008

Genetic diversity in *in vitro*-conserved germplasm of *Curcuma* L. as revealed by RAPD markers

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Abstract

A set of 30 accessions of five *Curcuma* species - *C. latifolia*, *C. malabarica*, *C. manga* and *C. raktakanta* and 13 morphotypes (identified on the basis of morphological markers) of *C. longa* conserved in the *In Vitro* Genebank at National Bureau of Plant Genetic Resources, New Delhi, were subjected to RAPD analysis. Of the 200 RAPD primers screened, 21 polymorphic primers were selected for further study. Mean genetic similarities based on Jaccard's similarity coefficient ranged from 0.18 to 0.86 in accessions of cultivated species, *i.e.*, *C. longa* and from 0.25 to 0.86 in wild species. The dendrogram derived from the RAPD data corroborated the morphological classification of the morphotypes. The efficiency of individual RAPD primers was also compared; primers OPC-20, OPO-06, OPC-01 and OPL-03 were adjudged highly informative in discriminating the germplasm of *Curcuma*.

Additional key words: conservation, *in vitro* genebank, marker informativeness, morphotypes, turmeric.

Introduction

The genus *Curcuma* (family *Zingiberaceae*) comprises more than 80 species of rhizomatous perennial herbs and has a widespread occurrence in the tropics of Asia and extends to Africa and Australia (Purseglove *et al*. 1981). Velayudhan *et al.* (1994) reported that a total of 40 species of *Curcuma* occur in India. Commonly known as turmeric, *Curcuma longa* L. (syn. *C. domestica* Val.) is a commercially important spice crop and curcumin is also used in medicine (Maheshwari *et al*. 2006 and references therein).

The conservation and utilization of plant genetic resources is facilitated and promoted if collections are properly characterized and evaluated. A large number of accessions (2 368) of wild and cultivated species of *Curcuma*, collected from different geographic regions, are being conserved largely in field genebanks and to some extent in *in vitro* genebanks in India (Ravindran *et al*. 2005). Currently, some 137 accessions of *Curcuma* species are being conserved in the *In Vitro* Genebank at National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India. At present, the most important task is to select and conserve a representative diversity of germplasm in the *in vitro* genebank from large number of accessions maintained in the field genebank.

The germplasm of *Curcuma* collected from various geographic regions of India has been characterized based on morphological traits and a considerable variation has been observed in it (Velayudhan *et al*. 1994, Sasikumar *et al*. 2005). Velayudhan *et al*. (1999) identified 21 distinct morphotypes of six taxonomic groups from 550 accessions of *C. longa* on the basis of morphological characterization. But morphological characters are usually controlled by many loci and may be influenced by environment which can complicate the assessment of genetic diversity and consequently their conservation and utilization. DNA markers allow the identification of accessions unambiguously to assess the relative diversity among and within the species. Results of RAPD-based markers compared with other classes of DNA-based markers in the same plant material have demonstrated reasonably high level of correspondence for inter- and intra-generic diversity analysis in soybean (Powell *et al*. 1996), *Vitis* (Tessier *et al*. 1999), *Vaccinium* (Garkava-Gustavsson 2005) or *Vigna* (Dikshit *et al*. 2007).

The main objective of the present study was to address the concerns related to the genetic diversity among the accessions of *Curcuma* species conserved in the *In Vitro* Genebank at NBPGR, New Delhi.

Received 20 September 2006, *accepted* 29 July 2007.

Abbreviations: D_J - discriminating power; RAPD - random amplified polymorphic DNA; UPGMA - unweighted pair group method with arithmetic mean.

Acknowledgment: The authors are thankful to the Director, NBPGR, New Delhi, for providing the facilities.

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The major concerns were: *1*) does the germplasm of *Curcuma* conserved in the *In vitro* Genebank at NBPGR possess significant genetic diversity among the accessions collected from different habitat, as it is clonally propagated crop, *2*) does the analysis of diversity revealed by RAPD markers correspond to that of the morphotypes identified on the basis of morphological markers by Velayudhan *et al*. (1999), so as to validate the conservation of representative germplasm, and *3*) evaluation of discrimination power of RAPD primers for analysis of the genetic diversity efficiently among and within the species, as very limited information about the application of DNA-marker is available in the literature for *Curcuma* species. Additionally, we also investigated the relationship among 30 accessions of five species of *Curcuma* collected from different geographical regions in India for effective conservation and utilization of *Curcuma* germplasm in crop improvement programs.

Materials and methods

Plant: The experimental material comprised 30 accessions of five *Curcuma* species *i.e.* 26 accessions of *C. longa* L. [including 13 morphotypes identified by Velayudhan *et al*. (1999)] and one each of *C. latifolia* Rosc.*, C. malabarica* Velayudhan*, C. manga* Val., and *C. raktakanta* Mangaly and Sabu. These accessions were initially collected from different geographical regions of India (Table 1). Subsequently, they were conserved as *in vitro* cultures in the *In Vitro* Genebank at NBPGR, New Delhi, India, following the protocols reported earlier by Tyagi *et al*. (2004).

DNA extraction and RAPD analysis: The leaves of *in vitro*-conserved plantlets were used for DNA extraction. Total genomic DNA was extracted from 1 g of leaves of each accession (from one culture vessel), using modified Saghai-Maroof *et al.* (1984) method. After RNAse treatment, the DNA concentrations were determined following the method of Brunk *et al.* (1979) using a fluorometer (*Amersham Biosciences*, Piscataway, USA) and bisbenzimide (Hoechst dye 33258) as the fluorescent dye. The DNA samples were stored at -20 °C till further use. Working solutions of genomic DNA (5 mg cm^{-3}) were prepared after dilution with sterile distilled water and stored at 4 °C for subsequent use in RAPD analysis.

PCR reaction was carried out in a DNA thermal cycler (*GeneAmp 9600* PCR system, *Perkin-Elmer,* Norwalk, GB). A total of 200 primers (*Operon Technologies*, Alameda, USA), were used for RAPD analysis. Of these, 21 primers were found to be polymorphic (Table 2) and were used for further analysis. The preparation of reaction mixture and PCR conditions were followed as described earlier (Tyagi *et al*. 2007). Methods for statistical analyses of the data of present study are described below:

Diversity analysis: The scoring was done in samples where the bands were clearly visible and amplified products were reproducible over two repeated amplifications. Amplified fragments were manually scored for presence (1) and absence (0) and binary matrices were subjected to statistical analyses using *NTSYS-pc* (Numerical Taxonomy and Multivariate Analysis System version. 2.1) by *Exceter* software (Rohlf 2000). To compute pair-wise genetic similarities, Jaccard's similarity coefficients (J_{ij}) were calculated as follows: $J_{ij} = a / (n - d)$, where *a* is the number of RAPD bands present in both *i* and *j* accessions, *d* is the number of bands absent in both *i* and *j* accessions, and *n* is the total number of RAPD bands. The similarity values were derived as genetic distance by subtracting each value from unit value.

The similarity matrices were computed and corresponding dendrograms of genetic relatedness were constructed by applying Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering algorithm.

Principal component analysis, Bootstrapping analysis and analysis of molecular variance: Principal components analysis (PCA) was performed in addition to cluster analysis using *NTSYS-pc 2.1* to generate a correlation matrix. This matrix was run on *EIGEN* to identify the eigenvectors, and the ordinations were displayed in three dimensions. The robustness and validity of clustering pattern was tested by Bootstrap analyses of 1000 bootstrap samples using the software *WINBOOT* (Yap and Nelson 1996). The variation in RAPD patterns was analyzed by analysis of molecular variance (*AMOVA*) using *GENALEX 6* (Genetic Analysis in Excel), cross-platform package for population genetic analysis (Flanagan 2005, Peakall and Smouse 2006).

Evaluation of discrimination powers of RAPD primers: To compare the efficacy of RAPD primers used in diversity analysis of the tested accessions of *Curcuma* species, the information obtained from profile data of each primer or assay-unit (U) was used for the estimation of number of polymorphic bands (n_p); number of monomorphic bands (n_{np}); average number of polymorphic bands per assay-unit (n_p/U); number of loci (L) = total number of bands *i.e.* $n_p + n_{np}$ obtained for each marker type; number of loci per assay-unit: $n_u = L/U$; total number of unique banding patterns (T_p); average number of patterns per assay-unit (I) were estimated following Belaj *et al*. (2003). Discriminating power (D_j) of each primer, *i.e.,* the probability that the two randomly chosen accessions from the sample of 30 accessions have different banding pattern and, thus, are distinguishable from one another, were estimated following the method described by Tessier *et al.* (1999).

Table 1. Passport data of 30 accessions of 5 species of *Curcuma* used in present study. *- Code comprised serial number of accession, abbreviated name of species, area of collection: lt - *C. latifolia*; ne - North-East; mb - *C. malabarica*; kl - Kerala; lg - *C. longa*; rt - *C. raktakanta*; hp - Himachal Pradesh; un - unknown; ap - Andhra Pradesh ; mg - *C. manga*; N-E - North-eastern; N-W - North-western.

Number	Code*	Indigenous collection number	Species	Area [region] of collection
1	1_lt_ne	IC313093	*C. latifolia*	North-East [N-E Himalayas]
2	2_mb_kl	IC088846	*C. malabarica*	Kerala [South India]
3	3_lg_kl	-	*C. longa*	Kerala [South India]
4	4_rt_kl	IC088862	*C. raktakanta*	Kerala [South India]
5	5_lg_kl	IC266522 [M3]	*C. longa*	Kerala [South India]
6	6_lg_kl	IC136980 [M4]	*C. longa*	Kerala [South India]
7	7_lg_ne	IC137010 [M6]	*C. longa*	North-East [N-E Himalayas]
8	8_lg_ne	IC137023 [M8]	*C. longa*	North-East [N-E Himalayas]
9	9_lg_ne	IC137046 [M9]	*C. longa*	North-East [N-E Himalayas]
10	10_lg_ne	IC406490 [M10]	*C. longa*	North-East [N-E Himalayas]
11	11_lg_hp	IC137068 [M11]	*C. longa*	Himachal Pradesh [N-W Himalayas]
12	12_lg_hp	IC137076 [M13]	*C. longa*	Himachal Pradesh [N-W Himalayas]
13	13_lg_kl	IC088749 [M14]	*C. longa*	Kerala [South India]
14	14_lg_kl	IC137109 [M15]	*C. longa*	Kerala [South India]
15	15_lg_un	IC137110 [M16]	*C. longa*	unknown
16	16_lg_kl	IC17177 [M17]	*C. longa*	Kerala [South India]
17	17_lg_ne	IC266539 [M18]	*C. longa*	North-East [N-E Himalayas]
18	18_lg_ap	IC360175	*C. longa*	Andhra Pradesh [South India]
19	19_lg_ap	IC360183	*C. longa*	Andhra Pradesh [South India]
20	20_lg_ap	IC088863	*C. longa*	Andhra Pradesh [South India]
21	21_lg_ap	IC137076	*C. longa*	Andhra Pradesh [South India]
22	22_lg_kl	IC088749	*C. longa*	Kerala [South India]
23	23_lg_kl	IC137109	*C. longa*	Kerala [South India]
24	24_lg_kl	IC137110	*C. longa*	Kerala [South India]
25	25_lg_kl	IC017177	*C. longa*	Kerala [South India]
26	26_lg-un	IC012450	*C. longa*	unknown
27	27_lg_un	-	*C. longa*	unknown
28	28_lg_ne	IC088910	*C. longa*	North-East [N-E Himalayas]
29	29_mg_kl	IC088814	*C. manga*	Kerala [South India]
30	30_lg_kl	IC069953	*C. longa*	Kerala [South India]

Results

RAPD analysis: Of the 200 RAPD primers screened initially, 21 were found to be polymorphic and a total of 172 amplification products were scored with an average frequency of 8.19 bands per primer (Table 2). The molecular size of the bands ranged from 500 to 3400 bp. The number of bands generated per primer ranged from five in primer OPC-10 to 10 in primers OPC-01, OPC-05, OPD-07 and OPK-19; all the bands generated by each primer were polymorphic (Table 2).

Diversity analysis: Pair-wise genetic similarities ranged from 0.18 to 0.86 in all the accessions, 0.25 to 0.86 in four wild species of *Curcuma* and 0.18 to 0.82 in 26 accessions of cultivated species *i.e. C. longa*. The mean values of pair-wise genetic similarities were 0.52 for all accessions considered together, 0.56 for wild species of *Curcuma* and 0.5 for accessions of *C. longa*.

The accessions were grouped by subjecting the similarity values to *UPGMA* clustering (Fig. 1). Based on the dendrogram, the 30 accessions grouped into two main clusters at 33 % similarity. Cluster I had only two accessions of *C. longa* for which geographical regions were unknown. Cluster II, being the major one, included 28 accessions comprising most of the accessions of *C. longa* collected from different geographical regions and four wild species. The cluster II could further be divided into two sub-clusters, *i.e.*, II-A and II-B at 35 % similarity, with 12 and 16 accessions, respectively. Three

Table 2. Data on polymorphism and discrimination power estimated for 21 RAPD primers used to assess the genetic diversity of *Curcuma* accessions.

Primers [sequence 5' to 3']	Size range [bp]	Number of polymorphic bands	Banding patterns	Discriminating power
OPA-03 [AGTCAGCCAC]	1500-3200	8	22	0.975
OPC-01 [TTCGAGCCAG]	1000-3200	9	25	0.986
OPC-05 [TGGACCGGTG]	1000-3300	10	22	0.961
OPC-09 [CTCACCGTCC]	1500-3000	8	23	0.977
OPC-10 [TGTCTGGGTG]	900-3000	5	09	0.867
OPC-13 (AAGCCTCGTC)	100-1000	8	14	0.826
OPC-20 [ACTTCGCCAC]	500-3000	9	27	0.993
OPD-07 [TTGGCACGGG]	700-3400	10	19	0.968
OPF-07 [CCGAATTCCC]	1100-3000	8	17	0.949
OPI-16 [TCTCCGCCCT]	1200-3300	7	21	0.965
OPI-19 [AATGCGGGAG]	700-3000	6	18	0.959
OPJ-19 [GGACACCACT]	500-3400	9	22	0.979
OPK-17 [CCCAGCTGTG]	400-2500	7	20	0.966
OPK-19 [CACAGGCGGA]	300-2000	10	24	0.974
OPL-03 [CCAGCAGCTT]	1500-3300	8	24	0.986
OPM-14 [AGGGTCGTTC]	100- 900	8	21	0.959
OPO-06 [CCACGGGAAG]	500-3300	10	26	0.991
OPO-10 [TCAGAGCGCC]	500-2500	8	21	0.970
OPO-11 [GACAGGAGGT]	500-2500	8	23	0.976
OPO-13 [GTCAGAGTCC]	1000-3300	8	15	0.945
OPO-16 [TCGGCGGTTC]	700-3000	8	23	0.979

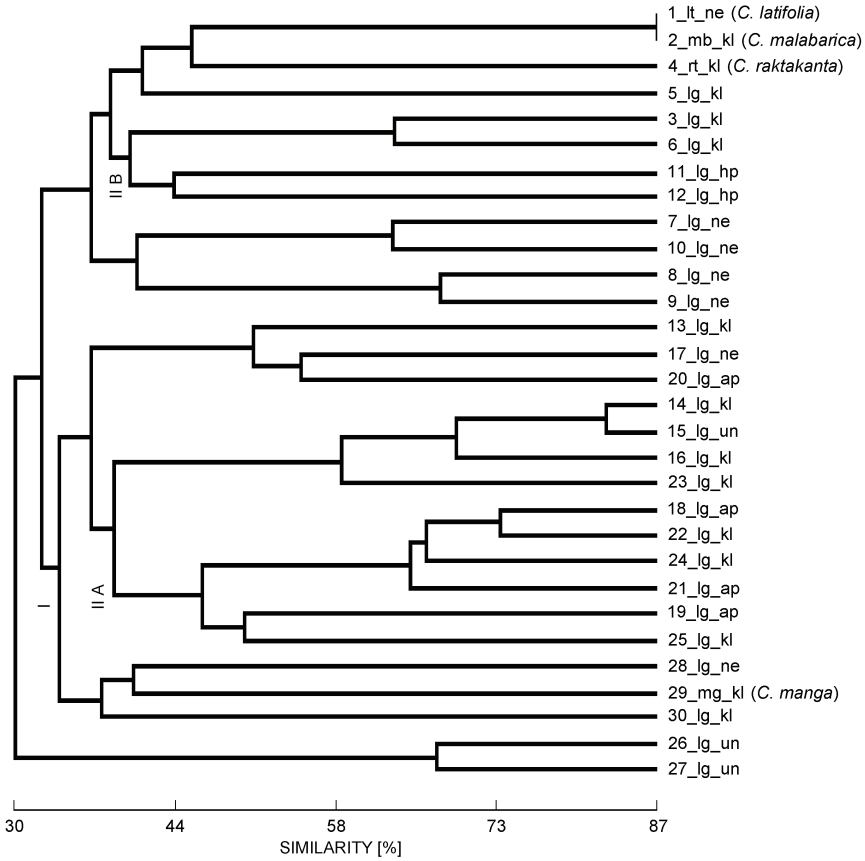

Fig. 1. Dendrogram based on UPGMA analysis of genetic similarities estimated among the 30 accessions of *Curcuma* species (all accessions with out botanical names belong to *C. longa*; for abbreviations see footnote of Table 1).

wild species, namely, *C. latifolia*, *C. malabarica* and *C. raktakanta* were grouped in II-A whereas and *C. malabarica* were found be similar at 87 % level whereas 48 % similarity was recorded with *C. raktakanta*. Of the 30 accessions of *Curcuma* species studied, 26 belong to *C. longa* collected from different geographical regions such as South India (18) *i.e.* from Andhra Pradesh (4) and Kerala (14); North-East Himalayan region (7), North-West Himalayan region, *i.e.*, Himachal Pradesh (2) and unknown (3). The accessions collected from Kerala were grouped together in cluster II (A and B). Majority of the accessions from North-East were grouped together in sub-cluster II-B only. All the four accessions collected from Andhra Pradesh and two accessions collected from Himachal Pradesh were grouped in sub-cluster II-A.

Considering the 13 morphotypes of *C. longa* together, the range of diversity was observed as 18 - 68 %. Among these, eight morphotypes (M3, M4, M6, M8, M9, M10, M11 and M13) collected primarily from Himalayan region were grouped together in sub-cluster II-B and five (M14, M15, M16, M17 and M18) collected mainly from South India were grouped in sub-cluster II-A. The ranges of diversity were 34 - 69 % and 32 - 69 % in accessions collected from South India and North-East Himalayan region, respectively.

We constructed the dendrogram of 14 accessions collected from South India, 7 accessions from North-East and 13 morphotypes of *C. longa*, separately. Considerable variability was observed in accessions collected from South India (34 - 69 %) and North-East (32 - 69 %), and relatively larger extent of diversity (18 - 68 %) was observed in 13 morphotypes identified on the basis of morphological characters.

Associations among the 30 accessions were also revealed by PCA. Overall, the grouping pattern from PCA corresponded well with the clustering pattern of the dendrogram. The PCA indicated that all the components were required to explain 100 % of the variation. Bootstrap analyses based on 1000 pseudosamples also validated the dendrogram based on Jaccard's similarity coefficient; classifying the 30 accessions in two major clusters. Bootstrap value, *i.e.*, 60 % was observed in 16 out of 30 branches. The results of the *AMOVA* partitioning of variance (mention the most important data from Table 3) showed that RAPD variation among accessions was significant based on 99 permutations (df = 85, estimated variance = 70 %, PhiPT value = 0.303). It was found that among accessions variance component accounted for 70 % while among species variance component accounted for only 30 % of the total variance.

Evaluation of discrimination power of RAPD primers: The number of banding patterns (Tp) ranged from 9 in primer OPC-10 to 27 in OPC-20 with an average (I) of 20.8. The discriminating power ranged from 0.826 in OPC-13 to 0.993 in OPC-20 (Table 2). Based on above parameters, the identified 21 RAPD markers were highly informative in discriminating the germplasm accessions of *Curcuma* among and within the species. Among the selected primers, highest discriminating powers were observed in OPC-20 (0.993), OPO-06 (0.991), OPC-01 and OPL-03 (0.986).

Discussion

For *Curcuma* species, efficacy of isozyme markers for diversity analysis has been reported among six early flowering species of *Curcuma* (Apavatjrut *et al*. 1999), eight populations of *C. alismatifolia* (Paisooksantivatana *et al*. 2001) and 15 accessions of *C. longa* (Shamina *et al*. 1998). However, only one recent report is available on analysis of 15 species of *Curcuma* using RAPD and ISSR markers (Syamkumar and Sasikumar 2007).

Variability was high in 25 accessions of *C. longa* as the similarity values were at par with those of all accessions including wild species. It implies that natural and conscious selection by breeders/farmers over the years helped to evolve the clones adapted to the local geographical regions. Considerable variability was also observed for polymorphic isozyme loci in 15 accessions of cultivated species *C. longa* by others (Shamina *et al*. 1998). Based on cytological studies, *C. longa* (2n = 32, 62, 64) originated as allotetraploid and developed overtime by selection and vegetative propagation of the wild diploid *C. aromatica* (2n = 42) and closely related tetraploid species; which is probably why long-established local germplasm/cultivars differ so much due to perpetuated variation (Weiss 2002). Variability observed in *C. longa* accessions can be questioned due to likely occurrence of somaclonal variation as *in vitro*-conserved accessions were used in present study. This possibility is ruled out as random accessions of *in vitro*-conserved germplasm of *Curcuma* have been periodically assessed for genetic stability using biochemical and molecular methods; no genetic variation was detected using the shoot tip explants in our laboratory . Grouping of *C. manga* in the cluster of the accessions of *C. longa* could not be resolved; one probable explanation could be the confusion in taxonomic identification based on single or few key characters in species identity in *Curcuma* (Syamkumar and Sasikumar 2007) of this species that needs to be confirmed.

In our study material, 13 morphotypes were included which were identified on the basis of morphological characters from 550 accessions of *Curcuma* species. Further, these morphotypes were classified in six infra-specific groups, *i.e.*, *C. longa* var. *typica*, *C. longa* var. *atypica*, *C. longa* var. *camphora, C. longa* var. *spiralifolia*, *C. longa* var. *musacifolia* and *C. longa* var. *platifolia* (Velayudhan *et al*. 1999). One of the objectives of present study was to verify if the diversity

revealed by the RAPD markers corresponds to that of the above 13 morphotypes conserved in the *In Vitro* Genebank. According to Velayudhan *et al*. (1999) greater similarities were observed in M3 and M4 (long duration and 'Alleppy' type) belonging to *C. longa* var. *typica*, M11 and M13 (medium duration) belonging to *C. longa* var. *atypica*, M6 and M10 (tall types) belonging to *C. longa* var. *musacifolia*, M8 and M18 (distinct plant types with horizontally disposed leaves) belonging to *C. longa* var. *camphora* and M9 belonging to *C. longa* var. *spiralifolia*. On the basis of RAPD analysis of the morphotypes, our results support the existing morphological classification at infra-specific level as proposed by Velayudhan *et al*. (1999), as fairly close correspondence was exhibited in most of the morphotypes.

Discriminating power (D_j) values of a given primer were calculated to assess the efficiency of the primers tested in the present study. The maximum D_j (0.993) for primer OPC-20 with maximum number of banding patterns (27) indicated at the orderly correspondence but the order was not followed for lowest value. The primer OPC-13 with lowest D_j (0.826) produced 14 banding patterns as compared to 9 by the primer OPC-10 having higher D_j (0.867). Our results show that discriminating power of the primers did not only depend on the number of banding patterns it generated but also on the frequencies of the unique banding patterns *e.g.* the primers OPA-03, OPC-05 and OPJ-19, all with 22 bands had different D_j (0.975, 0.961 and 0.979, respectively). On the contrary, the primers OPI-19 and OPM-14 having the same D_j (0.959) but produced 18 and 21 bands, respectively. This result can be explained by the differences in frequency between the banding patterns generated by the primer OPI-19 and OPM-14, as also observed Tessier *et al*. (1999). It was concluded that discriminating power of a given primer can be considered to be a reliable indicator of the efficiency of that primer in diversity analysis.

The RAPD analysis in the present study has proven to be successful in revealing the diversity within and among the species of *Curcuma* as also reported earlier in *Curcuma* spp. (Syamkumar and Sasikumar 2007), *Crocus* spp. (Grilli Caiola *et al.* 2004) and in *Vigna* spp. (Betal *et al.* 2004). The study indicated significant genetic diversity in *Curcuma* germplasm being conserved in the *In Vitro* Genebank at NBPGR, which also corresponds to that of the morphotypes identified on the basis of morphological markers (Velayudhan *et al*. 1999). This study will serve the purpose of germplasm curators to analyse the genetic diversity of large number of accessions of *Curcuma* germplasm maintained in field genebanks and thereafter to select the diverse accessions for cost-effective conservation in the *In Vitro* Genebank for further utilization in crop improvement programmes.

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