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Genetic diversity analysis by RAPD and ISSR markers in turmeric (*Curcuma longa* L.)

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Abstract
The present investigation was undertaken to study the extent of genetic diversity present among 15 genotypes of turmeric (*Curcuma longa* L.) collected from High Altitude Research Station, Pottangi, Koraput representing a wide range of geographic locations of India using the RAPD and ISSR markers. A total of 6 RAPD and one ISSR markers were amplified and scored for bands. Data analysis was done using NTSYS-pc version 2.0 software and dendrograms were constructed following UPGMA and bootstrap analyses. The primers resulted in the amplification of 65 loci and out of these, 64 bands (98.5%) were polymorphic. Among the polymorphic bands, 8 were found to be cultivar specific. Highest resolving power and RAPD primer index was found for the primer OPN 16. Maximum similarity (0.75) was found between cvs. PTS 43 and PTS 51 and cv. RH 5 was clearly distinguished from rest of the cultivars. The genetic variations existed among the cultivars as revealed by RAPD and ISSR analyses might expedite the crop improvement in turmeric.

Keywords: Genetic diversity, RAPD, ISSR, turmeric, *Curcuma longa*

Introduction
Turmeric (*Curcuma longa* L.) is a tropical perennial herbaceous spice crop used from time immemorial for its colour and aroma. It is cultivated extensively in India, Sri Lanka, parts of China and Pakistan for its underground rhizomes to be used as condiment, dye stuff and cosmetic (Rao and Rao, 1994). India is the largest producer and exporter of turmeric in the world. It occupies about 6% of the total area under spices and condiments in India (Palai, 2001). Turmeric powder and its main component, *curcumin* have a wide range of medicinal and culinary uses. It is a stomachic, carminative tonic, blood purifier, vermicide and an antiseptic. It is gaining importance world over as a potential source of new drug to combat a variety of ailments as the species contains molecules credited with anti-inflammatory, antimicrobial, insect repellent, anti-rheumatic, antifibrotic, antivenomous, antiviral, antidiabetic, antihepatotoxic as well as anticancerous properties (Narendran, 2005) [9]. The volatile oil derived from steam distillation of crushed turmeric tubers is of orange-yellow colour. The dried rhizome yields 5-6% of oil and fresh one gives 0.25% essential oil (Krishnamurthy *et al.*, 1975) [7].
Traditionally used morphological and biochemical markers are not reliable, because many characters of interest have low heritability and are genetically complex. Molecular markers are considered best for analysis of genetic diversity and varietal identification since there is no effect on stage of development, environment or management practices. Among many facets of DNA marker technology, DNA fingerprinting has the most pervasive application with increased power of resolution and potential for absolute objectivity. This is manifested with the advent of many reliable molecular markers like RFLP, RAPD, SSR, ISSR, SNP and SSCP etc. Among the various techniques available, RAPD (Random Amplified Polymorphic DNAs) and ISSR (Inter Simple Sequence Repeats) are potentially simple, rapid and reliable DNA fingerprinting methods (Sharma *et al.*, 2017) [17]. Keeping in view of the above facts, the present investigation was undertaken to study the genetic diversity in turmeric cultivars by using RAPD and ISSR markers for their utility in future plant breeding programmes.

Materials and Methods
Fifteen cultivars of turmeric (*Curcuma longa* L.) representing a wide range of geographic locations of India, collected from the High Altitude Research Station, Pottangi, Koraput were taken for the present study (Table 1). Genomic DNA was isolated from the tender leaves of 30 days old seedlings following CTAB (Cetyl trimethyl ammonium bromide) method of Doyle

and Doyle (1990) [2] with little modification. The isolated DNA from each accession was purified by RNAse-A (Bangalore Genei, India) and the quality of DNA was assessed on agarose gel (0.8%). The DNA was quantified through spectrophotometer (Thermoscientific UV-VIS).

PCR amplification was performed in a 25 µl reaction set up containing 1 ng/µl of template DNA, 200 µM each of dNTPs, 20 ng of primer, 1.0 U of *Taq* DNA polymerase and 1x polymerase chain reaction buffer. Six arbitrary decamer RAPD primers and one ISSR primer were used for amplification (Table 2). The amplification of target DNA was done following Williams *et al*. (1990) [22] in a thermal cycler (Perkin Elmer Model 9600) for 45 cycles using temperature profiles of 2 min. initial denaturation at 94ºC, 1 min. denaturation at 94ºC, 1 min. annealing at 37ºC, 2 min. polymerization at 72ºC and 5 min. final extension at 72ºC. PCR products were mixed with 2.5 µl of gel loading dye and electrophoresed on 1.5% agarose gel in 1x TAE buffer (Sambrook *et al*., 1989) [16]. The gel was stained in ethidium bromide (0.5 µg/ml) for 20 min. Then after destaining in distilled water, the gel image was viewed and stored in the gel documentation system (Bio-Rad). DNA fragment sizing and matching was done by directly scoring the images. Individual bands within lanes were assigned to a particular molecular weight comparing with the DNA molecular marker. Total number of bands within each lane and number of polymorphic bands were noted. The amplification products using each primer were checked at least twice for their reproducibility.

RAPD and ISSR banding patterns were scored as '1' for presence of a particular band and as '0' for its absence in each of the primer-genotype combinations. Jaccard's similarity coefficients for each pair-wise comparison between cultivars were calculated to construct the similarity matrix following Jaccard (1908) [5]. The matrix was subjected to unweighted pair group method for arithmetic averages (UPGMA) analysis to generate a dendrogram (Sneath and Sokal, 1973) [19]. The cluster analysis was carried out by using the computer package NTSYS-pc version 2.0 software (Rohlf, 1998) [15]. The computation of NJ (neighbour-joining) trees and robustness of the nodes in the dendrogram were tested by bootstrap analysis in the Free Tree program version 0.9.1.5 software (Pavileek *et al*., 1999) [11].

Resolving power (Rp) of the primers was calculated as $Rp = \Sigma I_b$, where I_b (band informativeness) = $1 - [2x (0.5-p_i)]$ and p_i was the proportion of the genotypes showing i^{th} band as per Prevost and Wilkinson (1999) [12]. Polymorphism information content (PIC) showed the primer efficiency in detecting polymorphism based on number of alleles identified and their frequency (Anderson *et al*., 1993) [1] and was calculated as $PIC = 1-\Sigma p_i^2$, where p_i was the frequency of i^{th} band amplified by the primer. Then the primer index (PI) was estimated as the sum of PIC of all the bands amplified by the same primer (Smith *et al*., 1997) [18].

Table 1: List of turmeric genotypes used in the study and their sources

Sl. No.	Genotypes	Source
1	Acc. 27	IISR, Kozhikode, Kerala
2	Acc. 29	IISR, Kozhikode, Kerala
3	Acc. 31	IISR, Kozhikode, Kerala
4	Acc. 35	IISR, Kozhikode, Kerala
5	Acc. 39	IISR, Kozhikode, Kerala
6	Acc. 40	IISR, Kozhikode, Kerala
7	Acc. 47	IISR, Kozhikode, Kerala
8	PCT 8	IISR, Kozhikode, Kerala
9	PTS 43	HARS, Pottangi, Koraput, Odisha
10	PTS 47	HARS, Pottangi, Koraput, Odisha
11	PTS 51	HARS, Pottangi, Koraput, Odisha
12	PTS 52	HARS, Pottangi, Koraput, Odisha
13	PTS 55	HARS, Pottangi, Koraput, Odisha
14	PTS 57	HARS, Pottangi, Koraput, Odisha
15	RH 5	IISR, Kozhikode, Kerala

Results and Discussion

Six RAPD and one ISSR primers were used for developing DNA profile and evaluating the genetic diversity among 15 cultivars of turmeric. All the primers successfully amplified a total of 65 reproducible bands, of which 64 (98.46%) were polymorphic. A similar polymorphism (95.10%) was reported by Raghunathachari *et al.* (2000) [13] using RAPD technique in 18 accessions of scented rice. The observed high promotion of polymorphic loci suggested that a profound intra-specific variation was existing among the turmeric cultivars. Greater genetic divergent populations revealed by RAPD markers was also reported by Hwang *et al.* (2001) [4] and Jagadev and Jena (2020) [6]. The amplification pattern of all the primers was summarized in Table 2. The number of bands per primer ranged from 5 in OPN 15 to 16 in OPN 16 (Fig. 1) with an average of 9.286 bands per primer. The average number of amplification fragments produced per primer was conforming to that recorded with certain other plants examined analogously (Ho *et al*., 1997) [3]. The size of amplified product ranged from 350 bp to more than 3000 bp. All the primers revealed polymorphism among the turmeric cultivars. The primer, OPN 16 amplified highest number of polymorphic bands (i.e. 16) and other primers, OPA 18, OPD 02, OPD 03, OPD 20 and OPN 15 amplified 8, 10, 11, 7 and 5 polymorphic bands, respectively. The ISSR 1 primer (GACA) 4 amplified 7 polymorphic bands (Fig. 2). Based on the level of polymorphism detected, all the primers except OPN 15 (due to poor amplification) were identified as the informative primers. All the RAPD primers amplified unique bands, but the ISSR primer could not amplify any unique band. The primer index varied from 1.60 (OPN 15) to 6.01 (OPN 16). The ISSR primer has a primer index of 2.24. The resolving power of the six RAPD and one ISSR primers ranged from 2.80 (OPN 15) to 10.99 (OPN 16) (Table 2). Besides high resolving power value, the primer, OPN 16 was able to distinguish all the 15 turmeric cultivars. Several DNA

fragments were amplified in each sample. While some of these segments were common among the samples, some segments were amplified from one individual (cultivar specific) band. For example, a 2900 bp DNA segment was only amplified by primer OPN 16 from Acc. 29 (Fig. 1). Most of the PCR products were in the size range of 900 bp to 1900 bp. The cultivar RH 5 could be identified from rest of the cultivars due to absence of a band (Fig. 2). A relatively high genetic variation was detected among the turmeric cultivars. The similarity indices estimated on the basis of both the RAPD primers and ISSR primer ranged from 0.031 (between RH 5 and ACC 40) to 0.750 (between PTS 51 and PTS 43 (Table 4c) with an average similarity of 0.306 between cultivars suggesting the existence of high level of genetic diversity due to the collection of genotypes from various parts of the country.

The RAPD data cluster analysis (Fig. 3) based on Jaccard's coefficients using UPGMA, classified all the 15 turmeric cultivars into two major groups *viz.*, I and II. The group I consisted of PTS 57 and RH 5 and the group II consisted of two sub-clusters *viz*., IIA and IIB. The sub-cluster IIA comprised of Acc. 40, Acc. 27 and Acc. 29 in which a well supported (96% bootstrap support) sister cluster including Acc. 27 and Acc. 29 was recognized. The sub-cluster IIB comprised of IIB1 and IIB2 and the IIB1 contained Acc. 31, PTS 47 and PTS 52, while IIB 2 contained 7 cultivars *viz.*, Acc. 47, Acc. 35, Acc. 39, PCT 8, PTS 55, PTS 43 and PTS 51 in which a well supported sister cluster (71% bootstrap support) including PTS 43 and PTS 51 was recognized.

Similarly the ISSR data cluster analysis (Fig. 4) classified all the cultivars into two groups *viz.*, I and II. The group I included only RH 5 and the group IIA included only PTS 47. The group IIB comprised of two sub-clusters *viz*., IIB1 and IIB2 and the sub-cluster IIB1 contained Acc. 27, PTS 52, Acc. 40, Acc. 39, Acc. 29 and Acc. 31, while IIB2 contained Acc. 35, PTS 55, PTS 57, PTS 51, PTS 43. Acc. 47 and PCT 8 in which a well supported (77% bootstrap support) sister cluster including PTS 51 together with PTS 43, Acc. 47 and PCT 8 was recognized. Similarly, in the RAPD and ISSR combined data analysis (Fig. 5), group I contained RH 5 and PTS 57 while IIA included Acc. 40, Acc. 27 and Acc. 29 in which a well supported (95% bootstrap support value) sister-cluster comprising Acc. 27 and Acc. 29 was recognized. The sub-cluster IIB1 contained Acc. 31, PTS 47 and PTS 52, while IIB2 contained Acc. 35, Acc. 39, PTS 55, PCT 8, Acc. 47, PTS 43 and PTS 51. We found RAPD profiling was efficient enough to reveal usable level of DNA polymorphism among the chosen genotypes, which was supported by various workers with earlier observations (Virk *et al.*, 1995; Mongkolporn *et al*., 2004, Srivastava *et al*., 2004 and Sharma *et al*., 2017) [17].

The considerable polymorphism detected in this study illustrated that genetic divergence could be found among turmeric cultivars of the same origin. Further, the investigation demonstrated that the high levels of genetic polymorphism and genetic differentiation as revealed by RAPD and ISSR analyses might play an important role in the dynamic genetic variations of turmeric cultivars. The consensus fingerprint profiling was useful for the estimation of genetic variability between the turmeric cultivars. Hence, the approaches used in the present study would make a valuable contribution to the conservation of crop genetic resources. The use of RAPD and ISSR markers in conjunction with appropriate statistical tools would provide a firm basis for molecular characterization in further improvement of turmeric genotypes.

Table 2: Details of RAPD and ISSR primers used for the molecular characterization of 15 cultivars of turmeric

Primer type	Primer code	Primer sequence (5' to 3')	Range of amplified product size (bp)	Total no. of bands	Monomorphic bands	Polymorphic bands	Polymorphism (%)	Unique band (s)	Genotype(s) with unique band	Resolving powder	Primer index
RAPD	OPA 18	SAGGTGACCGT	650-3000	8	0	8	100.0	1	PCT-8	6.13	3.25
	OPD 02	GGACCCAACC	1100-3000	10	0	10	100.0	1	PCT-8	5.60	3.18
	OPD 03	GTCGCCGTCA	500-2600	11	0	11	100.0	1	RH 5	6.00	3.68
	LPD 20	ACCCGGTCAC	900-2000	7	0	7	100.0	1	RH 5	6.67	2.49
	OPN 16	AAGCGACCTG	350-2900	16	0	16	100.0	2	ACC 29	10.99	6.01
	OPN 15	CAGCGACTGT	600-1400	5	0	5	100.0	2	ACC 47	2.80	1.60
ISSR	ISSR1	(GACA)4	700-1900	8	1	7	87.5	0	ACC 39	7.77	2.24

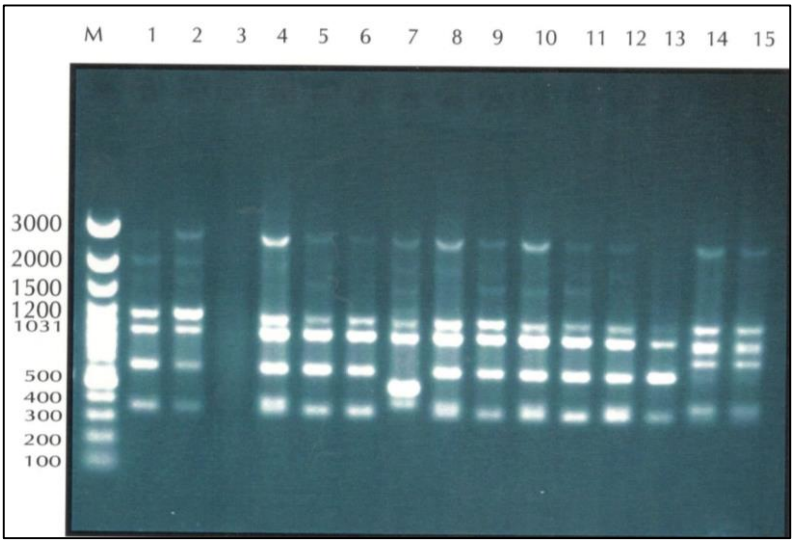

Fig 1: RAPD profile of turmeric generated using primer OPN 16. Lane numbers correspond to turmeric cultivars given in Table 1. M = Molecular weight marker, numbers on the left hand margin represent the molecular weight markers in bp.

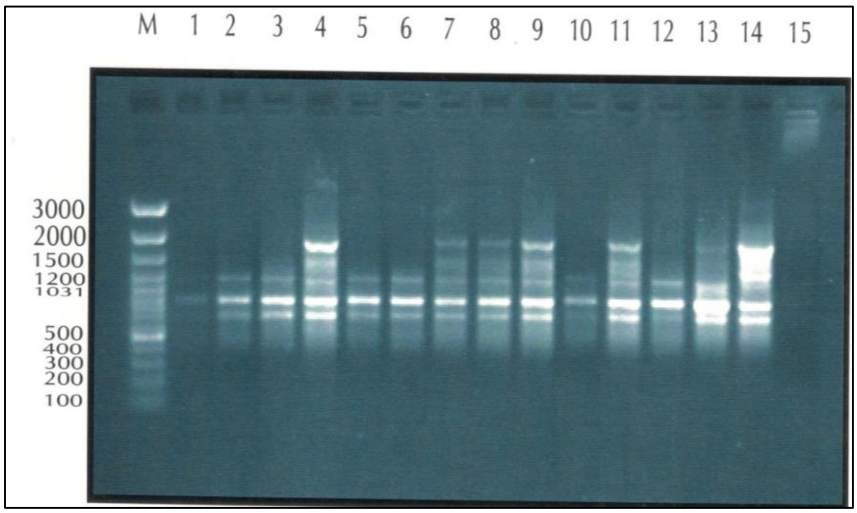

Fig 2: ISSR profile of turmeric generated using primer ISSR 1. Lane numbers correspond to turmeric cultivars given in Table 1. M = Molecular weight marker, numbers on the left hand margin represent the molecular weight markers in bp.

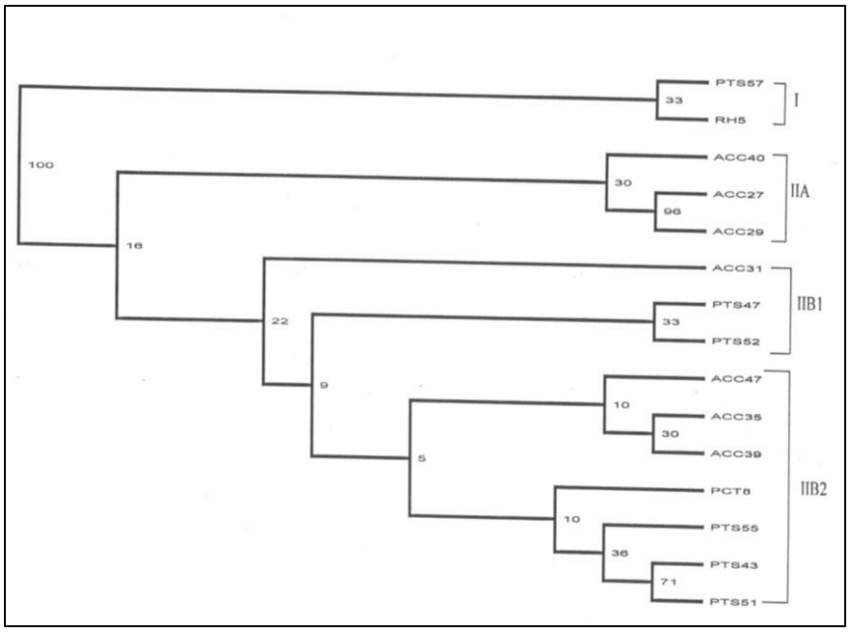

Fig 3: Dendrogram constructed on the basis of RAPD data. The numbers at the nodes indicate the bootstrap percentages for occurrence of the branches to the right of the nodes. The distances are reflected in the branch lengths

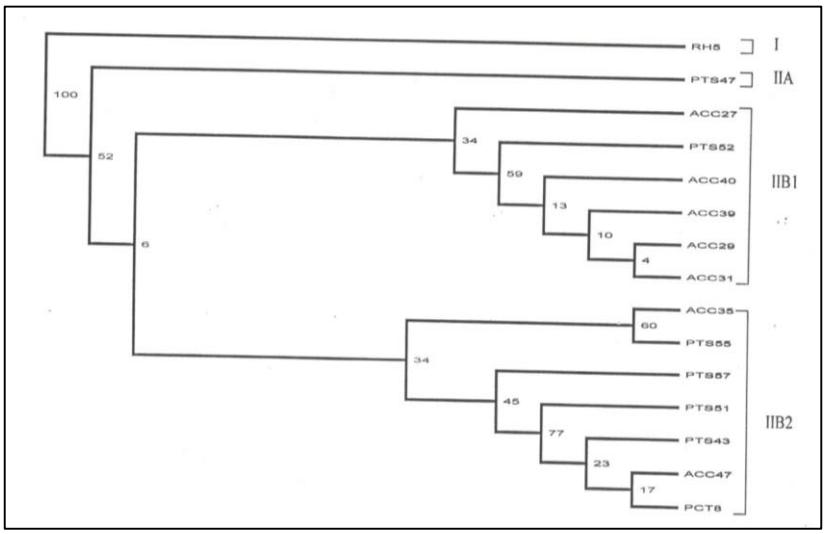

Fig 4: Dendrogram constructed on the basis of ISSR data. The numbers at the nodes indicate the bootstrap percentages for occurrence of the branches to the right of the nodes. The distances are reflected in the branch lengths

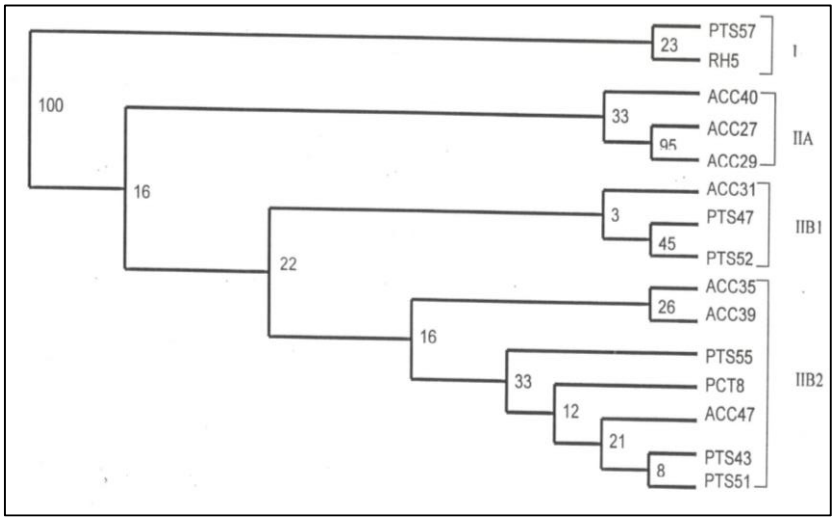

Fig 5: Dendrogram constructed on the basis of RAPD and ISSR data. The numbers at the nodes indicate the bootstrap percentages for occurrence of the branches to the right of the nodes. The distances are reflected in the branch lengths.

Table 3: Clustering pattern of 15 turmeric cultivars as per RAPD, ISSR and combined similarity matrix data

Major cluster No.	Sub-cluster No.	Name of genotypes		
		RAPD	ISSR	Combined (RAPD and ISSR)
I	I	PTS 57, RH 5	RH 5	PTS 57, RH 5
II	IIA	Acc. 27, Acc. 29, Acc. 40	PTS 47	Acc. 27, Acc. 29, Acc. 40
	IIB1	Acc. 31, PTS 47, PTS 52	Acc. 27, Acc. 29, Acc. 31, Acc. 39, Acc. 40, PTS 52	Acc. 31, PTS 47, PTS 52
	IIB2	Acc. 35, Acc. 39, Acc. 47, PTS 43, PTS 51, PTS 55, PCT 8	Acc. 35, Acc. 47, PTS 43, PTS 51, PTS 55, PTS 57, PCT 8	Acc. 35, Acc. 39, Acc. 47, PTS 43, PTS 51, PTS 55, PCT 8

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