

Detection and Evaluation of Genetic Variation in 17 Promising Cultivars of Turmeric (*Curcuma longa* L.) Using 4C Nuclear DNA Content and RAPD Markers

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Summary Rhizome yield, 4C nuclear DNA content and RAPD analysis were carried out of 17 promising cultivars of turmeric (*Curcuma longa* L.). The rhizome yield per plant varied significantly from 77.66 to 350 g among 17 cultivars of *Curcuma longa*. Significant variation of 4C DNA content was recorded at the intraspecific level with values ranging from 4.30 to 8.84 pg. The differential DNA content observed among 17 different cultivars of *Curcuma longa* comprising same ($2n=48$) chromosome number could be attributed to the loss or addition of highly repetitive sequences in the genome. Random amplified polymorphic DNA (RAPD) analysis clearly revealed genetic variation among 17 cultivars of turmeric showing differential polymorphism using 20 primers. The amplification fragments per primer ranged from 4 to 17 in 17 cultivars with fragment size ranging from 0.4 kb to 3 kb. Intraspecific polymorphism ranged from 35.6% to 98.6% among 17 cultivars studied. The RAPD primers OPN06 and OPA04 having strong resolving power were able to distinguish all 17 cultivars. The extent of genetic diversity among 17 cultivars was computed through Nei's genetic similarity and genetic distances. The genetic variations detected through 4C nuclear DNA content and RAPD analysis have significance for turmeric improvement programmes.

Key words Turmeric cultivars, Nuclear DNA content, RAPD.

Turmeric (*Curcuma longa* L.) of the family Zingiberaceae is an important spice; well known as condiment and colouring agent since time immemorial (Anonymous 1950). In Indian system of medicine turmeric is used as stomachic, tonic and blood purifier. It possesses unique combination of properties like antioxidant, hepatoreactive and anticancer effect (Roses 1999). Oil of turmeric has got antifungal, anti-inflammatory and antiarthritic activity and acts as antacid, carminative and appetisers (Anonymous 1950). India enjoys monopoly in production and export of this crop. Turmeric is an asexually propagated crop displaying great morphological diversity. More than 50 cultivars of *C. longa* are known by different trade names. Until recently, several promising cultivars released formally are characterized on the basis of morphological data, curcumin content and yield potential. Worldwide demand on turmeric (Roses 1999) necessitate work on conservation of turmeric germplasm and their further genetic improvement. Therefore precise characterization of promising cultivars and determination of genetic variation among those are felt necessary. Until recently, several promising cultivars released formally are characterized on the basis of morphological data, curcumin content and yield potential. These characters differ under varying environmental condition thereby posing problem in proper identification of cultivars. Unlike morphological mark-

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ers, cytological (chromosome numbers, nuclear DNA content) and molecular markers (RAPD, AFLP, ISSR etc.) are not prone to environmental influences and accurately characterize the plants portraying the extent of genetic diversity among taxa (Bennett 1987, Bennett and Smith 1991, Waugh and Powell 1992, Chalmers *et al.* 1994, Das *et al.* 1998, Rodriguez *et al.* 1999, Das *et al.* 2001). Of the different markers, RAPD has been widely used in the last decade in cultivar identification programme (Schnell *et al.* 1995) and in assessing genetic variations among different taxa at DNA level because of its cost effectiveness and simple operation without requiring prior knowledge of species DNA sequences (Williams *et al.* 1990, Frankel *et al.* 1995). RAPDs reveal similar patterns of genetic diversity when compared with other marker types and can be performed more rapidly than most other methods (Morell *et al.* 1995) and can provide vital information for the development of genetic sampling, conservation and improvement strategies (Waugh and Powell 1992, Chalmers *et al.* 1994). No report has yet been published so far either on the genetic characterization or on the extent of genetic variations existing among promising cultivars of turmeric using 4C DNA content and RAPD analysis.

The present study deals with the in situ DNA estimation and RAPD analysis of 17 promising cultivars of turmeric to identify and evaluate extent of genetic variation existing among these.

Materials and methods

Rhizomes of 17 cultivars of turmeric i.e. Acc29, Acc27, Acc39, Acc35, Acc43, Acc40, Acc31, Acc47, TU6, PTS43, PTS47, PTS52, PTS51, PTS55, PTS59, RH5 and PCT8 were collected from turmeric germplasm collection of Orissa University of Agriculture and Technology (O.U.A.T.), Bhubaneswar, Orissa, India. Of these 17 cultivars of turmeric, cultivars TU6, RH5 and PCT8 were initially brought from the state of Bihar, India, whereas rest were collected from the same state, Orissa. These cultivars of *Curcuma longa* comprised $2n=48$ chromosomes. Rhizomes of all cultivars were planted in the field at O.U.A. T, Bhubaneswar (29 m above MSL) during June, 2002 and were harvested after 8 months of growth i.e. during February, 2003. Mean rhizome yield per plant was calculated taking average of 3 clumps during harvest.

For Feulgen cytophotometric estimation of 4C DNA, ten fixed root tips from each cultivar were hydrolysed in 1N HCL for 12 min at 60°C, washed in distilled water and stained in Schiff's reagent for 2 h at 14°C; each root-tip squash was prepared in 75% acetic acid. Ten scorings were made from each slide and 4C DNA was estimated from metaphase chromosomes using NIKON Optiphot microscope with microspectrophotometer following the method of Sharma and Sharma (1980) with monochromatic light at 550 nm. In situ DNA values were obtained on the basis of optical densities which were converted to picograms (pg) by using Van't Hof's (1965) 4C nuclear DNA value (67.1 pg) for *Allium cepa* as standard. Cultivar N-53 of *A. cepa* was used in our experiment. ANOVA were performed among the nuclear DNA values to find out the differences at cultivar level.

Nuclear DNA extraction

Genomic DNA was isolated from fresh and young leaves. Leaves were harvested freshly and washed thoroughly with cold autoclaved water and then blotted to dry. About 2 g leaf was excised from the upper tip portion of the buds. DNA was isolated following the protocol of Doyle and Doyle (1990) with little modification. Insoluble polyvinyl polypyrrolidone was added to the leaf tissue prior to grinding. The crude DNA was purified with RNase A 60 $\mu\text{g ml}^{-1}$ of DNA solution followed by washing thrice with purified chloroform: isoamyl alcohol (24:1). To test the quality and quantity of the purified DNA, the samples were electrophoresed in 0.8% agarose gel along with known amount of uncut lambda DNA (Bangalore Genei pvt. Ltd, Bangalore, India) as standard. The sample DNA was diluted as 25 ng μl^{-1} for RAPD-PCR analysis.

Table 1. Rhizome yield, 4C nuclear DNA content and extent of DNA polymorphism in 17 cultivars of *Curcuma longa*

Cultivars	Rhizome yield (g) mean±SD	4C nuclear DNA content (pg)	Number of polymorphic bands*	% of polymorphic bands
ACC29	208.33±7.63	8.10±0.10	46	73.0
ACC27	244.33±5.13	8.58±0.06	31	39.7
ACC39	255.12±5.22	7.31±0.07	38	41.8
ACC35	305.00±5.00	6.81±0.05	60	65.2
ACC43	262.66±5.50	6.07±0.04	59	58.4
ACC40	185.23±6.31	7.57±0.05	52	61.9
ACC31	259.00±6.55	6.86±0.04	69	98.6
ACC47	235.66±5.13	7.16±0.09	40	55.5
TU6	232.33±7.02	5.73±0.08	71	60.7
PTS43	350.00±8.35	6.27±0.13	69	68.3
PTS47	157.00±5.56	5.30±0.05	36	52.9
PTS52	266.00±5.53	5.13±0.08	41	70.7
PTS51	250.00±8.00	5.53±0.10	26	35.6
PTS55	313.33±7.23	8.37±0.08	55	63.2
PTS59	77.66±5.50	8.84±0.04	40	55.5
RH5	266.33±6.02	4.83±0.06	81	71.7
PCT8	151.66±8.5	4.30±0.07	85	82.5

* SD value was zero since number of polymorphic bands in different cultivars remained constant in repeated experiments.

Table 2. ANOVA of rhizome yield and 4C DNA content in 17 cultivars of *Curcuma longa*

Source of variance	Degree of freedom	Sum of square	Mean square	Variance component
(a) Rhizome yield per plant				
Between cultivars	16	222455.3	13085.6	266.15**
Residual	34	1770.0	49.17	—
Total	50	224225.3		
(b) 4C nuclear DNA content				
Between cultivars	16	92.345	5.772	293.17**
Residual	34	0.669	0.0197	
Total	50	93.014		

** Significant at $p=0.01$ level of significance.

RAPD amplification

Twenty random decamer primers (Operon Tech., USA) from A, C, D and N series (OPA02, 03, 04, 06, 10; OPC02, 05, 06; OPD03, 07, 08, 18, 20 and OPN05, 06, 07, 08, 15) were used for RAPD analysis. RAPD assays were performed in a final volume of 25 μ l containing (10 mM Tris-HCL pH 9.0); 1.5 mM MgCl₂; 50 mM KCL and 0.01% gelatin; 200 μ M of each dNTPs; 0.4 M primer; 25 ng template DNA and 0.5 unit of Taq DNA polymerase (Bangalore Genei; Bangalore, India). The RAPD analysis was performed as per the methodology described by Williams *et al.* (1990) using Gene Cycler, Bio Rad, USA. The first cycle consisted of denaturation of template DNA 94°C for 5 min, primer annealing 37°C for 1 min and primer extension at 72°C for 2 min. In the next 42 cycles the period of denaturation was reduced to 1 min at 92°C while the primer annealing and primer extension time remained same as in the first cycle. The last cycle consisted of only primer extension (72°C) for 7 min. The reactions ended with an indefinite hold at 4°C.

Amplification products were electrophoresed in 1.5% agarose gels containing ethidium bromide 0.5 μ g ml⁻¹ in TAE buffer (40 mM Tris base, 20 mM sodium acetate, 20 mM EDTA, glacial acetic acid to pH 7.2) for 3 h at 60 volts. A total of 2.5 μ l loading buffer (1.0X TAE, 50% glycerol,

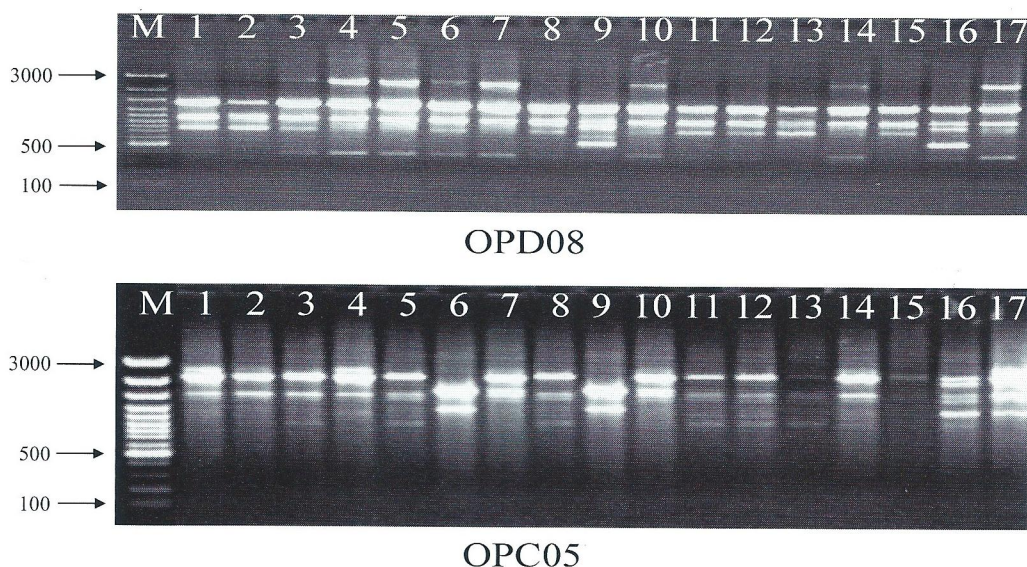


Fig. 1. RAPD amplification products obtained from the 17 cultivars studied: 1 Acc27, 2 Acc29, 3 Acc31, 4 Acc35, 5 Acc39, 6 Acc40, 7 Acc43, 8 Acc47, 9 Pts 43, 10 Pts47, 11 Pts51, 12 Pts52, 13 Pts55, 14 Pts59, 15 TU6, 16 RH5, 17 PCT8. M=the weight of molecular markers.

0.25% bromophenol blue and 0.25% Xylene Cyanol) was added to each reaction before electrophoresis. After electrophoresis, gels were observed under UV-illuminator, documented in Gel-Doc 2000 (Bio-Rad, USA) and photographed.

Resolving Power

According to Prevost and Wilkinson (1999) the Resolving power (R_p) of a primer is: $R_p = \sum IB$ where IB (band informative ness) takes the value of: $1 - [2 \times (0.5 - p)]$, p being the proportion of the 17 genotypes (turmeric cultivars analyzed) containing the band.

RAPD data analysis

In RAPD analysis, the presence or absence of the bands were taken into consideration, but the differences in their intensity were ignored. POPGENE software was used to calculate Nei's unbiased genetic distance between cultivars with all markers (derived from 20 random primers) (Nei 1973). Total gene diversity among 17 cultivars could be calculated using this software.

Results

Rhizome yield

The rhizome yield per plant of 17 cultivars of turmeric varied significantly from 77.66 g in PTS59 to 350 g in PTS43 after first harvest (Tables 1, 2).

4C nuclear DNA content

The 4C nuclear DNA amount of all 17 promising turmeric cultivars are mentioned in Table 1. The 4C nuclear DNA content was found to be lowest (4.3 pg) in PCT8 and highest (8.84 pg) in PTS59. The ANOVA test showed that the variation in the nuclear DNA content among 17 cultivars of turmeric was significant at 1% level (Table 2).

Table 3. Nei's original measures of genetic identity and genetic distance between different cultivars of *Curcuma longa*

pop ID	Acc27	Acc29	Acc31	Acc35	Acc39	Acc40	Acc43	Acc47	Pts43
Acc27	****	0.8883	0.8715	0.7933	0.7989	0.8715	0.7654	0.8436	0.7654
Acc29	0.1185	****	0.8603	0.7598	0.7542	0.8603	0.7095	0.8324	0.7430
Acc31	0.1375	0.1504	****	0.8212	0.8156	0.8883	0.7709	0.8715	0.8045
Acc35	0.2316	0.2747	0.1970	****	0.9497	0.8547	0.8939	0.8156	0.8380
Acc39	0.2245	0.2821	0.2038	0.0516	****	0.8492	0.9106	0.8324	0.8436
Acc40	0.1375	0.1504	0.1185	0.1569	0.1635	****	0.8268	0.8939	0.8156
Acc43	0.2674	0.3432	0.2601	0.1122	0.0936	0.1902	****	0.7989	0.8436
Acc47	0.1701	0.1834	0.1375	0.2038	0.1834	0.1122	0.2245	****	0.7877
Pts43	0.2674	0.2970	0.2176	0.1768	0.1701	0.2038	0.1701	0.2386	****
Pts47	0.1375	0.1375	0.1834	0.3122	0.2747	0.1970	0.3199	0.1504	0.3046
Pts51	0.1440	0.1311	0.1902	0.2747	0.2529	0.1902	0.3122	0.2107	0.2529
Pts52	0.1504	0.1375	0.1701	0.2821	0.2457	0.1834	0.3199	0.1248	0.3199
Pts55	0.1970	0.2245	0.1902	0.1635	0.1311	0.1635	0.1970	0.1440	0.1970
Pts59	0.1504	0.1504	0.1834	0.2245	0.1902	0.1440	0.2895	0.1504	0.2747
Tu6	0.3752	0.4779	0.3671	0.2747	0.2674	0.3354	0.2970	0.3752	0.1701
Rh5	0.4512	0.5053	0.5146	0.5527	0.5239	0.5146	0.5239	0.5239	0.4338
Pct8	0.5334	0.5146	0.4689	0.5053	0.5146	0.5239	0.4600	0.4960	0.4779

pop ID	Pts47	Pts51	Pts52	Pts55	Pts59	Tu6	Rh5	Pct8
Acc27	0.8715	0.8659	0.8603	0.8212	0.8603	0.6872	0.6369	0.5866
Acc29	0.8715	0.8771	0.8715	0.7989	0.8603	0.6201	0.6034	0.5978
Acc31	0.8324	0.8268	0.8436	0.8268	0.8324	0.6927	0.5978	0.6257
Acc35	0.7318	0.7598	0.7542	0.8492	0.7989	0.7598	0.5754	0.6034
Acc39	0.7598	0.7765	0.7821	0.8771	0.8268	0.7654	0.5922	0.5978
Acc40	0.8212	0.8268	0.8324	0.8492	0.8659	0.7151	0.5978	0.5922
Acc43	0.7263	0.7318	0.7263	0.8212	0.7486	0.7430	0.5922	0.6313
Pts47	0.8603	0.8101	0.8827	0.8659	0.8603	0.6872	0.5922	0.6089
Pts43	0.7374	0.7765	0.7263	0.8212	0.7598	0.8436	0.6480	0.6201
Pts47	****	0.8939	0.8883	0.7933	0.8324	0.6257	0.6536	0.5922
Pts51	0.1122	****	0.8715	0.8101	0.8603	0.6648	0.6145	0.5531
Pts52	0.1185	0.1375	****	0.8268	0.8771	0.6145	0.5754	0.5698
Pts55	0.2316	0.2107	0.1902	****	0.8045	0.7654	0.6369	0.5531
Pts59	0.1834	0.1504	0.1311	0.2176	****	0.6592	0.5866	0.5810
Tu6	0.4689	0.4083	0.4869	0.2674	0.4167	****	0.6480	0.5419
Rh5	0.4252	0.4869	0.5527	0.4512	0.5334	0.4338	****	0.5810
Pct8	0.5239	0.5923	0.5624	0.5923	0.5430	0.6127	0.5430	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

RAPD analysis

RAPD analysis was carried out of 17 cultivars using 20 RAPD primers. The amplification fragments per primer ranged from 4 to 17 in 17 cultivars with fragment size range from 0.4 kb to 3 kb (Fig. 1). Polymorphism was found to be highest in ACC31 showing 98.6% polymorphic bands and lowest in PTS51 showing 35.6% polymorphic bands (Table 1). Fig. 1 shows the amplification profiles obtained with two of the primers i.e. OPD08 (Rp 8.117) and OPC05 (Rp 6.294) which are used to amplify segments of genomic DNA from all 17 cultivars. The RAPD profiles were reproducible, and displayed no variation when studied in triplicate per species as well as per primer.

Analysis of genetic diversity

A relatively high genetic variation was detected among the turmeric cultivars (Table 3). The values of genetic similarities ranged from 0.551 between PCT8 and both PTS55 and TU6. Like-

wise, high genetic variation ranging from 0.6154 (in between PTS52 and TU6) to 0.9497 (in between Acc31 and Acc35) (Table 3).

Discussion

Differential rhizome yield

Seventeen promising cultivars of turmeric obtained from OUAT showed significant variation in their rhizome yield per plant when analysed after 8 months of growth in first harvest. The existing variation among 17 cultivars quantified through rhizome yield were proved to have genetic basis as revealed by diversification of DNA amount and differential polymorphism of DNA in different cultivars using RAPD markers.

Diversification in DNA amount

The 4C DNA values were reported for the first time in 17 cultivars of turmeric. Significant differences of 4C DNA were recorded between the cultivars showing minimum 4.3 pg to maximum 8.84 pg nuclear DNA content. Such intraspecific variations are in close agreement with the reports of other workers (Furuta *et al.* 1975, Price *et al.* 1980, Das *et al.* 1998). The constancy in the DNA amount at the cultivar level in the repeated experiments revealed the stable 4C DNA content in each cultivar of turmeric. Though all 17 cultivars studied comprised constant chromosome number ($2n=48$), the DNA amount differed significantly and the differences in the DNA content depend on the repetitive DNA amount (Flavell *et al.* 1977). The variability in the DNA content in different cultivars might be attributed to the loss or addition of many repeats in the genomes through alterations in the micro- and macro-environment during evolution in the selection of new cultivars (Price *et al.* 1980, Das *et al.* 1998). The variability of DNA amount could be attributed to loss or addition of highly repetitive DNA sequences rather than AT- or GC-rich sequences in a genome (Martel *et al.* 1997) which reached a certain level and got stabilized during micro-evolution and gradual selection.

Differential polymorphism of DNA

The result presented in the present study demonstrated the utility of using RAPD markers to characterize genetic diversity among 17 promising cultivars of *C. longa*. Differential polymorphism was noted in 17 cultivars of turmeric showing variation in percentage of polymorphic bands from 35.6% in PTS51 to 98.6% in Acc31 using 20 primers. The observed high proportion of polymorphic loci reveals profound intraspecific variation among the turmeric cultivars. Significant genetic variations by RAPD markers have also been reported in other species at cultivar level (Colombo *et al.* 1998, Das *et al.* 1998, Huang *et al.* 2003). Wide genetic distances determined by Nei's genetic distance reveals relatively high genetic variation among 17 cultivars. The considerable polymorphism detected in the present study also illustrated genetic diversity among turmeric cultivars of the same origin as reported among coffee cultivars (Sera *et al.* 2003). The observed intraspecific differences among 17 turmeric cultivars could be ascribed to the fluctuating micro and macro climatic conditions of habitat. Nei's analysis of RAPD data also reveals that all turmeric cultivars belonging to the state of Orissa are genetically more closer than the cultivars (TU6, RH5 and PCT8) originally belonging to the distant habitat in the state of Bihar. The greater sensitivity of RAPDs obtained in the result to cultivar diversity may be derived from rapid evolution of non-coding, repetitive DNA sequences detected by RAPDs. This hypothesis has been corroborated from Plomion *et al.* (1995).

Thus our results demonstrate that 4C DNA content and RAPD marker provide an effective tool for detection and evaluation of genetic variation existing among 17 promising cultivars of turmeric.

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