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Multivariate based marker analysis in turmeric (*Curcuma longa* L.)

K. R. Vijayalatha and N. Chezhiyan
Horticultural College and Research Institute
Tamil Nadu Agricultural University, Coimbatore –641003, India.
E-mail: viji_k_r@yahoo.com

ABSTRACT

Genetic diversity of 30 accessions of turmeric was assessed at the molecular level and compared to morphological traits for degree of divergence. The pattern of clustering of quantitative data based on D^2, K means and UPGMA revealed discrepancy among them. The cluster profile, based on quantitative data and RAPD markers exhibited considerable levels of congruence between them. Accessions studied for degree of divergence by RAPD profiles revealed 68.50% polymorphism for 21 primers. The highest number of fragments (10) was obtained with primer OPG 19 while OPC 18 was completely monomorphic. Primers OPB 08, OPC 20, OPE 09 and OPG 19 detected a high level of polymorphism (> 90%). Discrepancy observed at both morphological and molecular levels in the accessions emphasizes the need for specific morphological and molecular markers for discriminating these accessions.

Key words : Genetic divergence, turmeric, marker, polymorphism, primer

INTRODUCTION

The turmeric of commerce is dried rhizome of *Curcuma longa* L. (syn. *Curcuma domestica* Val.) belonging to the family Zingiberaceae and traces its origin to tropical rain forests of South East Asia. Turmeric, the Indian saffron, is mainly valued for its colouring constituent, 'curcumin', which is indispensable in food industry, confectionery, pharmaceuticals and cosmetics (Zachariah and Babu, 1992). In recognition of the importance of turmeric, emphasis on evolving new varieties with high yield and quality has been in the forefront of research.

Germplasm characterization is an important link between conservation and utilization of plant genetic resources. For any breeding programme, genetic diversity is raw material to the breeder since genetic variation determines the potential for selection and is useful in resolving phylogenetic relationships. However, existence of multiple local names and lack of authentic identity of materials have narrowed genetic resource base. Conventional taxonomic techniques in conjunction with molecular biology tools may help resolve taxonomic confusion prevailing in the genus. Though a few studies on morphological and anatomical characterization of *Curcuma* species have been attempted, not much has been done on molecular characterization (Sasikumar, 2005).

Recently, use of molecular markers has assumed great significance in germplasm characterization and assessment of genetic diversity. Universal acceptance of PCR-based markers has accelerated the use of molecular markers, more specifically DNA based markers, that are valid in assessing genetic diversity as these are genetically stable and detectable during all stages. Hence, an effort was made to understand quantitative relationship and genetic relation using multivariate statistical tools and RAPD markers.

MATERIAL AND METHODS

Two hundred and twenty three accessions of turmeric drawn from different states of India are maintained in the germplasm pool at Department of Spices and Plantation Crops, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore and were used as the experimental material.

Plant material : The number of accessions in the germplasm was too high to assess the divergence at a molecular level. The D^2 statistic was found to be a useful tool in grouping the accessions phenotypically. Therefore, all the 223 accessions were subjected to Mahalonobis D^2 statistic to cull out variable parameters. The accessions clustered into two groups, exhibiting a wide variability for eight parameters of

importance, among the 22 quantitative traits evaluated. Further D^2 statistic was performed based on eight parameters, to group the accessions into five clusters. Thirty accessions were selected to represent each cluster, based on growth and yield performance. The accessions were raised in randomized block design and replicated twice. Biometrical observations were recorded on five randomly tagged plants. Details of accessions are furnished in Table 1.

DNA extraction

Molecular profiling of selected accessions was done using RAPD marker. DNA from all the 30 accessions was extracted using the protocol described by McCouch *et al* (1988) from leaves frozen at –20°C.

RAPD analysis

Genomic DNA from 30 accessions was amplified using a set of 20 arbitrary oligonucleotide decamer primers (Operon Technologies, Alameda, Calif., USA) (Table 1). Amplification reactions were in volumes of 20 ml containing 10 mm Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 per cent gelatin, dNTPs each at 0.1 mM, 0.2 mM primer, 25-30 ng of genomic DNA and 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore). Amplifications were performed in 0.2 ml thin-walled PCR tubes in a PTC 100 thermal cycler (MJ Research Inc.) programmed for 35 cycles. After initial denaturation for 2 min at 92°C, each cycle consisted of 1 min at 92°C, 1 min at 36°C and 2 min at 72°C. These 35 cycles were followed by 7 min. final extension at 72°C.

Table 1. Accessions selected by D^2 statistic

S. No.	Accession number	Accession
1	2	BS - 2
2	15	BS - 16
3	22	BS -23
4	47	BS - 48
5	52	BS - 53
6	75	BS - 76
7	88	BS - 89
8	89	BS - 90
9	114	BS - 115
10	120	Kanthi
11	121	Shoba
12	122	VK 5
13	130	Sudarshana
14	131	Suguna
15	132	Suvarna
16	133	Roma
17	134	Kalimpong
18	146	Alleppey
19	148	PTS 12
20	151	Prabha
21	152	Prathibha
22	156	PTS 2
23	169	Erode local
24	175	Erode local
25	184	Erode local
26	189	CO 1
27	194	JTS 2
28	195	Rajendrasonia
29	198	PTS 55
30	209	Erode local

PCR amplified products (15 ml) were subjected to electrophoresis in 1.5% agarose gel in 1 x TBE buffer at 120 V for 3.5 h using Apelex electrophoresis unit.

Statistical analysis

Amplified DNA fragments detected upon electrophoretic separation in each genotype were scored for presence (1) or absence (0) of clear and unambiguous bands. A data matrix comprising '1' and '0' was formed and this data matrix was subjected to further analysis.

Two different sets of data gathered (quantitative and RAPD marker) were subjected to cluster analysis. The binary data generated from RAPD marker data set was subjected to Sequential Agglomerative Hierarchical Non-Overlapping (SAHN) clustering. UPGMA (Unweighted Pair Group Method with Arithmetic Average) dendrogram was constructed based on Jaccard's similarity coefficient matrix.

The quantitative data were also subjected to cluster analysis using D^2 statistic (Mahalonobis, 1936) and K means (Sneath and Sokal, 1973) based on Euclidean distance and UPGMA method based on squared Euclidean distance.

RESULTS AND DISCUSSION

The D^2 statistic identified eight variable parameters and clustered them into five groups. Thirty accessions were selected from the five clusters (Table 1). On ranking the characters, relative contribution of yield (65.65%), followed by girth of secondary rhizomes (10.46%), weight of primary and secondary rhizomes (8.57% and 7.77% respectively) and days to maturity (6.63%) were the highest contributors to total divergence, while plant height, number of leaves and number of tillers contributed the least to divergence. Hence, seventeen accessions of cluster 1, ten accessions of cluster 5 and single genotype from cluster 2, 3 and 4 were shortlisted as the experimental material and were compared at the morphological and molecular level (Table 2).

Table 2. Cluster mean values of eight characters for turmeric accessions

Cluster / character	1	2	3	4	5	% contribution
Number of accessions	143	2	2	2	74	
Plant height (cm)	37.36	34.07	32.61	32.46	34.06	0.08
Number of leaves	15.03	12.04	11.38	11.75	12.84	0.22
Number of tillers	3.68	1.67	1.75	1.71	1.74	0.60
Days to maturity	248.71	233.25	242.75	251.50	235.38	0.63
Weight of primary rhizomes (cm)	100.89	92.50	127.08	87.09	106.51	8.57
Weight of secondary rhizomes (cm)	67.44	71.67	75.00	61.25	71.38	7.77
Girth of secondary rhizomes (cm)	5.11	4.42	4.46	4.21	4.19	10.46
Yield (kg/plot)	3.77	2.96	2.78	2.70	3.65	65.65

Table 3. Cluster composition of turmeric genotypes based on D^2 and K means cluster analysis

Cluster No.	Cluster members based on D^2 statistic	Cluster members based on K means
1	BS 2, BS 76, Kalimpong	BS 2, BS 16, BS 23, BS 53, BS 89, BS 90, Prathibha, PTS 2, JTS 2, ER 209
2	CO1, JTS 2	BS 76, Shoba, VK 5, Prabha, ER 175, ER 184, CO1, Salem local
3	Bs 16, BS 23, BS 53, BS 89, BS 90, BS 115, Kanthi, Shoba, Roma, Salem local	Sudarshana, Suguna, Rajendrasonia
4	VK 5, Sudarshana, Suguna, Suvarna, Alleppey, PTS 12, Prabha, Prathibha, PTS 2, ER 169	BS 115, Kanthi, Allepey
5	ER 175, ER 184, Rajendrasonia, PTS 55, ER 209	Suvarna, Roma, Kalimpong, PTS 12, ER 169, PTS 55

Multivariate analysis

The accessions were subjected to different statistical tools : D^2 statistic, K means and UPGMA, based on 22 quantitative traits.

Using D^2 statistic

The accessions were grouped into five clusters based on D^2 statistic for 22 quantitative traits. The clustering pattern revealed that except for BS 2 and BS 76, accessions from Bhavanisagar clustered together. Among accessions from Erode, ER 169 was found to be distinct. Accessions having the same geographical origin, *viz*., Sudarshana and Suguna from Andhra Pradesh, VK 5, Prabha and Prathibha from Kerala, clustered together, while, PTS 55 was distinct among the accessions from Orissa (Table 3).

Using K means

A cluster tree was constructed with 22 quantitative traits using K means clustering, based on Euclidean distance. The K means grouped the accessions into five clusters (Table 3). The pattern of clustering revealed that the accessions from Bhavanisagar clustered together, except for BS 76, BS 89 and BS 115. Among the accessions from Erode, ER 209 and ER 175 clustered together, while, ER 184 clustered with CO 1. Accessions from the same geographical origin, Sudarshana and Suguna, VK 5, Prabha and Shoba were similar, while, Prathibha and Suvarna were distinct from other Kerala accessions. Among accessions from Orissa, PTS 2 was distinct from the others and clustered with Bhavanisagar genoytpes. The genotype Rajendrasonia was distinct from rest of the accessions.

Using UPGMA

A dendrogram was constructed with 22 quantitative traits using UPGMA, on the basis of squared Euclidean distance of standardized data. The pattern of clustering indicated two distinct groups among accessions. All the accessions, except Kalimpong, formed a single cluster. The remaining 29 accessions formed two subgroups, one with 17 accessions and the other with 12 accessions.

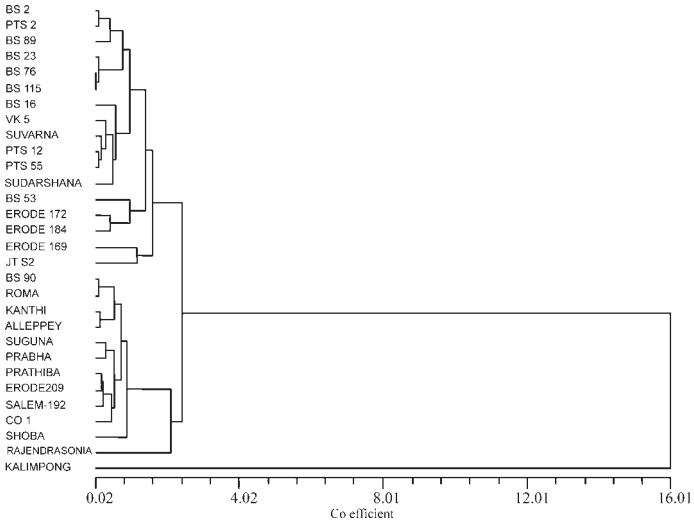

Fig 1. Dendrogram of thirty accessions using UPGMA based on squared Euclidean distances

Accessions from Bhavanisagar and Erode locations exhibited similarity except for BS 90 and ER 209, which were distinct. Among the accessions, BS 76 and BS 115 from Bhavanisagar and PTS 12 and PTS 55 from Orissa, exhibited higher level of homogenity between them. Accessions Sudarshana and Suguna from Andhra Pradesh, and, Prabha and Prathibha from Kerala were very distinct though these were from the same geographical area (Fig 1).

However, accessions from diverse geographical origin like PTS 2 and Roma (Orissa), and, VK 5 and Suvarna (Kerala) got clustered together with accessions from Bhavanisagar (Tamil Nadu).

RAPD polymorphism

RAPD profiles for the thirty accessions were generated to make genetic diversity analysis. Of the seventy-decamer primers used for RAPD analysis, 21 primers yielded scorable, unambiguous markers while 14 primers failed to amplify.

PCR amplification of template DNA produced a total of 89 markers of which 61 markers were found to be polymorphic (68.5%) and the rest were monomorphic (Table 4).

Table 4. Level of polymorphic loci detected by RAPD analysis

Number of primers	21
Total number of markers produced	89
Range of markers	2 – 10
Average number of markers	4
Number of monomorphic markers	27
Number of polymorphic markers	61
Per cent polymorphism	68.50

The number of markers produced per primer varied from two (OPB 11, OPB 14, OPC 01, OPC 16, OPC 18, OPE 03 and OPE 04) to ten (OPG 19). A higher number of polymorphic markers (10) were obtained with primer OPG 19 while primer OPC 18 produced monomorphic markers. Based on the level of polymorphism detected by individual primers, four primers (OPB 08, OPC 20, OPE 09 and OPG 19) revealed over 90% polymorphism (Table 5).

Cluster analysis was performed based on Jaccard's similarity coefficient and a dendrogram was constructed (Fig 2) involving all the accessions. The dendrogram based on RAPD profiles reflected considerable level of genetic considering the geographical distribution. Accessions were classified into two major groups. The first group was further sub-grouped into three (1A, 1B, 1C) clusters. This group consisted of accessions from Bhavanisagar, viz., BS 16, BS 23, BS 53, BS 89, BS 90 and BS 115, with higher level of similarity among those from the same geographical origin (Shamina *et al*, 1198). Other accessions grouped along with accessions of Bhavanisagar include PTS 12, Prabha, Roma, JTS 2 and Rajendrasonia.

Accessions ER 175 and ER 184 were grouped with CO1. Some accessions having the same geographical origin and higher level of similarity (based on quantitative data) were found in different clusters. Accessions Prabha,

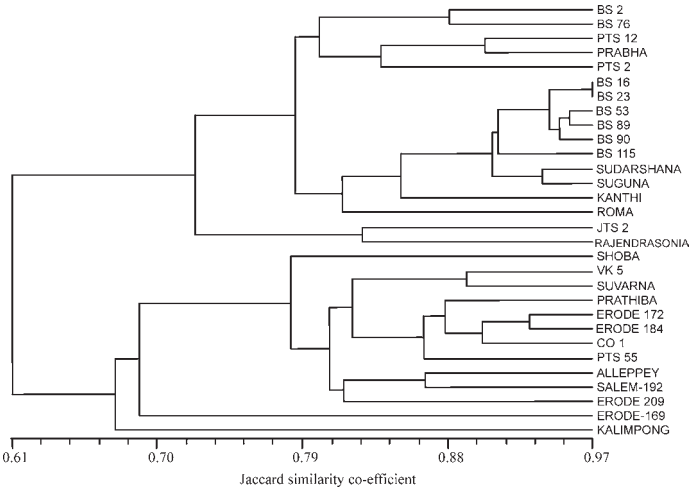

Fig 2. Dendrogram of thirty accessions using UPGMA based on Jaccard similarity coefficient for RAPD markers

Table 5. Details of random primers used for RAPD analysis

Random Primer	5 ' to 3' sequence	GC content (%)	Total markers	Poly-morphic markers	Poly-morphism (%)
OPB - 08	GTCCACACGG	70	4	4	100.00
OPB - 09	TGGGGGACTC	70	7	6	85.71
OPB - 11	GTAGACCCGT	80	2	1	50.00
OPB - 14	TCGGCTCTGG	70	2	1	50.00
OPB - 15	GGAGGGTGTT	60	5	3	60.00
OPC - 01	TTCGAGCCAG	70	2	1	50.00
OPC - 16	CACACTCCAG	60	2	1	50.00
OPC - 18	TGAGTGGGTG	60	2	0	0.00
OPC - 20	ACTTCGCCAC	60	3	3	100.00
OPE - 03	CCAGATGCAC	60	2	1	50.00
OPE - 09	CTTCACCCGA	60	4	4	100.00
OPF - 03	CCTGATCACC	60	5	3	60.00
OPF - 04	GGTGATCAGG	60	2	1	50.00
OPF - 09	CCAAGCTTCC	60	9	8	88.89
OPF - 13	GGCTGCAGAA	60	9	5	55.55
OPG - 10	AGGGCCGTCT	70	6	2	33.33
OPG - 13	CTCTCCGCCA	70	3	1	33.33
OPG - 14	GGATGAGACC	60X	3	2	66.67
OPG - 17	ACGACCGACA	60	4	3	75.00
OPG - 19	GTCAGGGCAA	60	10	9	90.00
OPG - 20	TCTCCCTCAG	60	3	2	66.67
Total			89	61	68.50
Average marker / primer				4.23	2.90

Pathibha, VK 5 and Kanthi from Kerala were found to be in different clusters. However, accessions of diverse geographical origin such as PTS 12 (Orissa), Prabha (Kerala), Suvarna (Andhra Pradesh) and VK 5 (also from Kerala) formed separate clusters.

Genetic diversity assessed using 22 quantitative traits among the 30 accessions was consistent with not all the methods used. The accessions exhibited considerable level of congruence between them. The probable reason could be variation inherent in the nature of algorithms involved. The inconsistency may be due to efficiency of the algorithm to eliminate bias in the quantitative data, which is invariably under the influence of environment. Discrepancy among the three tools studied shows that morphological traits alone are not sufficient for discriminating the accessions, as, turmeric lacks defined morphological descriptors, coupled with cultivar-specific characters. This meager morphological diversity exhibited among accessions may be due to their related pedigree, which make discrimination of accessions rather cumbersome. This also reinforces the belief that accessions collected from the same geographical area do not have any impact on genetic diversity.

In comparing clustering pattern of quantitative traits with RAPD markers, genetic dissimilarity was noticed. However, clustering of Bhavanisagar accessions showed that Sudarshana and Suguna, ER 175 and ER 184 were similar both at the morphological and molecular level. Credibility of genetic diversity analysis involving 22 quantitative traits and 89 RAPD markers could not be established beyond doubt, since; pedigree details of the accessions involved were not available. Though the accessions are known for their geographical origin, the latter could not be used as a criterion to establish relationship between accessions, since, accessions from the same geographical origin had clustered separately. Molecular profiling of *Curcuma* accessions had some similarity with morphological characterization, though, there were incongruities of the accessions of unidentical morphology falling in the same group, or *vice versa* (Syamkumar and Sasikumar, 2007).

Although RAPD markers are widely used for genetic analysis, the inherent problems in the RAPD technique do not make these markers a reliable system for reproduction of results. However, the technical simplicity and high throughput of this technique (Williams *et al*, 1990) helps to engage RAPD markers for large-scale survey. Marker systems such as SSR may help establish differences among accessions with greater accuracy compared to RAPD markers. Further, use of molecular markers should be combined with reliable morphological descriptors of qualitative nature and pedigree details of the genotype, to strengthen the diversity analysis and, in turn, aid germplasm management.

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