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Molecular marker based genetic diversity analysis of *Curcuma* species from India

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Received 4 May 2006; received in revised form 14 November 2006; accepted 6 December 2006

Summary

Molecular genetic fingerprints of 15 *Curcuma* species were developed using Inter Simple Sequence Repeats (ISSR) and Random Amplified Polymorphic DNA (RAPD) markers to elucidate the genetic diversity/relatedness among the species. Thirty-nine RAPD primers yielded 376 bands of which 352 were polymorphic and out of the 91 bands produced by the 8 ISSR markers, 87 were polymorphic. Dendrograms were constructed based on the unweighted pair group method using arithmetic averages. Cluster analysis of data using UPGMA algorithm placed the 15 species into seven groups that are somewhat congruent with classification based on morphological characters proposed by the earlier works. However, the study also warrants the limitations of the conventional taxonomic tools for resolving the taxonomic confusion prevailing in the genus and suggests the need of molecular markers in conjunction with morpho-taxonomic and cytologic studies while revising the genus, which is currently in progress. The maximum molecular similarity observed between two of the *Curcuma* species namely *Curcuma raktakanta* and *Curcuma montana* is suggestive of the need for relooking the separate status given to these two species. Further, the status of *C. montana* and *Curcuma pseudomontana*, the two species mainly discriminated based on the presence of sessile tubers also need to be reassessed.
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Keywords: *Curcuma*; DNA fingerprinting; ISSR; RAPD; Species diversity; Taxonomy

1. Introduction

Over 80 species are reported in the genus *Curcuma* (Zingiberaceae) from Indo Malayan region and about 40 of them are indigenous to India (Velayudhan et al., 1999). However, it is now believed that at least some of the species may be synonyms and there may not be 80 true *Curcuma* species as reported earlier (Sasikumar, 2005). Taxonomic confusion has been reported for Chinese *Curcuma* species as well (Liu and Wu, 1999). A comprehensive global taxonomic revision of the genus has not yet been attempted. The major problems in the taxonomic studies of the genus are lack of type specimens and illustrations of old species, lack of protologues with finer details in the earlier literature, absence of important floral parts in the herbarium specimens, incomplete description of the rhizome features in the herbarium sheets, fleshy and perishable aerial portions, etc. (Sasikumar, 2005).

Position of spikes in *Curcuma* is either terminal or lateral and is one on the major discriminatory traits of the species along with presence of coma bract, bract color. However, the position of the spikes and the color characters has been a subject of controversy (Larsen and Smith, 1978). Roxburgh (1910) pointed out that this difference is seasonal, the early spikes being lateral and the latter ones central. Santapau (1945, 1952) added that in *Curcuma pseudomontana* at the beginning of the rainy season the plant has a large spike coming out from the side of the leaves. Gradually by beginning of August, this lateral spike decays and the central one appears surrounded by leaves, resulting in both central and lateral spikes in the same plant. Santapau (1952) also reported that the color characters show many variations within the species. Bract color variation is also noted in *Curcuma ecalcarata* (Sasikumar, 2005; Sabu, 1991). Hence both these characters are undependable for the delimitation of species in the genus. Conventional taxonomic techniques in conjunction with molecular biology tools may go a long way in resolving the taxonomic confusion prevailing in the genus. Though a few studies on morphological and

Abbreviations: ISSR, inter simple sequence repeats; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA

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doi:10.1016/j.scienta.2006.12.021

anatomical characterization of *Curcuma* species and cultivars have been attempted, not much has been done on molecular characterization (Sasikumar, 2005).

Curcuma molecular biology studies, so far, are confined to few isozyme-based characterization of germplasm accessions/ species (Shamina et al., 1998; Apavatjrut et al., 1999; Paisooksantivatana et al., 2001).

Relying much on the morphological characters alone in species delimitation has its own limitations in the genus. Molecular biology techniques like ISSR/RAPD markers thus assume significance.

The present work is the first attempt in molecular characterization of 15 economically important *Curcuma* species and it adds relevance in the present ongoing context of the taxonomic revision of the genus.

2. Materials and methods

2.1. Plant material

The study was conducted at the Genetic Resources and Molecular Breeding Laboratory, Crop Improvement and Biotechnology Division, Indian Institute of Spices Research, Calicut. The experimental material comprised of 15 *Curcuma* species, maintained in the field gene bank of Indian Institute of Spices Research, Calicut (Table 1).

2.2. DNA extraction

Fully opened fresh tender leaves of the *Curcuma* species were used for the isolation of DNA. The genomic DNA was isolated by CTAB method (Doyle and Doyle, 1987). The extraction buffer contains 2% CTAB, 1.5 M NaCl, 100 mM Tris, 20 mM EDTA and 0.1% β mercaptoethanol.

2.3. PCR condition

RAPD reaction was carried out in 25 μl reaction volume containing 20 ng genomic DNA, 1 U Taq DNA polymerase (Biogene, USA), 200 μM dNTPs, 2 mM $MgCl_2$ and 10 pmoles of random decamer primer according to Williams et al. (1990). Amplification condition consisted of pre denaturation at 94 °C for 3 min, denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. Number of cycles was 35.

For ISSR reaction, the primer concentration used was 60 pmoles $reaction^{-1}$ and the annealing temperature in PCR condition was raised to 55 °C and number of cycle repeats was 32.

The reaction was carried out in a programmable thermal controller, PTC–100 (MJ Research Inc., USA).

2.4. Electrophoresis of PCR products

The amplified products were visualized in a 2% agarose gel containing 0.5 μg ml^{-1} of ethidium bromide and documented by a gel documentation system (Alpha Imager 2200, USA). The bands were scored based on the molecular weight marker (1 kb DNA ladder, New England Biolabs).

2.5. Data scoring and analysis

The electrophoretic patterns were visually analysed and DNA bands were scored as present (1) or absent (0). The matrix obtained was entered in to the NTSYS—pc programme (Rohlf, 1993) and Jaccard's similarity index (JSI) was calculated for each pair of samples. A UPGMA dendrogram was constructed based on JSI.

3. Results

A total of 39 random decamer primers were selected for RAPD analysis from Operon Technologies, Inc., Almeda, USA). Out of the 14 ISSR primers screened for polymorphism, 8 were selected for the characterization of the 15 *Curcuma* species. The sequence of the RAPD/ISSR primers used for the molecular genetic fingerprinting of the 15 *Curcuma* species and the total number of bands produced by each primer, number of polymorphic bands and percentage of polymorphism produced by each primer are presented in Table 2.

Thirty-nine random decamer primers produced a total of 376 scorable bands in the 15 species studied out of which 352 were polymorphic. The percentage of polymorphism ranged from a maximum of 100% showed by 22 random primers (i.e. 56.41% of the total RAPD primers used) to a minimum of 75% showed by OPA 11 (Table 2). Whereas in case of the eight ISSR primers studied, six of them (75%) gave 100% polymorphic bands among the species and two primers namely $(TCC)_5$ AG and $(GACA)_3$ gave 70 and 90.91% polymorphism, respectively (Table 2). Pooled over the primers a total of 467 markers were obtained out of which 439 (94.61%) were polymorphic.

3.1. Cluster analysis

The UPGMA dendrogram (Fig. 1) showed two main clusters. The first one included *C. ecalcarata* Mangalay & Sabu of the sub genus *Paracurcuma* Val. along with *Curcuma decipiens* Dalz. of the sub genus *Eucurcuma*. Valeton (1918) was the first to classify the genus *Curcuma* into *Eucurcuma* and *Paracurcuma* mainly based on the presence or absence of anther spurs. The two clusters split at a Jaccards Similarity Index (JSI) 0.570. The two nodes in the cluster one (*C. ecalcarata* and *C. decipiens*) showed 0.668 JSI between them. *Curcuma haritha*, *Curcuma aromatica*[a] and *Curcuma aromatica*[b] formed the first group in cluster two. *C. haritha* showed 0.800 JSI with the *C. aromatica*[a] and *C. aromatica*[b] in the group while the two *C. aromatica* entities showed 0.856 JSI between them. *Curcuma longa* syn. *Curcuma domestica* formed a single group and the node gave 0.642 JSI with the other species in the cluster II. Four species formed the third group in the cluster, the biggest group in cluster II. In this group, *Curcuma sylvatica* had 0.708 JSI with *C. pseudomontana* of the same group and the later one had 0.730 JSI with *Curcuma malabarica* and *Curcuma zedoaria* of the same group. The node of the *C. malabarica* and

Table 1
Curcuma species studied and their important morphological features

Species	Ploidy level ($2n$)	Floral characters			Rhizome characters			Aerial characters	
		Spike position	Color of calyx	Color of corolla	Color of rhizome	Aroma of rhizome	Taste of rhizome	Color of leaf sheath	Leaf midrib color
Curcuma amada Roxb.	42	Lateral	Light purple	Pale yellow	Pale yellow, White	Mango	Gingery	Green	Green
Curcuma aromatica[a] Salisb.*	42, 63, 86	Lateral	Purple	White	Pale yellow, White	Camphoraceous	Bitter	Green	Green
Curcuma aromatica[b] Salisb.*	42, 63, 86	Lateral	Purple	White	Pale yellow, White	Camphoraceous	Bitter	Green	Green
Curcuma aeruginosa Roxb.	63	Lateral	Purple	White	Virdis Green	Camphoraceous	Bitter	Dark purple	Purple brown
Curcuma caesia Roxb.	42	Central/lateral	purple	Purple	Blue	Camphoraceous	Bitter	Purple brown	Purple brown
Curcuma comosa Roxb.	–	Lateral	Light purple	Light purple	Yellow	Camphoraceous	Bitter	Greenish light purple	Light purple green
Curcuma decipiens Dalz.	32,42, 62	Central	Purple	Purple spot	White, Pale yellow	Camphoraceous	No taste	Greenish purple	Green
Curcuma ecalcarata Sivarajan & Indu	–	Central	White	White	White	Slight camphoraceous	Slight bitter	Green	Green
Curcuma haritha Mangalay & Sabu	42	Lateral	Light purple	White	Pale yellow	Camphoraceous	Slight bitter	Light purple brown	Light purple brown
Curcuma longa L.	62, 63, 64	Central	White, Green, Light purple	White	Orange yellow, Light orange yellow	Camphoraceous/ turmeric aroma	Slight bitter	Green	Green
Curcuma montana Wall.	–	Lateral/central	Light purple	White	Pale yellow	Camphoraceous	Bitter	Purple	Green
Curcuma malabarica Vela et al.	63	Lateral	Purple	Purple	Yellow	Camphoraceous	Bitter	Green	Light purple
Curcuma pseudomontana Grah.	–	Central	White, Green, Purple	Light purple	Pale yellow	Camphoraceous	No taste	Purple	Green
Curcuma raktakanta Mangalay & Sabu	63	Lateral	Purple	White, Light purple	Pale yellow	Camphoraceous	Slight bitter	Dark purple brown	Green
Curcuma sylvatica Val.	–	Lateral	Light purple	Light purple	White	Mango/camphoraceous	Slight bitter	Green	Light purple brown
Curcuma zedoaria Rosc	63, 64	Lateral	Purple	Light purple	Light orange yellow	Camphoraceous	Slight bitter	Green	Light purple green

* *Curcuma aromatica* collected from two locations namely *Curcuma aromatica*[a] (National Bureau of Plant Genetic Resources, Trissur, Kerala) and *Curcuma aromatica*[b] (Wynad, Kerala) is maintained in the gene bank separately.

Table 2
Sequence of RAPD/ISSR primers, number of bands generated by each primer, number of polymorphic bands and percent polymorphism

S. no.	Primer	Sequence (5′–3′)	Total no. of bands	No. of polymorphic bands	Polymorphism (%)
RAPD					
1	OPA 05	AGGGGTCTTG	10	10	100.00
2	OPA 07	GAAACGGGTG	12	10	83.33
3	OPA 08	GTGACGTAGG	13	11	84.62
4	OPA 10	GTGATCGCAG	11	10	90.91
5	OPA 11	CAATCGCCGT	12	9	75.00
6	OPA 12	TCGGCGATAG	11	10	90.91
7	OPA 14	TCTGTGCTGG	9	8	88.89
8	OPA 15	TTCCGAACCC	9	9	100.00
9	OPA 17	GACCGCTTGT	9	9	100.00
10	OPA 18	AGGTGACCGT	11	10	90.91
11	OPA 19	CAAACGTCGG	11	11	100.00
12	OPB 09	TGGGGGACTC	8	7	87.50
13	OPB 12	CCTTGACGCA	8	8	100.00
14	OPB 16	TTTGCCCGGA	6	6	100.00
15	OPC 07	GTCCCGACGA	9	9	100.00
16	OPC 08	TGGACCGGTG	13	13	100.00
17	OPC 09	CTCACCGTCC	11	10	90.91
18	OPC 10	TGTCTGGGTG	9	9	100.00
19	OPC 11	AAAGCTGCGG	6	6	100.00
20	OPC 12	TGTCATCCCC	4	4	100.00
21	OPC 13	AAGCCTCGTC	9	9	100.00
22	OPC 14	TGCGTGCTTG	5	5	100.00
23	OPC 16	CACACTCCAG	13	12	92.31
24	OPC 18	TGAGTGGGTG	8	8	100.00
25	OPC 20	ACTTCGCCAC	8	8	100.00
26	OPD 01	ACCGCGAAGG	11	11	100.00
27	OPD 03	GTCGCCGTCA	10	10	100.00
28	OPD 04	TCTGGTGAGG	9	9	100.00
29	OPD 05	TGAGCGGACA	9	9	100.00
30	OPD 07	TTGGCACGGG	13	10	76.92
31	OPD 08	GTGTGCCCCA	12	11	91.67
32	OPD 13	GGGGTGACGA	9	8	88.89
33	OPD 14	CTTCCCCAAG	10	9	90.00
34	OPD 15	CATCCGTGCT	14	12	85.71
35	OPD 16	AGGGCGTAAG	11	11	100.00
36	OPJ 10	AAGCCCGAGG	7	7	100.00
37	OPJ 16	CTGCTTAGGG	9	9	100.00
38	OPJ 17	ACGCCAGTTC	8	7	87.50
39	OPJ 18	TGGTCGCAGA	9	8	88.89
		Total	376	352	93.62
ISSR					
1	ISSR 1	(CT) 8 TG	17	17	100.00
2	ISSR 2	(CT) 8 AC	5	5	100.00
3	ISSR 3	(TCC) 5 AG	10	7	70.00
4	ISSR 4	(AGC) 4 GT	12	12	100.00
5	ISSR 5	(CAC) 3 GC	14	14	100.00
6	ISSR 6	(CTC) 3 GC	13	13	100.00
7	ISSR 7	(GACA) 3	11	10	90.91
8	ISSR 8	(GACA) 3 GC	9	9	100.00
		Total	91	87	95.60

C. zedoaria showed 0.772 JSI between them. *C. sylvatica* of the group three showed 0.669 JSI with *Curcuma caesia* and *Curcuma aeruginosa*, which formed the fourth group in cluster II. The two nodes in the fourth group showed 0.720 JSI between them. Going up in the dendogram the two species of *Eucurcuma*, *Curcuma raktakanta* and *Curcuma montana*, showed maximum similarity between them (0.91 JSI) forming the fifth group in cluster II. *Curcuma comosa* had 0.856 JSI with *C. montana* and *C. raktakanta*. Whereas the lone species *Curcuma amada* having JSI 0.721 with *C. comosa*, *C. raktakanta* and *C. montana* formed the sixth group in cluster II. This group in turn had 0.660 JSI with *C. sylvatica* of group three and the two nodes of the *C. caesia* and *C. aeruginosa* in the group four.

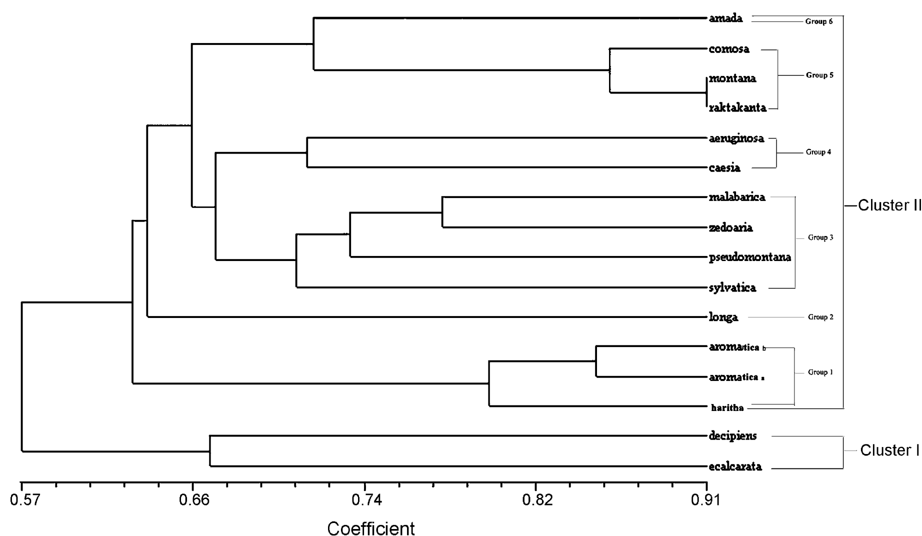

Fig. 1. Dendrogram of 15 *Curcuma* species from India resulting from a UPGMA cluster analysis obtained from 8 ISSR and 39 RAPD primers.

4. Discussion

It is interesting to note that the two species falling in cluster I, viz. *C. decipiens* and *C. ecalcarata*, belonged to the two separate sub genera, viz. *Eucurcuma* and *Paracurcuma*, respectively, of Valeton. In the genus *Paracurcuma*, Valeton had included only two species, viz. *C. ecalcarata* and *Curcuma aurantiaca* both lacking anther spur or with a very short spur. In fact, the presence/absence of anther spur was considered by Valeton as major criteria in classifying the genus into two subgroups based on his observation of Malaysian and Javan *Curcuma* species. However Santapau (1945, 1952) had cautioned about the wisdom of solely relying floral or vegetative traits for discriminating the *Curcuma* species. Larsen and Smith (1978) also further stressed the limitations of solely relying floral or vegetative features in discriminating *Curcuma* species. *C. decipiens* and *C. aurantiaca* (the other members of *Paracurcuma*) are cohabiting species having much similarity in floral, vegetative and rhizome characters, though anther spur length variation is there in these two species. Further, forms related to *C. decipiens* that set seeds have also been noticed in central Kerala, India. The infra specific variation noticed in both of these species is so high that some infra specific types may appear to be morphologically quite distinct. Both seed setting and non seed setting types are also noticed in these species. These two species are evolutionarily very active and turbulent species and hence they may be considered as very primitive ones (KC Velayudhan, Unpublished). Based on the past cytological literature, one can further elucidate the origin of genus *Curcuma* and cultivated species such as *C. longa* to amphidiploidy (allotetraploidy) and the triplodization (Raghavan and Venkatasubban, 1943; Sato, 1948; Chakravorti, 1948; Ramachandran, 1961). Thus the genus *Curcuma* as a whole is amphidiploid. More variation occurs in seed setting diploid (allotetraploid) species with $2n = 42$. Thus the inclusion of two morphologically distant primitive species in main cluster I irrespective of their morphological difference is possible. Likewise, all the 15 species studied also bifurcated into two major clusters at 0.57 JSI irrespective of the morphology indicating at least a 50% general similarity between the species falling in the two clusters based on the amphidiploid concept containing two genomes. The highly polymorphic nature of bands in essence tallies with the high polymorhism noticed in the entire genus, which accounts for the species diversity. Further, the grouping of two distant species together may also be attributed to the limitation of relying on a single or few key characters in species identity and taxonomic classification. Alternately one can argue that the deviation of marker based divergence from an observed morphological divergence will depend on the degree of association between the markers and expressed traits (Gupta and Varshney, 1999).

The two species in the cluster I, i.e. *C. decipiens* and *C. ecalcarata* share many common vegetative, rhizome, floral and traits. They also cohabitat at high altitudes in rocky patches (Sabu, 1991). Of course, wide distribution has been noticed in case of *C. ecalcarata* which occur from plains to hills as high as 700 m elevation.

The 13 other species included in the cluster II also revealed some interesting pattern. In the group one of cluster II, two species are included namely *C. haritha*, *C. aromatica*[a] and *C. aromatica*[b]. In this group, the two *C. aromatica* entities, collected from separate places in Kerala showed 85.6% similarity between them and morphologically also they resembled closely. Both species of this group also share similar ecological niches in the midlands and base of Western Ghats of India. *C. aromatica* is a seed setting species (George, 1981) and true seedling variation may be the reason for the observed variation in the two entities of *C. aromatica*. Both the species occur throughout Kerala in semi-shaded forests, grass lands and even in plantations and are reported to have a somatic chromosome number $2n = 42$ (Raghavan and Venkatasubban, 1943; Joseph et al., 1999). Rhizomes of both the species are rich in camphor, camphene, ar-turmerone, etc. (Bordoloi et al.,

1999; Dan et al., 2002). Between the two species they showed 79% similarity based on the molecular data. The second group of cluster II is represented by a lone species *C. longa*, a triploid ($2n = 63$) species, which is distinct from all other species in its morphological and biochemical characters and is the predominantly cultivated species throughout the world. Four species namely *C. sylvatica*, *C. pseudomontana*, *C. zedoaria* and *C. malabarica* formed the third group in cluster II. In this group *C. zedoaria* and *C. malabarica* having almost similar floral, underground and aerial characters showed 77% similarity between them. Both are triploid ($2n = 63$) too (Ramachandran, 1961; Joseph et al., 1999). *C. sylvatica* is a lateral spike producing species as *C. zedoaria* and *C. malabarica*, but it is having distinct floral and rhizome characters as compared to the three other species in the group. The entity *C. sylvatica*, earlier reported by Valeton (1918) as a separate species, due to mango flavour of the rhizome is now considered as a variant of *C. amada* (KC Velayudhan, personal communication) based on midrib color of the juvenile leaves. However, the DNA profiling of this entity does not agree with this view as the true *C. amada* grouped distinctly from this entity in the dendrogram (cluster II, group six). *C. caesia* and *C. aeruginosa* formed the fourth group in cluster II. The pairing of *C. caesia*, popularly known as 'black turmeric', possessing deep blue colored rhizome having camphoraceous aroma and bitter taste with *C. aeruginosa* which is also having the similar morphological and rhizome characters is very interesting. Between them they shared 72% similarity. Rhizomes of both the species are also rich in camphor (Sirat et al., 1998; Behura, 2000) and their leaf midrib is having purple color. Fifth group of cluster II consisted of three species namely *C. raktakanta*, *C. montana* and *C. comosa*. The two species, *C. raktakanta*, and *C. montana* in this group showed maximum similarity between them among the 15 species in the dendrogam (91% similarity). Characterized by dwarf stature, these two species share many common floral, vegetative and rhizome features (Velayudhan et al., 1996, 1999). However, they do differ in the position of the spike and color of corolla (Velayudhan et al., 1999) which are of course characters of controversy. Considering the very high degree of molecular similarity obtained in conjunction with the morphological similarity of the two species, it would be prudent to examine the existing separate status of the two species. Most probably they can also be synonyms like *C. zedoaria*–*Curcuma xanthorrhiza* (Indian species); *Curcuma albicoma*–*Curcuma sichuanensis*; *Curcuma chuanyujin*–*Curcuma kwangsiensis*; *Curcuma wenyugin*–*C. aromatica* and *C. zedoaria*–*Curcuma phaeocaulis* (Chinese species) (Liu and Wu, 1999).

C. amada (Mango ginger) distinct form all other species, maintained a distinct identity being the lone species in the sixth group of cluster II. *C. amada* characterized by mango plus ginger flavoured rhizome is next important *Curcuma* species under cultivation, after *C. longa*. The rhizome of *C. amada* is rich in camphor, ar-curcumene, α pinene, zingiberene, curzerenene, β elemene, isoborneol, etc. (Srivastava et al., 2001).

The group three in cluster II accommodates *C. pseudomontana* of section *Non Tuberosa* along with entities such as *C. malabarica*, *C. zedoaria* and *C. sylvatica* of the section *Tuberosa*. The inclusion of *C. pseudomontana* of section *Non Tuberosa* along with entities of *Tuberosa* of Valeton can be attributed to some extent the amphidiploid origin of the genus. *C. pseudomontana*, originally descried from Western Ghats, India had a confused taxonomy as it closely resembles *C. montana* except for the side corms. *C. pseudomontana* and *C. montana* share many common floral and vegetative characters and occur in similar habitat (Sabu, 1991; Velayudhan et al., 1999). *C. pseudomontana* is stated to have both central and lateral spike (Santapau, 1945). Thus a close relook into the morpho-taxonomic traits of *C. montana* and *C. pseudomontana* in the light of the earlier studies and present finding is warranted before according a permanent separate species status to them.

The molecular profiling of the 15 *Curcuma* species shared some similarity with the conventional taxonomic studies reported though there were dialectical situation of species of unidentical morphology falling in the same group or vice versa. The present study warrants that relying solely the floral, vegetative, rhizome features for taxonomic characterization of *Curcuma* may be ambiguous as there is lack of type specimens, protologues, missing floral parts in the herbarium specimens, etc. As taxonomic revision of the genus is now under way, it would be prudent to use ISSR/RAPD markers alongwith the morpho-taxonomic/qualitative, cytological parameters to resolve the identity of closely resembling *Curcuma* species when there is confusion with respect to their unequivocal identity.

Acknowledgements

The authors are thankful to the Director, Indian institute of Spices Research, Calicut, for providing the facilities and to Dr. K.C. Velayudhan, Principal Scientist, National Bureau of Plant Genetic Resources (Regional Station) Trissur, Kerala, India, for his valuable suggestions and critical review of the manuscript. We are also thankful to Mr. V.P. Sankaran, Technical officer, Experimental farm, IISR, Peruvannamuzhi, for field assistance.

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