EST-SSR marker revealed effective over biochemical and morphological scepticism towards identification of specific turmeric (*Curcuma longa* L.) cultivars

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Abstract Turmeric (*Curcuma longa* L., family Zingiberaceae) is one of the most economically important plants for its use in food, medicine, and cosmetic industries. Cultivar identification is a major constraint in turmeric, owing to high degree of morphological similarity that in turn, affects its commercialization. The present study addresses this constraint, using EST-SSR marker based, molecular identification of 8 elite cultivars and 88 accessions in turmeric. Fifty EST-SSR primers were screened against eight cultivars of turmeric (Suroma, Roma, Lakadong, Megha, Alleppey Supreme, Kedaram, Pratibha, and Suvarna); out of which 11 primers showed polymorphic banding pattern. The polymorphic information content (PIC) of these primers ranged from 0.13 to 0.48. However, only three SSR loci (CSSR 14, CSSR 15, and CSSR 18) gave reproducible unique banding pattern clearly distinguishing the cultivars ‘Lakadong’ and ‘Suvarna’ from other cultivars tested. These three unique SSR markers also proved to be effective in identification of ‘Lakadong’ cultivars when analysed with 88 accessions of turmeric collected from different agro-climatic regions. Furthermore, two identified cultivars (Lakadong and Suvarna) could also be precisely differentiated when analysed and based on phylogenetic tree, with other 94 genotypes of turmeric. The novel SSR

markers can be used for identification and authentication of two commercially important turmeric cultivars ‘Lakadong’ and ‘Suvarna’.

Keywords *Curcuma longa* · Expressed sequence tags (ESTs) · Simple sequence repeats (SSRs) · Cultivar identification · Phylogenetic analysis

Introduction

Turmeric (*Curcuma longa* L.), the golden spice, of the family Zingiberaceae is a perennial rhizomatous plant found all over Asia, South East Asia and Africa. It is one of the most important herbs cultivated in the tropical and subtropical countries. India is the largest producer, consumer, and exporter of turmeric in the world, followed by China, Indonesia, Bangladesh, and Thailand (Selvan et al. 2002). Turmeric rhizome has multipurpose use in medicine, aromatherapy, cosmetics, dye, and food industry (Sasikumar 2005; Singh et al. 2013). It possesses several activities such as anti-inflammatory, anti-oxidant, antimicrobial, antiviral, antifungal, insecticidal, nematocidal activity, anti-ageing, lipid lowering effects, antiallergic, immunomodulatory, antidiabetic, and anticancerous properties (Sasikumar 2005; Corcolon et al. 2015). Turmeric is predominantly vegetatively propagated and has been reported as triploid ($2n = 3x = 63$; $x = 21$) and nonaploid ($2n = 9x = 63$; $x = 7$) (Senan et al. 2013). Cultivars of turmeric are highly diversified and its taxonomy is quite confusing. Out of about 50 named cultivars reported in India (Rama Rao and Rao 1994), only few elite cultivars such as Suroma, Roma, Lakadong, Megha, Alleppey Supreme, Kedaram, Pratibha, and Suvarna are grown at different agro-climatic regions for commercial products of

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turmeric (Anandaraj et al. 2014). Elite cultivars of turmeric released formally were so far characterized on the basis of morphological and biochemical data. Morphologically, all cultivars of turmeric look similar. There is no clear-cut, morphological difference in leaves, flowers, and rhizomes often creating confusion over authentic identification and help adulteration in commercial market. Apart from varying morphological and biochemical indices such as curcumin, oleoresin, and essential oil content under different environmental conditions, predominance of local synonyms make the characterization of turmeric cultivars a more complicated task. Moreover, a renewed interest in the commercially important elite cultivars of turmeric as new spice crop has made it necessary to precisely characterize them for providing accurate genetic information. Thus, the need was realised for development of cultivar specific molecular markers in turmeric for identification and protection of registered cultivars. This would go a long way in preserving their germplasm-uniqueness uninfluenced by environmental factors unlike the cases in morphological and biochemical markers.

Most of the work on molecular characterization of turmeric is limited to isozyme studies (Shamina et al. 1998) and RAPD/ISSR analysis of cultivars and accessions (Nayak et al. 2006; Syamkumar 2008; Jan et al. 2011; Khan et al. 2013; Verma et al. 2015). These reports are based mostly on assessment of genetic diversity or relatedness among turmeric accessions/cultivars involving construction of phylogenetic tree from cluster analysis of molecular data. However, the phylogenetic tree can only group certain cultivars/accessions having close similarity but cannot identify a particular cultivar. Moreover, RAPD and ISSR markers are dominant, less reliable, and sensitive to reaction conditions interfering with the reproducibility of banding pattern between experiments (Wang and Chuang 2013). To overcome these limitations, simple sequence repeat (SSR) markers have been developed in many plants. Union for the Protection of New Varieties of Plants (UPOV) observes that SSR markers are more suitable for DNA profiling because of their multi-allelic nature, reproducibility, high polymorphism, easy automation, and co-dominant nature of inheritance (UPOV/INF/17/1 2010 guideline). SSR markers are widely applied in genotyping of species (Chrobokova et al. 2011; Behera et al. 2012; Koussao et al. 2014) and in cultivar identification (Liu et al. 2014; Basheer-Salimia et al. 2014). In turmeric, limited work is reported so far on molecular characterization, using SSR markers (Siju et al. 2010; Joshi et al. 2010; Khan et al. 2013; Singh et al. 2015) aiming largely at diversity assessment, accessions of cultivars, and not for cultivar identification. SSRs being present in both coding and noncoding regions (Kalia et al. 2011) and EST-SSRs especially have advantages over genomic SSRs as their

expressed sequence data can be easily retrieved from the public databases (Varshney et al. 2005). Moreover, EST-SSRs may increase the applicability of DNA markers by expressing the variation in transcribed genes (Scott et al. 2000). Several EST-SSR markers has been developed in different plants for cultivar identification (Wang and Chuang 2013; Sameh et al. 2014; Hong et al. 2015), but until date, not a single EST-SSR marker has been developed for authentic identification of turmeric (*Curcuma longa* L.) cultivars. The present study was aimed at development of novel EST-SSR markers to be used as a resource of reference information for rapid and authentic identification of specific turmeric cultivars.

Materials and methods

Plant materials and DNA extraction

Total eight registered varieties of *Curcuma longa* L. (Fig. S1) were collected from different research stations of Kerala, Meghalaya, and Odisha and all 88 accessions of turmeric were collected from different regions of Odisha (Table 1; Fig. S2). These genotypes were maintained in the green house of Centre for Biotechnology. Fully opened fresh tender leaves of turmeric were used for the isolation of DNA. The genomic DNA was isolated by Doyle and Doyle method Doyle and Doyle (1990) with required modification. The extraction buffer contained 2% CTAB (SRL, India), 1.4 M NaCl (SRL, India), 100 mM Tris-HCl (SRL, India) (pH 8.0), 20 mM EDTA (SRL, India) (pH 8.0), and 2% β -mercaptoethanol (SRL, India). The quality of purified DNA was analysed using 0.8% agarose gel (Himedia, India) and quantified using spectrophotometer by taking OD at 260/280 ratio. Purified DNA was diluted to final concentration of 25 ng/ μ L with Tris-EDTA buffer (pH 8.0) and stored at -20°C for further use.

Biochemical evaluation

Curcumin content of turmeric cultivars was determined following ASTA (1958), and oleoresin content and essential oil content of rhizome were estimated following methods of Singh et al. (2013).

For analysis of curcumin content, sliced rhizome was dried, powdered, and refluxed for 4 h using soxhlet apparatus, by taking 0.1 g of powdered rhizome in 250 ml of acetone. The solution was filtered and diluted and absorbance was taken at 420 nm by spectrophotometer. Curcumin percentage of the sample was estimated according to the ASTA (1958).

For analysis of oleoresin content, first oleoresin extraction was done by taking 100 g of turmeric rhizome (from

Table 1 Information of 8 cultivars and 88 accessions analysed in this study

Serial no	Cultivars/accessions	Accession number of registered cultivars	Place of collection	Latitude	Longitude
1	Suroma	PTS-24	High Altitude Research Station, Koraput, Odisha	18°55'48.6"	82°29'21.4"
2	Roma	PTS-10	High Altitude Research Station, Koraput, Odisha	18°55'48.6"	82°29'21.4"
3	Lakadong	RPRC-54	North Eastern Hill University, Shillong, Meghalaya	25°34'43.58"	91°53'35.71"
4	Megha	RPRC-62	North Eastern Hill University, Shillong, Meghalaya	25°34'43.58"	91°53'35.71"
5	Alleppey Supreme	Acc. 585	Indian Institute of Spice Research, Kerala	11°15'31.511"	75°46'49.47"
6	Kedaram	Acc. 126	Indian Institute of Spice Research, Kerala	11°15'1.511"	75°46'49.47"
7	Pratibha	Acc. 361	Indian Institute of Spice Research, Kerala	11°15'31.511"	75°46'49.47"
8	Suvarna	Acc. 98	Indian Institute of Spice Research, Kerala	11°15'31.511"	75°46'49.47"
9	TC1		Bhubaneswar	20°17'11.228"	85°46'27.553"
10	CBT 1		Kandhamal	20°17'735"	84°05'686"
11	CBT 2		Kandhamal	20°17'029"	84°04'050"
12	CBT 3		Kandhamal	20°18'287"	84°03'353"
13	CBT 4		Kalahandi	19°55'50.7"	83°12'16.1"
14	CBT 5		Kalahandi	19°55'56.5"	83°11'35.1"
15	CBT 6		Kalahandi	19°56'14.4"	83°12'16.8"
16	CBT 7		Kalahandi	19°56'39.7"	83°11'03.7"
17	CBT 8		Kalahandi	19°54'01.4"	83°11'19.5"
18	CBT 9		Koraput	18°55'02.7"	82°31'33.6"
19	CBT 10		Koraput	18°55'48.6"	82°29'21.4"
20	CBT 11		Koraput	18°56'32.0"	82°27'48.4"
21	CBT 12		Koraput	18°55'58.7"	82°31'27.7"
22	CBT 13		Koraput	18°54'32.0"	82°27'33.1"
23	CBT 14		Gajapati	19°04'308"	84°15'536"
24	CBT 15		Gajapati	19°05'393"	84°15'549"
25	CBT 16		Gajapati	19°04'505"	84°14'432"
26	CBT 17		Gajapati	19°03'820"	84°14'243"
27	CBT 18		Gajapati	19°03'759"	84°13'914"
28	CBT 19		Rayagada	19°29'545"	83°26'095"
29	CBT 22		Rayagada	19°29'488"	83°25'424"
30	CBT 23		Rayagada	19°31'234"	83°25'609"
31	CBT 20		Rayagada	19°29'133"	83°25'747"
32	CBT 21		Rayagada	19°29'775"	83°25'909"
33	CBT 24		Anugul	20°40'020"	85°05'223"
34	CBT 25		Anugul	20°39'555"	85°05'172"
35	CBT 26		Anugul	20°39'897"	85°05'880"
36	CBT 27		Anugul	20°39'087"	85°06'801"
37	CBT 28		Anugul	20°55'571"	84°46'952"
38	CBT 29		Dhenkanal	20°38'55.6"	85°36'6.0"
39	CBT 30		Dhenkanal	20°36'49.8"	85°31'17.5"
40	CBT 31		Dhenkanal	20°38'27.0"	85°31'46.6"
41	CBT 32		Dhenkanal	20°40'33.9"	85°31'12.8"
42	CBT 33		Dhenkanal	20°41'18.9"	85°33'04.04"
43	CBT 34		Cuttack	20°39'06.8"	85°55'16.4"
44	CBT 35		Cuttack	20°38'39.2"	85°54'59.9"
45	CBT 36		Cuttack	20°38'56.7"	85°54'59.1"
46	CBT 37		Cuttack	20°36'30.2"	85°58'28.3"

Table 1 continued

Serial no	Cultivars/accessions	Accession number of registered cultivars	Place of collection	Latitude	Longitude
47	CBT 38		Cuttack	20°37'40.8"	85°53'30.5"
48	CBT 39		Puri	19°53'54.8"	85°48'37.0"
49	CBT 40		Puri	19°48'33.0"	85°44'02.7"
50	CBT 41		Puri	19°47'52.3"	85°39'00.3"
51	CBT 42		Puri	19°47'49.4"	85°40'56.3"
52	CBT 43		Puri	19°48'40.2"	85°46'20.8"
53	CBT 44		Bhadrak	21°03'34.4"	86°29'39.4"
54	CBT 45		Bhadrak	21°5'17.4"	86°25'24.3"
55	CBT 46		Bhadrak	21°13'08.2"	86°23'48.8"
56	CBT 47		Bhadrak	21°15'09.2"	86°25'54.9"
57	CBT 48		Bhadrak	21°08'52.2"	86°23'41.6"
58	CBT 49		Mayurbhanj	22°13'51.2"	86°12'38"
59	CBT 50		Mayurbhanj	22°13'21.3"	86°13'09.6"
60	CBT 51		Mayurbhanj	22°13'48.1"	86°13'37.2"
61	CBT 52		Mayurbhanj	22°14'01.2"	86°14'34.9"
62	CBT 53		Mayurbhanj	22°13'53.4"	86°15'12.3"
63	CBT 54		Khurdha	19°50'466"	85°05'211"
64	CBT 55		Khurdha	19°51'370"	85°04'684"
65	CBT 56		Khurdha	19°52'290"	85°04'918"
66	CBT 57		Khurdha	19°52'120"	85°03'727"
67	CBT 58		Khurdha	19°52'887"	85°04'133"
68	CBT 59		Subarnapur	20°40'328"	83°39'512"
69	CBT 60		Subarnapur	20°40'024"	83°39'001"
70	CBT 61		Subarnapur	20°35'225"	83°37'597"
71	CBT 62		Subarnapur	20°35'113"	83°37'327"
72	CBT 63		Subarnapur	20°34'412"	83°38'426"
73	CBT 64		Sambalpur	21°06'463"	84°12'448"
74	CBT 65		Sambalpur	21°08'505"	84°15'012"
75	CBT 66		Sambalpur	21°27'59"	83°58'302"
76	CBT 67		Sambalpur	21°29'136"	83°51'232"
77	CBT 68		Sambalpur	21°35'193"	84°02'482"
78	CBT 69		Jharsuguda	21°45'065"	83°47'117"
79	CBT 70		Jharsuguda	21°47'216"	83°36'346"
80	CBT 71		Jharsuguda	21°49'427"	83°55'181"
81	CBT 72		Jharsuguda	21°48'432"	84°18'420"
82	CBT 73		Jharsuguda	21°49'183"	83°50'449"
83	CBT 74		Boudh	20°50'26.3"	84°18'09.2"
84	CBT 75		Boudh	20°51'08.8"	84°16'33.2"
85	CBT 76		Boudh	20°51'22.6"	84°16'15.5"
86	CBT 77		Boudh	20°50'09.4"	84°17'04.9"
87	CBT 78		Boudh	20°51'40.5"	84°13'58.6"
88	CBT 79		Nayagarh	20°09'33.5"	85°01'5.85"
89	CBT 80		Nayagarh	20°20'03.5"	84°50'13.9"
90	CBT 81		Nayagarh	20°09'33.7"	85°01'5.88"
91	CBT 82		Nayagarh	20°09'33.8"	85°01'58.8"
92	CBT 83		Nayagarh	20°13'47.4"	84°58'40.3"
93	CBT 84		Nabarangpur	19°15'26.6"	82°33'28.1"
94	CBT 85		Nabarangpur	19°17'14.0"	82°34'43.1"

Table 1 continued

Serial no	Cultivars/accessions	Accession number of registered cultivars	Place of collection	Latitude	Longitude
95	CBT 86		Nabarangpur	19°18'09.0"	82°35'23.0"
96	CBT 87		Nabarangpur	19°18'42.3"	82°36'49.5"

Serial no 1–8: registered cultivars; serial no 9–96: genotype accessions

which oil already extracted) in a soxhlet apparatus. About 250 ml of the acetone was added to it and extraction process was continued until the colour of the acetone becomes transparent. After extraction, evaporation of the acetone was done in water bath at 65 °C. For complete evaporation of acetone, extract was placed in hot air oven at 75 °C for 1 h. The final extract found from this process was oleoresin. The oleoresin percentage was calculated as:

$$\text{Oleoresin}\% = \frac{\text{Weight of the residue} \times 100}{\text{Weight of the sample}}.$$

For estimation of essential oil content, sliced rhizomes (100 g) were allowed to hydro distillation using a Clevenger's apparatus. A flask containing rhizomes was heated for 2–6 h and the condensed vapour was separated throughout an auto-oil/water separator. The oil present at the upper most layers was collected in a container and treated with a pinch of anhydrous sodium sulphate to make moisture free. Each essential oil extraction was run in triplicates. The oil samples were stored at 0 °C in air tight glass container for gas chromatographic mass spectrometric (GC–MS) analysis.

Gas chromatographic mass spectrometric analysis of essential oil of turmeric was carried out with a Clarus 580 series of GC instrument (Perkin Elmer), equipped with SQ8S MS and elite-5 fused silica capillary column (30 m × 0.25 mm internal diameter; film thickness 0.25 mm). GC–MS (70 eV) data were measured on coupled with MS detector. MS source temperature at 230 °C; MS Quadra pole temperature at 150 °C; injector temperature at 250 °C; mass scan, 50–600 amu; and 0.2 scan/s with 0.1 inter scan delay. The oven temperature was set initially at 60–120 °C with ramping 3 °C then increasing up to 215 °C at 5 °C/min. The NIST (2011) software was used for library search for identification of major chemical constituent of essential oil.

Designing of SSR primers from EST sequences of turmeric

In this present study, we have taken 12593 expressed sequenced tag (EST) sequences from our earlier turmeric transcriptome data. These EST sequences were screened for the presence of SSR repeat motifs using the MISA software (<http://pgrc.ipk-gatersleben.de/misa/misa.html>). A

microsatellite was explained as a sequence of DNA comprising of at least 12 repeated units for mononucleotides, seven units for dinucleotide repeats, and three units for tri-, tetra-, penta-, and hexa-nucleotide repeats. SSR primers flanking the repeat sequences were designed using the Primer 3 plus software program. A total of 50 pairs of EST-SSR primers have been synthesized (IDT, USA) and used in this study.

PCR amplification and SSR marker selection

PCR amplification was performed in a 25 µL reaction system containing 1 µL 25 ng genomic DNA, 1 µL 5 pM of each primer (forward and reverse) (IDT, USA), 0.17 µL 3U/µL Taq DNA polymerase (Bangalore Genie, INDIA), 2.5 µL 10× buffer mixed with 15 mM MgCl₂, and 0.2 µL 25 mM dNTPs mix (Merck, India). The amplification of the reaction was performed in an Applied Biosystems Veriti 96 Well Thermal Cycler using the following temperature cycling parameters: initial denaturation for 5 min at 94 °C followed by 36 PCR cycles of denaturation at 92 °C for 1 min, corresponding annealing temperature for 1 min and extension at 72 °C for 2 min, and a final extension step at 72 °C for 7 min. The PCR products were separated using 8% non-denaturing polyacrylamide gel electrophoresis in 0.5× TBE buffer at a constant power of 120 V for 3 h using Genei vertical gel electrophoresis apparatus. The gels were stained with Ethidium bromide (SRL, India) (0.5 µg/ml) and visualized under UV light using Bio-Rad gel documentation system. A100 bp plus DNA step ladder (Thermo Scientific) was used as the molecular size standard.

Data analysis and construction of phylogenetic tree

The number of alleles and polymorphic information content (PIC) was calculated for 11 SSR loci producing polymorphic banding patterns (Saal and Wricke 1999). Furthermore, molecular data of all 96 samples analysed here were grouped and tree was constructed by an unweighted neighbor-joining tree-based dissimilarity matrix with 1000 bootstrap replicates using the Darwin software package v5 Perrier and Jacquemoud-Collet (2013).

Validation of unique SSR markers

Primers producing unique PCR profile were further validated by taking 30 individuals of each cultivar, Lakadong and Suvarna, for matching of unique pattern in those. The unique profiles were also used for identification of cultivars i.e. Lakadong and Suvarna from 88 accessions collected from different regions of Odisha.

Results

Morphological characteristics

In this study, we have collected eight different varieties of turmeric such as Suroma, Roma, Megha, Alleppey Supreme, Kedaram, Pratibha, Suvarna, and Lakadong along with 88 accessions from different regions (Table 1), but not a single variety could be identified correctly through comparison of morphological characteristics only. The morphological characteristics were very closely similar in all cultivars collected for this study with minor difference in rhizome size (Fig. S1) making it unreliable for authentic identification.

Biochemical analysis for curcumin, oleoresin, essential oil content, and gas chromatography (GCMS) analysis of eight turmeric cultivars

In this study, we have also analysed major biochemical characters such as curcumin, oleoresin, and essential oil content of eight turmeric cultivars (Table 2). The curcumin content was found to be highest (8.1%) in Lakadong and lowest (4.3%) in Suvarna. In case of oleoresin, both Lakadong and Prativa were found to be highest (16%) and Roma along with Megha sharing the lowest percentage (13.0%). Essential oil content was found to be highest (0.9%) in Lakadong and lowest (0.5%) in Kedaram. Similarly, fresh rhizome yield was maximum (436 g/plant) in Suroma and minimum (300 g/plant) in Suvarna.

Gas chromatography and mass spectrometry (GCMS) analysis was also carried out by taking rhizome essential oils of eight turmeric cultivars. After the analysis, we found that in all eight cultivars, the major constituent is α -Turmerone (Table 2) having no significant variation in area percentage (35–43%). As all the cultivars exhibited the same major constituent, it was very difficult to identify these cultivars on the basis of major phytoconstituents.

Establishing unique DNA profile using SSR markers to discriminate turmeric cultivars

A total of 50 SSR-containing primers were designed from EST sequences of turmeric. Of these, 24 primer pairs were found to be monomorphic, 11 primer pairs such as CSSR 7, CSSR 9, CSSR 14, CSSR 15, CSSR 18, CSSR 27, CSSR 29, CSSR 35, CSSR 38, CSSR 41, and CSSR 44 (Table 3) showed polymorphism, whereas ten primer pairs failed to amplify any product among cultivars such as Suroma, Roma, Lakadong, Suvarna, Megha, Kedaram, Pratibha, and Alleppey Supreme. These 11 set of SSR markers detected 79 alleles, with varied amplicon size ranging from 170 to 900 bp (Table 3). Here, we perceived some differences between the expected and the observed amplification product size. The range of allele numbers varied from 5 to 9 alleles in 11 polymorphic primers used. The PIC value of SSR markers ranged from 0.13 to 0.48 (Table 3). Different PCR conditions, including the concentrations of template DNA, Mg^{2+} , and Taq DNA polymerase and repeat experiments, were tested to further confirm the PCR amplification. Only two genotypes, viz., Lakadong and Suvarna, gave reproducible banding patterns in CSSR 14, CSSR 15, and CSSR 18 under all conditions tested where as other genotypes could not. The gel picture of SSR amplification of eight cultivars of turmeric including reproducible unique banding pattern of Lakadong and Suvarna (each having two replicates) with CSSR 18, CSSR 15 and CSSR 14 primers was given after separating with 8% polyacrylamide gel electrophoresis (Fig. 1). The unique profile of Lakadong genotype could be amplified with nine, five, and seven numbers of distinct alleles in CSSR 18, CSSR 15, and CSSR 14 primers, respectively. Similarly, the unique profile of Suvarna genotype was also amplified with six, four, and nine numbers of distinct alleles in CSSR 18, CSSR 15, and CSSR 14 primers, respectively (Fig. 1). These three SSR markers could efficiently differentiate Lakadong and Suvarna cultivar on the basis of unique profile from all other six cultivars taken in this study.

Genetic relationship of turmeric cultivars based on phylogenetic tree

The assessments of the genetic relationship of the 8 cultivars along with 88 accessions of turmeric were based on 11 EST-SSR markers which were analysed by 8% polyacrylamide gel. For example, a gel picture of primer CSSR 18 bearing 96 samples is given (Fig. 2). Similarly, 11 SSR primers were used for development of phylogenetic tree. After getting the DNA profile of 11 SSR loci, scoring has been done on the basis of the presence and absence of

Table 2 Biochemical analysis of eight cultivars of turmeric

Cultivar name	Curcumin (%) (mean $\pm$ SD)	Oleoresin (%) (mean $\pm$ SD)	Essential oil (%) (mean $\pm$ SD)	Major constituent	Area percentage of ar-Turmerone (mean $\pm$ SD)
Suroma	5.1 $\pm$ 0.4	14.7 $\pm$ 0.8	0.8 $\pm$ 0.1	ar-Turmerone	42.35 $\pm$ 0.3
Roma	5.0 $\pm$ 0.8	13.0 $\pm$ 0.4	0.8 $\pm$ 0.09	ar-Turmerone	39.21 $\pm$ 0.5
Lakadong	8.1 $\pm$ 0.5	16.0 $\pm$ 0.8	0.9 $\pm$ 0.08	ar-Turmerone	43.42 $\pm$ 0.4
Megha	6.0 $\pm$ 0.9	13.0 $\pm$ 0.9	0.8 $\pm$ 0.03	ar-Turmerone	40.63 $\pm$ 0.2
Alleppey Supreme	5.5 $\pm$ 0.1	15.5 $\pm$ 0.1	0.7 $\pm$ 0.04	ar-Turmerone	38.24 $\pm$ 0.4
Kedaram	5.4 $\pm$ 0.4	14.0 $\pm$ 0.6	0.5 $\pm$ 0.08	ar-Turmerone	41.68 $\pm$ 0.2
Prativa	6.1 $\pm$ 0.7	16.0 $\pm$ 0.4	0.7 $\pm$ 0.03	ar-Turmerone	35.24 $\pm$ 0.3
Suvarna	4.3 $\pm$ 0.2	13.02 $\pm$ 0.3	0.6 $\pm$ 0.01	ar-Turmerone	36.24 $\pm$ 0.2

Each sample was analysed in triplicates

Table 3 Details of 11 SSR markers giving polymorphic banding patterns among turmeric genotypes

SSR markers	Contig/GenBank accession no	Repeat motif	Primer sequence 5'–3'	Expected size (bp)	Product size (bp)	Allele no	PIC
CSSR 7	gil87149121	(CCT) 4	F: CGC AGC TGA CAC TTC TTC CT R: AAG TCC GGG AGT TCT AAA GG	440	220–650	9	0.45
CSSR 9	gil87148886	(TATT) 3	F: AAC AAG CCT TTA TCA AAA TTG C R: GCC GTG AAA ATC GTG AAG AC	566	380–900	7	0.38
CSSR 14	Contig 4757	(GGC) 6	F: CTG CGG TCC AAG TAC AAG ATC R: CTA GCT GGT GGC GGT GGT	360	170–550	9	0.48
CSSR 15	Contig 4832	(GAG) 6	F: AAG TCC TCC AAG AAC AACAAC R: CTT TAG CCC AAG TGA TAG ACG	550	370–850	5	0.13
CSSR 18	Contig 4971	(AGA) 13	F: CTT TTG GCT GAT AAA TGG AAG G R: AAG AAA GAA CTG ACA TCC TCC G	570	350–850	9	0.43
CSSR 27	gil87150473	(GGAG) 3	F: TCT CCC GAG TGA TTC TTT GA R: TCT TCC TCC ATA TCC CCT GA	470	170–800	8	0.40
CSSR 25	gil87148161	(TGC) 8	F: AGG TTT TGA GTG TGG GTT GG R: ACT CAG CAA AAG CTG CAA CA	483	210–790	6	0.23
CSSR 35	gil87149923	(CACCTC) 3	F: ATT GCT TCC CAA CCC AAA AT R: GAG ACA GCT CGG ATC ATT CC	445	230–800	5	0.15
CSSR 38	gil87148698 (2)	(GAG) 7	F: TTC AGG TTT CCG AGG ACC TT R: GCT TGT CGG CGA AGT TCA	400	210–770	9	0.44
CSSR 41	gil87148239	(GGA) 6	F: ATC AGC GTT GCA TCA TCA AA R: CAG GTC ATA TGA TAG CGA GCA A	457	250–820	7	0.35
CSSR 44	gil87148003	(GTC) 4	F: CAA AAG ATG CTG GCA CTC AA R: CAT CAG GGA AAA ATG GGA TG	488	265–830	5	0.17

bands. A phylogenetic tree was constructed using the Darwin v5 software. All the 96 genotypes were grouped into two main clusters. In one cluster, Lakadong varieties along with seven accessions (CBT 20, CBT21, CBT39, CBT40, CBT54, CBT76, and TC1) were grouped, and in another cluster, rest seven varieties and 81 accessions were grouped (Fig. 3). The tree clearly separated Lakadong and Suvarna varieties from all other six varieties taken in this

study. Roma and Suroma varieties along with 18 accessions were present in one group. Prativa, Kedaram, Alleppey Supreme, and Megha were grouped nearby each other showing maximum similarity among them (Fig. 3). The unique DNA profile obtained for Lakadong and Suvarna (Fig. 1) was validated through this phylogenetic tree as these two cultivars were far separated from other six varieties.

Fig. 1 Unique reproducible DNA banding pattern (marked as circle) of Lakadong and Suvarna clearly discriminates them from other six known turmeric cultivars using three EST-SSR markers such as **a** CSSR 18, **b** CSSR 15, and **c** CSSR 14 SSR primers, Lane 1–2

Suroma, Lane 3–4 Roma, Lane 5–6 Lakadong, Lane 7–8 Megha, Lane 9–10 Alleppey Supreme, Lane 11–12 Kedaram, Lane 13–14 Pratibha, Lane 15–16 Suvarna. Ladder used-100 bp DNA ladder plus

Validation of unique SSR markers

Unique profile of cultivar Lakadong and cultivar Suvarna generated in the present study was further confirmed by taking 30 individuals of each cultivars with three primers CSSR 14, CSSR 15, and CSSR 18 (data not shown). Furthermore, Lakadong was identified from the 88 accessions which indicate the prevalence of cultivation of Lakadong cultivar in Odisha, whereas Suvarna was not found among the accessions.

Discussion

In the present study, we have established for the first time, a unique DNA profiling of two turmeric cultivars, Lakadong and Suvarna, for their authentic identification using SSR markers. Since the morphological characters were

inconsistent to demarcate these cultivars, molecular profiling through use of SSR markers could be useful to address the problem of genetic identification of cultivars. Efficiency of SSR marker for cultivars identification has been reported in several plants (Trujillo et al. 2014; Sameh et al. 2014; Chen et al. 2014).

The biochemical analysis revealed that the curcumin content was found to be highest in Lakadong (8.1%) and lowest in Suvarna (4.3%). In case of oleoresin, both Lakadong and Prativa found to be highest (16%) and Roma along with Megha sharing the lowest percentage (13.0%). Essential oil content was found to be highest in Lakadong (0.9%) and lowest in Kedaram (0.5%) followed by Suvarna (0.6%) (Table 2). The curcumin, oleoresin, and essential oil content showing difference between Lakadong and Suvarna cultivar were not sufficient and reliable for cultivar identification as these contents are not stable and vary from place to place (our unpublished data). However, of

Fig. 2 Validation of CSSR 18 marker by taking unknown cultivated 88 turmeric accessions (Lane 10–96) of Odisha against known Lakadong and Suvarna cultivar as control. Lane 1 Suroma, Lane 2

Roma, Lane 9 Lakadong, Lane 4 Megha, Lane 5 Alleppey Supreme, Lane 6 Kedaram, Lane 7 Pratibha, Lane 8 Suvarna, 88 accessions (Lane 10–96). Ladder used-100b DNA ladder plus

course, the positive correlation of these data along with molecular profile together could be reliable and useful towards proper authentication of turmeric cultivars. SSR profiles could be efficient for discriminating and fingerprinting different cultivars. Microsatellite markers have also been proved to be highly polymorphic and competent for cultivar identification in other plants like apple (Liu et al. 2014), grapevine (Basheer-Salimia et al. 2014), olive (Sameh et al. 2014), Anthurium (Wang and Chuang 2013), and walnut (Chen et al. 2014).

In this study, a total of 50 SSR-containing primers were designed from EST sequences of turmeric out of which 10 primer pairs did not amplify any product; this is relatively low compared to the rate found with EST sequences in other species (Nicot et al. 2004). Failure to amplify the product might be due to several reasons: (1) primers designed from EST sequences might overlay two exons; (2) sequence errors might occur during primer synthesis; and (3) primer pairs could circumscribe large introns.

With respect to polymorphism, 11 primer pairs showed polymorphic banding pattern in different cultivars such as Lakadong, Suvarna, Megha, kedaram, Pratibha, and Alleppey Supreme, whereas in all SSR markers, Suroma and Roma genotypes gave similar banding patterns. This is possibly because Roma and Suroma cultivars have been selected with some biochemical differences only without having any genetic basis. Many times, different non-specific bands appeared creating unnecessary bias towards establishing proper SSR fingerprints. Thus, repeat experiment under changing conditions could give clearer results. In this study, different PCR conditions, including the concentrations of template DNA, Mg^{2+} , and Taq DNA polymerase and repetition of experiment, were tested to further confirm the PCR amplification. Finally, only two genotype, viz., Lakadong and Suvarna, gave reproducible unique banding patterns with CSSR 14, CSSR 15, and CSSR 18 primers under all conditions tested, whereas other genotypes could not (Fig. 1). The reason behind getting

Fig. 3 Unweighted neighbor-joining dendrogram based on 11 SSR loci. Turmeric genotypes and accessions are indicated by names according to Table 1. Bootstrap values over 90 have been added to the corresponding branches

unique banding patterns only in Lakadong and Suvarna might be due to differences in their genetic makeup. These three SSR markers (Table 3) could efficiently differentiate Lakadong and Suvarna cultivars, on the basis of unique profiles from rest other cultivars taken for this study.

Furthermore, in the study, we observed that in some cases, the amplified product size was either larger or smaller than the expected product size. The increase in product size might be due to the amplification of small introns or presence of an INDEL and decrease in product size might be resulted from: (1) deletion of a small sequence within flanking regions; (2) non-specific annealing of primers; and (3) duplication of SSR repeats in the genome. The similar type of results showing differences in expected and observed product size has also been reported in other species such as anthurium (Wang and Chuang 2013) and wheat (Nicot et al. 2004; Sehgal et al. 2012).

The 11 polymorphic primer pairs used here were amplified multiple reproducible banding patterns with high intensity. Amplification of multiple bands through SSR marker in the present study could be due to the allopolyploidy nature of turmeric (Ravindran et al. 2007), where the basic chromosome number is suggested as $x = 21$, in turn which was originated by dibasic amphidiploidy from $x = 9$ and $x = 12$ or by secondary polyploidy (Nair et al. 2010; Leong-Skorhickova et al. 2007). Multiple bands produced by SSRs in this study is in agreement with other reports of turmeric (Siju et al. 2010; Senan et al. 2013; Singh et al. 2015) and other polyploid species such as potato (Rocha et al. 2010) and apple (Liu et al. 2014) including allopolyploid species such as wheat (Nicot et al. 2004; Sehgal et al. 2012).

Furthermore, these SSR markers could be used to establish the genetic relationship of eight turmeric cultivars along with 88 accessions based on phylogenetic tree. The assessments of the genetic relationship of the eight cultivars along with 88 accessions were based on 11 EST-SSR markers. All the 96 genotypes were grouped into two main clusters. In one cluster, Lakadong cultivar along with seven accessions was grouped, and in another cluster rest, seven cultivars and 81 accessions were grouped (Fig. 3). The tree clearly separated Lakadong and Suvarna cultivars from all six cultivars taken in this study which further validated our result revealing conspicuous genetic difference. Out of 88 accessions, 18 (20.45%) were very closely related to Suroma and Roma cultivars. This is because in Odisha, mostly Roma and Suroma varieties are cultivated widely. Similarly, a total of six accessions, out of 88, exhibited similar banding pattern with that of cultivar Lakadong establishing the fact that Lakadong variety is cultivated in the state. This result again supported our study. The unique DNA profile obtained for Lakadong and Suvarna (Fig. 1) was also validated through this phylogenetic tree and was far separated from other six turmeric varieties. Other

researchers have also established the fingerprint profiles of 53 major cultivated Chinese bayberries using ten SSR markers (Xie et al. 2011). Madhou et al. (2013) used 11 SSR markers to characterize molecular polymorphisms among 88 litchi accessions and revealed the existence of 42 different genetic profiles. These results revealed that SSRs are potent tool for identification and protection of cultivars. Thus, the novel SSR markers developed in present study would be useful for authentic identification of the Lakadong and Suvarna cultivars of turmeric.

Conclusion

In conclusion, the present paper, with its reliable and reproducible nature results, indicates that a standard set of SSR markers (CSSR 14, CSSR 15, and CSSR 18) can be efficiently used for authentic identification of the two commercially important turmeric cultivars, Lakadong and Suvarna, and for establishing genetic relationship among other cultivars. This study while promising enough significance in cultivar identification, germplasm conservation, and genetic improvement of turmeric also shows the importance of applications of SSR markers in unique profiling of rare cultivars and the protection of resultant intellectual property.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

References

- Anandaraj M, Prasath D, Kandiannan K, Zachariah TJ, Srinivasan V, Jha AK, Singh BK, Singh AK, Pandey VP, Singh SP, Shoba N (2014) Genotype by environment interaction effects on yield and curcumin in turmeric (*Curcuma longa* L.). Ind Crop Prod 53:358–364
- ASTA (1958) Official analytical methods. American Spice Trade Association: Englewood Cliffs, NJ, Method 18.0 (Color power of turmeric)
- Basheer-Salimia R, Lorenzi S, Batarseh F, Moreno-Sanz P, Emanuelli F, Grando MS (2014) Molecular identification and genetic relationships of Palestinian grapevine cultivars. Mol Biotech. 56:546–556
- Behera L, Patra BC, Sahu RK, Nanda A, Sahu SC, Patnaik A, Rao GJ, Singh ON (2012) Assessment of genetic diversity in medicinal rice using microsatellite markers. Aust J Crop Sci 6:1369

- Chen L, Ma Q, Chen Y, Wang B, Pei D (2014) Identification of major walnut cultivars grown in China based on nut phenotypes and SSR markers. *Sci Hort* 26:168–240
- Chrobokova E, Raddová J, Vachůn M, Krška B, Pidra M (2011) An analysis of apricot cultivars by random amplified polymorphic DNA and microsatellite primers. *Hort Sci (Prague)* 38:125–133
- Corcolon EA, Laurena AC, Dionisio-Sese ML (2015) Genotypic characterization of turmeric (*Curcuma longa* L.) accessions from Mindanao, Philippines using RAPD markers. *Procedia Chem* 14:157–163
- Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* 12:13–15
- Hong JH, Kwon YS, Mishra RK, Kim DH (2015) Construction of EST-SSR databases for effective cultivar identification and their applicability to complement for Lettuce (*Lactuca sativa* L.) distinctness test. *Am J Plant Sci* 6:113
- Jan HU, Rabbani MA, Shinwari ZK (2011) Assessment of genetic diversity of indigenous turmeric (*Curcuma longa* L.) germplasm from Pakistan using RAPD markers. *J Med Plant Res* 4:823–830
- Joshi RK, Kuanar A, Mohanty S, Subudhi E, Nayak S (2010) In silico mining and characterization of EST derived microsatellites from *Curcuma longa* L. *Bioinformation* 5:128–131
- Kalia RK, Rai MK, Kalia S, Singh R, Dhawan AK (2011) Microsatellite markers: an overview of the recent progress in plants. *Euphytica* 177:309–334
- Khan H, Sivalingam PN, Chauhan S, Awasthi OP, More TA (2013) Improved crossing technique and identification of true F₁ hybrids of *Ziziphus mauritiana* Lam. by molecular markers. *Sci Hort* 150:164–171
- Koussao S, Gracen V, Asante I, Danquah EY, Ouedraogo JT, Baptiste TJ, Vianney TM (2014) Diversity analysis of sweet potato (*Ipomoea batatas* [L.] Lam) germplasm from Burkina Faso using morphological and simple sequence repeats markers. *Afr J Biotechnol* 13:729–742
- Leong-Skorhickova J, Sida O, Jarolimova V, Sabu M, Fer T, Travnicek P, Suda J (2007) Chromosome numbers and genome size variation in Indian species of *Curcuma* (Zingiberaceae). *Ann Bot* 100:505–526
- Liu GS, Zhang YG, Tao R, Fang JG, Dai HY (2014) Identification of apple cultivars on the basis of simple sequence repeat markers. *Genet Mol Res* 13:7377
- Madhou M, Normand F, Bahorun T, Hormaza JJ (2013) Fingerprinting and analysis of genetic diversity of litchi (*Litchi chinensis* Sonn.) accessions from different germplasm collections using microsatellite markers. *Tree Genet Genomes* 9:387–396
- Nair RR, Shiva KN, Anchu S, Zachariah TJ (2010) Characterization of open-pollinated seedling progenies of turmeric (*Curcuma longa* L.) based on chromosome number, plant morphology, rhizome yield and rhizome quality. *Cytologia* 75:443–449
- Nayak S, Naik PK, Acharya LK, Pattnaik AK (2006) Detection and evaluation of genetic variation in 17 promising cultivars of turmeric (*Curcuma longa* L.) using 4C nuclear DNA content and RAPD markers. *Cytologia* 71:49–55
- Nicot N, Chiquet V, Gandon B, Amilhat L, Legeai F, Leroy P, Bernard M, Sourdille P (2004) Study of simple sequence repeat (SSR) markers from wheat expressed sequence tags (ESTs). *Theor Appl Genet* 109:800–805
- Perrier X, Jacquemoud-Collet JP (2013) Darwin software. <http://darwin.cirad.fr/>
- Rama Rao M, Rao DV (1994) Genetic resources of turmeric, advances in horticulture. Plantation and spice crops. Malhotra Publishing House, New Delhi
- Ravindran PN, Babu KN, Sivaraman K (2007) (eds) Turmeric: the genus *Curcuma*. CRC Press
- Rocha EA, Paiva LV, Carvalho HH, Guimarães CT (2010) Molecular characterization and genetic diversity of potato cultivars using SSR and RAPD markers. *Crop Breed Appl Biotechnol* 10:204–210
- Saal B, Wricke G (1999) Development of simple sequence repeat markers in rye (*Secale cereale* L.). *Genome* 42:964–972
- Sameh MR, Olfa SD, Saleh B, Ali F (2014) Microsatellite marker-based identification and genetic relationships of millennium olive cultivars in Tunisia. *Int J Agro Agric Res* 5:55–63
- Sasikumar B (2005) Genetic resources of Curcuma: diversity, characterization and utilization. *Plant Genet Resour* 3:230–251
- Scott KD, Eggler P, Seaton G, Rossetto M, Ablett EM, Lee LS, Henry RJ (2000) Analysis of SSRs derived from grape ESTs. *Theor Appl Genet* 100:723–726
- Sehgal SA, Tahir RA, Nawaz M (2012) Molecular characterization of wheat genotypes using SSR markers. *Int J Bioautomation* 16:119–128
- Selvan MT, Thomas KG, Manojkumar K (2002) Ginger (*Zingiber officinale* Rosc.). Indian spices-production and utilization. Coconut Development Board, India, pp 110–131
- Senan S, Kizhakayil D, Sheeja TE, Sasikumar B, Bhat AI, Parthasarathy VA (2013) Novel polymorphic microsatellite markers from turmeric, *Curcuma longa* L. (Zingiberaceae). *Acta Bot Croat* 72:407–412
- Shamina A, Zachariah TJ, Sasikumar B, George JK (1998) Biochemical variation in turmeric (*Curcuma longa* Linn.) accessions based on isozyme polymorphism. *J Hortic Sci Biotechnol* 73:479–483
- Siju S, Dhanya K, Syamkumar S, Sasikumar B, Sheeja TE, Bhat AI, Parthasarathy VA (2010) Development, characterization and cross species amplification of polymorphic microsatellite markers from expressed sequence tags of turmeric (*Curcuma longa* L.). *Mol Biotechnol* 44(2):140–147
- Singh S, Joshi RK, Nayak S (2013) Identification of elite genotypes of turmeric through agro climatic zone based evaluation of important drug yielding traits. *Ind Crop Prod* 43:165–171
- Singh AK, Nanda P, Singh A, Singh B (2015) Genetic diversity analysis in turmeric (*Curcuma Longa* L.) based on SSR markers. *JBERR* 2(1):20–24
- Syamkumar S (2008) Molecular Biochemical and morphological characterization of selected Curcuma accessions. Thesis, Calicut:Calicut University
- Trujillo I, Ojeda MA, Urdiroz NM, Potter D, Barranco D, Rallo L, Diez CM (2014) Identification of the Worldwide Olive Germplasm Bank of Córdoba (Spain) using SSR and morphological markers. *Tree Genet Genomes* 10:141–155
- UPOV/INF/17/1 (2010) Guideline for DNA-profiling: molecular marker selection and database construction “BMT Guideline”. Geneva
- Varshney RK, Graner A, Sorrells ME (2005) Genic microsatellite markers in plants: features and applications. *Trends Biotechnol* 23:48–55
- Verma S, Singh S, Sharma S, Tewari SK, Roy RK, Goel AK, Rana TS (2015) Assessment of genetic diversity in indigenous turmeric (*Curcuma longa*) germplasm from India using molecular markers. *Physiol Mol Biol Plants* 21:233–242
- Wang JY, Chuang KC (2013) Development of novel microsatellite markers for effective applications in *Anthurium* cultivar identification. *Euphytica* 189:421–431
- Xie RJ, Zhou J, Wang GY, Zhang SM, Chen L, Gao ZS (2011) Cultivar identification and genetic diversity of Chinese bayberry (*Myrica rubra*) accessions based on fluorescent SSR markers. *Plant Mol Biol Rep* 29:554–562