# Development, characterization and utilization of genomic microsatellite markers in turmeric (Curcuma longa L.)

**Article** *in* Biochemical Systematics and Ecology · August 2010

DOI: 10.1016/j.bse.2010.08.006

# Development, characterization and utilization of genomic microsatellite markers in turmeric (*Curcuma longa* L.)

S. Siju [a], K. Dhanya [a], S. Syamkumar [a], T.E. Sheeja [a], B. Sasikumar [a,*], A.I. Bhat [b], V.A. Parthasarathy [a]

[a] Division of Crop Improvement and Biotechnology, Indian Institute of Spices Research, Calicut, Kerala 673 012, India
[b] Division of Crop Protection, Indian Institute of Spices Research, Calicut, Kerala 673 012, India

*Keywords:*
*Curcuma longa*
Genetic diversity
Microsatellite marker
Polymorphism
Simple sequence repeat
Turmeric

A B S T R A C T

Development of a robust set of 18 genomic microsatellite markers from turmeric (*Curcuma longa* L.) and its effective utilization in estimating the genetic diversity of 20 turmeric accessions are described. A total of 103 alleles were detected with an average of 5.7 alleles per locus. These markers displayed varied levels of polymorphism as evident from its discriminating power ranging from 0.19 to 0.70. The UPGMA cluster analysis of genetic distance values resolved the 20 turmeric accessions into five main groups. Three sets of genetically identical accessions were detected within the analyzed accessions, suggesting a revisit of the germplasm collection strategy based on vernacular identity. The entire grouping pattern of the entities was loose and independent of their geographical origins. These polymorphic SSR markers would be useful for the population genetic studies and germplasm management of turmeric.

## 1. Introduction

Turmeric (*Curcuma longa* L. syn. *Curcuma domestica* Val.), family Zingiberaceae, is a pan tropical crop cultivated widely in South East Asia. Due to its multitude use as spice, natural dye, food preservative and therapeutic agent (Sasikumar, 2005), it is gaining high demand in food, cosmetic and pharmaceutical industries. Turmeric is considered to be a triploid [$2n = 3x = 63$; $x = 21$] (Ramachandran, 1961; Islam, 2004), though a recent report based on the flow cytometric data and chromosome counts suggested a new ploidy status [$9x$] to turmeric by defining a new basic chromosome number [$x = 7$], but without contradicting the triploid status (Skornickova et al., 2007). Though turmeric is propagated clonally, viable sexual reproduction is also reported (Sasikumar et al., 1996). Lack of clear cut morphological traits among turmeric cultivars coupled with vernacular identity of the germplasm collection results in accumulation of duplicates in the germplasm accessions (Shamina et al., 1998), taxing heavily on conservation cost and hampering the crop improvement work. Existence of synonymous/homonymous entities within the crop (Shamina et al., 1998; Syamkumar, 2008) and genus *Curcuma* have been reported (Liu and Wu, 1999; Syamkumar and Sasikumar, 2007; Syamkumar, 2008).

Molecular markers could complement the conventional morphological studies for the discrimination of turmeric cultivars by providing a genetic background for the observed phenotypic variability. Molecular markers employed for characterization studies of turmeric are limited to the application of RAPD (Salvi et al., 2001; Panda et al., 2007; Tyagi et al., 2007), RAPD and or ISSR markers (Nayak et al., 2006; Hussain et al., 2008; Syamkumar, 2008; Vijayalatha and Chezhiyan, 2008), isozyme markers (Shamina et al., 1998) and SSR markers (Sigrist et al., 2010).

* Corresponding author. Tel.: +91 495 2731410; fax: +91 495 2731187.
*E-mail address:* bhaskaransasikumar@yahoo.com (B. Sasikumar).

Among the robust class of molecular markers, microsatellites or SSRs (simple sequence repeats) are widely employed for the accurate genotyping in a variety of plant species. These markers are characterized by the presence of 1–6 nucleotide repeats (Gupta et al., 1996), found within the coding and non coding regions (Tautz and Renz, 1984; Toth et al., 2000) of the genome with co-dominant, hyper variable and multiallelic nature (Powell et al., 1996). The development of SSR markers for a new species is time consuming and labour intensive (Zane et al., 2002; Squirrell et al., 2003). However, once developed, these markers provide endless high-throughput applications in molecular breeding by providing accurate, cost effective and reliable genotyping. Genomic SSR markers have been developed in other economically important spice crops such as *Zingiber officinale* (Lee et al., 2007), *Vanilla planifolia* (Bory et al., 2008) and *Piper nigrum* (Menezes et al., 2009). The development of 17 EST-SSR (Siju et al., 2010) and 17 genomic SSR (Sigrist et al., 2010) markers has been recently reported in turmeric. The present study was conducted to generate robust sets of reproducible and polymorphic microsatellite markers from a small-insert genomic library for its effective utilization in the germplasm characterization of turmeric.

## 2. Materials and methods

### 2.1. Plant materials and genomic DNA isolation

Genomic DNA was extracted from the fresh leaves of 20 turmeric accessions (Table 1), collected from different geographical locations of India and maintained at the Indian Institute of Spices Research (IISR) Experimental farm, Peruvannamuzhi, Calicut, India following the protocol of Syamkumar et al. (2003). The purity and concentration of the isolated DNA was estimated using Biophotometer (Eppendorf, Hamburg, Germany). The final DNA concentration was adjusted to 25 ng/μl for PCR analysis.

### 2.2. Construction of a small-insert microsatellite enriched genomic DNA library

A small-insert genomic DNA library enriched for the microsatellite repeat (AG)*n* in turmeric was constructed following the protocol of Glenn and Schable (2005). Briefly, the genomic DNA was digested with *Rsa* I restriction enzyme and ligated into double stranded SuperSNX linkers (SuperSNX24 forward – 5′-GTTTAAGGCCTAGCTAGCAGAATC-3′ and SuperSNX24+4P reverse -5′-pGATTCTGCTAGCTAGGCCTTAAACAAAA-3′). Linker-ligated DNA was denatured and hybridized to 3′ biotinylated oligonucleotide probe – $(AG)_{12}$. The hybridized probe-microsatellite containing DNA fragments were captured on streptavidin coated magnetic beads (Dynal Biotech ASA, Oslo, Norway). The hybridized DNA fragments were eluted from the beads, amplified by PCR using SuperSNX24 forward primer, cloned into pTZ57R/T vector (Fermentas, UAB, Lithuania) and transformed into One shot® TOP10 chemically competent *Escherichia coli* cells (Invitrogen, USA). Recombinant clones were identified using blue/white screening on LB agar plates containing ampicillin, X-gal and IPTG. Presence of inserts in the positive clones was confirmed using colony PCR with vector specific M13 forward (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse (5′-CAGGAAACAGCTATGAC-3′) primers. Plasmid DNA of selected clones was isolated using Fastplasmid mini kit (5 prime, Hamburg, Germany) and sequenced at the Bioserve Biotechnologies, Hyderabad, India.

**Table 1**
List of turmeric accessions used for genetic diversity analysis indicating their accession number, identity and place of collection from India.

| Sl. no: | Accession number | Accession identity | Place of collection (state, location in India) |
|---|---|---|---|
| 1 | 307 | Agarthala | Tripura, North-East India |
| 2 | 1 | Alleppey | Kerala, South India |
| 3 | 2 | Amalapuram | Andhra Pradesh, South India |
| 4 | 203 | Arunachal | Arunachal Pradesh, North-East India |
| 5 | 17 | Avanigadda | Andhra Pradesh, South India |
| 6 | 52 | Ayur | Kerala, South India |
| 7 | 560 | Coimbatore | TamilNadu, South India |
| 8 | 11 | Dhagi | Assam, North-East India |
| 9 | 290 | Dibrugarh | Assam, North-East India |
| 10 | 71 | Gaspani | Nagaland, North-East India |
| 11 | 16 | Gorakhpur | Uttar Pradesh. North India |
| 12 | 10 | Jabedi | Orissa, East India |
| 13 | 75 | Jorhat | Assam, North-East India |
| 14 | 28 | Kasturi | Andhra Pradesh, South India |
| 15 | 12 | Katergia | Orissa, East India |
| 16 | 31 | Kuchipudi | Andhra Pradesh, South India |
| 17 | 326 | Lakadong | Meghalaya, North-East India |
| 18 | 304 | Lembucherra | Tripura, North-East India |
| 19 | 288 | Manipur | Manipur, North-East India |
| 20 | 32 | Mananthody | Kerala, South India |

### 2.3. Nucleotide sequence analysis and detection of SSRs

The presence of microsatellites in the sequenced clones was identified using MSATCOMMANDER (Faircloth, 2008). Redundant sequences were identified by clustering the sequences using CAP3 program (Huang and Madan, 1999).

### 2.4. Primer designing and synthesis

A selected set of primer pairs flanking the SSR sequences were designed using the Primer 3 (Rozen and Skaletsky, 2000) and also by manual selection criteria of 18–26 nucleotide length, Tm of 55–65 °C, G + C content of 40–65%, generated amplicon size of 100–280 bp. Oligonucleotides were custom synthesized at Sigma GenoSys, Bangalore, India.

### 2.5. Amplification conditions and product electrophoresis

Amplification of the SSR loci was carried out in a 25 μl PCR mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5.0 pmol each of primers, 50 ng genomic DNA and 1 U *Taq* DNA polymerase (Sigma, Missouri, USA). The amplifications were performed using a thermal cycler (Master Cycler EP Gradient S, Eppendorf, Germany) with the following profile: initial denaturation at 94 °C for 5 min, 35 cycles of [denaturation at 94 °C for 30 s, annealing at 62–65 °C for 45 s, (depending on primers used, see Table 2), extension at 72 °C for 1 min], and a final extension at 72 °C for 20 min.

The amplified products were mixed with an equal volume of denaturing dye [98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 10 mM EDTA] and denatured at 94 °C for 5 mins. The PCR products were resolved in 8.0% denaturing PAGE (7 M Urea; 1× TBE) and the banding patterns were visualized by silver staining (Benbouza et al., 2006). Molecular size of the amplicons was estimated using a 10-bp DNA ladder (Invitrogen, Carlsbad, CA) and or 20-bp DNA ladder (Bangalore Genei,

**Table 2**
Characteristics of 18 polymorphic microsatellite markers developed in *Curcuma longa* L.

| Marker | GenBank accession number | Forward primer (5′–3′)<br>Reverse primer (5′–3′) | Repeat motif | $T_a$[a] (°C) | Allele size range (bp) | $N_a$[b] | $D$[c] |
|---|---|---|---|---|---|---|---|
| CuMiSat-01 | HM124531 | AAACCGCAAGAAAACTGAAG<br>CTCTTCCCTGAACGATTCC | $(AG)_6$ | 62 | 238–218 | 4 | 0.69 |
| CuMiSat-02 | HM124532 | TATGTGATGGTTGGGACG<br>GTAGTGGAGGAAGACGCC | $(AG)_{16}$ | 62 | 146–118 | 9 | 0.62 |
| CuMiSat-03 | HM124533 | GCACTACTTCCTTCTCGTTCAA<br>CGTCGTAAAGATTAGCGTGTG | $(AG)_{19}$ | 65 | 200–140 | 6 | 0.66 |
| CuMiSat-04 | HM124534 | TCAGGTTTCAGGGTGTAGAAG<br>CCCAGCAAGATTTTACCAAG | $(AG)_9$ | 64 | 204–188 | 2 | 0.19 |
| CuMiSat-05 | HM124535 | AGCAGTGCGTCTTTCATC<br>CTCTTGTCACGGAACCTC | $(AG)_{13}$ | 63 | 186–160 | 6 | 0.66 |
| CuMiSat-06 | HM124536 | AAGAAACTCCAACCACAATCC<br>CTTGTCTTCCTCCTCCATTG | $(AG)_{12}$ | 62 | 120–92 | 5 | 0.66 |
| CuMiSat-07 | HM124537 | AGCATGTGTCTAGCTCTTTGC<br>AAGCAGTCGTTCCTCTACTGAC | $(AG)_{19}$ | 64 | 220–128 | 5 | 0.62 |
| CuMiSat-08 | HM124538 | CATTGCGTGCCCACTTCC<br>CCTCCCTGTCGCTCTCCTC | $(AG)_{17}$ | 65 | 178–132 | 9 | 0.66 |
| CuMiSat-09 | HM124539 | AGTTGTGAAAGGGATAGAGTAGTTG<br>AAGAAAGCAAATGCCAAGG | $(AG)_{21}$ | 62 | 202–178 | 4 | 0.66 |
| CuMiSat-10 | HM124540 | CACCCTATGAGTGCTAACTGAAG<br>ACCTGCACCACGATCAAC | $(AG)_9$ | 65 | 206–186 | 3 | 0.62 |
| CuMiSat-11 | HM124541 | ACAGTCCCCTTCCCACTC<br>TCTTGTTCCTATGCTCTACGC | $(AG)_{15}$ | 65 | 208–166 | 7 | 0.66 |
| CuMiSat-12 | HM124542 | AAGGTTGCTGCTTGTTGAGAA<br>GCATATTGCCTTACATGCCTAA | $(AG)_7$ | 62 | 162–154 | 4 | 0.62 |
| CuMiSat-13 | HM124543 | CCCGAAGCCATTTCTCAG<br>TCGTCTCTCCTCTGCCAAC | $(AG)_8$ | 65 | 268–238 | 5 | 0.66 |
| CuMiSat-14 | HM124544 | GCTGACTGTGGCAAAAGAGTC<br>GCTGCGCTTCTTCTTAATGAC | $(AG)_7$ | 64 | 158–146 | 6 | 0.66 |
| CuMiSat-15 | HM124545 | GCAGAACTCACCAAGTAATGGC<br>TTGAACAACCAACACCCTAACTG | $(AG)_{11}$ | 62 | 201–177 | 6 | 0.57 |
| CuMiSat-16 | HM124546 | CATTTGTTCTGCTCGCTTCTAC<br>CTGCTCCGCTGTCTCTCAC | $(AG)_{19}$ | 63 | 196–158 | 7 | 0.62 |
| CuMiSat-17 | HM124547 | ATGTGGTTGAGGAATGATGAGAC<br>CTATTTCCCATAGCCCTTGTAGC | $(AG)_{18}$ | 65 | 228–196 | 8 | 0.66 |
| CuMiSat-18 | HM124548 | GTTCACAGCTTTAGCAGGGACAA<br>CTCCTCTCCATATTCTCCATCTCG | $(CT)_{14}$ | 65 | 238–208 | 7 | 0.70 |

[a] $T_a$, optimized annealing temperature of the primer.
[b] $N_a$, total number of alleles.
[c] $D$, discriminatory power (Tessier et al., 1999).

Bangalore, India). The electrophoretic patterns of the PCR products were recorded digitally using Kodak Gel Logic 200 image analysis system (Eastman Kodak Company, Rochester, NY).

### 2.6. Data analysis and interpretation

The variability at each locus was characterized in terms of number of alleles (Na). For assessing genetic similarity among the turmeric accessions, only clear and prominent bands were scored. The presence (1) and absence (0) of bands were recorded for individual accessions and the data were entered into a binary matrix as discrete variables. This binary matrix was used to calculate the genetic similarity as Jaccards coefficient using SIMQUAL subroutine in the SIMILARITY routine of NTSYS-pc version 2.02i (Rohlf, 1999; Exeter Software, Setauket, NY) software package. The resultant similarity matrix was employed to construct dendrograms using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighted Pair-Group Method with Arithmetic Mean (UPGMA). To test the goodness of fit between the UPGMA clustering and the original similarity matrix, the cophenetic correlation coefficient was computed using the COPH and MXCOMP module of the NTSYS-pc version 2.02i. The robustness of the dendrogram was evaluated with a bootstrap analysis performed on the binary data set using 1000 permutations in Freetree software (Pavlicek et al., 1999). To compare the efficiency of the generated markers for differentiating turmeric accessions, the discriminating power ($D$) (Tessier et al., 1999) of each locus was estimated.

## 3. Results

### 3.1. Identification of SSRs from microsatellite enriched library and their sequence properties

The microsatellite enriched genomic DNA library were screened and characterized for microsatellite repeats. Sequence analysis of 104 clones showed the presence of one or more SSRs in 87 clones. Assembling of sequences using CAP3 revealed 62 unique clones harboring microsatellite repeats. The size of the repeats varied from 6 to 21. Among the repeats detected, 53 could be classified as perfect, 2 as compound and 7 as imperfect repeats.

### 3.2. Primer designing and evaluation

Out of the 62 unique microsatellite repeats identified from the enriched genomic library, 48 were amenable to primer designing. Primers could not be designed for the remaining sequences due to the unavailability of sufficient number of bases to meet the primer designing criteria or due to biased base compositions in the flanking nucleotide sequence. A set of 48 primer pairs were synthesized and tested for amplification in 20 turmeric accessions. Prominent bands of expected size were generated by 32 primers. Other primers showed either no amplification or multiple bands or pronounced stutters and were excluded from further study.

### 3.3. SSR polymorphism in turmeric accessions

The polymorphic potential of the amplified microsatellite markers were tested in a panel of 20 turmeric accessions. Among the 32 primers that produced amplicons, 18 polymorphic SSR loci were identified which displayed varied levels of polymorphism. The remaining markers were monomorphic as revealed by its amplification profile. The polymorphic markers were assigned the name "CuMiSat" (*Curcuma* MicroSatellite) followed by an arbitrary number. A total of 103 different alleles (bands) could be detected on these accessions. The number of allelic variants ranged from 2 (CuMiSat-04) to 9 alleles (CuMiSat-08) with an average of 5.7 alleles per locus. During genotyping, a banding pattern with a maximum of three alleles per individual accessions was observed. The details of the newly generated markers along with their sequences characteristics and polymorphism potentials are listed in Table 2.

The discriminating power of the generated SSR markers ranged from 0.19 (CuMiSat-04) to 0.70 (CuMiSat-18). The combined average discriminating power of all loci was highly effective (0.62). Moreover, the markers (CuMiSat-08, 17 and 18) were highly informative with high value of $D$, number of alleles and good quality amplification profiles.

### 3.4. Genetic diversity analysis

Clustering of the 20 turmeric accessions using the dominant scoring (presence/absence) of bands based on UPGMA separated them into five main groups at the Jaccards similarity coefficient – 0.79 (Fig. 1). The cophenetic correlation coefficient between the dendrogram and the original similarity matrix was significant and high ($r = 0.996$), indicating a good fit of the original data to the clustering. Group I comprised of eight accessions in which a set of identical (synonymous) accessions were detected (Jabedi, Jorhat, Katergia and Manipur, all from the north-eastern part of India). Mananthody and Alleppey (two accessions from Kerala) and Kuchipudi (Andhra Pradesh) were the sole members of the group II, III and IV respectively. Group V formed the major group within the dendrogram by accommodating nine accessions from diverse geographical origins, in which two sets of identical accessions were detected. The first set was constituted by Amalapuram (Andhra Pradesh) and Gorakhpur (Uttar Pradesh). The second set of identical accessions

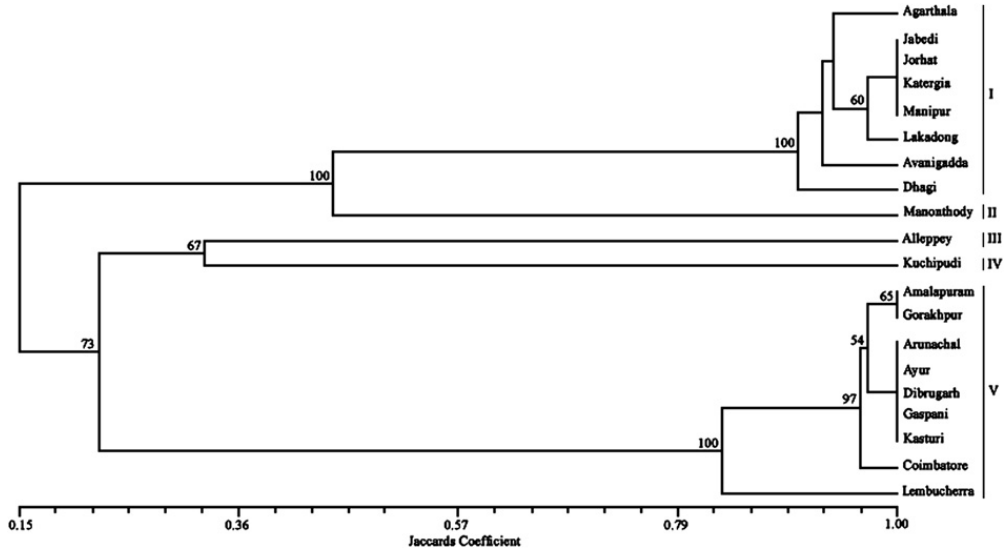


**Fig. 1.** UPGMA dendrogram showing the genetic divergence of 20 turmeric accessions based on the Jaccards similarity coefficients using 18 genomic microsatellite markers. Bootstrap values above 50 (in percentages) are indicated in the nodes.

within the group V were Arunachal (Arunachal Pradesh), Ayur (Kerala), Dibrugarh (Assam), Gaspani (Nagaland) and Kasturi (Andhra Pradesh). Lembucherra, an accession from Tripura (North-East India), formed a distant member within the same group V. An overview of the clustering pattern indicates that the grouping of the accessions was by and large independent of the place of collection.

The genetic similarity coefficients among the 20 accessions amplified using 18 SSR markers ranged from 0.10 to 1.0. The highest value (1.0) corresponded to [(Jabedi, Katergia, Jorhat and Manipur), (Amalapuram and Gorakhpur) and (Ayur, Arunachal, Dibrugarh, Gaspani and Kasturi)] accessions that generated identical fingerprints across the markers studied. Among the accessions, Avanigadda showed lowest similarity value (0.10) with seven accessions (Arunachal, Amalapuram, Ayur, Dibrugarh, Gaspani, Gorakhpur and Kasturi). Similarly, Alleppey too showed lowest similarity (0.10) with the Lakadong accession.

## 4. Discussion

### *4.1. Isolation of microsatellites and enrichment efficiency*

In the present study, a robust set of 18 polymorphic microsatellite markers were developed for use in turmeric. A microsatellite enrichment efficiency of 84% (87 repeats out of 104 sequenced clones) was observed using the protocol of Glenn and Schable, 2005. The results of the present study indicate the adequate distribution of $(AG)_n$ repeats in the *C. longa* genome thereby providing amble scope for generating more number of microsatellite markers. The abundance of the dinucleotide repeat – $(AG)_n$ in *C. longa* ESTs have been reported earlier (Siju et al., 2010).

### *4.2. Genetic polymorphisms as assessed by SSR markers*

The newly developed SSR loci were polymorphic and detected an average of 5.7 alleles per locus. In most of the genotyped markers, a maximum of three alleles per individual were observed, which further supports the triploid status of the *C. longa* L. (Ramachandran, 1961; Islam, 2004). This triploid type of amplification pattern is also in agreement with the recent reports on microsatellite markers isolated from turmeric (Siju et al., 2010; Sigrist et al., 2010). As it is difficult to ascertain the exact allelic dosage of individuals presenting two detected alleles (partial heterozygotes) for a given locus in triploids (Esselink et al., 2004; Nakagawa and Ito, 2009), calculation of heterozygosity [$H_E$], standard tests for deviations from Hardy-Weinberg equilibrium [HWE] and linkage disequilibrium [LD] were not possible. The inability of calculating these genetic parameters in turmeric was also reported earlier (Siju et al., 2010; Sigrist et al., 2010). However, discrimination power (Tessier et al., 1999) of all generated microsatellite markers was calculated. The discrimination power calculations have been previously applied to polyploids (Jungmann et al., 2009; Kiani et al., 2010) as a measure of estimating the efficiency of SSR markers in discriminating accessions. The average discriminating power of microsatellite markers (0.62) observed in the present study ensures the future utility of the generated microsatellite markers for genetic diversity studies in turmeric.

### 4.3. Germplasm characterization and analysis of genetic diversity

Accurate knowledge of genetic variability among turmeric accessions is important for establishing core collections of germplasm and in aiding breeding work. Instances of synonymous entities identified in the present study indicate the robustness of microsatellites markers for the precise assessment of cultivar type and genetic diversity in turmeric. Among the 20 studied accessions, three cases of synonymous entities were identified. The first one was contributed by members of Group I (Jabedi, Jorhat, Katergia and Manipur), all entities collected from the adjoining states in the north-east India based on vernacular identity rather than any genetic identity. The second set of identical accessions was composed of entities in the group V – Amalapuram (Andhra Pradesh) and Gorakhpur (Uttar Pradesh). The third set of accessions that showed identical patterns were from north-eastern [Arunachal (Arunachal Pradesh), Gaspani (Nagaland), Dibrugarh (Meghalaya)]; northern [Gorakhpur (Uttar Pradesh)] and southern [Ayur (Kerala), Kasturi (Andhra Pradesh)] states of India. Existence of synonymous turmeric accessions in Brazil using genomic SSR markers has been described recently (Sigrist et al., 2010).

Rapid germplasm movement across the length and breadth of the country by the settlers would have resulted in the spread of the cultivars to different parts of India. It is quite possible that the same genetic material would have been spread to different regions in the olden days and got acclimatized to the place under a different vernacular name in course of time. Clonal propagation would have resulted in preserving/fixing the genetic fidelity of the original material. Recollection of the same entities based on vernacular identity would have added to the build up of duplicates in the gene bank. The SSR analysis confirms the fact that collecting turmeric accessions based on vernacular identity could result in adding duplicates into the germplasm collections.

Thus the newly developed microsatellite markers exhibiting rich polymorphism could be used for future studies involving assessment of genetic diversity of turmeric for generating a reliable molecular data for assisting the crop improvement of turmeric.

## Acknowledgements

This work was supported by a research grant from the Department of Biotechnology (DBT), Government of India, New Delhi. The authors thank Distributed Information Sub Centre, Indian Institute of Spices Research, Calicut for providing the internet facility.

## References

Benbouza, H., Jacquemin, J.M., Baudoin, J.P., Mergeai, G., 2006. Biotechnol. Agron. Soc. Environ. 10, 77–81.
Bory, S., DaSilva, D., Risterucci, A.M., Grisoni, M., Besse, P., Duval, M.F., 2008. Sci. Hortic. 115, 420–425.
Esselink, G.D., Nybom, H., Vosman, B., 2004. Theor. Appl. Genet. 109, 402–408.
Faircloth, B.C., 2008. Mol. Ecol. Resour. 8, 92–94.
Glenn, T.C., Schable, N.A., 2005. Methods Enzymol. 395, 202–222.
Gupta, P.K., Balyan, H.S., Sharma, P.C., Ramesh, B., 1996. Curr. Sci. 70, 45–54.
Huang, X., Madan, A., 1999. Genome Res. 9, 868–877.
Hussain, Z., Tyagi, R.K., Sharma, R., Agrawal, A., 2008. Biol. Plant. 52, 627–633.
Islam, M.A., 2004. PhD Thesis, University of Hannover, Germany.
Jungmann, L., Sousa, A.C.B., Paiva, J., Francisco, P.M., Vigna, B.B.Z., do Valle, C.B., Zucchi, M.I., de Souza, A.P., 2009. Conserv. Genet. 10, 1873–1876.
Kiani, M., Zamani, Z., Khalighi, A., Fatahi, R., Byrne, D.H., 2010. Plant Breed.. doi:10.1111/j.1439–0523.2009.01708.x.
Lee, S.Y., Fai, W.K., Zakaria, M., Ibrahim, H., Othman, R.Y., Gwag, J.G., Rao, V.R., Park, Y.J., 2007. Mol. Ecol. Notes 7, 1009–1011.
Liu, N., Wu, T.L., 1999. J. Trop. Subtrop. Bot. 7, 146–150.
Menezes, I.C., Cidade, F.W., Souza, A.P., Sampaio, I.C., 2009. Conserv. Genet. Resour. 1, 209–212.
Nakagawa, S., Ito, M., 2009. J. Plant Res. 122, 581–584.
Nayak, S., Naik, P.K., Acharya, L.K., Pattnaik, A.K., 2006. Cytologia 71, 49–55.
Panda, M.K., Mohanty, S., Subudhi, E., Acharya, L., Nayak, S., 2007. Int. J. Integr. Biol. 1, 189–195.
Pavlicek, A., Hrda, S., Flegr, J., 1999. Folia Biol. (Praha.) 45, 97–99.
Powell, W., Machray, G.C., Provan, J., 1996. Trends Plant Sci. 1, 215–222.
Ramachandran, K., 1961. Curr. Sci. 30, 194–196.
Rohlf, F.J., 1999. NTSYS-pc Numerical Taxonomy and Multivariate Analysis System. Version 2.02i. Exeter Software, Setauket, New York.
Rozen, S., Skaletsky, H., 2000. In: Krawetz, S., Misener, S. (Eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, USA, pp. 365–386.
Salvi, N.D., George, L., Eapen, S., 2001. Plant Cell Tissue Organ Cult. 66, 113–119.
Sasikumar, B., 2005. Plant Genet. Resour: C&U. 3, 230–251.
Sasikumar, B., George, J.K., Zachariah, T.J., Ratnambal, M.J., Babu, N.K., Ravindran, P.N., 1996. J. Spices Aromatic Crops 5, 41–48.
Shamina, A., Zachariah, T.J., Sasikumar, B., George, J.K., 1998. J. Hortic. Sci. Biotechnol. 73, 479–483.
Sigrist, M.S., Pinheiro, J.B., Azevedo-Filho, J.A., Colombo, C.A., Bajay, M.M., Lima, P.F., Camilo, F.R., Sandhu, S., Souza, A.P., Zucchi, M.I., 2010. Plant Breed. doi: 10.1111/j.1439–0523.2009.01720.x.
Siju, S., Dhanya, K., Syamkumar, S., Sasikumar, B., Sheeja, T.E., Bhat, A.I., Parthasarathy, V.A., 2010. Mol. Biotechnol. 44, 140–147.
Skornickova, J.L., Sida, O., Jarolimova, V., Sabu, M., Fer, T., Travnicek, P., Suda, J., 2007. Ann. Bot. 100, 505–506.
Squirrell, J., Hollingsworth, P.M., Woodhead, M., Russell, J., Lowe, A.J., Gibby, M., Powell, W., 2003. Mol. Ecol. 12, 1339–1348.
Syamkumar, S., 2008. PhD Thesis, Calicut University, Calicut, India.
Syamkumar, S., Lowarence, B., Sasikumar, B., 2003. Plant Mol. Biol. Rep. 21 171a–171e.
Syamkumar, S., Sasikumar, B., 2007. Sci. Hortic. 112, 235–241.
Tautz, D., Renz, M., 1984. Nucleic Acids Res. 12, 4127–4138.
Tessier, C., David, J., This, P., Boursiquot, J.M., Charrier, A., 1999. Theor. Appl. Genet. 98, 171–177.
Toth, G., Gaspari, Z., Jurka, J., 2000. Genome Res. 10, 967–981.
Tyagi, R.K., Agrawal, A., Mahalakshmi, C., Hussain, Z., Tyagi, H., 2007. In Vitro Cell. Dev. Biol. Plant 43, 51–58.
Vijayalatha, K.R., Chezhiyan, N., 2008. J. Hortic. Sci. 3, 107–111.
Zane, L., Bargelloni, L., Patarnello, T., 2002. Mol. Ecol. 11, 1–16.