Innovare
Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 10, 2014

Original Article

ASSESSMENT OF GENETIC DIVERSITY IN *TINOSPORA CORDIFOLIA* BY INTER SIMPLE SEQUENCE REPEATS (ISSR) AND EXPRESSED SEQUENCE TAGGED- SIMPLE SEQUENCE REPEATS (EST-SSR)

KHUSHBOO SINGH, SANGEETA KADYAN, MANJU PANGHAL, JAYA PARKASH YADAV

Department of Genetics, M. D. University, Rohtak-124001, Haryana, India.
Email: yadav1964@rediffmail.com

Received: 11 Sep 2014 Revised and Accepted: 10 Oct 2014

ABSTRACT

Objective: In this study, assessment of genetic diversity was carried out using two kinds of molecular markers: Inter-Simple Sequence Repeats (ISSR) and Expressed Sequence Tag Simple Sequence Repeats (EST-SSR) in *T. cordifolia.*

Methods: A total of 20 primers/primer pairs were tested for the detection of polymorphism. For genetic diversity assessment, certain parameters such as Polymorphic Information Content (PIC), Marker Index (MI), effective multiplex ratio (EMR) and DDI (Diversity detecting Index) were used.

Results: The PIC, MI, EMR and DDI values ranges from 0.306-0.351, 0.76-1.18, 3.86-2.16 and 0.739-0.175 respectively. Cluster analysis based on Jaccard`s similarity coefficient using an Unweighted Pair Group Method with Arithmetic mean (UPGMA) classified all 24 accessions in to two major clusters respectively for both the marker system which demarcated the accessions according to different climatic zones. Similarity indices ranged from 0.68-1.0 for ISSR and 0.52-0.96 for EST-SSR.

Conclusion: Both marker systems ISSR and EST-SSR separate out the accessions from different climatic zones in to different groups. In addition, both have shown a high genetic diversity and a good consistency among different genotypes of *T. cordifolia*. Out of these two, EST-SSR proves more efficient as it directly correlates with the geographical distribution of the plant.

Keywords: *Tinospora cordifolia*, ISSR, EST-SSR, Genetic diversity.

INTRODUCTION

The ability of a species to adapt to different environmental conditions resides in their genetic diversity. Thus, genetic diversity plays an important role in survival and adaptability of the species. Methods of evaluation of genetic diversity include phenotypic, biochemical and molecular markers [1]. Among these markers, molecular markers are not affected by environmental conditions, are found in the entire genome. They are widely used in studies of genetic diversity. Microsatellites, also referred to, as simple sequence repeats (SSRs), are tandem repeated sequences and comprise mono-, di-, tri-, tetra-, penta or hexa-nucleotide units. SSRs possess a large number of advantages, such as high information content, co dominance, locus specificity and easy detection as PCR-based molecular markers. These have become important tools to study genetic diversity, construct genetic maps and analyze evolutionary processes of plant species [2,3]. Two different marker strategies have been used based on microsatellites; SSR and inter-simple sequence repeat (ISSR).

ISSR is a general term for a genome region between microsatellite loci. The complementary sequences to two neighbouring microsatellites are used as PCR primers; the variable region between them gets amplified. Inter-Simple Sequence Repeats (ISSR) are widely used in genetic diversity studies as they need no prior DNA sequence information, development cost is low, and laboratory procedures can easily be transferred to any plant species [4,5]. Traditional methods of developing Simple Sequence Repeats (SSR) markers are usually time-consuming and labour-intensive, but with the development of functional genomics, expressed sequence tag (EST)-SSR has become the latest aspect of SSR development. EST-SSR originates from transcribed regions of genomes, which may reflect information of specific genes. Consequently, EST databases have become an increasingly valuable resource for SSR marker or its development [6,7,8]. So far, the study of polymorphism, diversity and transferability of EST-SSRs has been performed in many plant species such as *Citrus* [9], soyabean [10], Jatropha [11], sorghum [12] etc.

Tinospora cordifolia is a diploid (2n=22), deciduous climbing shrub belonging to family Menispermaceae. According to ancient traditional Ayurvedic system of India, it is a constituent of several remediation used for various treatments such as general debility, dyspepsia and urinary diseases [13]. Detection of polymorphism in this plant, using the ISSR and EST-SSR are certainly better than other previously used marker systems (as best to our knowledge, this is the first study on EST-SSR and no one has used these two-marker systems simultaneously for estimation of genetic diversity in *T. cordifolia*). Hence, we report the development of EST SSR markers and an attempt was made to develop a better approach to analyze the genetic diversity of 24 different genotypes of *T. cordifolia* collected from the Northwest region of India by using the two marker systems i. e. ISSR and EST- SSR.

MATERIALS AND METHODS

Collection of different plant accessions of *T. cordifolia* & DNA Extraction: A total of 24 different genotypes of *T. cordifolia* were collected from 8 different ecological sites of 3 climatic zones of Northwest India for the analysis of genetic diversity by ISSR and EST-SSR (Table 1).

Primer designing for ISSR

A total number of 20 ISSR primers (Table 2), synthesized from Sigma Aldrich.

Design of EST SSR: A total of 500 *T. cordifolia* EST sequences were retrieved from NCBI database. For EST-SSR development, we analyzed all these sequences by tandem repeat finder. After pre-processing, SSR containing sequences were identified by Gramene, SSRIT - Simple Sequence Repeat Identification Tool [15]. 20 primer pairs were successfully developed on the basis of following standard parameters by using primer3 software [16] (a) the target amplicon size of 200–900 bp, (b) the optimal annealing temperature to 52.8-60.1°C, (c) average GC content 45-60% and (d) the primer length at 18-24 bp. These 20 EST-SSR primers (Table 3) were synthesized from Eurofins.

Table 1: Collection sites: 24 accessions from 8 ecological sites of 3 different climatic zones

S. No.	Accessions	Collection Site	Climatic Zone
1	JS1 JS2 JS3	Jaisalmer	Arid Western Zone Rajasthan
2	B1 B2 B3	Bikaner	Arid Western Zone Rajasthan
3	R1 R2 R3	Rohtak	Semi Arid Central Zone Haryana
4	J1 J2 J3	Jaipur	Semi Arid Eastern Zone Rajasthan
5	S1 S2 S3	Sirsa	Semi Arid North West Zone Haryana
6	M1 M2 M3	Mahendragarh	Semi Arid Southwest Zone Haryana
7	BH1 BH2 BH3	Bhopal	Humid Sub Tropical Zone Madhya Pradesh
8	U1 U2 U3	Udaipur	Sub Humid Southern Zone Rajasthan

Total genomic DNA was extracted using a modified CTAB method based on the protocol of Doyle & Doyle [14]. Quality and concentration of total DNA was verified by UV 2450 spectrophotometer (Shimadzu, Japan) at 260 and 280 nm. Further quality of DNA was tested by submerged horizontal agarose gel (0.8%) DNA electrophoresis (Biorad, USA) and visualized under UV light, Gel documentation system (Alpha Innotech, USA).

Table 2: List of ISSR Primers

S. No.	Primer Name	Tm (°C)	Sequence(5´→ 3´)
1	ISSR 1	35.1	GCACACACACAC(12)
2	ISSR 2	66.5	GGGTGGGGTGGGGTG(15)
3	ISSR 3	45	GACAGACAGACAGACA(16)
4	ISSR 4	44.1	ACTGACTGACTGACTG(16)
5	ISSR 5	56.4	GCGACACACACACACA(16)
6	ISSR 6	23.8	ATATATATATATATATC(17)
7	ISSR 7	46.8	AGAGAGAGAGAGAGAGC(17)
8	ISSR 8	46.6	AGAGAGAGAGAGAGAGG(17)
9	ISSR 9	42.9	GAGAGAGAGAGAGAGAT(17)
10	ISSR 10	43.3	GAGAGAGAGAGAGAGAC(17)
11	ISSR 11	50.3	GTGTGTGTGTGTGTGTC(17)
12	ISSR 12	53.3	ACACACACACACACACC(17)
13	ISSR 13	54.9	ACACACACACACACACG(17)
14	ISSR 14	53.2	TGTGTGTGTGTGTGTGA(17)
15	ISSR 15	59	CACACACACACACACACG(18)
16	ISSR 16	53.7	CACACACACACACACAGT(18)
17	ISSR 17	46	GAGAGAGAGAGAGAGATC(18)
18	ISSR 18	55.5	CACACACACACACACATG(18)
19	ISSR 19	55.3	GAGCAACAACAACAACAA(18)
20	ISSR 20	59.1	CTCGTGTGTGTGTGTGTGT(19)

Table 3: List of EST SSR Primer pairs

S. No.	Primer Name	SSR Motif	Tm (°C)	Forward Primer	Reverse Primer
1	T1	$(TC)_{34}$	55.9	ATCGATTCTTGCCACACTCC	TCAACACAAAGAGAAACAAACACA
2	T2	$(CAC)_{7}$	55.3	TTATCAGCGCATCGGTTACA	GAGGAATACGGGTTCGATCA
3	T3	$(AAG)_{11}$	57.3	CGCTTTCATTCTCCGACTTC	GCAATCTCATCCTTCCTTGC
4	T4	$(AT)_{12}$	52.8	CGTCGAGCCTTACATCAACA	ACAACCAAAACATCAAATGACA
5	T5	$(AAG)_{15}$	57.3	CAGAGAGGCAACATCGGAGT	TCCGTTCTTACGGATGGTTC
6	T6	$(TGA)_{7}$	55.3	CCGTTTATTCAGCCCTTTCA	ATCGTCCCATGGTTTCACAT
7	T7	$(CTT)_{10}$	57.3	TCTGTGGCTCCAATTTCTCC	GCGATCAGCAATACCCAAGT
8	T8	$(AAG)_{12}$	55.3	CGATTGTTCTTCGCGTTCTT	GGGAGCCATGTATCCAGTGT
9	T9	$(CTT)_{12}$	55.3	ACCGATCAACGCTTTTCAAC	GCACACACAATGCCATAAGC
10	T10	$(CT)_{16}$	55.3	TTCATGGAGACGAAGCCTCT	CATCCGCAGTCCATTTTCTT
11	T11	$(GAG)_{5}$	57.3	GCCGAGGTGCTCTTATTGAA	CCTTTCCACCTAGTGCTTCG
12	T12	$(GAG)_{5}$	57.3	GTTAAATCTGTGTCGCTGCTC	CGAAGCCGACATCTGAAATC
13	T13	$(AGA)_{7}$	54	TAACAAACCCAGCATCACCA	ATTGGAACTGTCCCTGTTGG
14	T14	$(TAT)_{4}$	54.7	ATTACCTCGCAGGGGAATC	TCCATCCACAAAAGAATGAATG
15	T15	$(CT)_{10}$	55.9	TCCGACGTCTTCTTCTTCGT	TGCAGCCTCAAAACTTTCATC
16	T16	$(CAC)_{6}$	59.3	TCATGTCCAACATTCCTAAAACA	TCAAAGTAATCGTCTGATCGTCAT
17	T17	$(CT)_{10}$	58.6	CCAAAATTGCAGTTCGTGAT	GGAGGGAGGGAGGGAGAG
18	T18	$(AAG)_{11}$	60	TAAAGACGGTGCAAGGAAGG	GTCTTGCAAGGGCTCTGTTC
19	T19	$(CAC)_{7}$	59.1	TCATGTCCAACATTCCTAAAACA	CATTCAAAGTAATCGTCTGATCG
20	T20	$(AT)_{10}$	60.1	CCTGGTGCGTTTGAGAAGAT	AATATAACGCAGGCCACTCG

Finally, 20 primer for ISSR and 20 primer pairs for EST-SSR were selected for validation of amplification and assessment of the polymorphism in *T. cordifolia.*

PCR Amplification for ISSR and EST SSR

A total number of 20 ISSR primers and 20 EST-SSR primer pairs were screened using DNA samples. PCR amplification was performed in a thermo cycler (BioRad).

An initial denaturation period of 10 min at 94 ºC was followed by 44 cycles at 94 ºC, 1 min at 33-53ºC, 2 min at 72 ºC, and then 10 min at 72 ºC for final extension. Reaction was carried out in a total volume

of 25 µl containing about 50 ng of template DNA, 200 µM of dNTPs, 2.5 µl of 10 × Taq buffer, 10 µM of Primer and 1.5 U Taq DNA polymerase. To reduce the possibility of cross contamination in the amplification reactions, a control reaction was used. It consisted of the reaction mixture excluding any DNA matrix. The amplification products were analyzed by electrophoresis on 1.5% agarose gel for ISSR and 2-2.5% for EST SSR in 1× TAE buffer (pH 8.3) and detected by ethidium bromide staining. The 500 bp DNA Ladder was used to determine the size of the ISSR fragments and 100 bp Ladder was used for EST-SSR product.

From the preliminary screening, primers that could amplify visible bands were selected for further examination. Different anneal temperature was examined to optimize the amplification condition for the selected primers. Eventually, primers that produced clear and reproducible bands were selected for the amplification of all samples. The gel was photographed under UV light in a gel documentation system (Alpha Innotech).

Data Analysis

The bands were scored using the binary scoring system that recorded the presence and absence of bands as 1 and 0, respectively. Typical bands scored were bright and well separated from other bands, and faint bands were not scored to avoid the scoring of artificial bands. Genetic similarity was calculated on the basis of Jaccard's similarity coefficient.

The resulting matrix of genetic similarity was used to construct the dendrogram through the unweighted pair group method with arithmetic mean (UPGMA) with the help of statistical package NTSYS-pc version 2.0.2e [17].

The ISSR and EST SSR markers were characterized by using **Marker Index (MI)**, which is obtained by multiplying **Polymorphic Information Content (PIC)** and **effective multiplex ratio (EMR).**

PIC was calculating by using formula;

$1-\sum P_i^2$ where *P* is the frequency of *i*-th allele [18].

EMR is the average number of polymorphic loci in a single analysis for a particular set of objects [19,20].

DDI (Diversity Detecting Index) was calculated from the proposed formula:

$DDI=PIC\times n_m/n_{OTU}$

Where n_m is the number of markers (polymorphic loci) and n_{OTU} is the number of examined objects (operational taxonomic units-OTU)

RESULTS

Assessment of genetic diversity in *T. cordifolia*

In case of ISSR, 15 primers have shown polymorphism and have generated 83 discernible DNA fragments with 58 being polymorphic. Each band indicated as a single locus, minimum loci shown by a primer was 3 by ISSR primer 13 and 15. Primer 17 showed the maximum 10 bands.

However, in case of EST SSR, each primer denotes a specific loci and a single band specifies as an allele. Therefore, out of 20 primer pairs, 12 were found to be polymorphic i. e. 12 loci. These 12 loci generated 26 alleles (1-6 alleles per locus) i. e. T1, T5, T10 and T14 have shown a single allele/locus and T15 have shown 6 alleles /locus with an average of 2.16 alleles /locus.

For assessment of genetic diversity certain parameters were used such as PIC, EMR, MI and DDI.

Polymorphism Information Content (PIC)

PIC (mean value) calculated for ISSR and EST SSR were 0.306 and 0.351 respectively. ISSR results showed 3.87 loci per primer and EST SSR showed average of 2.17 allele per locus. PIC was calculated for each polymorphic locus with a range of 0.13- 0.46 for ISSR and 0.10-0.46 for EST-SSR (Table 4).

Effective Multiplex Ratio (EMR)

Since EST SSR markers are usually locus specific, the 12 markers analyzed are considered equal to 12 loci (as one locus per primer pair) in the present study. In case of ISSR marker, 15 markers yielded 58 polymorphic loci. EMR is the average number of polymorphic loci in a single analysis for a particular set of objects. In case of ISSR and EST SSR, the EMR were 3.86 and 2.16 respectively (Table 4).

Marker Index (MI): ISSR had higher MI values (1.18) than that of EST SSR (0.76) because of polymorphism in many loci (Table 4).

Diversity Detecting Indexes (DDI): Diversity detecting indexes in our study were DDI_{ISSR} = 0.739 and $DDI_{EST\ SSR}$ =0.175 (Table 4).

Table 4: Characteristics of two types of molecular markers used for genetic similarity analysis of 24 accessions of *T. cordifolia*

Characteristics	ISSR*	EST SSR
Number of primer or primer pairs	15	12
Number of polymorphic product	58	26
EMR	3.86	2.16
PIC range of values	0.13-0.46	0.10-0.46
PIC mean	0.306	0.351
MI(EMR×PIC)	1.18	0.76
DDI	0.739	0.175

*for ISSR only polymorphic products were considered

Dendrogram of ISSR

The 24 genotypes were divided in to two main groups A and B on the basis of Unweighted Pair-Group Method with Arithmetic averages (UPGMA) cluster analysis. The cluster A including 6 genotypes (all genotypes of Udaipur, two of Jaisalmer and one genotype of Bhopal) and cluster B having remaining 18 genotypes. Further, the dendrogram divided in to two sub clusters B1 and B2. The B2 sub cluster including 2 genotypes (one each of Sirsa and Mahendragarh) and B1 having 16 genotypes (Figure 1).

Dendrogram of EST-SSR

The 24 genotypes were divided into two main groups A and B on the basis of Unweighted Pair-Group Method with Arithmetic averages (UPGMA) cluster analysis including 2 genotypes in cluster A and 22 genotypes in cluster B. The two genotypes of Bhopal were included in group A and remaining of the genotypes was in a separate group B. Further all genotypes of Sirsa, Rohtak and Udaipur separates out in sub cluster B1with one jaipur genotype. Whereas, all the genotypes of Mahendragarh, Bikaner and Jaisalmer separated in to sub cluster B2 with two genotypes of Jaipur (Figure 1).

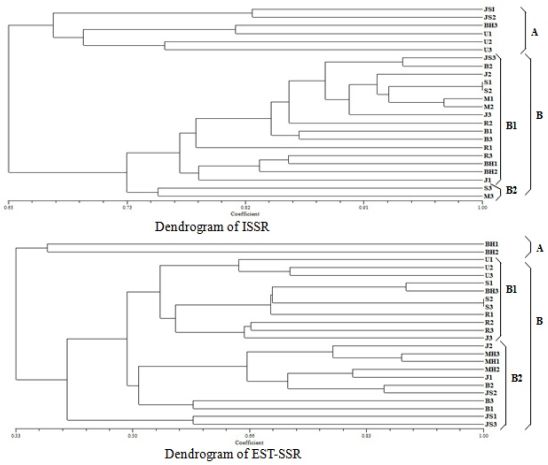

Fig. 1: shows dendrograms of 24 accessions of *T. cordifolia* based on Genetic Similarity calculated using ISSR and EST-SSR

DISCUSSION

T. cordifolia is an important medicinal plant used in traditional Indian system of medicine, Ayurveda. Therefore, it is important to develop a species-specific marker system like EST-SSR along with ISSR. In the present study, we mined the publically available EST database of *T. cordifolia* for SSRs, designed primers to amplify a subset of these loci, and screened them to detect polymorphism for genetic diversity. The various researchers have studied the genetic diversity of the plant in different regions of India with the help of different molecular markers mainly by RAPD and a single study of ISSR. Rout [21] studied the genetic diversity of 15 individual clones of *Tinospora cordifolia* collected from the reserve forests of Orissa by using RAPD markers. Their results showed the low level of genetic diversity. Shinde and Dhalwal [22] used the RAPD technique for determination of different components present in an Ayurvedic herbal formulation Rasayana churna, *T. cordifolia, T. terestris* and *E. officinalis* as its constituents. Rana and his coworkers [23] studied the genetic diversity and morpho-physiological features among *Tinospora* populations of northwestern Himalayan region by RAPD and ISSR. *T. cordifolia* is dioecious in nature and primarily propagated through vegetative mode. This condition preludes genetic recombination and reshuffling of genes that is manifested in the form of genetic variation [23]. Here in the present investigation, we have studied the genetic diversity of 24 accessions of *T. cordifolia*, collected from 8 different ecological sites of 3 different climatic zones of northwest India by using two microsatellite based molecular markers i. e. ISSR & EST-SSR. To access the genetic diversity of *T. cordifolia* from different ecological zones in India, 20 primers/primer pairs were used, of which 12 (for EST-SSR) and 15 (for ISSR) showed polymorphism. For EST-SSR, two genotypes of Bhopal clustered separately in a single group-A (sub humid tropical zone). Further clustering indicates that genotypes of Udaipur, Sirsa and Rohtak (semi arid zone) sub clusters in to B1 while genotypes of Bikaner and Jaisalmer separates out in a sub cluster group-B2 (arid zone). Whereas for ISSR, all genotypes of Udaipur, two of Jaisalmer and one genotype of Bhopal comes out in a separate cluster and remaining 18 genotypes separates out in a large cluster. This is understandable as these accessions are located far apart, at different altitudes and belong to different climatic zones. ISSR results showed slightly more variation in clustering as compared to EST SSR, the possible explanation for this may be due to human intervention or other ecological variation, which makes partitioning and distribution of variability complex. There are studies, which also cited these kinds of reasons [24]. However, results of EST-SSR clearly showed that climatic conditions and physical parameters might affect the plant genome as the plant is adapted and these changes are inherited through next generation.

Also there are various studies that show that there are a relationship between genetic diversity and geographic distribution and also the climatic similarity has been observed in several species of aromatic plants such as *Azadirachta indica* [25], *Artemisia annua* [26], *Tanacetum vulgare* [27], three *Achillea* species [28] and *Achillea millefolium* [29]. These studies also correlate with our study of EST SSR that climate in near place is generally same (with some limited variation), in term of genetical changes is one of the most important and effective agent on genetic information in different way (like mutation and natural selection). On the basis of particular marker types, 2 separate dendrograms were created for ISSR and EST SSR. Based on the similarity coefficient, two major groups were generated from all the accessions tested by UPGMA clustering analysis for each marker system. Genetic similarity calculations gave mean value of 0.84 (value ranges from 0.68 to 1.0) for ISSR and 0.66 (value ranges from 0.33 to 1.0) for EST SSR.

Since results shows significant value of genetic diversity with both the marker systems. For determining the overall utility of a given marker system and to detect the polymorphism, the MI was calculated for ISSR and EST SSR marker system. The average PIC, MI and the mean similarity coefficient analyses clearly indicated that the genetic diversity was considerably abundant. In addition, in these types of studies, it is important to find out the number of marker loci and calculation of number of loci is required for elucidation of genetic relationship among accessions, hence, a DDI parameter could be helpful in preliminary estimation of necessary number of loci. Moreover, the results of EST-SSR marker system correlated with their geographical distribution while ISSR results showed some variations. Therefore, according to our results, EST-SSR proved to be more efficient than ISSR based on the UPGMA results as it directly correlates with the different accessions collected from the different climatic zones. The accessions from different climatic zones were placed in separate groups indicating the presence of genetic diversity in the plant i. e. plants from different climatic zone were more genetically diverse as compared to the plants from the same climatic zone.

CONCLUSION

T. cordifolia is an important medicinal plant used against various ailments. An attempt was made to develop a better approach to analyze the genetic diversity of 24 different genotypes of *T. cordifolia* collected from the Northwest region of India by using the two marker systems i. e. ISSR and EST- SSR. Our results have revealed the higher level of genetic diversity. Furthermore, the study can be used for more advance genetic analysis and molecular studies.

COMPETING INTERESTS

There is no competing interest between the authors.

ACKNOWLEDGEMENT

The authors are thankful to National Medicinal Plant Board (Grant no & Date- Z.18017/187/CSS/R&D/HR-01/2011-12-NMPB/24/11/2011), New Delhi for the award of Major Research Project grant. Authors are also thankful to UGC, New Delhi for the award of UGC SAP grant.

REFERENCES

1. Sun M. Effects of population size, mating system and evolutionary origin on genetic diversity in *Spiranthes sinensis* and *S. hongkongensis*. Cons Bio 1996;10:10785-95.
2. Tuskan GA, Gunter LE, Yang ZK, Yin TM, Sewell MM, Di-Fazio SP. Characterization of microsatellites revealed by genomic sequencing of *Populus trichocarpa*. Can J For Res 2004;34:85-93.
3. Yin TM, DiFazio SP, Gunter LE, Zhang XY, Sewell MM, Woolbright SA, *et al*. Genome structure and emerging evidence of an incipient sex chromosome in *Populus*. Gen Res 2008;18:422-30.
4. Barth S, Melchinger AE, Lubberstedt TH. Genetic diversity in *Arabidopsis thaliana L. Henyh.* Investigated by cleaved amplified polymorphic sequence (CAPS) and inter simple sequence repeats (ISSR) markers. Mol Ecol 2002;11:495-505.
5. Huang CQ, Liu GD, Bai CJ, Wang WQ, Zhou SY, Yu DQ. Estimation of genetic variation in *Cynodon dactylon* accessions using the ISSR technique. Biochem Sys Ecol 2010;38:463-70.
6. Pashley CH, Ellis JR, McCauley DE, Burke JM. EST databases as a source for molecular markers: lessons from *Helianthus*. J Hered 2006;97:381-8.
7. Becher R. EST-derived microsatellites as a rich source of molecular markers for oats. Pl Breed 2007;126:274-8.
8. Heesacker A, Kishore VK, Gao WX, Tang SX, Kolkman JM, Gingle A, *et al*. SSRs and INDELs mined from the sun-Xower EST database: abundance, polymorphisms, and cross-taxa utility. Theor Appl Genet 2008;117:1021-9.
9. Luro FL, Costantino G, Terol J, Argout X, Allario T, Wincker P, *et al*. Transferability of the EST-SSRs developed on *Nules clementine (Citrus clementina* Hort ex Tan) to other *Citrus* species and their effectiveness for genetic mapping. BMC Gen 2008;9:287.
10. Mulato BM, Möller M, Zucchi MI, Quecini V, Pinheiro JB. Genetic diversity in soybean germplasm identified by SSR and EST-SSR markers. Pesq Agrop Brasil 2010;45:276-83.
11. Wen M, Wang H, Xia Z, Zou M, Lu C, Wang W. Developmenrt of EST-SSR and genomic-SSR markers to assess genetic diversity in *Jatropha Curcas L*. BMC Res Not 2010;3:42.
12. Ramu P, Billot C, Rami JF, Senthilvel S, Upadhyaya HD, Reddy LA, *et al*. Assessment of genetic diversity in the sorghum reference set using EST-SSR markers. Theor Appl Genet 2013;126:2051-64.
13. Ahmad SM, Hoot SB, Qazi PH, Verma V. Phylogenetic patterns and genetic diversity of Indian *Tinospora* species based on

chloroplast sequence data and cytochrome P450 polymorphisms. Pl Syst Evol 2009;281:87-96.
14. Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 1987;19:11-5.
15. Temnykh S, Lukashova A, Cartinhour S. Computational and experimental analysis of microsatellites in rice (*Oryza sativa L.*): frequency, length variation, transposon associations, and genetic marker potential. Gen Res 2001;11:1441-52.
16. Untergrasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, *et al*. Primer3-new capabilities and interfaces. Nucl Aci Res 2012;40:e115.
17. Rohlf FJ NTSYS-pc 2.0e. Exeter Software, Setauket, New York, USA; 1998.
18. Smith JSC, Chin ECL, Shu H, Smith OS, Wall SJ, Senior ML, *et al*. An evaluation of the utility of SSR loci as molecular marker in maize (*Zea mays L.*): comparison with data from RFLPS and pedigree. Theor Appl Genet 1997;95:163-73.
19. Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, *et al*. The comparison of RFPL, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breed 1996;2:225-38.
20. Milbourne D, Meyer R, Bradshaw JE, Baird E, Bonar N, Provan J, *et al*. Comparison of PCR-based marker system for analysis of genetic relationship in cultivated potato. Mol Breed 1997;3:127-36.
21. Rout GR. Identification of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. and Thoms using RAPD markers. J Biosci 2006;61:118-22.
22. Shinde VM, Dhalwal K. DNA fingerprinting of *Tinospora cordifolia* using RAPD analysis. J Glob Pharm Tech 2012;2:38–42.
23. Rana V, Thakur K, Sood R, Sharma V, Sharma TR. Genetic diversity analysis of *Tinospora cordifolia* germplasm collected from northwestern Himalayan region of India. J Genet 2012;91:99-103.
24. Deshwal RPS, Singh R, Malik K, Randhawa GJ. Assessment of genetic diversity and genetic relationships among 29 populations of *Azadirachta indica A.* Juss using RAPD markers. Genet Res Cr Evol 2005;52:285-92.
25. Singh A, Negi MS, Rajagopal J, Bhatia S. Assessment of genetic diversity in *Azadirachta indica* using AFLP markers. Theoret Appl Genet 1999;99:272-9.
26. Sangwan RS, Sangwan NS, Jain DC, Kumar S, Ranade AS. RAPD profile based genetic characterization of chemotypic variants of *Artemisia annua L.* Biochem Mol Bio Int 1999;47:935-44.
27. Keskitalo M, Pehu E, Simon JE. Variation in volatile compounds from tansy (*Tanacetum vulgare L.*) related to genetic and morphological differences of genotypes. Biochem Syst Ecol 2001;29:267-85.
28. Farajpour M, Ebrahimi M, Amiri R, Sadat-Noori SH, Sanjari S, *et al*. Study of genetic variation in yarrow using inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) markers. Afr J Biotechnol 2011;54:11137-41.
29. Farajpour M, Ebrahimi M, Amiri R, Golzari R, Sanjari S. Assessment of genetic diversity in *Achillea millefolium* accessions from Iran using ISSR marker. Bioch Syst Ecol 2012;43:73-9.