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Analysis of genetic diversity through AFLP, SAMPL, ISSR and RAPD markers in *Tribulus terrestris*, a medicinal herb

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Abstract *Tribulus terrestris* is well known for its medicinal importance in curing urino-genital disorders. Amplified fragment length polymorphism (AFLP), selective amplification of microsatellite polymorphic loci (SAMPL), inter-simple sequence repeat (ISSR) and randomly amplified polymorphic DNA (RAPD) markers were used for the first time for the detection of genetic polymorphism in this medicinal herb from samples collected from various geographical regions of India. Six assays each of AFLP and SAMPL markers and 21 each of ISSR and RAPD markers were utilized. AFLP yielded 500 scorable amplified products, of which 82.9% were polymorphic. SAMPL primers amplified 488 bands, 462 being polymorphic (94.7%). The range of amplified bands was 66 [$(TC)_8$G + M-CAG] to 98 [$(CA)_6$AG + M-CAC] and the percentage polymorphism, 89.9 [from $(CT)_4$C $(AC)_4$A + M-CTG] to 100 [from $(GACA)_4$ + M-CTA]. The ISSR primers amplified 239 bands of 0.4–2.5 kb, 73.6% showed polymorphism. The amplified products ranged from 5 to 16 and the percentage polymorphism 40–100. RAPD assays produced 276 bands, of which 163 were polymorphic (59%). Mantel test employed for detection of goodness of fit established cophenetic correlation values above 0.9 for all the four marker systems. The dendrograms and PCA plots derived from the binary data matrices of the four marker systems are highly concordant. High bootstrap values were obtained at major nodes of phenograms through WINBOOT software. The relative efficiency of the four molecular marker systems calculated on the basis of multiplex ratio, marker index and average heterozygosity revealed SAMPL to be the best. Distinct DNA fingerprinting profile, unique to every geographical region could be obtained with all the four molecular marker systems. Clustering can be a good indicator for clear separation of genotypes from different regions in well-defined groups that are supported by high bootstrap values.

Keywords *Tribulus terrestris* · Molecular markers · Genetic diversity · Medicinal plant

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Introduction

India being one of the 12 mega biodiversity centers of the world has tremendous natural wealth in the form of over 7,500 medicinal plant species. This accounts for 20% of its total flora, which is much higher than an average of 12.5%, reported of the world flora. The traditional plant based medicines have now become quite popular in both developing and developed countries. The ever-increasing demand of herbal drugs is causing loss of precious biodiversity and also creating shortage of raw material. Since, there have been few studies on genetic diversity on Indian medicinal plants (Singh et al. 2002; Negi et al. 2004; Bahulikar et al. 2004); for efficient conservation and management of genetic resources, it is imperative to analyze the genetic composition of species of different phytogeographical regions. For this purpose, we have selected *Tribulus terrestris* (Puncture-vine), a herb of Zygophyllaceae, endowed with various medicinal properties. For example, fruits are used in curing urinary discharges, cough, asthma, pain, spermatorrhoea, ophthalmia, anemia, dysentery, skin and heart diseases; leaves purify blood and

are used as aphrodisiac; and roots are good stomachic and appetizer. Its purported effects include increased luteinizing hormone release and thus testosterone production, increased sperm production, increased ejaculatory volume and increased libido. The original use for *T. terrestris* extract was as a 'tonic' to treat sexual dysfunction. It is an important constituent of various medicinal preparations like Dashmularishta, Tribestan etc. world-wide. The herb is a C_4 summer annual, distributed in the tropics as well as sub-tropics. In India it occurs in the tropics and warm temperate region, up to an altitude of 5,400 m. It possesses solitary yellow flowers and globose fruits with hard and sharp spines and is propagated through seeds. There are several native species in the genus *Kallstroemia* (*K. parviflora* and *K. californica*, family: Zygophyllaceae) that could be confused with *T. terrestris*, because of their similar morphology; especially when the plants have not begun to flower and fruits are not present. Such plants, often get accidentally mixed with *T. terrestris* while collecting raw material for herbal drug preparations, making them less efficient or some times toxic. This lack of authenticated raw material is a major problem facing the herbal pharmaceutical industry which have severe consequences, starting from decrease in drug efficacy to cardiac arrhythmias and poisoning. DNA fingerprinting techniques are very useful in correct identification of taxa, and thus preparation of authenticated and effective drugs. Another application of molecular markers is in carrying out genetic diversity studies. Multi-locus markers are more advantageous than single-locus markers, as they have a high mutiplex ratio. Randomly amplified polymorphic DNA (RAPDs) have been extensively used for the assessment of genetic diversity in a variety of plants like *Saxifraga cernua* (Kjølner et al. 2004), *Zea mays* (Garcia et al. 2004), *Ziziphus* spp. (Singh et al. 2006), *Saccharum* and *Erianthus* (Selvi et al. 2006), *Panax quinquefolius* (Lim et al. 2007) etc. Inter-simple sequence repeat (ISSR) markers are much more informative than RAPDs and have been used for the analysis of genetic diversity in *Cicer* sp. (Souframanien and Gopalakrishna 2004), *Morus alba* (Awasthi et al. 2004), *Pisum sativum* (Baranger et al. 2004), *Asparagus acutifolius* (Sica et al. 2005), *Corchorus* species (Roy et al. 2006) and others. Amplified fragment length polymorphism (AFLP) has helped unravel genetic diversity in *Azadirachta indica* (Singh et al. 2002), *Brassica nigra* (Negi et al. 2004), *Ranunculus acris* (Odat et al. 2004), *Nicotiana attenuate* (Bahulikar et al. 2004), *Brassica rapa* (Zhao et al. 2005), *Cicer* sp. (Shan et al. 2005), *Z. mays* (Menkir et al. 2005), *Cynodon* (Wu et al. 2006), *Glycine soja* (Ru et al. 2006), *Myricaria laxiflora* (Liu et al. 2006) *Gardenia jasminoides* (Han et al. 2007), *Chimonanthus* spp. (Zhou et al. 2007) and others. Selective amplification of microsatellite polymorphic loci (SAMPL) though a modification of AFLP, detects more polymorphic loci. It is therefore more useful in cases with expected low level of diversity. Inspite of having manifold medicinal uses and world-wide distribution there has been no report on the extent of the genetic diversity prevalent in *T. terrestris* to the best of our knowledge. Therefore, we have conducted this study to analyze genetic diversity present in this valuable medicinal plant from five different agro-climatic regions of India. We have used AFLP, SAMPL, ISSR and RAPD markers for this purpose. The potential of these molecular markers was also compared.

Materials and methods

Plant material

Leaf samples from each of the single plant were collected from natural populations growing in Karnataka (six samples from Mysore), Uttaranchal (ten samples from Dehradun), Maharashtra (four samples from Amravati) and Rajasthan (four samples from Udaipur). Fifteen plants growing in the herbal garden at Hamdard University (Jamia Hamdard) represent the genotypes from Delhi.

DNA extraction

Genomic DNA was extracted from lyophilized leaf material following the modified protocol of Doyle and Doyle (1990) and stored at −20°C till further use. The DNA was quantified using agarose gel electrophoresis and employing serial dilutions of uncut λ-DNA as marker.

RAPD and ISSR analyses

Randomly amplified polymorphic DNA assay was carried out in 15 µl reaction volume containing 50 ng DNA, 5 mM $MgCl_2$, 2 mM each of dNTP, 10 µM random decamers, 0.6 U *Taq* polymerase (Invitrogen). Amplification was performed with thermal cycler (Eppendorf Germany) using the cycling parameters of Das et al. (1999). One cycle of 94°C for 2 min, 37°C for 2 min, 72°C for 2 min followed by 30 cycles of 94°C for 1 min, 37°C for 1 min, 72°C for 1 min. The last cycle was followed by 10 min extension at 72°C. The amplified products were resolved in 1.2% agarose gel (0.5 × TBE), stained with ethidium bromide (10 µg/ml) and photographed under UV light. For ISSR analysis, 20 ng of template DNA was subjected to amplification with 7.5 µM of 3′- anchored microsatellite primers, other components remaining same as that of RAPD. Electrophoresis was performed on 1.2% agarose gel. The

annealing temperatures of the cycling parameter were re-adjusted for each microsatellite primers according to their calculated melting temperature (T_m) based on the sequence composition [$T_m = 4°(G + C) + 2°(A + T) - 3°C$].

AFLP and SAMPL analyses

Amplified fragment length polymorphism analysis was performed as per the technical instructions of the manufacturer (Invitrogen, Life Technologies). Genomic DNA (300 ng) was restricted with *Eco*RI and *Mse*I (2.5 U each) in a restriction buffer (50 mM Tris–HCl, pH 7.5, 50 mM Mg acetate, 250 mM K-acetate) in total volume of 25 µl. *Mse*I and *Eco*RI adapters were subsequently ligated to digested DNA fragments. The adapter ligated DNA was preamplified using the following parameters: 20 cycles of 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. The pre-amplified DNA was diluted 50 folds and used as a template for selective amplification reaction using *Eco*RI and *Mse*I primers with three selective nucleotides at 3′-end. The *Eco*RI adapter specific primer was end labeled at 3′ using -P32-ATP using PNK enzyme. Selective amplification was carried out initially at 94°C for 30 s, annealing at 68°C for 30 s and 72°C for 30 s; this was followed by a touch-down cycling protocol where the annealing temperature was reduced by 1°C in each cycle. This continued till the annealing reached 56°C. And finally 23 cycles of 94, 56 and 72°C for 30 s each. The reaction was stopped by addition of gel loading dye (98% formamide dye) and the samples were resolved on 6% denaturing polyacrylamide gel and autoradiographed (Sambrook et al. 2001). For SAMPL analysis, the *Eco*RI primer was replaced by 3′ anchored microsatellite primers, all other conditions and parameters remaining the same. Products were resolved on 6% denaturing PAGE and autoradiographed.

Data analysis

The amplification products were scored for the presence (1) and absence (0) of bands across the genotypes to generate a binary matrix. Co-migrating bands were assumed to be originating from the same genetic locus.

The binary matrix was analyzed using the NTSYS-pc version 2.11w software to calculate the similarity values and generate the phenogram. Jaccard's similarity coefficient was utilized for estimating the pairwise similarity between the operational taxonomic units (OTUs) using the formula $GS_{ij} = a/a + b + c$, where GS_{ij} represent the genetic similarity between the two individuals 'i' and 'j'. '*a*' is the number of polymorphic bands shared by 'i' and 'j', '*b*' is the number of bands in 'i' and absent in 'j' and '*c*' is the number of bands present in 'j' and absent in 'i' (Jaccard 1908). After obtaining the similarity matrix, clustering was performed by sequential agglomerative hiearchical nested clustering, a distance based method, where series of successive mergers are used to group individuals with similar characteristics. The graphical representation of the cluster (phenogram) was obtained by using the unweighted pair group method of mathematical averages (UPGMA). Mantel test (Mantel 1967) was used to test the correlation of coefficient between pairs of similarity matrices and for determining cophenetic correlation values using NTSYS-pc, version 2.11w.

The degree of confidence at the nodes of the phenogram was evaluated through WINBOOT software (Yap and Nelson 1995). The original binary matrix was bootstrapped between 100 and 1,000 replicates to generate variant matrices, which were then re-analyzed to generate phenograms with the bootstrap values at the nodes.

Polymorphic information content (PIC) or average heterozygosity was calculated as per the formula of Roldan-Ruiz et al. (2000): $PIC = 2f_i\ (1 - f_i)$, where f_i is the frequency of the amplified allele and $1 - f_i$ is the frequency of null allele. Average heterozygosity (H_{av}) is obtained by taking the average of PIC values obtained for all the markers and is calculated as:

$$H_{av} = \sum [2fi(1 - fi)]/N$$

Multiplex ratio (MR) for each assay was estimated by dividing the total number of bands (monomorphic—m, and polymorphic—p) amplified by the total number of assays (primer combinations employed—*n*) as per Powell et al. (1996).

$$MR = m + p/n$$

Marker index (MI) was obtained by multiplying the average heterozygosity (H_{av}) with MR (Powell et al. 1996).

$$MI = H_{av} \times MR$$

Results

AFLP analysis

Six AFLP primer combinations produced 500 scorable products. Table 1 summarizes the number of bands amplified for different primer combinations and percentage polymorphism detected in *T. terrestris*. The average number of bands per primer combination was 83.3 and percentage polymorphism ranged from a low of 74.7 in primer combination E-AAG + M-CTA to a high of 97.2 in primer combination E-ACT + M-CAG. Figure 1a is a representative AFLP profile generated by primer combination E-ACT + M-CAG. This proved to be the most

informative primer combination as it amplified maximum number of products (106) and detected highest polymorphism (97.2%). The monomorphic products are marked 'M' (Fig. 1a). The characteristic feature of this AFLP profile is the amplification product unique to the genotypes from Delhi (lanes 1–6, band marked as 'D'). In addition, some of the genotypes from Mysore (lanes 17–19) lack an amplification product indicated as 'H'. Additionally, the genotypes from Amravati has an amplification profile distinct from rest of the genotypes due to presence of unique bands (lane 23, bands marked 'U'). Other rare polymorphic bands are 'B-1' in the accessions from Mysore and 'C-1' in Dehradun.

SAMPL analysis

An average of 81.3 bands were amplified per primer combination and the percent polymorphism ranged between 89.9 [$(CT)_4C(AC)_4A$ + M-CTG] and 100 [$(GACA)_4$ + MCTA]. Of 488 bands obtained with six primer combinations, 462 were polymorphic (94.7% polymorphism). Figure 1b shows a SAMPL fingerprint obtained with primer combination $(CT)_4C(AC)4A$ + M-CTG. Out of 89 amplified products, nine are monomorphic, three are marked 'M' in Fig. 1b. A-1, A-2 and A-3 are rare polymorphic bands as they are present in only some of the genotypes from Mysore, Rajasthan and Delhi, respectively. The result is listed in Table 2.

ISSR analysis

Twenty-one ISSR primers generated a total of 239 bands. Several polymorphic products specific to a particular geographic region could be observed. A representative fingerprint pattern generated by primer S-13 is shown in Fig. 1c. Out of 13 amplicons generated by this primer (size range 0.45–2.3 kb), five were monomorphic marked as 'A'. A 0.8 kb amplicon 'B' is present only in genotypes from Mysore (lanes 1–4, Fig. 1c), and a 1.25 kb product is unique to genotypes from Delhi ('C', lanes 5–14). Several other bands were shared by genotypes from most geographical regions. Such bands, exemplified by 'C*', are shared by samples from Dehradun and Amravati, and 'M*' is shared by all genotypes except those from Mysore (0.8 kb; Fig. 1c). Another such product is indicated as 'D' which is also absent from genotypes originating from Mysore. The bright intensity of amplicon 'D' suggests its origin from a repetitive region such as a microsatellite locus. The results are summarized in Table 3.

As mentioned earlier, 21 ISSR primers generated 239 bands (size range 0.4–2.5 kb), an average of 11.4 amplifications per primer. The detected percent polymorphism was as low as 40 (primer UBC-2) to as high as 100 (UBC-4), an average of 73.6%.

Table 1 *Tribulus terrestris*: AFLP results

Primer combination	Polymorphic bands/total number of bands	% Polymorphism
E-ACT + M-CAG	103/106	97.2
E-ACT + M-CAT	81/103	78.6
E-AAG + M-CTA	62/83	74.7
E-AGC + M-CAC	44/56	78.6
E-AAG + M-CAG	80/95	84.2
E-ACA + M-CTG	48/57	84.2
Total (average)	418/500 (69.6/83.3)	82.9

RAPD analysis

Randomly amplified polymorphic DNA assays were performed with a total of 21 random decamers. In total, 276 amplicons were scored in the size range of 0.4–3.0 kb. This gives an average of 13.1 bands per primer. Of the 276 bands, 163 proved to be polymorphic (59.0%) and hence informative (Table 4). G-12 proved to be the most efficient with 90% of the 20 products being polymorphic. In contrast, G-11 primer could detect only 8.3% polymorphism though it generated 12 amplified products. A representative RAPD profile obtained by primer A-1 is shown in Fig. 1d. Of a total of 12 bands (0.4–2.3 kb), 7 are polymorphic (58.33%). Two distinct categories are visible. The first marked 'A' are monomorphic, and the second category marked 'B', a 0.55 kb band, is unique to genotypes from Dehradun (lanes 17–22, Fig. 1d).

Statistical analysis

The presence (1) and absence (0) of bands were scored across all genotypes. The binary matrix generated was used to calculate a similarity matrix based on Jaccard's coefficient. The phenograms based on genetic similarity data of AFLP and SAMPL are comparable. The major difference is in the clustering of genotypes of Amravati. AFLP presents them as sub-cluster of cluster II with similarity coefficients of 0.95–0.97 (Fig. 2a). In SAMPL analysis they cluster with similarity coefficients ranging from 0.64 to 0.8 (Fig. 2b). This reiterates that SAMPL markers detect more polymorphic loci. The phenograms based on ISSR and RAPD data indicate that genotypes from different geographical regions are clearly distinguishable as separate clusters (Fig. 2c, d).

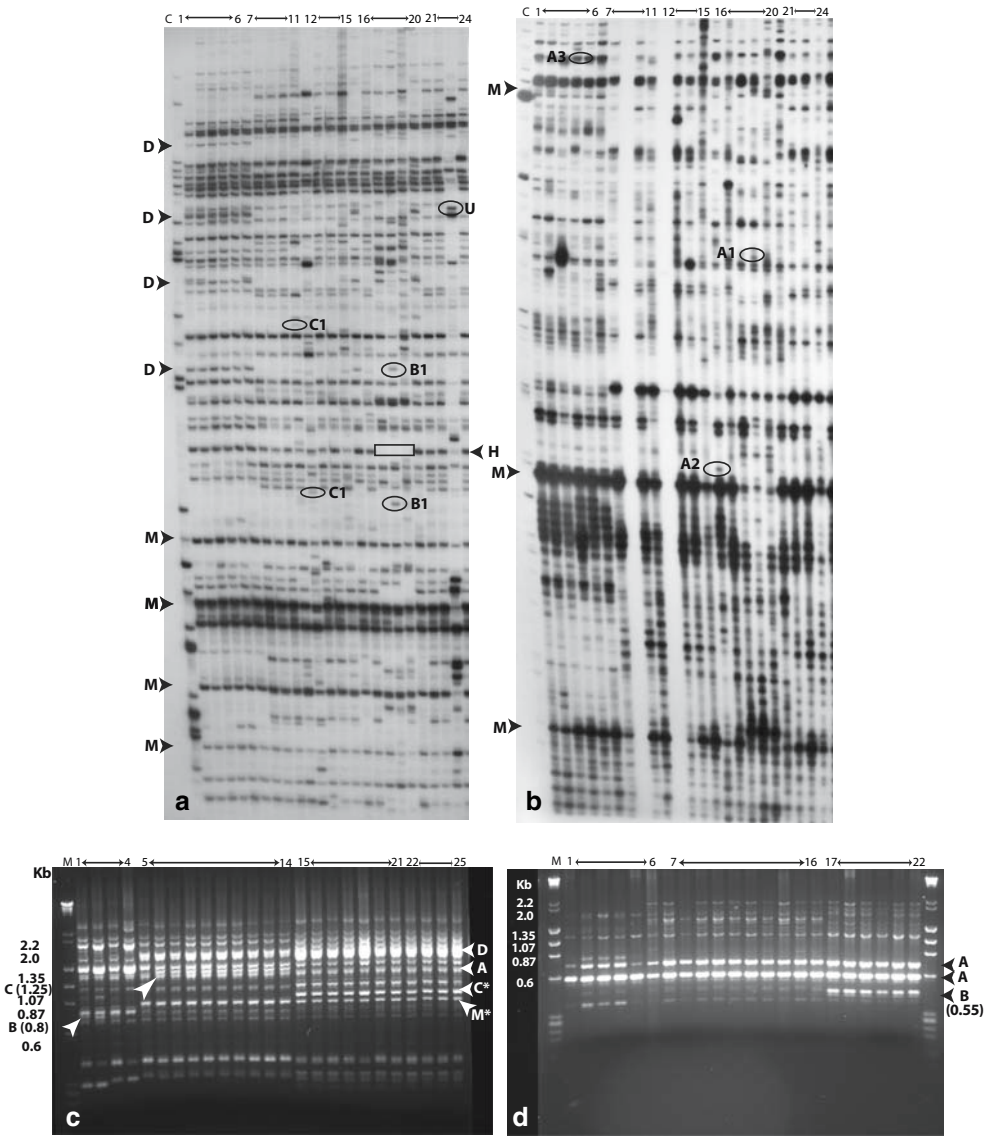

Fig. 1 AFLP profile showing the genetic polymorphism in *Tribulus terrestris* (**a**), detected with primer combination E-ACT + M-CAG. Samples in lanes *C* control (tomato DNA), *1–6* Delhi, *7–11* Dehradun, *12–15* Rajasthan, *16–20* Mysore, *21–24* Amravati. **b** SAMPL profile of 24 different accession lines obtained by the primer combination $(CT)_4C(AC)_4A$ + M-CTG. Lanes *C* control (tomato DNA), *1–6* Delhi, *7–11* Dehradun, *12–15* Rajasthan, *16–20* Mysore, *21–24* Amravati. **c** Inter-simple sequence repeat polymorphism using ISSR primer S-13. Lanes—*M* Marker (mix of λ DNA digested with *Hin*dIII and φX 174 DNA digested with *Hae*III), *1–4* Mysore, *5–14* Delhi, *15–21* Dehradun, *22–25* Amravati. **d** RAPD profile using primer A-1. Lanes—*M* Marker, *1–6* Mysore, *7–16* Delhi, *17–22* Dehradun, *M* Marker

Table 2 *Tribulus terrestris*: summary of SAMPL assays

Primer combination	Polymorphic bands/ total number of bands	% Polymorphism
$(CT)_4C\ (AC)_4A$ + M-CTG	80/89	89.9
$(GT)_4G(AG)_4A$ + M-CAT	74/76	97.4
$(TC)_8G$ + M-CAG	65/66	98.5
$(GACA)_4$ + M-CTA	74/74	100.0
$(CA)_6AG$ + M-CTA	79/85	92.9
$(CA)_6AG$ + M-CAC	90/98	91.8
Total (average)	462/488 (77/81.3)	94.7

All the phenograms confirm consistency of data. The principal correspondence analysis of the AFLP and SAMPL (data not shown) reveal genotypes from Mysore as being most diverse. They are not only different from other genotypes but also from each other, and hence, cannot be grouped together. In the case of ISSR and RAPD (data not shown), however, all the genotypes belonging to a particular geographic region were grouped together in PCA plots. This confirms the ability of AFLP and SAMPL markers to analyze more number of loci and thus differentiate closely related genotypes.

Table 3 *Tribulus terrestris*: interzonal genetic diversity through ISSR

Primer	Polymorphic bands/total no. of bands	% Polymorphism	Region-specific bands (kb)
S-2	5/7	71.4	–
S-3	9/10	90.0	Delhi—1.8 kb, Mysore—1.3 kb
S-4	5/6	83.3	–
S-5	7/10	70.0	Dehradun—1.5 kb
S-6	7/10	70.0	–
S-8	12/16	75.0	Delhi—0.55 kb
S-11	8/12	66.7	–
S-13	8/13	61.5	Mysore—0.84 kb, Delhi—1.25 kb
S-14	7/11	63.6	Delhi—0.55 kb
S-18	10/13	76.9	Delhi—1.0 kb
UBC-1	6/8	75.0	Dehradun—0.52 kb
UBC-2	4/10	40.0	Mysore—0.8 kb
UBC-4	16/16	100.0	–
UBC-5	13/16	81.2	Delhi—0.72 kb, Mysore—2 kb
UBC-6	10/13	76.9	Delhi—1.35 kb
UBC-7	11/13	84.6	Mysore—0.97 kb
UBC-8	5/10	50.0	–
UBC-9	8/12	66.7	Delhi—0.82 kb
UBC-10	8/13	61.5	Delhi—0.50 kb
UBC-11	14/15	93.3	Mysore—0.57 kb, Delhi—0.65 and 1.30 kb
UBC-12	3/5	60.0	Mysore—0.872 kb
Total (Av.)	176 (8.4)/239 (11.4)	73.6	–

Table 4 *Tribulus terrestris*: summary of interzonal genetic diversity study using RAPD

Primer	Polymorphic bands/ total no. of bands	% Polymorphism	Region-specific bands
A-1	7/12	58.3	Delhi—1.7 kb, Dehradun—0.55 kb
A-2	6/14	42.9	Dehradun—0.5 and 0.9 kb
A-3	7/11	63.6	Delhi—0.45 kb
A-5	12/15	80.0	Delhi—0.6 kb, Mysore—1.7 kb
A-7	9/15	60.0	–
A-8	13/16	81.2	Delhi—1.5 kb, Mysore—1.70 and 1.75 kb, Dehradun—1.45 and 1.40 kb
A-9	6/19	31.6	Delhi—0.56 kb
A-10	8/16	50.0	Delhi—0.87 kb, Dehradun—1.1 kb
G-2	10/15	66.7	–
G-4	8/10	80.0	Delhi—1.1 kb, Dehradun—1.2 and 1.5 kb
G-5	2/12	16.7	Dehradun—0.73 and 0.85 kb
G-6	6/10	60.0	Mysore—0.75 kb
G-7	7/10	70.0	Delhi—0.57 and 0.87 kb
G-8	12/14	85.7	Delhi—0.65 kb
G-9	8/11	72.7	–
G-10	6/11	54.5	–
G-11	1/12	8.3	Dehradun—0.75 kb
G-12	18/20	90.0	Mysore—0.45 and 2.3 kb, Delhi—0.93 kb, Dehradun—0.78, 0.9 and 3 kb
G-13	4/12	33.3	Delhi—0.75 kb
G-14	9/13	69.2	Dehradun—2.1 kb
G-15	4/8	50.0	–
Total (Av.)	163 (7.7)/276 (13.1)	59.0	–

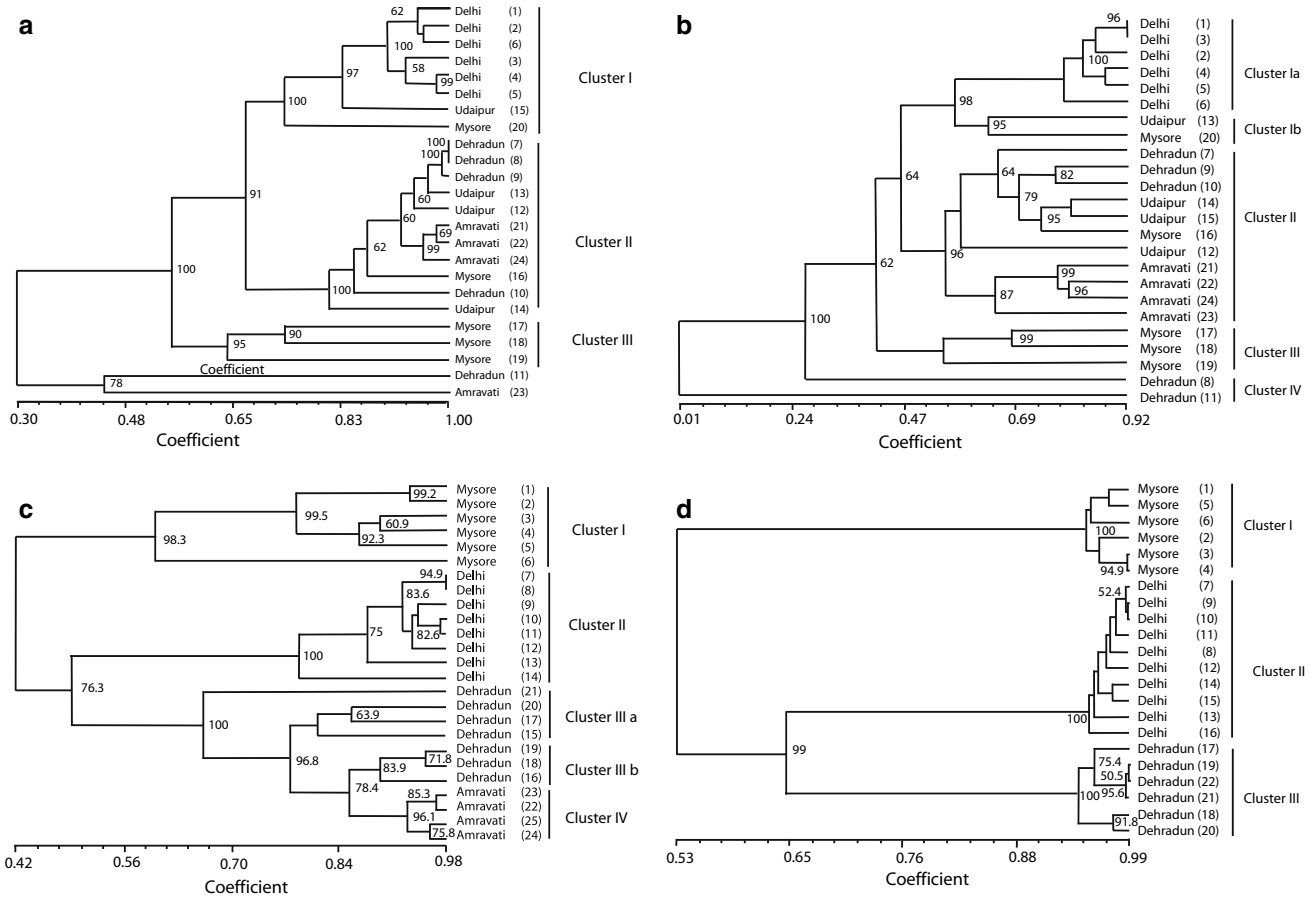

Fig. 2 Phenetic relationships among *Tribulus terrestris* genotypes as revealed by data from **a** AFLP, **b** SAMPL, **c** ISSR and **d** RAPD marker systems

The four molecular marker systems were compared on the basis of four different criteria (Table 5). AFLP revealed highest MR (83.3) followed by SAMPL, RAPD and ISSR. In case of polymorphism detection, SAMPL as a tool scored higher than AFLP, ISSR and RAPD. The fact that microsatellites are hypervariable and when assayed revealed higher heterozygosity, as both SAMPL and ISSR score over AFLP and RAPD in terms of average heterozygosity (H_{av}). Finally, 'MI', a measure of overall efficiency of a marker is highest for SAMPL, making it the most efficient marker for the genetic diversity studies in *T. terrestris*. Mantel test (Mantel 1967) was employed to determine the coefficient of correlation between the similarity matrices generated by these markers. The coefficient of correlation between AFLP and SAMPL was 0.793 ($P = 0.01$). Mantel test was also employed to analyze the 'goodness of fit' for the UPGMA dendrograms generated by each marker system (AFLP, SAMPL, ISSR and RAPD). This was done by making the cophenetic similarity matrices from each UPGMA phenogram and then comparing

Table 5 Comparison of various molecular markers in evaluating genetic diversity of *Tribulus terrestris*

Molecular marker	AFLP	SAMPL	ISSR	RAPD
No. of genotypes	24	24	25	25
Total no. of bands (n)	500	488	239	276
Polymorphic bands (p)	418	462	176	163
Total no. assays/primer combinations	6	6	21	21
Multiplex ratio (n/T)	83.3	81.3	11.4	13.1
Percentage polymorphic (%p)	82.9	94.7	73.6	59
Average heterozygosity (H_{av})	0.26	0.32	0.31	0.25
Marker index (MI) = $H_{av} \times$ MR	21.7	26	3.5	3.3

these cophenetic similarity matrices with the original similarity matrices (generated from binary data) for each marker technique. It revealed values higher than 0.95 for all the markers used [RAPD ($r = 0.998$, $P = 0.01$), AFLP ($r = 0.993$, $P = 0.01$), ISSR ($r = 0.973$, $P = 0.01$), SAMPL ($r = 0.957$, $P = 0.01$)], thus confirming their authenticity. Bootstrap analysis of this data was performed to determine the confidence values as percentages at each node. Very high bootstrap values were obtained from the major nodes. Hundred percent support value was obtained for the node at which cluster II separates from cluster III in the phenogram obtained from AFLP data. The support value for the Delhi cluster was also 100% (Fig. 2a). The bootstrap values for the RAPD phenogram are also 100% for each cluster (Mysore, Delhi and Dehradun) as evident from Fig. 2d.

Discussion

Geographically isolated populations of plant species tend to accumulate genetic variations during the course of environmental adaptations. The medicinal herb, *T. terrestris* chosen for the current study is an annual with propagation through seeds. However, the predominant mode of genetic material exchange, whether through selfing or out crossing needs to be unambiguously established. All four molecular markers, that rely on arbitrary and semi-arbitrary nature of amplification detected polymorphism percentage ranging between 60 and 95%. AFLP revealed an average of 82.9% polymorphism across all genotypes. Such high percentage polymorphism (79.5) is also reported in ten populations of two different habitat types of *R. acris* (Odat et al. 2004). In our studies the low region-wise diversity is in congruence with the study of Fu et al. (2004) on little blue stem (*Schizachyrium scoparium*), where AFLP revealed high (>91%) interpropulation and low (only 7.9%) intrapopulation genetic diversity. Natural populations propagating through seeds implicitly have higher level of variation. AFLP analysis of six natural populations of *Rubus arcticus* detected high level of interpopulation genetic variation (0.72–0.94) measured as Simpson index (D). In *T. terrestris* AFLP grouped Delhi genotypes into one tight cluster. Likewise, genotypes from Dehradun and Amravati formed distinct groups. But, genotypes from Jaipur appear diverse and showed relatedness to genotypes from Delhi, Dehradun and Amravati. Several reasons could be attributed to the low intra-zonal diversity detected in the present investigation. First, the genotypes have genetically adapted to local environment and have become homogenous as a result of 'inbreeding' or exchange of genetic material within the said population. Thus, in the present investigations, unique DNA amplification profile brought out a distinct genetic identity belonging to genotypes of separate geographical regions. However, such a specific genetic separateness has not created a hybridization barrier across zones, and therefore provides an opportunity to genotypes from different zones to exchange genetic material. The spines and warty protuberances on *T. terrestris* fruits enable them to easily attach to animals and humans and to stick onto vehicle tires (cars, farm, airplane), subsequently facilitating long distance dispersion and spread and thus exchange of genetic material between distant populations. Our results are comparable to white clover (*Trifolium repens*) where RAPD indicated closeness between population of Atlantic Canada to population from Georgia and those from West Virginia to the cultivar Sacramento (Gustine et al. 2002). Similarly, RAPD analysis of *Iris setosa* showed genotypes from one location sharing higher level of genetic relatedness with other geographic location (Artyukova et al. 2001). This corroborates the fact that geographically and genetically distinct populations can exchange genetic information and that these are not completely isolated. In the present study similar results were obtained with SAMPL, ISSR and RAPD markers as they unraveled higher level of interzonal but a much lower level of intra population genetic diversity. In the present investigation, various high intensity bands were amplified, like a 1.8 kb ISSR band amplified by primer S-13. Artyukova et al. (2001) have also reported RAPD bands of such high intensity in *Iris*. The high intensity bands could be a consequence of high copy number. The region-specific bands detected in our studies can be used for the authentication of this medicinal herb as the biochemical properties of the pharmaceutically active ingredients is known to be influenced by the geographic region. Wang et al. (2003) have already exploited RAPD for confirming the genuineness of *Bupleurum chinense*. Na et al. (2004) have detected three RAPD primers which can discriminate between Korean and Chinese *Astragali radix*. The comparison of the four molecular marker systems revealed SAMPL to be the best as it generated highest percentage polymorphism and high MI. The capability of SAMPL markers lies in the detection of hypervariability in the microsatellite region. Microsatellites detect high level of polymorphism (Teulat et al. 2000), which can be used to discriminate between closely related genotypes. The hypervariability of SSR is due to variable copy number of repeat units at a locus (Tautz and Renz 1984). The measure of overall efficiency of a marker system remains the 'MI' which is highest for SAMPL as also reported by Tosti and Negri (2002). Phenograms generated from AFLP, SAMPL, ISSR and RAPD data showed similarity in relative placement of genotypes. SAMPL and AFLP markers provided a more detailed idea on the genetic relationship. The coefficient of correlation between SAMPL and AFLP markers is high ($r = 0.793$) in

T. terrestris. Similar results have been obtained in *A. indica* by Singh et al. (2002), thereby implying that these marker techniques can be used judiciously and effectively for analyzing genetic diversity. The cophenetic correlation values of Mantel test are above 0.95 for each marker system. This validates the phenograms obtained after UPGMA analysis of binary matrices data. These findings can be used in authentication of the medicinally important herbs and perhaps in recognizing high yielding population in a particular area. Further advantages can be for planning conservation strategies, the population representing the maximum genetic diversity need to be conserved followed by the population which compliments the previous one.

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