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A comparative phylogenetic analysis of medicinal plant *Tribulus terrestris* in Northwest India revealed by RAPD and ISSR markers

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ABSTRACT

Kumar A, Verma N. 2012. A comparative phylogenetic analysis of medicinal plant Tribulus terrestris *in Northwest India revealed by RAPD and ISSR markers. Biodiversitas 13: 107-113.* Several DNA marker systems and associated techniques are available today for fingerprinting of plant varieties. A total of 5 RAPD and 8 ISSR primers were used. Amplification of genomic DNA of the 6 genotypes, using RAPD analysis, yielded 164 fragments that could be scored, of which 47 were polymorphic, with an average of 9.4 polymorphic fragments per primer. Number of amplified fragments with random primers ranged from 6 (AKR-1) to 10 (AKR-4) and varied in size from 200 bp to 2,500 bp. Percentage polymorphism ranged from 16% (AKR-4) to a maximum of 41% (AKR-4), with an average of 29.6%. The 8 ISSR primers used in the study produced 327 bands across 6 genotypes, of which 114 were polymorphic. The number of amplified bands varied from 7 (ISSR 7) to 12 (ISSR 1&3), with a size range of 250-2,800 bp. The average numbers of bands per primer and polymorphic bands per primer were 40.87 and 14.25, respectively. Percentage polymorphism ranged from 24% (ISSR 4) to 53.84% (ISSR 2), with an average percentage polymorphism of 35.59% across all the genotypes. The 3′-anchored primers based on poly (AC) and poly (AT) motifs produced high average polymorphisms of 53.84% and 40.81%, respectively. ISSR markers were more efficient than the RAPD assay, as they detected 35.59% polymorphic DNA markers in *Tribulus terrestris* as compared to 29.6% for RAPD markers. Clustering of genotypes within groups was not similar when RAPD and ISSR derived dendrogram were compared, whereas the pattern of clustering of the genotypes remained more or less the same in ISSR and combined data of RAPD and ISSR.

Key words: Genetic diversity, Phylogenetic analysis, *Tribulus terrestris*, RAPD, ISSR

Abbreviations: CTAB: Cetyltrimethylammonium bromide, ISSR: Inter simple sequence repeat, RAPD: random amplified polymorphic DNA, PCR: Polymerase chain reaction, PCA: principal component analysis, TT: *Tribulus terrestris*, UPGMA: unweighted pair group method

INTRODUCTION

Puncture vine (*Tribulus terrestris*) is an important medicinal weed found wildly in India, most parts of central and east Africa and in other Asian countries including Sri Lanka, China and Japan. *Tribulus terrestris* belonging to the Zygophyllaceae family grows naturally in moist places in woods, low mountains and hills. Its geographical distributions are wide in East Asia, particularly in China. This plant species, commonly called puncture vine, is a perennial herb highly valued in Chinese traditional medicine. In addition, the claimed therapeutic values of *T. terrestris* include treatment for dyspepsia, poor appetite, fatigue and psychoneurosis. Recent years have seen a highly accelerated demand for *T. terrestris*, fruits which has inevitably led to destructive over-harvesting and depletion of its natural resources (Van Valkenburg and Bunyapraphatsara 2001).

Molecular markers are highly heritable and exhibit enough polymorphism to discriminate genotypes. They can be very useful in identifying varieties at early stages of growth and characterize the genotype comprehensively since they are available in very high numbers and are distributed throughout the genome. Application of molecular markers as complementary approach for genetic characterization has been reported in many crops (Karp et al. 1998). ISSR (Gupta et al. 1994; Zietkiewicz et al. 1994; Bornet and Branchard 2001) markers are considered superior to RAPD (Qian et al. 2001). RAPD analysis provides high resolution and can be carried out on small amount of DNA (Carter and Sytsma 2001; Jolner et al. 2004). However, it is well known that RAPD markers can be sensitive to changes in reaction conditions, resulting in low reproducibility and inconsistencies in amplification efficiencies among samples (Weising et al. 1995). ISSR markers have been used to characterize gene bank accessions (Blair et al. 1999) as well as to identify closely related cultivars (Fang and Roose 1997). The potential supply of ISSR marker depends on the variety and frequency of microsatellites, which changes with species and the SSR motifs that are targeted (Depeiges et al. 1995). ISSR primers with a given microsatellite repeat should reflect the relative frequency of that motif in a given genome and would provide an estimate of the motif's abundance. A large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner (Santalla et al. 1998). This limits the use of morphological characters and isozymes,

which are few or lack adequate levels in *Tribulus terrestris*. Molecular genetic markers have developed into a powerful tool to analyze genetic relationships and genetic diversity. Both RAPD and ISSR remain attractive options despite availability of sophisticated techniques because they are easy, quick, simple and economical. Present study reports molecular characterization of 6 cytotypes of *T. terrestris*, from Northwest India employing RAPD and ISSR techniques. Our aims also were to: (i) provide a better understanding of the phylogenetic relationships between Tribulus population, and (ii) determine the degree to which PCR based markers such us as RAPDs or ISSRs are able to assess the variation and genetic relationship within *Tribulus terrestris* population.

MATERIALS AND METHODS

Several experiments were carried out, however, only the optimized protocol is described here.

Plant material and DNA preparation

Here, we used the set of 6 cytotypes of *Tribulus terrestris* (Table 1). The leaves of *Tribulus terrestris* were collected from naturally grown population of the six different districts (Patiala, Delhi, Meerut, Muzaffarnagar, Baghpat and Haridwar) of Northwest India in 2006, and their identity was confirmed and voucher specimens were deposit in the herbarium, Department of Botany, Punjabi University, Patiala, India.

Young and healthy leaves from single trees of each accession were used for DNA isolation by CTAB method with minor modifications (Doyle and Doyle 1990; Saghai-Maroof et al. 1984). Essentially, the extraction buffer composition was 4% w/v CTAB, 1.4MNaCl, 100 mM Tris-HCl (pH 8), 20 mM EDTA, 2% PVP w/v, and 0.2% 2-mercapto ethanol v/v. DNA was treated with bovine pancreatic RNAse and extracted once with phenol: chloroform (1:1) and twice with chloroform: iso-amyl alcohol (24:1). After precipitation with iso-propanol, a 70% ethanol wash was given. DNA was dissolved to appropriate dilution in TE buffer and quantified in a spectrophotometer.

Primer screening

A preliminary experiment on 6 randomly selected Tribulus accessions was carried out to select most suitable primers for identification. 6 random primers and 8 ISSR primers were screened for repeatability, scorability, and their ability to distinguish within varieties. Random primers AKR-1, AKR-2, AKR-3, AKR-4, AKR-5 and AKR-6 procured from (Imperial LifeSci Ltd. India); and primers ISSR1-8 from (custom made from Bangalore Genei) exhibited maximum efficiency of discrimination in terms of resolving power (Prevost and Wilkinson 1999). These primers were employed for varietal identification.

Amplification conditions for RAPD and ISSR-PCR and gel electrophoresis

In order to select optimal primers for effective use in RAPD-PCR analysis, 6 random primers (10 bp) of the Imperial biotech company were screened using TT population genomic DNA; 5 primers that generated clear bands and reproducible fragments were selected for further investigation (Table 2).

RAPD assay was carried out in 25 µl reaction mixture containing 2.5 µl 10x Taq DNA-polymerase PCR amplification buffer, 10 mM dNTPs, 1.0 U/µl of Taq DNA polymerase (Genei, India), 15 pmoles of 10-mer primer (Operon Technologies Inc, USA) and 50 ŋg of genomic DNA. Amplification was performed in PCR (Techne, India).

The sequential PCR steps involved: 1 cycle of 2 min at 93°C, 2 min at 35°C and 2 min at 72°C followed by 44 cycles of 1 min at 93°C, 1 min at 36°C and 2 min at 72°C. The last cycle was followed by 10 min extension at 72°C. The amplified products were resolved in 1.8% agarose gel (1xTBE) followed by Ethidium Bromide staining and the bands detected then photographed using a gel documentation system (BioRad, USA) (Figure 1).

A total of 8- primers were tested to amplify DNA banding patterns using the total genomic DNA (Table 2). These were: ISSR-1 $(GAGA)_4$ GAT, ISSR-2 $(GAGA)_4$GAAC, ISSR-3 $(GAGA)_4$ GAAT, ISSR-4 $(ACC)_4$Y, ISSR-5 $(GACA)_4$, ISSR-6 $(GATA)_4$, ISSR-7$(GA)_9$ C and ISSR-8 $(GA)_9$ A.

The PCR reaction mixture (25 µL) was composed of 50 ng of total cellular DNA (2 µL), 10 µM of primer (2.5 µL), 2.5 µL of *Taq* DNA polymerase reaction buffer, 0.35 µL of *Taq* DNA polymerase and 10 mM of each dNTPs (DNA polymerization mix). Each reaction mixture was overlaid with 25 µL of mineral oil to avoid evaporation during PCR cycling. Amplifications were performed in a DNA

Table 1. Localities and their geographical coordinates from which *Tribulus terrestris* samples were collected for the morphological and chromosomal characterization.

Coll. no.	Locality	Geographical coordinates	Chromo-some no. (2n)	Ploidy	Accession No.
01	New Delhi; Railway Line, Near Old Delhi railway station	28º36" N, 77º12" E	12	Diploid	**49318**
02	Uttaranchal, Haridwar; PAC Play ground, Jwalapur	29º48" N, 78º36" E	48	Octaploid	**49316**
03	Uttar Pradesh, Baghpat; Railway Line, Near Baraut railway station	28º00" N, 77º00" E	36	Hexaploid	**49320**
04	Punjab, Patiala; Road side, Punjabi Univ. Patiala Campus	30º09" N, 76º17" E	24	Tetraploid	**49321**
05	Uttar Pradesh, Meerut; Play ground, CCS Univ. Meerut Campus	29º00" N, 77º00" E	24	Tetraploid	**49319**
06	Uttar Pradesh, Muzaffarnagar; Waste Land of Titawi village	29º09" N, 77º43" E	24	Tetraploid	**49317**

Table 2. Analysis of banding patterns generated by RAPD and ISSR assay for the six *Tribulus terrestris* cultivars

Primer name and sequence	Total no. of amplified products	Polymorphic bands	% Polymorphism	Expected PCR product size (bp)	Annealing temperature (^{0}C)
RAPD					
AKR-1 (3'TGTGTGCCAC5')	23	6	26	200 to 2,500 for all products	36^0C for all 5 primers
AKR-3 (3'AGGCTGTGCT5')	25	7	28		
AKR-4 (3'GTGCCGTTCA5')	50	8	16		
AKR-5 (3'GGGTGGGTAA5')	27	10	37		
AKR-6 (3'CCGACAAACC5')	39	16	41		
	Total = 164	47	Average = 29.6		
ISSR					
ISSR-1 $(GAGA)_4$ GAT	49	20	40.81	300-1500	52
ISSR-2 $(GAGA)_4$GAAC	26	14	53.84	250-2500	52
ISSR-3 $(GAGA)_4$ GAAT	51	18	35.29	250-1500	52
ISSR-4 $(ACC)_4$Y	50	12	24	250-2800	60
ISSR-5 $(GACA)_4$	42	15	35.71	250-2000	52
ISSR-6 $(GATA)_4$	45	17	37.77	250-2000	42
ISSR-7 $(GA)_9$C	35	8	22.85	250-2000	55
ISSR-8 $(GA)_9$A	29	10	34.48	300-2000	55
	Total = 327	114	Average = 35.59		

amplification thermo cycler (Techne India). The apparatus was programmed to execute the following conditions: a denaturation step of 7 min at 94°C followed by 30 cycles each composed of 30 s at 94°C, 45 s at the primer's specific melting temperature (T_m) at 52-60°C and 2 min at 72°C. A final extension step of 7 min also at 72°C was run at the end of the last PCR cycle.

PCR reactions were electrophorezed on 1.4% agarose gels in 10xTBE buffer by loading 25 µL of the reaction mixture into prepared wells. Gels were run for 4-5 h at 90 V, stained with ethidium bromide (10 µg ml^{-1}) according to Sambrook et al. (1989), and ISSR banding patterns were visualized using a UV transilluminator (Figure 2).

Statistical data analysis

For RAPD and ISSR analysis, the banding patterns were recorded using a gel documentation system (Bio-Red Gel Doc 1000), and the image profiles and molecular weight of each band were determined by Molecular Analyst/pc (Version 1.2) software. The fragment size scored ranged from 250 to 1000 bp. Both weak bands with negligible intensity and smearing bands were excluded from final data analysis. The bands with the same molecular weight and mobility were treated as identical fragments. In the data matrices, Amplicons were scored as discrete variables, using 1/0 as presence/absence of homologous bands for all samples. The data matrices were analyzed by the SIMQUAL program of NTSYS-pc (Version 1.8), and similarities between accessions were estimated using the Jaccard coefficient. For phylogenetic analysis, Dendrograms were produced from the distance matrices using the unweighted pair group method with arithmetic averages (UPGMA). In order to compare the levels of genetic diversity between the *Tribulus* species (Sneath and Sokal 1973). Finally, a principal component analysis (PCA) was performed in order to highlight the resolving power of the ordination.

RESULT AND DISCUSSION

Marker profile and discrimination of genotypes

Molecular markers generated by RAPD and ISSR target different regions of the genome, though in a random manner. Theoretically, a combination of RAPD and ISSR markers would give a better coverage of genome. Primer wise and technique wise results are given in Table 2 Amplification of genomic DNA of the 6 TT genotypes, using RAPD analysis, yielded 164 fragments that could be scored, of which 47 were polymorphic, with an average of 9.4 polymorphic fragments per primer. Number of amplified fragments with random primers ranged from 6 (AKR-1) to 10 (AKR-4) and varied in size from 200 bp to 2,500 bp. Percentage polymorphism ranged from 16% (AKR-4) to a maximum of 41% (AKR-4), with an average of 29.6%. Polymorphism was high enough to enable discrimination of all the varieties, though none of the primers discriminated all the accessions independently. The 8 ISSR primers used in the study produced 327 bands across 6 genotypes, of which 114 were polymorphic. The number of amplified bands varied from 7 (ISSR 7) to 12 (ISSR 1&3), with a size range of 250-2,800 bp. The average numbers of bands per primer and polymorphic bands per primer were 40.87 and 14.25, respectively. Percentage polymorphism ranged from 24% (ISSR 4) to 53.84% (ISSR 2), with an average percentage polymorphism of 35.59% across all the genotypes. The 3′-anchored primers based on poly (AC) and poly (AT) motifs produced high average polymorphisms of 53.84% and 40.81%, respectively. ISSR markers were more efficient than the RAPD assay, as they detected 35.59% polymorphic DNA markers in *Tribulus terrestris* as compared to 29.6% for RAPD markers. This deduced that these primers employed in the study returned a high degree of confidence in identification.

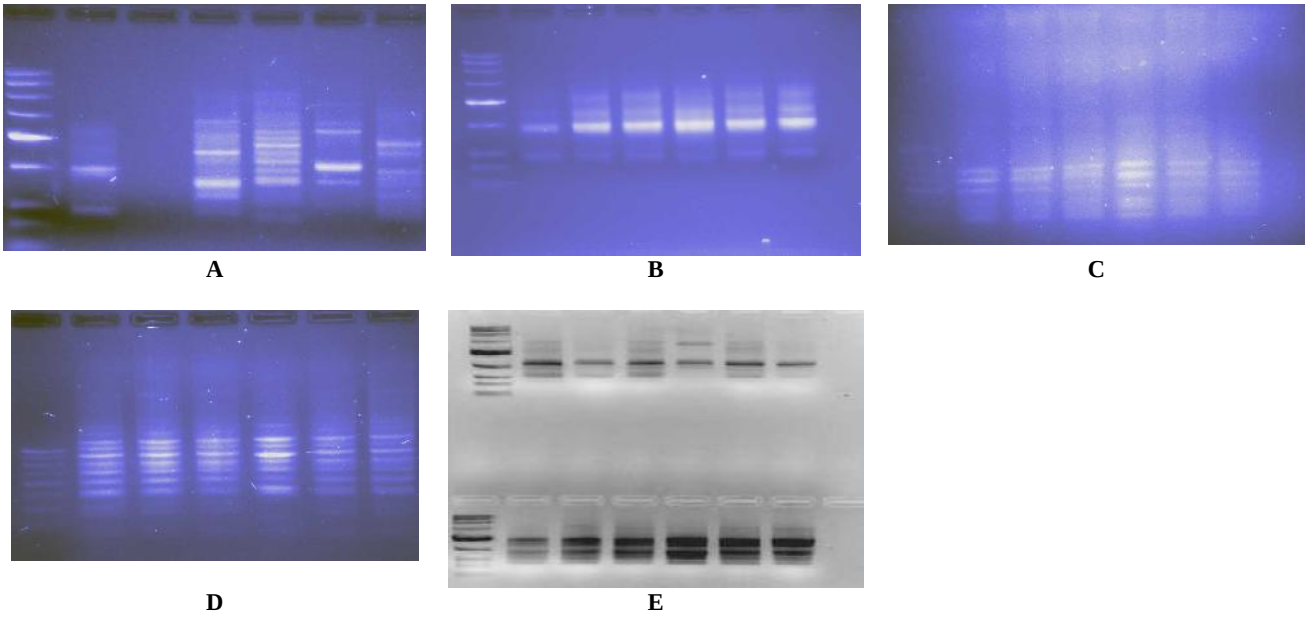

Figure 1. A. Screening of 6 RAPD primers for *Tribulus terrestris* genotypes DNA fingerprinting; and RAPD-PCR products of 6 different *Tribulus terrestris* DNA samples with random primers: B. AKR-1, C. AKR-2, D. AKR-3, E. AKR-4, F. AKR-5. From left to right: Lane M. 100bps DNA ladder, lane 1. Haridwar, 2. Meerut, 3. Delhi, 4. Baghpat, 5. Muzaffarnagar, and 6. Patiala

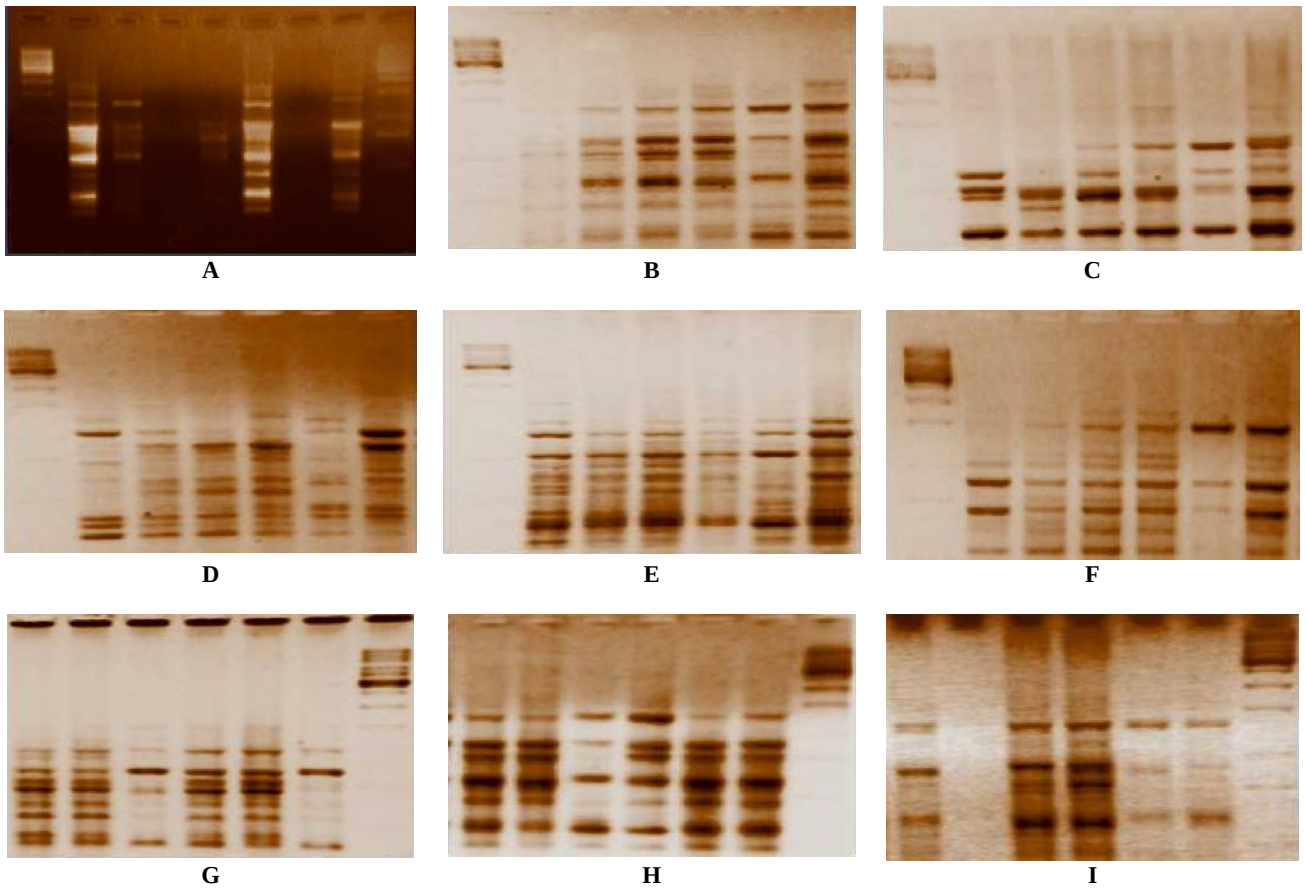

Figure 2. A. Screening of ISSR primers; and ISSR profile of 6 *Tribulus terrestris* genotypes with primer: B. ISSR-1, C. ISSR-2, D. ISSR-3, E. ISSR-4, F. ISSR-5, G. ISSR-6, H. ISSR-7, I. ISSR-8. From left to right: Lane M. 100bps DNA ladder, lane 1. Haridwar, 2. Meerut, 3. Delhi, 4. Baghpat, 5. Muzaffarnagar, and 6. Patiala

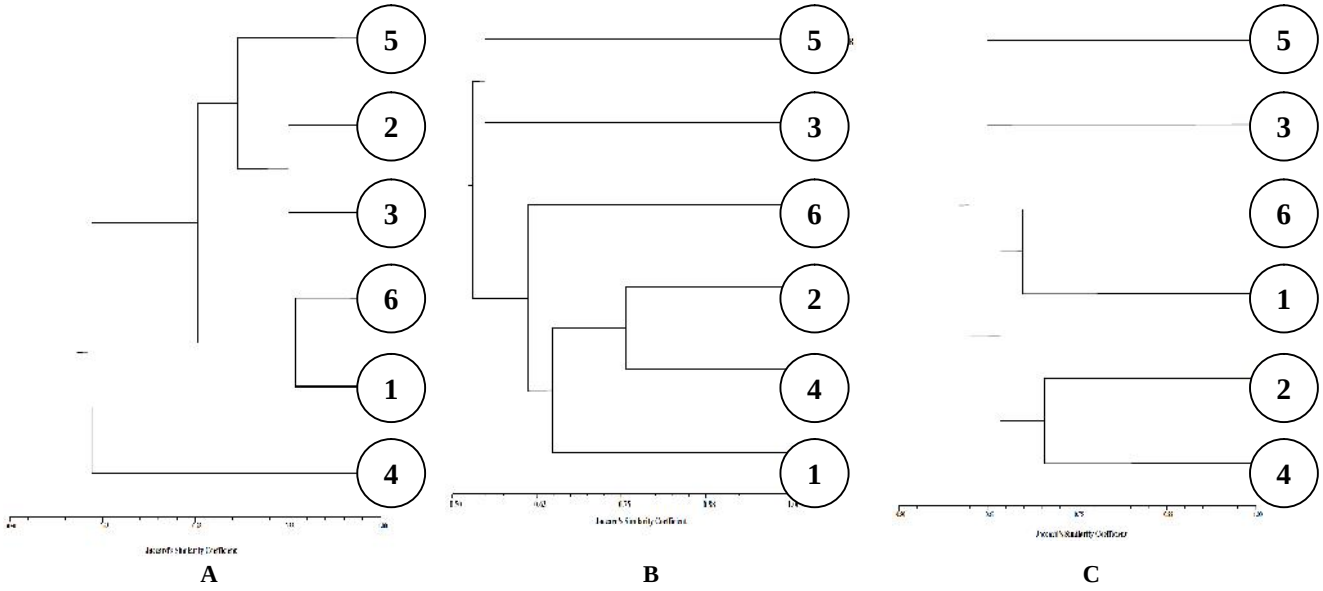

Figure 3. Dendrograms generated using UPGMA analysis, showing relationships between *Tribulus terrestris* genotypes, using: A. RAPD, B. ISSR, C. combining both RAPD and ISSR data. Note: 1. Haridwar, 2. Meerut, 3. Delhi, 4. Baghpat, 5. Muzaffarnagar, and 6. Patiala

Genetic relations and utilization of diversity

Although major emphasis of this work was to generate DNA profiles of the varieties, the marker data was also used to study genetic relations among varieties. Cluster analysis was used to generate three dendrograms based on the Jaccard coefficient of RAPD, ISSR and combined of both for all 6 plant samples (Figure 3.A, 3.B, 3.C respectively). The resultant dendrograms from RAPD and ISSR showed that the populations were divided into two groups but some differences were observed in the allocation of populations to these groups in the RAPD and ISSR data sets, while combined data represent three groups of *Tribulus* population. In the dendrograms derived from the RAPD data set the Jaccard coefficient ranged from 0.61 to 1.00. Populations from Muzaffarnagar, Meerut, Delhi, Patiala and Haridwar all formed one main group, and another second group was composed of Baghpat population. Within the latter group, populations of Meerut and Delhi constituted one sub-group and populations from Patiala and Haridwar clustered into the other subgroup. Populations from Patiala and Haridwar appeared to be closer to each other than clusters of the other populations. Dendrograms derived from the ISSR data set the Jaccard coefficient ranged from 0.525 to 1.00. Populations from Muzaffarnagar and Delhi formed one group, and another second main group was composed of Meerut, Patiala and Haridwar and Baghpat population. Within the latter group, populations of Meerut and Baghpat constituted one sub-group. Populations from Meerut and Baghpat also appeared to be closer to each other than clusters of the other populations

In the combined dendrogram derived from RAPD and ISSR analysis, the Jaccard coefficient ranged from 0.582 to 1.00. Populations from Muzaffarnagar and Delhi formed one group, Patiala and Haridwar formed the second group and remaining samples those from Meerut and Baghpat, formed the third group. Populations of Meerut and Baghpat also appeared to be closer to each other than another clustered populations of *Tribulus terrestris.* The dendrogram based on RAPD showed some variation with in the clustering of genotypes. The ISSR and combined dendrogram indicated a similarity that Meerut and Baghpat populations were closer to each other than other populations. All the *Tribulus* genotypes could be discriminated based on these total 13 RAPD and ISSR primers.

PCA output produced by NTSYS using the simple matching matrix for RAPD and ISSR combined data

In order to visualize the data, you may wish to present the PCA graph, which gives a good three-dimensional picture of the variation. Principal Component Analysis associated with the Minimum Spanning Tree, based on the six genotypes of *Tribulus* and provided complementary information to cluster analysis, as it allows a graphical presentation of the distribution of the cultivars in a three-dimensional plot (Figure 4.A, 4.B, 4.C). In this representation Populations from Meerut and Baghpat are the most similar cultivars similar like ISSR-PCA analysis. The Eigenvalues indicate that three components provide a very good description of the data, as account for 69.68% of the standardized variance. The analysis of Eigenvectors provides information about the traits responsible for the separations along the first three Principal Components. PC1 had 29.2124% of the total variation. This Principal Component is responsible for the individualization of Delhi and Muzaffarnagar populations. PC2 exhibited 21.6469% of the total variability, and is responsible for the separation of Meerut and Baghpat from Haridwar and Patiala populations. A part from the other cultivars PC3 had 18.9246% of the total variation. PC3 is responsible for the separation of Haridwar and Patiala population.

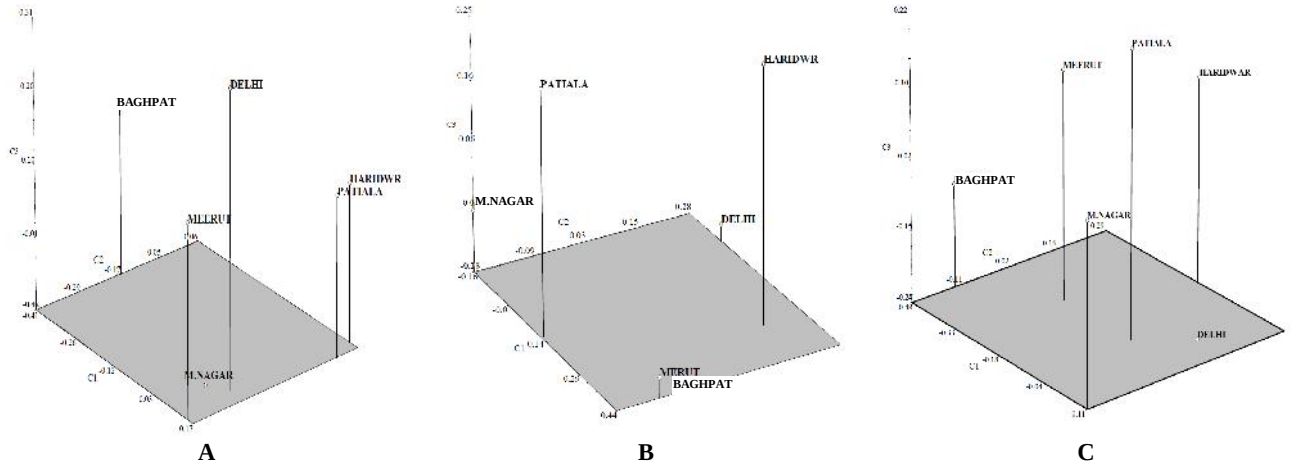

Figure 4. Three-dimensional plot of principal component analysis of using *Tribulus terrestris* genotypes using: A. RAPD, B. ISSR, C. combining both RAPD and ISSR analyses.

The evolution of varieties in distinct agro-climatic zones demonstrates significant levels of variation in response to the selection pressure in the zones (Singh et al. 1998). It is, therefore, not surprising to find significant levels of polymorphism among the 6 genotypes of puncturevine in RAPD (29.6%) and ISSR (35.59%) markers. The RAPD technique has been applied to assess molecular polymorphism in Vigna (Kaga et al. 1996), mung bean (Santalla et al. 1998; Lakhanpaul 2000). The success of our study in identifying polymorphism is due to the use of a number of randomly selected prescreened highly informative primers.

CONCLUSION

With the increasing use of DNA fingerprinting in plant and its potential use in herbal drug industry, the preparation of good quality and quantity DNA has become a major concern. The extraction from tissue needs to be simple, rapid, efficient and inexpensive when many samples are used, such as in population studies, molecular breeding and screening of raw herbal drug materials.

Here we report a study on the genetic variation of *Tribulus terrestris* populations of Northwest India by means of random amplified polymorphic DNA (RAPD) and ISSR fingerprinting. Our aim was to investigate the genetic variation within population of *Tribulus terrestris.* Accessions with the most distinct DNA profiles are likely to contain the greatest number of novel alleles. It is these accessions that are likely to uncover the largest number of unique and potentially agronomic useful alleles. This strategy has resulted in a high proportion (50%) of new and useful quantitative trait loci alleles in rice and tomato (Tanksley and McCouch 1997). This information may assist in the development of an effective cultivation strategy on the *Tribulus terrestris.* Further studies are envisaged to quantify the genetic gain in populations derived from genotypes with distinct DNA profiles. The study of a big collection should provide a better knowledge about genetic diversity and its relationship with geographical origin. This information could be very valuable in the management and cultivation of plant genetic resources for the herbal drug formulation research.

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