RESEARCH ARTICLE

ASSESSMENT OF GENETIC FIDELITY OF MOTHER PLANT AND *IN VITRO* RAISED MEDICINAL PLANT *EPHEDRA GERARDIANA* THROUGH MOLECULAR MARKERS

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Abstract: *Ephedra gerardiana* is an important medicinal gymnosperm shrub. It has been traditionally use for an assortment of medicinal purpose. Molecular markers analysis was conducted to screen genetic fidelity among *in vitro* raised plantlets compare with mother plant of *Ephedra gerardiana*. Genetic fidelity of regenerated plants was assessed using Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) Primers. A total of 50 RAPD primers and 30 SSR primers were utilized in the present study to analyze genetic fidelity of mother plant and among tissue culture raised plants of *Ephedra gerardiana*. Out of 50 RAPD primers, 19 primers exhibited DNA amplification in all the DNA samples and out of 30 SSR primers, 18 were show amplification. The amplified products of the regenerated plants showed similar banding patterns to that of the mother plant thus demonstrated the homogeneity of the micropropagated plants. The banding pattern ruled out presence of any kind of somaclonal variation. Thus, the results revealed that genetic fidelity between the micropropagated and mother plant in *Ephedra gerardiana* and supports the suitability of tissue culture technique for generation of genetically similar plants. Hence, the results obtained confirmed genetic stability of regenerated plants.

Keywords: *Ephedra gerardiana*, Micropropagation, Genetic fidelity, RAPD, SSR

INTRODUCTION

Ephedra is a gymnospermic genus of Gnetales and family Ephedraceae. Ephedra belongs to class Genetopsida having 3 different genera Ephedrales, Gnetales and Welwitschiales respectively (sharma *et al.,* 2012). *Ephedra*, also known as ma huang, contains the ephedrine alkaloid that stimulates the central nervous system (Tod and Stewart 1997). *Ephedra* have significant medicinal properties due to the presence of various keenly secondary metabolites. Ephedrine alkaloids have an adrenaline like effect on the body it excites the nervous system, opens blood vessels, and stimulates the heart (www.ephedrafacts.com). The *Ephedra sinica* plant contains ephedrine alkaloids that are used in the herbal supplements once they have been cultivated from the dried stems of the plant. Some of the main alkaloids in *Ephedra* are ephedrine and pseudoephedrine, which can be found in over the counter (OTC) drugs, but an important distinction is that the alkaloids in OTC drugs are produced synthetically (Paul 2001). Synthetic ephedrine is more potent than the ephedrine alkaloids found in ephedra used mainly as a bronchial decongestant, ephedrine is found in bronchodialators such as Primatene, while pseudoephedrine is commonly utilized in decongestants such as Sudafed. Physiologically, it acts to expand breathing passages, constrict blood vessels, and increase arterial blood pressure. It is the increase in arterial blood pressure that causes severe hypertension, stroke, or heart attack by Jody (1997). Plant tissue culture appears to be a reliable method for the production of *Ephedra* within a short time span. Among the various methods of *in vitro* propagation, shoot proliferation is considered to be the least susceptible to genetic modification (Shenoy *et al.,* 1992). Somaclonal variation has been reported to some extent at different levels i.e. morphological, cytological, cytochemical and molecular in micropropagated plants (Rani and Raina 2000). The genetic analysis of micropropagated plants using a multidisciplinary approach, especially at the DNA sequence level initially and at various cultural stages, it is essential to maintain genetic integrity of micropropagated plants. Several cytological, isozyme and molecular markers have been used to detect the variation and/or confirm the genetic fidelity in micropropagated plants (Gupta *et al.,* 2009; Kumar *et al.,* 2011). The sensitivity, reproducibility and strong discriminatory power of microsatellite and RAPD markers (Parida *et al.,* 2009; Sharma *et al.,* 2013) make them suitable for detecting somaclonal variation. However, their application in the study of somaclonal variation has been quite limited.

In the present study, *Ephedra gerardiana* were propagated using shoot buds as the explants. The

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micropropagated plants were successfully transferred to the field. The genetic fidelity of *in vitro* raised plants was assayed using RAPD and SSR markers to assess the genetic stability among the regenerated plantlets.

MATERIAL AND METHOD

Plant collection

Ephedra gerardiana were collected from Natural habitat of Chakrata forest division in the Uttarakhand state and were grown in the polyhouse of the K.L.D.A.V (PG) College Roorkee.

In vitro culture of *Ephedra gerardiana*

In vitro culture of *Ephedra gerardiana* was followed by sharma *et al* 2012. Regenerated plants genomic DNA were used for genetic fidelity analysis with mother plant.

Genomic DNA extraction

Stem tissue was used for DNA extraction of mother plant as well as regenerated plantlets. Initially cut stem tissue into small pieces and put into liquid nitrogen after that grind using mortar and pestle. DNA extraction was made using modified CTAB procedure (Doyle and Doyle 1990). The quantity of isolated DNA was determined by using Biophotometer (Eppendorf, Germany), visualized on 0.8% agarose gel stained with ethidium bromide and a final concentration of 80ng was used for PCR.

Development of SSR and RAPD primer

ESTs sequences of *Ephedra* were retrieved from available data base NCBI (http://www.ncbi.nlm.nih.gov/) and were screened for presence of microsatellites using CAP 3 and MISA tools (Thiel *et al*., 2003, downloaded from http://pgrc.ipk-gatersleben.de/misa/), Finally SSR primers were designed using Primer 3 software.

RAPD and SSR PCR amplification

The obtained DNA was diluted as required working concentration for PCR analysis using RAPD and SSR primers. Both primers amplification was performed using a thermal cycler (Veriti, ABI, Germany).

For RAPD analysis the reaction mixture composed of 100 ng DNA, 1X Taq buffer, varying the $MgCl_2$ concentration 1.5 mM to 3.0 mM, 10 pmoles primer, 0.2 mM dNTPs and 1 U of Taq DNA polymerase (Genei, Bangalore, India). The amplification program was as follows; initial denaturation at 95°C for 5 min followed by 45 cycles of denaturation at 94°C for 40s, annealing at 34-40°C for 1.5 min, extension at 72°C for 1 min and finally ending with one cycle at 72°C for 10 min. After amplification, PCR products were stored at −20°C till performing electrophoresis. Amplified products were run in 1.5% agarose gel and submerged in 1x Tris-Acetic acid- EDTA (TAE) buffer (pH 8.0) at a constant voltage (60 V).

For the SSR analysis the reaction mixture composed of 80 ng DNA, 1X Taq buffer, 1.5 mM $MgCl_2$, 10 pmoles each of forward and reverse primers, 0.2 mM dNTPs and 1 U of Taq DNA polymerase (Genei, Bangalore, India). The amplification program was as follows; initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30s, annealing at 50-58°C for 45s, extension at 72°C for 1 min and finally ending with one cycle at 72°C for 7 min. After amplification, PCR products were stored at −20°C till performing electrophoresis. Amplified products were run in 2.5% agarose gel and submerged in 1x Tris-Acetic acid- EDTA (TAE) buffer (pH 8.0) at a constant voltage (60 V). The profiles were documented by UV trans-illuminator equipped gel documentation system (Avegene, Taiwan). The molecular size of amplified fragments was compared with 100 bp DNA ladder (Genei, Bangalore, India).

Data analysis

The scorable bands were included in the analysis. Each band was considered a single locus and 1-0 matrices were formed for further analysis. Presence and absence of each locus was coded by 1 or 0 respectively. The binary data were used to compute pairwise similarity coefficients and the similarity matrix thus obtained was subjected to cluster analysis using UPGMA (unweighted par group method with arithmetic average) using NTSYS-PC version 2.01 program for cluster analysis (Nei, 1971).

RESULT AND DISCUSSION

Development of ESTs SSR markers from available data

Screening of 4,982 ESTs related to *Ephedra* to identification of 931 microsatellite sites. In the current analysis, mononucleotide repeats were not included because of the abundance of poly A/T repeats, arising due to the presence of Poly A/T tails in cDNA molecules and errors prevailing due to sequencing (Jain *et al.*, 2010; Jain *et al.*, 2014). In total 3.71% of the ESTs were found to carry microsatellites within them which are quite comparable to some of the previous studies conducted on other plants (Victoria *et al.*, 2011). The tri-nucleotide repeat motifs were the most prevalent (54.13%) class of microsatellites quickly followed by di-nucleotide (36.53%) and tetra-, penta- and hexa-nucleotide repeats together contributing 9.34% (Figure 1). However results observed in the current study contradicts the tri-nucleotide abundance observed by Parida *et al.* (2010). One possible reason behind this observation could be the small data set considered in the present study. Considering the length of microsatellite loci, 64.9 % microsatellites were grouped as class II microsatellite repeats (< 20 nucleotide long) and 35.1 % microsatellites as class I microsatellite repeats (≥ 20 nucleotide long). Similar preponderance of class II repeats has been reported in other studies also (Roorkiwal and Sharma, 2011; Jain *et al.*, 2014). The

inverse relationship between the length of microsatellites and their abundance holds true in the present study. This could be due to the fact that longer repeats have downward mutation bias (Katti *et al.*, 2001).

The total classified frequency numbers of dinucleotide repeats were observed to be 50.36% followed by AG repeats, 30.66% AT repeats and AC repeats 18.98% (Figure 2). Among the dinucleotide repeats, the most and least frequent motifs were GA and AT respectively. Similar abundance of GA dinucleotide repeats, has been observed in many other plant species in the recent past (Sharma, 2011; Victoria *et al.*, 2011). This repeat class is known to play an active role in gene regulation in both animal and plant species (Trivedi, 2004). In trinucleotide repeats, AAG repeat class was found to be the most prevalent trinucleotide repeat followed by ATC repeat class (Figure 3,4), which could be possibly due to their high AT content (Cordeiro *et al.*, 2003; Pinto *et al.*, 2004) and consequent usage bias in the coding sequences (Morgante *et al.*, 2002).

The assessment of the genetic stability of *in vitro* plant is vital step in the study of tissue culture technique for regeneration of genetically true-to-type plantlets (Katti *et al.*, 2001). Although the present scenario plant conservation is predominantly depends on micropropagation methods. However, genetic and phenotypic variants are reported during in this method, which may be the focal elucidation to originating somaclonal variants. Thus, the risks of genetic change induced by micropropagation and the important of assessing the genetic stability must be considered in the perspective of conservation.

The present study was conducted to screen genetic fidelity in *Ephedra gerardiana* regenerated plantlets through direct organogenesis using Nodal segment as explant. PCR based RAPD and SSR technique has been frequently used for genetic fidelity in this medicinal shrub plants.

In the present investigation 50 RAPD primers were used, amongst which only 19 primers show banding pattern (Figure 5). The number of scorable band for each primer varied from 6-15 with an average of 10.68 bands present per locus (Table 1.1, 1.2) and found 96% genetic stability with mother plant (Table 1.3 , Figure 7).

Out of 30 SSR primers 18 SSR primers show the scorable band pattern (Figure 6). Total 33 amplicon were amplified with average 1.83 per locus. That show low polymorphism on an average 0.09 and maintain more than 99 % genetic similarity with mother plants (Table 1.4, 1.5, 1.6, Figure 8). Markers results illustrate the high genetic similarity of tissue culture plant with mother plant. It is the sign of no somaclonal variation at genetic level. Similarity matrix based on Jaccard's coefficient revealed that pairwise value between the mother plant and its tissue cultured plants ranged from 0.86 to 0.95 with RAPD primers and among tissue cultured plants, it was 0.96 to 1.00 with SSR primers, thus indicating a high degree of genetic fidelity. Many factors are responsible for inducing variability during micropropagation such as explants source, time of culture, number of subcultures, phytoharmone, genotype, media composition and the level of ploidy.

Saini *et al*. (2004) have reported that RAPD analysis of sugarcane showed 90% of genetic purity among its micropropagated plants. Further, Jain *et al*. (2005) also found no significant variation among meristem derived RAPD analysis. Independent studies conducted by Devarumath *et al*. (2007), Lal *et al*. (2008) and Tawar *et al*. (2008) on plants propagated from apical meristems reported high rate of genetic fidelity. RAPD marker banding patterns have been the source behind these results. They found more than 97% genetic similarity between mother plant and micropropagated plant and concluded these tissue cultured plants were genetically stable. Sharma *et al*. (2007) evaluated genetic fidelity of micropropagated plantlets of medicinal plant using RAPD markers. They observed no genetic variation and no somaclonal variation between mother plant and micropropagated plantlets. Besides them, Suprasanna *et al*. (2006, 2007), Lal *et al*. (2008) and Pandey *et al*. (2012) in their respective studies also analyzed genetic fidelity in *Saccharum officinarum* regenerated plantlets through RAPD and SSR markers. Results obtained from banding pattern obtained from both the makers indicated no sign of somaclonal variation and confirmed genetic stability of regenerated plants.

SSRs are sensitive, reproducible and have strong discriminatory power (Parida *et al*., 2009) and thus, can be used to detect the genetic variations and somaclonal variation but their use in detecting the clonal fidelity is quite restricted. Though there are few reports of testing genetic fidelity in many plants using various molecular markers (Taylor *et al*., 1995; Saini *et al.*, 2004; Suprasanna *et al.*, 2006; Devarumath *et al*., 2007; Lal *et al.*, 2008), 90-100% genetic fidelity using SSR marker has been reported in medicinal plants such as *Centurea ultreiae*, (Pandey *et al.*, 2012).

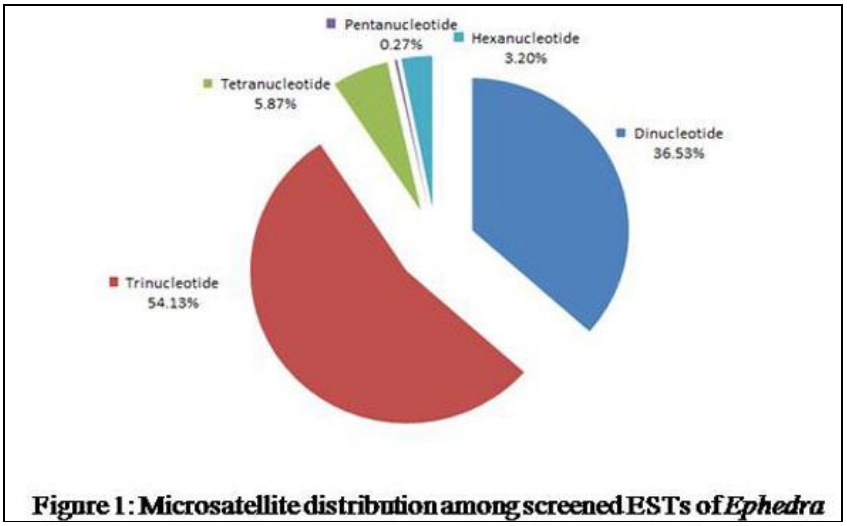

Figure 1: Microsatellite distribution among screened ESTs of *Ephedra*

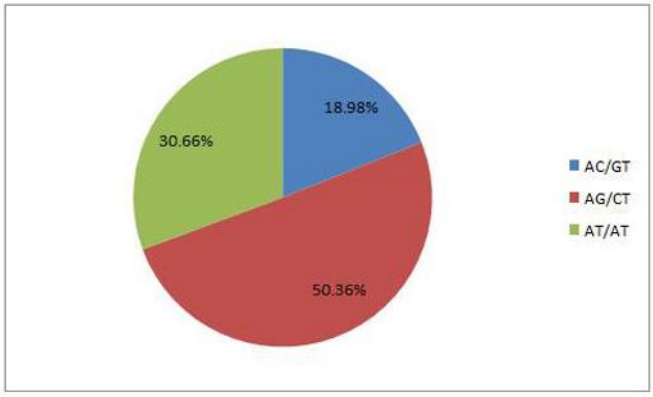

Figure 2: Individual motif abundance among di-nucleotide repeats in *Ephedra* ESTs

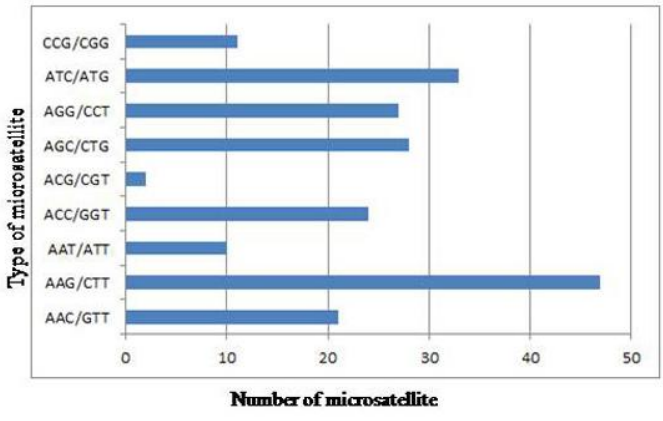

Figure 3: Individual motif abundance among Tri-nucleotide repeats in *Ephedra* ESTs

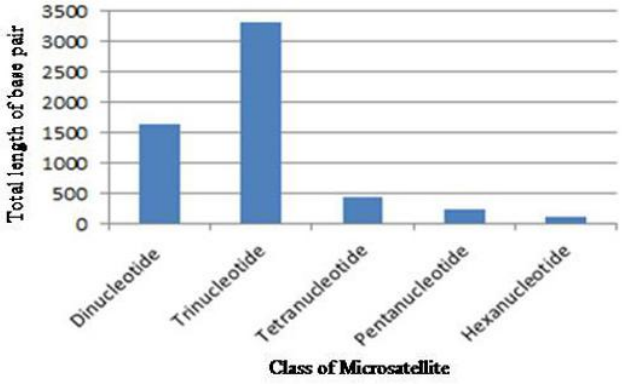

Figure 4: Length contribution by each class of Microsatellite

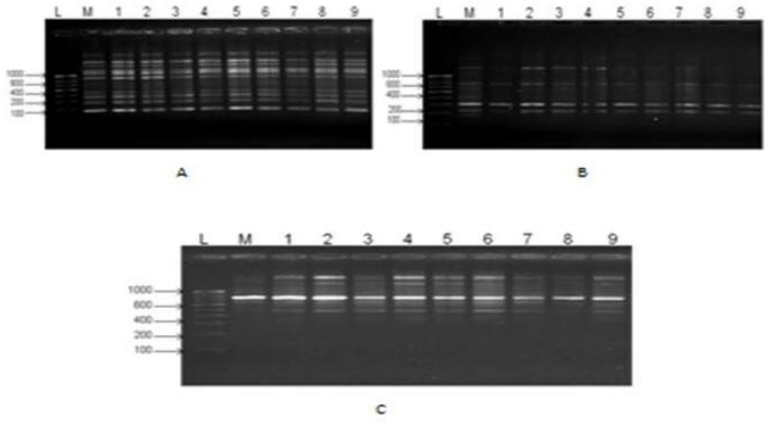

Figure 5: A,B,C RAPD primer number 2,14,16 (Table) on 1.5 % Agarose gel Lane M - Mother Plant; Lane 1 to 9 regenerated plantlets of *Ephedra gerardiana*; Lane L - 100 bp ladder.

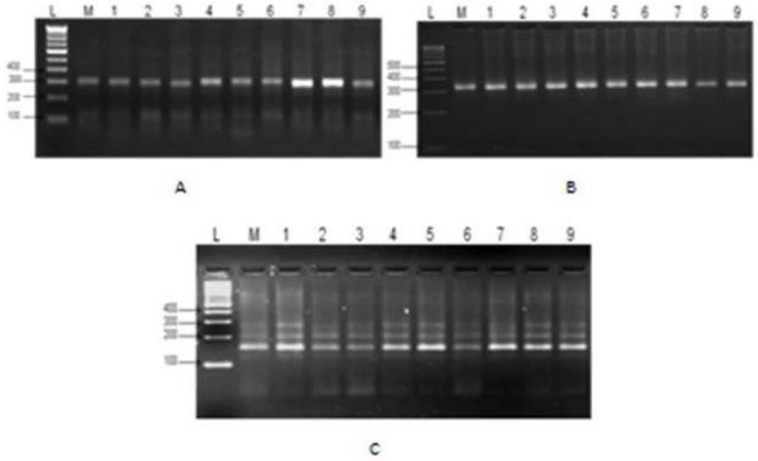

Figure 6: A,B,C SSR primer pair number 1,4,7 (Table) on 3% metaphor gel Lane M - Mother Plant; Lane 1 to 9 regenerated plantlets of *Ephedra gerardiana*; Lane L - 100 bp ladder.

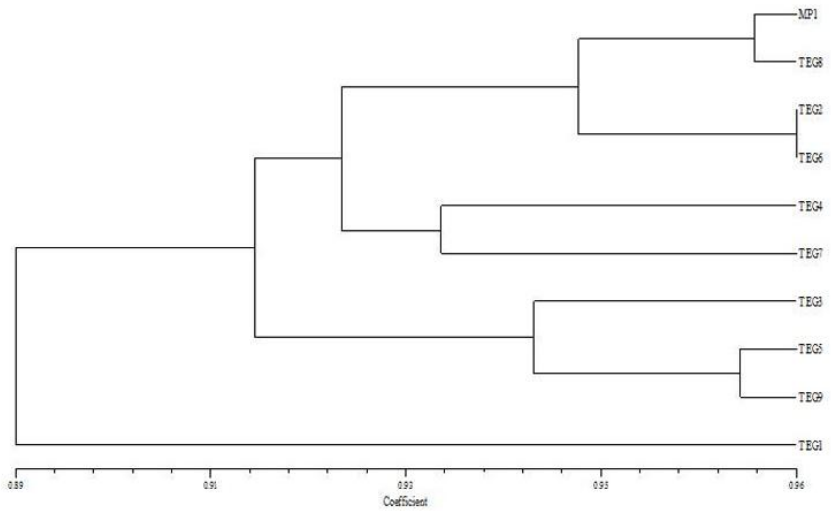

Figure 7: Dendrogram show the genetic similarity of the mother plant with tissue culture plantlets on the basis of jacard coefficient using RAPD primers

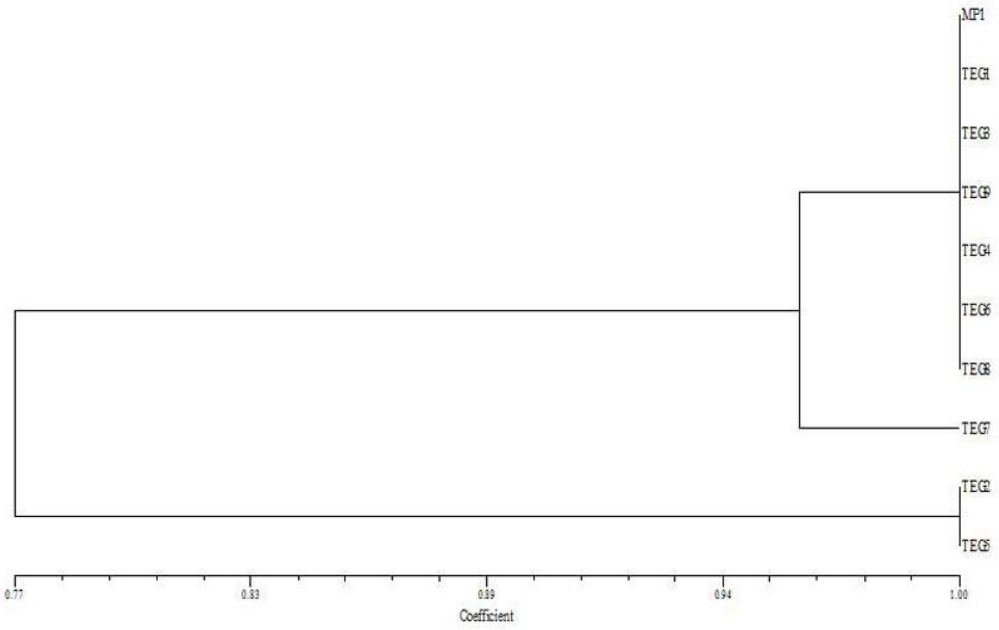

Figure 8: Dendrogram show the genetic similarity of the mother plant with tissue culture plantlets on the basis of jacard coefficient using SSR primers

CONCLUSION

From the study, it was concluded that the genetic stability of *Ephedra gerardiana* plantlets observed in our study indicates good genetic fidelity, when examined by two marker systems. A polymorphic rate was detected very low due to the point mutations or any changes occurs the primer binding side. It was also found more than 90% plantlets similar with the mother plant. Molecular marker is beneficial for the studies of phylogenetic analysis, cluster analysis and genetic fidelity of the plant.

Table 1. List of primers exhibiting DNA amplification in RAPD analysis of *Ephedra gerardiana*

S. No.	Primers	Sequence of primers -5'3'	Amplified Band	Polymorphic Band
1	OPP-01	**5'**-GATGCACTCC	8	1
2	OPP-02	**5'**-TCGGCACGCA	10	2
3	OPP-05	**5'**-CTGTATCGCC	13	3
4	OPP-07	**5'**-GTGTCTCAGG	7	1
5	OPP-11	**5'**-GTGGGCTGAC	15	2
6	OPP-12	**5'**-GTCCATGCCA	11	2
7	OEP-03	**5'**-CCAGTAGCAC	10	4
8	OEP-07	**5'**-AGATGCAGCC	9	2
9	OEP-11	**5'**-GAGTCTCAGG	13	5
10	OEP-12	**5'**-TTATCGCCCC	11	1

11	OEP-14	**5'**-TGCGGCTGAG	14	2
12	OEP-16	**5'**-GGTGACTGTG	15	4
13	OEP-17	**5'**-CTACTGCCGT	12	1
14	OPW-02	**5'**-ACCCGGCCAA	6	0
15	OPW-03	**5'**-GTCCGGAGTG	8	2
16	OPW-04	**5'**-CAGAAGCGGA	7	0
17	OPW-05	**5'**-GGCGGATAAG	9	2
18	OPW-06	**5'**-AGGCCCGATG	12	4
19	OPW-07	**5'**-CTGGACGTCA	13	3
Total No. of bands			**203**	**41**

Table 2. Data of RAPD analysis

S. No.	Parameters	Readings
1	Number of assay	19
2	Number of amplicon	203
3	Average Number of amplicon	10.68
4	Average number of polymorphic amplicon	2.16
5	Percentage polymorphism	21.6

Table 3. Nei's genetic similarity Jacard coefficient for *Ephedra gerardiana* on the basis of RAPD analysis.

	MP1	TEG1	TEG2	TEG3	TEG4	TEG5	TEG6	TEG7	TEG8	TEG9
MP1	1									
TEG1	0.9	1								
TEG2	0.96	0.86	1							
TEG3	0.92	0.88	0.88	1						
TEG4	0.94	0.9	0.9	0.87	1					
TEG5	0.93	0.88	0.91	0.92	0.87	1				
TEG6	0.95	0.87	0.96	0.91	0.9	0.89	1			
TEG7	0.95	0.89	0.92	0.9	0.92	0.88	0.9	1		
TEG8	0.96	0.89	0.94	0.89	0.92	0.95	0.91	0.91	1	
TEG9	0.95	0.87	0.92	0.94	0.9	0.95	0.92	0.91	0.91	1

Table 4. List of primers exhibiting DNA amplification on the basis of SSR analysis of *Ephedra gerardiana*

S. No.	Marker	SSR Motif	Amplified band	Polymorphic band
1	F-AAGGTTAGCTTCAACATGGA R- AGACTCCATGAGTCCACAAC	(TC)11	1	-
2	F-AGCCCTAAGTAAGGCTCCTA R- TTCTCATCCTCTGTTTCCTC	(GCT)7	2	-
3	F- CTGCGTTTGAGAAATATCGT R- CCCTGAAGGTCAAGGAAAT	(GA)8	1	-
4	F- GGCTGAGAAGAGATACGAGA R- GCCTTAGGAAGTTCAGCTTT	(GTG)6	1	-
5	F- TTACAGATGGTACAGCCACA R- TGAAGATTTGCCACTCTCTT	(GA)7	1	-
6	F- CCAACATGTTCAACAATCTC R- TATCAAGCTTCCTTGTCCAT	(AG)6	3	1
7	F- CGTATTCTCCAAATTGCATC R- CAGATCAAGAAAGCGAAATC	(CGC)6	3	-
8	F- TTACAGATGGTACAGCCACA R- TGAAGATTTGCCACTCTCTT	(GA)22	2	-
9	F- CTCCAATGATAGACCCAGAA R- AGGATAACCTGATTGCTGTG	(GAT)6	1	-
10	F- GCTTGAGAAGAATCCAGATG R- AGCATGAAGTCTCTTCGTTT	(AGC)6	2	-
11	F- GGAGAACTTTCTGTCTCAAA R- AGTGTTGCTCAAAGAGCCTA	(AG)8	2	1
12	F- TTGGAATGAATCAACCTGAC R- GGGACAGTGTATCTCCTTGA	(TAG)6	1	-
13	F- GTATTCTCCAAATTGCATCC R- CAGATCAAGAAAGCGAAATC	(CG)6	1	-
14	F- TCATCAGCAACAGCAATATG R- CAATAAAGCAGATTCGTCGT	(CA)6	2	-
15	F- GCAGCATCATACTCAAATCC R- CCGGGTTTGTATCGTATATC	(CCT)6	2	-
16	F- TTAGCATCTGGTGATCCTGT R- AGATCCACTCCCTACGAAAT	(AGC)6	2	-
17	F- TCTTGAAACCCTAGAAACCA R- CTCCAACCTGAAGAAGAAGA	(GAG)6	3	1
18	F- CGGTGAATTGAGAGTGAAGA R- CGAAATCTAGACCTTTGTGG	(CAG)6	3	-
Total Number of Band			**33**	**03**

Table 5. Data of SSR analysis

S. No.	Parameters	Readings
1	Number of primers assay	18
2	Number of amplicon	33
3	Average Number of amplicon	1.83
4	Average number of polymorphic amplicon	0.09
5	Percentage polymorphism	9.09

Table 6. Nei's genetic similarity Jacard coefficient for *Ephedra gerardiana* on the basis of SSR primers

	MP1	TEG1	TEG2	TEG3	TEG4	TEG5	TEG6	TEG7	TEG8	TEG9
MP1	1									
TEG1	1	1								
TEG2	0.76	0.76	1							
TEG3	1	1	0.76	1						
TEG4	1	1	0.76	1	1					
TEG5	0.76	0.76	1	0.76	0.76	1				
TEG6	1	1	0.76	1	1	0.76	1			
TEG7	0.96	0.96	0.80	0.96	0.96	0.80	0.96	1		
TEG8	1	1	0.76	1	1	0.76	1	0.96	1	
TEG9	0.96	1	1	1	1	0.76	1	0.96	1	1

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