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Molecular characterization of *Ephedra* species found in Pakistan

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ABSTRACT. *Ephedra*, also known as "ma huang", is a dioecious, drought- and frost-resistant, perennial, evergreen shrub with compelling medicinal value. The genus is represented by 42 species around the world, 9 of which were provisionally reported from Pakistan. Species of the genus have a controversial taxonomy due to their overlapping morphological features. Conventional tools alone are not sufficient for characterizing the species. The objective of present study was to assess the genetic variability present in different biotypes of *Ephedra* growing in Pakistan using molecular markers. A total of six genotypes collected from diverse geographic zones of Pakistan were used. The DNA of all genotypes was amplified using nine randomly amplified polymorphic DNA (RAPD) primers to study genetic variability at the molecular level. The dissimilarity coefficient matrix based on the data of 9 RAPD primers was used to construct a dendrogram which was then used to group the genotypes in clusters. Based on the dendrogram and dissimilarity coefficient matrix, the RAPD markers used here revealed a moderate to high level of genetic polymorphism (6 to 49%) among the genotypes. It was found that the collection of genotype accessions from Swat Valley in northwestern Pakistan was most distantly related to the other five collections. More molecular markers including functional genes and ribosomal spacer regions are suggested to find a better estimate of the genetic diversity present in *Ephedra* growing in Pakistan. The information provided here is useful for identifying valuable *Ephedra* variants which will be used for medicinal purposes and earning foreign currency.

Key words: *Ephedra*, Ephedrine, Molecular markers, Genetic distance, Diversity, Random amplified polymorphic DNA

INTRODUCTION

Ephedra, also known as "ma huang" (Anonymous, 1996), is a dioecious, drought- and frost-resistant, perennial, evergreen shrub. It is native to central Asia and distributed throughout China, Tibet, India, Japan, Southern Siberia, Spain, Sicily, and Afghanistan (Morton, 1977; Grieve, 1979; Budavari, 1996; Leung and Foster, 1996). *E. distachya* is found in Europe, and there are ten *Ephedra* species reported from North America which include *E. trifurca* or *E. viridis*, *E. nevadensis* and *E. americana* (Morton, 1977). Several *Ephedra* species have been cultivated experimentally in Australia, Kenya, England, and the US (Grieve, 1979; Budavari, 1996; Leung and Foster, 1996), but commercially important species are available mostly in China, northwestern India and northern Pakistan (Morton, 1977; Nasir and Ali, 1987; Tyler et al., 1988). *Ephedra* is quite rich in species and genetic diversity with extreme morphological proliferations. The high degree of overlapping traits does not allow the species to be identified using only conventional tools. Hence, only 9 species (*E. ciliate*, *E. regeliana*, *E. pacyclada*, *E. wallichii*, *E. gerardiana* Syns E. *distachya*, *E. przewalskyi*, *E. procera* Syn E. *Nebrodensis*, *E. sarcocarpa*, *E. monosperma*) were provisionally recognized from Pakistan (Nasir and Ali, 1987), which needs further confirmation. The use of DNA technology could be the most appropriate tools in this regard.

The plant is a sporophyte. It bears strobili, which are compound and unisexual. They are borne as small spikes at the nodes. The plants are dioecious, but there are occasional reports of monoecious plants bearing bisporangiate strobili with stamens at the base and ovules above. Male strobili, 2-4, arise in the axial of a leaf or clustered at the nodes and consist of a short axis bearing a number of rather thick bracts or scales arranged decussately in pairs (Figure 1A). The closely set pairs of bracts vary from four to sixteen. In the axial of each bract, the lower one or two, stands a single staminate or male flower consisting of an axis (microsporangiophore) bearing at its tip one to eleven anthers (microsporangia) which are porose and invested by two connate bracteoles below (perianth lobes). *Ephedra* has the polyplicate pollen type, characterized by a pointed-oval shape and longitudinal ridges. This pollen type is found in the living *Ephedra*. The key characters in distinguishing the pollen of species of *Ephedra* are the number of ridges, the shape of the ridges (rounded *vs* sharp), the branching pattern between the ridges, and the size of the grain (35-70 µm long) (Steeves and Barghoorn, 1959; King and Sigleo, 1973).

Figure 1. A. Female strobili. **B.** Male strobili.

Female strobili are also 2-4 per node (Figure 1B). Each female strobilus consists of a central axis with 2-4 pairs of decussate bracts which are fused to form cupules at the base. The bracts may be dry and winged or fleshy to sometimes brightly colored, depending on the modes of seed dispersal by wind or animals. Each strobilus ends in a group of female flowers 1 to 3 in number, each consisting of a short stalk and a terminal ovule (mega sporangium). The inner integument of the ovule fuses with the nucellus in the lower half, but its upper half remains free and is prolonged into a thin-walled micropilar tube, which is sometimes spirally twisted and opens at its tip into a tongue-like extension or the tent pole (tubillus). Here, the pollination drop is exuded by means of which the pollen is drawn down the micropylar tube into the

pollen chamber. The pollination is anemophilus. There is double fertilization (Chopra, 1987). The seeds, 1-2, are usually ovoid to triangular in outline. The testa is dark brown and bears a number of processes touching the micropylar tube formed by the inner papery layer. As the seed develops, it is surrounded by a third thick and fleshy layer formed by the fusion of the subtending bracts of the strobilus.

The seed in *Ephedra* has no obligatory dormant period and may germinate immediately. The two linear cotyledons grow steadily until they are several centimeters long. On germination, they come out (epigeal) and form the cotyledonary leaves which are responsible for photosynthesis in the early stages of growth (Chopra, 1987; Nasir and Ali, 1987). *Ephedra* is a gymnospermic genus of *Gnetales* and family Ephedraceae. The genus is represented by 42 species from around the world, nine of which are

Table 1. Medicinal properties of *Ephedra sinica* and its constituents.

Herb/extracted chemicals	Form	Uses/actions
Herb (stems and branches)	Decoction, powder, tincture	Decongestant, opens sinuses, increases perspiration, dilates bronchioles (antiasthmatic use), diuretic, central nervous system stimulant, raises blood pressure, alleviates aches and rheumatism, alleviates hay fever/colds, lowers fever
Ephedrine, Hydrochloride, Sulfate, Tannate	Tablets, suppositories	Antiallergenic, antiasthmatic, antispasmatic, decongestant, cough suppressant, stimulant, vasoconstrictor
Pseudoephedrine, Hydrochloride, Sulfate	Tablet, liquid	Decongestant, cough suppressant
Norpseudoephedrine (Amlodipine)	Tablet, liquid	Peripheral vasodilator used to treat angina

Source: Chevallier, 1996.

provisionally reported from Pakistan. The genus is recognized for 14 phyto-chemicals, of which ephedrine and pseudoephedrine are the most important (Table 1).

Traditionally they are used for respiratory tract diseases. Besides its use as medicine, some of its species are used as diet supplement for weight reduction. Locally, it is used to enhance the effect of snuff. Species of the genus have a controversial taxonomy due to their overlapping morphological features. Conventional tools alone do not help in the characterization of the species. Finally, studies are rare due to difficulties in accessing the area that is known as a habitat for this species, and also due to the lack of markers and well-characterized phenotypes. Therefore, the main objectives of the study were to collect *Ephedra* plants growing in various parts of Pakistan and fingerprint genetic variation present in the material using randomly amplified polymorphic DNA (RAPD) markers.

MATERIAL AND METHODS

Plant material

Plant specimens were collected from three different locations, including Hazar Ganji National Park (Baluchistan), Wali Tangi (Baluchistan) and an alpine zone of Utror (Kalam District Swat NWFP) based on the information on flora of Pakistan (Nasir and Ali, 1987; Table 2). These sites are reported to be rich sources for the *Ephedra* species in Pakistan. The specimens were preserved at -80°C using an ultra-low freezer at the IBGE, Peshawar.

Table 2. Collection of *Ephedra* specimens from different parts of Pakistan.

No.	Codes	Site of collection
1	2	Hazarganji National Park Baluchistan
2	3	Hazarganji National Park Baluchistan
3	4	Zob Baluchistan
4	5	Utror Swat (female plant)
5	6	Zob Baluchistan
6	7	Utror Swat (male plant)

DNA and polymerase chain reaction analyses

Since there was no available leaf tissue in the *Ephedra* species, stem tissue was therefore used for DNA extraction. The plant tissues were ground using either a mortar and pestle after -80°C treatment for 1 week or a coffee grinder for 20 min after a 12-h lyophilization at -40°C and at high vacuum. Modified CTAB DNA extraction protocol (Doyle and Doyle, 1987; Shah et al., 2000) was applied using ground tissue. The DNA obtained was air-dried for 4 h and dissolved in TE buffer. The DNA obtained was run on 0.8% agarose gels (in 1X TBE) containing ethidium bromide (1.5 µL ethidium bromide/100 mL gel). Initially, 2 µL DNA with 0.5 µL loading dye was used. All the specimens were run at once at 100 V (70 mA) for an appropriate time. The gel was visualized under a UV source and the amount of DNA per µL was estimated by relative comparisons of the DNA bands with each other and the λ-DNA ladder digested with restriction enzymes. An RNAse A treatment (0.2 µL per 50 µL) for each sample for 1 h at 37°C was performed in a shaking water bath.

The DNA obtained was diluted twice or thrice to working concentrations for polymerase chain reaction (PCR) analysis. For PCR, a master mix containing $MgCl_2$, BSA, dNTPs, RAPD primer, double-distilled water, Taq buffer, and Taq DNA polymerase (all molecular grade) was prepared on ice. The master mix was adjusted by volume according to the amount of DNA loaded into each PCR tube. RAPD primer sets were obtained from a commercial synthesizer Gene Link Technology (USA). Nine RAPD decamer primers were used to obtain an optimum accuracy in species identification. All the specimens were run in one reaction at specific temperatures set in PCR thermocycler (ABI Prism, USA) with 34°C annealing temperatures. Sequence information of the primers is presented in Table 3.

Table 3. Detailed information of randomly amplified polymorphic DNA primers used in characterizing *Ephedra* genotypes.

No.	Oligo name	Sequences 5'-3'	Size (nt)	MW (amu)	Tm (°C)	%GC
1	GLA02	TGCCGAGCTG	10	2044	33.6	70
2	GLA03	AGTCAGCCAC	10	2996	29.5	60
3	GLA05	AGGGGTCTTG	10	3099	29.5	60
4	GLA08	GTGACGTAGG	10	3108	29.5	70
5	GLB10	CTGCTGGGAC	10	3044	33.6	70
6	GLB19	ACCCCCGAAG	10	2981	33.6	60
7	GLB20	GGACCCTTAC	10	2987	39.5	70
8	GLC09	GTCCCGACGA	10	3012	33.6	70
9	GLD07	TTGGCACGGG	10	3084	33.6	70

Size (nt) = number of nucleotides in a primer; MW = molecular weight in atomic mass units (amu); Tm = melting temperature; GC% = percentage of guanine and cytosine nucleotides.

For a 25-µL reaction mix, the amounts of chemicals used in the PCR mix were as follows: 3 µL $MgCl_2$, 2.5 µL Taq buffer, 0.25 mM dNTP mix, 0.1 µL BSA, 1 µL primer, template DNA modified according to concentration of specimens ranging from 0.5 to 3 µL, 0.5 µL Taq DNA polymerase, and double-distilled, deionized and sterile water was added to make the volume up to 25 or 16 µL. A total of 40 cycles were run. The product was electrophoresed again on a 1.5% agarose gel mixed with ethidium bromide run for 3-4 h to resolve the bands clearly.

Data analysis

Only the scorable bands were included in the analyses. Every band was considered a single locus and bi-variate 1-0 matrices were formed for further analysis. Presence or absence of each single fragment was coded by 1 or 0, respectively, and was scored for a binary data matrix. Genetic distances (GD_{xy}) were calculated for each pair of lines using the method described by Nei and Li (1979) as follows: $GD_{xy} = 1 - d_{xy} / d_x + d_y - d_{xy}$, where d_{xy} is number of common bands (loci) in two genotypes, d_x is number of bands (loci) in genotype 1, d_y is number of bands (loci) in genotype 2. The binary data were used to compute pairwise similarity coefficients,

and the similarity matrix thus obtained was subjected to cluster analysis using the UPGMA (unweighted pair-group method with arithmetic average) algorithm on NTSYS-PC version 1.70 (Rohlf, 1993). The bivariate data (1 - 0) was adjusted in MS Excel before retrieving into NTSYS computer program for cluster analysis. The data were standardized, and the similarity matrix was calculated which was subjected to cluster analysis to generate a dendrogram using SAHN command in the software.

RESULTS AND DISCUSSION

A single plant was considered as a specimen and measures were taken to avoid mixing it with another plant that was collected from the same location. On this basis, six specimens were collected in which two specimens were from the Utror Swat area, which were confirmed to be *Ephedra gerardiana*. Further, it was confirmed that one of the specimens was a male plant and the other a female plant. This information was lacking for the other specimens due to the lack of available reproductive parts in *Ephedra*, and the specimens were numbered accordingly (Table 2).

Average genetic dissimilarity (estimated as genetic distance) among six *Ephedra* genotypes using RAPD primers GLA02, GLA03, GLA05, GLA08, GLB10, GLB19, GLB20, GLC09, and GLD07 are presented in Table 4. Range of genetic distances estimated was from 6 to 49%. Maximum genetic distances (49%) were estimated in one comparison (among genotypes 2 to 5) while 6% genetic distance was estimated for only one comparison (among genotypes 4 to 6).

Table 4. Average genetic distances for nine primers in all six genotypes of *Ephedra*.

Genotype	2	3	4	5	6
2	–				
3	0.38	–			
4	0.28	0.45	–		
5	0.49	0.43	0.34	–	
6	0.08	0.07	0.06	0.11	–
7	0.43	0.33	0.38	0.4	0.07

2: Collected from Hazarganji National Park Baluchistan (*E. nebrodensis*); 3: Collected from Hazarganji National Park Baluchistan (*E. nebrodensis*); 4: Collected from Zob Baluchistan (*E. pacyclada*); 5: Collected from Utror Swat (*E. gerardiana*); 6: Collected from Zob Baluchistan (*E. pacyclada*); 7: Collected from Utror Swat (*E. gerardiana*).

The dissimilarity coefficient matrix of six *Ephedra* genotypes based on the data of 9 RAPD primers using the UPGMA method was used to construct a dendrogram which is presented in Figure 2. Six genotypes were grouped into 2 groups (A and B). Group A comprised two genotypes, both of which belong to the same geographical region (collected from the Hazarganji National Park, Baluchistan). Genotype 5 did not match with any other genotype, and hence it is inferred that it is genetically most distinct from other genotypes used during the present study.

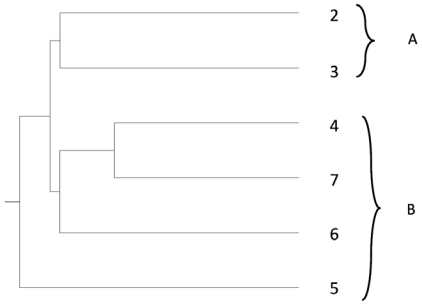

Figure 2. Dendrogram constructed for six *Ephedra* genotypes using bivariate data generated by nine randomly amplified polymorphic DNA primers. *2-7*, same collections as described in Table 3. *A* and *B* represent two distinct groups of the genotypes.

Genetic dissimilarity (estimated as genetic distance) among six *Ephedra* genotypes using RAPD primer GLA05 are presented in Figure 3. Range of genetic distances estimated was from 0 to 66%. Maximum genetic distances (66%) were estimated in one comparison (between genotypes 3 and 5), while 0% genetic distance was estimated for only one comparison (among 2-4).

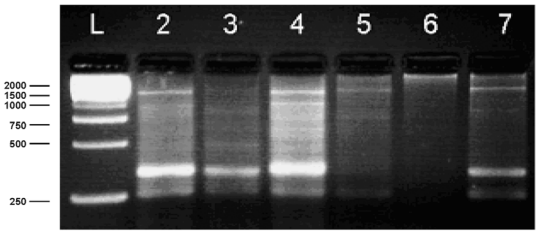

Figure 3. Polymerase chain reaction profile of six *Ephedra* genotypes using randomly amplified polymorphic DNA primer GLA05. *Lanes 2* to *7*, same collections as described in Table 3, whereas *L* is the molecular weight marker (1-kb ladder fragment size in bp is presented on the left of the image).

The *Ephedra* plant is a small woody, highly branched shrub, seldom exceeding 2 m in height; some species (*E. foliata*) have a straggling or climbing stem, which is green, fistular, fluted, long jointed phylloclade, yellowish green to olive-green when young and which carries on photosynthesis. A number of important species are native to Pakistan and can be observed in various forms on exposed, dry rocks (Figure 4) at altitudes ranging from 2500 to 5000 m (Nasir and Ali, 1987).

Figure 4. A view of *Ephedra* in its natural habitat.

The branches are slender and green and bear minute scale-like leaves in pairs at each node. Branches arise in the axial of the scaly leaves; they may be in pairs or threes according to the species. Branching of the side shoots has a broom-like appearance, confined to the lower three nodes, and these are close together giving the appearance of a bush. The root is a long tap root characteristic of xerophytes. In *E. gerardiana*, there are longitudinal geoclines on the diameter of the herbal stem and subepidermal fibers. In *E. pachyclada*, there are altitudinal geoclines where the plant growing at higher ground tends to have more fibers in a subepidermal fiber bundle with more pith fibers recognized (Mikage and Kondo, 1996). Annual rings are formed as growth proceeds, but the plant life is not more than 50 years. The outstanding feature of the stem histology is the presence of large vessels in the secondary wood at the beginning of growth in spring. The plant is of high herbal value (Walton and Manos, 2003) and contains products that may be used in curing several human diseases such as cardiovascular effects (White et al., 1997).

Mitochondrial RNA editing is present in cox3 mRNA *E. gerardiana* (Malek et al., 1996). Complete or partial nucleotide sequences of five different rRNA species, coded by nuclear (18S, 5.8S, and 5S) or chloroplast genomes (5S, 4.5S) from *Ephedra*, were determined (Troitsky et al., 1991). ChlB gene of *E. altissima* is sequenced (Boivin et al., 1996).

Ephedra is an important medicinal plant, but not much data on molecular analysis are available for this genus. Generally, much study has been done on the use of molecular markers in

important cereal crops such as wheat, maize, rice, etc. It is of prime importance to elucidate the genetic makeup of *Ephedra*, so that this genus can be better utilized for commercial and medicinal purposes. The present study is the first documented attempt to determine genetic polymorphism in *Ephedra* using RAPD primers. Six *Ephedra* genotypes collected from various locations throughout Pakistan were included in the study. The purpose of the present study was to determine the extent of genetic polymorphism (by estimating genetic distances) in *Ephedra* genotypes.

For overall genetic diversity studies, nine RAPD primers were used. The RAPD primers produced different levels of genetic polymorphism. Overall, genetic distances ranged from 6 to 49%. The present study reinforces earlier reports that RAPDs can be used for studying genetic polymorphism and tagging of useful genes in crop improvement programs.

It is evident from the present study that PCR-based assays such as RAPDs can be used effectively to estimate genetic variability in *Ephedra*, and considering easy handling of the technique, they are especially suitable for diagnostic and breeding programs where large number of lines/accessions have to be analyzed. It also should be noted that analyses that are more molecular are required to reach a better conclusion regarding genetic variability and more detailed mapping of the *Ephedra* genome.

The present study was aimed at identifying genetic diversity in a set of *Ephedra* genotypes collected from various geographical locations in Pakistan. *Ephedra* is a medicinal crop of commercial importance, but in the past, no molecular biology study had been done to classify and study different species/sub-groups of *Ephedra*. The present study is the first attempt to characterize six *Ephedra* genotypes based on differences in their DNA sequences using RAPD primers. Using nine RAPD primers, it was concluded that a PCR-based assay (principally RAPDs) could be used successfully to detect genetic diversity and estimation of genetic distances in different biotypes of *Ephedra*. It is, however, recommended that further study be done using more molecular markers, to achieve a better understanding about the structure and diversity of the *Ephedra* genome. In addition, it is recommended that extra-nuclear DNA methods (mainly mitochondrial and chloroplast DNA methods) should be explored for further understanding of the *Ephedra* genome and classification of this important medicinal plant. It is also anticipated that comparative genomic and association mapping techniques including ribosomal RNA (Liu and Schardl, 1994) may be employed to understand the genetic mechanisms underlying important traits and thereby biochemical compounds such as ephedrine in this important plant species.

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