Pak. J. Weed Sci. Res., 21(2): 163-172, 2015

GENETIC DIVERSITY OF *Ephedra procera* FROM HIGH ALTITUDES OF QUETTA VALLEY, BALOCHISTAN USING RAPD AND ISSR

Shazia Saeed[1]*, Younas Khan Barozai[1], Alia Ahmed[1], Rasool Baksh Tareen[1], Ghulam Muhammad Ali[2], Armaghan Shehzad[2] and Sania Begum[2]

ABSTRACT

Ephedra procera Fisch & Mey is an important wild medicinal, evergreen dioecious shrub found fragmentally at high altitudes of Balochistan, Pakistan. Published data regarding the E. procera is limited in the area under discussion. Therefore study was initiated to determine genetic diversity of this species from three different altitudes of Quetta valley, Balochistan through genetic polymorphism (by estimating genetic distances). The study was carried out during 2012-2014 using different molecular markers, ten-mer Random amplified polymorphic DNA (RAPD) and eighteen-merInter-Simple Sequence Repeat (ISSR). Out of total sampled plants, nine were selected for this study. Out of 35RAPD and 5 ISSRs, 6 RAPD and 3 ISSRs were able to characterize the studied genotypes. A total of 92 DNA fragments were amplified and 57 (61.95%) were polymorphic with an average of 10.2 amplified per primer. Unweighted pair group method of arithmetic means (UPGMA) method was used for cluster analysis indicating high genetic diversity in investigated population. Distance tree illustrated maximum genetic diversity between A2 and C1. It may be due to the altitudinal gradient, fragmented population and anthropogenic factors. Data analyses provided information to implement conservation strategy for this taxon.

Key words: Genetic diversity, *Ephedra procera,* high altitudes, RAPD, ISSR.

Citation: Saeed, S., Y.K. Barozai, A. Ahmed, R.B. Tareen, G.M. Ali, A. Shehzad and S. Begum. 2015. Genetic diversity of *Ephedra procera* from high altitudes of Quetta valley, Balochistan using RAPD and ISSR. Pak. J. Weed Sci. Res. 21(2): 163-172.

[1]Dept. of Botany, University of Balochistan, Quetta, Pakistan
[2]National Institute of Genomics and Advanced Biotechnology (NIGAB), NARC, Islamabad, Pakistan
*Corresponding author's email: shaz.saeed@yahoo.com

INTRODUCTION

Ephedra procera Fisch.&Mey. is one of the important specie of the genus *Ephedra* of the family Ephedracea (gymnospermae). It has been reported that 42 species represent this genus on the world leve. While the share of Pakistan is provisionally reported to be nine (Ghafoor *et al.,* 2007); whereas, five species of the genus *Ephedra* are found in Balochistan including *Ephedra procera, E. gerardiana, E. intermedia, E. sarcocarpa* and *E. ciliata.* It is a dioecious perennial, evergreen shrub found in different elevation zones of Balochistan including high altitudes of Quetta valley. This plant is important both ethnobotanically and commercially and is locally known as "Nari oman". This plant is used for treating asthma by the rural community. As the use of medicinal plants for different diseases is a common practice in rural communities of Pakistan (Khan *et al.,* 2009; Khan *et al.,* 2014) therefore, proper documentation of wild plants is helpful for further studies. Ephedrine is manufactured from this species by Merck Marker's, Quetta. Many reported have shown that the native regions of this genus is central Asia (Budavari, 1996; Leung and Foster, 1996). The plant is a sporophyte and bears strobili, which are compound and unisexual. Branches are slender, smooth striate; strobili solitary; bracts 4 pairs, connate. Fruit ovoid, sub globose. The extract from *E. procera* had the highest antibacterial properties. Phenolic compounds present in the plant are responsible for its effective free radical scavenging, antioxidant and antibacterial activities. Extract of *E. procera* could be an important source for antioxidant capacity and antibacterial activity (Dehkordi *et al.,* 2015). *Ephedra* can be subdivided into three groups according to geographic ranges of species. Ghafoor *et al.* (2007) stated that a PCR-based assay is always useful and user friendly to investigate and confirm genetic diversity in different biotypes of a plant species.

Genetic diversity is the level of diversity that refers to the total numbers of genetic characterizations in the genetic makeup of a species and plays an important role in the survival and adaptability of species (Volis *et al.,* 2001). Information about the genetic diversity and distribution of a plant is helpful for breeding and conservation strategies (Petit *et al.,* 1998; Bruschi *et al.,* 2003; Meloni *et al.,* 2006). Genetic diversity among individuals or populations can be assessed using different markers.

RAPD is highly suitable for quick fingerprinting and for analysis of genetic relationships among populations. The observed variations in the number of bands amplified by different random primers may be influenced by variable factors such as primer sequence, template quantity and less by the number of annealing sites in the genome (Kernodle *et al.,* 1993). ISSR do not require preliminary sequence

information and are less susceptible to laboratory conditions (Adams *et al.,* 2003).

The area under discussion is away from cities and there are limited human activities. Due to mountainous nature of the area such as Sra Ghurgai (Takatu mountain range), Zarghoon and Hazarganji), there is limited published data. Therefore the present study is first document to collect this specie from such hard areas. This species has complex morphology so molecular characterization for the first time using RAPD and ISSR was also accomplished.

MATERIALS AND METHODS

Study sites

A total of three geographic sites were selected for the present study (Fig. 1). All these sites are the natural habitat of the *Ephedra procera*. The detail of the three sites is provided in Table-1.

Figure 1. Locations of the studied sites.

Site 1

Sra Ghurgai is located at Koh-i-Takatu, and the altitude ranges from 1660 to 3000 meters above sea level. Latitude 30º 17′-39º towards North and Longitude = 67º 01′-02º towards East. Total area of SraGhurgai is 140 Sq.Km (Saeed *et al.* 2014).

Site 2

The Zarghoon range's highest peak is Loy Saar Naikan, at 3,578 meters, while the second highest peak is Kuchnai Saar, at 3,404 meters. At 3,578 metres, Zarghoon is the highest peak of Baluchistan. The base of the Zarghoon up to Ziarat is densely covered by Juniper trees, a forest of about (810 km^2). The area is made up of high hills, steep slopes and narrow valleys.

Site 3

Hazarganji National Park is covered with desert and forest habitats. The trees in the forests include Juniper, pistachio, almond and ash trees. The altitude of this site ranges from 1600-3000 m. This

park is located approximately 20 km south- west of Quetta, Balochistan (30 º 07'N, 66º 58'E) (Anon. 1998).

Table-1. Altitudinal description of the study sites.

Site No	Sampling site	Site Code	Latitude (N)	Longitude (E)	Elevation (m)
1	SraGurgai (Takatu Mountain Range (SG)	A.1	30.17	67.01	1660-3000
		A.2	30.29	67.06	
		A.3	30.33	67.08	
2	Zarghoon (Zr)	B.1	30.21	67.12	2200-3200
		B.2	31.05	67.18	
		B.3	31.22	68.01	
3	Hazarganji (HG)	C.1	30.21	66.54	1700-2600
		C.2	30.07	65.55	
		C.3	30.15	66.07	

Plant Material

Plants were sampled during 2012-2013 from nine different locations grouped in three geographic sites (Fig. 1 & 2), at different altitude and identified by a Taxonomist, Department of Botany, University of Balochistan, Quetta, Pakistan. The voucher specimens were submitted to herbarium of Botany department, University of Balochistan for future reference. Fresh and young stems were collected and stored at ultra-low freezer at NIGAB, NARC Islamabad for RAPD and ISSR.

Figure 2. *Ephedra procera* growing in its natural habitat (as A: Site-1 (Sra Ghuragi), B: Site-2 (Zarghoon) and C:Site-3 (Hazarganji).

DNA Extraction and Purification

Extraction of total DNA was performed at NIGAB, National Agriculture Research Center, Islamabad using CTAB (100 mM Tris-HCl (PH 8.0), 1.4 M NaCl, 20 mM EDTA) protocol according to Badr *et al.* (2012). The method was modified with phenolic extraction. The mixture was deprotenized once with Phenol:Chloroform:Iso-

amyalcohol (25:24:1,v/v) followed by twice with Chloroform: Isoamyalcohol (24:1, v/v). The DNA samples were electrophoresed in 1% agarose gel along with DNA marker (100bp Ladder). The estimation of DNA concentration in a given samples was further checked on Biospec-Nano at 260/280 nm.

PCR Reaction

A total 40 random 10-mer RAPD and 18-mer ISSR primers were used, 9 of them were able to detect the polymorphism (Table-2). The amplification reaction was carried out in 20*µl* reaction volume containing 5X Dream *Taq* buffer, 2mM dNTPs mix, 20 pmol primers from Operon and Gene Link USA, 10 ng DNA, 0.01% gelatin and 0.2 *µl* Dream *Taq* DNA polymerase (5 U/*µl*). PCR amplification was performed through 35 cycles after an initial denaturation for 3 minutes at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 45 seconds. Annealing temperature optimized differently ranged from 34 -55ºC for RAPD and ISSR, respectively. An extension step for 45 seconds at 72ºC for 2 minutes and an additional extension step for 5 minutes at 72ºC in the final cycle. The amplification products were then resolved by electrophoresis in 1.8% agarose gel containing ethidium bromide (0.5µg / ml) in 1X TAE buffer. The PCR products were visualized under UV light and photographed using Gel documentation and the size of markers were estimated by comparing to the standard marker included in the gel.

Data analysis

The amplified bands were scored as 1 (present) and 0 (absent), RAPD and ISSR data were clustered and dendrograms based on similarity matrices, and then were generated using the paired group method by using NTSYS 2.10j (Rohlf, 1998).

RESULTS AND DISCUSSION

This study is the first report of the *Ephedra procera* characterized by molecular markers and phylogenetic analysis. Molecular character and genetic diversity in different geographical zones at different elevation ranged from 1600m to 3500m a.s.l. Study was based by using two different molecular markers like RAPD and ISSR. RAPD was also reported earlier by Ghafoor *et al.* (2007) for cluster analysis in another species of *Ephedra* genus, whereas, ISSR is used for the first time for genus *Ephedra*. In this study, combined RAPD and ISSR amplified approach was used for cluster analysis. The use of two different markers could target different regions of genome and could help in removing error for the detection of polymorphism (Souframanien and Gopalakrishna, 2004; Phong *et al.* 2011). *E. procera* was not characterized earlier, so the present approach of two markers would give the preliminary characters of this plant.

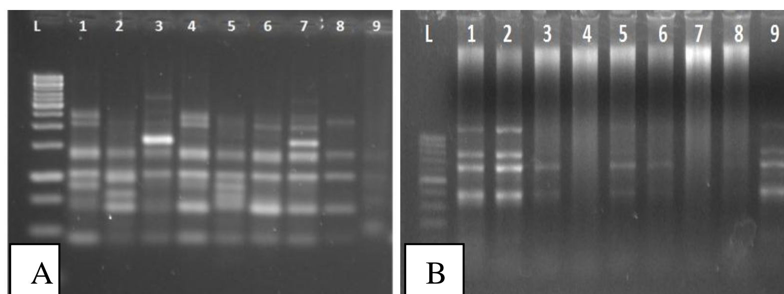

Figure 3. Genomic DNA amplification pattern in *Ephedra procera*, with two primers (A. OPA4 B. ISSR 856).

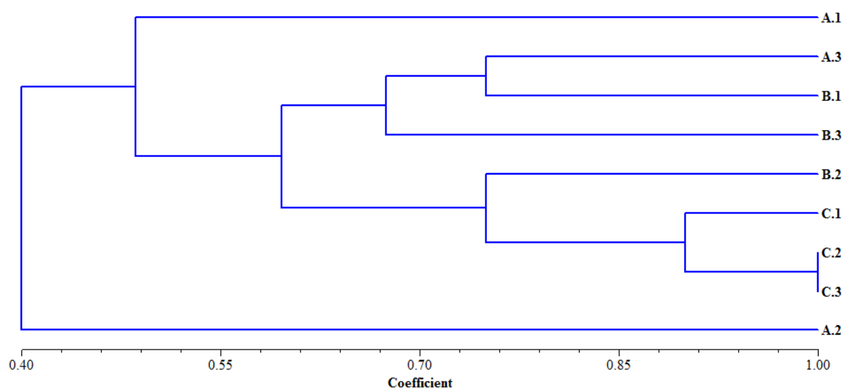

Figure 4. UPGMA dendrogram based on data generated from RAPD and ISSR primers, showing the genetic distance among the studied populations (listed in Table-1).

RAPD and ISSR markers analyses of *E. procera* revealed (Fig. 3) distinct genetic variation within and among specie (Table-3). A total of 92 bands of DNA amplified scored out of which 57 (62%) were polymorphic. Out of 35 RAPD and 5 ISSR, 6 RAPD and 3 ISSR showed clear amplification. Only polymorphic bands were scored for data analysis. There were few primers which show amplification but unable to show polymorphism. On the other hand some were not amplified. Earlier, Ghafoor *et al.* (2007) reported the genetic polymorphism in five *Ephedra* using RAPD primers from different regions of Pakistan. Fragment size of RAPD ranged from 300bp to 700bp and ISSR ranged from 90bp to 1500bp. OPA-4 gives 84% highest polymorphic bands, followed by OPA-13 and ISSR-856 which shows 71% polymorphism.

Table-2. Primers information used for DNA amplification

Primer	5`- 3` Sequence	Size nt
RAPD		
OPA-2	TGCCGAGCTG	10
OPA-3	AGTCAGCCAC	10
OPA-4	AATCGGGCTG	10
OPA-13	CAGCACCCAC	10
OPC-1	GTTTCGCTCC	10
OPC-2	TGATCCCTGG	10
ISSR		
UBC-808	$(AG)_8C$	17
UBC-856	$(AC)_8TG$	18
UBC-841	$(GA)_8TC$	18

Size (nt) = number of nucleotides in a primer

Table-3. Primers analysis and number of DNA polymorphic bands produced.

Primer	Annealing Temperatures (°C)	Total number of bands	Number of polymorphic bands	Polymorphic bands (%)
RAPD				
OPA-2	36 °C	9	4	44%
OPA-3	34 °C	7	2	22%
OPA-4	36 °C	25	21	84%
OPA-13	36°C	7	5	71%
OPC-1	34°C	3	1	33%
OPC-2	36°C	12	7	58%
ISSR				
UBC-808	52°C	7	3	42%
UBC-856	50°C	14	10	71%
UBC-841	55°C	8	4	50%
Total		92	57	62%

Genetic distances (Table-4) were estimated by dendrogram (Fig. 4) using NTSYS 2.10. Genetic distance values ranged from 0.25-0.90 between population A.2 and C1 characterized the closely related population of C.1, C.2 and C.3 (HG). The similarity coefficient indicated that the studied population was divided into two main groups A.1 and sub-cluster A.3 (SG). B.1, B.3 (Zr) whereas second cluster contained B.2 (Zr), C.1, C.3 (HG). While A.2 showed a different pattern cluster with A.1 (SG) and closely related with C.2 (HG) cluster.

Genetic variation was studied with and among population of *E. procera* present in different habitats such as altitudinal gradient, soil texture and slope that might be the reason of genetic variation. Earlier it was reported by Badr *et al.* (2012) that genetic variation may be due to difference in local geographic conditions and variation of locations along environmental heterogeneity. In analogous studies, the comparison sequences of this species was reported by others (Huang *et al.* 2005; Huang and Price 2003). They further added that *Ephedra*

species found with the greatest density of sampling from North America. More investigation revealed that classification is poorly related to other characters like cytology, micromorphology, chemistry and DNA sequences.

Table-4. Similarity index of nine *E. procera* based on RAPD and ISSR data analysis

Varia bles	A.1	A.2	A.3	B.1	B.2	B.3	C.1	C.2	C.3
A.1	1.00								
A.2	0.45	1.00							
A.3	0.45	0.50	1.00						
B.1	0.40	0.45	0.75	1.00					
B.2	0.55	0.40	0.40	0.55	1.00				
B.3	0.40	0.45	0.65	0.70	0.65	1.00			
C.1	0.60	0.25	0.55	0.60	0.75	0.70	1.00		
C.2	0.50	0.35	0.65	0.60	0.75	0.60	0.90	1.00	
C.3	0.50	0.35	0.65	0.60	0.75	0.60	0.90	1.00	1.00

Maximum genetic diversity observed within (SG) Takatu-mountain population, supported our hypothesis of altitudinal gradient that affects vegetation of an area. Lowest genetic diversity observed within C (HG) might be because of low elevation as compared to all other studied sites. Similar results were reported by Douaihy *et al.* (2011) that high levels of genetic diversity were observed at species and population levels. Highest genetic variation observed among population of A.2 (SG) and C.1 (HG) support this hypothesis that in disturbed population genetic diversity can be observed between and among population as earlier reported by (Adams *et al.* 2003; Meloni *et al.* 2006) that Population analysed at different elevations of Mount Lebanon were strongly differentthan populations from the rest of the range.

CONCLUSION

In light of the present study, it is concluded that *Ephedra procera* have diversity, dependent on the habitat. It seems that due to over collection, this species will be endangered species in the near future. Due to habitat disturbance, anthropogenic effects and over collection of specie for its significant medicinal and commercial value, fragmented population pattern have been observed. Further investigation on this genus using co-dominant marker is highly

recommended that will help in better understanding of this complex genus and implementation of conservation strategies.

ACKNOWLEDGEMENT

This study is a part of PhD dissertation and will be submitted to The University of Balochistan, Quetta, Pakistan. Authors are thankful to Taj Muhammad Rind, Chief Conservator Forest Department, Balochistan, for field surveys and Dr. G.M. Ali, Director National Institute of Genomics and Advanced Biotechnology NIGAB NARC Islamabad for their facilitation.

REFERENCES CITED

Adams, P.A., A.E. Schwarzbach, and P.R. Naresh 2003. The concordance of terpenoid, ISSR and RAPD markers, and ITS sequence data sets among genotypes: an example from Juniperus. Biochemical Systematics and Ecology. 31: 375–387.

Badr, A., Z. Abo-El-Khier, A. Ghada, A. Hegazi, Abd-El-Kawi and A. El-Sawy. 2012. Genetic variation in seven natural populations of *Artemisia judaica* L. in South Sinai using RAPD Markers. World Applied Sciences Journal 18 (10): 1475-1480.

Bruschi, P., G.G. Vendramin, F.Bussotti, and P.Grossoni. 2003. Morphological and molecular diversity among Italian populations of Quercus petraea (Fagaceae). Annals of Botany. 91: 707–716.

Budavari, S. 1996. The merck index: an encyclopedia of chemicals, drugs, and biologicals. 12th edn. Merck and Co. Inc., Whitehouse Station.

Dehkordi, N.V., M. A. Kachouie, A. G. Pirbalouti, F. Malekpoor and M. Rabei. 2015. Total phenolic content, antioxidant and antibacterial activities of the extract of *Ephedra procera* fisch. et mey. *Acta Poloniae Pharmaceutica and Drug Research,* 72(2): 341-345.

Douaihy, B., G. Giovanni, A. Vendramin, N. Boratynski, Machon and M. B., Dagher-Kharrat .2011. High genetic diversity with moderate differentiation in *Juniperus excelsa* from Lebanon and the eastern Mediterranean region. AoB Plants.1-14.

Ghafoor. S., M.M. Shah, H. Ahmad, Z.A. Swati, S.H. Shah, A. Pervez and U. Farooq. 2007. Molecular characterization of *Ephedra* species found in Pakistan. Genetics and Molecular Research 6 (4): 1123-1130.

Huang J., E. David, Giannasi and R.A Price. 2005. Phylogenetic relationships in *Ephedra* (Ephedraceae) inferred from chloroplast and nuclear DNA sequences Molecular Phylogenetics and Evolution 35: 48–59.

Huang, J. and R.A Price. 2003. Estimation of the age of extant *Ephedra*using chloroplast *rbcL*sequence data. Mol. Biol. Evol. 20, 435–440.

Kernodle, S.P., R.E. Cannon, and J.G. Scandalios. 1993. Concentration of primer and template qualitatively affects product in random amplified polymorphic DNA-PCR. Biotechniques. 14: 362-364.

Khan, M.A., R.E. Blackshaw and K.B. Marwat. 2009. Biology of Milk thistle and management options for growers in northwest Pakistan. Weed Biol. Manag. 9(2): 99-105.

Khan, M.A., K. B. Marwat, B. Gul, F. Wahid, H. Khan and S. Hashim. 2014. *Pistia stratiotes* L. (Araceae): Phytochemistry, use in medicines, phytoremediation, biogas and management options. Pak. J. Bot. 46(3): 851-860.

Leung, A.Y. and S. Foster. 1996. Encyclopedia of common natural ingredients used in food, drugs, and cosmetics. 2nd edn. John Wiley and Sons Inc., New York.

Meloni, M., D. Perini, R. Filigheddu, and G. Binelli. 2006. Genetic variation in five Mediterranean populations of *Juniper usphoenicea* as revealed by Inter-Simple Sequence Repeat (ISSR) markers. Annals of Botany. 97: 299–304.

Petit, R.J., A. Mousadik, and O. Pons. 1998.I dentifying populations for conservation on the basis of genetic markers. Conservation Biology. 12: 844–855.

Phong, D.T., V.T.T. Hien, T.T.V. Thanh, and D.V. Tang. 2011. Comparison of RAPD and ISSR markers for assessment of genetic diversity among endangered rare *Dalbergia oliveri* (*Fabaceae*) genotypes in Vietnam. Genet. Mol. Res., 10(4): 2382-2393.

Rohlf , J. 1998. NTSYSpc numerical taxonomy and multivariate analysis system version 2.0 User Guide F. Department of Ecology and Evolution State University of New York Stony Brook, NY 11794-5245.

Saeed, S., M.Y.K. Barozai, A. Ahmed and S.H. Shah. 2014. Impact of altitude on soil physical and chemical properties in Sra Ghurgai (Takatu mountain range) Quetta, Balochistan. International Journal of Scientific & Engineering Research 5(3): 730-735.

Souframanien, J. and T.A. Gopalakrishna. 2004. Comparative analysis of genetic diversity in blackgram genotypes using RAPD and ISSR markers.Theor. Appl. Genet., 109: 1687-1693.

Volis, S., S. Mendlinger, Y. Turuspekov, U. Esnazaov, S. Abugalieva, and N. Orlovsky. 2001. Allozyme variation in turkmenian populations of wild barley, *Hordum spontaneum* Koch. Ann Bot. 87:435–446.