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Utilization of RAPD marker to analyze natural genetic variation in *Calligonum polygonoides* L. - A key stone species of Thar desert

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Abstract
The species *Calligonum polygonoides* L. of Polygonaceae is one of the most economically important resources of the Indian desert, playing an important role in the lives of local populace. A great range of genetic diversity could be seen in diverse populations of this species which are spread all over western Rajasthan. DNA-based molecular markers are playing increasingly important role in the analysis of genetic diversity in wide range of plants. The present study is a first attempt to collect, compile and collate to define the existing natural genetic diversity at intra-specific and inter-populational levels in *C. polygonoides.* The present report deals with 22 genotypes of *C. polygonoides* collected from different districts of Rajasthan using random amplified polymorphic DNA (RAPD) marker. In total 100 fragments with 89 polymorphic bands amplified by 10 random primers, with the polymorphic rate of 89.30% were recorded. The dendrogram was constructed based on the matrix of Dice coefficients by unweighted pair-group mean analysis (UPGMA) using the NTSYS-PC version 1.8 exeter software. From the analysis of data it is imperative that predominantly obligate out breeding behavior of *C. polygonoides,* made these diverse accessions to spread and occupy specific geographical niches in the arid regions of Rajasthan.

Keywords: *Calligonum polygonoides*, Intra-specific variation, Genetic diversity, Genetic relatedness.

INTRODUCTION

Calligonum polygonoides, bestowed the status of key-stone species of Indian desert, is an important source of food for sustenance during frequently occurring famines and is also valued for commercial and medicinal purposes (Bhandari, 1995). *Calligonum polygonoides* with smooth stems and branches, bearing abortive flowers and small succulent fruits, is eaten when food is scarce during famines in arid regions of India. Flowers made into bread or cooked with clarified butter or coconut oil to make a local delicacy (Bewal, 2007). Roots and thick branching stems are used as fuel (Singh *et al*., 1996). The aqueous paste of plant acts as an antidote against the heavy doses of opium and also against poisonous effects of certain harmful plants (Singh *et al*., 1996). *Calligonum polygonoides* form communities with other dominating species at the top of sand dunes and stabilize the shifting sand dunes as it consists of dense network of roots.

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One of the basic requirements for the sustainable and optimal utilization of the available meager resources of Indian desert region is to characterize and estimate the existing genetic diversity in natural populations. However, *C. polygonoides* did not receive the due attention it deserves as a key stone species of Indian desert. From cytological point of view there is a single report by Valovich *et al*. (1973), who proposed 2n=54 as somatic chromosome number. No chromosome number reports for *C. polygonoides* or any other species of the genus *Calligonum* from India are available as yet. The studies on relationship and clustering of *Calligonum* species through isozyme analysis has been reported lately by Tao and Ren (2004). The quick perusal of published literature on molecular characterization indicates that the RAPD analysis to assess inter-specific relationship in *C. polygonoides* collections from China has been attempted by Ren *et al*. (2002). Realizing the importance and utility of RAPDs as ideal genetic markers, they have been used in the present investigation, to assess the genetic diversity and determine genetic relatedness/ distance among various accessions of *C. polygonoides*, collected from different regions of Rajasthan state of India.

MATERIALS AND METHODS

Twenty two accessions of *Calligonum polygonoides* (Table 1 *[Supplementary data]*) collected from six districts of Rajasthan (Jhunjhunu, Jodhpur, Jaisalmer, Bikaner, Barmer and Nagaur) were analyzed for molecular genetic variation among them. Fresh, young and small caducous leaves from these plants were bulked so as to make approximately 1-2g of leaf sample. Total genomic DNA was extracted following CTAB method described by Murray and Thompson (1980).

Analysis of DNA polymorphism was done in two phases. The first (preliminary) phase, done on a subset of three accessions, was an evaluation of 40 decamer primers (Operon Technologies, Alameda, California, USA). Ten primers, including Operon designated primers OPA-11, OPA-14, OPA-17, OPA-18, OPA-19, OPD-04, OPD-08, OPD-10, OPD-13 and OPD-17 showed consistent banding patterns and amplification were selected for use in the second phase of study. The second phase of experiments involved amplification of genomic DNA with selected primers in each reaction. The reaction mixture consisted of 10mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each dNTPs (Cinna. Gen. Inc.,Iran), 15 ng of each primer, 1.0 U *Taq* DNA polymerase (Banglore Genei, India) and 40 ng genomic DNA per 25 µl reaction volume. A negative control PCR tube containing all components except genomic DNA was included in all the runs. DNA amplification experiments were repeated three times to cofirm the authenticity of the results. Amplification reactions were performed in a PCR thermal cycler (Gene Amp PCR system 9700) - 40 cycles as 94^0C for 1 minute at start followed by denaturation at 94^0C for 1 minute, annealing at 37^0C for 1 minute, extension at 72^0C for 2 minutes and final extension at 72^0C for 10 minutes. The reactions ended with an indefinite hold at a temperature of 4^0C.

The amplification products were mixed with 2 µl of gel loading dye (0.25% bromophenol blue, 25% Xylane cyanol and 30% glycerol) and 10 µl of completed amplification products, were electrophoresed in 1% agarose gel with 0.5X TBE buffer (pH 8.0) for 3-4 hours supplying 50V of electricity. The 1 Kb ladder (Fermentas), was taken as the standard marker in each amplification reaction. The ethidium bromide stained agarose gels were visualized under ultra violet light and then photographed for documentation. The dendrogram was constructed based on the matrix of Dice coefficients by unweighted pair-group mean analysis (UPGMA) using the software NTSYS-PC version 1.8 (Exeter software).

RESULTS

Forty oligonucleotide primers with random sequences have been tested with three different concentration levels of genomic DNA (20ng, 30ng and 40ng) among 22 accessions of *C. polygoinoides.* Of these, 40ng has been found to be ideal for each PCR amplification reaction. Out of the forty oligonucleotide random primers tested, only 10 of them have yielded reproducible and discrete bands. Primers of OPA series gave many unique bands in various samples while primers of OPD series yielded mostly monomorphic bands in various samples (Table 2 *[Supplementary data]*). Genomic DNA was amplified in all the 22 genotypes of *C. polygonoides* with these ten selected primers producing a total of 100 bands representing the amplification product(s), which were scored for their presence (1) or absence (0). Of these 100 bands, 89 were polymorphic, 5 were monomorphic and 6 were unique bands. This yielded an average of 8.9 polymorphic, 0.5 monomorphic and 0.6 unique fragments per primer. Percentage of polymorphic bands among amplified bands ranged from 83.3% (OPA 19, OPD 10 and OPD 17) to a maximum of 100% (OPD 4, OPD 8) with an average of 89.3% polymorphism (Table 2). The number of amplified fragments per sample ranged from 6 (OPD 17) to 17 (OPA 11) (Fig. 1 & Fig. 2). The size of the fragments varied from 200-3050 bps in size.

A dendrogram based on UPGMA analysis grouped the 22 samples into three main clusters (Fig. 3 *[Supplementary data]*). Cluster I is comprised of 10 samples collected from Jaisalmer and Nagaur districts and a single accession i.e. *C. polygonoides* JNVU/RI/2005/33 from Barmer district. Cluster I could further be subdivided into 3 groups. The first subcluster (Ia) had four samples (2 from Jaisalmer and 2 from Nagaur), second subcluster (Ib) consists of 3 samples (all from Jaisalmer). The third subcluster (Ic) has all samples from Nagaur district with an exception of JNVU/RI/2005/33 which is from Barmer.

Cluster II grouped 2 samples, one from Jaisalmer and the one from Bikaner and appeared to be distinct from all others. The third main cluster i.e. Cluster III is comprised of samples collected from Bikaner and Barmer districts with one exception of JNVU/RI/2005/55 which is from Jaisalmer district. This cluster could further be resolved into three sub-clusters. First subcluster (IIIa) consists of two samples from Barmer and one from Jaisalmer. Second subcluster (IIIb) contains populations collected from Bikaner, while two accessions viz. JNVU/RI/2005/34

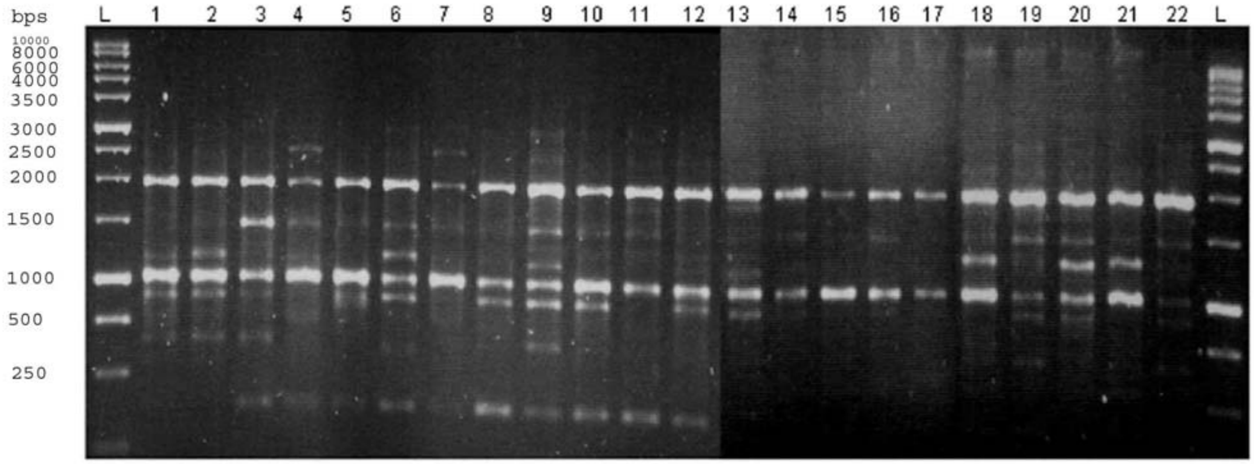

Figure 1: RAPD amplification profile of 22 accessions of *Calligonum polygonoides* using OPD 17 primer.

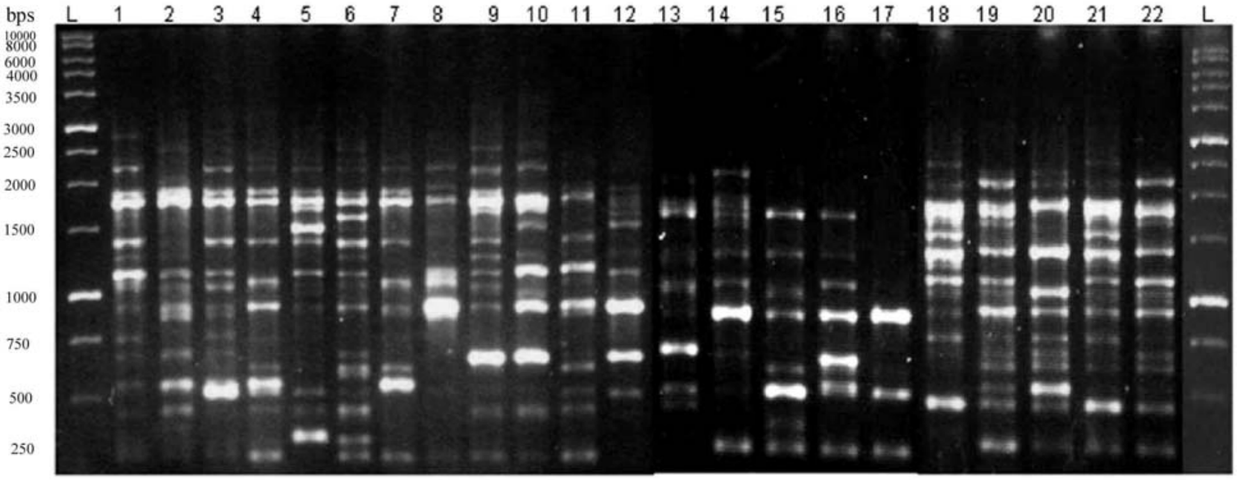

Figure 2: RAPD amplification profile of 22 accessions of *Calligonum polygonoides* using OPA 11 primer.

and JNVU/RI/2005/35 were grouped into third subcluster (IIIc).

DISCUSSION

The minimum similarity obtained by Dice's similarity coefficient was 0.51 among JNVU/RI/2005/35 and JNVU/RI/2005/36 while maximum similarity was 0.93 among JNVU/RI/2005/36 and JNVU/RI/2005/44.

The evolution of varieties in distinct climatic zones demonstrates significant levels of variations in response to the selection pressure in their native environment (Singh et al. 1996). It is therefore not surprising to find significant levels of polymorphism among 22 genotypes of *C. polygonoides* as revealed by RAPD markers. Clustering of genotypes was similar to some extant when RAPD dendrogram was compared with the morphological variation recorded in accessions collected from various locations (Bewal, 2007). Morphologically JNVU/RI/2005/35 collected from Barmer and JNVU/RI/2005/51 collected from Jaisalmer showed maximum variation with respect to plant height, bark color, internodal length, flower color and fruit color. The populations collected from Jaisalmer are found to be most heterogenous, however on the other hand populations from Bikaner seems to be homogenous with very limited morphological and cytological diversity being recorded in them (Bewal, 2007). Exceptionally JNVU/RI/2005/33 and JNVU/RI/2005/37 clustered away from other samples of their respective locations. This observation may be attributed to marker sampling error and/or the level of polymorphism detected, reinforcing again the importance of the number of loci probed and their coverage of the overall genome in obtaining reliable estimates of genetic relationships among cultivars (Loarce *et al*., 1996).

Calligonum polygonoides JNVU/RI/2005/35 was collected from Pachpadra in Barmer district produced many unique bands which was also reflected in morphological and cytological observations of this plant. This sample showed pink colored flowers and red colored wings on the fruit, which gives a reddish appearance rather than yellow color during flowering and fruiting. The location Pachpadra (Barmer) is known for its exceedingly rich alkalinity and soil salinity. From the present investigations it is amply clear that the genetic variation recorded in accessions collected from this particular region, may be the manifestation of stress response/tolerance, at genomic DNA level, expressed by the plants.

Dendrogram depicted in Fig.2, did indicate the pattern of clustering according to the location from which they were collected, although there were still exceptions to it. The species under study cross pollinates (*anemophily*), as reported in many members of family Polygonaceae (Raju *et al.*, 2001) which directly reflects in variations at morphological, cytological as well as molecular levels, not withstanding the fact that the populations of the species under study are located at tens to hundreds of kilometers of distance. Accessions with the most distinct DNA profiles are likely to contain the utmost number of novel alleles (Souframanien *et al*., 2004). It is these accessions that are likely to uncover the largest number of unique and potentially useful alleles of the genes like stress resistance/tolerance. Runo *et al*. (2004) used RAPD markers to assess genetic diversity within and between population of *Melia volkensii* in order to suggest appropriate conservation and management strategies. Study of Turkish grape germplasm by Karatas *et al*. (2008) suggests that the geographical and ecological distribution of the plants contributes to higher genetic variability. The study presented here does make an attempt to correlate the genetic variation with geographical distribution of plants with available range and quantum of natural variations in populations of *C. polygonoides*. The plants do seem to follow a trend and accessions collected from same district tend to group together. There are some accessions which deviate from this pattern which can be attributed to differential native environmental conditions. Predominantly obligate out breeding behavior of *C. polygonoides*beside certain non-comprehensible reasons prompted these accessions to spread and occupy specific geographical niches thereby enhancing their adaptational capabilities *vis-à-vis* native climatic conditions. The grouping may further improve if more primers are included in the study. The putatively similar bands originating for RAPDs in different individuals may not necessarily be homologous, although they may share the same size in base pairs (Liu *et al*., 1999; Runo *et al*., 2004). This situation may at times lead to inaccurate conclusions when calculating genetic relationships. Considering this fact, simple hybridization experiment using the polymorphic bands can confirm the extent of variation and give further insight into the relationship among different accessions (Latha *et al*., 2004; Santalla *et al*., 1998). Nevertheless the present study is a first attempt to compile, collate, analyze and define the existing natural genetic diversity at genomic level, among the populations of *Calligonum polygonoides* collected from different locations of Rajasthan (India).

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