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Molecular Analysis of Genetic Diversity in Wild Accessions of *Bunium persicum* (Boiss.) Fedtsch – A Critically Endangered Medicinal Plant of Temperate Himalayas

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ABSTRACT

Random amplified polymorphic DNA (RAPD) markers were used to assess genetic diversity in *Bunium persicum* (Boiss.) 'Fedtsch' (Apiaceae), a critically endangered medicinal plant collected from 15 locations in Himachal Pradesh and Jammu and Kashmir, India. A high level of genetic divergence was observed in the collected accessions. A total of 173 bands were generated by 36 random 10-mer primers, of which 168 were observed to be polymorphic indicating a high level (97.11%) of polymorphism among the accessions studied. Similarity, measurement using UPGMA followed by dendrogram analysis, resulted in two major clusters. The results of this preliminary investigation suggests that there is alot of genetic diversity among the wild accessions of this endangered medicinal plant species which needs to be documented, characterized, conserved and used for its improvement.

Keywords: DNA fingerprinting, genetic diversity, herbal spice, random amplified polymorphic DNA
Abbreviations: CITES, convention on international trade in endangered species of wild fauna and flora; **PCR**, polymerase chain reaction; **RAPD**, random amplified polymorphic DNA; **UPGMA**, unweighted pair group method with arithmetic mean

INTRODUCTION

Bunium persicum (Boiss.) 'Fedtsch', commonly known as "Kalazeera", is a spice-yielding member of the Apiaceae family. It grows wild in some parts of Himachal Pradesh (Lahaul-spiti, Chamba and Kinnaur), Jammu and Kashmir (Srinagar and its adjoining areas and Kishtwar) and in some parts of Uttranchal (Garhwal and Kumaon) at an altitude ranging from 2000-3500 masl in the Western Himalayan region, extending up to Baluchistan and Afghanistan (Bhartiya 1967).

Kalazeera is an indispensable condiment consumed all over India. It has found a significant place in the indigenous system of medicine, as the fruits are regarded as a stimulant and carminative and are useful in curing diarrhea, dyspepsia, fever, flatulence, stomache ache, haemorrhoids and obstinate hiccups (Chauhan 1999). The seeds (fruits) have, however, been replaced by their essential oil which is now widely used for seasoning pickles, meat sauce, soups, candies, etc.

B. persicum is a highly threatened plant species and is one of the 32 prioritized medicinal plants for development in India (http://www.cites.org).

Frankel (1974) opined that genetic variation is essential for the long-term survival of the species and it is critical feature in conservation. *Ex situ* conservation will help to maintain the population of the red listed species by facilitating their restoration back to nature.

For efficient conservation and management, the genetic composition of the species in different geographic regions needs to be assessed. RAPD markers have been used for the assessment of genetic diversity in a large number of plant species, including many red-listed plants (Li *et al*. 2002; Fu *et al*. 2003; Padmalatha and Prasad 2006b, 2007). Therefore, in the present investigation RAPD markers were employed for the molecular analysis of genetic diversity among collections of *B. persicum*.

MATERIALS AND METHODS

A total of 15 accessions were collected from Himachal Pradesh and Jammu and Kashmir. The accession codes and state names are given in **Table 1**. The bulbs were cultivated and plants were maintained in the Shilly nursery located at about 1500 m asl, in the fields of the Department of Forest Products of Dr Y. S. Parmar University of Horticulture and Forestry, Solan-Himachal Pradesh, India.

DNA isolation, amplification and data analysis

Young, fresh leaf tissue (500 mg) was collected from the lower nodes after 20-25 days of plant initiation. Total genomic DNA was isolated from this leaf tissue sample by following the protocol of Doyle and Doyle (1987) with some minor modifications. The leaf tissue was powdered in liquid nitrogen and immediately transferred to 50 ml polypropylene tubes containing 15 ml of preheated (65°C) extraction buffer. DNA quantity and quality was evaluated spectrophotometrically using a Perkin Elmer Lambda 35 UV/VIS spectrophotometer.

DNA amplification was carried out in an Eppendorf Mastercycler gradient using 10-mer RAPD primers obtained from Integrated DNA Technologies, USA. 50 ng of genomic DNA was used for each PCR amplification reaction. Each PCR reaction of 20 µl reaction volume contained 1X PCR buffer, 1.5 mm $MgCl_2$, 200 µM dNTP mix, 10 pmol of primer (Integrated DNA Technologies, USA) and 1U of *Taq* polymerase. All the chemicals used in the PCR reaction were procured from (Bangalore Genei, India) unless otherwise stated.

Amplification conditions were: initial denaturation of 3 min at 95°C followed by 35 cycles each of 30 s at 94°C, 1 min at 35°C and 1 min at 72°C and finally a 7 min extension at 72°C.

All the experiments were repeated twice to ensure reproduce-

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Table 1 List of accessions of *B. persicum* and their collection sites.

Location name	Sample codes	District (State)	Altitude (m)	Latitude*	Longitude*
Gurez	GZ-1, GZ-2, GZ-3	Baramulla (J&K)	1980	34° 38'N	74° 49'E
Khrew	KW-2, KW-3, KW-4	Pulwama (J&K)	1360	33° 54'N	75° 01'E
Harwan	HW-1, HW-6, HW-7	Srinagar (J&K)	1620	34° 10'N	74° 54'E
Sangla	SG-1, SG-3, SG-10	Kinnaur (H.P.)	2590	31° 25'N	78° 14'E
Kalpa	KA-2, KA-3, KA-4	Kinnaur (H.P.)	2774	31° 32'N	78° 15'E

*Encarta U.S. geological survey

Table 2 Jaccard's coefficient matrix.

	GZ-2	GZ-3	GZ-4	HW-1	HW-6	HW-7	KW-2	KW-3	KW-4	KA-2	KA-3	KA-4	SG-10	SG-1	SG-3
GZ-2	1.0000														
GZ-3	0.9415	1.0000													
GZ-4	0.9005	0.8888	1.0000												
HW-1	0.8362	0.8245	0.8304	1.0000											
HW-6	0.8187	0.8070	0.8011	0.9590	1.0000										
HW-7	0.8245	0.8128	0.8187	0.9298	0.9590	1.0000									
KW-2	0.8011	0.8011	0.7719	0.7543	0.7485	0.7660	1.0000								
KW-3	0.7894	0.8011	0.7602	0.7543	0.7368	0.7543	0.9532	1.0000							
KW-4	0.6374	0.6491	0.6783	0.5555	0.5146	0.5438	0.7426	0.7309	1.0000						
KA-2	0.6315	0.6081	0.6023	0.5497	0.5321	0.5380	0.6900	0.7251	0.7368	1.0000					
KA-3	0.5555	0.5438	0.5730	0.5087	0.4795	0.5087	0.6257	0.6608	0.7076	0.9005	1.0000				
KA-4	0.5789	0.5789	0.6081	0.5204	0.4912	0.5321	0.6140	0.6491	0.7192	0.8070	0.8128	1.0000			
SG-10	0.5614	0.5497	0.5906	0.4795	0.4619	0.5029	0.6081	0.6315	0.6900	0.7309	0.7719	0.7602	1.0000		
SG-1	0.5614	0.5497	0.5789	0.4444	0.4269	0.4678	0.5847	0.5964	0.6198	0.6491	0.6549	0.6783	0.8596	1.0000	
SG-3	0.5789	0.5555	0.5964	0.4619	0.4444	0.4853	0.6023	0.5789	0.6374	0.6432	0.6257	0.6725	0.8304	0.9473	1.0000

bility. Amplified DNA fragments were separated by electrophoresis at 80V in 1 X TAE for 3 hrs on 1.2% agarose gel. Gels were stained with ethidium bromide and photographed by the gel documentation system (Amersham Pharmacia).

Analysis of RAPD data

Amplified fragments were scored for the presence (1) and absence of (0) bands. The data matrices were analyzed by the SIMQUAL program of NTSYS-pc (ver. 2.02e; Rohlf 1998) and the similarities between genotypes were estimated using Dice's coefficient. A dendrogram was constructed from the resultant similarities matrices using the unweighted pair group mean averages (UPGMA) method (Sokal and Sneath 1963).

RESULTS

Out of 40 RAPD primers used in the present study, 36 were observed to be polymorphic whereas four did not amplify. A total of 173 bands were generated in the 15 accessions, of which 168 were found to be polymorphic indicating a high level (97.11%) of polymorphism among the accessions studied. The number of bands ranged from 2 to 9 with an average of 4.8 bands per primer.

The genetic similarity ranged from 0.9590 to 0.4269 (**Table 2**). Accessions, namely HW-6 and SG-1 collected from Harwan and Khrew, respectively, depicting a similarity value of 0.4269, were observed to be quite distant. On the other hand, accessions HW-1, HW-6 and HW-7 originating from Harwan appeared to be closely related. Maximum similarity value (0.9590) was exhibited by each of the accession sets namely HW-1 and HW-6, and HW-6 and HW-7. Based on Jaccard's similarity coefficient, the top 10 closely related accession sets included HW-1 and HW-6; HW-6 and HW-7 (each 0.9590); KW 2 and KW 3 (0.9532), SG 1 and SG 3 (0.9473); GZ 2 and GZ 3 (0.9415); HW 1 and HW 7 (0.9298); GZ 2 and GZ 4 ; KA 2 and KA 3 (each 0.9005); GZ 3 and GZ 4 (0.8888); and SG 1 and SG 10 (0.8596). It is interesting to note that the accessions within each of these 10 sets were collected from the same place. In the present study, primer D5 produced the highest (9) number of polymorphic bands, whereas only one band was amplified by D1. Overall, the average similarity coefficient was observed to be 0.75947.

A dendrogram was constructed using the similarity coefficient and UPGMA (**Fig. 1**). The 15 accessions were divided into two major clusters. Cluster 1 included all the accessions collected from Jammu and Kashmir (Gurez, Khrew and Harwan), whereas accessions originating from Himchal Pradesh (Sangla and Kalpa) were grouped into the second cluster. Accession KW-4 was observed to be most divergent in cluster 1. Inter- and intra-cluster placement of accessions was consistent with their geographical distribution. Accessions originating/collected from the same or nearby location were found to be close to each other, whereas accessions collected from geographically distant states clustered separately. Thus, in the present investigation, geographical diversity was observed to be an index of molecular genetic diversity in *B. persicum*.

DISCUSSION

Availability and assessment of genetic variation are central to the improvement of any crop species. Moreover, it has become critical to characterize elite germplasm lines for their accurate and unambiguous identification so that false claims can be contested against in a court of law.

Cluster analysis based on morphological and yield contributing attributes revealed that the populations under study could be grouped into two clusters (Sadiq *et al.* 2008). Populations of Gurez, Sangla and Khrew formed Cluster I and Kalpa and Harwan formed Cluster II. The distribution of populations in different clusters indicates that even though the samples were selected from different eco-geographical areas the genetic makeup along with breeding system, heterogeneity, genetic drift, natural and unidirectional selection pressure must be the cause of genetic diversity among different populations besides geographic variation to some extent. Therefore, selection of genotypes for hybridization may be made on the basis of genetic diversity rather than geographic diversity. However, due attention needs to be paid to geographic diversity, to accommodate sufficient genetic diversity in the germplasm.

An earlier study by Sadiq *et al*. (2008) on morphological variations supports the results of the present study demonstrating a wide genetic variability in the accessions of *B. persicum* as studied by RAPD. Singh (1993), while working with *Bambusa tulda* and Chauhan *et al.* (2000) in chirpine found no eco-geographical link with cluster pattern of the genotypes and the progenies, respectively. The present result supports the findings of Chartuvedi and Pandey (2001) in *Bombax cieba*, Mohapatra (1996) in *Acacia cate-*

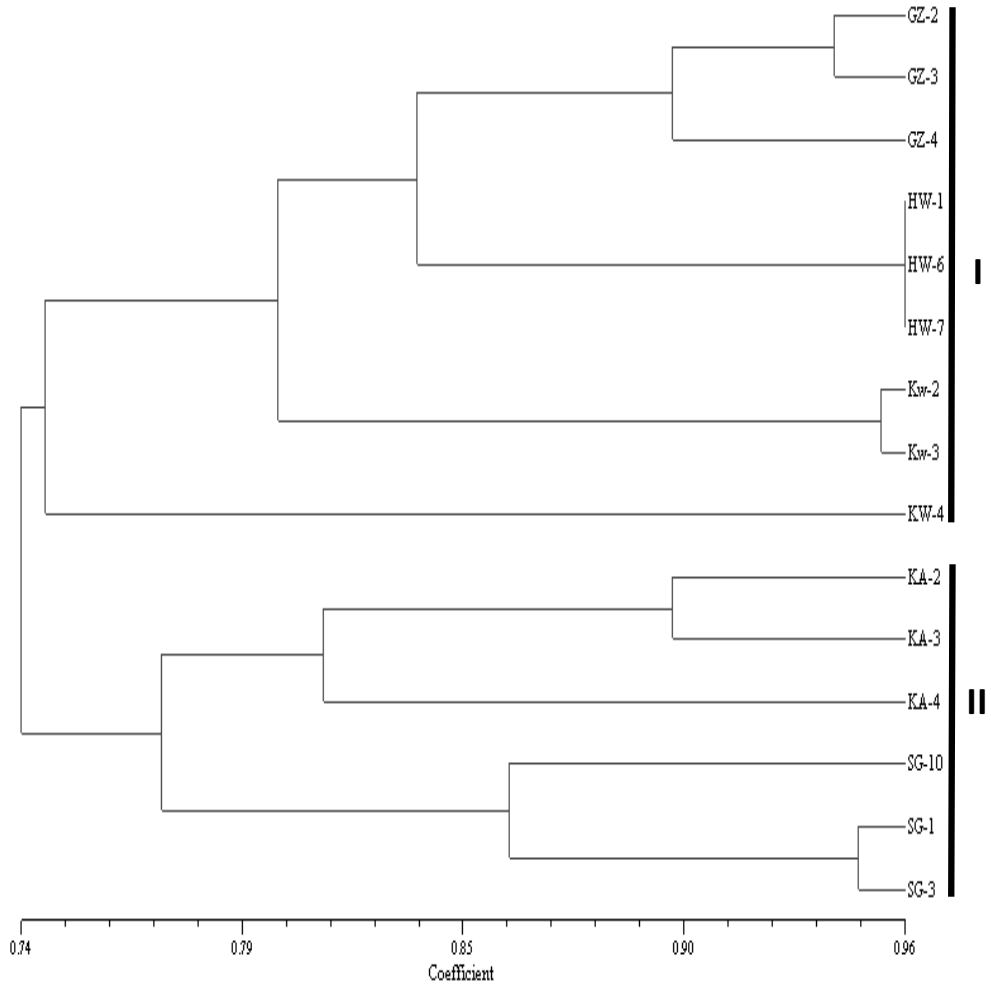

Fig. 1 Dendrogram depicting clustering pattern of 15 accessions of *Bunium persicum* based on 168 polymorphic bands generated by 36 RAPD markers, the Jaccard algorithm, and the UPGMA clustering method. Roman numerals I and II indicate clusters for grouping the *Bunium persicum* samples.

chu, Pandey *et al.* (1995) in *Populus deltoides*, Bhat (1999) in *Albizia lebbeck*, Thiru Selvan (1995) in *Dendrocalamus hamiltonii* and Surendran and Chandrasekharan (1988) in *Eucalyptus tereticornis*.

Genetic similarity for the morphological data of *B. persicum* was calculated using the Jaccard algorithm and a dendrogram was constructed using the UPGMA clustering method. The distribution of the various populations into clusters and within cluster was somewhat random. One clear sub-cluster having populations from sites Sangla and Khrew could be easily identified. The most similar populations were Sangla and Khrew. Morphological characters were also used to discriminate 25 Greek olive cultivars and the dendrogram constructed formed five clusters (Hagidimitriou *et al.* 2005).

PCR-based techniques have been successfully used to analyze DNA polymorphism in a number of plant species: *Panax ginseng* (Park *et al*. 1995), *Piper longum* (Banerjee *et al*. 1999), *Corylus avellana* (Galderisi *et al*. 1999). Among them, RAPD is a simple and relatively inexpensive technique to identify germplasm.

RAPD was found useful in revealing genetic variation both between and within different species of *B. persicum*. All the 36 primers clearly differentiated 15 *B. persicum* samples that showed 97.11% polymorphism. Among the 36 primers used, 15 primers namely C-1, C-2, C-5, C-11, C-13, C-15, C-16, C-19, C-20, D-2, D-5, D-8, D-10, D-13, and D-20 were informative in detecting variation among the 15 *B. persicum* samples. Of these, seven primers, C-2, C-5, C-11, C-13, C-16, D-5 and D-13 were most efficient in differentiating genotypes for major amplified products. These primers should be employed for assessment of intra-specific diversity and simple identification in *B. persicum*. A comparative study on the use of ISSR, microsatellites and RAPD markers for varietal identification of carrot genotypes was reported by Briard *et al.* (2001). RAPD analysis was also used to study the genetic structure of an endangered plant, *Antirrhinum microphyllum* (Torres *et al.* 2003), identification of *Melissa officinalis* subspecies (Wolf *et al.* 1999) and assessment of identity, diversity and quality of *Echinacea* as reported by Kapetyn and Simon (2002).

The high level of polymorphism based on RAPD analysis in accessions of *B. persicum* is primarily due to the wild species included in the present study. Polymorphism as high as 97.11% observed in accessions of *B. persicum* is very high compared to a number of other endangered plant species e.g. *Cactoris fernandeziana* (24.5%) by Brauner *et al*. (1992), *Paeonia suffraticosa* (22.5%) and *Paeonia rockii* (27.6%) by Pei *et al*. (1995), *Cathay argophylla* (32%) by Wang *et al.* (1996) and *Dacydium pierrei* (33.3%) by Su *et al.* (1997). Due to high polymorphism the species should be able to adapt to different environmental conditions. High diversity in accessions of *B. persicum* is expected since the species is cross pollinated.

The present investigation and similar studies on strawberry (Kashyap *et al.* 2005), *Costus specious* (Mandal *et al.* 2007), *Andrographis paniculata* (Padmesh *et al.* 1999) suggest that RAPD is more appropriate for analysis of intraspecific genetic divergence.

The dendrogram (**Fig. 1**) based on NYTSYS-pc version 2 showed distinct separations of the collected populations, which have a fair level of variation at the morphological level. The most divergent samples based on the RAPD analysis should be used in cross hybridization programmes.

From the above discussion, it is clear that there are enough data to say that our population of this species in the

two states of Himachal Pradesh and Jammu & Kashmir have reasonably enough genetic variability.

In essence, the data presented here reflects the utility of RAPD in the analysis of genetic divergence within this medicinally important herbal spice. On the basis of present investigation, it could be concluded that there is tremendous variability in different populations of *B. persicum* in Himachal Pradesh and Jammu & Kashmir based on morphological and RAPD markers. It is possible now to undertake long-term breeding and conservation programmes of this species in the two states. Practically better understanding of distribution of genetic variability at the intraspecific level would help identify superior genotypes to be further exploited for genetic enhancement, as well as to evolve strategies for the establishment of effective *in situ* and *ex situ* conservation strategies.

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