INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 3, No. 5, p. 35-42, 2013

RESEARCH PAPER OPEN ACCESS

Genetic characterization of *Berberis* species collected from Kunhar River catchment using morphological and molecular markers

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Key words: Morphological traits, *Berberies*, Kunhar, RAPD.

doi: http://dx.doi.org/10.12692/ijb/3.5.35-42 Article published on May 20, 2013

Abstract

The *Berberis* genus represents perennial spiny shrubs with reddish brown to pale yellowish stem bark. From Pakistan *Berberis* have been classified on morphological characters but no attempt was made to classify it on molecular traits. The first documented report of using molecular as well as numerical parameters for dissecting the genus from different localities of Kunhar River catchment. The assessment of *Berberis is* genus based upon 19 morphological characters. The data obtained from numerical analysis was computed for getting dendrogram, which classified 24 collections into two major groups i.e. group A and B. All the genotypes of group-A was having red fruits whereas the collections clustered into group-B produce black fruits. Molecular characterization was done with the help of 12 RAPD primes to elaborate genetic polymorphism in *Berberis* colletions. The primers based amplification in the collections revealed high level of genetic polymorphism, i.e. 6-86%. The genetic diversity estimated as genetic distances computed into dendrogram, separated the collections into 5 distinct groups A, B, C, D and E, disseminated as *Berberis parkeriana, B. lycium, B. pachyacantha, B. kunwarensis and B. orthobotrys*, respectively. However the collection B-8, which closely resembles *B. parkeriana* morphology was out grouped and needs future elaboration with more markers assorted discrimination.

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Introduction

Introcution

The Kunhar valley is one of the most beautiful places in Pakistan which is 150 kilometers long. This valley lies in the North of district Mansehra of Hazara Division, Khyber Pukhtoonkhawa, Pakistan (Ahmad, 1969). The valley lies between 34° 14/to 35° 11/ North latitude and 72° 49/ to 74° 08/ East longitude. The area is surrounded on the East and South side by Azad Jammu and Kashmir, on North by Chilas and Gilgit, and on the West side by district Shangla and Buner. Altitude of the area ranges from 915 m to 5280 m at Balakot to Malika Parbat respectively (Jan *et al.*, 2008). Map of the area is presented in figure 1.

Berberis, belong to Berberidaceae of order Rananculales (Cronquist 1981; Takhtajan 1987). Berberidaceae contains about 15 genera and 650 species worldwide, found in temperate region of the northern hemisphere (Bottini *et al.,* 2002). *Berberis* is represented by 20 species in Pakistan. Mostly the species is distributed in the mountainous parts of the country (Jafri, 1975).The species of *Berberis* reported from Kunhar river catchment established through classical taxonomy but offer potential for their reassessment though molecular research.

Numerical traits provide an estimate of genetic diversity. Numerical taxonomic techniques including principal components and cluster analysis have been successfully used to classify and measure the pattern of genetic diversity as in blackgram (Ghafoor *et al.,* 2001), chickpea (Naghavi and Jahansouz, 2005) and lentil (Sultana *et al.,* 2006).

Fingerprinting using molecular marker techniques such as AFLP, RAPD, and RFLP has proven especially valuable for inferring the origin and population structure of invasive plants (Mueller and Wolfenbarger, 1999). DNA markers can be used to detect variation in the DNA level and have proved to be extremely effective in distinguishing between closely related groups (Ayele *et al.,* 1999). The technique is being successfully used widely for the assessment of genetic diversity, identification and differentiation in various plant species, such as *Oryza* (Mackill, 1995), *Brassica* (Jain *et al.,* 1994), *Medicago* (Yu and Pauls, 1993), *Euterpeedulis* (Caroso *et al.*, 2000), Zea mays (Ajmone *et al.,* 1998), *Oxalis tuberose* (Tosto and Hopp, 2000),*Pinus sylvestris* (Lerceteau and Szmidt, 1999).

According to Bottini *et al.* (2002) AFLP can be used for elaborating DNA based variation in *Berberis* species. Where as sequence analysis of the Internal Transcribed Spacer (ITS) of the 18S (ITS1)-5.8S-26S (ITS2) rDNA region for phylogenetic relationships between species of the genus *Berberis* (Bottini *et al.*, 2007) was also successful.

A large number of studies have been performed on the genus *Berberis* but still the taxonomy of the genus is somewhat uncertain. The uncertainty of the *Berberis* genus may due to hybridization which produces intermediate forms in transitional zones that cause difficulties in taxonomy of *Berberis* (Bottini *et al.,* 1999b). Based on taxonomic studies Pakistani *Berberis* genus is compared with herbarium specimen and living one growing in Kew botanical garden. Though *Berberis* genus needs thorough experimental studies because the taxonomic studies of mostly species of *Berberis* is based on a single herbarium specimen (Jafri, 1975). The aims of the present studies are to reassess the *Berberis* species collected from kunhar valley through classical and molecular tools. Also to elaborating phylogenetic relationship of the species based on marker assisted technology.

Materials and methods

Plant specimens were collected from 24 different localities of Kunhar Valley Mansehra, KP Pakistan. Field data including locality, altitude and GPS coordinates were documented (table 4) and submitted into Herbarium Hazara University Pakistan (HUP) for future references. The specimens were provisionally identified with the help of Herbarium specimens and available literature

(Stewart, 1967 & 1972; Jafri, 1975 and Xi- Ven & Hedge, 1994). The Plant specimens were dried and then grinded with pestle and mortar. Genomic DNA was isolated with the help of modified protocols of Doyle & Doyle, 1990 and Lodhi *et al.*, 1994.

The isolated DNA was checked with Gel electrophoresis and was subjected to dilution according to the quality and quantity of the DNA. The DNA which is visually concentrated for PCR reaction was diluted twice or thrice as needed. For each sample a pre mix of 12.5 µl was prepared on ice for PCR as given in table 1.All the specimens were run independently with each primer for PCR amplification. The thermocycling conditions were as (1) 94°C denaturation for 5 minutes, (2) 94°C denaturation for 1 minute (3) annealing temperature 30°C for 1 minute and (4) extension temperature 72°C for 2 minutes. Step 2 to 4 was repeated for 35 times. Final extension temperature 72°C was provided for 10 minutes. For PCR amplification Eleven RAPD primers were used. Details of the primers are represented in table 3.

Molecular data analysis

Only the scoreable bands were included in the analyses. Every band was considered as single locus and bivariate 0, 1 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-dxy/dx+dy-dxy

The average linkage (UPGMA) algorithm was used as a clustering method. The dendrogram was constructed by using Popgene32 version 1.7 (http://www.popgene/ualberta/fhey).

Morphological characterization

Morphological characteristics include 12 quantitative traits i.e leaf width, leaf length, spine length, no leaves per internode, no of spine per leaf, internode length, no of flowers per inflorescence, fruit breadth, fruit length, no of seed per fruit, seed breadth, and seed length were selected, and 7 qualitative traits i.e. stem texture, stemcolor, leaf venation, leaf colour, leaf texture, fruit texture and fruit color were selected.Numerical analysis was also carried out leading to the recognition of different species and distinct varieties of species. Hierarchical clustering was performed using the Euclidean distance index and group average strategy with the computer package MINITAB Inc., 1996, version 17).

Results

The PCR amplification results showed that only three out of the 12 RAPD primers viz., Gt-2, Gt-5 and Gt-7 were amplified with PCR reaction. Gel samples of the PCR amplified DNA samples of 24 genotypes of *Berberis* collections are provided in Figures 2-4.

Average Genetic dissimilarity among 24*Berberis* genotypes using RAPD primer Gt-2, Gt-5 and Gt-7was determined. Range of average genetic distances estimated was from 06 to 86%. Maximum genetic distances (86%) was estimated among one comparison (between comparison 1 and 15) while 6% genetic distance was estimated among five one comparisons (between comparisons 2 & 5, 2 & 9, 6 & 8, 10 &12 and 11 & 16).

The dissimilarity coefficient matrix of 24 *Berberis* genotypes based on the data of 3 RAPDs primers obtained through POPGENE 32 computer program was used to construct a dendrogram which is presented in Figure 5**.**The 24 collection from Kunhar River catchment were assorted into 5 groups (A, B, C, Dand E). Group A comprised of nine genotypes; all of them belong to same species (*Berberis parkeriana*) but different localities, while genotype B-8. (*Berberis parkeriana)* was out grouped in dendrogram though which is morphologically closely related with all other genotypes of group A. Group B comprised of four genotypes of same species (*B. lycium*). Group C comprised of onegenotypes (*B. pachyacantha*). Group D comprised of sixgenotypes belongs to same species (*B. kunwarensis*). Group E

comprised of three genotypes of same species (*B. orthobotrys*).

Table 1. Basic information of RAPD primers used for DNA amplification.

S. NO	OLIGO NAME	PRIMER SEQUENCE (5'-3')	MOL. WT	%GC
1	GLA-11	CAATCGCCGT	2988	60
2	GLA-15	TTCCGAACCC	2948	60
3	GLA-18	AGGTGACCGT	3068	60
4	GLB-12	CCTTGACGCA	2988	60
5	GLB-14	TCCGCTCTGG	2995	70
6	GLC-20	ACTTCGCCAC	2948	60
7	Gt- 2	TGCGCGATCG	3044	70
8	Gt-3	ACGTGCCGAT	3066	60
9	Gt- 4	GCGAATTCCG	3028	60
10	Gt- 5	GTGCAATGAG	3092	50
11	Gt- 6	GGATCTGAAC	3052	50
12	Gt- 7	GGACTCCACG	3013	70

Source: Alpha DNA Company Canada

Table 2. Locality, Altitude and Coordinates of various *Berberis* species.

S. No.	Acc. No.	Genus	Species	Locality	Altitude (Feet)	Coordinates
1`	8	Berberis	B. parkerianaSchneid	Biyari (Kapigali)	5115	N: 34° 36' 652" E: 73° 24' 823"
2	3	Berberis	B. parkerianaSchneid	Kaghan	6793	N: 34° 47' 040" E: 73° 31' 305"
3	20	Berberis	B. parkerianaSchneid	Balakot	3590	N: 34° 33' 363" E: 73° 20' 587"
4	9	Berberis	B. lyciumRoyle	Biyari (Kapigali)	7150	N: 34° 35' 338" E: 73° 23' 744"
5	4	Berberis	B. parkerianaSchneid	Kaghan	6560	N: 34° 46' 281" E: 73° 31' 383"
6	22	Berberis	B. parkerianaSchneid	Balakot	3545	N: 34° 33' 329" E: 73° 20' 605"
7	6	Berberis	B. parkerianaSchneid	Mahandari	5501	N: 34° 41' 728" E: 73° 34' 684"
8	7	Berberis	B. parkerianaSchneid	Mahandari	5501	N: 34° 41' 728" E: 73° 34' 684"
9	16	Berberis	B. parkerianaSchneid	Darnundi (Kapigali)	4735	N: 34° 35' 346" E: 73° 22' 601"
10	21	Berberis	B. lyciumRoyle	Balakot	3570	N: 34° 33' 341" E: 73° 20' 593"
11	14	Berberis	B. kunwarensisRoyle	Danna (Kapigali)	8336	N: 34° 35' 205" E: 73° 24' 569"
12	12	Berberis	B. lyciumRoyle	Katta (Ganool)	5310	N: 34° 36' 282" E: 73° 25' 285"
13	1	Berberis	B. orthobotrys Bien. ex Aitch.	Naran	10678	N: 34° 52' 913" E: 73° 41' 513"
14	11	Berberis	B. parkerianaSchneid	Katta (Ganool)	5034	N: 34° 36' 635" E: 73° 25' 231"
15	19	Berberis	B. kunwarensisRoyle	Gali (Kapigali)	8398	N: 34° 35' 213" E: 73° 24' 581"
16	13	Berberis	B. kunwarensisRoyle	Danna (Kapigali)	8300	N: 34° 35' 602" E: 73° 24' 212"
17	2	Berberis	B. orthobotrysbien. ex aitch.	Naran	8676	N: 34° 54' 294" E: 73° 40' 191"
18	24	Berberis	B. pachyacanthaKoehne	SaifulMaluk	9134	N: 34° 57' 294" E: 73° 42' 191"
19	5	Berberis	B. lyciumRoyle	Mahandari	5560	N: 34° 41' 776" E: 73° 34' 647"
20	10	Berberis	B. parkerianaSchneid	Ganool	5457	N: 34° 36' 211" E: 73° 25' 321"
21	17	Berberis	B. kunwarensisRoyle	Cheeran (Kapigali)	7680	N: 34° 358' 682" E: 73° 38' 993"
22	15	Berberis	B. kunwarensisRoyle	Danna (Kapigali)		N: 34° 35' 659" E: 73° 24' 232"
23	23	Berberis	B. orthobotrys Bien. ex Aitch.	Shogra	730	N: 34° 45' 291" E: 73° 31' 183"
24	18	Berberis	B. kunwarensisRoyle	Gali (Kapigali)	8303	N: 34° 35' 141" E: 73° 24' 794"

Morphological similarity was estimated among 24collection based upon the numerical analysis through MINITAB program is provided in the dendrogram (Figure 6).Twenty four collections of *Berberis* were assorted into 2 major groups i.e. A and B. These two groups are separated from one another having 48.36% differences. Group A comprises of 3 subgroups, i.e. I, II and III. Subgroup I comprised of one species (*B. pachyacantha)*.Subgroup II comprised of six specimens which show maximum similarity 70% belong to same species (*B. kunwarensis*). Subgroup III comprised of three specimens which show maximum similarity 86% belong to same species (*B. orthobotrys*). While group B comprises of 2 subgroups. Subgroup I comprised of ten specimens which show maximum similarity 85% belong to same species (*B. parkeriana)* and subgroup II comprised of four specimens which show maximum similarity 84% belong to same species (*B. lycium*).

Discussion

DNA markers can be used to detect variation in the DNA level and have proved to be extremely effective in distinguishing between closely related groups (Ayele *et al.,* 1999).Amplified Fragment Length Polymorphism (AFLP)can be used for elaborating DNA based variation in *Berberis* species (Bottini *et al.*, 2002). In present study Randomly Amplified Polymorphic DNA (RAPD) primers has been used to documented genetics diversity and Phylogenetic relation among twenty-four *Berberis* genotypes collected from various locations of Kunhar river catchment.

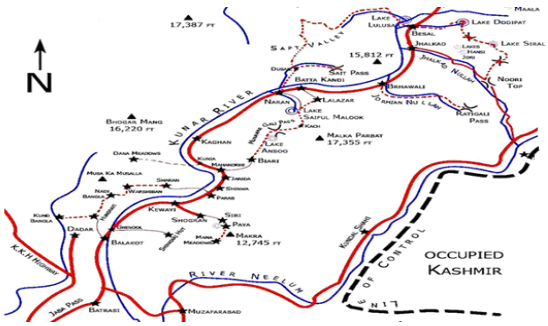

Fig. 1. Map shows different locality of Kunhar valley from which *Berberis* species have been collected.

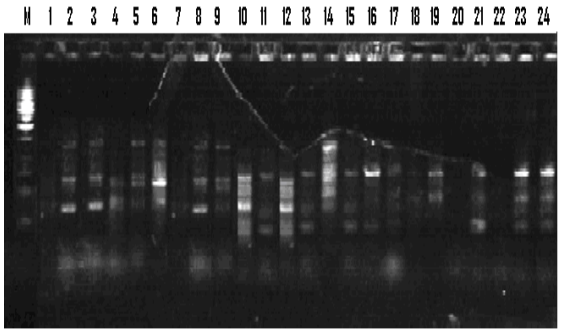

Fig. 2. PCR profile of twenty four *Berberis* genotypes using RAPD Primer Gt. 02.

The concerted attempt reassessment of finding genetic polymorphism in *Berberis* using numerical taxonomy as well as Randomly Amplified Polymorphic DNA (RAPD) tools showed that though the species clearly segregated from one another, there is a much more confusion in identification of *Berberis* species. Therefore for precise identification of various species, molecular and numerical taxonomy was used. Twenty four genotypes of *Berberis* collected from various localities of Kunhar river catchment, Pakistan were included in the study.

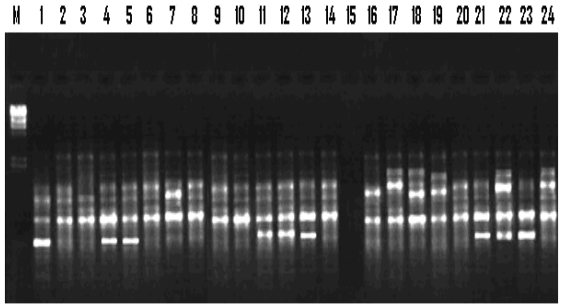

Fig. 3. PCR profile of twenty four *Berberis* genotypes using RAPD Primer Gt. 05.

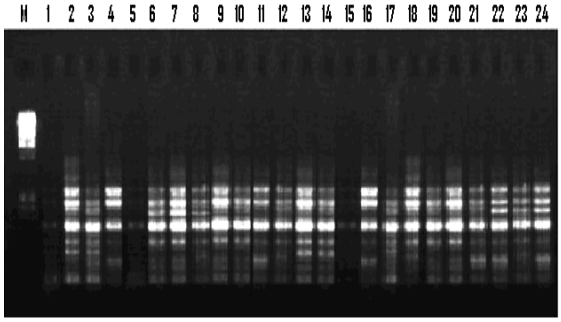

Fig. 4. PCR profile of twenty four *Berberis* genotypes using RAPD Primer Gt. 07.

For over all genetic diversity studies, twelve Randomly Amplified Polymorphic DNA (RAPD) primers were used (GLA-11, GLA-15, GLA-18, GLB-12, GLB-14,GLC-20, Gt-2, Gt-3, Gt-4, Gt-5, Gt-6 and Gt-7). Among them, nine primers (GLA-11, GLA-15, GLA-18, GLB- 12, GLB-14, GLC-20, Gt-3, Gt-4 and Gt-6) did not optimized PCR. The RAPD primers produced different levels of genetic polymorphism. Over all genetic distances ranged from 06 to 86%. The study strengthened earlier reports that RAPDs can be used for studying genetic polymorphism and tagging of useful genes. It is also evident from the PCR based assays,that RAPD-markers can be used effectively to estimate genetic variability in *Berberis* and could be considered as an easy diagnostic analysis for identifying the over lapping traits. It can also be noted that more molecular analysis is required to reach on a better conclusion regarding genetic variability and more detailed mapping of the *Berberis* genome.

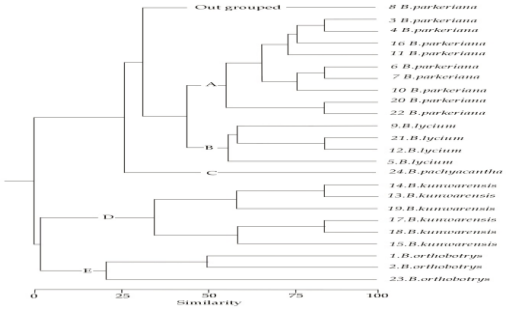

Fig. 5. Dendrogram using Pop Gene 32.

It is further evident from numerical analysis that numerical taxonomy can be used with limitations for identification of complicated species particularly species having continuous polyploidy andamong closely related taxa. Our experience with numerical taxonomy of *Berberis* revealed that the population No.8 verified as *B. parkeriana* was not conformed by DNA analysis. Dendrogram obtained from numerical data showed that all the collection were classified into two major groups i.e. group A and B corresponding to the different phylogenetic origins. These two groups are separated from one another on the bases of fruit color. All members of group-A produce red fruits, while that of group-B produce black fruits. Group-A is further divided into 3 subgroups i.e. I, II and III. All members of subgroup I produced 2 ovules and their leaves had 10-30 spinose-serration, (*Berberis pachyacantha* Koehne). All members of subgroup II also produced 2 ovules but their leaves had only 7-10 spinose-serration (*B. kunwarensis* Royle). All members of subgroup III produce 3 ovules and their leaves had 10-20 spinose-serration (*B.orthobotrys* Bien. ex. Aitch). Group-B was divided into 2 subgroups i.e. I and II. The leaves of subgroup I were green both on dorsal and ventral surfaces (*B.parkeriana* Schneid), where as that of subgroup II are green on dorsal surface and whitish on ventral surfaces (*B. lyceum* Royle).

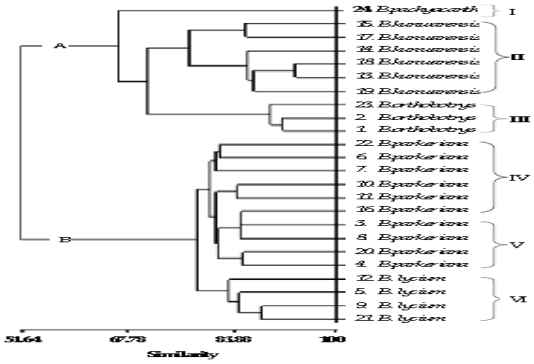

Fig. 6. Numerical Dendrogram using SPSS.

Details of numbering of samples of both dendrogram are available in table 1

Present study is first attempt in Pakistan to characterize *Berberis* genotypes of Kunhar river catchment (from 3545 – 9134 ft, Table 1) based on differences in DNA bands obtained through Randomly Amplified Polymorphic DNA (RAPD) primers. Total twelve RAPD primers were used for discrimination. Out of these, nine primers did not optimize for PCR reaction. It is therefore recommended that more molecular markers are required to achieve a better understanding about the structure and diversity of *Berberis* genome. Numerical characterization of the collection and computation of data into simple dendrogram was an interesting part of the study, it can be employed to other species also. A comparison of the clustering of DNA amplification and numerical data revealed that DNA analysis provide handy tool for precisely

supplementing the establishment of groups and taxons in plant Kingdom.

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