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Elaboration of phylogenetic studies in *Berberis* spp. from Karakorum Ranges. II. floral and morpho-pathological data

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Key words: Genetic diversity, phylogeny, *Berberis,* Karakoram Mountain Ranges, medicinal plant, morphology, floral characters, pathological data.

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Abstract

Berberis species are considered very important in Eastern, Ayurvedic, Unani and modern allopathic medicines for treatment of cancer, diabetes, bone fractures, jaundice, enlargement of spleen, AIDS, osteoporosis, heart problems, ocular trachoma, hypertension, infectious diseases, cholera, diarrhea, dysentery, eye troubles and leprosy. The root and the stem bark of *Berberis* spp. are the richest source of Berberine alkaloids. Overlapping characters across Berberis spp. make identification often difficult. Present research was undertaken to study phylogenetic relationships among various accessions of *Berberis* collected from Central Karakoram National Park using detailed morphological, floral and pathological characters. Genetic diversity was estimated in material using UPGMA procedure. Genetic Distances among all possible combinations were estimated and ranged from 1 % to 25%. Data regarding morphological, pathological and floral characters was also analyzed using dendrogram analysis. Dendrogram constructed using pooled data was found most informative. Eleven accessions of *Berberis* were grouped into three main groups. It has been concluded that 5 accessions included in subgroups A1 and A2 (low altitude, less than 2050 m) more likely falls in *B. pseudumbellata* subspecies *pseudumbellata;* instead of previously reported species *B. Lyceum.* Similarly, it has been concluded that accessions # 4, # 5 and # 6 (higher altitudes (more than 2050 m) are more closely related to *B. pseudumbellata* subspecies *gilgitica.* It has also been suggested that as classification of *Berberis* spp is still not clear, hence more work preferably based on DNA technology is needed to reach on better and concrete conclusion regarding taxonomic classification of *Berberis* species/subspecies.

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Introduction

Berberis is the most pronounced genus of family Berberidaceae having ca. 450 species. *Berberis* species (spp.) are perennially deciduous or evergreen thick woody spiny shrubs covered with a thin brittle bark erect up to 3.3 m high (Agrios, 1988; Herrera, 1984; Hooker, 1980; Kulkarni *et. al.,* 2012; Mehrhoff *et. al.,* 2003). Most of the members of *Berberis* are distributed across the globe in the Northern hemisphere (Chamberlain and Hu, 1975; Landrum, 1999), temperate and sub-tropical parts of Asia, Europe and America (Chandra and Purohit, 1980) and a few species scattered in Africa (Ahrendt, 1961). According to Li *et. al.* (2010) the genus (*Berberis)* originated in eastern Asia, and have migrated from eastern Asia to North America in the Oligocene period (33.9 million to 23 million years).

A large number of native wildlife including avifauna and mountain angulates are known to depend on *Berberis* fruits, leaves, bark and roots and use this species as wild-herbal-clinic (Khan *et. al.* 2014a; Khan *et. al.*, 2014b). Several spp. serve as the alternate hosts to two important rust pathogens (*Puccinia graminis* and *P. striiformis*) of small grains and grasses (especially wheat), sometimes generating large-scale epidemics (Negi, 2013; Jin, 2011; Naef *et. al.et. al.,* 2002; Agrios, 1988; Barbu-Diaconescu, 1961; Watson and Luig, 1958). Several are invasive in nature (USDA, 2011; Lubell *et. al.*, 2008a, b; Mehrhoff *et. al.*, 2003). Besides, its horticultural, economic and ecological value, they are considered highly medicinal around the world (Sing *et. al.*, 2009). It is commonly used in Eastern, Ayurvedic, Unani and modern system of medicines (Chopra *et. al.*, 1981; Chandra and Purohit, 1980). *Berberis* has been reported effective in treatment of cancer, diabetes, bone fractures, jaundice, enlargement of spleen, AIDS, osteoporosis, heart, ocular trachoma, hypertension, infectious diseases, cholera, diarrhea, dysentery, eye troubles and leprosy, (Khan *et. al.*, 2014a; Sing *et. al.,* 2009; Asif *et. al.*, 2007; Fatehi *et. al.*, 2005; Caraballo *et. al.*, 2004; Kuo *et. al.*, 2004; Villinski *et. al.*, 2003; Ivanoska and Philipov, 1996; Koo and Seang, 1996; Chopra *et. al.*, 1981; Chandra and Purohit, 1980).

The root (5%) and the stem bark (4.2%) of *Berberis* spp. are the richest source of Berberine alkaloids(Kulkarni *et. al.,* 2012; Janbaza and Gilanib, 2007).

Extensive systematic research on floral anatomy have been conducted to study evolutionary relationships among closely related genera within the family Berberidaceae (Hoot *et. al.*, 1999; Kosenko, 1980; Kim and Jansen, 1998) and it was conclude that some generic pairs including *Berberis*-Mahonia have close affinity and *Berberis* was probably derived from Mahonia by reducing leaflets to produce spines (Fig. 1). It is generally agreed that *Berberis* is a difficult and uncertain genus due to extremely high morpho-pathological and phytochemical variation. Which, probably due to environment and hybridization (Khan *et. al.* 2014a; Rao *et. al.* 1998a; Chapman, 1936; Ahrendt, 1961). Overlapping characters, especially in leaves, stem, bark and berry make field identification often difficult (Tiwari and Adhikari, 2011; Rao *et. al.,* 1998b; Chapman, 1936; Jafri 1975). *Berberis* is represented by 29 species in Pakistan, which are distributed in the Northern mountainous parts of the country (eFlora, 2014; Jafri, 1975). Stewart and Stewart (1939) were the first to report *Berberis* from Gilgit-Baltistan. Since then, 12 species have been reported from Gilgit-Baltistan by more than 11 researchers and only 8 species have been described from the study area (table 1). According to available literature, *Berberis pseudumbellata* Parker subsp. *gilgitica* Jafri is endemic to Gilgit-Baltistan (Alam and Ali, 2010; Jafri, 1975) province of Pakistan and has become critically endangered (Alam and Ali, 2010).

Most of the *Berberis* spp. reported from study area were previously (table 1) identified in the field following short surveys or quick visit of herbarium specimens. *Berberis* spp. reported from Central Karakoram National Park (CKNP) and Gilgit-Baltistan are based on classical taxonomy and potentially required to reassess through more

elaborate studies including numerical and molecular data.

Present research was aimed at study of phylogenetic relationships among various accessions of *Berberis* collected from CKNP area using detailed morphological, floral and pathological characters. These studies will help in more precise elaboration of phylogenetic relationships among *Berberis* spp. found in the area particularly and rest of the world in general, which is still somewhat uncertain (Bottini *et. al.*, 1999b, 2000, 2007).

Material and methods

Study area

CKNP is the largest protected area in Pakistan and stretches over an area of 10,000 km² along the Karakoram mountain ranges. It extends from 35°N to 36.5°N Latitude and from 74°E to 77°E Longitude. Park holds world's greatest glacial mass outside poles and harbors complex floro-faunal diversity. Anthropo-climatic changes have led species to greater vulnerability even critical endangered including *Berberis* spp. (Hussain *et. al.*, 2012; Alam and Ali, 2010; WWF Pakistan, 2009). Study was carried out during 2012-14 in four major valleys of the CKNP viz; Bagrot, Rakaposhi, Rahimabad-Guro and Naltar. Some geographic information about the study area and sampling sites are presented in table 2.

Climate

Climate is predominantly cold arid and temperate in the lower elevations. Prevalent season is winter, occupying the valleys eight to nine months a year. (WWF Pakistan, 2009). Area lacks significant rainfall, averaging in 120 to 240 millimeters (4.7 to 9.4 in) annually (Karrar and Iqbal, 2011). Most of rainfall occurs during winter and early spring.

Sample collection

Extensive surveys were conducted in aforementioned valleys and eco-climatic zones of CKNP. Eleven accessions were studied in the field (natural populations). For each selected representative accession, field notes were taken along with the voucher specimen following standard technique (Jain and Rau 1977). During the field survey, field notes, date, locality, habitat and brief identification features were also noted. Samples were studied using 30 morpho-pathological, floral and pathological characters (table 3).

Data were collected against 30 different parameters (see table 3) from 11 *Berberis* accessions. Details on flowers, leaves, spines, plant height and number of berries count were recorded in the field. For this purpose 100 leaves and 100 spines were measured from each representative accession. Moreover, 10 different plants from the same population were measured for height scale.

Berries and seed details were taken in the laboratory using scale and electric weight balance. For comparison of fresh and dried berries, berries were dried using thermal oven at 40°C (104 °Fahrenheit) for seven days (Bottini *et. al.*, 2000, 2002). 100 berries and 100 seeds of each accession were measured for length and width, however, for weight measurement 100 berries and 1000 seeds were weighed. Infection prevalence was recorded using berry infection rate out of 200 berries which were collected randomly from each accession. Global Positioning System (GPS-Garmin) was used to record geographical coordinates and elevations (mean height above sea level). Climatic data was retrieved from local weather stations.

Statistical analyses

Genetic diversity was estimated in material using UPGMA procedure (Nei and Li, 1979). Moreover, Statistical Package for the Social Sciences (SPSS ver. 16.0) and Microsoft Excel 2010 were also used for data analysis and construction of dendrograms.

Results and discussion

Characters/Parameters

Basic statistics including mean values, standard error and ranges for 30 characters studied during present research are presented in Table 3.

Mean number of leaves, length of lamina and width of lamina were 58.69, 2.98 cm and 1.05 cm, respectively. Mean number of berries per branch was 5.94 ±1.86while mean number of seeds per berry was 2.48 ± 0.21. Mean seed infection (%) was observed 23.36 ± 7.48. While mean plant height was 2.71 ± 0.18 meter.

Table 1. Summary of available literature regarding taxonomy of *Berberis* spp. from study area.

Year	Author	Area	*Berberis* Species							
			L	Br	OOr	OrC	PP	PGl	Ku	Pr
2013	Abbas *et. al.*	Naltar Valley		√		√	√			
2009	Alam and Ali	Naltar, Skoro La-Baltistan						√		
2009	Sherwali Khan	Bagrot, Haramosh (thesis)		√		√	√	√		
2007	Khan &Khatoon	Bagrot, Haramosh		√		√	√			
2006	Qureshi *et. al.*	Gilgit to Hunza, Gilgit, Nomal, Naltar	√						√	
2000	Kashif MS	Naltar Valley					√			√
1975	Jafri, SHM	Gilgit-Naltar						√		
1974	Ghafoor, A.	Naltar 1000 ft						√		
1954	Stewart, RR.	Naltar 3300 m			√					
1939	Stewart & ID.	Gilgit 2700 m			√					
		TOTAL REPORTS	2	3	2	3	4	4	1	1

L = *B. lyceum*; Br = *B. brandisiana*; Ku = *B. kunwarensis*; Pr = *B. parkeriana*; PP = *B. pseudumbellata pseudumbellata*; PGl = *B. pseudumbellata gilgitica*; OOr = *B. orthobotrys orthobotrys*; OrC = *B. orthobotrys capitata*.

Table 2. Some geographic information regarding study area and collection sites (CKNP).

S.No.	Collection Site	North	East	Altitude (m)	Slope Face
1	Rahimabad	36°06'25.29"	74°18'13.26"	1733.8	South facing
2	Goro	36°10'51.25"	74°17'24.96"	1708.2	South facing
3	Nomal	36°05'28.37"	74°16'55.78"	1639.3	North-East facing
4	NaltarMW	36°09'22.67"	74°11'57.19"	2723.8	South-East facing
5	NaltarEnd	36°10'23.71"	74°09'58.29"	2941.8	South facing
6	BagrotUp	36°02'23.34"	74°35'16.66"	2624.1	North facing
7	BagrotDwn	36°02'00.67"	74°34'00.51"	2572.9	West facing
8	Juglot	36°10'44.77"	74°18'40.81"	2033.5	South facing
9	Hupaye	36°14'10.06"	74°26'32.29"	2042.1	North facing
10	Ghulmet	36°14'22.96"	74°29'03.89"	1989.0	South facing
11	Thol	36°14'12.24"	74°26'04.24"	1924.4	North facing

Genetic distances

Genetic distances (GD) were estimated using UPGMA (Unweighted Pair Group of Arithmetic Mean) procedure as proposed by Nei and Li (1979). GD estimates are presented in Table 4. Genetic Distances among all possible combinations were estimated and ranged from 1 % to 25%. Low range (0.01-0.25) of GD estimates indicated close genetic relatedness among the accessions.

Data regarding morphological, pathological and floral characters was also analyzed using dendrogram analysis (computer program SPSS ver. 16.0 was used for the construction of dendrograms). Four different dendrograms were constructed (Figs. 2, 3 using morphological, pathological, floral and pooled (morphological, pathological and floral characters) data. Dendrogram constructed using pooled data (Fig. 3) was most informative among all and was used for further discussion. Eleven accessions of *Berberis* were grouped into three main groups viz A, B and C (Fig. 3). Group A was further subdivided into 2 subgroups viz; A1 and A2. Subgroup A1 (comprising accession # 9 from Hupaye and # 11 from Thol). Subgroup A2 comprised upon 3 accessions (# 8 from Juglot, # 10 from Ghulmet and # 1 from Rahimabad). Main group B was further divided in 2 subgroups B1 and B2. Subgroup B1 comprised 3 accessions (# 4 from Naltar MW, # 6 from Bagrot up and # 5 from Naltar E). Subgroup B2 comprised accession # 2 from Goro and # 3 from Nomal. Main group C comprised only 1 accession (# 7 collected from Bagrot Down). It is evident from Figure 3 that Main group A comprised 5 accessions all of them from relatively low altitude area (less than 2050 meter). While main groups B and C predominantly comprised accessions from relatively higher altitude (more than 2050 meter) except accessions (included in subgroup B2) # 2 and # 3 (collected from 1708 and 1639 meters, respectively).

Table 3. Basic statistics for 30 morpho-pathological and floral characters in *Berberis* spp.

		$\bar{x}$ ± SE		
1.	Avg. No. of leaves per branch	58.69 ± 4.60	84.700	40.100
2.	Avg. Lamina length (cm)	2.98 ± 0.130	3.850	2.480
3.	Avg. Lamina width (cm)	1.05 ± 0.120	2.000	0.630
4.	Avg. Leaf Size (cm)	3.23 ± 0.400	5.826	1.658
5.	Avg. No. of thorns per branch	49.74 ± 5.03	68.300	16.300
6.	Avg. Size (length) of spine (cm)	1.30 ± 0.060	1.670	0.980
7.	Avg. No. of berries per branch	5.94 ± 1.860	16.400	0.000
8.	Avg. No. of Seeds per berry	2.48 ± 0.210	3.500	1.500
9.	Avg. Wt. (g)per Berry	0.06 ± 0.010	0.130	0.035
10.	Avg. wt. (g) Dry Pulp per berry	0.04 ± 0.010	0.106	0.028
11.	Avg. Wt. (g) total Seeds per berry	0.02 ± 0.000	0.028	0.007
12.	Avg. Wt. (g) one seed	0.01 ± 0.000	0.012	0.004
13.	Avg. Wt. (g) per 1000 seeds	6.59 ± 0.660	11.831	4.013
14.	Seed Floating Percentage (%)	27.35 ± 5.42	57.692	5.714
15.	Seed Submerged Percentage (%)	72.65 ± 5.42	94.286	42.308
16.	Seed Infection (%)	23.36 ± 7.48	73.000	0.000
17.	No Seed (%)	11.82 ± 2.23	24.000	2.000
18.	1 Seeded (%)	17.27 ± 4.43	48.000	5.000
19.	2 Seeded (%)	18.82 ± 3.53	36.000	2.000
20.	3 Seeded (%)	18.00 ± 3.26	35.000	0.000
21.	4 Seeded (%)	9.18 ± 2.810	33.000	1.000
22.	5 Seeded (%)	1.45 ± 0.670	6.000	0.000
23.	6 Seeded (%)	0.09 ± 0.090	1.000	0.000
24.	Plant Height (m)	2.71 ± 0.180	3.370	1.750
25.	Number of Outer Whorl Sepals	3.74 ± 0.191	5.000	2.900
26.	Number of Inner Whorl Sepals	4.03 ± 0.199	5.000	2.900
27.	Number of Petals	5.26 ± 0.152	6.000	4.600
28.	Number of Stamens	5.37 ± 0.175	6.000	4.600
29.	Number of Carpals	1.00 ± 0.000	1.000	1.000
30.	Number of Flowers at each Axil	11.69 ± 0.845	16.80	7.900

Table 4. Genetic diversity estimates among 11 accessions of *Berberis*.

	1	2	3	4	5	6	7	8	9	10
2	0.10									
3	0.10	0.04								
4	0.10	0.09	0.09							
5	0.10	0.09	0.09	0.02						
6	0.10	0.09	0.09	0.01	0.02					
7	0.25	0.25	0.25	0.25	0.25	0.25				
8	0.03	0.10	0.10	0.10	0.10	0.10	0.25			
9	0.04	0.10	0.10	0.10	0.10	0.10	0.25	0.04		
10	0.03	0.10	0.10	0.10	0.10	0.10	0.25	0.02	0.04	
11	0.04	0.10	0.10	0.10	0.10	0.10	0.25	0.04	0.01	0.04

Fig. 1. Relationships of family Berberidaceae with related families. Source: Hoot *et. al.* (1999).

Previous claims challenged

Based on dendrogram (presented in Fig. 3, constructed using morphological, pathological and floral characters) and observation of floral characters (using present research and previously reported characteristics described in flora of Pakistan (eFlora, 2014; Jafri, 1975). It has been concluded that 5 accessions included in subgroups A1 and A2 more likely falls in *B. pseudumbellata* subspecies *pseudumbellata;* instead of previously reported species *B. Lyceum* (Qureshi *et. al.*, 2006). The observation is further strengthened by the fact that *B. Lyceum* has been reported previously native to Himalayan ranges and not Karakoram ranges. Two accessions collected from Goro and Nomal (both low altitude collection sites) which are included in subgroup B2 are also more closely related to *B. pseudumbellata* subspecies *pseudumbellata*.

Similarly, it has been concluded that accessions # 4, # 5 and # 6 are more closely related to *B. pseudumbellata* subspecies *gilgitica*. Present findings favor previous reports of Alam and Ali (2009), Jafri (1975) and Ghafoor (1974). But are in contrast to Stewart (1939, 1954), Sheikh (2000), Khan and Khatoon (2007) and Abbas *et. al.*, (2013). Details of review of literature regarding previous taxonomic classification and reports are presented in Table 2). It should be kept in mind that these authors (Stewart, Sheikh, Khan and Khatoon and Abbas *et. al.*) based their conclusion on description of morphological characters on a seen-and-passed-by technique (short survey) only and details regarding such morphological characters are missing. Moreover, these descriptions did not mention exact location (GPS coordinates) of their collection sites.

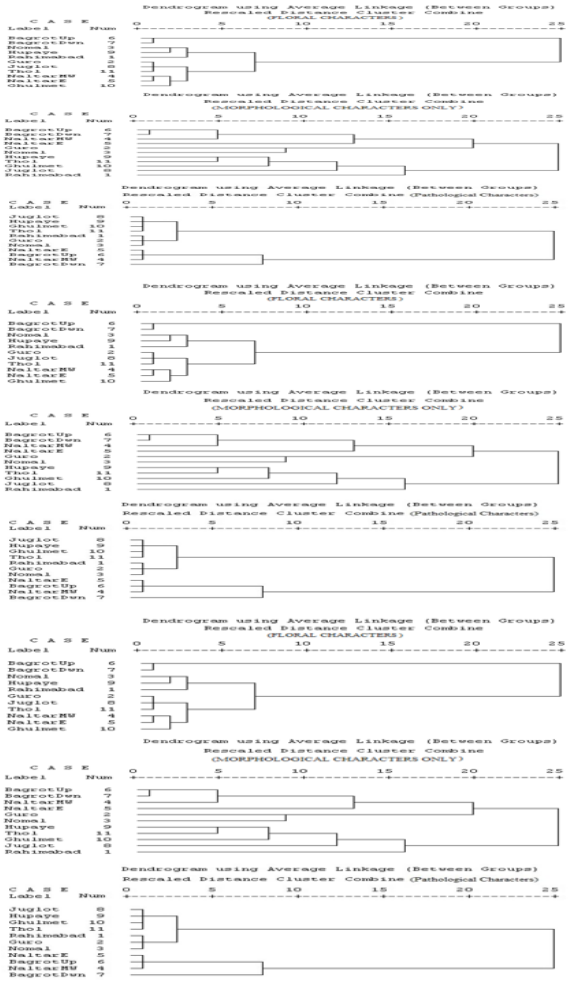

Fig 2. Dendrograms (floral, morphological and

pathological characters respectively) in *Berberis* spp. (Constructed using SPSS cluster analysis, Version 16.0).

Way forward

As it is clear from above discussion that taxonomic classification of *Berberis* is still not clear and much work (preferably based on DNA technology) is needed to reach on better and concrete conclusion regarding taxonomic classification of *Berberis* species/subspecies from the area.

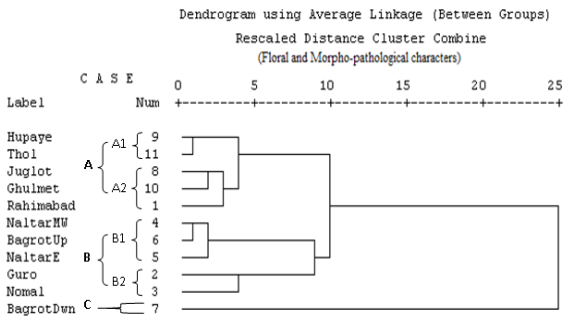

Fig. 3. A dendrogram based upon pooled data (floral, morphological and pathological characters) in *Berberis* spp. (Constructed using SPSS cluster analysis, Version 16.0).

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