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Inhibition of Poly Phenol Oxidation during Genomic DNA Isolation and Morphological Variations in *Berberis lycium* Royle Genotypes

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Abstract: *Berberis lyceum* is an economically and medicinally important genus distributed abundantly in the mountainous ranges of Pakistan. The color of hairs on the leaves of *B. lyceum* genotypes was pale to brown while the branch color was brown in all *B. lyceum* genotypes. Similarly, flower color was pale yellow while the plant height was ranged from 2.0 to 5.0 meters. Average length of lamina was 2.4 cm, average numbers of thorns per branch were 50.0 and average number of berries per branch was 18. Leaves tend to have comparatively high phenolic compounds that can interfere with DNA isolation. DNA-based markers are becoming popular for the identification of plants. Molecular genetic diversity studies of Berberidaceae family are almost lacking, which revealed the neglected status and the extent of the gap in the knowledge of this family. Mercaptoethanol being a reducing agent directly or indirectly causes damage to DNA. It is one of the limiting factors found in our study that can inhibit the oxidation process and at its low concentration brown viscous DNA pellet was extracted from fresh leaves while at high concentration intact whitish DNA pellet of good quality was observed that found suitable for PCR amplification. The DNA quantity and quality from dry leaves were quite low that was improved with the increase in the concentration of β 2-mercaptoethanol. It is concluded that a combination of 1% PVP and 0.01 to 0.06 % β mercaptoethanol was found satisfactory for extracting DNA from fresh and dry leaves of *B. lyceum* genotypes. Further, fresh and dry leaves were not able to exhibit any significant effects.

Keywords: *Berberis lyceum*, Barberry, Morphological variations, DNA isolation, RAPD

Introduction

Berberis is one of the important genus of Berberidaceae known for its health care usages and medicinal properties. In Pakistan, there are 29 different *Berberis*

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species that are mostly distributed in the Northern mountain ranges (Khan et al. 2014c). *Berberis* species are perennial, deciduous, evergreen, thick woody spiny shrubs covered with a thin, brittle bark and are erect up to 3.3 meters (Kulkarni et. al., 2012). *Berberis lyceum* is an economically and medicinally important genus distributed abundantly in major continents of the world such as America, Europe and Asia. In Pakistan, it is commonly known as Barberry and Ishkeen in Urdu widely found in the Western Himalaya and Nepal while in Pakistan, it is distributed in mountain ranges, especially in Kashmir and North West Himalayan regions (Sood et al., 2012; Khan et al., 2014c). The plant contains major alkaloid berberine (Khosla et al., 1992), which is an isoquinoline alkaloid known for its activity against cholera and diarrhea. It has antispasmodic property and is also used for treatment of Jaundice and internal wounds. The stem is said to be diuretic and laxative and are useful in rheumatism. The bark of its root is a valuable medicine in intermittent and remittent fevers. The dried mass of Barberry root bark after mixing with molten animal fat are being used as bandages for bone fractures while the fruit is used as a cure for renal disorder. The extract of the fruits is recommended against the stomachache, intestinal problems and diarrhea. The plant leaves are used as a tea substitute while on the whole Barberry plant is helpful for the treatment of swollen and sore eyes, broken bones, internal injuries, ulcer, jaundice and rheumatism (Kaur and Miani, 2001).

It is a subtractive, rigid, spiny shrub, 2.7 to 3.6 meters in height having small pale yellow flowers and small berries, occurs abundantly in the Himalayas, from Kashmir to Kumaun Hills at 2000-2,700 meters altitude. Flowering in the shrub starts from the mid of March and remain in progress up to the end of April. The fruits start ripening from the second week of May and the mature ripened fruits are relished by the villagers and children from the month of May to July (Sood et al., 2010).

Population studies and genetic diversity studies in the Berberidaceae is almost lacking. In spite of three major revisions (Rao et al., 1998), the taxonomy of *B. lyceum* Royle complex still remains, unclear and confusing. Berberis revealed morpho-pathological and phytochemical variations, especially in the leaves, stem, flower and berry size that make field identification difficult and challenging due to environmental and hybridization effects (Khan et al., 2014c). Overlapping characters make field identification often challenging such as leaf texture and serrations varied from season to season and with the age of the plant (Rao et al., 1998). The taxonomic standing of *Mahonia* and *Berberis* as distinct genera has been the subject of much debate among botanists and horticulturists. Morphological characters such as leaf and stem complexity, inflorescence structure, and floral anatomy had served distinctly to separate *Mahonia* from *Berberis.* Nevertheless, the obvious difference in physical appearance between the two genera, with compound leaves within *Mahonia* versus simple leaves within *Berberis,* makes a unified circumscription hard to reconcile (Yan-Jun et al., 2006).

The huge amount of variations that existed in their natural populations and their altitude preference from 2000 to 2,700 meters and habitat preferences makes its taxonomic identification further difficult. DNA-based markers are becoming popular for the identification of plants because genetic composition is less affected by age, physiological condition, environmental factors, harvest, storage and processing. In recent days, the sequence variations have been used to develop specific markers for the identification and authentication of raw

drugs and herbal formulations (Balasubramani et al., 2011). Nuclear ribosomal RNA genes and internal transcribed spacer (ITS) sequences have become favored markers in evolutionary studies. Conventional macro-morphology and microscopic examination were not helpful in critically distinguishing *Berberis* species. DNA markers were developed by amplifying and sequencing the complete internal transcribed spacer regions (ITS1, 5.8S rRNA and ITS2). These are useful as a molecular pharmacognostic tool in quality control of raw drugs (Kim et al., 2014; Balasubramani et al., 2011).

Higher contents of polyphenolic compounds and polysaccharides present in the cell as secondary metabolites usually coprecipitate with DNA can interfere with the activity of the DNA polymerase enzyme (Pandey et al., 1996; Memon et al., 2010) that interfere with DNA isolation and cause problems in the amplification with Random Amplified Polymorphic DNA (RAPD) markers. However, commercial kits for routine extraction are economically costly and their regular usage is difficult for large-scale genomic applications. In the present study, an effort has been made to analyze *B. lyceum* populations in Orkzai agency and compare them with populations of Karakorum ranges and the Himalayan mountains. Besides this, our main idea is to optimize the polyphenol oxidation during genomic DNA extraction and analyze them for RAPD-DNA assessment of *B. lyceum* genotypes.

Materials and Methods

Plant materials

Extensive field surveys were carried out in the southern extension of the Hindu Kush mountains that consisted of Sara Mela forests; situated in Orakzai agency, Khyber Paktunkhwa. Similarly, Central Karakarum National Park, CKNP that stretches over an area of 10,000 km^2 along the Karakoram mountain ranges were surveyed while the Margalla range as lesser Himalayas were also investigated. *B. lyceum* samples were collected population wise in the range of 9 km from five different places at a distance of 1.5 to 2 km in Sara mela forests, Orakzai Agency, Khyber Paktunkhwa as shown in Figure 1. In each population four plants were picked and from each plant, 4 to 5 branches were analyzed. Similarly from different regions of Margalla Hills National Park, Islamabad and Karakoram mountain ranges of Gilgit-Bilitistan, *B. lyceum* samples were collected and identified as reviewed in distributing literature for *B. lyceum* in Flora of Pakistan (Ahrendt, 1961).

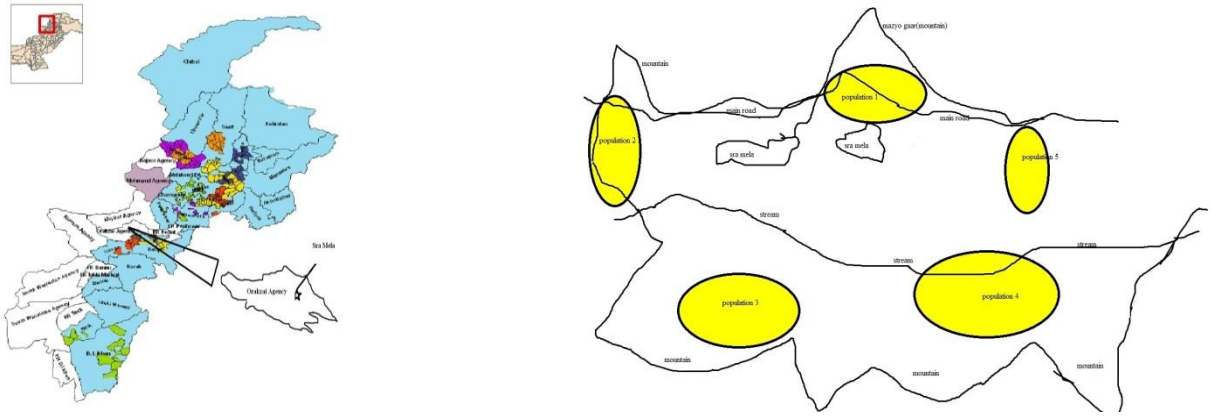

Figure 1. Sara mela (Orakzai Agency) marked as the potential region of *Berberris lycium* genotypes.

The leaves were collected and stored immediately in plastic zip bags containing silica gel. The morphologically closely related plants have been identified and collected from mixed wild populations. Various phenotypic characters were recorded on field observations that include vegetative characters like plant height, leaf size, presence or absence of hairs, and reproductive features i.e. color of flowers and inflorescence. The gross morphological attributes were taken into consideration for the identification of taxa that include stem, color of bark, color of leaf surface and nature of inflorescence, etc.

DNA Isolation

DNA isolation from *B. lyceum* is not always easy or routine procedure (Smith et al., 1991) as they contained large amount of primary and secondary metabolites (Sangwan et al., 1998). Although methods are available that yielded a high quantity of DNA, but they also coprecipitate a lot of secondary metabolites, which can hinder with PCR amplification. Existing inexpensive phenol-chloroform based methods are extensively used to deal with problems such as excessive polysaccharides in specific groups of plants (Ivanova et al., 2009). After trying a lot of published protocols and failing repeatedly to obtain DNA that was not contaminated with polyphenolic compounds. Fresh and dried leaf samples of *B. lyceum* (100 mg) was grounded in liquid nitrogen along with and without 1% Polyvinylpyrrolidone w/v and 0.005 % (v/w) β 2-mercaptoethanol using 900µL extraction buffer (100mM Tris-HCl pH 8.0, 50mM EDTA pH 8.0, 500 mM NaCl + 0.07% 2-marceptoethanol). After crushing 130µL 10% SDS and 300µL sodium acetate was added to the sample and shaken gently to form slurry. Whereas 0.01 and 0.06% β 2-mercaptoethanol was also employed in extraction buffer as reducing agent to avoid oxidation of polyphenols that can interfere with the extraction of genomic DNA. All these compounds were freshly added. The tubes were then incubated at 65^{o}C in an incubator for over-night. An equal volume (600µL) of chloroform iso-amyl alcohol (24:1 v/v) was added and centrifugation was preceded at 12000 rpm for 5-10 minutes at room temperature to separate the phases. As a result two layers were formed. The upper supernatant layer of around 500-600µL was carefully decanted and transferred to a new tube and treated with 1.5 volumes of isopropanol, 100µL of Potassium Iodide and 20µL of glass milk particles (silica particles) was also added. The precipitated nucleic acids were washed in 70% ethanol, air dried and suspended in TE buffer. The dissolved pellets were again incubated with 3 µL RNase A (10 mg/mL) at 65°C for 1 h. DNA was precipitated by adding 0.1 v/v of 3 M sodium acetate (pH 4.0) and 0.7 v/v isopropanol and left overnight at 4°C. DNA pellet was extracted and washed with 70% ethanol, air dried and dissolved in 100 µL TE buffer for further analysis. For preventing oxidation of phenolic compounds, a relative concentration of PVP (Sigma) (1 % w/w), and 2-mercaptoethanol (0.005-0.06v%; v/w) was used per g of leaf tissue while grinding in liquid nitrogen.

Quantification of Genomic DNA

Genomic DNA was quantified and purity was verified by measuring the absorbance at 260 and 280 nm with UV Visible Spectrophotometer. The extracted DNA (5 µL) was electrophorased on 1% agarose gels, stained with ethidium bromide, visualized and photographed under UV light in a molecular imager gel doc system.

Polymerase chain reaction (PCR) analysis

A total of three random primers from series A, E and F of Operon Technologies were used for amplification of template DNA as shown in Table 1.

Table 1. RAPD PCR markers used for amplification of DNA of *Berberris lyceum*.

S. No	RAPD-markers	Primer sequences 5'-3'	Amplified product (bp)
1	OPA-03	AGTCAGCCAC	1500-3200
2	OPE-07	AGATGCAGCC	1003-3200
3	OPF-07	CCGAATTCCC	1100-3000

A standard 25 µl reaction contained 50 ng to 100 ng template DNA, 1.0 U Taq DNA polymerase, 1 X PCR reaction buffer containing 1.5 mM $MgCl_2$, 10 picomoles primer and 100 µmoles of each dNTPs. DNA amplification was performed in Perkin Elmer DNA thermal cycler, according to Williams et al. (1990). PCR reactions without DNA were used as negative control. The thermal cycling protocol was programmed for an initial denaturation step of 3 min at 94°C, followed by 40 cycles of 45 seconds at 94°C, 1 minute at 37°C, extension at 72°C for 1 minute and final extension at 72°C for 7 minutes. The RAPD products were separated by electrophoresis according to their molecular weight on 1.5 % (w/w) agarose gels submerged in 1 X TBE buffer and then stained with ethidium bromide (100 µg ml-1) solution for 15 minutes. The DNAs were visualized on a UV-transilluminator and documented using the gel documentation system. The λ DNA digested by EcoRI and Hind III was used in the gel as a standard size marker.

Results and Discussion

Morphological characters of *B. lyceum* genotypes collected from different regions of Sara mela forests (Orakzai Agency), Central Karakorum National Park and Margalla Hills National Park are shown in Table 2 and Table 3. The color of hairs on the leaves of *B. lyceum* genotypes were pale brown and the branch color was brown in all *B. lyceum* genotypes while flower color was observed as pale to yellow.

Mean number of leaves per branch were 40.0 for *B. lyceum* genotypes collected from Central Karakorum National Park and Orakzai Agency while these were 65.0 for *B. lyceum* genotypes collected from Margalla Hills National Park. Average leaf size was highest for *B. lyceum* genotypes collected from Orakzai Agency while it was least for *B. lyceum* genotypes collected from Margalla Hills National Park. Average numbers of seeds per berry were 3.0 for *B. lyceum* genotypes collected from Margalla Hills National Park and 2.0 each for *B. lyceum* genotypes collected from the Central Karakorum National Park and Orakzai Agency, respectively. Plant height was ranged from 2.0 meters (Orakzai Agency, CKNP) to 5.0 (Margall Hills National Park) while the average length of lamina was 2.4 cm and average width of lamina was 1.0 cm. Similarly, average no of thorns per branch were 50.0 while the average number of berries per branch was 18. In the same way, average number of seeds per berry was 2.0 ranging from 1.0 to 5.0 respectively. Khan (2014b) analyzed the genetic diversity of medicinally important *Berberis* species from Karakoram mountain ranges and indicated their spatial distribution. In the same way, Khan et al. (2014a) elaborate phylogenetic relationships of various accessions of *Berberis* from CKNP area using morpho-pathological characters. Twenty four morpho-pathological characters were studied in 11 *Berberis* natural populations from Karakoram mountain ranges. Relatively lower range of genetic distance estimates was observed among 11 *Berberis* accessions that indicated close genetic relatedness among the accessions. Hussain et al. (1998) enumerates four varieties of *B. lyceum* using morphological, distributional,

Table 2. Quantitative characters of *B. lyceum* genotypes collected from different regions of Sara mela forests (Orakzai Agency), Central Karakorum National Park and Margalla Hills National Park.

Morphological characters	**Location**								
	Central Karakorum National Park (CKNP)			**Orakzai Agency, KP**			**Margalla Hills National Park**		
	Latitude: 35°N to 36.5°N			Latitude: 33°41'12.27"			Latitude: 33°45'0"		
	Longitude: 74°E to 77°E			Longitude: 70°57'24.16"			Longitude: 73°7'0.12"		
	Elevation: 1200m-6000m (asl)			Elevation: 1830 metres (asl)			Elevation: 732 metres (asl)		
	X± SE	Min	Max	X ± SE	Min	Max	X ± SE	Min	Max
Avg. No. of leaves per branch	40.0	85.0	62.0	40.0	50.0	45.0	65.0	50.0	80.0
Avg. Lamina length (cm)	2.0	4.0	3.0	2.0	4.0	3.0	2.4	1.6	3.3
Avg. Lamina width (cm)	1.0	2.0	1.0	1.0	2.0	1.5	1.0	1.0	2.0
Avg. Leaf Size (cm)	2.0	6.0	4.0	2.5	6.0	4.2	2.5	1.8	3.5
Avg. No. of thorns per branch	16.0	68.0	42.0	50.0	60.0	55.0	60.0	50.0	70.0
Avg. Size (length) of spine (cm)	1.0	2.0	1.0	1.0	2.0	1.5	1.3	1.0	1.9
Avg. No. of berries per branch	8.0	16.0	8.0	10.0	20.0	10.0	9.0	18.0	0.0
Avg. No. of Seeds per berry	2.0	4.0	3.0	2.0	4.0	3.0	3.0	1.0	5.0
Plant Height (m)	2.0	3.0	3.0	3.0	4.0	3.5.0	2.8	2.0	3.0 to 5.0

Table 3. Qualitative characters of *Berberis lyceum* genotypes collected from different regions of Sara Mela forests (Orakzai Agency).

S. No	Hairs on leaves	Spine color	Branch color	Flower color
Pop. 1	+	Brown	Brown	Pale-yellow
Pop.2	-	Brown	Brown	Pale-yellow
Pop.3	+	Pale	Brown	Pale-yellow
Pop.4	+	Pale	Brown	Pale-yellow
Pop.5	_	Pale	Brown	Pale-yellow

+ present, - Absent

leaf epidermal and pollen morphological data and revealed that in all the varieties of *B. lyceum*, the upper epidermis is papillose with convex periclinal cell walls and inconspicuous cell boundaries. They classified the sub varieties on the basis of stem, leaf and inflorescence characters. Both pollen morphology and leaf epidermal studies are good indicators for the identification of *Berberris* species. The isolation of high quality genomic DNA from plants containing secondary metabolites like polyphenols and polysaccharides is difficult in several plant species and fail to produce good quality DNA that found fit for PCR, as the polyphenols adhere and interfere with DNA amplification. During the present study β 2-mercaptoethanol, Polyvinylpyrrolidone, Potassium Iodide, Sodium Dodecyl sulfate and CTAB were employed in extraction buffer. At low concentration of β 2-mercaptoethanol (0.005), brown viscous DNA pellet was observed from fresh leaves as shown in Table 4. The quantity and quality of DNA pellet were also not satisfactory for PCR. At high concentration of β 2-mercaptoethanol (0.01), intact brown viscous DNA pellet was noticed and its quality was further improved with increasing concentration of β 2-mercaptoethanol (0.06) and good intact whitish DNA pellet was observed that was suitable for PCR. From dry leaves, the quantity of extracting DNA was quite low and at low concentration of β 2-mercaptoethanol, a brownish viscous DNA pellet was sighted and its quality was improved with the increase of β 2-mercaptoethanol concentration and found suitable for PCR. Mathew et al. (2014) suggested that CTAB based protocol with 2-mercaptoethanol yielded DNA that appeared to be brownish yellow in color revealing the oxidation of phenolic compounds. In their oxidized forms, polyphenols covalently bind to proteins and DNA, giving the DNA a brown color and making it useless for most research applications (Aljanabi et al., 1999). The use of a higher salt concentration and PVPP along with 2-mercaptoethanol are useful to remove polysaccharide contaminants and polyphenols. PVP has been used frequently in CTAB extraction protocols to counter polyphenol oxidation. However, PVP when used alone at different concentrations yielded very faint bands or sheared bands in agarose gels and there were no amplification products after PCR analysis (Mathew et al., 2014). Schneerman et al. (2002) have reported that PVP alone was not

Table 4. Inhibition of oxidation for polyphenols and removal of polysaccharides using different treatments in CTAB protocol.

Sample Texture	Treatments with extraction buffer					Polyphenol oxidation effects	DNA quantity µg/mL	DNA quality	PCR amplification
	β 2-mercaptoethanol conc. (%) w/v	Polyvinyl pyrrolidone conc. (%) w/v	Potassium Iodide conc. (%) w/v	Sodium Dodecyl sulfate conc. (%) w/v	CTAB conc. (%) w/v				
Fresh leaves (100 mg)	0.005	0	0.1	0.013	0	Brown viscous DNA pellet	1200±848	1.16±0.1	-
	0	1	0	0	2.5	Whitish DNA pellet	6450±7848	1.06±0.1	-
	0.06	0		0.013	0	Intact brown viscous DNA pellet	5250±6575	1.03±0.1	-
	0	1	0	0	3	Intact slightly brown DNA pellet	3300±2969	1.1±0.1	-
	0.01	1	0	0	3	Good intact whitish DNA	825±106	1.1±0.1	+
Dry leaves (10 mg)	0.005	0	0.1	0.013	0	Brown viscous DNA pellet	599.95±0.4	1.25±0.1	-
	0	1	0	0	2.5	Whitish DNA pellet	899.94±0.5	1.16±0.1	-
	0.06	0		0.013	0	Intact brown viscous DNA pellet	599.96±0.4	1.10±0.1	-
	0	1	0	0	3	Intact slightly brown DNA pellet	1199.92±0.4	1.0±0.1	-
	0.01	1	0	0	3	Good intact whitish DNA	749.94±0.3	1.1±0.1	+

able to increase the yield or prevent the contamination of DNA as polyphenol in the presence of PVP at 1-3% interfere with other antioxidants. Polysaccharides also interfere with biological enzymes such as, polymerases, restriction endonucleases and ligases, resulting in unsuccessful amplification (Michiels et al., 2003). Mercaptoethanol being a reducing agent inhibits oxidation processes that directly or indirectly cause damage to DNA (Weising et al., 2009). Of the various treatments, a combination of 1% PVP and 0.01 to 0.06 % β mercaptoethanol was found satisfactory for extracting DNA from fresh and dry leaves. Further, fresh and dry leaves were not able to exhibit any significant effects (Jorge et al., 2005).

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