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Estimation of molecular diversity of *Berberis lycium* in three districts of Azad Jammu and Kashmir

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Abstract

Research study was carried in the Pant Genetic Resources Institute (PGRI), NARC Islamabad for the assessment of molecular diversity of *Berberis lycium*in three districts of Azad Jammu and Kashmir. The present research describes the importance genetic diversity of medicinally important Berberis specie Berberberis lycium from great mountainous regions of AJK. Nine locations i.e; Hurnamaira, Datot, Topa, Tolipeer, Hullar, Morifurman Shah, Trarakhal, Baloch and Pullandri were selected on the basis of different altitudes and agroclimatic conditions and mature fruits were harvested from different barberry ecotypes. The matured harvested fruits were kept in the plastic pots and transported to Pant Genetic Resources Institute (PGRI), NARC Islamabad where they were subjected to -80 ˚C. Seeds were separated from the fruit pulp and used for protein analysis. SDS PAGE technique was used for the molecular characterization of selected ecotypes. Ecotypes Morifurman Shah and Tolipeer showed maximum genetic variation on the basis of molecular studies.

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Introduction

Genus Berberis exhibits a global distribution and almost all 450 species identified worldwide found across northern Hemisphere (Ahrendt 1961; Rao *et al.* 1998; Landrum 1999; Tiwari *et al.* 2012). It makes almost 70% composition of family Berberidaceae. Being one of the primitive angiosperm (Bruckner, 2000), it has a strong influence among various traditional and modern healthcare systems (Lewis and Ausubel 2006; Imanshahidi and Hosseinzadeh 2008; Kulkarni and Dhir 2010). Asia and South America are considered to be the primary and secondary centres for its diversity respectively (Landrum, 1999).

*Berberis lycium*is an evergreen shrub belongs to family Berberidaceae. It is also known as Indian barberry in English, kashmal or kasmal in Hindi, Ishkeen in Urdu and sumbal locally. It is a suberect, rigid, spiny shrub 2.7- 3.6 m in height. The genus Berberis is widely distributed in America, Europe and Asia.

It is well known medicinal plant with overall edibility rating 3 (1-5 scale) and medicinal rating 3 (1-5 scale). Every part of this plant has some medicinal value. Its root, bark, stem and fruits are used in various ayurvedic preparations (Bhattacharjee *et al.*, 1990). It is extensively used for the treatment of various diseases like liver disorders, abdominal disorders, skin diseases, cough, diabetes mellitus etc. (Ahmed *et al.*, 2009).

The plant was described under the name of Ambaribis by Al-Biruni. He also mentioned its Persian name as Zirkash. Its common name is Kashmal. However, it is called Ishkeen and Sumbal in the area of its collection (Said *et al.*, 1996). *Berberis lycium* was described in 1837 by John Forbes Royle. *Berberis lycium* commonly known as Barberry belongs to the genus Berberis of family Berberidacae. It sustains its leaves in all seasons. It produces flowers from May to June. Its flowers are bisexual and pollinated by insects. The plant is widely used for medicinal purposes.

*Berberis lycium*is native to Nepal, globally distributed in various parts of world. It occurs abundantly in the Himalayan region of India and Pakistan. Within India, it has been found in Jammu and Kashmir, Himachal Pradesh, Uttar Pradesh, Sikkim, Madhya Pradesh and Tamil Nadu between altitude ranges of 850 - 3500 metres. In Pakistan it is distributed in northern areas such as Gilgait, Baltistan, Ghizer, Astor, Diamer and Swat. It is also found in Nilgiris and Ceylon.

The plant is not fastidious but grows well in thin dry and shallow soil (Huxley *et al.*, 1992). It is a hardy plant but suffer severe damage in winters. It can be hybridized freely with the other members of the genus. They can be pruned severely but they easily resprout from the base. The plant is propagated by the mean of seeds. The seeds of the over ripe fruit will take longer time to germinate. The seedling can be raised in nursery and then transplanted when small plants are grown and are easy to handle. However, proper ventilation is required to prevent the damping off of the seeds.

*Berberis lycium*is one of the plant species being abundantly available, the whole plant especially root is extensively used for the treatment of several human diseases under local practices in Pakistan (Khan *et al.*, 2001). The various chemical constituent of *Berberis lycium*are berberine, berbamine, chinabine, karakoramine, palmatine balauchistana mine, gilgitine, jhelumine, punjabine, sindamine, chinabine acetic acid, maleic acid, ascorbic acid (Khare *et al.*, 2004). The plant contains major alkaloid berberine which is an isoquinoline alkaloid and umbellitine. This is usually taken from root or root bark of the *Berberis lycium*, and other Berberis species abundantly available in local forests. The fruits contain malic, tartaric, citric acids and tannins (Sharma *et al.*, 2003).

Ahrendt 1941 surveyed the Berberis spp. and published a detailed revision of Berberis. Rao and Hajra (1993) while treating the family for the flora of

India, included 54 species of Berberis in Indian region. The conclusion of most of the mentioned workers are primarily based on previous herbarium collections and often on solitary collections scattered in different herbaria. Study of the live plants in the natural habitat is rarely attempted. Molecular genetic diversity studies of this family were not attempted before, which revealed the neglect status and the extent of gap in the knowledge of family. Rao *et al.* (1998) solved the identification and taxonomy of *Berberis lycium* complex based on morphological basis. These identifications were based on extensive field studies as well as herbarium specimens. This helped extremely in solving the taxonomic problem of several species complexes. It is important to use DNA based markers to study genetic diversity in the species as they are expected to reveal results that are less affected by environmental factors.

DNA-based markers have now become a popular means for the identification and authentification of plants diversity because genetic composition is unique for each individual irrespective of the physical form and is less affected by age, physiological condition, environmental factors, harvest, storage and processing. Polyacrylamide gel electrophoresis is used for the qualitative characterisation of proteins in biological preparations, for variation and quantitative determinations (Yip *et al.*, 2007).

Objectives

To study genetic diversity in *Berberis lycium* using SDS-PAGE.

To elucidate genetic variations of *Berberis lycium* in Azad Jammu and Kashmir.

Materials and methods

This study was carried out in to investigate the biodiversity of *Berberis lycium* in three districts viz; Poonch, Bagh and Sudhnoti of Azad Jammu and Kashmir the plant populations were visited three times in selected locations Hurnamaira, Datot, Tolipeer, Topa, Mori Furman Shah, Hullar, Trarkhal, Baloch and Pullandri of Azad Jammu and Kashmir during flowering, fruiting and mature fruit harvesting and comprehensive data were collected. These locations were selected because of difference in altitude and diverse agro-climatic conditions. The research material was comprised of nine ecotypes of barberry. The ecotypes were checked for certain.

SDS-PAGE

SDS-PAGE technique was used to identify molecular diversity of available ecotypes of *Berberis lycium*. SDS-PAGE technique was used to identify molecular diversity of available ecotypes of berberis (*Berberis lycium* L.). Molecular analysis involves the use of molecular techniques for assessing genetic diversity of plant germplasm. Protein extraction was done from seeds in Pant Genetic Resources Institute (PGRI), NARC Islamabad.

Procedure of Protein Extraction

Polyacrylamide gel electrophoresis (PAGE) of seeds was used to describe the genetic structure of the ecotypes of *Berberis lycium* L. Seed protein was extracted using a modified version of Lammelli (1970). The procedure is as follows:

Preparation of Samples

The fully matured seeds were ground by pre-cooled (at -20 ^{0}C) pestle and mortar 0.15 gm of crushed tuber was suspended in 1 ml cold extraction buffer. Each sample was taken in the same ratio and suspended in cold extraction buffer. The homogenized mixture was incubated at 4^{0}C overnight. Next morning the mixture was vortexes and the samples were centrifuged at 15000 rpm for 15 minutes at 4 ^{0}C in order to remove the cell debris. In order to precipitate the proteins from the extract, 1 ml of acetone was added in each sample and the resultant suspension was left overnight at -20 ^{0}C.

Sample Solubilization for SDS-PAGE

Following overnight incubation in acetone at -20 ^{0}C, the proteins were pellet out by spining at 12,000 rpm for 10 minutes at 4 ^{0}C in an eppendorff centrifuge.

The supernatant acetone was discarded and pellet was air-dried. The pellet of each sample was resuspended in 400µl sample buffer. It was properly mixed by vortexing and with the help of a needle, teasing the pellet gently so as to aid in solubilzation. The solubilized samples were centrifuged at 10,000 rpm for 10 minutes and the supernatant was saved and stored at -20 ºC for further use. Next day the samples of protein extraction 10 µl was loaded in each well of the gel.

Separating Gel

The vertical slab gel unit was assembled in casting mode in a small flask, appropriate volumes of monomers solution, separating gel buffer, 10% SDS solution, glycerol and water were mixed leaving out the ammonium per sulfate and the TEMED. The flask was stopped and the solutions were mixed well. The APS and TEMED were added and the flask was shaked carefully avoiding the feneration of bubbles. The solution was pippet out in to the sandwich leaving sufficient space for stacking gel. The gel was allowed to solidify for more than half an hour.

Stacking Gel

In a small flask appropriate volume of monomer solution, stacking gel buffer, 10 % SDS solution, glycerol and water were mixed according to the protocol.

The APS and TEMED were left out and the solution was desecrated. After that the APS and TEMED were added and the solution was well mixed. 1-2 ml of distilled water was added to sandwich to rinse the surface of the gel. Then the sandwich was filled with stacking gel solution. At the same time a Teflon comb was inserted into the sandwich. Care was taken not to trap the bubbles below the teeth of the comb. Gel was allowed to solidify for not less than half an hour.

Loading and running the gel

The comb was slowly removed from the gel and was pulled straight up to avoid disturbing the well dividers. Each well was rinsed with distilled water. The casting stand was inverted to drain the wells. Each well was filled with tank water. Using a micropipette 10 µl of each sample was loaded in each well one by one. Upper and lower chambers were assembled. Lower buffer chamber was filled with tank buffer until the sandwich was immersed in buffer. Care was taken not to trap bubbles under the ends of sandwich. Upper chamber was also filled with tank buffer. The lid was put on the unit and connected to power supply. The power supply was set to a constant current. After 4-5 hours, the dye reached to the bottom of the gel. The power supply was turned off and power cables were disconnected.

Staining and De-staining of the gel

For staining the sandwich was disassembled. The gel was put into the stain kept in a small tray. The tray was shaken for 48 hours on a stirrer. For de-staining the gel was removed from the staining solution and was transferred to de-staining solution. It was shaken for about 6 hours. After de-staining the gel was shifted to distil

Led water. The analysis of gel was made and protein bands were visualized tran-illuminator and photographs were taken for further references. For the long term storage the gel was sealed in a plastic bag by using gel dryer. Then it was kept at room temperature.

Gel Photography

The photographs of the gel were taken at the end.

Statistical Analysis

The data thus obtained was analyzed by simply statistical techniques using the procedure of cluster analysis with the help of computer software “Statistica” 12.0 for Windows. Cluster analysis were conducted on the basis of average distance of k-means and accessions in each cluster were then analyzed for basic statistics. For SDS-PAGE all the monomorphic and polymorphic protein bands were scored. Only unambiguous bands were used in the analysis. Each band was given score of 1 for presence

of polymorphism and 0 for absence. The dendrogram of total seed protein, based on dissimilarity matrix were done using unweighted pair group method with arithmetic averages (UPGAMA) as used by (Rohlf, 2005).

Results and discussion

SDS-PAGE

The SDS-PAGE is a practical reliable method because seed storage proteins are largely independent of environmental fluctuation. For molecular characterization of barberry ecotypes SDS-PAGE has been used because of its reliability and simplicity for protein assessment and description of genetic structure of the germplasm.

Protein profile of different accession was estimated and their distinct banding pattern on running gel has been noted after staining and destaining.

Table 1. Scoring of SDS-PAGE of 9 barberry ecotypes.

Bands	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Hurnamaira	1	1	1	0	1	0	0	1	0	1
Datot	1	1	1	0	1	0	1	1	0	1
Topa	1	1	1	0	1	0	0	1	1	1
Tolipeer	1	1	1	0	1	1	1	1	0	1
Hullar	1	1	1	1	1	0	1	1	0	1
Morifurman shah	1	1	1	0	1	1	1	1	0	1
Trarkhal	1	1	1	0	1	0	1	1	1	1
Baloch	1	1	1	1	1	0	1	1	1	1
Pullandri	1	1	1	1	1	1	1	1	1	1

The results for SDS-PAGE of 9 barberry ecotypes are shown in Fig 1.Seed protein analysis by SDS-PAGE has proved to be an effective way of revealing the differences and relationship between ecotypes. The high stability of the seed protein profile and its additive nature make seed protein electrophoresis a powerful tool in elucidating the origin and the evoluation of plants.

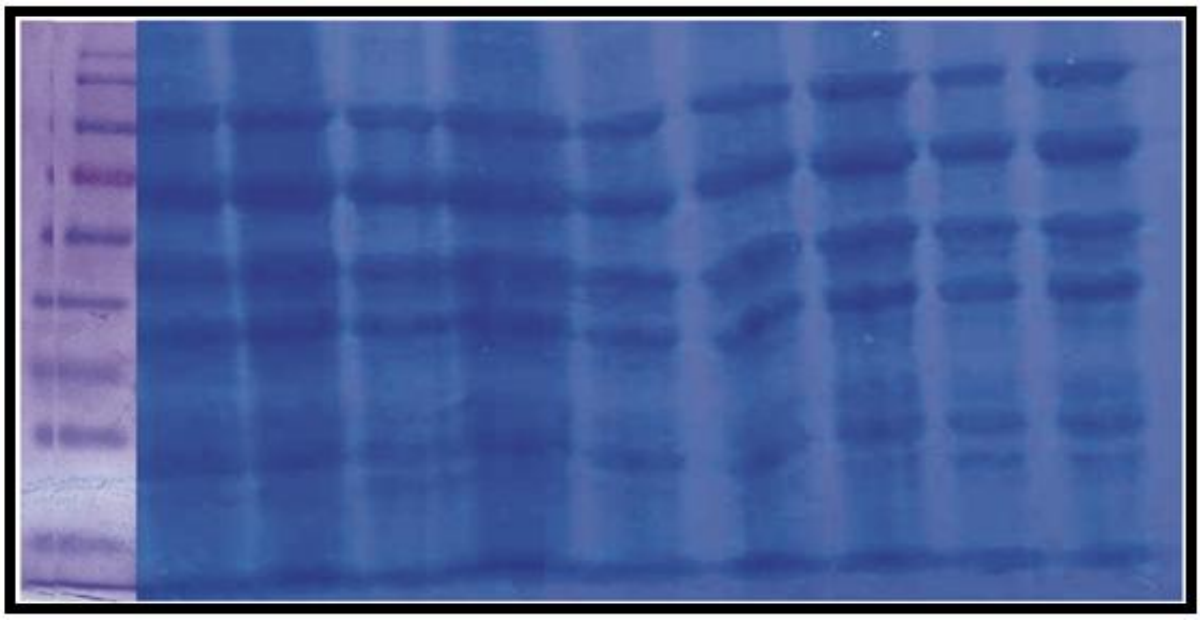

Fig. 2. Tree diagram of SDS-PAGE of 9 barberry ecotypes.

The SDS-PAGE techniques have more advantages in the classification of genotypes (Abd-El-Zaher*et al.*, 2006).The SDS-PAGE technique is also extensively used in taxonomic and assessment of genetic diversity (Abd El-Hady *et al.,* 2010). The present investigation revealed variation in the 9 barbery ecotypes. In our experiment we evaluated 9 barberry ecotypes having diverse ecological background through SDS-PAGE slab type method using 12.25% polyacrylamide concentration. A standard molecular weight marker (Page Ruler/ Prestained protein Ladder) were used for computing the molecular weights of the different protein bands. SDS-PAGE analysis revealed protein bands with different molecular weights. Some ecotypes possessed some bands which were absent in other varieties. The data was scored for the presence (1) and absence (0) of the bands. Based on the results of electrophoretic band spectra, similarity index was calculated for all possible pair of electrophoregrams.

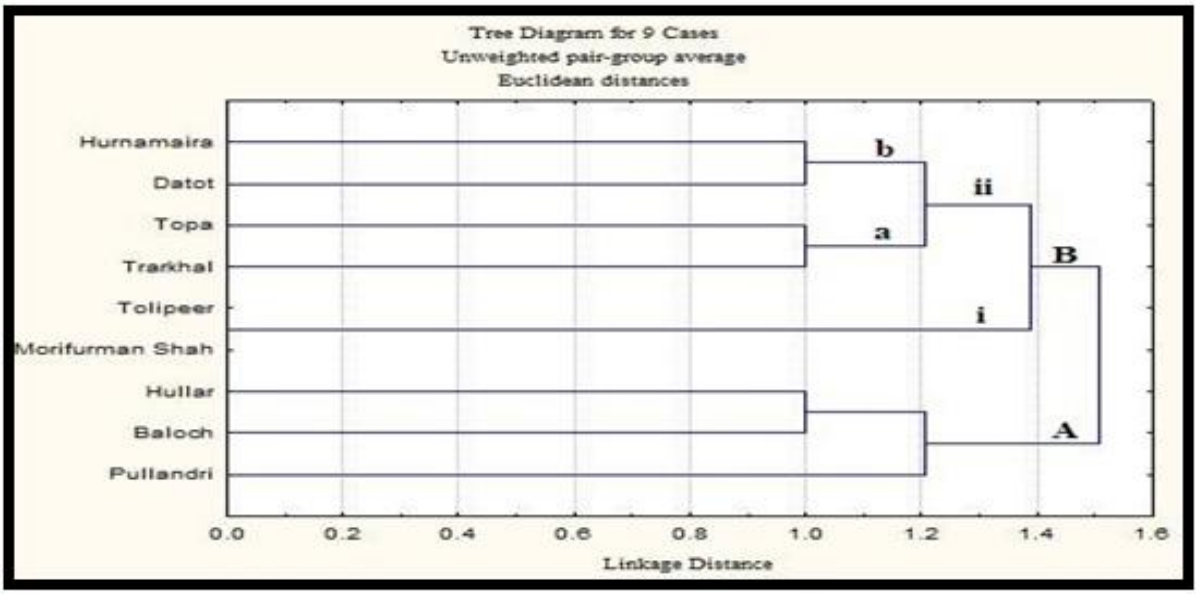

Fig. 2. Tree diagram of SDS-PAGE of 9 barberry ecotypes.

In Fig 1.photograph of the gel showed protein profiles of 9 barberry ecotypes. The banding pattern drawn from the photograph of gel, indicated variation among ecotypes on molecular level. Bands were visible at variable distance in the gel.

In Fig 2.two main clusters A and B were found on the basis of SDS-PAGE. Cluster A consists of three ecotypes i.e., Pullandri, Baloch and Hullar. From these three ecotypes Pullandri showed maximum variation and present as out lier in this cluster while Hullar and Baloch are interlinked. Main cluster B is further divided into two sub clusters I and II. Cluster I consists of consists of two ecotypes Tolipeer and Morifurman Shah they were genetically same and showed maximum diversity amongst other ecotypes. Cluster II is further divided into two sub sub clusters a and b. Sub sub cluster a consists of two ecotypes Trarkhal and Topa they are distinctly related. Sub sub cluster b consists of two ecotypes Datot and Hurnamaira that are distinctly related at the linkage distance of 1.0. Molecular studies greatly varied from the morphological and biochemical studies it may be due to that morphological and biochemical characters can be influenced by the environment. Molecular studies revealed that ecotypes Tolipeer, Morifurman Shah and Pullandri showed maximum variation.

Conclusion

SDS-PAGE technique was used to identify molecular diversity of available ecotypes of berberis (*Berberis lycium* L.). Molecular analysis involves the use of molecular techniques for assessing genetic diversity of plant germplasm. Protein extraction was done from

seeds. On the basis of molecular studies it was concluded that ecotypes Pullandri, Tolipeer and Morifurman Shah are genetically more diverse amongst the others and they can be further exploited in the future projects.

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