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MORPHOLOGY AND PHYLOGENY OF IMPORTANT MEDICINAL PLANT BERBERIS LYCIUM ROYLE BASED ON *MATK, RBCL, ITS* AND *TRNH-PSBA* FROM AZAD JAMMU AND KASHMIR

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Abstract

Berberis lyceum Royle has a long history of medicinal uses to treat different diseases. Dry fruits and roots of this species are medicinally important and are extensively used in many parts of the world. The samples of this species were randomly collected from five districts of Azad Kashmir, Pakistan, including 35 locations. In this study fruits, leaves, stem, roots, and thorn of *B. lyceum* were used. The morphological studies were conducted to evaluate its qualitative and quantitative traits. For genomic analysis, DNA was extracted from fresh leaves and confirmed on 1 % agarose gel electrophoresis. Fifteen samples were selected for phylogenetic analysis by using four markers including *matK, rbcL, ITS,* and *trnH-psbA*. Morphological data showed a difference in their values due to the variations in their altitude, climatic conditions, and soil texture. The phylogenetic study and sequence demarcation tool (SDT) analysis revealed that *B. lycium* Royle sequences identified in the current study are genetically very similar to each other and they developed the distinct clade with very close isolates previously reported and their pairwise sequence identity (PSI) score is more than 99% among themselves. All the genetic markers (*matK*, *rbcL*, *ITS* and *trnH-psbA*) successfully clustered the sequences and revealed that these markers can be used for species authentication of *B. lycium*. The 3D protein structural models for *matK* protein sequences were predicted through I-TASSER. Models having the highest C-score were selected for Ramachandran plot analysis and indicated that 3D protein models of selected samples of *B. lyceum* were satisfactory. The findings of the current study are very important for the future identification and conservation of this medically important species in the region.

Key words: *Berberis lycium*, Morphological attributes, Phylogenetic analysis, Soil texture, Altitude.

Introduction

Genus *Berberis* belongs to family *Berbaredaceae* (Bhattacharjee, 2001; Bhardwaj & Kaushik, 2012). It is spiny, semi-deciduous, and hermaphrodite plant found in Asia and other part of the world. The plant shows variations in the phytochemical and morpho-pathological parameters including stem, leaves, berry color and size due to environmental changes of specific area (Khan *et al*., 2014a; Neag *et al*., 2018). *B. lycium* is an economically and medicinally important species widely distributed Pakistan, Afghanistan, India (Himalayas) region (Chand *et al*., 2007; Asis *et al*., 2007; Gulfraz *et al*., 2007, 2008; Ahmed *et al*., 2009; Ahmad *et al*., 2011; Irshad *et al*., 2013; Khan *et al*., 2016). In Pakistan, it is found on the mountain ranges, especially in Kashmir and North West Himalayan area between 2000-2700 m altitude (Khan *et al*., 2014c). The plant starts flowering in March and fruits ripen in May (Sood *et al*., 2013). The inflorescence is a raceme with 8-15 and alternatively arranged on branches. The branches and stem are greyish in color, spines are 1cm long (Ahmed *et al*., 2009; Kulkarni *et al*., 2012). The species shows maximum morphological and phytochemical variations making it a taxonomically difficult species (Khan *et al*., 2014a). Overlapping characters, especially in leaves, bark thickness, flower color, and berry size cause ambiguity in the field identifications (Tiwari & Adhikari, 2011; Lucas *et al*., 2012). Many macro-morphological parameters, histological characteristics and microscopic examinations were carried out for authentication of species of this genus (Yan *et al*., 2007; Yip *et al*., 2007), but these were not as reliable as molecular investigations. DNA-based markers used in molecular genetic studies are becoming popular because genetic configuration is less affected by environmental factors, physiological conditions, age, harvest, processing and storage processes. Identification and phylogeny have been done by using the sequence variations to develop specific markers (Balasubramani *et al*., 2011). The DNA barcoding technique is used to identify the species with the help of small sequences of DNA. These molecular techniques prove useful in many applications comprising; large-scale biodiversity surveys and discriminating forest species with high confidence (Hollingsworth *et al*., 2011). The most promising DNA barcode loci ITS (nuclear genome) and *matK, trnH-psbA* and *rbcL* (plastid genome) have been used while investigating the Indian *Berberis* species and other genera (Kim *et al*., 2004). *ITS* and *trnH-psbA* has exhibited high authentication power for all species where as *matK* and r*bcL* are not applicable for all species (Roy *et al*., 2010).

Our aim in this study was to explore the morphological and phylogenetic parameters of *B. lycium* collected from different districts of Azad Kashmir. The data generated from this work can prove helpful for many other researchers and surveyors to achieve their research goals and similarly to the pharmaceutical industries for producing herbal medicines (Yeşilada & Küpeli, 2002; Srivastava *et al*., 2004; Rashmi *et al*., 2008; Singh *et al*., 2009; Rahimi *et al*., 2014; Pradhan & Saha, 2016; Ozturk & Hakeem, 2018, 2019 a,b; Ozturk *et al*., 2020).

Materials and Methods

This study was carried out for the assessment of morphological and phylogenetic parameters of *Berberis lycium* Royle from five districts of Azad Kashmir region namely; Muzaffarabad, Hattian, Bagh, Poonch and Neelum. Three surveys were conducted to collect comprehensive data regarding flowering season, and fruits in post harvesting season. The places were selected on the basis of differences in their altitude and climatic features.

Morphological study: Ten morphological parameters were investigated from five districts including 35 sites (ecotypes) of AJ&K; 7 sites were selected from District Muzaffarabad & Hattian, 3 from District Bagh & Poonch and 15 from District Neelum. Three plants were randomly selected from specific locations of five districts. These were identified and evaluated with the help of information published in the Flora of Pakistan (2011). The data was recorded in cm together with the colour of fruits and leaves as well as number of leaves. The morphological studies included; fruit colour, fruit size, leaf colour, leaf size, tiller length, tiller number, number of leaves and thorn, thorn size and root width.

Phylogenetic study: Four barcode loci (*matK, rbcL, ITS* and *trnH- psbA*) were used in the study and 15 accessions were collected from each barcode loci.

Primers designing for sequencing: Primer-3 software was used for the primer designing of four genes of *B. lycium*. The primer sequences were;
matK F: TCATGTATATGAATGCGAATCG
R: CCAATCAAAGTAATTATTGGG
rbcL F: AAGCAGGGGCCGCTGTAGCTG
R: AAATGGTTGGGAGTTCACGT
ITS F: AAAGACCCGCGAACTTGTGAAC
R: AGGTGAGTGCTAGATGCAAC
trnH-psbA F: ATTCAATTTTTTCTACTTGTAT
R: TACGAGTCATTGAACTTGCAG

DNA extraction, amplification, sequencing and phylogenetic and SDT analysis: Total genomic DNA was extracted from fresh leaves following "Thermo Scientific Kit" method and confirmation of DNA was held on 1% agarose gel. PCR amplification was done by using thermo cycler (Simpli Amp) and a total of four sequencing primers were used for the plant. In the case of DNA segments amplification, total volume of reaction mixture was 25µl, amplification of all optimized primers was done by using 0.5 mM µL dNTP's, 0.05 units/µL of Taq polymerase,1.5 mM $MgCl_2$ and 10 pico moles primer. Genetic analyser (ABI Prism 3100) was used for the bidirectional nucleotide sequencing of *B. lyceum* genes. BioEdit programme (http://www.mbio.ncsu.edu/BioEdit) was used for editing the sequences for nucleotides variations. The ClustalW in MegAlign programme of laser gene (DNA STAR Inc., Madison, WI, USA) was used to align the nucleotide sequences and the NCBI Genbank source was used for taking universal sequences (http://www.ncbi.nlm.nih.gov/genomes).

The morphological characters were studied before to know the morphological relationship of *Berberis* species (Bhat *et al*., 2010). The Basic Local Alignment Search Tool (BLAST) of Genbank/NCBI was used to identify the *Berberis* species. To determine the divergence of sequences among the species of AJ&K, multiple sequence alignments were done. Maximum likelihood and Neighbour-joining methods were used to produce phylogenetic tree. The indels and gaps in all positions were excluded. The MEGA 7.0.20 software (Kumar *et al*., 2016) was used to reconstruct the pgylogneric tree using bootstrap method (with 1000 replications). SDT (sequence demarcation tool) analysis was carried out by using the SDTv1.2 program (Muhire *et al*., 2014) with defult setting. The pairwise sequence identity (PSI) values based three colored matrix is presented here.

Protein structural analysis: The protein 3D structural models of *matk* gene were constructed, though I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/).

Results and Discussions

Morphological studies were carried out on the samples collected from 35 places (ecotypes – as morphologically they show some variations as shown in the Fig. 1) selected from five districts of AJ&K. It included two qualitative traits i.e., leaf color and fruit color, selected for this study (Fig. 1).

Fig. 1. Representative plants of *B. lycium* Royle located in different areas of AJ&K used in the current study.

Qualitative traits

1. Leaf color
   The color of *B. lycium* leaves collected from 5 districts ranged from light green to green.

2. Fruit color
   The fruit color of *B. lycium* collected from 5 districts was light purple, purple and black (sup Table 1).

Quantitative traits: In the present study 8 quantitative traits were evaluated; leaf length, root width, fruit size, thorn length, tiller length, number of leaves, number of thorns and tillers. The leaf length for 35 ecotypes showed variations in the range of 1.1±0.022 cm to 4.4±0.08cm. The maximum leaf length was recorded in the ecotypes of District Muzaffarabad (Dhaman Jholi 4.4±0.08 cm), and minimum leaf length in District Neelum (Keran 1.1±0.022 cm). The highest value of thorn size was found in District Muzaffarabad (Ranjhata 2.16±2.1 cm) while minimum value was found in District Neelum (Keran 0.76±0 cm). These findings confirmed the data published by Khan *et al*., (2014c). The maximum value of root width was observed in District Muzaffarabad (Chanjhal 3.049±0.94 cm) and the minimum root width in District Neelum (Neelum 0.71±0.27 cm). Sood *et al*., (2013) have recorded that the diameter of root is 3-8 cm and the fruit is 7 mm long. The size of fruits showed variations in the range of (0.507±0 cm to 1.778±0 cm) in District Neelum (Bagna-Kalis). The maximum value of tiller length was found in District Bagh (Bagh 770.4±5.78 cm) while minimum value was recorded in District Neelum (Keran 36.1±2.72 cm) (sup Table 2). These findings confirm those reported by Khan *et al*., (2014b). The maximum value for number of leaves was noted in District Muzaffarabad (Copra Gali 683±47.6), while minimum value was found in District Neelum (Keran 122±3.48). The maximum number of thorns was recorded in District Muzaffarabad (Garhi Dupatta 426±72.5) and minimum in District Neelum (Keran 22±1.8). These findings are in accordance with the previous published data (Hussain *et al*., 2015). The highest value of number of tillers was recorded in District Muzaffarabad (Serli Sacha 10±1.2) but minimum in District Neelum (Ethaie 3±0) (sup Table 3). The morphological parameters exhibited remarkable differences due to the variations in their altitude, climatic conditions and soil texture as suggested by Ibrar *et al*., (2007) as well.

Comparison between the morphological traits of *B. lycium* R using ANOVA: The positive and significant correlation was seen in leaf length, root width, fruit size, tiller length, leaf number and thorn number. An insignificant correlation was noted in thorn size and number of tillers in different samples collected from 35 ecotypes (Table 1).

Extraction of genomic DNA: Fifteen samples of *B. lycium* were selected, and total genomic DNA extracted from fresh leaves following Thermo Scientific Kit method. The confirmation of DNA was held on 1 % agarose gel (Fig. 2).

Supplementary Table 1. Qualitative traits of *B. lycium* Royle collected from five districts of AJ&K.

S. No	Places	Leaf color	Fruit color
1.	Serli Scha	Light green	Purple
2.	Copra Gali	Light green	Light purple
3.	Sadbun	Light green	Light purple
4.	Chanjhal	Light green	Purple
5.	Bakreyali	Light green	Purple black
6.	Ranjhata	Light green	Light purple
7.	Daman Jholi	Green	Purple
8.	Haryala	Light green	Purple
9.	Subhai Mali	Light green	Purple
10.	Sheesha Mali	Light green	Purple
11.	Grhi Duptta	Light green	Purple
12.	Kaalis	Light green	Light purple
13.	Sarran Chattian	Light green	Light purple
14.	Gori Syedan	Light green	Purple
15.	Bagh	Light green	Light purple
16.	Qadrabad	Light green	Light purple
17.	Arja	Light green	Purple
18.	Rawlakot	Light green	Purple
19.	Khrick	Light green	Purple
20.	Banjhosa	Green	Purple
21.	Neelum	Green	Purple
22.	Ziarat	Light green	Purple
23.	Thangar	Light green	Light purple
24.	Chinar Pura	Green	Purple
25.	Shahkot	Light green	Light purple
26.	Bagna	Green	Purple
27.	Medan Syedan	Green	Purple
28.	Lawat	Light green	Purple
29.	Kundal Shahi	Green	Purple
30.	Sathrian	Green	Light purple
31.	Laala	Green	Light purple
32.	Palang	Green	Purple
33.	Keran	Light green	Light purple
34.	Ethaie	Green	Light purple
35.	Slam Pura	Green	Purple

Phylogenetic and sequence demarcation tool (SDT) analysis: The phylogenetic analysis of *matK*, *rbcL*, *ITS* and *trnH-psbA* gene was done to infer the relationship of the current isolates with the previously reported isolates of *B. lycium*. Fifteen sequences of each gene of *B. lycium* were were analysed with the sequences retrieved from the NCBI after BLASTn of these sequences. The phylogenetic analysis of the sequences of each gene identified in the current study showed that they all made the same group that means all the sequences are very close to each other. On the basis of *matK* gene sequences, the phylogenetic tree is shown in Fig. 3(a) whereas their SDT analysis results in the form of three colored matrix is shown in Fig. 3(b). On the basis of *rbcL* gene, the phylogenetic tree and SDT matrix are shown in Fig. 4(a) and (b). On basis of *ITS* gene, the phylogenetic tree and SDT results are shown in the Fig. 5(a) and (b) respectively. On the basis of *trnH-psbA* gene, the phylogenetic tree and three colored matrix is shown in Fig. 6(a) and (b) respectively. The overall phylogenetic and SDT analysis of all these genes show very close relationship among all isolates of the current study as well as their close relatedness with *B. lycium* Royle isolates from Indian origin.

Supplementary Table 2. Quantitative attributes of *B. lycium* Royle collected from five districts of AJ&K.

S. No.	Places	Leaf length (cm)	Root width (cm)	Fruit size (cm)	Thorne size (cm)	Tiller length (cm)
1.	Serli Scha	3.43 ± 0.20	2.54 ± 0.43	0.762 ± 0	1.15 ± 0.025	163.9 ± 15.7
2.	Copra Gali	3.9 ± 0.22	1.38 ± 0.11	1.27 ± 0	1.53 ± 0.08	215.2 ± 8.11
3.	Sadbun	3.7 ± 0.22	1.94 ± 0.91	1 ± 0	1.32 ± 0.02	97.3 ± 4.88
4.	Chanjhal	3.4 ± 0.11	3.049 ± 0.91	1.778 ± 0	1.41 ± 0.14	132.3 ± 1.98
5.	Bakreyali	3.0 ± 0.42	2.14 ± 0.22	1.27 ± 0	1.26 ± 0.09	119.0 ± 20.79
6.	Ranjhata	3.3 ± 0.05	1.09 ± 0.05	1.016 ± 0	2.16 ± 2.1	123.9 ± 10.1
7.	Daman Jholi	4.4 ± 0.08	1.29 ± 0.14	1.27 ± 0	1.30 ± 0.31	84.9 ± 1.56
8.	Haryala	3.5 ± 0.05	1.24 ± 0.07	1.522 ± 0	1.32 ± 0.07	148.9 ± 9.1
9.	Subhai Mali	3.7 ± 0.155	1.35 ± 0.05	1.27 ± 0	1.29 ± 0.05	177.2±6.27
10.	Sheesha Mali	4.3 ± 0.24	1.29 ± 0.05	1.016 ± 0	1.52 ± 0.09	199.2±18.5
11.	Garhi Duptta	3.5 ± 0.24	1.32 ± 0.1	1.016 ± 0	1.43 ± 0.05	156.8±3.19
12.	Kaalis	3.9 ± 0.22	1.29 ± 0.12	1.524 ± 0	1.29 ± 0.15	154.0±41.3
13.	Sarran Chattian	2.8 ± 0.31	1.32 ± 0.1	1.016 ± 0	1.01 ± 0.13	73.9±4.39
14.	Gori Syedan	3.0 ± 1.04	1.74 ± 0.41	1.185 ± 0.084	1.015 ± 0.048	90±28.1
15.	Bagh	4.0 ± 0.19	0.95 ± 0.1	0.762 ± 0	0.87 ± 0.07	770.4±577.8
16.	Qadrabad	3.7 ± 0.07	1.07 ± 0.07	0.508 ± 0	1.07 ± 0.125	159.4±8.80
17.	Arja	3.7 ± 0.12	1.153 ± 0.15	0.762 ± 0	1.38 ± 0.20	185.8±95.2
18.	Rawlakot	3.8 ± 0.16	1.29 ± 0.09	1.523 ± 0	1.38 ± 0.16	365.1±28.6
19.	Khrick	3.7 ± 0.12	1.294 ± 0.06	0.509 ± 0	1.49 ± 0.14	372.8±27.6
20.	Banjhosa	3.6 ± 0.028	1.18 ± 0.09	1.016 ± 0	1.24 ± 0.07	188.8±20.5
21.	Neelum	3.4 ± 0.08	0.79 ± 0.21	1.016 ± 0	0.98 ± 0.07	101.3±5.9
22.	Ziarat	3.2 ± 0.05	1.10 ± 0.08	0.762 ± 0	0.98 ± 0.14	116.8±19.2
23.	Thangar	3.3 ± 0.15	1.26 ± 0.09	1.016 ± 0	1.00 ± 0.27	126.1±2.23
24.	Chinar Pura	3.2 ± 0	0.71 ± 0.27	1.012 ± 0	1.27 ± 0	157.5±1.74
25.	Shahkot	3.4 ± 0.13	2.79 ± 1.16	0.763 ± 0	1.18 ± 0.37	132.3±1.98
26.	Bagna	2.7 ± 0.61	1.66 ± 0.60	0.507 ± 0	1.26 ± 0.08	97.0±8.32
27.	Medan Syedan	3.8 ± 0.65	1.60 ± 0.33	1.016 ± 0	1.52 ± 0.254	110.2±17.0
28.	Lawat	3.1 ± 0.22	1.32 ± 0.06	1.016 ± 0	1.15 ± 0.07	97.6±10.7
29.	Kundal Shahi	2.7 ± 0.22	2.28 ± 0.25	1.28 ± 0	1.26 ± 0.13	105.8±24.5
30.	Sathrian	3.4 ± 0.08	1.26 ± 0.05	1.26 ± 0	1.25 ± 0	105.8±24.5
31.	Laala	3.2 ± 0.06	1.24 ± 0.03	1.015 ± 0	1.27 ± 0	89.1±2.76
32.	Palang	3.2 ± 0.05	1.01 ± 0	1.31 ± 0	1.26 ± 0	85.5±4.21
33.	Keran	1.1 ± 0.22	1.35 ± 0.05	0.931 ± 0.085	0.76 ± 0	36.1±2.72
34.	Ethaie	3.5 ± 0	1.35 ± 0.3	0.762 ± 0	0.85 ± 0.05	83.8±2.45
35.	Slam Pura	3.1 ± 0.12	1.2 ± 0.14	1.21 ± 0.037	1.24 ± 0.03	71.4±6.02

Table 1. ANOVA analysis of *B. lycium* Royle collected from five Districts of AJ&K.

S. No	Quantitative attributes	SS	DF	MS	F	P value
1.	Leaf length*	32.27	34	0.949	3.865	$p<0.0001$
2.	Root width*	28.44	34	0.8364	2.727	$p=0.0002$
3.	Fruit size *	9.622	34	0.283	209.7	$p<0.0001$
4.	Thorn size	13.4979	34	3970	0.9961	$p=0.4916$
5.	Tiller length*	1.66	34	48773	1.623	$p<0.0001$
6.	Leaf amount*	1.04	34	304397	2.991	$p<0.0001$
7.	Thorn amount*	4.74	34	139446	5.374	$p<0.0001$
8.	Tiller amount	4190	34	123.2	1.461	$p=0.0910$

*Correlation is significant ($p<0.05$)

Table 2. Base composition of Four Bar Code Loci by using *MEGA* 6.06.

Base composition (%)	A	T	G	C	AT contents	GC contents
matK	31.0	31	15.0	22.9	67.3	37.9
rbcL	25.1	31	22.9	20.9	56.1	43.8
ITS	22.0	25.9	27.2	24.8	47.9	51.7
trnH-psbA	42.6	30.0	15.3	12.1	72	27.4

Supplementary Table 3. Amount of leaf, thorn and tiller of *B. lycium* Royle collected from five districts of AJ&K.

S. No.	Places	Leaf amount	Thorne amount	Tiller amount
1.	Serli Scha	484±101.4	218±47.3	10±1.2
2.	Copra Gali	683±47.6	369±70.7	9±1.4
3.	Sadbun	572±39.5	230±18.2	7±1.1
4.	Chanjhal	594±115.5	215±22.1	8±2
5.	Bakreyali	424±58.4	207±30.5	6±0.8
6.	Ranjhata	296±19.9	145±64.2	6±0.3
7.	Daman Jholi	370±29.0	97±12.6	7±0.3
8.	Haryala	517±16.7	303±1.45	7±0.5
9.	Subhai Mali	547±16.7	265±24.0	9±1.5
10.	Sheesha Mali	611±83.1	350±53.2	9±1.6
11.	Garhi Duptta	615±79.8	426±72.5	7±0.6
12.	Kaalis	492±87.17	239±59.3	7±0.9
13.	Sarran Chattian	283.7±61.4	102±33.3	5±0.7
14.	Gori Syedan	250.3±65.7	122±54.5	7±0.6
15.	Bagh	358.3±2.90	170±5.04	8±0.3
16.	Qadrabad	454.3±69.1	227±49.3	6±0
17.	Arja	460.7±76.6	256±13.4	6±0.6
18.	Rawlakot	518.3±52.0	248±9.6	5±0.7
19.	Khrick	536±59.6	256±13.4	7±0.6
20.	Banjhosa	514±82.8	284±22.5	6±0.3
21.	Neelum	368.7±14.9	199±26.1	4±0
22.	Ziarat	333.3±83.6	211±36.1	5±0.6
23.	Thangar	406.3±32.8	229±49.1	7±0.3
24.	Chinar Pura	4587.9	289±5.2	7±0.2
25.	Shahkot	546±108.1	219±25.8	7±0.5
26.	Bagna	521±101.0	254±22.2	6±0.8
27.	Medan Syedan	163±7.5	155±1.5	4±0.3
28.	Lawat	391±55.4	84±15.6	5±0.3
29.	Kundal Shahi	455±116.3	210±24.0	6±0.3
30.	Sathrian	466±3.75	267±22.0	8±2
31.	Laala	171±10.4	90±30.8	6±0.3
32.	Palang	304±86.4	128±1.7	5±0.3
33.	Keran	122±3.48	22±1.8	5±0.7
34.	Ethaie	334±12.7	138±6.0	3±0
35.	Slam Pura	334±107.9	93±24.9	5±0.5

Supplementary Table. 4 Analysis of four barcode loci of *B. lycium* Royle by using *MEGA 6.06* and BLAST method for four barcode loci of *B. lycium* Royle.

Items	*mat-k*	*rbcL*	*ITS*	*psbA-trnH*
No. of sequences	15	15	15	15
Average length (bp)	486	433	567	404
Variables numbers	3	352	0	0
BLAST method (%)	99	99	99	99

The sequences of these genes identify *B. lycium* species and the results best matched with a similarity of more than 99 percent sequence similarity with the isolates of *B. lycium* species in databanks (Sup. Table 4). The NJ and ML, procedures showed almost similar associations. The number of nucleotides of *matK, rbcL, ITS* and *trnH-psbA* gene have shown similar base compositions in A, T, G, C percent in *B. lycium* species. The sequences were found generally AT rich as compared to GC contents (Table 2). The number of nucleotides in study sequences showed similar results with the study published by Iqbal *et al*., (2013). The detail of the sequences identified in the current study is mentioned in the Table 3.

The alignment of sequences studied was straight forwarded. The result showed similarity with the results of Roy *et al*., (2010). The mean length of *matK, rbcL, ITS* and *trnH-psbA* sequences were 486, 433, 567 and 404 bp respectively. The percentage variable sites were 3, 352, none for *ITS* and *trnH-psbA* (supp Table 4). A comparison was made for intraspecific distance of four barcode loci among *B. lycium* species. The intraspecific divergence of *matK* was higher (0.129) but lower in the case of *psbA-trnH* (0.000). The interspecific divergence of *psbA-trnH* was higher (0.447) and lower in the case of *matK* (0.002). In recent years many studies have used *ITS* sequences as genetic markers for various species at intra generic and generic levels (Dubouzet & Shinoda, 1999). *ITS* sequence-based is reliable and efficient DNA marker to classify *B. lycium* considered the relationships in Patagonian species of *Berberis* (*Berberidaceae*) based on the categorization of rDNA internal transcribed spacer sequences (Bottini *et al*., 2007).

Our results of *matK, rbcL, ITS* and *trnH-psbA* genes successfully clustered all the samples and showed that these genes can be used in for the identification of *B. lycium* species. Medicinal plants belonging to various geographical origins were effectively identified by using *matK* regions because of its high inter and intraspecific variability (Yan *et al*., 2008). According to CBOL Plant Working Group (2009), high altitude plants show successful PCR amplification rate because of another protein coding region of chloroplast genome *rbcL*. Less variation has been shown by *rbcL* region because of their less capacity to this region, when combined with *trnH-psbA,* it can give potential results (Kress & Erickson, 2007).

Protein structural analysis and validation: ExPASy (https://web.expasy.org/translate/) was used to translate nucleotide sequences into amino acid sequences. The protein 3D structural models of *matK* gene were constructed, though I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The protein model with higher C-score indicates high confidence and more reliable prediction. The structural models of (*matK* gene) protein of selected samples of *B. lycium* having highest C-score (shown in Fig. 7) were selected for further analysis. The Ramachandran plots were drawn through RAMPAGE (Fig. 8). Analysis of Ramachandran plots indicated that 3D protein models of selected samples of *B. lycium* species were satisfactory as they had ≥ 38.1 % to ≤ 43.1% amino acid residues that occurred in favored region, ≥ 26.9% to ≤ 31.9 % in allowed region and in outlier region ≤ 26.9% to ≤ 30.0% (Table 4).

Table 3. Detail of the sequences identified in the current study.

Sr. No.	Species	Gene name	Location of samples	Bp	Accession No.
1.	*Berberis lycium* Royle	*matK*	Ranjata, Muzaffarabad	488	MH198450
2.	*Berberis lycium* Royle	*matK*	Dhaman Jholi, Muzaffarabad	488	MH198451
3.	*Berberis lycium* Royle	*matK*	Chanjal, Muzaffarabad	488	MH198452
4.	*Berberis lycium* Royle	*matK*	Bakreyali, Muzaffarabad	488	MH198453
5.	*Berberis lycium* Royle	*matK*	Copra gali, Muzaffarabad	488	MH198454
6.	*Berberis lycium* Royle	*matK*	Serli Scha, Muzaffarabad	488	MH198455
7.	*Berberis lycium* Royle	*matK*	Patkai, Muzaffarabad	488	MH198456
8.	*Berberis lycium* Royle	*matK*	Bagna, Neelum	488	MH198442
9.	*Berberis lycium* Royle	*matK*	Thanger, Neelum	488	MH198443
10.	*Berberis lycium* Royle	*matK*	Shah Kot, Neelum	488	MH198444
11.	*Berberis lycium* Royle	*matK*	Lwat, Neelum	488	MH198445
12.	*Berberis lycium* Royle	*matK*	Haryala, Hattian	488	MH198446
13.	*Berberis lycium* Royle	*matK*	Sarran Chattian, Hattian	488	MH198447
14.	*Berberis lycium* Royle	*matK*	Garhi Dupatta, Hattian	488	MH198448
15.	*Berberis lycium* Royle	*matK*	Gori Syedan, Hattian	488	MH198449
16.	*Berberis lycium* Royle	*rbcL*	Ranjata, Muzaffarabad	448	MH142838
17.	*Berberis lycium* Royle	*rbcL*	Dhaman Jholi, Muzaffarabad	448	MH142839
18.	*Berberis lycium* Royle	*rbcL*	Chanjal, Muzaffarabad	448	MH142840
19.	*Berberis lycium* Royle	*rbcL*	Bakreyali, Muzaffarabad	448	MH142841
20.	*Berberis lycium* Royle	*rbcL*	Copra Gali, Muzaffarabad	448	MH142842
21.	*Berberis lycium* Royle	*rbcL*	Serli Scha, Muzaffarabad	448	MH142843
22.	*Berberis lycium* Royle	*rbcL*	Patkai, Muzaffarabad	448	MH142844
23.	*Berberis lycium* Royle	*rbcL*	Bagna, Neelum	448	MH142845
24.	*Berberis lycium* Royle	*rbcL*	Thanger, Neelum	448	MH142846
25.	*Berberis lycium* Royle	*rbcL*	Shah Kot, Neelum	448	MH142847
26.	*Berberis lycium* Royle	*rbcL*	Lwat, Neelum	448	MH142848
27.	*Berberis lycium* Royle	*rbcL*	Haryala, Hattian	448	MH142849
28.	*Berberis lycium* Royle	*rbcL*	Sarran Chattian, Hattian	448	MH142850
29.	*Berberis lycium* Royle	*rbcL*	Garhi Dupatta, Hattian	448	MH142851
30.	*Berberis lycium* Royle	*rbcL*	Gori Syedan, Hattian	448	MH142852
31.	*Berberis lycium* Royle	*ITS*	Ranjata, Muzaffarabad	536	MH198427
32.	*Berberis lycium* Royle	*ITS*	Dhaman Jholi, Muzaffarabad	536	MH198428
33.	*Berberis lycium* Royle	*ITS*	Chanjal, Muzaffarabad	536	MH198429
34.	*Berberis lycium* Royle	*ITS*	Bakreyali, Muzaffarabad	536	MH198430
35.	*Berberis lycium* Royle	*ITS*	Copra Gali, Muzaffarabad	536	MH198431
36.	*Berberis lycium* Royle	*ITS*	Serli Scha, Muzaffarabad	536	MH198432
37.	*Berberis lycium* Royle	*ITS*	Patkai, Muzaffarabad	536	MH198433
38.	*Berberis lycium* Royle	*ITS*	Bagna, Neelum	536	MH198434
39.	*Berberis lycium* Royle	*ITS*	Thanger, Neelum	536	MH198435
40.	*Berberis lycium* Royle	*ITS*	Shahkot, Neelum	536	MH198436
41.	*Berberis lycium* Royle	*ITS*	Lwat, Neelum	536	MH198437
42.	*Berberis lycium* Royle	*ITS*	Haryala, Hattian	536	MH198438
43.	*Berberis lycium* Royle	*ITS*	Sarran Chattian, Hattian	536	MH198439
44.	*Berberis lycium* Royle	*ITS*	Garhi Dupatta, Hattian	536	MH198440
45.	*Berberis lycium* Royle	*ITS*	Gori Syedan, Hattian	536	MH198441
46.	*Berberis lycium* Royle	*trnH-psbA*	Ranjata, Muzaffarabad	404	MK283650
47.	*Berberis lycium* Royle	*trnH-psbA*	Dhaman Jholi, Muzaffarabad	404	MK283651
48.	*Berberis lycium* Royle	*trnH-psbA*	Chanjal, Muzaffarabad	404	MK283652
49.	*Berberis lycium* Royle	*trnH-psbA*	Bakreyali, Muzaffarabad	404	MK283653
50.	*Berberis lycium* Royle	*trnH-psbA*	Copra Gali, Muzaffarabad	404	MK283654
51.	*Berberis lycium* Royle	*trnH-psbA*	Serli Scha, Muzaffarabad	404	MK283655
52.	*Berberis lycium* Royle	*trnH-psbA*	Patkai, Muzaffarabad	404	MK283656
53.	*Berberis lycium* Royle	*trnH-psbA*	Bagna, Neelum	404	MK283657
54.	*Berberis lycium* Royle	*trnH-psbA*	Thanger, Neelum	404	MK283658
55.	*Berberis lycium* Royle	*trnH-psbA*	Shahkot, Neelum	404	MK283659
56.	*Berberis lycium* Royle	*trnH-psbA*	Lwat, Neelum	404	MK283660
57.	*Berberis lycium* Royle	*trnH-psbA*	Haryala, Hattian	404	MK283661
58.	*Berberis lycium* Royle	*trnH-psbA*	Sarran Chattian, Hattian	404	MK283662
59.	*Berberis lycium* Royle	*trnH-psbA*	Garhi Dupatta, Hattian	404	MK283663
60.	*Berberis lycium* Royle	*trnH-psbA*	Gori Syedan, Hattian	404	MK283664

Table 4. Ramachandron scores of *matK* gene for three samples of *Berberis lycium* Royle.

Samples	Number of residues in Favoured region	Number of residues in Allowed region	Number of residues in Outlier region
B. lycium BI-1	43.1 %	26.9%	30.0 %
B. lycium BI-2	38.1 %	31.9 %	30.0 %
B. lycium BI-3	41.2 %	31.9 %	26.9 %

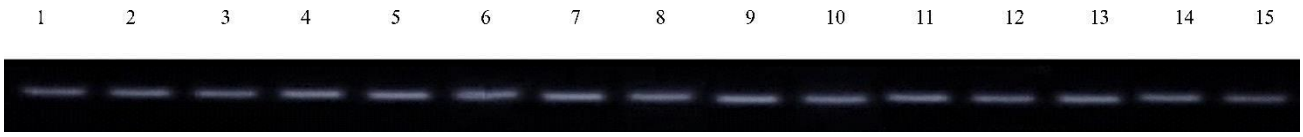

Fig. 2. The DNA isolated from fifteen samples of *B. lycium* Royle collected from five different districts of AJ&K. It was run on 1 % agarose gel.

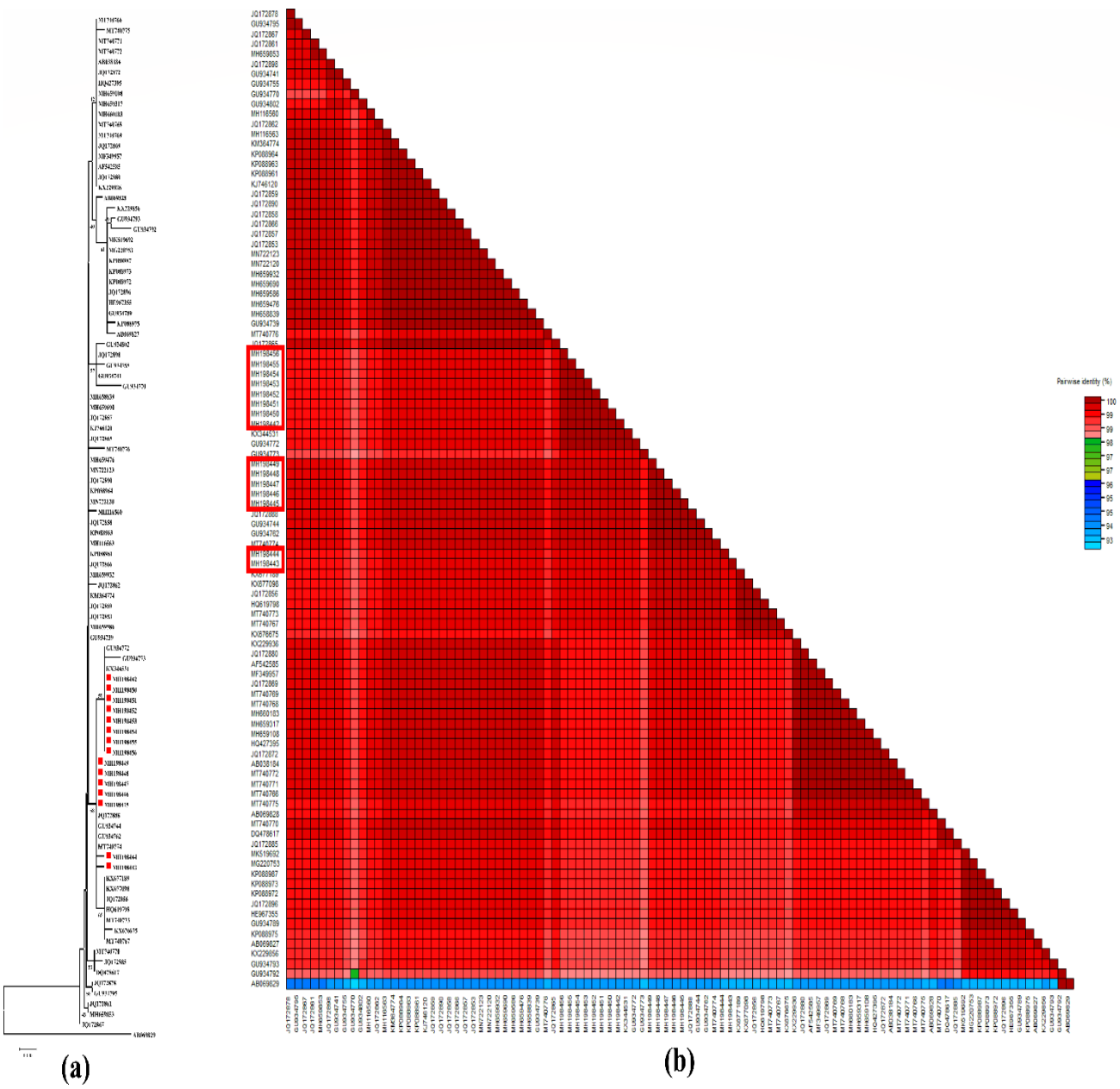

Fig. 3. The phylogenetic tree analysis and sequence demarcation tool (SDT) analysis of *matK* gene sequences. (a) The phylogenetic tree of fifteen *B. lycium* samples collected from study area of AJ&K. A neighbor-joining tree was reconstructed based on the *matK* gene using bootstrap method (with 1000 replicates) in MEGA 7.0.20 program. Isolates of the current study are labelled with red square and tree was rooted on an outgroup isolate (AB069829). Interestingly, all the isolates of the current study fell in the single major group with tree sub-groups (clades). (b) A three colored matrix was developed by sequence demarcation tool (SDT) by running the fasta sequences of all the isolates (that were used in the phylogenetic tree) using the default setting of the program (SDTv1.2). The color var is shown on the right side of matrix. The isolates of the current study are labelled with red rectangular on the lift side naming (only accession numbers) of the sequences.

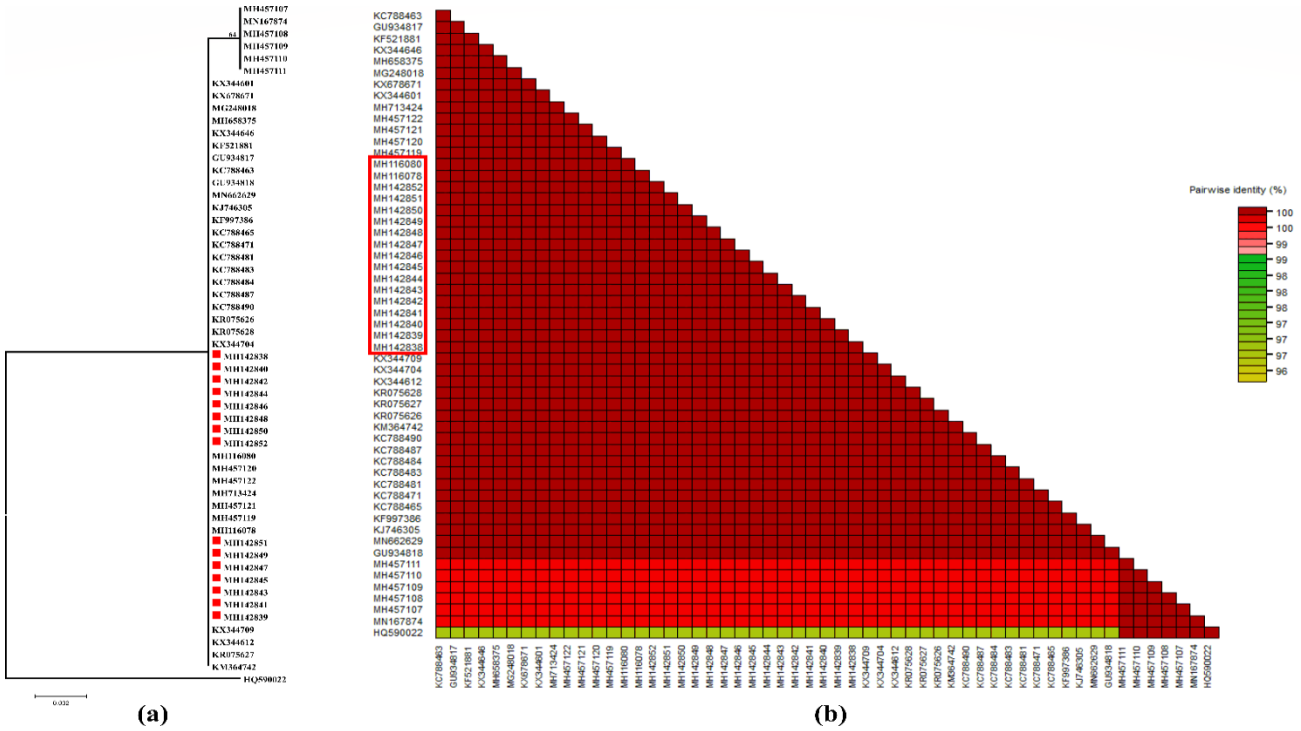

Fig. 4. The phylogenetic tree analysis and sequence demarcation tool (SDT) analysis of *rbcL* gene sequences. (a) The phylogenetic tree of fifteen *B. lycium* samples collected from study area of AJ&K. A neighbor-joining tree was reconstructed based on the *rbcL* gene using bootstrap method (with 1000 replicates) in MEGA 7.0.20 program. Isolates of the current study are labelled with red square and tree was rooted on an outgroup isolate (HQ590022). Interestingly, all the isolates of the current study developed a single clade with their very close sequences. (b) A three colored matrix was developed by sequence demarcation tool (SDT) by running the fasta sequences of all the isolates (that were used in the phylogenetic tree) using the default setting of the program (SDTv1.2). The color var is shown on the right side of matrix. The isolates of the current study are labelled with red rectangular on the lift side naming (only accession numbers) of the sequences.

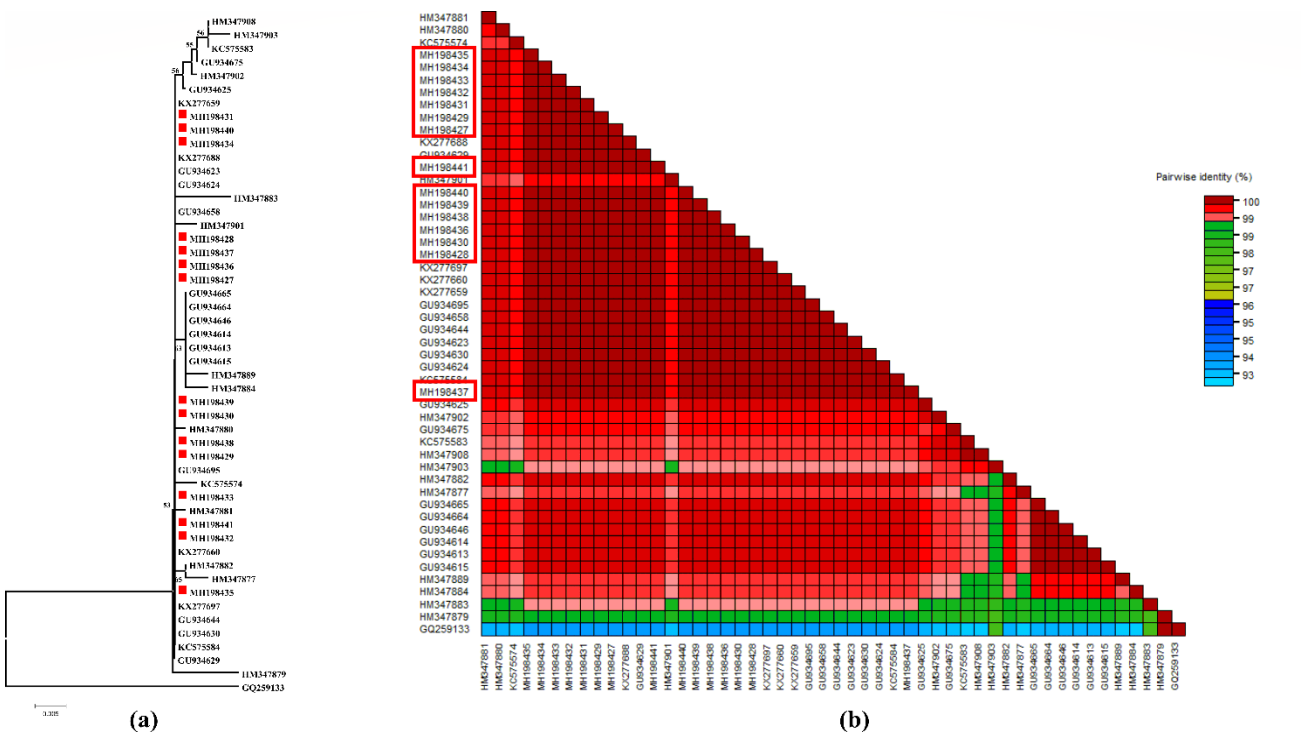

Fig. 5. The phylogenetic tree analysis and sequence demarcation tool (SDT) analysis of ITS gene sequences. (a) The phylogenetic tree of fifteen *B. lycium* samples collected from study area of AJ&K. A neighbor-joining tree was reconstructed based on the ITS gene using bootstrap method (with 1000 replicates) in MEGA 7.0.20 program. Isolates of the current study are labelled with red square and tree was rooted on an outgroup isolate (GQ259133). Interestingly, all the isolates of the current study fell in the single major group with many close isolates. (b) A three colored matrix was developed by sequence demarcation tool (SDT) by running the fasta sequences of all the isolates (that were used in the phylogenetic tree) using the default setting of the program (SDTv1.2). The color var is shown on the right side of matrix. The isolates of the current study are labelled with red rectangular on the lift side naming (only accession numbers) of the sequences.

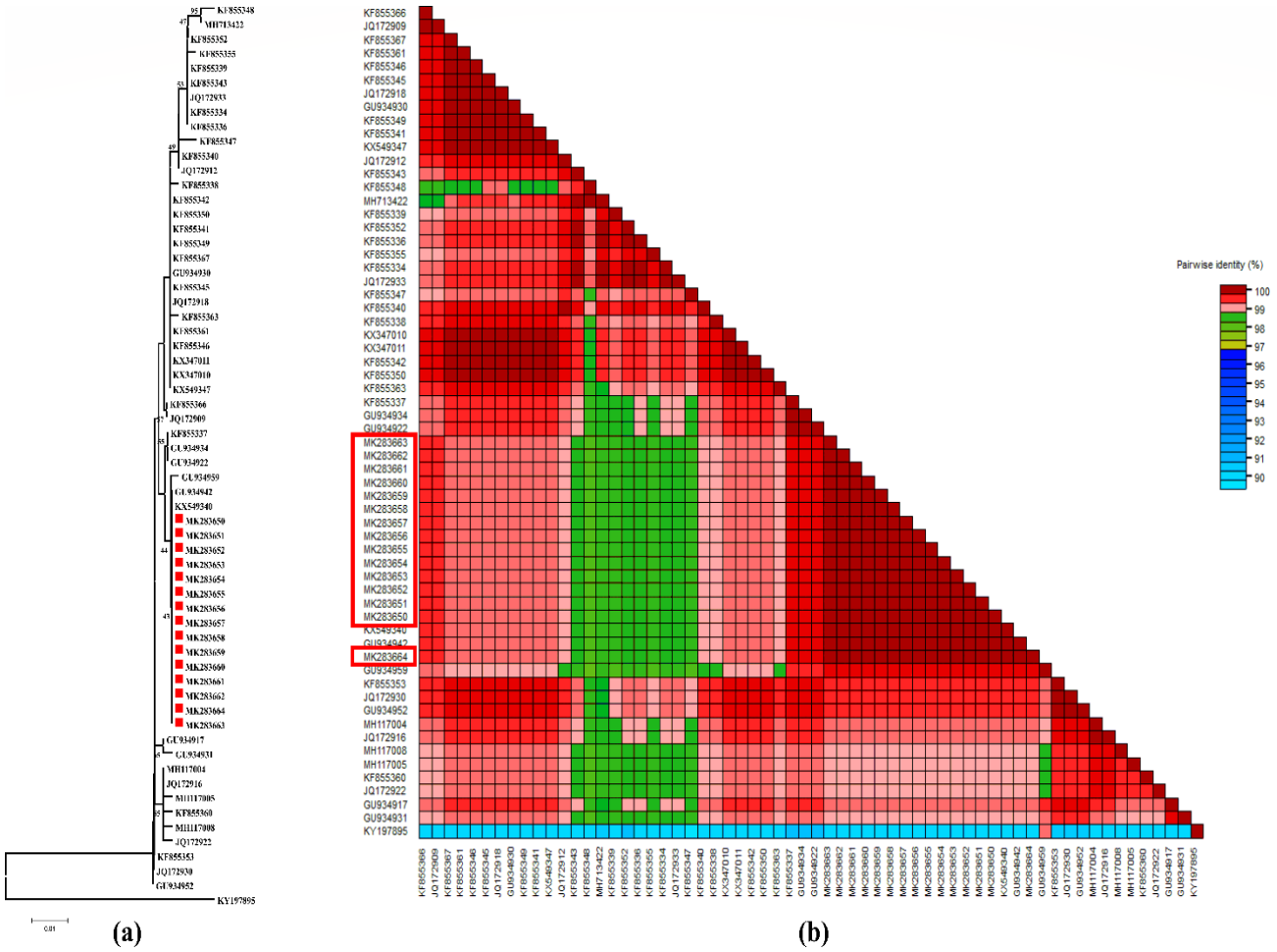

Fig. 6. The phylogenetic tree analysis and sequence demarcation tool (SDT) analysis of trnH-psbA gene sequences. (a) The phylogenetic tree of fifteen *B. lycium* samples collected from study area of AJ&K. A neighbor-joining tree was reconstructed based on the trnH-psbA gene using bootstrap method (with 1000 replicates) in MEGA 7.0.20 program. Isolates of the current study are labelled with red square and tree was rooted on an outgroup isolate (KY197895). Interestingly, all the isolates of the current study developed a single clade with two closest isolates. (b) A three colored matrix was developed by sequence demarcation tool (SDT) by running the fasta sequences of all the isolates (that were used in the phylogenetic tree) using the default setting of the program (SDTv1.2). The color var is shown on the right side of matrix. The isolates of the current study are labelled with red rectangular on the lift side naming (only accession numbers) of the sequences.

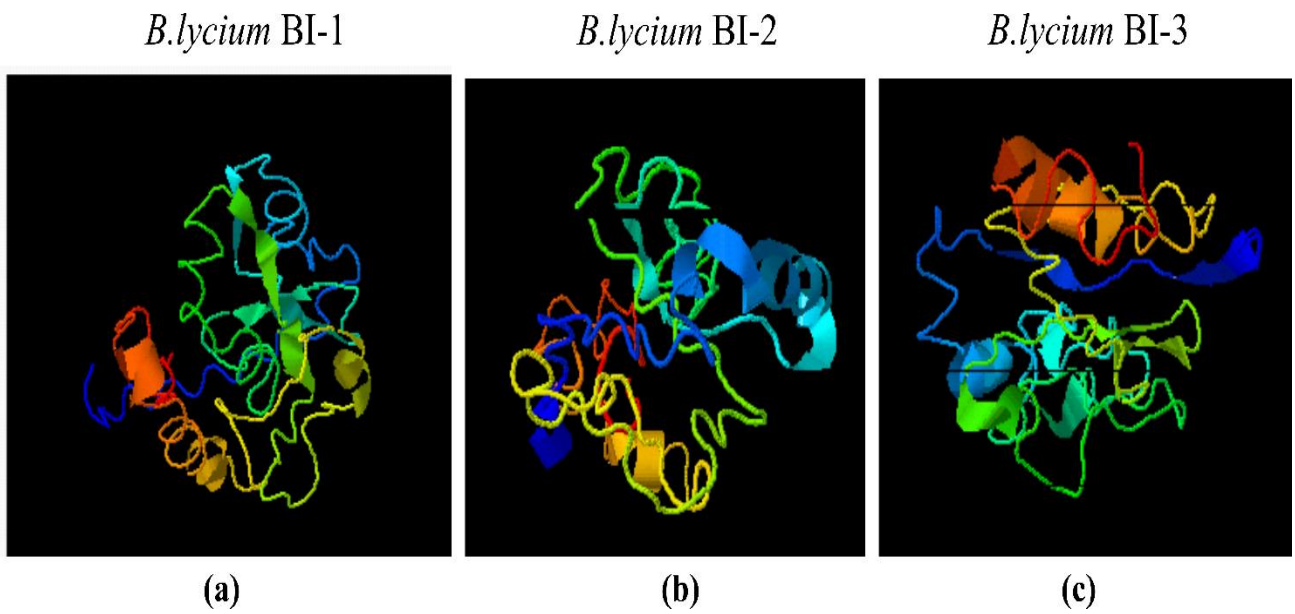

Fig. 7. The 3D protein structural models for *matK* protein of three sequences of *Berbris lycium* Royle predicted through I-TASSER.

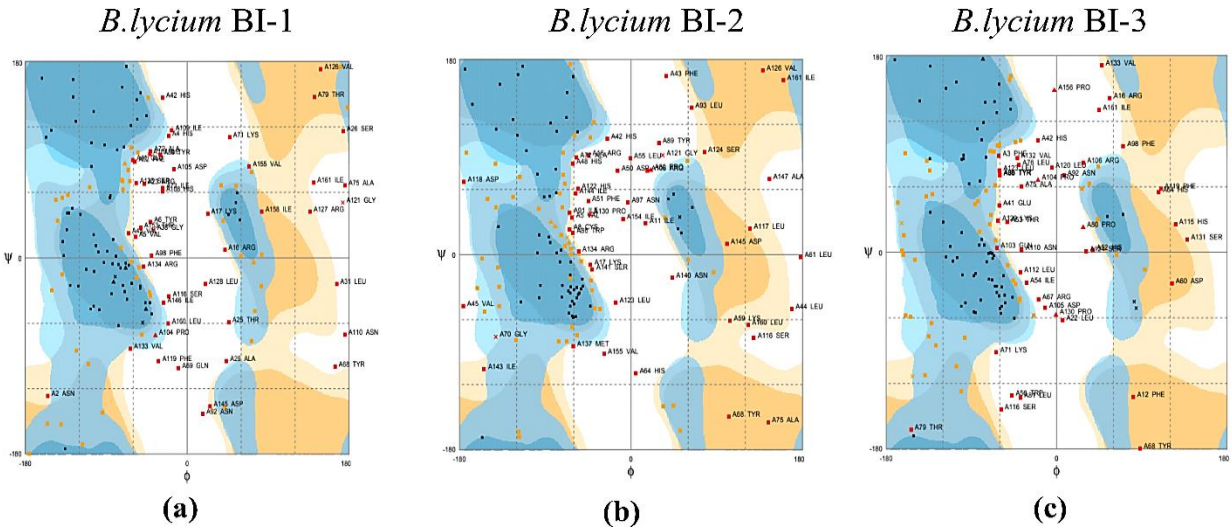

Fig. 8. Ramachandran plots of *matK* gene for same three sequences of *Berberis lycium* Royle. Models having highest C-score were selected for Ramachandran plot analysis.

Conclusion

Present investigation has shown that there is huge variation in their traits and mean values in the samples of the investigated species. The morphological parameters showed remarkable difference in the plant samples of *B. lycium* collected from five districts due to variations in their altitude, climatic conditions and soil texture. Phylogenetic study was conducted on 15 samples collected from the study area; 4 bar code loci were selected for this purpose. All four genes were successfully amplified. The 3D protein models constructed by I-TASSER and its validation through RAMPAGE predicted the good quality protein structural models for *matK* gene. The findings of the current study are very important for the future identification and conservation of this medically important plant species in the region.

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