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Biosystematic and molecular genetic study of *Berberis* (Berberidaceae) from North of Iran

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Keywords: epidermis, ITS, medicinal plant, pollen, SEM.

Summary. There are 500 species of *Berberis* in the Berberidaceae family, which are native to temperate and subtropical regions of Europe, Asia, Africa, North America, and South America. The present study investigates molecular relationships and micromorphology of pollen and epidermis of the *Berberis* in North of Iran, since it is a very important medicinal plant. This study examined 15 individuals from the Chahar Bagh region of Golestan province belonging to four species and one interspecific hybrid. Leaf epidermis and pollen grains were studied using light microscopy and scanning electron microscopy. In order to analyze the sequences of the ITS nuclear genome, various software programs were used. In the studied species, irregular, rounded, and polygonal epidermal cells were observed, along with anticline, smooth, and wavy wall patterns. In all species, the pollen is spherical and the pollen ornamentation are smooth in *B. integerrima*, granular in *B. vulgaris*, microperforate in *B. orthobotrys*, and microechinate in *B. crataegina*. Phylogenetic analysis shows high support for monophyly of *Berberis* species in the nrDNA ITS tree (PP = 1, ML BS = 100, MP BS = 100). Molecular and micromorphological features were found to be able to separate the five species in Chahar Bagh region. These results provide researchers with basic information to explore this valuable genus based on the findings of this study.

Биосистематика и молекулярно-генетическое исследование *Berberis* (Berberidaceae) на севере Ирана

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Ключевые слова: лекарственное растение, пыльца, СЭМ, эпидермис, ITS.

Аннотация. В семействе Berberidaceae насчитывается 500 видов *Berberis*, произрастающих в умеренных и субтропических регионах Европы, Азии, Африки, Северной и Южной Америки. В настоящем исследовании изучаются генетические взаимоотношения и микроморфология пыльцы и эпидермиса листьев видов рода на севере Ирана, поскольку это очень важное лекарственное растение. В ходе исследования были изучены 15 особей из региона Чахар-Баг провинции Голестан, принадлежащих к четырём видам и одному межвидовому гибриду. Эпидермис листьев и пыльцевые зерна *Berberis* изучали методами световой и сканирующей электронной микроскопии. Для анализа ITS последовательностей ядерного генома использовались различные программы. У изученных видов наблюдались клетки эпидермиса не-

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правильной, круглой и полигональной формы, а также антиклинальные, гладкие и волнистые структуры стенок. У всех видов пыльца сферическая, орнаментация пыльцы: гладкая у *B. integririma*, зернистая у *B. vulgaris*, микроперфорированная у *B. orthbotrys* и микроэхинированная у *B. crataegina*. Филогенетический анализ показывает высокую поддержку монофилии видов *Berberis* в дереве ITS нрДНК (PP = 1, ML BS = 100, MP BS = 100). Было обнаружено, что молекулярные и микроморфологические особенности позволяют разделить пять видов в регионе Чахар-Баг. Результаты работы предоставляют исследователям важную информацию для изучения этого ценного рода.

Introduction

In the Berberidaceae family, *Berberis* L. has 500 species and is native to temperate and subtropical regions of Europe, Asia, Africa, North America, and South America (Judd et al., 1999; Rahimi-Madiseh et al., 2016). *Berberis* sp. is a shrub with a height of one to three meters, with red, brown, or yellow wood, oval leaves, and sour fruit. *Berberis* gives flowers in late spring and early summer (Ahrendt, 1961; Behrad et al., 2023). Several *Berberis* species are reported in Iran, according to botanists, *Berberis crataegina* DC., *B. integerrima* Bunge, *B. khorasanica* Browicz et Ziel., *B. orthobotrys* Bien. ex B. D. Jacks., and *B. vulgaris* L. (Browicz, Zielinski, 1975; Rezaei et al., 2011). *Berberis* is a medicinal plant, with its leaves, fruit, bark, stems and roots all having medicinal properties. All parts of these plants have been reported to have medicinal properties such as: antimicrobial, antiemetic, antipyretic, antioxidant, antiseptic, antiarrhythmic, and sedative. Different types of barberry contain berberine and berbamine as their main compounds (Rahimi-Madiseh et al., 2016). Many sources have examined this plant's pharmacological properties, including exhibiting antibacterial properties, reducing smooth muscle contraction, reducing inflammation, preventing platelet accumulation, stimulating bile secretion, lowering blood pressure and preventing tachyarrhythmias (Bhardwaj et al., 2012).

Stomata are one of the leaf structures that provide adaptation to dry conditions through their size, density, and distribution, so studying them across taxa can be helpful (Zarinkamer, 2007). Using mature leaves of *B. microphylla* G. Forst. grown in a variety of environmental conditions, Radice et al. (2015) studied their morphology and anatomy. Leaf anatomy of *B. ruscifolia* Lam was studied by Arambri et al. (2006) and show that sclerenchyma is abundant and mesophyll is dorsiventral. Light microscopy was used by Saeed-Ur et al. (2019) to study the cuticles of *Berberis* leaflets in Pakistan and provide a taxonomic key based on micromorphological characters. One of the research is description of the histological characteristics related to the medicinal properties of *B. aristata* DC., *B. lyceum*, and *B. asiatica* Roxb ex DC. (Mokhber-Dezfuli et al., 2014). Other studies have examined the therapeutic properties of leaf compounds (Mazandarani et al., 2013; Sabir et al., 2013).

In today's world, pollen grains play a very important role in plant classification, genealogy, and understanding the past. There may be similarities and differences in the structure of pollen grains due to the fact that pollen grains must disperse to fulfill their reproductive function (Furness, 2008). To complement basic biological data studies describing genotypes, pollen grain characterization is an important step in genetic resource conservation and improvement programs (de Castro Nunes et al., 2012). Anatomical and morphological features of *Berberis microphylla* pollen grains were described by Radice and Arena (2016). As an indicator of pollen grain yield, they used the correlation between size and germination percentage. According to Perveen and Qaiser (2010), four types of pollen grains have been identified in Pakistan's Berberidaceae family. A study conducted by Saeed-Ur et al. (2018) examined *Berberis* pollen grains and their taxonomic importance in Pakistan and found that palynological features can be used to identify *Berberis* species at the species level. According to Blackmore and Heath (1984), *Mahonia aquifolium* and *Berberis vulgaris* have syncolpate, psilate, or punctate pollen. As a result, morphological traits were often used in the past to identify species, but this method has problems, such as inability to identify the morphology in all seasons (Pourmeidani et al., 2023). A molecular technique can be used to identify and protect endangered species or to introduce new species, when species identification has moved from morphological traits to genetic traits. Plant populations have been studied genetically and ecologically using molecular markers. The identification and introduction of medicinal plants in different habitats are of particular importance. A great deal of attention has been paid to the phylogenetic relationships within *Berberis*, despite the well-established relationships within Berberidaceae (Kim, Jansen, 1996; Kim et al., 2004a).

A phylogenetic study using ITS nuclear sequence data was conducted by Kim et al. (2004b) on 79 species of *Berberis*. AFLP molecular markers were used by Heidari et al. (2009) on species from the Khorasan region. A relatively low level of diversity was found within the wild *Berberis* populations of this province, and there was a significant difference between them. Using ITS and RAPD markers, Sodagar et al. (2012) studied *Berberis* species in Khorasan from a biosystematic and molecular perspective. There are many medicinal benefits associated with *Berberis* plant.

This study had the following specific goals:

1) Determining the taxonomic identity of pollen micromorphological characters; 2) Analyzing epidermal variations in four species and one interspecific hybrid; 3) Examining affinities and relationships among taxa; 4) Investigating the molecular characteristics of Iranian *Berberis* species.

Materials and Methods

Morphological methods

In Golestan province, the studied area is in Chahar Bagh forest region (Fig. 1). This study area is located in Irano-Turanian vegetation zone. Its height varies from 2150 to 3700 meters. Within the northern latitude of 34°36' to 40°36' and longitude of 54°23' to 54°44', this area is between the provinces of Semnan and Golestan. A total of four species and one interspecific hybrid of *Berberis* were examined in this study (Table 1).

The Flora of Iran was used to identify these populations collected from a variety of habitats (Azadi, 2008) (Fig. 2). Gonbad Kavous university herbarium (GKUH) preserves all studied specimens.

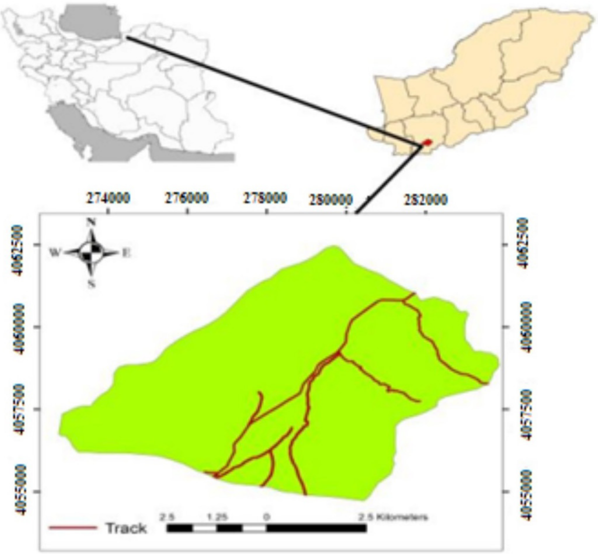

Fig. 1. Location of the studied area in Golestan province.

Table 1. List of studied populations of *Berberis* and outgroup

No.	Taxon	**Altitude**	**Longitude and latitude**	**Voucher number**	**Accession number**
1	*B. vulgaris* L.	2649	36˚63'1"E, 54˚50'5"N	804026 GKUH	PV430737
2	*B. vulgaris*	2252	36˚64'0"E, 54˚53'0"N	804034 GKUH	–
3	*B. vulgaris*	2248	36˚63'8"E, 54˚51'9"N	804035 GKUH	–
4	*B. orthobotrys* Bien. ex B. D. Jacks.	2254	36˚63'1"E, 54˚50'5"N	804027 GKUH	PV430738
5	*B. orthobotrys*	2278	36˚38'1"E, 54˚38'0"N	804032 GKUH	–
6	*B. orthobotrys*	2256	36˚64'1˚E, 54˚510"N	804033 GKUH	–
7	*B. crataegina* DC.	2279	36˚63'8˚E, 54˚36'3"N	804028 GKUH	PV430740
8	*B. crataegina*	2220	36˚62'1˚E, 54˚50'3"N	804038 GKUH	–
9	*B. crataegina*	2252	36˚68'4˚E, 54˚51'7"N	804041 GKUH	–
10	*B. integerrima* Bunge	2260	36˚38'9˚E, 54˚52'8"N	804029 GKUH	PV430739
11	*B. integerrima*	2249	36˚63'1˚E, 54˚50'5"N	804036 GKUH	–
12	*B. integerrima*	2239	36˚68'4˚E, 54˚58'6"N	804039 GKUH	–
13	*B. integerrima × vulgaris*	2293	36˚63'8˚E, 54˚51'4"N	804043 GKUH	PV430741
14	*B. integerrima × vulgaris*	2253	36˚63'1E, 54˚50'8"N	804044 GKUH	–
15	*B. integerrima × vulgaris*	2248	36˚63'9˚E, 54˚50'7"N	804030 GKUH	–
16	*Alloberberis higginsiae*	-	-	–	KX549408.1

Epidermal cells morphology

The first step is to select fresh leaves and put them in a flask. Then, by adding distilled water, the resulting solution is boiled until the leaf is soft. Carnoy's solution (a 3 : 1 mixture of ethanol and acetic acid) is added to the sample and placed in the refrigerator for 24 to 72 hours. To stabilize the samples, they were washed with distilled water and

70 % alcohol was added to the test tube. Samples may be preserved in fixatives (FAA: Formalin-Acetic Acid-Alcohol). In order to observe the structure, some of the fixed leaf is placed in a container based on its size (watch glass is used for this sample). The extractant should now be added to this container so that the color of the leaf cells (chloroplasts and other parts) disappears and the structure is more visible. A light microscopy was used to examine the slides prepared from the studied samples. At the Research Institute of Razi Tehran, Iran, images of their overall appearance and ornamentation were examined and photographed with a scanning electron microscope model VEGA// TESCAN-LMU under voltages of 22KV and 15KV.

Palynological study

In this study, pollen grains from *Berberis* species were examined using a Light Microscopy (LM) (VANOX AHBS3) and a Scanning Electron Microscopy (SEM) (VEGA//TESCAN-LMU) and examined the features such as polar, equatorial measurements, P/E ratio, pollen shape, and exine ornamentation. Most of the pollen samples were obtained from fresh and herbarium specimens. LM samples were acetolyzed according to Erdtman's method (Erdtman, 1952). Approximately 30 pollen grains per population were measured. Digital cameras, such as the DP12, were used to take these images using an Olympus light microscope. Gold plates were sputter-coated on double-sided tape affixed stubs prior to SEM analysis. At Research Institute of Razi, Tehran, Iran, photomicrographs were taken with a VEGA//TESCAN-LMU Electron Microscopy at an accelerating voltage of 15–22 KV. The applied terminology is based on Punt et al. (2007). Table 4 summarizes the characteristics of pollen grains of the studied *Berberis* species.

Molecular study

Taxon sampling

Sampling includes plants from specimens of *B. vulgaris* L., *B. orthobotrys* Bien. ex B. D. Jacks., *B. crataegina* DC., *B. integerrima* Bunge and *B. integerrima* Bunge × *vulgaris* chosen as ingroup for nrDNA ITS. Based on previous molecular phylogenetic studies (Kim et al., 2004b), *Alloberberis higginsiae* (Munz) C. C.Yu et K. F.Chung with compound-leaves was chosen as an outgroup.

DNA extraction, PCR and sequencing

The DNA of dried leaf materials deposited in the Gonbad Kavous University herbarium (GKUH) was extracted using the Kit method. Primers ITS5m of Sang et al. (1995) and ITS4 of White et al. 1990 were used to amplify the nrDNA ITS region (Table 2). DNA regions were amplified by PCR following procedures described in detail by Amini et al. (2018). By electrophoresis in 1 % agarose gels in 1-TAE buffer (pH = 8), PCR products were assessed for quality and photographed (UVItec, Cambridge, United Kingdom). Through Pishgam Inc., Tehran-Iran, PCR products and primers were sent to Macrogen (Seoul, South Korea) for Sanger sequencing.

Table 2. Sequence of primers used for polymerase chain reactions

Primer	Direction	Sequence
ITS5m	Forward	5'–GGAAGGAGAAGTCGTAACAAGG–3'
ITS4	Reverse	5'–TCCTCCGCTTATTGATATGC–3'

Sequence alignment

We aligned a single dataset using the web-based version of MUSCLE (Edgar, 2004, at http://www.ebi.ac.uk/Tools/msa/muscle/) under default parameters, and then manually adjusted it. It was necessary to introduce numerous single and multiple base indels (insertions/deletions) to align the dataset. For the ITS dataset, indels were treated as missing data.

Phylogenetic inferences

Parsimony method

We conducted maximum parsimony analyses with PAUP* version 4.0a157 (Swofford, 2002). Using the heuristic search option, 1000 replications of random addition sequence were conducted with an automatic increase in maximum number of trees for nuclear dataset using tree bisection-reconnection branch swapping (TBR). The analysis excluded characters that were uninformative. Using a full heuristic search with 100000 bootstrap replicates (Felsenstein, 1985), branch support values (MP BS) were estimated.

Bayesian inference

Based on the Akaike information criterion (AIC) (Posada, Buckley, 2004), Bayesian inference (BI) models of sequence evolution were selected using the program MrModeltest version 23 (Nylander, 2004). Nucleotide substitution was best modeled with a GTR+G model for nrDNA ITS. On the CIPRES Science Gateway (Cyber infrastructure for Phylogenetic Research cluster) (Miller et al., 2010; https://www.phylo.org) for the dataset, BI analyses were conducted using MrBayes version 3.2 (Ronquist et al., 2012). By saving trees every 100 generations, Bayesian analyses were conducted with default priors (uniform priors) and the best-fit sequence evolution model for the dataset, with two runs of ten million generations and four chains (one cold and three heated with a heating parameter of 0.2). To calculate posterior probability values (PP), the trees sampled after discarding 25 % were used to build a consensus phylogram using the 50 % majority rule. The Tree View version 1.6.6 (Page, 2001) was used to visualize the trees.

Phylogenetic networks

As a distance-based network construction method, NeighborNet (NN) was implemented in SplitsTree4, version 4.1.4.3 (Huson, Bryant, 2006) based on the ITS sequence-based uncorrected p-distance (Bryant, Moulton, 2004). Prior to analysis, the outgroup was excluded from the ITS matrix.

Results

Epidermal cell description

The epidermal and stomatal characteristics of the leaves were examined, such as cell shape, anticline wall patterns, stomata index, density, size, and types (Table 3). In this study, irregular, rounded, and polygonal epidermal cells were observed. Wavy and straight walls were observed in epidermal cells. In the leaves of *B. integerrima* and *B. vulgaris* species, irregular cells with straight walls were observed. A rounded cell shape with wavy walls was observed in the leaves of *B. crataegina* and *B. orthobotrys* species, while a polygonal cell shape with straight walls was observed in the leaves of *B. integerrima* × *vulgaris*. The stomata of all studied species are anomocytic (Fig. 3). *Berberis crataegina* had the largest stomata observed under the SEM, while *B. orthobotrys* had the smallest. According to the measurements obtained from the light microscopy, these species have the longest and shortest stomata, respectively. *Berberis crataegina* species had the highest stomatal density. The lower part of the studied samples had a greater number of stomata than the upper part (Fig. 4). The results of the examination of epidermis and stomata in the studied species summarizes in the Table 3.

Table 3. Leaf epidermal anatomical features of *Berberis*

Taxon	**Cell shape**	**Anticlinal walls**	**Stomata type**	**Stomata index (mm2)**	**Stomata size (μm)**
B. vulgaris	Irregular	Straight	Anomocytic	2.96	8.55 × 5.79
B. orthobotrys	Rounded	Wavy	Anomocytic	3.11	8.22 × 4.91
B. integerrima	Irregular	Straight	Anomocytic	3.23	10.34 ×5.90
B. crataegina	Rounded	Wavy	Anomocytic	4.33	10.69 × 8.17
B. integerrima × vulgaris	Polygonal	Straight	Anomocytic	3.93	9.77 × 9.36

Pollen morphology

Four species and one interspecific hybrid of *Berberis* were separated based on the pollen grains of the studied species (Fig. 5). In Table 4, all palynological structures and measurements for the examined species concerning pollen type from polar view, polar (P) and equatorial (E) measurements, P/E ratio, pollen shape, and exine ornamentation are shown. The Figure 6 shows selected SEM micrographs of pollen surfaces and pollen grains. *Berberis crataegina* had the smallest pollen grain, while *B. integerrima* had the largest. Pollen in all species is spherical, with smooth ornamentation in *B. integerrima*, granular ornamentation in *B. vulgaris,* microperforate ornamentation in *B. orthobotrys* and *B. integerrima* × *vulgaris* and microechinate in *B. crataegina.*

Table 4. Pollen grain Characters (SEM)

Species	Px(μm)	Ex(μm)	P/E	Exin(μm)	Ornamentation
B. vulgaris	15.59 ± 0.36	14.08 ± 0.38	1.1	1.88	granulate
B. orthobotrys	17.09 ± 0.51	15.7 ± 0.28	1.08	2.05	microperforate
B. integerrima	28.35 ± 2.15	26.73 ± 0.73	1.06	2.11	smooth
B. crataegina	13.00 ± 0.32	11.5 ± 1.3	1.1	1.98	microechinate
B. integerrima × vulgaris	15.47 ± 0.13	13.33 ± 0.73	1.1	2.04	microperforate

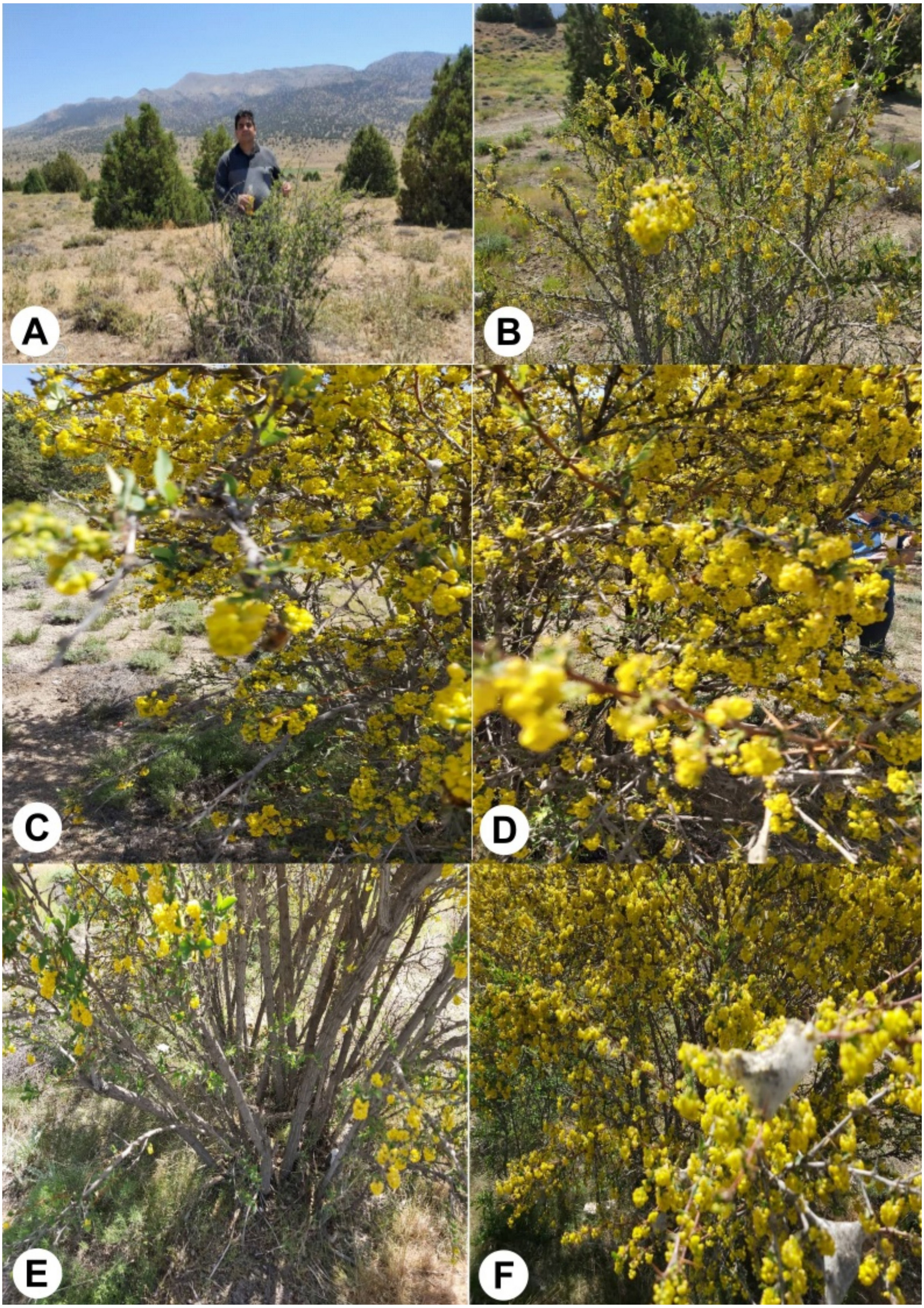

Fig. 2. The natural habitat of *Berberis*: A, B – *Berberis vulgaris*; C, D – *B. orthobotrys*; E, F – *B. crataegina* (Golestan Province, Chahar Bagh).

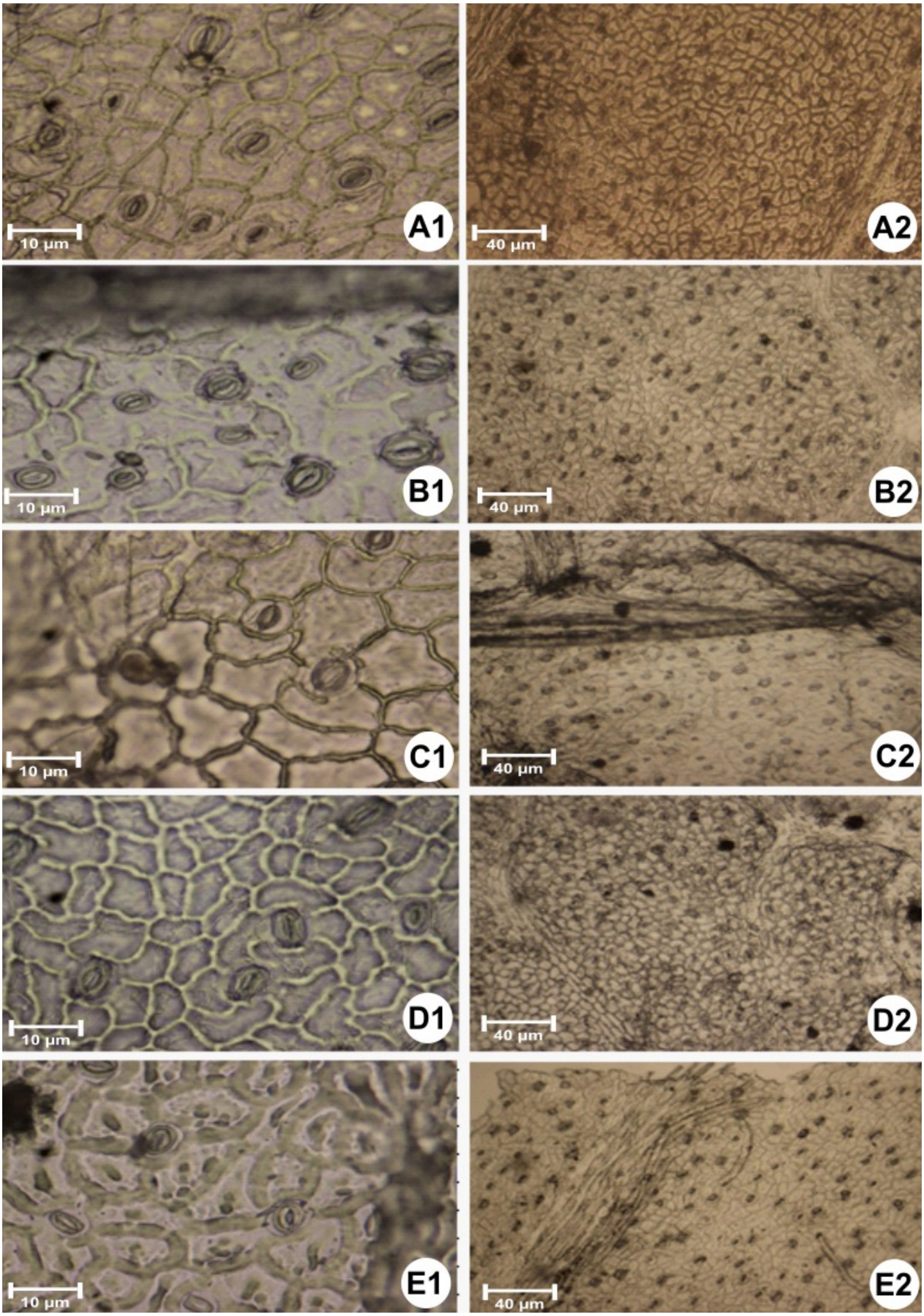

Fig. 3. Epidermal cells on the leaf surface: shape, size, anticlinal wall and stomata by light microscopy. 40× (right) and 10× (left): A1, A2 – *Berberis crataegina*; B1, B2 – *B. vulgaris*; C1, C2 – *B. integerrima* × *vulgaris*; D1, D2 – *B. integerrima*; E1, E2 – *B. orthobotrys.*

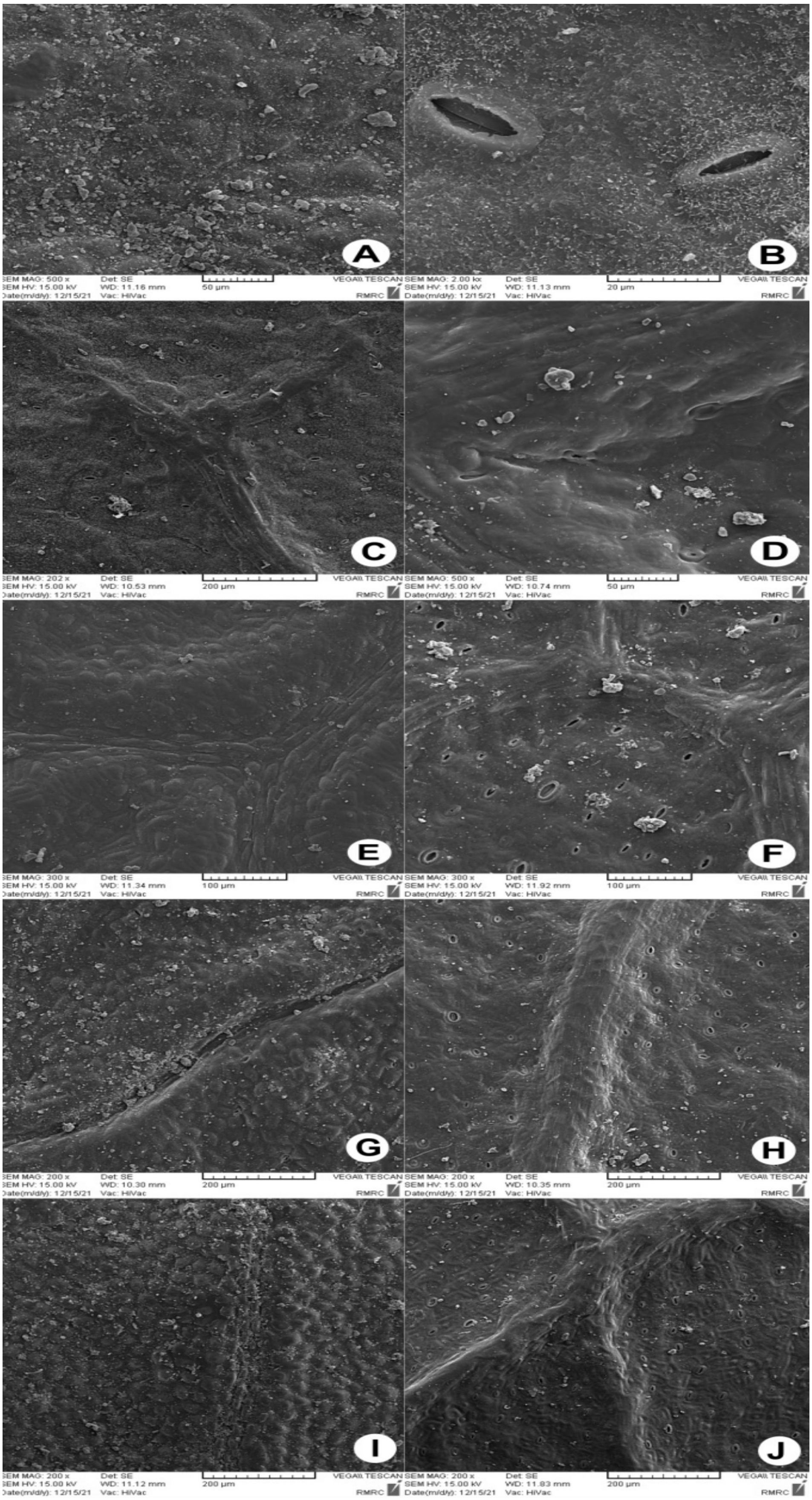

Fig. 4. Epidermal cells on the leaf surface: shape, size, anticlinal wall and stomata by scanning electron microscopy. 20, 50, 100, 200 Micrometer. A, B – *Berberis integerrima*; C, D – *B. vulgaris*; E, F – *B. integerrima* × *vulgaris*; G, H – *B. crataegina*; I, J – *B. orthobotrys.*

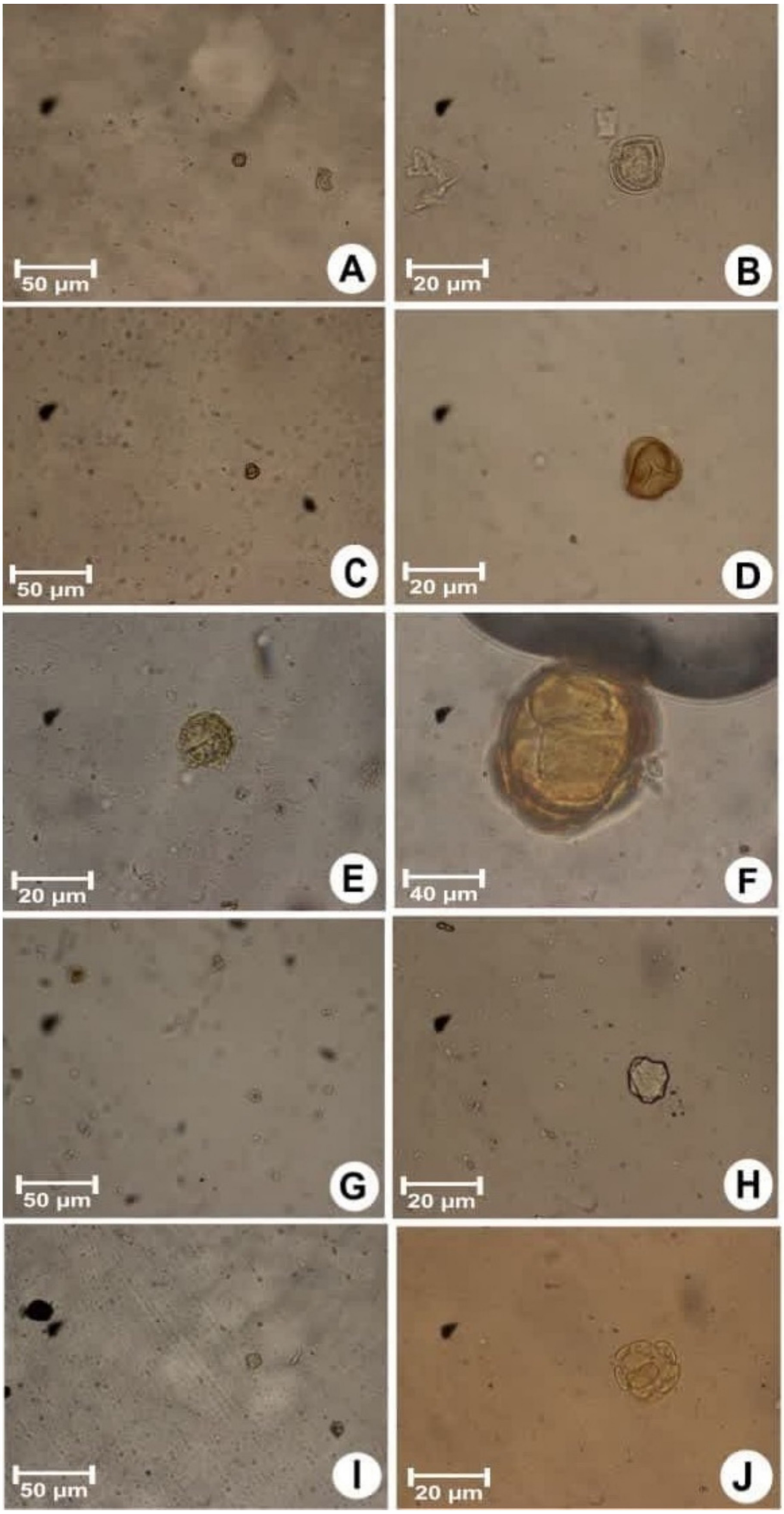

Fig. 5. The pollen surface by light microscopy: A, B – *Berberis integerrima*; C, D – *B. vulgaris*; E, F – *B. integerrima* × *vulgaris*; G, H – *B. crataegina*; I, J – *B. orthobotrys.* 10× (right) and 40× (left).

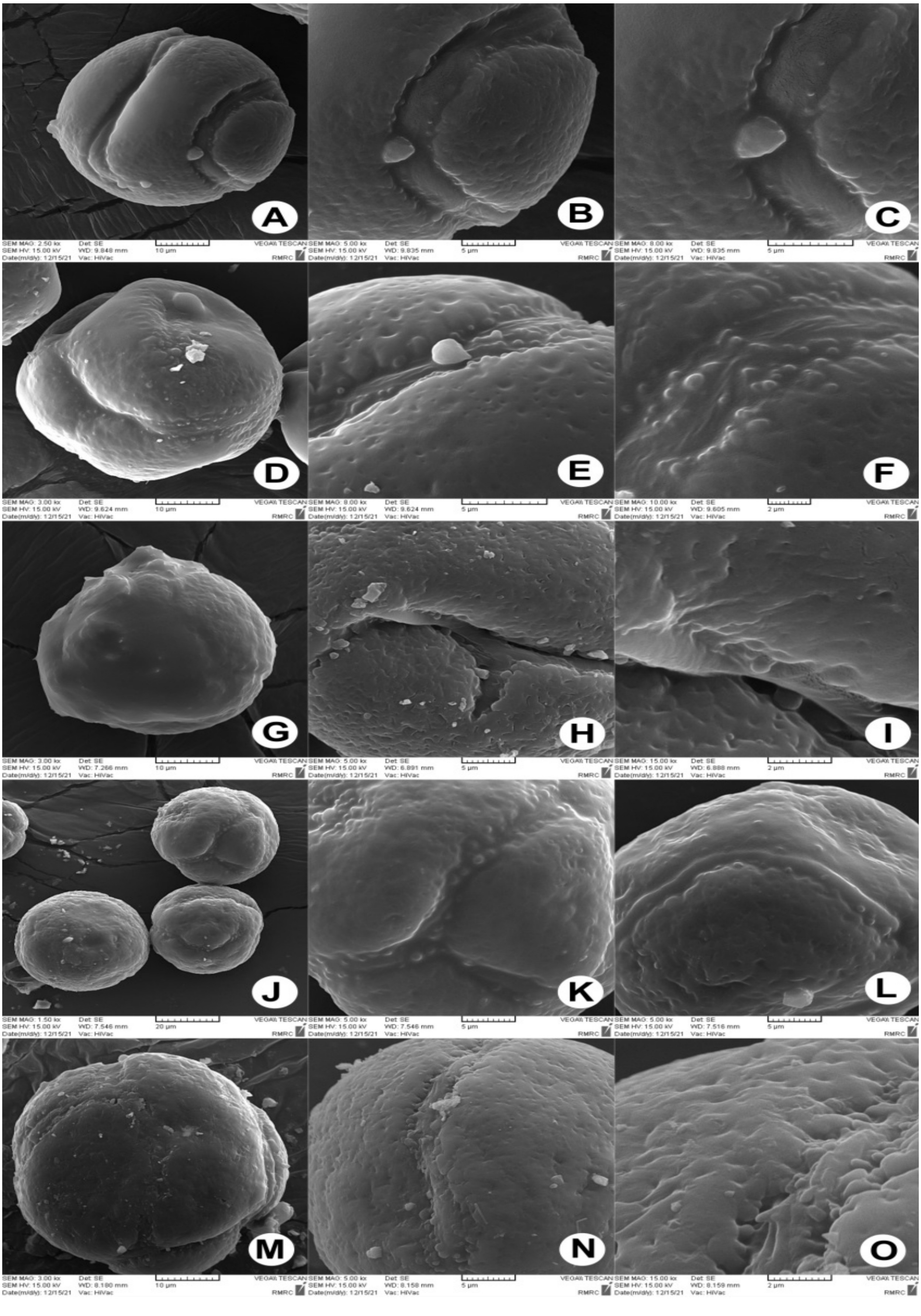

Fig. 6. The pollen surface by scanning electron micrographs. 5, 10 Micrometer. A, B, C – *Berberis integerrima*; D, E, F – *B. vulgaris*; G, H, I – *B. integerrima* × *vulgaris*; J, K, L – *B. crataegina*; M, N, O – *B. orthobotrys*.

Phylogenetic analysis

From the single analysis of the nrDNA ITS region, Table 5 summarizes information about alignment characteristics, selected model of nucleotide substitution, and tree statistics. There are 652 characters in the aligned nrDNA ITS matrix. A maximum parsimony analysis of the number of 153 shortest trees with 86 steps produced a consistency index (CI) of 0.87 and a retention index (RI) of 0.79 based on the nrDNA ITS data. In both Bayesian and maximum parsimony trees, branching patterns are similar, but in the Bayesian tree, species relationships are more resolved and clades have higher support. Figure 7 shows the nrDNA ITS sequence tree based on bootstrap values. Based on the results of the analysis, *Berberis* species are monophyletic in this phylogram (PP = 1, ML BS = 100, MP BS = 100). Molecular results also show a close relationship between *B. vulgaris*, *B. integerrima*, and *B. integerrima* × *vulgaris*, which indicates polytomy between these three species. Among the three species listed above, *B. crataegina* is considered a sister taxon.

Table 5. Dataset and tree statistics from single analysis of the nuclear region

Total sample	nrDNA ITS
Number of sequences	6
Number of ingroup sequences	5
Alignment length [bp]	652
Number of parsimony-informative characters	95
Number of MPTs	153
Length of MPTs	86
Consistency index (CI)	0.87
Retention index (RI)	0.79
Evolutionary model selected (under AIC)	GTR + G + I

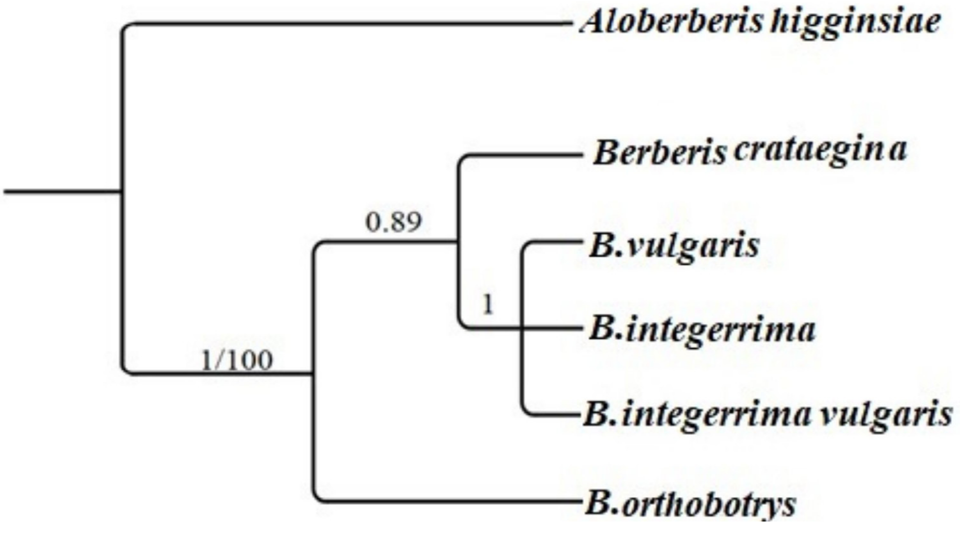

Fig. 7. 50 % majority rule consensus tree resulting from the Bayesian phylogenetic analysis of the nrDNA ITS dataset. Numbers of the branches are posterior probability (PP) from the BI and bootstrap support (BS) values from a MP, respectively (values < 50 % were not shown).

Phylogenetic networks

The NeighborNet diagram confirms the phylogenetic results that the *Berberis* is composed of two clades, indicating almost complete separation of the studied populations. There were two main groups in the ITS split graph (Fig. 8). A population of *B. crataegina* and *B. orthobotrys* (4 and 5) is part of lineage I. Lineage II includes *B. vulgaris*, *B. integerrima*, and *B. integerrima* × *vulgaris* (1, 2 and 3).

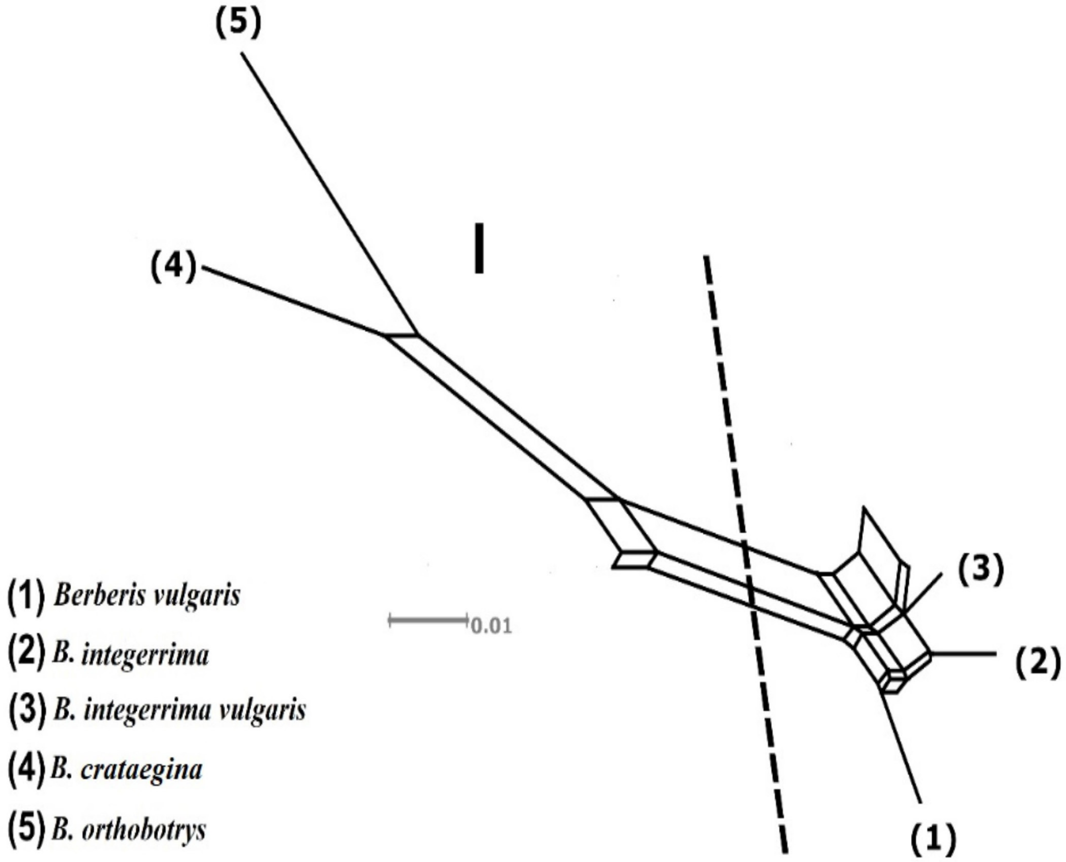

Fig. 8. NeighborNet for ITS sequences of *Berberis*. Two major groups were recovered (i. e., lineage I and lineage II).

Discussion

The importance of plants in the treatment of diseases has led researchers to search for 21st century medicines in plants and believe they may be the solution to future medical issues. There has been a long tradition of using barberry (*Berberis* sp.) as a medicinal plant in Iran and many other great civilizations throughout history, and now, with the widespread recognition of its effective medicinal substances such as berberine, it has become widely used in pharmaceutical industries (Rahimi-Madiseh et al., 2016). It is important for plants to adapt to changing environmental conditions, for example, physiological and anatomical mechanisms to survive. The morphology of a leaf is a miniature representation of the plant's functions and properties; In order to preserve resources and adapt to environmental changes, their shape, size, and structure seem necessary (Arena et al., 2011). Stomata have a variety of accompanying cells, which can be used to identify different plant species as one of their morphological traits, for example, parenchymatous or keratinized subsidiary cells. Different species have been studied to determine the relative abundance of stomata on different surfaces of leaves based on their response to environmental conditions (Redmann, 1985; Khazaei, 2010). Results showed that the lower part of the examined barberry leaves had more stomata than the upper part, indicating hypostomatous status. This feature allows the plant to absorb more CO_2 gas without losing excessive water. A high degree of phenotypic flexibility was observed in the leaves of *Berberis* species. Plants may adjust their morphology to new environmental conditions based on changes in leaf morphology and structure. In Radice et al. (2015), morphology and anatomy of mature leaves of *B. microphylla* grown under different environmental conditions were compared. They also found that the leaves of this species had thick cuticles and anomocytic pores. The observed changes in leaf morphology and structure may indicate plants trying to adapt to a new environment (Radice et al., 2015). According to Arambarri et al. (2006), raphids and crystals were absent in *B. ruscifolia* leaves. *Berberis* is taxonomically a very complex genus with a variety of species with different characteristics. It is difficult to distinguish between species due to polyploidy and hybridization. *Berberis* is essentially a diploid genus

($2n = 28$) (Cadic, Decourtye, 1987). The exterior of mature pollen grains exhibits slits that indicate where the spiral opening (syncolpot) is located. Pollen grains are fully expanded and spherical in shape. The structures of *Berberis* pollen are variable (Furness, 2008). All examined pollen grains were spherical and were decorated with smooth, granular, or porous surfaces. Finally, using the palynological data obtained from these samples, it was possible to separate the five taxa in this region to some extent. In accordance with our results, the evidence of pollen grains of barberry genus in Khorasan provinces by Sodagar et al. (2011) showed that the pollen grains were single, clypeate, spherical, and lacked any symmetry, and since the study only used a light microscopy, their statistical analysis did not show a significant difference in the size of the pollen grains, and they recommended more detailed investigation with SEM (Sodagar et al., 2011). A remarkable amount of progress has been made in molecular studies of plants, and phylogenetic research changed evidence of how organisms are related (Soltis D. E., Soltis P. S., 2000).

The molecular method has been successful in studying subspecies relationships in different genera so far (Amini et al., 2018, 2019). In the *Berberis* genus, species identification requires appropriate molecular methods due to polyploidy and hybridization. According to this study's molecular marker, *Berberis* species are monophyletic in this phylogram with high support (PP = 1, ML BS = 100, MP BS = 100). Due to low sequence divergence among these taxa, no phylogenetic resolution can be obtained, so chloroplast markers must be used to resolve the relationships between populations. According to Adhikari et al. (2015), using nuclear ITS and chloroplast *ndh*F sequences to analyze 68 *Berberis* individuals, the monophyly of *Berberis* s. l. was confirmed. However, species with compound leaves (which are not in our samples) were shown to be paraphyletic. In the NN method of distance-based network construction, conflicting phylogenetic signals can be visualized and evolutionary histories that do not follow tree-like patterns can be interpreted (Bryant, Moulton, 2004). According to the NeighborNet diagram (Fig. 8), the studied populations are almost completely separated within the network, supporting the phylogenetic results. Reticulation is evident from the splits graph, which reveals extensive internal network structure. Phylogenies generally show good correlation between splits graph groups and clades recovered in the phylogenies (with minor exceptions). According to Ahrendt (1961), simple leaf species descend from compound leaf species. It is likely that hybridization is responsible for polytomy within a subclade as well as close relationships between *B. vulgaris*, *B. integerrima*, and *B. integerrima* × *vulgaris* species. Sodagar et al. (2011) found a high proportion of polyploidy and hybridization in the barberry genus in Khorasan provinces. This is confirmed by karyological studies. For the genus, $2n = 2x = 28$ has been reported in previous studies (Bottini et al., 2007). According to chromosome studies, *B. vulgaris, B. crataegina*, *B. orthobotrys*, and *B. integerrima* have four sets of chromosomes that are tetraploids (Sodagar et al., 2011).

According to a study of barberry simple and compound leaf species in Nepal, all species are diploids with $2n = 28$, and polyploidy is not an important factor in barberry diversity (Adhikari et al., 2010). Polytomy can be caused by phenomena such as hybridization, polyploidy, apomixis, lineage sorting, and reticulate evolution (Nasrollahi et al., 2019). According to our hypothesis, the relationships between the species studied in subclade B are not well resolved because of variable levels of polyploidy and low gene flow. Iranian plants, especially horticultural products, have favorable genetic reserves due to their diversity. Because wild populations exist throughout the country, barberry is an ideal micro-fruit for primary research in a variety of fields. Despite the fact that a number of researches have been conducted on the barberry plant, most of them have focused on its medicinal properties, only a few have focused on its identification, diversity investigation, and morphological study. As morphological diversity is an obvious and valuable requirement for plant breeding, investigating the morphological and molecular characteristics of barberry seemed to be essential and necessary.

Conclusions

For the first time, this study examined the epidermal and stomatal characteristics of leaves of *Berberis* species in north of Iran, such as cell shape, anticline wall patterns, stomata index, density, size, and types. A nuclear marker nrDNA ITS was used to examine the molecular characteristics of the collected samples because of multiple copies, high degree of sequence variability and universal marker**.** These characteristics and molecular results allowed us to distinguish to some extent the four species and one interspecific hybrid in Chahar Bagh region from each other, based on the results of our study. It can be said that this study provides researchers with the basic information they need to study and investigate this important genus to the fullest extent possible.

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