

ARTICLE

## Micromorphological, anatomical and molecular study of *Hedera* species (Araliaceae) in Iran

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**ABSTRACT** *Hedera*, with 12 extant species, is a genus of evergreen climbers native to Europe, north Africa, and south Asia. In this study, the micromorphological, anatomical structure and molecular evidences of 11 populations from two species of *Hedera* (*H. helix* and *H. pastuchovii*) have been considered to evaluate the relationships in *Hedera*. In total, seven quantitative and qualitative characters of pollen were selected and measured. Based on this study, the anticlinal wall and surface sculpturing of seed support for separation of two species of *Hedera*. Micromorphology of epidermis illustrated two types of epidermal cells: puzzle-shaped and polygonal cells. Using nuclear (nrDNA ITS) marker, we reconstructed phylogenetic relationships within two species of *Hedera*. This data set was analyzed by phylogenetic methods including Bayesian inference, maximum likelihood, and maximum parsimony. In phylogenetic analyses, all members of two species formed a well-supported clade (PP = 1; ML/BS = 100/100) and divided into two major clades (A and B). Neighbor Net diagram demonstrated separation of the studied populations. The results showed that these taxa differ in taxonomically important micromorphological, anatomical and molecular characteristics and these data provide reliable evidence for separation of these two species.

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### Introduction

*Hedera* L. (Araliaceae), with 12 extant species, is a genus of evergreen climbers native to Europe, north Africa, and south Asia (Vargas et al. 1999; Grivet and Petit 2002; Ackerfield and Wen 2003; Valcárcel et al. 2003a; Valcárcel and Vargas 2010; Valcárcel et al. 2014) and occupies forest under stories and riparian vegetation in temperate latitudes throughout Europe, N Africa and Asia (Meusel et al. 1965; Mabberley 1997). *Hedera* is a considerable element in Asian and European woodlands, comprising a large component of the forest understory.

The taxonomical treatments of *Hedera* published through the second half of the 20th century recognized between three and 19 species (Seemann 1868; Tobler 1912; Lawrence and Schulze 1942; Pojarkova 1951). More recently authors have accepted the 12 entities proposed by McAllister and Rutherford (McAllister 1982, 1988; Rutherford 1984, 1989; McAllister and Rutherford 1990; Rutherford et al. 1993) with slight changes at the subspecies level (Ackerfield and Wen 2002; Valcárcel 2008; Valcárcel and Vargas 2010). Since McAllister and Rutherford's

treatment, the identification and delimitation of *Hedera* species have mainly been based on a combination of trichome morphology (stellate-multiangulate, stellate-rotate, and scale like hairs; Seemann 1868; Lum and Maze 1989; McAllister and Rutherford 1990; Ackerfield and Wen 2002; Valcárcel and Vargas 2010), juvenile leaf morphology (from entire to 3–7 lobate; Rutherford et al. 1993; Ackerfield and Wen 2002; Valcárcel 2008; Valcárcel and Vargas 2010), and ploidy level (from 29 to 89; Vargas et al. 1999).

Micromorphological characters are good diagnostic value to recognize many taxa, fundamentally in the species level. The pollen morphological analysis is successfully used as an additional document for delimitation of the taxa (Amini et al. 2018; 2019). Van Helvoort and Punt (1984) in the Northwest European Pollen Flora mentioned that pollen class of *Hedera* is 3-zonocolporate, sub erect to semi-erect with reticulate ornamentation. In Scandinavian pollen flora, apocolpium diameter of pollen grains is about 10 p. Colpi is narrow; exine thickness is about 2.5 p and sometimes distinctly thicker at poles (Erdtman et al. 1961).

The anatomical structure of some *Hedera* species

show that anatomical features can be valuable in species delimitation especially about similar taxa. Taxonomic value of epidermis anatomical features is well documented in botanical literature (Dilcher 1974; Metcalf 1985). Savulescu and Luchian (2009) studied the diagnostic value of *Hedera* epidermis and illustrated that epidermis is made up of only one cells layer with polygonal cells with thin lateral walls and corrugated.

Molecular data can provide extra criteria for systematic classification of the species studied that have been only based on the morphological characters (Chase et al. 1993). Nuclear ribosomal DNA provides highly informative variation that has been used to infer phylogenetic relationships in angiosperms. Its value stems from the high rate of divergence at the specific, and sometimes, the populational level (Baldwin 1993; Vargas et al. 1998; Vargas et al. 1999). The internal transcribed spacer (ITS) is the region of the 18S-5.8S-26S nuclear ribosomal cistron (Baldwin et al. 1995) and have often been used for inferring phylogeny at the generic and infrageneric relationships of genera (e.g., Baldwin 1992; Baldwin et al. 1995; Amini et al. 2018; Nasrollahi et al. 2019). Previous molecular and cytogenetic studies identified two main centers of diversity for *Hedera*, the eastern and western parts of the Mediterranean region. Molecular phylogenetic reconstructions of 37 of the 41 Araliaceae genera clearly placed *Hedera* within the Asian Palmate group (Lowry et al. 2001; Wen et al. 2001; Valcárcel et al. 2003; Plunkett et al. 2004).

Vargas et al. (1999) applied variation in chromosome number and internal transcribed sequences (ITS) of nrDNA to infer phylogenetic relationships of a wide range of *Hedera* species. Polyploidy was found to be frequent in *Hedera*, with diploid, tetraploid, hexaploid and octoploid populations being detected. Valcárcel and Vargas (2012) studied the phylogenetic reconstruction of key traits in the evolution of ivies (*Hedera* L.) and the analyses of the nrDNA ITS and plastid *trnT-L* sequences revealed multiple connections between the Mediterranean region and Asia and suggest recurrent colonization between these two areas. Valcárcel et al. (2014) studied the origin of the early differentiation of ivies (*Hedera*) and the radiation of the Asian Palmate group (Araliaceae). Genome incongruence and hard nuclear and plastid basal polytomies are detected for the Asian Palmate group where the lineage of *Hedera* is placed.

Despite ecological impacts and economic significance of *Hedera* to the horticultural industry, the taxonomy of *Hedera* is controversial and historical relationships are poorly defined.

The specific goals of this study were as follows: (1) to examine pollen and seed micro-morphological characters that could be useful for the diagnosis of taxa; (2) to

determine the patterns of variation in epidermal characteristics in two species; (3) to evaluate the affinities and relationships of taxa; (4) to investigate molecular properties of *Hedera* in Iran.

## Materials and Methods

### Morphological methods

In the present study, 11 populations of two *Hedera* species were collected from different locations in North Iran and preserved in the Gonbad Kavous University Herbarium (GKUH). Identification of populations was carried out based on Flora Iranica (Browicz 1973). The list of voucher populations and details of localities are given in Table 1.

Palynological studies on pollens of *H. helix* L. and *H. pastuchovii* Woron. Ex Grossh was made using a light microscope (LM) (Olympus, Vanox AHBS3) with a DP12 digital camera and a scanning electron microscope (SEM; Tescan, Vega-3 LMU). For SEM investigations, the pollen grains were transferred directly to double-sided tape affixed stubs and were sputter-coated with gold plates. The applied terminology based on Punt et al. (2007). For LM studies, the samples were acetolyzed following Erdtman's technique (Erdtman 1952). The pollen samples were obtained mostly from freshly collected herbarium populations. The measurements were based on at least 30 pollen grains per population. The characters of pollen grains of the studied *Hedera* species are summarized in Table 2.

In order to detect significant differences in the studied characters among the investigated species, an analysis of variance (ANOVA) was done. To determine the species relationships, we have used cluster analysis and principal component analysis (PCA; Ingrouille 1986). For multivariate analysis, the mean of the quantitative characters was calculated. Qualitative characters were coded as binary or multistate. Variables were standardized for multivariate statistical analysis. Average taxonomic distances and squared Euclidean distances were calculated as dissimilarity coefficient in the cluster analysis of pollen data. In order to determine the most variable characters among the studied species, factor analysis based on principal components analysis was performed. SPSS ver. 20 and PAST ver. 2.17c (2013) softwares were performed for statistical analysis.

Seeds of the two species of *Hedera* were taken from herbarium samples: before mounted directly on aluminum stubs using double-sided adhesive, they were examined under a stereomicroscope to ensure the normal size and development. After having been coated with a thin layer (ca. 25 nm) of gold they were analyzed using a SEM (Tescan, Vega-3 LMU) at an accelerating voltage of 15-22 kV at

**Table 1.** List of species used in the study along with localities and vouchers.

Taxa	Collection data	GenBank accession no./ITS
<i>H. helix</i> L.	Tehran: Research Institute of Forests and Rangelands, Haji Moradkhani, 803298, GKUH	LC508655
<i>H. helix</i>	Golestan: Gonbad Kavous, Haji Moradkhani, 803297, GKUH	LC508656
<i>H. helix</i>	Fars: Kamfiruz, Tang-e Bostan, Haji Moradkhani, 803299, GKUH	LC508657
<i>H. pastuchovii</i> Woron. Ex Grossh.	Golestan: Gorgan, Naharkhoran forest, Haji Moradkhani, 803270, GKUH	LC508658
<i>H. pastuchovii</i>	Golestan: Gorgan, Ziarat village, Haji Moradkhani, 803273, GKUH	LC508659
<i>H. pastuchovii</i>	Gilan: Gisum forest, Haji Moradkhani, 803289, GKUH	LC508660
<i>H. pastuchovii</i>	Gilan: Fuman forest, Mirmahaleh, Haji Moradkhani, 803292, GKUH	LC508661
<i>H. pastuchovii</i>	Gilan: Somee Sara, Haji Moradkhani, 803290, GKUH	LC508662
<i>H. pastuchovii</i>	Mazandaran: Nur forest, Haji Moradkhani, 803279, GKUH	LC508663
<i>H. pastuchovii</i>	Mazandaran: Najardeh, Haji Moradkhani, 803278, GKUH	LC508663
<i>H. pastuchovii</i>	Mazandaran: Sari, Haji Moradkhani, 803295, GKUH	LC508665
<i>Trevesia palmata</i> Vis.	GenBank	KF591487
<i>Brassaiopsis mitis</i> Clarke	GenBank	AY304801

GKUH: Gonbad Kavous University Herbarium

Research Institute of Razi (Tehran, Iran). For recording gross morphology and size parameters, at least 10 seeds were measured.

#### Anatomical methods

All materials were boiled for 15 min, and then fixed in Carnoy solution (alcohols to acetic acid in proportion 3:1). The epidermis was separated with H<sub>2</sub>O<sub>2</sub> and acetic acid (1:1), in order to prepare the leaves. Materials were kept warm in a tube with previous lotions (the above-mentioned solutions) for 4 hours, then the leaves were cleaned. After cleaning, the materials were washed in distilled water. Epidermis separation followed. Epidermal samples were stained with 2% aceto-carmin and were mounted on microscopic glass slides. Slide sections were studied and

photographed with the help of an Olympus light microscope by using a Olympus DP12 Digital Camera. Some characters (stomata length/weight, number of stomata, number of epidermal cells) were measured with Image Tools ver. 3.0 and Axio Vision 4.8. (Table 5).

#### Molecular methods

##### Taxon sampling

Sampling includes plants from 3 populations of *H. helix* and 8 populations *H. pastuchovii* were chosen as ingroup for nrDNA ITS. *Trevesia palmata* Vis. and *Brassaiopsis mitis* Clarke were chosen as outgroup following previous molecular phylogenetic studies (Wen et al. 2001). A list of all the taxa used in this study and the sources,

**Table 2.** Pollen morphological characters for the examined taxa of *Hedera*.

Taxon	Length of polar axis (µm ± SD)	Length of equatorial axis (µm ± SD)	P/E	Shape	Colpus length (µm ± SD)	Colpus width (µm ± SD)	Ornamentation
<i>H. helix</i>	21.55 ± 1.48	18.77 ± 0.43	1.14	Subprolate	18.60 ± 0.12	1.23 ± 0.05	Microperforate
<i>H. helix</i>	21.05 ± 1.17	19.65 ± 0.27	1.07	Subprolate	18.80 ± 0.09	1.14 ± 0.01	Microperforate
<i>H. helix</i>	22.05 ± 1.17	18.65 ± 0.27	1.07	Subprolate	17.80 ± 0.09	1.25 ± 0.01	Microperforate
<i>H. pastuchovii</i>	38.75 ± 2.03	27.37 ± 0.32	1.41	Prolate	24.70 ± 0.15	2.05 ± 0.09	Reticulate
<i>H. pastuchovii</i>	33.65 ± 1.19	24.43 ± 0.35	1.37	Prolate	25.16 ± 0.16	2.74 ± 0.04	Reticulate
<i>H. pastuchovii</i>	36.79 ± 1.18	25.67 ± 0.29	1.43	Prolate	23.96 ± 0.07	2.63 ± 0.01	Reticulate
<i>H. pastuchovii</i>	38.73 ± 1.31	27.35 ± 0.34	1.41	Prolate	24.36 ± 0.21	2.85 ± 0.03	Reticulate
<i>H. pastuchovii</i>	34.65 ± 1.36	22.43 ± 0.31	1.54	Prolate	25.10 ± 0.16	2.70 ± 0.01	Reticulate
<i>H. pastuchovii</i>	36.75 ± 0.41	24.47 ± 0.44	1.50	Prolate	23.98 ± 0.12	2.34 ± 0.07	Reticulate
<i>H. pastuchovii</i>	37.64 ± 0.34	25.35 ± 0.17	1.48	Prolate	25.49 ± 0.28	2.45 ± 0.04	Reticulate
<i>H. pastuchovii</i>	35.44 ± 0.38	23.65 ± 0.28	1.49	Prolate	25.70 ± 0.19	2.78 ± 0.06	Reticulate

voucher information and GenBank accession numbers are given in Table 1.

#### *DNA extraction, PCR and sequencing*

Total genomic DNA was extracted from dried leaf materials deposited in Gonbad Kavous University Herbarium (GKUH), using Kit method. The nrDNA ITS (Nuclear ribosomal DNA Internal Transcribed Spacer) region was amplified using the primers ITS5m of Sang et al. (1995) and ITS4 of White et al. (1990). PCR amplification of the DNA regions followed procedures described in detail by Naderi Safar et al. (2014). The quality of PCR products was checked by electrophoresis in 1% agarose gels in 1 × TAE (pH 8) buffer and were photographed with an UV gel documentation system (UVItc, Cambridge, UK). PCR products along with the same primers were sent for Sanger sequencing at Macrogen (Seoul, South Korea) through Pishgam (Tehran-Iran).

#### *Sequence alignment*

Single dataset was aligned using the web-based version of MUSCLE (Edgar 2004, at <http://www.ebi.ac.uk/Tools/msa/muscle/>) under default parameters followed by manual adjustment. The alignment of dataset required the introduction of numerous single and multiple-base indels (insertions/deletions). Positions of indels were treated as missing data for the ITS dataset.

### **Phylogenetic inferences**

#### *Parsimony method*

Maximum parsimony (MP) analyses were conducted using PAUP\* version 4.0a157 (Swofford 2002). The heuristic search option was employed for nuclear dataset using tree bisection-reconnection (TBR) branch swapping, with 1000 replications of random addition sequence and an automatic increase in the maximum number of trees. Uninformative characters were excluded from the analyses. Branch support values (MPBS) were estimated using a full heuristic search with 1000 bootstrap replicates (Felsenstein 1985) each with simple addition sequence.

#### *Likelihood method*

Maximum likelihood (ML) analyses were carried out using the RAxML-HPC2 on XSEDE (8.2.8) at the CIPRES Science Gateway. Bootstrap values (LBS) were calculated in RAxML-HPC2 based on 1000 replicates with one search replicate per bootstrap replicate.

#### *Bayesian inference*

For Bayesian inference (BI) analyses, models of sequence evolution were selected using the program MrModeltest version 2.3 (Nylander 2004) based on the Akaike

information criterion (AIC) (Posada and Buckley 2004). This program indicated a GTR+G model for nrDNA ITS. BI analyses were performed using MrBayes version 3.2 (Ronquist et al. 2012) on the CIPRES Science Gateway (Cyber infrastructure for Phylogenetic Research cluster) (Miller et al. 2010, <https://www.phylo.org>) for the dataset. Bayesian analyses were performed, with default priors (uniform priors) and the best-fit model of sequence evolution for dataset, with two runs of ten million generations and four simultaneous chains (one cold and three heated with a heating parameter of 0.2), by saving trees every 100 generations. The trees sampled after discarding 25% as “burn-in” were collected to build a 50% majority rule consensus phylogram were used to calculate posterior probability values (PP). Tree visualization was carried out using Tree View version 1.6.6 (Page 2001).

#### *Phylogenetic networks*

NeighborNet (NN) a distance-based network construction method (Bryant and Moulton 2004) was implemented in SplitsTree4, version 4.14.4 (Huson and Bryant 2006) based on the uncorrected *p*-distance between populations that was calculated from the ITS sequence data. The ITS matrix was modified prior to analysis by excluding the outgroup.

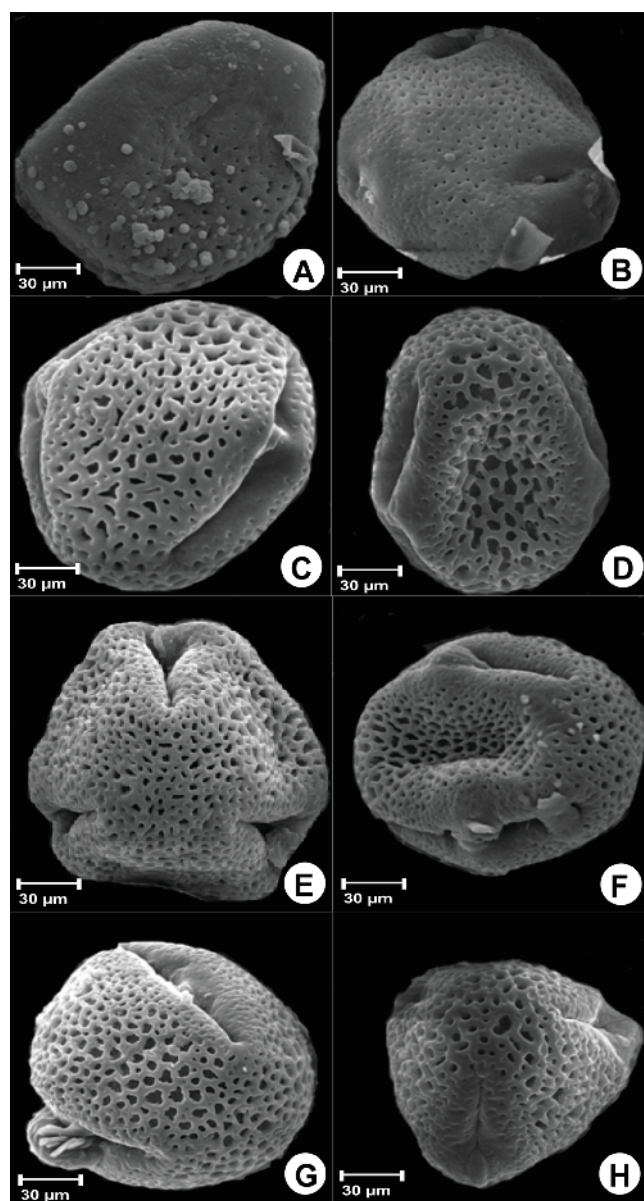
## **Results**

### **Pollen morphology**

The pollen grains of the studied species revealed some variations and separated two species of *Hedera*. All palynological structures and measurements for the examined species concerning pollen type from polar view, polar (P) and equatorial (E) measurements, P/E ratio, colpus length and width, pollen shape and exine ornamentation were shown in Table 2. Selected SEM micrographs of the pollens and their surfaces are shown in Fig. 1. Generally, type of pollen grain aperture is observed tricolporate among studied species (Fig. 1). Length of polar and equatorial axis were found useful in separating two species. Polar axis (P) length of pollen grains ranging from the smallest size for *H. helix* (21.05 μm) to the largest size for *H. pastuchovii* (38.75 μm). Equatorial axis (E) length of pollen grains ranged from the smallest size in *H. helix* (18.65 μm) to the largest size in *H. pastuchovii* (27.37 μm). The shape classes are based on the ratio between the length of polar axis (P) and equatorial diameter (E) (Erdtman 1952). The P/E ratio ranged from 1.07 to 1.54, therefore the pollen shape is subprolate in *H. helix* but prolate in *H. pastuchovii*. The ornamentation of tectum is microperforate in *H. helix* (Fig. 1B) and is reticulate in *H. pastuchovii* (Fig. 1C).

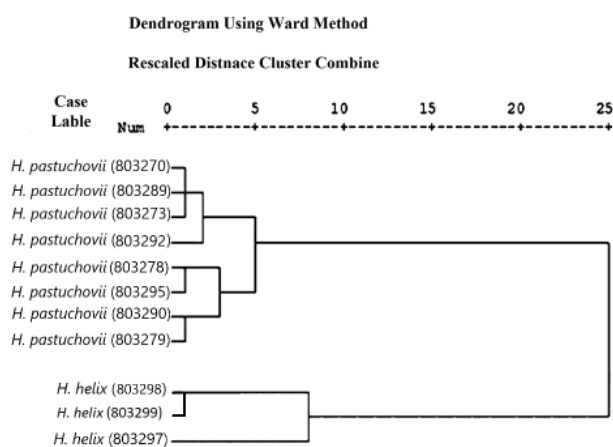
In order to define the diagnostic value of pollen grains



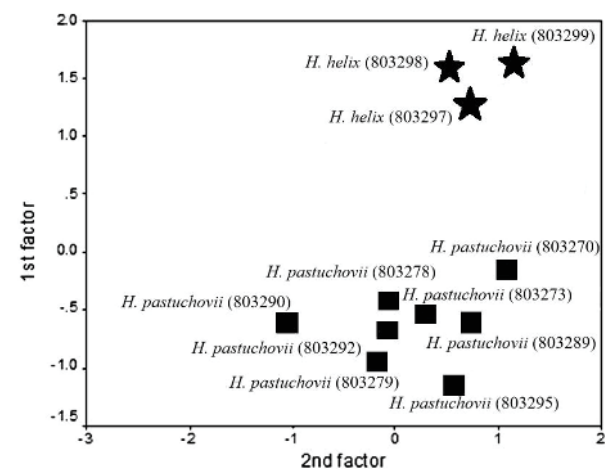


**Figure 1.** Scanning electron micrographs (SEM) of pollen surface in *H. helix* and *H. pastuchovii*. (A, B) *H. helix*, (C, D) *H. pastuchovii*, (E, F) *H. pastuchovii*, and (G, H) *H. pastuchovii*.

in species delimitations in studied *Hedera* species, cluster analysis by Ward's method was performed on the base of seven qualitative and quantitative features (Fig. 2). Ward's dendrogram showed two main clusters (Fig. 2). First cluster composed of populations of *H. helix*. Second cluster composed of two subclusters and contained populations of *H. pastuchovii*. Principal component analysis revealed that there were two components providing more than 76% of total observed variation in studied pollen grains. Studying the component loading was evident that shape and ornamentation of tectum are most important features



**Figure 2.** Cluster analysis (Ward's method) based on pollen features of *Hedera*.



**Figure 3.** PCA plot of *Hedera* species based on observed pollen data.

in the first factor and P/E ratio and length of polar axis are most significant in the second factor. PCA confirmed the results of cluster analysis by Ward's method based on qualitative and quantitative features of pollen grains (Fig. 3).

### Seed shape and size

Taxonomic importance of selected seed features in the examined taxa are summarized in Table 3. Selected SEM micrographs of the seeds and their surfaces are shown in Fig. 4. Seed size differs significantly between two species of *Hedera*. Generally, seeds of *H. helix* are slightly smaller than those of *H. pastuchovii*. The length of the seeds ranged from 3.18 mm in *H. helix* to 5.45 mm in *H. pastuchovii* (column 2 in Table 3), and their width varied from 2.08 mm in *H. helix* to 4.87 mm in *H. pastuchovii* (column 3 in Table 3). In general, the seeds shapes have been observed

**Table 3.** Some diagnostic seed micromorphological features in species of *Hedera*.

Taxon	Length (mm)	Width (mm)	Length/Width	Shape	Anticlinal wall	Sculpturing
<i>H. helix</i>	3.40 ± 0.01	2.08 ± 0.04	1.63	Almond to Circular	Shallowly undulate	Smooth
<i>H. helix</i>	3.38 ± 0.04	2.18 ± 0.05	1.55	Almond to Circular	Shallowly undulate	Smooth
<i>H. helix</i>	3.18 ± 0.02	2.28 ± 0.04	1.39	Almond to Circular	Shallowly undulate	Smooth
<i>H. pastuchovii</i>	5.15 ± 0.06	4.28 ± 0.04	1.20	Almond to Circular	Deeply undulate	Polygonal
<i>H. pastuchovii</i>	5.25 ± 0.02	4.47 ± 0.06	1.17	Almond to Circular	Deeply undulate	Polygonal
<i>H. pastuchovii</i>	5.15 ± 0.03	4.87 ± 0.05	1.05	Almond to Circular	Deeply undulate	Polygonal
<i>H. pastuchovii</i>	5.30 ± 0.02	4.37 ± 0.06	1.21	Almond to Circular	Deeply undulate	Polygonal
<i>H. pastuchovii</i>	5.05 ± 0.01	3.67 ± 0.07	1.37	Almond to Circular	Deeply undulate	Polygonal
<i>H. pastuchovii</i>	5.18 ± 0.04	4.27 ± 0.06	1.21	Almond to Circular	Deeply undulate	Polygonal
<i>H. pastuchovii</i>	5.20 ± 0.01	4.17 ± 0.06	1.24	Almond to Circular	Deeply undulate	Polygonal
<i>H. pastuchovii</i>	5.45 ± 0.07	4.16 ± 0.02	1.31	Almond to Circular	Deeply undulate	Polygonal

almond to circular in two species (Figs. 4A and 4G).

### Seed Sculpturing

In terms of exomorphology, the surface of the seed in *H. helix* is smooth and the anticlinal walls are shallowly undulate (Figs. 4B, 4D and 4F). In *H. pastuchovii*, the seeds have a polygonal surface and the anticlinal walls are observed deeply undulate (Figs. 4H, 4J and 4L).

### Epidermal cell description

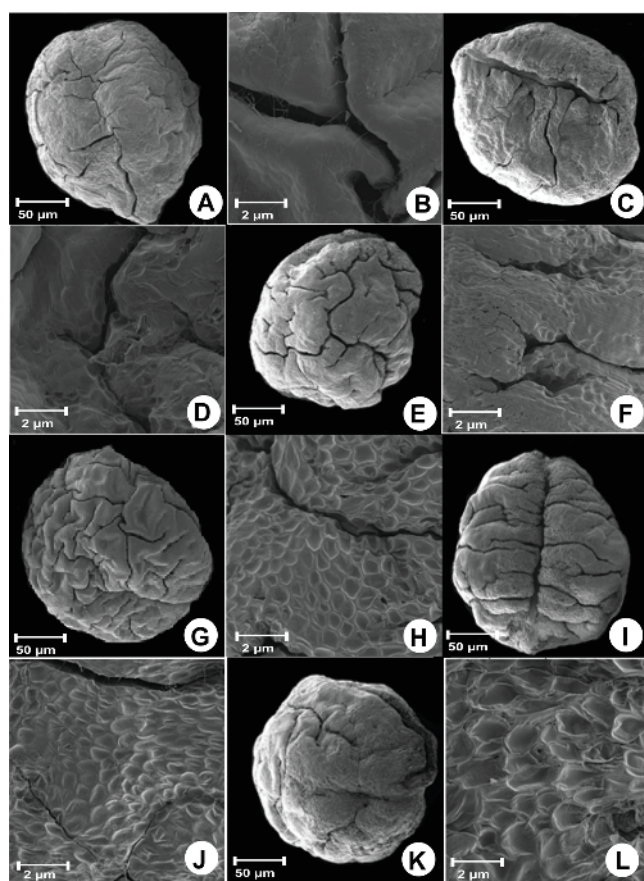
Epidermal and stomata characters of the leaves, such as cell shape, anticlinal wall patterns, stomata index, density, size, and types were examined (Table 4). There were two types of epidermal cells: puzzle-shaped and polygonal cells could be seen, and anticlinal walls have

been observed the wavy and sinuous shapes. There are polygonal cells with wavy anticlinal walls on the adaxial leaf side of *H. helix* (Figs. 5A). Puzzle-shaped cells with sinuous cell walls were seen in *H. pastuchovii* (Figs 5C, 5E and 5G). Abaxial leaf epidermal cells were irregular, with wavy anticlinal walls in *H. helix* (Fig. 5B). No stomata were seen on the adaxial surface of the examined species (Figs. 5A and 5C). All studied species had stomata on the abaxial leaf surface. All treated populations were of the anemocytic stomata type (Figs. 5B and 5D). The largest in size stomata were observed in *H. pastuchovii* (Figs. 5F and 5H) and the smallest were observed in *H. helix* (Fig. 5B). The maximum stomatal index and density were registered in *H. helix* (Table 4).

**Table 4.** Leaf epidermal anatomical features of *Hedera*.

Taxon	Adaxial epidermis			Abaxial epidermis				
	Cell shape	Anticlinal walls	Stomatal apparatus cell	Anticlinal walls	Stomata index (mm <sup>2</sup> )	Stomata density (mm <sup>2</sup> )	Stomata size (µm)	Stomata type
<i>H. helix</i>	Pol	Wa	-	Irr	7 ± 0.01	191.70 ± 2.3	49.62 × 42.24	Anemocytic
<i>H. helix</i>	Pol	Wa	-	Irr	9 ± 0.04	198.50 ± 4.3	46.31 × 40.43	Anemocytic
<i>H. helix</i>	Pol	Wa	-	Irr	7 ± 0.02	189.32 ± 3.3	52.41 × 46.34	Anemocytic
<i>H. pastuchovii</i>	Puz	Sin	-	Sin	11 ± 0.09	120.25 ± 2.1	71.32 × 66.24	Anemocytic
<i>H. pastuchovii</i>	Puz	Sin	-	Sin	13 ± 0.02	107.18 ± 1.4	62.45 × 53.14	Anemocytic
<i>H. pastuchovii</i>	Puz	Sin	-	Sin	12 ± 0.03	132.23 ± 1.2	61.41 × 54.32	Anemocytic
<i>H. pastuchovii</i>	Puz	Sin	-	Sin	10 ± 0.04	121.16 ± 3.2	72.43 × 65.30	Anemocytic
<i>H. pastuchovii</i>	Puz	Sin	-	Sin	12 ± 0.01	95.19 ± 2.5	65.25 × 51.20	Anemocytic
<i>H. pastuchovii</i>	Puz	Sin	-	Sin	10 ± 0.04	108.14 ± 4.2	60.42 × 52.33	Anemocytic
<i>H. pastuchovii</i>	Puz	Sin	-	Sin	13 ± 0.07	135.28 ± 1.7	73.40 × 62.30	Anemocytic
<i>H. pastuchovii</i>	Puz	Sin	-	Sin	12 ± 0.09	140.19 ± 3.5	65.41 × 55.34	Anemocytic
<i>H. pastuchovii</i>	Puz	Sin	-	Sin	14 ± 0.04	153.14 ± 4.2	72.35 × 66.30	Anemocytic
<i>H. pastuchovii</i>	Puz	Sin	-	Sin	13 ± 0.03	109.24 ± 6.5	66.40 × 58.34	Anemocytic

Irr: Irregular; Pol: Polygonal; Puz: Puzzle-shaped; Sin: Sinuous; Wa: Wavy.



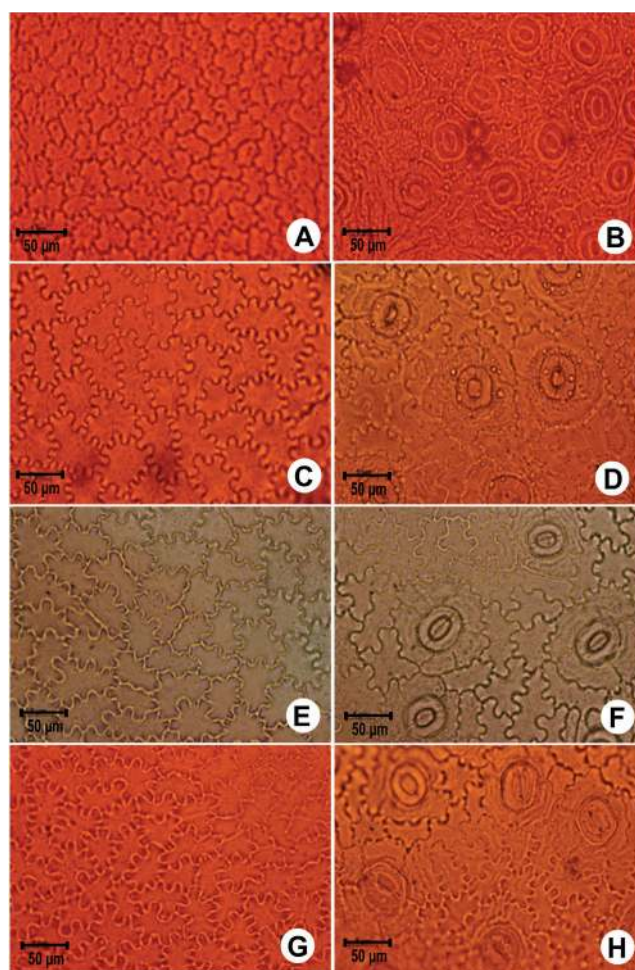
**Figure 4.** Scanning electron micrographs (SEM) of seed surface in *H. helix* and *H. pastuchovii*. For each taxon, the first micrograph shows the outline of the seed indicating its general shape, and the second micrograph is a close view of the seed surface. (A, B) *H. helix*, (C, D) *H. helix*, (E, F) *H. helix*, (G, H) *H. pastuchovii*, (I, J) *H. pastuchovii*, and (K, L) *H. pastuchovii*.

### Phylogenetic analysis

Detailed information about alignment characteristics, selected model of nucleotide substitution, as well as tree statistics from the single analysis of the nrDNA ITS region, are summarized in Table 5. The aligned nrDNA ITS matrix comprises 602 characters. The maximum parsimony, maximum likelihood and Bayesian analyses of the nrDNA ITS produced congruent trees and gave similar results. All members of this genus form a well-supported clade (PP = 1, ML/BS = 100/100) (Fig. 6). The *Hedera* clade is composed of two clades. Clade A includes the populations of *H. helix* (PP = 0.96, ML/BS = 98/100) and the clade B (PP = 0.95, ML/BS = 100/100) comprises the rest of the species of *Hedera* (*H. pastuchovii*) (Fig. 6).

### Phylogenetic networks

The NeighborNet diagram (Fig. 7) revealed almost complete separation of the studied populations within the



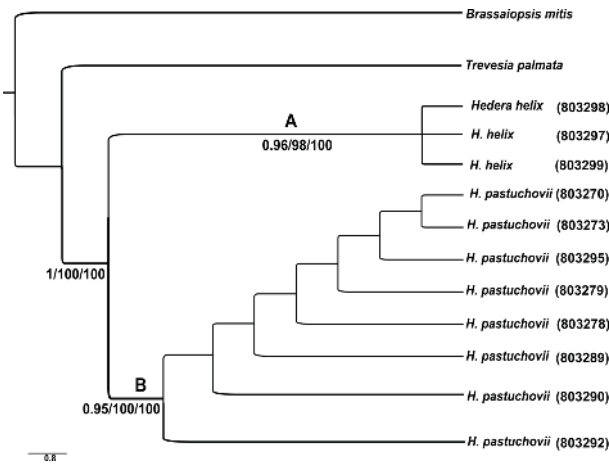
**Figure 5.** Epidermal cells on the adaxial and abaxial side of the specimens' leaves: shape, size, anticlinal wall and stomata under light microscopy. (A, B) *H. helix*, (C, D) *H. pastuchovii*, (E, F) *H. pastuchovii*, and (G, H) *H. pastuchovii*.

network, supporting the phylogenetic results that the *Hedera* is composed of two clades. Populations of *H. helix* (1, 2 and 3) are distinct and stand separately from the other

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

Total sample	nrDNA ITS
Number of sequences	13
Number of ingroup sequences	11
Alignment length [bp]	602
Number of parsimony-informative characters	56
Number of MPTs	16
Length of MPTs	74
Consistency index (CI)	0.98
Retention index (RI)	0.87
Evolutionary model selected (under AIC)	GTR+G





**Figure 6.** Consensus tree (50% majority rule) 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 and ML analysis, respectively (values <50% were not shown).

populations at a major distance. The groups formed in the splits graph are readily correlated to the clades recovered in the phylogenies. We use the term “lineage” to refer to groups of populations in the NN diagram (Fig. 7), and “clade” to refer to groups in the phylogenies (Fig. 6). The ITS splits graph revealed two main groups (Fig. 7). One of these, lineage “I” correlates to clade “A” in Fig. 6 and is composed of populations of *H. helix*. The later, lineage “II” that includes the populations of *H. pastuchovii*, corresponding to clade “B” in Fig. 6. Populations of *H. pastuchovii* (4-11) show a more near genetic affinity and are placed close to each other (Fig. 7).

**Discussion**

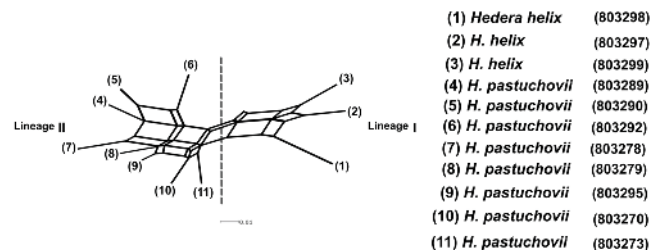
*Hedera* has gained little attention in previous micromorphological and phylogenetic studies, hence, this study presents the first comprehensive investigation of this genus in Iran. Our achievement provides statistical support for the recognition of two species as well as the two major groups traditionally recognized in *Hedera* based on trichomes: the stellate group (*H. helix*) and the scale-like group (*H. pastuchovii*) in agreement with Valcárcel and Vargas (2010). Natural distribution of *H. helix* is Europe and ploidy level and somatic chromosome number of it is  $2n = 2x = 48$ . Whereas *H. pastuchovii* distributed mainly in Caucasus, Iran and Afghanistan with  $2n = 6x = 144$  somatic chromosome number. In general, it can be concluded that, reproductive traits such as pollen and seed traits have achieved the end of their evolution and are valuable in systematic studies. Pollen grains of *H. helix*

are smaller rather than *H. pastuchovii*. This is consistent with their different ploidy level that *H. helix* is diploid ( $2n = 2x = 48$ ), whereas *H. pastuchovii* is hexaploid ( $2n = 6x = 144$ ) (Valcárcel and Vargas 2010).

In consistence with finding of Savulescu and Luchian (2009), our results show that epidermis is made up of only one cells layer with polygonal cells with thin lateral walls and corrugated. Stomata are present on the lower (abaxial) surfaces of leaves only. Locality and habitats of the species also significantly affect the stomata density. *Hedera helix* occupies forest and open woodlands with high humidity. In woodland habitats, it frequently forms a dense ground cover occupying large areas and made up of many individuals (Metcalfé 1958). So high stomata density was observed in *H. helix* and low density in *H. pastuchovii*. Two species demonstrated an anemocytic type of stomata. Stomata have a significant role as valuable differentiating characters at different levels of plant ecology, taxonomy and physiology. Furthermore, the stomata type, density and structure may affect plant physiology, water efficiency and biomass (Luo and Zhou 2001). Humidity in the forest areas can affect the stomata density because the plants do not suffer from drought in such areas. Generally, plants have different strategies to cope with ecological factors. The findings in the current study are in accord with those in other studies relating to the stomata and structure (Miller 1983).

Plant molecular studies chiefly advanced in the recent years and molecular phylogenetic investigation has dramatically alternated our views of organismal relationships (Soltis and Soltis 2000). Nuclear molecular technique has been successfully used for research of infraspecific variations in different genera (Sheidai et al. 2013, 2014). Therefore, we decided to use the molecular approach for research of infra-specific variations between *Hedera* species. The results of molecular studies based on nuclear DNA sequence data are congruent with taxonomy.

Phylogenetic analyses indicated the monophyly of *Hedera* with strongly support (PP = 1.00, ML/BS = 100/100) and divided into two major clades. Our result is also in concordance with Vargas et al. (1999) and that



**Figure 7.** NeighborNet for ITS sequences of *Hedera*. Two major groups were recovered (i.e., lineage I and lineage II).



in their analysis *H. helix* is belonging to diploid clade and *H. pastuchovii* is nested in polyploid clade. Molecular study (nrDNA ITS and *trnT-L* sequences) conducted by Valcárcel and Vargas (2012), did not confirm any close relationship between *H. helix* and *H. pastuchovii*. Thus, it is maybe due to variable levels of polyploidy and low gene flow between these two species. Even though, we confirm the monophyly of the genus *Hedera* relationships among populations of each species in clades A and B, were poorly resolved and form a polytomy. NeighborNet analysis of data set displayed a clear correlation between genetic clusters and geographical groups as natural distribution of *H. helix* is Europe, Whereas *H. pastuchovii* distributed mainly in Asia. Geographical distribution of genetic diversity is also consistent with this cytotoxic diversity pattern as the highest genetic diversity is found in the Mediterranean basin while other areas have lower levels of genetic diversity (Vargas et al. 1999; Grivet and Petit 2002; Ackerfield and Wen 2003; Valcárcel et al. 2003; Valcárcel 2008; Green et al. 2011).

In conclusion, present study was carried out to provide additional evidence for taxonomists. These taxa differ in taxonomically important micromorphological and molecular characteristics. Generally, statistical and bioinformatics tests revealed the great difference between two species. In fact, sequence divergence among populations of each species was generally low, resulting in a lack of phylogenetic resolution, so it is necessary to use chloroplast markers and some other molecular techniques useful at the population level for example AFLP, ISSR, microsatellites to better resolve the relationships between the populations of one species. A number of fast evolving genes will be necessary for resurrecting subspecies or varieties in these two species.

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