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ARTICLE

Molecular, micromorphological and anatomical study of rangeland species of *Atriplex* (Chenopodiaceae) in Iran

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ABSTRACT *Atriplex*, as the largest genus of the Chenopodiaceae, is well known for its taxonomic complexity resulting from overlapping morphological characters. This halophytic perennial is distributed in salty and dry soils of Eurasia, America and Australia. *Atriplex* is one of the most widely cultivated rangeland species in Iran, which improves and revitalizes the rangelands. These unique characteristics of *Atriplex* make it a valuable plant. The present study is the first micromorphological investigation of this genus in Iran. In this study, the molecular evidence, micromorphological and anatomical structure of four species of *Atriplex* have been considered to evaluate their relationships. The basic shape of the pollen grains in most taxa is subprolate, however prolate and spheroidal pollen grains were recorded for *A. lentiformis* and *A. canescens*. One type of trichome (glandular) is described. Here, among the glandular trichomes, density and size of trichomes are considered as valuable characteristics. Micromorphology of epidermis illustrated three types of epidermal cells including puzzle-shaped, polygonal and irregular. Stem cross sections showed rounded shape, but the margins are different between four species. Using nuclear and plastid markers (nrDNA ITS and *rpl*32-*trn*$L_{(UAG)}$), we reconstructed phylogenetic relationships within four species of *Atriplex*. This data set was analyzed by phylogenetic methods including Bayesian inference, maximum likelihood and maximum parsimony. In phylogenetic analyses, all members of four species formed a well-supported clade (PP = 1; ML/BS = 100/100), divided into three major subclades (I, II and III). The results of the present study showed the usefulness of micromorphological, anatomical and molecular characteristics in taxon delimitation at specific levels.

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Introduction

Chenopodiaceae, as the largest family of the Caryophyllales (Cuenoud et al. 2002), contains 110 genera with 1700 species. They are predominantly found in arid to semiarid, saline and agricultural habitats in temperate and subtropical regions (Grigore 2012; Grigore et al. 2014). However, this family is very problematic from a taxonomical point of view and several attempts were recorded to clarify its position within Caryophyllales, and especially the phylogenetic relationships between Amaranthaceae and Chenopodiaceae (Kadereit et al. 2003). Angiosperm Phylogeny Group II (2003), APG III (2009) and APG IV (2016) do not recognize Chenopodiaceae as a separate family from Amaranthaceae, instead only the second is being maintained. Although all morphological characters seem to overlap in Chenopodiaceae and Amaranthaceae s. str., the familial status of the Chenopodiaceae is accepted in recent taxonomic treatments (Sukhorukov 2014; Hernández-Ledesma et al. 2015; Sukhorukov et al. 2019).

Atriplex L. (Atripliceae) can complete its life cycle in saline soils and can be used optimally if there are proper management plans. *Atriplex* species are known for complex genetics, rapid evolutionary rates and high tolerance to xeric, saline, and contaminated soils. It contains several distinguishable species by their morphology, cycle of development and ecological adaptation (Barrow and Osuna 2002). *Atriplex* is the largest genus of the Chenopodiaceae including 260 species in the world (Sukhorukov and Danin 2009) with 18 species in the Flora Iranica region (Hedge 1753). It is mainly distributed in arid and semiarid regions of Eurasia, America and Australia (Sukhorukov and Danin 2009). It is crucial that *Atriplex* species are endowed with an aerial and root-like biomass in the arid and semi-arid regions. The species of *Atriplex* are annual, perennial herbs, subshrubs, or shrubs. The species are often covered with bladder-like hairs that later

Figure 1. Natural habitat of studied species in East North of Iran.

collapse, and rarely with long trichomes. They constitute an efficient tool and relatively little expensive in the struggle against erosion and the desertification (Essafi et al. 2006). In South Africa, the genus seems to be less diverse, however in this region and in South America, it has not been extensively studied so far.

The evolution of C4 photosynthesis has played an important role in the evolutionary success of the genus because the bulk of *Atriplex* species perform C4 photosynthesis. *Atriplex* has typical Kranz anatomy with a layer of bundle sheath cells surrounding each vascular bundle and radially arranged palisade cells (Kadereit et al. 2010). This Atriplicoid leaf type (Carolin et al. 1975) occurs in two variants, viz. the *Atriplex halimus* L. and the *A. dimorphostegia* Kar. & Kir. types, respectively (Khatib 1959; Kadereit et al. 2003). Mozafar and Goodin (1970) studied vesiculated hairs in *A. halimus* as a mechanism for salt tolerance. Troughton and Card (1973) examined the anatomy of *A. buchananii* (Kirk) Kirk ex Cheeseman leaves in New Zealand. The previous phytochemical analyses of some *Atriplex* spp. reported the presence of several classes of secondary metabolites such as saponins, glycosides, flavonoids, tannins, terpenoids, alkaloids, and proteins (Ksouri et al. 2012). Khaniki et al (2012) examined anatomical structure of leaves and stems in *Chenopodium* L. and *Atriplex* in South Khorasan Province. Foliar anatomy of three species of Chenopodiaceae family including *Chenopodium album* L., *Kochia prostrate* L. and *Noaea mucronata* (Forssk.) Asch. & Schweinf. were studied by Zarinkamar (2006). Lu et al. (2018a) investigated the pollen morphological characters of 13 genera and 24 species of the Chenopodiaceae. Molecular phylogeny of Atripliceae (Chenopodioideae, Chenopodiaceae) including *Atriplex* and its implications for systematics, biogeography, flower and fruit evolution and the origin of C4 photosynthesis has been done by Kadereit et al. (2010). *Atriplex* is a rather polymorphic genus with fruiting bract morphology that has many transitional character states, the delimitation from its relative genera has always been problematic (Wilson 1984).

Due to the structural adaptations of this genus to environmental conditions (Kadereit et al. 2010), it was decided to examine the rangeland species of *Atriplex*. Proper knowledge of plant species is essential in the ecosystem and the type of management that should be applied in rangelands.

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

Taxa	Collection data (all samples are from Iran)	GenBank accession no. ITS/rpl32-trnL(UAG)
A. canescens (Pursh) Nutt.	Golestan: Gonbad Kavous, Chapar Qoymeh, Tahmasebi, 803298, GKUH	LC631597/ LC631609
A. canescens	Golestan: Gonbad Kavous, Chapar Qoymeh, Tahmasebi, 803297, GKUH	LC631598 / LC631610
A. canescens	Golestan: Gonbad Kavous, Chapar Qoymeh, Tahmasebi, 803299, GKUH	LC631599 / LC631611
A. lentiformis (Torr.) S.Wats.	Golestan: Gonbad Kavous, Agh Abad, Tahmasebi, 803292, GKUH	LC631588 / LC631600
A. lentiformis	Golestan: Gonbad Kavous, Agh Abad, Tahmasebi, 803290, GKUH	LC631589 / LC631601
A. lentiformis	Golestan: Gonbad Kavous, Agh Abad, Tahmasebi, 803279, GKUH	LC631590 / LC631602
A. halimus L.	Golestan: Gonbad Kavous, Chapar Qoymeh, Tahmasebi, 803270, GKUH	LC631594 / LC631606
A. halimus	Golestan: Gonbad Kavous, Chapar Qoymeh, Tahmasebi, 803273, GKUH	LC631595 / LC631607
A. halimus	Golestan: Gonbad Kavous, Chapar Qoymeh, Tahmasebi, 803289, GKUH	LC631596 / LC631608
A. leucoclada Boiss.	Golestan: Gonbad Kavous, Agh Abad, Tahmasebi, 803278, GKUH	LC631591 / LC631603
A. leucoclada	Golestan: Gonbad Kavous, Agh Abad, Tahmasebi, 803295, GKUH	LC631592 / LC631604
A. leucoclada	Golestan: Gonbad Kavous, Agh Abad, Tahmasebi, 803296, GKUH	LC631593 / LC631605

GKUH: Gonbad Kavous University Herbarium

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

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
A. canescens	19.23 ± 0.32	18.52 ± 0.21	1.11	Spheroidal	19.23 ± 0.16	1.31 ± 0.09	granulate
A. canescens	19.15 ± 0.72	18.42 ± 0.34	1.03	Spheroidal	18.42 ± 0.13	1.16 ± 0.04	granulate
A. canescens	20.41 ± 0.32	18.94 ± 0.72	1.13	Spheroidal	17.27 ± 0.82	1.38 ± 0.07	granulate
A. lentiformis	28.75 ± 1.02	18.37 ± 0.22	1.56	Prolate	20.70 ± 0.12	1.72 ± 0.09	microperforate
A. lentiformis	23.65 ± 1.17	19.43 ± 0.31	1.21	Prolate	19.16 ± 0.18	1.74 ± 0.02	microperforate
A. lentiformis	26.79 ± 1.13	19.67 ± 0.19	1.36	Prolate	21.96 ± 0.04	1.63 ± 0.05	microperforate
A. halimus	37.32 ± 0.72	26.31 ± 0.39	1.41	Subprolate	26.36 ± 0.21	2.93 ± 0.07	microechinate
A. halimus	32.43 ± 1.02	25.78 ± 0.52	1.25	Subprolate	22.16 ± 0.19	2.62 ± 0.04	microechinate
A. halimus	32.71 ± 0.58	24.12 ± 0.35	1.35	Subprolate	24.98 ± 0.12	2.20 ± 0.04	microechinate
A. leucoclada	35.19 ± 1.04	23.61 ± 0.13	1.49	Subprolate	24.42 ± 0.37	2.53 ± 0.06	Granulate-microechinate
A. leucoclada	33.38 ± 0.47	24.40 ± 0.31	1.36	Subprolate	24.21 ± 0.44	2.31 ± 0.11	Granulate-microechinate
A. leucoclada	33.21 ± 0.34	23.54 ± 0.49	1.41	Subprolate	24.72 ± 0.11	2.61 ± 0.09	Granulate-microechinate

There is no comprehensive micromorphological, anatomical and molecular study covering rangeland species of *Atriplex* in Iran. Therefore, the main objective of this study is to provide a detailed investigation and description of trichomes, pollen and epidermis micromorphology of the rangeland species of *Atriplex*. This has been mainly investigated by scanning electron microscopy (SEM) to determine whether this data is valuable in the taxonomy of the genus and delimitation of the species. Furthermore, micromorphology of epidermis, trichomes, and pollen of these species are described for the first time. The specific objectives of this study were as follows: (1) to find morphological characters that could be useful for the diagnosis of taxa; (2) to study the species relationship; (3) to assess the value of micromorphological, anatomical and molecular characters in rangeland species of *Atriplex* in Iran.

Materials and methods

Morphological methods

In the present study, 12 specimens of four *Atriplex* species were collected from different locations in North Iran (Fig. 1) and preserved in the Gonbad Kavous University Herbarium (GKUH). Identification of specimens was carried out based on Flora Iranica (Hedge 1753). The list of voucher specimens and details of localities are given in Table 1. Palynological studies on pollens of *Atriplex canescens* (Pursh) Nutt., *A. lentiformis* (Torr.) S.Wats., *A. halimus* and *A. leucoclada* Boiss. were 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 from freshly collected herbarium specimens. The measurements were based on at least 30 pollen grains per specimen. The characters of pollen grains of the studied species are summarized in Table 2. Trichome micromorphological characters of four species of *Atriplex* were investigated. Samples were removed from Gonbad Kavous University Herbarium (GKUH): To check the consistency of trichome types in different parts of a certain taxa, trichomes of stem and leaves were first investigated with stereomicroscope. Scanning electron microscopic studies were only made on leaf samples. Small pieces of leaves were fixed on aluminum stubs using double-sided adhesive and coated with a thin layer of gold-palladium. The SEM micrographs were taken in a SEM (Tescan, Vega-3 LMU) at an accelerating voltage of 15-22 kV at Research Institute of Razi (Tehran, Iran). The descriptive terminology mainly follows Salmaki et al. (2009); Zamfirache et al. (2009); Osman (2012), with some modifications. A list of voucher specimens used in this study is presented in Table 1.

Anatomical methods

The materials for anatomical studies were fixed in the field with formalin-acetic acid-alcohol (FAA). Four cross-sections were measured for each sample to assess the consistency of anatomical characters. All materials were boiled for 15 min and then fixed in Carnoy solution (alcohols to acetic acid in proportion 3:1). Handmade cross-sections were obtained from the stem using commercial razor blades. The cross-sections were obtained using carmine and methylene green double staining methods. Subsequently, the materials were washed in distilled water

Table 3. Leaf epidermal anatomical features of *Atriplex*.

Taxon	Cell shape*	Anticlinal walls	Stomata index (mm^2)	Stomata density (mm^2)	Stomata size (μm)	Stomata type
A. canescens	Puz	Zip	8 ± 0.19	189.52 ± 4.7	45.73 × 40.31	Anomocytic
A. canescens	Puz	Zip	8 ± 0.11	195.42 ± 2.5	41.24 × 38.72	Anomocytic
A. canescens	Puz	Zip	8 ± 0.09	173.42 ± 5.3	44.31 × 40.51	Anomocytic
A. lentiformis	Puz	Str	10 ± 0.16	117.31 ± 3.8	69.21 × 62.37	Anomocytic
A. lentiformis	Puz	Str	10 ± 0.73	112.21 ± 1.1	61.19 × 48.62	Anomocytic
A. lentiformis	Puz	Str	12 ± 0.08	118.25 ± 1.8	58.32 × 35.85	Anomocytic
A. halimus	Pol	Sin	15 ± 0.06	151.16 ± 3.2	82.43 × 62.30	Anomocytic
A. halimus	Pol	Sin	12 ± 0.09	147.19 ± 2.5	75.25 × 53.20	Anomocytic
A. halimus	Pol	Sin	14 ± 0.17	158.14 ± 3.2	80.42 × 50.33	Anomocytic
A. leucoclada	Irr	Wa	13 ± 0.04	125.28 ± 1.8	53.40 × 61.37	Anomocytic
A. leucoclada	Irr	Wa	12 ± 0.13	110.19 ± 3.3	55.41 × 52.31	Anomocytic
A. leucoclada	Irr	Wa	12 ± 0.15	123.14 ± 3.2	52.35 × 56.30	Anomocytic

*Irr: Irregular; Pol: Polygonal; Puz: Puzzle-shaped; Sin: Sinuous; Str: Straight; Wa: Wavy; Zip: Zip-shaped.

(1 minute) and dehydrated through an ethyl alcohol (70%) and were mounted on microscopic glass slides. Slide sections were studied and photographed.

Epidermis studies on leaves of *A. canescens*, *A. lentiformis*, *A. halimus* and *A. leucoclada* were made using a scanning electron microscope (SEM; Tescan, Vega-3 LMU). Small pieces of leaves were fixed on aluminum stubs using double-sided adhesive and coated with a thin layer of gold-palladium. The SEM micrographs were taken in a SEM (Tescan, Vega-3 LMU) at an accelerating voltage of 15-22 kV at Research Institute of Razi (Tehran, Iran). Some characters (stomata length/weight, number of stomata, number of epidermal cells) were measured with Image Tools ver. 3.0 and AxioVision 4.8. (Table 3).

Molecular methods

Taxon sampling

A sampling includes plants from 12 specimens and four species of *Atriplex* were chosen as an ingroup for nrDNA ITS and *rpl*32-*trn*$L_{(UAG)}$. *Halimione verucifera* Aellen was chosen as an outgroup following previous molecular phylogenetic studies (Kadereit et al. 2010). A list of all the taxa used in this study and the sources, 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 the 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). The *rpl*32-*trn*$L_{(UAG)}$ spacer was amplified using the rpl32-F and trn$L_{(UAG)}$ primers described in Shaw et al. (2007). 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 a UV gel documentation system (UVItec, 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

Combined 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 the dataset required the introduction of numerous single and multiple-base indels (insertions/deletions). Positions of indels were treated as missing data for the ITS and *rpl*32-*trn*$L_{(UAG)}$ datasets.

Phylogenetic inferences

Parsimony method

Maximum parsimony (MP) analyses were conducted using PAUP* version 4.0a157 (Swofford 2002). The heuristic search option was employed for the combined 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.

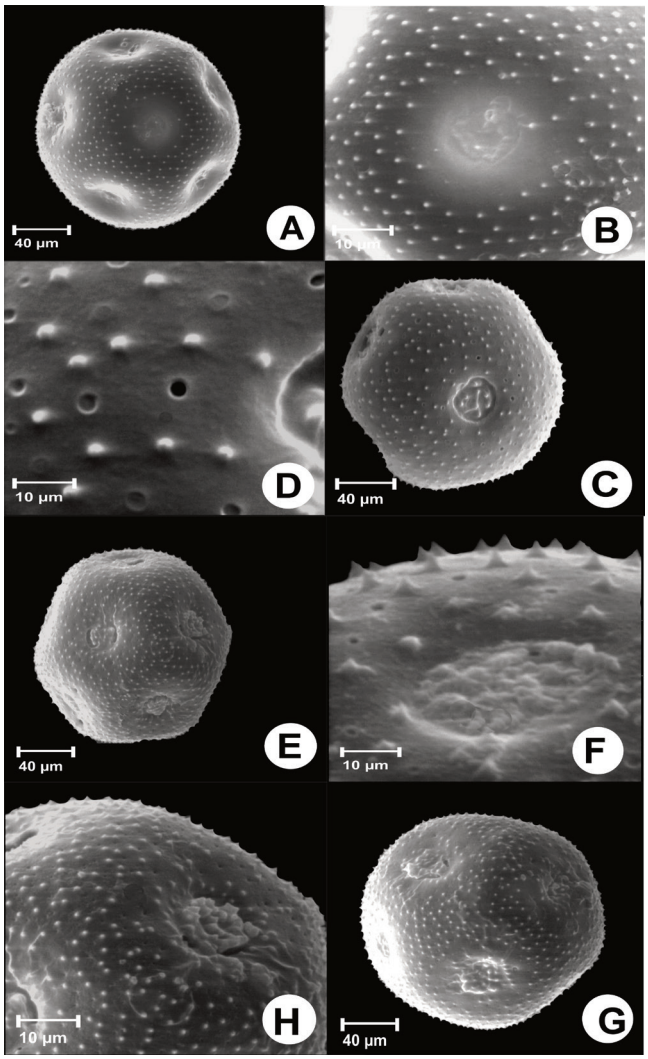

Figure 2. Scanning electron micrographs (SEM) of pollen surface in *Atriplex*. (A, B) *A. canescens*, (C, D) *A. lentiformis*, (E, F) *A. halimus*, (G, H) *A. leucoclada*.

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+I+G model for the combined dataset. 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).

Results

Pollen morphology

The pollen grains of the studied species revealed some variations and separated four species of *Atriplex*. 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 are shown in Table 2. Selected SEM micrographs of the pollens and their surfaces are shown in Fig. 2. Generally, type of pollen grain aperture is observed subprolate among studied species (Fig. 2). The length of the polar and equatorial axis was found useful in separating four species. Polar axis (P) length of pollen grains varied from the smallest size for *A. canescens* (19.15 µm) to the largest size for *A. halimus* (37.32 µm). The equatorial axis (E) length of pollen grains ranged from the smallest size in *A. lentiformis* (18.37 µm) to the largest size in *A. halimus* (26.31 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 1.03 to 1.56, therefore the pollen shape is subprolate in *A. leucoclada* and *A. halimus* but prolate in *A. lentiformis* and spheroidal in *A. canescens* were also seen. The ornamentation of tectum is granulated in *A. canescens* (Fig. 2B), microperforate in *A. lentiformis* (Fig. 2D), microechinate in *A. halimus* (Fig. 2F) and is granulate-microechinate in *A. leucoclada* (Fig. 2H).

Trichome morphology

One basic type of trichome can be distinguished on the leaf surface of the studied taxa including glandular. Selected SEM micrographs of common trichome types are presented in Fig. 3. Some trichome characters which provide appropriate variation for taxa discrimination including the length of stalk, number of trichome cells (unicellular, bi-cellular or multi-cellular) and the density of trichome cells. A considerable variation is observed among the glandular trichomes. Based on the observed

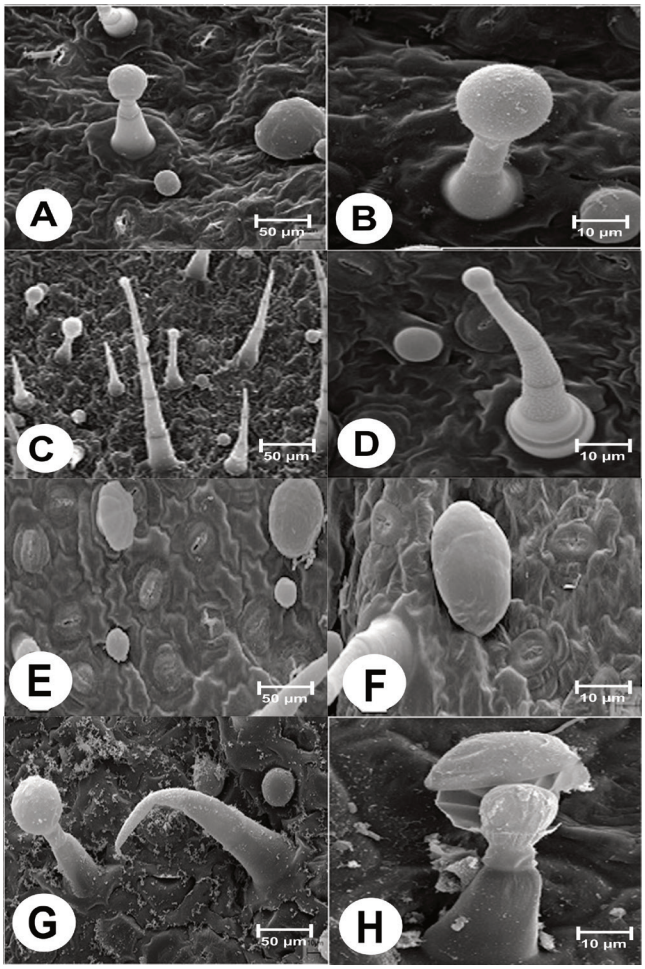

Figure 3. Scanning electron micrographs (SEM) of trichome in *Atriplex*. (A, B) *A. canescens*, (C, D) *A. lentiformis*, (E, F) *A. halimus*, (G, H) *A. leucoclada*.

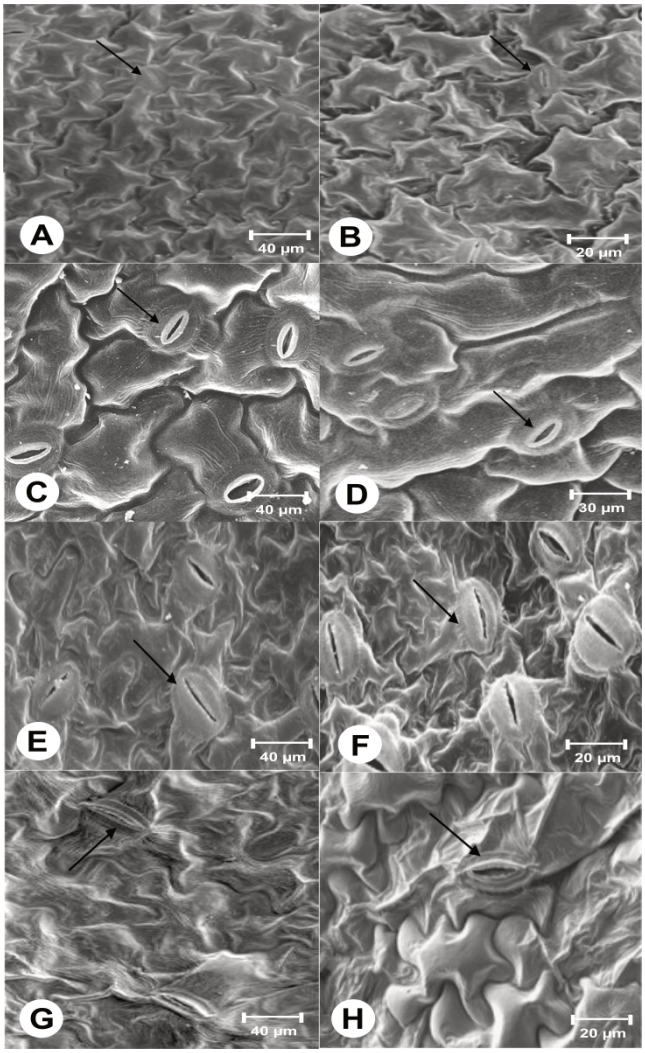

Figure 4. Epidermal cells on leaves: shape, size, anticlinal wall and stomata under Scanning electron micrographs (SEM) in *Atriplex*. (A, B) *A. canescens*, (C, D) *A. lentiformis*, (E, F) *A. halimus*, (G, H) *A. leucoclada*.

variations, glandular trichomes can be divided into two subtypes covering short stalked (e.g., *A. halimus*; Figs. 3E and 3F) and long stalked (e.g., *A. lentiformis*; Figs. 3C and 3D). Stalks of trichomes can be unicellular (e.g., *A. leucoclada*, Figs. 3G and 3H), bi-cellular (e.g., *A. canescens*, Figs. 3A and 3B) or multi-cellular (e.g., *A. lentiformis*, Figs. 3C and 3D). All the glandular trichomes in our study were unbranched. Some features of unbranched glandular trichomes including size, shape and number of cells in trichome provide useful diagnostic characters for recognizing examined taxa. The size of unbranched glandular trichomes varied from short (up to 100 µm, e.g., *A. halimus*; Figs. 3E and 3F) to long (200 to 600 µm, e.g., *A. lentiformis*, Figs. 3C and 3D).

Epidermal cell description

Epidermal and stomata characters of the leaves, such as cell shape, anticlinal wall patterns, stomata index, density, size and type were examined (Table 3). Three types of epidermal cells including puzzle-shaped, polygonal and irregular cells can be seen. Anticlinal walls have been observed the wavy, sinuous, straight and zip-shaped. Puzzle-shaped cells with zip-shaped and straight anticlinal walls on the adaxial leaf side of *A. canescens* and *A. lentiformis* were seen (Figs. 4B and 4D). Polygonal cells with sinuous cell walls were seen in *A. halimus* (Fig. 4F). Abaxial leaf epidermal cells were irregular, with wavy anticlinal walls in *A. leucoclada* (Fig. 4H). All studied specimens were of the anomocytic stomata type (Figs. 4C). The largest stomatal size was observed in *A. halimus* (Figs. 4E and 4F) and the smallest was observed in *A. canescens* (Figs. 4A and 4B). The maximum stomatal density was registered in *A. canescens* (Table 3).

Table 4. Dataset and tree statistics from single and combined analysis of the regions.

Total sample	nrDNA ITS	*rpl*32-*trn*L$_{(UAG)}$	ITS+*rpl*32-*trn*L$_{(UAG)}$
Number of sequences	13	13	13
Number of ingroup sequences	12	12	12
Alignment length [bp]	627	1278	1905
Number of parsimony- informative characters	106	140	324
Number of MPTs	16	23	29
Length of MPTs	74	87	94
Consistency index (CI)	0.68	0.67	0.64
Retention index (RI)	0.78	0.88	0.91
Evolutionary model selected (under AIC)	SYM+I+G	GTR+G	GTR+I+G

Anatomical study

Selected LM micrographs of cross-sections of the stem are presented in Figure 5. Most characters show significant variability between four species but were constant among different specimens of each studied species.

Stem anatomy

Stem cross sections showed rounded shape with wavy and straight margins (e.g., *A. halimus* in Fig. 5E and *A. canescens* in Fig. 5A). The epidermis is composed of single layered cells. The collenchyma tissue is located under the epidermis of the stem. They are 4-5-layered in four species. The cortex tissue is composed of 4-5-layered parenchyma cells. Vascular bundles are arranged in a single circle. The shape and number of vascular bundles are different. The number of vascular bundles is 10-14 with U-shaped and V-shaped outline (e.g., *A. canescens* in Fig. 5A and *A. halimus* in Fig. 5F). The xylem is surrounded by sclerenchymatous cells. Druse crystals were mainly distributed in the mesophyll and were densely distributed in *A. halimus* (Fig. 5F), but sparsely in *A. canescens* (Fig. 5A).

Phylogenetic analysis

Detailed information about alignment characteristics, selected model of nucleotide substitution, as well as tree statistics from the combined analysis of the nrDNA ITS and *rpl*32-*trn*L$_{(UAG)}$ regions, are summarized in Table 4. The aligned nrDNA ITS and *rpl*32-*trn*L$_{(UAG)}$ matrix comprised 627 and 1278 characters, respectively. The maximum parsimony, maximum likelihood and Bayesian analyses of the combined data 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 *Atriplex* clade is composed of three subclades. Subclade I includes the specimens of *A. canescens* (PP = 0.98, ML/BS = 99/100) and the subclade II (PP = 0.89, ML/BS = 100/94) comprises the specimens of *A. lentiformis* and the subclade III (PP = 0.97, ML/BS = 84/86) contains the rest of the species of *Atriplex* (*A. halimus* and A. *leucoclada*) (Fig. 6).

Discussion

About 15% of Iranian lands are affected by salinity. The rangeland species of *Atriplex*, such as *A. canescens*,

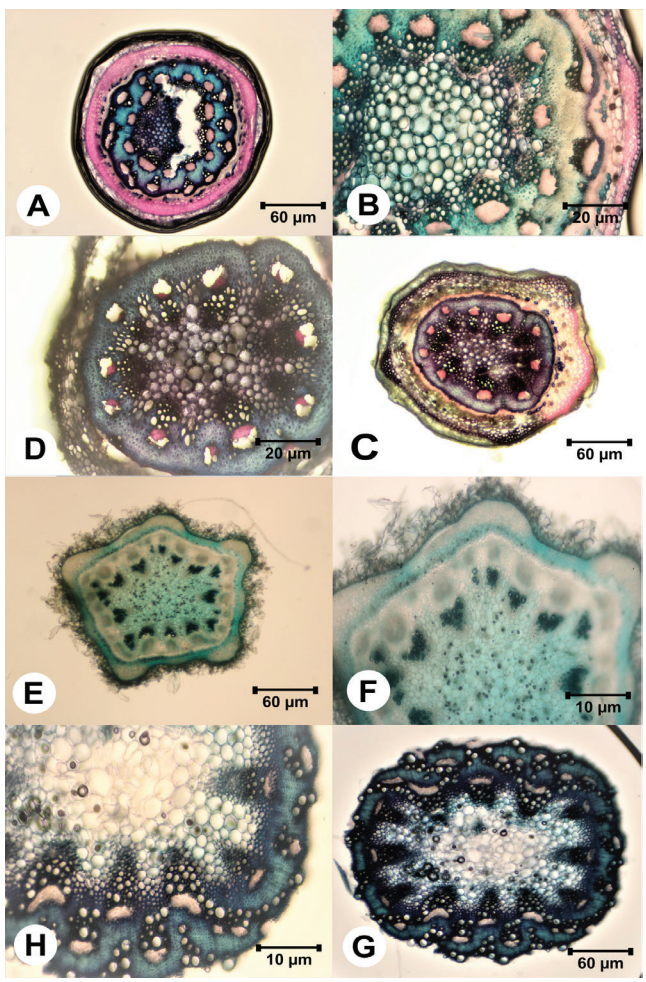

Figure 5. Transverse sections of stem in *Atriplex*. (A, B) *A. canescens*, (C, D) *A. lentiformis*, (E, F) *A. halimus*, (G, H) *A. leucoclada*.

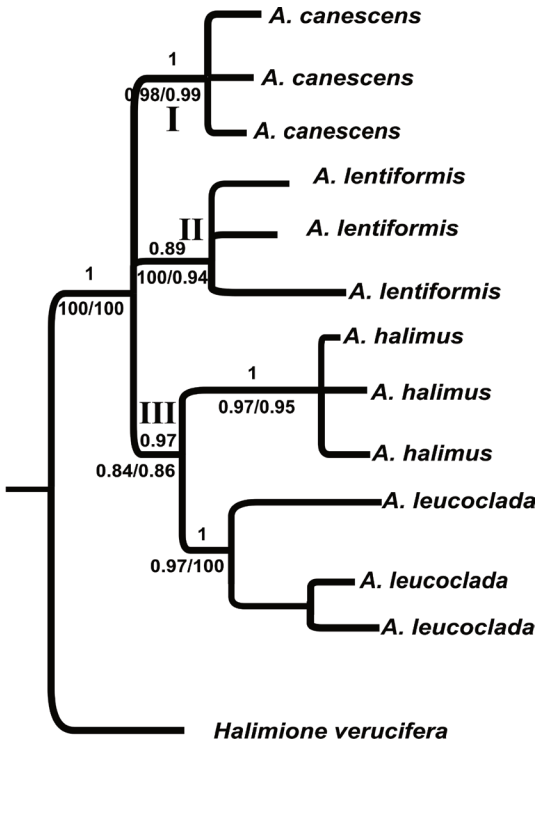

Figure 6. 50% majority rule consensus tree resulting from the Bayesian phylogenetic analysis of the nrDNA ITS and *rpl*32-*trn*L$_{(UAG)}$ datasets. Numbers of the branches are posterior probability (PP) from the BI and bootstrap support (BS) values from a MP and ML analysis, respectively (values <50 % were not shown).

A. halimus, A. lentiformis and *A. leucoclada* are notable for planting in saline soils. *Atriplex* is a halophyte plant distributed in dry and saline regions of different parts of Iran. While there are studies on cultivation (Moghadam 1973), cultivation methods (Henteh 1990), nutritional value (Ranjbar Fardiee 1991), growth (Eskandari 1995) and karyotype (Amoiee and Ahmadian 1995), *Atriplex* has gained little attention in previous micromorphological and phylogenetic studies. Hence, this study presents the first comprehensive investigation of rangeland species of *Atriplex* in Iran. Micro-morphological evaluation of the *Atriplex* species has shown the diagnostic value of these characters. Most of the pollen micromorphological features, such as pore diameter, pore number and pore membrane ornamentation, have been used to separate the different taxa of Chenopodiaceae at the generic level by SEM (scanning electron microscopy), although pore number and diameter are visible by LM (light microscopy) (Hao et al. 1989; Olvera et al. 2006; Perveen and Qaiser 2012; Lu et al. 2018a, 2018b). In previous studies, pollen size was used as a criterion to differentiate pollen types (Hao et al. 1989; Perveen and Qaiser 2012; Lu et al. 2018a). However, pollen size is excluded from Chenopodiaceae pollen classification, as Pan (1993) pointed out that it could be influenced by humidity in different habitats. Despite the general similarity of the pollen throughout Atripliceae (Olvera et al. 2006), our observations indicate that pollen micromorphology is a valuable source in taxonomic re-evaluations within the genus.

So far, no micromorphological study has been performed on trichomes of leaf surface in *Atriplex* species and this study is the first study to investigate the characteristics of trichomes in studied species. In a study by El Ghazali et al. (2016) on the stem epidermis in Chenopodiaceae, salt sacs were found in all species of *Atriplex*, *Obione*, *Halimione* Aellen, and in some species of *Salsola* L. and *Chenopodium*. The trichomes in these plants show a great variety in terms single-celled or multi-celled, tuberous and dense, and therefore this trait can be beneficial in the distinction of *Atriplex* species. *Atriplex canescens* species have medium density tuberous multicellular trichomes. Different specimens of *A. lentiformis* have very long density hairs with small glands at the end of them. *Atriplex leucoclada* species have single-celled hairs as glandular hairs with low density. The hairs in *A. halimus* are very short and have a very large salt gland. These hairs have no base and possess a medium density, and these results are in accordance with the study of Mozafar and Goodin (1970). They concluded that the salt concentration in vesicle hairs of *A. halimus* is higher than leaf saponin. These vesicles hairs have played a very crucial role in removing toxins and salts from the leaves, parenchyma and vascular bundles. In all *Atriplex* species, salt bladders occur (Schirmer and Breckle 1982; Carolin 1983; Reimann and Breckle 1988) and by a specialized mechanism of salt removal from leaves, they prevent dangerous accumulation of toxic salt levels (Mozafar and Goodin 1970; Osmond et al. 1980). The leaves of the most *Atriplex* species, particularly from field plants, are replete with bladders. In mature leaves, the salt bladders collapse and produce a thick layer all over the leaf surface. According to Smaoui (1971), salt bladders in *A. halimus* are numerous and their development occurs during leaf life. This thick layer of salt bladders constitutes a nearly impenetrable surface and often shows crystal deposits identified as sodium chloride (Osmond et al. 1980; Bennert and Schmidt 1983).

So far, no comprehensive study has been performed on the epidermis of *Atriplex* species and the current study is the first research on the characteristics of the epidermis and stomata in this genus. The characteristics of the epidermis examined by scanning electron microscopy in *Atriplex* species show a variety of micromorphological features. The epidermis acts as a barrier against mechani-

cal damage, insects, excessive light and lack of water (El Ghazali et al. 2016). The type of stomata in all species of this genus is anomocytic, which is consistent with the study of Khaniki et al. (2012). Locality and habitats of the species also significantly affect the stomata density. In woodland habitats, it mostly forms a dense ground cover occupying large areas and comprising many individuals (Metcalfe 1950). High stomata density was observed in *A. canescens* and *A. halimus* and low density in *A. lentiformis* and *A. leucoclada*. Stomata have a considerable role as valuable differentiating characters at various levels of plant ecology, taxonomy and physiology (Amini et al. 2019). Furthermore, the stomata type, density and structure have been affected by plant physiology, water efficiency and biomass (Luo and Zhou 2001). Generally, plants have different strategies to cope with ecological factors. The findings in the current study are in accordance with other studies relating to the stomata and structure (Miller 1983). The limited anatomical studies have been performed in the *Atriplex* genus, including the study of Khaniki et al. (2012). The cross section of the stem in the species of *Atriplex* is round and the non-woody pericycle is seen as a layer. In accordance with the studies of Khaniki et al. (2012), the vascular bundles are scattered in parenchymal tissue.

Kaderiet et al. (2003) studied the phylogenetic relationships of Amaranthaceae and Chenopodiaceae families based on *rbcl* chloroplast marker, but this marker could not distinct between the two families. In this study, phylogenetic analysis showed studied species with very high support are monophyletic (PP = 1, ML/BS = 100, MP/BS = 100). Studies on *Atriplex* showed that, specimens of *A. canescens* were isolated from specimens of *A. lentiformis* with high support (PP = 99, MLBS = 98, MPBS = 100). *Atriplex canescens* has a highly variable form and readily hybridizes with several other species in the *Atriplex* genus. This species is adapted to variable edaphical and environmental conditions (Mansen *et al.* 2004). In current study, this species is placed in a distinct subclade. Molecular study (nrDNA ITS, *atpB-rbcL* and *rbcl* sequences) conducted by Kaderiet et al. (2010), confirm close relationship between *A. halimus* and *A. leucoclada* and our results agreed with them. Relationships between specimens remained unresolved.

Conclusion

In conclusion, the present study was carried out to provide additional evidence for taxonomists and rangeland specialists. *Atriplex* is one of the mostly planted rangeland species in Golestan province (Iran), which improves and revitalizes the rangelands of the province. *Atriplex* is a valuable plant which tolerates fluctuation in salinity and stands higher levels of temperature. In some *Atriplex* species, such as *Atriplex canescens*, a great variety of vegetative forms has been observed. Also, these species have mixed with several other species in the genus *Atriplex*, which has caused the polyploidy phenomenon (increasing the number of chromosomes) and has created a very high adaptation to ecological and environmental conditions. These taxa differ in taxonomically important micromorphological and molecular characteristics. Due to low sequence divergence at the population level, AFLP or SSR markers, or RADs will be necessary for shedding further light on the relationships with more sampling at the population level. So micromorphological, molecular and anatomical studies will be aid for further research to identify and select suitable species of *Atriplex* in rangeland ecosystems.

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