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Macro- and micro-morphological, and anatomical investigation of achenes in the Iranian *Anemone* and *Pulsatilla* species

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Abstract

Anemone L. (*Ranunculaceae*) with ca. 150 morphologically variable species is distributed all over the world. Morphological characters are widely used to discriminate species. In the present study, therefore, the Iranian species of *Anemone* and *Pulsatilla* are investigated by their achene macro- and micro-morphological and anatomical features. The micro-morphological and anatomical studies are performed employing the scanning electronic microscope (SEM). Multivariate statistical analyses of various quantitative and qualitative characters of the achene as well as their anatomy, resulted in distinguishing four different achene types. Although, obvious differences are observed in the achene body surface; number of the endocarp and mesocarp layers, and in the shape and length of the style, it is also revealed that, features like the number of epicarp layers and margin surface, have no diagnostic values. The analyses are carried out by SPSS software, using the Ward's and the Average linkage methods. Both the dendrograms showed that, the *P. albana* is separated from other species which is consistent with systematic studies and the achene features. Besides, in the present study, *A. blanda* is also rediscovered.

Keywords: Cross section, dendrogram, fruit, pericarp, *Ranunculaceae*, taxon

بررسی ریخت‌شناسی، ریزریخت‌شناسی و تشریحی فندقه گونه‌های ایرانی *Anemone* و *Pulsatilla**

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چکیده

جنس *Anemone* متعلق به تیره آلاله‌ییان دارای ۱۵۰ گونه با پراکنش در سرتاسر دنیا می‌باشد. ویژگی‌های ریخت‌شناسی همواره برای تشخیص و تمایز مرز گونه‌ها مورد استفاده قرار گرفته است. در این مطالعه، گونه‌های ایرانی *Anemone* L. و یک گونه *Pulsatilla* تحت بررسی‌های ریخت‌شناسی، ریزریخت‌شناسی و تشریحی فندقه قرار گرفته‌اند. بررسی‌های ریزریخت‌شناسی و تشریحی با استفاده از میکروسکوپ الکترونی نگاره (SEM) انجام شد. صفات متعدد کمی و کیفی تعریف و در نمونه‌های مورد مطالعه اندازه‌گیری شدند. براساس شکل کلی فندقه و ویژگی‌های تشریحی، چهار تیپ برای فندقه‌ها تشخیص داده شد. تفاوت‌های آشکار در الگوی بدنه فندقه (achene body)، تعداد لایه‌های اندوکارپ و مزوکارپ و همچنین طول و شکل خامه نشان داد که این صفات دارای ارزش تشخیصی بوده و صفاتی نظیر تعداد لایه‌های اپی‌کارپ و سطح حاشیه فندقه، فاقد تفاوت‌های شاخص در بین آرایه‌های مورد مطالعه می‌باشند. آنالیزهای آماری با استفاده از نرم‌افزار SPSS به دو روش Ward و Average linkage بر پایه کلیه صفات کمی و کیفی انجام شد که در هر دو دندروگرام جدایی *P. albana* Stev. از آرایه‌های *Anemone* و جدایی آرایه‌های *Anemone* از یکدیگر کاملا مشهود است. به علاوه در این مطالعه، گونه *A. blanda* Schott & Kotschy به عنوان یک گونه بازکشف شده گزارش می‌شود.

واژه‌های کلیدی: آرایه، پریکارپ، آلاله‌ییان، دندروگرام، سطح مقطع عرضی، میوه

* مستخرج از رساله دکتری نگارنده نخست به راهنمایی دکتر فریبا شریف‌نیا و دکتر مصطفی اسدی ارایه شده به واحد علوم و تحقیقات دانشگاه آزاد اسلامی

Introduction

Anemone L. (*Ranunculaceae*) consists of 150 species, although exists in both hemispheres but mainly distributed in the northern hemisphere (Tamura 1995). The number of species varies in different studies, e.g., Ziman *et al.* (2008) introduced 118 species with 15 subgenera while Hoot *et al.* (2012) reported 200 species with two subgenera. *Anemone* species live in a wide range of habitats including arctic region, alpine tundra, prairies, Mediterranean areas, and semi-deserts (Schuettpelz *et al.* 2002, Ziman *et al.* 2006). The genus possesses great morphological variation due to the wide geographical distribution (Ziman 1998).

Seven species of *Anemone* are identified in Iran so far, among which four species are mentioned in Flora Iranica (Rechinger & Riedl 1992). These species are: *A. coronaria* L., *A. biflora* DC., *A. petiolulosa* Juz., and *A. caucasica* Willd. ex Rupr. The other three species are: *A. gortschakowii* Kar & Kir. (recorded by Mobayen 1985), *A. tschernjaewii* Regel. (recorded by Joharchi & Akhani 2006, Joharchi & *et al.* 2011), and *A. narcissiflora*, (recorded by Akrami *et al.* 2011). It is worth to note that, although *A. petiolulosa* is introduced as a distinct species in Flora Iranica (Rechinger & Riedl 1992) and the Soviet Union flora (Komarov 1937), it is classified in variety level in the studies of Ziman *et al.* (1998) and Heidary Baladehi (2007). The same is true for *A. gortschakowii* which is considered as a distinct species in Soviet Union flora (Komarov 1937), and flora of Iran (Mobayen 1985), but it is classified in variety level in Sinno-Saoud *et al.* (2007) and Parsa (1951). It should be noted that, in flora of Iran (*l.c.*) and Flore de l' Iran (Parsa 1951), in addition to *A. gortschakowii*, other taxa such as *A. biflora*, *A. coronaria*, *A. narcissiflora*, and *A. blanda* are also mentioned.

Fruits are usually considered to have stable and higher taxonomic values (Liu *et al.* 2002). In the classification system of *Ranunculaceae*, the fruit types and the external morphology together with the characteristic of pollen grains were emphasized, while the fruit anatomy was usually overlooked (Wang & Wang 1979, Johansson 1995, Tamura 1993, Takhtajan 2009). Based on the fruit characters, Hutchinson (1923), divided *Ranunculaceae* into two subfamilies, i.e., *Helleboroideae* (ovaries with more than one ovule, fruits follicular or baccate) and *Ranunculoideae* (ovaries with one ovule, fruit a bunch of dry achenes, very rarely baccate) but later, other researchers focused on fruit features in their studies.

According to Wang & Ren (2008), apocarpous gynoecium that contain anatropous and unitegmic ovules, with the body almost parallel to the funicle and entirely fusing with it, improve into one-seeded achenes having a head (fruitlets).

Cheng *et al.* (2015) studied the achene morphology and anatomy in 27 genera of *Ranunculaceae* and introduced four groups based on the epidermal surface, vascular bundle and mesocarp as well as endocarp cell structure characteristics. According to this, the *Anemone*, *Pulsatilla* Mill., and *Clematis* L. were placed in the 4th group with non-branching vascular bundle and one-layer fiber endocarp. Tamura (1993) and Takhtajan (2009), based on fruit characters (e.g., achene with no obvious longitudinal veins), pointed out that, these three genera should be placed in one subfamily (*Rannculoideae*).

Tang *et al.* (2008) explained the carpel fusion patterns of *Ranuculaceae* fruits that could provide morphological evidences for the molecular systematics.

Chaudhary & Trifonaova (1988) showed fruit anatomy importance in taxonomical classification by reviewing 11 Nepalese *Anemone* species. Ziman *et al.* (2008) demonstrated achene characters with diagnostic potential values. Maciejewska-Rutkowska & Antkowiak (2013) considered four Polish *Anemone* and described their pericarp anatomy. They showed the achene body, the achene style, the number of endocarp layers, and outline of its cells as diagnostic characters. Rasmussen in southern Africa (1979) divided some species of *Anemone* based on having fleshy fruit in *Knowltonia* Salisb., while Manning & Goldbaltt (2013) reclassified the fleshy fruit species in *Anemone*.

Anemone biflora was first recognized as a taxonomic complex by Juzepchuk (1937), and included it under subsection *Biflora* Juz. within the *Oriba* Spach. a section of the genus *Anemone*. Ziman *et al.* (1998) studied this complex in central and south-west Asia and, on the basis of their morphology (length of achene hair, sepal size, and petiolule size), and the anatomical structure of petioles of *A. biflora* and ten segregate species, distinguished one subsection, *Biflora* P. Popov, with three series and the following six species: *A. biflora* DC., with three varieties [var. *biflora*, var. *petiolulosa* (Juz.) Ziman, and var. *eranthioides* (Regel) Ziman], *A. gortschakowii* Kar. & Kir., *A. bucharica* (Regel) Juz. ex Komarov, *A. baissunensis* Juz., *A. serawschanica* Kom., and *A. tschernjaewii* Regel.

The present study thus aims to: 1. Precisely classify *Pulsatilla albana* Stev., the only species of *Pulsatilla* genus in Iran which some researchers believed that, it should be classified under *Pulsatilla* genus (Mobayen 1985, Rechinger & Riedl 1992, Yaprak *et al.* 2011), while others suggested to consider it as a species of *Anemone* (Boissier 1867, Parsa 1951, Davis 1965), 2. Exploring the morphological and anatomical characteristics of the fruit of Iranian *Anemon*e (except for *A. tschernjaewii*) as well as *P. albana*, and 3. Investigate the classification level and the relation of *A. biflora* complex taxa of Iran.

Materials and Methods

In this study, more than 130 plant samples from four different herbaria are investigated. Among this, samples from the herbarium (TARI) of Research Institute of Forests and Rangelands (Tehran, Iran), and also some specimens collected from different areas in Iran (Table 1) were selected for micro-morphological studies.

In order to compare the achenes, different quantitative and qualitative characters are defined and measured (Tables 2–3). The outer surface of the achenes is also investigated employing a light microscope together with a Hitachi scanning electronic microscope (SEM) at Shahid Beheshti University (Tehran, Iran). The samples were coated with a 550-Å-thick layer of gold in a Polaron SC7610 vacuum coating apparatus for 180s and then examined by the above-mentioned electron microscope with three different magnifications (x70, x200, and x400).

- Cross section study

The samples were kept in the fixative solution for two to three days, and afterwards the cutting was done under the loop with the help of blade. Each sample was prepared using the same method. At least three specimens from different localities were studied on average using Carnoy, a digital measurement tool and some characters were measured (Tables 2–3). The qualitative and quantitative data were prepared as a matrix, and the data were standardized before performing the cluster analyses. The analyses were carried out by SPSS software, using the Ward's and the average linkage methods.

Results

The morphological and anatomical characters of the *Anemone* fruits taxa showed some similarities and different between taxa (Table 2 & Figs 1–3), length of the studied achenes varied between 1.5 to 8 mm and observed to be ovate-obovate (*A. narcissiflora*), biconvex boat-shaped (*A. biflora, A. coronaria*, and *P. albana*), semispherical-ovate-shaped (*A. caucasica* and *A. blanda*) with persistent and non-persistent styles.

Anemone narcissiflora possesses the biggest achene which is 2–5 times longer than the other species both in length and width. *Anemone blanda* and *A. caucasica* possess the shortest length while *P. albana* has the shortest width. The size of *A. biflora* var. *biflora* achenes were almost 1.3–2.6 times than the size of other achenes (except for *A. narcissiflora*), while *A. biflora* var. *gortschakowii*, *A. biflora* var. *petiolulosa*, and *A. coronaria* achenes have just had almost the same size.

Pericarp was consisting of three layers and in the considered taxa, the epicarp had one-layer, mesocarp 1–4 layers and endocarp 1–2 layers.

In the present study, we therefore, specify four types based on the shape of achenes and pericarp characteristics such as the cross-section, body shape and the number of pericarp layers as follows:

Type 1: Including *Anemone narcissiflora* only with large size, inversely urceolate/obovate-shaped achenes, curved style and flattened wings. The body of achenes were completely glabrous, striate-rugose, and reticular in the wing area with the thickest pericarp structure. The achene cross-section were narrow and ellipsoid with two spherical tubercles on the dorsal and vertical areas. Endocarp was in the form of 2–3 fiber layers with thick ellipsoid cell walls and had fiber-shaped cells around non-branched vascular bundles (Figs 3 & 5).

Type 2: Comprising three varieties from *Anemone biflora* and *A. coronaria*. The three varieties of *A. biflora* generally showed biconvex boat-shape achenes with a curved beaked style. The achene body was striate-rugose with lanate and rugulose dense hairs. They have got the thinnest pericarp structure (ca. 25 µm) and their cross-section was flattened ellipsoid-almost spherical, with two tubercles on both sides and also fiber cells around two non-branched vascular bundles. It should be noted that, in spite of their similarities, these taxa show some differences as well. For instance, some features observed on the surface of *A. biflora* var. *biflora*, such as surface concavities or styles which exclusively were 3–4 times longer than the other two taxa styles and not seen in the other two taxa. The hair basal of this taxon was bulbously swelled, while for the other two taxa it was normal with no thickness.

In this taxon, endocarp cells were quadrangular, while in others it was ellipsoid-spherical in shape. *Anemone biflora* var. *gortschakowii* had the longest hair length between the taxa, while the shortest style was observed in *A. biflora* var. *petiolulosa* (Figs 1, 2 & 4).

Anemone coronaria achene was almost ellipsoid-spherical in shape with a curved beaked style. The achene body was rugose with lanate and deflated dense long hairs. Pericarp was thin consisting up to 3–4 layers, Its cross-section it was flattened ellipsoid with tubercles comprising thick-wall cavities or cells comprising of one layer epicarp, 1–2 compact layer(s) mesocarp, one layer endocarp, and quadrangular cells with thick walls (Figs 2 & 4).

Table 1. Studied samples along with their related data in Iran

Taxon	Locality	Altitude (m)	Date	Collector	Herbarium No.
Anemone biflora var. *biflora*	Khorasan prov., Bejnourd-Gorgan road, before Robat-qharabil village	1050	09.02.1991	Assadi & Shahsavari	69127
	Khorasan prov., 9 km from Birjand road	1780	24.04.1986	Assadi & Maasoumi	55647
	Ilam prov., Abdanan, Anaran toward Gorazan	1520	20.04.1996	Mozaffarian	93161
A. biflora var. *petiolulosa*	Khorasan prov., Mashhad toward Qhoochan, Chenaran, Binalood slopes	1500–2000	19.06.1996	Jamzad, Mazhary, Paryab & Amirabadi	75777
	Semnan prov., Shahpasand toward Shahrood	1800	03.05.1974	Mendel, Foroughi, Sanni & Shirdelpoor	11144
	Golestan prov., Gorgan, 3 km from E of Maraveh-tapeh	250	07.04.1976	Hewer	3596
A. biflora var. *gortschakowii*	Khorasan prov., 45 km from N of Shirvanm	2300–3000	23.04.1986	Assadi & Maasoumi	50413
	Golestan prov., Gorgan, 9 km from E of Maraveh-tapeh	300	24.06.1986	Assadi & Maasoumi	55476
	Yazd prov., Ardakan, Toot-va-Anjirvand	1800	25.04.1996	Mozaffarian	77401
A. narcissiflora	Azerbaijan prov., Maku toward Khoy, SW mountain of Kennedy church	2400–2650	29.06.1978	Assadi & Mozaffarian	30313
	Azerbaijan prov., Marand, Soltan-Jahangir Mountain	2500	25.06.2008	Akrami & Mozaffarian	93815
	Azerbaijan prov., about 30 km from NE of Marand, Kooh Kamar village	2000–2600	17.06.1988	Assadi & Shahsavari	65627
A. blanda	Kermanshah prov., 150 km from NW of Kermanshah, Babayadegar	1300	10.04.1990	Shooshtary & Hatami	2572
	Kermanshah prov., 150 km from W of Karand, Rijab village	900	09.04.1990	Hatami	2561
	Khozestan prov., Shooshtar, 37 km from Ahvaz road	380	08.03.1972	Foroughi	3089
	Mazandaran prov., Siahbisheh, Chalous road	2120	11.05.1972	Sabeti	3688
A. caucasica	Mazandaran prov., Pol-e-sefid, Sang-deh village forest	1700–2200	24.05.1995	Assadi	73287
	Mazandaran prov., Siahbisheh, Chalous road, 39 km toward Marzan-abad	2050	05.08.1991	Khatamsaz & Rahmanpour	69241
	Azerbaijan prov., Kalibar toward Makeidy	1300–1700	13.05.1975	Wendelbo & Assadi	17007
P. albana	Azerbaijan prov., Asalem toward Khalkhal	2300	29.05.1978	Wendelbo & Assadi	27801
	Azerbaijan prov., Arasbaran protected area	2300	09.06.1976	Assadi & Maasoumi	20263
	Azerbaijan prov., Asalem toward Khalkhal, Almas-col	2350–2400	17.07.1975	Wendelbo & Assadi	18512

Table 2. The achene character and quantitative features of the studied *Anemone* and *Pulsatilla* species

Quantitative Characters Taxon	Length range of the achene	Width range of the achene	Achene length mean to width mean ratio	Number of Epicarpe layers	Number of Mesocarp layers	Number of Endocarp layers	Style length range	Tricome length range
A. biflora var. *biflora*	4–4.2	1.2–1.3	3.28	1	1–2	1	2.5–3	2–2.2
A. biflora var. *gortschakowii*	2.7–3	1.3–1.5	2.03	1	3–4	1	1	2–2.5
A. biflora var. *petiolulosa*	2.7–2.8	0.7	3.92	1	1–2	1	0.5–0.7	1
A. blanda	1.5–1.6	1.1–1.2	1.34	1	1–2	1	0.25	0.16–0.18
A.caucasica	1.5	1.3	1.15	1	3–4	2–3	0.16	0.12
A. coronaria	2.1–2.3	1.3–1.4	1.62	1	1–2	1	1.4–1.6	2.7–3
A. narcissiflora	7.5–8	4.5	1.72	1	2–3	2–3	1.4–1.5	0
P. albana	1.8–2.3	0.9–1.1	2.05	1	2–4	1–2	18	0.4–0.5

Type 3: Including *Anemone blanda* and *A. caucasica* which had the smallest achene size among the species. The achenes were semispherical-ovate shape with an inclined warty style. The achene surface was completely rugose with short dense hairs and deflated base. The differences of these two species were: 1) style and hair length in *A. blanda* were longer, its cross-section was ellipsoid with tubercles on the middle area, one layer epicarp, 1–2 layer(s) mesocarp and one layer endocarp with spherical cells possessing equal thickness in all the cell walls, while the *A. caucasica* cross-section was quadrangular with tubercles on the angles, 2–4 layers mesocarp with compact cell, and 1–2 layers endocarp with thick narrow U-shaped cylindrical cells (Figs 2, 3 & 5).

Type 4: This type was solely consisting of the *Pulsatilla albana* species. The achenes were biconvex boat-shaped with an extremely long, pubescence persistent style. Cavities were observed on the achene surface and the longest style belonged to this species (16–18 mm). Surface of the achene body was striate-rugose or striate with straight trichome, non-rugulose with deflated base. The cross-section was rugulose-ellipsoid, compared to other species, the pericarp thickness was 40 µm (average) consisting of 4–7 layers, one layer epicarp, 2–4 layers mesocarp with compact cells, 1–2 layers endocarp with spherical cells possessing walls with equal thickness (Figs 1 & 4).

- Rediscovered species

Anemone blanda Schott & Kotschy in Oest. Bot. Wochenbl. 4: 129 (1854)

Syn.: *Anemone appenina* L. subsp. *blanda* (Sch. & Ky.) Hayek; *A. appenina* L.; *A. blanda* var. *parvula* (DC.) Boiss.

Specimen examined: Iran: Mazandaran province, Siahbisheh, Chalous road, 2120 m, Sabeti (3588).

Plant perennial; stem 18–20.5 cm high, 3 mm width, striate, pubescent; radical leaves with long petioles, pilose; segments sessile, short-petioluled, trifid to the middle with incised-dentate lobules and with few subobtuse or obtuse teeth; leaf blade trisected to base, few pilose, three involucral leaves long-petioled, trisected to base; inflorescence solitary with one flower, long peduncles covered with hairs; flowers 2.4–3 cm in diameter, tepals 8, linear-oblong, blue, glabrous on the outside and inside; anther global and yellow; fruitlets with 1.5–1.6 mm length and 1.1–1.2 mm width, semispherical-shaped with flat bed possessing a tubercle-like appendix in the head zone (Fig. 8).

Distribution: Central Asia, Balkan, Cyprus, west of Syria, Caucasus, Georgia, Turkey and Iran.

During the study of *A. caucasica* specimens, we have observed that, one specimen showed some noticeable differences from the others. This had motivated us to investigate for further references such as Soviet Union flora (Komarov 1937), Flora Iranica (Rechinger & Riedl 1992), Flora of Turkey (Davis 1965), Flora of Iran (Mobayen 1985), and Flore de l' Iran (Parsa 1951). Since the closest

species to *A. caucasica* is *A. blanda*, we concluded that, the considered specimen may probably be *A. blanda*. It is important to notice that, in spite of some similarities between *A. blanda* and *A. caucasica*, there exists distinguishing differences between these two species. For instance, flora of Turkey highlights some of these differences as follows: "*A. caucasica* differs from *A. blanda* in being smaller and more delicate, stems 6–13 cm, involucral leaves glabrescent above, green beneath, tepal 8–11, blue or white, usually 7–13 mm, on cliff ledges, 50–700 m or more". Finally, through a detailed procedure of investigation and comparison between these two species, we have reached the conclusion that, this specimen is actually *A. blanda*.

Table 3. Comparison of the anatomical and morphological features of *Anemone* and *Pulsatilla* species

Feature	*A. caucasica*	*A. blanda*	*A. coronaria*	*A. gortschakowii*	*A. biflora* var. *petiolulosa*	*A. biflora* var. *biflora*	*A. narcissiflora*	*P. albana*
Achene shape	Ovate, surface concavities with short inclined warty appendix	Semispherical-shaped with flat bed, possessing tubercle-like appendix in head zone	Ellipsoid, spherical-shaped	Biconvex boat-shaped, flattened with no concavity	Biconvex boat-shaped, hair normal, without thickness, having curvature in upper half with no concavity	Biconvex boat-shaped, surface concavities, hair basal is bulbously swelled	Inversely urceolate or obovoid, sometimes ellipsoid, laterally flattened	Biconvex boat-shaped, surface concavities with extremely long, pubescence persistent style
Style	Upright warty style ± 0.17 mm	Short, curved warty style, ± 0.25 mm	Curved-beaked style, 1.4–1.6 mm	Curved- beaked style, ± 1 mm	Beaked style, upright, 0.5–0.7 mm	Curved- beaked style, 2.5–3 mm	Curved- beaked style, 1.4–1.5 mm, non-persistent	Extremely long, pubescence persistent style, 16–18 mm
Achene basal	Semicircular	Flattened	Taper	Taper with blunt tip	Taper	Cone-shaped, taper with blunt tip	Flattened, bottle neck-like, taper	Taper, flattened
Margin	With 4 bulges on 4 corners	Semispherical	With wings, curved-oval	Curved-semispherical	Curved cone-shaped	Cone-shaped, curved	Narrow with pellicle	Semispherical
Indumentum	With short dense hairs, deflated basal ± 0.12–0.3 mm	With short dense hairs, deflated basal ± 0.16–0.18	With long dense hairs with deflated basal, lanate, deflated, 2.7–3 mm	With dense hairs with deflated basal ± 2.5 mm	With dense hairs with deflated basal ± 1 mm	Hairy with dense, long, waved hairs ± 2.2 mm with deflated trichome basal	Completely glabrous	Straight trichome, non-rugulose with deflated basal ± 0.5 mm
Surface of achene body	Completely rugose	Completely rugose	Rugose	Rugose	Rugose	Striate-rugose	Reticulate	Striate-rugose
Surface of margin	Completely rugose	Completely rugose	Rugose	Rugose	Rugose	Striate-rugose	Mainly reticulate	Striate-rugose
Structure of pericarp	± 35 µm, 4–7 layers	± 20 µm, 3–4 layers	± 20 µm, 3–4 layers	± 20 µm, 3–4 layers	± 25 µm, 3–4 layers	± 24 µm, 3–4 layers	55–60 µm, 4–7 layers	± 40 µm, 4–7 layers
Epicarp	1 layer	1 layer	1 layer	1 layer	1 layer	1 layer	1 layer	1 layer
Mesocarp	2–4 layers with cell strongly compressed	1–2 layers with cell strongly compressed	1–2 layers	1–2 layers	1–2 layers	1–2 layers with cell strongly compressed	2–3 layers with cell strongly compressed	2–4 layers
Endocarp	2 layers endocarp with thick narrow U-shaped cylindrical cells	1 layer, spherical endocarp cells with equal thickness in wall (lignified)	1 layer, quadrangular endocarp cells with equal thickness in wall (lignified)	1 layer, ellipsoid-spherical endocarp cells with equal thickness in wall (lignified)	1 layer, ellipsoid-spherical endocarp cells with equal thickness in wall (lignified)	1 layer, quadrangular endocarp cells with equal thickness in wall (lignified)	3 layers, elliptic cells with high uniform thickness in wall (lignified)	2 layers, spheric endocarp cells with equal thickness in wall (lignified)
Achene cross section	Quadrangular with tubercles on angles sides, fiber cells around 2 non-branched vascular bundles	Ellipsoid with tubercles on middle area	Flattened ellipsoid with tubercles comprising thick wall cavities or cells	Spherical with 2 tubercles on both sides, fiber cells around 2 non-branched vascular bundles	Spherical with 2 tubercles on both sides, fiber cells around 2 non-branched vascular bundles	Flattened ellipsoid-almost spherical with 2 tubercles on both sides, fiber cells around 2 non-branched vascular bundles	Narrow, ellipsoid with 2 spherical tubercles on dorsal and vertical areas	Ellipsoid, without 2 non-branched vascular bundles

Table 4. Comparison of *Anemone caucasica* and *A. blanda* features

Character	*A. caucasica*	*A. blanda*
Stem height (cm)	9–17	18–20.5
Leaf length	Shorter	Longer
Similarities between radical and involucral leaves	Similar	Dissimilar
Presence of petiolule in radical leaves segments	Absent	Present
Trichome of involucral leaves	Absent	Present
Leaf shape	Trisected with obovate segments	Trisected with flabellate segments
Shape of leaf tip	Obtuse	Acute
Peduncle height	1/4 of stem height or shorter	1/2 or 1/3 of stem height
Radical leaves status during blossom	Absent	Present
Tepal length (mm)	1.2–1.5	0.6–1
Tepal shape	Monomorphic	Dimorphic
Achene shape	Ovate	Semispherical
Achene trichome	All over except at top	All over
Style shape	Upright warty	Curved warty
Achene cross section	Quadrangular with tubercles on angle sides	Ellipsoid with a tubercles on middle area
Average style length (mm)	0.17	0.25
Number of endocarp layers	2	1
Thickness of endocarp cell walls	U-shaped	Uniform

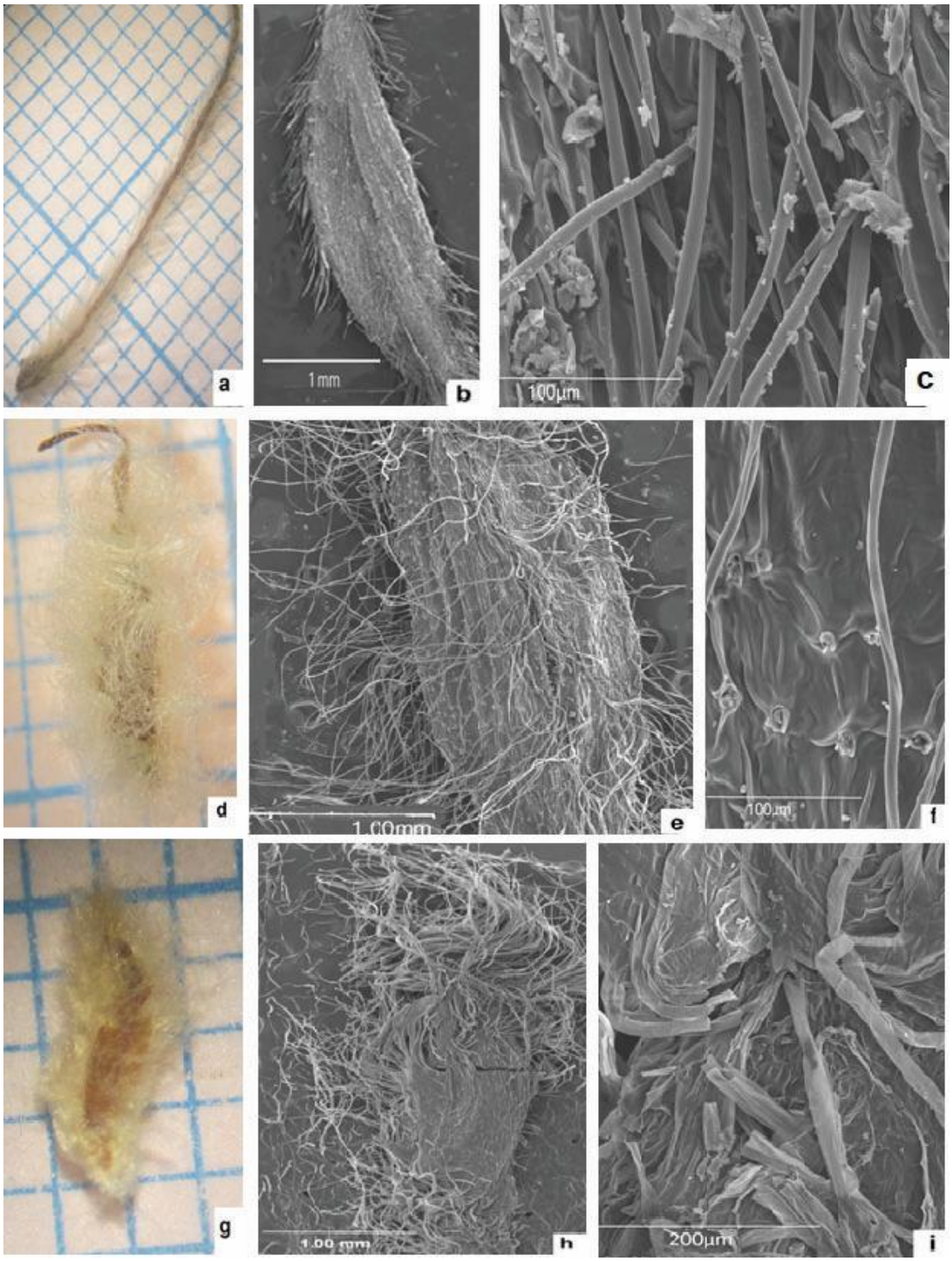

Fig. 1. Morphology and details of achene surface in studied species: a-c. *P. albana*, d-f. *A. biflora* var. *biflora*, g-i. *A. biflora* var. *petiolulosa*.

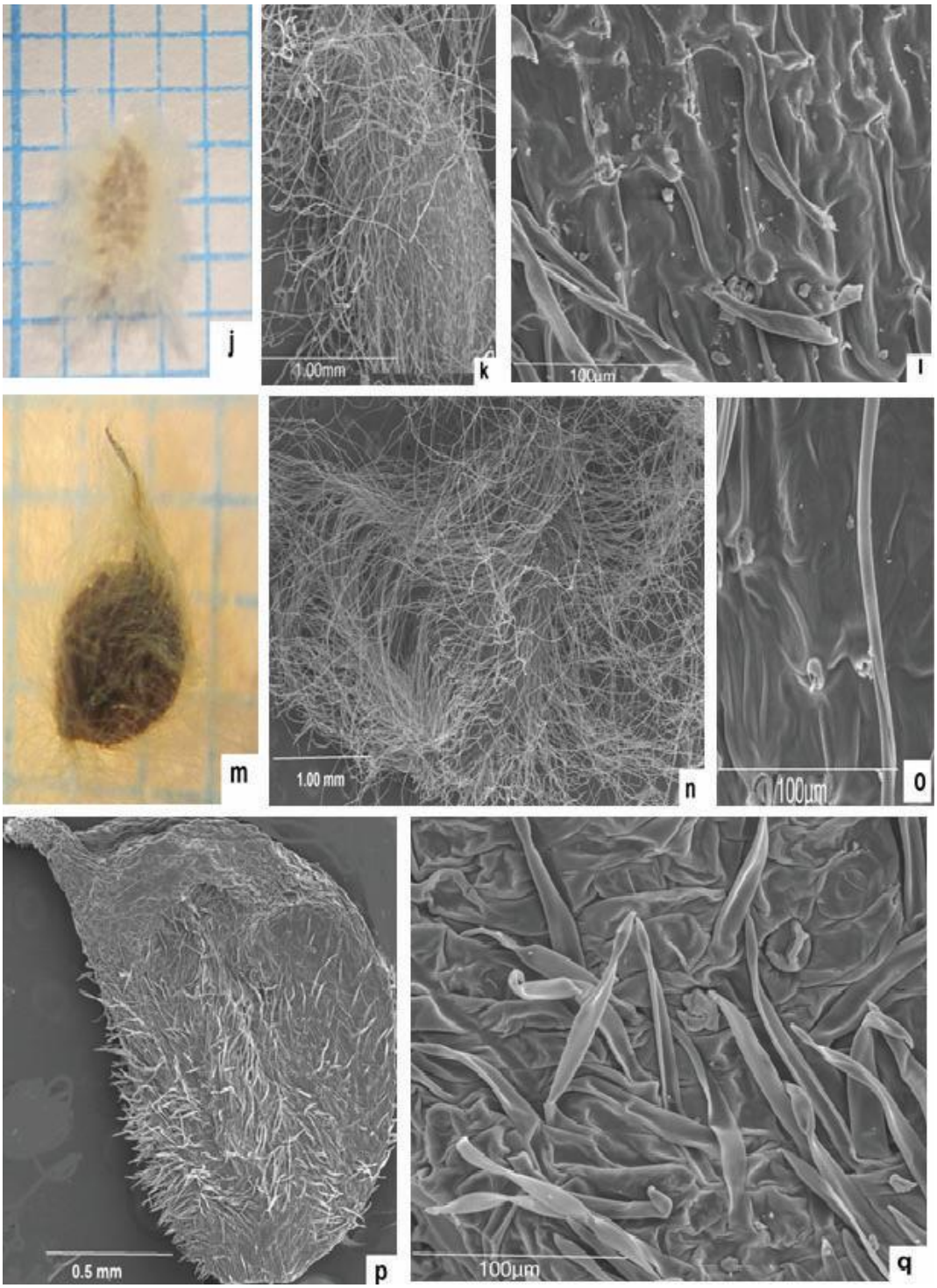

Fig. 2. Morphology and details of achene surface in studied species: j-l. *A. biflora* var. *gortschakowii*, m-o. *A. coronaria*), p-q. *A. blanda*.

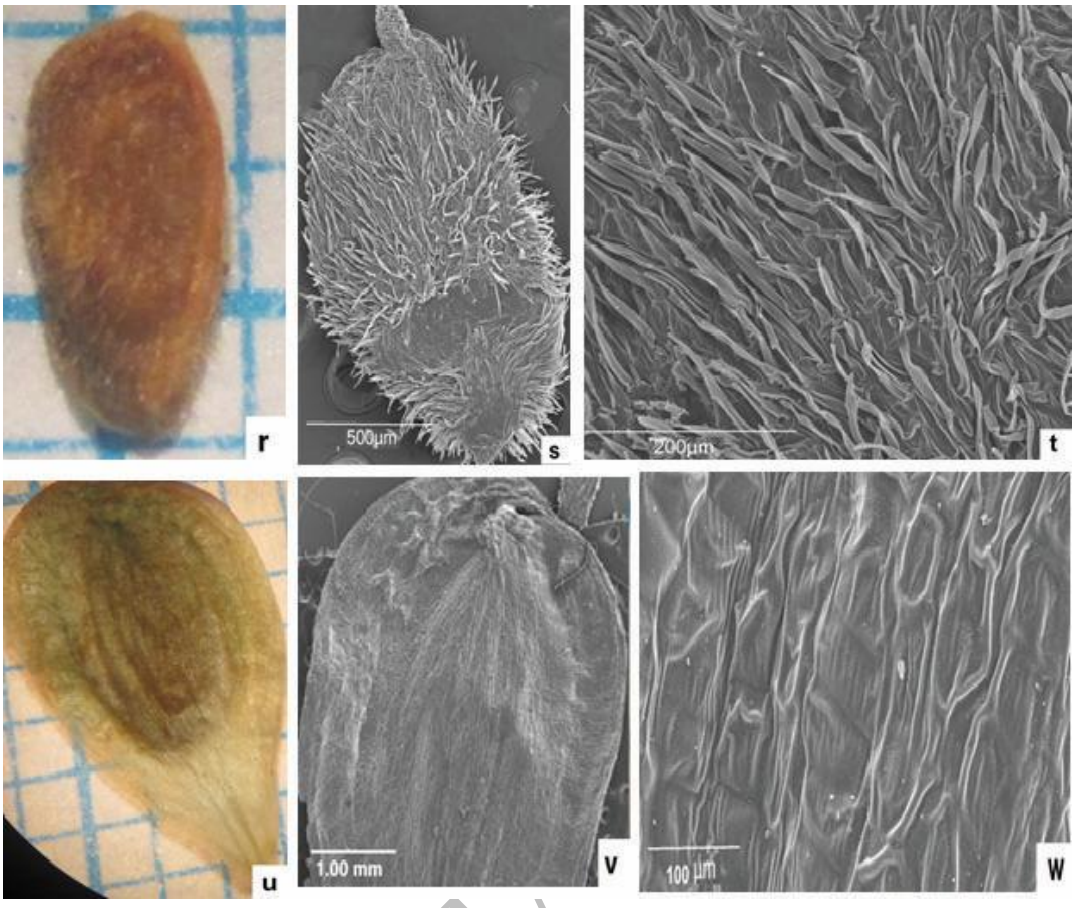

Fig. 3. Morphology and details of achene surface in studied species: r-t. *A. caucasica*, u-w. *A. narcissiflora*.

Discussion

The results derived from the present study are almost consistent with taxonomical classification of *Anemone* and *Pulsatilla* species and their varieties. Previous studies on achenes of these taxa are mostly concentrated on macro morphology (Ziman *et al.* 2008 & 2011, Sinno-Saoud *et al.* 2007, Tamura 1993 & 1995); however, we tried to study micro-morphological features as well. Four types are introduced based on the surface of achene body and micro- morphological features. Two of these types (types 1 and 4) comprised of only one species while other two types included several species. This is worth mentioning that, some of the included taxa show distinguishing differences despite of being morphologically similar. This fact is thoroughly explained in the "Results" section.

Conventionally, typical macroscopic features of achenes such as size, shape and style shape, are used to diagnose the *Anemone* species (Ziman *et al.* 2008, Tamura 1993 & 1995), as these features possess diagnostic values indeed. On the other hand, some researches recommend employing the micro-morphology of achene as a diagnostic criterion. For instance, it is noted that, micro-morphological features are capable of diagnosing the subgenera (Maciejewska-Rutkowska & Antkowiak 2013). In this regard, some characteristics such as the achene body and the achene style are the best features that can be employed while the achene surface (indumentum) possesses the least diagnostic value.

There are some conflicting opinions about the possibility of discrimination between different layers of pericarp. Ziman *et al.* (2008) emphasize on the taxonomical value of pericarp structure, while Kuzniewski (1964) believed that, the endocarp is not observable and diagnosable in *Anemone* achene cross-sections. Chaudhary & Trifonova (1988) are also stated

that, different layers of pericarp can be discriminated and recognized the epicarp, mesocarp and endocarp layers.

In this study, evident differences are observed in the achene body surface, cross-section, number of mesocarp and endocarp layers, shape, and style length. However, it should be noted that, features such as the number of epicarp layers and margin surface do not have much diagnostic value. Statistical methods are also employed in the present study. Based on the qualitative and quantitative features of the considered species of achenes; dendrograms are depicted using Ward and Average Linkage methods (Fig. 7).

Both dendrograms show that, the *P. albana* species is separated from other species, which is consistent with the all systematic studies and the achene features. This fact also supports the classification of this species in *Pulsatilla* and is consistent with the results of Heidary Baladehi (2007); where the anatomic study of petiole, stem and pollen is done for five *Anemone* species and one *Pulsatilla* species. Their results show that, the anatomical features are appropriate features and possess taxonomical value. Features such as the number and arrangement of petiole vascular bundle, the number of fiber layers, the type of vascular bundle surrounding tissue and the type of the cells between vascular bundles are capable of discriminating the studied taxa, and confirm the separation of *P. albana* from other *Anemone* species (Figs 6–7) (Heidary Baladehi 2007).

In the next step, the *A. narcissiflora* is separated from other taxa, a result which is expected considering the size, body structure and the achene anatomical structure, i.e., existence of the wings, and lack of non-branched vascular bundles and fiber cells (Figs 6–7).

In the third step, two branches are observed. The first branch including two species which are *A. caucasica* and *A. blanda*. This phenetical result is completely consistent with the visual results and taxa classification in different types (Figs 6–7). In the second branch, *A. biflora* var. *biflora*, *A. biflora* var. *petiolulosa*, *A. biflora* var. *gortschakowii*, and *A. coronaria* are observed. Although, both *A. biflora* var. *gortschakowii*, and *A. biflora* var. *petiolulosa* are considered as two distinct species in some studies (Komarov 1937, Rechinger & Riedl 1992, Mobayen 1985), they are introduced as a variety of *A. biflora* in other references such as Ziman 1998 and Sinno-saoud *et al.* (2007) due to their morphological similarities and common habitat. In the current investigation, it is observed that, the similarities of these taxa are more noticeable than their differences. Therefore, we confirm the second viewpoint and consider them as a variety of *A. biflora*, as well. It should be noted that, the dendrograms resulting from the above-mentioned two methods are completely similar, except for the taxa gaps and the position of *A. narcissiflora* (Figs 6–7).

Our observations show a meaningful relation between the sections classification and the introduced types. For instance, type 1 (comprising *A. narcissiflora* which is distinctively different from other species in features such as shape of achene, pericarp characteristic, cross section, lack of trichome, presence of wing etc.) belongs to sect. *Homalocarpus* with X=7 (Table 5), and it is totally different from other *Anemone* species. This fact is completely consistent with the statistical analyses (Figs 6–7) which believe *A. narcissiflora* is maximally different from all other *Anemone* species.

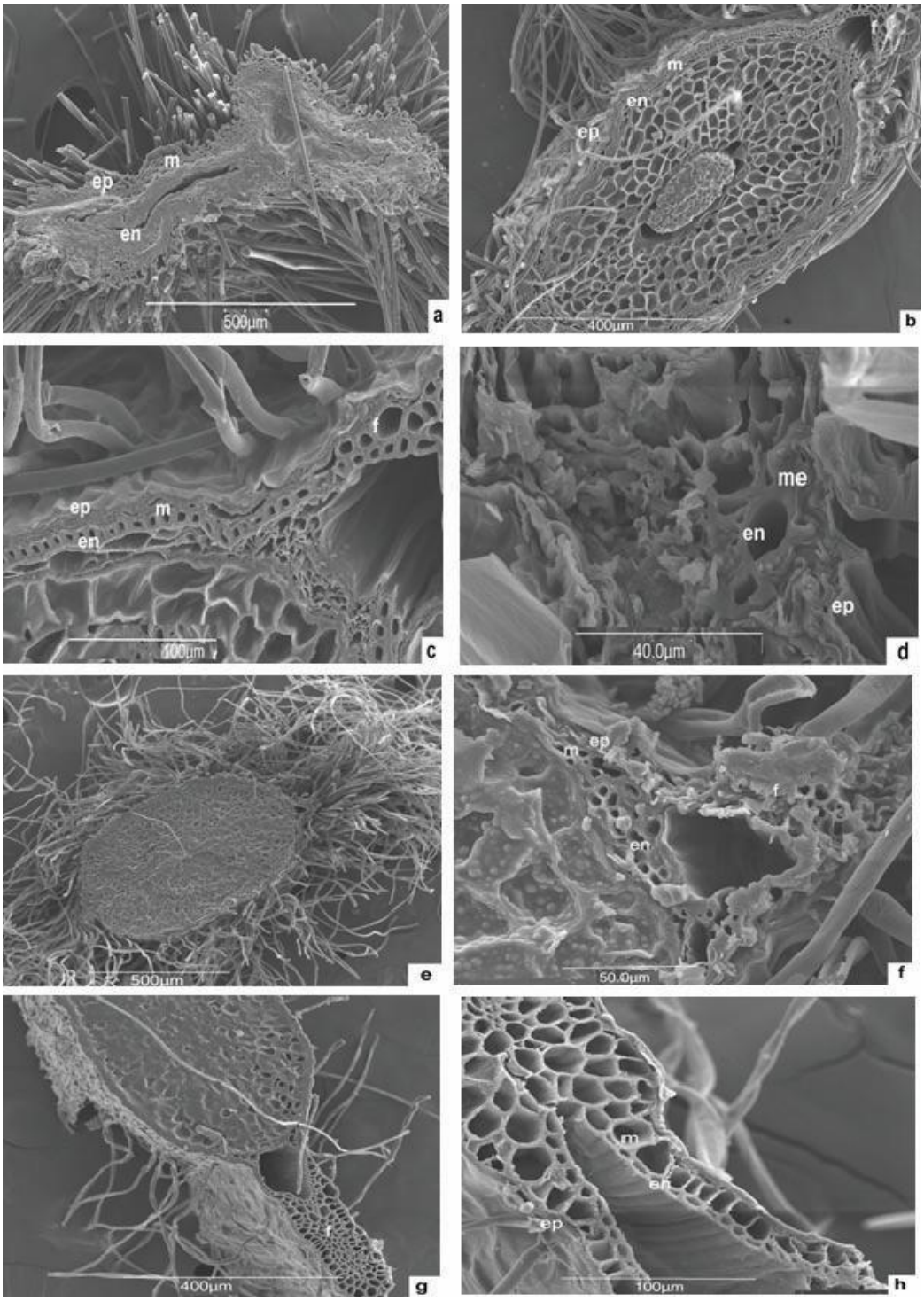

Fig. 4. Anatomical structure of the achene: a. *P. albana*, b, c. *A. biflora* var. *biflora*, d. *A. biflora* var. *petiolulosa*, e, f. *A. biflora* var. *gortschakowii*, g, h. *A. coronaria*. ep: epicarp, m: mesocarp, en: endocarp, f: fiber cells.

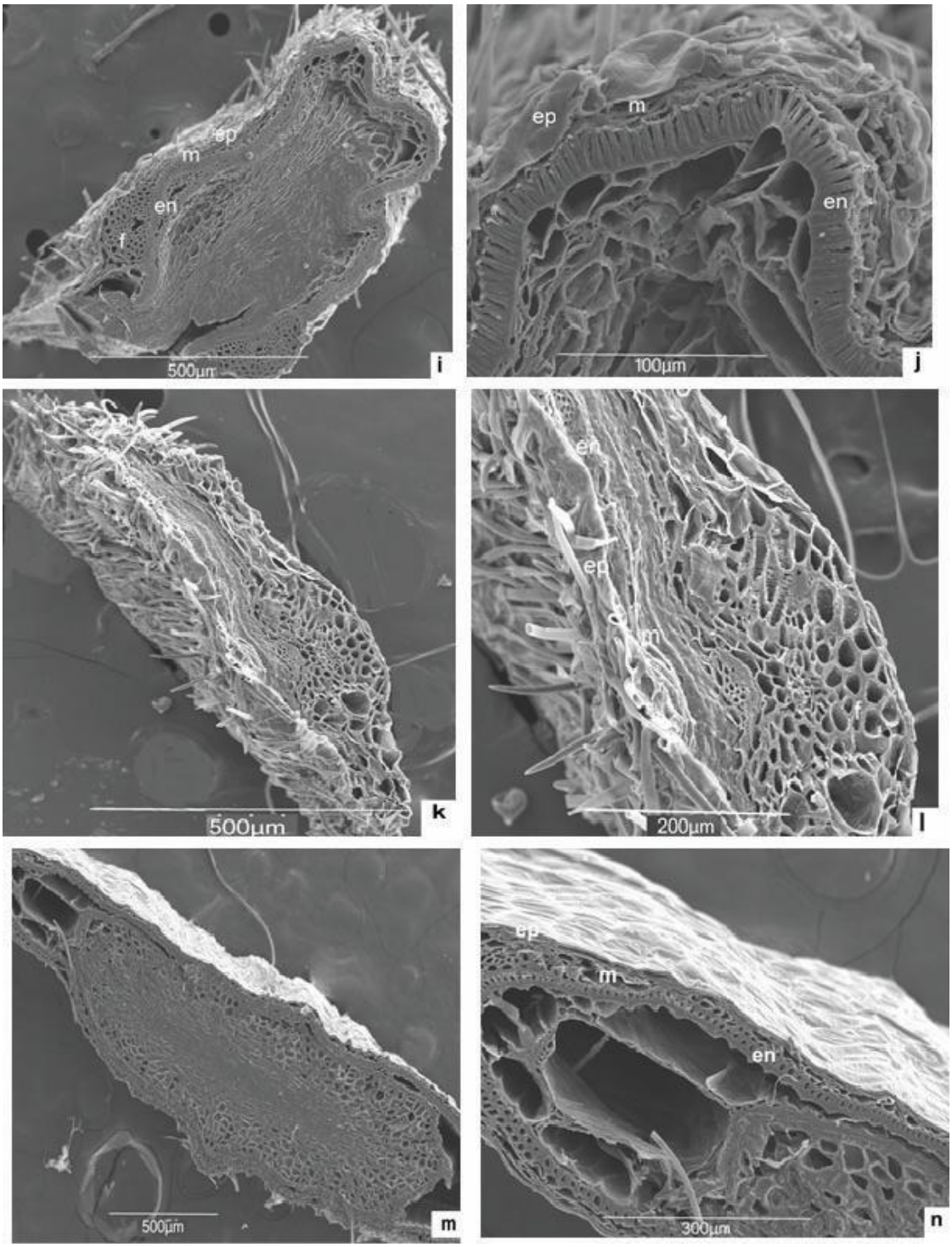

Fig. 5. Anatomical structure of the achene: i, j. *A. caucasica*, k, l. *A. blanda*, m, n. *A. narcissiflora*. ep: epicarp, m: mesocarp, en: endocarp, f: fiber cells.

Type 3, including two species of *A. blanda* and *A. caucasica* which belongs to sect. *Tuberosa* with X=8. The most essential characters of the sect. *Tuberosa* are tuberous rhizomes, stipule-like bases of basal leaf petioles, dimorphic perianth and ellipsoid, and sparsely puberulent fruits (Ziman *et al.* 2008). Within this section, Ziman *et al.* (2008) described two series i.e., ser. *Tuberosae* and ser. *Caucasicae* Ziman, Bulakh & Kadota. Ser. *Tuberosae* differs from ser. *Caucasicae* in length of fruit styles, shape of carpel stigmas, number of tepals, tepal length, and length of involucral leaf petioles. *Anemone blanda* is a member of the ser. *Tuberosae* which is characterized by globose or irregular-shaped rhizomes, involucral leaves having distinct petioles, solitary flowers with 8–20 tepals, sessile, and hardly compressed, shortly pubescent fruit (Tamura 1991).

It is worthful to note that, *A. blanda* is not mentioned in Flora Iranica; however, other floras such as Soviet Union flora (Komarov 1937), flora of Turkey (Davis 1965), flora of Iran (Mobayen 1985), and Flore de l' Iran (Parsa 1951) recorded this species from Qara gul mountain (Azerbaijan province, Iran). Based on flora of Turkey (Davis 1965), some characters such as tepals length, color and trichome of involucral leaves, altitude of distribution region, and tepals number are the diagnostic characters between *A. blanda* and *A. caucasica.* On the other hand, in Soviet Union flora (Komarov 1937) characters like radical leaves trichome, present of petiolule in radical leaves segments, peduncle length, similarities between radical and involucral leaves, tepals length, achene trichome, and style shape are introduced as diagnostic characters.

This investigation shows that, the characters which have diagnostic value for the considered taxa, i.e., *A. caucasica* and *A. blanda* (Figs 8–9), are stem height, leaf length, similarities between radical and involucral leaves, presence of petiolule in radical leaves segments, trichome of involucral leaves, leaf shape, shape of leaf tip (Fig. 10), peduncle height, presence of radical leaves during blossom, tepal length and shape (Fig. 10), achene shape & trichome (Figs 2–3), achene cross section, style shape, style length, number of endocarp layers, and thickness of endocarp cells wall (Fig. 5).

One of the remarkable features of this study is the utilization of micro-morphological methods in addition to the macro-morphological approach stated above. The micro-morphological investigation is carried out on the achene of the considered species. Meaningful differences have been observed between *A. caucasica* and *A. blanda* taxa during this investigation. Remarkably, the observations show that, the achene micro-morphological features are consistently reliable and they are independent of the environmental conditions.

Type 2, including *A. coronaria* and the members of *A. biflora* complex, belongs to sect. *Anemone* with X=8 (Table 5). In spite of common characters such as long dense trichome, biconvex boat-shaped achene, and other features mentioned in table 3, these taxa have some individual differences which distinguish them from each other. This fact is also confirmed by dendrogram (Fig. 6).

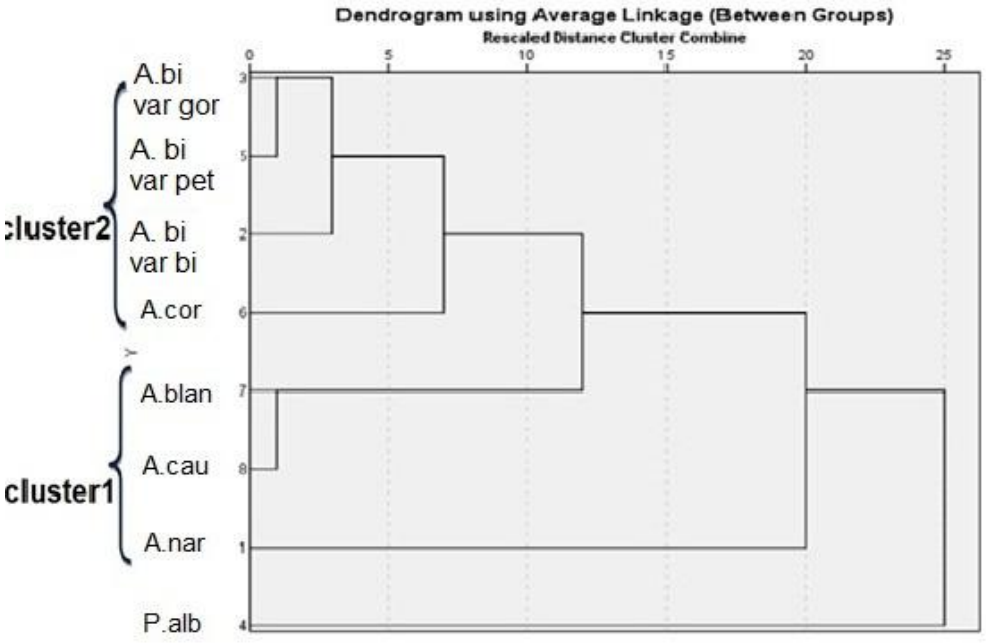

Fig. 6. The dendrogram resulted from Average linkage method based on quantitative and qualitative characteristics of the Iranian *Anemone* and *Pulsatilla albana* achenes (A. bi var. gor: *A. biflora* var. *gortschakowii*, A. bi var. pet: *A. biflora* var. *petiolulosa*, A. bi var. bi: *A. biflor* var. *biflora*, A. cor: *A. coronaria*, A. bla: *A. blanda*, A. cau: *A. caucasica*, A. nar: *A. narcissiflora*, P. alb: *P. albana*).

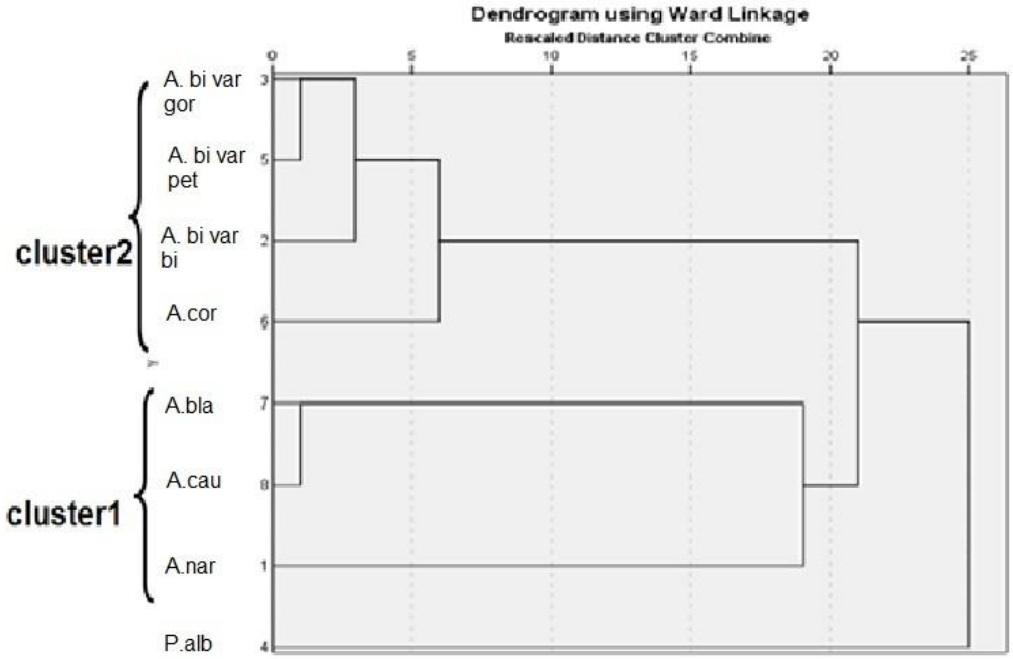

Fig. 7. The dendrogram resulted from Ward linkage method based on quantitative and qualitative characteristics of the Iranian *Anemone* and *Pulsatilla albana* achenes (A. bi var. gor: *A.biflora* var. *gortschakowii*, A. bi var. pet: *A. biflora* var. *petiolulosa*, A. bi var. bi: *A. biflor* var. *biflora*, A. cor: *A. coronaria*, A. bla: *A. blanda*, A. cau: *A. caucasica*, A. nar: *A. narcissiflora*, P. alb: *P. albana*).

Fig. 8. *Anemone blanda.*

Fig. 9. *Anemone caucasica.*

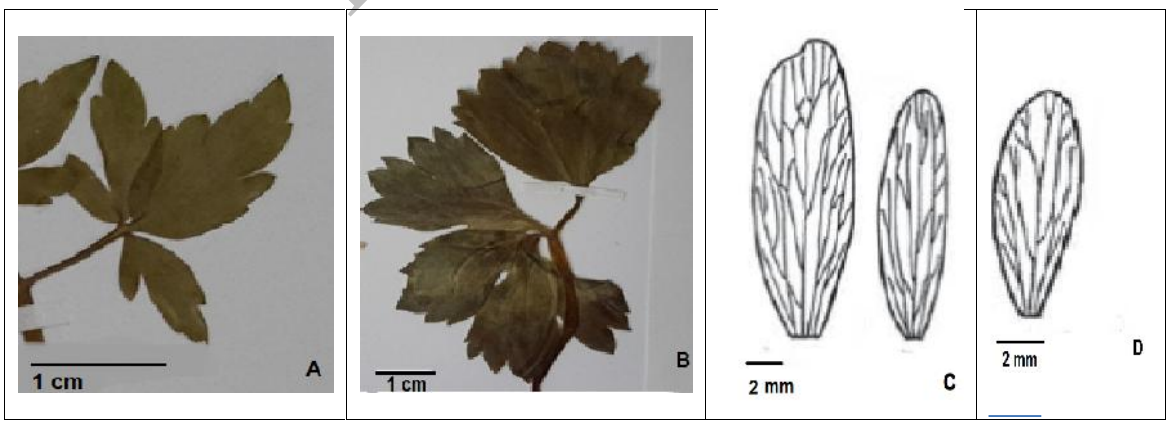

Fig. 10. Comparison of *Anemone blanda* (B, C) and *A. caucasica* (A, D) leaves and tepal.

Table 5. The *Anemone* Iranian taxa position in different classifications

Taxon	Ziman (2008)	Tamura (1995)	Hoot *et al*. (2012)
A. caucasica *A. blanda*	Subgen. *Anemonanthea* sect. *Tuberosa*	Subgen. *Anemonanthea* sect. *Tuberosa*	Subgen. *Anemone* sect. *Anemone*
A. biflora var. *biflora* *A. biflora* var. *petiolulosa* *A. biflora* var. *gortschakowii* *A. coronaria* *A. tschernjaewii**	Subgen. *Anemone* sect. *Anemone*	Subgen. *Anemone* sect. *Anemone*	Subgen. *Anemone* sect. *Anemone*
A. narcissiflora	Subgen. *Homalocarpus* sect. *Homalocarpus*	Subgen. *Homalocarpus* sect. *Homalocarpus*	Subgen. *Anemonidium* sect. *Homalocarpus*

* This species is not studied in the present paper; however, it is among the species which are distributed in Iran.

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