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THE IMPORTANCE OF QUALITATIVE AND QUANTITATIVE ANATOMICAL TRAITS IN DISTINGUISHING SPECIES OF THE SERIES CRATAEGUS SUBSERIES ERIANTHAE IN IRAN

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Abstract

Anatomical traits (leaf and epidermal cells) of 10 *Crataegus* species belonging to the series *Crataegus* subseries *Erianthae* were examined for a taxonomic evaluation. Anatomical characteristics were categorized into qualitative and quantitative traits. All data were analyzed using PCA and clustering methods. The results showed that qualitative such as anticlinal walls of lower epidermal cells, the shape of the vascular bundle of the midrib, the ventral shape of midribs, the stomatal type, and the mesophyll type are more effective than quantitative traits in species identification. Some quantitative traits such as the thickness of the middle vein, the ratio of length to width of the first layer of palisade parenchyma, and the ratio of length to width of the second layer of palisade parenchyma can also be used in species separation. In the species related to high altitudes, the width of the first layer of palisade parenchyma was much less than the species of low altitudes. *Crataegus babakhanloui* and *C. aminii*, which did not differ significantly in terms of qualitative traits, differed in several quantitative traits such as the length of the first layer of palisade parenchyma and the length of the long axis of stomata. Among various species and specimens, the highest length of the long axis of stomata was observed in *C. aminii* and the lowest belonged to specimens in *C. meyeri*. *Crataegus khatamsazae* and *C. hatamii*. These species were described as new species based on morphological and micromorphological differences. Our results showed differences in their quantitative and qualitative anatomical traits.

Keywords: altitude; clustering; Leaf anatomy; PCA; taxonomy

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اهمیت صفات تشریحی کمی و کیفی در تمایز گونه‌های سری **Crataegus** زیر سری **Erianthae** در ایران

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چکیده: صفات تشریحی (سلول‌های برگ و اپیدرم) ۱۰ گونه زالزالک متعلق به زیرسری *Erianthae* برای ارزیابی تاگزونومیکی مورد بررسی قرار گرفت. ویژگی‌های تشریحی به دو گروه صفات کمی و کیفی تفکیک شدند. تمامی داده‌ها با استفاده از روش‌های PCA و خوشه‌بندی تجزیه و تحلیل شدند. نتایج نشان داد که صفات کیفی مانند شکل دیواره آنتی‌کلینال اپیدرم تحتانی، شکل دسته‌های آوندی رگبرگ میانی، شکل سطح شکمی رگبرگ میانی، تیپ روزنه‌ای و تیپ مزوفیل در تشخیص گونه‌ها موثرتر از صفات کمی هستند. همچنین از برخی از صفات کمی مانند ضخامت رگبرگ میانی، نسبت‌های طول به عرض اولین و دومین لایه پارانشیم نردبانی، می‌توان در جداسازی گونه‌ها استفاده کرد. عرض اولین لایه پارانشیم نردبانی در گونه‌های مربوط به ارتفاعات بالا بسیار کم‌تر از گونه‌های ارتفاعات پایین بود. دو گونه *C. babakhanloui* و *C. aminii* که از نظر صفات کیفی اختلاف چندانی نداشتند، در تعدادی از صفات کمی مانند طول اولین لایه پارانشیم نردبانی و طول محور بلند روزنه بسیار متفاوت بودند. طول محور بلند روزنه در گونه *C. aminii* با اختلاف بسیار زیاد بیشترین مقدار و در جمعیت‌های گونه *C. meyeri* کم‌ترین مقدار را داشت. دو گونه *C. khatamsazae* و *C. hatamii* که با داشتن اختلافاتی از نظر صفات مورفولوژیکی و میکروفولوژیکی به‌عنوان گونه جدید به فلور ایران معرفی شده بودند، از نظر صفات کیفی و کمی آناتومی نیز اختلاف داشتند.

INTRODUCTION

The genus *Crataegus* L., with a variable number of species ranging from 150 to 1200, is distributed in temperate regions of the Northern Hemisphere, including Europe, Asia, North America, and to a lesser extent, North Africa and southern regions (Dickinson & Phipps 1985; Christensen 1992; Potter & al. 2007). The distribution of this genus in Iran covers most parts of the country (Riedl, 1969; Khatamsaz, 1992; Christensen, 1992). According to the latest studies conducted on *Crataegus* in Iran, the genus consists of 39 taxa, including 23 species, 7 subspecies, 6 varieties, and 3 forms (Hamzeh & al. 2014). From a taxonomic point of view, the genus *Crataegus* has always been subject to complexities and ambiguities, leading to extensive studies of various aspects of this genus. The identification of species in this genus is challenging due to numerous factors, including overlap in morphological characteristics, variable distinguishing traits within a species (such as the number of seeds in the fruit), the need for samples in flowering and fruiting stages for accurate identification and the presence of interspecific hybrids. The effect of environmental factors on leaf morphological changes has long been the focus of attention of botanists, ecologists, and paleobotanists (Royer & Wilf 2006; Zarafshar & al. 2009; Anten & al. 2010; Guerin & al. 2012; Katarzyna 2012; Chitwood 2016; Tonggui & al. 2016). Although morphological traits vary under the influence of different climatic conditions, some undergo fewer changes (Jones & Wilkins 1971). According to Christensen (1992), the morphological traits of the *Crataegus*, ser. *Crataegus*, subser. *Erianthae* changes under the influence of the environment. Plant Anatomy, like other biosystematic sciences, has always been used as a complementary tool in taxonomy. Many studies have been conducted on the taxonomic values of morphological traits, karyology, apomixis, polyploidy, and molecular studies in the genus *Crataegus*, but little information is available about the anatomical features of this genus (Dickinson & Phipps 1985; Campbell & al. 2007; Gladkova 1968; Talent & Dickinson 2007; Dickinson & al. 2007; Albarouki & Peterson 2007; Lo & al. 2007; 2009; 2010; Nieto-Ángel & al. 2009). Therefore, it seems necessary to investigate the genus *Crataegus* from different perspectives and utilize various biosystematic methods. Few studies have been done on the anatomical structure of this genus using light microscopes (Metcalf & Chalk 1950; Saribas & Yaman 2005; Demiray 2007; Novruzova & al. 1990; Jia & al. 2024) and electron microscopy (Shahbaz 2009; Ganeva & al. 2009; Chwil & al. 2006; Hamzeh'ee & al. 2016). In the present study, for the first time, the anatomical characteristics of species in the *Crataegus* section *Crataegus* series *Crataegus* subseries *Erianthae* (Pojarkova) Christensen in Iran was examined to explore the taxonomic application of anatomical traits and their role in distinguishing species.

MATERIALS AND METHODS

Anatomy

Anatomical studies of 16 accessions of 10 species from the *Crataegus* section *Crataegus* series *Crataegus* subseries *Erianthae* were made from the herbarium specimens deposited in TARI and TUH (Acronyms according to Holmgren & Holmgren 1990). The studied species include *Cartaegus ambigua* Meyer ex Becker subsp. *ambigua*, *C. aminii* Khatamsaz, *C. babakhanloui* Khatamsaz, *C. caucasica* K. Koch, *C. hatamii* Hamzehʹee K.I. Christensen & Attar, *C. khatamsazae* Hamzehʹee, K.I. Chr. & Attar, *C. kurdistanica* Hadac & Chrtek, *C. meyeri* Pojark., *C. sakranensis* Hadac & Chrtek, and *C. songarica* K. Koch (Table 1). To obtain certain traits, except for species that had only one sample (*C. aminii*, *C. babakhanloui*, *C. caucasica*, *C. hatamii*, and *C. khatamsazae*) two or three specimens of each species were selected from different altitudes. All leaves were selected at full maturity from fruit-bearing branches. Fifteen sections were cut from each leaf sample and traits were measured from eight appropriate sections on each slide. Then, their averages for each trait were analyzed (Table 5).

Leaf sample fixation, Cross sectioning, and staining

Leaf samples were soaked in warm water (60 degrees Celsius) for 30 minutes to fix the tissues and facilitate cutting. Then, they were transferred to FAA fixative solution (37% formaldehyde, 95% ethyl alcohol, concentrated acetic acid, distilled water) for 48 hours, followed by a transfer to 70% ethanol (Ruzin, 1999). Manual cross-sectioning was performed using commercial blades, starting from the middle third region of the leaf midrib. The prepared sections were stained using Sodium hypochlorite solution (commercial bleach) at concentrations of 30%, 40%, and 50% for 30-40 minutes. After several washes with distilled water, the sections were briefly immersed in 3% acetic acid solution to neutralize the alkaline effect of sodium hypochlorite. They were then rinsed with distilled water. The sections were subjected to double staining using Methyl green and Bismarck brown stains. To examine the epidermal structure and stomatal pattern in leaves, herbarium samples were first heated in distilled water for 15 minutes. Then, the epidermis of the samples was separated using a NaOH solution by the Stürm method (Dilcher, 1974). After rinsing with distilled water, the separated epidermal tissue was subjected to staining by immersing it in sodium hypochlorite solutions of 50%, 60%, 70%, and 80% concentrations for 30 minutes to four hours. The stained samples were then immersed in safranin dye for 20 to 60 seconds. After staining, the cuticle present in the epidermal tissue appears light pink to slightly dark. To semi-permanently fix the stained leaf and epidermal tissue, the heated gelatin-glycerin fixative was used (Sass, 1951). The prepared slides were observed and photographed using a Leitz Wetzler light microscope equipped with a Nikon Coolpix S10 digital camera.

Statistical analysis

All studied traits were categorized into qualitative and quantitative traits. Quantitative traits were measured using Microstructure Measurement Software version 1.0. All measurements were recorded in micrometers. Anatomical terminology followed by Metcalf and Chalk (1950) (Fig. 1).

The anatomical traits used in data analysis (midrib, blade thickness, and leaf epidermis) are listed in Table 2. Quantitative data were processed by the statistical variation method (one-way ANOVA). The mean values of quantitative traits were used for multivariate analyses (PCA), while qualitative characteristics were coded as binary characters i. e. 0 and 1 representatives of absence or presence. The Single Linkage and Euclidean Distance methods were used as similarity levels in cluster analysis of qualitative traits. All data were analyzed by Minitab software ver. 14.

RESULTS AND DISCUSSION

Qualitative traits

Analysis of 23 qualitative traits using PCA and Cluster method showed relatively similar species grouping. The first three PCA axes represented ca. 53% of the total variation in the dataset (Table 3). Of these, 19 traits had greater value in the first three axes than the others. These traits included: mesophyll type (MeTy0, MeTy1), dorsal shape of midrib (DorM3), anticlinal walls of lower epidermal cells (AWC0, AWC3) and shape of the vascular bundle of midrib (SVbM2) in PCA1, AWC1, AWC2, stomata type (StTy2, StTy3 and StTy7) in PCA2 and DorM2, ventral shape of midrib (VenM1, VenM2, VenM3), SVbM0, SVbM1, SVbM2, StTy1, StTy4, StTy5 and StTy6 in PCA3 axis. In total, three main groups were observed, including 1) *C. ambigua*, 2) *C. khatamsazae*, *C. caucasica*, *C. babakhanloui*, *C. aminii*, *C. hatamii*, *C. sakranensis*, and 3) *C. songarica*, *C. kurdistanica*, and *C. meyeri* in different axes of PCA (Fig. 2) and species cluster (Fig. 3). In different species, the anticlinal wall of lower epidermal cells (AWC) was observed in four states: smooth, wavy, relatively sinuous and sinuous (Figs. 4A-D). The smooth form was observed only in three specimens of *C. ambigua* collected under different environmental conditions (Table 1), giving rise to a distinct cluster of this species (Fig. 3). Morphologically, this species is distinguished from the other species by traits such as the number of pyrenes, shape, size, and number of leaf lobes.

Table 1. Localities and abbreviations of 16 accessions of *Crataegus* species section *Crataegus* series *Crataegus* subseries *Erianthae* (Pojarkova) Christensen.

Taxon	**Abbreviations**	**Localities**
Crataegus ambigua subsp. *ambigua*	*C. ambig*1	Kurdestan: ca 40 Km from Bane to Marivan around Shipanchu village, 1700 m, Ghahreman & Mozaffarian, 18189 (TUH).
C. ambigua subsp. *ambigua*	*C. ambig*2	Kurdestan: 25 Km from Baneh to Sanandaj, Nekerouz route, around Mirdeh Village, 1560 m, Hamzeh'ee, Attar, Alavi 95293 (TARI).
C. ambigua subsp. *ambigua*	*C. ambig*3	Charmahal va Bakhtiari: Road from western to Naghan, 1900m, Mozaffarian 57350 (TARI).
C. aminii	*C. aminii*	Esfahan, 8 Km Zob- Ahan Road, 1500 m, Amin 33157 (holotype: TARI).
C. babakhanloui	*C. babakh*	Markazi: Karaj- Chalus pass, Aderan, Arangeh, 1700 m, Khatamsaz 47505 (holotype: TARI).
C. caucasica	*C. caucas*	Azerbaijan: Kaleybar, Arasbaran Protected Area, Aynelou forest, 1500 m, Attar, Zamani, Raei, Maleki 40450 (TUH).
C. hatamii	*C. hatam*	Fars: Road of Shiraz-Sepidan, Komeir, 4 Km after Komeir to Sepidan, ca. 2000 m, Hamzeh'ee & Hatami 91713 (holotype: TARI).
C. khatamsazae	*C. khatam*	Fars: Road of Shiraz-Sepidan, Komeir to Abshar- e Margun, 6 Km to Abshar- e Margun, Cheshmerizi, 2000 m, Hamzeh'ee & Hatami 91711 (holotype: TARI).
C. kurdistanica	*C. kurdis*1	Azerbayjan: 17 Km after Ahar to Tabriz, 1360 m, Attar, Zamani 40420 (TUH).
C. kurdistanica	*C. kurdis*2	Azarbayejan West: Sardasht to Pyranshahr, 2 Km of Shiveh Mardan village, 1200 m, Hamzeh'ee, Attar, Alavi 95306 (TARI).
C. meyeri	*C. meyer*1	E. Azarbaijan: 2 Km to Kaleybar from Ahar, left of the road, 1360 m, Attar, Zamani, 37831 (TUH).
C. meyeri	*C. meyer*2	Kurdestan: Beginning of Avihang road from Sanandaj, Left side, 1800 m, Hamzeh'ee, Attar, Alavi, Maroofi 95263 (TARI).
C. sakranensis	*C. sakran*1	Fars: Shiraz to Kazeroun, Track of Ghorogh-e Kotal-e Dokhtar, Dasht-e Arzhan, beginning of Ghorogh, 2000 m, Hamzeh'ee & Hatami 91710 (TARI).
C. sakranensis	*C. sakran*2	Fars: Khafr, Kuh-e Dena, 2350 m, Riazi 8160 (TARI).
C. songarica	*C. songar*1	Semnan: Turan Protected Area, Nahar valley at N. foot of Kuh-e Peyghambar, Gardens above the village, 1300-1350 m, H. Freitag 13792 (TARI).
C. songarica	*C. songar*2	Khorasan: Road from Tayebad to Rashkhar, Dardewoy village to Kuh-e Maadane Ahan-e Sangan, 1476 m, Mozaffarian 93800 (TARI).

Table 2. List of anatomical traits used in data processing in the studied *Crataegus* species.

	Abbreviation	Quantitative traits		Abbreviation	Qualitative traits
1	BT	Blade thickness	1	MeTy	Mesophyll type: 0=Dorsiventral, 1=Isolateral
2	TLCB	Thickness of the lower cuticle of the blade	2	DorM	Dorsal shape of midrib: 1=Flat, 2=Apiculate, 3=Appendiculate
3	TUCB	Thickness of the upper cuticle of the blade	3	SVbM	Shape of the vascular bundle of midrib: 0=Elliptical, 1=Reniform, 2=Circular/ Elliptical
4	LSP	Length of the spongy layer	4	VenM	Ventral shape of midrib: 1=Concave, 2=Flat, 3=Convex
5	LFrLPP	Length of the first layer of palisade parenchyma	5	StTy	Stomata Type: 1=Incomplete cyclocytic bicyclic, 2=Actinocytic, 3=Tetracytic, 4=Cyclocytic bicyclic, 5=Anomocytic, 6=Cyclocytic-Actinocytic, 7=Actinocytic-Stephanocytic
6	LSeLPP	Length of the second layer of palisade parenchyma	6	AWC	Anticlinal walls of lower epidermal cells: 0=Wavy, 1= Shallow sinuous, 2= Smooth, 3= Sinuous
7	WFrLPP	Width of the first layer of palisade parenchyma	7	UTr	Upper surface trichome: 0=Absent, 1= Present
8	WSeLPP	Width of the second layer of palisade parenchyma	8	LTr	Lower surface trichome: 0=Absent, 1= Present
9	ML	Midrib length			
10	MW	Midrib width			
11	TLCM	Thickness of the lower cuticle of midrib			
12	TUCM	Thickness of the upper cuticle of midrib			
13	TLEM	Thickness of the lower epidermis of midrib			
14	TUEM	Thickness of the upper epidermis of midrib			
15	TLCoM	Thickness of the lower collenchyma of midrib			
16	TUCoM	Thickness of the upper collenchyma of midrib			
17	TLPM	Thickness of the lower parenchyma of midrib			
18	TUPM	Thickness of the upper parenchyma of midrib			
19	TXyScM	Thickness of the xylem sclerenchyma of midrib			
20	TPhScM	Thickness of the phloem sclerenchyma of midrib			
21	PhLM	Phloem length of the midrib			
22	PhWM	Phloem width of the midrib			
23	XyLM	Xylem length of the midrib			
24	XyWM	Xylem width of the midrib			
25	LFrLPP /WFrLPP	Length of the first layer/Width of the first layer of palisade parenchyma			
26	LSeLPP/WSeLPP	Length of the second layer /Width of the second layer of palisade parenchyma			
27	ML/MW	Midrib length / Midrib width			
28	StD	Stomatal density (300x 300 µm2)			
29	LSub	Lower subsidiary cell size			
30	LLASt	Length of long axis of stomata			

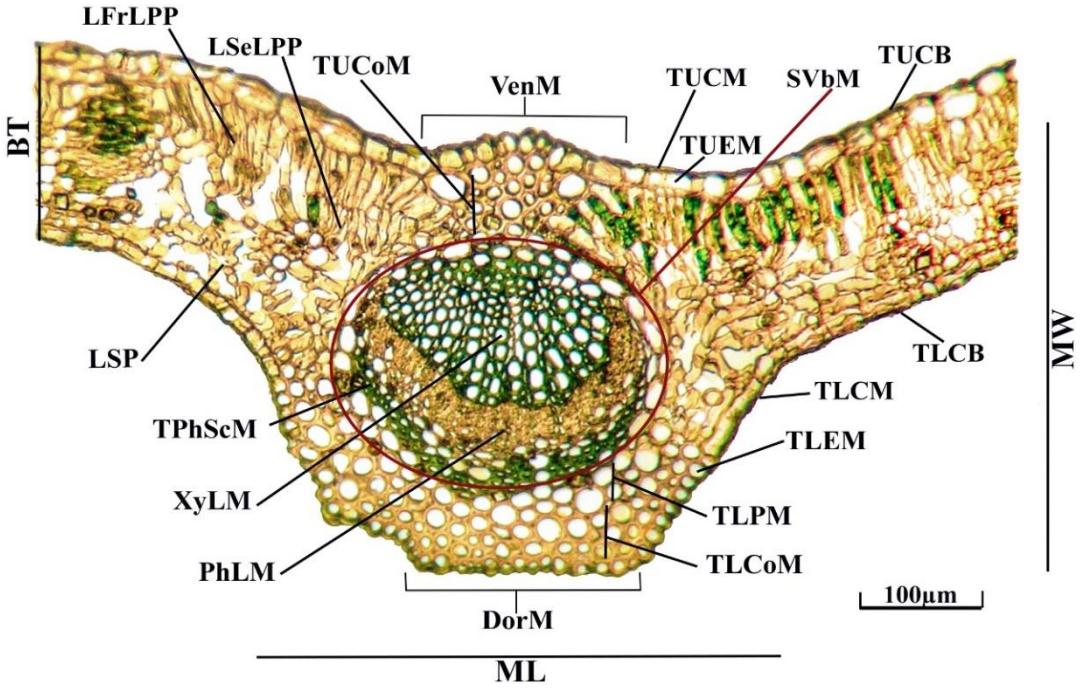

Fig. 1. Transverse section of leaf of *Crataegus songarica*: BT= Blade thickness; LSP= Length of the spongy layer; LFr & SeLPP= Length of the first and second layer of palisade parenchyma; TL & UCB= Thickness of the lower and upper cuticle of blade; ML & W= Midrib Length and width; TL & UCM= Thickness of the lower and upper cuticle of midrib; TL & UEM= Thickness of the lower and upper epidermis of midrib; TL & UCoM= Thickness of the lower and upper collenchyma of midrib; TL & UPM= Thickness of the lower and upper parenchyma of midrib; TPhScM= Thickness of phloem sclerenchyma; PhLM= Phloem width of the midrib; XyLM= Xylem length of the midrib; DorM= Dorsal shape of midrib; VenM= Ventral shape of midrib; SVbM= Shape of the vascular bundle of midrib; TPhScM= Thickness of the phloem sclerenchyma of midrib.

The presence of a sinuous anticlinal wall in *C. meyeri*, *C. songarica*, and *C. kurdistanica* placed them in the positive part of the PCA1 axis. In the negative part of PCA1, specimens of *C. aminii*, *C. babakhanloui*, *C. caucasica*, *C. hatamii*, *C. sakranensis*, and *C. khatamsazae* with a shallow sinuous anticlinal cell wall (Fig. 4C) formed a large cluster. These species, which have some morphological similarities such as fruit color (red to purple) and number of pyrenes (2-4), are also placed together based on anatomical characteristics.

Crataegus aminii and *C. babakhanloui* were introduced as new species in the Flora of Iran (Khatamsaz 1991; 1992). In addition to the similar morphological traits of these two taxa, the qualitative micromorphological traits of the lower leaf epidermis as well as features of seed surface, stomata density, parallel striae and scattered wax granules, isodiametric/elongate cells, depressed anticlinal cell wall and wrinkled cell surface have placed these two species very close to each other (Hamzehee & al. 2014; 2016). Many kinds of epicuticular wax crystalloids are of great systematic significance in angiosperms (Barthlott & al. 1998). Qualitative characteristics of the leaf epidermis have a taxonomical role in the identification of *Crataegus* species (Ganeva & al. 2009). The qualitative anatomical features also showed that the elliptical shape of the vascular bundle of midrib and the cyclocytic stomatal type were observed only in these two species (Figs. 4G & H and 5C & D).

Based on morphological and micromorphological traits, *C. hatamii* and *C. khatamsazae*, were introduced as new species and *C. caucasica* as a new report from Iran (Hamzehee & al. 2014). Morphologically, the two new species were compared with *C. sakranensis* and *C. ambigua*. In the ordination analysis of qualitative anatomical traits, *C. hatamii* and *C. sakranensis* were close together (Fig. 2), and in the cluster analysis, these two species were placed next to *C. khatamsazae* (Fig. 3). Renal vascular bundles in the midrib and the anomocytic stomatal type, which was observed only in *C. khatamsazae* (Figs. 4K & 5G), created a subcluster

distinct from all other species (Fig. 3), confirming the separation of this species from others in the subser. *Erianthae*. *Crataegus hatamii* and *C. caucasica* were collected from two different geographical areas far from each other (Table 1). These two species are very different in terms of morphological and micromorphological traits (Hamzehee & al. 2014). In the species cluster analysis, the concave shape of the midrib in ventral surface, which was observed only in these two taxa (Figs. 5E & F), separated them from other species and placed them in a distinct subcluster (Fig. 3). *Crataegus caucasica* was distinguished from *C. hatamii* and other species by the cyclocytic bicyclic stomatal type (Fig. 4I & J) (Figs. 2 & 3). *Crataegus sakranensis* with the cyclocytic actinocytic stomatal type, which was observed only in specimens of this species (Fig. 4Q), formed a distinct cluster alongside the species *C. hatamii* and *C. caucasica* (Figs. 2 & 3). In addition to morphological and anatomical traits, micromorphological characteristics such as the shape of the seed epidermal surface, the type of cell surface ornamentation, and the anticlinal walls of the seed cells distinguish this species from other species (Hamzehee & al. 2014).

Two specimens of *C. kurdistanica* were collected, in a steppe area in Ahar in East Azerbaijan Province, and in West Azerbaijan Province, near Oshnavieh, with a nearly forested habitat. The qualitative anatomical traits of these two accessions showed 100% similarity. Also, two specimens of *C. meyeri* collected from two different regions at elevations i.e. 1360 m and 1800 m a.s.l. (Table 1), were placed next to each other and in the *C. kurdistanica* group (Figs. 2 & 3). These two species are also very close in terms of morphological traits (Hadaq & Chrtek, 1980; Christensen, 1992). Isolateral mesophyll type was observed only in *C. meyeri* (Fig. 6H). The mesophyll type in the other species was dorsiventral.

Two specimens of *C. songarica* were collected in two areas with almost similar habitats, with an altitude difference of about 100 m (Table 1). The actinocytic stephanocytic stomatal type was observed only in these two specimens (Fig. 5R). Taxonomically, *C. songarica* is a related species to *C. ambigua* (Christensen, 1992), but qualitative anatomical features place it alongside two species i.e. *C. meyeri* and *C. kurdistanica* (Figs. 2 & 3). The different states of the anticline wall of lower epidermal cells, including wavy, shallow sinuous, and smooth in *C. ambigua*, shallow sinuous and sinuous in *C. meyeri* (Fig. 6D), and sinuous in *C. songarica* and *C. kurdistanica*, have caused the proximity of these species in the positive parts of PCA1, PCA2, and one of the three main clusters (Figs. 2 & 3).

Table 3. Eigenvector scores of qualitative anatomical traits on three main PCA axes.

Variable	PCA1 (20%)	PCA2 (19%)	PCA3 (14%)
MeTy0	-0.329	-0.186	-0.016
MeTy1	0.329	0.186	0.016
DorM1	0.136	-0.009	0.029
DorM2	0.049	0.131	-0.226
DorM3	0.260	0.143	0.016
VenM1	-0.162	-0.147	0.406
VenM2	-0.096	-0.004	-0.264
VenM3	0.162	0.147	-0.406
SVbM0	-0.168	0.109	-0.296
SVbM1	-0.161	0.011	0.278
SVbM2	0.242	-0.104	0.366
AWC0	-0.253	0.238	0.040
AWC1	-0.181	0.293	0.112
AWC2	-0.107	0.350	0.082
AWC3	0.386	-0.135	-0.089
UTr	0.181	-0.193	-0.125
StTy1	0.195	0.152	0.287
StTy2	0.145	0.427	0.081
StTy3	0.145	0.427	0.081
StTy4	-0.149	-0.83	0.237
StTy5	-0.161	0.11	-0.278
StTy6	-0.021	-0.122	0.243
StTy7	0.159	-0.219	-0.012

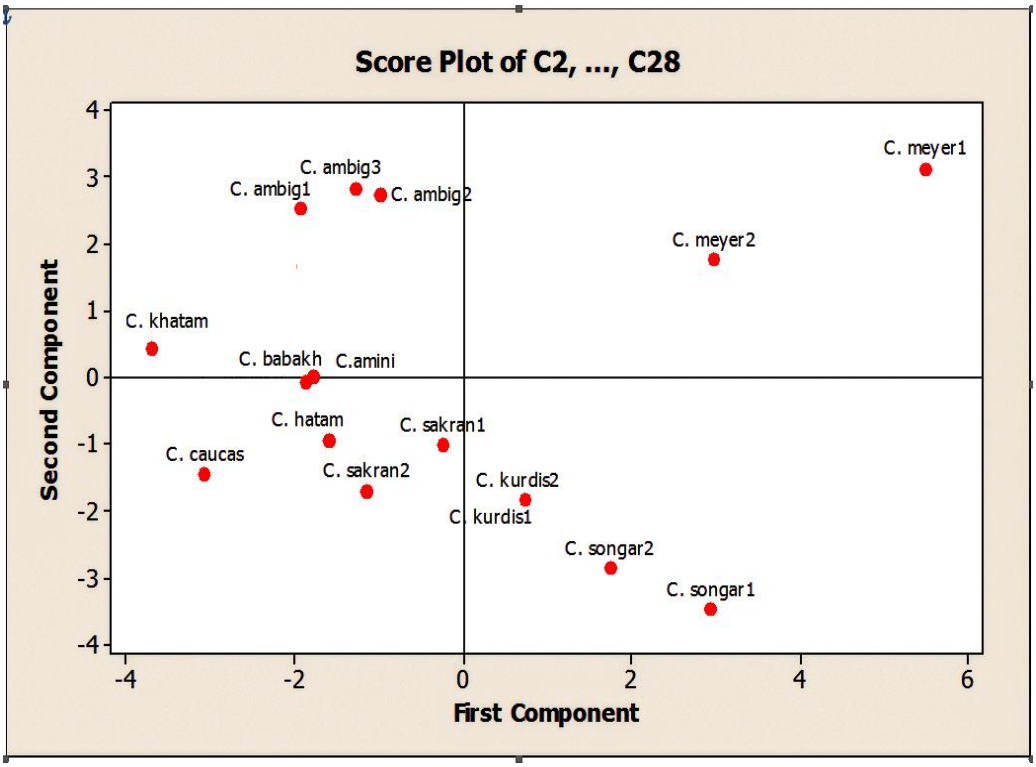

Fig. 2. PCA ordination of 10 *Crataegus* taxa based on qualitative anatomical traits.

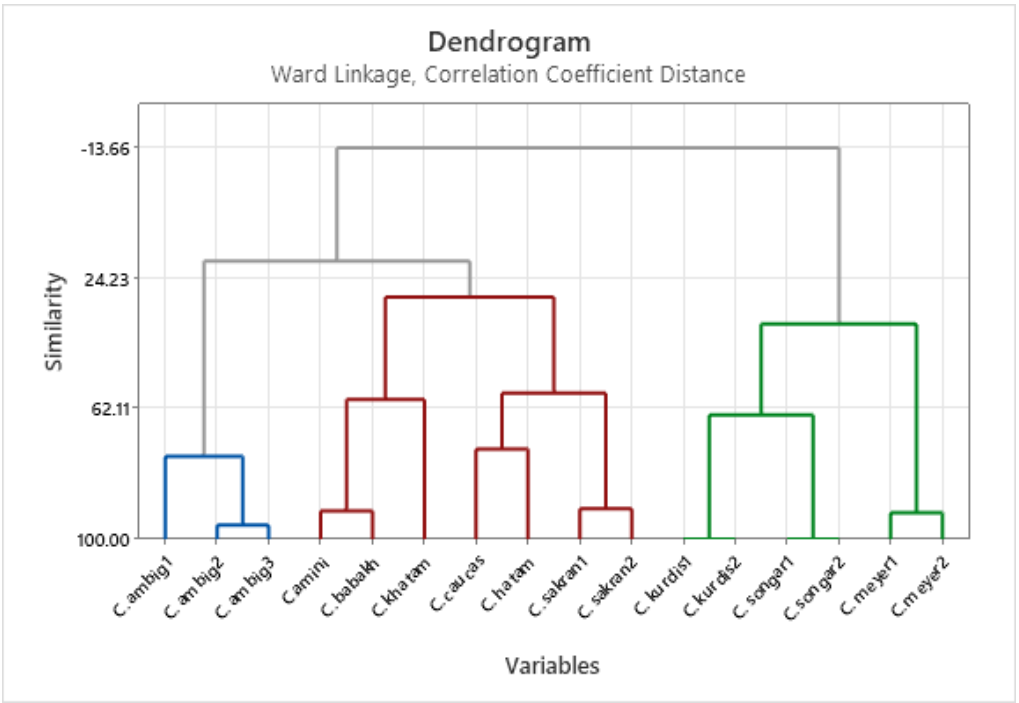

Fig. 3. Cluster analysis of 10 *Crataegus* taxa based on qualitative anatomical traits.

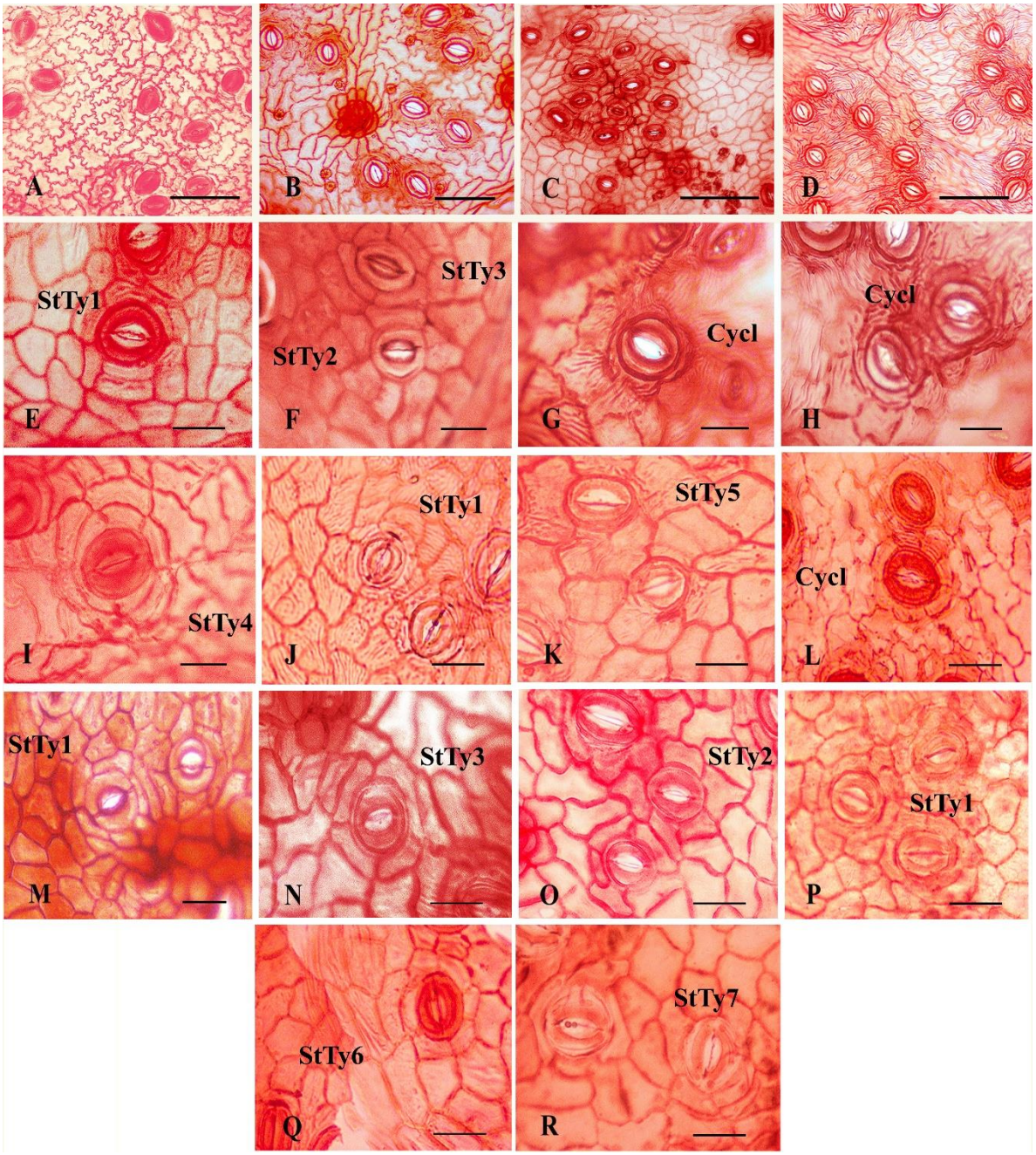

Fig. 4. (A- R): Anticlinal walls of cells and type of stomata on the lower surface of leaves: A, *Crataegus caucasica* (AWC0= Wavy); B, *C. kurdistanica* (AWC3=Sinuous); C, *C. khatamsazae* (AWC1=Shallow sinuous); D, *C. ambigua* subsp. *ambigua* (AWC2=Smooth). E-F, *C. ambigua* subsp. *ambigua* (StTy1= Incomplete cyclocytic bicyclic; StTy2= Actinocytic; StTy3= Tetracytic); G, *C. aminii* (Cycl=Cyclocytic); H, *C. babakhanloui* (Cycl=Cyclocytic); I, *C. caucasica* (StTy4= Cyclocytic bicyclic); J, *C. hatamii* (StTy1= Incomplete cyclocytic bicyclic); K, *C. khatamsazae* (StTy5= Anomocytic); L, *C. kurdistanica* (Cyclocytic); M-O, *C. meyeri* (StTy1= Incomplete cyclocytic bicyclic; StTy3= Tetracytic; StTy2= Actinocytic); P & Q, *C. sakranensis* (StTy1= Incomplete cyclocytic bicyclic; StTy6= Cyclocytic-Actinocytic); R, *C. songarica* (StTy7= Actinoctyic-Stephanocytic). Scale bars: A-D=100 µm & E-R=30 µm.

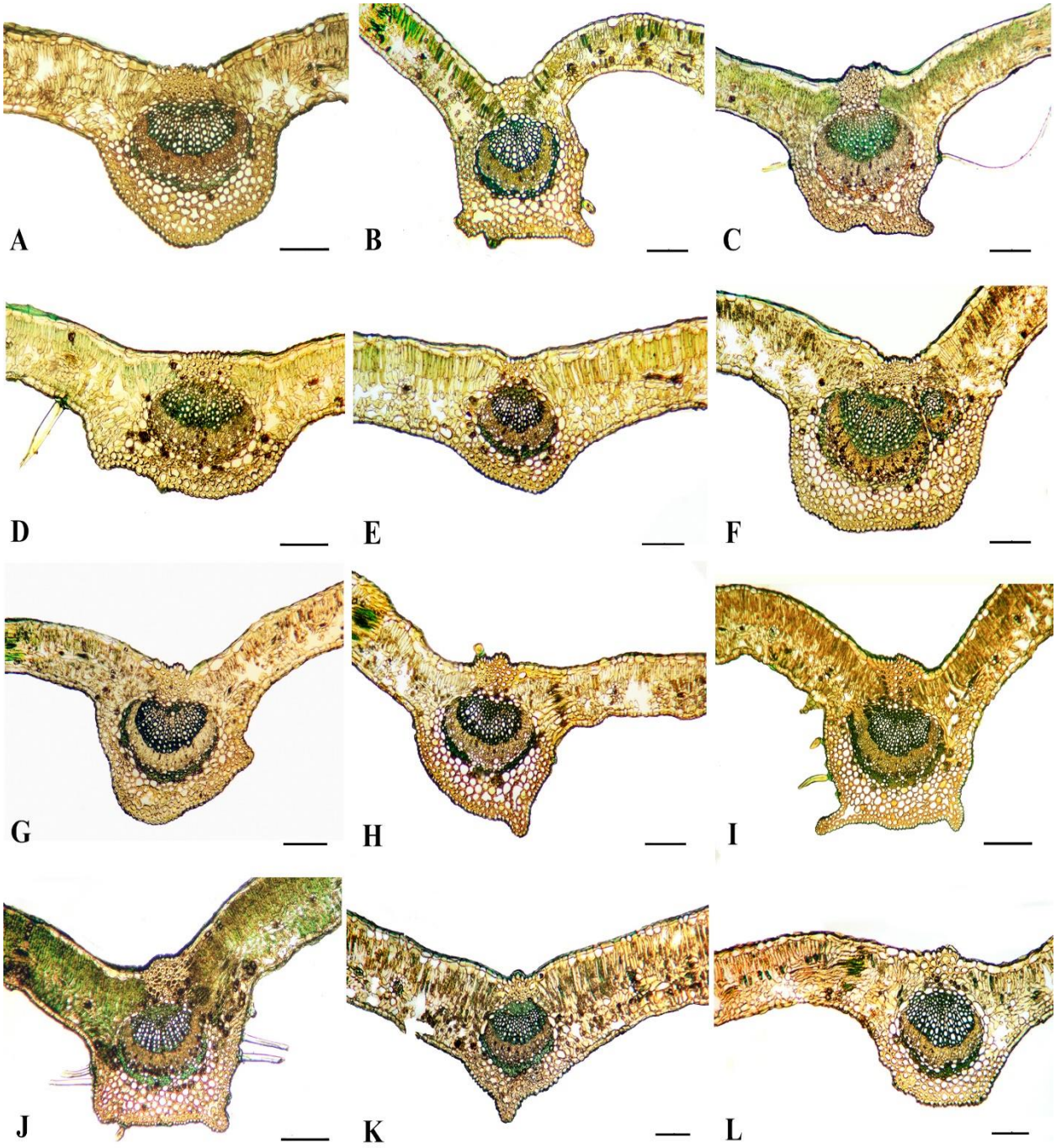

Fig. 5. (A-L) Diagnostic anatomical traits of midrib cross sections: A & B, *Crataegus ambigua* subsp. *ambigua* (VenM3; SVbM0 & SVbM2; DorM1 & DorM2); C, *C. aminii* (VenM3; SVbM0; DorM2); D, *C. babakhanloui* (VenM2; SVbM0; DorM2); E, *C. caucasica* (VenM1; SVbM2; DorM); F, *C. hatamii* (VenM1; SVbM2; DorM1); G, *C. khatamsazae* (VenM3; SVbM1; DorM2); H, *C. kurdistanica* (VenM3; SVbM2; DorM2); I & J, *C. meyeri* (VenM3; SVbM2; DorM3 & DorM2); K, *C. sakranensis* (VenM3; SVbM2; DorM 2); L, *C. songarica* (VenM3; SVbM2; DorM1). Scale bars=100 µm. Abbreviated attributes are presented in Fig. 1 and Table 2.

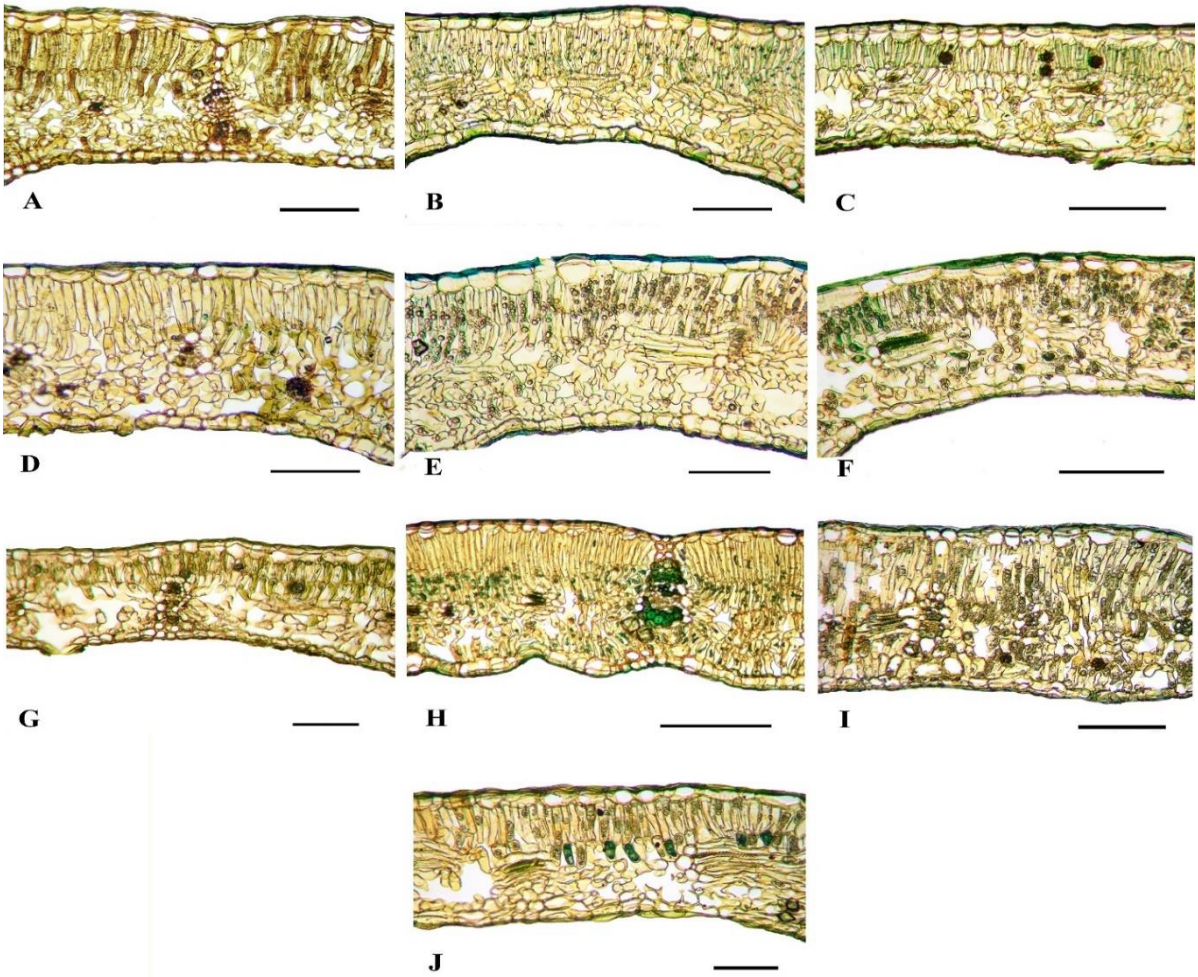

Fig.6. (A-J) Diagnostic anatomical traits of blade cross sections: A, *Crataegus ambigua* subsp. *ambigua*; B, *C. aminii*; C, *C. babakhanloui*; D, *C. caucasica*; E, *C. hatamii*; F, *C. khatamsazae*; G, *C. kurdistanica*; H, *C. meyeri* (MeTy1= Isolateral mesophyll); I, *C. sakranensis*; J, *C. songarica*. Scale bars=100 µm.

Quantitative traits

To determine the significance of quantitative trait differences between species, 30 traits were analyzed using ANOVA at the 99% level. All traits were significantly different among species. The PCA method was used to determine the correlation of traits with species. The first three axes of PCA accounted for 61% of the total variance. In the positive part of PCA1, the most important quantitative traits that had higher values than other traits included midrib length and width (ML and MW), phloem length and width of the midrib (PhLM and PhWM), xylem length and width of midrib (XyLM and XyWM), and thickness of the upper parenchyma of midrib (TUPM), which together constitute the midrib size (Table 4). The midrib was the thickest in *C. aminii*, distinguishing this species from other species (Table 5). These traits had significant differences in accessions and species at different altitudes. It seems that the altitude does not affect its changes. *Crataegus aminii* and *C. babakhanloui*, which did not differ significantly in qualitative traits, exhibited significant differences in these quantitative features (Table 5). The type specimens of these two species were collected in two completely different habitats. *Crataegus aminii* was collected on the Isfahan Zob Ahan highway at an altitude of 1500 meters in a dry environment, and *C. babakhanloui* was collected on the Chalus-Arangeh Road at an altitude of 1700 meters in a mountainous relatively dry environment.

Usually, the temperature of the environment decreases with increasing altitude. In the study by Abbas Azimi & al. (2020), a negative correlation was

observed between the thickness of the midrib of *Alnus subcordata* C. A. Mey. and temperature. In the present study, no correlation was observed between increasing altitude and midrib thickness.

In the negative part of the PCA2 axis, the blade thickness (BT) traits, thickness of the upper cuticle of blade (TUCB), length of the spongy layer (LSP), length of the first layer of palisade parenchyma (LFrLPP), length of the second layer of palisade parenchyma (LSeLPP), length to width ratio of the first layer of palisade parenchyma (LFrLPP/WLFrLPP), length to width ratio of the second layer of palisade parenchyma (LSeLPP/WSeLPP), and length to width ratio of the midrib (ML/MW) had higher values than other traits (Table 4). These traits separated *C. meyeri*1, *C. caucasica*, *C. ambigua*1, *C. ambigua*2, *C. ambigua*3, *C. sakranensis*1, and *C. sakranensis*2 from other species located in the positive PCA2 axis (Fig. 7). The trend of increasing altitude was from the positive part of PCA2 axis at an altitude of 1200 m (*C. kurdistanica*2) to the negative part of PCA2 at an altitude of 2350 m (*C. sakranensis*2) (Fig. 7, Table 1).

Leaf thickness was positively correlated with increasing altitude. The lowest leaf thickness was observed in *C. babakhanloui* and increased with increasing altitude in the *C. ambigua*2 (1560 m a.s.l.), *C. hatamii* and *C. ambigua*3 (1900-2000 m a.s.l.), respectively. Two specimens of *C. sakranensis*1 and *C. sakranensis*2 (2000-2350 m a.s.l.) had the highest leaf thickness.

In other studies, the increase in blade thickness was positively correlated with altitude (Velízquez-Rosas & al., 2002; Abbas Azimi & al., 2020; Jia & al., 2024).

According to Velízquez-Rosas & al. (2002), the increase in epidermal cell and cuticle thickness correlates with altitude, although in some cases no correlation was observed. In this study, the thickness of the upper cuticle of the blade did not correlate significantly with altitude. The highest thickness of the upper cuticle was observed in *C. caucasica* and *C. aminii* at an altitude of 1500 meter a.s.l., followed by *C. ambigua*3 at an altitude of 1900 m a.s.l., *C. hatamii* and *C. sakranensis*1 at 2000 m a.s.l., and *C. meyeri*2 at 1800 m a.s.l. respectively (Table 5). The cuticle thickness in *C. khatamsazae* (3.72 µ) at 2000 m a.s.l. was very different from *C. hatamii* (4.20 µ) at the same altitude.

In the measurements performed, the greatest length of the spongy layer was observed in *C. sakranensis*, *C. hatamii*, and *C. ambigua* respectively at high altitudes in the range of 1900 to 2350 m a.s.l. (Table 1). The length of these layers decreased in other species with decreasing altitude (Table 5). However, there was a significant decrease in two species related to high altitude, namely *C. khatamsazae* at an altitude of 2000 m a.s.l., and *C. meyeri*2 at an altitude of 1800 m a.s.l. The length of this layer also had variable values among specimens of the same species (Table 5).

The highest length of the first and second layers of palisade parenchyma was observed in the species *C. ambigua*3 (1900 m a.s.l.) and *C. sakranensis*2 (2350 m a.s.l.), respectively. Species in the range of 1600 m to 2350 m a.s.l. (at the negative part of PCA2 axis) had the greatest length compared to other species (Fig. 7). The length of these traits in the two species *C. hatamii* and *C. khatamsazae* differed significantly even though they were collected at the same altitude, 2000 m a.s.l. The length of these layers in *C. hatamii* was several times higher than in *C. khatamsazae* (Table 5). These traits in addition to having different amounts between species, also showed significant differences between specimens of the same species at different altitudes. For example, the length of the first layer of palisade parenchyma in *C. ambigua*1 and *C. ambigua*2 at an altitude of 1600 m a.s.l. was 54.75µ and 55.84µ, respectively, and in *C. ambigua*3 at an altitude of 1900 m a.s.l. was 73.89µ. In *C. meyeri*1 at an altitude of 1360 m a.s.l. it was 58.60µ and in *C. meyeri*2 at an altitude of 1800 m a.s.l. it was 52.69µ.

The length of the second layer of palisade parenchyma in *C. ambigua*3 was 45.77µ, in *C. ambigua*2 and *C. ambigua*1 it was 38.89µ and 32.25µ, respectively (Table 5).

Two length-to-width ratios of the first and second layers of palisade parenchyma in the negative part of PCA2 axis separated species and specimens.

The highest ratio of the first layer of palisade parenchyma was in *C. ambigua*3 at an altitude of 1990 m, *C. meyeri*1 at 1360 m, *C. sakranensis*2 at 2350 m, and *C. ambigua*1 at 1700 m a.s.l., respectively. These traits also had significant differences in different specimens of the same species and at different altitudes (Table 5). The highest ratio of the length to width of the second layer of palisade parenchyma was in *C. sakranensis*2 at 2350 m, *C. ambigua*3 at 1990 m, and *C. meyeri*1 at 1360 m a.s.l., respectively. While in other species, this ratio decreased at different altitudes (Table 5).

Table 4. Eigenvector scores of quantitative anatomical traits on three main PCA axes

Variable	PCA1 (25%)	PCA2 (23%)	PCA3 (13%)
BT	-0.032	-0.362	0.076
TLCB	0.086	-0.155	-0.365
TUCB	0.156	-0.202	-0.087
LSP	-0.046	-0.326	0.065
LFrLPP	-0.101	-0.311	0.099
LSeLPP	-0.027	-0.319	0.157
WFrLPP	0.006	-0.078	0.213
WSeLPP	0.103	0.202	0.214
ML	0.324	0.016	-0.024
MW	0.337	-0.083	0.006
TLCM	0.188	-0.172	-0.013
TUCM	-0.130	-0.193	-0.341
TLEM	-0.052	-0.073	-0.254
TUEM	0.208	0.106	0.180
TLCoM	0.060	-0.108	0.192
TUCoM	0.233	-0.100	0.194
TLPM	0.182	-0.043	0.268
TUPM	0.287	-0.032	0.022
TXyScM	-0.023	-0.016	0.355
TPhScM	0.220	-0.011	0.150
PhLM	0.324	-0.009	-0.139
PhWM	0.294	-0.129	0.008
XyLM	0.286	0.046	-0.184
XyWM	0.291	-0.008	-0.129
LFrLPP /WFrLPP	-0.055	-0.314	-0.026
LSeLPP/WSeLpp	-0.072	-0.328	0.038
ML/MW	-0.063	0.245	-0.090
StD	-0.035	0.122	0.278
LSub	-0.110	-0.194	0.102
LLASt	0.148	-0.014	-0.216

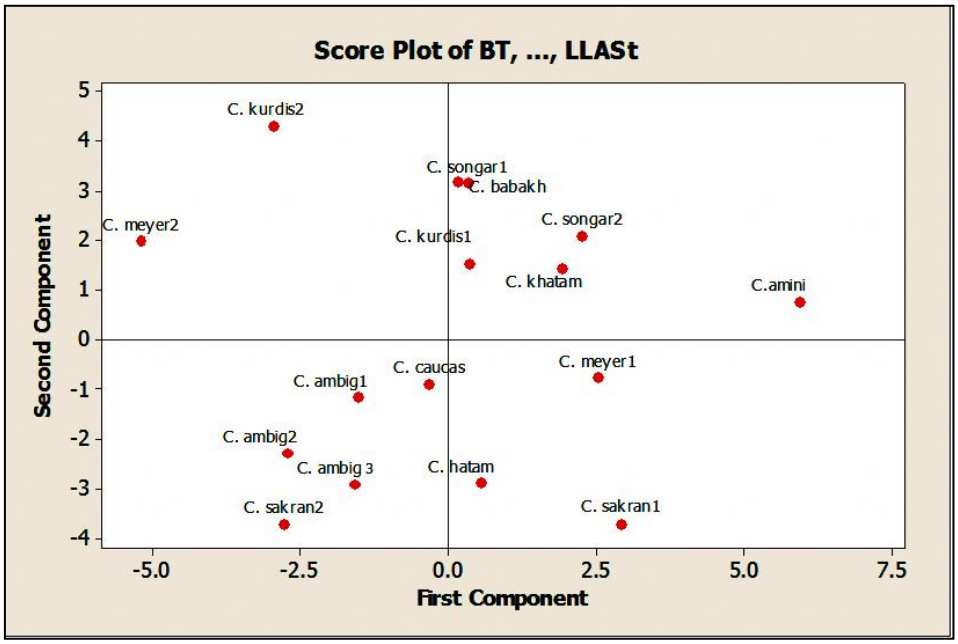

Fig. 7. PCA ordination of 10 *Crataegus* taxa based on quantitative anatomical traits.

Table 5. Mean and standard error of quantitative anatomical traits in *Crataegus* spp.

Sample	*C. ambig1*	*C. ambig2*	*C. ambig3*	*C. amini*	*C. babakh*	*C. caucas*	*C. hatam*	*C. khatam*
BT (μm)	194.63±9.37	227.30±6.40	242.53±15.14	173.96±7.06	149.81±12.14	225.39±7.12	236.21±14.44	178.84±16.58
TLCB (μm)	4.19±1.09	3.83±0.97	3.00±0.43	4.47±0.99	3.40±0.59	3.63±0.75	3.71±0.74	3.45±0.67
TUCB (μm)	4.12±0.69	3.61±0.72	4.21±0.70	4.37±1.35	3.96±0.46	4.83±0.77	4.20±0.76	3.72±0.56
LSP (μm)	70.59±6.48	77.64±5.41	85.84±7.50	59.08±4.92	55.89±10.50	76.25±8.69	97.92±14.44	71.45±9.29
LFrLPP (μm)	54.75±6.86	55.84±9.66	73.89±10.22	37.35±3.17	31.34±3.47	58.95±7.54	51.62±8.24	36.22±4.34
LSeLPP (μm)	32.25±5.53	38.89±4.55	45.77±4.42	30.92±3.42	25.74±4.47	33.36±5.34	43.33±4.52	26.65±4.13
WFrLPP (μm)	8.81±3.24	9.39±1.90	10.20±0.98	9.83±0.68	7.54±1.33	11.57±1.14	11.14±1.34	10.79±2.78
WSeLPP (μm)	7.88±1.35	8.46±1.35	10.20±1.31	10.87±1.71	11.28±0.75	11.07±1.16	10.37±1.72	9.29±1.00
LM (μm)	429.13±43.04	333.40±15.50	373.54±13.59	529.73±11.21	437.98±23.03	403.35±31.55	410.63±31.57	483.30±45.19
WM (μm)	356.54±21.37	330.15±18.06	332.52±13.87	467.26±10.94	329.75±21.63	345.52±22.79	382.24±21.96	421.13±14.16
TLCM (μm)	2.89±0.39	2.84±0.92	3.44±0.37	3.02±0.59	3.90±1.11	3.31±0.89	3.87±0.41	3.47±0.68
TUCM (μm)	6.75±1.07	4.92±1.33	4.32±0.90	3.92±0.32	4.07±0.88	4.61±1.05	4.68±0.77	4.06±1.31
TLEM (μm)	17.48±1.82	13.74±2.34	20.57±3.90	15.60±4.95	18.61±1.31	16.27±2.88	16.21±3.93	16.83±2.33
TUEM (μm)	15.41±3.77	14.93±7.47	19.44±2.28	19.53±3.25	17.94±2.60	19.63±3.21	17.96±4.36	17.60±3.18
TLCoM (μm)	20.00±5.44	29.57±6.64	21.43±7.68	19.58±4.54	18.20±3.11	17.26±3.28	25.15±10.72	22.12±6.12
TUCoM (μm)	49.01±8.29	42.44±4.62	53.43±16.35	63.39±5.27	34.10±3.10	36.42±7.72	66.23±2.66	65.94±5.32
TLPM (μm)	46.94±9.14	58.02±11.53	55.19±12.58	68.77±4.21	50.24±5.84	66.85±15.03	87.02±14.61	66.57±18.48
TUPM (μm)	7.71±4.14	22.71±6.92	16.66±3.27	36.07±6.27	16.13±1.64	23.98±5.63	22.80±3.90	22.86±4.61
TXyScM (μm)	22.78±7.70	23.84±8.32	25.27±6.84	21.04±4.76	21.24±1.95	39.27±8.08	33.88±7.75	19.39±6.64
TPhScM (μm)	19.99±4.69	21.40±6.10	17.96±3.04	24.28±9.18	23.13±7.07	22.37±4.96	24.25±5.17	20.35±4.82
PhLM (μm)	279.39±1.96	196.26±6.91	237.95±14.26	338.83±10.81	269.23±8.36	217.76±10.45	238.26±13.63	302.46±8.41
PhWM (μm)	51.91±15.39	55.14±4.59	63.44±16.22	81.87±9.85	57.30±6.94	47.20±6.10	61.04±4.61	57.43±6.67
XyLM (μm)	200.37±30.62	138.59±14.37	179.21±15.87	275.28±9.22	217.40±14.06	161.58±8.31	175.79±6.95	227.66±5.42
XyWM (μm)	88.61±7.16	64.91±8.20	77.59±13.03	131.70±10.21	82.77±5.99	107.58±51.10	86.03±11.59	103.18±5.58
ML/MW	1.20±2.01	1.01±0.86	1.12±0.98	1.13±1.02	1.33±1.06	1.17±1.38	1.07±1.44	1.15±3.19
LSeLPP /WSeLPP	4.09±4.10	4.60±3.37	4.49±3.37	2.84±2.00	2.28±5.96	3.01±4.60	4.18±2.63	2.87±4.13
LFrLPP /WFrLPP	6.21±0.47	5.95±0.20	7.24±0.10	3.80±0.21	4.16±0.38	5.10±0.15	4.63±0.16	3.36±0.64
StD (no. mm^2)	17.20±2.49	15.80±1.92	15.20±2.68	15.80±5.70	19.20±4.44	16.20±2.07	24.40±1.30	25.20±2.07
LSub (μm)	267.85±71.72	295.07±126.25	313.36±46.21	179.13±37.20	155.50±36.79	338.17±70.22	301.95±58.81	123.41±55.38
LLASt (μm)	51.53±13.71	53.31±11.29	45.88±2.19	70.35±4.55	43.97±9.50	53.07±5.40	49.39±3.69	44.50±8.62

Table 5. Continued.

Sample	*C. kurdis1*	*C. kurdis2*	*C. meyer1*	*C. meyer2*	*C. sakran1*	*C. sakran2*	*C. songar1*	*C. songar2*
BT (μm)	162.70±5.97	156.66±7.16	220.85±24.68	188.15±14.42	267.26±13.66	256.31±15.71	150.97±14.41	166.85±12.82
TLCB (μm)	3.63±0.97	2.17±0.87	2.78±1.03	3.47±1.20	3.61±0.63	3.57±0.63	2.99±0.54	2.92±0.78
TUCB (μm)	4.16±0.77	3.24±1.19	3.99±1.18	3.44±1.06	4.20±0.68	4.15±0.97	3.68±0.74	3.86±0.57
LSP (μm)	56.42±11.31	63.23±4.16	70.48±8.31	68.96±6.91	112.70±11.72	107.03±16.90	58.31±9.25	51.97±3.93
LFrLPP (μm)	43.48±6.32	34.04±4.55	58.60±8.41	52.69±13.15	59.60±9.28	70.25±15.19	43.02±5.16	49.92±8.57
LSeLPP (μm)	27.07±5.42	24.69±3.14	43.00±4.14	27.16±5.02	33.40±3.34	47.84±8.68	28.36±1.01	30.52±5.48
WFrLPP (μm)	8.82±1.50	10.70±1.25	8.60±2.20	9.14±1.31	10.03±2.63	10.36±2.06	10.60±0.63	9.84±0.94
WSeLPP (μm)	10.02±1.45	10.77±0.91	10.12±0.99	10.42±2.07	10.16±0.43	9.29±1.71	12.46±2.40	11.54±2.13
LM (μm)	469.20±24.16	359.94±28.61	534.92±43.58	330.65±36.24	486.89±6.28	372.13±18.01	425.68±25.34	425.74±71.04
WM (μm)	387.29±16.49	299.47±35.68	442.57±24.25	260.44±20.24	446.58±28.52	325.01±16.24	347.54±12.44	382.12±51.59
TLCM (μm)	2.69±0.85	2.14±0.68	3.39±1.18	2.35±0.37	3.93±0.94	3.02±0.82	2.64±0.49	3.39±0.17
TUCM (μm)	4.72±1.56	3.08±0.73	2.99±0.70	5.05±0.58	4.78±0.42	4.74±0.47	2.71±0.28	2.55±0.47
TLEM (μm)	14.35±2.59	13.58±3.59	12.20±1.56	17.74±4.18	16.81±1.17	16.86±1.89	14.38±3.67	17.62±2.99
TUEM (μm)	15.09±3.16	17.69±2.74	19.74±6.22	12.46±3.32	14.93±4.11	13.05±1.55	19.44±4.62	22.36±4.14
TLCoM (μm)	16.41±2.07	15.57±2.43	19.99±8.46	13.57±2.40	32.30±6.68	32.81±18.24	40.61±10.78	30.35±6.56
TUCoM (μm)	69.77±10.12	51.08±13.57	100.68±0.49	29.57±6.75	90.97±8.91	45.23±7.30	54.23±6.94	54.02±7.07
TLPM (μm)	86.83±8.05	57.83±14.40	98.16±10.14	50.86±9.28	70.10±7.39	51.10±22.83	69.50±13.40	54.41±7.53
TUPM (μm)	28.21±3.75	10.94±3.53	18.38±6.68	10.45±1.87	33.75±4.92	11.05±1.92	22.27±6.43	27.88±8.87
TXyScM (μm)	29.74±14.29	27.45±7.67	28.60±7.07	23.52±6.70	25.26±4.84	31.05±4.18	40.12±2.96	30.26±11.42
TPhScM (μm)	17.78±1.16	17.55±6.32	26.80±6.40	17.67±6.56	21.58±5.30	21.08±5.31	21.93±4.99	30.38±10.58
PhLM (μm)	265.55±18.28	201.74±7.33	282.73±20.11	184.46±12.38	322.00±7.45	220.89±9.46	252.73±25.30	276.51±61.79
PhWM (μm)	56.49±6.52	47.48±3.32	75.10±11.84	40.74±1.06	70.72±4.25	58.17±5.74	52.54±4.95	59.84±11.82
XyLM (μm)	201.06±10.80	168.53±5.28	163.44±44.36	145.59±6.09	259.03±8.55	174.86±9.18	206.72±19.57	220.72±40.93
XyWM (μm)	71.29±6.15	76.68±8.59	84.56±5.33	61.74±5.11	97.82±8.04	73.89±3.84	75.72±9.24	98.23±7.73
ML/MW	1.21±1.47	1.20±0.80	1.21±1.80	1.27±1.79	1.09±0.22	1.14±1.11	1.22±2.04	1.11±1.38
LSeLPP /WSeLPP	2.70±3.74	2.29±3.45	4.25±4.18	2.61±2.43	3.29±7.77	5.15±5.08	2.28±0.42	2.64±2.57
LFrLPP /WFrLPP	4.93±0.24	3.18±0.27	6.81±0.26	5.76±0.10	5.94±0.28	6.78±0.14	4.06±0.12	5.07±0.11
StD (no. mm^2)	23.80±3.19	29.80±3.96	26.00±1.87	22.00±1.58	20.00±1.87	24.80±3.49	20.40±4.77	22.80±1.30
LSub (μm)	282.13±58.47	219.34±50.67	214.03±76.14	232.66±88.92	260.07±39.60	215.49±59.20	222.25±65.24	237.36±51.18
LLASt (μm)	46.71±5.92	41.72±3.31	36.63±3.52	38.22±5.14	39.10±3.38	43.71±2.23	46.92±3.19	48.99±12.07

In the positive part of PCA2 axis, the ratio of midrib length to width is the only trait that has a higher value than other quantitative traits. The highest ratio was measured in *C. babakhanloui* (1.33µ) and the lowest was in *C. ambigua*2 (1.01µ). This trait was not related to altitude and variable ratios were observed in different specimens (Table 5).

In the positive part of PCA3 axis, the traits width of the first and second layers of palisade parenchyma, thickness of the lower parenchyma of midrib, thickness of the xylem sclerenchyma of midrib, stomatal density had higher values than other traits. In the negative part of this axis, the traits thickness of the lower cuticle of blade, thickness of the upper cuticle of midrib, thickness of the lower epidermis of midrib, and length of long axis of stomata had higher values than other traits.

The highest width of the first layer of palisade parenchyma was observed in *C. caucasica* at 1500 m a.s.l., then in *C. hatamii* and *C. khatamsazae* at 2000 m a.s.l. The width of the first layer of palisade parenchyma in species such as *C. sakranensis*2 at 2350 m and *C. sakranensis*1 at 2000 m a.s.l., as well as *C. ambigua*3 at 1900 m a.s.l., was much lower than other species at similar altitudes or species at lower altitudes.

The two species *C. babakhanloui* and *C. aminii*, which did not differ significantly in qualitative traits, were very different in a number of quantitative traits (Table 5). The maximum width of the second layer of palisade parenchyma was measured in two specimens of *C. songarica* in the altitudinal range of 1350 to 1470 m and then in *C. babakhanloui* at 1700 m a.s.l. This trait had different values in different species and specimens at different altitudes and was not related to elevation (Table 5).

The species *C. meyeri*1, *C. hatamii* and *C. kurdistanica*1 had the highest thickness of the lower parenchyma of midrib compared to other species and specimens with thicknesses of 98.16, 87.02 and 86.83µ, respectively. The thickness of the lower parenchyma of midrib in *C. meyeri*2 and *C. kurdistanica*2 was 50.86 and 57.83µ, respectively. This trait also had very different and significant values in other species and specimens (Table 5). In the PCA1 axis, other midrib-related traits including phloem length and width of the midrib, xylem length and width of the midrib in *C. aminii* were significantly different from other species (Table 4, Fig. 7).

One of the midrib segments is the xylem sclerenchyma, the greatest thickness of which was observed with a large difference in *C. songarica*1, *C. caucasica*, and *C. hatamii* compared to other species. This trait also had different values in other species and specimens at different elevations. The thickness of the xylem sclerenchyma was measured as 30.26µ in *C. songarica*2 and 40.12µ in *C. songarica*1. The lowest thickness was observed in *C. khatamsazae* with a thickness of 19.39µ (Table 5).

Stomata density is mentioned in the literature as an important indicator for species, which is directly related to photosynthesis, respiration, and leaf area (Körner & al. 1989; Velízquez-Rosas & al. 2002; Meier & Leuschner 2008; Xu & al. 2009; Paridari & al. 2012; Jia & al. 2024). In this study, stomatal density did not show any correlation with species and altitude. The highest stomatal density was counted in the *C. kurdistanica*2 (30), *C. meyeri*1 (26), and *C. khatamsazae* (25). The stomatal density was recorded as 24 in *C. kurdistanica*1, 22 in *C. meyeri*2, 17, 16, and 15 in three specimens of *C. ambigua*, 20.4 and 22.8 in two specimens of *C. songarica* (Table 5). In the study by Abbas Azimi & al. (2020), stomata density in the species *Alnus subcordata* C. A. Mey. decreased from west to east and increased from low to high elevations in the Hyrcanian forests. In other studies, various types of stomata and their densities have been reported (Zhi-Duan & Zhi-Yun, 1991; Paridari & al. 2012; Nisa & al. 2019). Stomata diversity has been reported in *Betula papyrifera* Marshal (Pyakurel & Wang 2014) and *Fagus orientalis* Lipsky (Bayramzadeh 2011) due to ecological and habitat differences. It seems water deficiency leads to increased stomatal density (Ichie & al. 2015).

In the negative part of PCA3, the lower cuticle of the blade had different thicknesses in specimens at various altitudes. The highest thickness was measured in *C. aminii* (4.47µ) and the lowest in *C. kurdistanica*2 (2.17µ). The lower epidermis of the midrib also had different thicknesses in different specimens and altitudes. The two specimens *C. songarica*1 and *C. songarica*2 had the lowest thickness of upper cuticle midrib among other species and specimens.

The longest stomatal axis was measured in *C. aminii* (70.35µ) and the shortest length was measured in *C. meyeri* specimens (38.22µ and 36.63µ). The elevation gradient had no effect on the decrease or increase of the longest stomatal axis, but due to its great variation in species, it can be used to distinguish *C. aminii* and *C. meyeri* from other species.

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