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Assessment of leaf stomatal morphological diversity in grape cultivars (*Vitis vinifera* L.) and introduction of cultivars adapted to semi-arid conditions of Qazvin, Iran

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Abstract

Leaf stomatal morphology is a key determinant of plant adaptation to environmental stress, particularly drought. This study conducted a comprehensive assessment of stomatal traits across 26 grape cultivars (*Vitis vinifera* L.) cultivated in the semi-arid region of Qazvin, Iran. Over two growing seasons (2022–2023), epidermal impressions were prepared using the nail polish technique, and parameters including stomatal density, dimensions, complex type, surface coefficient, shape coefficient, and percentage of occupied area were quantified using light microscopy and image analysis software. Multivariate analyses (MANOVA, PCA) and univariate tests (ANOVA, FDR-corrected correlations) were employed. Results revealed considerable diversity in all measured traits. Cultivar 'Ahmadi' exhibited the largest stomata (polar length: 41.29 μm , equatorial width: 29.25 μm) and the lowest density (39 stomata per unit area), whereas 'Karreh Ruye' showed the highest density (124 stomata). PCA identified two independent axes of variation: stomatal size (explaining 69.6% of variance) and density-coverage (17.7%). Although an inverse trend between size and density was observed, the correlation was not statistically significant after FDR correction. Cluster analysis grouped cultivars into two main clusters, reflecting distinct morphological strategies: large stomata with low density versus small stomata with high density. The predominant stomatal complex type was anomocytic. The findings indicate that stomatal traits, particularly the size-density combination, could serve as preliminary morphological markers for screening cultivars with putative drought adaptation. Cultivars such as 'Ahmadi', which exhibit a morphology often associated with water conservation strategies, warrant further physiological evaluation under water-limited conditions.

Keywords: Abaxial epidermis, Anomocytic stomata, Drought adaptation, Iran, Multivariate analysis, Phenotypic plasticity, Principal component analysis (PCA)

Introduction

Stomata, microscopic pores on plant epidermal surfaces, serve as critical gatekeepers for gas exchange and transpiration while simultaneously representing potential entry points for pathogens (Boso et al., 2016). In grapevines (*Vitis vinifera* L.), these structures are integral to maintaining physiological balance, particularly under water-limited conditions, where transpirational water loss primarily occurs through the leaves (Gribaudo et al., 2001; Peccoux, 2011). At the molecular level, stomatal development is orchestrated by conserved signaling pathways involving Epidermal Patterning Factor (EPF) peptides, receptor kinases such as Too Many Mouths (TMM) and ERECTA (ER), and basic Helix-Loop-Helix (bHLH) transcription factors including SPEECHLESS (SPCH),

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Mute, and Fama (Torii, 2021). Furthermore, phytohormones, particularly abscisic acid (ABA), act as central mediators of stomatal closure in response to drought and can influence developmental plasticity (Chen et al., 2020). Drought adaptation in grapevines involves both genetic programming of stomatal patterning and dynamic physiological responses. Stomatal regulation plays a central role in this process (Gomez del Campo et al., 2003). Considering the crop's economic importance and sensitivity to water stress, understanding the genetic and environmental determinants of stomatal morphology is essential for breeding resilient cultivars. Stomatal density appears to be predominantly affected by water status during the vegetative growth period, as reported for apple trees. (Ellas, 1995). Stomatal traits, including density, dimensions, and complex type, vary considerably depending on genetic background, rootstock, and environmental factors. Genetic control is evident, for example, from studies showing ploidy effects on stomatal size and number (Motosugi et al., 2002). Environmental modulation is also important, as stomata respond to light, CO₂, and water availability (Shim et al., 2003; Gokbayrak et al., 2008; Saeidi et al., 2020). The community-weighted mean of stomatal size increased linearly with latitude, whereas those of stomatal density and stomatal area fraction showed humpbacked relationship. The community-weighted mean of stomatal area fraction was correlated with climatic aridity, consistent with the adaptation strategies of plant species to achieve high maximum rates of gas exchange in arid regions when water is available. (Liu et al., 2017). In grapevines, stomatal characteristics vary significantly among cultivars and are affected by rootstock genotype, cultivation ecology, and management practices. (Shiraishi et al., 1996; Kara & Ozeker, 1999; Boso et al., 2016). This variation is not random; angiosperm stomatal morphology has evolved along spatially optimal allometric relationships, providing a wide range of trait combinations that confer flexibility in gas exchange (De Boer et al., 2016). Phenotypic plasticity enables further adjustment to seasonal and climatic cues, as seen in apple trees where spring water status strongly influences stomatal density (Elias, 1995). The first leaves on shoots had the fewest stomata per unit leaf area, while the number increased in subsequent leaves. In contrast, stomatal size, including length and width, did not differ (Bodor P. et al., 2019). Such phenotypic plasticity will be crucial under climate change, as water scarcity may favor stomatal traits that conserve water (Chen, 2022). Despite extensive research on grapevine physiology, comprehensive studies simultaneously examining quantitative (density, dimensions) and qualitative (complex types) stomatal traits across diverse genetic backgrounds remain limited. Previous work has often focused on specific traits, particular cultivars, or controlled conditions, leaving a gap in understanding the integrated morphological diversity available in germplasm collections, especially under semi-arid field conditions (Rogiers et al., 2009; Candar et al., 2021). Grapevine orthologs of *WOX9*, *TCP4*, and *GRF1-9* are involved in leaf outgrowth and expansion. Understanding these genes can inform further studies on molecular regulation of leaf development and the relationship between leaf structure and function, potentially aiding grape production improvement (Li et al. 2023). Furthermore, the relationships between key traits, such as the hypothesized trade-off between stomatal size and density, require validation using robust multivariate approaches and appropriate statistical corrections for multiple comparisons. This study aimed to address these gaps by conducting a comprehensive morphological analysis of leaf stomata across 26 grape cultivars (including native and introduced varieties) grown in the semi-arid region of Qazvin, Iran. Specific objectives were to: (1) quantify the diversity in stomatal density, dimensions, complex types, and derived coefficients; (2) investigate correlations among these traits using rigorous statistical corrections; (3) classify cultivars based on multivariate stomatal phenotypes; and (4) identify cultivars with morphological signatures suggestive of drought adaptation potential, thereby providing a practical, trait-based screening tool for local breeders and farmers to prioritize cultivars for cultivation or breeding programs under the increasing water scarcity in Qazvin province.

Materials and Methods

Study Area and Plant Materials

This study was conducted over two consecutive growing seasons (2022-2023) on 26 cultivars of *Vitis vinifera* L. (Table 1). Leaf samples were collected from commercial vineyards located in the Takestan, Qazvin, and Buin-Zahra counties of Qazvin Province, Iran (geographical coordinates: 48°45' to 50°50' E longitude and 35°37' to 36°45' N latitude). The samples had been identified by the Grapevine Research Center of Takestan, Qazvin Province. Voucher specimens are deposited in the Herbarium of Payame Noor University, Tehran, Iran. The selected cultivars included both traditional local varieties cultivated in the region and introduced cultivars to capture a broad spectrum of genetic diversity. All sampled vineyards were homogeneous in terms of cultivation conditions (soil type, irrigation regime, and orchard management practices) to minimize the environmental impact on the investigated traits.

For each of the 26 cultivars, three mature and healthy vines were randomly selected. From each vine, three fully expanded leaves were collected from the middle part of the shoot (internodes 7–8). For stomatal characterization, six non-overlapping microscopic fields were randomly examined on the abaxial epidermis of each leaf using a light microscope. Stomatal density was calculated by counting all stomata within each field of view, while stomatal dimensions were measured for a minimum of 15 stomata per field. The final value for each trait per cultivar was derived from the mean of all observations across plants, leaves, and fields. To maintain cellular turgor and prevent wilting, the leaf samples were immediately placed in labeled plastic bags containing a moist cloth after excision and transported to the laboratory in a cool box. Sampling was conducted during periods of the day (7-9 AM or 6-7 PM) when temperature and light intensity were minimal to reduce the effect of transient environmental stress on stomatal characteristics. The samples were stored in a refrigerator (4°C) in a humid environment until analysis.

Table 1. List of grapevine (*Vitis vinifera* L.) cultivars, their corresponding local names, and collection sites in Qazvin province, Iran.

Voucher number	Cultivars	Local name	Locality	Geographical localities
253	Ahmadi	Ahmadi	Qazvin, Rashteghon	36° 19' E 50° 09' N
254	Perlete France	Perletsefid	Qazvin, Rashteghon	36° 19' E 50° 09' N
255	Angor syah	Syah angoreh	Takestan, Qorogh	36° 02' E 49°40' N
256	Bidaneh safid	Spyehbidoneh	Takestan, Qorogh	36° 02' E 49°40' N
257	Mish pestan	Mish pestan	Takestan, Doalloraah	36° 04' E 49° 45' N
258	Syah shoneh zodras	Syaeshshoneh	Takestan, Qorogh	36° 02' E 49°40' N
259	Shani pykami	Pykamisyeshshoneh	Takestan, Doalloraah	36° 04' E 49° 45' N
260	Sorkhak or yaghoti	Sorkhak	Takestan, Doalloraah	36° 04' E 49° 45' N
261	Shast aroos	Shast aroos	Qazvin, Rashteghon	36° 19' E 50° 09' N
262	Shelehangor	Shelehangoreh	Qazvin, Rashteghon	36° 19' E 50° 09' N
263	Sabi gerd	Sabi gerd	Takestan, Doalloraah	36° 04' E 49° 45' N
264	Asgari safid	Asgari Syaden	Takestan, Qorogh	36° 02' E 49°40' N
265	Perletghermezfr	Perletsorkh	Takestan, Doalloraah	36° 04' E 49° 45' N
266	Fakhrii	Fakhrii	Takestan, Qorogh	36° 02' E 49°40' N
267	Karehroyeh	Karehroyeh	Qazvin, Rashteghon	36° 19' E 50° 09' N
268	Kondori	Kondorii	Qazvin, Rashteghon	36° 19' E 50° 09' N
269	Molaii	SorkhehMolii	Takestan, Doalloraah	36° 04' E 49° 45' N
270	Yazandaii	Yazandaii	Qazvin, Rashteghon	36° 19' E 50° 09' N
271	Khoshnav	Khoshnav	Takestan, Qorogh	36° 02' E 49°40' N
272	Ash syaeh	Ash syaeh	Takestan, Qorogh	36° 02' E 49°40' N
273	Bidanehghermez	Sorkhehbidoneh	Takestan, Qorogh	36° 02' E 49°40' N
274	Chafteh	Chafteh	Qazvin, Rashteghon	36° 19' E 50° 09' N
275	Taftisyah.R	Taftisyah.R	Takestan, Qorogh	36° 02' E 49°40' N
276	Sabi pykami	Sabi pekami	Takestan, Doalloraah	36° 04' E 49° 45' N
277	Yazondiighermez	Sorkhehyazondaii	Takestan, Qorogh	36° 02' E 49°40' N
278	Phlem seedless	Phelm	Qazvin, Rashteghon	36° 19' E 50° 09' N

Preparation of Epidermal Impressions and Microscopy

For the examination of stomatal characteristics, the epidermal impression technique using clear nail polish was employed (Mehmet & Sadettin 2019). A thin layer of colorless nail polish was applied to two areas each on the abaxial (lower) and adaxial (upper) leaf surfaces, specifically in the interveinal regions. After the polish had completely dried, the resulting polymer film was carefully peeled off using transparent adhesive tape and mounted onto glass microscope slides. Separate slides were prepared for each sample (Figure 1 and 2).





Figure 1. Sampling locations on the abaxial (A) and adaxial (B) epidermis of grapevine



Figure 2. Steps for preparing slides using the clear nail polish method

Microscopic Observation and Imaging

Observations and imaging were conducted using an Optika B-383PLi light microscope. For each sample, images were captured at different magnifications (40×, 100×, 400×), and the images with optimal clarity and focus were selected for subsequent analysis.

Measurement of Morphological Parameters

The obtained digital images were analyzed using ImageJ (version 1.53) and Digimizer (version 5.4.9) software. The standard field of view area was calibrated to 319 μm^2 for all samples. The parameters measured or calculated for each sample are presented in Table 2 (Mehmet & Sadettin 2019).

Table 2. Descriptions and codes of qualitative and quantitative stomatal traits measured in the study.

Trait	Code	Description
Stomatal Density	N	Number of stomata per calibrated unit area.
Stomatal Dimensions	P&E	Polar length (P) and equatorial width (E), measured in micrometers.
Stomatal Perimeter	Perimeter	Perimeter of each stoma, measured in micrometers.
Stomatal Area	$P \times E$	Calculated as the product of polar length and equatorial width.
Percentage of Area Occupied by Stomata	-	Calculated using the formula $[(N \times P \times E) / \text{Total Sample Area}] \times 100$.
Stomatal Surface	SS	Calculated as $(\text{Polar Length} \times \text{Equatorial Width}) / 4$.
Stomatal Shape Coefficient	SSC	Calculated as $(\text{Equatorial Width} / \text{Polar Length}) \times 100$.
Stomatal Complex Type		The type of stomatal complex determined based on standard classification systems (e.g., anomocytic, anisocytic, etc.)

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics (version 27; IBM Corp., Armonk, NY, USA). Analyses were conducted on cultivar-level means, obtained by averaging measurements across leaves and sampling fields for each cultivar. Descriptive statistics were calculated for all stomatal morphological traits, including pore length (P), pore width (E), pore size (PS), stomatal size (SS), stomatal density (N), stomatal shape coefficient (SSC), and percentage of stomatal coverage. Hierarchical cluster analysis was performed to classify grape cultivars based on stomatal density (N), stomatal area ($P \times E$), and percentage of stomatal coverage (%). Prior to clustering, all variables were standardized (z-scores) to eliminate scale effects. Clustering was conducted using the average linkage (between-groups) method with squared Euclidean distance as the similarity measure. The resulting dendrogram was used to identify major cultivar clusters. To assess the contribution of individual traits to cluster separation, one-way ANOVA was applied to the same variables used in the clustering procedure. Normality and homogeneity of variances were assessed at the cultivar-mean level. Univariate normality was evaluated using the Shapiro–Wilk test, and homogeneity of variances among cultivars was assessed using Levene’s test. Although univariate tests indicated no strong deviation from normality ($p > 0.05$), Multivariate Analysis of Variance (MANOVA) was primarily justified by its robustness to moderate departures from normality, particularly when using Pillai’s Trace as the multivariate test statistic. MANOVA was applied to examine overall differences in stomatal morphological traits among grape cultivars. MANOVA was conducted using cultivar means as observational units. Following a significant MANOVA result, univariate analyses of variance (ANOVA) were conducted for individual traits to identify variables contributing to multivariate differences among cultivars. Effect sizes were quantified using partial eta-squared (partial η^2). Principal Component Analysis (PCA) was performed on standardized variables (z-scores) to explore patterns of covariation among stomatal traits and to reduce data dimensionality. Components with eigenvalues greater than one (Kaiser criterion) were retained for interpretation, and trait loadings and communalities were examined to assess the contribution of individual variables to each principal component. Pearson correlation coefficients were calculated to evaluate pairwise relationships among stomatal morphological traits. To account for multiple comparisons, p-values were adjusted using the False Discovery Rate (FDR) method according to the Benjamini–Hochberg procedure. All statistical tests were evaluated at a significance level of $\alpha = 0.05$.

Results

Variability in Stomatal Dimensions and Shape

All observations were focused on the abaxial leaf epidermis, as preliminary examinations confirmed that the adaxial epidermis is devoid of stomata (Figure 3), consistent with the hypostomatic nature of grape leaves (Figures 4–7). Consequently, only representative images of the adaxial surface are presented to illustrate this anatomical feature, while comprehensive imaging and analysis were directed to the stomata-bearing abaxial epidermis.

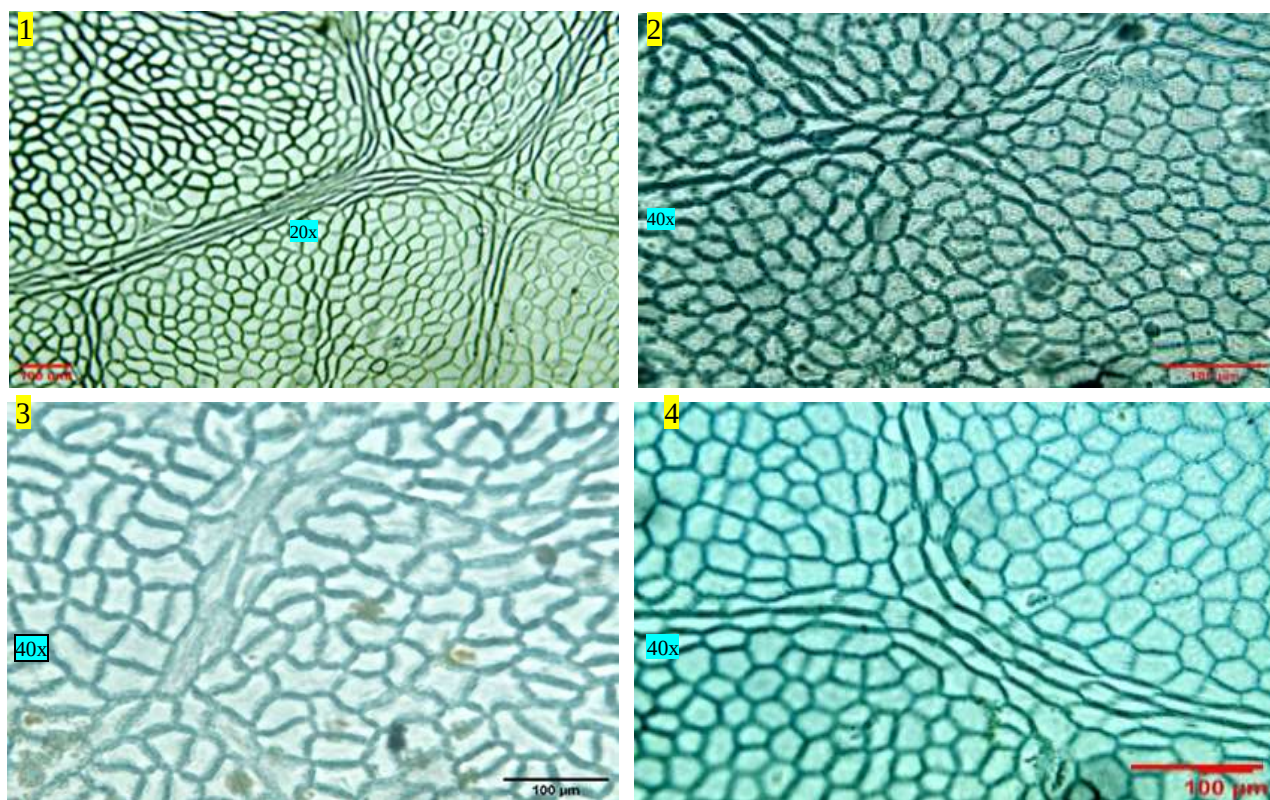


Figure 3. Morphology of the adaxial leaf epidermis in various grapevine cultivars. (1) Bidaneh Safid, (2) Ahmadi, (3) Perlete France, (4) Chafteh

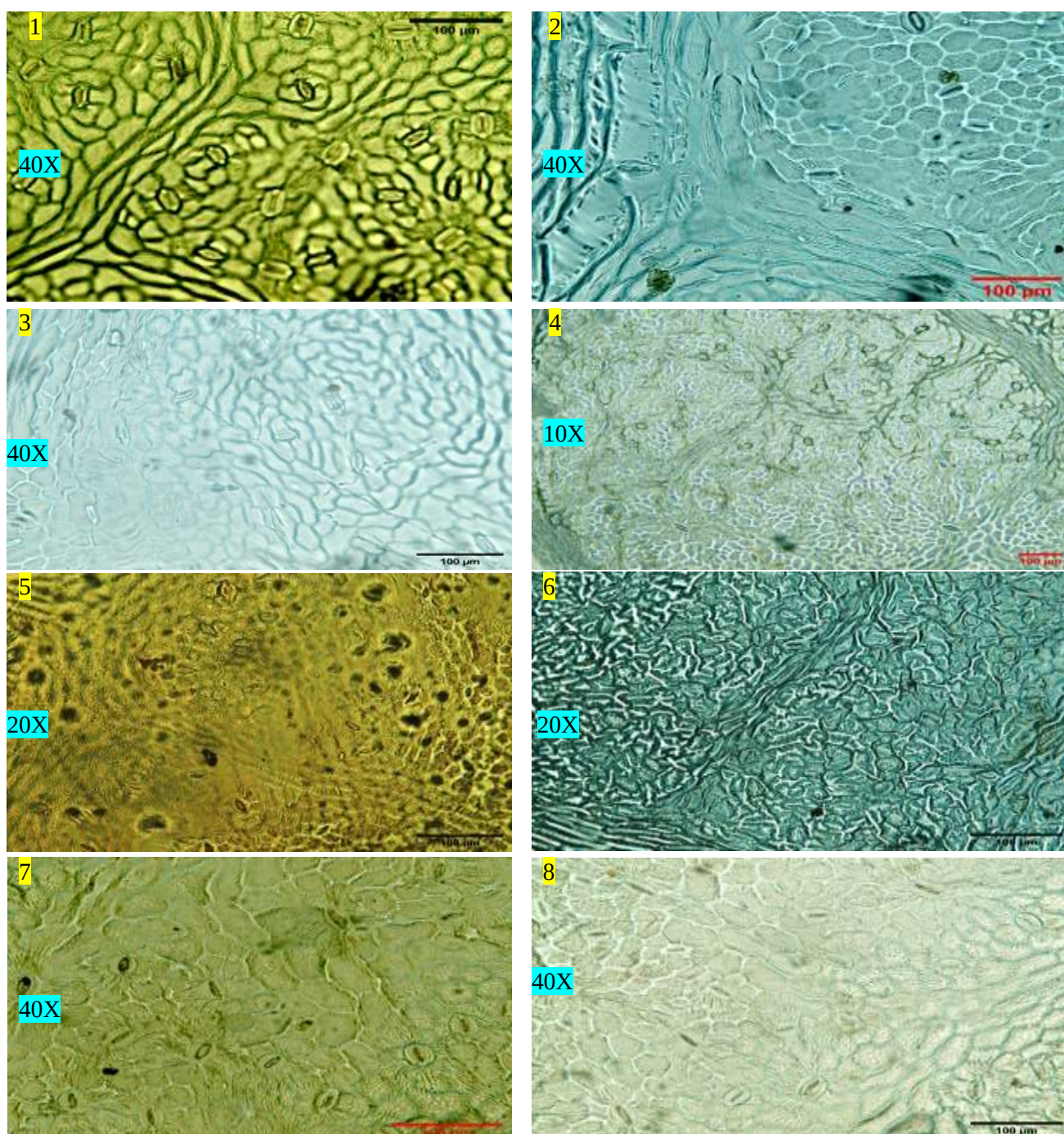


Figure 4. Morphology of the abaxial leaf epidermis in various grapevine cultivars. (1) Bidaneh Safid, (2) Ahmadi, (3) Agh Siah, (4) Flame Seedless, (5) Tafti, (6) Bidaneh Ghermez, (7) Chafteh, (8) Khoshnav.

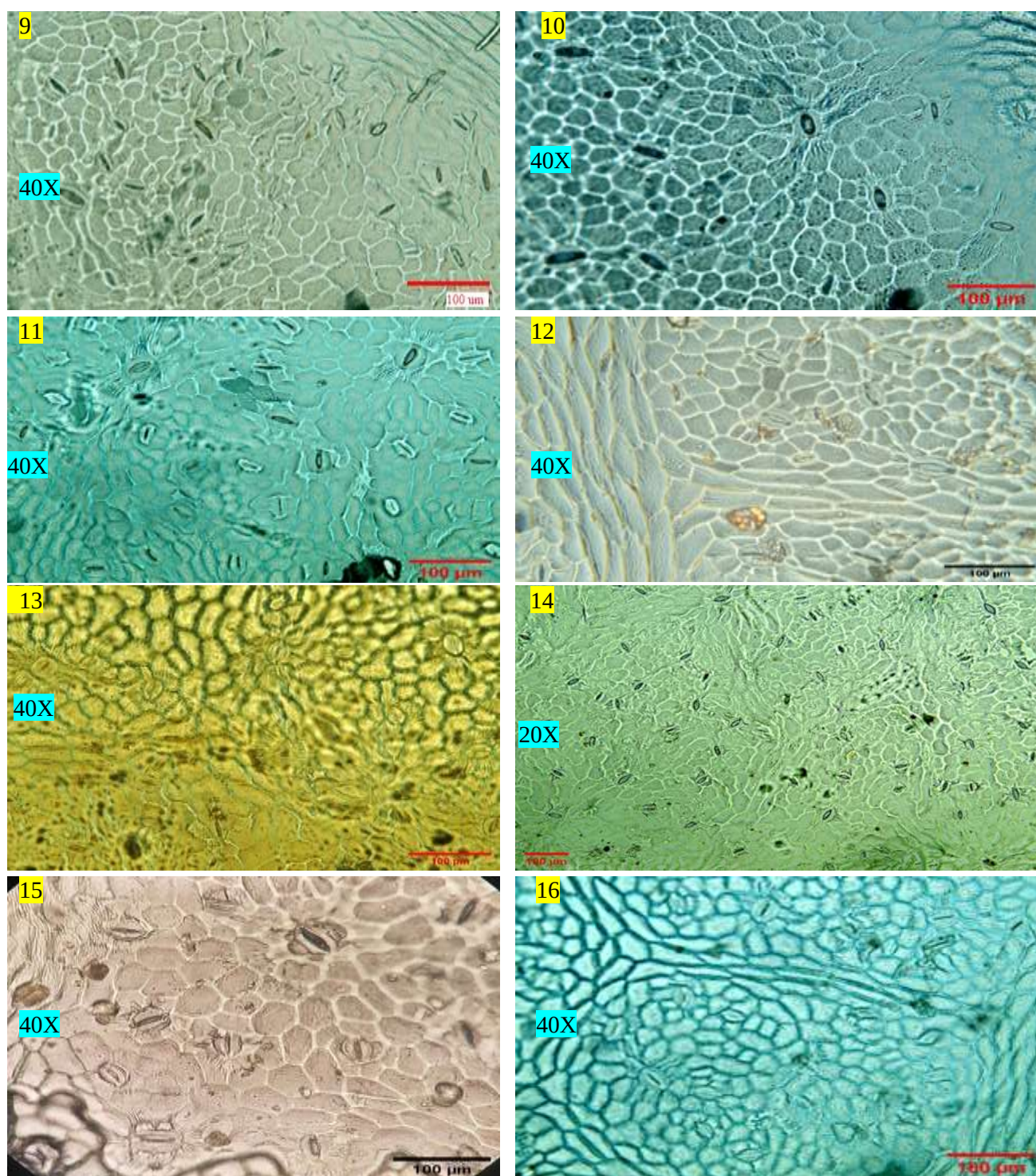


Figure 5. Morphology of the abaxial leaf epidermis in various grapevine cultivars. (9) Mish Pestan, (10) Sabi Gerd, (11) Sabi Pykami, (12) Sorkhak, (13) Siah Angur, (14) Siah Shuneh Zoodras (early-ripening), (15) Siah Shuneh Pikami, (16) Shast Aroos.

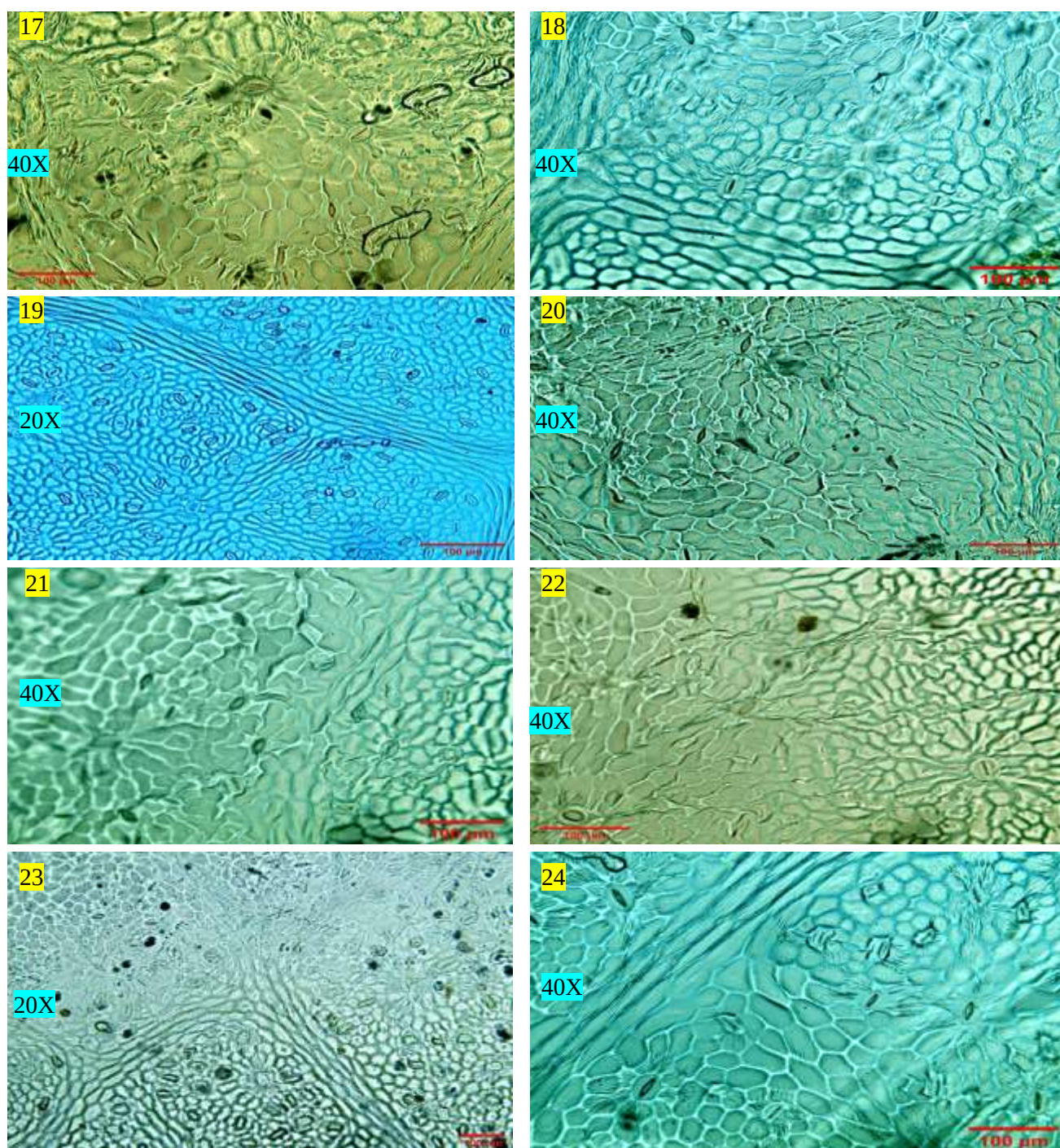


Figure 6. Morphology of the abaxial leaf epidermis in various grapevine cultivars. (17) Shel Angur, (18) Asgari, (19) Farkhi, (20) Kareh royeh, (21) Kondori, (22) Molaii, (23) Perlete France, (24) Perlte ghermez.

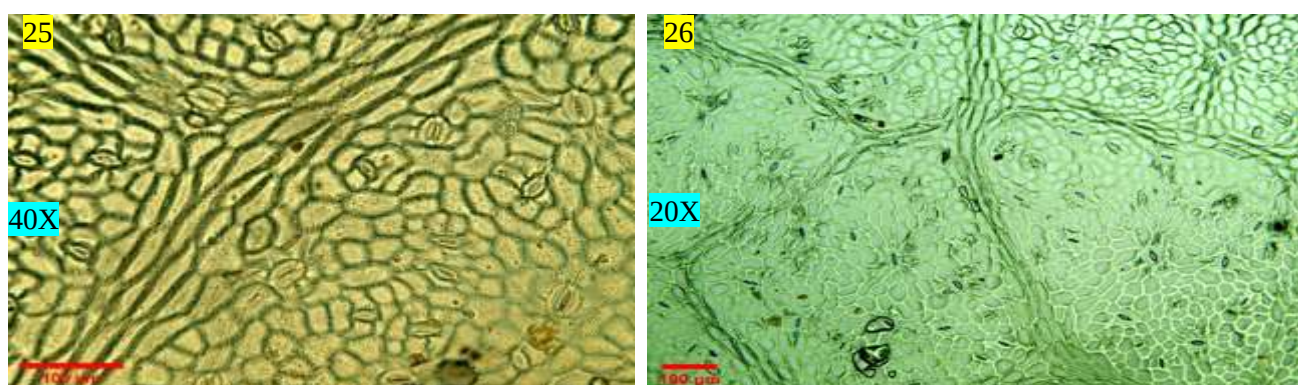


Figure 7. Microscopic view of the abaxial leaf epidermis. (25) Cultivar Yazandaii Sefid (white), (26) Cultivar Yazandaii Ghermez (pink/red)

Data analysis revealed considerable diversity in stomatal dimensions among the 26 studied cultivars. As presented in Table 3 and Figure 8, the polar length (P) of stomata varied significantly between cultivars. The 'Ahmadi' cultivar exhibited the greatest polar length with a mean of 41.29 µm, while the 'Bidaneh Ghermez' cultivar showed the smallest with a mean of 24.78 µm. The equatorial width (E) of stomata followed a similar pattern of diversity. The 'Ahmadi' cultivar had the greatest mean equatorial width (29.25 µm), whereas the 'Sorkhak' cultivar possessed the smallest mean equatorial width (16.89 µm) (Table 3).

Table 3. Mean polar length, mean equatorial width, length-to-width ratio, and perimeter of stomata in different grapevine cultivars.

V.N	P	E	P/E	NT	PS um
253	41.29	29.25	1.41	1	111.88
254	34.50	24.81	1.39	2	93.97
255	31.93	20.99	1.52	3	84.27
256	37.18	23.98	1.50	4	97.36
257	33.62	23.96	1.40	2	91.25
258	33.59	24.16	1.39	2	91.57
259	35.68	24.50	1.45	2	95.52
260	26.20	16.89	1.55	2	68.63
261	33.98	22.82	1.48	2	90.40
262	35.06	25.19	1.39	2	95.48
263	38.64	26.51	1.45	2	103.56
264	33.45	23.46	1.42	1	88.94
265	31.25	21.74	1.43	2	84.20
266	40.06	24.65	1.62	3	103.40
267	26.25	17.93	1.46	4	70.23
268	28.84	20.73	1.39	1	78.86
269	31.68	21.99	1.44	2	85.25
270	32.07	20.86	1.53	3	84.29
271	24.41	18.49	1.32	3	67.94
272	24.80	17.59	1.40	1	67.24
273	24.78	18.56	1.33	3	68.58
274	28.32	18.90	1.49	2	75.14
275	28.64	20.73	1.38	1	78.30
276	37.23	27.28	1.36	3	102.09
277	31.83	24.80	1.28	3	89.46
278	33.35	24.91	1.34	2	92.00

Abbreviations: V.N = Voucher number; P = Polar length; E = Equatorial width; P/E = Length-to-width ratio; NT = Number of stomatal types; PS = Perimeter of stoma

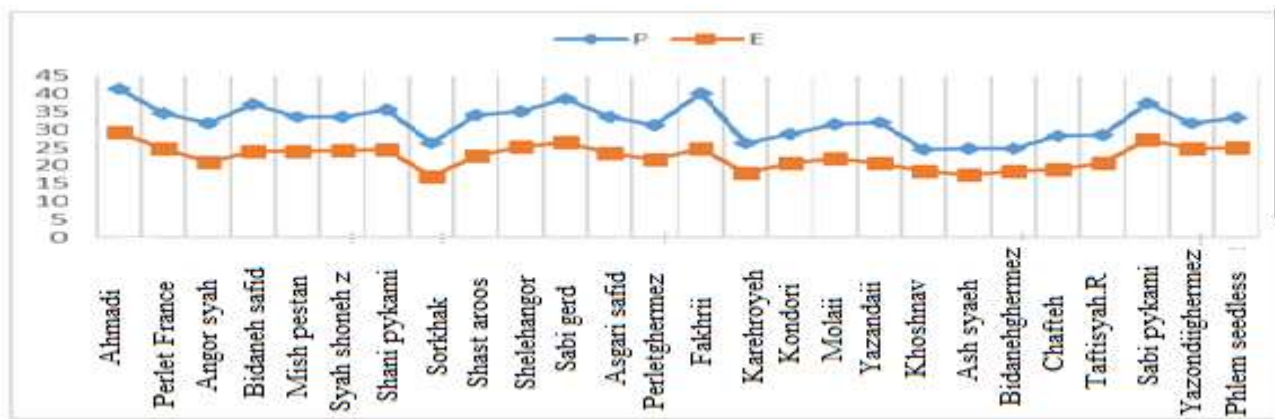


Figure 8. Comparison of polar length (P, μm) and equatorial width (E, μm) of stomata in 26 grapevine cultivars from Qazvin province

Based on these measurements, the polar length to equatorial width ratio (P/E) was calculated. This index, indicative of stoma elongation and shape, was highest in the 'Farokhi' cultivar (1.62) and lowest in the 'Yazandaii Ghermez' cultivar (1.28) (Table 3 and Figure 9). Furthermore, measurements of the stoma perimeter showed that the 'Ahmadi' cultivar had the largest mean perimeter (111.88 μm), while the 'As Siah' cultivar had the smallest mean perimeter (67.24 μm) (Table 3).

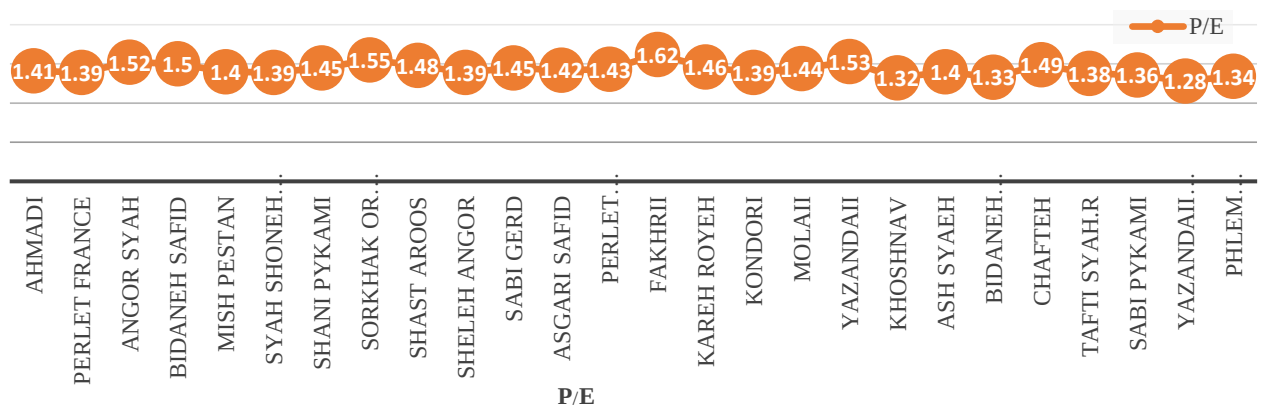


Figure 9. Polar length to equatorial width ratio (P/E) of stomata in 26 grapevine cultivars.

Variability in Stomatal Density and Surface Coverage

In contrast to the trends observed for stomatal dimensions, stomatal density (number per unit area) showed an inverse pattern. According to the data in Table 4, the 'Karreh Ruye' cultivar had the highest density with 124 stomata per standard area ($\sim 319 \mu\text{m}^2$), while the 'Ahmadi' cultivar had the lowest density with 39 stomata in a standard area ($\sim 319 \mu\text{m}^2$). This finding indicates an inverse relationship between stomatal size and density across the different cultivars. The calculation of the percentage of leaf area occupied by stomata, using the formula $[(N \times P \times E) / \text{Total Area}] \times 100$, revealed that the 'Fakhrii' and 'Molaii' cultivars each had the highest coverage, at approximately 23%. They were followed by the 'Bidaneh Safid' and 'Shast Aroos' cultivars, each with approximately 22% coverage. In contrast, the 'Khoshnav' and 'Ash Syah' cultivars had the lowest percentage of surface coverage, at approximately 7% each (Table 4).

Table 4. Analysis of stomatal density and stomatal area to leaf area ratio in the lower epidermis of different grapevine cultivars.

V.N	N	P×E	N×P×E	N×P×E /Area	N×P×E /Area×100
253	39	1207.73	47101.56	0.17	17
254	46	855.94	39373.47	0.14	14
255	39	670.21	26138.21	0.10	10
256	71	891.57	63301.47	0.22	22
257	68	805.53	54776.40	0.19	19
258	46	811.53	37330.58	0.13	13
259	56	874.16	48952.96	0.18	18
260	58	442.51	25666.04	0.09	09
261	73	775.42	56605.92	0.22	22
262	67	883.16	59171.81	0.20	20
263	44	1024.34	45071.24	0.18	18
264	49	784.73	38452.11	0.15	15
265	40	679.37	27175.00	0.11	11
266	64	987.47	63198.65	0.23	23
267	124	470.66	58362.15	0.21	21
268	65	597.85	38860.45	0.15	15
269	90	696.64	62697.88	0.23	23
270	98	668.98	65560.05	0.20	20
271	49	451.34	22115.7	0.07	07
272	48	436.23	20939.13	0.07	07
273	46	459.91	21156.17	0.09	09
274	72	535.24	38537.85	0.12	12
275	43	593.70	25560.10	0.08	08
276	41	1025.63	41641.01	0.13	13
277	45	789.38	35522.28	0.13	13
278	74	830.74	61475.38	0.20	20

Abbreviations: V.N = Voucher number; N = Stomatal density; P×E = Mean stomatal area; N×P×E = Total stomatal area; Stomatal Area Occupancy = Percentage of leaf area occupied by stomata

Analysis of Stomatal Surface and Shape Coefficients

To better understand the relationship between stomatal dimensions and potential function, the Stomatal Surface coefficient (SS) and Stomatal Shape Coefficient (SSC) were calculated. As expected and based on the data in Table 5, the 'Ahmadi' cultivar, possessing the largest dimensions, had the highest SS value (301.93), while the 'As Siah' cultivar had the lowest (109.05). On the other hand, the 'Farokhi' cultivar, with the highest P/E ratio, had the highest SSC value, and the 'Yazandaii Ghermez' cultivar, with the lowest P/E ratio, had the lowest SSC value (Table 5).

Table 5. Stomatal surface area (SS) and stomatal shape coefficient (SSC) values in different grapevine cultivars.

Voucher number	SS	SSC
253	301.93	141
254	213.98	139
255	167.55	152
256	222.89	150
257	201.38	140
258	202.88	139
259	218.54	145
260	110.62	155
261	193.85	148
262	220.79	139
263	256.08	145
264	196.18	142
265	169.84	143
266	246.86	162
267	117.66	146
268	149.85	139
269	174.16	144
270	167.24	153
271	112.83	132
272	109.05	140
273	114.97	133
274	133.24	149
275	148.42	138
276	256.40	136
277	197.34	128
278	207.74	134

Calculation formulas:

(1) Stomatal Surface Area (SS) = (Stomatal length × Stomatal width) / 4

(2) Stomatal Shape Coefficient (SSC) = (Stomatal width / Stomatal length) × 100

Diversity and Frequency of Stomatal Complex Types

Microscopic observations revealed diversity in stomatal complex types among the cultivars. Based on common classification systems, four main types were identified: anomocytic, anisocytic, tetracytic, and actinocytic (Figure10). The identification of stomatal complex types was based on the characteristic number, arrangement, and relative size of the subsidiary cells surrounding the guard cell pair,



following established anatomical descriptions (Metcalf & Chalk, 1979). The anomocytic type was observed as the most prevalent pattern across most cultivars. (Table 6). The number of stomatal complex types observed per cultivar varied from 1 to 4. In the 'Bidaneh Safid' and 'Karreh Ruye' cultivars, the arrangement of cells surrounding the stomata in the vein region did not conform to the standard patterns and was classified as an "Unspecified Type." (Table 6 & Figure 11).

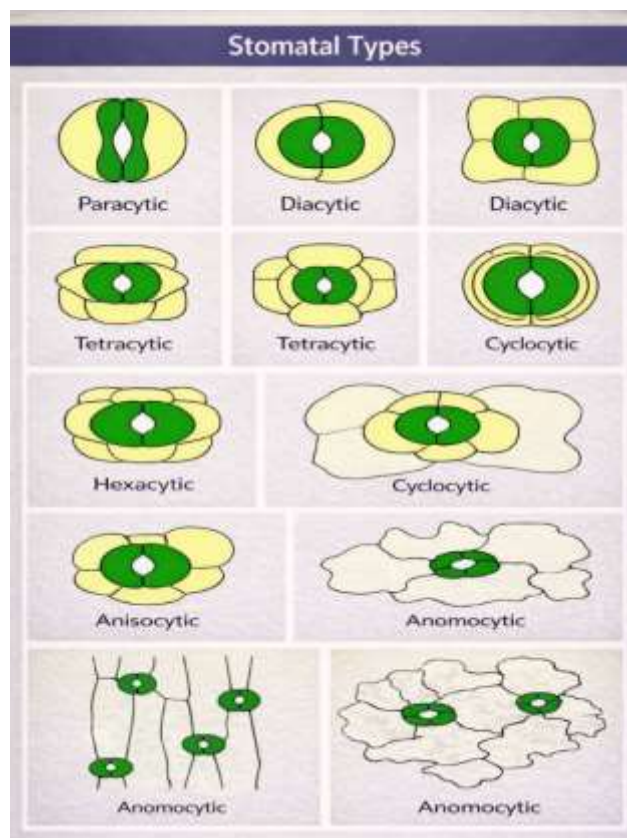


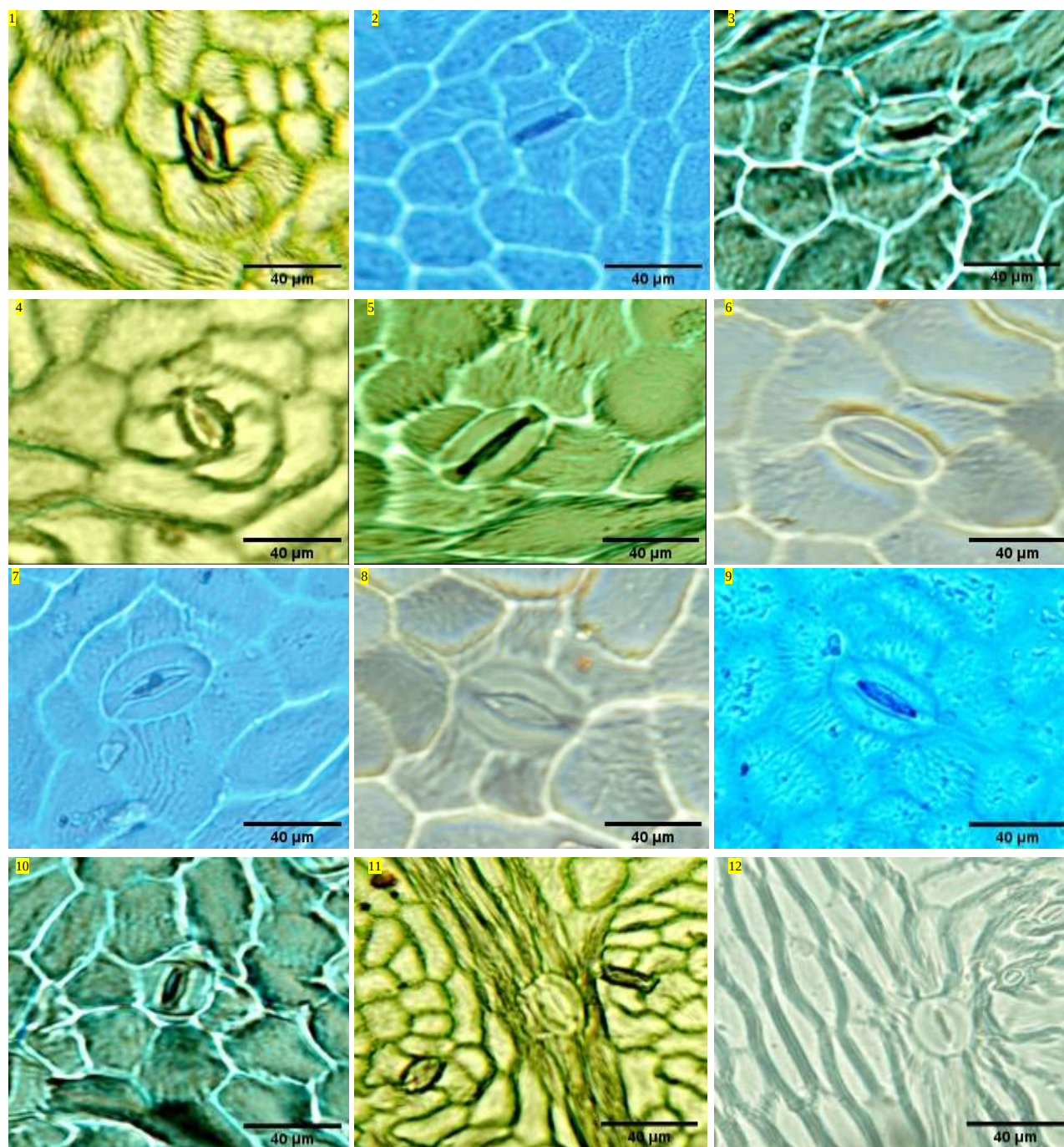
Figure 10. Stomatal complex types (Nguyen & Blatt, 2024).

Table 6. Stomatal types and their frequency in different grapevine cultivars. All measurements are in micrometers (μm).

V.N	Type stomata					N.S
	Anomocytic	Tetracytic	Actinocytic	Anisocytic	Uncertain	
253	39	0	0	0	0	39
254	46	0	9	0	0	57
255	35	0	3	1	0	39
256	33	11	0	29	1	71
257	64	0	4	0	0	68
258	40	0	6	0	0	46
259	51	0	5	0	0	56
260	46	0	14	0	0	58
261	54	0	19	0	0	73
262	55	0	12	0	0	67
263	16	0	28	0	0	44
264	49	0	0	0	0	49
265	32	0	8	0	0	40
266	11	0	34	16	0	64
267	94	0	15	14	1	124
268	65	0	0	0	0	65
269	82	0	8	0	0	90
270	50	0	18	20	0	98
271	30	0	8	11	0	49
272	48	0	0	0	0	48
273	23	4	0	19	0	46
274	54	18	0	0	0	72
275	43	0	0	0	0	43
276	34	0	5	2	0	41
277	29	0	7	9	0	45
278	63	11	0	0	0	74

Voucher Abbreviations: V.N = Voucher number; N.S = Number of stomata.





Relationships between traits and cluster analysis

Correlation analysis revealed no significant relationship between stomatal area ($P \times E$) and stomatal density (N). However, as stomatal size increased, the number of stomata per unit area tended to decrease, and vice versa (Tables 7 & 8) and (Figure 12). Pearson correlation analysis among stomatal morphological traits revealed strong positive associations among size-related variables (P , E , PS , and SS), whereas stomatal density (N) showed no significant correlation with size traits after FDR correction. In contrast, stomatal density was positively correlated with stomatal coverage percentage (Table 9). A comparison of the maximum percentage of leaf area occupied by stomata ($P \times A \times T$ or $[(N \times P \times E \text{ } \mu\text{m}^2) / \text{Area } \mu\text{m}^2] \times 100$), the minimum stomatal area coefficient ($P \times E / 100$), and the mean density showed that in the studied cultivars, as the number of stomata increased, the maximum $P \times A \times T$ value decreased relative to the minimum area coefficient ($P \times E / 100$) (Figure 12).

Table 7. Results of simple linear regression analysis examining the relationship between stomatal density (dependent variable) and stomatal area (independent variable) across 26 grape cultivars.

ANOVA ^a					
Model	Sum of Squares	df	Mean Square	F	Sig.
Regression	649.769	1	649.769	1.563	0.223 ^b
Residual	9980.270	24	415.845		
Total	10630.038	25			

^aNote: df for regression = number of predictors; df residual = n - predictors - 1; df total = n - 1.

Table 8. Regression coefficients analysis for the relationship between stomatal density (dependent variable) and stomatal area (independent variable)

Coefficients ^a					
Model		Unstandardized Coefficients B	Std. Error	Standardized Coefficients Beta	t
1	(Constant)	78.184	15.235		5.132
	P.E	-.025	.020	-.247	-1.250

Dependent Variable: Stomatal density (Nu)

Significant at $p < 0.05$

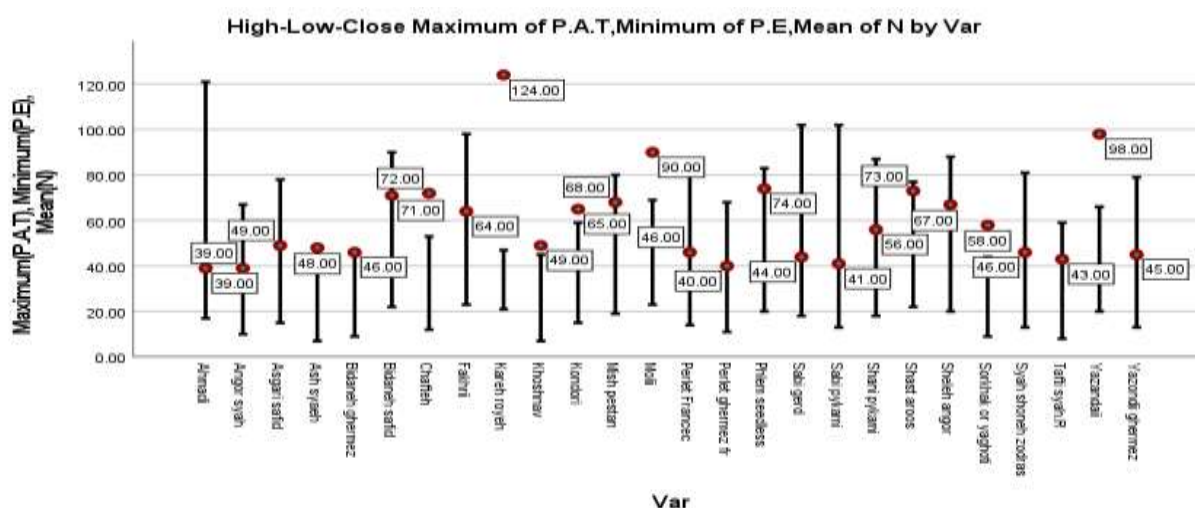
Table 9. Pearson correlation matrix of stomatal morphological traits (Based on cultivar-level means, $n = 26$; SPSS v27; p -values adjusted using FDR)

Trait	P	E	PS	SS	N	Coverage	SSC
P	1	0.92***	0.99***	0.98***	-0.16	0.61*	0.24
E	0.92***	1	0.97***	0.98***	-0.30	0.47	-0.14
PS	0.99***	0.97***	1	0.99***	-0.21	0.57	0.11
SS	0.98***	0.98***	0.99***	1	-0.25	0.53	0.05
N	-0.16	-0.30	-0.21	-0.25	1	0.63**	0.34
Coverage	0.61*	0.47	0.57	0.53	0.63**	1	0.37
SSC	0.24	-0.14	0.11	0.05	0.34	0.37	1

Significance levels (after FDR correction):

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

This analysis examined the relationship between the mean of the smallest stomatal size ($P \times E$), the highest stomatal area coverage coefficient, and the mean stomatal density within a defined area. The corresponding graph revealed a significant correlation between stomatal size, stomatal density, and the percentage of area occupied by stomata. The study demonstrated that as stomatal density increases, the mean percentage of area occupied by stomata shows a greater decrease. Indeed, a comparative plot of the relationship between stomatal density and the area occupied by stomata revealed an inverse correlation (Table 8 and Figure12).

**Figure 12.** Comparison of maximum stomatal area occupancy ($P \times A \times T$), minimum stomatal area ($P \times E \mu\text{m}^2/100$), and mean stomatal density among different grapevine cultivars

Based on the dendrogram derived from stomatal density (N), stomatal area ($P \times E$), and the percentage of leaf area occupied by stomata (Coverage), using the average linkage method and squared Euclidean distance, the following results were obtained. The dendrogram

for the aforementioned results was generated using SPSS software (version 27). The dendrogram, based on stomatal density (N), stomatal area ($P \times E$), and the percentage of area occupied by stomata Coverage, after Z-score standardization of variables, divided the cultivars into two main clusters (Figure 13). According to the dendrogram in Figure 13, the grape cultivars were categorized into two major groups with several subgroups and sub-divisions. Cluster I included: 'Karreh Ruye', 'Molaii', and 'Yazandaii Sefid'. Cluster II consisted of two main subgroups: Subgroup II-a: 'Kandari', 'Chofteh', 'Khoshnav', 'As Siah', 'Bidaneh Ghermez', and 'Sorkhak'. Subgroup II-b: 'Ahmadi', 'Sabi Gerd', and 'Sabi Pykami'. Other cultivars were placed within a further sub-branch of Subgroup II-b, including: 'Perlete France', 'Angoor Siah', 'Bidaneh Safid', 'Mish Pestan', 'Siah Shuneh Zoodres', 'Shani Pikami', 'Shast Aroos', 'Shal Angoorre', 'Asgari Safid', 'Perlette Ghermez', 'Fakhrii' ('Farkhi'), 'Bidaneh Ghermez', 'Tafti', 'Yazandaii Ghermez', and 'Flame Seedless' (Figure 13).

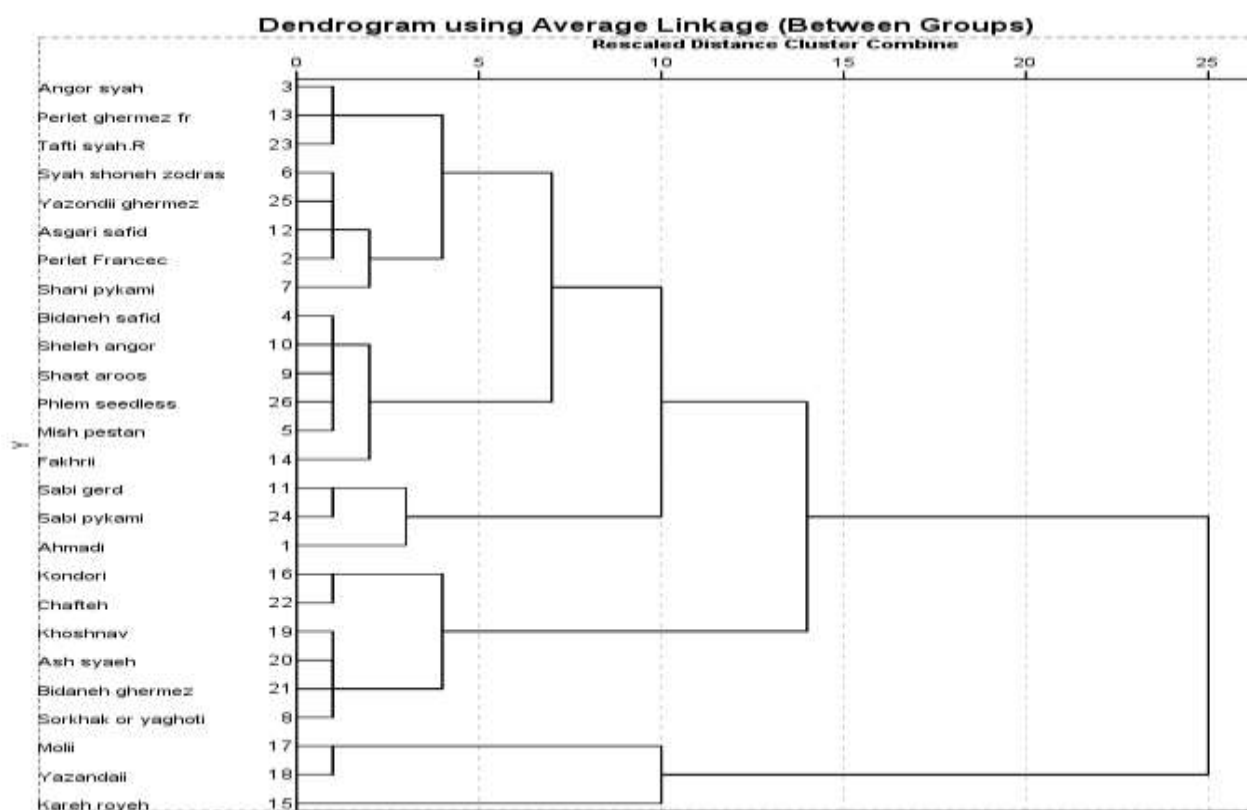


Figure 13. Cluster dendrogram of 26 grape cultivars based on stomatal traits. The dendrogram was generated using the average linkage method with squared Euclidean distance, following z-score standardization of three variables: stomatal density (N), stomatal area ($P \times E$), and the percentage of leaf area occupied by stomata (Coverage %).

To assess trait contributions to cluster formation, we performed one-way ANOVA comparing the same variables used in the hierarchical clustering, namely stomatal density (N), stomatal area ($P \times E$), and Coverage (%) between the two main clusters. Significant differences were detected for stomatal density (N) and Coverage (%) with Coverage showing the strongest effect; $F = 38.92$, $p < 0.001$, whereas stomatal area ($P \times E$) did not differ significantly between clusters ($p = 0.076$). Discriminant analysis further identified Coverage as the primary variable driving cluster separation (standardized coefficient = 0.891), with an overall classification accuracy of 92.3%. To further explore the size–density relationship, we calculated the stomatal length-to-density ratio (P/N) as an integrative descriptive index. This ratio, analyzed separately using MVSP 7.21 software, grouped cultivars into two clusters that primarily reflected variation in this composite trait. Cultivars with higher P/N ratios (indicating relatively larger stomata per unit density) tended to cluster separately from those with lower ratios. This complementary analysis illustrates the heterogeneity of size–density relationships among cultivar subsets, but does not constitute independent evidence for or against a stomatal trade-off. Instead, it highlights the complexity and context-dependence of the relationship between stomatal size and density (Figure 14).

Table 10. Mean ($\pm$ SD) values of stomatal density (N), stomatal area ($P \times E$), and stomatal coverage (%) for the two main clusters identified by hierarchical cluster analysis. Differences between clusters were evaluated using one-way ANOVA based on the same variables employed in clustering. Coverage percentage showed a highly significant difference between clusters ($F = 38.92$, $p < 0.001$), stomatal density also differed significantly between clusters ($p < 0.01$), whereas stomatal area ($P \times E$) did not show a significant difference ($F = 3.45$, $p = 0.076$).

Cluster	n	N (count)	Coverage (%)	$P \times E$ (μm^2)	Key differentiating trait
I	3	104.0 $\pm$ 17.8	21.3 $\pm$ 1.5	612.1 $\pm$ 123.3	High density & coverage
II	23	54.0 $\pm$ 12.4	14.6 $\pm$ 5.0	757.1 $\pm$ 209.8	Variable density & area

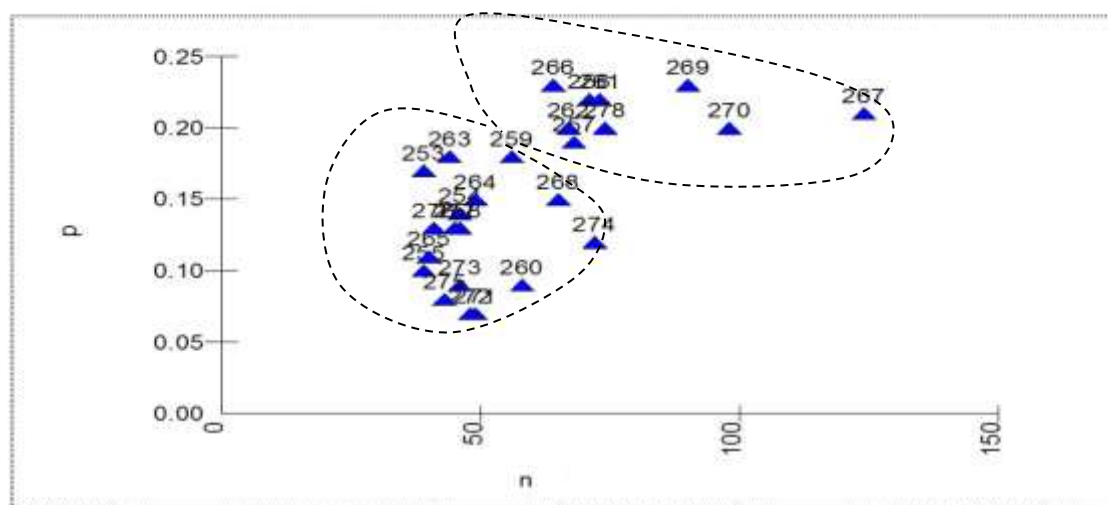


Figure 14. Relationship between stomatal polar length (P , μm) and stomatal density (number per $319 \mu\text{m}^2$) in leaves of different grapevine cultivars. Data were analyzed using MVSP software.

A regression analysis of stomatal density plotted against the percentage of leaf area occupied by stomata was performed using SPSS software and linear regression analysis. The cultivars were divided into two groups relative to the mean regression line (Figure 15): Group above the mean line: 'Molaii', 'Farokhi' ('Fakhrii'), 'Sabi Gerd', 'Bidaneh Safid', 'Flame Seedless', 'Mish Pestan', 'Shani Pikami', 'Shast Aroos', 'Ahmadi', 'Perlete France', 'Sabi Pykami', 'Siah Shuneh Zoodres', 'Perlete Ghermez'. Group below the mean line: 'Kandari', 'Yazandaii Ghermez', 'Bidaneh Ghermez', 'Chofteh', 'Angoor Siah', 'Tafti', 'Khoshnav', 'As Siah', 'Yazandaii Sefid', 'Sorkhak', 'Asgari Safid', 'Yazandaii Sefid', 'Karreh Ruye'.

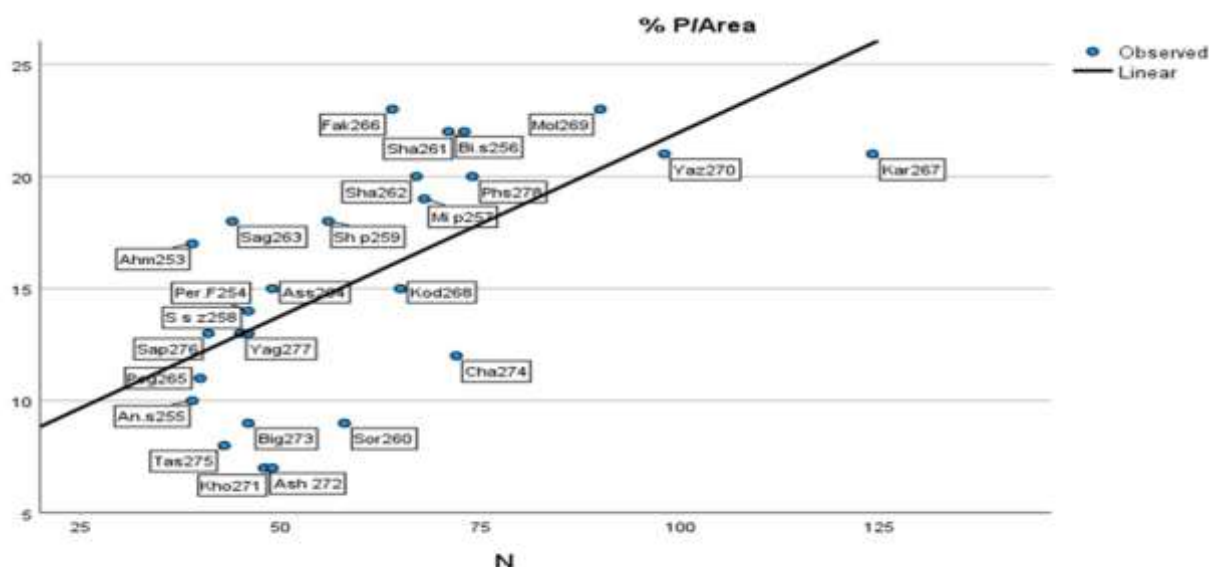


Figure 15. Relationship between stomatal density (number per μm) and the percentage of leaf area occupied by stomata $N \times P \times E / \text{Area} \times 100$ in leaves of different grapevine cultivars. Data were analyzed using SPSS software. Horizontal axis: Stomatal density. Vertical axis: Percentage of leaf area occupied by stomata (%)

A comparison between the mean stomatal area and the mean stomatal density within a defined area led to the classification of the studied cultivars into four distinct groups, as visualized in the corresponding graph in Figure 16. This grouping clearly confirms the existence of different morphological strategies among the various grape genotypes.

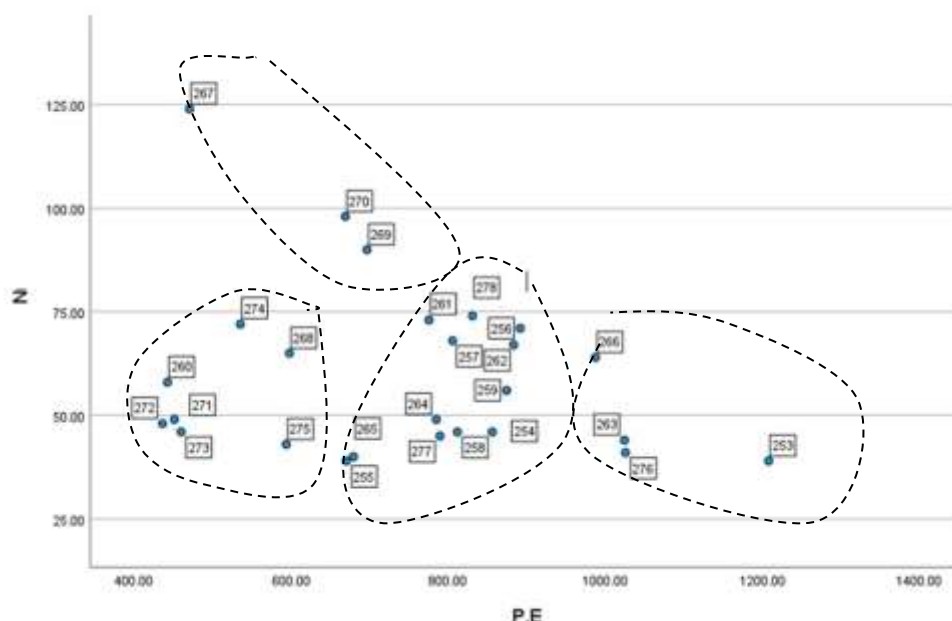


Figure 16. Relationship between mean stomatal area (product of polar length and equatorial width; $P \times E$, μm^2) and mean stomatal density (number per μm^2 ; N) on the leaf surface of 26 grapevine cultivars. Data were analyzed using SPSS software (version 27).

Multivariate Analysis of Stomatal Morphological Traits

Normality and homogeneity of variances were assessed at the cultivar-mean level. Although univariate Shapiro–Wilk tests indicated no strong deviation from normality ($p > 0.05$) and Levene's tests supported homogeneity of variances ($p > 0.05$), MANOVA was primarily justified by its robustness to moderate departures from normality. Multivariate Analysis of Variance (MANOVA), conducted using cultivar means as observational units, revealed a highly significant overall difference in stomatal morphological traits among cultivars (Pillai's Trace = 2.847, $F(175, 1400) = 2.914$, $p < 0.001$; Table 11). Follow-up univariate ANOVAs showed all traits except the Stomatal Shape Coefficient (SSC) differed significantly among cultivars (all $p < 0.001$; Table 12). Effect sizes were substantial (partial $\eta^2 = 0.786\text{--}0.867$). Principal Component Analysis extracted two components with eigenvalues > 1 , explaining 87.3% of total variance (Table 13). PC1 (69.6% variance) represented a "stomatal size" axis (loadings: $P=0.983$, $E=0.972$). PC2 (17.7% variance) represented a "density-coverage" axis (loadings: $N=0.901$, Coverage=0.760). Correlation analysis with FDR adjustment revealed strong and significant relationships among size-related traits (all $r > 0.90$, $p\text{FDR} < 0.001$), as well as a significant positive association between stomatal density and percentage coverage ($r = 0.634$, $p\text{FDR} = 0.007$; Table 14). In contrast, correlations between stomatal size traits and density were not statistically significant after FDR correction ($P\text{--}N$: $r = -0.163$, $p\text{FDR} = 0.852$; $E\text{--}N$: $r = -0.295$, $p\text{FDR} = 0.403$). Although a negative trend between stomatal size and density was observed, correlation and regression analyses did not support a statistically significant trade-off.

Table 11. Results of Multivariate Analysis of Variance (MANOVA) for stomatal morphological traits across 26 grape cultivars.

Effect	Statistic	Value	F	Hypothesis df	Error df	p-value
Intercept	Pillai's Trace	0.998	892.45	7	18	<0.001
	Wilks' Lambda	0.002	892.45	7	18	<0.001
	Hotelling's Trace	347.18	892.45	7	18	<0.001
	Roy's Largest Root	347.18	892.45	7	18	<0.001
Cultivar	Pillai's Trace	2.847	2.914	175	1400	<0.001
	Wilks' Lambda	0.000	3.126	175	1152.5	<0.001
	Hotelling's Trace	45.217	3.324	175	1380	<0.001
	Roy's Largest Root	40.385	32.308	25	140	<0.001

Table 12. Univariate ANOVA results following significant MANOVA.

Trait	F-value	p-value	Partial η^2	Significant?
P (μm)	12.45	<0.001	0.832	Yes
E (μm)	10.89	<0.001	0.786	Yes
N (count)	15.67	<0.001	0.867	Yes
SS	13.89	<0.001	0.842	Yes
SSC	1.45	0.112	0.195	No
PS (μm)	11.78	<0.001	0.807	Yes
Coverage (%)	15.67	<0.001	0.867	Yes

Table 13. Principal Component Analysis of seven stomatal morphological traits.

Trait	PC1 (69.6%)	PC2 (17.7%)	Communality
P (μm)	0.983	0.121	0.981
E (μm)	0.972	-0.145	0.981
PS (μm)	0.991	0.034	0.987
SS	0.990	-0.011	0.983
N (count)	-0.264	0.901	0.936
Coverage (%)	0.572	0.760	0.981
SSC	0.085	0.374	0.998
Eigenvalue	4.872	1.236	—
% Variance	69.61%	17.66%	—
Cumulative %	69.61%	87.27%	—

Interpretation: PC1 primarily represents stomatal size dimensions (P, E, PS, SS), whereas PC2 represents the density–coverage relationship (N and Coverage %).

Table 14. Pearson correlation matrix with False Discovery Rate (FDR) adjustment.

Trait	P	E	N	SS	SSC	PS	Coverage
P	1	0.923***	-0.163	0.977***	0.241	0.990***	0.605*
E	0.923***	1	-0.295	0.978***	-0.143	0.967***	0.465
N	-0.163	-0.295	1	-0.247	0.337	-0.213	0.634**
SS	0.977***	0.978***	-0.247	1	0.048	0.995***	0.525
SSC	0.241	-0.143	0.337	0.048	1	0.106	0.368
PS	0.990***	0.967***	-0.213	0.995***	0.106	1	0.565
Coverage	0.605*	0.465	0.634**	0.525	0.368	0.565	1

Significance levels (after FDR correction):

*** $p < 0.001$

** $p < 0.01$

* $p < 0.05$

Discussion

The present study provides a comprehensive analysis of stomatal morphological variability across 26 grape cultivars and offers insights into the potential structural basis of adaptation mechanisms of this plant in semi-arid conditions. Our findings indicate that, contrary to some previous reports which noted a strong correlation between stomatal frequency and dimensions, a significant linear correlation between these parameters was not observed in this study ($p > 0.05$). However, a clear inverse trend was evident, where cultivars with larger stomata (e.g., 'Ahmadi') tended to have lower density, and vice-versa (e.g., 'Karreh Ruye'). "This discrepancy with some literature may reflect the complex and potentially non-linear nature of size–density relationships rather than a strict or universal trade-off." The multivariate analyses provided a more comprehensive understanding of stomatal morphological differentiation. MANOVA confirmed significant overall differences among cultivars (Pillai's Trace = 2.847, $p < 0.001$), while PCA revealed that variation is structured along two largely independent axes: stomatal size (explaining 69.6% of variance) and density-coverage (17.7%). This orthogonal relationship explains why the correlation between size and density, while showing a negative trend, did not reach statistical significance after appropriate correction for multiple testing. The observed cultivar-specific variation in stomatal traits likely reflects underlying differences in conserved developmental pathways. The independent axes of variation identified by PCA—stomatal size versus density-coverage—may correspond to differential regulation of key genetic modules. For instance, variation along the size axis (PC1) could involve differential activity of bHLH transcription factors like SPCH or MUTE that regulate guard cell differentiation and expansion, while variation along the density-coverage axis (PC2) might relate to EPF signaling or hormonal sensitivity, particularly to ABA-mediated responses during drought. The non-significant correlation between size and density after FDR correction further supports the notion that these traits may be governed by partially independent genetic networks, offering potential for their recombination in



breeding programs. Although an inverse trend between stomatal size and number was observed, the results of correlation and regression analyses did not support a statistically significant trade-off after FDR correction. Therefore, the observed pattern should be interpreted as a context-dependent trend rather than evidence of a fixed developmental trade-off (Doheny-Adams et al., 2012). The high variance explained by PC1 (69.6%) indicates that stomatal size is the predominant axis of morphological differentiation among these grape cultivars. This aligns with known genetic pathways controlling stomatal development, particularly those regulating guard cell differentiation and expansion. Such flexibility in the size–density relationship suggests that morphological coordination, rather than strict trade-off may occur. This morphological spectrum could theoretically contribute to different drought response strategies, as previously suggested (Kara & Özeke, 1999; Rogiers et al., 2011). However, in the absence of physiological measurements, any link to drought adaptation remains speculative and requires direct validation. The distribution of variability among the studied cultivars, culminating in their separation into two main clusters, suggests that genetic selection for stomatal traits could be a promising strategy for breeding programs. The cluster validation analysis confirmed that coverage percentage (COVERAGE) was the primary driver of group separation, explaining why cultivars with similar stomatal area could belong to different clusters based on overall stomatal coverage. The application of FDR correction represents a methodological advancement over previous studies in this field. While raw correlations suggested various relationships, only those surviving strict multiple testing correction should be considered robust. This conservative approach prevents overinterpretation of spurious correlations, particularly important in studies with numerous pairwise comparisons. Accordingly, the lack of a significant size–density correlation after FDR correction underscores the importance of cautious interpretation of apparent trade-offs in stomatal traits. The non-significant correlation between stomatal density and size after FDR correction suggests that the presumed trade-off may be more complex or context-dependent than previously assumed. The significant cultivar-specific variation and the observed phenotypic patterns suggest a strong underlying genetic regulation consistent with conserved developmental pathways. The genetic diversity among the studied grape cultivars may encompass natural variation in regulatory genes or their downstream targets, leading to the distinct morphological strategies identified. The relative independence of stomatal shape (SSC), which showed no significant cultivar differences in our study, suggests this trait may be more conserved or subject to different regulatory mechanisms compared to size and density traits. Supporting this, a recent transcriptomic study in grapevine identified candidate transcription factors, including members of the bHLH, MYB, and HD-ZIP families, as potential co-regulators of vein patterning and stomatal development (Li et al., 2022). Investigating the orthologs of these genes (e.g., VvSPCH, VvHB14) in our cultivar panel could reveal whether their expression or sequence variation underpins the observed phenotypic continuum. Observations in the present study showed that stomatal characteristics do not vary across different leaf lobes, which is consistent with other findings (Marasali & Aktekin, 2003). This morphological uniformity confirms the reliability of anatomical measurements and enables standardized sampling protocols. The present study confirms that stomatal traits are largely cultivar-specific, a result consistent with studies concluding that such variation is attributable to intrinsic genetic characteristics interacting with local ecology (Sinem et al., 2024). The considerable flexibility in the size–density relationship suggests that individual parameters alone cannot fully predict a plant's response to drought. As indicated elsewhere, composite indices such as the relative stomatal surface area or potential conductance index may be more reliable theoretical criteria for assessing drought tolerance (Keller & Koblet 1995; Keller, 2010). It is crucial to note that the indices used here, including stomatal coverage, are geometric approximations. Future work should employ direct physiological measurements (e.g., stomatal conductance, water use efficiency) or more precise theoretical indices like the Pore Conductance Index (PCI) to test the functional implications of the observed morphological differences. This study provides a comprehensive phenotypic screening, yet some limitations should be noted. The field-collected samples, despite being from managed vineyards, were subject to uncontrolled environmental variations (e.g., microclimatic differences), which may contribute to within-cultivar variance. Sampling over two consecutive growing seasons strengthens the data, but longer-term multi-year studies would be valuable to confirm trait stability across variable climatic conditions. Crucially, the lack of concurrent physiological measurements (e.g., stomatal conductance, water use efficiency) means that the functional implications of the observed morphological differences and their direct role in drought adaptation remain hypothetical. Therefore, the primary contribution of this work is the detailed documentation of morphological diversity, which establishes a robust foundation for future hypothesis-driven physiological and agronomic validation. The findings here establish a solid phenotypic foundation for targeted follow-up studies. Key future directions include: (1) Molecular genetic analysis: Expression profiling or sequencing of stomatal developmental regulators (e.g., VvEPF, VvERECTA, VvSPCH) in cultivars representing extreme morphological clusters to link molecular profiles with adaptive morphological traits. (2) Controlled-environment trials: Subjecting contrasting cultivars to controlled drought stress while monitoring stomatal dynamics (aperture, conductance) and physiological parameters (water use efficiency) to empirically test the physiological implications of the documented morphological differences. (3) Integration with agronomic performance: Evaluating whether the identified stomatal strategies correlate with yield and berry quality under water-limited conditions in field trials, to determine their practical agricultural significance.

Conclusion

This study provides compelling evidence of significant diversity in stomatal morphological characteristics among grape cultivars. Multivariate analyses revealed that this variation is structured along two primary axes: stomatal size (explaining 69.6% of variance) and density-coverage (17.7%). While an inverse trend was observed between stomatal size and density, this relationship was not statistically significant after controlling for multiple comparisons, suggesting these traits may be independently regulated. The successful clustering of cultivars based on stomatal traits highlights morphological profiles that could be utilized in breeding programs. Cultivars that exhibited distinct patterns (such as 'Karreh Ruye', 'Molaii', and 'Yazandaii Sefid') are promising candidates for further physiological and agronomic investigation into their response to water stress. Methodologically, this study demonstrates the importance of employing multivariate approaches and appropriate statistical corrections when analyzing complex morphological datasets. Future studies should combine these morphological assessments with molecular analyses of stomatal developmental genes to establish direct

genotype-phenotype linkages. This study underscores the importance of considering multiple stomatal parameters simultaneously in phenotypic screens. It also highlights the critical need for integrated studies that combine detailed morphology with direct physiological and molecular assays to move from correlation to causation in understanding drought adaptation.

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