



Original Scientific Paper

Diversification of yellow-flowered *Sempervivum* (Crassulaceae) species from the Balkan Peninsula: evidence from the morphometric study of the epidermal structures of rosette leaves

Maja JOVANOVIĆ^{1*}, Dmtitar LAKUŠIĆ², Branislava LAKUŠIĆ³ and Bojan ZLATKOVIĆ¹

¹ University of Niš, Faculty of Sciences and Mathematics, Department of Biology and Ecology, Niš, Serbia

² University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden Jevremovac, Belgrade, Serbia

³ University of Belgrade, Faculty of Pharmacy, Department of Botany, Belgrade, Serbia

* Correspondence: maja.jovanovic1@pmf.edu.rs

ABSTRACT:

Several related yellow-flowered houseleek species which occur on the Balkan Peninsula are divided into two complexes: *Sempervivum ciliosum* (*S. ciliosum*, *S. jakucsii*, *S. klepa*, *S. octopodes*, and *S. galicicum*) and the *S. ruthenicum* complex (*S. ruthenicum*, *S. leucanthum*, *S. kindingeri*, and *S. zeleborii*). Due to strong phenotypic plasticity and a limited number of studies, it is difficult to assert at this point whether all the above species are well defined in the taxonomic sense. Detailed studies of the epidermal structures have not been conducted for any of the species in either complex. The aim of this study was to investigate the degree of variability of the epidermal structures together with their potential usefulness for the taxonomic characterization of the species studied. A total of 18 quantitative characters of the epidermal structures of the adaxial and abaxial surfaces of the rosette leaves were analysed within 16 populations. In all species, the epidermal cells are polygonal or irregularly shaped, with straight or sinuous anticlinal walls, while the rosette leaves are amphistomatic with anisocytic stomata. Simple biseriate multicellular glandular trichomes were found on the adaxial and abaxial surfaces and the margins of the rosette leaves. The results of the descriptive statistics, univariate (ANOVA) and multivariate statistical analysis (CDA, AHC) showed low to high variability in the epidermal cells, guard cells and trichomes. The multivariate analysis showed diversification among the complexes and species. The length of the marginal and apical trichomes of the rosette leaves contributed most to diversification.

Keywords:

Sempervivum ciliosum complex,
Sempervivum ruthenicum complex,
epidermal cells, stomata, trichomes

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INTRODUCTION

Sempervivum L. is a member of the family Crassulaceae J.St.-Hil., comprising between 1300 and 1500 leafy and stem succulent herbaceous plants and subshrubs (BERGER 1930; 'T HART 1997; 'T HART *et al.* 2003; THIEDE & EGGLI 2007; MORT *et al.* 2010). Most species inhabit the temperate and subtropical regions of the Northern Hemisphere and Africa (VAN HAM & 'T HART 1998). Although representatives are easily recognized morphologically,

their diversity of life form, the ability to hybridize intragenerically in natural habitats, as well as cytological and chromosomal variability (UHL 1992; MORT *et al.* 2010), is reflected in the unresolved taxonomic status of many genera and species.

The genus *Sempervivum* L. is mainly distributed in semi-arid or arid rocky habitats in the mountain and alpine belts of the mountain massifs of the Iberian Peninsula, the Apennines and the Balkan Peninsula, Asia Minor and the Caucasus (LIPPERT 1995; THIEDE & EGGLI

2007; KARAER *et al.* 2011; KLEIN & KADEREIT 2015). The species of this genus are herbaceous, perennial succulents with monocarpic rosettes which have the outstanding capacity for vegetative reproduction. From sessile, open or partially closed rosettes, a single stem bearing an inflorescence develops in the flowering stage, the scorpion-like cyme often forming panicles, with 9- to 14-petalled flowers (HAGEMANN 1986). The strong phenotypic plasticity has led to insufficient knowledge of this genus. Despite the recognizable habitus, little is known of the aspects of anatomy, morphology, taxonomy, and phylogeny. Based on the latest conspectus of this genus, the reported number of species varies between 40 and 60 (‘T HART *et al.* 2003; THIEDE & EGGLI 2007). In addition, several heterotypic infraspecies taxa and natural hybrids are described (‘T HART *et al.* 2003; KLEIN & KADEREIT 2016). Contrary to previous claims, some recent studies report the exact number of 46 *Sempervivum* species (‘T HART *et al.* 2003; KLEIN & KADEREIT 2015).

Although the Balkan Peninsula may be considered one of the centres of diversification of the genus *Sempervivum*, the species found in this region are still insufficiently studied from the morpho-anatomical point of view (‘T HART *et al.* 2003). In this study, the yellow-flowered *Sempervivum* species from the Balkans are divided into two informal groups, the *Sempervivum ciliosum* and *Sempervivum ruthenicum* complexes. The species within the *S. ciliosum* complex are characterized by comparatively small, closed rosettes, predominantly yellow or greenish petals, yellow-green or purple filaments and long, sometimes interwoven marginal trichomes of rosette leaves (HAGEMANN 1986; MICEVSKI 1998; ‘T HART 2002; JOVANOVIĆ *et al.* 2018) (Fig. 1). This complex includes *S. ciliosum* Craib, *S. jakucsii* Pénzes, *S. klepa* Micevski, *S. octopodes* Turrill and *S. galicicum* (Sm.) Micevski, all of which are endemic species for the Balkan Peninsula. The *Sempervivum ruthenicum* complex includes *S. ruthenicum* Schnittsp. & C. B. Lehm., *S. leucanthum* Pančić, *S. kindingeri* Adamović and *S. zeleborii* Schott., with some of the species distributed outside the Balkan Peninsula. They are recognizable by large, open rosettes, greenish-yellow or white petals (sometimes tinged purple at the base), purple or white filaments, and short (less than 2 mm long) marginal trichomes of rosette leaves (HAGEMANN 1986; ‘T HART 2002) (Fig. 1). Characterized by a wide range of morphological variability resulting from broad phenotypic plasticity as well as the possibility of spontaneous hybridization and discrepancies in the interpretation of the status of certain species by different authors, the intrageneric taxonomy of these groups is still confusing. Thus, apart from descriptions in regional floras and non-detailed information on their distribution (VELEV 1970; HAGEMANN 1986; MICEVSKI 1998; ‘T HART 2002) and a limited number of studies on morphological variability (JOVANOVIĆ *et al.* 2019a, b), no detailed studies on trait variability have been conducted for these species. The phenotypic plasticity has resulted in variable taxo-

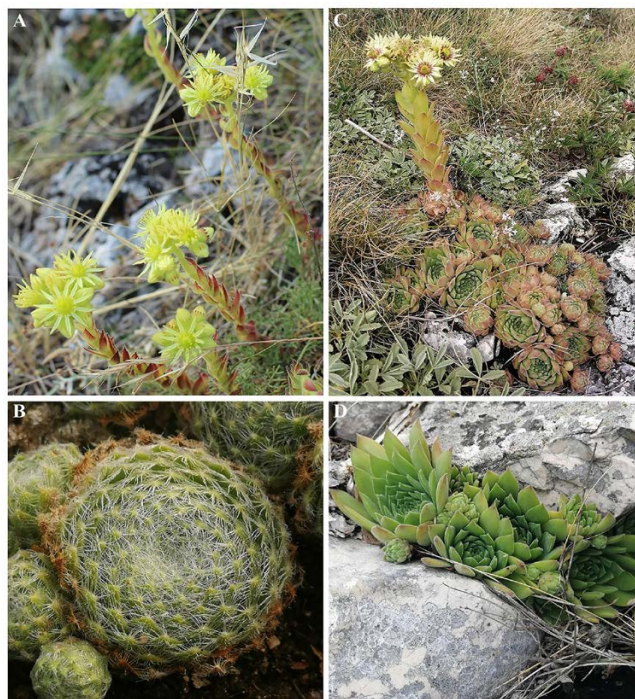


Fig. 1. Habitus and rosette of: (A-B) *S. ciliosum*- representative of the *S. ciliosum* complex and (C-D) *S. zeleborii* - representative of the *S. ruthenicum* complex.

nomic recognition in the different treatment of the genus by various authors. In the current floristic literature and relevant check-lists (FAVARGER & ZÉSIGER 1964; PARNELL & FAVARGER 1993; EURO+MED 2006-; WFO 2021), *S. ciliosum*, *S. octopodes*, *S. jakucsii*, *S. zeleborii*, *S. ruthenicum*, *S. kindingeri* and *S. leucanthum* are accepted at species rank. In contrast, the taxonomic position of *S. klepa* and *S. galicicum* remains unresolved since many authors consider them as synonyms or varieties of *S. ciliosum*. *Sempervivum galicicum* is often recognized only as an *S. ciliosum* var. *galicicum* (‘T HART *et al.* 2003), although MICEVSKI (1998) considers it as a well-separated species, distinguished from *S. ciliosum* by smaller rosettes, longer rosette leaves, a higher stem, smaller flowers and flower parts. As a well-defined species, *S. klepa* is recognized only by MICEVSKI (1998), differentiated from *S. ciliosum* and related taxa by smaller rosettes, a shorter stem, smaller flowers and flower parts. Nowadays *S. jakucsii* is considered a well-defined species (WFO 2021), but in the past was given as a synonym of *S. ciliosum* (PARNELL 1988) since, based on the original description provided by PÉNZEZ (1965), there are no major differences between these taxa. Regarding *S. octopodes*, it is still not clear whether this taxon deserves species rank or should be considered as a subspecies of *S. ciliosum* since the plants from the western part of the range are morphologically similar to *S. ciliosum* (‘T HART 2002). Thus, it is often referred to as *S. ciliosum* subsp. *octopodes* (ZONNEVELD 1999; ‘T HART *et al.* 2003), recognizable by pale yellow petals, purple or lilac at the base, and purplish filaments, features absent in *S. ciliosum*. The taxonomic position of

the species within the *S. ruthenicum* complex is less vague. For a long time, *S. ruthenicum* and *S. zeleborii* were considered synonyms, but *S. zeleborii* is distinguished from *S. ruthenicum* by the smaller size of all its parts, particularly the nectary scales (PARNELL & FAVARGER 1990). Although the taxonomy of these groups at the intrageneric level remains questionable, a phylogenetic analysis based on two nuclear markers shows that the representatives of these two complexes belong to different well-supported intrageneric clades, indicating their genetic specificity (KLEIN & KADE-REIT 2015).

The epidermal structures of the species within the genus *Sempervivum* have not been extensively studied. The available studies refer to a limited number of epidermal traits (KEAN 1927; CODIGNOLA *et al.* 1990; KIRILENKO 2018). However, studies on leaf epidermis structures have been largely conducted in other genera of Crassulaceae such as *Kalanchoe*, *Crassula*, *Rhodiola*, and *Sedum*, etc., demonstrating the taxonomic importance of these structures (WERYSZKO-CHMIELEWSKA & CHERNETSKYY 2005; LI & ZHANG 2008; ABDEL-RAOUF 2012; CHERNETSKYY 2012; ZLATKOVIĆ *et al.* 2017). In various representatives of the family Crassulaceae, the stomata are mostly superficial and anisocytic (THIEDE & EGGI 2007), while the presence and type of trichomes prove to be comparatively important for taxonomy (WERYSZKO-CHMIELEWSKA & CHERNETSKYY 2005). For many species of *Sempervivum*, the position and length of the trichomes covering the rosette leaves, stems and stolons, as well as all parts of the inflorescences, play an essential role in their identification (VELEV 1970; HAGEMANN 1986; MICEVSKI 1998; 'T HART 2002; JOVANOVIĆ *et al.* 2018).

As mentioned, the number of studies concerning the variability of the epidermal structures of the representatives of the genus *Sempervivum*, especially of the species within the *S. ciliosum* and *S. ruthenicum* complexes, are very limited and usually incomplete. Since they are morphologically similar, the aims of this study were: a) to provide insight into the qualitative and quantitative characteristics of the epidermal structures of the rosette leaves; b) to determine the level of diversification between the analysed complexes and their representatives on the basis of the epidermal structures; c) to provide information as to whether the characteristics of the epidermal structures could be used for taxonomic purposes; d) to verify whether the morphological relationships based on the characteristics of the epidermal structures coincide with the results of phylogenetic studies of the genus *Sempervivum*.

MATERIALS AND METHODS

Plant Material. For this study, 210 individuals from 14 populations of yellow-flowered *Sempervivum* species collected from the area of the Balkan Peninsula were analysed. The analysis also includes individuals of yellow-flowered *S. ruthenicum* from the Dnieper Lowland in

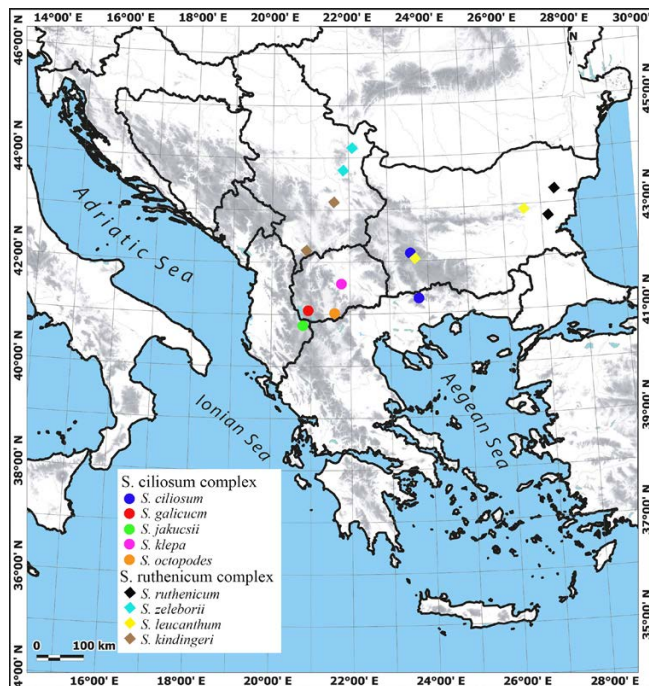
Ukraine (9 individuals) and *S. wulfenii* from the Alps (5 individuals), considered here as an outgroup. The information on the voucher specimens deposited in the herbarium of the Department of Biology and Ecology, Faculty of Natural Sciences and Mathematics, University of Niš (HMN), localities and habitats are given in Table 1 and Fig. 2. Prior to the analysis, the individuals were subjected to an acclimation period in the greenhouse of at least one year. To ensure uniform conditions during the cultivation period, all of the collected individuals were planted on Floradur® substrate (FloraGard, Vertriebs GmbH fuer Gartenbau, Germany) mixed with sand (ratio 1:4) in containers of the same diameter.

Epidermal structures. A total of 18 quantitative characteristics of the epidermal structures of the adaxial and abaxial surfaces of the rosette leaves of 224 individuals (including outgroup populations) were analysed. To clarify the analysis process of the epidermal structures, the analysed characteristics were divided into two groups: a) characteristics of the epidermal cells and stomata, and b) the characteristics of the trichomes. For the analysis of the features of the epidermal cells and stomata, one leaf was taken from the middle part of the rosette of each individual. Without prior treatment, the epidermis from the adaxial and abaxial surfaces from the upper third of the leaves, manually separated from the parenchyma, was used for the preparation of temporary slides (RUZIN 1999). The number of stomata and epidermal cells was determined as the average of three randomly selected areas (1 mm²) on each epidermal peel. The prepared epidermal peels were photographed with a Leica DM 1000 light microscope (Leica Microsystems ©, Wetzlar, Germany) at 100× magnification. To measure the length of the trichomes on the apical and marginal leaf parts, whole leaves, without separation of the epidermis, were photographed with a Leica MZ-16A stereomicroscope (Leica Microsystems©, Wetzlar, Germany). The measurements of all the studied epidermis structures (Tables 2 and 3) were taken using Digimizer Image Analysis Software (MedCalc Software ©, Belgium).

Statistical Analysis. The statistical processing of the obtained data was done using STATISTICA 8.0 software (StatSoft, Inc., Tulsa, USA). The degree of variability of the analysed traits (at the level of each species) was analysed by parameters of descriptive statistics. Multivariate statistical analysis was performed with transformed data (log) and included canonical discriminant analysis (CDA) and agglomerative hierarchical clustering based on the Mahalanobis distance (AHC). These analyses were conducted to test whether the analysed species differed from each other based on the selected traits. The significance of the differences among the analysed traits was determined by the univariate analysis of variance (ANOVA) with the addition of Tukey's HSD *post hoc* test and t-test.

Table 1. Collection data, the habitat characteristics and voucher numbers of the examined taxa.

| | Taxon | No. Ind. | Locality | Substratum | Altitude (m) | Legator | Voucher |
|------------------------------|--|----------|---|------------|--------------|---|-----------|
| <i>S. ciliosum</i> complex | <i>S. ciliosum</i> | 15 | Bulgaria: Mt. Rila, Rila | silicate | 570 | Zlatković, B., Gussev, Ch. | HMN 13989 |
| | <i>S. ciliosum</i> | 15 | Greece: Mt. Orvilos | limestone | 2170 | Lakušić, D. | HMN 13990 |
| | <i>S. galicicum</i> | 15 | North Macedonia: Mt. Galičica, Crven kamen | limestone | 1770 | Zlatković, B. | HMN 13991 |
| | <i>S. jakucsii</i> | 15 | Albania: Mt. Mal Ivan, Burimas | limestone | 1380 | Zlatković, B., Lazarević, P. | HMN 14366 |
| | <i>S. klepa</i> | 15 | North Macedonia: Mt. Klepa, Podles | silicate | 1080 | Jovanović, M., Zlatković, B. | HMN 14412 |
| <i>S. ruthenicum</i> complex | <i>S. octopodes</i> | 15 | North Macedonia: Mt. Kajmakčalan | silicate | 2450 | Zlatković, B., Tomović, G., Niketić, M. | HMN 14361 |
| | <i>S. ruthenicum</i> | 15 | Bulgaria: Aytos | silicate | 307 | Zlatković, B., Gussev, Ch. | HMN 14363 |
| | <i>S. ruthenicum</i> | 15 | Bulgaria: Varna, Slanchevo | sands | 105 | Zlatković, B., Gussev, Ch. | HMN 14413 |
| | <i>S. ruthenicum</i> | 9 | Ukraine: Kyiv, Khodosivka | sands | 112 | Iakushenko, D. | HMN 14414 |
| | <i>S. zeleborii</i> | 15 | Serbia: Mt. Beljanica | limestone | 1320 | Zlatković, B., Anačkov, G. | HMN 14362 |
| | <i>S. zeleborii</i> | 15 | Serbia: Mt. Veliki krš | limestone | 1110 | Zlatković, B., Popović, M. | HMN 14415 |
| | <i>S. leucanthum</i> | 15 | Bulgaria: Mt. Rila, Yakoruda | silicate | 1890 | Zlatković, B., Gussev, Ch. | HMN 14364 |
| | <i>S. leucanthum</i> | 15 | Bulgaria: Mt. East Stara planina, Krumova vana | limestone | 300 | Zlatković, B., Gussev, Ch. | HMN 14416 |
| | <i>S. kindingeri</i> | 15 | Serbia: Mt. Sokolovica, Prolom Banja | silicate | 750 | Zlatković, B., Bogosavljević, S. | HMN 14365 |
| | <i>S. kindingeri</i> | 15 | Serbia: Mt. Ošljak, Popovo Prase | limestone | 1908 | Zlatković, B. | HMN 14417 |
| | <i>S. wulfenii</i> subsp. <i>juvanii</i> | 5 | Slovenia: Ljubljana Botanic Garden (Donačka gora) | - | - | Jogan, N., Bavcon, J. | HMN 14418 |

**Fig. 2.** Location of analysed populations from the Balkan Peninsula. For the description of the location and habitats of the species cf. Table 1.

RESULTS

With regard to the general appearance of the epidermal structures, especially the stomata and trichomes, no significant differences were found between the adaxial and abaxial surfaces of the rosette leaves, nor between the species. The difference was found in the epidermal cells, with respect to their anticlinal walls.

Epidermal cells. The epidermal cells were polygonal or irregularly shaped, with straight, slightly curved or sinuous (with U-shaped undulations) anticlinal walls. The type of anticlinal walls varied between the species of the *S. ciliosum* and *S. ruthenicum* complexes (Fig. 3, Supplementary Fig. 1). The epidermal cells of the leaves of the species within the *S. ciliosum* complex have mostly sinuous anticlinal walls or, less commonly, curved walls, especially on the abaxial surface. To a lesser extent, and specifically in certain individuals of *S. ciliosum* and *S. galicicum*, the epidermal cells of the leaves have almost straight walls. In contrast, the species of the *S. ruthenicum* complex contain epidermal cells with slightly curved or straight anticlinal walls, with no differences in the appearance of the adaxial and abaxial leaf surfaces. Based on the descriptive statistics, the quantitative characteristics of the epidermal cells are high to moderately variable in all the studied species (Tables 2 and 3). Characteristics such as the number of

Table 2. Descriptive statistics for the epidermal structures of the rosette leaves of the species within the *S. ciliosum* complex.

| Acr. | Trait | <i>S. ciliosum</i> | | <i>S. galiticum</i> | | <i>S. jakucsii</i> | | <i>S. klepa</i> | | <i>S. octopodes</i> | |
|------|---|--------------------|-------|---------------------|-------|--------------------|-------|-----------------|-------|---------------------|-------|
| | | \bar{x} | SD | \bar{x} | SD | \bar{x} | SD | \bar{x} | SD | \bar{x} | SD |
| Elad | Epidermal cell length (ad.)* | 164.05 | 25.62 | 178.97 | 22.45 | 136.54 | 18.39 | 131.01 | 15.42 | 141.92 | 16.99 |
| ELab | Epidermal cell length (ab.)* | 174.77 | 24.36 | 185.78 | 25.90 | 146.95 | 14.51 | 129.15 | 17.79 | 137.16 | 14.17 |
| EWad | Epidermal cell width (ad.)* | 73.57 | 11.89 | 82.30 | 14.82 | 67.81 | 6.15 | 72.53 | 7.82 | 66.71 | 6.16 |
| EWab | Epidermal cell width (ab.)* | 71.41 | 11.04 | 78.62 | 10.14 | 60.24 | 9.38 | 58.02 | 8.27 | 66.94 | 5.29 |
| GLad | Guard cell length (ad.)* | 28.38 | 3.06 | 33.61 | 3.57 | 28.39 | 2.49 | 35.41 | 2.64 | 27.87 | 1.64 |
| GLab | Guard cell length (ab.)* | 28.71 | 3.18 | 32.12 | 2.49 | 29.18 | 3.10 | 34.71 | 2.85 | 27.68 | 1.68 |
| GWad | Guard cell width (ad.)* | 8.14 | 1.41 | 10.39 | 1.30 | 7.58 | 1.22 | 10.32 | 1.94 | 8.06 | 1.47 |
| GWab | Guard cell width (ab.)* | 7.92 | 1.50 | 9.28 | 1.33 | 8.04 | 1.19 | 8.93 | 1.17 | 7.75 | 1.17 |
| NEad | No. epidermal cells/mm ² (ad.) | 166.51 | 44.95 | 131.96 | 29.48 | 175.49 | 44.24 | 154.18 | 26.88 | 178.54 | 38.46 |
| NEab | No. epidermal cells/mm ² (ab.) | 155.92 | 37.85 | 126.23 | 21.92 | 184.02 | 33.21 | 155.46 | 21.29 | 192.13 | 40.65 |
| NSad | No. stomata/mm ² (ad.) | 29.02 | 9.26 | 34.07 | 15.24 | 31.51 | 12.94 | 24.87 | 9.21 | 37.68 | 11.66 |
| NSab | No. stomata/mm ² (ab.) | 29.47 | 10.40 | 34.26 | 9.66 | 32.84 | 10.91 | 26.75 | 7.47 | 35.88 | 9.98 |
| TLad | Trichome length (ad.)* | 146.81 | 28.88 | 181.34 | 33.77 | 192.75 | 15.56 | 158.52 | 24.63 | 193.50 | 19.22 |
| TLab | Trichomes length (ab.)* | 141.60 | 27.80 | 158.34 | 24.56 | 210.78 | 22.37 | 198.32 | 21.74 | 202.91 | 21.36 |
| TLma | Trichomes length (marginal)** | 1.82 | 0.43 | 1.17 | 0.23 | 2.03 | 0.33 | 3.10 | 0.47 | 0.98 | 0.16 |
| TLap | Trichome length (apical)** | 0.71 | 0.18 | 0.70 | 0.15 | 1.69 | 0.33 | 2.08 | 0.49 | 0.56 | 0.12 |
| NTad | No. trichomes/mm ² (ad.) | 29.22 | 8.14 | 21.93 | 5.30 | 16.92 | 3.17 | 34.43 | 7.78 | 57.17 | 15.46 |
| NTab | No. trichomes/mm ² (ab.) | 30.24 | 7.35 | 23.18 | 3.15 | 22.57 | 8.26 | 44.22 | 8.00 | 49.33 | 7.23 |

Note: \bar{x} - mean; SD- standard deviation; *- expressed in μm , ** - expressed in mm. The trichome traits refer only to glandular multicellular biseriolate trichomes

Table 3. Descriptive statistics for the epidermal structures of the rosette leaves of the species within the *S. ruthenicum* complex from the Balkan Peninsula.

| Acr. | Trait | <i>S. ruthenicum</i> | | <i>S. zeleborii</i> | | <i>S. leucanthum</i> | | <i>S. kindingeri</i> | |
|------|---|----------------------|-------|---------------------|-------|----------------------|-------|----------------------|-------|
| | | \bar{x} | SD | \bar{x} | SD | \bar{x} | SD | \bar{x} | SD |
| Elad | Epidermal cell length (ad.)* | 121.44 | 18.54 | 120.75 | 18.41 | 138.72 | 34.95 | 124.76 | 15.68 |
| ELab | Epidermal cell length (ab.)* | 120.99 | 15.54 | 120.39 | 21.45 | 147.51 | 36.25 | 137.11 | 13.77 |
| EWad | Epidermal cell width (ad.)* | 57.93 | 6.00 | 56.96 | 10.89 | 64.09 | 17.45 | 59.46 | 6.74 |
| EWab | Epidermal cell width (ab.)* | 55.58 | 8.25 | 55.49 | 10.86 | 65.58 | 14.32 | 63.67 | 6.20 |
| GLad | Guard cell length (ad.)* | 31.12 | 2.76 | 31.24 | 3.34 | 32.00 | 2.83 | 31.55 | 3.17 |
| GLab | Guard cell length (ab.)* | 31.20 | 2.50 | 30.32 | 3.82 | 32.39 | 3.51 | 32.75 | 4.16 |
| GWad | Guard cell width (ad.)* | 7.30 | 0.94 | 8.54 | 2.03 | 8.89 | 1.82 | 8.00 | 1.00 |
| GWab | Guard cell width (ab.)* | 7.63 | 1.22 | 7.90 | 1.67 | 8.52 | 1.56 | 7.98 | 1.19 |
| NEad | No. epidermal cells/mm ² (ad.) | 212.00 | 32.11 | 179.67 | 30.27 | 199.25 | 60.93 | 158.60 | 24.05 |
| NEab | No. epidermal cells/mm ² (ab.) | 217.07 | 39.02 | 164.78 | 36.25 | 185.39 | 58.98 | 148.90 | 21.34 |
| NSad | No. stomata/mm ² (ad.) | 44.19 | 16.34 | 41.07 | 8.79 | 40.12 | 16.19 | 29.20 | 8.26 |
| NSab | No. stomata/mm ² (ab.) | 39.34 | 12.70 | 37.85 | 9.07 | 37.51 | 12.47 | 28.23 | 7.05 |
| TLad | Trichome length (ad.)* | 183.57 | 28.38 | 198.44 | 41.73 | 175.18 | 27.80 | 174.87 | 23.03 |
| TLab | Trichomes length (ab.)* | 187.40 | 26.40 | 186.50 | 38.82 | 181.86 | 29.79 | 184.80 | 30.16 |
| TLma | Trichomes length (marginal)** | 0.46 | 0.12 | 0.40 | 0.16 | 0.48 | 0.11 | 0.37 | 0.07 |
| TLap | Trichome length (apical)** | 0.36 | 0.12 | 0.35 | 0.12 | 0.39 | 0.08 | 0.34 | 0.08 |
| NTad | No. trichomes/mm ² (ad.)* | 26.90 | 7.84 | 33.58 | 12.26 | 24.63 | 34.95 | 35.28 | 15.14 |
| NTab | No. trichomes/mm ² (ab.)* | 27.68 | 7.84 | 37.62 | 13.21 | 23.00 | 36.25 | 34.67 | 15.80 |

Note: \bar{x} - mean; SD- standard deviation; *- expressed in μm , ** - expressed in mm. The trichome traits refer only to glandular multicellular biseriolate trichomes.

epidermal cells per mm² on the adaxial/abaxial leaf surfaces (NEad, NEab) and the length of the epidermal cells on the adaxial/abaxial leaf surfaces (ELad, ELab) show the highest degree of variability. The species of the *S. ciliosum*

complex generally have longer and wider epidermal cells both on the adaxial and abaxial surfaces when compared to the species of the *S. ruthenicum* complex, based on the differences in the mean values of the analysed characteris-

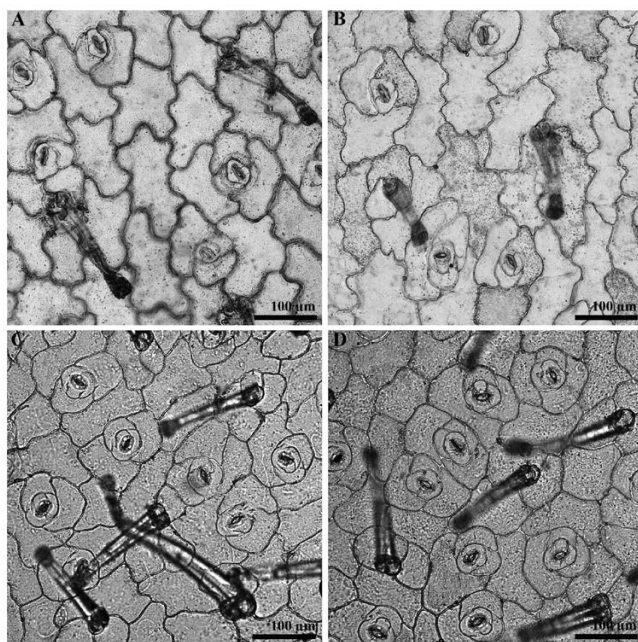


Fig. 3. Epidermal structures of the rosette leaf in: (A, B) *S. ciliosum* - representative of the *S. ciliosum* complex and (C, D) *S. leucanthum* - representative of the *S. ruthenicum* complex. (A, C) - adaxial surface; (B, D) abaxial surface.

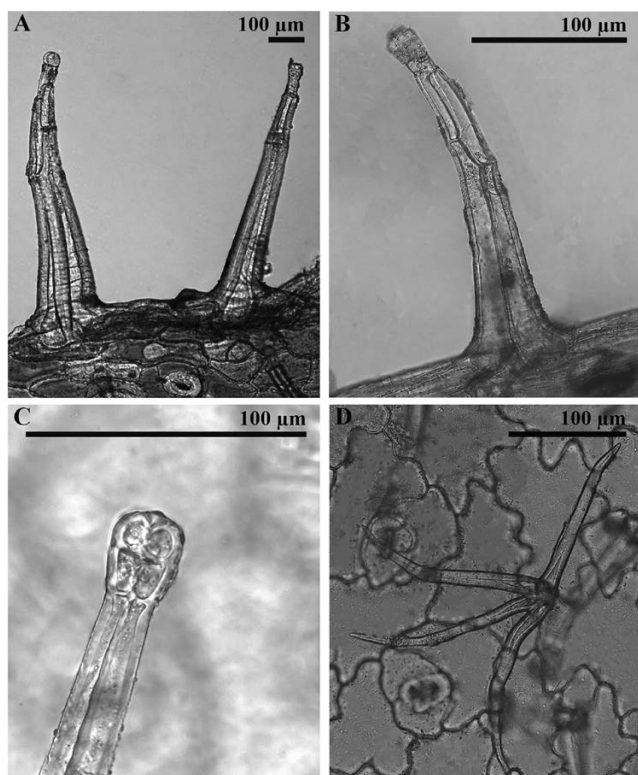


Fig. 4. Glandular multicellular biseriate trichomes in: (A) *S. kindingeri*, and (B) *S. leucanthum*; (C) head of glandular trichomes in *S. klepa*, and (D) stellate non-glandular trichome in *S. ciliosum*.

tics at the complex level, as determined by the t-test (Table 4). From all the species studied, *S. galicicum* has the largest epidermal cells with a length of 178.97 and 185.78 μm and a width of 82.30 and 78.62 μm , respectively, while *S. zeleborii* has the smallest cells with a length of 120.75 and 120.39 μm and a width of 56.96 and 55.49 μm (adaxial and abaxial, respectively). Regarding the density ratio of each analysed feature between the adaxial and abaxial surfaces of the rosette leaves, no clear pattern can be observed. In the species of the *S. ciliosum* complex, the number of epidermal cells per mm^2 is generally higher on the abaxial surface, whereas in the leaves of *S. ciliosum* and *S. galicicum* the situation is reversed. In contrast, in the species of the *S. ruthenicum* group from the Balkans, the epidermal cells are more numerous on the adaxial surface, with the exception of the individuals of *S. ruthenicum*. *Sempervivum ruthenicum* is simultaneously characterized by the highest number of epidermal cells (NEad, 212.00 ± 32.11 ; NEab, 217.07 ± 39.02). Conversely, *S. galicicum* has the lowest number of epidermal cells (NEad, 131.96 ± 29.48 ; NEab, 126.23 ± 21.29) per unit area.

Stomata. The leaves of all the species studied are shown to be amphistomatic, with anisocytic, scattered stomata, without any clear distribution pattern (Fig. 3). As for the variability of the stomatal quantitative characteristics, the standard deviation showed a moderate degree of variability in the length (GLad, GLab) and width (GWad, GWab) of the guard cells, while the traits pertaining to the number of stomata (NSad, NSab) were more variable (Tables 2 and 3). In terms of the length and width of the guard cells, it is noticeable that there are no significant differences in their length on the adaxial and abaxial surfaces between all the species in this study. Conversely, the width of the guard cells on the adaxial surface is generally greater than that on the abaxial surface. The leaves from the species within the *S. ruthenicum* complex contain longer guard cells than in the *S. ciliosum* complex (Table 4), with the exception of *S. klepa* from the *S. ciliosum* complex, whose largest guard cells have a length of 35.41 μm on the adaxial surface and 34.71 μm on the abaxial surface. In contrast, the guard cells of *S. octopodes* are the shortest with a length of 27.87 μm adaxially and 27.68 μm abaxially. Similarly, *S. galicicum* characteristically has adaxial guard cells of 10.39 μm and abaxial ones of 9.28 μm in width, while *S. ruthenicum* has the narrowest guard cells on both the adaxial (7.30 μm) and abaxial (7.63 μm) surfaces. The stomata are more numerous in the species of the *S. ruthenicum* complex, with more stomata on the adaxial surface. An opposite tendency is observed in the species of the *S. ciliosum* complex, with the abaxial surface being richer in stomata (Table 4). *Sempervivum ruthenicum* has the highest number of stomata per mm^2 among all the species (NSad, 44.19 ± 16.34 ; NSab, 39.34 ± 12.70), while *S. klepa* has the lowest (NSad, 24.87 ± 9.21 ; NSab, 26.75 ± 7.47).

Table 4. Mean values of the analysed epidermal structures of the rosette leaves at the complexes level with t-test results ($p \leq 0.05$).

| Acr. | Trait | S. ciliosum complex | | S. ruthenicum complex | | t-test | |
|------|---|---------------------|-----------|-----------------------|------------------------|--------|--|
| | | \bar{x} | \bar{x} | t-value | p | | |
| ELad | Epidermal cell length (ad.) [*] | 153.76 | 126.42 | 7.83 | 0.0000 ^{***} | | |
| ELab | Epidermal cell length (ab.) [*] | 158.10 | 131.50 | 6.94 | 0.0000 ^{***} | | |
| EWad | Epidermal cell width (ad.) [*] | 72.75 | 59.61 | 8.28 | 0.0000 ^{***} | | |
| EWab | Epidermal cell width (ab.) [*] | 67.77 | 60.08 | 4.84 | 0.0000 ^{***} | | |
| GLad | Guard cell length (ad.) [*] | 30.34 | 31.48 | -2.32 | 0.0213 ^{***} | | |
| GLab | Guard cell length (ab.) [*] | 30.18 | 31.67 | -2.91 | 0.0040 ^{***} | | |
| GWad | Guard cell width (ad.) [*] | 8.77 | 8.18 | 2.46 | 0.0148 ^{***} | | |
| GWab | Guard cell width (ab.) [*] | 8.31 | 8.01 | 1.50 | 0.1351 ^{n.s.} | | |
| NEad | No. epidermal cells/mm ² (ad.) | 162.20 | 187.38 | -4.22 | 0.0000 ^{***} | | |
| NEab | No. epidermal cells/mm ² (ab.) | 161.61 | 179.04 | -2.82 | 0.0053 ^{***} | | |
| NSad | No. stomata/mm ² (ad.) | 31.03 | 38.65 | -4.15 | 0.0000 ^{***} | | |
| NSab | No. stomata/mm ² (ab.) | 31.44 | 35.73 | -2.83 | 0.0051 ^{***} | | |
| TLad | Trichome length (ad.) [*] | 169.95 | 183.01 | -2.91 | 0.0040 ^{***} | | |
| TLab | Trichomes length (ab.) [*] | 175.59 | 185.14 | -2.00 | 0.0469 ^{***} | | |
| TLma | Trichomes length (marginal) ^{**} | 1.82 | 0.43 | 19.40 | 0.0000 ^{***} | | |
| TLap | Trichome length (apical) ^{**} | 1.08 | 0.36 | 11.99 | 0.0000 ^{***} | | |
| NTad | No. trichomes/mm ² (ad.) | 31.48 | 30.10 | 0.74 | 0.4618 ^{n.s.} | | |
| NTab | No. trichomes/mm ² (ab.) | 33.30 | 30.74 | 1.47 | 0.1439 ^{n.s.} | | |

Note: \bar{x} - mean; * - expressed in μm , ** - expressed in mm, *** - the differences between the complexes are significant, n.s. - non-significant differences

Table 5. ANOVA (F - Fisher's coefficient, p - the level of significance) and the values of the standardized coefficients for the first three canonical axes (CA) of variation in the epidermal structures.

| Acr. | Trait | ANOVA | | CDA | | |
|-----------------------|---|---------------|----------|---------------|---------------|---------------|
| | | F | p | CA 1 | CA 2 | CA 3 |
| ELad | Epidermal cell length (ad.) | 17.05 | 0.0000* | -0.125 | -0.366 | -0.067 |
| ELab | Epidermal cell length (ab.) | 23.16 | 0.0000* | -0.132 | -0.633 | -0.189 |
| EWad | Epidermal cell width (ad.) | 12.80 | 0.0000* | 0.243 | 0.252 | 0.265 |
| EWab | Epidermal cell width (ab.) | 12.59 | 0.0000* | 0.154 | -0.067 | -0.345 |
| GLad | Guard cell length (ad.) | 12.85 | 0.0000* | -0.002 | 0.296 | 0.059 |
| GLab | Guard cell length (ab.) | 8.99 | 0.0000* | 0.182 | 0.262 | 0.062 |
| GWad | Guard cell width (ad.) | 9.82 | 0.0000* | 0.000 | -0.083 | 0.012 |
| GWab | Guard cell width (ab.) | 3.10 | 0.0026** | -0.119 | -0.027 | 0.125 |
| NEad | No. epidermal cells/mm ² (ad.) | 8.40 | 0.0000* | -0.097 | -0.230 | 0.354 |
| NEab | No. epidermal cells/mm ² (ab.) | 11.75 | 0.0000* | -0.214 | -0.605 | 0.231 |
| NSad | No. stomata/mm ² (ad.) | 6.80 | 0.0000* | 0.235 | 0.021 | -0.170 |
| NSab | No. stomata/mm ² (ab.) | 4.89 | 0.0000* | 0.044 | 0.044 | -0.004 |
| TLad | Trichome length (ad.) | 8.31 | 0.0000* | 0.240 | 0.069 | -0.015 |
| TLab | Trichomes length (ab.) | 12.33 | 0.0000* | 0.135 | 0.346 | 0.253 |
| TLma | Trichomes length (marginal) | 282.04 | 0.0000* | -0.908 | 0.028 | -0.292 |
| TLap | Trichome length (apical) | 190.25 | 0.0000* | -0.471 | 0.134 | 0.465 |
| NTad | No. trichomes/mm ² (ad.) | 20.85 | 0.0000* | -0.110 | 0.433 | -0.614 |
| NTab | No. trichomes/mm ² (ab.) | 17.59 | 0.0000* | 0.012 | 0.608 | -0.191 |
| Eigenvalue | | | | 15.552 | 2.208 | 1.390 |
| % explained variation | | | | 0.728 | 0.831 | 0.896 |

Note: Significant values of coefficients are bolded; * - extremely high statistical significance; ** - high statistical significance.

Trichomes. Trichomes are present on the leaf surfaces of both the examined complexes (Fig. 4). In the species from the *S. ruthenicum* complex, the indumentum is tomentose to velvety, greyish or without specific colouration. It consists of short hairs of uniform length which are some-

what more prominent abaxially, as well as in the upper parts on both sides of the rosette leaves. In the representatives from the *S. ciliosum* complex, the indumentum of the leaves is more villous, with a layer of short hairs of uniform length, intermixed with less dense, long hairs

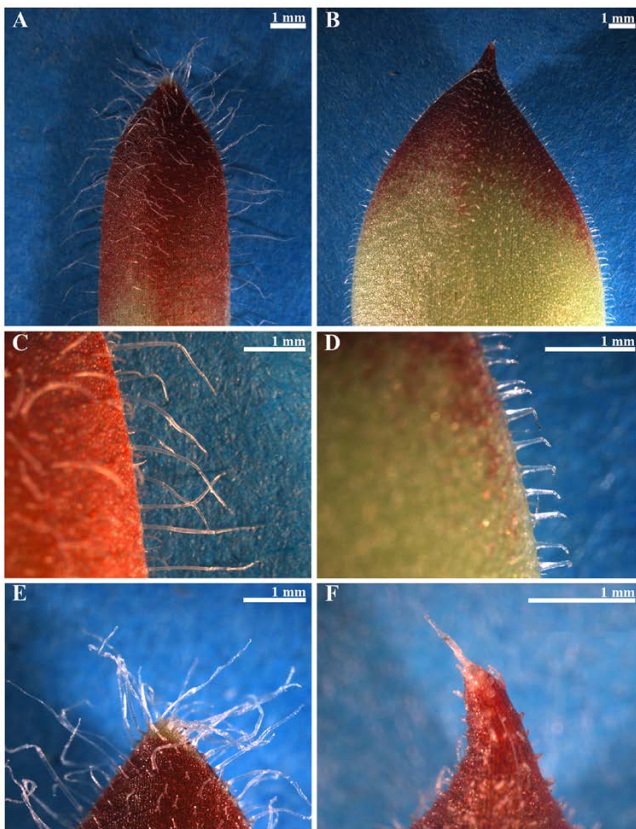


Fig. 5. Different types of indumentum on the rosette leaves in: (A, C, E) *S. galicicum* - representative of the *S. ciliosum* complex and (B, D, F) *S. kindingeri*- representative of the *S. ruthenicum* complex; (A, B) Characteristic of indumentum type; (C, D) glandular multicellular biseriata marginal trichomes and (E, F) apical trichomes.

(Fig. 5). The long hairs are unequal and gradually become longer towards the apex. They are mainly present on the outer and upper parts, but are usually absent from the lowest third of the leaf. The upper surfaces of the rosette leaves are greyish to almost white in colour, with long, soft hairs, some of which project beyond neighboring leaves or are sometimes partially intertwined. Glandular, multicellular, biseriata trichomes were found on the rosette leaves of all the examined species. The stalks of the trichomes usually consist of two to three pairs of long cells, while the heads are spherical or cuneiform in shape, composed of several, at least four secretory cells. In the individuals of *S. ciliosum*, *S. octopodes*, *S. zeleborii*, and *S. kindingeri*, the presence of non-glandular, multicellular, uniseriate stellate trichomes are sporadically noted (a total of 1-2 trichomes in all individuals analysed per species), in the upper part of the rosette leaves (Fig. 4). The trichomes on the marginal leaf part are structurally indistinguishable from those on the adaxial and abaxial surfaces. The rosette leaves are characterised by a gradually to abruptly narrowing apex and the presence of several to only one

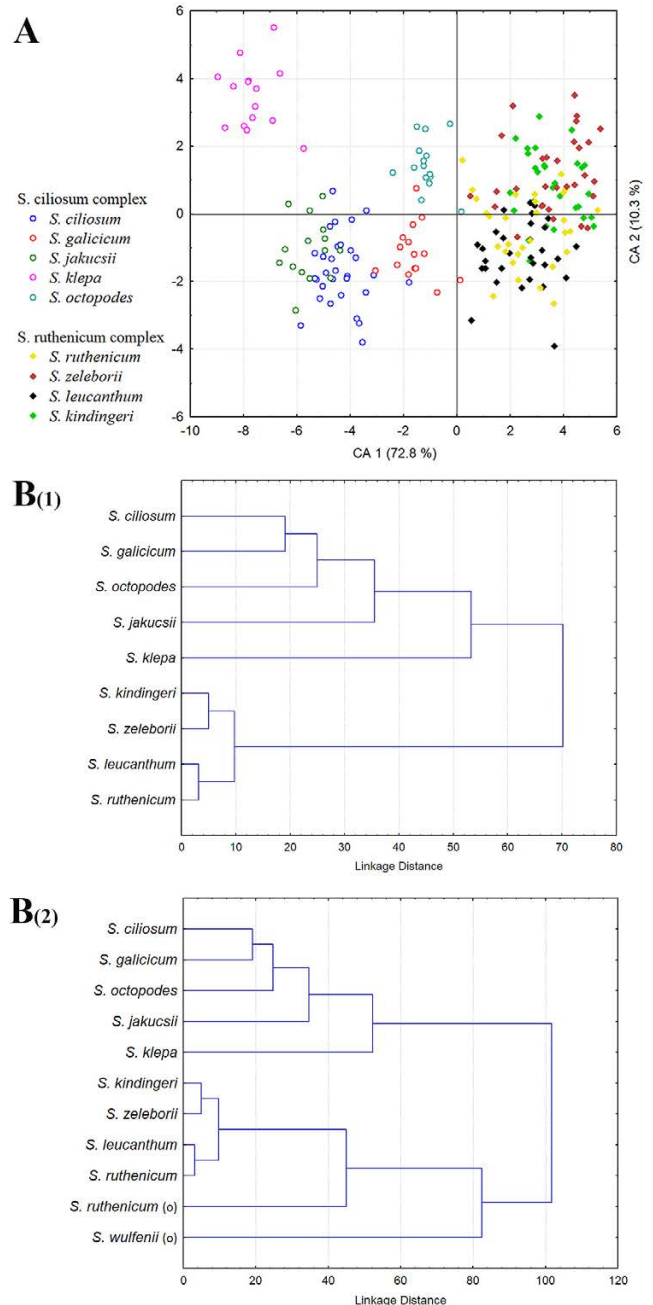


Fig. 6. Multivariate statistical analysis based on rosette leaf epidermal structures: (A) canonical discriminant analysis (CDA); (B) agglomerative hierarchical clustering (AHC) based on the Mahalanobis distance. **B**₍₁₎ – a dataset with ingroup taxa only; **B**₍₂₎ – a dataset with ingroup and outgroup (o) taxa.

trichome in the apical part (Fig. 5). The trichomes on the marginal and apical leaf parts are of the same type, but for easier interpretation of the results, in the further text they will be referred to as marginal and apical trichomes. Although the trichomes differ markedly in overall surface appearance, the same type of trichome is present in all the analysed species. In addition to the specific features of the

indumentum, significant differences were also observed, especially between the two complexes studied, in terms of their quantitative characteristics. The quantitative characteristics of all the measured trichomes show high to moderate or low variability (Tables 2 and 3). The high degree of variability is characteristic of the trichome length (adaxial/abaxial). The trichomes are longer on the adaxial leaf surface, but this is not necessarily the general rule. The deviation from this rule is best seen in the following comparison: *S. octopodes* has the longest trichomes adaxially (TLad, 193.50 ± 19.22), while *S. jakucsii* has the longest trichomes on the abaxial surface (TLab, 210.78 ± 22.97). The lowest values of trichome length are found in *S. ciliosum* (TLad, 146.81 ± 28.88 ; TLab, 141.60 ± 27.80). The number of trichomes per mm^2 on the adaxial/abaxial surface shows a moderate degree of variability, with a higher number of trichomes on the adaxial surface. Trichomes are most numerous in *S. octopodes*, ranging from 51.17 trichomes per mm^2 on the adaxial surface to 49.33 on the abaxial surface. *Sempervivum jakucsii* has the lowest number of adaxial 16.92 and abaxial 22.57 trichomes per mm^2 . Within all the analysed traits, the lengths of the marginal and apical trichomes (TLma and TLap) have the lowest standard deviation values, and can thus be considered as the least variable traits. Moreover, there are significant differences in these traits among the complexes, i.e. the species of the *S. ciliosum* complex have significantly longer apical trichomes, and even longer marginal trichomes than the species of the *S. ruthenicum* complex (Table 4). *Sempervivum klepa* is characterised by the largest marginal (TLma, 3.10 ± 0.47) and apical trichomes (TLap, 2.08 ± 0.49). Conversely, *S. kindingeri* can be highlighted as the species with the shortest marginal and apical trichomes (TLma, 0.37 ± 0.07 ; TLap, 0.34 ± 0.08).

Univariate analysis of variance (ANOVA). According to the ANOVA results (Table 5), the analysed species differ significantly from each other, based on the differences in the mean values of all of the analysed characteristics. The analysed traits of the epidermal cells, stomata and trichomes are extremely statistically significant ($p < 0.001$), except for the width of the abaxial guard cells (GWab), which is considered highly statistically significant ($p < 0.01$) (Table 5). According to the value of Fisher's coefficient (F), the length of the marginal trichomes (TLma, $F_{8,209} = 282.04$) and that of the apical trichomes (TLap, $F_{8,209} = 190.25$) stand out, indicating their considerable importance in distinguishing between the analysed species. In contrast, the guard cell dimension and the number of stomata per mm^2 are characterized by the lowest values of Fisher's coefficient (F) and are therefore less suitable for explaining the differences in the epidermal structures between the analysed species.

Canonical discriminant analysis. According to the canonical discriminant analysis (Table 5), the first axis

(CA1) was involved in 72.8% of the differentiation and was mainly determined by the length of the marginal trichomes (TLma) and the length of the apical trichomes (TLap). The second axis (CA2), which was mainly determined by the length of the abaxial epidermal cells (ELab) and the number of trichomes per mm^2 abaxially (NTab), contributed 10.3% of the total variability. CDA showed an almost complete and clear differentiation between the complexes along the first axis. In this sense, the species within the *S. ciliosum* complex are positioned on the negative side, while those within the *S. ruthenicum* complex are on the positive side of the same axis (Fig. 6A). *Sempervivum klepa* shows the most significant degree of differentiation, as the species are completely separated along the first and second axes. *Sempervivum octopodes* is separated from related species along the second axis. *Sempervivum ciliosum*, *S. jakucsii* and *S. galicum* were concentrated to a greater extent in the negative part of both axes, with individuals of *S. ciliosum* and *S. jakucsii* showing a general similarity, while *S. galicum* showed a greater tendency to separate from the previous species. As for the species within the *S. ruthenicum* complex, all the analysed individuals are positioned in the positive part of the first axis. Within this complex, the differentiation between the species is not as pronounced as in the previous complex. *Sempervivum ruthenicum* and *S. leucanthum* form a more or less homogeneous group with a slight tendency to differentiate along the second axis with respect to the group composed of *S. zeleborii* and *S. kindingeri* individuals. Subsequently, the mean values of the traits which contribute most to the differentiation along the first axis were tested using Tukey's HSD of homogeneous groups for the unequal N *post hoc* test. The length of the marginal trichomes (TLma) separated all species within the *S. ciliosum* complex with the exception of *S. ciliosum* and *S. jakucsii*, and *S. octopodes* and *S. galicum*. Thus, the same feature did not prove significant in separating *S. zeleborii* from *S. kindingeri* and *S. ruthenicum*, and *S. leucanthum* from *S. ruthenicum*. The length of the apical trichomes (TLap) showed a clear differentiation between the complexes. Within the *S. ciliosum* complex, the apical trichome length statistically indicates the differentiation of *S. klepa* and *S. jakucsii* from the rest of the taxa. Within the *S. ruthenicum* complex, the same feature did not imply a separation of species.

Agglomerative hierarchical cluster analysis (AHC). The phenogram obtained by agglomerative hierarchical cluster analysis based on the Mahalanobis distance (AHC) sorted the studied species similarly to CDA (Fig. 6B₍₁₎). The only deviation from CDA is in the relationship between *S. ciliosum* and *S. galicum*, as they are more closely related here than in CDA. Conversely, *S. jakucsii* is more distinct in its relationship to *S. ciliosum*. To establish the relationships with non-Balkan populations of *S. ruthenicum* and *S. wulfenii*, additional AHC analysis

was performed. Based on the phenogram, populations of outgroup species (*S. ruthenicum* and *S. wulfenii*) are not closely related to ingroup populations (Fig. 6B₍₂₎). Interestingly, the populations of *S. ruthenicum* from the ingroup and outgroup datasets are not directly related as expected. This suggests differences in epidermal structures between populations from the Balkan Peninsula and populations outside this area.

DISCUSSION

Although the variability of some epidermal structures (e.g. the indumentum, stomatal density, the shape of the anticlinal walls) may depend on habitat conditions, epidermal structures have been shown to be taxonomically important for species differentiation in many studies and, accordingly, have clarified certain taxonomic issues (MILAN *et al.* 2006; WANG *et al.* 2009; MAVI *et al.* 2011; MENG *et al.* 2016; LI *et al.* 2017). *Sempervivum* species have not been included in detailed anatomical studies in the past, and therefore little is known about their variability. Comprehensive data on the variability of epidermal structures are also not available for species within the *S. ciliosum* and *S. ruthenicum* complexes, with the exception of limited data on the length of the marginal trichomes of the rosette leaves (HAGEMANN 1986; MICEVSKI 1998; 'T HART 2002).

The epidermal cells, stomata, and trichome types do not differ significantly in general appearance among the species examined in this study. All the species are characterized by amphistomatic rosette leaves, aniscocytic stomata and glandular multicellular biseriate trichomes, which are very common features in Crassulaceae (GREGORY 1998; DUARTE & ZANETI 2002; THIEDE & EGLI 2007; ZLATKOVIĆ *et al.* 2017). The non-glandular, multicellular, stellate trichomes registered on the leaves of *S. ciliosum*, *S. octopodes*, *S. zebeborii* and *S. kindingeri* have been reported only for *Kalanchoe* (BOITEAU & ALLORGE-BOITEAU 1995). However, their importance for species differentiation in this study is unlikely to be very great, as they are sporadic and extremely rare. In addition, HAGEMANN (1986) and 'T HART (2002) noted that non-glandular trichomes can be found below the apex of the rosette leaves within the species of the *S. ruthenicum* complex (*S. ruthenicum*, *S. kindingeri*, and *S. leucanthum*), but their presence was not confirmed in our study.

In a quantitative sense, the anatomical structures show a clearer separation of complexes compared to single species. The separation between the complexes is best seen in the lengths of the epidermal cells, marginal and apical trichomes, which are significantly larger in the species of the *S. ciliosum* complex (Fig. 5). In contrast, the stomatal traits can be used with less confidence to separate the complexes and species. In the succulent species of the family Crassulaceae, stomatal density is expected to be low with the average number in the range 5-80 per mm² (STROBEL

& SUNDBERG 1984; THIEDE & EGLI 2007). Also according to TING & GIBBS (1982), succulent plants usually have between 10 and 65 stomata per mm², without specific information about their position on the plant. The values obtained in this study are within the reported data and thus reflect their succulent nature. As for the difference in the number of stomata between the adaxial and abaxial surfaces, one would expect there to be more of them on the adaxial surface, which is common for most Crassulaceae species (THIEDE & EGLI 2007). However, this cannot be accepted as *a priori* correct, since MOREIRA *et al.* (2012) and CHERNETSKYY (2012) have shown that the abaxial surface of *Kalanchoe* leaves is richer in stomata. The assumption that stomata are more numerous on the adaxial surface cannot be generalized and does not contribute significantly to the diversification of the species analysed in this study. In contrast, according to ZLATKOVIĆ *et al.* (2017), differences in the number of stomata adaxially and abaxially proved to be significant traits for detecting the differences between the species of the *Sedum album* complex. However, the size of the epidermal and guard cells can also be ploidy-dependent (MELARAGNO *et al.* 1993; KATAGIRI *et al.* 2016). Based on the available data, the species within the *S. ciliosum* and *S. ruthenicum* complexes are cytologically different. Specifically, *S. ciliosum* and *S. octopodes* are characterized by $2n = 34$, while the species from the *S. ruthenicum* complex, *S. zebeborii*, *S. ruthenicum* and *S. leucanthum* contain $2n = 64$ (FAVARGER & ZÉSIGER 1964; PARNELL & FAVARGER 1993; 'T HART 2002; 'T HART *et al.* 2003). According to the results of this study, the epidermal cell length of the species within the *S. ciliosum* complex is generally higher than that of the *S. ruthenicum* complex, regardless of the lower ploidy level. In contrast, the guard cell length is usually higher in the species of the *S. ruthenicum* complex, which can be correlated with the higher ploidy level (BEAULIEU *et al.* 2008). While a negative correlation is noticed regarding ploidy and stomatal density (BEAULIEU *et al.* 2008), stomatal density is higher in the species of the *S. ruthenicum* complex. Although the chromosome numbers for the analysed populations from the Balkan Peninsula have not been determined and could deviate from those given in the literature, previous statements lead us to believe that the level of ploidy in this study may not always be positively related to cell size in the species. Therefore, the size of the epidermal and guard cells, as well as the density of the stomata, cannot be determined by ploidy alone, but may also be under the control of environmental factors.

The characteristics of the trichomes, especially the role of the length of the marginal trichomes, proved to be the most appropriate to explain the difference between the analysed complexes. In various identification keys, their length is often presented as one of the most important features for distinguishing between the species of the complexes *S. ciliosum* and *S. ruthenicum*. *Sempervivum ciliosum* and related species from the Balkan Peninsula

are recognized in the literature by 2–4 mm long marginal trichomes (HAGEMANN 1986; MICEVSKI 1998; 'T HART 2002), but the results of the current study showed that the studied species of the *S. ciliosum* complex develop marginal trichomes which are mostly shorter than 2 mm. An exception is *S. klepa* with the obtained length of marginal trichomes within the proposed range (3.10 mm). In contrast, the species from the *S. ruthenicum* complex are recognizable by marginal trichomes shorter than 2 mm (HAGEMANN 1986; 'T HART 2002), while our results showed that all the species from this complex have marginal trichomes much shorter than 2 mm, i.e. their length does not exceed 0.50 mm. The discrepancies between the values reported in the literature and those obtained in this study probably arose due to differences in the sample size, methods and instruments used for measurement, or even the original habitat in relation to greenhouse growing conditions. As a comparatively variable trait, the length of marginal trichomes in itself should be used with caution for taxonomic purposes, both for complexes and at species level. In addition to the length of the marginal trichomes, their qualitative characteristics, reflected in the type of indumentum, could be used more freely in identification keys.

Multivariate analysis showed a clear difference between the two groups of yellow-flowered *Sempervivum* species from the Balkan Peninsula. This separation is also supported by phylogenetic studies, as *S. ciliosum* and *S. ruthenicum* belong to different well-supported intrageneric clades which are clearly distant from each other (KLEIN & KADEREIT 2015). Within the groups studied here, the species of the *S. ciliosum* complex are more separated than those of the *S. ruthenicum* complex. *Sempervivum klepa* shows the most significant segregation in the group of related species to which it belongs, based on most of the analysed epidermis characteristics. It is interesting to note the relationship of *S. octopodes* with *S. ciliosum*, which are well separated according to CDA. The taxonomic position of *S. octopodes* is controversial. ZONNEVELD (1999) considers it a subspecies of *S. ciliosum*, in contrast to TURRILL (1937) and MICEVSKI (1998), who assign the rank of a species to this taxon. The distinction between *S. octopodes* and *S. ciliosum* was previously based only on morphological characteristics, while the results of the analyses of the epidermal characteristics from this study could be used as additional confirmation of their taxonomic identity. On the other hand, the similarity in epidermal traits between *S. ciliosum* and *S. jakucsii* was more expected, as many authors now consider *S. jakucsii* a conspecific taxon and synonym of *S. ciliosum* (PARNELL 1988; BARINA *et al.* 2013). The position of *S. galicicum* is rather surprising, since 'T HART (2003) considers this taxon as a variety of *S. ciliosum*, in contrast to MICEVSKI (1998), who claims that *S. galicicum* is a well-defined species. In a recent study, JOVANOVIĆ *et al.* (2019a, b) pointed out the existence of morphological differences in the floral parts of *S. ciliosum*

subsp. *ciliosum* from Mt. Galičica (= *S. ciliosum* var. *galicicum*) and the rest of the analysed populations in the species area. It should be emphasized that the higher degree of pronounced differentiation between the species of the *S. ciliosum* complex may be a consequence of sample size, since all the species within this complex are represented with a single population, except for *S. ciliosum*. This may lead us to believe that the explained separation may represent differences between the specific population used in this study, rather than the species. It is possible that in a sample with more populations per species the distribution of the mean values for the epidermal characteristics would vary, which in turn could result in different relationships between the analysed species. The reason behind the disproportionate number of populations for the analysed species is the consequence of the ability of hybridization with other species from the genus, which were excluded from this study. Furthermore, erroneous distribution data, presumably arising from a misinterpretation of related *Sempervivum* or *Jovibarba* taxa with the species of the *S. ciliosum* complex, have resulted in a significantly smaller number of localities where these species can be found, which was confirmed during the fieldwork. Considering the established relationships between the species of the *S. ruthenicum* complex, the epidermal structures influenced the separation of *S. ruthenicum* and *S. zeleborii*. This indicates the importance of the epidermal structures in the diversification, as some authors consider that the morphological differences between these species are unclear ('T HART 2002).

For comparison, non-Balkan yellow-flowered species *S. ruthenicum* and *S. wulfenii* were also used as outgroups in some of the analyses in this study. It can be seen that the populations of these taxa differ in most epidermal characteristics from the yellow-flowered *Sempervivum* species found on the Balkan Peninsula. Thus, it is interesting to note that populations of *S. ruthenicum* from the Balkan Peninsula and populations from the Dnieper Lowland in Ukraine are not directly related as expected. To a certain extent, this can also refer to the relationship between the analysed populations, and not the species itself, due to the differences in the sample size and the number of populations of the analysed species. This suggests differences in epidermal structures, e.g. in the length and number of trichomes (adaxial/abaxial), the length of marginal and apical trichomes, the number of epidermal cells per mm² and their length on both surfaces of the rosette leaves. Similarly, *S. wulfenii* stands out as the most distantly related species, even more closely related to the *S. ruthenicum* complex. This separation is mainly influenced by differences in guard cell length, the number of epidermal cells per mm² and trichome characteristics. However, in addition to genetic differences, it is also possible that these differences are simply reflected by the long-term adaptation of epidermal structures to different ecological conditions in geographically distant areas.

CONCLUSION

Here we provide the first insights into the qualitative and quantitative characteristics of the epidermal structures (epidermal cells, stomata, trichomes) of the rosette leaves of yellow-flowered *Sempervivum* species from the Balkan Peninsula. Although the general characteristics of the epidermal cells, stomata and trichome types did not differ significantly between the species examined in this study, the morphometric analysis revealed an almost complete and clear differentiation between the *S. ciliosum* and *S. ruthenicum* complexes. The revealed differentiation between the complexes was mainly determined by the length of the marginal and apical trichomes.

The anatomical segregation of the species studied is congruent with the genetic segregation revealed in phylogenetic studies of the genus *Sempervivum*. Our results confirm the opinion of those authors who indicated that epidermal structures can be used for taxonomic purposes. However, since only features of epidermal structures were used, no final taxonomic conclusions can be drawn at this level. More detailed studies are needed in the future, combined with data on the morphological, karyological, molecular, and biochemical characters of these taxa, to determine their taxonomic relationships and phylogeny on the Balkan Peninsula.

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REZIME

Različitość żutocvetnih vrsta roda *Sempervivum* (Crassulaceae) Balkanskog poluostrva: rezultati morfometrijskog istraživanja epidermalnih struktura listova rozete

Maja JOVANOVIĆ, Dmtar LAKUŠIĆ, Branislava LAKUŠIĆ i Bojan ZLATKOVIĆ

Balkansko poluostrvo nastanjuje nekoliko sličnih, žutocvetnih vrsta čuvaruča svrstanih u dva kompleksa: *Sempervivum ciliosum* (*S. ciliosum*, *S. jakucsii*, *S. klepa*, *S. octopodes*, *S. galicicum*) i *S. ruthenicum* (*S. ruthenicum*, *S. leucanthum*, *S. kindingeri*, *S. zeleborii*). Usled izrazite fenotipske plastičnosti trenutno je teško utvrditi da li su sve vrste pomenutih kompleksa dobro definisane u taksonomskom smislu. Detaljna istraživanja epidermalnih struktura do sada nisu sprovedena ni za jednu od navedenih vrsta. Cilj ove studije bio je da se ispita stepen varijabilnosti epidermalnih struktura i mogućnost njihove primene u taksonomskoj karakterizaciji navedenih vrsta. Ukupno 18 kvantitativnih osobina epidermalnih struktura adaksijalne i abaksijalne površine listova rozete je analizirano na nivou 16 populacija. Kod svih vrsta, epidermalne ćelije su poligonalnog ili nepravilnog oblika, sa ravnim ili vijugavim antiklinalnim zidovima, dok su listovi amfistomatični sa anizocitnim tipom stoma. Prosti biserijadni višćelijski žlezdani trihomi pronađeni su na adaksijalnoj i abaksijalnoj površini, kao i na marginama listova rozete. Rezultati deskriptivne statistike, univarijantnih (ANOVA) i multivarijantnih statističkih analiza (CDA, AHC) ukazuju na nizak do visok stepen varijabilnosti epidermalnih ćelija, ćelija zatvaračica i trihoma. Multivarijantne analize ukazuju na raznolikost kompleksa i vrsta, dok dužine marginalnih i apikalnih trihoma listova u najvećoj meri doprinose njihovoj različitosti.

Ključne reči: *Sempervivum ciliosum* kompleks, *Sempervivum ruthenicum* kompleks, epidermalne ćelije, stome, trihomi, Balkansko poluostrvo