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Morphometric analyses and species delimitation in *Legousia* (*Campanulaceae*)

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Abstract: In order to evaluate the traditional morphological characters commonly used to distinguish the taxonomically problematic species of *Legousia*, we carried out a morphometric study with univariate analysis, principal component analysis (PCA), discriminant analysis (DA) and cluster analysis (UPGMA). The analyses were based on data from 436 individuals on 207 herbarium specimens from 18 countries in Europe, Africa and Asia representing all recently accepted species of the genus. *Legousia falcata* was found clearly distinct based on binary characters, *L. falcata* and *L. hybrida* were separated using refined binary and continuous characters, whereas *L. pentagonia*, *L. snogerupii* and *L. speculum-veneris* were all mixed in a single cluster, as were *L. castellana*, *L. falcata* and *L. scabra*. In a further refined sampling, *L. snogerupii* was separated by PCA, DA and UPGMA, but *L. pentagonia* and *L. speculum-veneris* were still indistinguishable and formed a single mixed cluster. Based on the results, we propose to reduce *L. castellana* and *L. scabra* to synonyms of *L. falcata*, and *L. skvortsovii* to a synonym of *L. hybrida*, while subspecific rank is proposed for *L. pentagonia* as *L. speculum-veneris* subsp. *pentagonia*, comb. & stat. nov. A key and nomenclatural synopsis of accepted taxa is provided and their geographic distribution is outlined.

Key words: cluster analysis, discriminant analysis, herbarium specimens, *Legousia*, morphometry, principal component analysis, *Specularia*

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Introduction

Legousia Durande is one of 84 genera in the family *Campanulaceae* (*Campanulaceae* Working Group 2018). The genus is distributed in the Old World from Macaronesia in the west to Central Asia in the east and from North Africa to NW and Central Europe. All its representatives grow in open fields, sparse forests, grasslands and on ruderal land. Sales & Hedge (2001) enumerated five species for the Iberian Peninsula, Tutin (1976) five species for all of Europe, while Lammers (2007) and Euro+Med (2006+) accepted seven species in the entire distribution of the genus (Table 1). The latter includes *L. perfoliata* (L.) Britton, here considered as *Campanula perfoliata* L. At the generic level, recent molecular works have demonstrated the affinity of *Legousia* to *Triodanis* Raf. (Americas), *Asyneuma* Griseb. & Schenk (North Africa and Eurasia) and *Phyteuma* L. (Europe) (Cosner & al. 2004; Mansion & al. 2012; Jones & al. 2017), but also to *Campanulastrum* Small (North America) (Eddie & al. 2003; Roquet & al. 2008; Haberle & al. 2009; Mansion & al. 2012, as *Campanula americana* L.; Crowl & al. 2014). No extensive molecular studies have been performed on relationships within the genus *Legousia*. However, the consensus phy-

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Table 1. Traditionally accepted species in the genus *Legousia*, with authorship, references and chromosome numbers. × = accepted; – = not treated.

Nomenclature		**References**							**Chromosome number**
Species	Authorship	Euro+Med (2006+)	Tutin (1976)	Damboldt (1978)	Sales & Hedge (1978)	Strid (2016)	Lammers (2007)	Quézel & Santa (1963)	Tan & al. (2015)
Legousia speculum-veneris	(L.) Chaix	×	×	×	×	×	×	×	$2n = 20$
Legousia pentagonia	(L.) Druce	×	×	×	–	×	×	–	$2n = 20$
Legousia snogerupii	Biel & Kit Tan	–	–	–	–	×	–	–	$2n = 20$
Legousia hybrida	(L.) Delarbre	×	×	×	×	×	×	×	$2n = 20$
Legousia falcata	(Ten.) Fritsch	×	×	×	×	×	×	×	–
Legousia scabra	(Lowe) Gamisans	×	×*	–	×	×	×	–	–
Legousia juliani	(Batt.) Briq.	×	–	–	–	–	×	×**	–
Legousia skvortsovii	Proskur.	–	–	–	–	–	×	–	–

* as *Legousia castellana* (Lange) Samp.; ** as *Specularia juliani* Batt.

logram based on *rbcL* sequence data in Haberle & al. (2009) and the tree from the combined plastid and PPR phylogeny in Crowl & al. (2014) both place *L. pentagonia* (L.) Druce as sister to *L. hybrida* (L.) Delarbre and *L. speculum-veneris* (L.) Chaix. On the contrary, Mansion & al. (2012) showed a paraphyletic relation in the genus *Legousia*, with *L. pentagonia* and *L. speculum-veneris* in one clade and *L. falcata* (Ten.) Fritsch in another clade sister to the American genera *Triodanis* and *Campanulastrum* (as *Campanula*). The monophyly in *Legousia* has therefore not been clearly confirmed, and there are no studies with sufficient sampling to draw definite conclusions. Mansion & al. (2012) suggested a single dispersal to the Americas from a Mediterranean *Legousia* ancestor during the Late Miocene. This single introduction was quickly followed by the diversification of several lineages now representing *Campanulastrum* and *Triodanis* (Mansion & al. 2012). Furthermore, in Haberle & al. (2009), Mansion & al. (2012) and Crowl & al. (2014), *Legousia* species were represented by only a single specimen each, and these studies are therefore inconclusive as far as interspecific variation and the distinctness of the species is concerned. The chromosome number of four *Legousia* species has uniformly been reported as $2n = 20$ (Table 1).

The genus *Legousia* was described by Durande (1782) and later by Candolle (1830) as *Specularia* Heist. ex A. DC., a name nowadays synonymized under *Legousia*. McVaugh (1948) argued for separation of *Legousia* (as *Specularia*) from the American *Triodanis*, a standpoint also maintained by Gadella (1966). However, the species-level taxonomy of the genus has received only little attention by recent authors and is generally based on a few traditional morphological characters. Tutin (1976) separated five species mainly by inflorescence arrangement, calyx lobe : ovary ratio, calyx lobe : corolla ratio and capsule shape. Damboldt (1978) distinguished four species for the Turkish flora by calyx lobe : corolla ratio, calyx lobe shape, calyx lobe : ovary ratio and capsule shape. Sales & Hedge (2001) distinguished the species by calyx lobe direction, capsule shape, inflorescence arrangement and capsule surface morphology. Similarly, Strid (2016) followed previous authors and used inflorescence arrangement and calyx lobe : corolla ratio, but also added corolla shape and colour as diagnostic characters.

The need for the present study became apparent when the first author worked at LD (Biological Museum, Lund University, Sweden) with identification of vascular plants collected in northern Morocco. In this material, the species of the small genus *Legousia* appeared surprisingly difficult to distinguish based on the characters used in the literature, and comparison with herbarium material from throughout the range of the genus appeared not to solve these issues. Therefore, a more thorough morphometric analysis of all taxa of this genus appeared warranted. During this work the following questions were formulated:

- Are the established morphological characters appropriate/sufficient for distinguishing the accepted species?
- Can the subordinate taxa of *Legousia* be distinguished by morphological characters?
- Which taxa within *Legousia* do morphometrics reveal?

The main aim of this study is to critically test the delimitation of the traditionally recognized species based on selected morphological characters (including all characters suggested by previous authors). We are aware that molecular data may be needed to fully resolve the taxonomy and interrelationships of the species concerned. However, since all taxa currently recognized in *Legousia* have been described based on morphology alone, we consider it warranted to test their distinctness based on morphological characters. Furthermore, such an overview of the morphological variation will be needed as a basis for future molecular work.

Material and methods

This study is based on 205 herbarium specimens stored at LD, two specimens borrowed from MHA (Herbarium of the Main Botanical Garden, Moscow) and two pictures from MPU (Herbier de l'Universite Montpellier II). The *Legousia* collection at LD ranges from the early 19th century to present times and represents the entire distributional area of the genus. The Mediterranean and East Mediterranean area is particularly well represented at LD, thus covering the diversity centre of *Legousia*. The specimens used for this study are selected to represent diversity and geographical distribution in a broad sense. Each specimen typically comprised several individuals that could be measured independently, and therefore the sampled entities are referred to as individuals in this study. A list of all specimens and their individuals is provided in the Appendix. Measurements were made using a Leitz Elvar stereo microscope with an ocular micrometre. All specimens were identified to species level, either by previous staff or by the first author. Some obviously wrongly identified specimens were re-identified after the first sampling (S1, see below), the specimens were resampled and the results evaluated again.

All species of the genus accepted by recent authors were included in the study, except for *Legousia juliani* (Batt.) Briq., which was excluded because of lack of herbarium specimens suitable for analysis. This species has not been re-collected since the type was gathered at Djebel Ouahch, outside Constantine in Algeria in 1889 (Battandier 1905: 222, as *Specularia juliani* Batt.). The type gathering consists of two specimens at MPU (MPU010143 and MPU010144). We examined high-resolution images of these specimens, kindly made available by the curators of MPU, and found that they comprise five rather complete and two damaged individuals. The individuals are all small, poorly developed, appear to be stunted due to habitat conditions and are badly suited for taxonomic evaluation. The individuals are similar to small individuals of *L. falcata* in most respects, but differ from normally developed *L. falcata* in their single terminal flowers, long pedicels, erect calyx lobes and, according to the protologue, the corolla should have a relatively pale colour (not observable on herbarium specimens).

The two most recently described species, *Legousia skvortsovii* Proskur. (Proskuryakova 1980) and *L. snogerupii* Biel & Kit Tan (Tan & al. 2015), are represented in this study by their types. *Legousia hybrida*, *L. pentagonia* and *L. speculum-veneris* all have a pre-Linnaean history and their types were not analysed in the study. *Legousia speculum-veneris* was mentioned by Linnaeus (1753, as *Campanula*) from southern Europe, *L. pentagonia* from Thrace and *L. hybrida* from England and France. At LD, these species are well represented with specimens from the respective areas. *Legousia falcata* was described by Tenore (1811) as *Prismatocarpus falcatus* Ten. and *L. scabra* (Lowe) Gamisans was originally published as *Prismatocarpus scaber* Lowe (1838), both without explicit types.

The taxonomy and nomenclature of *Legousia castellana* (Lange) Samp., *L. falcata* and *L. scabra* are complex. Sales & Hedge (2001) proposed both *L. falcata* and *L. scabra* as accepted names with *L. castellana* as a synonym of *L. scabra*. Lammers (2007) proposed *L. scabra* as an accepted name with *L. castellana* and *L. falcata* var. *scabra* (Lowe) Meikle as synonyms. Dobignard & Chatelain (2011) proposed *L. falcata* subsp. *castellana* (Lange) Jauzein as an accepted name with *L. castellana* and *L. scabra* as synonyms. Tison & al. (2014) proposed *L. falcata* subsp. *castellana* as an accepted name with *L. castellana* and *L. scabra* as synonyms.

The study was divided into three parts: (1) a broad sampling of 436 individuals of *Legousia falcata*, *L. hybrida*, *L. pentagonia*, *L. scabra* (syn. *L. castellana*), *L. snogerupii* and *L. speculum-veneris* from 17 countries (Supplemental Table 1; see supplemental content online) here referred to as S1; (2) a more focused sampling of 110 individuals from 12 countries of the same six species and *L. skvortsovii* (S2); and (3) a sampling of 48 individuals of *L. pentagonia*, *L. snogerupii* and *L. speculum-veneris* from three countries (S3). In each part of the project, characters presumably useful for separating the included species were gathered from the literature and subjected to statistical analyses. Two sample t-tests were used to analyse differences in single characters between pairs of species and ANOVAs were used to analyse differences in single characters among several species. The multivariate method Principal Component Analysis (PCA) was used to analyse matrices of several characters and species to get a general overview of the variation independent of earlier species identifications. Before PCA analyses, all characters were normalized by division by their standard deviations using the software's correlation matrix. Cluster analysis was performed by the paired group algorithm (UPGMA, unweighted pair-group method with arithmetic mean, Michener & Sokal 1957) and Gower similarity index (Gower 1971). When appropriate, Discriminant Analyses (DA) was also performed to find potentially useful characters to separate predefined taxa. All analyses were run in PAST 3.19 (Hammer & al. 2001).

Sampling 1

In the first analyses (S1), presumably diagnostic key characters were selected from Tutin (1976), Damboldt (1978), Sales & Hedge (2001) and Strid (2016). In addition, some potentially diagnostic characters not used by earlier authors were included (Supplemental Table 2).

Non-floral characters were preferred, because *Legousia falcata*, *L. hybrida* and *L. scabra* were almost exclusively available as fruiting specimens. Some authors (Gadella 1966; Damboldt 1978) emphasized the presence of cilia at the filament base to separate *L. speculum-veneris* from *L. pentagonia*. This trait could unfortunately

not be evaluated in the samped specimens and therefore had to be excluded. Most characters were binary since they were collected from dichotomous keys; numeric, continuous characters were grouped into categories according to intervals given in the literature.

Sampling 2

Based on the results of S1, and the analyses of additional characters for *Legousia falcata* and *L. scabra* from Tison & al. (2014) as described below, a second sampling (S2) with fewer but more complete individuals and refined characters was performed by PCA, DA and cluster analysis. Now, the sampling was restricted to complete and well-developed specimens in which also floral characters were scorable. Characters without a significant impact on species discrimination as revealed by S1 were removed and new characters better supporting discrimination between some species were added (Supplemental Table 3).

Tison & al. (2014) used the shape of trichomes on the capsule ("papilles") to separate *Legousia falcata* from *L. scabra* (*L. falcata* subsp. *falcata* versus *L. falcata* subsp. *castellana*, sensu Tison & al. 2014). *Legousia falcata* is characterized by rounded trichomes ("papilles arrondies") and *L. scabra* by pointed hairs ("papilles coniques"). We collected data for this trait and analysed with a t-test with species as groups. Tison & al. (2014) also suggested the combination of the characters rounded trichomes with calyx lobes longer than half the length of the mature capsule to distinguish *L. falcata*. To distinguish *L. scabra*, pointed hairs with calyx lobes shorter than half the length of the mature capsule are suggested. The treatment in Sales & Hedge (2001) is mostly similar, but differs in nomenclature. With type of trichomes as groups (pointed vs rounded), we analysed the calyx lobe : capsule ratio with a t-test. Also a PCA was performed with seven characters and 29 individuals of *L. falcata* and *L. scabra*.

Based on the analysis of S1 and S2, t-tests were run for critical characters in the *Legousia speculum-veneris* complex. According to Damboldt (1978) and Tutin (1976), a critical key character separating *L. speculum-veneris* from *L. pentagonia* is the calyx lobe : ovary length ratio, described as 1:1 in *L. speculum-veneris* and 1:2 in *L. pentagonia*. We collected data for this character as calyx lobe length divided by ovary length (both in mm); the quotients were analysed with a test for equal means (t-test) with species as groups. Tison & al. (2014) suggested the length of the hairs on the calyx lobes and ovary to separate the two species and described it as 0.5 mm or shorter in *L. speculum-veneris* and longer than 0.5 mm in *L. pentagonia*. Also corolla length should separate the two species, with 6–13 mm in *L. speculum-veneris* and 12–20 mm in *L. pentagonia.* We recorded hair and corolla length and analysed them with a t-test with predetermined species as groups. Also a t-test was performed with short hairs (≤0.5 mm) as one group and long hairs (>0.5 mm) as another, suggesting differences between the two species, and with corolla length as variables. In another t-test, corolla length (≤10 mm and ≥14 mm, respectively) made two groups, with hair length as variables. Finally, we performed a PCA with species and hair length combined with corolla length as groups and hair length and corolla length as variables.

Furthermore, we tested the utility of the character calyx lobes : ovary length for separating the six species using a univariate analysis of variance (ANOVA) followed by Tukey's HSD pairwise test.

According to Damboldt (1978) and Strid (2016), the calyx lobes : corolla length ratio separates the genus into two groups. The calyx lobes are longer than the corolla in *Legousia falcata*, *L. hybrida* and *L. scabra* and shorter or equal to the corolla in *L. pentagonia*, *L. snogerupii* and *L. speculum-veneris*. To test this, we ran a separate t-test for this character separating the *L. speculum-veneris* complex from the other species.

Proskuryakova (1980) described *Legousia skvortsovii* as a new species distinct from the very similar *L. hybrida* based on capsule length and overall hairiness. To compare these characters between the two species, we recorded capsule length, hair length and hair density (number of hairs per millimetre in transect) and analysed them with t-tests with species as groups.

Sampling 3

The final sampling (S3) included only the species *Legousia pentagonia*, *L. snogerupii* and *L. speculum-veneris* (hereafter referred to as the *L. speculum-veneris* complex), which were not clearly separated in S1 and S2. The analysis was restricted to the characters reported to be diagnostic for them with new characters selected (Supplemental Table 4) mainly from Tan & al. (2015).

Special care was taken to find specimens with well-preserved floral parts including corolla and filaments. This sampling also focused on continuous characters, better suited for statistical analyses. All sampled specimens of *Legousia snogerupii* were listed in Tan & al. (2015), i.e. they are paratypes of the species name. As S1 and S2 were found insufficient for distinguishing between *L. pentagonia* and *L. speculum-veneris* and most of the specimens at LD had been repeatedly re-determined by earlier staff, the names were regarded as putative but retained in order to run ANOVA and post hoc tests.

The rather arbitrary character if the capsule is narrowed at the apex (Tutin 1976; Damboldt 1978; Tan & al. 2015; Strid 2016) was calculated as:

$$\text{capsule constriction ratio} = \frac{\text{capsule diameter at apex}}{\text{capsule diameter at widest}}$$

Difference between seed shape (orbicular for *Legousia pentagonia* sensu Damboldt [1978] versus ellipsoid for *L. speculum-veneris*) was calculated as:

$$\text{seed shape ratio} = \frac{\text{seed width}}{\text{seed length}}$$

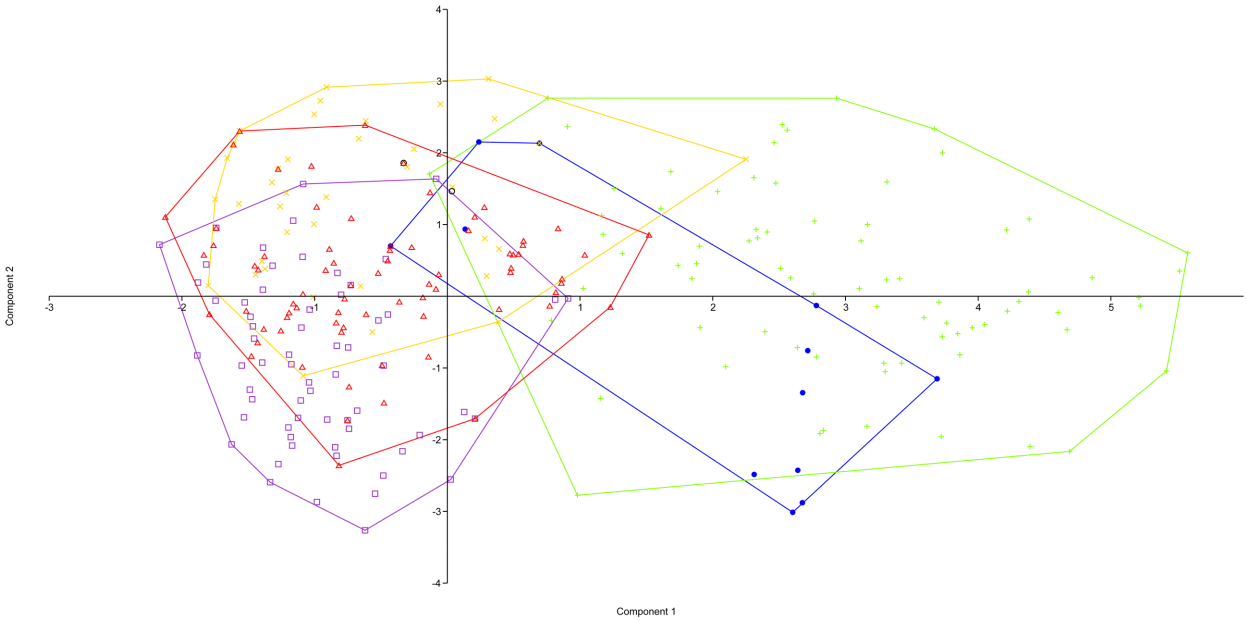


Fig. 1. PCA scatter plot based on 13 morphological characters and 31 variables, 436 individuals and six taxa of *Legousia*, here surrounded by convex hulls. PC 1 explains 24% of variance and PC2 15% of variance. + *L. falcata* (green), ● *L. scabra* (blue), □ *L. hybrida* (lilac), × *L. pentagonia* (yellow), △ *L. speculum-veneris* (red), ○ *L. snogerupii* (black).

The amount of hairs on the stem, calyx lobes and ovary were estimated by counting the number of hairs per millimetre in transect.

The resultant data matrix with 48 individuals of three species and 13 characters was subjected to PCA ordination and discriminant analysis (DA). In the latter, the individuals were grouped a priori into putative species and missing data were supported by column average substitution. Furthermore, differences among the means of each continuous character were tested by ANOVA. If differences among means were statistically significant ($p<0.05$), then Tukey's pairwise was run.

Results

Sampling 1

The PCA produced five components with eigenvalues greater than one (Supplemental Table 5). The first and second component explained 24% and 15% of the variance, respectively. A scatterplot of the two first components (Fig. 1) shows rather good separation of *Legousia falcata* + *L. scabra* from the other species, but only a weak tendency to separate *L. hybrida* on the negative side along PC2. *Legousia pentagonia*, *L. snogerupii* and *L. speculum-veneris* appeared mixed on the negative side of PC2. The character loadings (Supplemental Table 5) showed that calyx shape, capsule length and calyx direction were the most influential characters in this analysis, but several other characters also contributed.

The UPGMA dendrogram (Supplemental Fig. 1) separated *Legousia falcata* and most samples of *L. scabra* from the other species. Also *L. hybrida* formed a separate cluster but included some samples of *L. pentagonia*. Between these rather well-delimited clusters, *L. pentagonia* formed a cluster also including some samples of *L. snogerupii* and *L. speculum-veneris*. Most *L. speculum-veneris* samples formed another separate cluster, although with the inclusion of some samples of *L. hybrida* and *L. scabra*.

Sampling 2

The t-test for trichome shape between *Legousia falcata* and *L. scabra* revealed no significant difference between group means (p=0.89, F=1.15), nor did the relation between hair shape and calyx lobes : capsule length ratio (p= 0.37, F=1.34). The PCA produced three components with eigenvalues greater than one (Supplemental Table 6). The first and second component explained 30% and 20% of the variance, respectively. The scatterplot of the two first components (Fig. 2) shows individuals with rounded hairs combined with calyx lobes longer than half the length of the capsule (green dots; *L. falcata* sensu Tison & al. 2014) scattered over most of the area, individuals with pointed hairs and calyx lobes longer than half as long as the capsule (red squares; not classified by Tison & al. 2014) mainly in the lower right area, and finally two individuals with the combination of rounded hairs and calyx lobes shorter than half the length of the capsule (blue diamonds). According to Tison & al. (2014) only the two latter individuals should match the description of *L. scabra* sensu stricto, but as shown with the t-test the combination of the two characters is most likely a coincident. The results suggest that there are no significant differences within the group formed by *L. falcata* and *L. scabra* and therefore these two taxa were treated as one in subsequent analyses.

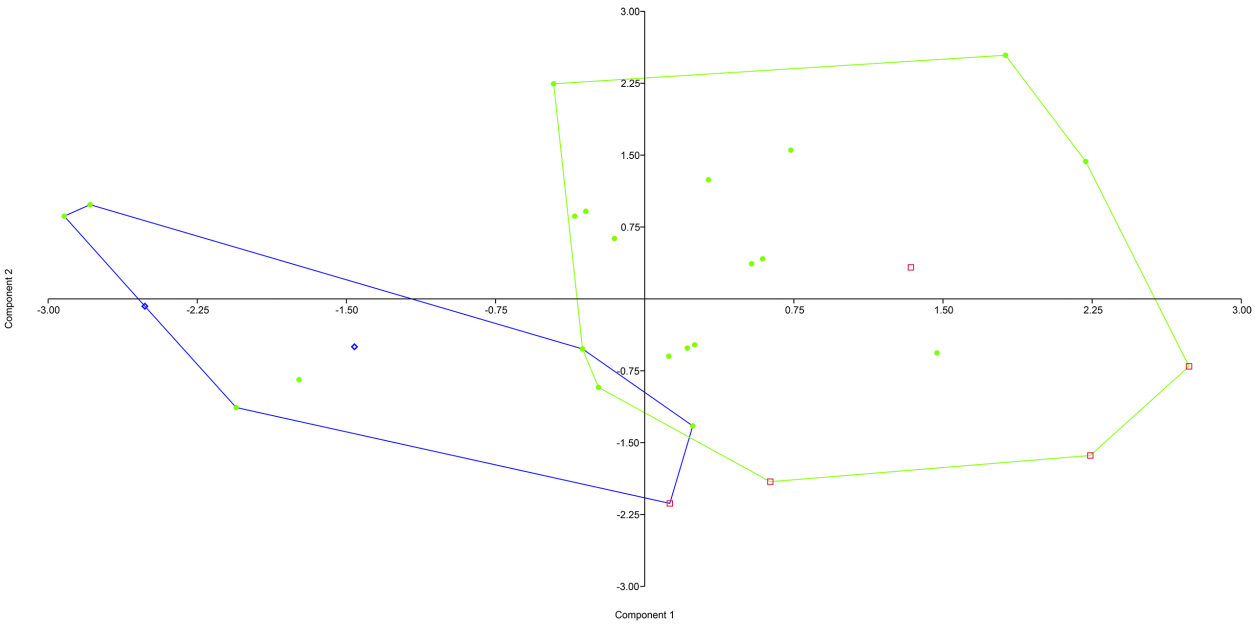


Fig. 2. PCA scatter plot on seven variables, 29 individuals and the two taxa *Legousia falcata* and *L. scabra*. PC 1 explains 30% of variance and PC2 20% of variance. ● (green) individuals with rounded hairs combined with calyx lobes longer than half of capsule, □ (red) individuals with pointed hairs and calyx lobes longer than half of capsule, ◊ (blue) individuals with rounded hairs and calyx lobes shorter than half of capsule. Left convex hull (blue) represents predetermined *L. scabra* and right *L. falcata*. Only the two blue diamonds (◊) have combined characters matching *L. castellana* sensu Tison & al. (2014).

The PCA performed for all species (*Legousia scabra* excluded) produced five components with eigenvalues greater than one (Supplemental Table 7). The first and second component explained 30% and 14% of the variance, respectively. A scatterplot of the two first components (Fig. 3) shows full separation of all *L. hybrida* + *L. skvortsovii* and almost total separation of *L. falcata*. In contrast, *L. pentagonia*, *L. snogerupii* and *L. speculum-veneris* all mix in a group on the positive side of PC 1.

The DA revealed four main groups; *Legousia falcata* and *L. snogerupii* were well separated, whereas *L. pentagonia* and *L. speculum-veneris* were mixed together, and so was *L. hydrida* with *L. skvortsovii*. The biplot showed that the result was ruled mainly by calyx lobe shape, calyx lobe/corolla shape, capsule shape and capsule hairs (Supplemental Fig. 2).

The UPGMA dendrogram (Supplemental Fig. 3) revealed three main clusters with *Legousia falcata* well

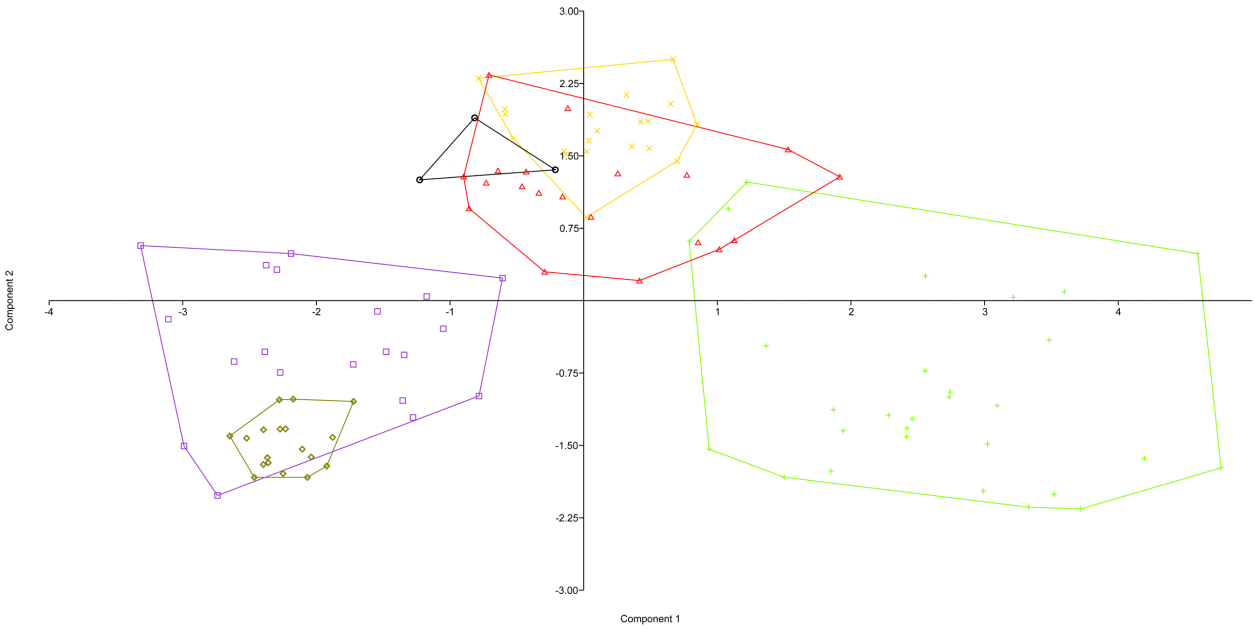


Fig. 3. PCA scatter plot based on 13 characters, 29 variables, 110 individuals and six taxa of *Legousia*, here surrounded by convex hulls. PC 1 explains 30% of variance and PC2 14% of variance. + *L. falcata* (green), □ *L. hybrida* (lilac), ◊ *L. skvortsovii* (brown), × *L. pentagonia* (yellow), △ *L. speculum-veneris* (red), ○ *L. snogerupii* (black).

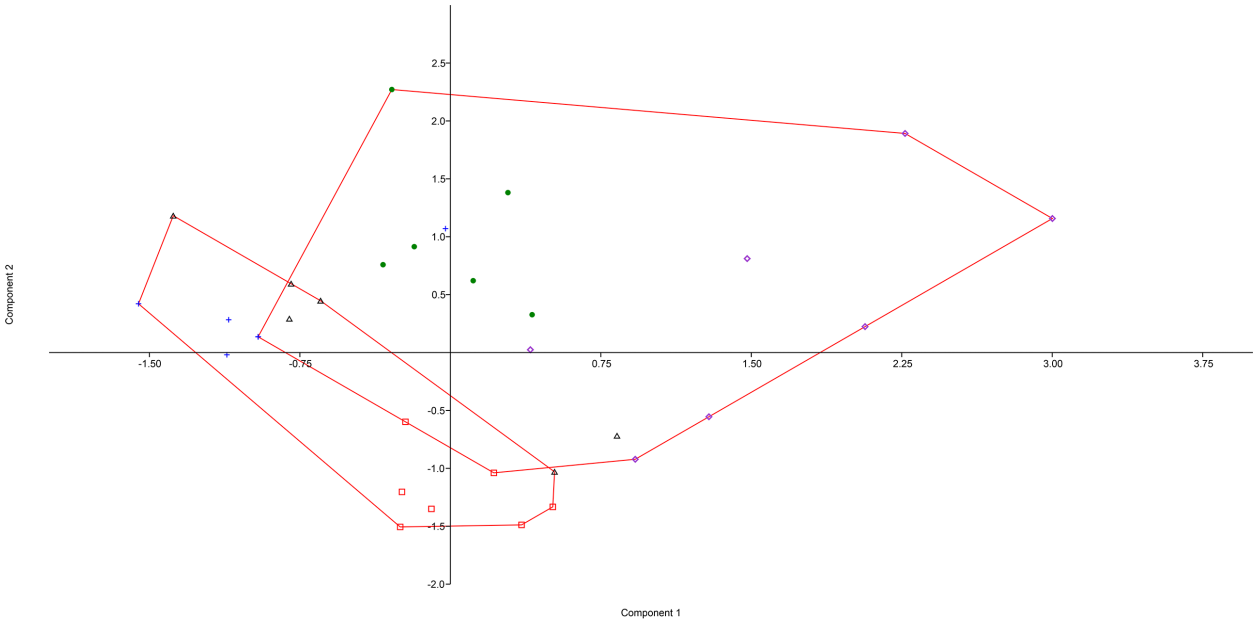


Fig. 4. PCA scatter plot based on two morphological characters, 40 individuals and the two taxa *Legousia speculum-veneris* and *L. pentagonia*. □ (red) individuals with short hairs and short corollas (= *L. speculum-veneris* sensu Tison & al. 2014), ◊ (lilac) individuals with short hairs and long corollas, ● (green) individuals with long hairs and long corollas (= *L. pentagonia* sensu Tison & al. (2014), + (blue) individuals with long hairs and short corollas, △ (black) intermediate individuals. Convex hulls indicate predetermined species; left polygon = *L. speculum-veneris*, right polygon = *L. pentagonia*.

separated with no mix of other species. *Legousia hybrida* and *L. skvortsovii* formed a cluster of their own and, finally, *L. pentagonia* and *L. speculum-veneris* were combined with *L. snogerupii* in a third cluster.

Several t-tests revealed no significant difference between group means: calyx lobe : ovary length between *Legousia pentagonia* and *L. speculum-veneris* (p=0.91, F=1,23), hair length (p=0.97, F=1.25), hair length as groups and corolla length as variables (p=0.66, F=4.16); corolla length as groups and hair length as variables (p=0.93, F=1.03).

However, corolla length showed significant difference if the two predetermined species were groups (p=<0.001, F= 11.86). The PCA scatter plot (Fig. 4) with species and hair length combined with corolla length as groups and hair length and corolla length as variables showed a great mixture of characters within the plot. *Legousia speculum-veneris* sensu Tison & al. (2014) with short hairs and short corolla appears on the negative side of the two axes, and *L. pentagonia* sensu Tison & al. (2014) with long hairs and long corolla on the positive side of the PC2 axis. However, as the convex hulls show, there are several individuals identified as each species not corresponding to these characters.

The ANOVA performed for the calyx lobe : ovary length ratio showed significant differences between *Legousia falcata* and *L. hybrida* (p=<0.001) and *L. hybrida* and *L. pentagonia* (p=<0.001).

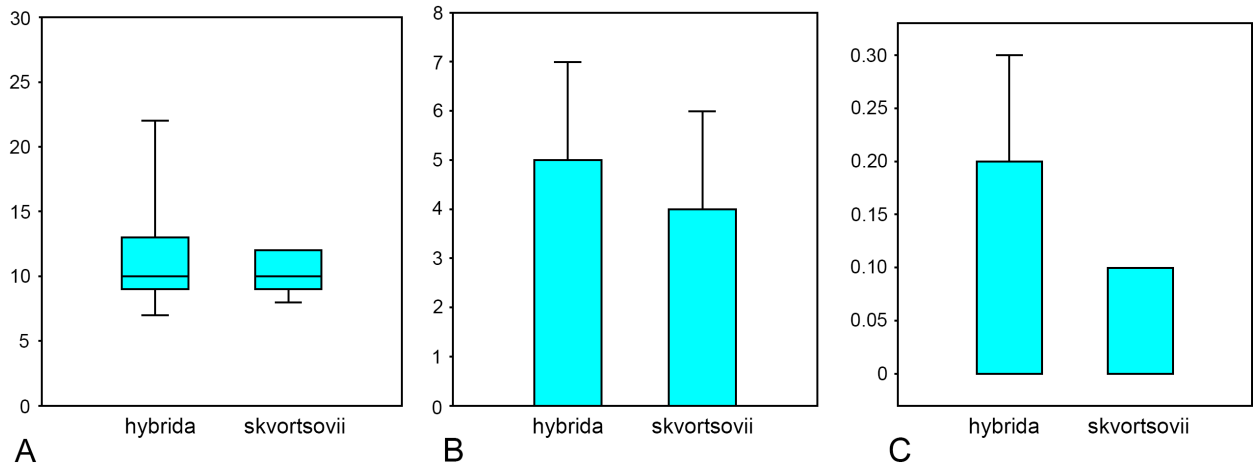


Fig. 5. Morphological variation in *Legousia hybrida* and *L. skvortsovii*. A: capsule length in mm; B: hair density in number of hairs per mm; C: hair length in mm.

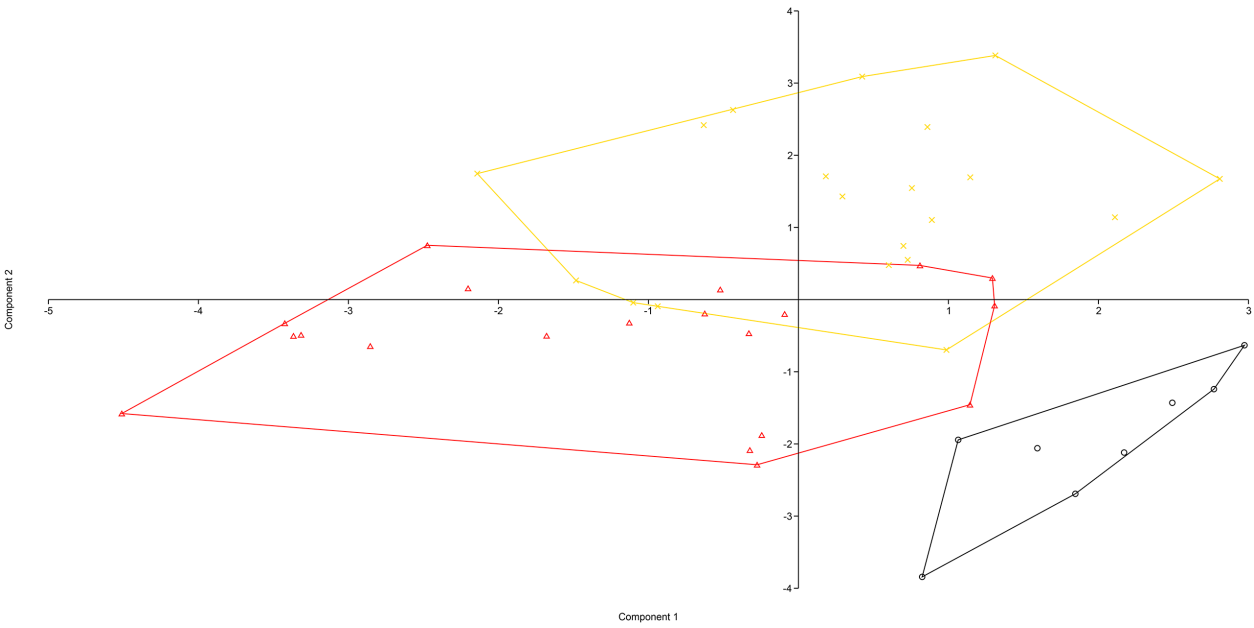


Fig. 6. PCA scatter plot on 13 variables, 48 individuals and three taxa of *Legousia*, surrounded by convex hulls. PC 1 explains 23% of variance and PC2 19% of variance. × *L. pentagonia* (yellow), △ *L. speculum-veneris* (red), ○ *L. snogerupii* (black).

The t-test for calyx lobe : corolla length between the *Legousia speculum-veneris* complex and the other species showed significant differences (p=<0.001, F=3.94) between the groups; the mean ratio for the *L. speculum-veneris* complex was 0.72 (calyx lobes shorter than corolla) as compared to 1.53 (calyx lobes longer than the corolla) for the other species.

The t-test for hair density between *Legousia hybrida* and *L. skvortsovii* revealed no significant difference between group means (p=0.27, F=1.34), nor did the capsule length (p=0.23, F=5.72). Hair length was significantly different between the two species (p=0.006, F=4.78): mean length of 0.1 mm in *L. hybrida* and 0.07 mm in *L. skvortsovii* (most individuals of the latter were glabrous) (Fig. 5).

Sampling 3

The PCA on this sample of only members of the *Legousia speculum-veneris* complex produced five components with eigenvalues greater than one (Supplemental Table 8). PC1 and PC2 explained 23% and 19% of the variance, respectively. A scatterplot of the two first components (Fig. 6) revealed full separation of samples of *L. snogerupii* on the negative side of PC1 and the positive side of PC2, while there was a major overlap between *L. speculum-veneris* and *L. pentagonia* around zero along PC2. As shown by the character loadings (Supplemental Table 8), most of the characters included contributed relatively equally to this analysis.

The UPGMA cluster analysis (Fig. 7) clearly separated *Legousia snogerupii* in its own cluster, but did not show any separation between *L. pentagonia* and *L. speculum-veneris*.

The discriminant analysis (DA) run with the three putative species as predetermined groups resulted in 96% correctly classified individuals. A confusion matrix showed that one *Legousia pentagonia* was classified as *L. speculum-veneris* and one *L. speculum-veneris* was classified as *L. snogerupii* (Supplemental Table 9). However, the character loadings showed that the results were almost completely based on the single character capsule length (discriminant function 1: 2.42, discriminant function 2: 2.48), with weak influence of corolla length (discriminant function 1: 1.11, discriminant function 2: 1.29); see also Fig. 8B & D.

The results of ANOVAs on characters for species within the *Legousia speculum-veneris* complex revealed significant differences between several group means: Stem hairs *L. pentagonia*/*L. snogerupii* (p=0.002), *L. snogerupii*/*L. speculum-veneris* (p=0.007); capsule length *L. pentagonia*/*L. snogerupii*, (p=0.003), *L. pentagonia*/*L. speculum-veneris* (p<0.001); calyx lobes length *L. pentagonia*/*L. speculum-veneris* (p=0.012); capsule constriction ratio *L. pentagonia*/*L. speculum-veneris* (p=<0.001), *L. snogerupii*/*L. speculum-veneris* (p=0.011) (Fig. 8). However, there was no significant difference in seed size ratio (Damboldt 1978) between *L. pentagonia* and *L. speculum-veneris* (p=0.22, F=1.55). Descriptive statistics for characters in the *L. speculum-veneris* complex are presented in Supplemental Table 10.

Discussion

The results of this study suggest that several morphological characters repeatedly used for species delimitation in *Legousia* are generally unsuitable or are not stable over the range of species. The results also reveal that *L. hybrida* is the most distinct species, fol-

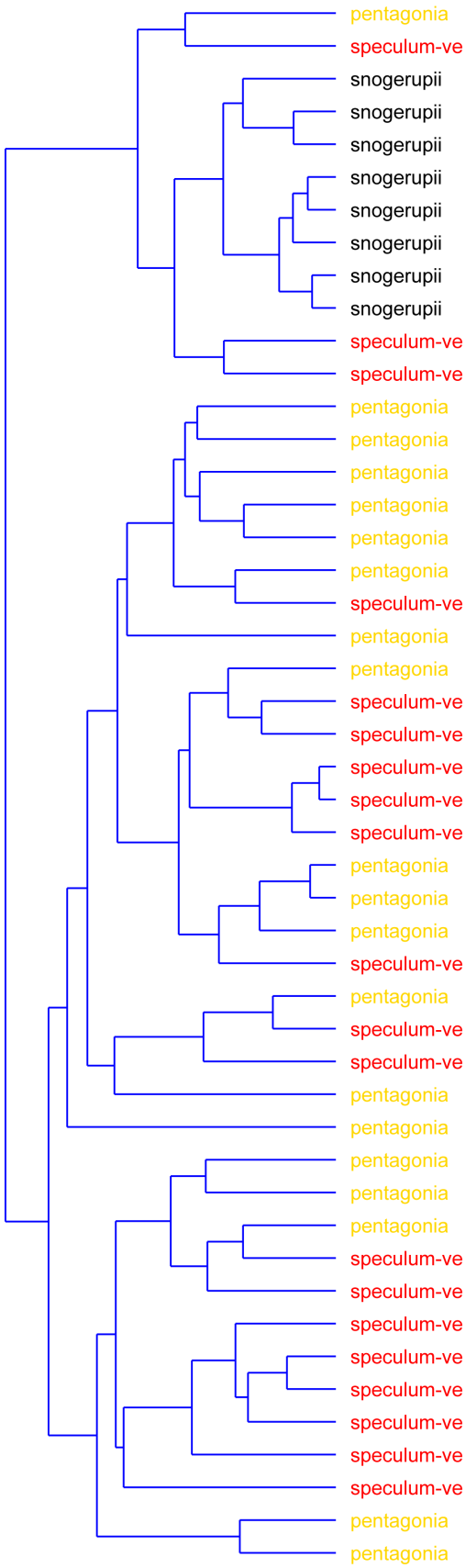


Fig. 7. Hierarchical clustering of individuals of *Legousia pentagonia* (yellow), *L. speculum-veneris* (red) and *L. snogerupii* (black) based on 13 characters using paired group algorithm (UPGMA) and Gower similarity index.

lowed by *L. falcata* (including *L. scabra*) and *L. snogerupii*, while several other species that have been accepted by previous authors turned out to be difficult to define and characterize.

As a consequence of this study, we propose to synonymize *Legousia skvortsovii* with *L. hybrida*, with the latter as the accepted name following the rule of priority. We found no significant differences in the characters suggested to be diagnostic for these species (Proskuryakova 1980). The PCA and DA performed with 13 characters and 29 variables plotted *L. skvortsovii* as enclosed by *L. hybrida*. The Kopet Dag population is rather distinct, but it fits within the variation of *L. hybrida* (Fig. 3, 5 & Supplemental Fig. 2). *Legousia skvortsovii* was described from 16 individuals on the type specimen and about 20 individuals on a paratype. From these, 19 individuals (53% of the type material) were selected and analysed together with 25 individuals of *L. hybrida*. The number of selected individuals of *L. skvortsovii* seemed justified since the variation in the type material is very limited. An addition of more individuals should not influence the results.

We also propose to synonymize *Legousia scabra* (= *L. falcata* subsp. *castellana* sensu Dobignard & Chatelain 2011) with *L. falcata*, with the latter as the accepted name. The characters suggested to differ between these taxa by previous authors (Sales & Hedge 2001; Tison & al. 2014) showed no significant differences in our sample, and the PCA plotted the characters and combination of characters randomly, with no clear separation (Fig. 2).

Unfortunately, the only extant specimens of *Legousia julianii* are poorly developed, small and unbranched plants that are not well suited for a taxonomic analysis. Based on such meagre material it is impossible to clearly state whether the deviant characters shown by the individuals are environmentally induced phenotypic variations or genetically determined. The fact that the taxon has never been re-collected may suggest that the type collection consists of atypical individuals, or that the population has gone extinct. In any case, its taxonomic status has to remain unsolved until the taxon is re-collected. *Legousia julianii* in most respects is similar to *L. falcata*, a common species in the same area in Algeria, although the five individuals on the type specimens of *L. julianii* differ from *L. falcata* by having single terminal flowers, long pedicels, and erect calyx lobes.

The second PCA (Fig. 3) well distinguished clusters with *Legousia falcata*, *L. hybrida* and the *L. speculum-veneris* complex, and this pattern was corroborated by the UPGMA cluster analysis on the same data. The latter complex, comprising the species *L. pentagonia*, *L. snogerupii* and *L. speculum-veneris*, is distinct in so far as its members form a separate group in most of the analyses performed here, thus confirming their close affinity as suggested in the literature (Tutin 1976; Damboldt 1978; Tan & al. 2015; Strid 2016). However, within the *L. speculum-veneris* complex, only *L. snogerupii* appears

reasonably distinct based on a combination of multiple characters (Fig. 6 & 7). *Legousia snogerupii* is distinguishable in both the second and the third sampling and is rather well separated from the other species in the *L. speculum-veneris* complex (Fig. 6, 7 & Supplemental Fig. 2). In contrast, with the exception of the single character capsule length (Fig. 8B), the characters given in the literature and included in this study are apparently insufficient for differentiating *L. pentagonia* and *L. speculum-veneris* (Fig. 6, 7 & Supplemental Fig. 2).

That we were unable to find a clear distinction between *Legousia pentagonia* and *L. speculum-veneris* may be controversial because these species may be the most well known in the genus, have a long pre-Linnaean history and have been accepted by numerous authors over the centuries. However, already Sales & Hedge (2001) pointed out that, despite apparent differences between the two species, intermediate forms frequently appear in the East Mediterranean area. In addition, most of the specimens representing these taxa at LD have undergone repeated re-identification by different experts over the years, indicating the problematic differentiation of the two species based on morphology and a lacking consensus about their delimitation.

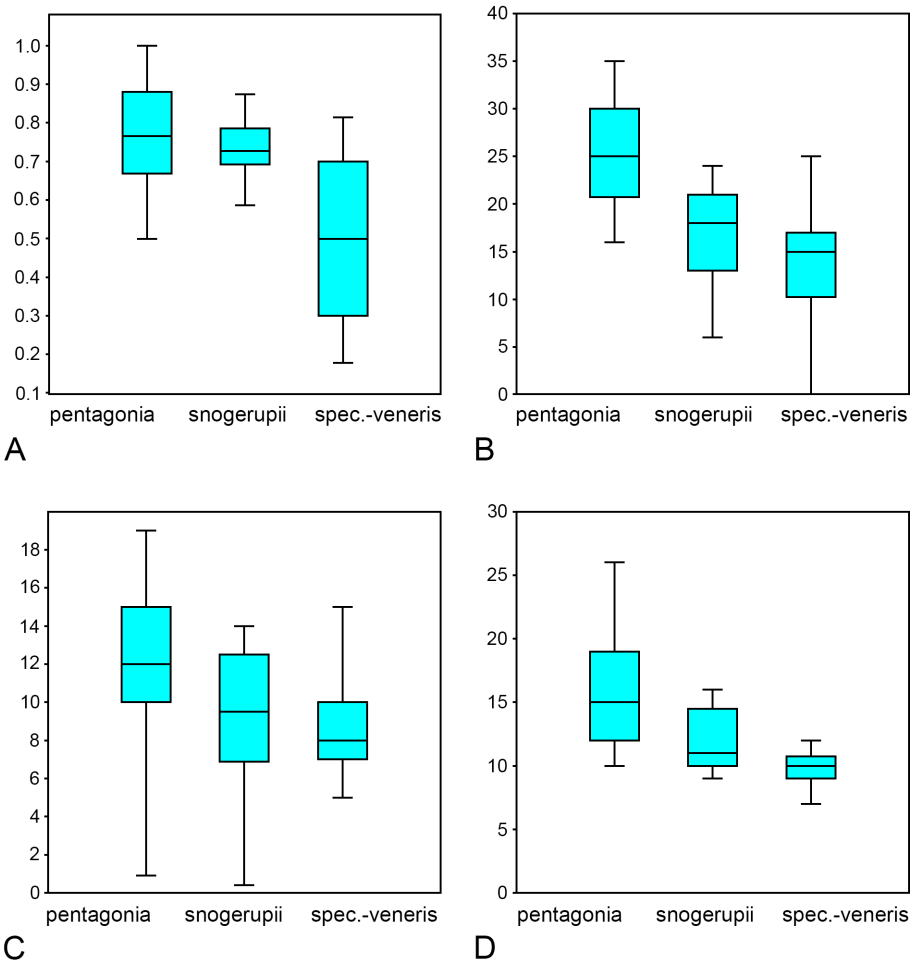
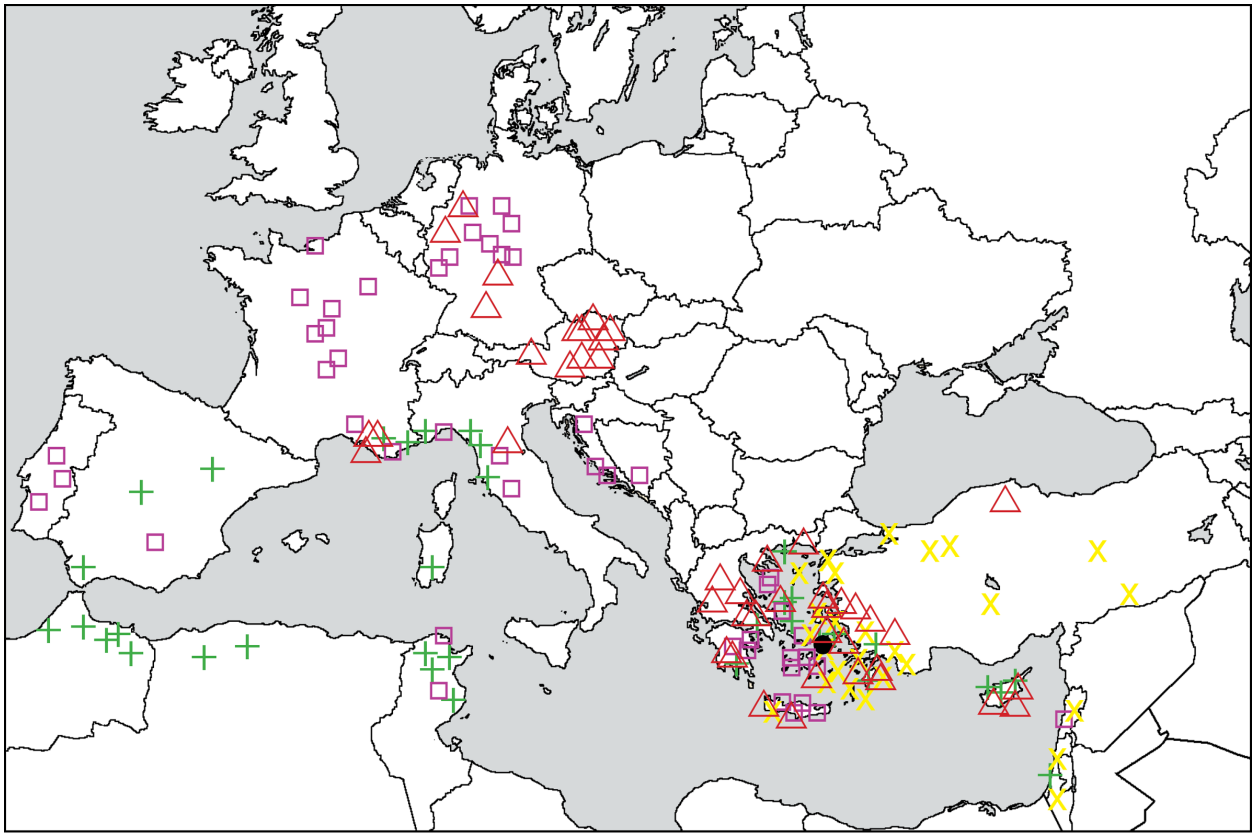


Fig. 8. Morphological variation in *Legousia pentagonia*, *L. snogerupii* and *L. speculum-veneris*. A: calyx constriction ratio in %; B: capsule length in mm; C: calyx lobe length in mm; D: corolla length in mm. Boxes represent 25–75 quartiles; horizontal line represents median; short horizontal lines represent minimal and maximal values.

The strength of PCA when evaluating morphometric data is that there is no need to predefine any groups. Thus, the analysis is unbiased and the investigated entities are not forced into potentially misinterpreted groups as may be the case with e.g. discriminant analyses. Discriminant analysis of the *Legousia speculum-veneris* complex, however, identified 96% of the species correctly (Supplemental Fig. 2) and the resulting scatterplot showed only a relatively small overlap between *L. pentagonia* and *L. speculum-veneris*. However, the separation here is apparently almost exclusively based on a single character (capsule length), suggesting that the characters are largely mutually uncorrelated and that the separation may be considered artificial. The calyx lobe : ovary length ratio used by Tutin (1976) and Damboldt (1978) got no support in this study and did not qualify as a diagnostic character, nor did the comparison of the seed shape between the two species as claimed by Damboldt (1978).

According to the results of the ANOVA in S3, the most reliable characters separating *Legousia speculum-veneris* and *L. pentagonia* are capsule length (≤25 mm vs ≥20 mm, respectively); calyx lobes length (≤15 mm vs ≥10 mm, respectively) and capsule constriction ratio (30–70% vs 65–90%, respectively). However, the two taxa overlap in all individual characters and a sharp species boundary is not to be found. It should also be noted that the chromosome numbers of both taxa are the same (as for *L. hybrida* and *L. snogerupii*), which is why the cytology is not particularly helpful in separating the species.

We also tested the correlation between corolla length and calyx lobe hair length. This character was proposed by Tison & al. (2014) to be most useful to distinguish between *Legousia pentagonia* and *L. speculum-veneris*. Because the characters are unweighted, contrary to identified herbarium specimens, the result should give



□ *L. hybrida*, + *L. falcata*, ● *L. snogerupii*, △ *L. speculum-veneris* subsp. *speculum-veneris*, × *L. speculum-veneris* subsp. *pentagonia*.

Fig. 9. Geographic distribution of accepted taxa of *Legousia* based on specimens in LD.

an unbiased view of the relation between the two species if these two characters are useful for a separation. However, we found no significant correlation between the combination and re-combination of characters in the t-tests, and the PCA scatter plot showed a great mix of character combinations and predetermined species (Fig. 4).

It may therefore be argued that the two taxa are only arbitrary subdivisions of a single large but morphologically variable species. Notably, the distribution area of *Legousia pentagonia* is included within the wide distributional range of *L. speculum-veneris*, suggesting that *L. pentagonia* may be considered as the eastern morphotype (Greece, Cyprus, Turkey, Israel and Lebanon) of a largely clinal east-west variation gradient. For these reasons, we propose to reduce *L. pentagonia* to subspecies level under *L. speculum-veneris*.

Taxonomic treatment

1. Calyx lobes longer than corolla (1.2–2.4 × longer) **2**
– Calyx lobes shorter, or as long as corolla (0.3–1 × longer) **3**
2. Calyx lobes erect, lanceolate-ovate . . . . ***L. hybrida***
– Calyx lobes erecto-patent or recurved, linear . . . . . ***L. falcata***
3. Flowers solitary; stem flexuous, trailing; calyx lobes glabrous . . . . . ***L. snogerupii***
– Flowers in a panicle, stem erect; calyx lobes with hairs **4**
4. Capsule <20 mm long, constriction ratio 30–70%; calyx lobes <15 mm long . . . . . ***L. speculum-veneris*** subsp. ***speculum-veneris***
– Capsule >20 mm long, constriction ratio 65–90%; calyx lobes >10 mm long . . . . . ***L. speculum-veneris*** subsp. ***pentagonia***

Legousia speculum-veneris (L.) Chaix in Villars, Hist. Pl. Dauphiné 1: 338. 1786 ≡ *Campanula speculum-veneris* L., Sp. Pl. 1: 168. 1753 ≡ *Specularia speculum-veneris* (L.) A. DC., Monogr. Campan.: 346. 1830.

Legousia speculum-veneris subsp. ***speculum-veneris***
Distribution (Fig. 9): C Europe, N and E Mediterranean area.

Legousia speculum-veneris subsp. ***pentagonia*** (L.) Wahlsteen, **comb. & stat. nov.** ≡ *Campanula pentagonia* L., Sp. Pl. 1: 169. 1753 ≡ *Specularia pentagonia* (L.) A. DC., Monogr. Campan.: 344. 1830 ≡ *Legousia pentagonia* (L.) Druce, List Brit. Pl.: 46. 1908.
Distribution (Fig. 9): E Mediterranean area; elsewhere introduced.

Legousia snogerupii Biel & Kit Tan in Phytotaxa 201: 64. 2015.
Distribution (Fig. 9): Greece (Kyklades).

Legousia hybrida (L.) Delarbre, Fl. Auvergne, ed. 2: 47. 1800 ≡ *Campanula hybrida* L., Sp. Pl. 1: 168. 1753 ≡ *Prismatocarpus hybridus* (L.) L'Hér., Sert. Angl.: 3. 1789 ≡ *Specularia hybrida* (L.) A. DC., Monogr. Campan.: 348. 1830.
= *Legousia skvortsovii* Proskur. in Byull. Moskovsk. Obshch. Isp. Prir., Otd. Biol. 85(4): 95. 1980.
Distribution (Fig. 9): NW and C Europe, all of the Mediterranean area including N Africa, SW Asia and eastward to C Asia.

Legousia falcata (Ten.) Fritsch in Mitt. Naturwiss. Vereins Univ. Wien, ser. 2, 5: 100. 1907 ≡ *Prismatocarpus falcatus* Ten., Fl. Napol. 1(Prodr.): xvi. 1811 ≡ *Specularia falcata* (Ten.) A. DC., Monogr. Campan.: 345. 1830.
= *Prismatocarpus scaber* Lowe in Trans. Cambridge Philos. Soc. 6: 538. 1838 ≡ *Legousia scabra* (Lowe) Gamisans, Cat. Pl. Vasc. Corse: 100. 1985 ≡ *Legousia falcata* var. *scabra* (Lowe) Meikle, Fl. Cyprus 2: 1055, 1897. 1985.
= *Specularia castellana* Lange, Ind. Sem. Hort. Haun.: 25. 1855 ≡ *Legousia castellana* (Lange) Samp., Herb. Port.: 127. 1913 ≡ *Legousia falcata* subsp. *castellana* (Lange) Jauzein, Fl. Champs Cultiv.: 131. 1995.
Distribution (Fig. 9): all of the Mediterranean area including Madeira, N Africa and SW Asia.

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