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## Taxonomic Status of the Neglected *Ophrys sphegodes* subsp. *grammica* in the Balkan Peninsula

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**ABSTRACT:** Since its description, the taxon *Ophrys sphegodes* subsp. *grammica* has been considered endemic to Greece. The morphological and chorological data of this taxon have been overlooked because the name has been used as a synonym for *O. sphegodes* subsp. *taurica* in most publications and online databases. Recently discovered *Ophrys* populations in Serbia were identified as *O. sphegodes* subsp. *grammica*. As these populations represent the northernmost point of distribution of this taxon, we provided data on the morphology, flowering season, and ecology. To determine the taxonomic status of this taxon, we performed comparative morphological analyses, comparing them to other populations of affined taxa (*O. sphegodes* subsp. *grammica*, *O. sphegodes* subsp. *taurica*, and *O. sphegodes* subsp. *sphegodes*). The analysis included 23 morphometric characters, performed on a total of 120 individuals. Examined populations of *O. sphegodes* subsp. *grammica* are closely associated with populations of *O. sphegodes* subsp. *sphegodes*, with near overlap in the values of the characteristics evaluated, indicating that this subspecies is more morphologically similar to the typical one than to *O. sphegodes* subsp. *taurica*. Although *O. sphegodes* subsp. *grammica* and *O. sphegodes* subsp. *sphegodes* are morphologically similar, the flowers of the first taxon are smaller, as evidenced by the length and width of the stigmatic cavity, as well as the length of the petals and labellum. The labellum of *O. sphegodes* subsp. *grammica* is shorter than the dorsal sepal, and this feature distinguishes it from both the typical subspecies and *O. sphegodes* subsp. *taurica*, where it is longer. The results of this study clearly show that the name *O. sphegodes* subsp. *grammica* should not be equated with *O. sphegodes* subsp. *taurica*, since it is a morphologically distinct and well-defined taxon.

**KEYWORDS:** Balkan Peninsula; morphometrics; *Ophrys sphegodes* complex

### 1 Introduction

Given its unique relationship with pollinators, the genus *Ophrys* L., a member of the Orchidaceae family, has caught the interest of numerous scientists, including Darwin himself [1], as a model organism for the study of floral evolution. The species of this genus are perennial herbaceous geophytes with a predominant distribution in the Mediterranean area, and one of the centers of diversity is the Balkan Peninsula [2]. The genus *Ophrys* is one of the genera that have had several taxonomic treatments and the most significant increase in the number of species in recent decades [3–6]. Depending on the methodology applied in the circumscription of the genus and the applied species concepts, the number of species varies significantly. Molecular methods, primarily sequencing different parts of the nuclear and plastid genomes, recognize up to ten species [6–9]. In contrast, there are 350 species [5], of which a considerable number have been recognized and described mostly based only on morphology. Some researchers have argued that



the interactions between *Ophrys* species and their pollinators offer more valuable insights than neutral markers due to their crucial role in the process of speciation [10,11]. *Ophrys* species are known to attract a limited number of pollinator species [12] by emitting floral scents that mimic sex pheromones [13,14]. Variations in these floral scents can lead to shifts in pollinator species, potentially facilitating reproductive isolation among populations of the same species and promoting speciation [15]. Taxonomic confusion arising from conflicting views regarding which criteria should be used to delimit species in this genus could have significant consequences for conservation [16]. *Ophrys sphegodes* complex is one of the most taxonomically difficult groups within the genus, with the number of species varying from 3 to 48 [4,17]. Many of the newly proposed taxa seem to be artificial and merely extremes in the cline of variation [18]. On the other hand, several recently recognized taxa do appear to be isolated by flowering time and pollinator specificity [18]. Since the distribution and morphology of these species are not sufficiently documented, some authors have decided to treat this entire complex of species as several subspecies of *O. sphegodes* [19,20]. *Ophrys sphegodes* subsp. *grammica* (B. Willing & E. Willing) Kreutz was described as a subspecies of *O. mammosa* Desf. [21]. Delforge et al. [22] elevated it to the species status. Antonopoulos [23] and Delforge [4] also recognized this taxon as a species. In the Catalogue of European Orchids, Kreutz [24] introduced a new nomenclatural combination: *O. sphegodes* subsp. *grammica*. However, this name is more commonly used as a synonym for *O. sphegodes* subsp. *mammosa* (Desf.) Soo ex E. Nelson [19,20]. In Niketić et al. [25], the name *O. sphegodes* subsp. *grammica* is treated as a synonym of *O. sphegodes* subsp. *taurica* (Aggeenko) Soó ex Niketić & Djordjevic, which is a new nomenclatural combination for *O. sphegodes* subsp. *mammosa*. In Serbia seven taxa have been recorded from the genus *Ophrys* [26], including two from the *O. sphegodes* complex. Morphometric studies are traditionally used in species delimitation of orchids [27–30] as well as in genus *Ophrys* [31–34]. Phenotypic differentiation among taxa is often more significant than genetic data [35], but using morphometric characters for taxonomic purposes is relevant in the genus *Ophrys* [35]. This may be attributed to the fact that morphometric traits, which are likely subject to selection pressures to align with the morphology of pollinators [36], may undergo more rapid evolutionary changes and be less affected by hybridization compared to neutral genetic markers [15].

The aims of the study were: a) to clarify the taxonomic status of *Ophrys sphegodes* subsp. *grammica* based on comparative morphological and morphometric analyses, and to compare it with *O. sphegodes* subsp. *taurica* and the type subspecies, b) to provide a morphological description and habitat preferences for *O. sphegodes* subsp. *grammica* from the northernmost point of distribution (Serbia) and c) to estimate the IUCN threatened status of this taxon, new to Serbia.

## 2 Material and Methods

*Ophrys sphegodes* subsp. *grammica* was discovered for the first time in Serbia in April 2023, on Svrljiške Mts., and data on its distribution, habitat preference, flowering period, population size, and morphology were obtained. One specimen from each site, without underground organs for vegetative propagation, was collected and deposited in the general collection of the Herbarium of the University of Novi Sad (BUNS) [37].

### 2.1 Morphological Analysis

Morphological analyses were carried out on a total of nine populations and 120 individuals (Table 1). The location of the populations is given with less precise coordinates due to the high risk of endangerment of these species in nature and to prevent their exploitation. Aboveground vegetative organs of 8–15 plants from each population were measured in the field, and one well-developed flower was removed from each of them and placed in an alcohol:glycerol:water solution (4:4:2) for morphological and morphometric description. The flowers were dissected and photographed with a Leica DFC 290 HD camera (Leica Microsystems

GmbH, Wetzlar, Germany). To preserve the three-dimensional shape of the dissected flowers, they were measured using a calibrated sliding scale immediately after dissection. After photographing, finer details were measured using Leica LASV software (Leica Microsystems GmbH, Wetzlar, Germany). Character selection and measurement methodology followed Bateman [6] and Paušić [38] with the exclusion of speculum characters due to a different method of flower preservation, as well as the additions of new characters made by the authors of this study. In total, 23 floral traits were measured with an accuracy of 0.1 mm.

**Table 1:** Sampled taxa and populations of the *Ophrys sphegodes* subspecies in the Balkan Peninsula. Abbreviations: n—number of individuals used for morphometric analyses, N and E—geographical coordinates

Population code	Taxon	Locality	n	N (°)	E (°)
ERD	<i>sphogodes</i>	Serbia, Erdelj	15	45.190	19.708
HOR	<i>sphogodes</i>	Serbia, Horgoš	15	46.147	19.913
GOR	<i>sphogodes</i>	Croatia, Goranec	10	45.899	16.080
DOR	<i>grammica</i>	Serbia, Donji Rinj	15	43.300	22.295
DOL	<i>grammica</i>	Serbia, Dolac	15	43.307	22.198
SKA	<i>grammica</i>	Greece, Skaloti	8	41.419	24.283
AYL	<i>taurica</i>	Greece, Aisymi-Leptokarya	15	41.034	25.944
TAX	<i>taurica</i>	Greece, Taxiarches	12	41.257	24.207
NSG	<i>taurica</i>	Greece, Nestos Gorge	15	41.118	24.752
Total			120		

Material identification was done according to Willing and Willing [21] and Delforge [4]. The material was also compared with the digitized holotype specimen in the herbarium of the Botanic Garden and Botanical Museum Berlin-Dahlem (B) (<http://herbarium.bgbm.org/object/B100178004>) (accessed on 22 May 2025) [39]. The morphological characteristics of the *O. sphegodes* subsp. *grammica* populations from Serbia were compared with literature data [4,17,19,20,23] for *O. sphegodes* subsp. *grammica*, *O. sphegodes* subsp. *sphogodes*, and *O. sphegodes* subsp. *taurica* (Table 2). The morphological description of *O. sphegodes* subsp. *grammica* from Serbia is based on our study and obtained results.

## 2.2 Statistical Analysis

Measurement data for each plant (Table 1) were summarized in an Excel spreadsheet (Microsoft Corporation, Redmond, WA, USA). The data collected were analysed using the software Statistica for Windows, version 13 (TIBCO Software Inc., Santa Clara, CA, USA). The mean, standard deviation, and coefficient of variation values were obtained for each morphometric trait. Statistical analyses included descriptive statistics, testing of statistical significance (ANOVA), and multivariate analyses (discriminant and principal component analysis).

**Table 2:** Comparative morphological characteristics of *O. sphegodes* subsp. *sphegodes*, *O. sphegodes* subsp. *taurica*, *O. sphegodes* subsp. *grammica*, and populations from Serbia. Descriptions according to Nelson [17], Delforge [4], Pedersen and Faurholdt [19], Antonopoulos [23], Kühn et al. [20]

Character/ Taxon	subsp. <i>sphegodes</i>	subsp. <i>taurica</i>	subsp. <i>grammica</i>	Serbian populations
Number of flowers	(2-)5-9(-12)	(2-)5-12(-18)	2-11	3-16
Sepals	Bright green, white-green, yellowish, occasionally with rose tinge; oval-lanceolate; 8-14 × (2-)3-7 mm	Olive green to pale green; lateral sepals bicolor (green/purplish); oval lanceolate; 9-19 × 4-9 mm	Green to white, sometimes speckled pink, relatively pale; oval-lanceolate; 9-12 × 5-7 mm	Pale green, sometimes tinted pink around edges and central nerve; ovate 9.5-14.5 × 4.8-7.8 mm
Petals	Spreading, glabrous or with finely hairy margins; greenish-yellow, olive green or pale green; when green often edged red; oblong to linear-lanceolate; margins flat to strongly undulate; 4-10(-11) × 2.5-4.5(-5) mm	Glabrous or velvety; yellowish green, ochre, olive green or purple, sometimes suffused with red rust; narrowly triangular to lanceolate; margins flat to undulate; 5-13 × 1.5-4.5 mm	Glabrous to velvety; green to ochre-brown; narrowly triangular to lanceolate; 5-8 × 1.5-3 mm	Velvety; yellow-green, green, pink-green, edges sometimes red; margins flat to slightly undulate; lanceolate to triangular; 5.7-10.3 × 2.3-3.9
Lip	Entire to occasionally more or less three-lobed; blackish, medium brown to pale reddish-brown, sometimes with 1mm wide yellow margins; longer than dorsal sepal; 9.5-16 × 9-18 mm	Entire, rarely slightly three-lobed; orbicular to broadly oboval; reddish brown or dull purplish brown, with a thin paler brownish, purple, rarely yellow hairless margins; approximately as long as the dorsal sepal; 9-20 × 9-22 mm	Entire, rarely slightly three-lobed; orbicular to near oboval; reddish brown, olive green, yellowish-brown, rather pale; margins paler yellow, pale orange-green 9-13 × 9-13 mm	Entire to strongly three-lobed; elliptic, sometimes obovate; olive-green to red-brown; with wide (up to 2 mm) yellow to green margins; 8.3-12.9 × 7.3-12 mm
Lip margins	Brownish submarginal band of hairs, basal part more or less shaggy, sometimes slightly shorter on the distal half	Submarginal band of hairs velvety to shaggy, greyish on basal part, the distal part velvety to subglabrous	Submarginal hairs on basal half dense, greyish on shoulders, short on distal half	Submarginal band of hairs on basal half dense and long, white to grey; short and velvety on distal half
Front edge	Emarginate, rarely rounded around a small terminal point (appendage) or lacking	Rounded to acuminate with a small terminal point, pendant to near horizontal	Acuminate (prolonging margins of lip); terminal point small to absent	Acuminate, rarely rounded, terminal point small
Basal swellings (bulges)	Absent to obliquely conical (rarely more than 1/4 as large as the stigmatic cavity)	Obliquely conical; at least half as large as the stigmatic cavity	Small, rounded	Rounded; almost as large as the stigmatic cavity 1.1-3 mm

(Continued)

Table 2 (continued)

Character/ Taxon	subsp. <i>sphogodes</i>	subsp. <i>taurica</i>	subsp. <i>grammica</i>	Serbian populations
Speculum (mirror)	Greyish to bluish, sometimes finely edged white; H-shaped	Grey, bluish grey or lilac; sometimes finely edged white; H-shaped or two parallel longitudinal bands	Greyish, rarely edged white; H-shaped	White to grey, H-shaped or two parallel longitudinal bands
Stigmatic cavity	Rounded, constricted at base; external walls drab, tinted green, ochre to reddish	Blackish; constricted at base	Pale, olive-green to pale orange-green	Rounded, constricted at base, external walls white with hints of brow; 2.2–4 mm
Basal field	Reddish brown to pale olive green, paler than the centre of the lip	Blackish	Pale, olive-green to pale orange-green	Pale green to orange-green, lighter than the rest of the labellum
Pseudo- eyes	Greenish–grey, sometimes encircled with pale greenish	Whitish to pale blue; with a greyish central spot	Greenish, shiny, rather drab	Pale green, green to green-grey
Flowering period	(March–) April–May (–June)	March–April (–May)	(Late March–) April–June	Late April
Number of flowers	(2–)5–9(–12)	(2–)5–12(–18)	2–11	3–16

### 2.3 Distribution and Ecology

The distribution of the new taxon for Serbia is presented on a map generated in QGIS 3.42 (QGIS Association, Bern, Switzerland) [40] with a 10 × 10 km grid using the Universal Transverse Mercator (UTM) projection.

The geological substrate was determined using a 1:100,000 scale geological map of the study area. Habitat type was determined using the EUNIS habitat classification [41]. The total number of individuals at each site was used to assess population size. The IUCN Red List categories and criteria [42] were used to determine the endangerment status of *O. sphogodes* subsp. *grammica* in Serbia.

## 3 Results

### 3.1 *Ophrys sphogodes* subsp. *grammica* in Serbia

*Ophrys sphogodes* subsp. *grammica* (B. Willing & E. Willing) Kreutz, Kompend. Eur. Orchid.:116 (2004), Fig. 1.

Homotypic Synonyms: *Ophrys mammosa* subsp. *grammica* B. Willing & E. Willing, Mitteilungsbl. Arbeitskreis Heimische Orchid. Baden-Württemberg 17(4):523 (1985); *Ophrys grammica* (B. Willing & E. Willing) Devillers-Terschuren & Devillers, Naturalistes Belges 72(3):101 (1991).

Holotype: GREECE, Kastoria, 2,8 km O Ptelea (Kas 87), Erodierte, fast bewuchsfreie Sand-steinhänge, Sa, 740 m, UTM:EK 0879, 18 May 1985, leg. B. & E. Willing 1694 (B 10 0178004!).



**Figure 1:** *Ophrys sphegodes* subsp. *grammica*–Svrljiške Mts. (Eastern Serbia, 23 April 2023). Photo B. Radak

### 3.1.1 Morphological Description of *Ophrys sphegodes* subsp. *grammica* from Serbia

Plants are slender, 13–63 cm tall, with 3–16 small flowers in a loose inflorescence. Sepals pale green, lateral sepals sometimes tinged pink at margin and central nerve, ovate,  $9.5\text{--}14.5 \times 4.8\text{--}7.8$  mm. Dorsal sepal longer than the labellum. Petals velvety, yellow–green, green to pinkish–green, margins sometimes red, lanceolate to triangular, margins flat to slightly undulate,  $5.7\text{--}10.3 \times 2.3\text{--}3.9$  mm. Lip entire to strongly trilobed, elliptic to obovate; olive green to reddish brown; with broad yellow to green margin, acuminate, rarely rounded, terminal point small,  $8.3\text{--}12.9 \times 7.3\text{--}12$  mm. Basal part of the lip and basal swelling with long and dense grey to white hairs. Basal swellings rounded; almost as large as the stigmatic cavity,  $1.1\text{--}3.0 \times 2.2\text{--}4.1$  mm. Speculum white to grey, H-shaped or two parallel longitudinal bands. Stigmatic cavity rounded,

constricted at base, external walls white with a hint of brown. Basal field pale green to orange–green, lighter than the rest of the lip. Pseudo–eyes pale green, green to green–grey (Table 2).

### 3.1.2 Habitat and Ecology of *Ophrys sphegodes* subsp. *grammica* from Serbia

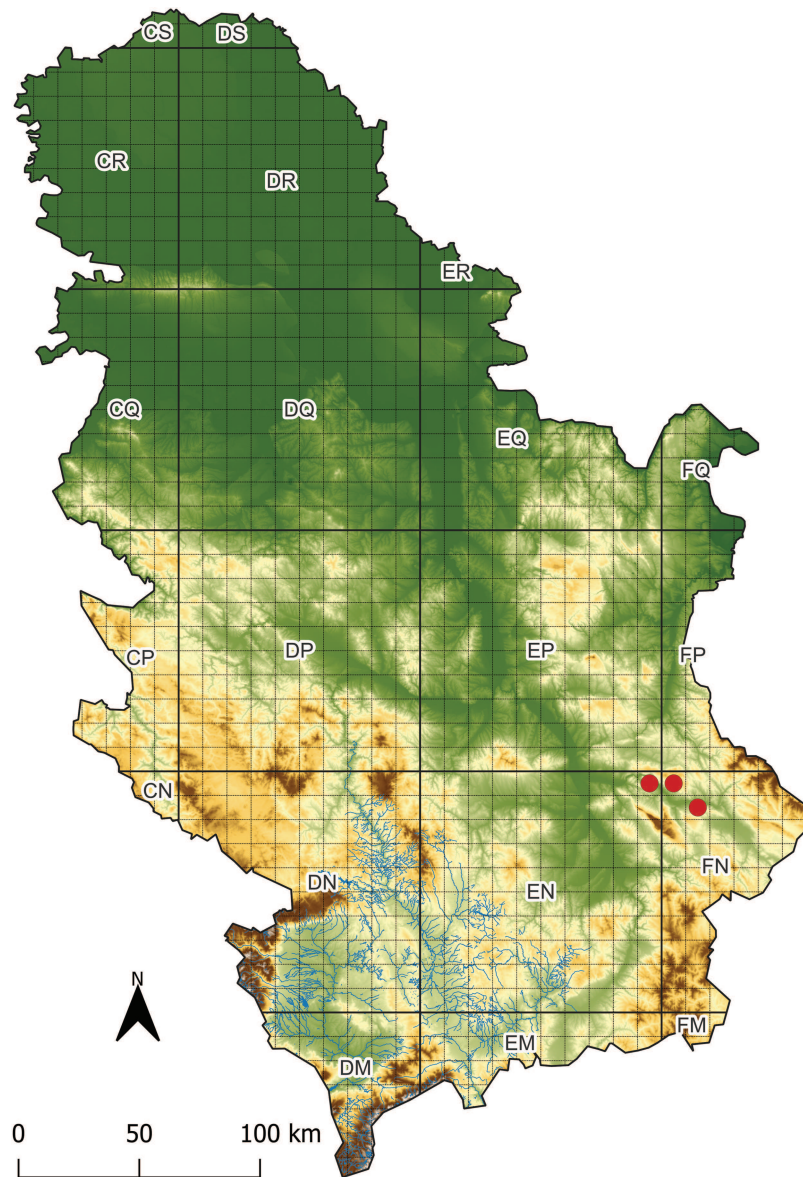
In Serbia, this taxon was found on a substrate predominantly composed of layered limestones, between 294 and 884 m a.s.l. At the Donji Rinj site, the herbaceous layer covered up to 95%, dominated by the following species: *Artemisia alba* Turra, *Carex humilis* Leyss., *Chamaecytisus hirsutus* (L.) Link, *Festuca valesiaca* Schleich. ex Gaudin, *Fragaria vesca* L., *Globularia cordifolia* L., *Hieracium pilosella* L. *Plantago media* L., *Potentilla incana* P. Gaertn., B. Mey. & Scherb., and *Satureja kitiaibelli* Wierzb (ass. *Potentillo-Caricetum humilis* R. Jov. 1955). Individual dwarf specimens of *Crataegus monogyna* Jacq and *Juniperus communis* L. have been recorded. According to the EUNIS classification, this habitat type belongs to Continental dry grassland (true steppe) (Code R1B). The herbaceous layer cover at sites Dolac and Ljubatovica was less substantial than the previous location (about 30%), and according to EUNIS this habitat belongs to Temperate and submediterranean thorn scrub (Code S35). The following species were identified at the site: *Acer monspessulanum* L., *Carpinus orientalis* Mill, *Cornus sanguinea* L., *Genista tinctoria* L., *J. communis*, *Pyrus communis* L., *Prunus spinosa* L., *Quercus pubescens* Willd., and *Rosa canina* L. The herbaceous layer consisted of: *Agrimonia eupatoria* L., *Campanula lingulata* Rchb., *Clematis vitalba* L., *Clinopodium vulgare* L., *Euphorbia cyparissias* L., *Galium verum* L., *G. cordifolia*, *H. pilosella*, and *Teucrium chamaedrys* L (ass. *Carpinetum orientalis serbicum* Rudski 1940 emend. B. Jovanović 1953).

### 3.1.3 Distribution of *Ophrys Sphegodes* subsp. *grammica* in Serbia

*Ophrys sphegodes* subsp. *grammica* has been recorded at three locations in the Svrljiške Mts. in Eastern Serbia (Fig. 2): Dolac, N 43.317, E 22.194, UTM EN99, limestone, 294 m a.s.l., Exp E 26°, 23 April 2023, leg. B. Radak, A.Vlku, J. Peškanov s.n. (BUNS 2-0043); Donji Rinj, N 43.300, E 22.295, UTM FN09, limestone, 865 m a.s.l., Exp S 24°, 23 April 2023, leg. B. Radak, A. Vlku, J. Peškanov s.n. (BUNS 2-0044); Ljubatovica, N 43.237, E 22.394, UTM FN18, limestone and dolomite, 350 m a.s.l., Exp S 25°, 24 April 2023, leg. B. Radak, A. Vlku, J. Peškanov s.n. (BUNS 2-0045).

### 3.1.4 Population Size and Conservation Status of *Ophrys sphegodes* subsp. *grammica* from Serbia

In April 2023, 265 specimens of *O. sphegodes* subsp. *grammica* were found in full bloom on an area of 100 m<sup>2</sup> near the village of Dolac. Another smaller group of 38 specimens was found not far from the previous population. Between the villages of Donji Rinj and Gornji Rinj, a total of 134 specimens were found, 34 of which had just begun to flower. The remaining specimens were found in rosettes. At the Ljubatovica site, only two specimens were found, and they were in full bloom. At all sites combined, 339 flowering specimens were found. Applying the categories and criteria of the IUCN Red List, the current threatened status of *O. sphegodes* subsp. *grammica* on the territory of Serbia is classified as Endangered (EN): B2ac(iv)+C2b. Based on the number of mature individuals and the fact that individuals of this species were found in only three locations, its area of occupancy (AOO) is estimated to be less than 500 km<sup>2</sup>. As far as we know, the threatened status of *O. sphegodes* subsp. *grammica* in Greece has not yet been determined. At all three sites, the expansion of the surrounding shrub vegetation, occurring to varying degrees, poses a potential threat to the continued existence of this species' habitat. There is a quarry near the Dolac site, and its expansion, as well as the related operations (construction and traffic), could completely degrade the habitat at this site.



**Figure 2:** Distribution of *Ophrys sphegodes* subsp. *grammica* in Serbia. Map created using the Free and Open Source QGIS [40]

### 3.2 Morphometric Analysis

#### 3.2.1 Descriptive Statistics

Basic statistical parameters were processed at both the taxon and population levels (Table 1). The coefficient of variation analysis reveals that the entire sample at the taxon level exhibits moderate variability (CV = 10%–30%) for the majority of the examined morphometric features (Table 3).

**Table 3:** Descriptive statistics for analysed morfometric traits of 120 individuals from 9 populations of three *a priori* defined *Ophrys sphegodes* subspecies. Abbreviations: M—arithmetic mean, SD—standard deviation, CV—coefficient of variation (%), TAUR—subsp. *taurica*, SPHE—subsp. *sphgodes*, GRAM—subsp. *grammica*

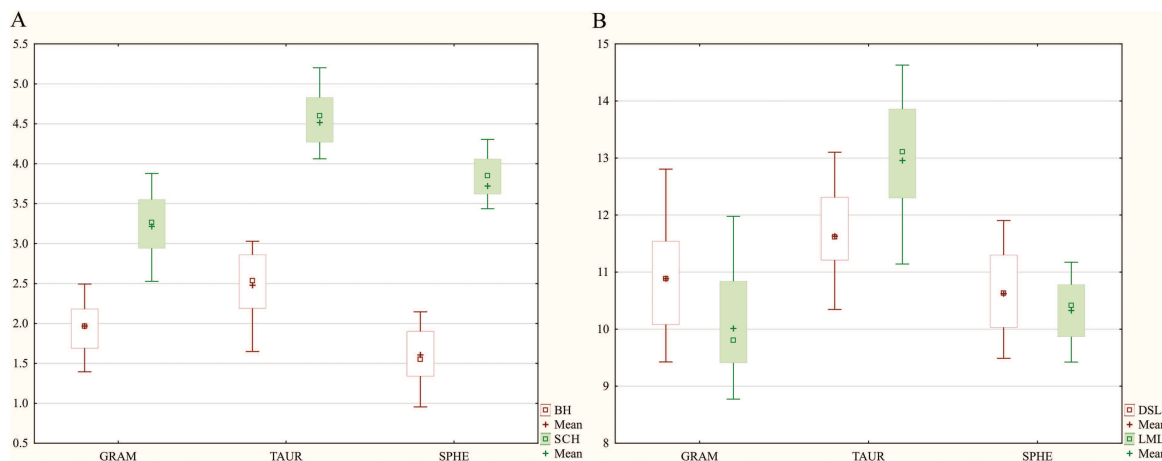
Traits	Descriptive parameter	TAUR	SPHE	GRAM	Traits	Descriptive parameter	TAUR	SPHE	GRAM
BL	M	15.028	20.961	21.982	LML	M	12.956	10.328	10.013
	SD	2.988	3.632	2.988		SD	1.191	0.659	0.945
	CV%	19.881	17.328	17.569		CV%	9.194	6.382	9.437
BMW	M	5.056	5.959	6.337	AL	M	0.723	0.273	0.421
	SD	0.904	0.984	0.904		SD	0.293	0.211	0.214
	CV%	17.881	16.514	17.218		CV%	40.468	77.531	50.730
OL	M	15.230	12.414	13.182	BH	M	2.479	1.607	1.967
	SD	1.932	2.880	1.932		SD	0.514	0.395	0.419
	CV%	12.684	23.203	23.093		CV%	20.736	24.606	21.314
DSL	M	11.630	10.621	10.884	LMW	M	12.166	9.059	9.406
	SD	1.091	0.817	1.091		SD	1.304	1.025	0.968
	CV%	9.379	7.697	12.816		CV%	10.722	11.316	10.296
DSMW	M	5.582	4.923	5.052	LBW	M	11.678	8.524	9.087
	SD	0.638	0.490	0.638		SD	1.349	1.002	0.913
	CV%	11.439	9.950	13.394		CV%	11.549	11.752	10.043
DSW	M	5.493	4.845	4.958	AW	M	1.510	0.658	1.130
	SD	0.615	0.488	0.615		SD	0.473	0.436	0.427
	CV%	11.205	10.081	13.708		CV%	31.323	66.184	37.798
PL	M	7.540	7.242	7.225	SCW	M	4.091	4.190	3.914
	SD	0.771	0.689	0.771		SD	0.430	0.505	0.423
	CV%	10.219	9.517	13.589		CV%	10.512	12.058	10.815
PMW	M	3.415	3.090	2.951	NW	M	2.614	2.924	3.152
	SD	0.451	0.477	0.451		SD	0.622	0.426	0.539
	CV%	13.191	15.451	13.535		CV%	23.792	14.564	17.092
PW	M	2.260	2.610	2.315	WB	M	7.402	5.589	5.395
	SD	0.504	0.372	0.504		SD	1.626	0.851	0.875
	CV%	22.318	14.237	14.389		CV%	21.961	15.233	16.222
LSL	M	11.904	11.457	11.475	GL	M	6.740	6.635	6.914
	SD	1.115	1.071	1.115		SD	0.723	0.567	0.745
	CV%	9.363	9.352	11.222		CV%	10.732	8.549	10.774
LSMW	M	6.459	5.247	5.651	SCH	M	4.516	3.721	3.215
	SD	0.667	0.382	0.667		SD	0.412	0.500	0.457
	CV%	10.327	7.275	10.507		CV%	9.115	13.428	14.231

(Continued)

**Table 3 (continued)**

Traits	Descriptive parameter	TAUR	SPHE	GRAM	Traits	Descriptive parameter	TAUR	SPHE	GRAM
	M	6.203	4.897	5.334					
LSW	SD	0.659	0.419	0.659					
	CV%	10.617	8.562	11.665					

The apical appendage features are the only variables in the zone of increased variability (CV = 30%–50%). Individual populations of each species exhibit a similar pattern of variability in the examined morphometric features, with the majority of characters falling into the zone of moderate variability. Populations of *O. sphegodes* subsp. *sphegodes* has a somewhat higher proportion of characters in the low variability zone than the other two. According to the ANOVA results, the most statistically significant differences between the examined taxa occur for four characters: lip maximum length (LML), labellum width at the level of the bulges (LBW), lip maximum width (LMW), and stigmatic cavity height (SCH). For all four characters, *O. sphegodes* subsp. *taurica* has higher mean values, on average 1.5 to 3 mm higher than the other two subspecies. In comparison to *O. sphegodes* subsp. *sphegodes*, *O. sphegodes* subsp. *grammica* populations (SKA, DOR, DOL) have lower mean values for lip maximum length and stigmatic cavity height, but greater values for labellum width (Table 3). Comparing the height of bulges to stigmatic cavity height, we observed that in *O. sphegodes* subsp. *taurica* populations, bulges are always larger than half the height of the stigmatic cavity, whereas in *O. sphegodes* subsp. *sphegodes* populations, they are always smaller (Fig. 3A). *Ophrys sphegodes* subsp. *grammica* populations (SKA, DOR, DOL) have bulges that are two-thirds the height of the stigmatic cavity, with lower mean values compared to *O. sphegodes* subsp. *taurica*. Comparing the length of the labellum and the dorsal sepal, *O. sphegodes* subsp. *taurica* shows a longer labellum than the dorsal sepal; instead, *O. sphegodes* subsp. *grammica* shows the opposite pattern. The labellum length in *O. sphegodes* subsp. *sphegodes* populations have slightly higher mean values than the dorsal sepal (Fig. 3B).



**Figure 3:** Box Plot of multiple variables grouped by taxon, based on 120 individuals from 9 populations of three *a priori* defined *Ophrys sphegodes* subspecies. (A) Comparative overview of morphometric characters; SCH—stigmatic cavity height and BH—bulges height; (B) Comparative overview of morphometric characters; DSL—dorsal sepal length and LML—labellum maximum length. Abbreviations: GRAM—subsp. *grammica*, TAUR—subsp. *taurica*, SPHE—subsp. *sphegodes*

### 3.2.2 Analysis of Variance

A one-factor analysis of variance was conducted on two levels: between all sampled populations at the whole sample level and between three *a priori* defined taxa. Each morphometric feature examined revealed considerable interpopulation variability among all populations in the total samples (Table A1). The characters that statistically differ the most between the analysed populations are: lip maximum length (LML), labellum width at the level of the bulges (LBW), lip maximum width (LMW), and stigmatic cavity height (SCH). At the taxa level, a large number of characters show statistically significant differences between the observed subspecies. The same characteristics between taxa, as at the population level, exhibit high F values. Furthermore, characters that differ statistically among the investigated taxa are: lateral sepal maximum width (LSMW), appendage length (AL), appendage width (AW), bract length (BL), and bulges height (BH) (Table 4).

**Table 4:** Analysis of variance (ANOVA) for 23 morphometric characters when a taxon is used as a grouping variable. All morphometric characters that show statistically significant variability between taxa for  $p < 0.05$  are marked in bold

Traits	F	p
Bract length	<b>46.939</b>	<b>0.000</b>
Bract maximum width (BMW)	<b>17.782</b>	<b>0.000</b>
Ovary length (OL)	<b>12.443</b>	<b>0.000</b>
Dorsal sepal length (DSL)	<b>8.963</b>	<b>0.000</b>
Dorsal sepal maximum width (DSMW)	<b>13.623</b>	<b>0.000</b>
Dorsal sepal width at the half-length (DSW)	<b>13.731</b>	<b>0.000</b>
Petal length (PL)	1.911	0.153
Petal maximum width (PMW)	<b>11.655</b>	<b>0.000</b>
Petal width at the half-length (PW)	<b>8.418</b>	<b>0.000</b>
Lateral sepal length (LSL)	1.956	0.146
Lateral sepal maximum width (LSMW)	<b>49.534</b>	<b>0.000</b>
Lateral sepal width at the half-length (LSW)	<b>54.562</b>	<b>0.000</b>
Lip maximum length (LML)	<b>115.301</b>	<b>0.000</b>
Appendage length (AL)	<b>36.490</b>	<b>0.000</b>
Bulges height (BH)	<b>94.166</b>	<b>0.000</b>
Lip maximum width (LMW)	<b>39.381</b>	<b>0.000</b>
Labellum width at the level of the bulges (LBW)	<b>95.730</b>	<b>0.000</b>
Appendage width (AW)	<b>37.368</b>	<b>0.000</b>
Stigmatic cavity width (SCW)	<b>3.678</b>	<b>0.028</b>
Neck width (NW)	<b>10.176</b>	<b>0.000</b>
Width between bulges (WB)	<b>35.512</b>	<b>0.000</b>
Gynostemium length (GL)	1.659	0.195
Stigmatic cavity height (SCH)	<b>82.841</b>	<b>0.000</b>

### 3.2.3 Canonical Discriminant Analysis (CDA)

In the *a priori* classification, when a taxon is used as a grouping variable, only three individuals (2.50%) were determined differently; that number is slightly greater when the population is utilized and is 11.67%,

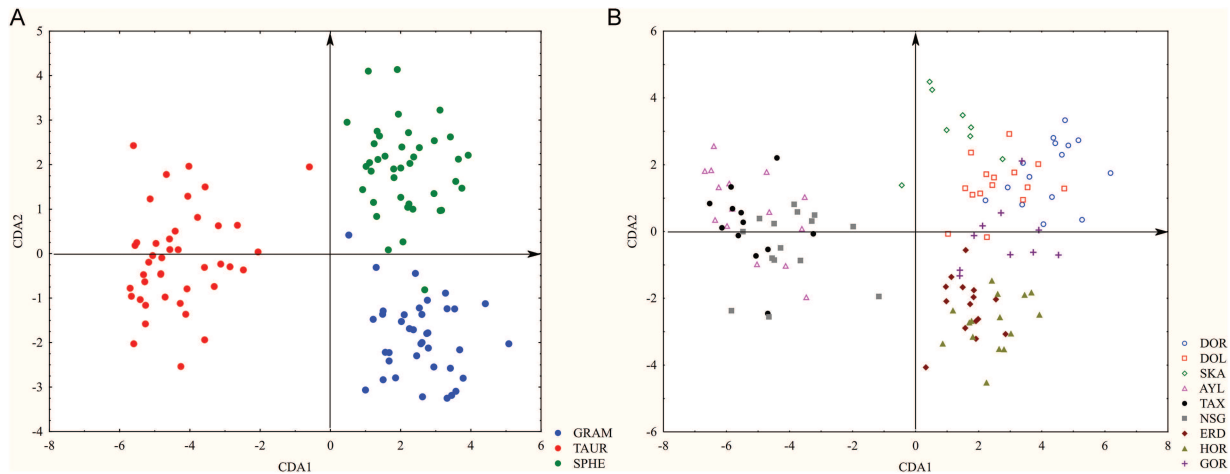
i.e., 14 individuals. The first two axes defined more than 79.64% of the group discrimination (CDA 1, 65.17% and CDA 2, 14.47%) (Table 5).

**Table 5:** Canonical discriminant analysis (CDA)—standardized coefficients for canonical variables on the first two canonical axes, derived from discriminant function analysis on 23 morphometric characters from 9 populations (120 individuals)

Traits	CDA1	CDA2	CDA3
Bract length	0.710	0.022	-0.356
Bract maximum width (BMW)	0.316	-0.159	0.493
Ovary length (OL)	-0.162	0.339	-0.625
Dorsal sepal length (DSL)	-0.243	0.180	-0.811
Dorsal sepal maximum width (DSMW)	0.484	-0.459	-1.696
Dorsal sepal width at the half-length (DSW)	-0.555	0.471	1.628
Petal length (PL)	0.210	0.066	0.404
Petal maximum width (PMW)	-0.263	0.109	-0.140
Petal width at the half-length (PW)	0.440	-0.159	-0.502
Lateral sepal length (LSL)	0.013	-0.496	0.611
Lateral sepal maximum width (LSMW)	0.002	0.827	-0.026
Lateral sepal width at the half-length (LSW)	-0.342	-0.106	0.273
Lip maximum length (LML)	-0.712	-0.502	-0.139
Appendage length (AL)	0.161	0.246	-0.331
Bulges height (BH)	-0.091	0.426	0.175
Lip maximum width (LMW)	-0.203	-0.513	0.228
Labellum width at the level of the bulges (LBW)	0.223	0.783	-0.100
Appendage width (AW)	-0.219	0.280	0.670
Stigmatic cavity width (SCW)	0.134	-0.778	-0.156
Neck width (NW)	0.481	0.361	0.505
Width between bulges (WB)	-0.063	0.128	-0.161
Gynostemium length (GL)	0.286	0.309	0.156
Stigmatic cavity height (SCH)	-0.466	-0.847	0.362
Eigenval	14.199	3.154	1.627
Cum. prop	0.652	0.796	0.871
Cum. prop (%)	65.17	79.64	87.11

The morphological space of the first two CDA axes clearly separates the three investigated taxa. *Ophrys sphegodes* subsp. *taurica* (Aisymi-Leptokarya, Greece (AYL), Taxiarches, Greece (TAX), Nestos Gorge (NSG)) and populations of the other two taxa separate along the first axis (Fig. 4A). The following characteristics contribute the most to differentiation with respect to the first axis: lip maximum length (LML), bract length (BL), dorsal sepal width at the half-length (DSW), neck width (NW), and dorsal sepal maximum width (DSMW). The features that contribute the most to discriminating along the second axis are stigmatic cavity height (SCH), lateral sepal maximum width (LSMW), labellum width at the level of the bulges (LBW), stigmatic cavity width (SCW), and lip maximum width (LMW). Regarding this axis, the populations of *O. sphegodes* subsp. *grammica* (Donji Rinj, Serbia (DOR), Dolac, Serbia (DOL), Skaloti, Greece (SKA)) restricted to the positive space of this axis are distinguished from the populations of *O. sphegodes* subsp.

*sphogodes* (Erdelj, Serbia (ERD), Horgoš, Serbia (HOR), Goranec, Croatia (GOR)). Separation between *O. sphogodes* subsp. *sphogodes* and *O. sphogodes* subsp. *grammica* was considerably more evident when a taxon was employed as the grouping variable. However, in the analysis of populations, the mixing of individuals of these two taxa appears to be restricted to a narrow zone (Fig. 4B).



**Figure 4:** Canonical discriminant analyses of 120 individuals from 9 populations belonging to three *Ophrys sphogodes* subspecies, based on 23 morphometric characters. (A) The first two discriminant axes when a taxon is applied as a grouping variable; (B) the first two discriminant axes when a population is applied as a grouping variable. Abbreviations: GRAM—subsp. *grammica*, TAUR—subsp. *taurica*, SPHE—subsp. *sphogodes*

#### 4 Discussion

The taxon *Ophrys mammosa* subsp. *grammica* was discovered in the area of Kastoria Prefecture in northwestern Greece by Willing and Willing [21], and since then it has been considered endemic to northwestern Macedonia, with Mount Grammos as the center of its range [23]. More recently, populations of this taxon have also been recorded in other regions of Greece, on mountain ranges in eastern Macedonia [43] and in southern Pindos, the Ionian Islands, and Crete [2,23]. Although Delforge [4] suggests that this species may also occur in Albania, to our knowledge, its occurrence has not been documented in Albania, North Macedonia, or Bulgaria. Its range outside Greece has been found only in the territory of Montenegro [44,45]. The finding of the subspecies *O. sphogodes* subsp. *grammica* in the Svrljiške Mts. is its first record for Serbia. Moreover, this record represents the northernmost point of range for this taxon. According to Antonopoulos [23], *O. sphogodes* subsp. *grammica* flowers throughout May and the first half of June, where Delforge [4] gives the flowering period from late March to June. All three populations in Serbia were found in late April (23rd and 24th). The populations at Dolac and Ljubatovica, located at lower altitudes (294–350 m a.s.l.), were in full bloom, while the population at Donji Rinj (884 m a.s.l.) was just beginning to flower. The habitats where this subspecies has been discovered in Serbia are usually warm and dry, with substrates that have a slightly acidic to slightly basic reaction and are low in nutrients. The occurrence of *O. sphogodes* subsp. *grammica* in such habitats is consistent with the statements in the literature about this taxon and the genus in general. In Greece, *O. sphogodes* subsp. *grammica* grows mainly on skeletal soils formed by erosion of limestone or serpentine. It can occur on short grasslands, in shrublands, oak forests, pine forests, pine forest clearings, and alpine meadows at elevations up to 1200 m above sea level [4,23,43]. Despite having somewhat differing perspectives on the *O. mammosa* complex, Devillers and Devillers-Terschuren [3] and Delforge [4] both identify *O. sphogodes* subsp. *grammica* as a species within it. Although some authors split

*O. mammosa* into several species, Pedersen and Faurholdt [19] treat the whole complex as one subspecies of *O. sphegodes*. Various forms of data, such as molecular, morphometric, and chemical, often do not align [35], resulting in differing interpretations of species boundaries. Methods based on genetic data are then judged as more reliable; however, speciation sometimes leaves no signature at the level of neutral markers, and in that case, neutral marker-based methods may fail to detect species boundaries by excluding the data that are the most informative [35]. Although some floral features may resemble the body forms of female wasps, other floral features most likely serve other purposes, such as effective pollinium transfer [46]. Rakosy et al. [47] proposed that there is strong pollinator-mediated selection for the labellum's morphological characteristics, which pollinators use for functional interaction. On the other hand, relaxed selection and other stochastic factors are more likely to have an impact on features that are not utilized in effective interaction. Collecting data on the range of morphological variability, as well as their pattern of variability, is crucial to understand which traits are involved in population divergence and to establish the evolutionary scenario underlying the speciation process [48]. According to Delforge [4] pattern of coloration of *O. grammica* (basal field green-orange, lighter than centre of lip) could define a group consisting of *O. grammica*, *O. herae* M. Hirth & H. Spaeth, *O. cretensis* (H. Baumann & Künkele) Paulus, and *O. caucasica* Woronow ex Grossh. There is confusion in identification between *O. grammica* and *O. herae*, especially in the coastal parts of Greece [23]. The main difference according to Paulus [49] between these two species is their pollinators, *O. grammica* is pollinated by *Andrena nigroaenea*, while *O. herae* is pollinated by *Andrena thoracica*. Identification of *O. grammica* is more certain in higher altitudes of continental Greece [2]. Apart from the different pollinators, *O. herae* has a larger and darker labellum and flowers earlier than *O. grammica* [23]. *Ophrys cretensis* is considered endemic to the Greek islands, occurring mainly in Crete [19]. According to Antonopoulos [23], this species flowers later than *O. herae* and has a different pollinator (*Andrena vachali* subsp. *creticola*). On the other hand, the presence of *O. grammica* in Crete was also confirmed. This calls into question the separation of these taxa, and suggests the possibility of a single widespread taxon. From the results of this study, it is evident that the populations of *O. sphegodes* subsp. *taurica* are morphologically significantly different from those of *O. sphegodes* subsp. *grammica*, and it is clear that the names of these two taxa should not be used as synonyms. Despite their geographical proximity, no *O. sphegodes* subsp. *grammica* populations from our sample, including one from Greece, were grouped with *O. sphegodes* subsp. *taurica* in the morphospace of CD analysis. The fact that a greater number of studied characters fall into the zone of intermediate variability, with values closer to the lower limits of the zone, suggests that certain morphological characteristics are rather stable. The height of *O. sphegodes* subsp. *grammica* specimens from Serbia varies considerably, as does the number of flowers, and differ from the values given in the literature for this taxon. The lateral sepals are never distinctly bicoloured, as in *O. sphegodes* subsp. *taurica*, although pink pigment occasionally occurs along the margins and central nerve, which also occurs in the type subspecies. The flower of *O. sphegodes* subsp. *grammica* are smaller than those of the other two taxa. It has a pale, predominantly olive-green lip and long, dense grey to white hairs on the shoulders and basal swellings (Fig. 1). The lip has a broad yellow-green margin and is shorter than the dorsal sepal, both characteristics that distinguish it from the other two taxa. In addition, this subspecies differs from the type subspecies by the front edge, which is acuminate. The general coloration of the flower differs markedly from *O. sphegodes* subsp. *taurica*, both in the colour of the sepal and lip, and in the colour of the basal field and pseudoeyes. The basal field is pale green or orange-green and always lighter than the rest of the lip, while the colour of the pseudoeyes is often the same as the basal field.

## 5 Conclusion

The results of this study show that the *O. sphegodes* subsp. *grammica* is a morphologically well-defined taxon, which is best seen from the size and shape of the labellum, as well as the colour of the basal field and labellum. This taxon should not be equated with *O. sphegodes* subsp. *taurica*, since there is a greater number of morphological characters that clearly separate them. The near overlap in the values of analysed morphological characters with *O. sphegodes* subsp. *sphegodes* indicates that this taxon should not be treated as a species. This study provides a foundation for future research on the distribution, ecology, and morphology of the *Ophrys sphegodes* complex in the Balkan Peninsula. A more thorough analysis of the morphology of these taxa should reveal additional features that distinguish similar taxa in this region and help to clarify the taxonomy of this complex.

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**Conflicts of Interest:** The authors declare no conflicts of interest to report regarding the present study.

## Appendix A

**Table A1:** Analysis of variance (ANOVA) for 23 morphometric characters. All morphometric characters show statistically significant interpopulation variability for  $p < 0.05$

Traits	F	<i>p</i>
Bract length	17.737	0.000
Bract maximum width (BMW)	11.621	0.000
Ovary length (OL)	13.470	0.000
Dorsal sepal length (DSL)	6.09	0.000
Dorsal sepal maximum width (DSMW)	4.854	0.000
Dorsal sepal width at the half-length (DSW)	4.691	0.000
Petal length (PL)	3.91	0.000
Petal maximum width (PMW)	4.215	0.000
Petal width at the half-length (PW)	2.804	0.000
Lateral sepal length (LSL)	4.39	0.000
Lateral sepal maximum width (LSMW)	14.11	0.000
Lateral sepal width at the half-length (LSW)	15.71	0.000
Lip maximum length (LML)	31.72	0.000
Appendage length (AL)	10.345	0.000

(Continued)

**Table A1 (continued)**

Traits	F	p
Bulges height (BH)	<b>23.427</b>	<b>0.000</b>
Lip maximum width (LMW)	<b>9.946</b>	<b>0.000</b>
Labellum width at the level of the bulges (LBW)	<b>23.320</b>	<b>0.000</b>
Appendage width (AW)	<b>10.6281</b>	<b>0.000</b>
Stigmatic cavity width (SCW)	<b>5.20</b>	<b>0.000</b>
Neck width (NW)	<b>8.196</b>	<b>0.000</b>
Width between bulges (WB)	<b>13.541</b>	<b>0.000</b>
Gynostemium length (GL)	<b>3.12</b>	<b>0.000</b>
Stigmatic cavity height (SCH)	<b>36.05</b>	<b>0.000</b>

Note: The values that are statistically significant are presented in bold.

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