

Morphological characterization of three natural hybrid orchid taxa, new for Serbia, Montenegro and North Macedonia

Boris Đ. Radak^{1,*}, Aleksa Z. Vlku¹, Jovan M. Peškanov¹, Vlado S. Matevski^{2,3} and Goran T. Anačkov¹

¹Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia

²Institute of Biology, Faculty of Natural Sciences and Mathematics, Ss Cyril and Methodius University in Skopje, Arhimedova 3, 1000 Skopje, North Macedonia

³Macedonian Academy of Sciences and Arts, Bul. Krste Petkov Misirkov 2, 1000 Skopje, North Macedonia

*Corresponding author: boris.radak@dbe.uns.ac.rs

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Abstract: During floristic investigations of Serbia, Montenegro and North Macedonia from 2011-2018, orchid specimens from the genus *Anacamptis*, possible hybrids, with characteristics intermediary to species already described for these countries, were discovered. These specimens, together with all potential parent species, were subjected to morphometric analysis in order to determine their hybrid status and characters that distinguished hybrids from parent taxa. Taxonomic studies have included the processing of quantitative and qualitative characters. A total of 60 characters were analyzed, of which 45 quantitative and 15 qualitative. Analysis of quantitative and qualitative characters included 82 specimens – 60 parents and 22 hybrids. Statistical analyses included descriptive and discriminate statistics and multivariate analyses. Hybrid specimens in general had intermediate values of measured characters with different degrees of similarity with one of the parent species. On the other hand, they have higher mean values of some floral characters that may have an evolutionary potential. The possible taxonomic importance of hybrid characters is discussed. Results confirmed that the analyzed specimens are natural orchid hybrids (*A. × parvifolia*, *A. × timbali* and *A. × gennarii*) and represent new plant taxa for the flora of investigated countries.

Keywords: *A. × parvifolia*; *A. × timbali*; *A. × gennarii*; morphometry; multivariate analysis

INTRODUCTION

Interspecific natural hybridization among vascular plants is a common phenomenon and has played a significant role in their speciation [1,2]. Thus, it is regarded as one of the leading mechanisms in plant evolution. It is likely that hybridization might be frequent among still divergent taxa and within the more recent and advanced angiosperms families, such as Orchidaceae [3]. The main force in the evolution of Orchidaceae is allopolyploid speciation via hybrid formation. This process is well documented for the Euro-Mediterranean genera *Dactylorhiza* Neck. ex Nevski, *Spiranthes* Rich. and *Serapias* L., but it does not seem to be involved in *Anacamptis* Rich. speciation processes [4].

The genus *Anacamptis*, with eleven species, is widespread in Europe, western Asia, and North Africa

[5,6]. Regarding pollination ecology, members of the genus *Anacamptis* can be divided into two unequal groups of species: the first contains members whose flowers have large quantities of nectar and heavy fragrance (*A. coriophora* (L.) R. M. Bateman, Pridgeon & M. W. Chase and *A. sancta* (L.) R. M. Bateman, Pridgeon & M. W. Chase), and a second group that includes all of the remaining species which are nectarless and with a weak scent [7]. The second group, which is a food-deceptive group of species, attracts pollinators by mimicking the color of rewarding species growing in the same habitats [8]. They are mostly pollinated by generalist pollinators and show considerable overlap in their pollinator community [9]. As a result of this, many species of *Anacamptis* can hybridize with each other, but their specialized floral biology prevents this from more frequent events [10]. Hybridization

is, in most cases, restricted to the F1 generation, with a small number of hybrid offspring [11]. Genetic and/or ethological barriers, mainly intrinsic postzygotic isolation, prevent the occurrence of extensive hybridization and introgression and maintain species boundaries among these orchids [9,11,12]. In many cases, hybrids have been consistently found when parent species are present but are often restricted to a few plants, which is an indication of the efficiency of isolation mechanisms. Hybrid swarms have been detected, but are rare [7,13]. The mechanism by which pair of species produce hybrids in small numbers or in large numbers is still unclear [14]. In the case of hybrid swarm formation, the majority of hybrid specimens show morphological intermediacy between parents, but in some individuals, parental characters are expressed in full [7].

Hybrid assessment in orchids has been carried out using several different criteria and techniques, such as assessing their morphology, distribution, crossing experiments, karyology, enzyme electrophoresis, various protein, and DNA characters [7,10,13-18]. However, for most hybrid taxa from the genus *Anacamptis*, except original descriptions, detailed morphological studies that could be used for their morphological characterization and their positioning among other members of this genus in regional floras, is still lacking.

In this paper, three natural orchid hybrids from the genus *Anacamptis* were examined by employing morphological techniques. In two cases (*A. × parvifolia* (Chaub.) H. Kretzschmar, Eccarius & H. Dietr. and *A. × timbali* (Velen.) H. Kretzschmar, Eccarius & H. Dietr.), one of the parent species is from *Laxiflorae* (Soó & G. Keller) H. Kretzschmar, Eccarius & H. Dietr. (*A. laxiflora* (Lam.) R. M. Bateman, Pridgeon & M. W. Chase and *A. palustris* (Jacq.) R. M. Bateman, Pridgeon & M. W. Chase, respectively) section, while the other one is from *Coriophorae* (Parl.) H. Kretzschmar, Eccarius & H. Dietr. (*A. coriophora* (L.) R. M. Bateman, Pridgeon & M. W. Chase subsp. *fragrans* (Pollini) R. M. Bateman, Pridgeon & M. W. Chase and *A. coriophora* (L.) R. M. Bateman, Pridgeon & M. W. Chase subsp. *coriophora*, respectively) section. The last one, *A. × gennarii* (Rchb. F.) H. Kretzschmar, Eccarius & H. Dietr., is a hybrid of species from *Moriones* (Parl.) H. Kretzschmar, Eccarius & H. Dietr. (*A. morio* (L.) R. M. Bateman, Pridgeon & M. W. Chase subsp.

caucasica (K. Koch) H. Kretzschmar, Eccarius & H. Dietr.) and *Papilionaceae* (Parl.) H. Kretzschmar, Eccarius & H. Dietr. (*A. papilionacea* (L.) R. M. Bateman, Pridgeon & M. W. Chase) sections. These hybrid taxa have not been registered for the area of Serbia, Montenegro and North Macedonia [19-21]. Such hybrid individuals often go unnoticed by botanists and their characteristics are included in the range of variability of one of the parent species. Since these taxa are of great conservation importance, knowing the limits of their variability is crucial in the selection of conservation priorities, such as whether to protect parent, hybrid or both taxa [22-25]. Therefore, the main goal of this study was to provide a morphological description of three hybrid orchid taxa new to three Central Balkan states and to explore patterns of variability of specific characters in the hybrids relative to the parent species.

MATERIALS AND METHODS

Plant material

During floristic investigations of Serbia, Montenegro and North Macedonia between 2011 and 2018, three hybrid orchid taxa (*A. × parvifolia*, *A. × timbali* and *A. × gennarii*), new for the investigated countries, were registered. Identification of hybrid specimens and parent species was done according to Flora Europaea and selected orchid monographs [5,26-30]. The first orchid hybrid taxon, *A. × gennarii*, was registered in North Macedonia in 2016 on the Pletvar Mountain Pass in the vicinity of the village of Pletvar. *A. × parvifolia* was registered in Montenegro in 2011, in Ulcinj Long Beach. The third hybrid taxon *A. × timbali* was registered in the vicinity of the villages Hajdukovo and Bački Vinogradi (N. Serbia) in a meadow alongside the road to the village Horgoš, in May 2018. For the purpose of morphometric analyses, 60 parent (10 of each parent species) and 22 hybrid specimens were sampled. Hybrid specimens were collected as follows: *A. × parvifolia* (3), *A. × gennarii* (10) and *A. × timbali* (9). Only the aboveground parts of the plants were sampled. The collected specimens of hybrids and their respective parents were herbarized and deposited in the Herbarium of the University of Novi Sad (BUNS) [31].

Morphology

Aboveground vegetative organs (stem and leaves) and inflorescence were measured with a caliper on the herbarized plant material. One flower per plant, together with the corresponding bract, was harvested from 1/3 to 1/2 of the distance from the base to the apex of the inflorescence in order to minimize the effect of the substantial diminution in flower size along this axis [32]. The flowers were rehydrated by thermal treatment in an ethanol solution, dissected and then scanned and measured in the software Digimizer ver. 5.3.2. In order to delineate differences in morphological trait expression between parent and hybrid taxa, 60 characters were used. Character and measurement methodology was followed [15,33] with some modifications as well as additions of new characters made by the authors of this study. These characters described stem and inflorescence (12), leaves (8), bract (8), ovary (2), labellum (15), spur (5), petals (4), lateral sepals (3), dorsal sepal (3). Of these characters, 45 were quantitative and 15 were qualitative. Quantitative characters can be divided into 33 morphometric, 4 meristic and 8 characters that represent indices calculated on the basis of proper linear morphometric characters.

Data analysis

Measurement data for individual plants were summarized in an Excel spreadsheet. Means, sample standard deviations and coefficients of variation were calculated for every morphometric character and morphometric index for each taxon (Supplementary Tables S1-S3). Meristic and qualitative characters and morphometric indices were not subjected to multivariate analyses. Meristic character values were presented for each analyzed taxon (Supplementary Tables S1-S3) while character states for 15 qualitative characters were grouped into seven plant parts and presented in order to describe differences among parent and hybrid taxa (Table 5). Morphometric characters were analyzed by multivariate methods using the software Statistica for Windows ver. 13.5. Statistical analyses included descriptive statistics, testing of statistical significance (ANOVA) and multivariate analyses (discriminant and principal component analysis) [34,35]. Characters that are not applicable to all taxa (lip median lobe base width; lip right lateral lobe width; lip right lateral lobe length;

length to the lip median lobe base and lip median lobe length), were excluded from the multivariate analyses. Also, the character "plant height" was excluded because of the extremely high values compared to other characters. Two other characters (stem diameter 1 and stem diameter 2) were excluded in order to prevent possible collinearity between them, which can alter the results of the analyses. Thus, multivariate analyses were performed on 25 morphometric characters.

RESULTS

Geographical (location, latitude, longitude and elevation), ecological (vegetation) and biological (phenophase) data for newly recorded hybrid orchid taxa, as well as for their parent species, for Serbia, Montenegro, and North Macedonia, are given in Table 1.

Analysis of variance (ANOVA)

All analyzed morphometric characters show statistically significant interpopulation variability (Table 2).

Comparative morphological analysis of hybrids and their parents

Compared to parent taxa, all three hybrid taxa have intermediate values for the largest number of investigated characters. Taxon *A. × gennarii* is intermediate for 28 of 36 characters, and *A. × parvifolia* and *A. × timbali*, for 31 and 32 of 41, respectively (Supplementary Tables S1-S3). The values of these characters in all three hybrids were closer to the parent species, which had larger dimensions. On the other hand, for several characters hybrid taxa had higher values than their parents: *A. × parvifolia* for six, and *A. × gennarii* and *A. × timbali* for seven, or lower values for three, one and two characters, respectively (Supplementary Tables S1-S3). *A. × gennarii* and *A. × timbali* had four characters in the zone of increased variability (CV 30-50%) while *A. × parvifolia* had only one. The vast majority of characters, in all investigated taxa, belong to a group of low variable (CV<10%) or moderately variable characters (CV 10-30%). Also, *A. × timbali* and *A. × parvifolia* had a higher number of low variable characters compared to their respective parents. The exception is *A. × gennarii*, which had an intermedi-

Table 1. Analyzed *Anacamptis* taxa: geographical, ecological and biological data of studied populations, number of sampled individuals, collector names and voucher numbers.

Taxon	CN ¹	Locality ²	Vegetation	Phenophase	Indiv. ³	Coll./Date ⁴	V ⁵
<i>A. coriophora</i> subsp. <i>coriophora</i>	CorC	Hajdukovo SRB	ass. <i>Rhinantho borbasii</i> - <i>Festucetum pratensis</i> , drier areas	full bloom	10	Radak B, Vlku A, 13.05.2018.	2-1456
<i>A. palustris</i> subsp. <i>palustris</i>	PalP	N46°07'12.8" E19°53'31.7"	ass. <i>Rhinantho borbasii</i> - <i>Festucetum pratensis</i> , wetter areas	beginning of flowering	10		2-1457
<i>A. × timbali</i>	Timb	88 m a.s.l.	ass. <i>Rhinantho borbasii</i> - <i>Festucetum pratensis</i> , drier areas	full bloom	9		2-1458
<i>A. coriophora</i> subsp. <i>fragrans</i>	CorF	Ulcinj Long Beach MNE	<i>Onobrychis caput-galli</i> community	full bloom	10	Radak B, 02.06.2012.	2-1459
<i>A. laxiflora</i>	LaxL	N41°53'19.8" E19°18'25.7"	ass. <i>Juncetum maritime-acuti</i>	full bloom	10		2-1460
<i>A. × parvifolia</i>	Parv	1 m a.s.l.	<i>Onobrychis caput-galli</i> community	full bloom	3		2-1461
<i>A. morio</i> subsp. <i>caucasica</i>	MorC	Pletvar NM	ass. <i>Astragalo-Helianthemetum marmorei</i>	beginning of flowering	10	Radak B, 19.05.2016.	2-1462
<i>A. papilionacea</i>	PapP	N41°22'02.8" E21°39'24.2"		beginning of flowering	10		2-1463
<i>A. × gennarii</i>	Genn	1008 m a.s.l.		full bloom	10		2-1464

¹CN – code name²SRB – Serbia; MNE – Montenegro; NM – North Macedonia³Indiv. – number of sampled individuals⁴Coll./Date – collector names/date⁵V – voucher number, Herbarium BUNS

ate number of characters with respect to parents, and more characters in the zone of increased variability than them (Supplementary Table S1-S3).

The higher values for the characters “number of flowers” and “number of other leaves” in *A. laxiflora*

represent the lower limit in the variability of these characters in its hybrid *A. × parvifolia*. Values for these two characters in hybrid specimens have a broad overlapping zone with the second parent, *A. coriophora* subsp. *fragrans*, which has higher values (Table S2). *A. × timbali* has a very similar variability pattern. The maximum number of leaves in *A. palustris* represents the lower value for this character in the hybrid, which has overlapping values for this character with *A. coriophora*, whose

Table 2. Analysis of variance (ANOVA) for 33 morphometric characters. All morphometric characters show statistically significant interpopulation variability for $p < 0.05$.

Characters	F	p
Bract length	18.984	0.000000
Bract maximum width	50.018	0.000000
Bract width at the half-length	28.154	0.000000
Ovary length	30.513	0.000000
Ovary maximum length	32.514	0.000000
Spur length	25.168	0.000000
Spur width	21.108	0.000000
Lateral sepal length	15.514	0.000000
Lateral sepal width at the half-length	33.761	0.000000
Lateral sepal maximum width	25.930	0.000000
Petal length	56.089	0.000000
Petal width at the half-length	68.835	0.000000
Petal maximum width	73.961	0.000000
Dorsal sepal length	24.451	0.000000
Dorsal sepal width at the half-length	57.959	0.000000

Characters	F	p
Dorsal sepal maximum width	51.112	0.000000
Lip maximum width	61.037	0.000000
Lip median lobe base width	22.2205	0.000000
Lip right lateral lobe width	25.916	0.000000
Lip maximum length	57.446	0.000000
Lip right lateral lobe length	75.526	0.000000
Length to the lip median lobe base	88.785	0.000000
Lip median lobe length	19.188	0.000000
Length to the top of lip median lobe	47.396	0.000000
Stem length	12.250	0.000000
Plant height	19.386	0.000000
Stem diameter 1	3.8025	0.003067
Stem diameter 2	6.2291	0.000049
Stem diameter 3	4.6631	0.000672
Leaf length	31.2226	0.000000
Leaf maximum width	3.7502	0.003368
Inflorescence length	9.8085	0.000000
Inflorescence width	11.077	0.000000

Table 5. Synopsis of 15 analyzed qualitative characters grouped into seven plant parts for all studied *Anacamptis* taxa.

Plant parts	<i>A. palustris</i>	<i>A. × timbali</i>	<i>A. coriophora</i> subsp. <i>coriophora</i>
Bract	entire; apex mostly subobtuse, rarely rounded; widest at the lower third, rarely at the lower quarter or at middle	entire; apex (rarely) subobtuse to acute; widest at the base, rarely at the lower third	entire; apex (mostly) subobtuse to acute; widest at the base, rarely at the lower third
Spur	cylindrical; apex rounded	intermediate form, rarely conical; apex mostly rounded, rarely subobtuse	conical; apex subobtuse
Lip	divided; clearly trilobate, rarely obscurely 3-lobed; sinuses shallow to (mostly) deep	divided; clearly trilobate, very rare obscurely 3-lobed; sinuses shallow to deep, mostly medium	divided; clearly trilobate; sinuses mostly deep, rarely medium
Lip median lobe	bilobed; mostly longer, rarely slightly longer or near equal as lateral lobes	mostly entire, rarely retuse, emarginate or bilobed; longer than lateral lobes	entire; longer than lateral lobes
Sepals	free, unfused	partially fused, only in the lower part	entirely fused
Leaves	basal rosette + arranged along the stem; longest leaf widest at the lower third, rarely at the middle, apex acute	basal rosette + arranged along the stem; longest leaf widest at the lower third, rarely at the middle, apex acute	basal rosette + arranged along the stem; longest leaf widest somewhere between lower third and middle, apex acute
Inflorescence	cylindrical, short to elongated	cylindrical to elongated cylindrical	short cylindrical to cylindrical
Plant parts	<i>A. laxiflora</i>	<i>A. × parvifolia</i>	<i>A. coriophora</i> subsp. <i>fragrans</i>
Bract	entire; apex acute or subobtuse; widest (mostly) at the lower third, rarely at the lower fifth or at the middle	entire; subobtuse; widest from the lower fifth to the lower third	entire; mostly subobtuse, very rare acute; widest (mostly) at the base, rarely at the lower fifth, lower quarter or lower third
Spur	cylindrical; apex mostly bilobed, rarely rounded	conical; apex rounded	conical; apex subobtuse
Lip	divided; clearly trilobate; sinuses mostly shallow, rarely medium or deep	divided; clearly trilobate; sinuses medium to deep	divided; clearly trilobate; sinuses mostly deep, rarely medium
Lip median lobe	truncated and obscurely bilobed, rarely entire; mostly far shorter than lateral lobes, rarely as long as or longer than lateral lobes	entire, rarely truncated and obscurely bilobed; longer than lateral lobes	entire; longer than lateral lobes
Sepals	free, unfused	free, unfused	entirely fused
Leaves	basal rosette + arranged along the stem; longest leaf widest at the middle, rarely at the lower third, apex acute	basal rosette + arranged along the stem; longest leaf widest around the lower third, apex acute	basal rosette + arranged along the stem; longest leaf widest (mostly) at the lower third, rarely at the middle or upper third, apex acute
Inflorescence	cylindrical to elongated cylindrical	elongated cylindrical	mostly cylindrical to elongated cylindrical
Plant parts	<i>A. morio</i> subsp. <i>caucasica</i>	<i>A. × gennarii</i>	<i>A. papilionacea</i>
Bract	entire; apex mostly rounded, rarely subobtuse; widest (mostly) at the lower third, rarely at the base or around the middle	entire; apex rounded, subobtuse or acute; widest at the lower third or middle	entire; apex mostly subobtuse, rarely acute or rounded; widest (mostly) at the middle, rarely at the lower third
Spur	cylindrical; apex rounded	cylindrical; apex mostly rounded, rarely truncated and obscurely bilobed	cylindrical, spur entrance wide; apex bilobed, rarely rounded
Lip	divided; clearly trilobate; sinuses shallow, rarely medium to deep	entire; lobes fused	entire; lobes fused
Lip median lobe	bilobed; longer than lateral lobes	NA	NA
Sepals	free, unfused	free, unfused	free, unfused
Leaves	basal rosette + arranged along the stem; longest leaf widest (mostly) at the middle, sometimes at the lower third, apex acute	basal rosette + arranged along the stem; longest leaf widest (mostly) at the lower third, rarely at the middle, apex acute	basal rosette + arranged along the stem; longest leaf widest at the middle, rarely at the lower third, apex acute
Inflorescence	cylindrical, sometimes short	ovoid, elongated, wide	ovoid, short to elongated

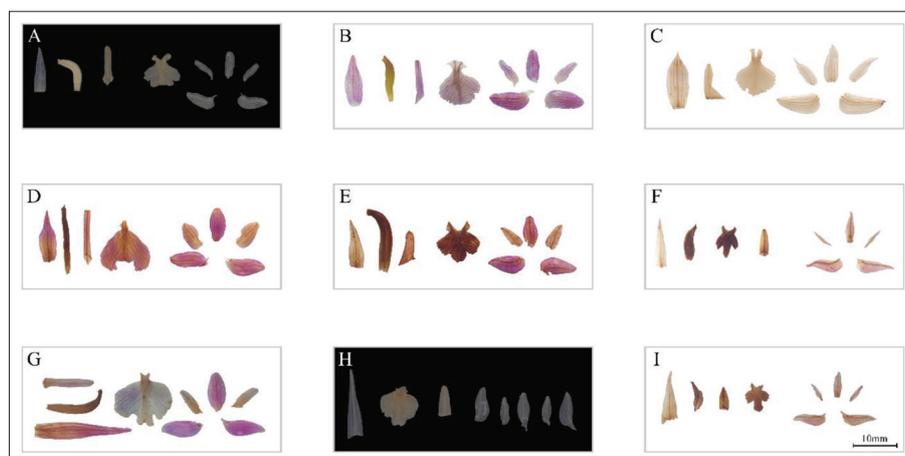


Fig. 1. Dissected flowers of nine analyzed *Anacamptis* taxa: A. *morio* subsp. *caucasica* (A), *A. x gennarii* (B), *A. papilionacea* (C), *A. laxiflora* (D), *A. x parvifolia* (E), *A. coriophora* subsp. *fragrans* (F), *A. palustris* (G), *A. x timbali* (H), *A. coriophora* subsp. *coriophora* (I). Scale bar represents a length of 10 mm.

values for this character are the highest. This hybrid is also characterized by the largest number of flowers as compared to the parents, but with a broad overlapping value with both of them (Supplementary Table S3).

Hybrid specimens often possess all or most of the qualitative character states that exist in parent taxa, but with a different frequency (Table 5). Most of the observed qualitative characters are either uniform or have the same character states in all analyzed taxa. On the other hand, the spur shape in *A. x gennarii* (Fig. 1B) is the same as in the parent taxon *A. morio* subsp. *caucasica* (Fig. 1A), but with larger dimensions, while the lip shape is very similar to that of another parent, *A. papilionacea* (Fig. 1C), with hardly visible indications of the existence of lobes. *A. x parvifolia* (Fig. 1E) resembles *A. coriophora* subsp. *fragrans* (Fig. 1F) in four qualitative characters (position of the broadest part of the leaf, lip shape, spur shape and shape of spur apex). However, unlike this parent, which has completely or almost completely fused lateral and dorsal sepals, the hybrid and *A. laxiflora* (Fig. 1D) do not possess this character. *A. x timbali* (Fig. 1H) specimens often have an emarginated or bilobed lip median lobe, similar to those in *A. palustris* (Fig. 1G). The bracts in *A. x timbali* are the broadest at the base, similar to those in *A. coriophora* (Fig. 1I).

Principal component analysis

The results of principal component analysis (PCA) showed that the first two axes describe a very large proportion of the total sample variability (74.62%), which is a necessary prerequisite if the obtained re-

Table 3. Principal component analysis (PCA) for 25 morphometric characters from all analyzed taxa – factor coordinates of the variables for the two first principal axes and the eigenvalues of the correlation matrix.

Characters	PCA 1	PCA 2
Bract length	-0.740001	0.179525
Bract maximum width	-0.857061	-0.279109
Bract width at the half-length	-0.786219	-0.502231*
Ovary length	-0.852340	0.017937
Ovary maximum length	-0.879517	0.056911
Spur length	-0.811822	0.173758
Spur width	-0.466108	-0.484610*
Lateral sepal length	-0.797060	-0.393942
Lateral sepal width at the half-length	-0.919890*	-0.130740
Lateral sepal maximum width	-0.875975	-0.374895
Petal length	-0.896842	-0.298925
Petal width at the half-length	-0.897734	0.157522
Petal maximum width	-0.908253*	0.152481
Dorsal sepal length	-0.838034	-0.318910
Dorsal sepal width at the half-length	-0.897033	0.245815
Dorsal sepal maximum width	-0.918554*	0.185248
Lip maximum width	-0.855229	0.295737
Lip maximum length	-0.928592*	-0.139717
Length to the top of lip median lobe	-0.901324*	-0.254950
Stem length	-0.506598	0.595860*
Stem diameter 3	-0.640299	0.161736
Leaf length	-0.442901	0.719315*
Leaf maximum width	-0.651775	-0.071383
Inflorescence length	-0.406877	0.735578*
Inflorescence width	-0.625564	0.444371
Eigenvalue	15.561924	3.091899
% Total variance	62.247696	12.367597
Cumulative % variance	62.247696	74.615293

* – characters that contributed most to the overall variability

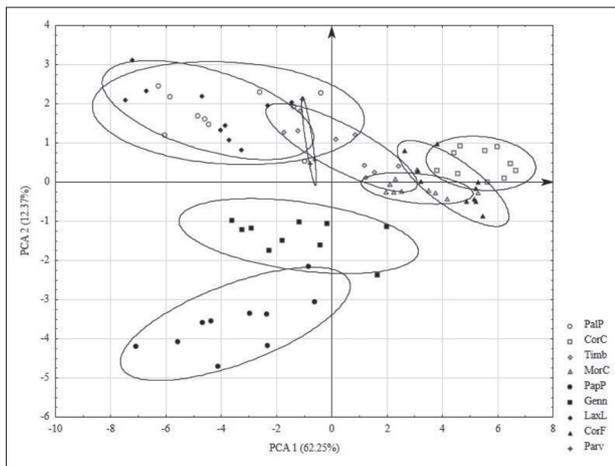


Fig. 2. Principal coordinate plot of the first two axes for 25 morphometric characters from all analyzed taxa (MorC – *A. morio* subsp. *caucasica*; Genn – *A. × gennarii*; PapP – *A. papilionacea*; LaxL – *A. laxiflora*; Parv – *A. × parvifolia*; CorF – *A. coriophora* subsp. *fragrans*; PalP – *A. palustris*; Timb – *A. × timbali*; CorC – *A. coriophora* subsp. *coriophora*).

sults are to be used to interpret the boundaries of the morphological variability of the investigated taxa. The characters that contributed most to the variability of the total sample on the first axis (PCA 1, 62.25%), were lateral sepal width at the half-length, petal maximum width, dorsal sepal maximum width, lip maximum length and length to the top of the lip median lobe; on the second axis (PCA 2, 12.37%) they were the length of the longest leaf, inflorescence length, stem length, bract width at the half-length and spur width (Table 3). The first axis separates on its negative side representatives of the *Laxiflorae* (LaxL and PalP) and *Papilionaceae* (PapP) sections from *Moriones* (MorC) and *Coriophorae* (CorC and CorF), which are on the positive side. The second axis separates species within the same sections. In the morphospace of the first two PCA axes, all three hybrid taxa occupy intermediate positions between the corresponding parent taxa (Fig. 2).

Canonical discriminant analysis

The first two axes defined more than 70% of the group discrimination (CDA 1, 42.15% and CDA 2, 30.20%). The characters that contributed most to the discrimination on the first axis were lip maximum length, dorsal sepal width at the half-length, petal maximum width, petal width at the half-length and lateral sepal length, whereas on the second axis they were bract

Table 4. Canonical discriminant analysis (CDA) – standardized coefficients for canonical variables on the first two canonical axes, derived from discriminant function analysis on 25 morphometric characters from all analyzed taxa.

Characters ¹	CDA 1	CDA 2
Bract length	0.50261	-0.49437
Bract maximum width	0.33336	-0.36937
Bract width at the half-length	0.02957	0.87285*
Ovary length	-0.88626	-0.27624
Ovary maximum length	0.38043	0.61918*
Spur length	0.82822	-0.07829
Spur width	-0.41386	0.24570
Lateral sepal length	-1.55305*	0.71105*
Lateral sepal width at the half-length	0.22377	0.48005
Lateral sepal maximum width	-0.30349	-0.46878
Petal length	0.80561	0.91397*
Petal width at the half-length	-1.29831*	0.08272
Petal maximum width	1.43402*	0.55774
Dorsal sepal length	0.02134	-0.23533
Dorsal sepal width at the half-length	1.35397*	-0.22730
Dorsal sepal maximum width	-0.95592	-0.75978*
Lip maximum width	0.03497	-0.52768
Lip maximum length	1.25023*	-0.00866
Length to the top of lip median lobe	-0.23567	-0.04254
Stem length	0.08983	-0.59205
Stem diameter 3	0.21854	0.18041
Leaf length	0.37108	-0.27491
Leaf maximum width	-0.18797	0.07489
Inflorescence length	-0.78624	-0.48199
Inflorescence width	-0.04089	-0.23318
Eigenvalue	31.73500	22.74037
Cumulative proportions	0.42145	0.72346
Cumulative proportions (%)	42.14542	72.34561

* – characters that contribute most to the discrimination of analyzed taxa

width at the half-length, ovary maximum length, dorsal sepal maximum width, petal length and lateral sepal length (Table 4). As with the PCA, this analysis discriminated between members of the sections *Laxiflorae* (LaxL and PalP) and *Papilionaceae* (PapP) along the positive side of the first axis, and *Moriones* (MorC) and *Coriophorae* (CorC and CorF) on the negative side. On the second axis, *A. papilionacea* (PapP) and *A. × gennarii* (Genn) showed clear separation from all other taxa. Also, along the negative side of the same axis, the two subspecies of *A. coriophora* (CorC and CorF) were separated, as well as the species from section *Laxiflorae*, *A. laxiflora* (LaxL), and *A. palustris* (PalP). Hybrid taxa were positioned in the morphospace between their respective parents (Fig. 3).

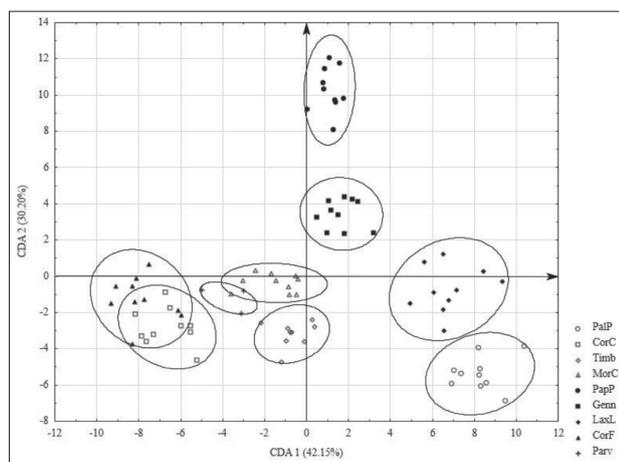


Fig. 3. Scatter plot of the first two canonical axes obtained by the discriminant analysis applied to 25 morphometric characters from all analyzed taxa (MorC – *A. morio* subsp. *caucasica*; Genn – *A. × gennarii*; PapP – *A. papilionacea*; LaxL – *A. laxiflora*; Parv – *A. × parvifolia*; CorF – *A. coriophora* subsp. *fragrans*; PalP – *A. palustris*; Timb – *A. × timbali*; CorC – *A. coriophora* subsp. *coriophora*).

DISCUSSION

All three newly registered hybrids for the region of Serbia (*A. × timbali*), Montenegro (*A. × parvifolia*) and North Macedonia (*A. × gennarii*) are known in botanical literature for some time [26–28], and there is plentiful evidence for their presence in Europe [5,16,36–40]. Hybridization between the parent species that resulted in these hybrids reflected their similar geographical distribution, overlapping habitat preferences and similar periods of flowering, but also the degree of genetic similarity [18]. One of the parent taxa is *A. morio* subsp. *caucasica*. There is insufficient knowledge about the distribution of this subspecies in the Balkans [5], so this is the first confirmation of its presence in the area of North Macedonia in the last 80 years [41].

A. papilionacea and its hybrid, *A. × gennarii*, are clearly separated from all the other analyzed taxa in the morphospace of both CDA axes, as confirmed by PCA, where a similar variability pattern was noted. This is the result of the specific floral morphology of these two taxa compared to all other analyzed – the absence of lip lobes. However, *A. × gennarii* inflorescence has intermediate characteristics in relation to parents. It resembles in shape *A. papilionacea* (ovoid, elongated), but the hybrid inflorescence is wider, and together with the different appearance of hybrid flow-

ers (smaller in all floral parts, except the lips, which are wider), it has a diagnostic importance in relation to this parent taxon. Additionally, the leaves of both parents are the widest at the middle of the leaf blade, whereas they are on the lower third in the hybrid.

Hybridization between *A. papilionacea* and *A. morio*, which results in the formation of *A. × gennarii*, is common and often results in the formation of hybrid swarms [5,30]. Hybrid specimens have wider inflorescences with more flowers with wider lips compared to their parents. Since these are characters with potential evolutionary significance from the standpoint of pollinator attraction [42–44], it could be assumed that in this way the hybrid individuals become more susceptible to pollinators than the parent specimens. However, regardless of the wider inflorescence with a higher number of flowers, the proportion of inflorescence length in the overall length of hybrid plants is lower than in the species *A. morio* subsp. *caucasica*, and the inflorescence has a lower flower density than in the other parent, *A. papilionacea*. However, the wider lips present in hybrids potentially represent a larger landing surface for pollinator insects. It is clear that the hybrid specimens have the highest values of vegetative characters, such as stem length, plant height and one of the characters of stem diameter. They also have the largest number of leaves, but the size of leaves is of intermediate value. This may be a consequence of microecological differences in habitat, but also of differences in ontogeny; at the time when both parent taxa were just beginning to bloom, the hybrid specimens were already in full flower.

Hybrids *A. × timbali* and *A. × parvifolia* have different pairs of parent species and subspecies, but all of them belong to two same sections: *Laxiflorae* (*A. palustris* and *A. laxiflora*) and *Coriophorae* (*A. coriophora* subsp. *coriophora* and *A. coriophora* subsp. *fragrans*). In addition to these two hybrid taxa, several others that have parents from the same groups of species and a similar combination of parental characteristics have been described [5,18,28,45]. Based on the large number of described hybrids and high frequency of their occurrence in nature, the phylogenetic similarity of these two groups was proposed [30]. The analyzed hybrid taxa were placed very close to each other in the morphospace of the first two discriminant axes, which indicates their morphological

similarity in the general appearance of the flowers, or it may be indirectly a consequence of a similar pattern of inheritance of characters or a set of characters from parents to hybrid progeny; however, our analyses cannot confirm this. Both hybrid taxa have a wider spur and a larger inflorescence relative to the total plant height than their parent taxa. Such changes can potentially influence modifications in pollinator specificity and have an impact on the hypothetical evolutionary future of hybrids [18]. In addition, *A. × parvifolia* has a longer inflorescence than its parent taxa and *A. × timbali* has a larger number of flowers than its parents. All of these characters have potential importance in pollinator attraction, which could lead to the isolation of hybrid individuals from parents. However, in the case of both hybrids, parents from the *Coriophorae* group have twice as many flowers per cm of inflorescence length than the hybrid taxa, and *A. coriophora* subsp. *fragrans* has 50% more flowers than its respective hybrid *A. × parvifolia*, which undermines the importance of registered changes in hybrids. Unfortunately, the presence of nectar in the flowers of both hybrid taxa was not checked in the field. The existence of such a character in hybrids may be very significant, since the representatives of the *Coriophorae* section are characterized by nectar production, and the *Laxiflorae* section are not.

The qualitative character that separates *A. × parvifolia* from both parents is the position of the widest part of the bract. *A. laxiflora* bracts are widest at the lower third of their length, in *A. coriophora* subsp. *fragrans* are widest at the base, whereas *A. × parvifolia* has bracts that are widest somewhere between these two positions. Some *A. × timbali* specimens have a spur that is intermediate in shape and size in relation to those of the parents. However, this spur shape is not stabilized in the hybrids, as some hybrid individuals have spurs that are identical to those of *A. coriophora*. *A. palustris* sepals are free, in *A. coriophora* they are entirely or almost entirely fused, while in *A. × timbali* they are only partially fused.

Individuals of all the analyzed hybrid taxa grew in the immediate vicinity (up to 0.5 m) of one parent species, while others grew at a distance of several to up to several tens of meters. In the case of *A. × gennarii* hybrids, there was no difference in the habitat conditions between the place where the hybrid,

together with one of the parents (*A. papilionacea*) lived, with regard to other parent taxa. Both parent taxa, *A. morio* subsp. *caucasica* and *A. papilionacea*, as well as the hybrid, were observed in thermophilous grassland (ass. *Astragalo-Helianthemetum marmorei*) on dolomite limestone at 1008 m a.s.l. [46]. Various orchid species grew syntopically at the same locality, such as *A. pyramidalis*, *A. laxiflora* subsp. *laxiflora*, *A. coriophora* subsp. *coriophora*, *Cephalanthera damasodium*, *Neotinea tridentata* subsp. *tridentata*, *Ophrys scolopax* subsp. *cornuta*, *O. sphegodes* and *Platanthera chlorantha*.

On the other hand, two other hybrids were found exclusively among individuals of the taxon from the *Coriophorae* section. Specimens of *A. × parvifolia* and those of the parent taxon *A. coriophora* subsp. *fragrans* (about one hundred instances) lived on the fixed coastal dunes with herbaceous vegetation (*Onobrychis caput-galli* community). About 20 m away from the location of the hybrid specimens, the second parent, *A. laxiflora* subsp. *laxiflora*, also a population of about 100 specimens, lived in a different habitat – Mediterranean salt meadows (ass. *Juncetum maritime-acuti*) [47]. Besides these three orchid taxa, specimens of *Serapias lingua* and *Serapias vomeracea*, were also registered on this locality, with dozens of specimens.

Eight individuals of *A. × timbali* lived among specimens of *A. coriophora* subsp. *coriophora*, in meadow vegetation (ass. *Rhinantho borbasii-Festucetum pratensis*). The second parent (*A. palustris* subsp. *palustris*) lived nearby (a few meters away), in the same vegetation, but on places with higher groundwater levels. On this locality, only one other orchid species, *Orchis militaris* subsp. *militaris* was registered, with only one specimen. All this indicates that despite some changes that took place in the morphology of the hybrids, there were no key differences in their ecological preferences in relation to both parents. In addition to reproductive isolation, this is a prerequisite for their formation as separate evolutionary units, independent from parent taxa [48,49].

Three registered *A. × parvifolia* individuals grew a few meters away from each other, but almost all individuals of the other two hybrid taxa were found growing in dense groups. This indicates that they originated from the same rootstock, which suggests

their recent clonal development from one or a few individuals. It can be assumed that all observed *A. × timbali* and *A. × gennarii* individuals are the products of only one hybridization event and that most of the hybrid individuals were formed by the clonal development of a few individuals that originated through hybridization of different parent species. This way of development and expansion of hybrid individuals was already recorded in other European orchids [3,18]. Considering the foregoing observation, the registered high morphological similarity among hybrid specimens was to be expected.

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Author contributions: BR designed the study, collected and performed the analysis of the plant material, performed dissection and measurement of flowers, statistical analyses and wrote the manuscript. AV participated in field research and data collection. JP performed measurements of vegetative plant organs and took part in data collection. VM reviewed the manuscript. GA supervised all activities and participated in field research, data analysis and manuscript preparation. All authors have revised and approved the final manuscript.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org/NewUploads/Uploads/Radak%20et%20al_Supplementary%20Information_4277.pdf