VERSITA

Biologia **66**/1: 55—63, 2011
Section Botany
DOI: 10.2478/s11756-010-0132-5

Morphometric and molecular (RAPD) analysis of six *Serapias* taxa from Croatia

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Abstract: The morphometric analyses and genetic variability assessed by RAPD markers have been used to analyse relations among six *Serapias* taxa from Croatia (*S. istriaca*, *S. pulae* originally described as hybrid, *S. ionica*, *S. vomeracea*, *S. lingua* and *S. cordigera*). *S. istriaca* distributed in southern Istria and the island of Lošinj and *S. pulae* stenoendemic taxon distributed only in southern Istria *S. ionica* is endemic to the Ionian and Dalmatian islands, while the remaining taxa are more widely distributed. The obtained results shows that the endemic *S. istriaca* is a well characterised taxon, that *S. pulae* is a hybrid between *S. istriaca* and *S. lingua* and that the hybrid is morphologically and genetically more similar to *S. lingua* than the second parental species *S. istriaca*. The division into the subsections *Steno-*, *Medio-* and *Platypetalae* is founded based on the floral morphology while the division into the sections *Serapias* and *Bilamellaria* is not evident in the quantitative morphological and genetic analyses. Furthermore, considerable genetic resemblance between *S. vomeracea* and *S. ionica* was established.

Key words: discriminant analysis; endemic orchids; hybridization index; molecular taxonomy; Istria

Introduction

The distribution of the *Serapias* genus is essentially Mediterranean: its range extends from the Caucasus in the east, to the Canary Islands and the Azores in the west, and to Brittany (France) in the north (Delforge 2006). *Serapias* is a monophyletic genus, isolated taxonomically and well characterised. Hence, it holds a special position within the *Orchidinae* subtribus (Baumann & Künkele 1989). Taxa within the genus are morphologically poorly defined, there are a number of very similar species, and thus their separation varies among authors. The problems lie in the difficulties in defining the species, which show an overlap in the variation of characteristics (Delforge 2006). Systematic studies based on morphology have utilized both quantitative and qualitative traits (Gölz & Reinhard 1980; Baumann & Künkele 1989; Martine & Gerbaud 1998; Lorenz 2001). A detailed study of the morphological variation using multivariate analysis of many morphological traits has rarely been undertaken Lorenz 2001; Pellegrino et al. 2005; Venhuis et al. 2007). *Serapias* has also been poorly studied at the molecular and genetic level (Venhuis et al. 2007).

The genus is taxonomically divided into two sections: the *Serapias* section and the *Bilamellaria* section (Baumann & Künkele 1989; Delforge 2006). The *Serapias* section, characterised by a rounded blackish boss at the base of the lip, comprises 6 taxa including *S. lingua*. This species is clearly isolated and is the unique true representative of the section (Baumann & Künkele 1989; Lorenz 2001; Delforge 2006). The remaining five species from the section are intermediate in varying degrees towards the taxa from the *Bilamellaria* section. It is difficult to determine if these are of hybrid origin or if they have the primitive characteristics of a common ancestor (Delforge 2006). *Serapias lingua* is also unique within its genus due to its chromosome polyploidy ($2n = 72$) while all other species of the genus have the basic chromosome number ($2n = 36$) (D'Emerico et al. 1997).

The second section, the *Bilamellaria*, is a monophyletic assemblage of species with two lamellae at the base of the lip (Baumann & Künkele 1989, Delforge 2006). These two sections have different pollination strategies: while the *Serapias* section uses a pseudocopulation strategy, the *Bilamellaria* adhere to a shelter strategy (Delforge 2006). Baumann & Künkele (1989) claim that these strategies play a crucial role in the tendency to create hybrids between members of the different sections.

A small area at the very southern tip of the Is-

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trian peninsula called Cape Kamenjak, a proclaimed protected landscape, has been recognised as a true orchid heaven due to findings of a greater number of orchid species (Perko 1998; Kranjčev 2005; Vuković 2005). Perko (1998) also described two stenoendemic species, *Serapias istriaca* M.L. Perko and *Serapias pulae* M.L. Perko, which is assumed, in terms of morphological characteristics, a hybrid between *S. istriaca* and *S. lingua.* The taxonomic position and relation of the *S. istriaca* taxon with other species of the genus was unclear to a certain extent, as its morphology most closely resembled that of the species *S. ionica,* which is endemic to the Ionian and some Dalmatian islands (the islands of Hvar, Korčula, and Brač), and *S. apulica,* which is endemic to the southern Italy from Monte Gargano to Tricase (Lecce) (Delforge 2006; Baumann et al. 2006). Both species are located more than 300 kilometres of air distance from the *S. istriaca* distribution area (Perko 1998) and it is hardly conceivable that they might have any reproductive connection. Furthermore, the taxonomic treatment of the entire related groups of *S. istriaca* is unclear and features several different interpretations. *Serapias ionica* and *S. apulica* (H. Baumann & Künkele) P. Delforge are classified into the *S. neglecta* group, or the *S. orientalis* or *S. vomeracea* group at the subspecies or the species level (Nelson 1968; Baumann & Künkele 1989; Buttler 1986; Gölz & Reinhard 1989; Delforge 1995, 2006; Baumann et al. 2006). Due to such an interesting and challenging taxonomic position, and in light of the endemic characteristics and hybridisation, the objective of this study was to investigate the morphology and genetic similarity of the six taxa found in the region of Croatia: *Serapias istriaca* M.L. Perko, *Serapias pulae* M.L. Perko, *Serapias ionica* E. Nelson ex H. Baumann & Künkele, *Serapias lingua* L., *Serapias vomeracea* (N.L. Burman) Briquet, and *Serapias cordigera* L., in order to clarify the related groups among the taxa.

Material and methods

Plant material

Plant material sampling was carried out in such a way that the first flower in full blossom was picked from the base of the spike. The samples were taken in the same way for all the researched taxa.

Serapias pulae, *S. istriaca*, *S. lingua* and *S. cordigera* were sampled by V. Hršak and S. Brana at the southernmost tip of the Istrian peninsula at Cape Kamenjak, a protected landscape, on April 24-th, (Fig. 1). This area is the *locus classicus* for *S. istriaca* and *S. pulae* (Perko 1998).

S. ionica was sampled on the island of Korčula, about 1.5 kilometres south of village of Čara on June 09-th 2004 by V. Hršak while *S. vomeracea* was sampled on the island of Krk near village of Soline on May 24–th 2004 by V. Hršak (Fig. 1) because they are not present in Cape Kamenjak area.

The plant specimens were taken from 15 plant individuals of each species at accessible sites. Three to four young leaves of each individual were taken in the spring and were transported fresh to the laboratory, instantly lyophilized and frozen at −80 °C until required.

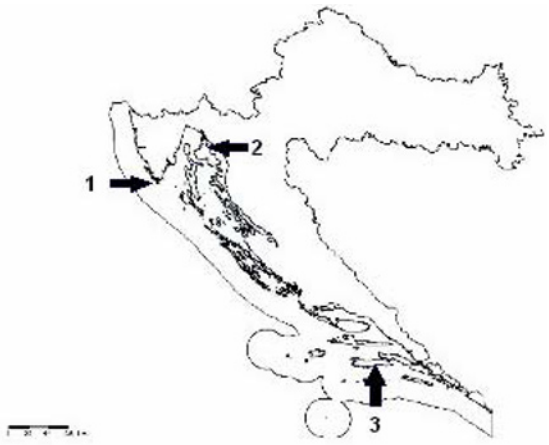

Fig. 1. Sampling localities (1 – Cape Kamenjak; 2 – Soline – island of Krk, Čara – island of Korčula).

Morphology

A total of 13 quantitatively measured morphological traits were recorded for each specimen. Eight traits were floral: epichile length, epichile width, hypochile length, hypochile width, sepal length, lateral sepal width, petal length, and petal width. Five traits were vegetative: stem height, spike length, number of flowers, number of leaves, and length of the longest leaf. Qualitative characters were used only for identifying the species. All measurements were made using a ruler.

DNA extraction, RAPD amplification and Electrophoresis

The DNA extraction method according to Doyle & Doyle (1990) was conducted using 40 mg of lyophilized tissue and 2% CTAB. DNA quality and concentration were estimated by visual comparison of orchid DNA sample smears with intensity and migration of the known concentration λ DNA in 0.8% agarose gel.

PCR amplification for RAPD analysis was carried out in 25 µL of reaction mix containing 10 ng of template DNA, PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.8), 0.2 µM of primer, 0.1 mM of each dNTP, 1.5 mM $MgCl_2$, 1U of Taq polymerase (Sigma) and stabilized with 20 µg BSA. The sequences of nine oligonucleotide primers (of 30 screened) are depicted in Table 3. RAPD fragments were amplified in a PTC-100 thermal cycler (MJ Research) with one step of 92 °C for 60 seconds, followed by 40 cycles of 60 s at 92 °C for denaturation, 60 s at 36 °C for annealing and 120 s at 72 °C for extension. The amplification products were separated in horizontal electrophoresis and 1.2% agarose gels at 120V for 2.5 hours. Gels containing amplified bands were stained with ethidium bromide (Sigma) and photographed using Polaroid® film type 667.

Data scoring and statistical analysis

Morphology

In order to determine the differences between the mean values of the morphological traits among species, a one-way ANOVA was used. In the case of a significant difference ($p < 0.05$) a Tukey HSD post hoc test for multiple comparisons was used.

Factor Analysis was used to determine the relationship structure among variables, i.e. to classify the variables.

Discriminant Analysis was applied to determine whether the measured morphological characters could signifi-

Table 1. Means of measured morphological traits ± standard deviations.

	S. ionica	*S. vomeracea*	*S. pulae*	*S. lingua*	*S. istriaca*	*S. cordigera*
Epichile length	21.1 ± 3.1	24.9 ± 2.0	17.1 ± 3.2	15.4 ± 1.5	22.4 ± 1.9	24.7 ± 3.2
Epichile width	13.1 ± 1.5	10.3 ± 1.3	11.8 ± 2.1	8.6 ± 0.8	13.0 ± 1.3	18.3 ± 2.3
Hypochile length	10.7 ± 1.1	14.1 ± 1.1	11.8 ± 2.2	11.0 ± 1.1	13.5 ± 0.9	11.4 ± 1.3
Hypochile width	15.8 ± 1.4	19.6 ± 2.5	16.6 ± 2.1	16.1 ± 1.1	18.1 ± 3.7	20.4 ± 1.8
Sepal length	19.4 ± 1.9	28.7 ± 2.8	18.6 ± 2.0	16.9 ± 2.2	22.6 ± 4.8	22.3 ± 2.2
Sepal width	6.2 ± 0.8	8.0 ± 0.8	6.1 ± 0.8	5.6 ± 0.8	7.4 ± 0.8	7.4 ± 0.9
Petal length	17.8 ± 1.2	25.8 ± 2.4	16.8 ± 2.3	16.0 ± 1.7	22.0 ± 1.5	19.6 ± 2.2
Petal width	6.3 ± 0.9	8.6 ± 1.0	4.2 ± 1.0	3.9 ± 0.6	7.8 ± 1.0	7.5 ± 1.1
Stem height	84.8 ± 10.6	484.5 ± 76.3	198.5 ± 42.6	236.6 ± 37.1	244.6 ± 32.3	296.6 ± 87.9
Spike length	42.9 ± 11.5	183.7 ± 62.7	64.5 ± 20.3	81.3 ± 19.1	96.3 ± 30.4	97.5 ± 35.3
Flower number	3.2 ± 0.9	5.8 ± 0.9	3.3 ± 1.1	4.0 ± 0.8	5.3 ± 1.0	5.4 ± 1.9
Leave number	4.3 ± 0.8	4.9 ± 0.5	4.3 ± 1.0	5.3 ± 1.0	5.4 ± 0.8	5.5 ± 0.8
Leave length	53.0 ± 8.7	178.8 ± 35.7	78.6 ± 15.7	98.5 ± 28.1	105.3 ± 16.5	103.9 ± 27.3

cantly discriminate among the taxa. This approach maximises the variance relative to predefined groups and tests which of the measured characters contributes significantly to the discrimination functions. Statistical analyses were performed using the Statistica 7.0 software.

Genetic analysis

The polymorphis m was recorded as presence/absence (1/0) of homologous fragments for all samples and one binary data matrix was assembled (data not shown).

Molecular diversity within each species sample was assessed by computing the Simpson diversity index (D) (Simpson 1949) and the Shannon-Wiener diversity index (H') (Shannon & Weaver 1949).

Principal Component Analysis (PCA) on variance/covariance matrix of the RAPD data was carried out to establish whether the given set contains discrete genetic groups. PCA was performed using PAST software version 1.74 (Hammer et al. 2001). The binary matrix was converted into a similarity matrix using the Nei (1972) genetic index and a nearest neighbour clustering dendrogram was constructed using NTSYSpc version 2.2 software (Rohlf 2005). In order to determine the significance of clustering, a cophenetic values matrix was computed and tested by Mantel test with 250 random permutations.

A hybrid index estimate was computed using a maximum likelihood (ML) approach in order to quantify the genetic contribution of *S. istriaca* and *S. lingua* as parental species to *S. pulae* hybrid individuals. The normality of the frequency distribution was tested using the Shapiro-Willk's test. Computing was carried out using HINDEX software (Buerkle 2005).

Results

Morphology

Means and standard deviations of the measured variables are shown in Table 1. Results of the ANOVA and Tukey post-hoc test (not shown) showed statistically significant differences between species in all measured variables. Of those species assumed to be the parentals of the hybrid *S. pulae*, it was shown that *S. istriaca* differs from *S. lingua* and from *S. pulae* in 9 morphological traits. In contrast, *S. lingua* and *S. pulae* differ in only 2 morphological traits.

Based on results of Factor Analysis (not shown), discriminant analysis was conducted separately for floral and vegetative traits.

Table 2. Pooled within-group correlations between the quantitative morphological characters and standardized canonical discriminant functions. The largest absolute correlations between each variable and any discriminant function are indicated in bold.

	root1	root2
Petal length	**0.542**	0.447
Epichile width	−0.336	**0.781**
Petal width	0.385	**0.751**
Epichile length	0.196	**0.627**
Sepal width	0.241	0.401
Hypochile length	0.301	0.109
Hypochile width	0.090	0.305
Sepal length	0.390	0.385
Eigenvalue	7.973	4.408
%variance explained	60.5	94.0
Stem height	**0.934**	0.350
Leave length	**0.677**	0.187
Flowers number	0.286	**0.720**
Leaves number	0.079	**0.714**
Spike length	0.541	0.131
Eigenvalue	5.884	0.566
%variance explained	87.6	96.0

Discriminant Analysis – floral traits.

The overall discrimination between *Serapias* species based on floral traits is highly significant (Willk's lambda = 0.011; $p < 0.000$). All variables have tolerance greater than 0.01, meaning there are no redundant variables in the matrix. The result of the canonical analysis shows that the first two discriminant functions have eigenvalues exceeding one and together explain 94% of the total morphological variance (Table 2). Table 2 shows that there are several discriminant variables. The first function distinguished species on the basis of hypochile length and petal length and width. Epichile width contributes to the discriminant function with the negative sign. The second function distinguished species based on epichile width and petal width.

Results of the discriminant analysis based on floral traits are shown in the scatterplot (Fig. 2). The first discrimination function best discriminates *S. vomeracea*

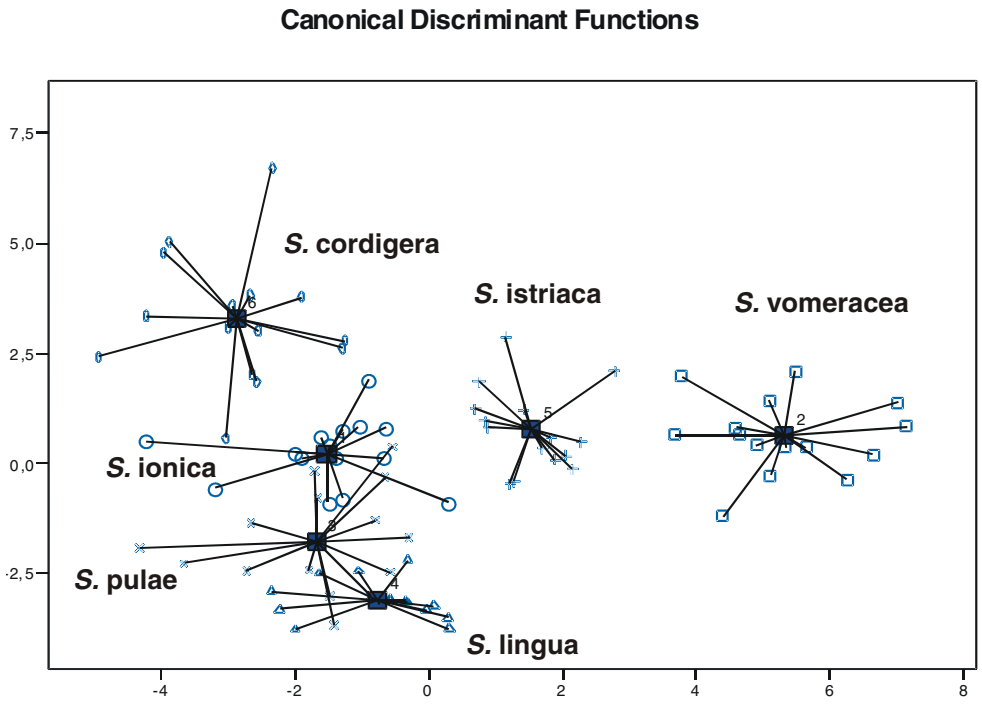

Fig. 2. Scatterplot of the Discriminant Analysis based on floral traits.

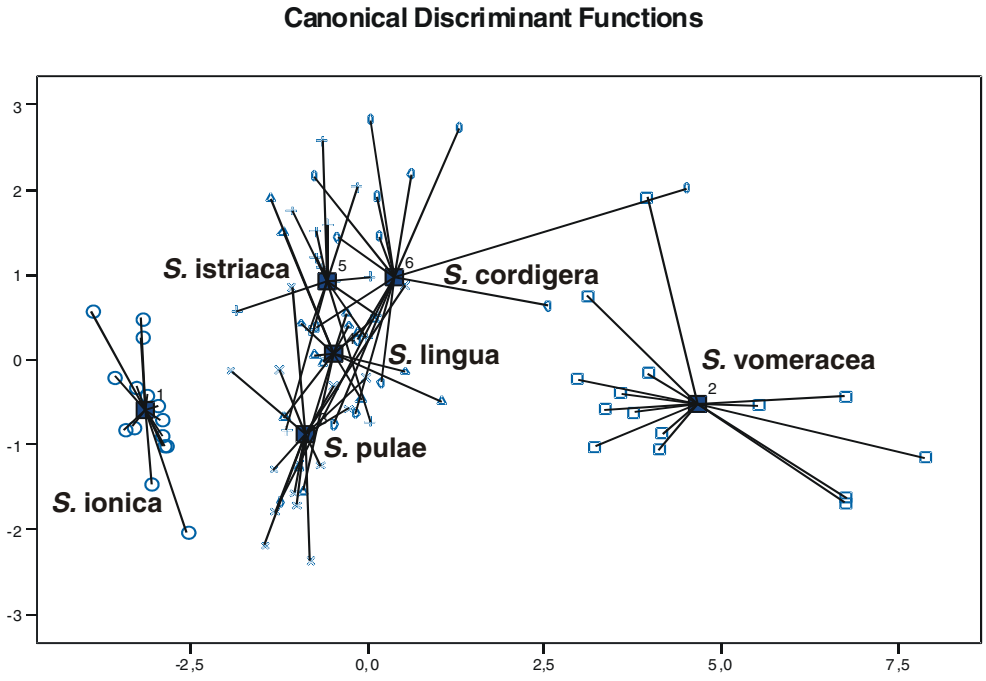

Fig. 3. Scatterplot of the Discriminant Analysis based on vegetative traits.

from the other species while second function discriminate S. lingua and *S. cordigera* from the other species. This analysis shows the close resemblance between *S. lingua* and *S. pulae*.

Discriminant Analysis – vegetative traits.
The overall discrimination of the *Serapias* species based on the vegetative traits is highly significant (Willk's lambda = 0.072; $p < 0.000$). Willk's lambda is somewhat larger compared to those in the discrimination analysis of floral traits, which indicates that discrimination based on vegetative traits was, to a certain extent, weaker than discrimination based on floral traits. The first three functions are significant, though only the first has an eigenvalue exceeding one. Also in this discriminant analysis, the tolerance of all variables exceeded 0.01, meaning there was no matrix ill conditioning, i.e. there were no redundant variables.

The first three discriminant functions are statistically significant ($p < 0.05$), though only the first func-

Table 3. List of RAPD primers used, total number of bands scored for each primer and each marker are indicated, together with the size range of the amplified products.

RAPD primer	Sequence (5′ – 3′)	No of recorded bands	Size range (bp)
P2	GGGTAACGCC	10	540–2300
P4	TTCCGAACCC	10	760–1800
P6	CAAACGTCGG	11	410–2500
P12	GGTGATCAGG	10	320–1320
P13	CCGAATTCCC	9	670–2100
P17	TGCCCGTCGT	11	480–2200
P18	CTCTCCGCCA	11	740–2250
P19	CTGCATCGTG	14	530–2500
P28	CTGACCAGCC	6	900–1850
Total		92	

tion has an eigenvalue exceeding one. Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions are shown in Table 2. The first two discriminant functions account for about 96 percent of the discriminatory power. The first discriminant function is marked most heavily by stem height. The second discriminant function is marked mostly by spike length. Number of flowers and number of leaves contributes to the discriminant function, but with a negative sign.

Means of canonical variables (not shown) showed that the first discrimination function best discriminates *S. vomeracea* and *S. ionica* from the other species. The second discriminant function discriminates *S. pulae* and *S. ionica* from other species. This is depicted in Fig. 3 where *S. vomeracea,* the tallest plants, and *S. ionica,* the shortest plants, are clearly discriminated from the other species.

Classification

Percentage of the correct classification based on floral traits were (not shown) 92.2. The most incorrectly classified specimens were in the hybrid species *S. pulae*. Furthermore, two plants of the species *S. ionica* and only one of the species *S. cordigera* were incorrectly classified. Classification based on vegetative traits showed that 73.3% of the originally grouped cases were correctly classified. These results suggest that classification based on floral traits was more successful than that of vegetative traits.

RAPD markers

The nine RAPD primers originated between 6 and 14 products each, giving a total of 92 polymorphic bands ranged in size from 320 to 2500 bp (Table 3). The average number of the amplification products obtained with one primer was 10.2.

Molecular diversity

Obtained Simpson diversity index (D) within each species sample was high ($D > 0.9$) and did not differ much among the species since D was ranged between 0.926–0.971 (Table 4). Nevertheless, the lowest diversity was established with *S. cordigera* and the highest diversity was found with *S. istriaca*. *S. pulae*, *S. lingua*. *Serapias vomeracea* gave similar results, such that *S. pulae* showed values between those of its assumed parental species.

Table 4. Diversity indices: D – Simpson diversity index; H' – Shannon-Wiener diversity index.

Species	*D*	*H′*
S. cordigera	0.926	2.978
S. ionica	0.953	3.160
S. istriaca	0.971	3.550
S. lingua	0.967	3.410
S. pulae	0.968	3.447
S. vomeracea	0.964	3.416

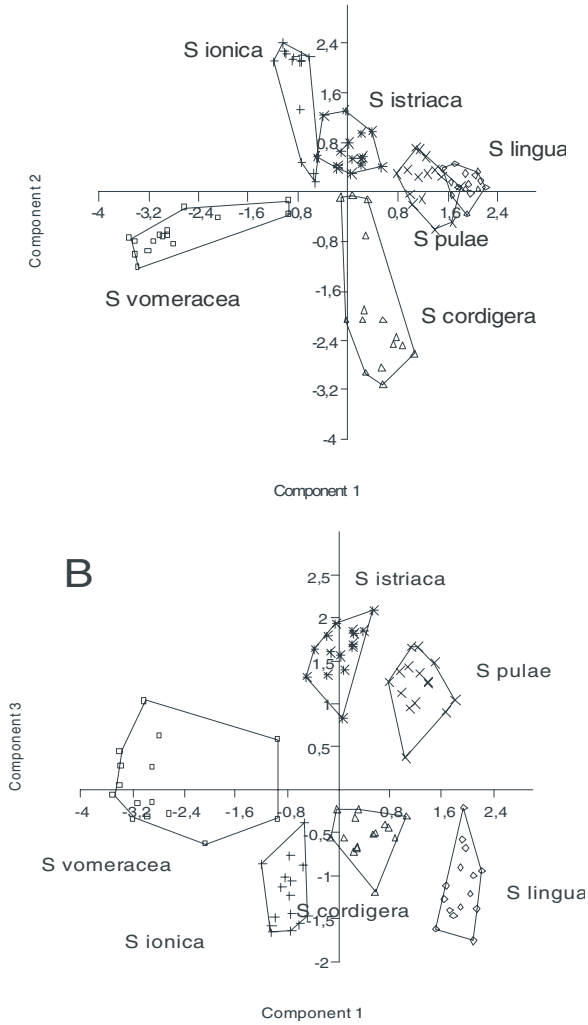

Fig. 4. Two dimensional distribution of *Serapias ionica*, *S. istriaca*, *S. lingua*, *S. pulae*, *S. vomeracea* and *S. cordigera* individuals obtained by PCA based on the variance-covariance matrix for the presence/absence of RAPD fragments. A – components 1 and 2; B – components 1 and 3.

The partitioning of molecular variability rendered in the plot generated by the PCA of the RAPD data set accounted for 68.3% of the observed variance with the first 10 components (Figs 4A, 4B). In plot A of the in-

dividual component scores along axis 1 (accounting for 18.73% of total variance), *S. lingua* and *S. pulae* were not identified as discrete groups. In plot B showing axis 1 and axis 3, the groups are identified as clearly discrete groups, which points to the conclusion that they clearly differ as genetic groups.

The nearest neighbour joining tree obtained from the Nei (1972) genetic similarity matrix inferred from 92 RAPD markers is shown in Fig. 5. Three main clusters were found. The first main cluster consists of 2 subclusters: *S. ionica* and *S. vomeracea*. The second main cluster consists of the *S. istriaca* subcluster and a subcluster consisting of *S. pulae* which also includes *S. lingua*. *S. lingua* did not separate from the remaining species, even though it is a representative of the section *Serapias*. Such a division into sections is not indicated in this genetic analysis. The third main cluster consists of *S. cordigera*. The cluster joining tree suggests that *S. pulae* as their putative hybrid of *S. istriaca* and *S. lingua* is genetically closer to the second

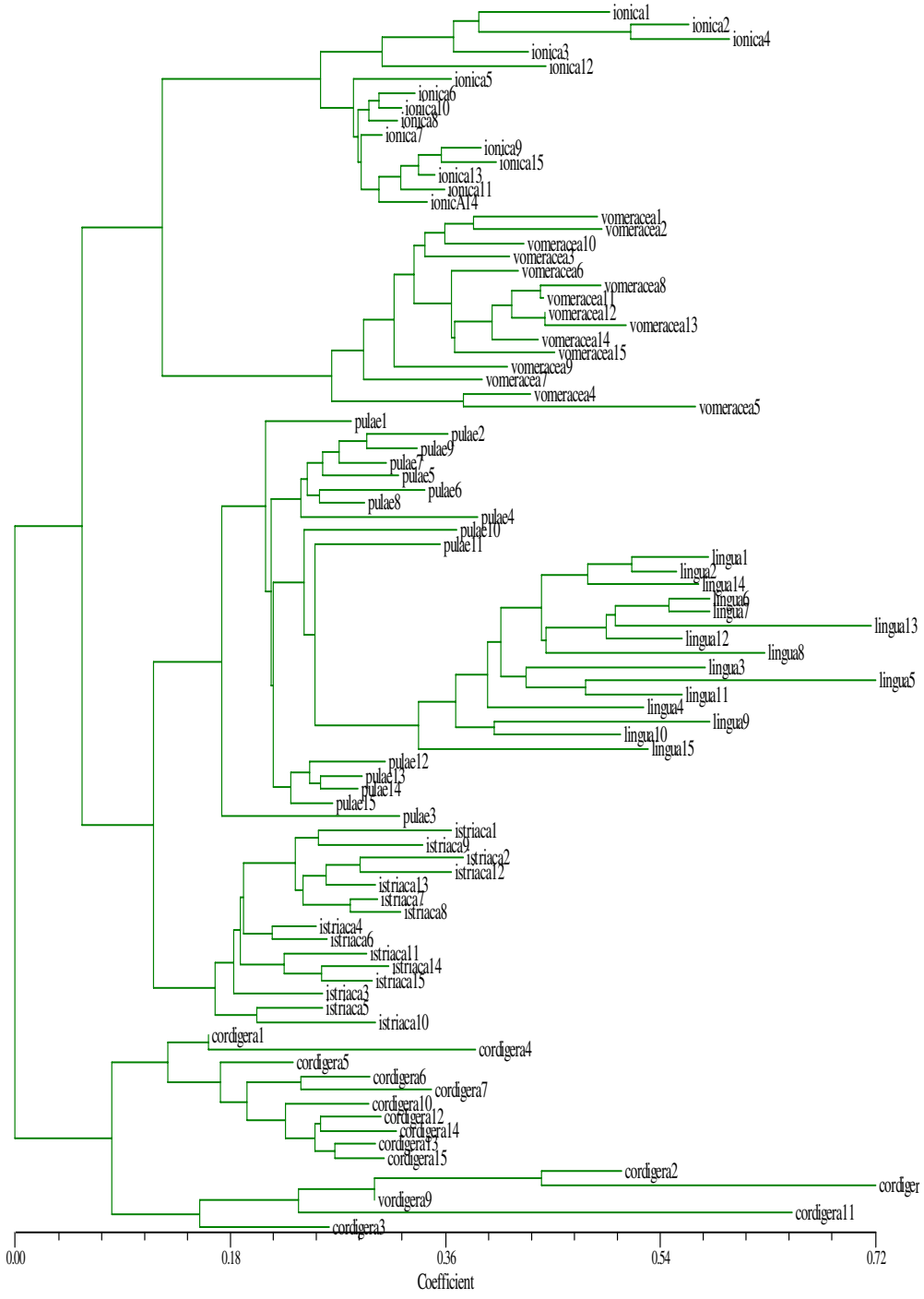

Fig. 5. Nearest neighbour clustering of *S. ionica*, *S. vomeracea*, *S. pulae*, *S. lingua*, *S. istriaca*, and *S. cordigera* using the Nei (1972) genetic similarity matrix.

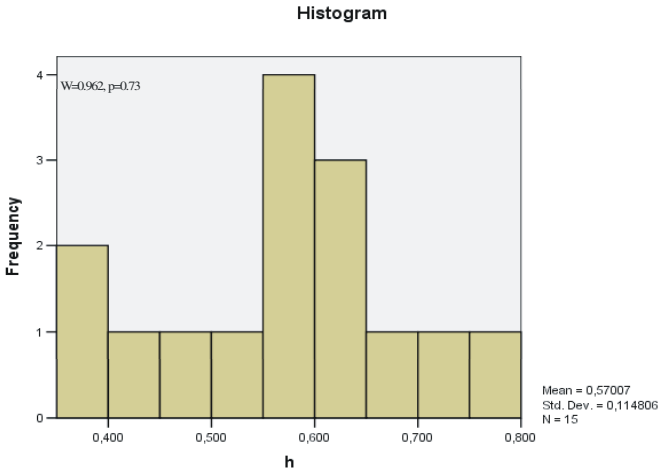

Fig. 6. Frequency distributions of 15 *Serapias pulae* individuals vs. maximum likelihood hybrid index based on RAPD data. Low hybrid index scores are indicative of *S. istriaca*-like individuals and high scores of *S. lingua*-like individuals. The mean score and standard deviation are given within the graph along with W- and p-values for the Shapiro-Willk's test.

parent, in this case *S. lingua*. In addition, *S. ionica* and *S. vomeracea* make up one of the main clusters indicating their genetic resemblance. *Serapias cordigera* represents a species that is completely separate from all those analysed in this research. The Mantel test between the cophenetic values matrix and values matrix of the Nei similarity index based on RAPD data gave r = 0.622 (999 random permutations, $p = 0.004$) indicating statistical significance of the clustering.

Hybridisation

The histogram relative to the distribution of ML hybrid index scores was set up so that *S. istriaca* would have low scores and *S. lingua* high scores at the end points on a linear scale between 0 and 1.

Hybrid index scores of *S. pulae* based on RAPD markers (Figure 6) ranged from 0.381 to 0.769 (mean 0.57 ± 0.03). Such a value range of the hybrid index suggests the hybrid origin of *S. pulae*. Although the frequency distribution did not differ significantly from normality according to the Shapiro-Willk's test ($W = 0.962$, $p = 0.73$), it was somewhat shifted to the right of the graph. The hybrid index scores higher than 0.5 were identified with 10 individuals, two scored approximately 0.5, while only 3 individuals scored less than 0.5. This indicates a more pronounced genetic similarity of *S. pulae* to *S. lingua*.

Inspection of the DNA fragments confirms a higher similarity of *S. pulae* and *S. lingua*. Namely, the *S. istriaca, S. pulae* and *S. lingua* complex has the total of 68 fragments, 16.2% (11) of which are shared with *S. pulae* and *S. lingua*, while 10.3% (7) of them are shared with *S. pulae* and *S. istriaca*.

Discussion

Morphological differences

Many authors stated that the differences in floral traits among the taxa in the genus *Serapias* are small and that the species are morphologically similar (Delforge 1995, 2006; Baumann & Künkele 1989; Lorenz 2001; Gölz & Reinhard 1989). Therefore the number of recognized species varies considerably by various authors, e.g. from 10 according to Nelson (1968) to 28 according to Delforge (2006).

However, according to the results of the ANOVA, this morphological study showed that some of the investigated species showed statistically significant differences in all quantitative morphological traits. Among the morphological traits, the vegetative traits were clearly separated from the floral traits. The present study indicated that the floral traits have greater discriminatory power than the vegetative traits, meaning that species can more successfully be differentiated according to their floral traits. The majority of other Mediterranean orchids have shown similar results, and are not easily distinguished on the basis of vegetative traits (Pellegrino et al. 2005).

The discriminant analysis showed that there are several morphological variables that can discriminate between the investigated species. Among those with the greatest discriminatory power is petal size, i.e. petal length and width, followed by epichile width and length and hypochile length. Virtually the same set of variables displayed the highest disciminatory power in the morphometrical analyses on the southwest European *Serapias* taxa (Venhuis et al. 2007). To a certain extent, this supports the division into the subsections *Steno-*, *Medio-* and *Platypetalae* as described by Baumann & Künkele (1989). The foundation for this division into subsections is the size of these floral elements that determine the size of the flowers and their parts.

According to the results of the present study, *S. vomeracea*, *S. cordigera* and *S. istriaca* would belong to the section *Platypetalae*. This is in agreement for *S. cordigera* (Baumann & Künkele 1989; Lorenz 2001), but not for *S. istriaca* and *S. vomeracea*. According to Perko (1998), *S. istriaca* lies at the transition between the subsection *Platypetalae* and *Mediopetalae*, and according to Baumann & Künkele (1989), *S. vomeracea* belongs to the subsection *Mediopetalae*. These differences can be explained in the fact that the obtained means in the present study are very near to the numerical borders between the subsections, and that measurements were made only a relatively small number of plants and, as such, deviations are possible that would mean a shift into another subsection.

Among the vegetative traits (characters), plant height had the highest discrimination power, while spike length had significantly lower power. This puts *S. vomeracea* and *S. ionica* on the opposite ends of the vegetative morphological gradient, as *S. vomeracea* is the tallest while *S. ionica* is the shortest species. Among the species that have hybridized and given hybrid taxa, *S. pulae*, in terms of quantitative morphological traits, is most similar to *S. lingua,* as they statistically differ in only one vegetative and one floral trait. As the second parental species, *S. istriaca* differs from the hybrid

S. pulae in 9 morphological traits. This suggests that, in terms of quantitative morphological traits, *S. pulae* is more similar to one parent (*S. lingua*) than the other (*S. istriaca*).

Genetic differences

It has been established that the average genetic distances between the species are not considerable, and that the genetic diversity within the species is rather even. This confirms the data on genetic similarity of the species within genus and indicates the recent origin of the species within the genus (D'Emerico et al. 2000; Bateman et al. 2003). The results suggest that *S. cordigera* represents a separate species clearly different from the other researched species. This is also supported by the fact that it is the only species among the studied ones that belongs taxonomically to the *Platypetalae* subsection (Baumann & Künkele 1989; Lorenz 2001).

Serapias ionica and *S. vomeracea* have proved to be closely genetically related according to results of the cluster analysis in this study. This can be expected since, they taxonomically belong to the same section, *Mediopetalae*, although they show different phytogeographical distributions. *Serapias vomeracea* is a widely spread species, while *S. ionica* is endemic to the Ionian and Dalmatian islands (Delforge 2006). *Serapias vomeracea* is considered to be the originating species for all other taxa from its group, with the flowers of some members ranging in size from small to large (Lorenz 2001). According to the present results, *S. istriaca* is a well-defined separate species that likely belongs to the *S. vomeracea* group as suggested by Delforge (2006). *Serapias istriaca* is located taxonomically at the crossing between the *Mediopetalae* and the *Platypetalae* subsections (Perko 1998).

Based on conclusion that variation in morphology and molecular markers correspond closely (Aceto et al. 1999), the taxonomically closer plants and those with more similar morphologies are also expected to be more similar genetically. Of all the plants researched *Serapias istriaca* is most similar to *S. ionica* by its habitus and morphology (Perko 1998). Hence it may be assumed that these two species are genetically most similar. However, the results of our research have not confirmed the high genetic similarities between these two species. In contrast, our research suggests that *S. ionica* is genetically closer to *S. vomeracea* although it is morphologically more similar to *S. istriaca*. It seems that the principal reason for this result lies in the fact that *S. istriaca* is between two subsections *Mediopetalae* and *Platypetalae*, while *S. ionica* is a true member of the *Mediopetalae* subsection together with*S. vomeracea.* This also suggests that the methods applied in this research seem to be best fitted for determining the genetic differences among the subsections.

In terms of morphology, another species similar to these two is *S. apulica,* whose taxonomical status is treated differently by various authors (Baumann & Künkele 1989; Perko 1998; Baumann et al. 2006). Since this species is not widespread in Croatia (Hršak 2000; Kranjčev 2005), the material was not available for analysis.

Furthermore, the pattern of cluster analysis in the present research has confirmed the hybrid origin of *S. pulae*. According to its qualitative and quantitative morphological characteristics, it was assumed to be a hybrid between *S. istriaca* and *S. lingua* (Perko 1998). These three species are syntopic in the area of Cape Kamenjak and belong to different sections. Although they are not closely related, they hybridise. It is common knowledge that the *Serapias* genus features most common hybridisation between the species from different sections and subsections (Lorenz 2001). Isolation mechanisms are much stronger among closely related species, which seems to be connected with the shelter pollination strategy (Baumann & Künkele 1989) or reproductive barriers act postzygotically (Pellegrino et al. 2005). It has also been established that the most common partner in the hybridisation within the genus is *Serapias lingua,* merely because it is the single true representative of the *Serapias* section (Baumann & Künkele 1989). This species hybridises with the members of all subsections in the *Bilamellaria* section (Baumann & Künkele 1989). Thus, in the Cape Kamenjak area, *S. lingua* hybridises with the stenoendemic species *S. istriaca*. According to the hybridization index, it has been established that the *S. pulae* hybrid genome is somewhat closer to the *S. lingua* genome than to the second parent, the *S. istriaca* species. It seems that *S. lingua* is a frequent and popular hybridisation partner due to its higher polyploidy level ($2n = 72$) in comparison with other species of its genus (Ehrendorfer 1980). The higher genetic similarity between *Serapias pulae* and *S. lingua* rather than with any other parental species may be connected with a higher polyploidy level of *S. lingua*. Although there is no data on the number of chromosomes presently for the *S. istriaca* and *S. pulae* it can be assumed that *S. istriaca* has the same number of chromosomes as the other members of its section *Bilamellaria* ($2n = 36$). If that is truly so it is to be expected that the hybrid between *S. lingua* and *S. istriaca* inherits by 1/3 more genetic material from *S. lingua* than from *S. istriaca*. The research has established that the ratio between the number of the shared fragments with the hybrid and those with each of the parent species almost identical to this ratio.

There is another reason why *Serapias lingua* is such a desirable partner for hybridisation. It is the only species within the *Serapias* genus characterised by vegetative reproduction inheritable by hybrids (Baumann & Künkele 1989), which gain a selective advantage in populating variable environments.

It is important to note that although the *S. cordigera* and the *S. lingua* species are considered to result in the putative hybrid species *S. olbia* (Delforge 2006), the said hybrid has not yet been found at Cape Kamenjak (Freyn 1877; Tommasini 1873; Starmühler 2003; Perko 1998; Topić & Šegulja 2000; Kranjčev 2005; Vuković 2005), nor anywhere in the territory of the Republic of Croatia (Hršak 2000; Kranjčev 2005). This suggests

that these two species do not hybridise in the area of Croatia or that *S. olbia* is not their hybrid at all.

Although this research has confirmed that the genetic differences among species in the *Serapias* genus are minor, which is in agreement with the current knowledge (Bateman et al. 2000; Lorenz 2001; Bateman et al. 2003), the hybrid origin of the *S. pulae* taxon has also been confirmed. The study suggests that *S. istriaca* deserves the status of a separate species.

Finally, based on the morphological discriminant analysis, it can be concluded that the division into the subsections *Platy-*, *Medio-*, and *Stenopetalae* is justified with respect to the floral morphology. The division into the sections *Serapias* and *Bilamellaria* is based on a single qualitative trait, and is not evident in the analysis of the morphological quantitative traits, the RAPD analysis of the current study, or in any other molecular analyses (Bellusci et al. 2008). As such, this study does not confirm the complete isolation of *S. lingua* from the remaining species, neither based on morphological nor genetic traits, as suggested by Baumann & Künkele (1989).

Acknowledgements

The authors would like to thank Sara Mareković and Aleš Vokurka for their technical assistance in collecting and preparing the plant material for analysis, and Ivan Radosavljević for RAPD analyses. The research was financed by the Ministry of Education, Science and Sports of the Republic of Croatia (Project 110 143) and supported by Natura Histrica, the Public Institution for Managing the Protected Natural Areas in Istria County.

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Received March 30, 2009
Accepted July 19, 2010