Disentangling the kinship of *Serapias* × *todaroi* Tin. (Orchidaceae) along the eastern Adriatic using chromosome count and morphometry

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ABSTRACT

The aim of the study was to confirm the hybridogenous status of several populations of putative *Serapias* × *todaroi* along the eastern Adriatic coast, and identify the most useful morphological traits for recognizing the hybrid in the wild. We have performed a karyological study, and compared the morphology of the hybrids with the assumed parental species *S. lingua* and *S. parviflora*. The intermediate features of the studied populations, particularly chromosome number and shape of the base of the labellum, strongly support its hybridogenous origin. Although morphometric analysis found certain traits of the hybrid to be intermediate with respect to the parental species, in general the hybrid plants closely resembled *S. lingua*. The flowers of the hybrid were mostly influenced by *S. lingua*, while the vegetative traits were mostly influenced by *S. parviflora*. To distinguish the hybrid in the field successfully, most attention should be paid to the shape of the base of the labellum and plant height, while flower size (particularly of labellum) can also be useful to some extent.

1. Introduction

Serapias L. is a genus of essentially Mediterranean distribution, ranging from the Azores to the Caucasus, extending northwards to Brittany. Since the genus is monophyletic, comprising approximately 30 species of similar morphology, the exact number of species given by authors varies. Although more recent phenetic studies based on morphometry (Venhuis et al., 2007) and phylogenetic analysis based on chloroplast genome (Bellusci et al., 2008) suggest differently, the genus has been traditionally divided into groups based mainly on the shape of the base of the labellum (Baumann and Künkele, 1989; Delforge, 2006). The main characteristic of the members of the *Serapiaria* group (e.g. *S. lingua* L.) is the presence of a round swelling at the base of the labellum, in some cases more or less grooved, whereas the members of the *Bilammelaria* group (e.g. *S. parviflora* Parl.) are characterized by two lamellae at the base of the labellum.

Serapias lingua (Fig. 1) is widespread in Europe and often occurs abundantly. It is a rather variable species, but can be distinguished by a dark, glossy swelling at the base of the lip, acting as a deception feature for insects, mostly males of *Ceratina cucurbitina* (Rossi, 1792) (Delforge, 2006). The chromosome number of *S. lingua* previously reported in the literature is 2n = 72 (Bianco et al., 1991; Brullo et al., 2014; Del Prete, 1978; D'Emerico et al., 2000). *S. parviflora* (Fig. 1) is a small-flowered, usually autogamous plant, not very morphologically variable and easily distinguished by its small, pale flowers and yellow pollinia. It is characterized by a chromosome number of 2n = 36 (Bianco et al., 1991; Del Prete, 1977; D'Emerico et al., 2000). Although rather widespread in Europe, *S. parviflora* usually occurs in small numbers. *S. lingua* and *S. parviflora* are very much sympatric, both of Mediterranean-Atlantic distribution. They occupy various habitats, but mostly grasslands, meadows, garrigues or open woodland, i.e. sunny to mid-shade habitats. Like many other terrestrial orchids, they bloom during spring and early summer, mostly from March to June (Delforge, 2006).

Hybridization among *Serapias* species is very frequent; moreover, the members of the genus appear to hybridize whenever they are syntopic, i.e. when they co-occur (Delforge, 2006). Many hybrid taxa within the genus have been reported in the literature (e.g. Baumann and Künkele, 1989; Borovečki-Voska, 2016; Cristaudo et al., 2009;

Fig. 1. Inflorescences of *Serapias lingua* (a), *S.* × *todaroi* (b) and *S. parviflora* (c).

Galesi et al., 2004; Perko, 1998; Sardaro et al., 2012). Hybrids can often be found in mixed populations with one or both parental species; however, independent populations of hybrids are also common. Along with the great morphological similarity among taxa, this can make their identification uncertain.

In 1846, a species named *Serapias todari* Tin. was described from Sicily, without any indication as to its hybridogenous origin (Tineo, 1846). Approximately 60 years later, a hybrid between *S. lingua* and *S. parviflora* from Italian mainland near Sicily was described by Camus et al. (1908) as *S.* × *semilingua* E.G. Camus. While performing a taxonomic overview of the genus, 80 years after the description of the hybrid, Baumann and Künkele (1989) concluded that the plants described by Tineo (1846), according to their description, are in fact hybrids between *S. lingua* and *S. parviflora*, and introduced the valid name *S.* × *todaroi* Tin. (=*S.* × *semilingua*), which is in use today.

Serapias × *todaroi* (Fig. 1) has been recorded in several countries within the distribution range of the parental species, including Portugal (Tyteca, 1997), Italy and France (Camus, 1927-1929, Camus et al., 1908; Lorenz, 2001), as well as on the Mediterranean islands Sicily (Galesi et al., 2004; Lorenz, 2001; Tineo, 1846), Sardinia (Lorenz, 2001), Corfu and Zakynthos (Renz, 1928); however, compared to the parental species, the overall number of findings is relatively low.

In spite of the frequent hybridization among orchids, hybrids are less studied and often neglected or marginally present in the literature. Identification keys for hybrid taxa are generally lacking and the identification mainly relies on presumptions, based on the co-occurrence with the parental taxa and somewhat intermediate morphology. This applies to *S.* × *todaroi*, which has not been included in any identification key. Unlike the better-studied parental species, the hybrid is poorly known regarding chromosomes, as only one count of 2n = 54 is available (Bianco et al., 1991). To our knowledge, no study combining karyological and morphological approach comparing *S.* × *todaroi* with the parental taxa in mixed populations exists up to date.

The aim of this study was to confirm the hybridogenous origin of putative populations of *S.* × *todaroi* found in Croatia (eastern Adriatic coast), using karyological evidence. We furthermore aimed to present a detailed comparison of morphology between the hybrid and the parental species, with a special emphasis on finding the most useful traits for hybrid identification.

2. Materials and methods

2.1. Fieldwork

In the period from 2008 to 2016 we repeatedly found populations of dubious *Serapias* specimens along the eastern Adriatic coast, and suspected them to be *S.* × *todaroi*. These populations, and the assumed parental species *S. lingua* and *S. parviflora* were ultimately studied on four islands in Croatia (Fig. 2), in order to test the origin of the putative hybrid. The populations were regularly small, with no more than 30 individuals per taxon. In cases where less than 20 individuals were found, the entire or nearly entire population was sampled. All three taxa were sampled for the purpose of morphometric analysis on the islands Dugi Otok (*S.* × *todaroi* = 21, *S. lingua* = 20, *S. parviflora* = 8), Molat (*S.* × *todaroi* = 11, *S. lingua* = 10, *S. parviflora* = 4) and Mljet (*S.* × *todaroi* = 15, *S. lingua* = 15, *S. parviflora* = 15), where they were found in mixed populations. *S.* × *todaroi* was additionally found on the island of Vir, growing in the absence of plants from the two parental species in a small population of only ten individuals which were all sampled. During fieldwork, flowers of all taxa were examined to determine the shape of the base of the labellum. Parental species were identified according to Delforge (2006), while the putative hybrid did not correspond to any species within the key.

2.2. Chromosome counts

Twenty individuals of *Serapias* × *todaroi* were sampled for the analysis, on the islands of Dugi Otok (10 individuals) and Mljet (10 individuals). The chromosomes were analysed using fully developed, unfertilized ovaries, following examples from the literature (Bellusci and Aquaro, 2008; Brullo et al., 2014; Cozzolino et al., 2004; D'Emerico et al., 2000). The ovaries were carefully removed from the spike, cut in half, and placed in 0.3% colchicine for 3–4 hours. Afterwards, the ovaries were rinsed with distilled water and fixed with a mixture of ethanol and acetic acid (3:1 v/v) for 1 h at 4 °C. The pre-treated ovaries were stained with Schiff's reagent or with fluorescent dye 4,6-diamidino-2phenylindol (DAPI; Sigma). They were immersed in 1 N HCl, heated for approximately 7 min at 60 °C and transferred to Schiff's reagent for 2 h. DAPI staining was performed according to a standard protocol (Mlinarec et al., 2006).

Haploid chromosome number was counted in 10 metaphase plates (five per population). Chromosome photographs were captured with an Olympus BX51 fluorescent microscope equipped with a highly sensitive
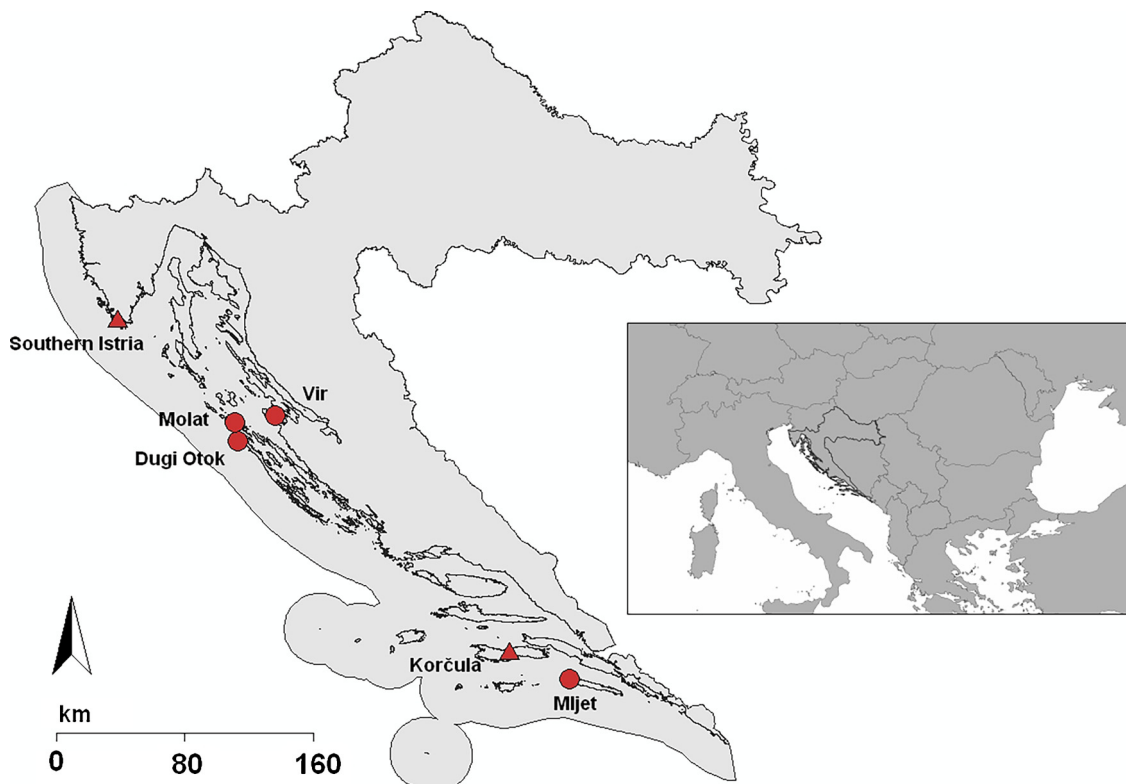
Fig. 2. Distribution map of *Serapias* × *todaroi* in Croatia. Circles point to our sampling sites; triangles point to records from the literature.

digital camera (Olympus DP70).

2.3. Morphology

The measurements were carried out on 45 individuals of *S. lingua*, 27 individuals of *S. parviflora*, and 57 individuals of putative *S.* × *todaroi*. Altogether 27 traits were measured, out of which 13 were floral (Fig. 3): petal length and width (PL, PW), lateral sepal length and width (LSL, LSW), middle sepal length and width (MSL, MSW), labellum length (LL), hypochile length and width (HL, HW), length of the lateral lobe of the labellum (LLLab), epichile length (EL), maximum and basal epichile width (MEW, BEW). The remaining vegetative traits (14) were: bract length and width (BL, BW), plant height (PH), spike length (SL), number of flowers (NF), number of leaves (NL), number of rosette and stem leaves (NRL, NSL), length and width of the longest leaf (LLLe, WLL), length and width of the longest rosette leaf (LLRL, WLRL), length of the uppermost leaf (LUL), length of the middle internode (LMI). All traits were measured directly in the field on fresh material using a digital calliper. In rare cases where direct measurements were not feasible, whole plants (without the underground parts) were transported into the laboratory, and measured within the next 48 h. In these cases, plants were kept in moist and cold conditions prior to the measurements, to prevent dehydration. For measurements of the floral traits, the first fully developed flower (located at the base of the spike) was sampled; floral parts were isolated and subsequently measured. All parts of measured flowers were prepared as part of the vouchers deposited in ZA – Herbarium Croaticum (Thiers, 2018).

2.4. Statistical analysis

The normality of distribution of the variables was tested using the Kolmogorov-Smirnov test at p ≤ 0.05. Since the variables were mostly not normally distributed, non-parametric tests were used in further analysis. To test the differences in the medians and distributions of the variables, the median test and Kruskal-Wallis test were used, while the

significance of the differences between pairs of taxa was tested with the Mann-Whitney test (p ≤ 0.05 for all tests).

Discriminant analysis (with simultaneous input of independent variables) was applied to determine the extent to which the measured variables could discriminate among the three taxa, with raw data transformed into Z-scores and used as input. Only the variables with statistically significant differences according to both the median test and the Kruskal-Wallis test were used in the discriminant analysis. In addition, variables containing missing values were excluded from the analysis and finally 13 variables were used (PL, PW, LSL, LSW, MSL, MSW, LL, HL, HW, LLLab, EL, MEW, BEW). In addition, the proportion of correctly classified individuals into each studied taxon was determined using classificatory discriminant analyses (Oksanen et al., 2017; Poljak et al., 2018). All statistical analyses were performed using the SPSS 22 software package, except for the classificatory discriminant analyses, for which "MorphoTools" R scripts in R v.3.2.2 (R Core Team, 2017) were used following the manual of Koutecký (2015).

3. Results

We recorded *Serapias lingua*, *S.* × *todaroi* and *S. parviflora* in four islands of Croatia: Molat, Vir, Dugi Otok, and Mljet (Fig. 2). All three taxa were growing together in mixed populations, except for the population on the island of Vir, where only the hybrid was found.

The inspection of flowers revealed a markedly different shape of the base of the labellum among parental species, while the putative hybrids showed intermediate forms (Table 1). We have confirmed the presence of a dark, glossy swelling at the base of the labellum of *S. lingua*, rather variable in shape. In the case of *S. parviflora*, two lamellae constant in shape were found at the base of the labellum. As for the putative hybrid, the structure at the base of the labellum was clearly intermediate between the swelling and the lamellar forms.

Chromosome count of the haploid chromosome set resulted in n = 27 (2n = 54) for *S.* × *todaroi* (Fig. 4).

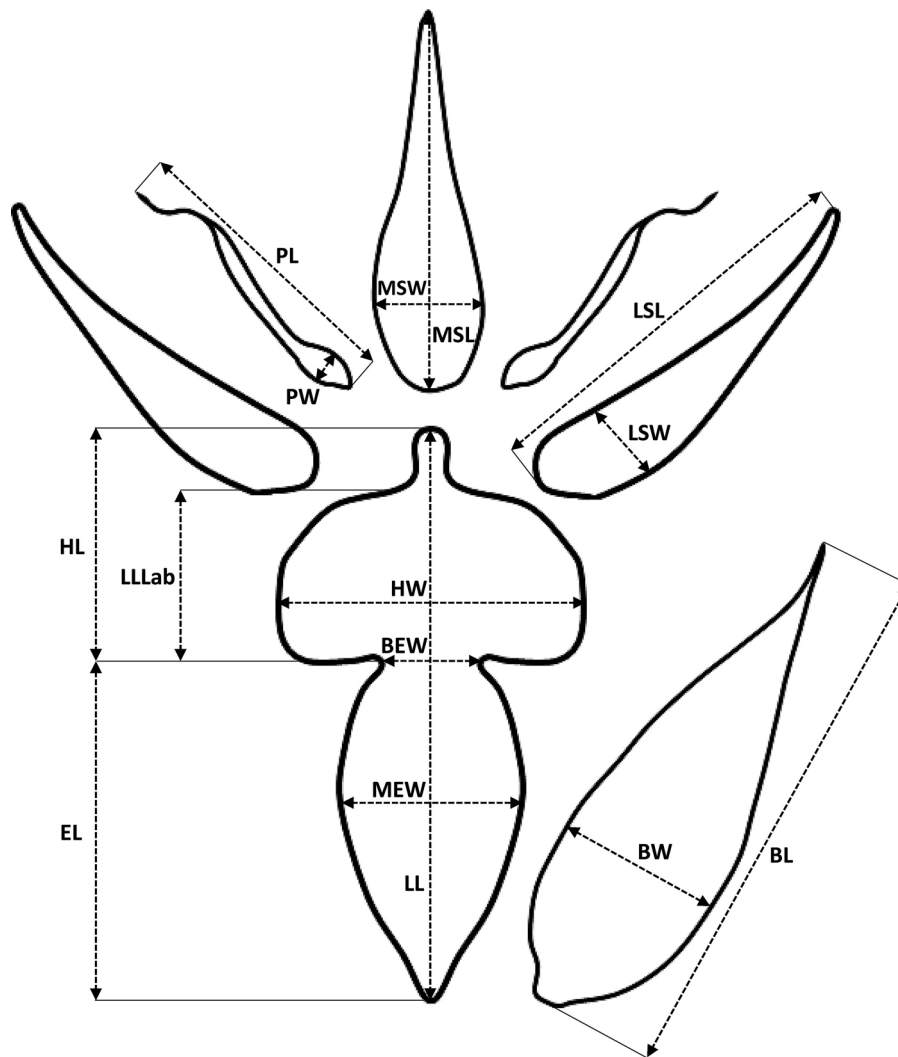Both median test and Kruskal-Wallis test showed statistically

Fig. 3. Diagram of the *Serapias* flower, with measured floral and bract (lower right) traits. For full description of the traits, see Section 2.3 of the Material and Methods.

Table 1
Morphological traits selected as important for identification based on statistically significant differences among taxa according to Mann-Whitney test, shown as rounded values of: (minimum) interquartile range (maximum). [1]Traits differentiating all three taxa. [2]Traits differentiating *Serapias lingua* from *S.* × *todaroi* and *S. parviflora*. All measures are given in mm. Scale bar on the photographs: 10 mm. For full description of the traits, see Section 2.3 of Material and Methods.

|	*S. lingua*	*S.* × *todaroi*	*S. parviflora*
PW[1]	(1) 2 (3)	(1) 2–3 (5)	3–4
HW[1]	(12) 14–15 (19)	(9) 13–15 (17)	(7) 9–10 (11)
MEW[1]	(6) 7–8 (11)	(4) 5–8 (9)	3–4
BEW[1]	4–5 (6)	(2) 4–5 (6)	2–3
PH[2]	(82) 135–181 (230)	(125) 160–238 (383)	(75) 161–271 (455)
NSL[2]	(1) 2–3 (4)	(2) 3–4 (7)	3 (4)
LLLe[2]	(50) 64–97 (150)	(53) 98–127 (183)	(55) 90–144 (213)
Chromosomes (2n)	72	54	36
Base of the labellum in the transverse section	Rounded swelling	Deeply grooved swelling	Two narrow lamellae
Dissected flowers			
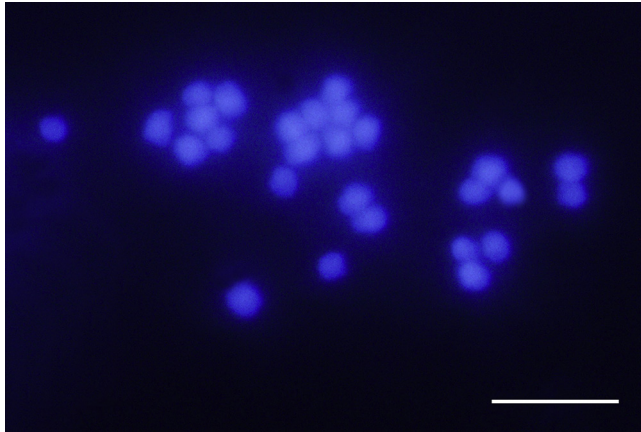
Fig. 4. Metaphase plate with the haploid chromosome set of *Serapias × todaroi*. Scale bar: 10 μm.

significant differences among taxa in 19 measured traits. Non-significant traits were: spike length, number of flowers, number of rosette leaves, width of the longest leaf, width of the longest rosette leaf, and length of the uppermost leaf. Additionally, the result of the Kruskal-Wallis test showed statistically significant differences among taxa regarding length of the middle internode, while the result of the median test showed statistically significant differences among taxa regarding bract length.

According to the Mann-Whitney test, pairs of taxa mainly differed in floral traits, out of which four were significantly different among taxa in all scenarios: petal width, hypochile width, maximum epichile width, and basal epichile width; as a result, these traits are selected as important for identification (Table 1). Moreover, the putative hybrid *Serapias × todaroi* exhibited intermediate values of these traits when compared to the parental species (Tables 1, S1–S2, Figs. S3 and S4). The highest similarity was found between *S. lingua* and *S. × todaroi*, which differed in only eight traits, half of them vegetative (plant height, number of stem leaves, length of the longest leaf and length of the middle internode). Three of these traits also differed *S. lingua* from *S. parviflora*, being selected as important for identification (Table 1). The most distinctive taxa was *S. parviflora*, significantly differing from each *S. lingua* and *S. × todaroi* in 18 traits, with an overlap in 14 traits.

Table 2 shows 13 discriminant variables and their correlation with

Table 2
Pooled within-group correlations between discriminating variables and standardized canonical discriminant functions. Variables ordered by absolute size of correlation within function. Asterisk (*) indicates highest absolute correlation between each variable and any discriminant function. For full description of the variables, see Section 2.3 of Material and Methods.

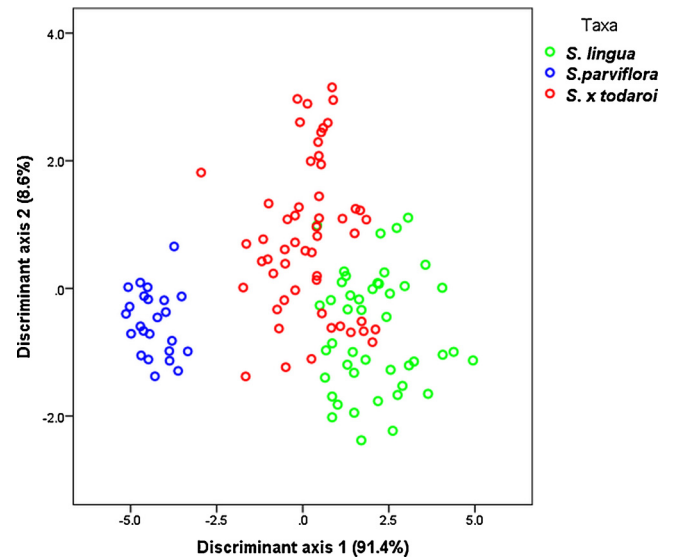|	Function	
	1	2
HW	0.546*	0.339
MEW	0.532*	−.176
EL	0.459*	0.400
BEW	0.444*	−.004
MSW	0.261*	0.191
MSL	0.226*	0.201
PL	0.197*	0.147
LLLab	0.274	0.585*
HL	0.255	0.495*
LL	0.443	0.486*
LSW	0.290	0.416*
LSL	0.303	0.372*
PW	−.283	0.343*
Eigenvalue	5.398	0.507
%variance explained	91.4	8.6

Fig. 5. Scatterplot of the discriminant analysis based on 13 floral traits (PL, PW, LSL, LSW, MSL, MSW, LL, HL, HW, LLLab, EL, MEW, and BEW). Wilk's lambda = 0.104; p < 0.000. For a full description of the traits, see Section 2.3 of Material and Methods.

discriminant functions. The first discriminant function is highly correlated with hypochile width, maximum epichile width, epichile length and basal epichile width while the second function is strongly correlated with length of the lateral lobe of the labellum, hypochile length, labellum length and lateral sepal width.

Results of the discriminant analysis are graphically presented in Fig. 5. The first function discriminates *S. parviflora* from the other two, while the second function discriminates between *S. lingua* and *S. × todaroi*. Overall, the analysis shows that *S. × todaroi* morphologically differs from both parental species (Fig. 5), but closely resembles *S. lingua*.

The results of the classificatory discriminant analyses are shown in Fig. 6. *Serapias parviflora* is clearly distinguished from the other two, with 100% posterior probabilities of the classification of each individual. However, the morphological relationship of *S. lingua* and *S. × todaroi* is more complex due to their higher overall similarity and the overlapping of the measured values. The lowest posterior classification probability (66.7%) was found for *S. × todaroi* sampled on the island of Mljet, followed by the same taxon sampled on the islands of Vir (70%) and Dugi Otok (76.2%). The remaining populations showed classification probabilities of at least 85%, indicating high probability of correct identification based on the measured traits.

In summary, *Serapias × todaroi* and *S. lingua* are morphologically very much alike, especially regarding their flowers. However, *S. × todaroi* has smaller flowers and conspicuously larger height, and the base of its labellum is characterized with a deeply grooved, dark swelling, being clearly intermediate between a round swelling and two lamellae (Table 1).

4. Discussion

Since the chromosome numbers for *Serapias lingua* and *S. parviflora* previously reported in the literature are 2n = 72 and 2n = 36, respectively (Bianco et al., 1991; Brullo et al., 2014; Del Prete, 1977, 1978; D'Emerico et al., 2000), we would expect an intermediate number of 2n = 54 within our hybrid populations. This was confirmed by our results, corroborating the previous report from Bianco et al. (1991). Furthermore, we have finally provided chromosomal evidence for such a number, whereas the only count by Bianco et al. (1991) merely provided the number. The intermediate chromosome number of
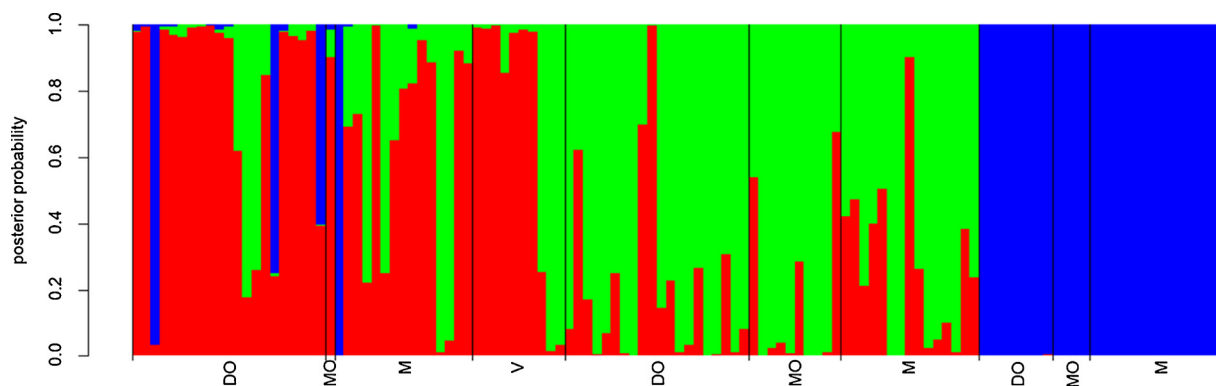
Fig. 6. Barplot with posterior probabilities of classification of each individual into each group from the results of the classification discriminant analysis. Red bars, *Serapias × todaroi*; green bars, *S. lingua*; blue bars, *S. parviflora*. Populations: DO: Dugi otok; MO: Molat; M: Mljet; V: Vir (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

S. × todaroi, collected from mixed populations, strongly supports that *S. lingua* and *S. parviflora* are the parental species of this hybrid.

Only few recent records of the putative hybrid on the eastern Adriatic coast (Bogdanović and Ljubičić, 2013; Jeričević and Jeričević, 2016) have been published since it was first found during our field work in 2008 (Šegota et al., 2012), increasing to altogether six known localities in this area so far. The area extends across the whole coast, ranging from the southern Istrian peninsula, across the islands Molat, Vir, Dugi otok, Korčula and Mljet. Considering the distribution of the parental species, it is likely that the hybrid is more frequent than currently recorded, but mostly overlooked due to its morphological resemblance to *Serapias lingua*, one of the most frequently recorded orchids along the Croatian coast (Kranjčev, 2005; Nikolić, 2018). Nevertheless, the stability of its populations requires further confirmation given that the other parental species *S. parviflora* is mainly autogamous (Bellusci et al., 2009; Delforge, 2006), with limited ability to participate in the hybridization process. The hybrids are probably sterile due to the seemingly triploid chromosome number, although vegetative propagation could potentially be contributing to the population stability. Clonality has been recognized as a common reproductive strategy in *S. lingua* (Pellegrino et al., 2015), however to our knowledge, no studies regarding the fertility and/or vegetative propagation of *S. × todaroi* have been performed yet. In our opinion, the hybrid is most probably not widespread, but rather rare or occasional.

Not surprisingly, we have found that floral traits are critical to distinguish the examined species, and appear to be more important than vegetative traits. Pairwise comparisons have pointed to the same, whereas non-significant traits were, in most cases, vegetative. Floral traits that were significantly different in all combinations of species pairs were petal width, hypochile width, maximum and basal epichile width. In other words, every taxon significantly differed from the other two in these four characteristics, which is a clear implication of their importance in distinguishing between the taxa. The traits related to the labellum (hypochile width, maximum and basal epichile width) have at the same time shown the strongest correlation with the first discriminant function, along with epichile length. The importance of the labellum is evident from the overall results of the discriminant analysis as well. Namely, out of the eight traits most strongly correlated with the first and second discriminant function, seven were associated with the labellum.

The importance of floral traits (particularly those associated with the labellum) in distinguishing members of the genus *Serapias* was previously stressed by Venhuis et al. (2007), who have studied morphological variations among members of the genus in southwestern Europe, including *S. lingua* and *S. parviflora*, and found hypochile and epichile size to be most strongly correlated with discriminant functions. Moreover, Hršak et al. (2011), who performed a morphological study of

six *Serapias* taxa in Croatia, including *S. lingua*, concluded that the floral traits have greater discriminatory power than the vegetative traits in distinguishing the studied taxa.

The only previous morphological comparison between *Serapias × todaroi* and its parental species was carried out by Lorenz (2001), in his study of the genus *Serapias* in Italy. Although Lorenz (2001) did not provide the information about the particular traits that best distinguish among these taxa, he had already concluded that, although the hybrid is roughly intermediate between *S. lingua* and *S. parviflora*, *S. × todaroi* is more similar to *S. lingua*. Notably, a comparison with the measurements of Lorenz (2001) performed on all three taxa shows that our plants from the eastern Adriatic are on average smaller. It is likely that such difference in size may be caused by the climatic differences given that populations studied by Lorenz (2001) were sampled on Malta, Sicily and Sardinia.

When the hybrid was first described, a note was already made about its similarity with *S. lingua* (Camus et al., 1908). During the revision of its name, Baumann and Künkele (1989) briefly discussed the influence of both parental species on the hybridogenous nature of *S. × todaroi*. According to their observations, the main trait confirming its hybrid origin was the intermediate shape of the base of labellum (Baumann and Künkele, 1989), a feature repeatedly observed in our study as well. Regarding the eastern Adriatic populations, our comparison of morphology has shown a great resemblance between *S. × todaroi* and *S. lingua*, especially regarding floral traits, strongly supporting the parenthood of *S. lingua* over the hybrid. On the other hand, *S. parviflora* significantly differed from both *S. × todaroi* and *S. lingua* in as many as 14 shared traits, most of them floral. The pattern of *S. parviflora* strongly differing from both *S. lingua* and *S. × todaroi* was also evident from discriminant analysis.

The floral "segregation" of *Serapias parviflora* and "mixing" between *S. lingua* and *S. × todaroi* are clearly shown in the results of the classification discriminant analysis, illustrating the dominant influence of a single parental species on the floral morphology of the hybrid. The distinction of *S. parviflora* regarding floral traits was expected, given that the species bears the smallest flowers in the genus. In spite of floral differences between *S. parviflora* and *S. × todaroi*, the parenthood of *S. parviflora* is supported by the similarity in plant height, intermediate shape of the base of labellum, intermediate chromosome number, and the fact that the hybrid was mainly syntopic with both parental species.

Although *S. lingua* and *S. × todaroi* have similar flower morphology, we have found some vegetative diagnostic traits to key them apart. Notably, *S. lingua* individuals were on average shorter, had a lower number of stem leaves, and their longest leaves were shorter than those of *S. × todaroi* and *S. parviflora*. The significance of plant height was previously indicated by Lorenz (2001). In his work, he provided a remark about *S. × todaroi* describing these plants as rather large and

robust, which sometimes even leads to it being confused with *S. vomeracea* (Lorenz, 2001). The hybrid was clearly intermediate between the parental species, and overall evidence suggests that *Serapias lingua* has mostly influenced its floral traits, while *S. parviflora* has mostly influenced vegetative traits, particularly plant height.

5. Conclusions

Morphological traits and chromosome counts of the studied eastern Adriatic hybrid populations confirm the assumed parenthood of *Serapias lingua* and *S. parviflora* of the putative hybrid. *S. lingua* is florally similar to the hybrid, while *S. parviflora* has distinctively small flowers, but its parenthood is also supported by the similarity in habitus, particularly plant height. Moreover, intermediate chromosome number, intermediate shape of the base of the labellum and the fact that *S. × todaroi* was mostly syntopic with the assumed parental species also strongly suggest its hybridogenous origin. The main diagnostic traits between these taxa are the plant height, the shape of the labellar base and, to some extent, the flower (particularly the labellum) size.

Conflicts of interest

None

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.flora.2018.09.006.

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