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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;

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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