Molecular Phylogenetics and Evolution 47 (2008) 986–991

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Phylogenetic relationships in the orchid genus *Serapias* L. based on noncoding regions of the chloroplast genome

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ARTICLE INFO

Article history:
Received 10 July 2007
Revised 10 March 2008
Accepted 11 March 2008
Available online 20 March 2008

Keywords:
cpDNA
Floral size
Orchidaceae
Phylogeny
Serapias

ABSTRACT

A molecular phylogenetic analysis was performed on 14 species of the Mediterranean unrewarding orchid genus *Serapias* using sequences of four noncoding regions of chloroplast DNA. This study has led to a new interpretation of the evolutionary relationships in this genus. The well-defined phylogenetic tree supports a division of taxa into two main clades, each including two minor groups. The molecular relationships found in this study differ from those defined by traditional systematic morphological assessments. By comparing the variation in sequence to variations in floral traits, we propose that the split in the two main lineages reflects an early differentiation of flower size, perhaps due to the shift from allo- to self-pollination. Conversely, the relationships within each minor group do not reflect floral size variation; therefore, we presume that this diversification resulted from genetic drift, local selection forces, and multiple, independent transitions towards self-pollination and polyploidy.

1. Introduction

The orchid genus *Serapias* L. is found primarily around the Mediterranean basin, and the center of its diversity lies in southern Italy and the Greek islands (Baumann and Künkele, 1989). Its distribution area is bordered by Northern Brittany in the north, northern Africa in the south, the Azores and Canaries in the west, and the Caucasus and Israel on the east. Like other terrestrial Euro-Asiatic Orchidinae, members of *Serapias* are perennial geophytes, producing a short vegetative shoot with few alternate leaves in early spring. Subsequently, plants elongate a reproductive scape bearing an inflorescence that is more or less dense depending on the taxon (Baumann and Künkele, 1989).

The floral shape and the pollination system are almost peculiar. The flower is subtended by a bract of a similar colour. The apical portion of the labellum, referred to as the epichile, is oriented downwards. The basal portion (hypochile) and two lateral lobes are arranged with the perigonium in a small, dark tube that varies in size among taxa (Baumann and Künkele, 1989). The pollination system is both generalistic, in that several insects may transfer pollinia (van der Cingel, 2001), and deceptive, in that plants do not offer energetic rewards, though pollinators can use the corolla as a nest hole (Dafni et al., 1981) or as a shelter on cold or rainy days (Gumprecht, 1977). Reproduction has long been assumed to be mediated by insects of appropriate dimensions. In this way, the size of the corolla may function as a pre-zygotic barrier, leading to diversification of the plant species (Dafni et al., 1981).

Doubt still surrounds the taxonomic arrangement of the *Serapias* genus as it is based essentially on quantitative, rather than qualitative, variations in floral traits (Baumann and Künkele, 1989). Given the considerable polymorphism that exists within populations and taxa, species boundaries often overlap. Consequently, authors have expressed different opinions on both the number of recognizable species—ranging from 10 (Nelson, 1968) to 17 (Baumann and Künkele, 1989) or even 27 (Delforge, 2005)—as well as their evolutionary relationships (Pridgeon et al., 1997; Bateman et al., 2003).

Molecular systematic studies conducted with nuclear ribosomal internal transcribed spacers (nrITS) have supported the monophyly of the *Serapias* genus among the Euro-Asiatic Orchidinae (Bateman et al., 2003; Pridgeon et al., 1997). However, they have failed to resolve the phylogenetic relationships of the *Serapias* taxa, a fact that indicates a very recent diversification. A potential alternative source of phylogenetic information is noncoding regions of chloroplast genome (cpDNA), which can be useful because of their high rate of random, independent mutations due to the absence of selective pressure (Böhle et al., 1994). Although the evolution of cpDNA has proven to be more complicated than initially assumed, prompting concerns about the use of cpDNA, many studies have supported the use of noncoding cpDNA regions in phylogenetic analysis (Shaw et al., 2005; Small et al., 2005). Moreover, cpDNA regions show low levels of homoplasy, making them particularly suitable for the comparison of closely related taxa that still exhibit a high gene flow (Clegg et al., 1994).

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doi:10.1016/j.ympev.2008.03.019

In this study, we have carried out a phylogenetic analysis of the genus *Serapias* by sequencing four discrete, noncoding cpDNA regions. We have compared the evolutionary relationships suggested by our phylogenetic tree to the current taxonomic arrangement. In light of the new information gathered in this study, we discuss potential mechanisms of speciation that may explain the present-day diversification.

2. Materials and methods

2.1. Plant material

To reconstruct the phylogenetic relationships of the *Serapias* genus, we analyzed sequences of chloroplast genome (cpDNA) from a total of 345 specimens, representing 14 species according to the Delforge (2005) classification. In addition, *Ophrys incubacea* Bianca and *O. bertolonii* Moretti, two taxa strictly related to *Serapias* genus (Bateman et al., 2003), were chosen as outgroups.

For each species, leaf material from several populations scattered throughout the Mediterranean basin (Table 1) was collected and stored in silica gel. Since we sampled leaf material from populations growing in legally protected areas, no herbarium vouchers were prepared, in order to protect local botanical heritage and to minimize environmental impact.

2.2. DNA extraction, PCR, and sequencing

Total genomic DNA was extracted from 1 g of leaf tissue according to the protocol of Doyle (1991). We amplified two intergenic regions and two introns of cpDNA. PCR primer pairs were designed by Taberlet et al. (1991) for the intergenic spacer (*trn*L–*trn*F) between the 3′exon of *trnL* (UAA) and *trnF* (GAA), by Hamilton (1999) for the intergenic spacer (*trn*S–*trn*G) between the tRNA–Ser (GCU) and tRNA–Gly (UCC), by Oxelman et al. (1997) for the *rps*16 intron and by Weising and Gardner (1999) for the *atp*F intron.

PCRs were performed in a total reaction (100 μL) containing 10–20 ng of cpDNA template, 10 μL of 10 × reaction buffer, 2 mM $MgCl_2$, 100 mM of each dNTP, 2.5 U of BioTaq™ DNA Polymerase (Bioline Inc., Boston, MA, USA), and 0.2 mM of each primer (MWG-Biotech AG, Ebersberg, Germany). The thermocycling procedure consisted of an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C. PCR reactions were performed on a PTC-100 Thermal Cycler (MJ Research Inc., Watertown, MA, USA). PCR fragments were purified with a QIAquick PCR purification kit (Qiagen S.p.A., Milan, Italy) to remove unincorporated primers and dNTPs.

Automatic DNA sequencing was performed on an ABI PRISM310 Genetic Analyzer (PE Biosystems, Foster City, CA, USA). Sequencing reactions were set up according to the manufacturer's recommendations, and purified by ethanol precipitation at room temperature. Both strands were sequenced at least once for each sample.

Sequence files were imported separately for each specimen into BioEdit 7.0.4.1 software (Hall, 1999), and complementary strands were aligned using the Clustal V option provided in the sequencing software. Ambiguous sites were checked manually and corrected by comparing electropherograms from both strands. Consensus sequences were obtained for each specimen, and 5′ and 3′ borders

Table 1
Serapias sequences for four noncoding regions of chloroplast DNA (*atp*F and *rps*16 intron, *trn*L–*trn*F and *trn*S–*trn*G intergenic spacers)

Taxon	Collection location		No. of Populations	GenBank accessions			
				*atp*F intron	*rps*16 intron	*trn*L–*trn*F spacer	*trn*S–*trn*G spacer
S. apulica (Baumann & Künkele) Delforge	Apulia	Italy	3	EU531518	EU549758	EU045538	EU420754
S. cordigera L.	Campania, Calabria, Apulia, Sardinia	Italy	7	EU531520	EU531537	EU045537	EU420756
	Zakinthos, Peloponnese	Greece	2	EU531520	EU531537	EU045537	EU420756
S. elsae Delforge	Algarve	Portugal	3	EU531527	EU549764	EU045545	EU420763
S. gregaria Godfery	Provence	France	3	EU531528	EU549765	EU045546	EU420764
S. levantina Baumann & Künkele		Israel	2	EU531531	EU549767	EU045540	EU420767
S. lingua L.	Apulia, Calabria, Sardinia	Italy	7	EU531522	EU549760	EU045542	EU420758
	Corfù	Greece	1	EU531522	EU549760	EU045542	EU420758
	Provence	France	1	EU531522	EU549760	EU045542	EU420758
	Andalusia–Extremadura	Spain	2	EU531522	EU549760	EU045542	EU420758
S. neglecta De Notaris	Liguria	Italy	1	EU531530	EU531534	EU045539	EU420766
	Provence	France	3	EU531530	EU531534	EU045539	EU420766
S. nurrica Corrias	Sardinia	Italy	2	EU531519	EU549759	EF690287	EU420755
S. olbia Verguin	Provence	France	3	EU531525	EU531535	EU045544	EU420761
S. parviflora Parlatore	Apulia, Calabria, Campania, Sicily, Sardinia	Italy	7	EU531521	EU531536	EU045541	EU420757
	Corfù	Greece	2	EU531521	EU531536	EU045541	EU420757
	Provence	France	1	EU531521	EU531536	EU045541	EU420757
	Extremadura	Spain	1	EU531521	EU531536	EU045541	EU420757
	Algarve	Portugal	1	EU531521	EU531536	EU045541	EU420757
S. perez-chiscanoi Acedo	Extremadura	Spain	3	EU531526	EU549763	EU045536	EU420762
S. politisii Renz	Apulia	Italy	3	EU531523	EU549761	EU045543	EU420759
	Corfù	Greece	3	EU531524	EU549762	EU045535	EU420760
S. olbia Verguin	Provence	France	3	EU531525	EU531535	EU045544	EU420761
S. strictiflora Welwitsch ex Veiga	Andalusia	Spain	2	EU531529	EU549766	EU045547	EU420765
	Evora	Portugal	1	EU531529	EU549766	EU045547	EU420765
S. vomeracea (N.L. Burman) Briquet	Piedmont, Lombardy, Liguria, Venetia, Emilia Romagna, Tuscany, Marche, Latium, Campania, Calabria, Sicily	Italy	28	EU531517	EU549757	AY883088	EU420753
	Zakinthos, Corfù, Chios, Crete, Peloponnese	Greece	8	EU531517	EU549757	AY883088	EU420753
	Provence	France	3	EU531517	EU549757	AY883088	EU420753
		Turchia	2	EU531517	EU549757	AY883088	EU420753
	Akrotiri, Mandria, Kedares	Cyprus	3	EU531517	EU549757	AY883088	EU420753
	Corfù	Greece	1	EU531517	EU549757	AY883088	EU420753
Ophrys bertolonii Moretti	Calabria	Italy	1	EU531515	EU531532	EU045548	EU420751
O. incubacea Bianca	Calabria	Italy	1	EU531516	EU531533	EU045549	EU420752