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NUCLEAR RIBOSOMAL DNA ITS PARALOGS AS EVIDENCE OF RECENT INTERSPECIFIC HYBRIDIZATION IN THE GENUS *OPHRYS* (ORCHIDACEAE)

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Ophrys holubyana Andrasovszky (Orchidaceae), distributed in the Carpatho-Pannonian region, is generally believed to be of hybrid origin. Although its hybrid origin is broadly accepted by many authors, no molecular evidence has been found to support the hypothesis. The nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) region was sequenced from *Ophrys holubyana*, and from the presumed progenitor taxa: *Ophrys fuciflora* (Cr.) Rchb. and *Ophrys bicornis* Sadler ex Nendtvich. Nearly all the known populations in the Carpatho-Pannonian region were sampled, and the first data on the nrDNA ITS sequence of *O. holubyana* and *O. bicornis* are presented. Aligning the ITS sequences revealed no differences among the ten samples. After cloning the amplified ITS regions, eight discrete ITS paralogs with regularly appearing nucleotide differences could be partitioned, differing in only 6 base pairs. Paralog sequences were detected not only in *O. holubyana* but also in some populations of the two parent species, suggesting that *O. fuciflora* and *O. bicornis* in the Carpatho-Pannonian region are also partially of hybrid origin themselves, or influenced by introgression. The study suggests that nrITS regions can be generally useful in the study of *Ophrys* systematics and phylogeography and for analyzing the hybrid zones of related *Ophrys* species groups.

Key words: *Ophrys*, Carpatho-Pannonian region, cloning, sequencing, hybridization, hybrid speciation, nrITS paralogs.

INTRODUCTION

The genus *Ophrys* is distributed from the Canary Islands in the west to Iran in the east, and from Scandinavia in the north to the Sahara in the south (Soliva et al., 2001; Bernardos et al., 2003). Its highest diversity is reached in the Mediterranean region.

The number of species within the genus has been estimated at anything between 21 (Nelson, 1962) and 215 (Delforge, 2001), showing that the taxonomy of the genus is controversial. Because many species are thought to be of hybrid origin (Nelson, 1962; Danesch and Danesch, 1972; Baumann and Künkele, 1982; Delforge, 1994), and the floral morphology is highly variable, it is obviously difficult to identify the species boundaries and to deduce the phylogenetic relationships of the genus.

The genus *Ophrys* is commonly divided into two sections, *Pseudophrys* and *Ophrys*, on the basis of pollination type (abdominal or cephalic) (Bernardos et al., 2003). The sections are further subdivided into species complexes and species groups (Delforge, 2001) based primarily on floral morphological characters (mainly the shape, color, hairiness and size of the labellum), which play a great role in the classification of the genus.

The *Ophrys fuciflora* complex in section *Ophrys* contains Atlanto-Mediterranean, Adriato-Mediterranean and Ponto-Mediterranean elements (Delforge, 2001). Two species from the complex are encountered in the Carpatho-Pannonian region and its surroundings: *Ophrys fuciflora* (Cr.) Rchb., possessing rather flat, broad lips with slightly emerging lateral lobes; and

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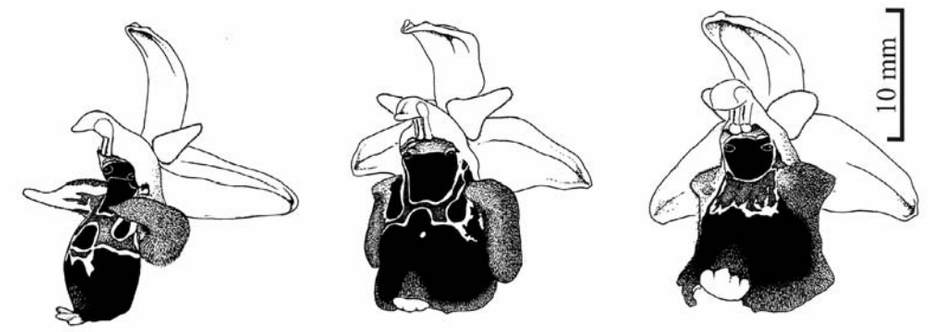

Fig. 1. Typical flowers of the studied *Ophrys* species. From left to right: *O. bicornis*, *O. holubyana*, *O. fuciflora*. Redrawn after photographs taken at Pécs (pop. 3), Balatonfüred (pop. 10) and Maróc (pop. 1) by A. Molnár V.

Ophrys bicornis Sadler ex Nendtvich, possessing transversely vaulted lips with conical, long lateral lobes (Fig. 1). The third member of the complex in the Carpatho-Pannonian region, *Ophrys holubyana*, was found and described by the Hungarian botanist Andrasovszky in 1915 (Andrasovszky, 1917) from the Republic of Slovakia in the former Austro-Hungarian Empire. This taxon has been interpreted as a hybrid derivative of *O. fuciflora* and *O. bicornis* expressing floral morphological features intermediate between its presumed progenitors (Fig. 1). This is very widely accepted in the orchid literature (Keller and Soó, 1930–1940; Soó, 1959; Kümpel, 1977; Procházka and Velísek, 1983; Baumann and Künkele, 1986; Buttler, 1986; Delforge, 1994; Potuček and Čačko, 1996; Procházka et al., 1999; Delforge, 2001; Vlčko et al., 2003), probably on the basis of the morphological and chorological features of the species involved. In addition to the floral morphological characters described above, *O. fuciflora* and *O. bicornis* are the only members of the *Ophrys fuciflora* species complex occurring in the Carpatho-Pannonian region, where *O. holubyana* is considered to be an endemic species. However, the distribution areas of the presumed parental species are parapatric here, and *O. holubyana* occurs in the contact zone of the areas (Molnár and Gulyás, 2005).

Diploid chromosome counts (2n = 36) are very frequent in sect. *Ophrys* (Löve and Kjellqvist, 1973; Greilhuber and Ehrendorfer, 1975; Queirós, 1983; Silvestre, 1983; Chiscano et al., 1990; Bianco et al., 1991; Bernardos and Amich, 2002). Polyploidy has been reported only once (Balayer, 1984). Bernardos et al. (2003) found the *O. scolopax* group to be karyologically very stable and claimed that "the speciation of the *O. scolopax* group has not led to changes either in chromosome numbers or chromosome morphology."

The morphological diversity of *Ophrys* species may be partly the result of interspecific hybridization and introgression, as suggested by morphological evidence from Anderson (1953), Stebbins and Ferlan (1956), Danesch and Danesch (1976), Vöth and Ehrendorfer (1976), Ehrendorfer (1980), and Pedersen and Faurholdt (1997). As has long been recognized by a number of authors (e.g., Stebbins, 1950; Grant, 1971), hybridization between species and subsequent polyploidization and gene duplication are important factors in adaptive divergence (Soltis et al., 2003). Hence, it is interesting that in the genus *Ophrys* polyploidy can only be detected in the primitive sect. *Pseudophrys*, while sect. *Ophrys* seems to contain only homoploid hybrids. Corresponding homoploid hybrid speciation in other groups of flowering plants has been studied in detail by Rieseberg (1991, 1997), Ungerer et al. (1998), Buerkle et al. (2000) and Lexer et al. (2003), who claim that it is a very rapid speciation process.

ITS sequencing is a very valuable tool for understanding the phylogeny of diverse groups of angiosperms (Baldwin et al., 1995). It has been suggested that ITS amplicons should be cloned to detect paralogs, because they could bear evidence of past hybridization; the importance of obtaining ITS paralogs from all members of the studied species lineage is emphasized as a way of revealing species relationships (Suh et al., 1993; Ritland et al., 1993). A growing number of studies have reported intra-individual ITS paralogs (e.g., Wendel et al., 1995; Fenton et al., 1998; Weiblen, 2000; Gernandt et al., 2001 ; Zhang et al., 2002; Wei et al., 2003; Behnke et al., 2004; Razafimandimbison et al., 2004), suggesting that the main cause of heterogeneity is slow concerted evolution due to hybridization and polyploidy. Incomplete concerted evolution is attributable to recent interspecific hybridization, high rates of mutation, loss of sexual recombination, or location of nrDNA loci on nonhomologous chromosomes (Campbell et al., 1997). It has recently become clear that complete concerted evolution of nrDNA is not as widespread among angiosperms as previously assumed (Bailey et al., 2003). High levels of ITS heterogeneity were found, for example, in Pinaceae, mainly due to the especially long ITS of this family (Gernandt et al., 2001), and in

TABLE 1. *Ophrys* species and codes of populations studied. Vouchers are deposited at the Herbarium of Debrecen University (DE).

Code	Taxon	Site	Voucher	nrITS type	GenBank accession no.
1.	*O. fuciflora*	Maróc, Hungary	Molnár, 782-1 DE	A, B, C, D	AJ972925-28
2.	*O. fuciflora*	Perchtoldsdorf, Austria	Molnár, 782-2 DE	A, B	AJ972929-30
3.	*O. bicornis*	Pécs, Hungary (loc. class.)	Molnár, 784-5 DE	A, G, I, L	A J972933, AM039522-24
4.	*O. bicornis*	Litohoron, Greece	Molnár, 784-6 DE	A	AJ972937
5.	*O. bicornis*	Siklós, Hungary	Molnár, 784-7 DE	A	AJ972938
6.	*O. bicornis*	Kunpeszér, Hungary	Molnár, 784-8 DE	A	AJ972939
7.	*O. bicornis*	Budapest, Hungary	Molnár, 784-9 DE	A	AJ972940
8.	*O. holubyana*	Šípkov, Slovakia	Molnár, 782b-3 DE	A, B, J	AJ972941-42, AM039525
9.	*O. holubyana*	Krásna Veš, Slovakia	Molnár, 782b-4 DE	A, B	AJ972945-46
10.	*O. holubyana*	Balatonfüred, Hungary	Molnár, 782b-5 DE	A, B	AJ972949-50
11.	*O. apifera*	Balatonszölös, Hungary	Molnár, 783-3 DE	-	AJ973253
12.	*O. sphegodes*	Cegléd, Hungary	Molnár, 780-11 DE	-	AJ973254
13.	*O. sphegodes*	Kunpeszér, Hungary	Molnár, 780-12 DE	-	AJ973255

Xylaria hypoxylon isolates (Fungi) (Platas et al., 2003). In *Ficus*, however, the nucleotide differences between the clones were irregular, though this may have been due to DNA polymerase errors (Weiblen, 2000).

The main objective of this study is to find molecular evidence of past hybridization in the studied *Ophrys* taxa. Hybridization might be combined with polyploidization (though highly unlikely in sect. *Ophrys*; see above), but chromosome counting was impossible because collecting the samples would have threatened the populations.

MATERIALS AND METHODS

PLANT MATERIAL

The study examined two populations of *O. fuciflora*, three of *O. holubyana* and five of *O. bicornis*, collected from ten different locations (Tab. 1; Fig. 2). The populations were classified by floral morphological characters according to the descriptive nomenclature of Delforge (2001). Two individuals were sampled from each population. All samples were fresh leaves stored in absolute alcohol, dried completely before extraction.

In addition, two samples of *O. sphegodes* from two different locations and one sample of *O. apifera*, taxa from two phylogenetically distantly related species groups (Soliva et al., 2001), were used as an outgroup in order to present the variability of ITS in *Ophrys*.

Fig. 2. Sampling sites of *O. fuciflora* complex: *O. fuciflora* (triangles), *O. holubyana* (squares) and *O. bicornis* (circles), mentioned in text and tables.

DNA EXTRACTION AND PCR

In this study we used sequences from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA). The DNA extraction procedure was carried out according to Doyle and Doyle (1987). Dried leaves (1–30 mg) were thoroughly ground in liquid nitrogen and then resuspended in lysis buffer (2% CTAB, 20 mM EDTA pH 8, 100 mM Tris-HCl pH 9, 1.4 mM NaCl). After incubation at 65˚C for 60 min, the samples were centrifuged at 20,000 g for 10 min; then the supernatant was extracted with an equal volume of chloroform and centrifuged for 15 min at 20,000 g. The extraction procedure was done twice. The DNA was precipitated with two volumes of 100% ethanol and stored at -20˚C or below for 1 h. DNA was pelleted by centrifugation at 20,000 g for 30 min. The pellet was washed twice with 70% ethanol, dried, and redissolved in 70 µl 0.1 M Tris (pH 7.5).

For amplifying ITS, the PCR reaction mixture contained 0.1 volume 10× PCR buffer (Zenon), 200 µM each of dNTPs (Fermentas), 2 mM $MgCl_2$, 0.2 µM of each primer, 1.25 U Taq DNA polymerase (Zenon) and 5 ng/µl genomic DNA extract. The amplifications were performed with a Perkin Elmer PCR System 2400

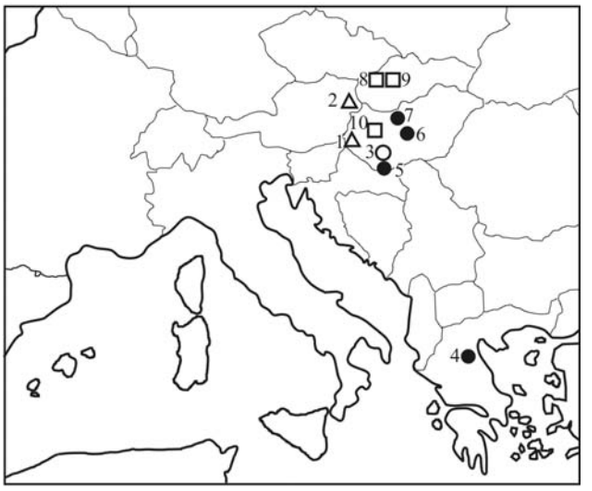

Fig. 3. Distribution of paralogs in *O. fuciflora* complex: *O. fuciflora* (triangles), *O. holubyana* (squares) and *O. bicornis* (circles) populations with (empty symbols) and without (filled symbols) paralogs.

programmed for a denaturation step at 94˚C for 4.30 min, followed by 33 cycles of denaturation for 30 sec at 94˚C, annealing for 30 sec at 51˚C and extension for 30 sec at 72˚C, the extension time being increased by one second in every cycle; the thermal cycling was ended by a final extension for 7 min at 72˚C.

PRIMERS AND SEQUENCING

The entire nrITS region was amplified by the newly devised plant-specific ITS1A (5'-GACGTCGCGAGAAGTCCA) and ITS1P (5'-CCGTACCATTTAGAGGAAGGAG) primers and the universal primer ITS4 (White et al., 1990), and applied in the PCR reaction to specifically amplify the plant nrITS.

For direct sequencing, the PCR products were purified using a Montage centrifugal unit (Millipore). An ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit V. 3.1 (Applied Biosystems) was used for cycle sequencing, and electrophoresis was carried out with an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) according to the manufacturer's instructions. The sequences were aligned with the ClustalW (Thompson et al., 1994) program with default parameters.

CLONING ITS

PCR products from *O. fuciflora*, *O. bicornis* and *O. holubyana* were cloned and sequenced for comparison with the results of direct sequencing. Ten ITS clones from one individual per species were sequenced to look for the presence of heterologous ITS copies within species. ITS PCR products were ligated and transformed using the pGEM-T Easy Vector System II (Promega Corp., U.S.A.). Transformed cells were screened with ampicillin, and recombinant plasmid DNA was isolated using the Wizard Plus Miniprep DNA purification system (Promega, U.S.A.).

TABLE 2. Composition of cloned ITS sequences from three paralog-bearing populations of *O. fuciflora*, *O. holubyana* and *O. bicornis*. Note that identical sequences of paralogs are not shown.

nrITS type	Nucleotide position					
	19	97	142	220	453	558
A	C	T	C	A	C	G
B	T	G	T	T	T	T
C	C	T	C	A	T	T
D	T	G	T	T	C	G
G	C	G	T	A	C	G
I	C	T	C	A	C	T
J	T	T	C	T	T	T
L	C	G	T	T	T	T

RESULTS

Direct sequencing of the nrITS region resulted in identical 624 bp sequences in *O. fuciflora*, *O. holubyana* and *O. bicornis*. As for the outgroup, sequence alignment revealed differences of only 1.44% (9 bp) for *O. sphegodes* and 2.24% (14 bp) for *O. apifera*.

The alignment of the cloned ITS sequences resulted in a matrix with 624 positions with single nucleotide polymorphism (SNP) at six nucleotide sites, which distinguished eight regular sequence variations (Tab. 2). The eight nrITS sequence variations were treated as nrITS paralogs, since intra-individual nrDNA polymorphism is indicative of sequence paralogy (Bailey et al., 2003).

Four paralogs (A, B, C, D) were cloned from *O. fuciflora* (population 1), three (A, B, J) from *O. holubyana* (pop. 8) and four (A, G, I, L) from *O. bicornis* (pop. 3). Two paralogs (A, B) were cloned from population 2 (*O. fuciflora*) and populatons 9 and 10 (*O. holubyana*). The most frequent paralog, A, was found in the majority (66.6%) of the clones. Paralog B was less frequent (10.0%), whereas the additional paralogs exhibited smaller frequencies (6.6% for C and 3.3% for the remaining ones). No polymorph nrITS sequences were found in the samples of populations 4, 5, 6 and 7 (*O. bicornis*), where only sequence type A could be detected. Another aspect is that *Ophrys bicornis* samples from Greece (pop. 4) and Hungary (pop. 5, 6, and 7) possessed only nrITS type A, while the samples of the same species from Pécs, Hungary (pop. 3) exhibited paralogs (Fig. 3).

DISCUSSION

ITS paralog sequences may emerge due to polymerase errors or PCR artefacts (see Introduction), but the regularity of differential nucleotides in our study excludes this possibility. Alternatively, the nrDNA polymorphism found in the studied *Ophrys* taxa could be due to a high mutation rate in the genus, the loss of sexual recombination, or location of the screened multigene family on nonhomologous chromosomes. However, asexual reproduction, which could lead to loss of sexual recombination, is a very rare phenomenon in *Ophrys* (van der Cingel, 1995), where it is only known from *Ophrys apifera*, a taxon not included in the studied species group. There are no reports of high mutation rates of nrDNA or of location of nrDNA on nonhomologous chromosomes in *Ophrys*, but these phenomena cannot be ruled out completely when interpreting nrDNA polymorphism. Still, it seems more likely that the intra-individual ITS sequence variation originated from crossing of different ITS lineages, in which case the polymorphic loci represent parental sequences and recombinants. Similarly, Campbell et al. (1997) interpreted the presence of polymorphic intra-individual nrITS in *Amelanchier "erecta"* to be an indication that this taxon originated through hybridization between taxa representing two main phylogenetic clades.

Samples of *O. holubyana* exhibited paralog sequences. This supports the previous assumption, based on floral morphological characters, that this taxon is of hybrid origin. However, the presence of paralog sequences in populations 1 and 2 (classified as *O. fuciflora*) as well as in population 3 (classified as *O. bicornis*) suggests a complex hybridization pattern.

Our pending Europe-wide survey suggests that paralogs A and B are original sequence variants belonging to the progenitor taxa (*O. bicornis* and presumably *O. fuciflora*), and that the remaining paralogs are recombinant sequences. Considering the present findings, the studied populations of the *Ophrys fuciflora* complex in the Carpatho-Pannonian region (*O. bicornis*, *O. fuciflora* s.str. and *O. holubyana*) could be divided into two groups: (i) populations without nrITS paralogs (pop. 4, 5, 6, 7, all classified as *O. bicornis*) should be regarded as possessors of one progenitor sequence; (ii) taxa with nrITS paralogs (pop. 1, 2, 3, 8, 9, 10, belonging to all three species) probably contain hybrids and introgressants. Taxonomy is beyond the scope of this paper, but it is worth mentioning that samples from the *locus classicus* of *Ophrys bicornis* Sadler ex. Nendtvich (pop. 3) contain nrITS paralogs.

Since paralog sequences are quickly homogenized due to concerted evolution (Baldwin et al., 1995; Soltis et al., 2003), the presence of paralogs in the present data set may imply that the hybrid zone in the Carpatho-Pannonian region has been formed recently (in postglacial times). Moreover, the dominant nrITS types are presumed to be connected to lineages isolated in Mediterranean refugia during previous glaciations, while the less frequent nrITS types are produced by recombination. Studies of nrITS paralogs could have some value in revealing the phylogenetic relationships and postglacial phylogeography of the genus *Ophrys*.

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