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Molecular identification of mycorrhizal fungi in *Dactylorhiza sambucina* (Orchidaceae)

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Abstract: We amplified and sequenced internal transcribed spacer (ITS) region of the nuclear ribosomal repeat from fungi in roots of *Dactylorhiza sambucina* (Orchidaceae) and used the extended database to identify the mycorrhizal fungi. We molecularly identified three ITS recovered from *D. sambucina* roots, one belonging to *Rhizoctonia* group, and two to ascomycetes, for the first time in Orchidoidae. In many cases, two sequence types were found from the same orchid root, providing that two taxa may be involved in mycorrhizal formation (multiple mycobiont colonization). Moreover, we demonstrated that *D. sambucina* plants, irrespective of their colour polymorphism, possess roots containing several fungi belonging to both asco- and basidiomycetes.

Key words: *Dactylorhiza sambucina*; ITS; mycorrhizal fungi; Orchidaceae

Introduction

The Orchidaceae is one of the largest plant families on Earth, including almost 10% of all flowering plant species (Dressler 1981). Members of this family grow in a wide range of habitats and have a substantial variety of life history strategies ranging from epiphytic to terrestrial, and from evergreen to nongreen species. All orchids are initially myco-heterotrophic (Leake 1994) but most eventually produce leaves and become photosynthetic. Fungal specificity in photosynthetic orchids remains contentious (Otero et al. 2002). The specificity of the interaction between orchids and their mycorrhizal fungi has long been controversial, with some authors claiming that orchids are generalists (Masuhara & Katsuya 1991; Masuhara et al. 1993), but others arguing that they are specialists (Clements 1988; McKendrick et al. 2002; Selosse et al. 2002). It was suggested that fungal specificity found in some photosynthetic orchids could be a function of the fungi that are available in the narrow range of habitats where the orchids occurred (ecological specificity; Perkins & McGee 1995) and might not reflect absolute specificity in fungal associations (Curtis 1939). The orchid mycorrhiza has been recognized as a distinct type (Smith & Read 1997), with the mycobiont forming pelotons (hyphal coils) in the cortical tissue of protocorms, roots, tubers, and rhizomes (Rasmussen 1995). Orchid mycorrhizal fungi have traditionally been studied via isolation and establishment of pure cultures from colonized root tissue (Currah et al. 1997). These methods usually involve surface sterilization of the orchid root and dissection of the mycobiont from the infected tissue, followed by a series of rinsings in sterile water before transfer to suitable growth media. Unfortunately, not all isolated fungi grow willingly, and cessation of growth after some time often makes the culture useless and methods independent of this should be preferred. Polymerase chain reaction (PCR) methods have been used to directly identify fungi within roots using fungal specific primers (Gardes & Bruns 1993; Kjøller & Rosendahl 2000). Such methods have been used to characterize mycobionts of Orchidaceae, eliminating the time-consuming culture step (Taylor & Bruns 1999). In particular, the nuclear ribosomal internal transcribed spacer (ITS) is the most often examined locus. Thus, molecular methods that can identify individual fungi when multiple fungal species infect a single plant are needed.

The aims of the present paper were to determine mycorrhizal fungi in *Dactylorhiza sambucina* roots using PCR to amplify specific nucleotide sequences and to test whether mycorrhizal specificity varies in relation to position along orchid root or across three colour polymorphs.

Material and methods

Study species

Dactylorhiza sambucina is a widespread rewardless orchid, occurring throughout the Mediterranean, central Europe, and into southern Scandinavia (Tutin et al. 1980), showing a flower colour polymorphism, with both yellow- and

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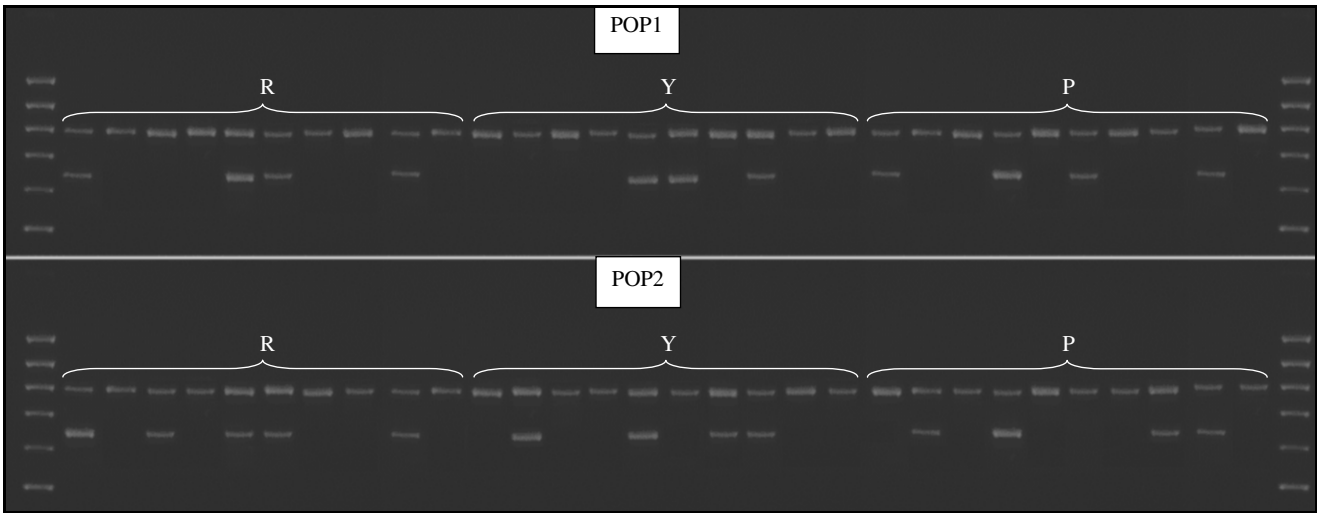

Fig. 1. Gel electrophoresis of internal transcribed spacer (ITS) region of the nuclear ribosomal from mycorrhizal orchid tissue of 60 specimens of *Dactylorhiza sambucina*. POP 1 and POP 2 indicate two populations, R, Y and P indicate three different coloured morphs, red, yellow and purple respectively.

purple-flowered individuals present in the same natural populations, although in different proportions. The flowers' lip provides a landing place for bees and bears a basal long empty spur. The labellum and the entrance to the spur have pigmented spots, which form a false nectar guide; inflorescences emit terpene hydrocarbons (Nilsson 1980). Pollination occurs mainly by generalized food deception of naïve bees. The main pollinators of *D. sambucina* are bumblebees (*Bombus* spp.), principally queens of *B. lapidarius*, *B. terrestris* and *B. lucorum*, *B. pascuorum*, cuckoo bumblebees (*Psithyrus* spp.) and bees of other genera, i.e. *Apis* spp. and *Osmia* spp. (Nilsson 1980). Each flower has a pollinarium with pollen packed into two pollinia, as it is in all the Orchidoideae (Dressler 1981) that can be removed by a single successful visit of a pollinator.

Populations of *D. sambucina* with three different coloured morphs (yellow, red and pink) localized in two sites (39°18′ N, 16°25′ E; 39°20′ N, 16°22′ E) on the Sila plateau (Calabria, Southern Italy) were chosen. Root material for ten specimens for each colour morph from each population was collected for molecular analysis. All roots were surface-sterilized using 10% to 20% bleach solution (Taylor & Bruns 1997). Using a compound microscope, we divided *Dactylorhiza* roots into 1 cm segments resulting in five mycorrhizal root samples per plant.

Molecular analysis

Characterization of *Dactylorhiza* mycorrhizae involved: (i) extraction of fungal and plant DNA from mycorrhizal plant tissue, (ii) amplification of fungal genomic regions useful in determining fungal identity, (iii) DNA sequencing for identification of mycorrhizal fungi and assessment of specificity.

We extracted DNA from each piece of root (total 180) according to Doyle (1991) protocol. To discriminate among fungal taxa colonizing *Dactylorhiza* roots, we amplified the highly variable fungal internal transcribed spacer (ITS) region of the nuclear ribosomal repeat directly from mycorrhizal orchid tissue using the eukaryotic universal primer pair ITS1-OF/ ITS4-OF (Taylor & McCormick 2008), which has been shown to be effective across all tested Basidiomycota. PCRs were carried out in a total reaction volume of 100 μL, containing approx. 10–20 ng of DNA, 100 µL of reaction buffer1X, 2mM $MgCl_2$, 100 mM of each dNTP, and 2.5 Units of BioTaq^{TM} DNA Polymerase (Bioline Inc., Boston, MA, USA), and 0.2 mM of each primer (MWG-Biotech AG, Ebersberg, Germany). The thermocycling profile consisted of an initial denaturation step at 94 ℃ for 3 min, followed by 30 cycles with 30 s at 94 ℃, 30 s at 55 ℃, and 2 min at 72 ℃. PCRs were performed on a PTC-100 Thermal Cycler (MJ Research Inc., Watertown, MA, USA). PCR fragments were purified by QIAquick PCR purification kit (Qiagen S.p.A., Milan, Italy) to remove unincorporated primers and dNTPs.

Sequencing reactions were set up according to Perkin-Elmer indications (Foster City, CA, USA) and purified using ethanol precipitation at room temperature. Automatic DNA sequencing was performed on an ABI PRISM310 Genetic Analyzer (PE Biosystems, Foster City, CA, USA). Both strands were sequenced at least once for each specimen.

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 as provided in the program. 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 were identified using mycorrhizal sequences already available in GeneBank (http://www.ncbi.nlm.nih.gov). Multiple alignments based on consensus sequences were carried out in BioEdit 7.0.4.1, using Clustal V. All sequences were submitted to a Blast search in GenBank as a first step to determine whether they contained the expected sequences.

Results

Successful double-stranded amplifications and complete sequences were obtained for all the orchid roots examined. PCR amplification of the ITS1F/ITS4 region resulted in DNA bands of two lengths, approx. 550bp and 700bp (Fig. 1). In many cases (23 out of 60), both products were amplified from a single fragment of root (Table 1). The amplification of the two sequence types from one root indicates that two taxa may be involved in mycorrhizal formation (multiple colonization). Moreover, while in 37 cases (61.67%) a unique longer PCR fragment was amplified and directly sequenced, in 23 cases (38.33%) a smaller frag-

Table 1. Number of PCR amplification in three colour morphtypes of *Dactylorhiza sambucina*.

		Plants		
		Red $n = 20$	Yellow $n = 20$	Pink $n = 20$
POP1	DS1	6	7	6
	DS1/DS2	2	2	3
	DS1/DS3	2	1	1
POP2	DS1	5	6	6
	DS1/DS2	2	3	2
	DS1/DS3	3	1	2

ment was present only in samples co-occurring with other fragments (Fig. 1). In reality, the sequence analysis showed that the smaller fragments were two kinds of sequences. Thus, we molecularly identified three ITS recovered from *D. sambucina* roots. The Blast search in GenBank showed that most samples produced an ITS (DS1=EF100192) of a mycorrhiza related to *Rhizoctonia* group, while other two rDNA sequences (DS2=EF100193, DS3=EF100194) exhibited an ascomycete-like ITS (Table 2). There were no significant differences among sites, among colour morphs and among root fragments in the kind of fungal sequences. Mycorrhizal colonization was observed at sparse, regular intervals along the length of the root.

Discussion

The major goals of this work were, first, to determine the mycorrhizal fungi present in *Dactylorhiza sambucina*, and, to determine if mycorrhizal specificity is structured among position along orchid root, and among orchid colour polymorphism.

We demonstrated that *D. sambucina* plants, irrespective of their colour polymorphism, possess roots containing several fungi belonging to both asco- and basidiomycetes (Table 2). As in many orchids (*Goodyera pubescens*, *Liparis lilifolia*, *Tipularia discolor*, McCormick et al. 2004; *Cypripedium* spp. Shefferson et al. 2005; *Orchis militaris* Shefferson et al. 2008) the sequence DS1 is related to taxa known as orchid mycorrhizal fungi falling within the anamorphic *Rhizoctonia* complex (Table 2). Recent molecular phylogenetic and ultrastructural analyses show that this complex falls into three distantly related families in teleomorph, Tulasnellaceae, Sebacinaceae, and Ceratobasidiaceae (Peterson et al. 1998; Taylor et al. 2002). In particular, previous molecular analyses revealed that *Dactylorhiza majalis* (Kristiansen et al. 2001) and *D. baltica* (Shefferson et al. 2008) roots were colonized by fungi belonging to *Ceratobasidium* spp. These groups have been noted as primary mycorrhizal symbionts of members of the Apostasioideae (Kristiansen et al. 2004), subfamily basal to the Cypripedioideae and sister of the Neottioidae, Epidendroidae and Orchidoidae, suggesting that *Rhizoctonia* complex may be the ancestral family of mycorrhizal fungi in the Orchidaceae. Based on ITS analysis it has been estimated that orchids can be associated with many different Basidiomycetes (McCormick et al. 2004; Shefferson et al. 2005; Shefferson et al. 2008) while in our study we isolated just one mycorrhizal fungi belonging to basidiomycetes.

Intriguingly, approx. one-half of *D. sambucina* roots contains ascomycetes. Some ascomycetes have been isolated form orchid roots (Selosse et al. 2004),

Table 2. Molecular identification of the fungal ITS recovered from *Dactylorhiza sambucina* roots.

	The closest sequences (above 91% similarity) found in GenBank by Blast analysis of the ITS sequence	GB accession number	percent identity
DS1 (EF100192)	*Rhizoctonia* sp.	AJ419931	96%
	Rhizoctonia aurim		96%
	uncultured Russulaceae	DQ061931	91%
	Ceratobasidium sp.	AB290022	91%
	Rhizoctonia sp.	AJ242895	91%
DS2 (EF100193)	*Gyoerffyella rotula*	AY729937	96%
	uncultured Ascomycete	DQ093761	95%
	Gyoerffyella aurim	DQ093677	95%
	Spirosphaera carici-graminis	EF029225	94%
	Helotiales sp.	EF093150	94%
	Naevola minutissima	AY853218	93%
	Anguillospora filiformis	AY148105	91%
DS3 (EF100194)	*Gyoerffyella* sp.	EF601602	100%
	uncultured Ascomycete	DQ093761	100%
	Spirosphaera carici-graminis	EF029225	98%
	Gyoerffyella rotula	AY729937	97%
	Helotiales sp.	EF093150	96%
	Naevola minutissima	AY853218	95%
	Anguillospora filiformis	AY148105	93%

but their mycorrhizal status is debated. This is, to our knowledge, the first evidence for ascomycetes forming mycorrhizae in Orchidoidae, while it is not an exception at least among Neottieae since *Epipactis microphylla*, *E. helleborine*, *E. atrorubens* and *E. distans* are also colonized by *Tuber* spp., *Wilcoxina* spp. and other ectomycorrhizal ascomycetes (Bidartondo et al. 2004; Selosse et al. 2004).

Limited data are so far available on the influence that mycobionts have on the morphological polymorphism. Taylor et al. (2003) showed that floral variation correlates with the high specificity toward single *Sebacina* taxa in *Hexalectris spicata*. Sequence similarity was found among the mycorrhizal fungi isolated from different root fragments and from morphologically (colour polymorphism) separated plants. These data suggest that there are no differences in fungi colonization along orchid root and that there is no relationship between mycorrhiza and the flowers' colour. Some investigations have shown that mycoheterotrophic plants maintain high specificity also when surrounded by numerous fungal species, contrary to the hypothesis that specificity may be simply due to an absence of alternative symbionts. Moreover, specificity in mycorrhiza for green orchids is still debated. In our case, the photosynthetic orchid *D. sambucina* is highly specific due to a strong dependence on fungal carbohydrates in case of low photosynthetic efficiency in field conditions (Girlanda et al. 2006). Indeed, other recent papers have reported high specificity in *Goodyera pubescens* and *Liparis lilifolia* (McCormick et al. 2004) such as in *Limodorum abortivum* (Girlanda et al. 2006). In contrast, *Tipularia discolor* (McCormick et al. 2004) showed association with at least four groups of tulasnelloid fungi and *Cephalantera damasonium* (Bidartonto et al. 2004) with many different basidiomycetes. A strong influence of the environment may, at least in part, explain the contrasting results in mycobiont specificity.

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