Molecular Ecology (2001) **10**, 2089–2093

SHORT COMMUNICATION

Identification of mycorrhizal fungi from single pelotons of *Dactylorhiza majalis* (Orchidaceae) using single-strand conformation polymorphism and mitochondrial ribosomal large subunit DNA sequences

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Abstract

The mitochondrial ribosomal large subunit (Ls) DNA was used to identify the orchid mycorrhizal fungi found in roots of *Dactylorhiza majalis*. The gene was amplified using DNA extracted from single pelotons obtained from fresh and silica gel dried roots. Furthermore, sequencing a variety of well-characterized orchid isolates expanded the fungal database of the mitochondrial ribosomal LsDNA. Polymerase chain reaction product length variants present in *D. majalis* were sequenced and identified using the expanded database. These analyses revealed two different peloton-forming fungi in samples from *D. majalis*, which sometimes occurred together as a single two-taxa peloton within the same cortex cell. The first taxon belonged to the genus *Tulasnella* and the second taxon was distantly related to *Laccaria*.

Keywords: *Dactylorhiza majalis*, mitochondrial ribosomal large subunit DNA, Orchidaceae, orchid mycorrhizal fungi, single pelotons

Received 10 November 2000; revision received 19 February 2001; accepted 8 March 2001

Introduction

Mycorrhizal associations are found throughout the orchid family. The fungal endosymbionts are found in the root cortex as intracellular coils, also known as pelotons (Burgeff 1936). It is generally accepted that the fungal hyphae within the cortical cells are digested by the plant, and because of this contribute to the orchids nutrition (Burgeff 1936; Rasmussen 1995). Smith (1966) showed that *Rhizoctonia solani* was able to supply orchid seedlings with sufficient nutrients for growth to occur. Most putative orchid mycorrhizal symbionts belong to the form genus *Rhizoctonia*, which includes the teleomorphs *Thanatephorus*, *Ceratobasidium*, *Serendipita*, *Tulasnella* and *Oliveonia* (Roberts 1999).

Orchid mycorrhizal fungi have traditionally been studied via the isolation and establishment of pure cultures from colonized root tissue (Warcup 1981; Currah *et al*. 1997). However, some fungi may not be able to grow on the media used 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; Cullings *et al*. 1996; Kjøller & Rosendahl 2000). This technique has also been used to characterize fungi in mycorrhizal orchid roots, thus circumventing the fungal isolation step (Taylor & Bruns 1997, 1999). The method employed by these authors involved extraction of total DNAs from sections of mycorrhizal tissue approximately 0.5 cm^3 in volume. They amplified several fungal gene regions from each sample, using three different primer combinations, compared fungal isolation with direct PCR-based identifications, and found that individuals of the highly specialized nonphotosynthetic (myco-heterotrophic) orchids *Cephalanthera austinae*, *Corallorhiza maculata* and *Corallorhiza mertensiana* were very rarely colonized by more than one fungal species. However, there is evidence that green orchids are often colonized by a variety of fungi,

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some of which may not interact as true mycorrhizae (Warcup 1981; Bayman *et al*. 1997). Thus, molecular methods are needed that can identify individual fungi when multiple fungal species infect a single plant. Molecular methods can help to distinguish putative mycorrhizal from nonmycorrhizal symbionts, when the fungi are already physiologically characterized (Taylor & Bruns 1997).

The aim of the present paper was to PCR-amplify specific nucleotide sequences from single pelotons, and to characterize these sequences with respect to their phylogenetic relationship to known fungal sequences.

Materials and methods

Plant and fungal material

Roots of *Dactylorhiza majalis* were collected at the Copenhagen Botanical Garden and DNA was isolated from single pelotons, which were obtained from each of four plants. Morphologically characterized fungi isolated from a variety of orchids were obtained from culture collections (Table 1).

Extractions

A total of 150 single pelotons were dissected from the outer cortex of fresh or rehydrated silica gel dried *D. majalis* roots following Rasmussen (1995). The pelotons were recovered in 2 μL water and transferred to 0.5 mL Eppendorf-tubes containing 6 μL ddH_2O and 2 μL 10× PCR buffer. The mix was either heated at 95 °C for 10 min in a heat block or boiled for 2 min in a microwave oven. DNA from fungal isolates was extracted using a chelix extraction method (Kjøller & Rosendahl 2000) or a CTAB/chloroform/isopropanol method (Gardes & Bruns 1993).

PCR

From the extracted single pelotons, 8 μL extract was used as template for PCR amplification, 73 pelotons were successfully amplified giving rise to two distinct groups, the longer amplicons and the shorter amplicons. The ML5/ML6 region (White *et al*. 1990) of the mitochondrial large submit (mt-Lr) RNA gene was amplified using a PCT100 thermocycler (MJ Research Inc., MA). The PCR mix used for each peloton contained: 2 μL 10× PCR buffer, 2 μL (10 mM) of each of the primers, 6 μL ddH_2O, 8 μL dNTP (2 mM) mix and 0.1 μL *Taq* polymerase. The PCR conditions were as described by Gardes & Bruns (1993). Some fungal cultures could not be amplified using the forward primer ML5 (CTCGGCAAATTATCCTCATAAG), and MLIN3 (CGACACAGGTTCGTAGGTAG) was used instead (Table 1).

Single-strand conformation polymorphism (SSCP)

Sixty-nine orchid peloton samples were analysed with SSCP leaving the samples that were sequenced, in which case the entire PCR product was used for the sequencing reaction. The SSCP gels were run for 2 h at 5 °C using the

Table 1 Sources of orchid fungi added to the ML5-6 database. Fungi from Canadian Collection of Fungal Cultures (CCFC) have DAOM accession numbers. CBS is Centraalbureau voor Schimmelcultures (CBS) Baarn & Delft, The Netherlands. UAMH is University of Alberta Microfungus Collection & Herbarium. AF is GenBank accession numbers

Fungal taxon	Source/Accession	Forward primer	Orchid source and reference to fungal description
Tulasnella irregularis	CBS574.83 AF345560	ML5	*Dendrobium dicuphum*; Warcup & Talbot (1980)
Tulasnella violea	DAOM222001 AF345562	ML5	Not isolated from an orchid
Epulorhiza anaticula	UAMH5428 AF345559	ML5	*Calypso bulbosa*; Currah & Sherburne (1992)
Tulasnella pruinosa	DAOM17641 AF345561	ML5	Not isolated from an orchid
Tulasnella deliquiscens A	D47-7 AF345852	ML5	*Dactylorhiza majalis;* Andersen (1990)
Tulasnella calospora B	D47-7 AF345853	ML5	*Dactylorhiza majalis;* Andersen (1990)
DM 1 (*Dactylorhiza mycorrhiza*)	AF345854	ML5	*Dactylorhiza majalis*
DM 2 (*Dactylorhiza mycorrhiza*)	AF345855	ML5	*Dactylorhiza majalis*
DM 3 (*Dactylorhiza mycorrhiza*)	AF345856	ML5	*Dactylorhiza majalis*
Ceratorhiza goodyerae-repentis	UAMH6440 AF345556	MLIN3	*Platanthera obtusata*; Currah & Sherburne (1992)
Moniliopsis anomala	UAMH6451 AF345557	MLIN3	*Coeloglossum viride*; Currah & Sherburne (1992)
Sistotrema sp.	UAMH5437 AF345558	MLIN3	*Piperia unalascensis*; Currah & Sherburne (1992)

Hoefer Mighty Small (SE250) vertical electrophoresis unit (Pharmacia Biotech Inc., CA) following Kjøller & Rosendahl (2000). The bands were visualized by silver staining following the procedure from the DNA Silver Staining Kit provided by Pharmacia Biotech.

Sequencing

The PCR products were purified using the QIAQuick PCR extraction kit following the manufacturer's instructions (QIAgen gmbh GE). The DNA was eluted with 30 µL of sterile H_2O and sequenced using the ML5/ML6 or the MLIN3/ML6 primers. Four samples, two from each group, were cycle sequenced, three successfully, using a kit from Perkin Elmer (402079) following manufacturer's instructions, and run on an ABI 377 automatic sequencer (Perkin Elmer). A 360-bp and 530 bp amplicons were obtained from single pelotons. Amplicon sizes of cultivated fungi ranged between 530 bp and 618 bp. Amplicons of each size were sequenced.

Data analyses

The obtained root-derived sequences were added to the updated 175 taxa basidiomycete sequence database (Bruns *et al*. 1998; Horton 2000) and a phylogram was generated using PAUP 4.0TM software (Swofford 1999). The phylogram is based on heuristic search with parsimony settings, 100 replicates with random seed and no root defined, using the complete 175 taxa updated sequence database with our own sequences added to it. A 75-bp gap was inserted from position 127 in the original matrix in order to align the sequence of DM3.

Results and Discussion

The use of PCR on single pelotons made it possible to discriminate among multiple mycorrhizal fungi infecting a single plant and to identify these fungi. Discrimination among multiple fungi would be more difficult using previous bulk DNA extraction approaches (Taylor & Bruns 1997, 1999).

Table 2 Total number of pelotons included in this study. Numbers of pelotons amplified, and the percentage of total amplified. The table also shows numbers of pelotons from fresh and dried roots, and the amplification success rates

Treatment	Total number of pelotons	Amplified	Percentage amplified
Fresh root	68	42	61.8%
Dried root	81	31	37.8%
Total	150	73	48.7%

In this study, about half of the pelotons had started to degrade due to digestion by the orchid cortical cells. Endophytes cannot be isolated and grown from such pelotons (Clements 1988; Rasmussen 1995), but it was possible to obtain PCR products from both fresh/active pelotons and pelotons that had started to degrade. This means that it is not necessary to harvest roots shortly after they have been colonized and that roots can be collected over a longer period of time. It was also possible to obtain fungal PCR products from dried roots although the percentage of pelotons that gave positive amplification were lower than from fresh roots (Table 2). This is important if the roots are collected under conditions where it is not possible to store frozen material.

PCR amplification of the ML5/ML6 region resulted in DNA bands of two lengths. In some pelotons a 360-bp fragment was amplified as previously reported (White *et al*. 1990; Bruns *et al*. 1998) but from other pelotons the PCR resulted in a 530-bp product. In two cases, both products were amplified from a single peloton. The amplification of the two sequence types from one peloton indicates that two taxa may be involved in formation of single pelotons. The two sequences of different length were confirmed by SSCP. The SSCP patterns obtained from the two amplicon lengths demonstrated that no significant variation existed within each of the sequence types (Fig. 1).

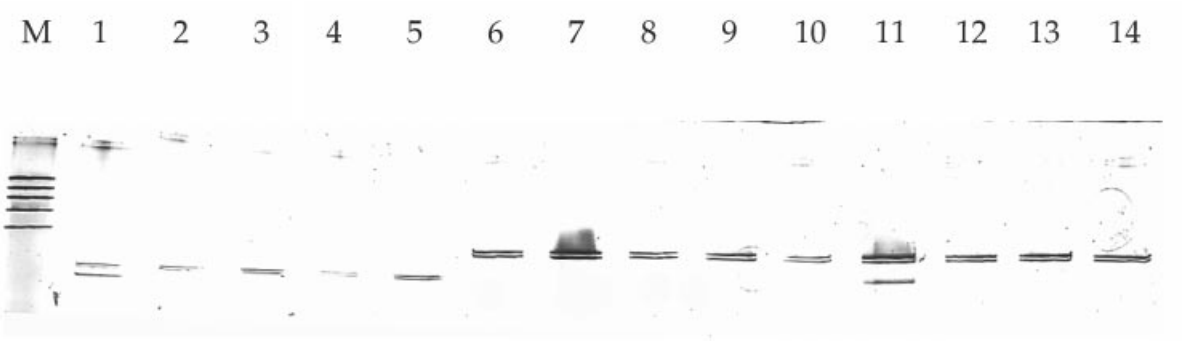

Fig. 1 Single-strand conformation polymorphism detection of sequence variation in ML5-6 fragments. All polymerase chain reaction (PCR) products were of equal size. Lanes 1–5 show fast-migrating fragments; sequence is labelled DM2 in Fig. 2. Lanes 6–14 show slow-migrating fragments; sequences are labelled *Tulasnella deliquiscens* in Fig. 2. Note lane 11 with both fast and slow PCR products.

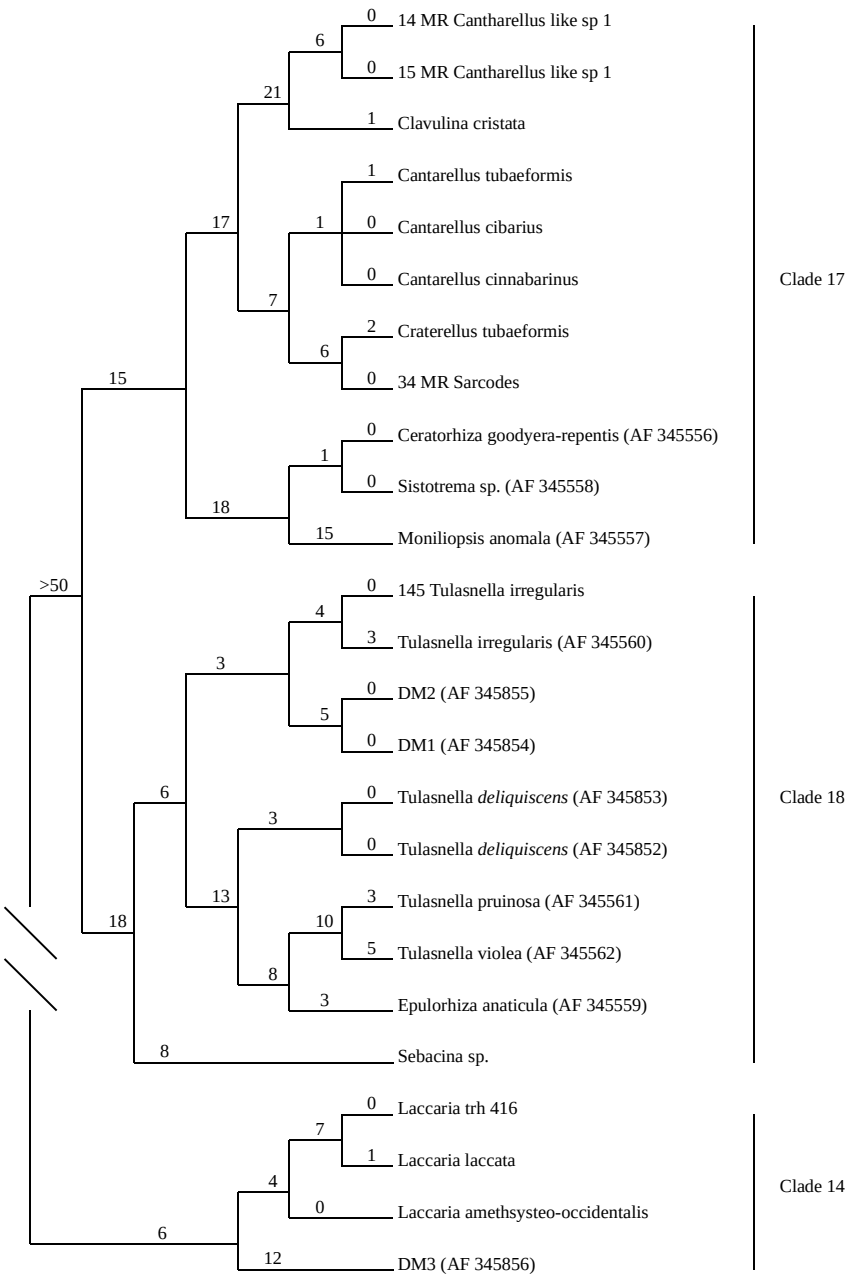

Fig. 2 Placement of orchid fungal sequences in a 50% majority rules strict consensus tree of 1536 most parsimonious trees based upon the updated 175 taxa basidiomycete database (Bruns *et al*. 1998; Horton 2000). Shown are clades 14, 17 and 18 as they appear in the original Bruns *et al*. (1998) paper, expanded with the added orchid mycorrhiza sequences. The shown clades were found not to be in conflict with the overall tree. Branch lengths are shown for each branch. GenBank accession numbers for each of the added sequences are shown in parentheses.

Different cultures grown from the same peloton have usually been treated as contamination (Clements 1988; Rasmussen 1995). However, as earlier studies have shown that green orchids are capable of forming mycorrhizal associations with more than one fungal species (Warcup 1981), we do not find it unlikely that more than one fungus can be found in one peloton. In contrast to the green orchids, Taylor & Bruns (1999) showed that some non-photosynthetic orchids are more specific in having only one mycorrhizal fungus present.

The heuristic search in PAUP 4.0™ generated 1536 equally parsimonious trees 706 steps long. A semistrict consensus tree was drawn and it showed the clades of interest are not in conflict with the overall tree. Clades elsewhere in the tree performed poorly, but had no direct influence on the area of the tree in interest. The 360 bp sequence from DM1 and DM2 both related to taxa within the expanded clade 18 (Bruns *et al*. 1998) (Fig. 2). All taxa in this group are known as peloton forming orchid mycorrhizal fungi falling within the *Rhizoctonia*-complex. (Warcup 1981; Rasmussen 1995).

The 530 bp DM3 sequence placed in clade 14 (Bruns *et al*. 1998) with *Laccaria*. DM3 do not associate closely with *Laccaria* but closer to this genus than other genera presented in the database. Furthermore, a 75-bp additional sequence fragment from position 127 found in DM3 separated

this sequence from the existing *Laccaria* sequences. The nearest neighbour outside clade 14 is *Armillaria albolanaripes*, which are 15 steps away from the *Laccaria* clade (Fig. 2). This result tells us that fungi performing as orchid mycorrhiza are perhaps more diverse than previously believed.

Further taxa sampling in clade 17 and 18 are needed to expand our knowledge concerning the relationship among these basidiomycetes. Likewise further taxa sampling in clade 14 would be useful to verify the phylogenetic placement of the peloton sequence DM3 in this clade.

The addition of sequences from a variety of well-characterized orchid fungi has increased the utility of the ML5-6 database (Bruns *et al*. 1998) for orchid mycorrhizal research. The new sequences demonstrate that a variety of orchid isolates in *Epulorhiza* belong to a distinct monophyletic clade related to *Tulasnella*. The remaining orchid isolates we have analysed belong to a second distinct clade (clade 17, Bruns *et al*. 1998) that is phylogenetically distant from *Tulasnella*. This clade is centred on the teleomorph *Thanatephorus* and includes the anamorphic isolates included in this analysis assigned to *Ceratorhiza*, *Moniliopsis* and *Sistotrema*.

Sequencing of DNA from single pelotons allows precise discrimination of orchid mycorrhizal associates because it helps to discriminate mycorrhizal fungi from nonmycorrhizal root endophytes, and allows the identification of each fungus when mixed infections occur. This method should assist in resolving controversies concerning specificity in green orchids (Hadley 1970; Clements 1988). The identification of the true mycorrhizal symbionts will be of critical importance to understanding the ecology of these orchids and to conservation efforts.

Acknowledgements

We thank Charlotte Hansen and Kenn Kristiansen for help in the laboratory, and David Hibbett for two *Tulasnella* DNA samples. We thank Tom Bruns, Michael Milgroom and three anonymous reviewers for comments and suggestions.

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This work was part of the MSc work of Kim Kristiansen on orchids and orchid mycorrhizas. The work was conducted at the Department of Mycology, University of Copenhagen under supervision of Soren Rosendahl and Rasmus Kjoller who are both working on molecular approaches to mycorrhizal ecology.
