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A PCR-based method for detecting the mycelia of stipitate hydnoid fungi in soil

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ABSTRACT

To reduce the reliance on sporocarp records for conservation efforts, information on the below-ground distribution of specific fungal species, such as stipitate hydnoid fungi, is required. Species-specific primers were developed within the internal transcribed spacer (ITS1 and ITS2) regions for 12 hydnoid fungal species including *Bankera fuligineoalba*, *Hydnellum aurantiacum*, *H. caeruleum*, *H. concrescens*, *H. ferrugineum*, *H. peckii*, *Phellodon confluens*, *P. melaleucus*, *P. niger*, *P. tomentosus*, *Sarcodon glaucopus* and *S. squamosus*. The specificity of the primer pairs was tested using BLAST searches and PCR amplifications. All primers amplified DNA only of the target species with the exception of those designed for *P. melaleucus*. In order to assess the ability of the primers to detect DNA from mycelium in soil, DNA extracted from soil samples taken from around solitary *H. peckii* sporocarps was amplified with the *H. peckii* primer 1peck and ITS2. *H. peckii* DNA was detected in 70% of all soil samples and up to 40 cm away from the base of individual sporocarps. The development of these species-specific primers provides a below-ground alternative for monitoring the distribution of these rare fungi.

1. Introduction

Stipitate hydnoid fungi are a polyphyletic group within the Basidiomycota with a hymenium of teeth as opposed to the more common gills or pores. Sporocarp production by stipitate hydnoid fungi has been declining across Europe over the last four decades (Arnolds 2003; Hrouda, 1999a; Otto, 1990) resulting in their inclusion on European Red Data Lists (Senn-Irlet et al., 2007). In parts of Europe, many species have not been recorded since the 1970s and are possibly extinct (Arnolds, 2003; Walleyn and Verbeken, 1999). In the UK, the situation was less clear, mainly because of the limited data on the abundance and distribution of hydnoid species (Newton et al., 2002a,b), however a grouped species action plan for 14 rare species from the genera *Bankera*, *Hydnellum*, *Phellodon* and *Sarcodon* was developed to provide a management framework for their future conservation (Anon, 1999). Most of these species are ectomycorrhizal (ECM) associates of *Pinus sylvestris*.

The mycelia of ECM fungi have a below-ground mutualistic association with the roots of a host plant (Smith and Read, 1997). Up to the mid 1990s, studies on the distribution of ECM fungi relied on sporocarp records (Bruns, 1995; Dahlberg, 2001). However, this has drawbacks. ECM fungi do not necessarily produce sporocarps every year and, when they do, trained fungal taxonomists are needed to distinguish species. Moreover, the absence of sporocarps of a species is no indication for the absence of its mycelium in the soil (Cairney, 2005).

An alternative approach which focuses on the presence of ECM fungal species below-ground is called 'morphotyping', and involves the identification of the composite plant root/fungal structure which is called an ectomycorrhiza. The morphological characteristics of these ectomycorrhizas can allow identification of fungi up to species level (Agerer, 1991a). However, morphotyping requires significant experience and is very time consuming. In addition, many closely related species often display similar morphology and cannot reliably be distinguished by morphotyping. Moreover, this approach does not take fully into account the mycelium which extends from the ectomycorrhizas into the surrounding substrate.

To date, the ecology of stipitate hydnoid fungi has mainly been studied by means of sporocarp records (e.g. Gurney, 2007; Holden et al., 2001; Newton et al., 2002a,b) andc the ectomycorrhizas of only four species have been described (Agerer, 1991b, 1992, 1993; Agerer and Otto, 1997). In order to further develop a UK conservation strategy for stipitate hydnoid fungi we need a better understanding of their below-ground distribution and persistence. The development of molecular techniques based on soil DNA extraction has made it possible to investigate the distribution of ECM fungal mycelium in soil (for review see Anderson and Cairney, 2007). Recently, primers were designed for the discrimination of sporocarps of two *Phellodon* species (Parfitt et al., 2007), however they were not intended for ecological studies, hence their ability to detect DNA from each target species in soil has not been tested. The aim

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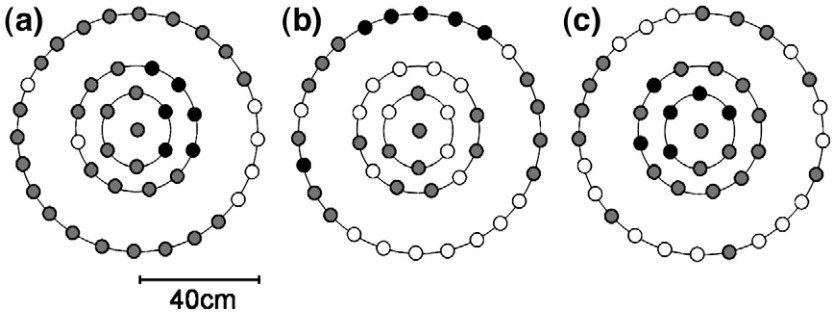

Fig. 1. Amplification of *H. peckii* DNA from soil extracts using species-specific ITS primers. Soil cores were taken around solitary sporocarps at three different locations in Culbin forest. Black circle = *H. peckii* amplicon detected and confirmed by sequencing; grey circle = *H. peckii* amplicon detected; open circle = no *H. peckii* amplicon.

of the present study was to develop species-specific PCR primers for all 12 stipitate hydnoid fungi thought to form ECM associations with Scots Pine (*P. sylvestris*) in the UK, and to assess the ability of the primers to detect the presence of the DNA of *H. peckii*, a representative species, in soil extracts.

2. Materials and methods

2.1. Sporocarp collection

Fresh sporocarps were collected from beneath Scots pine in 2004 and 2005 at Mar Lodge (56°59′32′′N, 03°29′28′′W), Rothiemurchus (57°09′00′′N, 03°47′12′′W) and Culbin (57°38′08′′N, 03°42′07′′W) in NE Scotland. Additional dried herbarium material was obtained from E. Holden (personal collection) and from the Royal Botanic Gardens Edinburgh, UK. Fresh sporocarp material was also collected in Uppland, Sweden (60°25′N, 17°49′E) in 2006 and two additional dried Swedish collections from Nåsten (59°48′N, 17°40′E) were obtained from AFS Taylor (personal collection). Sporocarps were identified using keys and descriptions from Pegler et al. (1997), Arnolds (2003) and Maas Geesteranus (1975). Details of all sporocarp collections are summarised in Supplementary Tables 1 and 2. A sample (2 mm^3) of fresh sporocarp material was stored in 300 µl 2% cetyltrimethylammonium bromide (CTAB) buffer (100 mM Tris–Cl, 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.2% β-mercaptoethanol, pH 8.0) at −20 °C until further processing.

2.2. DNA extraction and PCR amplification

DNA was extracted by adding a further 300 µl 2% CTAB and 500 µl phenol–chloroform–isoamyl alcohol (25:24:1) to the samples in a FastPrep lysing matrix-E tube (Qbiogene, Cambridge, UK). The samples were then lysed twice for 15 s at 5000 rpm in a Precellys 24 lyser (Stretton Scientific Ltd, Stretton, UK) and cooled on ice for 60 s in between. The samples were centrifuged for 5 min at 18,000 ×*g*, the aqueous layer was then further extracted using an equal volume of chloroform–isoamyl alcohol (24:1) and centrifugation at 20,100 ×*g* for 5 min before the nucleic acids were precipitated using one tenth volume of 3 M sodium acetate and one volume isopropanol. The precipitated nucleic acids were pelleted by centrifugation at 20,100 ×*g* for 30 min and washed with cold 70% ethanol before being air dried and resuspended in 50 µl Tris–EDTA buffer.

PCR amplifications were conducted in a 50 µl reaction volume containing 1 µl DNA template (35–65 ng/µl), 2.0 mM $MgCl_2$, 250 µM dNTPs (Bioline Ltd, London, UK), 10× buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCL (pH 8.0 at 25 °C), 0.01% Tween-20], 10 pmol ITS1F and ITS4B primers (Gardes and Bruns, 1993) and 2.5 U BIOTAQ polymerase (Bioline Ltd, London, UK). PCR was performed on a PTC-220 DYAD™ Thermal Cycler (MJ Research Inc, Waltham, MA, USA) and consisted of an initial denaturing step of 5 min at 95 °C, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s followed by a final extension at 72 °C for 10 min. Negative controls (reaction containing no DNA) were included in all PCRs. The PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide and visualised under UV light.

2.3. DNA sequencing and alignments

PCR products were purified using a magnetic bead ChargeSwitch PCR clean-up kit (Invitrogen, Paisley, UK) following the manufacturer's protocol. Purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit v3.1 on an ABI PRISM™ 3130xl genetic analyser (Applied Biosystems, Warrington, UK). Sequencing reactions were performed with the primers ITS1F and ITS4 (White et al., 1990). Sequences were manually checked, and where necessary edited using the SEQUENCHER software package (version 3.0; Gene Codes Corporation, Ann Arbor, MI USA).

Sequence alignments were produced using Clustal W (Thompson et al., 1994) in BioEdit (Hall, 1999) and T-Coffee (Notredame et al., 2000) using the DNA sequences generated in this study along with additional reference sequences obtained from both the UNITE (Kõljalg et al., 2005) and GenBank databases (Supplementary Table 3). Where required, the alignments were manually edited. A single alignment was initially produced using all DNA sequences of species occurring in Scotland but high interspecific variation made alignment difficult. Candidate primer sites were therefore initially selected from single genus alignments (except for *Bankera* which was aligned with all *Phellodon* species). The level of intraspecific variation was also assessed by aligning sequences of the same species from different sporocarps.

2.4. Development of species-specific primers and optimisation of PCR conditions

For each species, up to five locations (18–30 nucleotides in length) within the ITS1 and ITS2 spacer regions were identified that appeared to be sufficiently variable to use as the target site for designing species-specific primers. An internet based Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html, Northwestern University, Chicago, USA) was used to calculate the percentage GC content, melting temperature and the self-complementarity of each

Table 1
Average sequence similarities between ITS1 and ITS2 sequences of hydnoid species from the same genus

Genus	Average sequence similarity (%)	
	ITS1	ITS2
Bankera	N/A[a]	N/A[a]
Hydnellum	67.7	70.6
Phellodon	80.7	83.2
Sarcodon	65.0	73.0

[a] Data for the genus *Bankera* is not available since only one species was included in that genus.