Molecular identification of the primary root fungal endophytes of *Dipodium hamiltonianum* (Orchidaceae)

J.D.W. Dearnaley [A, B] *and A.F. Le Brocque*[A]

[A]Department of Biological and Physical Sciences, The University of Southern Queensland, Toowoomba 4350, Australia.

[B]Author for correspondence: Email: dearns@usq.edu.au
Phone: 07 4631 2804, Fax: 07 4631 1530

Abridged Title: Fungal endophytes of *Dipodium hamiltonianum.*

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Abstract. We have identified the primary root fungal endophytes of *Dipodium hamiltonianum* F.M. Bailey, a species of orchid endangered in Victoria and uncommon in New South Wales and Queensland. Genomic DNA was extracted from whole colonised root portions from four orchid individuals and PCR amplified with ITS1F and ITS4 primers. Cloning and sequencing of the main amplicons produced from the PCR analysis revealed that the primary root fungal endophytes were *Gymnomyces* and *Russula* spp., both members of the Russulaceae. The implications of this finding are discussed in terms of conservation of the orchid species.

Introduction

Dipodium hamiltonianum or yellow hyacinth orchid is a large terrestrial orchid species found in woodlands throughout eastern Australia (Jones 1988; Riley and Banks 2002). The plant grows to almost 1 m, and is distinct from other *Dipodium* species in that its leafless pale stems bear up to 35 red-purple spotted yellow flowers (Fig. 1; Bishop 1996). The distribution range of *D. hamiltonianum* includes Victoria, where its status is endangered (Conservation and Natural Resources 1994), to New South Wales and Queensland; where it is uncommon (Riley and Banks 2002). Threats to the species appear

to include collecting, grazing, altered fire regimes and the fact that few seeds are produced from plants annually (Department of Sustainability and Environment 2004).

D. hamiltonianum is an example of a myco-heterotrophic orchid species in that, as it is non-photosynthetic, it relies on a fungal endophyte to provide a source of carbon for growth. Indirect evidence suggests that the ultimate sources of this carbon are tree species, typically members of the *Eucalyptus* genus. Bishop (1996) reports that *Dipodium* species only grow in the presence of *Eucalyptus*. In addition, Bougoure and Dearnaley (2005) have recently shown that the primary root fungal endophyte of the related *D. variegatum* R. Br. are members of the *Russula* genus, common ectomycorrhizal fungi of *Eucalyptus* within Australia (Bougher 1995).

The survival of *D. hamiltonianum* requires identification of its fungal endophyte (Department of Sustainability and Environment 2004). Such information will assist in the conservation of the species through cultivation trials using fungal seed germination as well as growth of mature plants under horticultural conditions. Long term management of the species will also be facilitated by recognition of the other hosts of the fungus. Identification of possible tree host species will enable the determination of ecosystems suitable for reintroductions of the orchid and will highlight those ecosystems that should be preserved to maintain current orchid populations.

The primary objective of this research was to identify the major root fungal endophyte (s) of *D. hamiltonianum*. As previous studies of the root fungal endophytes of a *Dipodium* species (Bougoure and Dearnaley 2005) and North American myco-heterotrophic orchid species (Taylor and Bruns 1997) have shown such fungi to be difficult to isolate into pure culture, we extracted total DNA from whole colonised orchid roots and used the techniques of polymerase chain reaction (PCR), cloning and sequencing to answer this question.

Materials and methods

Acquisition of orchid and fungal material

Two to three roots were collected from four *D. hamiltonianum* plants at Coonabarabran (one plant) in northern New South Wales, and Sundown National Park (one plant) and Amiens State Forest (two plants) in southern Queensland (Table 1). Only roots were collected and plant voucher specimens were not obtained because of the conservation status of the species. One-centimetre long root portions were peeled and transversely sectioned to identify fungal colonised root regions. Sections were photographed with a Micropublisher 5.0 digital camera (QImaging, Canada) on a Nikon E600 upright microscope (Nikon Corporation, Tokyo, Japan). At each collection site, the trees species that occurred within an approximately 10 m radius of each plant were recorded.

(insert Fig. 1 and Table 1 here)

Molecular analysis of fungal endophytes

Two root samples from each plant were mechanically ground in microcentrifuge tubes with a plastic micropestle. Total DNA was extracted from root samples with a DNeasy Plant mini kit (Qiagen, Doncaster, VIC, Australia) following the manufacturer's instructions. Fungal ITS regions of each sample were PCR amplified in 50 μl reaction volumes, each containing 38 μl sterile distilled H_2O, 5 μl 10X buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100; Invitrogen Australia, Mt Waverley, VIC, Australia), 2.5 μl 50 mM $MgCl_2$ (Invitrogen Australia), 1 μl 10 mM dNTP (Invitrogen Australia), 1 μl of each of the fungal specific ITS1F primer (Gardes and Bruns 1993) and ITS4 (White *et al.* 1990), 0.5 μl of *Taq* DNA polymerase (Invitrogen Australia) and 1 μl of extracted genomic DNA. Amplifications were performed in a Thermo Hybaid PCR Express thermocycler

(Integrated Sciences, Willoughby, NSW, Australia) with 35 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min, with a final incubation at 72°C for 10 min. Reactions were performed in duplicate and negative controls were included without DNA. The resulting PCR products were electrophoresed in 2% (w/v) agarose gels with ethidium bromide, and visualized under UV light.

ITS-PCR products were purified with a DNA purification kit (Roche Applied Science, Castle Hill, NSW, Australia) before cloning with the pGEM-T Easy vector system (Promega, Annandale, NSW, Australia), both conducted as per the manufacturer's instructions. Sequencing reactions of clones representative of the major PCR products initially present were performed in 10 µl volumes containing approximately 400 ng of purified plasmid DNA, 6.4 pmoles of T7 promotor primer at the Brisbane laboratory of the Australian Genome Research Facility (AGRF). ITS sequences were analyzed using BLAST searches through GenBank (http://www.ncbi.nlm.nih.gov/).

Results

The orchids occurred in dry woodland communities with varying tree species present including three species of *Eucalyptus* and one *Callitris* (Table 1).

The majority of the sampled *D. hamiltonianum* roots were heavily colonized throughout although the brown coloured fungal pelotons were restricted to the inner cortex (Fig. 2). Root 2b was exceptional in that it was only lightly colonized (not shown).

PCR amplification of extracted root DNA with ITS1F and ITS4 primers produced either single or double banding patterns around 800bp (Fig. 3). Sequencing of the cloned ITS regions corresponding to these bands revealed that the primary fungal endophyte for roots 1a and 1b was a fungus with close identity to *Gymnomyces fallax* (92% over 723-725 bp)

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while the primary fungal endophyte of root 2a was a fungus with close identity to *Gymnomyces fallax* (95% over 510 bp) and *Gymnomyces fragrans* (95% over 510 bp) (Table 2). The main fungal endophyte of root 3a and 3b was a fungus with identity to *Russula leptidicolor* (85% over 632 bp) and *Russula lilacea* (87% over 484 bp) (Table 2). We were unable to identify the second lower and weaker band in root 3b even though 8 different clones were examined. Sequencing revealed that the higher molecular weight bands observed in root samples Dh1b, Dh2b, Dh4a and Dh4b were orchid ITS regions (results not shown) while the lower band observed in root Dh4a corresponded to a fungus with identity to a *Penicillium* sp. (99% over 444 bp) and *Eupenicillium reticulisporum* (97% over 444 bp) (Table 2).

(Insert Figures 2, 3, Table 2 here)

Discussion

The extent of fungal colonisation observed in the *D. hamiltonianum* roots was not surprising as most members of the *Dipodium* genus are highly dependent on soil fungi for their nutrition (Jones 1988). The close occurrence of these orchids to *Eucalyptus* may imply that organic food sources are channelled from tree to orchid via a fungal conduit, as has been suggested in other mycoheterotrophic orchids (Warcup 1985; Taylor and Bruns 1999; McKendrick *et al.* 2000; Selosse *et al.* 2002). Indentification of the fungal endophytes of the neighbouring tree species would be a further avenue to pursue, and would have management implications for the orchid in that it would confirm the link between the two plants. However there did not appear to be a tree host specific relationship in this study as the orchids were observed growing with three *Eucalyptus* species, including some with restricted ranges (*E. prava*, *E. youmanii*) and one with a wide range (*E. rossii*) in eastern Australia (Brooker and Kleinig 1999). Bishop (1996) has

previously reported *D. hamiltonianum* occurring in association with *Callitris* spp., reinforcing that the orchid has the capability to be linked to a wide variety of tree species.

These results suggest that *D. hamiltonianum* may have quite specific relationships with soil fungi in the family Russulaceae. Although orchid fungal specificity has been a contentious issue for many years (Warcup 1971; Perkins *et al.* 1995, Zelmer *et al.* 1996), molecular analysis of many autotrophic and mycoheterotrophic orchids have now suggested that orchids do have a narrow fungal endophyte range (Shefferson *et al*. 2005; Bougoure *et al.* 2005; McCormick *et al.* 2004; Selosse *et al.* 2002; Taylor and Bruns 1997; 1999). The *Penicillium* sp. identified in the first root sample from plant 4 is likely to be a soil contaminant as members of this group are not considered mycorrhizal (Rasmussen 2002). Surface sterilisation of orchid roots should be used in future studies of this type to eradicate such fungi.

The failure to obtain a fungal endophyte sequence from two of the eight root samples perhaps related to the low colonisation level of the tissue (root sample 2b) or inefficient fungal DNA extraction (root samples 2b and 4b). Amplification of orchid DNA in some samples suggest that the ITS1F and ITS4 primer combination is not always completely specific for fungal ITS regions. Although ITS1F was designed to discriminate fungal ITS regions from mixed DNA pools, Gardes and Bruns (1993) have shown that the ITS1F/ITS4 primer combination can sometimes result in plant ITS amplification. Such a problem here may have been exacerbated by the large size of the *D. hamiltonianum* roots.

The identification of a *Gymnomyces* species as an endophyte of *D. hamiltonianum* has parallels with the work of Taylor and Bruns (1997, 1999) who identified *Gymnomyces abietis* and other Russulaceae species in roots of the North American mycoheterotrophic orchid, *Corallorhiza maculata*. Bougoure and Dearnaley (2005) recently showed that the

main fungal endophytes of *Dipodium variegatum* are *Russula* spp. but this, to our knowledge, is the first record of a *Gymnomyces* sp. occurring as an endophyte in an Australian orchid. *Gymnomyces fallax* is not previously known to occur in Australia (Lebel 2003) and comparisons would be necessary to confirm that the sequences obtained in this study do not have identity with any of the 13 known Australian species. *Gymnomyces* fungi are hypogeous and ectomycorrhizal on *Eucalyptus* in Australia (Bougher 1995). An interesting aspect of the biology of *Gymnomyces* is that the fruiting bodies are eaten by fungivorous marsupials such as Bettongs and Pottoroos (Claridge and May 1994) which enhance fungal spore dispersal (Johnson 1996) and we could conjecture that these animals have an indirect role in the ecology of the orchid species. Investigation of this could be the subject of further conservation efforts.

We have intentionally used a small sample size (number of plants and roots) in this study because of the conservation status of the species and the fact that interference with the plant may have detrimental effects (Department of Sustainability and Environment 2004). The results presented here suggest that the crucial aspect to the conservation of the orchid species may be perpetuation of the Russulaceae fungi that are the main root endophytes of the plant. *Ex situ* conservation approaches, such as maintenance of the fungi in pure culture, may be achievable as, although members of the Russulaceae have previously been shown to be recalcitrant to laboratory growth (Taylor and Bruns 1997), there are now techniques available for culturing such fungi (Taylor *et al*. 2000; Sangtiean and Schmidt 2002). Continued studies will involve sampling of the endophytes of other *Dipodium* species and, after determining methods of culture, confirming the mycorrhizal status of isolated fungi.

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Fig. 1. The distinctive red-purple spotted yellow flowers of *D. hamiltonianum*. Scale bar = 5mm.

Table 1. Date of collection, location, plant code, tree species present.

Date collected	Location	Plant code[A]	Trees species present
December 2004	Sundown N.P. (Qld)	Dh1	*Eucalyptus prava*
			Eucalyptus youmanii
January 2005	Coonabarabran (NSW)	Dh2	*Eucalyptus rossii*
January 2005	Amiens State Forest (Qld)	Dh3	*Callitris endlicheri*
			Eucalyptus prava
January 2005	Amiens State Forest (Qld)	Dh4	*Eucalyptus youmanii*

[A] Codes for root samples are thus Dh1a, Dh1b etc.

**Fig. 2. Cross section of root of *D. hamiltonianum*
showing large numbers of brown-coloured
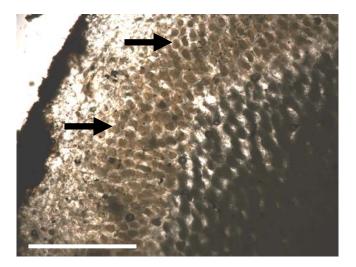fungal structures (arrows). Scale bar = 1000μm.**

Fig. 3. 2% agarose gels showing results of PCR with ITS1F and ITS4 primers of DNA isolated from two roots from each of 4 *Dipodium hamiltonianum* plants. a) root samples Dh1a, Dh1b, Dh2a, Dh2b. b) Root samples Dh3a, Dh3b, Dh4a, Dh4b. C = distilled water control.
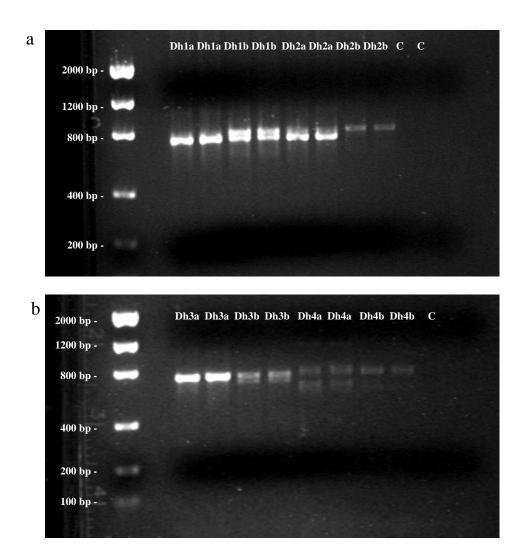
Table 2. Closest two matches from BLAST searches of fungal sequences amplified from the four *D. hamiltonianum* plants. Included are the deposited accession codes, the two closest GenBank matches and accession codes, sequence identity and overlap of each match.

Root sample no.	GenBank accession code	Closest species match & accession code		Sequence identity (%)	Sequence overlap (bp)
Dh1a	DQ178932	*Gymnomyces fallax*	AY239329.1	92	724
		Gymnomyces fallax	AY239349.1	92	723
Dh1b	DQ178933	*Gymnomyces fallax*	AY239329.1	92	725
		Gymnomyces fallax	AY239349.1	92	724
Dh2a	DQ178934	*Gymnomyces fallax*	AY239349.1	95	510
		Gymnomyces fragrans	AY239331.1	95	510
Dh3a	DQ178935	*Russula lepidicolor*	AY061687.1	85	632
		Russula lilacea	AY061731.1	87	484
Dh3b	DQ178936	*Russula lepidicolor*	AY061687.1	85	632
		Russula lilacea	AY061731.1	87	484
Dh4a	DQ178937	*Penicillium daleae*	AF033442	99	444
		Eupenicillium reticulisporum	AF033437.1	97	444