

FULL PAPER

Mathew John Irwin · Jeremy James Bougoure
John David William Dearnaley

Pterostylis nutans (Orchidaceae) has a specific association with two *Ceratobasidium* root-associated fungi across its range in eastern Australia

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Abstract In this study, we have identified the root-associated fungi of a common species of terrestrial orchid across its range in eastern Australia. We have amplified and cloned fungal ITS DNA extracted from roots of 15 *Pterostylis nutans* R. Br. plants from six separate geographic localities. Sequencing and GenBank comparison demonstrated two species of *Ceratobasidium* fungi as the main fungal partners of the orchid. Uncommon fungal associates included homobasidiomycete species such as a *Gymnomyces* sp. and a *Tricholoma* sp., *Leptodontidium orchidicola*, and an unidentified soil fungus. These results demonstrate that specificity for fungal partners occurs in *P. nutans* and reinforces the idea that conservation measures for endangered Australian orchids must include *ex situ* perpetuation of fungal symbionts as well as plant material.

Key words Australian terrestrial orchids · *Ceratobasidium* · Internal transcribed spacer (ITS) · Orchid mycorrhizas

Introduction

All members of the family Orchidaceae require colonization by fungi to provide both organic and inorganic nutrients in the early stages of development (Smith and Read 1997). At maturity, the majority of orchids are photosynthetic but appear to be dependent on root-associated fungi to provide a source of inorganic nutrition (Alexander et al. 1984). A recent discovery is that adult photosynthetic orchids can supply their fungal partner with carbon in return for their services (Cameron et al. 2006), and thus photosyn-

thetic orchid–fungal interactions can now be categorized as true mycorrhizal associations.

Australian orchids are generally associated with two main groups of root-associated fungi. The fully photosynthetic species associate mostly with *Rhizoctonia*-like fungi in the genera *Sebacina*, *Tulasnella*, *Ceratobasidium*, and *Thanetophorus* (Warcup 1971, 1981; Perkins and McGee 1995; Perkins et al. 1995; Bougoure et al. 2005). Nonphotosynthetic orchids appear to associate mostly with homobasidiomycete fungi such as members of the Russulaceae (Bougoure and Dearnaley 2005; Dearnaley and Le Brocq 2006; Dearnaley 2006).

Specificity, or association of an orchid species with a narrow fungal range, is a complex issue in Australian orchid research. Warcup (1971, 1981) surveyed a large number of Australian orchids and demonstrated that many species contained only a single species of fungus [e.g., members of the *Caladenia* and *Glossodia* genera all contained *Sebacina vermifera* Oberw. whereas *Diuris* orchids always contained *Tulasnella calospora* (Boudier) Juel] or were associated with a number of species from the same fungal genus [e.g., *Vanda hindsii* Lindl. and *Rhinerrhiza divitiflora* (F. Muell. ex Benth.) Rupp both contained several species of *Ceratobasidium* fungi]. However, Warcup (1971, 1981) also showed that some Australian orchids could be colonized simultaneously by a number of different fungal genera such as *Caladenia reticulata* R.D. Fitzg., *Eriochilus cucullatus* (Labill.) Rchb. f., and *Acianthus caudatus* R. Br. each contained both *Sebacina* and *Tulasnella* fungi. Recent molecular studies of Australian orchid fungi, although focusing largely on those present in nonphotosynthetic orchids, have shown orchid–fungal specificity in *Dipodium* spp. (Bougoure and Dearnaley 2005; Dearnaley and Le Brocq 2006) and a lack of specificity in the vine-like *Erythrorchis cassythoides* (A.M. Cunn.) Garay (Dearnaley 2006).

Bougoure et al. (2005) previously studied the root-associated fungi of six common terrestrial orchids in southeastern Queensland, Australia. Using internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP), cloning, and sequencing, two main *Ceratobasidium*-like fungi were identified in three *Pterostylis* species. The

M.J. Irwin · J.D.W. Dearnaley (✉)
Department of Biological and Physical Sciences, The University of
Southern Queensland, Toowoomba 4350, Australia
Tel. +617-4631-2804; Fax +617-4631-1530
e-mail: dearns@usq.edu.au

J.J. Bougoure
School of Plant Biology, Faculty of Natural and Agricultural
Sciences, The University of Western Australia, Crawley, Australia

fungus isolated from *Pterostylis longifolia* R. Br. and *P. nutans* was designated PN1-1; a closely related fungus isolated from *Pterostylis obtusa* R. Br. was named PO1-1. As very few plants were used in these analyses and these were obtained from a restricted geographic locality, assessment of fungal specificity patterns in these species was not possible.

In this study, we aimed to address the concept of fungal specificity in a common species of Australian photosynthetic terrestrial orchid. We have sampled roots of *P. nutans* at six different localities in eastern Australia and identified the fungal community of each plant via fungal ITS-PCR, cloning, and sequencing. The results suggest that the main fungal partners of the orchid species are two closely related species of *Ceratobasidium* fungi. This observation demonstrates that fungal specificity occurs in this species of orchid.

Materials and methods

Acquisition of plant material and DNA extraction

Pterostylis nutans plants were obtained by permit from two sites in Victoria (4 plants), one site in the Australian Capital Territory (3 plants), one site in New South Wales (3 plants), and two sites in Southern Queensland (5 plants) (Fig. 1, Table 1). Roots were hand sectioned to confirm fungal colonization, and photomicrographs of sections were recorded with a Nikon upright E600 light microscope (Nikon, Tokyo, Japan) (see Fig. 3). One-centimeter portions of plant roots were rinsed in tap water and placed in 100% bleach (White King, 4% available chlorine) for 30s. Root portions were then rinsed three times in sterile distilled water. Total DNA was extracted from the two regions of fungal colonization in each plant, i.e., just below the soil surface (A) and a region close to the tuber (B) (Fig. 2) with a Qiagen DNeasy plant DNA extraction kit (Doncaster, VIC, Australia) following the manufacturer's instructions.

Fungal ITS amplification

Fungal ITS DNA was PCR amplified in a 50- μ l reaction volume, with each containing 38 μ l sterile distilled H₂O, 5 μ l 10 \times buffer (50mM KCl, 10mM Tris-HCl, 0.1% Triton X-100; Invitrogen Australia, Mt. Waverley, VIC, Australia), 2.5 μ l 50mM MgCl₂ (Invitrogen Australia), 1 μ l 10mM dNTP (Invitrogen Australia), 1 μ l of each of the fungal-specific ITS1F primer (Gardes and Bruns 1993) and ITS4 (White et al. 1990), 0.5 μ l *Taq* DNA polymerase (Invitrogen Australia), and 1 μ l extracted genomic DNA. PCR amplifications were performed in a Thermo Hybrid 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.



Fig. 1. Map of Australia indicating the collection sites for *Pterostylis nutans*

Table 1. List of *Pterostylis nutans* samples obtained for this study, including plant designation, collection sites, date of collection, and Global Positioning System (GPS) positions

Plant no.	Site collected	Date collected	GPS position
Pn1-3	Stanthorpe QLD	September 2005	28°43'01.3" S 151°57'31.7" E
Pn4-5	Goomburra State Forest QLD	July 2005	27°59' S 152°21' E
Pn6-8	Blue Mountains NSW	June 2005	33°46'04" S 151°00'16" E
Pn9-11	Canberra ACT	August 2005	35°16'16.5" S 149°05'03.2" E
Pn12-14	Anglesea VIC	October 2005	38°25'10.36" S 144°10'21.95" E
Pn15	Grampians VIC	October 2005	37°37'45.9" S 142°20'05.4" E

QLD, Queensland; NSW, New South Wales; ACT, Australian Capital Territory; VIC, Victoria

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.



Fig. 2. Photograph of *Pterostylis nutans* indicating the two areas of fungal colonization (A and B). S, soil surface; T, tuber. Bar 2 cm

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), conducted as per the manufacturer's instructions. Sequencing reactions of 29 clones representative of the major fungal ITS-PCR products present in the original gel (i.e., some plant portions had a number of fungal ITS bands present, whereas others had no fungal ITS amplicons) were performed in 8- μ l volumes containing approximately 400 ng purified plasmid DNA, 6.4 pmoles of T7 promotor primer (Quantum Scientific, Brisbane, Australia) at the Brisbane laboratory of the Australian Genome Research Facility.

ITS sequence analysis

Combined ITS1, 5.8S, and ITS2 sequences were analyzed using BLAST (Altschul et al. 1997) and FASTA (version 3.4t21) (Pearson and Lipman 1988) searches of GenBank through ANGIS (<http://www.angis.org.au>). For construction of the phylogenetic tree, closest sequence matches to the PN1-1 sequence were obtained via FASTA and BLAST searches of GenBank (<http://www.angis.org.au>). These sequences were then aligned with Clustal W (Thompson et al. 1994) using default settings and edited using JALVIEW (Clamp et al. 2004) through ANGIS (<http://www.angis.org.au>). The resulting file was realigned using Clustal X (Thompson et al. 1997) using a gap opening penalty of 10 and varying the gap extension penalty between 1, 5, and 10. The resulting alignments were checked by eye with the alignment, using gap extension penalty of 5 chosen as the best representative and used for further analysis. This consensus alignment was loaded into PAUP (version 4.0b10) (Swofford 2002) and a neighbor-joining analysis run with bootstrapping of 1000 replicates conducted. Note sequence 10A was omitted from the analysis because of alignment difficulties.

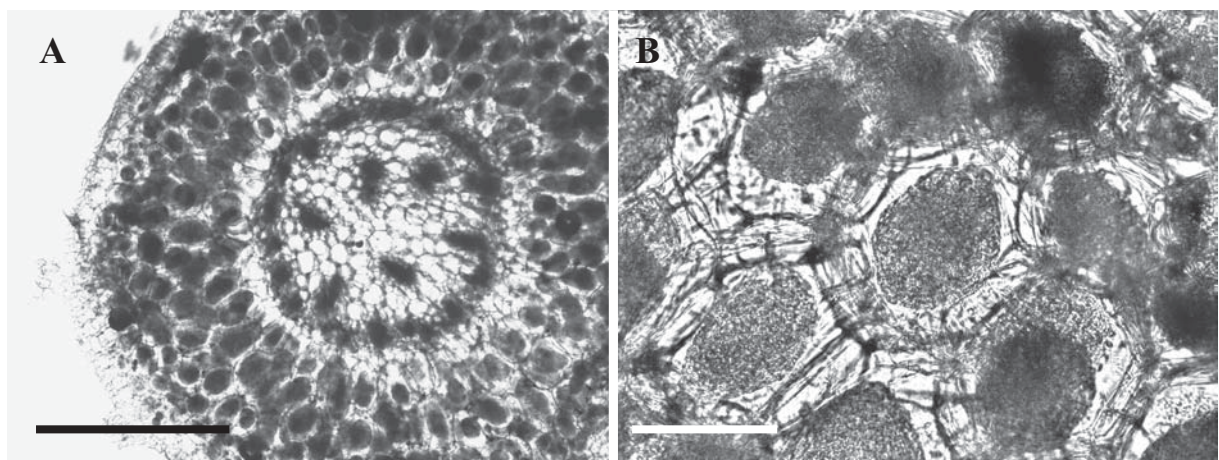


Fig. 3. Transverse section of a *Pterostylis nutans* root showing fungal colonization of cortex tissue (A). High-magnification micrograph of a section of orchid root showing detail of pelotons (B). Bars A 1000 μ m; B 110 μ m

Results

Fungal identification: ITS sequencing

Of the 28 fungal ITS PCR products that were cleanly sequenced, 19 had a closest match to the PN1-1 fungus originally isolated from *P. nutans* by Bougoure et al. (2005) (Table 2). Three of the sequenced fungal PCR products were most closely related to the PO1-1 fungus originally isolated from *P. obtusa* by Bougoure et al. (2005) (Table 2). There were a number of homobasidiomycetes present in the roots of *P. nutans*, a fungus with close identity to a putative *Tricholoma* sp. (96% over 419bp) (Pn15B; Table 2), and two sequences had closest sequence matches to a previously uncultured *Gymnomyces* sp. from the myco-heterotrophic orchid *Dipodium hamiltonianum* F.M. Bailey (Dearnaley and Le Brocque 2006) (Pn10B and Pn12B; Table 2). There were two unidentified soil fungi in *P. nutans* roots (Pn1A.1, Pn7A.1, Pn7B.3; Table 2), but one of these sequences (Pn1A.1) had a second best match to the ascomycete *Lepidotodidium orchidicola* Sigler et Currah (99% over 591 bp) (Table 2). The second best match for the Pn4 and Pn12A *Ceratobasidium*-like sequences was a sequence designated “uncultured Russulaceae,” suggesting that this is a misidentification by the submitting authors.

Neighbor-joining analysis separated non-orchid-derived *Ceratobasidium*-like fungal sequences from GenBank (Table 3) from the sequences that were similar to the PN1-1 and PO1-1 fungi of Bougoure et al. (2005) (99% bootstrap support; Fig 4). Within the latter, there were two major groups of sequences. The majority of PN1-1-related sequences from this study grouped together with the original PN1-1 (AY643803) from Bougoure et al. (2005) as well as a number of fungi isolated from orchids in the Pterostylidinae including *P. nutans* (DQ028797, DQ028799, DQ028800, DQ028804-6, DQ028808), *Linguella* sp. (DQ028802), *Diplodium grandiflorum* (R.Br.) D.L. Jones et M.A. Clem. (DQ028801, DQ028803), *Bunochilus macrosepala* D.L. Jones (ined.) (DQ028807), and *Taurantha ophioglossa* (R.Br.) D.L. Jones et M.A. Clem. (DQ028798) (Clements and Otero, unpublished results; in Table 3, these accession numbers are noted as DQ028***) (63% bootstrap support; Fig 4). A second group consisted of the original PO1-1 fungal sequence (AY643805) from Bougoure et al. (2005), the PO1-1-related sequences from this study, and sequences from fungi originally isolated by Clements and Otero from the Pterostylidinae orchids: *Pharochilum daintreanum* (Benth.) D.L. Jones et M.A. Clem. (DQ028809-11, DQ028816-17), *Pterostylis baptistii* Fitzg. (DQ028812-13), *Speculantha* sp. (DQ028814), *Speculantha rufescens* D.L. Jones (ined.) (DQ028818), *Speculantha parviflora* (R.Br.) D.L. Jones et M.A. Clem. (DQ028819), and *Plumatichilos tasmanicum* (D.L. Jones) D.L. Szlachetko (DQ028820) (53% bootstrap support; Fig 4).

Discussion

Pterostylis nutans appears to have a specific relationship with the PN1-1 and PO1-1 fungi originally isolated by Bougoure et al. (2005) as each of the 15 plants sampled always had one of these fungi present. These two fungi have previously been shown to be closely related, but not identical, species through ITS-RFLP analysis (Bougoure et al. 2005). GenBank BLAST searches suggest that the PN1-1 or PO1-1 fungi are probably members of the *Ceratobasidium* genus, an important group of plant pathogens (Johanson et al. 1998; Hietala et al. 2001) but also common mycorrhizal partners in a diversity of orchids worldwide (Filipello Marchisio et al. 1985; Currah et al. 1990; Perkins et al. 1995; Otero et al. 2004).

As outlined in the Introduction, specificity in Australian orchids is a complex issue. Absolute specificity or a “one orchid–one fungal species” relationship has been documented in some orchid species such as *Glossodia major* R. Br., *Diurus maculata* Smith, and *Acianthus exsertus* R. Br. (Warcup 1971, 1981). Taxon-level specificity or relationship of one orchid species with more than one member of the same fungal genus/family has been documented in *Rhinerrhiza divitiflora*, *Vanda hindsii*, *Microtis parviflora* R.Br., *Dipodium variegatum* M. Clements et D. Jones, and *Dipodium hamiltonianum* (Warcup 1981; Perkins et al. 1995; Bougoure and Dearnaley 2005, Dearnaley and Le Brocque 2006). Nonspecificity, or the presence of a range of unrelated fungal taxa in a single orchid species, has also been reported in a diversity of species including *Caladenia reticulata*, *Lyperanthus nigricans* R. Br., and *Erythrorchis cassythoides* (Warcup 1971, 1981; Dearnaley 2006). Combined with the data from the current study that demonstrate taxon-level specificity, it appears that Australian orchids vary considerably in the nature of their root–fungal associations. Girlanda et al. (2006) has suggested that specificity occurs in green orchid species that have extended periods of adult dormancy or in species that occur in shaded forest environments. *Pterostylis nutans* typically occurs in moist, sheltered areas (Jones 2006), so it is possible that limited irradiance is the reason that this orchid has a close association with the two *Ceratobasidium* fungi which may extend to supplementation of carbon nutrition for the orchid.

Absolute and taxon-level specificity would place constraints on the distribution of an orchid if the fungal partners were rare. In conservation activities involving release of ex situ symbiotically grown plants to the wild, it is critical that fungal partners are monitored in the long term, in addition to plant survival (Perkins and McGee 1995; Brundrett et al. 2003). Seed germination and protocorm growth of subsequent generations would conceivably be hindered if fungal populations were to disappear from an orchid habitat. Batty et al. (2001) have outlined methods for long-term ex situ storage of appropriate fungal partners of endangered orchids. Such approaches would provide a reservoir of fungal inocula that could be accessed for continued reintroduction to the natural state.

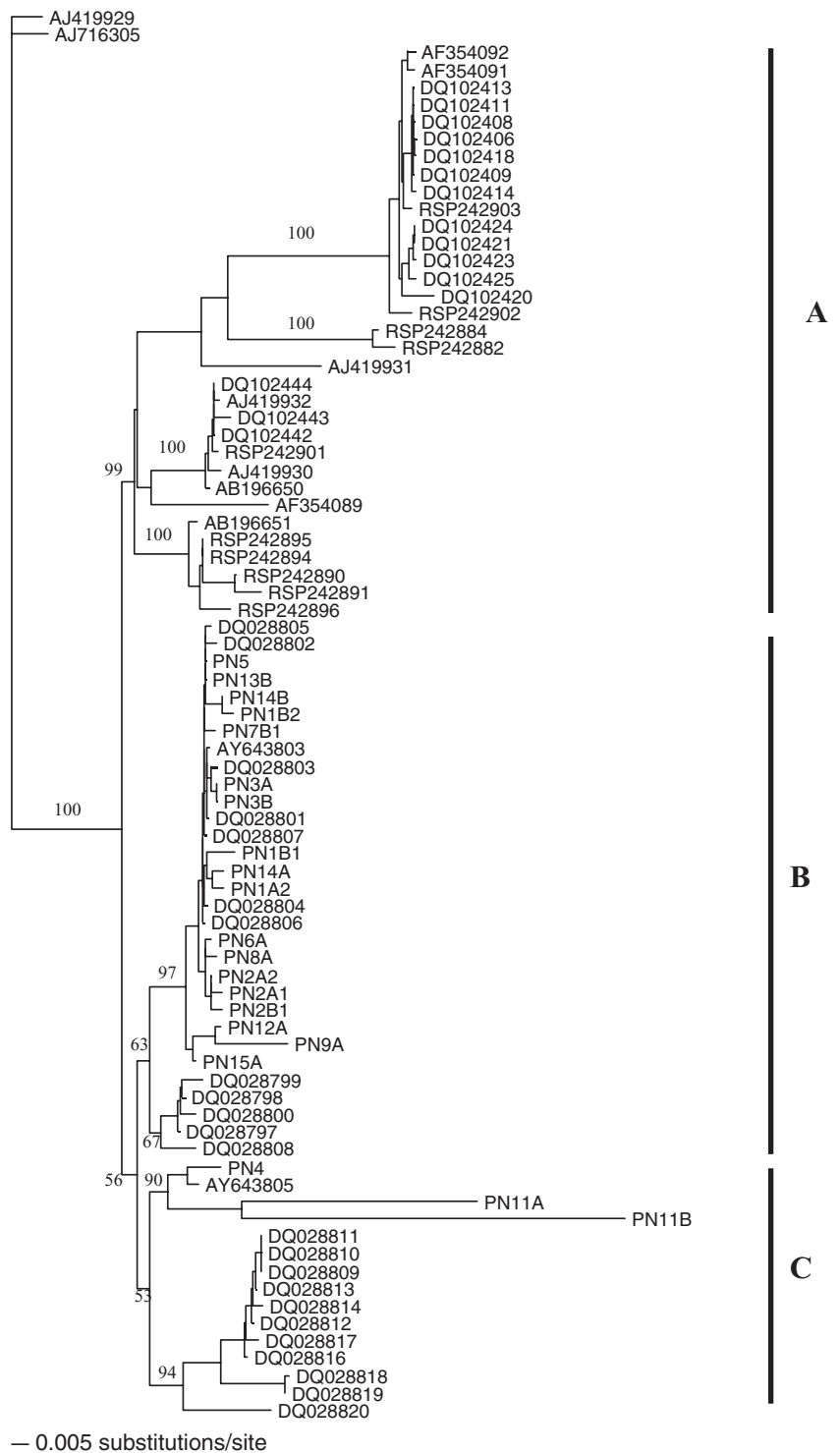
Table 2. List of identified fungal internal transcribed spacer (ITS)-DNA samples including their site origin, sequence number, deposited GenBank code, GenBank closest matches, and percentage matches

Site	Sequence	GenBank code	Closest species matches and accession codes	% Match	
Stanthorpe, QLD	Pn1A.1	EF090490	Soil fungal species	DQ914424.1	587/589 (99%)
			<i>Leptodontidium orchidicola</i> strain	AF486133.1	588/591 (99%)
Stanthorpe, QLD	Pn1A.2	EF090491	PN1-1	AY643803.3	605/610 (99%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	570/574 (99%)
Stanthorpe, QLD	Pn1B.1	EF090492	PN1-1	AY643803.3	556/567 (98%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	546/556 (98%)
Stanthorpe, QLD	Pn1B.2	EF090493	PN1-1	AY643803.3	596/606 (98%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	565/574 (98%)
Stanthorpe, QLD	Pn2A.1	EF090494	PN1-1	AY643803.3	602/610 (98%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	567/574 (98%)
Stanthorpe, QLD	Pn2A.2	EF090495	PN1-1	AY643803.3	606/610 (99%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	570/574 (99%)
Stanthorpe, QLD	Pn2B.1	EF090496	PN1-1	AY643803.3	603/610 (98%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	567/574 (98%)
Stanthorpe, QLD	Pn3A	EF090497	PN1-1	AY643803.3	607/610 (99%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028803.1	571/574 (99%)
Stanthorpe, QLD	Pn3B	EF090498	PN1-1	AY643803.3	608/611 (99%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028803.1	571/575 (99%)
Goomburra QLD	Pn4 ^a	EF090499	PO1-1	AY643805.3	598/612 (97%)
Goomburra QLD	Pn5 ^a	EF090500	Uncultured Russulaceae	DQ061931.1	651/691 (94%)
			PN1-1	AY643803.3	609/610 (99%)
Blue Mountains, NSW	Pn6A	EF090501	Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	573/574 (99%)
			PN1-1	AY643803.3	606/610 (99%)
Blue Mountains, NSW	Pn7A.1	EF090502	Uncultured soil fungus clone	DQ420771.1	580/585 (99%)
			Uncultured soil fungus clone	DQ420792.1	579/585 (98%)
Blue Mountains, NSW	Pn7B.1	EF090503	PN1-1	AY643803.3	605/613 (98%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	570/577 (98%)
Blue Mountains, NSW	Pn7B.3	EF090504	Uncultured soil fungus clone	DQ420771.1	581/582 (99%)
			Uncultured soil fungus clone	DQ420792.1	580/582 (99%)
Blue Mountains, NSW	Pn8A	EF176581	PN1-1	AY643803.3	599/605 (99%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	569/575 (98%)
Canberra, ACT	Pn9A	EF090505	PN1-1	AY643803.3	585/611 (95%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	506/518 (97%)
Canberra, ACT	Pn10A	EF090507	PN1-1	AY643803.3	427/476 (89%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	447/506 (88%)
Canberra, ACT	Pn10B	EF090508	Uncultured fungus clone	DQ178933.1	671/691 (97%)
			Uncultured fungus clone	DQ178932.1	671/692 (96%)
Canberra, ACT	Pn11A	EF090509	PO1-1	AY643805.3	498/565 (88%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028800.1	445/498 (89%)
Canberra, ACT	Pn11B	EF090510	PO1-1	AY643805.3	290/333 (87%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028800.1	285/331 (86%)
Anglesea, VIC	Pn12A	EF090511	PN1-1	AY643803.3	599/610 (98%)
			Uncultured Russulaceae isolate	DQ061931.1	660/696 (94%)
Anglesea, VIC	Pn12B	EF090512	Uncultured fungus clone	DQ178933.1	707/722 (97%)
			Uncultured fungus clone	DQ178932.1	706/722 (97%)
Anglesea, VIC	Pn13B	EF090513	PN1-1	AY643803.3	608/610 (99%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	573/574 (99%)
Anglesea, VIC	Pn14A	EF090514	PN1-1	AY643803.3	605/610 (99%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	570/574 (99%)
Anglesea, VIC	Pn14B	EF090515	PN1-1	AY643803.3	606/610 (99%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	570/574 (99%)
Grampians, VIC	Pn15A	EF090516	PN1-1	AY643803.3	604/610 (99%)
			Vouchered (<i>Ceratobasidium</i>)	DQ028807.1	569/574 (99%)
Grampians, VIC	Pn15B	EF090517	Uncultured cf. <i>Tricholoma</i> sp.	AY254876.1	403/419 (96%)
			Uncultured fungus isolate	DQ093748.1	372/385 (96%)

Note: Sequences designated A are fungi from the soil surface region of plants; sequences designated B are from the tuber region of plants; multiple sequences for some root portions reflect multiple fungal ITS products present in the original PCR gel

^aGoomburra soil surface and tuber samples were combined

Fig. 4. Internal transcribed spacer (ITS) rDNA phylogenetic tree constructed using the neighbor-joining method (NJ) showing relatedness of the sequences obtained from this study to other *Ceratobasidium*-like sequences in GenBank. Note the non-orchid-derived fungal sequences (**A**) separate from the orchid-derived fungal sequences (**B, C**). Within the latter there are two main groups: those sequences related to PN1-1 (AY643803) from Bougoure et al. (2005) (**B**) and those sequences related to PO1-1 (AY643805) from Bougoure et al. (2005) (**C**)



A previous investigation of the root-associated fungi of *P. nutans* was restricted to two plants at two geographic localities (Bougoure et al. 2005). Recent studies of orchid-fungal interactions have demonstrated that wide species sampling (where feasible) can give clearer assessments of specificity between orchid and fungal species. Girlanda et al. (2006) sampled *Limodorum abortivum* (L.) Swartz over a wide range in France and Italy and showed colonization largely by fungi in the Russulaceae. Selosse

et al. (2002) sampled *Neottia nidus-avis* (L.) L.C. Richard from multiple French populations and showed a preference for fungal species within the Sebacinaceae. Taylor et al. (2004) studied *Corallorhiza maculata* Raf. at various sites in the western United States and showed associations solely with Russulaceae fungi. The broader sampling approach used in this study has thus enabled more thorough assessment of the nature of the fungal associations in *P. nutans*.

Table 3. List of *Ceratobasidium*-like fungal ITS sequences used in neighbor-joining (NJ) tree

Accession no.	Fungal sequence name	Source	Authors
AJ419929	<i>Rhizoctonia</i> sp.	<i>Pinus sylvestris</i>	Gronberg et al. 2003
AJ716305	<i>Sclerotinia spermophila</i> ^a	Not known	Staats et al. 2005
AF354092	<i>Ceratobasidium</i> sp. AG-A	Soil	Gonzalez et al. 2001
AF354091	<i>Ceratobasidium</i> sp. AG-B (o)	Sweet potato	Gonzalez et al. 2001
DQ102413	<i>Ceratobasidium</i> sp. AG-A	Strawberry	Sharon et al., unpublished
DQ102XX	<i>Ceratobasidium</i> sp. AG-A	Strawberry	Sharon et al., unpublished
RSP242903	<i>Rhizoctonia</i> sp.	Not known	Salazar et al., unpublished
RSP242XX	<i>Rhizoctonia</i> sp.	Not known	Salazar et al., unpublished,
AJ419931	<i>Rhizoctonia</i> sp.	<i>Pinus sylvestris</i>	Gronberg et al. 2003
AJ419932	<i>Rhizoctonia</i> sp.	<i>Pinus sylvestris</i>	Gronberg et al. 2003
AB196650	<i>Ceratobasidium</i> sp. AG-I	<i>Artemisia</i> sp.	Hyakumachi et al., unpublished
AB196651	<i>Ceratobasidium</i> sp. AG-I	Soil	Hyakumachi et al., unpublished
AF354089	<i>Ceratobasidium</i> sp. AG-H	Soil	Gonzalez et al. 2001
DQ028***	<i>Ceratobasidium</i> sp.	Pterostylidinae	Clements and Otero, unpublished
AY643803	PN1-1	<i>Pterostylis nutans</i>	Bougoure et al. 2005
AY643805	PO1-1	<i>Pterostylis obtusa</i>	Bougoure et al. 2005

^aProbably misidentified species because it shows homology with *Rhizoctonia* sp.

The identification of *Ceratobasidium* spp. as the main fungal partners in *P. nutans* concurs with previous studies on this orchid. Warcup and Talbot (1967) isolated *Ceratobasidium cornigerum* (Bourdot) D.P. Rogers from the species growing in South Australia, and Perkins et al (1995) isolated a *Ceratohiza* sp. from a plant growing near Sydney. We had previously isolated a fungus designated PN1-1 from a single *P. nutans* plant growing at Crow's Nest National Park in Queensland (Bougoure et al. 2005). GenBank searches at the time had closest matches to *Rhizoctonia solani* J.G. Kühn, the anamorph of *Thanetophorus cucumeris* (A.B. Frank) Donk, but subsequent GenBank submissions and BLAST search similarities suggest that the PN1-1 fungus is likely a *Ceratobasidium* sp.

Neighbor-joining phylogenetic analysis clearly separated the non-orchid- and orchid-associated *Ceratobasidium*-like fungal taxa, suggesting that the putative pathogenic and symbiotic *Ceratobasidium* species are distinctly different species. Although pathogenic species of *Ceratobasidium* such as *C. cereale* D.I. Murray et Burpee have been described as orchid root-associated fungi (e.g., Peterson and Currah 1990), it is possible that these identifications may need reassessment at a molecular level. As further revisions of the form genus *Rhizoctonia* are conducted (Gonzalez et al. 2006), it appears that much of the classical taxonomy of this group may be revised. Phylogenetic analysis in this study reinforced that the PN1-1 and PO1-1 fungal isolates likely represent two separate species, and also that other members of the Pterostylidinae such as *P. baptistii*, *Pharochilum daintreeanum*, *Plumatochilus tasmanicum*, and *Speculantha* spp. may be colonized solely by a fungus with identity to the PO1-1 isolate.

The occurrence of *Leptodontidium orchidicola* in the root of one *P. nutans* plant is interesting. Although basidiomycetes are the most important group of orchid fungi (Rasmussen 2002), Selosse et al. (2004) have shown through ITS sequencing of root fungi and ultrastructural evidence that *Tuber* spp. can form mycorrhizal associations with *Epipactis microphylla* (Ehrh.) Swartz. *Leptodontidium orchidicola* is a common fungal endophyte that has been

found in many orchids worldwide (Currah et al. 1990; Bidartondo et al. 2004; Julou et al. 2005); thus, further physiological characterization of this fungus in orchids appears warranted.

Molecular studies of photosynthetic orchids in Europe and North America have shown that homobasidiomycetes can occasionally be present in plant roots in addition to the more dominant heterobasidiomycete genera (Kristiansen et al. 2001; McCormick et al. 2004; Shefferson et al. 2005). Homobasidiomycete fungal genera are the usual fungal partners of nonphotosynthetic orchids (Taylor and Bruns 1997, 1999; Bougoure and Dearnaley 2005; Dearnaley and Le Brocque 2006; Girlanda et al. 2006), and there is evidence that these fungi provide a carbon conduit from neighboring trees to orchid species (McKendrick et al. 2000; Selosse et al. 2002; Girlanda et al. 2006). It is tempting to speculate that the putative *Tricholoma* and *Gymnomyces* fungi present in some *P. nutans* roots provide evidence for alternative carbon sources for the species, but it is more likely that these fungi, along with the unidentified soil fungus, are surface contaminants or nonmycorrhizal fungi in the orchids.

Australia has more than 1300 native species of orchid (Jones 2006). A sizable proportion of these are endangered or vulnerable, and measures must be put in place to guard against permanent loss of species. A crucial first step in the conservation of rare orchid species is to store plant material, such as seed, in herbaria or similar institutions (Batty et al. 2001). Plants are then grown under artificial conditions before release to the wild (Scade et al. 2006). The role of mycorrhizal fungi in such conservation activities is starting to receive some attention (Batty et al. 2001, 2006; Brundrett et al. 2003).

In conclusion, we have demonstrated that *P. nutans* associates specifically with two closely related *Ceratobasidium* fungi across its range in eastern Australia. Given that there is evidence that some Australian orchids do not have specific associations with soil fungi (Warcup 1981; Dearnaley 2006), it appears important to treat each orchid species individually with respect to the identity of the mycorrhizal

fungi present. The fact the pattern of root-associated fungus occurrence is not consistent between members of the Pterostylidinae suggests that unstudied taxa may have unique fungi. Isolation and preservation of the root-associated fungi of rare members of the group such as *Petrorchis bicornis* (D.L Jones et M.A Clements) D.L Jones et M.A Clements and *Oligochaetochilus woollsi* (Fitzg.) D.L. Szlachetko is thus a priority before such orchid species are permanently lost from natural situations.

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