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

Mathew John Irwin, Jeremy James Bougoure, John David William Dearnaley

Mathew John Irwin, John David William Dearnaley (author for correspondence)
Department of Biological and Physical Sciences, The University of Southern Queensland, Toowoomba 4350, Australia.
Phone +617 4631 2804, Fax +617 4631 1530, Email: dearns@usq.edu.au.

Jeremy James Bougoure
School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia, Crawley 6009, 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 fifteen *Pterostylis nutans* plants from 6 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 homobasidomycete spp. 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 that conservation measures for endangered Australian orchids must include ex situ perpetuation of fungal symbionts as well as plant material.

Keywords: 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 photosynthetic orchid-fungal interactions can now be categorised 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 1981; 1971; Perkins and McGee 1995; Perkins et al. 1995; Bougoure et al. 2005). Non-photosynthetic orchids appear to associate mostly with homobasidiomycete fungi such as members of the Russulaceae (Bougoure and Dearnaley 2005; Dearnaley and Le Brocque 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 (eg. members of the *Caladenia* and *Glossodia* genera all contained *Sebacina vermifera* Oberwinkler while *Diuris* orchids always contained *Tulasnella calospora* (Boudier) Juel) or were associated with a number of species from the same fungal genus (eg. *Vanda hindsii* Lindl. and *Rhinerrhiza divitiflora* (F. Muell. ex Benth.) Rupp both contained several species of *Ceratobasidum* fungi). However, Warcup (1971; 1981) also showed that some Australian orchids could be colonized simultaneously by a number of different fungal genera eg. *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 non-photosynthetic orchids, have shown orchid-fungal specificity in *Dipodium* spp. (Bougoure and Dearnaley 2005; Dearnaley and Le Brocque 2006;) and a lack of specificity in the vine-like *Erythrorchis cassythoides* (Cunn.) Garay (Dearnaley 2006).

Bougoure et al. (2005) previously studied the root associated fungi of six common terrestrial orchids in south-eastern Queensland, Australia. Using ITS-RFLP, cloning and sequencing two main Ceratobasidiales-like fungi were identified in three *Pterostylis* species. The fungus isolated from *P. longifolia* and *P. nutans* was designated PN1-1 while a closely related fungus isolated from *P. obtusa* 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 *Pterostylis nutans* R. Br. 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 demonstrates that fungal specificity occurs in this species of orchid.

Materials and methods

Acquisition of plant material & DNA extraction

P. nutans plants were permit-obtained 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 colonisation and photomicrographs of sections were recorded with a Nikkon upright E600 light microscope (Nikkon Corporation, Japan) (Fig 3). 1cm 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 colonisation in each plant ie. 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 50µl reaction volume, with each containing 38µl sterile distilled H_2O, 5µl 10X buffer (50mM KCl, 10mM Tris-HCl, 0.1% Triton X-100; Invitrogen Australia, Mt Waverley, VIC, Australia), 2.5µl 50mM $MgCl_2$ (Invitrogen Australia), 1µl 10mM dNTP (Invitrogen Australia), 1µl of each of the fungal specific ITS1F primer (Gardes and Bruns 1993) and ITS4 (White et al. 1990), 0.5µl of *Taq* DNA polymerase (Invitrogen Australia) and 1µl of extracted genomic DNA. PCR amplifications were performed in a Thermo Hybaid PCR Express thermocycler (Integrated Sciences, Willoughby, NSW, Australia) with 35 cycles of 95°C for 1min, 50°C for 1min and 72°C for 1min, with a final incubation at 72°C for 10min. 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.

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 (ie. some plant portions had a number of fungal ITS bands present, while others had no fungal ITS amplicons) were performed in 8µl volumes containing approximately 400ng of purified plasmid DNA, 6.4pmoles 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 (version3.4t21) (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 re-aligned 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 neighbour-joining analysis run with bootstrapping of 1000 replicates conducted. Note sequence 10A was omitted from the analysis due to alignment difficulties.

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 *Pterostylis obtusa* R. Br. by Bougoure et al. (2005) (Table 2). There were a number of homobasidomycetes 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, *Leptodontidium orchidicola* Sigler and Currah (99% over 591bp) (Table 2). The second best match for the Pn4 and Pn12A *Ceratobasidium*–like sequences was a sequence designated "uncultured Russulaceae" suggesting that this 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) (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

P. 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 & D.Jones and *Dipodium hamiltonianum* (Warcup 1981; Perkins et al. 1995; Bougoure and Dearnaley 2005, Dearnaley and Le Brocque 2006). Non specificity, 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 which demonstrates 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. *P. 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.

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 colonisation largely by fungi in the Russulaceae. Selosse et al. (2002) sampled *Neottia nidus-avis* (L.) L.C.M. Rich from multiple French populations and showed preference for fungal species within the Sebacinaceae. Taylor et al. (2004) studied *Corallorhiza maculata* Rafinesque 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*.

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* from the species growing in South Australia while Perkins et al (1995) isolated a *Ceratorhiza* 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, the anamorph of *Thanetophorus cucumeris*, but subsequent GenBank submissions and BLAST search similarities suggest that the PN1-1 fungus is likely a *Ceratobasidium* sp.

Neighbour-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* have been described as orchid root associated fungi (eg. 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 (eg. 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. *L. 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 characterisation 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 non photosynthetic 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 neighbouring trees to orchids 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 non mycorrhizal fungi in the orchids.

Australia has over 1300 native species of orchid (Jones 2006). A sizeable 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 woollsii* (Fitzg) D.L. Szlachetko is thus a priority before such orchid species are permanently lost from natural situations.

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Tables

Table 1. List of *P. nutans* samples obtained for this study including plant designation, collection sites, date of collection and GPS positions

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

Table 2. List of identified fungal ITS-DNA samples including their site origin, sequence number, deposited GenBank code, GenBank closest matches and percentage matches. Note: sequences designated A are fungi from the soil surface region of plants while 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.

Site	Sequence	GenBank Code	Closest Species Matches & Accession Codes		% Match
Stanthorpe, QLD	Pn1A.1	EF090490	Soil fungal sp.	DQ914424.1	587/589 (99%)
			Leptodontidium orchidicola strain	AF486133.1	588/591 (99%)
Stanthorpe, QLD	Pn1A.2	EF090491	PN1-1	AY643803.3	605/610 (99%)
			Vouchered (Ceratobasidium)	DQ028807.1	570/574 (99%)
Stanthorpe, QLD	Pn1B.1	EF090492	PN1-1	AY643803.3	556/567 (98%)
			Vouchered (Ceratobasidium)	DQ028807.1	546/556 (98%)
Stanthorpe, QLD	Pn1B.2	EF090493	PN1-1	AY643803.3	596/606 (98%)
			Vouchered (Ceratobasidium)	DQ028807.1	565/574 (98%)
Stanthorpe, QLD	Pn2A.1	EF090494	PN1-1	AY643803.3	602/610 (98%)
			Vouchered (Ceratobasidium)	DQ028807.1	567/574 (98%)
Stanthorpe, QLD	Pn2A.2	EF090495	PN1-1	AY643803.3	606/610 (99%)
			Vouchered (Ceratobasidium)	DQ028807.1	570/574 (99%)
Stanthorpe, QLD	Pn2B.1	EF090496	PN1-1	AY643803.3	603/610 (98%)
			Vouchered (Ceratobasidium)	DQ028807.1	567/574 (98%)
Stanthorpe, QLD	Pn3A	EF090497	PN1-1	AY643803.3	607/610 (99%)
			Vouchered (Ceratobasidium)	DQ028803.1	571/574 (99%)
Stanthorpe, QLD	Pn3B	EF090498	PN1-1	AY643803.3	608/611 (99%)
			Vouchered (Ceratobasidium)	DQ028803.1	571/575 (99%)
Goomburra QLD	Pn4*	EF090499	PO1-1	AY643805.3	598/612 (97%)
			Uncultured Russulaceae	DQ061931.1	651/691 (94%)
Goomburra QLD	Pn5*	EF090500	PN1-1	AY643803.3	609/610 (99%)
			Vouchered (Ceratobasidium)	DQ028807.1	573/574 (99%)
Blue Mountains, NSW	Pn6A	EF090501	PN1-1	AY643803.3	606/610 (99%)
			Vouchered (Ceratobasidium)	DQ028807.1	570/574 (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 (Ceratobasidium)	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 (Ceratobasidium)	DQ028807.1	569/575 (98%)
Canberra, ACT	Pn9A	EF090505	PN1-1	AY643803.3	585/611 (95%)
			Vouchered (Ceratobasidium)	DQ028807.1	506/518 (97%)
Canberra, ACT	Pn10A	EF090507	PN1-1	AY643803.3	427/476 (89%)
			Vouchered (Ceratobasidium)	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 (Ceratobasidium)	DQ028800.1	445/498 (89%)
Canberra, ACT	Pn11B	EF090510	PO1-1	AY643805.3	290/333 (87%)
			Vouchered (Ceratobasidium)	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 (Ceratobasidium)	DQ028807.1	573/574 (99%)
Anglesea, VIC	Pn14A	EF090514	PN1-1	AY643803.3	605/610 (99%)
			Vouchered (Ceratobasidium)	DQ028807.1	570/574 (99%)
Anglesea, VIC	Pn14B	EF090515	PN1-1	AY643803.3	606/610 (99%)
			Vouchered (Ceratobasidium)	DQ028807.1	570/574 (99%)
Grampians, VIC	Pn15A	EF090516	PN1-1	AY643803.3	604/610 (99%)
			Vouchered (Ceratobasidium)	DQ028807.1	569/574 (99%)
Grampians, VIC	Pn15B	EF090517	Uncultured cf. *Tricholoma sp.*	AY254876.1	403/419 (96%)
			Uncultured fungus isolate	DQ093748.1	372/385 (96%)

*Goomburra soil surface and tuber samples were combined

Table 3. List of *Ceratobasidium*-like fungal ITS sequences used in NJ tree. Included are accession numbers, fungal sequence name, source of fungus and authors.

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

*probably misidentified sp. as shows homology with *Rhizoctonia* sp.

Figures

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

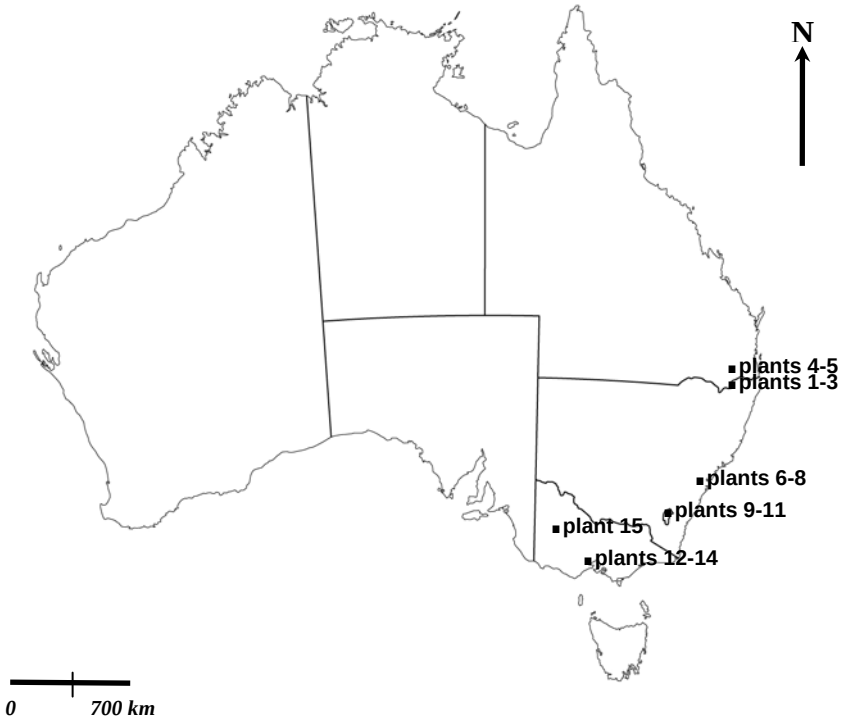

Fig. 2. Photograph of *P. nutans* indicating the two areas of fungal colonisation A & B, where S = soil surface and T = tuber (scale bar is 2 cm)

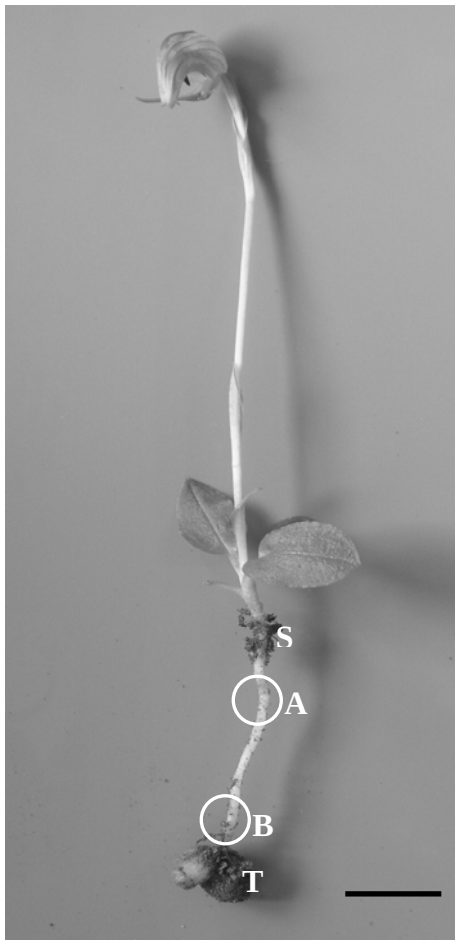

Fig. 3. Transverse section of a *P. nutans* root showing fungal colonisation of cortex tissue (A). High magnification micrograph of a section of orchid root showing detail of pelotons (B) (scale bars are 1000 μm and 110 μm)

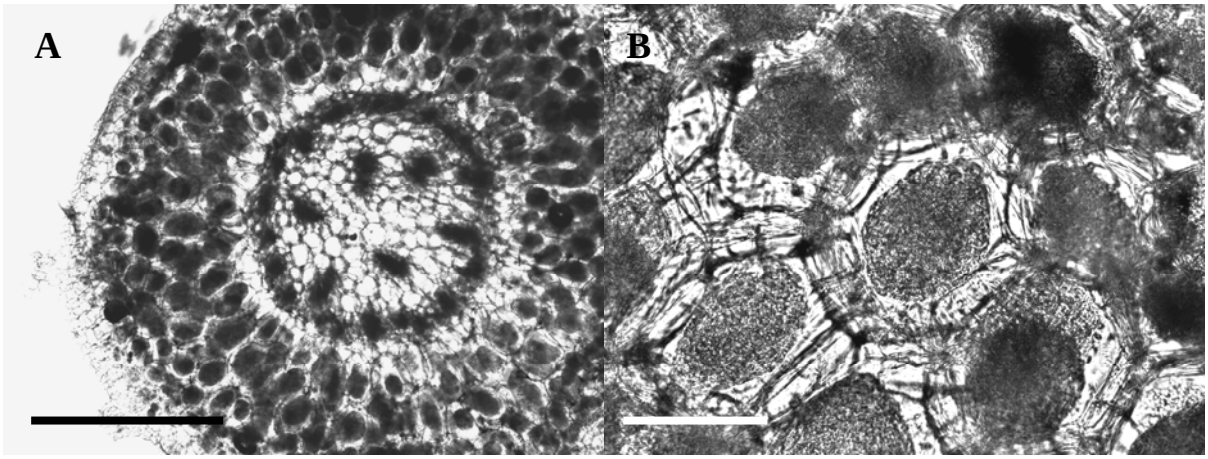

Fig. 4. ITS rDNA phylogenetic tree constructed using the neighbour-joining method (NJ) showing relatedness of the sequences obtained from this study to other *Ceratobasidium*–like sequences in GenBank. Note the non orchid-derived fungal sequences (A) separate from the orchid-derived fungal sequences (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).