The rare Australian epiphytic orchid *Sarcochilus weinthalii* associates with a single species of *Ceratobasidium*

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Abstract *Sarcochilus weinthalii* is a rare, epiphytic orchid largely restricted to the dry rainforests of eastern Australia. Part of the conservation procedures for the orchid includes isolation and identification of the associated mycorrhizal fungus. In this study the mycorrhizal fungal partner of the species was determined through extraction and sequencing of DNA from both colonised orchid roots and pure fungal cultures grown out from orchid roots. A single species of *Ceratobasidium* predominated in the orchid suggesting that the orchid displays narrow fungal specificity. This mycorrhizal fungus can now be used in conservation procedures involving *ex situ* growth and the reintroduction of plants to the natural state.

Key words *Sarcochilus weinthalii*, epiphytic orchids *Ceratobasidium* mycorrhizal specificity

Introduction

All orchids are completely nutritionally dependent on mycorrhizal fungi at the earliest stages of the life cycle. In adult photosynthetic orchids, mycorrhizal associations are often maintained to assist with water and mineral uptake (Yoder et al. 2000; Cameron et al. 2007). Recent evidence suggests that the adult orchid-fungal association may be mutualistic in nature as mycobionts receive photosynthate in return for their services (Cameron et al. 2006; 2008). The fungi that form orchid mycorrhizas are usually basidiomycete taxa (Rasmussen 2002; Dearnaley 2007) and largely belong to the *Rhizoctonia* form genus (Bougoure et al. 2005; Waterman and Bidartondo, 2008). This is an artificial grouping of unrelated fungi based on anamorphic life stage, and usually includes members of the *Ceratobasidium, Sebacina,* and *Tulasnella* genera (Smith and Read 2008).

There are three main growth forms for orchids. Terrestrial species are soil dwelling species, while lithophytes occur on rocks and epiphytes grow on other plants. Globally, the majority of orchids are found growing epiphytically (Jones 2006). Despite this, basic mycorrhizal studies of epiphytes have lagged behind terrestrial species. In studies that have been carried out, representatives of the three major *Rhizoctonia* genera outlined above, have been found as orchid associates (eg. Otero et al. 2007; Suarez et al. 2006; 2008; Pereira et al. 2003; Kottke et al. 2008).

Like terrestrial orchids, epiphytic species retain mycorrhizal partners throughout the life cycle (Pereira et al. 2005; Suarez et al. 2008). Given that epiphytic orchids grow under drought conditions for periods of time (Zotz and Schmidt 2006), mycorrhizal fungi would increase the surface area for water uptake, and so such dependency is not surprising. In addition, since the roots of epiphytic orchids have little to no contact with soil, mycorrhizal fungi may increase access to mineral nutrients (Osorio-Gil et al. 2008).

Sarcochilus weinthalii (F.M. Bailey) Dockrill is a rare Australian epiphytic orchid restricted to south-east Queensland and northern New South Wales (Jones 2006). It is a distinctive member of the *Sarcochilus* genus possessing cream flowers with purplish blotches on the sepals and petals (Fig. 1a). The species grows largely in inland areas of dry rainforest occurring high up on trees that are exposed to cool, moist breezes (Graham and Dearnaley unpublished data). Main threats to *S. weinthalii* appear to be illegal harvesting and clearing of habitat (Department of Environment and Resource Management 2009). An important component of conservation procedures for the orchid is isolation and identification of the associating mycorrhizal fungus. This would ensure that plants can be successfully grown *ex situ* and would enable the detection of suitable habitat for re-introductions.

The aim of this study was to determine the mycorrhizal fungal partner of *Sarcochilus weinthalii* by sampling the orchid at three structurally different sites in south-east Queensland, Australia. DNA was isolated from roots of the orchid and from pure fungal cultures grown out from roots. Fungal DNA was PCR-amplified with ITS1F and ITS4 primers, cloned and sequenced.

Materials and Methods

Sample collection

Root samples were collected from three different habitats in south-east Queensland, Australia - these were, Redwood Park (undisturbed dry rainforest) near Withcott (11 plants from 11 different dry rainforest trees), Mount Kynoch (disturbed dry rainforest) near Toowoomba (Four plants from two *Ligustrum* sp. and a single *Pittosporum* sp.) and Main Range National Park (Wet sclerophyll forest) near Killarney (Six plants from three *Cuttsia* sp.). Root samples were immediately placed on ice and then stored at -20^{o}C upon arrival back at the University of Southern Queensland.

Fungal DNA extraction, cloning and sequencing

A section of a thawed collected root sample was ground using a plastic pestle in a 1.5 ml microfuge tube with a small amount of sterilised sand, prior to the addition of lysis buffer, as per the manufacturer's instructions (Qiagen, Doncaster, Victoria, Australia). DNA was extracted by means of a DNeasy Plant Mini Kit (Qiagen, Doncaster, Victoria, Australia) using the manufacturer's instructions. PCR amplification involved adding 0.5 µL of the extracted DNA to 14 µL sterile milli-Q H_2O, 2 µL 10 X buffer (Scientifix, Cheltenham, Victoria, Australia), 2 µL 10 mM dNTP (Scientifix) and 0.5 µL of each of the fungal specific primer ITS1F (Gardes and Bruns 1993) and the ITS4 primer (White et al. 1990) and 0.5 µL of Hot Start DNA polymerase (Scientifix). Amplification reactions were duplicated and negative controls (distilled H_2O substituted for DNA) included and PCR reactions were performed in a Thermo Hybaid-PCR Express Thermal Cylinder (Thermo Hybaid, Ashford, Middlesex, UK) with 35 cycles of 94ºC and 58ºC for 1 min each followed by 72ºC for 2 min and a concluding incubation period of 10 min at 72ºC. After electrophoresis, PCR products were viewed in 2% (w/v) agarose gels containing ethidium bromide under UV light. Amplicons were cleaned using a DNA purification kit (Macherey-Nagel, Cheltenham, Australia) following the manufacturer's instructions. Cloning of the purified DNA samples was carried out with the pGEM-T Easy Vector System (Promega, Annandale, NSW, Australia) using the manufacturer's instructions. A Fast Plasmid Mini Kit (Eppendorf, Nth Ryde NSW, Australia) was used to separate plasmid DNA from *E. coli* cultures grown overnight in Luria Bertani nutrient broth (Difco, Detroit Michigan, USA). Plasmid DNA samples were PCR-amplified with the ITS1F and ITS4 primers to confirm cloning of fungal ITS regions. Sequencing of fungal ITS-DNA was performed at the Australian Genome Research Facility (AGRF) Brisbane.

Analysis of fungal sequences

Sequences were edited to eliminate vector sequence and to make sure of correct orientation and analysed with BLAST searches against the National Centre for Biotechnology Information (NCBI) sequence database (GenBank; http://www.ncbi.nlm.nih.gov/) to establish closest sequence matches. Phylogenetic analysis was performed with MEGA version 4 (Tamura et al. 2007). Closest sequences matches plus three representative sequences from the three sites were aligned using Clustal W (Thompson et al. 1994) and a neighbour-joining tree (Saitou and Nei 1987) was constructed from the alignment file using the Maximum Composite Likelihood method (Tamura et al. 2004) and bootstrapping of 1000 replicates (Felsenstein 1985).

Fungal isolation procedure

Fungal isolation from orchid roots was carried out using the methods based on Yamato et al. (2005). Root samples were either sprayed with 70% ethanol and left for 30 s before being washed in sterile distilled H_2O three times or were rinsed in tap water before the sterile distilled H_2O wash. Approximately 5 mm of each root was crushed in a 9 cm diameter Petri dish and 1 ml of sterile H_2O was added. Next, 20 ml of sterile potato dextrose agar (PDA; Sigma, Castle Hill, NSW, Australia), cooled to 45^{o}C, was then poured over the top and swirled gently to mix. The agar was then allowed to set before sealing the petri dish with parafilm and storing in the dark at 21^{o}C.

After three days, colonies that had been observed to grow from pelotons were cut out of the agar and grown on fresh PDA. Following several days of growth, colonies were subcultured again twice to ensure isolate purity. A portion of the fungal culture was next transferred to fungal isolation media (FIM; Clements and Ellyard 1979) and incubated at 25^{o}C to promote optimum growth. Cultures were left to grow for a week before a section of the colony was taken for identification through DNA extraction and sequencing (as outlined above).

Results

Molecular analysis of fungal DNA in plant roots

Fungal DNA was successfully extracted from 19 of the 21 root samples collected. PCR amplification gave multiple fungal ITS amplicons for most roots, however a band of approximately 700bp was common to all samples (not shown). BLAST searches found that the closest species match for this sequence was a *Ceratobasidium* sp. previously isolated from the Western Australian underground orchid *Rhizanthella gardneri* ((Rogers); Table 1; Bougoure et al. 2009). Bands other than the 700bp amplicon returned closest matches to a variety of different ascomycetes with no apparent commonality (Table 1). In total, 25 clones were sequenced, 12 of which all returned the same closest match in Genbank (Table 1). When these 12 sequences were aligned using the BLAST search multiple alignment tool, they were found to be 98% identical. Phylogenetic analysis of these *Ceratobasidium*-like sequences showed that they formed a clade (92% bootstrap support) with the *Ceratobasidium* taxa isolated from *R. gardneri* and the eastern Australian underground orchid *Rhizanthella slateri* ((Rupp) MA Clements and PJ Cribb; Fig. 2).

Molecular analysis of fungal DNA from pure cultures

Examination of root sections collected from each site revealed fungal colonisation on the outer part of the cortex (Fig. 1b). Fungal cultures were successfully isolated using the pour plate method. After two days, hyphae with distinct right angular branching were observed growing from pelotons (Fig. 1c). Seven fungal isolates were obtained, three from root samples taken at Main Range NP and four from samples taken at Redwood Park. Colonies grew well on the FIM plates and were white in colour. When viewed under a light microscope, distinctive monilioid cells were observed to be produced within four to five days after isolation (Fig. 1d), a characteristic common of *Ceratobasidium* spp. (Warcup and Talbot 1971). DNA was extracted from the colonies after pure cultures had been obtained. ITS-DNA was successfully PCR amplified for all but one of the samples

and all remaining samples produced bands that were 700bp in length. When these 700bp bands were sequenced and compared via BLAST sequence similarity analysis, they were shown to be 98-99% identical to the *in planta Ceratobasidium* sp. sequences which is within the single species parameter of Tedersoo et al. (2008).

Discussion

This study found that *S. weinthalii* associates with a single, previously undescribed species of *Ceratobasidium*. This identification is not surprising as members of the *Ceratobasidium* genus are common orchid mycobionts worldwide, having been detected in both terrestrial and epiphytic orchids previously (Irwin et al. 2007; Otero et al. 2002), including other *Sarcochilus* spp. (Gowland et al. 2007). *Ceratobasidium* species also include saprotrophic and plant pathogenic taxa (Gonzalez Garcia et al. 2006) and interestingly, a recent observation is that some *Ceratobasidium* species are capable of ectomycorrhizal associations (Collier and Bidartondo 2009). It is unlikely that the fungus identified here is ectomycorrhizal, in spite of the sequence similarity with the fungal partner of *R. gardneri* (which is ectomycorrhizal), as the *S. weinthalii* mycobiont occurs high up in the canopy and is not in contact with tree roots.

Isolation and identification of the mycorrhizal partner of *S. weinthalii* will assist in conservation procedures for the species. A number of studies have used field-isolated fungi for the *ex situ* propagation of rare orchids (eg. Zettler et al. 2005; Batty et al. 2006; Stewart and Kane 2007). Asymbiotic growth of *S. weinthalii* is problematic and seed germination is greatly enhanced with suitable mycorrhizal fungi (Clements 1987). Acquisition of the mycorrhizal partner of *S. weinthalii* will also assist in the reintroduction of plants back into the natural state. For example it will be possible to survey for the presence of the mycorrhizal fungus of *S. weinthalii* either via seed baiting (Hollick et al. 2007) or by molecular detection (Landeweert et al. 2003) and thus locate potential reintroduction sites.

Fungal specificity is a phenomenon often observed in terrestrial orchids (McCormick et al. 2004; Irwin et al. 2007; Bougoure et al. 2009). In epiphytic orchids, fungal specificity has also been recorded by Otero et al. (2002; 2004) who showed that in *Ionopsis utricularioides* (Swartz) Reichenbach f., mycorrhizal interactions were restricted to a single *Ceratobasidium* clade. Fungal specificity in orchids may have developed from a need for a more efficient physiological association between fungus and orchid, particularly in low light habitats (Otero et al. 2004; Girlanda et al. 2006; Bonnardeaux et al. 2007). An implication of fungal specificity is that it might lead to orchid species rarity if the mycobiont is not evenly or widely dispersed in the environment (Otero et al. 2007). A recent study by Phillips et al. (2011) however, have shown that specificity has not led to rarity in *Drakaea* spp. as the associated *Tulasnella* fungus is widely distributed in the environment. Therefore threats to the *S. weinthalii* may not be a reliance on a single species of an equally rare *Ceratobasidium* but may come principally from over collecting and clearing of suitable habitat.

Lack of endosperm in orchid seeds has resulted in complete dependency on outside sources of carbon to promote germination (Smith and Read 2008). Studies have suggested that fungal specific associations may be reduced at the seedling stage of development for some orchids (Masuhara and Katsuya 1994; Otero et al. 2004) or maintained for other species (Warcup 1971; Bonnardeaux et al. 2007). Additional studies examining the seed germination of *S. weinthalii* uninoculated, and with the *Ceratobasidium* sp. isolated here as well as closely related *Ceratobasidium* taxa, will aid in determining the extent of fungal specificity throughout the orchid life cycle.

Molecular studies of orchid endophytic fungi have typically focused on terrestrial orchid species (eg. Taylor and Bruns 1999; Bougoure et al. 2005; Irwin et al. 2007; Roy et al. 2009). Recently, molecular studies of fungi from some epiphytic orchids have been carried out in Ecuador (Suarez et al. 2006; 2008), Puerto Rico (Otero et al. 2002, 2004), Brazil (Pereira et al. 2003, 2005) and Singapore (Ma et al. 2003). In Australia, Boddington and Dearnaley (2008) reported molecular

identification of fungal endophytes of *Dendrobium speciosum* Smith while Gowland et al. (2007) documented the mycorrhizal partners of *Sarcochilus hillii* (F. Muell.) F. Muell., *Sarcochilus parviflorus* Lindl. and *Plectorrhiza tridentata* (Lindl.) Dockr., three other rainforest epiphytic species. Our study thus makes an important contribution to a growing field of orchid mycorrhizal research which is certain to expand given that epiphytic orchids predominate worldwide (Jones 2006).

In summary we have examined the mycorrhizal association of a rare, epiphytic orchid species at three sites in eastern Australia. Analysis of DNA *in planta* and from isolated fungal cultures suggests that the adult *S. weinthalii* plants associate with a single species of *Ceratobasidium*. Additional studies examining the seed germination with and without this fungus will aid in determining the extent of fungal specificity throughout the orchid life cycle. This fungus may also be used in future *ex situ* and *in situ* conservation procedures for the orchid.

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Fig. 1 *S. weinthalii* and its mycorrhizal partner. **a** The orchid in flower at Main Range National Park, bar = 3 cm. **b** Pelotons (arrows) in the root of the orchid, bar = 100 µm. **c** Isolated peloton growing in culture – note the right angle branching of hyphae, bar = 150 µm. **d** Monilioid cells produced from the *Ceratobasidium* isolate, bar = 50 µm

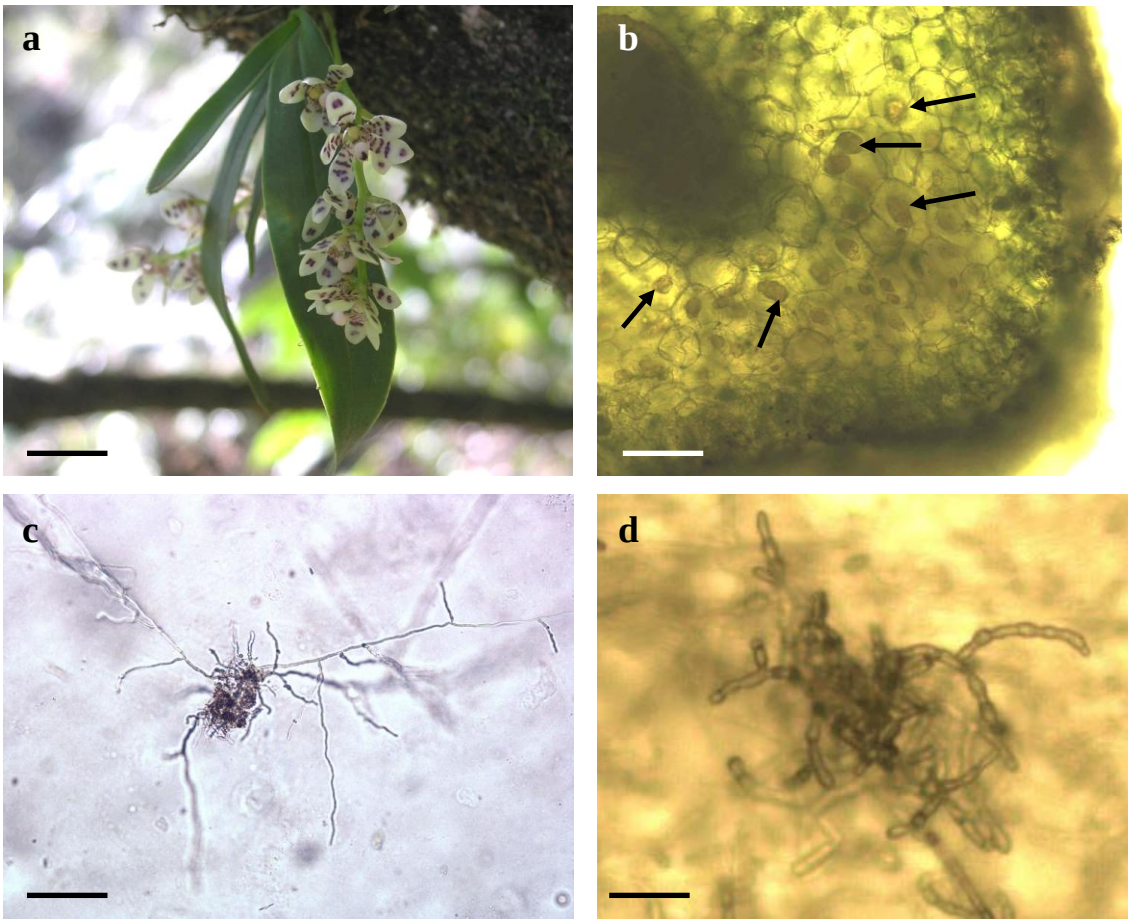

Table 1 Fungal ITS rDNA sequences identified in roots of *S weinthalii* and their closest GenBank match. Note: two clones were sequenced if root samples had multiple PCR amplicons. Lower expected (E) values indicate closer sequence matches

Site	Root sample No.	GenBank accession no.	Closest match	GenBank accession no.	E-value	Max identity
Redwood Park	RP-T1.1	GU991815	*Ceratobasidium* sp.	GQ175313.1	0_0	90%
	RP-T1.2	GU392001	Uncultured soil fungus	DQ420802.1	1E_175	85%
	RP-T2.3	GU391994	Uncultured soil fungus	DQ420996.1	0_0	97%
	RP-T4.4	GU991817	*Cladosporium langeronii*	DQ780380.2	2E_66	84%
		GU391998	*Ceratobasidium* sp.	GQ175313.1	0_0	90%
	RP-T4.5	GU391996	Uncultured endophyte	EF505554.1	0_0	98%
	RP-T5.6	GU991816	*Pestalotiopsis sp*.	EF451804.1	0_0	99%
	RP-T7.7	GU391995	Uncultured soil fungus	DQ420802.1	0_0	98%
		GU391999	*Ceratobasidium* sp.	GQ175313.1	0_0	91%
	RP-T8.8	GU392000	Uncultured soil fungus	DQ420996.1	0_0	97%
	RP-T9.9	GU991818	Fungal endophyte	FJ449979.1	2e_05	82%
		GU991820	*Ceratobasidium* sp.	GQ175313.1	1E_109	83%
	RP-T10.10	GU391997	*Ceratobasidium* sp.	GQ175313.1	0_0	91%
Mount Kynoch	MK-T1.11	GU391991	Uncultured fungus	EF635649.1	1E_145	82%
	MK-T2.12	GU391992	*Exserohilum turcicum*	FJ182235.1	2E_102	82%
	MK-T2.13	GU391993	*Ceratobasidium* sp.	GQ175313.1	0_0	91%
Main Range NP	MR-T1.14	GU392005	Fungal endophyte	FJ449695.1	5E_139	89%
		GU991821	*Ceratobasidium* sp.	GQ175313.1	0_0	91%
	MR-T1.15	GU392002	*Ceratobasidium* sp.	GQ175313.1	0_0	91%
	MR-T2.16	GU392006	*Heterodermia casarettiana*	DQ337305.1	0_0	96%
		GU991819	*Ceratobasidium* sp.	GQ175313.1	0_0	91%
	MR-T2.17	GU392007	Uncultured fungus	EU516891.1	3E_175	93%
		GU392008	*Ceratobasidium* sp.	GQ175313.1	0_0	90%
	MR-T3.18	GU392003	*Ceratobasidium* sp.	GQ175313.1	0_0	90%
	MR-T3.19	GU392004	*Ceratobasidium* sp.	GQ175313.1	0_0	91%

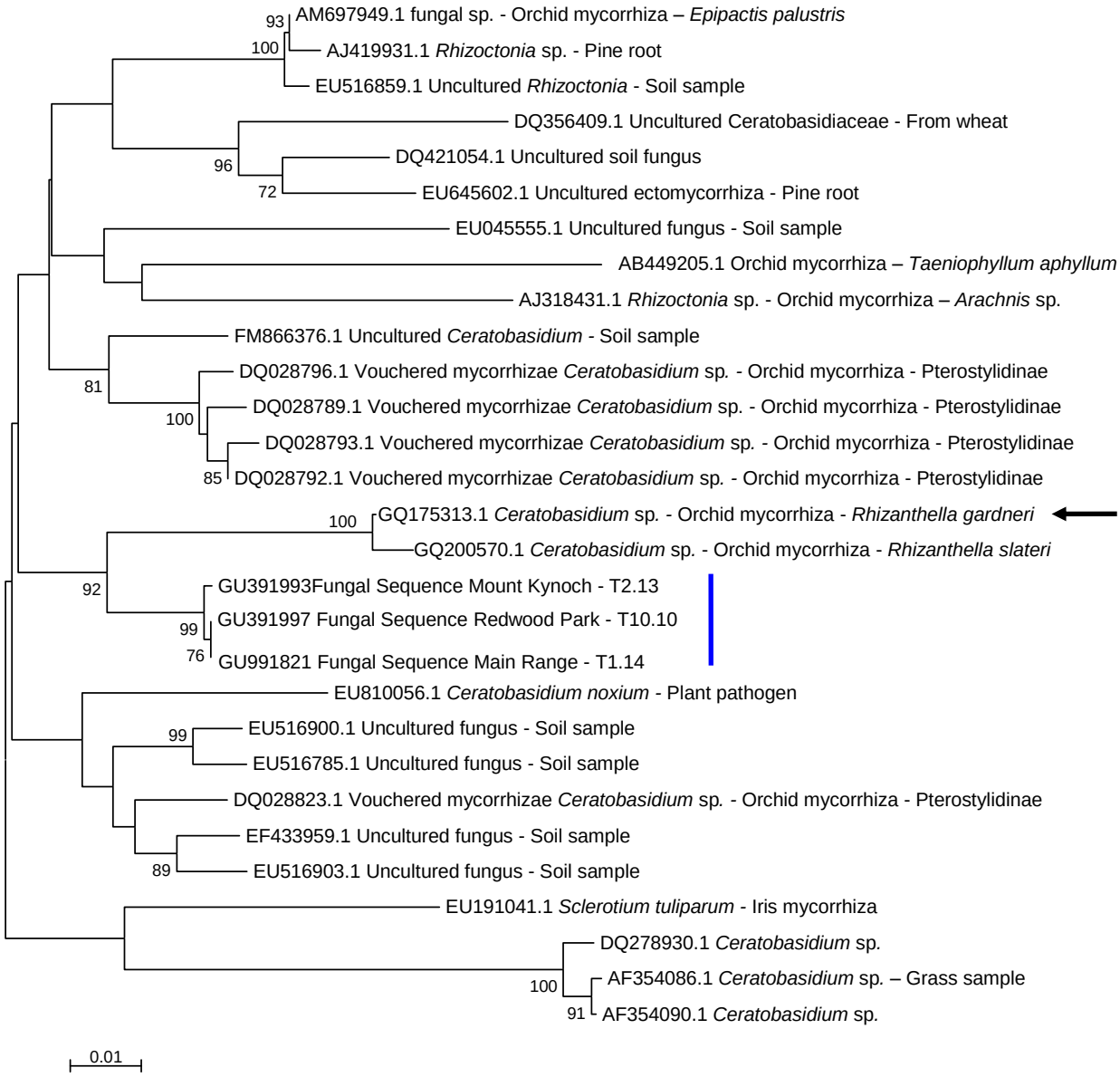

Fig. 2 Neighbour-joining phylogenetic tree of ITS rDNA sequences obtained from fungal symbionts of *S. weinthalii*. Vertical line = *Ceratobasidium* sequences from *S. weinthalii* (representative sequences shown for clarity), Arrow = closest GenBank match (*Ceratobasidium* sp. isolated from *R. gardneri*)