Molecular identification of a mycorrhizal Sebacinaceae from the endangered *Caladenia atroclavia* (black clubbed spider orchid)

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Abstract

Caladenia atroclavia D. Jones & M. Clements is an endangered orchid found only in the Stanthorpe region of south east Queensland. As part of a recovery process for the species we aimed to isolate and identify the mycorrhizal fungus from the plant. Fungal coils were isolated from the orchid and grown into pure culture. DNA was extracted from these cultures and also from colonised plant stem collar regions. Following fungal ITS-DNA PCR amplification, cloning and BLAST similarity searches, we identified the main fungal endophyte within the orchid as a Sebacinaceae sp. Co-inoculation trials showed that isolates of the fungus were capable of germinating seed of the orchid. Neighbour-joining phylogenetic analysis of the ITS-DNA of this mycorrhizal fungus showed that it was related to Sebacinaceae spp. that have been isolated from other Australian *Caladenia* spp. This fungus will be used in *ex situ* propagation procedures to ultimately increase the number of plants in the natural state.

Introduction

The family Orchidaceae is intriguing in that all of its species (approximately 25,000–35,000) are nutritionally supported by mycorrhizal fungi in the non-photosynthetic early stages of development (Smith and Read 1997). Hyphal coils or pelotons are also found in the roots of mature orchid species demonstrating the dependence of most orchids on fungi throughout their life cycle. There is much accumulated evidence that individual orchid species associate with a narrow range of mycorrhizal fungi, even across wide geographic areas (reviewed in Dearnaley 2007).

There are over 1300 species of orchid in Australia with most occuring as deciduous terrestrial plants. A large number of Australian species are at risk due to anthropogenic disturbance (e.g., land clearing, weed invasion, altered fire regimes) and climate change. The "spider orchids" of the genus *Caladenia* are some of the most striking of Australia's terrestrial orchids. The majority of the group are small, spring flowering species that occur in woodlands throughout much of southern Australia. There are 132 species of spider orchid in Australia (Jones 2006) and a number of these are under threat. The Environment Protection and Biodiversity Conservation Act 1999 (Department of Environment, Water, Heritage and Arts 2008) currently lists 4 spider orchid species as critically endangered, 20 species as endangered and 10 species as vulnerable. The study of the conservation biology of plants in this group is thus of national concern.

Caladenia atroclavia (Fig. 1A) is only found in the Stanthorpe region of south-east Queensland (Jones 2006). The plant grows to almost 40 cm, and is distinct from other spider orchid species in that its pale spidery flowers bear short black/purple clubs at the ends of the sepals and exude a scent described as an overheated electric motor (Jones 2006). The species exists at three locations within a National Park but recent estimates number the total plant population at less than 120 individuals (Dearnaley & Mathieson unpublished data). Threats to the species are thought to include illegal collection, inappropriate fire regimes, weed invasion and feral pigs (Department of Environment, Water, Heritage and Arts 2008). A recovery process for *C. atroclavia* requires isolation and identification of the main mycorrhizal partner of the species so that more plants can be produced *ex situ* and then be released to bolster the dwindling natural populations.

In this paper we report investigations into the mycorrhizal biology of *C. atroclavia*. Our approach was to identify the main fungal endophyte(s) of the orchid through sequencing of fungal DNA from peloton-derived cultures and plant stem collar regions. We also aimed to confirm the mycorrhizal status of the fungal isolates through seed germination experiments. Our results indicate that like other studied Australian *Caladenia* spp., the mycorrhizal partner of *C. atroclavia* is a Sebacinaceae sp.

Materials and methods

Sample collection and fungal isolation

Tissue samples were obtained from stem collar regions of three *C. atroclavia* plants (Figs 1A–C) growing at separate localities in south-east Queensland. Portions of the tissue samples were treated with 5% commercial bleach (4% available chlorine) for 5 min, rinsed in sterile distilled water then crushed to release pelotons. Individual pelotons were transferred to potato dextrose agar (pH 5.5; Sigma-Aldrich, Castle Hill, NSW, Australia) and incubated in the dark for several days at 20°C. Colonies were inspected regularly and sub cultured to ensure culture purity (Fig. 1D). Growing pelotons were photographed with a Micropublisher 5.0 digital camera (QImaging, Canada) on a Nikon E600 upright microscope (Nikon Corporation, Tokyo, Japan).

Molecular identification of *C. atroclavia* endophytic fungi

Fungal colonies (plant three) and stem collar portions (plants one and two) were ground with plastic pestles in micro-centrifuge tubes. DNA was extracted with a DNeasy Plant Mini Kit (Qiagen, Doncaster, Victoria, Australia) following the manufacturers' instructions. PCR amplification of the DNA

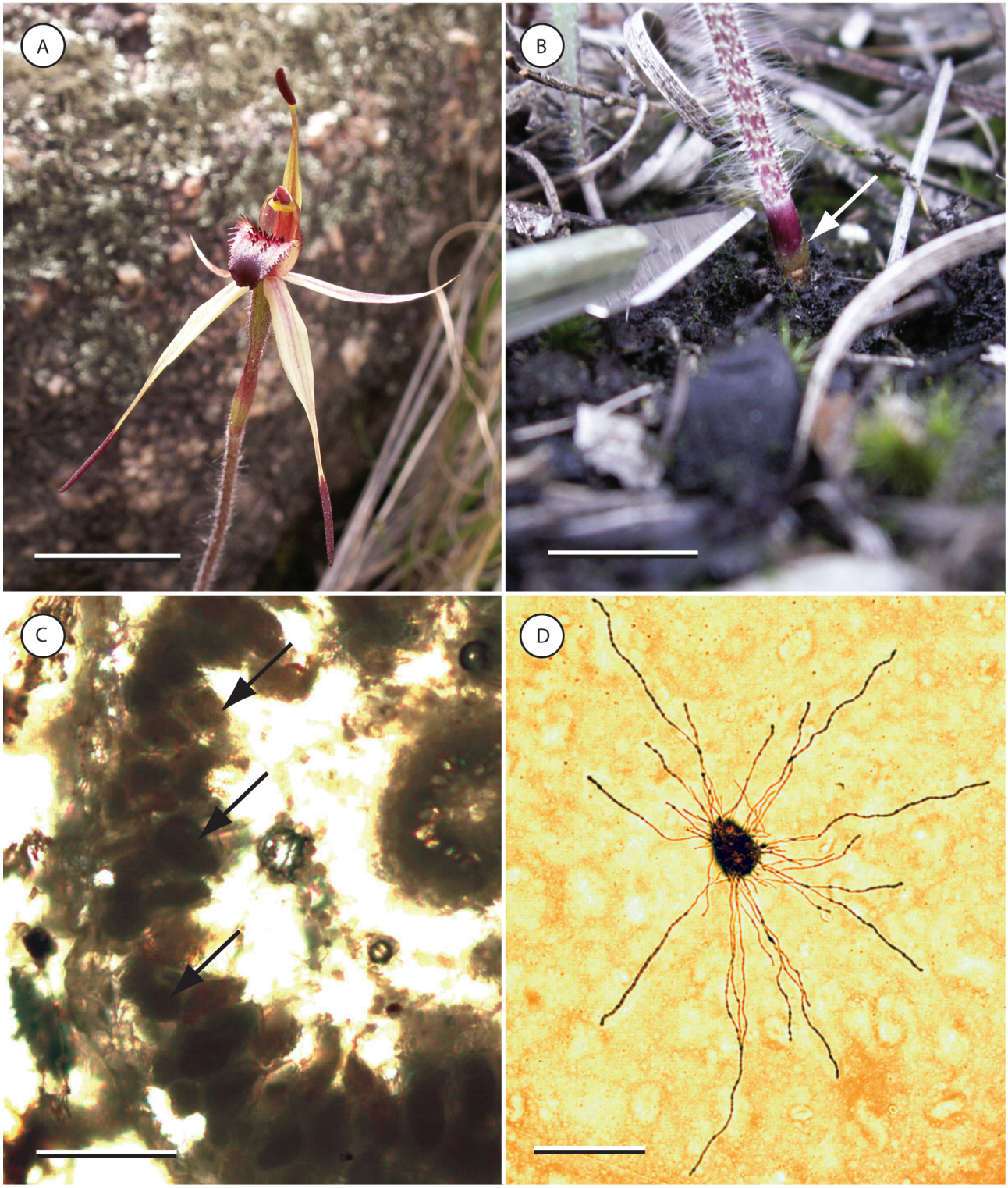

Fig. 1 A. *C. atroclavia* in flower September 2008. Scale bar = 5 mm. **B**. Sampling of orchid tissue occurred at the collar region of plant stems (arrow). Scale bar = 5 mm. **C**. Cross section of stem collar of *C. atroclavia* showing large numbers of fungal pelotons (arrows). Scale bar = 190 µm. **D**. Isolated peloton from *C. atroclavia* growing in pure culture. Scale bar = 230 µm.

samples involved adding 0.5 µL of the extracted genomic DNA to 14 µL sterile milli-Q water, 2 µL 10 × buffer (Scientifix, Cheltenham, Victoria, Australia), 2 µL 10 mM dNTP (Scientifix), 0.5 µL of each of the fungal specific ITS1-F primer (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) and 0.5 µL of Hot Start DNA polymerase (Scientifix). Amplification reactions were completed in duplicate sets with a negative control (distilled water instead of genomic DNA) included. All PCR reactions were carried out with 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 final incubation period of 10 min at 72ºC. Amplicons were visualised in 2% (w/v) agarose gels containing ethidium bromide under UV light, after electrophoresis. PCR samples were purified using a DNA purification kit (Macherey-Nagel, Cheltenham, Australia) following the manufacturers' instructions. Cloning of the purified DNA samples was carried out with the pGEM-T Easy Vector System (Promega, Annandale, NSW, Australia) following the manufacturers' instructions. A Fast Plasmid Mini Kit (Eppendorf, Nth Ryde NSW, Australia) was utilized to isolate plasmid DNA from *E. coli* cultures previously grown overnight in Luria

Table 1 Fungal ITS DNA sequences isolated from *C. atroclavia* plants showing the two closest GenBank matches, accession codes and sequence overlap.

Plant no.	Clone no.	GenBank code	GenBank closest species matches	Accession codes	Sequence identity and overlap
1*	1b	FJ611952	Uncultured soil fungus	DQ421243	90% 680 bp
			Uncultured ectomycorrhiza (*Phialophora*)	AY634154	89% 661 bp
2*	2a	FJ611953	*Sebacina vermifera*	DQ983816	97% 631 bp
			Mycorrhizal fungal sp.	AY328888	97% 510 bp
2*	2b	FJ611954	*Rhodotorula glutinis*	AB038069	90% 211 bp
			Rhodotorula glutinis	EU871506	90% 211 bp
2*	2c	FJ611955	*Sebacina vermifera*	DQ983816	97% 631 bp
			Mycorrhizal fungal sp.	AY328888	97% 510 bp
2*	2d	FJ611956	Uncultured fungus clone	EF090516	94% 694 bp
			Uncultured fungus clone	EF090500	94% 693 bp
3**	3a	FJ611949	*Sebacina vermifera*	DQ983816	97% 631 bp
			Mycorrhizal fungal sp.	AY328888	97% 511 bp
3**	3b	FJ611950	*Sebacina vermifera*	DQ983816	97% 631 bp
			Mycorrhizal fungal sp.	AY328888	97% 510 bp
3**	3c	FJ611951	*Sebacina vermifera*	DQ983816	97% 631 bp
			Mycorrhizal fungal sp.	AY328888	97% 510 bp

*Fungal DNA sequenced from extracted plant stems. **Fungi isolated from plant 3 and sequenced.

Bertani (Difco, Detroit Michigan, USA) nutrient broth solution. Plasmid samples were PCR-amplified with ITS primers to confirm cloning of fungal ITS regions. Sequencing of fungal DNA was carried out at the Brisbane node of the Australian Genome Research Facility (AGRF). Sequences were edited to remove vector sequence and to ensure 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 ascertain closest sequence matches. Phylogenetic analysis was conducted with MEGA version 4 (Tamura *et al.* 2007). All sequences were aligned using Clustal W (Thompson *et al.* 1994) and a neighbour-joining tree (Saitou & Nei 1987) was created from the alignment file using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and bootstrapping of 1000 replicates (Felsenstein 1985).

Seed germination tests

Seed collected from three *C. atroclavia* plants was surface sterilised in 25% commercial bleach for 15 min. Following rinsing in sterile distilled water, seed was placed onto NDY-6 agar plates with 0.5% sucrose (Warcup 1959) with or without inoculum of the three fungal isolates. Plates were incubated in the dark at 20°C for approximately 3 weeks. Seeds were recorded as germinated if embryos had swollen, ruptured the seed coat and epidermal hairs were being produced.

Results

Molecular identification of *C. atroclavia* endophytic fungi

The three fungal cultures successfully obtained from plant three likely represent the same fungal taxon as they were 98%-99% identical to each other when analysed through two sequence BLAST alignment. This fungus has 97% sequence similarity to a member of the *Sebacina vermifera* species complex originally isolated from *Caladenia tessellata* Fitzg. (Warcup 1988) (Table 1). As no fungal isolates were obtained from site one and two plants, total DNA was extracted from stem collar regions of these plants and PCR amplified with ITS1-F and ITS4 primers. Fungal sequence analysis showed that the stem collar sample from plant two contained the same Sebacinaceae sp. as plant three (98%–99% similar), as well as two unidentified fungi, one with a closest GenBank match to the non orchid-mycorrhizal *Rhodotorula glutinus* (Fresen.) F.C. Harrison and the other, an unidentified fungal taxon (Table 1). The stem collar sample from plant one contained an ascomycetous fungus (Table 1).

Phylogenetic analysis shows that the Sebacinaceae species obtained in this study is related to the Sebacinaceae fungi originally isolated from *Caladenia tessellata,* the fungi isolated from *Caladenia formosa* G.W. Carr and a single plant of *Caladenia tentaculata* Schltdl (Fig. 2). It is noteworthy that the *Sebacinaceae* spp. isolated from other Australian spider orchids such as *Caladenia tentaculata* (the majority of isolates) and *Caladenia dilatata* R. Br. form a separate clade with isolates from more distantly related Caladeniinae as well as the non-photosynthetic vine-like orchid *Erythrorchis cassythoides* (A. Cunn.) Garay and the Japanese orchid *Stigmatodactylus sikokianus* (Maxim. ex Makino) Rauschert (Fig. 2).

Seed germination tests

As seed from plants one and two lacked embryos, fungal inoculum (Fig. 1D) was added to plant three seed only. After four weeks co-inoculation, all 3 fungal isolates had successfully germinated seed although the overall percentage of germinated seed was low (26% of a total of 57 seeds). Seed without fungal inoculum remained ungerminated.

Discussion

The data presented here, although restricted by the endangered status of the orchid to a small sample size, indicate that the mycorrhizal fungus in *C. atroclavia* is a representative of the highly variable *Sebacina vermifera* (synonym *Serendipita vermifera* (Oberw.) P. Roberts 1993) complex (Warcup 1988; Weiß *et al.* 2004). This observation correlates with investigations of other Australian Caladeniinae (Warcup 1971, 1981, 1988; Bougoure *et al.* 2005; Bonnardeaux *et al.* 2007; Huynh *et al.* 2009; Wright unpub.), which have also been shown to be colonised by members of this fungal complex.

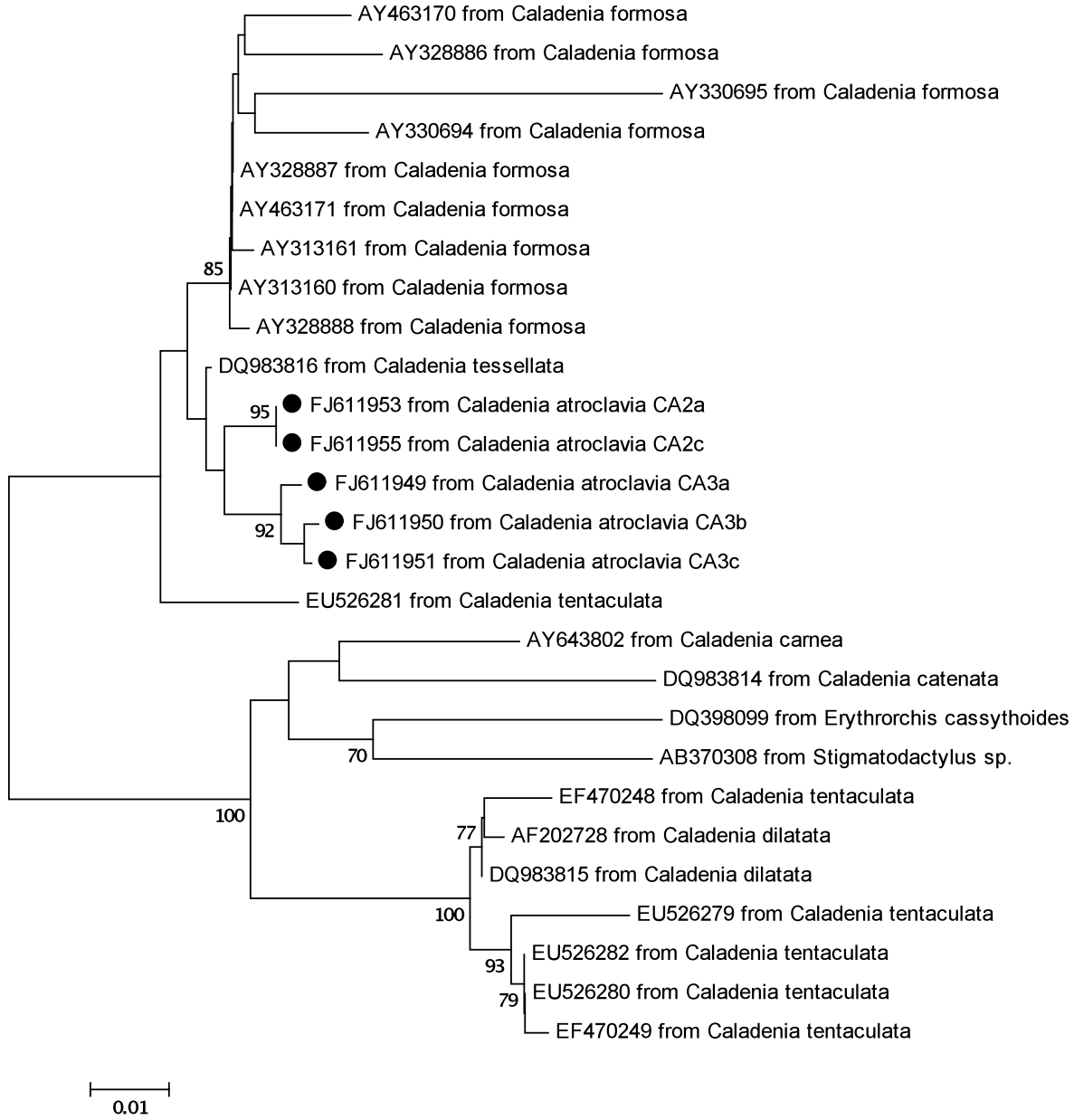

Fig. 2 Neighbour-joining phylogenetic tree of Sebacinoid ITS-DNA sequences obtained from a range of orchid hosts. Note the fungus from *C. atroclavia* (circles) groups with *Sebacinaceae* from *C. tessellata*, *C. formosa* and the endophyte from a single plant of *C. tentaculata*. Bootstrap values (above 70% values shown) are based on 1000 replicates.

Phylogenetic analysis grouped the fungus most closely with the Sebacinaceae from other Australian spider orchids including *C. tessellata*, *C. formosa* and a plant from the Maldon population of *C. tentaculata* (Magali Wright, pers comm.). The phylogenetic separation of the endophytes of other spider orchids such as *C. dilatata* and the majority of *C. tentaculata* plants demonstrates the diversity within this fungal complex and highlights the need for a more detailed taxonomic investigation of the group.

Sebacinoid fungi play a critical role in the health of natural systems worldwide. Sebacinoid members of the group A clade (Weiß *et al.* 2004) form ectomycorrhizal associations with tree species (Selosse *et al.* 2002a; Glen *et al.* 2002) as well as tripartite associations with trees and non-photosynthetic orchids (Taylor & Bruns 1997; McKendrick *et al.* 2000; Selosse *et al.* 2002b, 2004; Julou *et al.* 2005). Members of the group B clade (Weiß *et al.* 2004) form mycorrhizas with photosynthetic orchids (Bougoure *et al.* 2005; Suarez *et al.* 2008), liverworts (Kottke *et al.* 2003) and members of the Ericaceae (Bougoure & Cairney 2005; Selosse *et al.* 2007). It is interesting that the fungal endophyte of the non-photosynthetic *Erythrorchis cassythoides* groups with group B Sebacinaceae (Yagame & Yamato 2008; this study) raising the hypothesis that this fungus may not be the main mycorrhizal partner of the orchid.

Our ability to obtain fungal isolates from plant one and two samples was hampered by contaminating yeasts, bacteria and faster growing deuteromycete fungi. Such microbes are a common problem in research on orchid mycorrhizas (Warcup 1985; Clements 1988) particularly when source tissues are moist (Warcup 1981). The absence of a Sebacinaceae fungal sequence from plant one suggests that *C. atroclavia* may not exclusively associate with this mycorrhizal fungal species. An

alternative explanation is that the initial PCR step of extracted DNA may have exclusively amplified DNA from a more plentiful contaminating ascomycete fungus and masked the presence of other fungal taxa present. Although ascomycetes have recently been observed as forming mycorrhizas in orchids (Selosse *et al.* 2004), Australian orchids associate almost exclusively with basidiomycetes (Bougoure *et al.* 2005; Bougoure & Dearnaley 2005; Dearnaley & Le Brocque 2006; Dearnaley 2006; Bonnardeaux *et al.* 2007; Irwin *et al.* 2007).

The seed germination rate observed in this study is lower than the 52–66% germination rate achieved by Huynh *et al.* (2004) for *Caladenia formosa* but higher than the average <10% germination rate that has been reported for a range of Western Australian Caladeniinae by Ramsay *et al*. (1986). As per Warcup (1985) and Perkins and McGee (1995) we have used protocorm formation as an indicator of fungal compatibility. We will continue to monitor the development of protocorms to green leaf stage, which has been considered by many researchers to be a more accurate indicator of germination capacity of fungal isolates (Huynh *et al.* 2004).

The absence of seed embryos in plant capsules from sites one and two was a surprising discovery in this work and is of considerable concern in our conservation effort on this species. Embryo-less seed may be a function of inbreeding depression (Wallace 2003) and might suggest that the population sizes at these two sites are becoming restrictive (approximately three plants at site one and approximately 30 at site two, Dearnaley & Mathieson unpublished data). Broadhurst *et al.* (2008) however, has demonstrated that small populations of spider orchids *do* retain genetic diversity suggesting other causes of the embryo loss observed here. Light and MacConaill (1998) have shown that drought causes embryo abortion in seed of *Cypripedium calceolus* var. *pubescens* (Willd.) Correll and, as 2007 was a year of below average rainfall in the Stanthorpe region (Australian Bureau of Meteorology 2009), climate change may need to be added to the list of threats for *C. atroclavia*.

The main objective for this research was to isolate and identify the main mycorrhizal fungus of *C. atroclavia*. This has been achieved via DNA sequencing of isolated pelotons, *in planta* fungal DNA sequencing and via seed germination experiments. A number of threatened Australian orchid species have been conserved through symbiotic *ex situ* growth and transplantation (e.g., Batty *et al.* 2006; Scade *et al.* 2006; Smith 2006). Batty *et al.* (2006) demonstrate that seedling and dormant tuber transfer to the wild is superior to the release of seed to field sites for re-establishment of orchid populations. The Sebacinaceae sp. isolated here will now be used in the horticultural production of seedlings and tubers that can be introduced to sites to bolster the dwindling numbers of this endangered orchid species.

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