

## THE FUNGAL ENDOPHYTES OF *ERYTHRORCHIS CASSYTHOIDES*— IS THIS ORCHID SAPROPHYTIC OR PARASITIC?

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### Abstract

*Erythrorchis cassythoides* is a common climbing orchid in Eastern Australia. The plant lacks chlorophyll and typically is rooted at the base of mature trees suggesting the orchid receives its carbon supply via root fungi from either rotting vegetation or indirectly from living tree roots. We have analysed the fungal DNA within roots of the orchid using ITS-PCR analysis, cloning and molecular sequencing to gain insight into the mode of nutrition of this orchid. Fungal ITS rDNA sequences were successfully amplified and cloned from roots of three orchid plants occurring at different localities in SE Queensland. Comparison of these sequences with ITS rDNA in GenBank revealed that the fungal community of *E. cassythoides* roots consists of a saprotrophic homobasidiomycete and ectomycorrhizal fungal species thus suggesting that the orchid is potentially capable of both saprophytic and parasitic modes of nutrition.

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### Introduction

*Erythrorchis cassythoides* (Cunn.) Garay is a common climbing orchid of *Eucalyptus* forests occurring from south-east Queensland to southern NSW (Jones 1988). The plant lacks leaves and its wiry stems climb up to six metres on tree trunks (Bishop 2002). Being non-photosynthetic, the orchid relies on root-dwelling fungi to provide a source of carbon for growth (Williams 1979). As the plant is typically rooted in the leaf litter at the base of its tree host, common opinion is that the ultimate source of carbon for the orchid is decaying vegetation (Bishop 2002, Jones 1988, Nicholls 1969).

The fungi that colonise the roots of the family Orchidaceae can essentially be categorised into two main groups. The fully photosynthetic orchid species appear to rely on heterobasidiomycete fungi for seed germination and early (and sometimes adult)

growth (e.g. Bougoure *et al.* 2005, Perkins *et al.* 1995, Warcup 1981, Zelmer *et al.* 1996). The non-photosynthetic orchids are typically colonised by homobasidiomycetes with genera including *Thelephora-Tomentella*, *Russula*, *Erythromyces* and *Armillaria* (Bougoure and Dearnaley 2005, Cha and Igarishi 1996, Dearnaley and Le Brocque 2006, Girlanda *et al.* 2006, Hamada and Nakamura 1963, Taylor and Bruns 1997, 1999). It is believed that these fungi supply carbon from living tree roots to orchids (Taylor and Bruns 1997).

We have previously studied the fungal endophytes of plants in the *Dipodium* genus of orchids (Bougoure and Dearnaley 2005, Dearnaley and Le Brocque 2006). In two non-photosynthetic species examined using molecular biology techniques, the primary fungal endophytes of plant roots were members of the homobasidiomycete family

Russulaceae. As these fungi are commonly ectomycorrhizal on *Eucalyptus* (Bougher 1995) and *Dipodium* spp. typically occur at the base of mature *Eucalyptus* (Riley and Banks 2002) we conjectured that the orchids had an indirect parasitic mode of nutrition.

To gain insight into the mode of nutrition of *E. cassythoides* we aimed to identify the fungal community of roots of plants from four different locations in SE Queensland. Fungal ITS-PCR analysis, cloning and molecular sequencing were used to identify the primary fungal endophytes of these orchids. These results suggested that *E. cassythoides* is colonised by both saprotrophic and ectomycorrhizal fungi indicating that the orchid can derive its carbon both saprophytically and parasitically.

## Materials and Methods

### Acquisition of orchid material

Roots were collected from seven *E. cassythoides* plants at White Mountain State Forest, Helidon Hills, Geeham State Forest and Stanthorpe in SE Queensland Australia (Table 1) in September 2005. The host tree species (including dead/living status) was noted for each specimen. Underground root portions were gently removed from plants and cold stored until return to the University of Southern Queensland where they were stored at -70°C. Before DNA extraction, transverse sections were cut from the root samples to locate areas of fungal colonisation and these

were photographed using a Micropublisher 5.0 digital camera (QImaging, Canada) on a Nikon E600 upright microscope (Nikon Corporation, Tokyo, Japan).

### Molecular analysis of *Erythrorchis* endophytes

DNA was extracted from whole colonised orchid roots and fungal ITS DNA amplified using ITS1F and ITS4 primers (Gardes and Bruns 1993, White *et al.* 1990). After purification, amplicons were ligated into pGEM-T Easy plasmids (Promega, Annandale, NSW) and transformed into JM109 *E. coli* (Promega). Following blue-white selection, recombinant colonies were bulked with overnight growth in Luria Bertani broth. After plasmid extraction, inserts were sequenced at the Brisbane laboratory of the Australian Genome Research Facility. Sequences were BLAST searched in GenBank to determine closest species matches. For full details see Bougoure and Dearnaley (2005).

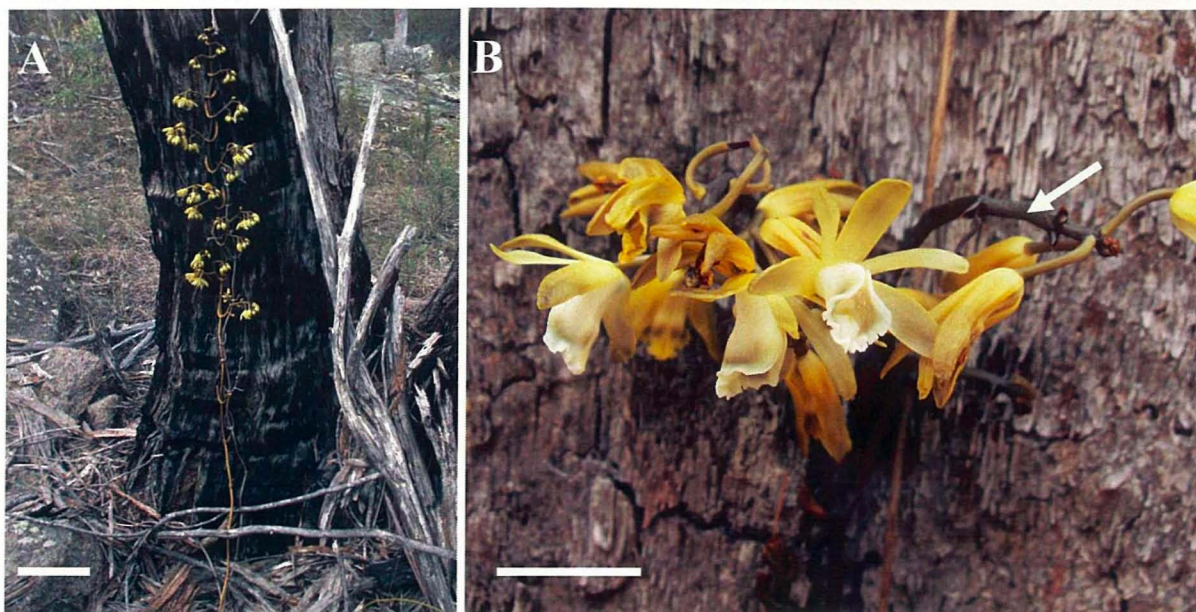
## Results

### Orchid collection details

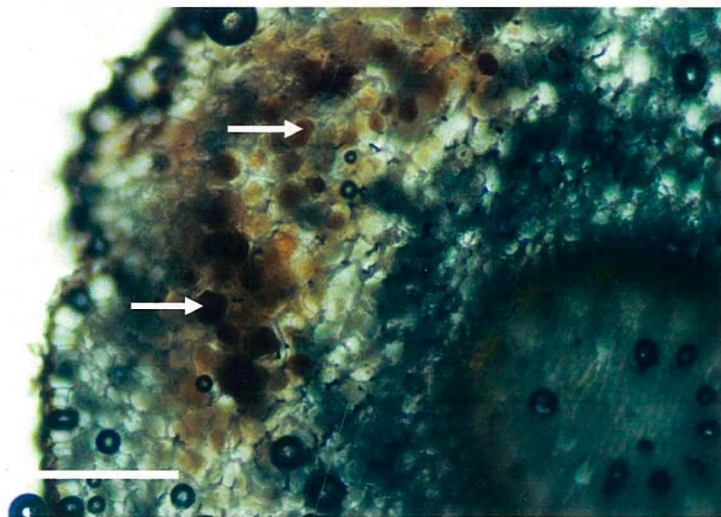
Orchids were typically located growing on the trunks of a variety of living and dead tree hosts (Table 1, Fig. 1A) and all seven orchids were in flower at the time of sampling (Fig. 1B). Of the seven orchid roots obtained, only three had obvious colonisation when viewed in cross section (Fig. 2).

**Table 1.** Collection sites for the seven *E. cassythoides* plants. Included are the plant number, whether or not fungal colonisation was apparent in hand sectioning of roots and identity and living/dead status of host plant.

Location	Plant No.	Colonisation apparent	Tree host (D)=Dead host (L)=Living host
White Mountain State Forest	EC1		<i>Eucalyptus acmenoides</i> (L)
Geeham State Forest	EC2	Colonised	<i>Corymbia intermedia</i> (L)
Stanthorpe	EC3		<i>Eucalyptus banksii</i> (L)
	EC4	Colonised	<i>Allocasuarina torulosa</i> (D)
Helidon Hills	EC5		<i>Eucalyptus microcorys</i> (D)
	EC6		<i>Allocasuarina torulosa</i> (D)
	EC7	Colonised	<i>Angophora woodsiana</i> (L)



**Figure 1.** A. Typical habit of *E. cassythoides*. B. Close up of *E. cassythoides* and non-chlorophyllous stem (arrow). Scale bars = approximately 4 cm and 2 cm respectively.



**Figure 2.** Cross section of root of *E. cassythoides* showing fungal pelotons (arrows). Scale bar = 500 µm.

**Molecular identification of *Erythrorchis* endophytes**

The initial PCR amplification produced a number of bands for plant samples EC2 and EC4; however, plant sample EC7 appeared to have a single amplified band (not shown). Sequencing of five clones from plant EC2 showed that the fungal community consisted of a number of species. These included a fungus with close affinity to an endophyte previously isolated from *Epacris pulchella* Cav. (99% identity over 517 bp), a fungus with similarity to *Russula* spp. (93% identity over 364 bp, 91–92% over 378 bp), a fungus with

similarity to *Metarhizium album* Petch (94% identity over 183 bp) and a fungus with identity to *Coltricia perennis* (L.) Murrill (99% over 175 bp; Table 2). Although three clones were examined for plant sample EC4, these all had affinity to *Gymnopus* spp. (95% identity over 724–762, 94% identity over 768 bp; Table 2). The single clone examined from plant EC7 represented a fungus with similarity to a *Sebacina* sp. (89% identity over 612–643; Table 2).

## Discussion

Previous studies of the fungal endophytes of another *Erythrorchis* sp. contrast with the observations made here. In *Erythrorchis altissima* (Blume) Blume, the main endophyte is the saprotrophic *Erythromyces crociareas* (Berk. & Br.) Hjortst. & Ryv. (Hamada and Nakamura 1963, Umata 1995). This fungal species was not found in the current investigation or *vice versa*, but it is pertinent that these previous studies were based on endophytes capable of being grown from orchid roots not DNA analysis of intact roots which is often more useful in identifying difficult to culture orchid fungal species (Bougoure and Dearnaley 2005, Girlanda *et al.* 2006, Taylor and Bruns 1997).

The fact that plant two (EC2) contained an ascomycete fungus (Table 2) previously isolated from the roots of the heath, *Epacris pulchella* (Bougoure and Cairney 2005) is interesting as the endophytes of orchids are usually basidiomycetes (Leake 2005). An exception to this is the European orchid *Epipactis microphylla* (Ehrh.) Swartz which has recently been shown to be colonised by ectomycorrhizal *Tuber* species (Selosse *et al.* 2004). *Metarhizium* spp. are well known insect parasites (Alexopoulos *et al.* 1996) and not previously known as orchid endophytes (Rasmussen 2002) so this sequence either represents a soil contaminant or an undescribed fungal species without a GenBank record.

It was not surprising to find *Russula* species as partners of this orchid. We have recently found members of the Russulaceae to be the major fungal endophytes of two species of non-photosynthetic *Dipodium* (Bougoure and Dearnaley 2005, Dearnaley and Le Brocq 2006). Taylor and Bruns (1997, 1999) have shown the major fungal endophytes of the North American, non-photosynthetic *Corallorhiza maculata* Rafinesque and *Corallorhiza mertensiana* Bongard to be members of the Russulaceae. Girlanda *et al.* (2006) underlines the global importance of this fungal group to the ecology of non-photosynthetic orchids with the recent observation that European *Limodorum* spp. also form exclusive partnerships with Russulaceae. As outlined by Taylor and Bruns

(1999) it is likely these fungi, which are well known ectomycorrhizal symbionts of mature forest trees worldwide, provide a conduit for carbon from host tree to orchid partner.

*Coltricia* is an ectomycorrhizal fungal species of conifers (Visser 1995) but has not previously been reported in orchids. *Sebacina* spp. are well known endophytes of orchids and hosts include non-photosynthetic genera such as *Neottia* and *Hexalectris* (McKendrick *et al.* 2002, Selosse *et al.* 2002a, Taylor *et al.* 2003) as well as a number of photosynthetic orchid species (Bougoure *et al.* 2005, Shefferson *et al.* 2005, Warcup 1988). Although some members of the Sebacinaceae are considered saprotrophic (Weiss and Oberwinkler 2001) recent evidence suggests that these fungi can be ectomycorrhizal on tree species and that they can play important roles in relaying carbon to orchids from host trees (Selosse *et al.* 2002a, b).

To my knowledge this is the first record of *Gymnopus* spp. as an endophyte of orchids. Although other members of the Tricholomataceae are important ectomycorrhizal species (Bougher 1995), *Gymnopus* is known as a saprotrophic fungus (Fuhrer 2005). Plant four (EC4) occurred at the base of a dead *Allocasuarina* suggesting that the fungus was supplying carbon derived from decaying tree material to the orchid. This conclusion is reinforced by the occurrence of a saprotrophic fungus in a close relative of the orchid (Hamada and Nakamura 1963, Umata 1995) as well as Jones (1988) who documents *E. cassythoides* seed germinating and growing in piles of *Eucalyptus* sawdust. *E. cassythoides* thus joins the small minority of myco-heterotrophic orchids that can obtain their carbon via fungal saprotrophs (Leake 2005).

It is uncertain as to why a large proportion of orchids studied were not obviously colonised. It is likely that all orchids did contain fungal endophytes but the region of colonisation was restricted and easy to miss in hand sectioning. Previous work on *Dipodium* spp. (Bougoure and Dearnaley 2005, Dearnaley and Le Brocq 2006) have shown most subterranean parts of the plants to be heavily colonised by endophytic fungi. In *Dipodium*, roots were short, thick and starch-filled representing an advanced state of myco-heterotrophy (Leake 1994) unlike the case in *E. cassythoides*.



**Table 2.** Closest two matches from BLAST searches of fungal ITS sequences amplified from the three colonised *E. cassythoides* plants. Included are the sample GenBank accession codes, GenBank matches and corresponding GenBank accession codes, sequence identity and overlap of each match.

Plant & clone no	GenBank Accession Code	Closest species match and accession code	Sequence identity (%)	Sequence overlap (bp)
Plant EC2 clone 1	DQ398091	<i>Epacris pulchella</i> endophyte AY627817.1	99	517
		<i>Epacris pulchella</i> endophyte AY627816.1	99	517
Plant EC2 clone 2	DQ398092	<i>Russula mustelina</i> AY061693.1	93	364
		<i>Russula parazurea</i> AY061704.1	92	378
Plant EC2 clone 3	DQ398093	<i>Metarhizium album</i> AY375446.1	94	183
		<i>Metarhizium album</i> AF137067.1	94	183
Plant EC2 clone 4	DQ398094	<i>Russula mustelina</i> AY061693.1	93	364
		<i>Russula parazurea</i> AY061704.1	91	378
Plant EC2 clone 5	DQ398095	<i>Coltricia perennis</i> DQ234560.1	99	175
		<i>Coltricia perennis</i> DQ234559.1	99	175
Plant EC4 clone 1	DQ398096	<i>Gymnopus luxurians</i> AY256709.1	95	762
		<i>Gymnopus gibbosus</i> AY263437.1	94	768
Plant EC4 clone 2	DQ398097	<i>Gymnopus luxurians</i> AY256709.1	95	757
		<i>Gymnopus luxurians</i> AF505765.1	95	724
Plant EC4 clone 3	DQ398098	<i>Gymnopus luxurians</i> AY256709.1	95	761
		<i>Gymnopus gibbosus</i> AY263437.1	94	768
Plant EC7 clone 1	DQ398099	Uncultured Sebacinaceae AY634116.1	89	643
		<i>Sebacina vermifera</i> AF202728.1	89	612

Although based on analysis of fungal DNA from three orchids, these results demonstrate a lack of fungal endophyte specificity for *E. cassythoides*. This differs from molecular studies of other non-photosynthetic orchids which have shown quite specific relationships between plants and fungi (Bougoure and Dearnaley 2005, Dearnaley and Le Brocque 2006, Girlanda *et al.* 2006, Taylor and Bruns 1997, 1999). In this study, it is noteworthy that the two orchids which occurred on living hosts were colonised by ectomycorrhizal fungal whereas the major fungal endophyte of plant four (EC4) (which was found growing at the base of a dead host) was a saprotrophic fungus. This information suggests that the orchid is potentially capable of both parasitic and saprophytic modes of nutrition and is possibly able to alter its nutritional mode depending on the state of the tree host. In North America, the non-photosynthetic *Hexalectris* is colonised by both ectomycorrhizal Sebacinaceae fungi as well as

potentially saprotrophic fungi in the genus *Thanatephorus* (Taylor *et al.* 2003) (although the authors ascribe a pathogenic role to the latter) suggesting that this phenomenon may not be unique to *E. cassythoides*.

Further evaluation of the mode of carbon nutrition of this orchid species would involve *in vitro* colonisation experiments with orchid seed, tree seedlings and pure cultures of the fungi identified here. Additionally a larger sample size for *in situ* studies should be utilised as these conclusions are based on a small number of orchids—only three of which were obviously colonised. Such a low colonisation rate is intriguing for such a fully achlorophyllous species and does not appear to relate to temporal factors (i.e. sample time) or environmental factors (e.g. preceding rainfall) (unpublished results).

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