

MOLECULAR INVESTIGATION OF STERILE ROOT-ASSOCIATED FUNGI FROM *EPACRIS MICROPHYLLA* R. BR. (ERICACEAE) AND OTHER EPACRIDIS AT ALPINE, SUBALPINE AND COASTAL HEATHLAND SITES

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Abstract

Sterile fungi were isolated from the roots of five epacrid species in Kosciuszko National Park, Australia: *Epacris microphylla* (alpine feldmark, alpine bog, subalpine and coastal sites), *E. paludosa*, *E. petrophila*, *Leucopogon montanus* and *Richea continentis* (alpine bog). Fifty-two isolates were used in sequencing studies of the ITS1, 5.8s and ITS2 regions of nuclear rDNA using the primers ITS1 and ITS4. All sequences had closest GenBank matches (78.8–99.6%) with a diverse group of ascomycetes. Forty-two per cent had high sequence similarity (>93%) with either other Australian epacrid isolates or ericoid fungi from northern hemisphere Ericaceae, suggesting a global distribution for this group of fungi. Neighbour-joining analysis of the ITS sequences indicated that, although none of the isolates were closely related to *H. ericae sensu stricto*, several may be related to *H. ericae* aggregate isolates.

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Introduction

Although formerly classified as Epacridaceae, epacrids are now regarded as a lineage within Ericaceae (Crayn & Quinn 2000, Kron *et al.* 1999). In common with many other Ericaceae, epacrids form ericoid mycorrhizas (see Cairney & Ashford 2002), a feature regarded as critical to their success in habitats that are characterised by extremely nutrient-poor sandy or humic acid soils and other edaphic stresses (Cairney & Meharg 2003, Read 1996). Structurally, ericoid mycorrhizas of epacrids are broadly similar to those of other Ericaceae and, from what is currently known, the mycorrhizas appear to be functionally analogous (Cairney & Ashford 2002).

Most ericoid mycorrhizal fungi have been isolated from host hair roots as sterile mycelia, with only a handful of taxa from northern hemisphere Ericaceae having been positively identified so far. These include the widely distributed taxa *Hymenoscyphus ericae* (Read) Korf & Kernan and *Oidiodendron maius* Barron, along with *Acremonium strictum* W. Gams and *Phialophora finlandia* C.J.K. Wang & H.E. Wilcox from *Gaultheria shallon* Pursh on Vancouver Island, Canada (Berch *et al.* 2002). The taxonomic status of other ericoid mycorrhizal endophytes from northern hemisphere Ericaceae has been inferred from rDNA sequence comparisons. These studies suggest that most northern hemisphere ericoid mycorrhizal fungal endophytes either form part of a *H. ericae* complex, have affinities with other Helotiales taxa or are related to the Onygenales taxon *O. maius* (Berch *et al.* 2002, Monreal *et al.* 1999, Sharples *et al.* 2000, Vrålstad *et al.* 2002). In addition, some appear to be related to Xylariales, Chaetothyriales or Sordariales ascomycetes (Berch *et al.* 2002).

Less is known regarding the taxonomic status of ericoid mycorrhizal endophytes from Australian Ericaceae. Isolates with >90% ITS sequence identity to *H. ericae* have been obtained from *Epacris impressa* Labill. and *Woollisia pungens* (Cav.) F. Muell. (McLean *et al.* 1998, Midgley *et al.* 2002). A single endophyte with c. 96% ITS sequence similarity to *O. maius* has also been obtained from *W. pungens* (Chambers *et al.* 2000), as has an

isolate with closest sequence identity (82%) to *Thielavia* sp. (Midgley *et al.* 2002), a Sordariales taxon known to form mycorrhizas with *G. shallon* (Berch *et al.* 2002). On the basis of ITS sequence comparisons, however, most mycorrhizal endophytes from epacrids appear to form part of a poorly-defined Helotiales group (Berch *et al.* 2002, Cairney & Ashford 2002, Chambers *et al.* 2000, McLean *et al.* 1999). Our knowledge of the taxonomic richness of endophytes that form mycorrhizas with Australian Ericaceae is currently constrained by the fact that endophytes from only a limited range of epacrid taxa, mainly from sclerophyll forest habitats, have been investigated. In order to increase our understanding of endophyte diversity, we have isolated root-associated fungi from *Epacris microphylla* R. Br. in three habitats, along with several epacrid species from alpine plant communities and used ITS sequence comparisons to infer their broad taxonomic affinities.

Materials and methods

Isolation of root-associated fungi

Roots of *E. microphylla* were collected from a coastal heathland site in Brisbane Waters National Park, NSW during May 1997, along with a subalpine grassland site and an alpine feldmark-like site in Kosciuszko National Park during January 1998 (Figure 1, Table 1). Roots from two additional *E. microphylla* plants, along with *E. paludosa* R. Br., *E. petrophila* Hook. f., *Leucopogon montanus* (R. Br.) J.H. Willis and *Richea continentis* B.L. Burt. were obtained from an alpine bog site in Kosciuszko National Park during January 2002 (Figure 1, Table 1). Roots were wrapped in wet paper towel and stored at 4°C (<24 h) prior to processing.

Two methods were used for endophyte isolation. In method I, hair roots were surface sterilised in a 37.5% solution of commercial bleach (Zixo, 4.5% available chlorine) with a drop of Tween 20 for one minute, followed by 10 changes (five minutes each) of sterile dist. H₂O. Endophytes were isolated from sterilised root pieces using a method based on the direct plating method of Pearson & Read (1973). Sterilised hair root pieces (2.0–3.0 mm long) were thus placed in 9.0 cm diam. petri dishes containing 2.0% malt agar (Oxoid), adjusted to pH 5.5 and containing 50 mg l⁻¹ streptomycin sulphate (Sigma, St Louis, Mo, USA). Petri dishes were incubated at 25°C in the dark and observed daily for hyphal emergence. Root pieces from which fast-growing fungi or rapidly sporulating fungi emerged were discarded, while slow-growing fungal colonies were subcultured on to 2.0% malt agar without antibiotics. Cultures were maintained by subculturing on 2.0% malt agar.

In method II, the hair roots were surface sterilised in 0.2% sodium hypochlorite for 5 minutes, washed three times in sterile dist. H₂O and cut into 1.0 mm long sections. Each root piece was placed into a separate well of a micro-titre plate containing bovine serum albumin and antibiotics as described by Steinke *et al.* (1996) and incubated in the dark at 25°C. Plates were checked daily for fast-growing hyphae that were killed by the addition of a crystal of copper sulphate. After 10 days the remaining root pieces were transferred to potato dextrose agar (PDA) (Oxoid) and incubated in the dark at 20°C for six to eight weeks, after which isolates were subcultured onto PDA.

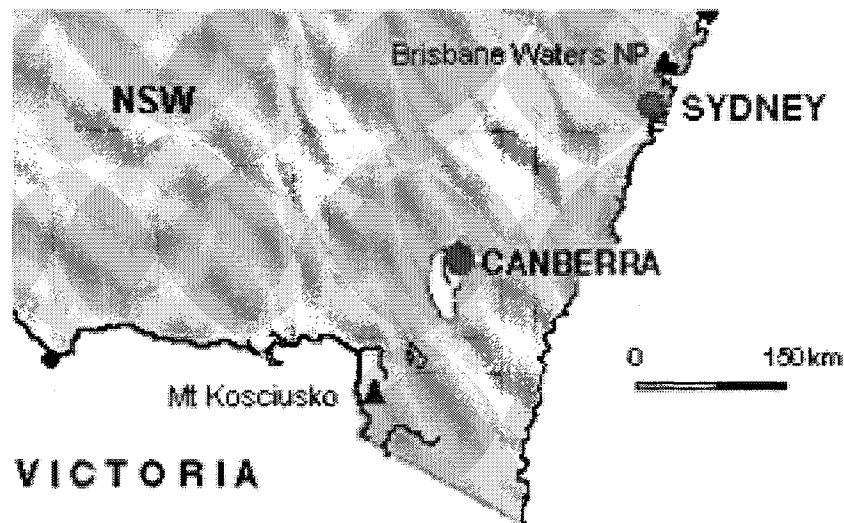


Figure 1. Map of NSW showing collection sites at Brisbane Waters NP and Mount Kosciuszko.

Sterile isolates from alpine bog plants were grouped according to culture morphology after six weeks growth on PDA and representatives from the most common morphological groups from each plant (13 in total) selected for DNA sequencing. Isolates from the *E. microphylla* plants (excluding alpine bog plants) were initially grouped on the basis of culture morphology. Representatives of each culture morphotype were then sorted on the basis of comparative restriction fragment length polymorphism (RFLP) analysis of the ITS region using the methods described by Midgley *et al.* (2002). This process identified 39 ITS-RFLP types (11 from coastal heathland, 18 from subalpine grassland, 10 from alpine feldmark), and single representative isolates of each type were selected for sequencing.

DNA was extracted from isolates obtained from the alpine bog plants using a DNeasy® Plant Mini Kit (Qiagen, Clifton Hill, Victoria, Australia) in accordance with the manufacturer's instructions, and from those from *E. microphylla* (excluding alpine bog plants) using the method of Gardes & Bruns (1993). The ITS region was amplified using the primers ITS1 and ITS4 (White *et al.* 1990). For isolates from *E. microphylla* (excluding alpine bog plants) PCR reactions were conducted using a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA, USA) with reaction mixtures and cycling conditions as described by Chambers *et al.* (2000). For the remaining isolates, PCR amplification was conducted in 25 µl reaction mixtures as described by McLean *et al.* (1999). Reactions were performed on a Biometra personal thermocycler (Pathtech, Victoria, Australia) with 30 cycles of: 94°C for 30 seconds; 55°C for 30 seconds and 72°C for one minute. All PCR reactions were performed in duplicate and a negative control containing no fungal DNA was included in each reaction run. ITS products were electrophoresed in agarose gels, stained with ethidium bromide and visualised under UV light.

For sequencing, ITS products from the isolates from *E. microphylla* (excluding alpine bog plants) were purified with Wizard PCR preps (Promega) prior to cloning with the pGEM-T Easy vector system (Promega). Clones were sequenced using the ABI Big-Dye reaction kit with an ABI 373-A automated fluorescent DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were performed with the primers T7 and SP6 (Promega). ITS products from the other isolates were purified using a QIAquick PCR Purification kit (Qiagen, Clifton Hill, Victoria, Australia). Sequencing reactions were performed on a Biometra thermocycler using ABI PRISM Big Dye Terminator kit (Qiagen) and either ITS1 and ITS4. Sequencing was performed using an ABI automated sequencer model 375A.

Table 1. Species of plant, plant number, location, habitat and date of collection.

Species of Ericaceae	Number of plants	Location	Lat/Long	Isolation/ID method*	Date of collection	Habitat
<i>Epacris microphylla</i>	2	Brisbane Waters National Park, NSW	33° 32', 151° 16'	I	May 1997	Coastal heathland
<i>E. microphylla</i>	3	Kosciuszko National Park, NSW	35° 52', 148° 29'	I	January 1998	Subalpine grassland
<i>E. microphylla</i>	2		36° 23', 148° 18'			Alpine feldmark-like
<i>E. microphylla</i>	2	Kosciuszko National Park, NSW	36° 27', 148° 15'	II	January 2002	Alpine bog
<i>E. paludosa</i>	3					
<i>E. petrophila</i>	3					
<i>Leucopogon montanus</i>	2					
<i>Richea continentis</i>	2					

*See text for details of each method.

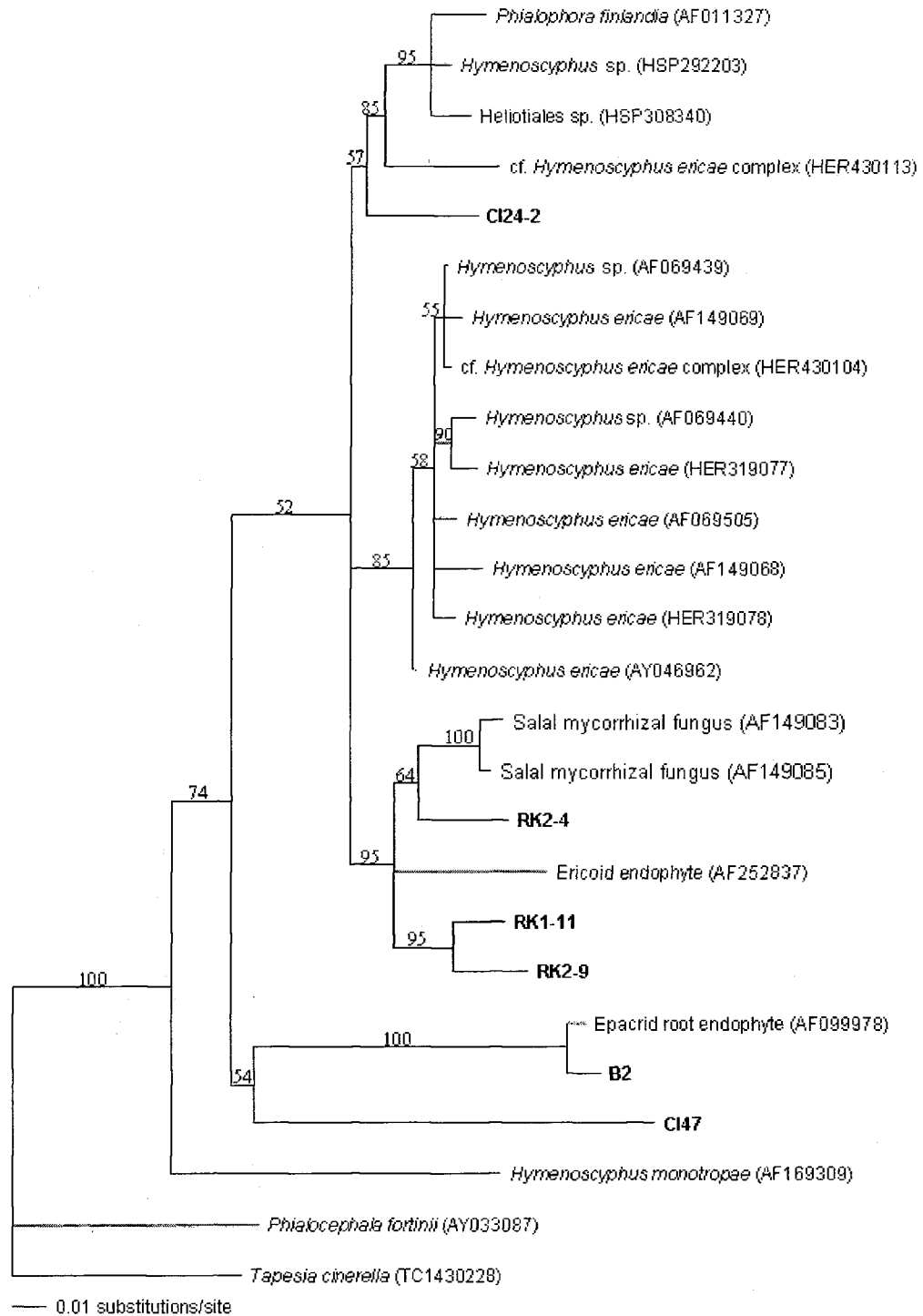


Figure 2. Neighbour-joining tree based on ITS sequence data for six endophytes from epacrid hair roots (**bold**) and *Hymenoscyphus*-like fungi from the GenBank nucleotide database. Numbers above branches indicate bootstrap support (%).

Table 2. Closest matches from FASTA searches between full length ITS sequences for endophytes isolated from hair roots of epacrids from alpine, subalpine and coastal heathland sites and sequences from the GenBank nucleotide database.

Isolate	GenBank Accession Code	Habitat	Host	Closest match (and GenBank accession code)	Description	Nucleotide Overlap	% similarity	E value	
CI24-2	AY268197	alpine feldmark	<i>E. microphylla</i>	<i>Hymenoscyphus ericae</i> 021*	(AF069439)	Helotiales	516	93.6	10 ⁻¹²¹
				<i>Hymenoscyphus ericae</i> 101*	(AF069505)	Helotiales	516	93.4	10 ⁻¹²¹
CI3	AY268195	alpine feldmark	<i>E. microphylla</i>	<i>Aporospora terricola</i>	(AF049088)	Ascomycota inc. sed.	477	89.1	10 ⁻⁹⁴
				<i>Paraphaeosphaeria pilleata</i>	(AF250821)	Pleosporales	415	88.7	10 ⁻⁸¹
CI11-1	AY268196	alpine feldmark	<i>E. microphylla</i>	ericoid endophyte DGC25*	(AF252841)	sterile isolate	614	95.4	10 ⁻¹⁵⁶
				ericoid endophyte GU36*	(AF252840)	sterile isolate	483	89.2	10 ⁻¹⁰⁶
CI47	AY268198	alpine feldmark	<i>E. microphylla</i>	<i>Hymenoscyphus ericae</i> 021*	(AF069439)	Helotiales	505	82.8	10 ⁻⁸⁸
				salal mycorrhizal fungus*	(AF149085)	sterile isolate	463	86.6	10 ⁻⁸⁸
CI50-2	AY268199	alpine feldmark	<i>E. microphylla</i>	<i>Solenopezia solenia</i>	(SSU57991)	Helotiales	489	85.5	10 ⁻⁹⁰
				<i>Piceomphale bulgarioides</i>	(PBZ81441)	Helotiales	483	83.4	10 ⁻⁸⁵
CII12	AY268201	alpine feldmark	<i>E. microphylla</i>	<i>Ophiosphaerella herpotricha</i>	(OH04861)	Pleosporales	462	83.1	10 ⁻⁷³
				<i>Rhizopycnis vagum</i>	(AF022786)	mitosporic ascomycete	460	82.4	10 ⁻⁷³
CII13-2	AY268202	alpine feldmark	<i>E. microphylla</i>	salal root associated fungus*	(AF149078)	sterile isolate	492	98.2	10 ⁻¹³¹
				<i>Colpoma quercinum</i>	(CQU293880)	Rhytismatales	486	89.7	10 ⁻¹⁰⁷
CII4	AY268200	alpine feldmark	<i>E. microphylla</i>	salal root associated fungus*	(AF149078)	sterile isolate	492	97.6	10 ⁻¹²⁹
				<i>Colpoma quercinum</i>	(CQU293880)	Rhytismatales	479	90.4	10 ⁻¹⁰⁶
CII42-2	AY268203	alpine feldmark	<i>E. microphylla</i>	<i>Phialocephala fortinii</i>	(AF360740)	Helotiales	525	97.1	10 ⁻¹³⁸
				<i>Phialocephala fortinii</i>	(AF214580)	Helotiales	495	96.8	10 ⁻¹²⁹
CII70	AY268204	alpine feldmark	<i>E. microphylla</i>	<i>Phialemonium dimorphosporum</i>	(PDI012299)	mitosporic ascomycete	521	93.9	10 ⁻¹¹²
				<i>Phialemonium dimorphosporum</i>	(PDI012300)	mitosporic ascomycete	516	93.9	10 ⁻¹¹¹
EMK1-29	AY279189	alpine bog	<i>E. microphylla</i>	salal mycorrhizal fungus*	(AF149077)	sterile isolate	505	93.1	10 ⁻¹¹⁶
				ericoid mycorrhizal fungus C28*	(AF072298)	sterile isolate	506	91.7	10 ⁻¹¹⁴
EMK2-23	AY279182	alpine bog	<i>E. microphylla</i>	ericoid mycorrhizal fungus C28*	(AF072298)	sterile isolate	517	93.4	10 ⁻¹²⁴
				salal mycorrhizal fungus*	(AF149077)	sterile isolate	516	93.2	10 ⁻¹²²
EPK1-1	AY279180	alpine bog	<i>E. paludosa</i>	<i>Phoma</i> -like coelomycete	(UCO310559)	Pezizomycotina	479	87.5	10 ⁻⁹²
				<i>Dermea viburni</i>	(AF141164)	Helotiales	482	86.1	10 ⁻⁹¹
EPK1-7	AY286401	alpine bog	<i>E. paludosa</i>	salal mycorrhizal fungus*	(AF149085)	sterile isolate	395	93.7	10 ⁻⁸⁸
				salal mycorrhizal fungus*	(AF149084)	sterile isolate	397	93.2	10 ⁻⁸⁷
EPK2-15	AY279190	alpine bog	<i>E. paludosa</i>	<i>Dactylella lobata</i>	(U51958)	Orbiliales	490	98.4	10 ⁻¹²⁷

Isolate	GenBank Accession Code	Habitat	Host	Closest match (and GenBank accession code)	Description	Nucleotide Overlap	% similarity	E value
				<i>Phialophora botulispورا</i> (AF083198)	mitosporic ascomycete	498	86.9	10 ⁻⁹⁹
EPTK1-26	AY279188	alpine bog	<i>E. petrophila</i>	salal mycorrhizal fungus* (AF149077)	sterile isolate	505	94.5	10 ⁻¹¹⁹
				ericoid mycorrhizal fungus C01* (AF072296)	sterile isolate	506	93.7	10 ⁻¹¹⁹
LMK3-31	AY279185	alpine bog	<i>L. montanus</i>	ericoid mycorrhizal sp. Sd9* (AF269067)	sterile isolate	512	96.5	10 ⁻¹²²
				epacrid root endophyte AP-1* (AF099089)	sterile isolate	459	96.5	10 ⁻¹¹⁸
LMK3-32	AY279184	alpine bog	<i>L. montanus</i>	ericoid mycorrhizal sp. Sd9* (AF269067)	sterile isolate	520	95.7	10 ⁻¹³³
				epacrid root endophyte AP-1* (AF099089)	sterile isolate	465	95.9	10 ⁻¹¹⁹
LMK3-7	AY279187	alpine bog	<i>L. montanus</i>	ericoid mycorrhizal fungus C01* (AF072296)	sterile isolate	507	96.5	10 ⁻¹²⁸
				ericoid mycorrhizal fungus A16* (AF072292)	sterile isolate	507	96.3	10 ⁻¹²⁸
RK1-11	AY279179	alpine bog	<i>R. continentis</i>	salal mycorrhizal fungus* (AF149085)	sterile isolate	476	92.6	10 ⁻¹⁰⁶
				salal mycorrhizal fungus* (AF149084)	sterile isolate	476	92.4	10 ⁻¹⁰⁵
RK2-1	AY279186	alpine bog	<i>R. continentis</i>	ericoid endophyte DGC25* (AF252841)	sterile isolate	602	94.7	10 ⁻¹⁴⁴
				<i>Phialocephala fortinii</i> (AF360740)	Helotiales	506	89.7	10 ⁻¹⁰⁷
RK2-4	AY279181	alpine bog	<i>R. continentis</i>	salal mycorrhizal fungus* (AF149085)	sterile isolate	483	94.6	10 ⁻¹¹³
				salal mycorrhizal fungus* (AF149084)	sterile isolate	483	94.4	10 ⁻¹¹²
RK2-9	AY279178	alpine bog	<i>R. continentis</i>	salal mycorrhizal fungus* (AF149085)	sterile isolate	477	92.5	10 ⁻¹⁰⁵
				salal mycorrhizal fungus* (AF149084)	sterile isolate	477	92.0	10 ⁻¹⁰⁴
DI16	AY268205	subalpine	<i>E. microphylla</i>	iceman fungus clone (ASCSP58SR)	sterile isolate	496	93.8	10 ⁻¹⁰⁶
				dark septate endophyte (AF168783)	sterile isolate	498	81.9	10 ⁻⁷⁴
DI18	AY268206	subalpine	<i>E. microphylla</i>	dark septate endophyte (AF168783)	sterile isolate	590	99.5	10 ⁻¹⁵⁹
				<i>Leptodontidium orchidicola</i> (AF214577)	mitosporic ascomycete	553	99.6	10 ⁻¹⁴⁹
DI24	AY268207	subalpine	<i>E. microphylla</i>	<i>Cryptosporiopsis radicola</i> (AF141193)	Helotiales	512	99.6	10 ⁻¹⁴⁰
				<i>Cryptosporiopsis melanigena</i> (AF141196)	Helotiales	509	99.0	10 ⁻¹³⁸
DI30	AY268208	subalpine	<i>E. microphylla</i>	<i>Pezicula</i> sp. (AF141173)	Helotiales	522	95.8	10 ⁻¹³⁴
				<i>Pezicula sporulosa</i> (AF141166)	Helotiales	522	95.8	10 ⁻¹³⁴
D144-2	AY268209	subalpine	<i>E. microphylla</i>	<i>Phialocephala fortinii</i> (AF360740)	Helotiales	525	99.1	10 ⁻¹⁴²
				<i>Phialocephala fortinii</i> (AF214580)	Helotiales	495	99.2	10 ⁻¹³⁴
DI47	AY268210	subalpine	<i>E. microphylla</i>	<i>Phialophora</i> sp. (AF083200)	mitosporic ascomycete	519	89.8	10 ⁻¹¹¹
				<i>Cistella acuum</i> (CAU57492)	Helotiales	520	88.3	10 ⁻¹⁰⁷
DI51-1	AY268211	subalpine	<i>E. microphylla</i>	<i>Phialocephala fortinii</i> (AF011362)	Helotiales	471	92.4	10 ⁻¹⁰⁹

Isolate	GenBank Accession Code	Habitat	Host	Closest match (and GenBank accession code)	Description	Nucleotide Overlap	% similarity	E value
				<i>Botryosphaeria stevensii</i> (BST293881)	Pezizomycotina	503	89.1	10 ⁻¹⁰⁹
DII17	AY268213	subalpine	<i>E. microphylla</i>	ericoid mycorrhizal fungus A16* (AF072292)	sterile isolate	523	98.9	10 ⁻¹⁴¹
				ericoid mycorrhizal fungus C07* (AF072297)	sterile isolate	523	98.9	10 ⁻¹⁴¹
DII23	AY268214	subalpine	<i>E. microphylla</i>	<i>Curvularia trifolii</i> (AF212310)	Pleosporales	568	99.5	10 ⁻¹⁵³
				<i>Curvularia gudauskasii</i> (AF071338)	Pleosporales	543	98.5	10 ⁻¹⁴³
DII29-2	AY268215	subalpine	<i>E. microphylla</i>	<i>Lachnum virgineum</i> (LVU59004)	Helotiales	516	94.0	10 ⁻¹²⁸
				<i>Lachnum controversum</i> (LCU58683)	Helotiales	517	91.7	10 ⁻¹²⁰
DII30-2	AY268216	subalpine	<i>E. microphylla</i>	<i>Ophiosphaerella herpotricha</i> (OH04861)	Pleosporales	462	82.7	10 ⁻⁷¹
				<i>Rhizopycnis vagum</i> (AF022786)	mitosporic ascomycete	460	81.9	10 ⁻⁷¹
DII48-1	AY268217	subalpine	<i>E. microphylla</i>	ericoid mycorrhizal fungus C21* (AF072296)	sterile isolate	524	93.7	10 ⁻¹²³
				ericoid mycorrhizal fungus C07* (AF072297)	sterile isolate	524	93.3	10 ⁻¹²²
DII7-1	AY268212	subalpine	<i>E. microphylla</i>	ericoid mycorrhizal sp. Sd9* (AF269067)	sterile isolate	514	95.7	10 ⁻¹³⁰
				epacrid root endophyte AP-1* (AF09989)	sterile isolate	466	95.5	10 ⁻¹¹⁶
DIII32	AY268218	subalpine	<i>E. microphylla</i>	<i>Verticillium leptobactrum</i> (AF108481)	Hypocreales	511	96.9	10 ⁻¹¹²
				unknown ascomycete (AA2279475)	sterile isolate	441	99.3	10 ⁻¹⁰³
DIII39-1	AY268219	subalpine	<i>E. microphylla</i>	unidentified ascomycete (A5279473)	sterile isolate	503	95.6	10 ⁻¹¹⁹
				unidentified ascomycete (A6279443)	sterile isolate	503	95.6	10 ⁻¹¹⁹
DIII44	AY268220	subalpine	<i>E. microphylla</i>	<i>Phialocephala fortinii</i> (AF360740)	Helotiales	527	99.2	10 ⁻¹³⁹
				<i>Phialocephala fortinii</i> (AF214580)	Helotiales	495	99.8	10 ⁻¹³¹
DIII49	AY268221	subalpine	<i>E. microphylla</i>	<i>Cryptosporiopsis radicola</i> (AF141193)	Helotiales	512	99.0	10 ⁻¹⁴²
				<i>Cryptosporiopsis melanigena</i> (AF141196)	Helotiales	509	98.4	10 ⁻¹³⁹
DIII62	AY268222	subalpine	<i>E. microphylla</i>	<i>Cylindrocarpon</i> sp. (CSP279482)	Hypocreales	506	96.8	10 ⁻¹²⁶
				<i>Cylindrocarpon</i> sp. (CSP279490)	Hypocreales	504	98.4	10 ⁻¹²⁵
A103	AY268189	coastal	<i>E. microphylla</i>	ericoid endophyte GU36* (AF252840)	sterile isolate	459	88.2	10 ⁻⁹³
				ericoid endophyte GU37* (AF252843)	sterile isolate	459	88.0	10 ⁻⁹³
A13	AY268185	coastal	<i>E. microphylla</i>	salal root endophyte (AY219880)	Hypocreales	541	90.9	10 ⁻¹⁰⁸
				<i>Phialophora fastigiata</i> (AF083192)	Helotiales	545	90.3	10 ⁻¹⁰⁵
A27	AY268186	coastal	<i>E. microphylla</i>	ericoid mycorrhizal fungus C28* (AF072298)	sterile isolate	523	96.9	10 ⁻¹³⁵
				Salal root associated fungus* (AF149076)	sterile isolate	514	96.0	10 ⁻¹²²
A37	AY271825	coastal	<i>E. microphylla</i>	<i>Cladosporium</i> sp. (CSP279487)	Pezizomycotina	512	99.6	10 ⁻¹²⁷

Isolate	GenBank Accession Code	Habitat	Host	Closest match (and GenBank accession code)		Description	Nucleotide Overlap	% similarity	E value
				<i>Cladosporium oxysporium</i>	(COX300331)	Pezizomycotina	512	99.6	10 ⁻¹²⁷
A40-1	AY268187	coastal	<i>E. microphylla</i>	<i>Phialophora</i> sp.	(AF083199)	mitosporic ascomycete	528	86.7	10 ⁻¹⁰⁵
				<i>Myxotrichum setosum</i>	(AF062815)	Myxotricaceae	521	87.1	10 ⁻¹⁰¹
A58	AY268188	coastal	<i>E. microphylla</i>	ericoid mycorrhizal fungus B17*	(AF072295)	sterile isolate	527	94.7	10 ⁻¹²⁶
				ericoid mycorrhizal fungus D33*	(AF072303)	sterile isolate	526	93.5	10 ⁻¹²⁴
B16	AY268191	coastal	<i>E. microphylla</i>	<i>Chaunopycnis alba</i>	(AF389195)	Hypocreales	533	93.6	10 ⁻¹¹²
				<i>Tolyocladium inflatum</i>	(AB044645)	Hypocreales	532	94.2	10 ⁻¹¹²
B19-6	AY268192	coastal	<i>E. microphylla</i>	<i>Hypoxylon papillatum</i>	(AF201710)	Xylariales	586	78.8	10 ⁻⁸³
				<i>Xylaria cornudamae</i>	(AF163031)	Xylariales	493	84.2	10 ⁻⁸¹
B2	AY268190	coastal	<i>E. microphylla</i>	Epacrid root endophyte E1-9*	(AF099978)	sterile isolate	445	99.6	10 ⁻¹¹⁹
				<i>Hymenoscyphus ericae</i> *	(AF069439)	Helotiales	402	90.3	10 ⁻⁸³
B22	AY268193	coastal	<i>E. microphylla</i>	<i>Chaunopycnis alba</i>	(AF389192)	Hypocreales	511	96.9	10 ⁻¹²⁴
				<i>Chaunopycnis alba</i>	(AF389191)	Hypocreales	488	98.4	10 ⁻¹²²
B31	AY268194	coastal	<i>E. microphylla</i>	<i>Cenococcum geophilum</i>	(CGPILITS)	Dothidiomycete	493	92.7	10 ⁻¹¹²
				<i>Cenococcum geophilum</i>	(CGR26ITS)	Dothidiomycete	493	92.5	10 ⁻¹¹¹

* indicates ericoid mycorrhizal fungi

Sequences were compared to other sequences in the GenBank and EMBL databases using the default parameters of the FASTA 3.0 program (Pearson & Lipman 1988) to identify closest matches. Where *H. ericae* appeared as one of the five closest sequence matches in the FASTA search, ITS sequences for those isolates were aligned with sequences for *H. ericae*/*P. finlandia* complex and related isolates available in the GenBank nucleotide database, in the BioEdit program [Version 5.0.6 (Hall 1999)] using the ClustalW option and the alignment refined manually. Neighbour-joining analysis was then conducted using the patristic distance matrix (1000 bootstraps re-sampling replicates) in PAUP (v. 4.01b9) (Swofford 2002) with *Tapesia cinerella* Rehm as outgroup. Parsimony analysis (with 1000 replicates) was also conducted using the heuristic search option of PAUP.

Results

ITS sequences were obtained from a total of 52 sterile isolates from the four habitats, with FASTA searches revealing closest identities to database ascomycete sequences that ranged from 78.8% to 99.6% (over 395–614 nucleotides) (Table 2). All sequences have been submitted to the GenBank nucleotide database (see Table 2 for accession codes). Forty-two per cent of the sequenced isolates had highest sequence similarity to ericoid mycorrhizal or putative ericoid mycorrhizal isolates from Ericaceae hosts. This proportion, however, varied with habitat, being highest (70%) for isolates from the alpine sites and lowest (17%) for the subalpine site (Table 2). Two isolates from *E. microphylla* at the alpine site (CI24-2 and CI47) had closest matches with *H. ericae*. Although they had highest sequence identities with unidentified ericoid mycorrhizal fungi, isolates EPK1-7, RK1-11, RK2-4 and RK2-9 from the alpine site, along with B2 from the coastal site also had >90% similarity (over 371–472 bp) to *H. ericae* (data not shown).

Neighbour-joining analysis separated the six isolates from the present study, along with *H. ericae* complex isolates, from *Hymenoscyphus monotropae* Kernan & Finocchio as a group that had moderate (74%) bootstrap support (Figure 2). The topologies of the neighbour-joining and parsimony trees were similar, hence only the neighbour-joining tree is shown. Within this large group, isolates B2 and C147, along with an ericoid mycorrhizal fungal isolate from *Epacris impressa* Labill. at a heathland site (AF099978), were separated from the other taxa as a group. However, bootstrap support for this was poor (54%) (Figure 2). The remaining isolates were separated as a large group that contained three sister groups. However, bootstrap support for the large group was weak (52%). One group (95% bootstrap support) comprised three isolates from *R. continentis* (RK2-4, RK1-11 and RK2-9), along with isolates from *G. shallon* (AF149083 and AF149085) and *Calluna vulgaris* L. (AF252837) (Figure 2). The second contained *H. ericae* and *Hymenoscyphus* spp. isolates (85% bootstrap support), while isolate CI24-2 formed part of the third, weakly-supported group, along with *H. ericae*-like isolates (HSP292203 and HER430113), an unknown Helotiales isolate (HSP308340) and *P. finlandia* (AF011327) (Figure 2).

Ten of the sequenced isolates had highest sequence similarities to *Phialocephala fortinii* Wang & Wilcox, *Phialophora* spp. and other dark septate endophytes from plant roots. *Phialocephala fortinii*-like isolates were only present amongst isolates from the alpine site and, in particular, the subalpine site. Isolate B31 from the coastal heathland site was most similar (92% over 493 nucleotides) with the ectomycorrhizal ascomycete *Cenococcum geophilum* Fr. (Table 2). The remainder of the isolates from the three sites had closest sequence matches with saprotrophic or pathogenic ascomycetes. Sequence identities for some of these matches were relatively high (for example isolate DI23 and A37 had strong similarity with *Curvularia* spp. and *Cladosporium* spp. respectively), suggesting that they may be congeneric. Sequence identities for others were <90% rendering taxonomic inference difficult (Table 2).

Discussion

ITS sequence comparisons indicated that a diverse array of ascomycetes was isolated from hair roots of epacrid taxa at the three field sites. Forty-two per cent of the sequenced isolates had closest sequence matches to ericoid mycorrhizal or putative ericoid mycorrhizal fungi from epacrids at other Australian heathland and sclerophyll forest sites, or to northern hemisphere Ericaceae, suggesting that these isolates, at least, probably represent ERM endophytes. The data thus confirm the conclusions of other investigations, that multiple ERM fungal taxa associate with Ericaceae at a given field site (Chambers *et al.* 2000, Perotto *et al.* 1996, Sharples *et al.* 2000, Xiao & Berch 1996). They further emphasise previous assertions that most ERM endophytes are ascomycetes with affinities to the *H. ericae*/*P. finlandia* complex or other Helotiales taxa (Berch *et al.* 2002, Monreal *et al.* 1999, Sharples *et al.* 2000, Vrålstad *et al.* 2002). The Onygenales taxon *O. maius* is also a common ERM endophyte of several northern hemisphere Ericaceae taxa and may be the dominant endophyte at some field sites (*e.g.* Perotto *et al.* 1996). None of the isolates from the current study, however, had significant sequence identity to *O. maius*.

Six isolates from the alpine sites and one from the coastal heathland had significant ITS sequence similarity to *H. ericae*. In the neighbour-joining analysis, however, none of the isolates from the present study formed part of the well-supported group that comprised *H. ericae* and other *Hymenoscyphus* sp. isolates. Isolates RK1-11, RK2-4 and RK2-9 were grouped strongly with three ericoid mycorrhizal fungi, one of which (AF149085) was regarded as part of the *H. ericae* aggregate by Vrålstad *et al.* (2002). Similarly, isolate C124-2 was grouped, albeit weakly, with *Hymenoscyphus*-like isolates and an isolate of *P. finlandia* that Vrålstad *et al.* (2002) included in the *H. ericae* aggregate. Thus, while none of the isolates from the present study represents *H. ericae sensu stricto*, clarification of their relationships with other fungi in the *H. ericae* aggregate will require further molecular analysis.

The five isolates that were suggested by the neighbour-joining analysis to have closest affinities with *H. ericae* complex isolates were obtained at the alpine sites. The fact that four of the five *H. ericae* complex isolates were obtained from *E. petrophila* and *R. continentis* at the alpine bog site may indicate that this group of fungi has a relatively limited habitat range. Equally, however, it might reflect the use of different isolation procedures at the alpine bog site compared with the other sites. More extensive, systematic sampling from the various habitats will be required to resolve this.

Four isolates from *E. microphylla* at the subalpine and alpine sites had close sequence matches to *P. fortinii*. This taxon is a common root endophyte from northern hemisphere Ericaceae and a range of other plant taxa worldwide and has been reported to occur in a broad range of habitats, including alpine zones (Addy *et al.* 2000, Jumpponen & Trappe 1998). *Phialocephala fortinii*, along with a range of other taxa, forms part of a poorly-defined group of root-inhabiting fungi known as 'dark septate endophytes' (DSE) (Jumpponen 2001). Although the extent to which most DSE form mutualistic or other forms of symbiosis with their plant hosts remains unclear, *P. fortinii* has been shown to confer growth and phosphorus acquisition benefits upon *Pinus contorta* Dougl. which were akin to those bestowed by mycorrhizal infection (Jumpponen 2001, Jumpponen *et al.* 1998). In contrast, Stoyke & Currah (1993) found that *P. fortinii* increased seedling mortality in *Menziesia ferruginea* J.E. Smith (Ericaceae). However, the authors acknowledged that this might reflect the rather artificial nature of the culture system used. Although the functional status of *P. fortinii* thus remains unclear, our data indicate that this fungus is a common endophyte of Australian Ericaceae, at least in alpine and subalpine habitats. Several isolates had closest sequence matches to taxa that are normally regarded as saprotrophs or pathogens. Further investigation would, however, be required to resolve the nature of their interactions with epacrid roots.

This study has improved our knowledge of epacrid root endophytes by expanding the area of collection to include alpine and subalpine habitats. We have shown that epacrid hair roots are colonised by a range of putative ericoid mycorrhizal endophytes that are taxonomically broadly similar to those from northern hemisphere Ericaceae. Furthermore, we have shown for the first time that fungi that have affinities with the *H. ericae*/*P. finlandia* complex and DSE, that are regarded as predominantly northern hemisphere endophytes, form root associations with epacrids in Australia. Such commonality between endophytes of epacrids and other Ericaceae supports the recent re-classification of the Epacridaceae from a separate family to a lineage within the Ericaceae and the probable evolutionary radiation of ericoid mycorrhizas from a common ancestral symbiosis (Cairney 2000, Cullings 1996).

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