Chapter 1: Literature review

1.1 Introduction

Mycorrhizas are mutualistic symbioses between plants and fungi. The formation of mycorrhizal symbiosis is a widespread strategy conferring a number of advantages to the host plants in natural ecosystems (Smith & Read, 1997). Mycorrhizal associations are found in a broad range of habitats including aquatic, desert, tropical rain forest, high altitudes and high latitudes (Allen, 1991). The majority of land plants form mycorrhizas; the range of host plants including most species of angiosperms, gymnosperms, pteridophytes and some bryophytes (Smith & Read, 1997).

Ericoid mycorrhizal roots show considerable uniformity in structure. The finest roots consist of a stele, a cortex of two layers and an epidermis, the epidermis being a layer of longitudinal, swollen cells extending back from the apex (Read, 1996; Smith & Read, 1997). The diameter of the roots that become infected by mycorrhizal fungi is generally less than 100 μm, and consequently the roots are referred to as 'hair roots' (Smith & Read, 1997). Such 'hair roots', that lack root hairs and contain swollen epidermal cells, are characteristic of the Ericales.

Many epidermal cells are usually colonised by ericoid mycorrhizal fungi; these hyphae forming a loose network over the zone of the mature epidermis (Read, 1996; Smith & Read, 1997). Hyphae generally penetrate the outer wall of the epidermal cells at right angles with, typically, one penetration point per cell. Adjacent epidermal cells may have fungal complexes at different stages of development (Duddridge & Read, 1982; Read, 1983; Smith & Read, 1997). Within the epidermal cells, dense hyphal complexes (coils), characteristic of ericoid mycorrhizas, are produced. When the infection is mature, the
epidermal cells are almost filled by the fungal hyphae (Smith & Read, 1997). The active life span of the individual infected cells is believed to be less than five to six weeks with deterioration of organelles in both the plant and fungal cells occurring subsequently (Smith & Read, 1997). The roots then shed these mature epidermes and the hypodermis of the cortex come to form the outer surface of the root (Smith & Read, 1997).

While most research on mycorrhizal associations of the Ericales has concentrated on the Ericaceae, this family is virtually absent from Australia. Only a few genera such as Agapetes, Gaultheria, Pernettya and Rhododendron exist in the subalpine heathlands (Specht, 1991). The closely related family Epacridaceae, however, with 25 genera, is widespread in lowland heath in Australia (Specht, 1991).

Epacrid plants have been used in amenity horticulture and land rehabilitation programs because of their attractive flowers and an ability to colonise adverse sites (Hutton, Dixon & Sivasithamparam, 1994). Most epacrids are difficult to propagate by tip-cutting procedures, however, cuttings inoculated with soil containing mycorrhizal propagules may significantly increase survival rates (McLean, Lawrie & Blazé, 1994). A number of surveys have shown that epacrid plants have a general tendency to form ericoid mycorrhizas in natural systems. For example, in an investigation in Western Australia, all 14 species surveyed, representing more than half of the Epacridaceae species native to Western Australia, were shown to be mycorrhizal (Hutton et al., 1994). McLean & Lawrie (1996) also found ericoid mycorrhizas in the hair roots of all ten Epacridaceae species surveyed in Victoria. It may thus be concluded that like northern heaths, epacrid plants are normally mycorrhizal in natural ecosystems.

Early observations revealed that the structure of hair roots of the Epacridaceae and Ericaceae was essentially similar. Thus the hair roots were shown to be composed of a stele, a cortex and a layer of epidermis, with no root hairs (McNabb, 1961). More recent
observations on the structure of hair roots of the Ericaceae using light and transmission electron microscopy have confirmed previous observations (Allen et al., 1989; Allaway & Ashford, 1996; Steinke, Williams & Ashford, 1996). More detailed structural characteristics have been revealed by virtue of the high resolution of electron microscopy. In the hair roots of Lysinema ciliatum R. Br., Allaway & Ashford (1996) found that the cortex comprises two layers of cells and has Casparian strips on the radial walls, while the epidermis has four to five longitudinal rows of cells, some being thick-walled. The thick-walled epidermal cells appear to be preferentially colonised with mycorrhizal fungi and readily detach in soil (Ashford, Allaway & Reed, 1996). These authors suggested that the infected thick-walled cells may survive dry seasons to infect new roots when conditions are more favourable.

1.2 Functional aspects of ericoid mycorrhizas

Plant species in the Ericaceae mainly occur on humus-type soils with low pH, low available nutrients and high organic matter content (Read, 1991). In these soils, the net rates of mineralisation are very low, thus most nitrogen and phosphorus exists in organic forms. Such organic nutrients are generally unavailable to higher plants, limiting the establishment of the plants without ericoid mycorrhizas (Read, 1983, 1991). For example, in a typical mor humus soil supporting ericaceous plants in England, hydrolysable organic nitrogen accounts for 71% of the total nitrogen, while only 0.4% is present as exchangeable or non-exchangeable nitrogen (Stibley & Read, 1974).

Extensive research has indicated that ericoid mycorrhizal fungi have the ability to utilise complex organic nitrogen sources in soil and to transfer derived nitrogen to the host plants (Leake, 1992). Mycorrhizal plants of Vaccinium macrocarpon Ait. under axenic conditions, were shown to utilise several amino acids (glycine, alanine, aspartic acid,
glutamic acid and glutamine) as sole nitrogen sources (Sibley & Read, 1980). Further work using the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf & Kernan has indicated that *H. ericae* can also use more complex nitrogen substances. Bajwa & Read (1985) demonstrated that the endophyte in axenic culture has the ability to utilise peptides of 2-6 amino acid residues in length as sole nitrogen sources and that nitrogen absorbed from these sources can be transferred to the host. More recent work using bovine serum albumin has shown that *H. ericae*, whether in pure culture or during symbiosis with the host, has the ability to utilise soluble protein as both nitrogen and carbon sources by the production of extracellular proteinase activity (Bajwa, Aburghub & Read, 1985; Leake & Read, 1989). Axenic culture experiments indicate that *H. ericae* can also utilise chitin and fungal necromass as sole nitrogen sources, while plants inoculated with the fungus can utilise nitrogen present in fungal cell walls (Kerley & Read, 1995). Read (1996) has emphasised the significance of chitin as a naturally occurring nitrogenous polymer in soils dominated by the Ericaceae, since a significant proportion of the organic nitrogen in mor humus soils exists in this form, derived largely from senescent fungal biomass.

Recently, Michelsen *et al.* (1996) used nitrogen isotope analysis to reveal the extent to which plant species with different types of mycorrhizal associations use various soil nitrogen sources under field conditions. Their results indicate that ericoid mycorrhizal shrubs appear to use organic nitrogen in fresh litter, strongly reinforcing the conclusions from laboratory-based experiments described above.

Ericoid mycorrhizas may also have the potential to improve the phosphorus status of their ericaceous host plants. Endophytes of *Calluna vulgaris* (L.) Hull, *V. macrocarpon* and *Rhododendron ponticum* L. (Ericaceae) have been shown to have the ability to utilise both soluble and insoluble organic phytate salts, the latter being the major source of the element in acid organic soil (Pearson & Read, 1975; Mitchell & Read, 1981).
Aside from increasing nutrient uptake of the host plants, ericoid associations have also been shown to ameliorate the toxicity of some metals. In the acid soils dominated by plants in the Ericaceae, the solubility and toxicity of toxic metal ions are relatively high (Read, 1983). Aluminium is widely recognised as the major toxic metal in heathlands, resulting in the exclusion of some plant species from the habitat (Read, 1991; 1996). It is known that the ericoid mycorrhizal fungus, *H. ericae* has significant resistance to aluminium and some other common metal pollutants such as copper, zinc and iron (Bradley, Burt & Read, 1981, 1982; Shaw & Read, 1989; Shaw *et al.*, 1990).

To date, a great deal of progress has been made towards understanding functional aspects of ericoid mycorrhizas in the Ericaceae. Previous work has clearly shown that the association provides host plants with access to organic nitrogen and phosphorus otherwise inaccessible to higher plants along with conferring resistance to toxic metals, both of which appear to have contributed to the success of ericaceous plants in stressful conditions of heathland soil (Read, 1991, 1996).

By contrast, there have been relatively few studies of functional aspects of ericoid mycorrhizas in epacrids. In a recent investigation of the response of mycorrhizal seedlings of four epacrid species [*Astroloma xerophyllum* (DC.) Sond., *Andersonia gracilis* DC., *Leucopogon conostephoides* DC. and *Leucopogon kingeanus* (F. Muell.) C. A. Gardner], to added nitrogen, phosphorus and complete nutrients, Bell, Pate & Dixon (1994) found that seedlings of three species showed significant positive responses in shoot height and shoot dry weight to added nitrogen added as NH$_4$NO$_3$, but no positive responses to added phosphorus as phosphate. The results indicate that the association may play a role in the uptake of nitrogen for host plants. Indeed in some subtropical heathlands colonised by epacridaceous species (Schmidt & Steward, 1997), soluble proteins and amino acids can constitute the major nitrogen fractions. $^{\delta^{15}}$N
enrichment studies sufficiently support the likely utilisation of soil organic nitrogen sources by ericoid mycorrhizal associations of Epacridaceae species in these habitats (Schmidt & Steward, 1997).

Recently, an apparent seasonality in mycorrhizal infection has been observed in hair roots of Western Australian eaprids. Hutton et al. (1994) found that from April to November, 20-40% of hair roots of *Astroloma xerophyllum* D. C. (Sand.) were infected, while during the dry summer months (December to March), no ericoid mycorrhizal infection was observed. Equally, Bell & Pate (1996) also reported a clear seasonality in hair root density and mycorrhizal infection within juvenile plants of four eaprid species [A. xerophyllum, A. gracilis, L. conostephioides and Croninia kingiana (F. Muell) J. Powell]. During March to December, 30-50% of hair roots were infected while plants were almost devoid of hair roots during the remainder of the year. Interestingly, 17-85% of the yearly increment in total plant nitrogen, phosphorus and dry matter occurred during the non-mycorrhizal season, implying that uptake of nitrogen and phosphorus and plant growth is independent of the mycorrhizal association (Bell & Pate, 1996), not consistent with the results in the Ericaceae that mycorrhizal endophytes improve nutritional status of host plants. Thus, to understand and elucidate the functional role of mycorrhizal endophytes of the Epacridaceae and the influence of the endophytes on fitness of host plants, more information on the ability of endophytes to absorb and assimilate simple and complex organic nitrogen and phosphorus is required.

1.3 *Fungi forming ericoid mycorrhizas*

The first ericoid mycorrhizal endophyte to fulfil Koch's postulates was isolated from *C. vulgaris* using a root maceration method (Pearson & Read, 1973). These workers plated out one or a group of cortical cells from successively washed hair roots, with hyphae
growing out from root cells being subcultured. Ninety-seven percent of fungal isolates obtained were sterile, dark-coloured and slow growing. Almost all selected isolates formed typical ericoid mycorrhizas when reinoculated onto the original host plant species under sterile conditions. Although the isolates were, in general, sterile, some isolates were stimulated to produce apothecia and were described as the ascomycete *Pezizella ericae* Read (Read, 1974), subsequently, this fungus has been renamed as *Hymenoscyphus ericae* (Read) Korf & Kernan (Kernan & Finocchio, 1983).

*H. ericae* has now been isolated from several different ericaceous plant species from different soil conditions. Dalpé, Litten & Sigler (1989) isolated two endophytes from roots of *Vaccinium angustifolium* Ait growing in North America. One endophyte was identified as *H. ericae*, the other was described as *Scytalidium vaccini* Dalpé, Litten & Sigler, which had the typical cultural characteristics of *H. ericae*. Bagger & Sigler (1993) speculated that, based on phenotypic similarities, *S. vaccini* and *H. ericae* were anamorph and teleomorph states of a single taxon. They used polymerase chain reaction (PCR) techniques to amplify and sequence the small ribosomal unit and the 5' internal transcribed spacer. The sequencing information revealed that sequence divergence between *S. vaccini* and *H. ericae* was very low (1.2-3.5%), confirming the presumed anamorph/teleomorph relationship. The relationship is further confirmed by recent work of Hambleton & Currah (1997), who obtained an isolate of *S. vaccini* which produced arthroconidia that were morphologically consistent with those produced by *H. ericae*.

In addition, some isolates morphologically similar to *H. ericae* have been reported. Kernan & Finocchio (1983) isolated a discomycete associated with the roots of *Monotropa uniflora* L. (Ericaceae). The endophyte was suggested to be congeneric with *H. ericae* and has been described as *Hymenoscyphus monotropae* Kernan & Finocchio based on morphology of the apothecium. Perotto et al. (1990) isolated five ericoid fungal strains from *C. vulgaris* and *Rhododendron ferrugineum* L. growing in a European alpine
zone. These strains had similar ultrastructural and biochemical characteristics with *H. ericae*. However, an antibody raised against *H. ericae* only weakly labelled hyphae of one of these isolates. The relationship between the isolates and *H. ericae* thus remains unclear.

Several *Oidiodendron* spp. have been identified as mycorrhizal with ericoid plant species in different ecological conditions. *Oidiodendron griseum* Robak has been obtained from *Vaccinium corymbosum* L. (Couture, Fortin & Dalpé, 1983), *Loiseleuria procumbens* (L.) Desv. (Stoyke & Currah, 1991) and *Gaultheria shallon* Pursh (Xiao & Berch, 1996). These species formed typical ericoid mycorrhizas under axenic conditions, confirming their mycorrhizal status. Other *Oidiodendron* spp. that have been subsequently shown to form ericoid mycorrhizas include *Oidiodendron rhodogenum* Robak, *Oidiodendron cerealis* (Thüm.) Barron (Dalpé, 1986), *Oidiodendron maius* Barron (Douglas, Heslin & Reid, 1989; Perotto *et al.*, 1996; Hambleton & Currah, 1997), *Oidiodendron periconioides* Morrall (Currah, Tsuneda & Murakami, 1993) and an unidentified *Oidiodendron* sp. (Pearson & Read, 1973). However, the physiological roles of *Oidiodendron* spp. as mycorrhizal endophytes are at present unclear, since positive host plant responses to infections have yet to be demonstrated.

In addition to the above identified endophytes, a few sterile, dark mycelial isolates have also been obtained from hair roots of ericaceous plants and have been shown to form ericoid mycorrhizas under aseptic conditions (Couture *et al.*, 1983; Perotto *et al.*, 1996; Xiao and Berch, 1996, Hambleton & Currah, 1997). Due to the absence of sexual and asexual reproductive bodies, these isolates remain unidentified.

There are other reports that a basidiomycete, *Clavaria* sp. could be an ericoid endophyte (Seviour, Willing & Chilvers, 1973), with immunocytochemical data indicating the presence of hyphae of *Clavaria* sp. in roots of *Rhododendron* sp. (Muller, Tessier &
Arbuscular mycorrhizas have also been found in ericaceous and epacridaceous plants (McGee, 1986; Koske, Gemma & Englander, 1990), however, there is no consistent agreement in this respect (Leake & Read, 1991).

In contrast, very little is known about the fungal endophytes forming mycorrhizas with the Epacridaceae (Steinke et al., 1996). Several workers have obtained endophytes from epacrid plants, most of which have been described as dark-coloured, sterile and slow-growing cultures, similar in morphology to *H. ericae* (Reed, 1989; Hutton et al., 1994; McLean & Lawrie, 1996; Steinke et al., 1996). Such similarity has been taken to suggest that the epacrid mycorrhizal endophytes may be closely related to *H. ericae* (Steinke et al., 1996). Microscopic observations indicate that the endophytes are ascomycetes (Hutton et al., 1994; Steinke et al., 1996), however due to the absence of fruiting bodies, the taxonomic position of those isolates remains unknown.

Hutton et al. (1994) carried out a comparison of pectic isozyme characteristics of mycorrhizal endophytes isolated from Australian Epacridaceae and known endophytes from the Ericaceae. They obtained over 400 isolates from 14 species of native Epacridaceae. Single isolates, representing eight groups that were separated based on morphological characteristics of the cultures, were selected for further investigation. Infection tests showed that six of the isolates formed typical ericoid mycorrhizas in cuttings of *L. ciliatum*. Pectic zymogram analysis revealed that none of the selected isolates had identical zymogram patterns to those of *H. ericae* or *Oidiodendron* spp., suggesting that there may be considerable genetic diversity among the isolates. It is at present not clear whether the fungi forming ericoid mycorrhizas in epacrid plant species are genetically related to *H. ericae* or *Oidiodendron* spp.. Molecular analysis of the endophytes may help to clarify the genetic relatedness between the endophytes of the Ericaceae and Epacridaceae.
1.4 Genetic diversity of ericoid mycorrhizal fungi

In a number of investigations more than one fungus has been obtained from the root systems of single or separate host plants. Pearson & Read (1973) obtained two distinct groups of slow-growing, dark endophytes from *C. vulgaris* plants with or without segmenting hyphae respectively. The isolate with segmenting hyphae was later identified as *H. ericae*. Dalpé et al. (1989) obtained two isolates from the roots of *V. angustifolium*. The two isolates were identified as *H. ericae* and *S. vaccinii*. Muller et al. (1986) found three distinct fungal endophytes in the same root tissue of *Rhododendron* sp., while Xiao & Berch (1996) obtained four fungal species from roots of *Gaultheria. shallon* Parsh plants collected from two field sites. Of the latter, *O. griseum* and two unidentified species formed typical ericoid mycorrhizas with the host under axenic conditions. These results lead the authors to suggest that many fungi form mycorrhizas with a single ericaceous plant species in the field.

With the advance of molecular approaches, it has become possible to assess the genetic diversity of ericoid mycorrhizal endophytes within the root systems of a single plant taxon. Perotto et al. (1996) first carried out a detailed investigation of the diversity of mycorrhizal endophytes associated with *C. vulgaris* using random amplified polymorphism DNA (RAPD) analysis (see section 1.5.4). Over one hundred and fifty isolates were obtained from the roots of ten plants. Inoculation experiments showed that 73 isolates were mycorrhizal endophytes. Of the mycorrhizal endophytes, 39 isolates produced conidia and were identified as *O. maius*. The other 34 isolates were classified into three groups based on colony morphology and microscopic characteristics. Those isolates were further subjected to RAPD analysis to investigate the degree of genetic polymorphisms in the groups. Three to five distinct subgroups were recognised within *O. maius* isolates from a single plant. Fewer polymorphisms were detected for the other three groups of isolates obtained from the same plant. The results confirm that several
ericoid mycorrhizal fungi (perhaps encompassing more than one species) and different individuals of the same fungus (O. maius) may infect a single host plant. These authors suggested that the diversity in the ericoid mycorrhizal community associated with the root system of a single plant was very high and is possibly underestimated due to the recalcitrance of some endophytes to isolation.

That more than one ericoid mycorrhizal fungal taxon coexists in the root system of a single host plant has been confirmed in a number of ericaceous plant species recently. Hambleton & Currah (1997) consistently isolated O. maius and S. vaccinii from 19 Ericaceae species collected in three different North American habitats. O. griseum was also obtained from several plants, providing evidence that two or more endophytes may colonise root systems of a single plant simultaneously.

The identity of the endophytes of plants in the Epacridaceae, the number of fungal taxa involved and the diversity of the ericoid mycorrhizal fungi have been little investigated. Recent work of Hutton et al. (1994), however, indicates that diversity exists in the endophyte population in the Epacridaceae. Hutton et al. (1994) isolated two sterile endophytes from a Western Australian epacrid L. ciliatum. Each endophyte displayed a distinct pectic zymogram pattern, suggesting that the two belong to different taxa. More recent work examining water stress tolerance of mycorrhizal endophytes from Western Australian epacrids indicates that a range of physiologically distinct endophytes may be associated with a single host plant, implying that genetic heterogeneity exists in the endophyte population. The taxonomic relationships between the isolates with differential water stress tolerance, however, remain unclear (Hutton et al., 1996).

Microscopic observations on endophytes have provided some information about the likely taxonomic position and diversity of the mycorrhizal endophytes in epacrids. Steinke et al. (1996) observed two morphologically different mycorrhizal endophytes in roots of
*Leucopagon parviflorus* (Andr.) Lindl. plants. One type was a thick-walled, brown-coloured fungus with hyphae 1.3 μm in diam. The other was a thin-walled hyaline fungus with smaller hyphal diam. (0.9 μm), suggesting them to be two different fungal taxa. Both types appeared to be ascomycetes on the basis of occurrence of simple septa with pores and Woronin bodies. In ultrastructural studies of mycorrhizas of *Dracophyllum secundum* R. Br., Allen *et al.* (1989) found two structural types of fungal endophytes. One isolate had simple septa and appeared to be an ascomycete. The other isolate had dolipore septa and appeared to be a basidiomycete. Unfortunately, observations of morphological features of the mycorrhizal endophytes *in planta* do not allow the characterisation of the endophytes. In recent years, molecular based techniques have been used extensively in fungal taxonomy and potentially provide a more accurate method for assessment of genetic diversity in fungal communities. By applying such techniques to mycorrhizal endophytes of plants in the Epacridaceae, it should thus be possible to obtain more precise taxonomic information, allowing detailed analysis in population of community structure to be undertaken.

1.5 Molecular methods for identification of mycorrhizal fungi

There are two general groups of molecular methods currently used for identification of fungi. One group is based on analysis of molecular variations at the protein level, while the other is based on variations at the nucleic acids level. In the former, proteins are separated by gel electrophoresis, followed by an *in situ* assay of enzyme activity. It provides useful information on protein variants (isozyme and allozyme). Such variants are potentially useful as genetic markers for studies of systematics and phylogenetics (Kohn, 1992; Goldman & O’Brien, 1993). This technique has been successfully applied for example to differentiate synthesised ectomycorrhizas between Scots pine and two closely related ectomycorrhizal basidiomycete species, *Suillus variegatus* (Fr.) O. Kuntze.
and *Suillus bovinus* (Fr.) O. Kuntze (Sen, 1990). Comparisons of banding patterns of esterase and peptidase isozymes revealed that ectomycorrhizas of *S. variegatus* and *S. bovinus* displayed distinct bands, which enabled the identification of ectomycorrhizas with each fungal species (Sen, 1990). More recently, Dodd et al. (1996) used esterase and malate dehydrogenase profiles to discriminate between the morphologically similar arbuscular mycorrhizal fungi *Glotus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe and *Glotus coronatum* Giovannetti. Isolates of the two species exhibited their own specific isozyme banding patterns, enabling characterisation of the species. However, isolates of the same species from different geographical locations (eg. isolate BEG24 from UK and BEG51 from Venezuela) showed identical banding patterns and could not be separated. Equally, Hutton et al. (1994) used pectic zymogram (pectin depolymerase and pectin methyl esterase) analysis to infer the genetic differences in mycorrhizal endophytes of Western Australian epacrids and known mycorrhizal fungi associated with the Ericaceae. The results indicate that isozyme analyses may be useful as an aid to taxonomy at the species level. Recently, with the development of DNA techniques, many molecular markers based on polymorphisms of nucleic acids eg. RFLP and microsatellite alleles (see below) have been developed for the fungi (Bruns, White & Taylor, 1991; Kohn, 1992).

1.5.1 Restriction fragment length polymorphism (RFLP) analysis
A widely used tool for fingerprinting in fungi is hybridisation-based restriction fragment length polymorphism (RFLP) analysis (Rosewich & McDonald, 1994). In this technique, genomic DNA is digested with endonucleases and separated in an agarose gel by electrophoresis. The DNA fragments on the gel are Southern blotted onto a membrane and then hybridised with a labelled specific probe, usually cloned from the library of the species under study or a rDNA gene (Rosewich & McDonald, 1994). The labelled-probes only hybridise to the species that has homology, thus identifying the targeted species. Marmelisse, Debaud & Casselton (1992) differentiated eleven *Hebeloma* species along
with ten strains of the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* Romagnési using this technique. Similarly, Sweeney, Harmey & Mitchell (1996) produced a repetitive DNA probe from an isolate of an ectomycorrhizal basidiomycete [*Laccaria proxima* (Bond.) Pat.]. This probe could separate *Laccaria* from other ectomycorrhizal fungi as it did not hybridise with DNA of isolates of the other species examined. Banding patterns of hybridisation clearly distinguished five *Laccaria* spp., five *Laccaria bicolor* (Maire) Orton isolates and three *L. proxima* isolates, strongly demonstrating the high sensitivity of RFLP analysis in fungal individual typing. Ribosomal DNA, in most cases, was used to generate probes because of the abundance of rRNA genes in eukaryotes and their conserved encoding sequences, permitting DNA hybridisation with DNA from a broad range of taxa. Armstrong, Fowles & Rygiewich (1989) used a labelled basidiomycete ribosomal DNA probe to distinguish *Laccaria laccata* (Scop., Fr.) Berk. & Broom and *L. bicolor* as well as three *L. laccata* isolates and six *L. bicolor* isolates. In this case, probe hybridised RFLP patterns were isolate-specific for the nine *Laccaria* isolates of the two species. Egger, Danielson & Fortin (1991) also adopted this technique along with the analysis of polymorphisms in the mitochondrial DNA to differentiate E-strain mycorrhizal fungi into two taxa. Rogers *et al.* (1989) also discriminated over 50 out of 63 basidiomycetous species, including some mycorrhizal fungi, using RFLP hybridisation with rDNA probes. Mitochondrial DNA polymorphisms have also been used in RFLP analysis for characterisation of mycorrhizal fungi (Egger *et al.*, 1991). Gardes *et al.* (1991a) examined the polymorphisms in the mitochondrial DNA of 38 *Laccaria* isolates belonging to four different species. RFLPs in mitochondrial DNA separated the four species and the majority of isolates tested.

In general, RFLP analysis in conjunction with Southern hybridisation techniques have considerable potential in the identification and classification of mycorrhizal fungi at the generic, specific and isolate level. One of the disadvantages of RFLP analysis is the laborious procedures needed to develop homologous probes for hybridisation, limiting its
application at large scale (Kohn, 1992; Resewich & McDonald, 1994). A further
disadvantage is the requirement of much high DNA concentrations for Southern blotting
(Rosendahl & Taylor, 1997)

1.5.2 DNA fingerprinting analysis

Hybridisation-based DNA fingerprinting uses procedures similar to RFLP except that the
probes are designed to hybridise with hypervariable repetitive sequences referred to as
variable number of tandem repeats (VNTRs) or minisatellite DNA. The VNTRs consist of
short motifs, usually at a length of 10-40 nucleotides. Because of the variability in the
number of tandem repeats at different loci from individual to individual, the banding
patterns in the Southern autoradiograms are highly individualistic, providing information
for individual identification (Jeffreys et al., 1991). Human minisatellite probes and the
core sequence of the phage M13, which detect minisatellites in mammalian systems, have
been used as probes in fungi (Meyer et al., 1993b). However, most fungi had only a
small number of hybridising fragments, suggesting that probes may not be useful for
fingerprinting analysis of many fungi (Resewich & McDonald, 1994).

The other kind of repeated motifs, only one or a few nucleotides long, found ubiquitously
in eukaryotes, are referred to as simple sequence repeat or microsatellites (Gupta et al.,
1994). A number of investigations have confirmed the occurrence of this kind of
repetitive DNA in fungi (Groppe et al., 1995). DeScenzo & Harrington (1994) revealed
that the synthetic oligonucleotide probe (CAT)$_5$ was sensitive to genetic variation in a
variety of fungi examined. These authors found each of 30 single basidiospore progeny
of a dikaryon of Heterobasidion annosum (Fr. : Fr.) Bref exhibited unique fingerprints,
providing evidence for the usefulness of the probes in studies of fungal ecology and
taxonomy. A few simple oligonucleotides i.e. (GTG)$_5$, (GACA)$_4$, (CT)$_8$ and the core
sequence of M13 have been used in the molecular identification of several fungi including
Saccharomyces sp. (Lieckfeldt, Mayer & Börner, 1993), Leptosphaeria sp., Penicillium
sp., Trichoderma sp., Candida sp. and Cryptococcus sp. (Meyer et al., 1993a). In most cases, polymorphisms allowed the identification of individual fungal strains within the species.

There are a few advantages to use DNA fingerprinting with oligonucleotide probes rather than RFLP. They are relatively easy to use because they eliminate the time-consuming process of developing probes specific to the fungus studied. The same oligonucleotide probe can also be applied to a wide variety of species (DeScenzo & Harrington 1994; Rosewich & McDonald, 1994). DNA fingerprinting along with related approaches are thus useful for the identification of fungal strains and as tools for the establishment of genetic relationships at and below species level (Lieckfeldt et al., 1993). Some researchers, however, have criticised RFLP and DNA fingerprinting as laborious and thus not easily applied in the routine identification of fungi (Meyer et al., 1993a). Secondly, significant quantities of DNA are required for these analyses, limiting their application in the characterisation of arbuscular mycorrhizal fungi where only a few spores are available for DNA extraction (Hadrys, Balick & Schierwater, 1992). In these respects, PCR-based molecular approaches demonstrate great advantages.

1.5.3 PCR-RFLP analysis of ribosomal RNA genes (rDNA)

Comparative studies of the nucleotide sequences of rRNA genes provide much information for the identification, characterisation and phylogenetic analysis of fungi over a wide range of taxonomic levels (White et al., 1990; Kohn, 1992). A number of investigators have adopted molecular techniques for mycorrhizal fungal identification and systematics, using either sequence variations of the nuclear or mitochondrial ribosomal RNA genes.

In most eukaryotes, including fungi, the ribosomal RNA gene occurs in the form of tandemly repeated units, each unit consisting of an encoding region and an intergenic
spacer. The encoding region comprises three rRNA genes, the 18S, 5.8S and 28S rRNA. Between the 18S and 28S rRNA genes lies the internal transcribed spacer (ITS) region including the two non-coding spacers (ITS1 and ITS2). The 5.8S rRNA gene lies between the ITS1 and ITS2 regions. The intergenic spacer region (IGS) exists between the 3' end of 28 rRNA gene and 5' end of the 18S rRNA gene (White et al., 1990).

Encoding regions of the rRNA gene are highly conserved. Oligonucleotides flanking the conserved regions have been synthesised as universal primers to amplify the ITS and IGS regions of fungi (White et al., 1990; Erland et al., 1994). PCR amplified products are subjected to endonucleases to detect restriction fragment length polymorphisms. The mutations of nucleotides at the recognising site of the endonucleases as well as insertions and deletions will result in an altered pattern of restriction fragments and produce polymorphisms between different genotypes (Weising et al., 1995). The ITS and IGS regions are highly hypervariable and may vary significantly in closely related taxa, and are thus useful for the identification of fungi at inter- and intra-specific levels (White et al. 1990). Many studies have illustrated the application of genetic variation of the ITS and IGS regions for identification of mycorrhizal fungi (e.g. Bruns et al., 1991; Gardes et al., 1991b; Erland et al., 1994; Henrion et al., 1994a).

Erland et al. (1994) clearly separated Tylospora fibrillosa Donk from isolates of a large number of other ectomycorrhizal fungi using endonucleases to detect polymorphisms in the ITS region. Erland (1995) further applied the approach to screen single mycorrhizas collected in a Swedish forest. It was found that 98% of mycorrhizas randomly collected in a Norway spruce forest had the macroscopical features of T. fibrillosa. Further RFLP analysis of the ITS region showed that the T. fibrillosa RFLP pattern was present in only 21% mycorrhizas amplified. There were four other distinct RFLP patterns, indicating the usefulness of PCR-RFLP as a rapid alternative to the identification of mycorrhizas based on microscopical characters. More recently, Anderson, Chambers & Cairney (1998) used
the technique to separate two isolates from 60 other *Pisolithus* isolates collected in New South Wales, Australia. Combined with the further sequencing of the ITS region and phylogenetic analysis, these authors confirmed the two isolates represented a *Pisolithus* species distinct from *Pisolithus tinctorius* (Pers.) Coker & Couch. Similar successful identifications of mycorrhizal fungi at the species level have been achieved in nine truffle species. (Henrion, Chevalier & Martin, 1994) and three arbuscular mycorrhizal fungal species *Glomus mosseae* (Nicol. & Gerd.) Gerdesmann & Trappe (BEG12), *Glomus caledonium* (Nicol. & Gerd.) Trappe & Gerdesmann (BEG20) and *Acaulospora laevis* Gerdesmann & Trappe (BEG13) (Sanders *et al.*, 1995), demonstrating the technique to be a versatile tool for mycorrhizal fungus characterisation at the interspecific level.

The intergenic spacer (IGS) is also hypervariable and can be used as a molecular marker (Henrion, Tacon & Martin, 1992). Henrion *et al.* (1994a) used IGS polymorphisms to separate an isolate of *L. bicolor*, introduced to nursery-grown Douglas fir, from other indigenous ectomycorrhizal fungi. Full-length nuclear 18S and 28S ribosomal RNA genes have also been used as target sequences for RFLP analysis (Henrion *et al.*, 1992). However, it is generally accepted that the encoding regions of rDNA are highly conserved. Genetic variations of the full rDNA gene are thus thought insufficient to distinguish different isolates within a fungal species.

As a variation of the method above, some taxon-specific primers have been designed based on available sequences, to exclusively amplify a region from a targeted taxon. For example, Simon, Lalonde & Bruns (1992) designed oligonucleotide primers to specifically amplify a portion of 18S rDNA of arbuscular mycorrhizal fungi. Gardes & Bruns (1993) designed a basidiomycete-specific primer. Glass & Donaldson (1995) constructed five primers that only amplify conserved genes from filamentous ascomycetes. More recently, Zézé *et al.* (1996) reported a species-specific primer for the detection of *Sentellospora castanea* Walker (BEG1) in arbuscular mycorrhizas. In
combination with other molecular techniques, these primers facilitate the rapid detection and identification of mycorrhizal fungi. However, due to the paucity of sequence data, it is impossible to design specific primers for every mycorrhizal fungal species.

PCR-RFLP provides an easy and efficient tool for the identification and detection of ectomycorrhizal as well as arbuscular mycorrhizal fungi at inter- and intraspecific levels. The resolution of the approach, however, is not always satisfactory when comparing different fungal isolates within the same species (Henrion et al., 1992) or in some cases, closely related species (Gardea et al., 1991a). More powerful and versatile methods are required to type fungal individuals.

1.5.4 Random amplified polymorphic DNA (RAPD) analysis

The RAPD method was developed by Williams et al. (1990). It is a modification of the PCR procedure. RAPD PCR amplifies DNA with single primers of arbitrary nucleotide sequence, while a pair of primers with specific sequence is used in PCR. RAPD primers are usually 10 bp long, with a GC content of at least 50%. High-GC primers have been shown to yield more amplification products in RAPD analysis of fungi (Kubelik & Szabo, 1995). Another difference between RAPD and PCR is the lower annealing temperature used in RAPD amplification to allow for mismatches between the primers and genomic DNA.

It has been postulated that primers can anneal to many DNA sequences with a variety of mismatches at low annealing temperatures. Some of those primers will be on the opposite strand and in the opposite direction to each other. Sequences between these positions, usually not exceeding a few kilobases, will be amplified. The resulting fragments are separated by agarose gels and visualised by ethidium bromide staining under ultraviolet light. The banding patterns are useful for genetic analysis (Williams et al., 1990) and identification (Hadrys et al., 1992).
Many applications have illustrated the higher resolution of RAPD over ITS-RFLP as a molecular marker in fungal identification. Jacobson, Miller & Turner (1993) used RAPD to distinguish different genotypes in natural populations of the ectomycorrhizal fungus *Suillus granulatus* (L. ex Fr.) Kuntze. Somatic incompatibility was previously used to discriminate different genotypes, somatically compatible isolates being interpreted as genetically identical. The authors, however, found that compatible *S. granulatus* isolates had different RAPD fingerprints, implying they are not genetically identical. This demonstrated the sensitivity of RAPD. Similarly, Wyss & Bonfante (1993) used RAPD to reveal inter- and intra-specific differences in the arbuscular mycorrhizal fungi *Glomus versiforme* (Karst.) Berch (HC/F-E01) and *Gigaspora margarita* Becker & Hall (HC/F-E10). RAPD fingerprints clearly separated isolates of the same species from different geographical regions as well as different individuals of the same isolate from four different locations. Lanfranco et al. (1993) also successfully used the RAPD technique to differentiate six *Tuber* spp. and six isolates of *Tuber magnatum* Pico.

RAPD analysis has become a widely used molecular method to identify fungi below the species level because of its simplicity in technological procedure, high resolution and the low cost of producing fingerprints (Hardys et al., 1992). However, RAPD analysis has some technical limitations. The main concerns are that its sensitivity to reaction conditions may affect the reproducibility of amplification products. Tommerup, Barton & O'Brien (1995) found that RAPD patterns were altered by reaction components, i.e. concentrations of MgCl₂, primers and DNA polymerase types and reaction temperature profile. Although identical RAPD patterns were reproduced by Tommerup et al. (1995) in different thermocyclers for a particular set of reaction conditions and a given taxon, it is suggested that only strictly standardised conditions will guarantee reproducibility (Hardys et al., 1992), making it difficult to compare fingerprints from different laboratories.
1.5.5 Microsatellite-primed PCR analysis

More recently, a combination of RAPD and DNA fingerprinting has been suggested for fingerprinting analysis. The probes that were previously used as hybridisation probes in conventional DNA fingerprinting have been used as single specific primers in PCR to amplify hypervariable DNA sequences in DNA genomes (Meyer et al., 1993a; Morgante & Olivieri, 1993; Gupta et al., 1994). Fingerprints generated using microsatellite primers were shown to be very polymorphic and potentially useful for identification of individuals because of the occurrence of high mutation rates in microsatellite regions (Meyer et al., 1993a; Morgante & Olivieri, 1993; Meyer & Mitchell, 1995; Weising et al., 1995). A few recent surveys of fungal DNA sequences deposited in the GenBank and BMBL database have revealed that microsatellite sequences are widespread in fungi, suggesting the method is applicable in genetic fingerprinting of fungi (Groppe et al., 1995; Hantula, Dusabenyasani & Hamelin, 1996).

Microsatellite-primed PCR combines both the advantages of DNA fingerprinting and speed of RAPD (Meyer et al., 1993b; Hantula & Müller, 1997). Compared to RAPD analysis, microsatellite-primed PCR offers two advantages. The method appears to be more reliable than RAPD analysis because of the longer primers, higher homology between primers and target DNA sequence and higher annealing temperatures (Meyer et al., 1993b). One further advantage is additional information provided by microsatellite-primed PCR. Microsatellite-primed PCR markers are of three types, i.e. a null allele, a dominant allele and co-dominant marker within a single genetic locus. RAPD has only two alleles, i.e. a null allele (absence of an amplified band) and a dominant allele (an amplified band) (Hantula & Müller, 1997). A particular amplified band can be in either homologous (AA) or heterozygous states (Aa). RAPD analysis does not distinguish the two different dominant genotypes. A co-dominant marker, on the other hand, can discern heterozygous from both homozygous states (AA & aa), thus providing additional
information to the on/off polymorphisms in RAPD markers (Weising et al., 1995; Hantula & Müller, 1997).

Several terms, i.e. directed amplification of microsatellite-region DNA (DAMD) (Costa & Martin, 1994), random amplified microsatellites (RAMS) (Hantula et al., 1996), PCR-amplified microsatellites (Morgante & Olivieri) and microsatellite-primed PCR (Buscot et al., 1996; Longato & Bonfante, 1997) have been suggested for this technique. In this project, the term microsatellite-primed PCR will be used.

This technique has been used in a variety of mycorrhizal fungi for identification and characterisation at the inter- and intra-specific levels. Costa & Martin (1994), for example, used the microsatellite primer (GTG)$_5$ to amplify DNA from basidiocarps of the ectomycorrhizal fungi Laccaria amethystina (Bolt. ex Hooker) Murr. and Xerocomus chrysenteron (Bull. ex St. Am.) Quél. Two and nine distinct fingerprints were obtained in X. chrysenteron and L. amethystina fruiting bodies respectively, collected from a single site of 100 m$^2$. This indicates the potential of PCR with microsatellite primers to reveal intraspecific variation. Recently, Haudek et al. (1996) applied the microsatellite primer (GACA)$_4$ and the core sequence of M13 as well as random oligonucleotide primers to differentiate ectomycorrhizal basidiomycetes belonging to three genera: Boletus, Russula and Amanita. Both micro- and minisatellite primers produced highly variable amplified DNA fragments between different strains of the same species. Buscot et al. (1996) detected interspecific differences in Morchella using the microsatellite primer (GTG)$_5$. Similarly, Longato & Bonfante (1997) separated seven species within the order Glomales, and 11 Tuber spp. using the microsatellite primer (GTG)$_5$. Isolates of Tuber maculatum Vittad from different regions of Italy and isolates of Tuber indicum Cooke & Massee of different geographical origin were also differentiated. Equally, Zézé et al. (1997) revealed genetic variation in spores of the arbuscular mycorrhizal fungus Gigaspora margarita (BEG34) from single-spore cultures using the minisatellite primer
M13. All these experiments show that microsatellite-primed PCR is efficient, reliable and sensitive, and can provide general molecular markers for the molecular identification of mycorrhizal fungi.

For arbuscular mycorrhizal fungi, which cannot be readily grown in axenic culture, the use of hybridisation-based fingerprinting approaches are difficult since large amount of DNA are required for analysis (Rosendahl & Taylor, 1997). Two alternative PCR-based methods have thus been developed to detect polymorphisms at inter- and intraspecific levels for these fungi. Simon, Lévesque & Lalonde (1993) developed a rapid approach to characterise arbuscular endophytes in colonised roots. Several pairs of taxon-specific primers were synthesised and used to amplify small subunit rRNA (18S) fragments by PCR. The amplified fragments were then subjected to single-strand conformation polymorphism (SSCP) analysis to detect DNA sequence differences. The sensitivity of the method allowed the identification of endophytes inside field-collected host roots at the species level. More recently, another PCR-based fingerprinting method AFLP (Amplified Fragment Length Polymorphism) has been used to characterise arbuscular mycorrhizal fungi at the strain level. The DNA of individual mycorrhizal spores was digested using endonucleases, ligated with common linkers, then amplified using primers matching with sequences in the linkers. Genetic variation among different individuals was reflected in differences in the length of amplified fragments. The method was successfully used to reveal genetic variation in spores from single isolates of mycorrhizal fungi *Glomus mosseae* BEG84 and *Glomus caledonium* BEG86 (Rosendahl & Taylor, 1997). However, compared to microsatellite-primed PCR, this method is time-consuming, limiting its application on a large scale.
1.6 Aims

Culture morphology in endophytes isolated from the Ericaceae suggests the mycorrhizal endophyte population may be complex (Allen et al., 1989; Hutton et al., 1994; Steinke et al., 1996). However, no thorough investigations of the genetic diversity of such fungi have been attempted. Although a recent investigation using pectic isozyme comparison has shown fungal endophytes from eucalyps were different with the known ericoid mycorrhizal taxa from Ericaceae (Hutton et al., 1994), the relationships of the endophytes of eucalyps with ericoid mycorrhizal endophytes from Ericaceae remain unclear. The aims of the current work were to (i) use microsatellite-primed PCR to assess the level of genetic diversity present in mycorrhizal fungal endophytes isolated from hair roots of Woollsia pungens (Cav.) F. Muell plants collected at a field site in Sydney, Australia; (ii) to investigate the distribution of individual endophytic genets within root systems of separate W. pungens plants; and (iii) by ITS sequence comparison, to infer possible taxonomic relationships among the endophytes isolated from W. pungens, an isolate of H. ericae and other fungi for which ITS sequences are available in the GenBank and EMBL nucleotide databases.
Chapter 2: Materials and Methods

2.1 Isolation of putative fungal endophytes

Five *Woollsia pungens* (Cav.) P. Muell plants were collected from a dry sclerophyll forest site at Lovers Jump Creek, Turramurra, NSW, Australia, in June 1996. Plants C and D were collected in close proximity to each other (root systems co-located), while plants A, B and E were located approximately 10 m from C and D and from each other. Upon collection, each plant was packed in moist paper towels and transferred to the laboratory within 24 h of collection. The root system of each plant was placed in a beaker and rinsed gently under running tap water for 4 h. Any remaining soil and debris were carefully removed using forceps. Hair roots from each plant were excised, wrapped in a piece of muslin and surface-sterilised in a solution of 37.5% commercial bleach (1.5% available chlorine) with a drop of Tween 20 for 1 min and then separately rinsed in 10 changes of sterile distilled water, each for 5 min in a laminar flow cabinet. Sterilised roots were then cut into segments ca 2-3 mm long.

Isolation of fungal endophytes from *W. pungens* roots was attempted using two methods; the incubation medium method and the direct plating method. The incubation medium method was modified from the method of Williams (1990). Only hair roots from plant E were incubated in this method. One sterilised root segment was placed in a drop of incubation medium containing streptomycin sulfate 500 mg l\(^{-1}\), gentamycin sulfate 500 mg l\(^{-1}\), tetracycline-HCl 500 mg l\(^{-1}\) and bovine serum albumin (BSA) 20 g l\(^{-1}\). Four drops of medium were placed on an inverted Petri dish lid and the bottom of the Petri dishes (containing 0.75% water agar) placed inverted upon the lid. Petri dishes were sealed with Parafilm™ and placed in an inverted position at 25°C in the dark and root segments
checked for hyphal growth every other day using an inverted microscope (Olympus, CK 2). Root segments with slow growing fungi were transferred to 9.0 cm diam. Petri dishes containing 2% malt agar (Oxoid).

The direct plating method was based on the method of Pearson & Read (1973). Sterilised root segments were placed in 9.0 cm diam. Petri dishes containing 2% malt agar adjusted to pH 5.5, containing 15.0 mg l⁻¹ streptomycin sulfate, 12.5 mg l⁻¹ gentamycin sulfate, 15.0 mg l⁻¹ tetracycline-HCl and 10.0 mg l⁻¹ benomyl. Hair roots were randomly selected and four root segments were put onto each plate with a total of 100 plates for plant A, 50 plates for plant B, 100 plates for plant C and 70 plates for plant D. Petri dishes were incubated in the dark at 25°C and the appearance of hyphae growing out of the root segments noted daily. Root pieces with fast growing fungi were aseptically removed and discarded. After 10 d, root segments from which a slow growing fungal colony had developed were subcultured onto 9.0 cm diam. Petri dishes containing 2% malt agar without antibiotics. Cultures were maintained by subculturing on to fresh 2% malt agar every 6-8 weeks. After repeated subculture, mycelia obtained were used for DNA extraction.

2.2 Mycorrhiza synthesis

2.2.1 Development of sterile seedlings

To confirm the mycorrhizal status of the putative fungal endophytes, one isolate from each genet (mycorrhizal individual) separated by microsatellite-primed PCR fingerprints was tested for its ability to infect both Vaccinium macrocarpon Ait (Ericaceae) and Dracophyllum secundum R. Br. (Empetraceae) hair roots under axenic conditions.
*D. secundum* seeds were collected at a site at Lovers Jump Creek, Turramurra, NSW, Australia during the period from December 1996 to January 1997 by placing seed pods in nylon stockings during their senescing period. Collected seed was stored in the dark at room temperature for six weeks before use. Seeds were treated with Kirstenbosch™ Seed Primer (National Botanical Institute, South Africa) according to the manufacturer's instructions prior to surface sterilisation. The primer contains some ingredients of smoke that can overcome dormancy and stimulate seed germination of some native Australian plants. Treated *D. secundum* seeds were wrapped in a piece of muslin (in batches of ca 300) and surface sterilised in a solution of 25% commercial bleach (1% available chlorine) with a drop of Tween 20 (Sigma) for 10, 15 or 20 min, followed by rinsing in three changes of sterile distilled water, each for 5 min. Sterilised seeds were placed onto 9.0 cm diam. Petri dishes (25-35 seeds per plate) containing 2% nutrient agar (Oxoid). Plates were incubated in a Thermoline (Gro-cabinet 10259) plant growth chamber with a 20°C, 16h/8h day/light cycle ca 350 μmol m⁻² s⁻¹. After 3 d, seeds observed by eye to be sterile were transferred to new 9.0 cm Petri dishes containing 0.75% water agar and incubated in the plant growth chamber (as described above) for germination. The plates were checked for germinated seeds at intervals of seven days. Seedlings observed to be contaminated were carefully removed in a laminar flow cabinet. Sterile seedlings with a true leaf (approximately 1.0 cm in length) were transferred to a new 9.0 cm diam. Petri dish containing 0.75% water agar and incubated as above until used for synthesis of mycorrhiza.

*V. macrocarpon* seeds were extracted from commercially-obtained frozen fruits and washed in running tap water. The seeds were surface sterilised for 20 min in a solution of 25% commercial bleach (1% available chlorine) and germinated following the procedure for *D. secundum* seeds.
2.2.2 Synthesis of mycorrhizas

Agar was initially used as culture medium for mycorrhizal synthesis in *D. secundum*. Ten g agar (Difco) was added to 1.0 l basal nutrient solution, autoclaved at 121°C for 15 min, and aseptically dispensed in approximately 25 ml volumes into sterile 70 ml plant tissue culture jars (Techno-plas) (Myers & Leake, 1996). A plug of fungus (1-2 cm²) was suspended in 500 μl sterile distilled water in a 1.5 ml sterile Eppendorf tube using a sterile blue pipette tip, vortexed for 20-30 s and a 100 μl suspension of mycelium of the putative endophytes was spread on the surface of the agar. Aseptically reared seedlings were then transferred individually into the jars and incubated in the growth chamber (as described above). Four weeks later, the main roots of *D. secundum* seedlings were ca 12-15 cm long, however, no hair roots were produced. Previous observations have confirmed that ericoid mycorrhizal endophytes only infect epidermal cells of delicate hair roots. In an attempt to stimulate hair root development, mix of river sand, peat and vermicultile (2:1:1) was used as culture medium. Approximately 30 ml of medium were dispensed into 70 ml tissue culture jars, 10 ml basal nutrient solution (same as for the agar medium) was added and jars were autoclaved at 121°C for 30 min. Two to three plugs (ca 1-2 cm²) of fungus were mixed well aseptically with the culture medium using a spatula and seedlings for the agar medium were transferred to the corresponding jars inoculated with same fungal isolates. The jars were incubated in the plant growth chamber (as described above). Due to the time limitation of the project, these seedlings were not examined for infection.

One isolate from each of 50 genets separated by microsatellite-primed PCR (Table 4) were randomly selected for mycorrhiza synthesis. Infection of aseptic *V. macrocarpon* seedlings with putative fungal endophytes was attempted using two methods. In the first method, sterile seedlings were placed individually in 150 ml test tubes containing approximately one third volume of river sand dried overnight at 120°C and 15 ml sterile one-eighth strength modified Hoagland solution containing (l⁻¹) 147.6 mg
Ca(NO₃)₂·4H₂O; 61.6 mg MgSO₄·7H₂O; 63.2 mg KNO₃; 17.0 mg KH₂PO₄; 0.625 mg ferric-EDTA; 0.3575 mg H₂BO₃; 0.2263 mg MnCl₂·4H₂O; 0.0275 mg ZnSO₄·7H₂O; 0.01 mg CuSO₄·5H₂O; 0.003 mg H₂MoO₄·H₂O (Stegner, 1971). Two to three plugs ca 1-2 cm² of fungus were mixed well with sand using a spatula in a laminar flow cabinet. Aseptically reared seedlings were transferred individually into test tubes. After eight weeks incubation in a plant growth chamber under the conditions described above, the roots were removed and examined for infection.

The second method involved infection with the putative mycorrhizal endophytes using the procedure described by Myers & Leake (1996). Sterile V. macrocarpon seedlings were grown on 9.0 cm diam. Petri dishes containing 1% basal nutrient agar. The basal nutrient solution contains (l⁻¹) 55.1 mg CaSO₄·2H₂O; 296 mg (NH₄)₂SO₄; 31.2 mg NaH₂PO₄·2H₂O; 39.4 mg MgSO₄·7H₂O; 23.9 mg KCl; 0.45 mg ferric-EDTA, 0.5 mg B (H₃BO₃); 0.5 mg Mn (MnCl₂·4H₂O); 0.1 mg Zn (ZnSO₄·7H₂O); 0.1 mg Cu (CuSO₄·5H₂O); 0.1 mg Mo (H₂MoO₄·H₂O). When seedlings were ca 2 cm long, a plug of fungus cut from the edge of a growing colony was placed near the root systems of the seedlings, two seedlings per plate. The plates were sealed with Parafilm™ and incubated in a plant growth chamber (as above) for 32 weeks before being examined for any infection.

To assess mycorrhizal infection by the putative endophytes, root pieces were excised from V. macrocarpon seedlings and stained following the procedure of McLean & Lawrie (1996) with some modifications. Root pieces were cleared in 10% KOH for 30 min at 90°C and stained in 0.4% Trypan blue (Sigma) at 90°C for 30 min. Roots were then destained using a lactic acid, glycerol and water mixture as described by Hutton et al. (1994) at 90°C for 1 h. Destained root pieces were finally mounted on new slides in 50% glycerol, observed and photographed using a Zeiss AxioLab light microscope. Although
D. secundum seedling eventually produced hair roots in the mix of river sand, peat and vermiculite, time did not allow the assessment of infection.

2.3 DNA extraction

DNA was extracted using a method modified from Gardes & Bruns (1993). Mycelium was ground into a fine powder using liquid nitrogen, transferred into a sterile 1.5 ml Eppendorf tube, and 750 μl CTAB extraction buffer (100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 2% hexadecyltrimethylammonium bromide, 1% polyvinylpyrrolidone and 0.2% β-mercaptoethanol) added. Samples were incubated in a hot block at 65°C for 1 h, then proteins were extracted with phenol /chloroform/isoamyl alcohol (PCI, 25:24:1, Sigma). The tubes were centrifuged at 14000 rpm for 5 min in a Hettich BBA 12R microcentrifuge and the supernatant extracted again with PCI by centrifugation at 14000 rpm for 3 min. The aqueous phase was pipetted into new Eppendorf tubes, 750 μl chloroform added, and the tubes centrifuged at 14000 rpm for 3 min. One tenth volume of 3 M sodium acetate (pH 5.2) and one volume isopropanol were then added to the aqueous phase and tubes centrifuged at 14000 rpm at 4°C for 1 min to precipitate the DNA. The DNA pellet was washed twice with 70% (v/v) ethanol and centrifuged at 14000 rpm for 5 min. The pellet was then dried in a vacuum desiccator for approximately 20 min and resuspended in 50 μl TE buffer (10 mM Tris pH 8.0; 1 mM EDTA, pH 8.0). DNA concentrations were measured using a spectrophotometer (Beckman DU 640) at 260 nm.
2.4 Microsatellite-primed PCR

Concentrations of primers, MgCl₂, DNA template and thermocycling conditions were optimised using primer (GACA)₄ and the genomic DNA of isolate D22 for use in microsatellite-primed PCR. Three primer [(GACA)₄, (GTG)₅ and (AT)₁₂] were screened and two primers (GACA)₄ and (GTG)₅ (Pharmacia Biotech) were used for subsequent PCR analysis. Amplifications were in final volumes of 50 μl containing 50-200 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM MgCl₂, 1.5 μM primer, 0.2 mM nucleotides (containing equal volumes of dATP, dCTP, dGTP and dTTP), and 2.5 units of Taq DNA polymerase (Promega).

Amplifications were performed for 30 cycles in an MJ Research PTC-100 thermal cycler using a method modified from Groppe et al. (1995). Each cycle consisted of the following steps: 1 min at 94°C for denaturing, 1 min at 48°C [for primer (GACA)₄] or at 52°C [for primer (GTG)₅] for annealing, 2 min at 72°C for extension, followed by a final extension at 72°C for 10 min. PCR amplification of each isolate was performed twice to confirm the reproducibility of the banding patterns. A negative control (no DNA template) was included in every set of PCR amplifications.

Amplification products were analysed by electrophoresis through a 1.5% (w/v) agarose gel in TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA, pH 8.0) at 150 V for approximately 2 h. The gels were stained with 1 μg ml⁻¹ ethidium bromide, rinsed in distilled water for 1 min, observed and photographed under ultraviolet light using a Gel Doc 1000 imaging system (Bio-Rad). Photographs were converted to negative images using the program Molecular Analyst® 1.4 on the imaging system.
Isolates having identical fingerprints using both primers were grouped as mycelial genets (Anderson, 1996). The molecular size of each PCR amplification fragment of a single representative isolate from each genet was estimated using a standard curve of migration distance versus log molecular size of pGEM DNA marker. Data from these isolates were then analysed on the basis of presence/absence of PCR amplification fragments (denoted by 1 or 0 respectively) and a dissimilarity (1-D) matrix constructed using the RAPDistance program 1.04 (Armstrong et al., 1994). Similarity coefficients were thus calculated using the formula of \( D = 2N_{xy}/(N_x+N_y) \), where \( N_{xy} \) is the number of common bands in both isolates, \( N_x \) and \( N_y \) are the number of bands present for each isolate (Nei & Li, 1979). The program Kitsch from the PHYLIP package 3.5c (Felsenstein, 1993) was used to cluster isolates based on (1-D) from 1000 replicates. Data were viewed as an unrooted consensus tree generated using the TREEVIEW program (Page, 1996).

2.5 PCR amplification of the ITS region

To determine phylogenetic relatedness of the mycorrhizal endophytes isolated from \textit{W. pungens} and to the ericoid mycorrhizal fungus \textit{H. ericae}, fourteen mycorrhizal endophytes were selected for sequence analysis based on microsatellite-primed PCR fingerprints. Isolates A01, C01 and D02 were selected from the three largest genets (genets 1, 32 & 33) inferred from fingerprinting data (Table 3). Isolate A16 and isolate D02 had identical fingerprints with (GTG)$_5$ and slightly different fingerprints with (GACA)$_4$. Both of these isolates were selected for sequence analysis. Isolate D01, classified as the same genet with isolate C01 (genet 32, Table 3) on the basis of identical fingerprints with the both primers (GACA)$_4$ and (GTG)$_5$, was also selected to see any genetic variations between isolates of same genet. The remaining isolates were selected to represent different branches in the phylogenetic tree generated from the fingerprinting
data. A non-confirmed putative endophyte A53 and a European isolate of the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Dorf & Kernan (Read 101) were included for comparison in this analysis.

The ITS regions of the selected isolates were amplified using the primers ITS1 and ITS4 (White *et al.*, 1990). Amplifications were conducted in 50 µl reaction volumes containing 50-200 ng DNA template, 50 mM KCl, 10 mM Tris-HCl (pH 9.0) and 0.1% Triton X-100, 2.5 mM MgCl₂, 25 pmol each primer and 1.25 units of *Taq* DNA polymerase (Promega).

Amplifications were carried out in an MJ Research PTC-100 thermal cycler for 30 cycles using 94°C for 30 s (denaturing), 60°C for 30 s (annealing) and 72°C for 30 s (extension), followed by a final extension at 72°C for 5 min. Negative controls without DNA template were included in every set of amplifications.

### 2.6 Sequencing of ITS PCR products

ITS products were cloned using the pGEM-T easy vector system (Promega) following the manufacturer's instructions. Two or three positive clones were randomly selected and screened by PCR to confirm the presence of inserts. Amplifications were carried out in a final volume of 15 µl, using bacterial colonies as templates. All the reaction conditions were the same as for ITS PCR described above.

Plasmid DNA was isolated using the Wizard™ plus mini prep DNA Purification System (Promega), following the manufacturer's instructions. Two or three plasmid preparations for each isolate were sequenced using an ABI automatic sequencer (373-A, Applied
Biosystems, Inc.) using the Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.). Sequencing reactions were performed using the primers T7 and SP6 (Promega). The ITS sequences from the 14 isolates and the sequence of *H. ericae* were analysed with the FASTA 3.0 program (Pearson & Lipman, 1988) and sequences aligned using PILEUP and PRETTY programs [in the EGCG extensions to the Wisconsin Package, Version 8.1.0 (Rice, 1996)]. The program HOMOLOGIES was used to compare pair-wise differences between sequences [in the EGCG extensions to the Wisconsin Package, Version 8.1.0 (Rice, 1996)]. Sequence data were bootstrapped 1000 times using SEQBOOT and a distance matrix constructed using DNADIST. Following KITCH analysis, with the Kimura 2-parameter distance method, a strict consensus tree was produced using CONSENSE [all programs in PHYLIP version 3.5c (Felsenstein, 1993)] and viewed using TREEVIEW (Page, 1996).
Chapter 3: Results

3.1 Isolation of putative endophytes

The two isolation methods yielded a total of 251 fungal isolates from the root systems of five *Woollsia pungens* (Cav.) F. Muell plants (Table 1). The incubation method produced only eight isolates from 100 root segments. More isolates were obtained from the direct plating method with 90 seconds surface sterilisation, with 243 isolates being obtained from 1280 root pieces taken from four plants. The fungal isolates ranged in colour from a light grey through dark grey to black when grown on 2% malt agar, being either waxy or velutinous in texture. All isolates produced sterile, slow-growing cultures. Based on the colour of the cultures, 175 dark, slow-growing isolates were selected for microsatellite-primed PCR analysis and mycorrhizal synthesis to assess genetic diversity of fungal endophytes isolated from four *W. pungens* plants.

**Table 1.** The number of slowing growing putative fungal endophytes obtained from portions of the root systems of five *W. pungens* plants by the incubation and direct plating methods.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Isolation method</th>
<th>Number of root pieces inoculated</th>
<th>Number of fungal isolates obtained</th>
<th>% root pieces yielding fungal isolates</th>
<th>Number of isolates selected for PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>direct plating</td>
<td>400</td>
<td>82</td>
<td>20.5%</td>
<td>60</td>
</tr>
<tr>
<td>B</td>
<td>direct plating</td>
<td>200</td>
<td>35</td>
<td>17.5%</td>
<td>28</td>
</tr>
<tr>
<td>C</td>
<td>direct plating</td>
<td>400</td>
<td>76</td>
<td>19.0%</td>
<td>47</td>
</tr>
<tr>
<td>D</td>
<td>direct plating</td>
<td>280</td>
<td>50</td>
<td>17.9%</td>
<td>40</td>
</tr>
<tr>
<td>E</td>
<td>incubation</td>
<td>100</td>
<td>8</td>
<td>8%</td>
<td>0</td>
</tr>
</tbody>
</table>
3.2 Synthesis of mycorrhizas

Both sterile \textit{Vaccinium macrocarpon} Ait and \textit{Dracophyllum secundum} R. Br. seedlings were used as host plants to test the ability of putative mycorrhizal endophytes to form mycorrhizas. Seeds of \textit{D. secundum} took approximately four weeks to begin germinating and the germination period lasted four to five weeks. Germination percentages of seeds with 20 min surface sterilisation was much higher than those of 5 and 10 min, with 73.7\% sterilised seeds producing sterile seedlings (Table 2). \textit{V. macrocarpon} seeds began to germinate in two weeks. Over 95\% of the \textit{V. macrocarpon} seeds yielded sterile seedlings after surface sterilisation using 1\% available chlorine for 20 min.

\textbf{Table 2. Effect of surface sterilisation time (5, 10 and 20 min), using 25\% commerial bleach (1\% available chlorine), on germination of \textit{D. secundum} seeds.}

<table>
<thead>
<tr>
<th>sterilisation time</th>
<th>5 min</th>
<th>10 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mean $\pm$ S. D.)</td>
<td>(mean $\pm$ S. D.)</td>
<td>(mean $\pm$ S. D.)</td>
<td></td>
</tr>
<tr>
<td>% germination</td>
<td>14.6 $\pm$ 9.4</td>
<td>22.7 $\pm$ 5.7</td>
<td>73.7 $\pm$ 14.7</td>
</tr>
<tr>
<td>number of seeds</td>
<td>251</td>
<td>266</td>
<td>278</td>
</tr>
</tbody>
</table>

Microsatellite-primed PCR separated the 175 selected isolates into 50 genetically distinct genets using primers (GACA)$_4$ and (GTG)$_5$ (Table 3; see section 3.4). Isolates having different fingerprints with (GTG)$_5$ or different fingerprints with (GACA)$_4$ were regarded as belonging to genetically distinct mycelial individuals (genets). Where isolates displayed identical fingerprints with (GTG)$_5$ and identical fingerprints with (GACA)$_4$, they were considered to belong to the same genet and grouped accordingly. A total of 50 genets were identified within the endophyte population isolated from the four \textit{W. pungens} plants.
(Table 3). A single isolate from each of the 50 genets was randomly selected for mycorrhiza synthesis.

Initially, mycorrhiza synthesis between *V. macrocarpon* and putative endophytes was attempted in sterile sand moistened with one-eighth strength modified Hoagland solution (Stegner, 1971) in 150 ml test tubes. However, no infections were observed in hair roots of *V. macrocarpon* using this method. Subsequently, basal nutrient agar (Myers & Leake, 1996) was used as a synthetic medium for the infection test. Of the 50 isolates tested, each representing a fungal genet, 43 isolates formed typical ericoid mycorrhizal structures in hair roots of *V. macrocarpon* seedlings grown on agar (Table 4), confirming their status as mycorrhizal endophytes. Some epidermal cells of hair roots were heavily colonised by hyphae (Fig. 1.). Control seedlings without inoculation remained uninfected.

Due to the time constraints associated with this work, although *D. secundum* seedlings eventually produced hair roots in mix of river sand, peat and vermiculite, it was not possible to examine these for infection before the end of the project. The ability of the isolates selected to infect *V. macrocarpon*, however, clearly demonstrated their status as ericoid mycorrhizal endophytes of *W. pungens*.
Table 3. Grouping of putative mycorrhizal endophytes isolated from four *W. pungens* plants based on the microsatellite fingerprints generated using primer (GACA)$_4$.

<table>
<thead>
<tr>
<th>Genets</th>
<th>Isolates*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>A03, A12, A18</td>
</tr>
<tr>
<td>3</td>
<td>A08, A16</td>
</tr>
<tr>
<td>4</td>
<td>A37, A47</td>
</tr>
<tr>
<td>5</td>
<td>A38</td>
</tr>
<tr>
<td>6</td>
<td>A39, A40, A41</td>
</tr>
<tr>
<td>7</td>
<td>A42</td>
</tr>
<tr>
<td>8</td>
<td>A43</td>
</tr>
<tr>
<td>9</td>
<td>A44, A46, A50, A54, A57, A58, A59</td>
</tr>
<tr>
<td>10</td>
<td>A45, A52</td>
</tr>
<tr>
<td>11</td>
<td>A48</td>
</tr>
<tr>
<td>12</td>
<td>A49, A55</td>
</tr>
<tr>
<td>13</td>
<td>A51</td>
</tr>
<tr>
<td>14</td>
<td>A53</td>
</tr>
<tr>
<td>15</td>
<td>A56</td>
</tr>
<tr>
<td>16</td>
<td>A60</td>
</tr>
<tr>
<td>17</td>
<td>B01, B02, B03, B04</td>
</tr>
<tr>
<td>18</td>
<td>B05, B06, B16, B21, B25, B28</td>
</tr>
<tr>
<td>19</td>
<td>B07, B19</td>
</tr>
<tr>
<td>20</td>
<td>B08</td>
</tr>
<tr>
<td>21</td>
<td>B09, B11</td>
</tr>
<tr>
<td>22</td>
<td>B10</td>
</tr>
<tr>
<td>23</td>
<td>B12, B13</td>
</tr>
<tr>
<td>24</td>
<td>B14</td>
</tr>
<tr>
<td>25</td>
<td>B15</td>
</tr>
<tr>
<td>26</td>
<td>B17, B23, B26</td>
</tr>
<tr>
<td>27</td>
<td>B18</td>
</tr>
<tr>
<td>28</td>
<td>B20</td>
</tr>
<tr>
<td>29</td>
<td>B22</td>
</tr>
<tr>
<td>30</td>
<td>B24</td>
</tr>
<tr>
<td>31</td>
<td>B27</td>
</tr>
<tr>
<td>32</td>
<td>C01, C02, C03, C04, C05, C06, C08, C09, C10, C11, C13, C14, C15, C16, C18, C19, C23, C24, C27, C31, C32, D01, D03, D04, D05, D06, D07, D08, D09, D10, D11, D12, D13, D14, D16, D17, D19, D25, D26, D32, D39</td>
</tr>
<tr>
<td>33</td>
<td>C20, C21, C22, D02, D15, D18, D20, D21, D22, D23, D24, D27, D28, D29, D30, D36, D37, D38</td>
</tr>
<tr>
<td>34</td>
<td>C07, C12, C17</td>
</tr>
<tr>
<td>35</td>
<td>C25</td>
</tr>
<tr>
<td>36</td>
<td>C26</td>
</tr>
<tr>
<td>37</td>
<td>C28</td>
</tr>
<tr>
<td>38</td>
<td>C29, C30</td>
</tr>
<tr>
<td>39</td>
<td>C33</td>
</tr>
<tr>
<td>40</td>
<td>C34</td>
</tr>
<tr>
<td>41</td>
<td>C35, C39</td>
</tr>
<tr>
<td>42</td>
<td>C36</td>
</tr>
<tr>
<td>43</td>
<td>C37</td>
</tr>
<tr>
<td>44</td>
<td>C38</td>
</tr>
<tr>
<td>45</td>
<td>C40, C41, C42, C43, C44, C45, C46, C47</td>
</tr>
<tr>
<td>46</td>
<td>D31</td>
</tr>
<tr>
<td>47</td>
<td>D33</td>
</tr>
<tr>
<td>48</td>
<td>D34</td>
</tr>
<tr>
<td>49</td>
<td>D35</td>
</tr>
<tr>
<td>50</td>
<td>D40</td>
</tr>
</tbody>
</table>

*Letters indicate from which of the four *W. pungens* plants (A-D) each isolate was obtained.
Fig. 1. Hair roots of *V. macrocarpon* seedlings showing ericoid-like mycorrhizal infection in epidermal cells formed by endophytes isolated from *W. pungens*. Fig. 1a-c isolate A56, Fig. 1d isolate D34. Some epidermal cells were extensively colonised by hyphal coil (arrows), typical of those produced by known ericoid mycorrhizal fungi. Bar = 20 μM.
Table 4. The mycorrhizal status of selected isolates of the 50 fungal genets obtained from four *W. pungens* determined by infection test on *V. macrocarpon* seedlings under axenic conditions.

<table>
<thead>
<tr>
<th>Genet</th>
<th>Isolates tested</th>
<th>Infection test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A01</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>A03</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>A16</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>A37</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>A38</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>A39</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>A42</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>A43</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>A44</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>A45</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>A48</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>A49</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>A51</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>A53</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>A56</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>A60</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>B01</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>B06</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>B07</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>B08</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>B09</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>B10</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>B12</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>B14</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>B15</td>
<td>+</td>
</tr>
<tr>
<td>26</td>
<td>B17</td>
<td>+</td>
</tr>
<tr>
<td>27</td>
<td>B18</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>B20</td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td>B22</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td>B24</td>
<td>+</td>
</tr>
<tr>
<td>31</td>
<td>B27</td>
<td>+</td>
</tr>
<tr>
<td>32</td>
<td>C01</td>
<td>+</td>
</tr>
<tr>
<td>33</td>
<td>D02</td>
<td>+</td>
</tr>
<tr>
<td>34</td>
<td>C07</td>
<td>+</td>
</tr>
<tr>
<td>35</td>
<td>C25</td>
<td>-</td>
</tr>
<tr>
<td>36</td>
<td>C26</td>
<td>+</td>
</tr>
<tr>
<td>37</td>
<td>C28</td>
<td>+</td>
</tr>
<tr>
<td>38</td>
<td>C29</td>
<td>+</td>
</tr>
<tr>
<td>39</td>
<td>C33</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>C34</td>
<td>+</td>
</tr>
<tr>
<td>41</td>
<td>C35</td>
<td>+</td>
</tr>
<tr>
<td>42</td>
<td>C56</td>
<td>+</td>
</tr>
<tr>
<td>43</td>
<td>C37</td>
<td>-</td>
</tr>
<tr>
<td>44</td>
<td>C38</td>
<td>+</td>
</tr>
<tr>
<td>45</td>
<td>C40</td>
<td>+</td>
</tr>
<tr>
<td>46</td>
<td>D31</td>
<td>+</td>
</tr>
<tr>
<td>47</td>
<td>D33</td>
<td>+</td>
</tr>
<tr>
<td>48</td>
<td>D34</td>
<td>+</td>
</tr>
<tr>
<td>49</td>
<td>D35</td>
<td>+</td>
</tr>
<tr>
<td>50</td>
<td>D40</td>
<td>+</td>
</tr>
</tbody>
</table>

+ indicates fungal coils were observed in epidermal cells, - indicates no infection observed.
3.3 Optimisation of microsatellite-primed PCR

3.3.1 Optimisation of reaction conditions for microsatellite-primed PCR
To obtain reproducible banding patterns, the concentration of primers, MgCl₂ and DNA template used in microsatellite-primed PCR were optimised using the primer (GACA)₄ (annealing temperature 48°C) and the genomic DNA of putative fungal endophyte D22 (see Table 4). Reaction conditions were modified from Groppe et al. (1995), using 2.5 units Taq DNA polymerase (Promega).

Three different concentrations of primers were tested (0.375 μM, 0.75 μM and 1.5 μM). All three concentrations resulted in similar banding patterns, however, 1.5 μM primer produced the best amplification. For 0.375 and 0.75 μM primer concentrations, some bands became fainter and bands less than 520 bp disappeared (Fig. 2). Therefore, 1.5 μM primer was used in all subsequent amplifications.

![Image]

Fig. 2. Effects of different concentrations of the primer (GACA)₄ (μM) on amplification of genomic DNA of putative fungal endophyte D22. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.
The concentration of MgCl$_2$ was also optimised. Six different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM) were tested. No amplification was achieved using the MgCl$_2$ concentrations of 0.5 and 3.0 mM. All other concentrations resulted in amplification with similar fingerprints. Fewer polymorphisms were detected, however, using 1.0 mM than higher concentrations (Fig. 3). Thus, 1.5 mM MgCl$_2$ was selected as the optimal concentration.

![Fingerprinting of putative fungal endophyte D22 using the primer (GACA)$_4$. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.](image_url)
The concentration of DNA template was also optimised. Six concentrations of DNA template ie 10, 20, 40, 80, 100, 200 ng 50 µl⁻¹ were tested. All concentrations facilitated amplification, however, the intensity of some bands amplified using 10 and 20 ng DNA was faint and some products were not amplified (Fig. 4). The fingerprints and band intensity were identical for the other four treatments. For all subsequent work, the concentration of DNA template used for amplification was in the range 50-200 ng 50 µl⁻¹.

**Fig. 4.** Effect of the concentration of DNA template (ng 50 µl⁻¹) on fingerprinting of putative fungal endophyte D22 using the primer (GACA)₄. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.
Three different programs were tested to optimise thermocycling conditions. The first was a modification of the PCR program used by Groppe et al. (1995) which was 30 cycles of 1 min at 94°C, 2 min at 48°C and 3 min at 72°C (program 1). Two shorter programs were also tested. These were 30 cycles of 30 s at 94°C, 30 s at 48°C and 2 min at 72°C (program 2); and 30 cycles of 1 min at 94°C, 1 min at 48°C and 2 min at 72°C (program 3).

All the three programs resulted in the same fingerprints being produced (Fig. 5), however, differences in band intensity were observed. Program 2 produced much fainter fingerprints. Programs 1 and 3 showed little difference in band intensity. Program 3 was completed in 2.5 h, 1 h less than program 1 thus saving considerable time. Program 3 was therefore used for all subsequent amplifications.

![Image](image.png)

**Fig. 5.** The effect of different PCR cycling programs on fingerprints of putative fungal endophyte D22 using primer (GACA)_4. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.
3.3.2 Screening of microsatellite primers

In preliminary experiments using the genomic DNA of putative endophyte D22 as template, primer (GACA)$_4$ produced successful amplifications and reproducible banding patterns. Several factors eg concentrations of the primer, MgCl$_2$, DNA template and thermocycling conditions were optimised. Using the conditions optimised above, two microsatellite primers: (GTG)$_5$ (annealing temperature 52°C) and (AT)$_{12}$ (annealing temperature 48°C) were tested for fingerprinting of putative endophyte D22. Primer (AT)$_{12}$ resulted in only a smear of products between 50 and 150 bp being produced while the primer (GTG)$_5$ generated highly reproducible and reliable banding patterns (Fig. 6). Thus primers (GACA)$_4$ and (GTG)$_5$ were used for fingerprinting of all 175 selected putative fungal isolates in this study.

![PCR fingerprinting of fungal isolate D22 using primers (GTG)$_5$ and (AT)$_{12}$.](image)

**Fig. 6.** PCR fingerprinting of fungal isolate D22 using primers (GTG)$_5$ and (AT)$_{12}$. Each primer was used for duplicate amplifications. Primer (AT)$_{12}$ did not produce any fingerprints and was excluded from subsequent amplification. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.
3.4 Microsatellite-primed PCR analysis

3.4.1 Microsatellite-primed PCR using primer (GACA)$_4$

Microsatellite-primed PCR using primer (GACA)$_4$ consistently produced reproducible fingerprints for all isolates. The number of DNA fragments generated per isolate varied between 5 and 14, with fragment size ranging between 250-3000 bp (Fig. 7-10). Faint or inconsistent bands and bands below 250 bp in size were discarded from the analysis.

Fingerprints from primer (GACA)$_4$ (Fig. 7) separated the 60 fungal isolates obtained from plant A into 16 different genets (Table 3). The two largest genets, (genets 1 and 9) comprised 31 and 7 isolates respectively. Genets 2 and 6 each contained three isolates, with the remaining genets containing either one or two isolates (Table 3).

Fingerprints generated using primer (GACA)$_4$ (Fig. 8) separated the 28 fungal isolates obtained from plant B into 15 distinct genets having identical fingerprints (Table 3). Genet 18 contained the largest number of isolates (six), followed by genet 17, which had four isolates and genet 26, which contained three isolates. All the other genets contained either one or two isolates (Table 3).

Fingerprints from primer (GACA)$_4$ (Fig. 9) separated the 47 fungal isolates obtained from plant C into 14 genets (Table 3). The two largest genets (genets 32 and 45) comprised 21 and 8 isolates respectively. Genets 33 and 34 each contained three isolates, genets 38 and 41 each contained two isolates and the remaining genets each had one isolate (Table 3).

The 40 fungal isolates obtained from plant D were separated into seven distinct genets by primer (GACA)$_4$ (Fig. 10; Table 3). The two largest genets, genets 32 and 33, were represented by 20 and 15 isolates respectively. These two genets (32 & 33) comprised
isolates from both plants C and D. The remaining five genets each comprised a single isolate (Table 3).

Overall, the 175 putative endophytes obtained from four *W. pungens* plants were separated into 50 separate genets based on fingerprints generated using primer (GACA)$_4$ (Table 3). While the majority of genets comprised only single or a few isolates, three genets (1, 32 and 33) were represented by 18-41 isolates (Table 3). In all but two cases, genets were confined to individual plants, with each plant yielding up to 7-16 genets. Genets 32 and 33, however, contained isolates obtained from both plants C and D (Table 3).
Fig. 7. Microsatellite-primed PCR fingerprints of putative fungal endophytes A01-A60 isolated from a single *W. pungeas* plant (plant A), using primer (GACA)$_4$. Ctrl = control. Isolate names are listed above each lane. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.
Fig. 8. Microsatellite-primed PCR fingerprints of putative fungal endophytes B01-B28 isolated from *W. pungens* plant B using primer (GACA)$_4$. Isolate names are listed above each lane. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.
Fig. 9. Microsatellite-primed PCR fingerprints of putative fungal endophytes C01-C47 isolated from *W. pungens* plant C using primer (GACA)$_4$. Ctrl = control. Isolate names are listed above each lane. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.
**Fig. 10.** Microsatellite-primed PCR fingerprints of putative fungal endophytes D01-D40 isolated from *W. pungens* plant D using primer (GACA)$_4$; Ctrl = control. Isolate names are listed above each lane. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.
3.4.2 Microsatellite-primed PCR using primer (GTG)$_5$

To test the reliability of genotyping of selected endophytes on the basis of microsatellite fingerprints using primer (GACA)$_4$, a second primer, (GTG)$_5$, was used for microsatellite-primed PCR. Reproducible fingerprints were obtained for all isolates amplified using primer (GTG)$_5$. The number of DNA fragments generated per isolate varied between 7 and 16, with fragment size ranging between 300-2350 bp (Fig. 11-14). Faint or inconsistent bands and bands below 300 bp in size were discarded from the analysis.

The 60 fungal isolates obtained from plant A were separated into 16 genets based on fingerprints generated using primer (GTG)$_5$ (Fig. 11). These genets were identical to those derived using primer (GACA)$_4$ (see Table 3).

Fingerprints from (GTG)$_5$ confirmed the 14 genets identified by primer (GACA)$_4$ within the 28 fungal isolates obtained from plant B (Table 4), but failed to separate genet 19 (isolates B07 & B19) and genet 24 (isolate B14). Genets 19 and 24 had identical fingerprints with (GTG)$_5$ (Fig. 12), but showed clear differences with (GACA)$_4$ (Fig. 8).

The 47 fungal isolates obtained from plant C were separated into 14 genets based on fingerprints generated using primer (GTG)$_5$. These genets were identical to those derived using primer (GACA)$_4$ (Fig. 13) (see Table 3).

Fingerprints generated using primer (GTG)$_5$ confirmed the genets identified by primer (GACA)$_4$ within the 40 fungal isolates obtained from plant D (Table 4), with the exception of genet 47 (isolate D33) and genet 49 (isolate D35), which it failed to separate (Fig. 14 c). The two isolates had slightly different fingerprints with primer (GACA)$_4$ (Fig. 10 c), however, no difference was detected with (GTG)$_5$ (Fig. 14 c).

54
In summary, amplification with the primer (GTG)$_5$ genetted most isolates in an identical fashion to (GACA)$_4$. In a few instances, however, isolates which were not separated by (GTG)$_5$ were separated from each other using (GACA)$_4$ (Table 3).

**Table 5.** Differences in geneting of fungal isolates based on fingerprints generated using primers (GACA)$_4$ and (GTG)$_5$.

<table>
<thead>
<tr>
<th></th>
<th>(GACA)$_4$</th>
<th>(GTG)$_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1$^+$ (A)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 (B)</td>
<td>1, 18, 32</td>
<td></td>
</tr>
<tr>
<td>32 (C &amp; D)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (A)</td>
<td>3, 33</td>
<td></td>
</tr>
<tr>
<td>33 (C &amp; D)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19 (B)</td>
<td>19, 24</td>
<td></td>
</tr>
<tr>
<td>24 (B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47 (D)</td>
<td>47, 49</td>
<td></td>
</tr>
<tr>
<td>49 (D)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Letters in parenthesis indicate from which plants (A-D) the fungal isolates were obtained.

$^+$Numbers indicate to which genet isolates belonged.
Fig. 11. Microsatellite-primed PCR fingerprints of putative fungal endophytes A01-A60 isolated from *W. pungens* plant A using primer (GTG)$_5$. Ctrl = control. Isolate names are listed above each lane. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.
Fig. 12. Microsatellite-primed PCR fingerprints of putative fungal endophyte B01-B28 isolated from *W. pungens* plant B using primer (GTG)_5. Isolate names are listed above each lane. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.
Fig. 13. Microsatellite-primed PCR fingerprints of putative fungal endophytes C01-C47 isolated from W. pungens plant C using primer (GTG)5. Ctrl = control. Isolate names are listed above each lane. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179.
Fig. 14. Microsatellite-primed PCR fingerprints of putative fungal endophytes D01-D40 isolated from W. pungens plant D using primer (GTG)$_5$. Ctrl = control. Isolate names are listed above each lane. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.
3.5 Phylogenetic analysis based on microsatellite-PCR fingerprints

One isolate from each of the 50 genets distinguished by microsatellite-primed PCR using primers (GACA)_4 and (GTG)_5 (Table 3) was included in a phylogenetic analysis. DNA fragments obtained for each isolate using microsatellite-primed PCR were scored as either present or absent and a dissimilarity (1-D) matrix (Table 6) calculated based on the combined data of the two primers.

Based on the presence/absence of fingerprint fragments, genets displayed 0-94% similarity to each other (Table 6), suggesting that considerable genetic diversity exists within isolated endophyte population. Clustering of isolates revealed four general genets within the phylogram (Fig. 15).

Group I contained 13 genets including 108 isolates, representing the largest general group. The three genets which were represented by most isolates (genets 1, 32 and 33), were co-located in this group. Groups II and III comprised 28 genets (49 isolates in total) and 8 genets (16 isolates in total) respectively, while group IV contained only one group (2 isolates). The genets constituting group I displayed between 9% (genets 3 and 33) to 67% dissimilarity (genets 18 and 28) to one another. The genets in group II had 10% (genets 11 and 12) to 82% dissimilarity (genets 2 and 37). The isolates in group III had 6% (genets 48 and 49) to 68% dissimilarity (genets 40 and 47). Group IV, group 38, had 48% (with genet 50) to 100% dissimilarity (with genet 35) with the genets in the other groups. Group I comprised genets obtained from each of the four plants. Indeed, microsatellite fingerprinting data suggested that some genets from different plants were closely related to each other, for instance, genets 1 and 32 (in group I) which were 86% similar based on the presence of shared bands (Table 6).
Table 6. Matrix of dissimilarity between the 50 putative endophyte genets isolated from four *W. pungens* plants based on combined data for microsatellite-primed PCR fingerprinting using primers (GACA)$_4$ and (GTG)$_5$.

| Genet | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  | 20  | 21  | 22  | 23  | 24  |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1     |     | 0.72|     |     | 0.76|     | 0.76|     | 0.76|     | 0.76|     | 0.81|     | 0.77|     | 0.46|     |     |     |     |     |     |     |
| 2     | 0.26| 0.76|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3     |     |     | 0.76| 0.76|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 4     |     |     |     |     | 0.81| 0.77| 0.46|     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 5     |     |     |     |     |     |     |     |     | 0.78| 0.78| 0.39| 0.35|     |     |     |     |     |     |     |     |     |     |
| 6     |     |     |     |     |     |     |     |     |     |     |     |     | 0.82| 0.80| 0.76| 0.25| 0.45| 0.29|     |     |     |     |     |
| 7     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 8     |     |     |     |     |     |     | 0.81| 0.77| 0.83| 0.65| 0.43| 0.52| 0.54|     |     |     |     |     |     |     |     |     |     |     |
| 9     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 10    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 11    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 12    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 13    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 14    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 15    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 16    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 17    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 18    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 19    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 20    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 21    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 22    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 23    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 24    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

61
Fig. 15. Phylogroupic relationships of the 50 putative endophyte genets isolated from four *W. pungens* plants inferred from a dissimilarity coefficient (J-D) from microsatellite-primed PCR fingerprints using primers (GACA)$_4$ and (GTG)$_5$. Groupings (I-IV) were assigned based on the major branches within the phylogram. Numbers behind hyphen indicate the number of isolates in each group. Scale bar represents 10% dissimilarity.
3.6 ITS rDNA sequence analysis of selected isolates

PCR using primers ITS1 and ITS4 amplified a single product for each of the selected isolates. The size of each PCR product was estimated against the DNA marker pGEM, and ranged from approximately 530 to 580 bp (including primers) for the 15 isolates amplified (Fig. 16). The length of the amplification product was 551 to 568 bp (including primers) or 512 to 529 bp (excluding primers) based on DNA sequences. Isolate A03 had the largest ITS region (529 bp) while isolates C40 and *Hymenoscyphus ericae* (Read) Korf & Kernan (Read 101) had the smallest ITS region (512 bp).

![Image](image_url)

Fig. 16. Amplified ITS region of selected fungal endophytes isolated from four *W. pungens* plants (A-D). He and Ctrl represent *H. ericae* and negative control (without DNA template) respectively. The size of the fragments of the molecular marker pGEM are 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179 bp.

An alignment of nucleotide sequences for the 14 isolates from *W. pungens* and *H. ericae* is presented in Fig. 17. The ITS sequences of isolates C01 and D01 were identical while all other sequences had some differences to each other. The majority of base differences
between the isolates were found in the ITS1 and ITS2 regions and were generally base substitutions. Indeed, in most isolates there were five indels in the sequence of the ITS1 region and four indels in the ITS2 region (Fig. 17). Isolate A03 had a 15 bp insertion at the beginning of the ITS1 region.

Isolates, A01, A16, C01, C07, D01 and D02 had a high degree of sequence similarity, ranging from 98.1% (between A01 and D02) to 99.6% (between A01 and C01) (Table 7). Isolates A49, B17 and D33 had a high degree of sequence similarity to each other (98.1% to 98.5%) (Table 7). Isolates C29 and C40 had 96.5% sequence similarity while both isolates displayed a relatively low degree of sequence similarity to all other isolates, ranging, for example, from 59.7% (between A03 and C29) to 68.3% (between A53 or C40). Similarly, isolate A03 had a low degree of sequence similarity to all other isolates, ranging from 59.7% (with isolate C40) to 71.4% (with H. ericae). H. ericae had a low degree of sequence similarity with all isolates from W. pungens, ranging from 66.5% (with C29) to 80.8% (with isolates B17 or D33) (Table 7).

Table 7. Percentage similarity (%) matrix of nucleotide sequences of ITS regions of the 14 fungal endophytes isolated from four W. pungens plants (A-D) and the ericoid mycorrhizal fungus H. ericae (He).

<table>
<thead>
<tr>
<th></th>
<th>A01</th>
<th>A03</th>
<th>A16</th>
<th>A49</th>
<th>A53</th>
<th>B17</th>
<th>C01</th>
<th>C07</th>
<th>C28</th>
<th>C29</th>
<th>C40</th>
<th>D01</th>
<th>D02</th>
<th>D33</th>
<th>He</th>
</tr>
</thead>
<tbody>
<tr>
<td>A01</td>
<td>100</td>
<td>69.6</td>
<td>98.3</td>
<td>90.2</td>
<td>84.3</td>
<td>91.3</td>
<td>99.6</td>
<td>98.3</td>
<td>90.8</td>
<td>67.4</td>
<td>67.8</td>
<td>99.6</td>
<td>98.1</td>
<td>90.5</td>
<td>79.8</td>
</tr>
<tr>
<td>A03</td>
<td>100</td>
<td>69.6</td>
<td>70.5</td>
<td>70.7</td>
<td>71.0</td>
<td>69.8</td>
<td>69.2</td>
<td>70.9</td>
<td>59.7</td>
<td>59.7</td>
<td>69.8</td>
<td>69.6</td>
<td>70.4</td>
<td>71.4</td>
<td></td>
</tr>
<tr>
<td>A16</td>
<td>100</td>
<td>90.3</td>
<td>84.4</td>
<td>91.1</td>
<td>98.7</td>
<td>98.9</td>
<td>90.4</td>
<td>67.8</td>
<td>68.2</td>
<td>98.7</td>
<td>98.7</td>
<td>90.7</td>
<td>79.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A49</td>
<td>100</td>
<td>83.2</td>
<td>97.7</td>
<td>90.5</td>
<td>90.3</td>
<td>88.4</td>
<td>66.7</td>
<td>66.3</td>
<td>90.5</td>
<td>90.9</td>
<td>98.5</td>
<td>80.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A53</td>
<td>100</td>
<td>83.1</td>
<td>84.4</td>
<td>84.4</td>
<td>83.9</td>
<td>67.9</td>
<td>68.3</td>
<td>84.4</td>
<td>84.3</td>
<td>83.5</td>
<td>75.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B17</td>
<td>100</td>
<td>91.7</td>
<td>91.1</td>
<td>89.6</td>
<td>67.6</td>
<td>66.9</td>
<td>91.7</td>
<td>91.7</td>
<td>98.1</td>
<td>80.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C01</td>
<td>100</td>
<td>98.7</td>
<td>91.0</td>
<td>67.8</td>
<td>68.2</td>
<td>100</td>
<td>98.5</td>
<td>90.9</td>
<td>79.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C07</td>
<td>100</td>
<td>90.4</td>
<td>67.6</td>
<td>68.0</td>
<td>98.7</td>
<td>98.7</td>
<td>90.7</td>
<td>79.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C28</td>
<td>100</td>
<td>67.0</td>
<td>67.2</td>
<td>91.0</td>
<td>90.4</td>
<td>88.6</td>
<td>78.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C29</td>
<td>100</td>
<td>67.8</td>
<td>67.8</td>
<td>67.2</td>
<td>67.2</td>
<td>66.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C40</td>
<td>100</td>
<td>68.2</td>
<td>68.2</td>
<td>66.9</td>
<td>66.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D01</td>
<td>100</td>
<td>98.5</td>
<td>90.9</td>
<td>79.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D02</td>
<td>100</td>
<td>91.3</td>
<td>79.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D33</td>
<td>100</td>
<td>80.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>He</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>
Fig. 17. Alignment of the ITS nucleotide sequences of the 14 fungal endophytes isolated from four W. pungens plants (A-D) and the ericoid mycorrhizal fungus H. ericae generated using the EGCG programs PILEUP and PRETTY. The names of isolates are indicated in the left-hand column, He represents H. ericae and Cons represents the consensus sequence. Asterisks indicate positions identical across all sequences. Hyphens indicate position not identical across the sequences.

Since the ITS sequences of isolates C01 and D01 were identical, only isolate D01 was used in the KITSCH analysis. The unrooted phyllogroupic tree inferred from nucleotide sequences of the ITS regions is presented in Fig. 18. Three general groups (I-III) were defined in the phyllogroupic tree (Fig. 18). Group I comprised six isolates (A01, A16, C01, C07, D01 and D02) from three different host plants. The six isolates within group I had < 1.9% sequence divergence. The second group comprised three isolates (A49, B17 and D33) with < 2.3% sequence divergence between them. Group III had isolates C29 and C40 with 3.5% sequence divergence between them. The remaining three isolates from W. pungens (A03, A53 and C28) were grouped individually. H. ericae was not clustered together with any of the isolates from W. pungens, being only 66.5-80.8% similar to the other isolates. Isolate C28 was 67.6-91.0% similar to the other isolates, A53 was 67.9-84.4% similar to the other isolates, while A03 was 59.7-71.4% similar to the other isolates.
Of the 14 isolates sequenced, six presumably distinct groups of isolates were identified across the root systems of four *W. pungens* plants. Four of these groups occurred in the root system of plant A (out of 5 isolates sequenced from this plant), three groups occurred in the root system of plant C (of 4 isolates sequenced), two groups occurred in plant D (of three isolates sequenced) and only one group was identified in plant B (although only one isolate from this plant was sequenced).

Bootstrap resampling was used to estimate the reliability of the inferred phylogroupic tree by assigning a statistical confidence to each branch. While the branches separating A16 and C07 was poorly supported (32.2%), most branches were strongly supported (92.5%-100%) (Fig. 18). The sequences of the ITS regions of the 14 isolates and *H. ericae* were compared to those available in the GenBank DNA sequence database to search for sequences having the highest degree of similarity to the selected isolates. The three most closely related sequences retrieved for each isolate are listed in Table 8. Results of the FASTA searches showed 13 of the isolates to be similar to ascomycetes in the Hyaloscyphaceae i.e. *Trichopezizella* (*Lachunm*) sp. and *Lachnella* sp. (75-86% identity over 519-534 nucleotides; Table 8). Isolate A03 was most similar to *Phialophora gregata* (Allington & Chamberlain) W. Gams (86% identity over 481 nucleotides). None of the isolates sequenced was similar enough to any of the sequences in the database to have genus or species names assigned to them.
Table 8. Sequences that had the highest degree of identity with sequences of 14 endophytes from *W. pungens* and the mycorrhizal fungus *H. ericae* retrieved from the GenBank database using the EGCG program FASTA.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>GenBank accession number</th>
<th>Closest species</th>
<th>Identity (%)</th>
<th>nucleotides</th>
<th>Overlap (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A01, C01/D01</td>
<td>TNU57813</td>
<td><em>Trichopezizella nidulans</em></td>
<td>84.7 - 85.1</td>
<td>522</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAY72611</td>
<td><em>Gelatinipulvinella astraeca</em></td>
<td>83.9 - 84.3</td>
<td>527</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LCU59145</td>
<td><em>Lachnellula calyciformis</em></td>
<td>83.7 - 84.1</td>
<td>528</td>
<td></td>
</tr>
<tr>
<td>A03</td>
<td>PGU66727</td>
<td><em>Philophora gregata</em></td>
<td>86.3</td>
<td>481</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PGU66729</td>
<td><em>Philophora gregata</em></td>
<td>86.3</td>
<td>481</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PGU66731</td>
<td><em>Philophora gregata</em></td>
<td>86.1</td>
<td>481</td>
<td></td>
</tr>
<tr>
<td>A16, C07</td>
<td>TNU57813</td>
<td><em>Trichopezizella nidulans</em></td>
<td>84.7 - 84.9</td>
<td>522</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAY72611</td>
<td><em>Gelatinipulvinella astraeca</em></td>
<td>84.3 - 84.8</td>
<td>527</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LCU58640</td>
<td><em>Lachnum enterpes</em></td>
<td>82.7 - 83.7</td>
<td>527</td>
<td></td>
</tr>
<tr>
<td>A49, D33</td>
<td>LCU59145</td>
<td><em>Lachnellula calyciformis</em></td>
<td>84.8 - 85.3</td>
<td>527</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAY72611</td>
<td><em>Gelatinipulvinella astraeca</em></td>
<td>84.3 - 84.8</td>
<td>530</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LPU58635</td>
<td><em>Lachnum pteridophyllum</em></td>
<td>83.6 - 83.7</td>
<td>530</td>
<td></td>
</tr>
<tr>
<td>A53</td>
<td>LCU58638</td>
<td><em>Lachnum controversum</em></td>
<td>82.2</td>
<td>522</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LSU58639</td>
<td><em>Lachnum spartinace</em></td>
<td>82.0</td>
<td>527</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAY72611</td>
<td><em>Gelatinipulvinella astraeca</em></td>
<td>82.4</td>
<td>528</td>
<td></td>
</tr>
<tr>
<td>B17</td>
<td>LCU59145</td>
<td><em>Lachnellula calyciformis</em></td>
<td>86.3</td>
<td>527</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAY72611</td>
<td><em>Gelatinipulvinella astraeca</em></td>
<td>84.8</td>
<td>527</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNU57813</td>
<td><em>Trichopezizella nidulans</em></td>
<td>84.7</td>
<td>523</td>
<td></td>
</tr>
<tr>
<td>C28</td>
<td>TNU57813</td>
<td><em>Trichopezizella nidulans</em></td>
<td>84.4</td>
<td>526</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LCU59145</td>
<td><em>Lachnellula calyciformis</em></td>
<td>84.6</td>
<td>534</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAY72611</td>
<td><em>Gelatinipulvinella astraeca</em></td>
<td>82.7</td>
<td>527</td>
<td></td>
</tr>
<tr>
<td>C29</td>
<td>LSU58639</td>
<td><em>Lachnum spartinace</em></td>
<td>75.3</td>
<td>530</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNU57813</td>
<td><em>Trichopezizella nidulans</em></td>
<td>74.7</td>
<td>525</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAU57492</td>
<td><em>Cistella acumin</em></td>
<td>73.9</td>
<td>532</td>
<td></td>
</tr>
<tr>
<td>D02</td>
<td>TNU57813</td>
<td><em>Trichopezizella nidulans</em></td>
<td>84.9</td>
<td>522</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAY72611</td>
<td><em>Gelatinipulvinella astraeca</em></td>
<td>84.1</td>
<td>527</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LCU58638</td>
<td><em>Lachnum controversum</em></td>
<td>83.4</td>
<td>524</td>
<td></td>
</tr>
<tr>
<td>C40</td>
<td>LSU58639</td>
<td><em>Lachnum spartinace</em></td>
<td>75.3</td>
<td>523</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNU57813</td>
<td><em>Trichopezizella nidulans</em></td>
<td>75.3</td>
<td>519</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LCU58638</td>
<td><em>Lachnum controversum</em></td>
<td>74.6</td>
<td>523</td>
<td></td>
</tr>
<tr>
<td><em>H. ericae</em></td>
<td>LLAF6599</td>
<td><em>Laccaria laccata</em></td>
<td>91.7</td>
<td>480</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGU57089</td>
<td><em>Cistella grevillei</em></td>
<td>82.1</td>
<td>515</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LCU58640</td>
<td><em>Lachnum enterpes</em></td>
<td>81.3</td>
<td>520</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 18. The unrooted phylogroupic tree of 14 fungal endophytes isolated from four *W. pungens* plants (A-D) and an isolate of the ericoid mycorrhizal fungus *H. ericae* based on nucleotide sequences of the ITS regions. Groupings (I-III) were assigned on the basis of the major branches within the phylogram. The numbers beside branches are bootstrap values from 1000 replicates. He represents *H. ericae*. The length of the scale bar equals a bootstrap value of 100.
Chapter 4: Discussion

4.1 Isolation of ericoid mycorrhizal endophytes

A total of 243 isolates were obtained from the four \textit{Woollsia pungens} (Cav.) F. Muell plants using the direct plating method. Fifty genets were identified within this endophyte population using microsatellite-primed PCR. Isolates representative of 43 genets were shown to produce typical ericoid mycorrhizal structures, confirming that the isolation/sterilisation method is useful for isolation of mycorrhizal endophytes from \textit{W. pungens}.

Isolation of putative mycorrhizal endophytes from \textit{W. pungens} was attempted using two methods: the incubation medium method and the direct plating method. The incubation medium method has recently been successfully used to isolate putative ericoid fungal endophytes from mycorrhizal roots of the epacrid species \textit{Leucopegon parviflorus} (Andr.) Lindl (Steinke \textit{et al.}, 1996). Of the 812 root pieces Steinke \textit{et al.} (1996) incubated, 512 root pieces produced growing hyphae. Two hundred and sixty-seven root pieces with growing hyphae were randomly selected and transferred onto modified Melin Norkrans agar (MMN), 1/4 strength potato dextrose agar (PDA) or water agar. A total of 149 fungal isolates were obtained with 49 of the isolates identified as non-mycorrhizal endophytes. The remaining 100 isolates were sterile and slow growing. It was suggested that a number of these isolates might be mycorrhizal endophytes based on morphological similarities to the known ericoid mycorrhizal fungi \textit{H. ericae} and \textit{Oidiodendron} spp. However, since Steinke \textit{et al.} (1996) did not conduct mycorrhizal synthesis experiments, it is not clear how many of the isolates were actually mycorrhizal endophytes. It is thus difficult to assess the efficiency of the method in the isolation of mycorrhizal endophytes from \textit{L. parviflorus}. In the present work, only eight isolates were obtained from 100 root
pieces incubated, suggesting that the incubation medium method may not suitable for the isolation of mycorrhizal endophytes from *W. pungens*.

In preliminary experiments, direct plating of root pieces of *W. pungens* sterilised using a 37.5% commercial bleach solution (1.5% available chlorine) for 30, 60 or 90 seconds produced a number of fungal mycelia, with the longer sterilisation time proving most effective. For all three treatments, 20-30% of the sterile root pieces yielded dark-coloured, and slow growing fungal colonies.

Directing plating of surface sterilised mycorrhizal root pieces has been used previously for isolation of mycorrhizal endophytes from a number of ericaceous plant species. For example, Stoyke & Currah (1991) isolated *Oidiodendron griseum* Robak from fine roots of several ericoid species following sterilisation in a 20% solution of household bleach for 1 min. More recently, Hambleton & Currah (1997) used an increased sterilisation time of 3 min, successfully isolating *Oidiodendron mainus* Barron and *Scytalidium vaccinii* Dalpé, Litten & Sigler from hair roots of 19 ericaceous plant species. Several other chemicals have also been successfully used for surface sterilisation of roots during endophyte isolation. Xiao & Berch (1992) used 30% H2O2 to sterilise roots of *Gaultheria shallon* Pursh for 30 s prior to plating onto 1/3 strength PDA. Twenty four of the 83 isolates obtained were shown to be mycorrhizal. More recently, these authors increased the sterilisation time to 1 min, the modified protocol yielding 175 mycorrhizal endophytes from 1120 root pieces (Xiao & Berch, 1996). Couture *et al.* (1983) surface sterilised roots of *Vaccinium angustifolium* Ait and *V. corymbosum* L. using 0.01% OsO4. Approximately half of the root pieces yielded fungal isolates and about 150 fungal isolates were obtained, however, only three isolates from each of the two plant species were shown to be mycorrhizal. The results described above indicate that direct plating of mycorrhizal root pieces surface sterilised using either NaOCl, H2O2 or OsO4 is a useful
method for isolation of ericoid mycorrhizal endophytes from both the Ericaceae and

A further method that has been successfully used in isolation of ericoid mycorrhizal
endophytes is the maceration method (Pearson & Read, 1973). In this method, root
pieces, either surface sterilised or without surface sterilisation are serially washed 20 to
40 times in sterile water, macerated or homogenised to detach the epidermal cells from the
stele and a small volume of cell suspension is placed on water agar in a Petri dish
(Pearson & Read, 1973; Perotto et al., 1996). The method has proven successful for
isolating mycorrhizal endophytes from a number of mycorrhizal plant species in the
Ericales including Calluna vulgaris (L.) Hull, Vaccinium myrtillus L., V. angustifolium,
R. ferrugineum L., Leucopogon juniperinus B. Br., Lysinema ciliatum R. Br.,
Andersonia caerulea R. Br. and Astroloma ciliatum (Lindl.) Druce (Pearson & Read,
1973; Dalpé et al., 1989; Reed, 1989; Perotto et al., 1990, 1996; Hutton et al., 1994).

The incubation, the direct plating and maceration methods have been proven to be useful
for isolation of mycorrhizal endophytes from eparidaceous plant species (Reed, 1989;
Hutton et al., 1994; Steinke et al., 1996). However, the methods may have variable
efficiency depending on the host plant material in question.

4.2 Germination of D. secundum seeds

Both V. macrocarpon and D. secundum seedlings were used as host plants to test the
abilities of fungal isolates obtained from W. pungens to form ericoid mycorrhizas. Sterile
seedlings were produced from surface sterilised seeds. High germination percentages
(73.7%) were achieved for D. secundum seeds following surface sterilisation and
treatment with Kirstenbosch™ Seed Primer. Eparid plants are usually propagated by tip
cuttings, however, strike rates are not always satisfactory, in some cases, below 10% (McLean et al., 1994). Furthermore, the delicate root systems of plants rooted by this method make planting out very difficult. The present data indicate that treatment of *D. secundum* seeds with the Seed Primer coupled with surface sterilisation using 1% sodium hypochlorite is an efficient way to stimulate seed germination, providing a useful means of propagation of *D. secundum*.

Seeds of the Epacridaceae are usually not used for propagation because of their very long dormancy period (McLean et al., 1994). Several studies have investigated the effects of smoke on seed germination of epacridaceous plants. These indicate that exposure to smoke from burning native Australian vegetation can promote seed germination in a few epacrid species (Dixon, Roche & Pate, 1995). Furthermore, even smoke-fumigated filter papers can significantly stimulate germination in some epacrid species (Hutton et al., 1997). The results of the present study are consistent with this, indicating that smoke-fumigated filter papers are effective in stimulating germination of *D. secundum* seeds.

### 4.3 Mycorrhizal synthesis

Over two hundred fungal isolates were obtained from four *W. pungens* plants using the direct plating method. One hundred and seventy five dark-coloured isolates were selected for microsatellite-primed PCR, which grouped these into 50 genets. Forty-three isolates, each representing an individual genet, formed typical ericoid mycorrhizal structures in hair roots of *V. macrocarpon* under axenic conditions, demonstrating the mycorrhizal status of the fungal isolates. Furthermore, this is consistent with the observations of Reed (1989) and Read (1996) that endophytes from the Epacridaceae can infect ericaceous hosts. Isolates from the remaining seven genets did not infect *V. macrocarpon* under the conditions used in the experiment. While this may suggest that these isolates are not
mycorrhizal endophytes, it is possible that under different conditions, infection of *V. macrocarpon* or other epacrids may be successful.

Sand moistened with water has been successfully used as a potting medium for mycorrhiza synthesis on roots of cuttings of *Vaccinium darrowii* Camp and *L. ciliatum* with endophytes from the Epacridaceae (Reed, 1989; Hutton *et al.*, 1994). This method was not successful in *V. macrocarpon* seedlings in the present study. No mycelium was observed on hair roots of the seedlings under the light microscope. Due to the difficulties of dispersing the fungal plugs into the depth of sand in test tubes, it is possible that hyphae were mainly concentrated on the top of the sand while the hair roots were sampled from the bottom of the test tubes. Glucose, pectin, activated charcoal and autoclaved soil have been shown to be efficient in stimulating mycorrhiza formation *in vitro* (Pearson & Read, 1973; Couture *et al.*, 1983; Duclos & Fortin, 1983) and it may be that infection of *V. macrocarpon* by the isolates in question may have been stimulated by the addition of exogenous carbon. A carbon source was not, however, included in the medium since there is clear evidence that exogenous carbon can result in apparent mycorrhiza formation between normally incompatible host/endophyte combinations (Duddridge, 1987).

Water agar with incorporated basal nutrients is also commonly used as a synthesis medium for ericoid mycorrhiza synthesis, with basal nutrients containing macro- and micro-elements that plants require (Leake & Read, 1991). Interestingly, the basal nutrient agar was not successful when used for *D. secundum* seedlings. No hair roots were produced in the seedlings growing in the medium. Leake & Read (1991) have suggested that hair root development in the Ericales may be completely inhibited in the absence of fungal endophytes. In some instances, the addition of sterilised heathland soil or activated charcoal on the surface of agar have been shown to increase the amount and uniformity of hair roots and mycorrhizal infection (Pearson & Read, 1973; Couture *et al.*, 1983; Perotto *et al.*, 1996). In the present study, when a mix of sand, peat and vermiculite was used as
a synthesis medium, roots of *D. secundum* seedlings developed normally and hair roots were produced. Although *D. secundum* seedlings formed normal hair-roots in this modified medium, these seedling were not examined for infection due to the limitation of time in the project. However, the status of the isolates as mycorrhizal endophytes was clearly demonstrated by their ability to infect *V. macrocarpon*.

### 4.4 Cultural characteristics of mycorrhizal fungal endophytes

Mycorrhizal endophytes from *Woollsia pungens* (Cav.) F. Muell varied from smoke grey through dark grey to black in colour when grown on 2% malt agar. Isolates produced colonies that were either waxy or velutinous. These descriptions are broadly similar to those of known ericoid mycorrhizal fungi from the Ericales (Read, 1974, Dalpé, 1986; Reed, 1989, Hutton *et al.*, 1994; Perotto *et al.*, 1996; Steinke *et al.*, 1996). These studies have further shown that the ericoid mycorrhizal fungal endophytes isolated from a single host plant species may display a range of morphological characteristics, suggesting that a diverse population of endophytes may exist in the root system of a single plant species. Recently, Hutton *et al.* (1994) isolated both smoke-grey and black endophytes (when grown on PDA) from a few Epacridaceae species, while Reed (1989) isolated dark grey to black endophytes from *L. juniperinus* on PDA and water agar. In contrast, Steinke *et al.* (1996) found the majority of putative endophytes that they isolated from *Leucopagon parviflorus* B. Br. were dark brown or olivaceous green on MMN agar.

While a few ericoid mycorrhizal fungal endophytes have been observed to produce reproductive bodies in axenic culture (Pearson & Read, 1973; Dalpé *et al.*, 1989; Xiao & Berch, 1995), most endophytes isolated from the Ericaceae (Perotto *et al.*, 1990, 1996; Xiao & Berch, 1996) and Epacridaceae (Reed, 1989; Hutton *et al.*, 1994; Steinke *et al.*, ...
1996) remain sterile in axenic culture. Under the cultural conditions adopted in the present study, all endophytes from *W. pungens* remained sterile, even when cultured for over six months. The absence of apothecia hampered delimitation of the taxonomic position of the endophytes by conventional morphological characteristics.

### 4.5 Genetic diversity of mycorrhizal fungal endophytes

Microsatellite-primed PCR revealed that many fungal genets colonised the root system of a single *W. pungens* plant at the field site. Seven to 16 fungal genets were present in a single root system, with a total of 50 genets identified in roots of four plants. These data strongly indicate that diversity within the fungal population is high. It is not possible from the fingerprints generated by microsatellite-primed PCR to discern with certainty the extent to which this reflects intraspecific variation, or the existence of different taxa within the isolated population. The very low degree of fingerprint similarity between some of the isolates (between genets 35 and 38), however, strongly suggests that the population comprises mycelia of several taxa along with several genets of some of these. Indeed, since these genets were isolated from plant C, it further implies that several taxa may inhabit the root system of a single *W. pungens* plant (confirmed by sequence data; see section 4.8).

Ultrastructural observations also suggest that at least two distinct endophytes can exist in a single ericoid mycorrhizal root system of *Rhododendron* sp. (Peterson *et al.*, 1980), while serological evidence further indicates a more complex endophyte diversity in single root systems of the genets (Muller *et al.*, 1986). Similar observations have also been reported in the epacrid *D. secundum* (Allan *et al.*, 1989).
Recently, Perotto et al. (1996) conducted a detailed investigation of the genetic diversity in mycorrhizal endophytes in C. vulgaris at a European heathland field site using molecular methods. These authors obtained 73 mycorrhizal endophytes from ten individual C. vulgaris plants. Thirty nine isolates were identified as Oidiodendron maius Barron based on the conidia produced, while the remaining sterile isolates were distinguished into three distinct groups based on morphology and microscopic characteristics. RFLP patterns and RAPD fingerprints were used to differentiate isolates belonging to the same groups and isolates obtained from individual plants. It was found that at least two mycorrhizal mycelia with different morphological characteristics and distinct RAPD fingerprints were isolated from each of the ten plants. Genetic diversity of endophytes of some plants was high. Thus in one plant, thirteen fungal mycelia were obtained and separated into seven groups based on RAPD fingerprints. The data from the present study indicate that diversity in the root system of a single W. pungens plant may be greater, with up to sixteen genets identified from roots of an individual plant. It must be noted, however, that there were obvious differences in the number of root pieces used for isolation from the two plants species. Perotto et al. (1996) used only a few pieces of hair roots for isolation of mycorrhizal endophytes. In the present work, 280-400 root pieces per plant were used for isolation of fungal endophytes. Twenty eight to 60 isolates were obtained from each of four plants. In contrast, Perotto et al. (1996) isolated a total of 11-17 endophytes from two plants collected from five different sampling sites for the two consecutive years.

It is important to stress that the estimates of diversity made in the present study should be considered as minimum estimates. Only juvenile plants which had relatively small root systems that could be removed form the field intact were sampled. As Hutton et al. (1994) have highlighted, mature epacrid plants can have extensive root systems, and this may house a greater diversity of endophytes. Only one single sterilisation/isolation protocol was employed and it is likely that some endophytes may not have been isolated
by this method. Perotto et al. (1996) also suggested the taxonomic diversity of natural ericoid mycorrhizal fungi community was likely to be underestimated due to some endophytes being recalcitrant to isolation. Finally, sampling was carried out on a single occasion and there is evidence of clear seasonality in hair root density and mycorrhizal infection of Western Australian epacrids (Hutton et al., 1994; Bell & Pate, 1996). Further sampling at different times of the year might yield still further endophytes.

4.6 Efficiency of microsatellite-primed PCR in identification of fungal genets

Microsatellite motifs were initially used to design probes in hybridisation-based DNA fingerprinting to detect DNA polymorphisms. Recently, they have been used to design single primers in PCR to investigate genetic variation within fungi (Buscot et al., 1996). The sensitivity of the technique has been well evidenced by the work with Pisolithus tinctorius (Anderson, 1996).

In the present work, most isolates from W. pungens which have identical fingerprints with (GTG)₅ also had identical fingerprints with (GACA)₄, however, several genets (eg 47 and 49) which produced identical fingerprints with (GTG)₅ had clearly different fingerprints with (GACA)₄ (Fig. 10c & 14c). This implies that primer (GACA)₄ may be more effective than primer (GTG)₅ in revealing DNA polymorphisms in the endophytes investigated in the present study. A similar situation was reported by Meyer et al. (1993b). These authors found isolates of different serotypes of Cryptococcus neoformans had a higher degree of dissimilarity with primer (GACA)₄ than with primer (GTG)₅. An investigation of the effectiveness of microsatellite primers by Gupta et al. (1994) suggested that tetranucleotide-based simple sequence repeats were more effective in amplifying the polymorphic patterns than di- and trinucleotide-based repeat primers. In a study of the ectomycorrhizal fungus Tuberc, however, (GTG)₅ was found to be more
successful than (GACA)$_4$ in amplifying polymorphisms (Longato & Bonfante, 1997). It seems there is no correlation between the effectiveness and the length of primers. More than one primer needs to be tested to detect genetic variation in ericoid mycorrhizal endophytes.

Primer (AT)$_{12}$ did not generate any fingerprints in the present work. Only smears between 50 and 150 bp in size were produced. Similarly, no amplification or only smears were produced in previous studies with the dinucleotide primers (CT)$_8$, (CA)$_8$, (CA)$_{10}$ and (AT)$_{10}$ (Gupta et al., 1994; Longato & Bonfante, 1997). A survey of the occurrence of microsatellites in fungal genomes indicated that the AT repeat is the most common microsatellite motif in fungal genomes (Hantula et al., 1996). It may be that this microsatellite is present in repeats of less than 12 in the fungal genomes studied here and was not amplified for this reason using the primer (AT)$_{12}$.

Microsatellite-primed PCR has also been used in investigations of phylogeny. Some workers have used fingerprinting data to cluster individual isolates of species from three genera Boletus, Amanita and Lactarius into the same species (with one exception) (Haudek et al., 1996) or isolates of C. neoformans into the same serotypes (Meyer & Mitchell, 1995). However, no information is available on the consistency and accuracy of using microsatellite-primed PCR data for phylogenetic analysis. In the present study, the phylogenetic relationships of the 14 isolates inferred from DNA sequence of the ITS regions were not fully consistent with those inferred from fingerprinting data. For example, the phylogenetic tree generated using sequence data indicates that isolate D33 was closely related to isolates A49 and B17. These three isolates had high sequence similarities with each other (Table 7). However, in microsatellite fingerprinting-based analysis, D33 was not grouped with A49 or B17. Isolates C29 and C40 were also grouped together based on sequence analysis while the two isolates were not grouped together in the phylogenetic tree based on microsatellite fingerprinting data.
Some isolates, however, were grouped similarly using both methods. Using microsatellite data, for example, isolates A01, A16, C01, C07, D01 and D02 were located in group I with A01, C01 and D01 in one subgroup and A16, C07 and D02 in another subgroup (Fig. 15). Sequence analysis indicated the six isolates had very high sequence similarities (98.1-100%) (Table 7), also suggesting the six isolates were in two closely related subgroups. Secondly, isolate B17 was shown to be closely related to isolate A49 in both phylogenetic trees. In the present study, fingerprinting data were only used for preliminary identification and initial grouping of isolates before some isolates were selected for sequence analysis. It appears that caution is required in the interpretation of microsatellite-primed PCR data when inferring phylogenies.

4.7 Distribution of individual mycorrhizal endophytes

While the majority of genets were represented by 1-8 isolates, three genets comprised up to 41 isolates, suggesting that in some cases root systems may be extensively colonised by individual mycelia. A more systematic study, based on accurate mapping of root systems and root pieces used for isolations would, however, be required to confirm genet distribution patterns. The existence of two genets (32 & 33), present in the root systems of two adjacent plants, raises the possibility that the two root systems were interconnected by endophyte mycelia. Such a situation is already well documented in some ectomycorrhizal (Brownlee et al., 1993) and arbuscular mycorrhizal associations (Newman, 1988), however the extent to which it occurs in ericoid mycorrhizal associations is less clear. Unlike the fungi involved in ectomycorrhizal or arbuscular mycorrhizal associations, mycorrhizal endophytes of the Ericaceae are known to grow for only a few mm from the hair root surface (Read, 1992; Smith & Read, 1997). The fact that little external mycelium is apparent in field-collected epacrid mycorrhizal roots (McLean & Lawrie, 1996) argues for a similar situation in the Epacridaceae. The two W.
*pungens* plants that bore the same genets were collected in juxtaposition to each other. It is thus possible that individual hair roots of the two may have been in contact, or at least in close proximity and that the two plants may indeed have been interconnected by mycelium from a single fungal genet. The possibility that the mycelial genets had fragmented into genetically identical, but spatially separated, ramets (*sensu* Dahlberg & Stenlid, 1995) which could have arisen due to fungivore activity or dispersal of asexual propagates such as arthrospores, however, cannot be excluded. Interestingly, Perotto *et al.* (1996) reported that endophytic mycelia forming a single genet were isolated from roots of individual *C. vulgaris* plants separated by ca 25 m of grassland. This further suggests that mycelial fragmentation and/or arthrospore production can effect the spread of ramets of ericoid mycorrhizal endophytes over considerable distances.

4.8 ITS rDNA sequence comparisons of selected isolates

Fourteen isolates from *W. pungens* were selected for sequence analysis and thirteen of them (excluding isolate A53) were shown to form mycorrhizas on hair roots of *V. macrocarpon* seedlings. Of the 14 isolates that were sequenced, 6 putative taxa were identified. Six isolates (A01, A16, C01, C07, D01 and D02) in group I (Fig. 18) had a high degree of sequence similarity (with < 1.9% sequence divergence) (Table 7). The low level of sequence divergence between them strongly suggests that the 6 isolates are probably conspecific. Egger & Sigler (1993) suggested the two mycorrhizal isolates *Hymenoscyphus ericae* (Read) Karf & Kernon and *Scytalidium vaccinii* Dalpé, Litten & Sigler to be anamorph and teleomorph states of a single taxon based on < 3.5% sequence divergence in a 292 bp portion of rDNA sequence. This is supported by the fact that low levels of intraspecific sequence divergence have been reported within the ascomycete species *Gaeumannomyces graminis* var. *avenae* (1%) (Goodwin *et al.*, 1995) and *Colletotrichum acutatum* Simmonds (< 3%) (Sreenivasprasad, Mills & Brown, 1994).
The three isolates A49, B17 and D33, had sequence divergence of < 2.3% and were clustered together (group II in Fig. 18), supported by bootstrap values of 100%, again suggesting that these three isolates belonged to another species. Isolates C29 and C40 also appear to be closely related, with 3.5% sequence divergence (group III in Fig. 18), and a bootstrap value of 100%, and therefore probably belong either to the same species or same genus. The remaining three isolates (A03, A53 and C28) had sequence similarities ranging from 70.7-83.9% with each other and each was placed on a different branch in the phylogenetic tree (Fig. 18), indicating that they probably represent different taxa.

In the present study, 6 tentative taxa were identified based on nucleotide sequence comparisons on the root systems of four *W. pungens* plants, with four of these taxa (represented by isolates A01/A16, A03, A49 and A53) identified on the root system of plant A (where five isolates from plant A were sequenced) and a smaller number of taxa (1-3) found on other plants (where 1-5 isolates per plant were sequenced). The same fungal taxon was present on more than one *W. pungens* plant. Thus the isolates in group I (presumed to be cospecific) were found in the root system of plants A, C and D. Group II was found in the root systems of plants A, B and D. Further taxa would probably be identified from each plant if more isolates had been sequenced. It is thus possible that the phylogenetic relations of ericoid mycorhizal endophytes may be much more complex than the morphological similarities of sterile cultures suggest (Steinke *et al.*, 1996). This again confirms that Ericales roots have a high diversity of mycorrhizal endophytes, a finding of both Perotto *et al.* (1996) who identified four different RFLP groups of endophytes in *C. vulgaris* plant in Europe, and also Hambleton & Currah (1997) who identified two to three different mycorrhizal fungi from 19 ericaceous species in Canada.

Groupings of the endophytes obtained via analysis of ITS sequence data are broadly supportive of the groupings of the isolates based on microsatellite-primed PCR
fingerprinting data. An obvious exception, however, is isolate D33, which was shown to have >98% identity with isolates A49 and B17 (Group II, Fig. 18), but was grouped with isolate C40 (Fig 15), based on microsatellite-primed PCR data. ITS sequence data have revealed that isolates D33 and C40 have <67% identity. Although microsatellite-primed PCR has been shown to be useful in interspecific comparisons of ascomycetes (Longato & Bonfante, 1997), ITS sequences are regarded as robust phylogenetic indications (eg Hershkovits & Lewis, 1996). The latter suggests that the ITS sequence analysis is more likely to reflect the true relationships between these isolates than microsatellite-primed PCR analysis.

None of the 14 isolates were positively identified to genus or species level by sequence comparison with the GenBank database; sequence similarities for 14 of the isolates ranging from 74.6-86.3% over approximately 530 nucleotides with the sequence for the genera *Trichopezizella* (=*Lachnum*), *Lachnellula* and *Geotalinipulvinella*. These two former genera belong to the family Hyaloscyphaceae and the latter to Leotiaceae: both families being assigned to the order Leotiales (Ascomycotina) (Hawksworth *et al*., 1996). The level of sequence similarity, between the *W. pungens* isolates and the two families in the Leotiales suggests that these isolates may belong to this order. This is perhaps significant, since *H. ericae* is assigned to the Leotiales. Isolate A03 was 86% similar to *Phialophora gregata* (Allington & Chamberlain) W. Gams (a genus known to inhabit plant roots, see Smith & Read, 1997) but over only 480 nucleotides and 83% similar over 478 nucleotides to *Trichopezizella* sp. This isolate is clearly quite different to all other isolates studied. Interestingly, a search of the GenBank database for closest matches to the full ITS sequences for a known isolate of the ascomycete *H. ericae*, indicated a similarity of 91% over 480 nucleotides with the basidiomycete *L. laccata* (Table 8). These results highlight the fact that little sequence data is available for mycorrhizal fungi and that more data need to be submitted to the database. Indeed, of the 13 recognised families within the
Leotiales, there are current ITS sequences for only five in the GenBank and EMBL databases (as of June 1998), and two of these were represented by single species only.

Sequence data can be very important in assigning taxonomic groupings to known fungi, especially for ericoid endophytes which are normally sterile in pure culture (Hutton et al., 1994; Perotto et al., 1995, 1996; Steinke et al., 1996). Egger & Sigler (1993), for example, identified a sterile mycorrhizal endophyte to be the anamorphic state of *H. ericae* on the basis of comparison of nucleotide sequence in a portion of rDNA. More recently, Chambers, Sharples & Cairney (1998a) used direct ITS sequence comparison to identify the previously unidentified mycobiont from *Pisonia grandis* R. Br. (Nyctaginaceae) as belonging to the Thelephoraceae, further emphasising the usefulness of the technique.

Similarly, Harney, Rogers & Wang (1997) used restriction site mapping of ITS region to characterise sterile dematiaceous fungi isolated from different hosts and locations. Two unknown sterile isolates were identified as *Phialophora finlandica* C. J. K. Wang & H. E. Wilcox.

None of the isolates from *W. pungens* have so far formed sexual fruiting structures in axenic culture, it is thus impossible to identify these fungi based on morphological characters. Previous work, however, indicates that sequence analysis of the ITS regions provides reliable determinations of taxonomic position. For example, Anderson et al. (1998) were able to identify two separate *Pisolithus* species among 62 isolates collected in a defined region in New South Wales using ITS sequences. The groupings of isolates were supported by basidiospore morphological characteristics. In some cases, molecular data have been shown to support the groupings of taxa based on morphological characteristics. For example, Liu, Rogers & Ammirati (1997) used ITS sequence analysis to investigate taxonomic and phylogenetic relationships among some species of *Dermocybe* and *Cortinarius*. Their results showed that the groups based on ITS sequence
data are consistent with previous morphological groupings of the taxa. In some cases, however, molecular data do not agree with conventional groupings and have suggested re-organisation of genus boundaries. Thus, Kretzer & Bruns (1997) used ITS sequence analysis to clarify the secatoid genus *Gastrosuillus* and its relationships to *Suillus*. The results showed that *Gastrosuillus laricinus* Singer & Both and *Suillus grevillei* (Klotzsch: Fr.) Singer were genetically indistinguishable. The two taxa were thus synonymised. Furthermore, all known *Gastrosuillus* species were transferred to *Suillus* based on sequence data. Similarly, Kretzer *et al.* (1996) found isolates of *Suillus granulatus* (Fries) Kuntze derived from different locations (North American, Europe and Asia) represented three different species.

While the taxonomic status of the sterile isolates from *W. pungens* remains unclear, the results of sequence comparisons show that none of the 14 isolates was identical to the known mycorrhizal fungus *H. ericae*. A similar situation has been reported in some ericaceous and epacridaceous plants. For example, ITS-RFLP analysis suggests that some sterile endophytes from *C. vulgaris* are taxonomically distinct from *H. ericae* (Perotto *et al.*, 1995, 1996). It thus maybe that, as suggested by Steinke *et al.* (1996), the range of fungi that form ericoid mycorrhizas is far greater than the number of taxa that have already been identified.

*H. ericae* is a widely distributed mycorrhizal fungus and has been isolated from a number of ericaceous plants collected at different habitats in different geographical locations including Europe and North America (Pearson & Read, 1973; Dalpé *et al.*, 1989; Egger & Sigler, 1993; Hambleton & Curnah, 1997). Recently it was identified from rhizoids of a liverwort collected from the Blue Mountains, New South Wales, Australia (Chambers Williams & Cairney, 1998b) so has a known southeastern Australian distribution. It is interesting that *H. ericae* was not identified within the endophyte population from *W. pungens*. It may be that further sampling would identify this species in epacrid roots,
since only juvenile plants that could be removed from the field with intact root systems were sampled. Mature epacrid plants can have extensive root systems, which would be expected to house a greater diversity of endophytes (Hutton et al., 1994). Also, only two isolation methods were attempted in the present study and it is likely that some endophytes may not have been isolated using this method (see Perotto et al., 1996). The direct plating isolation method used, however, was similar to that used by Pearson & Read (1973) for the isolation of H. ericae from ericaceous host plants, suggesting that if the fungus was present it would probably have been isolated in the present study. Alternatively, it may be that H. ericae does not form mycorrhizas with W. pungens or has a restricted distribution in the Blue Mountains, a region of New South Wales, Australia. Since H. ericae has also been identified in liverwort rhizoids from Antarctica (Chambers et al., 1998b), however, the latter seems unlikely. While the 14 isolates from W. pungens were most closely related to known ascomycetes, fungi resembling other taxonomic groupings including basidiomycetes, have been reported to be present on roots of plants of the Ericaceae and Epacridaceae based on microscopic examinations (Peterson et al., 1980; Allen et al., 1988; McLean & Lawrie, 1996). Further studies will be required to investigate their presence as mycorrhizal endophytes of W. pungens.

4.9 Conclusions

Microsatellite-primed PCR and sequence analysis of ITS regions revealed that there is a high level of diversity in ericoid mycorrhizal endophytes associated with W. pungens plants. Most genets were confined in individual plants and two genets were found in two adjacent plants. Root system colonisation by some endophytic mycelia was extensive. Sequence data suggested that six putative taxa were present within the endophyte population, with 1-4 taxa being found on the root system of each plant, indicating a few different taxa along with several genets of some of those exist in the root system of a
single plant. No isolates were positively identified as H. ericae. Closest matches with database ITS sequences suggested that many of endophytes probably belonged to the family Hyaloscyphaceae. Further work is necessary to stimulate conidiogenesis in pure culture and enable the delimitation of taxonomic status along with an investigation of the physiological role of the endophytes in the nutritional status of host plants.
References


Felsentein, J. 1993. PHYLIP (Phylogeny inference package) version 3.5c. Department of Genetics, University of Washington, Seattle.


Hutton, B. J., Sivasithamparam, K., Dixon, K. W. & Pate, J. S. 1996. Pectic
zymograms and water stress tolerance of endophytic fungi isolated from Western

polymorphic DNA markers are superior to somatic incompatibility tests for discriminating
genotypes in natural populations of the ectomycorrhizal fungus *Suillus granulatus.*
*Proceedings of the National Academy of Sciences of the United States of America* 90,
9159-9163.

recent advances in human DNA fingerprinting. In: *DNA Fingerprinting: Approaches and
Applications*, eds. Burke, T., Dolf, G., Jeffreys, A. J. & Wolff, R., Birkhauser Verlag,

Chitin degradation by *Hymenoscyphus ericae* and transfer of chitin-nitrogen to the host

Kernan, M. J. & Finocchio, A. F. 1983. A new discomycete associated with the roots of

approach for determining the rank of resolution. *Mycologia* 84, 139-153.


*Mycologia* 89, 586-589.

sequences from 38 recognised species of *Suillus sensu lato*: Phylogenetic and taxonomic


Molecular investigation of genetic diversity in ericoid mycorrhizal endophytes associated with *Woollsia pungens* (Cav.) F. Muell (Epacridaceae)

Guangwu Liu

Thesis submitted to School of Sciences,
University of Western Sydney, Nepean
as part of the requirement for
Master of Science (Honours)

1998
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
Declaration

I hereby certify that the submission of the thesis is my own work undertaken at University of Western Sydney, Nepean. No part of the work has been submitted to any other institutions for a degree.

Signature: Guangwu Liu

Guangwu Liu
# Table of contents

Acknowledgements .................................................. iii
Summary ........................................................................ 1

## Chapter 1: Literature review

1.1 Introduction ......................................................... 3
1.2 Functional aspects of ericoid mycorrhizas .................. 5
1.3 Fungi forming ericoid mycorrhizas ......................... 8
1.4 Genetic diversity of ericoid mycorrhizal fungi .......... 12
1.5 Molecular methods for identification of mycorrhizal fungi
  1.5.1 Restriction fragment length polymorphism (RFLP) analysis 15
  1.5.2 DNA-fingerprinting analysis .................................. 17
  1.5.3 PCR-RFLP analysis of ribosomal RNA genes (rDNA) .... 18
  1.5.4 Random amplified polymorphic DNA (RAPD) analysis .. 21
  1.5.5 Microsatellite-primed PCR analysis ..................... 23
1.6 Aims .................................................................... 26

## Chapter 2: Materials and methods

2.1 Isolation of putative fungal endophytes .................... 27
2.2 Mycorrhiza synthesis ............................................ 28
  2.2.1 Development of sterile seedlings ....................... 28
  2.2.2 Synthesis of mycorrhizas .................................... 30
2.3 DNA extraction .................................................. 32
2.4 Microsatellite-primed PCR .................................. 33
2.5 PCR amplification of the ITS region ....................... 34
2.6 Sequencing of ITS PCR products ......................... 35
Chapter 3: Results

3.1 Isolation of putative endophytes 37
3.2 Synthesis of mycorrhizas 38
3.3 Optimisation of microsatellite-primed PCR 43
  3.3.1 Optimisation of reaction conditions for microsatellite-primed PCR 43
  3.3.2 Screening of microsatellite primers 47
3.4 Microsatellite-primed PCR analysis 48
  3.4.1 Microsatellite-primed PCR using primer (GACA)4 48
  3.4.2 Microsatellite-primed PCR using primer (GTG)5 54
3.5 Phylogenetic analysis based on microsatellite-primed PCR fingerprints 60
3.6 ITS rDNA sequence analysis of selected isolates 63

Chapter 4: Discussion

4.1 Isolation of ericoid mycorrhizal endophytes 71
4.2 Germination of D. secundum seeds 73
4.3 Mycorrhiza synthesis 74
4.4 Cultural characteristics of mycorrhizal fungal endophytes 76
4.5 Genetic diversity of mycorrhizal fungal endophytes 77
4.6 Efficiency of microsatellite-primed PCR in identification of fungal genets 79
4.7 Distribution of individual mycorrhizal endophytes 81
4.8 ITS-rDNA sequence comparison of selected isolates 82
4.9 Conclusions 87

References 89
Acknowledgments

I thank Ku-ring-gai Municipal Council for permission to collect *Woollsia pungens* (Cav.) F. Muell (Epacridaceae) plants at Lovers Jump Creek, Turramurra (NSW). I am grateful to Germaine Beattie of Childrens Medical Research Institute (CMRI) in Sydney, Australia for performing automated DNA sequencing. I gratefully acknowledge my supervisors Dr. John W. G. Cairney and Dr. Susan M. Chambers for all of their guidance, assistance and support through the project and writing up of the thesis. In particular, I thank Dr. Susan M. Chambers for her assistance with the ANGIS computer analysis. I thank my wife Hui for her moral support and encouragement. I would also like to thank our parents for their care of our daughter during the time we are here in Australia.
Summary

Two hundred and forty three fungal isolates were obtained from roots of four *Woollsia pungens* (Cav.) F. Muell plants collected from a field site in New South Wales, Australia. One hundred and seventy five sterile isolates were slow growing and dark-coloured on 2% malt agar and were selected for further analysis. Microsatellite-primed PCR using the primers (GACA)$_4$ and (GTG)$_5$ separated these isolates into 50 genets. Isolates representative of 43 genets (including 168 isolates in total) formed typical ericoid mycorrhizal structures when inoculated onto roots of *Vaccinium macrocarpon* Ait (Ericaceae), confirming their status as mycorrhizal endophytes. It was estimated that a minimum of 43 genetically-distinct mycorrhizal mycelial genets were present in the root systems of the sampled *W. pungens* population with 7 to 15 distinct endophytic genets identified in each host plant, indicating that considerable genetic diversity exists within the endophyte population. While most genets were represented by less than eight isolates, three genets contained up to 41 isolates, suggesting that root system colonisation by some mycelia may be extensive. Most fungal genets were shown to be confined to individual plants, two genets (genets 32 & 33), however, were present within the root systems of two adjacent plants (plants C & D), suggesting that the two root systems were interconnected by the endophyte mycelia. The ITS region of thirteen mycorrhizal endophytes and a non-mycorrhizal isolate selected from the endophyte population were sequenced and compared to the sequence of *Hymenoscyphus ericae* (Read) Korf & Kernan as well as sequences from the GenBank database. A phylogenetic tree was generated from the nucleotide sequence data. This analysis revealed that six putative taxa were present in the root systems of four host plants. Four taxa were shown to be present in the root system of plant A, one in plant B, three in plant C and two in plant D, indicating that the endophyte population of a single *W. pungens* plant comprises mycelia of several taxa along with several genets of some of these. No isolates were positively identified to genus or species level. Closest matches with fungal sequences in the
database indicated that most isolates probably belonged to the order Leotiales. Cluster analysis on the basis of the ITS sequences indicated that *H. ericae* was not clustered together with any endophytes from *W. pungens*, suggesting that endophytes of *W. pungens* are not identical to the known ericoid mycorrhizal fungus *H. ericae*. *H. ericae* had a low degree of sequence similarity with isolates from *W. pungens*, with similarities ranging from 68.3-80.6%. Cluster analysis based on DNA sequences of the ITS region did not fully support the groupings inferred from microsatellite-based fingerprinting.