Interspecific and intraspecific variation of ectomycorrhizal fungi associated with *Eucalyptus* ecosystems as revealed by ribosomal DNA PCR–RFLP

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Gondwanan vegetation, and the Australian region in particular, is species rich for ectomycorrhizal fungi in epigeous and hypogeous forms with over 100 species recorded in small (1 ha) patches of forests. Distinguishing co-occurring ectomycorrhizal fungi as root associations in native (natural or wildlands) vegetation or plantations and discriminating them from other larger basidiomycetes, e.g. wood and leaf litter decomposer fungi, places large demands on molecular identification, especially if interspecific similarities and intraspecific variation occur in target sequences. One hundred and nine species of larger basidiomycetes from a single forest location were characterised by PCR–RFLP profiles of two genomic regions (nuclear rDNA ITS and mtLSU). Over one-third of the species for which multiple isolates were tested showed intraspecific variation in either one or both genomic regions. This remarkably high variation questions previous assumptions about intraspecific ITS sequence variation and highlights the value of integrated molecular and morphological databases including voucher specimens. It also emphasises the value of molecular investigations that use more than one genomic region. Interspecific similarities were common among the *Cortinariaceae*, especially in the ITS region. Discrimination of most *Cortinariaceae* species was achieved using variation in the mtLSU region in conjunction with the ITS. This new information raises the possibilities that the ITS sequence is more conserved and the mtLSU more variable than among species of the other 23 families. In the other families, interspecific ITS variation was greater and the mtLSU profiles grouped species within families. The high variation in the two genomic regions indicated possible differences in the fungal population structure between two adjacent, differently managed blocks of *Eucalyptus marginata* forest. The significance of this variation to ecology, biodiversity assessment and ecosystem management are discussed.

INTRODUCTION

PCR–RFLP (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) detects DNA sequence variation by amplifying a target genomic region then digesting it with restriction enzymes which recognise and cleave the DNA strand at a specific, short (usually 4–6 base pairs) DNA sequence. Electrophoresis results in a profile of fragment sizes, depending on the number and location of restriction sites in the amplified product. Appropriately targeted regions can provide resolution at the species level.

PCR–RFLP identification of fungi has created a surge of interest in applying this technique to the analysis of ectomycorrhizal fungal communities present on root tips, i.e. the functional part of the association from a nutrient cycling perspective, rather than relying on the spasmodic, sometimes cryptic and often unpredictable production of basidiomata as an indication of the presence or absence of particular species of these symbiotic organisms. Distinguishing which ectomycorrhizal species are present and whether or not they are forming basidiomes in Australian forests, woodlands and woody heaths is a challenge due to the high mycorrhizal diversity, evanescent basidiome development and a large diversity of hypogeous forms (Tommerup *et al.* 1994, Bougher 1995, Bougher & Tommerup 1996, Bougher & Syme 1998, Tommerup & Bougher 2000). Understanding the ecology and measuring ectomycorrhizal basidiomycete communities is hampered by the cryptic nature of these fungi, many of which are unculturable, have hypogeous basidiomata or only produce them rarely or under certain conditions (Trappe 1977, Luoma & Frenkel 1991, Johnson 1994, Tommerup & Bougher 2000).

A decline in the occurrence of ectomycorrhizal fungal sporocarps has preceded visible forest deterioration throughout Europe (Arnolds 1991). Hence the use of ectomycorrhizal fungi as bioindicators of forest sustainability has been proposed (Fellner & Pešková 1995, Tommerup & Bougher 2000). However, correlation between the abundance of basidiomata and the presence of that species on root tips has been shown to be low for some fungal species in pine forests (Gardes & Bruns 1996, Gehring *et al.* 1998) and is unknown for eucalypt forests. The detection of the fungus in the mycelial phase avoids the complex factor of environmental variables which stimulate basidiomata production.

PCR–RFLP identification of the fungal partner of mycorrhizas requires a database of PCR–RFLP profiles from identified material, cultures or basidiomes. In small areas of low diversity, this is not a major undertaking (Erland, 1995; Gardes & Bruns, 1996), but if the study area has a high level of fungal diversity, or the database is to be used over a wide geographical area, the discrimination of a greater number of species is necessary. Preliminary studies have demonstrated a potential for fungal discrimination among many species using PCR–RFLP of the rDNA ITS (internal transcribed spacers of ribosomal DNA) (Kårén *et al*. 1997, Farmer & Sylvia 1998). These studies were based in the Northern Hemisphere and included fungi from a broad geographical area, with only a single collection of many species. This presumes a low level of intraspecific variability, though some intraspecific variability in ITS sequence has been found, mainly in geographically separated populations (Gardes *et al*. 1991, Liu, Rogers & Ammirati 1997).

The biota of the Southern Hemisphere is extremely diverse, especially in the south-west corner of Western Australia (Hopper *et al*., 1996). The geological and climatic history has probably contributed to this condition, as the south-west corner of the continent has been above sea-level and unglaciated for the last 200 Myr. The natural vegetation is an ecological mosaic with a high degree of endemism in the plant, animal and fungal biota. Woody plant communities have higher diversity, degree of endemism and often more complex ecosystems than the Northern Hemisphere systems previously studied for ectomycorrhizal fungi (Bougher & Tommerup 1996, Hopper *et al*. 1996, Specht 1996, Tommerup & Bougher 2000). In an Australian context, the south-west region has particularly high endemism in the 9000 plant species and ecosystems have high diversity, high species richness with Gondwanan origins and often high intraspecific variation (Hopper *et al*., 1996). In one forest type, *Eucalyptus marginata* (jarrah), basidiomata of over 100 ectomycorrhizal species had been collected at one 3 ha study site (Bougher, Tommerup & Bolsenbroek, unpubl.). In addition to this high species richness, many Australian fungi are, as yet, unnamed. This high diversity and taxonomic shortfall place greater demands on the tools to be used in fungal identification. An accurate assessment of interspecific and intraspecific discrimination among the fungi at any study site is needed.

In view of this high level of diversity, we considered it was necessary to characterise as thoroughly as possible the fungal community from identified material at our study site before attempting to use the PCR–RFLP profiles to identify fungi in ectomycorrhizas. Our main concern was the ability to discriminate the large number of species, previous studies having compared a maximum of 44 species (Kårén *et al*. 1997). In addition, with a large number of reference species, it is necessary to be able to distinguish species by comparing patterns across gels, rather than running samples side-by-side on the same gel. Allowance must therefore be made for sizing inaccuracies, which may result in the grouping of similar profiles that may be distinguishable when electrophoresed on the same gel. We doubted whether one genomic region would provide enough characters to distinguish over 200 species. Kårén *et al*. (1997) found species in the genera *Cortinarius* and *Dermocybe* to be the most difficult to separate, with ten of the 17 morphospecies producing only three PCR–RFLP profiles.

Cortinarius is a large and diverse assemblage of fungi for which taxonomic discrimination of individual species is complex, as they exhibit plasticity of morphological characters (Bougher & Hilton 1989, Horak & Wood 1990) and may lead to misidentification of collections, particularly if all the developmental stages are not represented in the collection. The genus is well represented in Australia with many species, both named and as yet un-named, known from *E. marginata* forest (Hilton, Malajczuk & Pearce 1989; CSIRO Forestry and Forest Products Mycology Herbarium, Bougher & Tommerup, pers. comm.). While it may be possible to distinguish ten species from each other by PCR–RFLP (Chambers, Sawyer & Cairney 1999), it must be shown that they are also distinguished from all other species at the site before identification of the fungal partner of an ectomycorrhiza can be regarded as positive.

We also wished to check, where possible, the degree of intraspecific variability which might be found as we expected to find unidentified profiles from root-tips, as have most other workers (Gardes & Bruns 1996, Gehring *et al*. 1998, Jonsson *et al*. 1999). Gardes & Bruns (1996) produced 20 RFLP profiles from mycorrhiza in a patch of pine forest, twelve of them matched profiles of morphologically identified fungi, and eight which did not. These eight profiles were regarded as each representing an unknown taxon, which was considered likely to be a species or group of species. Jonsson *et al*. (1999) produced 69 unmatched PCR–RFLP profiles from root-tips in Swedish mixed forests, each of which they regarded as representing a different taxon.

To distinguish a wide array of Australian fungi and to discriminate them from plants Glen *et al*. (2001) developed sensitive larger basidiomycete specific primers for rDNA ITS. Importantly, they did not also amplify plant, ascomycete or bacterial DNA as had previous commonly-used primers (Gardes & Bruns 1993, Kårén *et al*. 1997) and this is critical if the target DNA is in complex mixtures of plant, fungal (including soil ascomycete), bacterial and possibly insect and other DNA. The new primers amplified DNA from a larger number of species than previous basidiomycete-specific primers, and from diverse orders of basidiomycetes (Glen *et al*. 2001).

This paper highlights the problems we encountered in establishing a PCR–RFLP database for eucalypt-associated ectomycorrhizal fungi, and discusses the possible reasons and repercussions of these difficulties. We analysed 197 identified basidiome collections of 109 species of fungi from *E. marginata* forest. In addition to developing identification tools the data provided information of taxonomic significance with implications for diversity at the species and subspecies levels. Variation in the ITS sequence has been used to infer phylogenetic relationships among fungi (Liu *et al*. 1997, Chambers *et al*. 1999). Often a single isolate of each species is analysed and further examination of the validity of this for a wide diversity of fungal species is essential to taxonomic theory and practical applications in ecology, biology and ecosystem management. An initial assessment of intraspecific variation by PCR–RFLP of the chosen region may reinforce

the validity of the analysis or indicate the need for inclusion of more specimens.

MATERIALS AND METHODS

Site

The study site had two blocks of the same type of eucalyptus forest, separated by an unsealed road and firebreaks. It was approximately 15 km east of Dwellingup, 100 km south-south-east of Perth, Western Australia. The dominant tree was *Eucalyptus marginata*, with occasional *Corymbia calophylla* (marri, formerly *E. calophylla*), *Banksia grandis* and *Persoonia longifolia*, and an understorey of shrubs including *Acacia pulchella*, *Macrozamia reidlei*, *Pteridium esculentum* and *Dryandra nivea*, which is type T vegetation of Havel (1975a, b). Vegetation graded to a small strip of type S 100 m from the northern margin. *Allocasuarina fraseriana* occurred occasionally towards this strip. One block (block A), had not been burnt for 66 years, and the other (block P), was spring burnt every 6–7 years using standard prescribed burning (S. Crombie, WA Department of CALM, pers. comm.). Prior to 66 yr ago, the blocks had the same fire regime. Pristine forest was selectively logged at the end of the 19th and early in the 20th century.

Sample collection

Both fresh collections and dried material from the CSIRO Forestry and Forest Products Mycology Herbarium, Perth, WA, were used as sources of DNA from identified basidiomata. Fresh basidiomata were collected from the study site every week between May and September 1995 and on less frequent occasions in 1996 to 1998, making a total of four years' collections of fresh basidiomata. Collections from elsewhere in the locality were also included to obtain as complete a set of reference species as possible. Two herbarium collections from *E. marginata* forest at Walpole, 300 km south of Dwellingup, were included to augment the single collections found in 1995–98. Unless identification from a single basidiome was unequivocal, only specimens including the full range of development of basidiomata were collected, to ensure positive identification from a complete set of taxonomic characters, including microscopic characters. For many species, two to twelve separate collections were analysed to assess the consistency of the data. The species were identified by characteristics of the basidiomata and spores with dried specimens deposited at the CSIRO mycology herbarium. Saprotrophs, wood and litter decaying fungi, were collected as well as ectomycorrhizal species because some genera have putatively mycorrhizal and saprotrophic species, and it is necessary to be able to differentiate these from the symbiotic fungi in environmental samples. Fresh basidiome samples for DNA extraction were stored on ice and freeze-dried as soon as possible after collection.

In some instances, DNA was extracted from two basidiomata of a herbarium collection for double-checking of collection integrity and PCR–RFLP results. A list of the collections of 109 species of larger basidiomycetes from the study site is given in Table 1. PCR–RFLP profiles for 41 of these species (Table 1) are given in Glen *et al*. (2001).

DNA extraction, primers, PCR amplification, restriction digestion and PAGE

DNA was extracted, amplified, digested, electrophoresed and the fragments sized as described in Glen *et al*. (2001). Primers ITSF and ITSR (Glen *et al*. 2001) for the rDNA ITS and ML3 and ML4 (White *et al*. 1990) for the mitochondrial LSU rDNA were used with the conditions specified in Glen *et al*. (2001) to selectively amplify basidiomycete DNA. For *Hydnum rufescens*, HRITSF was used in place of ITSF, and for *Austrogautieria manjimupana*, AMITSR was used in place of ITSR (Glen *et al*. 2001). After electrophoresis on 6% acrylamide gels, fragments were sized from a digital gel image using Molecular Analyst (Biorad).

Integration of molecular and morphological characters

PCR–RFLP profiles for each collection were stored in a Microsoft Access database as a series of fragment sizes. Repeated electrophoresis and sizing indicated that sizing of a particular fragment could vary by up to 3%, so searches for a particular profile incorporated a 5% margin for sizing error. These searches revealed similarities among species which would prevent unambiguous identification of fungi. For the purposes of this paper, the sizes have been translated into letter codes to improve visual ease of comparison. Morphological examination of collections was conducted independently. If intraspecific variation in PCR–RFLP profiles was found, all collections attributed to the same species were re-examined and a second basidiome sample was taken for verification of PCR–RFLP profiles.

RESULTS

PCR–RFLP

The PCR product sizes mostly ranged from 850 to 950 bp for the ITS fragment, and from 790 to 1640 bp for the ML3/ML4 fragment with a few larger and one smaller product of 216 bp for *Leucopaxillus lilacinus*. Codes for the sizes of the restriction fragments produced by digestion of the ITS fragment with *Alu*I, *Taq*I, *Hin*fI and *Hae*III and of the ML3/ML4 fragment digested with *Sau*3AI and *Hae*III are given in Table 2. The sum of fragment sizes may not be the same as the length of the original PCR product, as fragments smaller than 100 bp were not included, and where two fragments co-migrated on the gel, they are recorded as one fragment. In a few cases, it appeared that more than one fragment was amplified by the ML3/ML4 primer pair and this resulted in a fragment sum greater than the original product.

Fragments between 100 and 500 bp were reproducibly sized with an error of $+/-3\%$, however some codes, e.g. code CJ for *Alu*I and BA for *Taq*I, cover a broad range of sizes. This is because a large number of fungi, mainly in the family *Cortinariaceae*, with similar profiles produce a continuum of fragment sizes without clear demarcation of groups. For instance, CJ represents one fragment between 474 and 581 bp and another between 148 and 170 bp when digested with *Alu*I (Table 2). These sizes cover a broad range and some discrimination between the extremes of these ranges was

Table 1. Fungal collections used to assess RFLP of nuclear rDNA ITS and mt LSU PCR products. Herbarium nos are accession codes, E and H numbers from the CSIRO Forestry and Forest Products Mycology Herbarium, G numbers are Glen collections.

Family	Species	Herbarium nos
Amanitaceae	*Amanita eucalypti*	E5451, E5385
	A. sp. sect. *Lepidella*	E5584
	A. xanthocephala	E5445, G9509, G9510, G9511
Bankeraceae	*Phellodon* sp.	E5778[a]
Bolbitiaceae	*Conocybe* sp.[b] subgen. *Pholiotina*	E6028, E5920, E5773
Boletaceae	*Austroboletus occidentalis*	E5450, G9567
	Boletellus obscurecoccineus	E6001[a]
	Boletoid hypogeous	H7399[a]
	Boletus prolinius cfr sect. luridi	E5642, E5480, G9531
	B. sinapecruentus	E5444[a]
Clavulinaceae	*Clavulina vinaceo-cervina*	E5911, E5777, E5776, E5539, E5775
Coniophoraceae	*Podoserpula pusio*	E5542[a]
Cortinariaceae	*Cortinarius australiensis*	E5391, E5419
	C. austroalbidus	E5997, E5933
	C. cfr bellus	E5921, E5989
	C. fiveashianus	E5636
	C. globuliformis	H7327, H7260
	C. lavendulensis	E5517, E5985
	C. microarcheri	E5995, E5994, G9527
	C. rotundisporus	E5540
	C. sinapicolor	E5538, G9521,G9522, G9523, G9524
	C. sp. A	E5903
	C. sp. B	E5593
	C. sp. C	E6026, E6029
	C. sp. D	E5441
	C. sp. E	E5930, E5883
	C. sp. F	E6031
	C. sp. G	E5592
	C. sp. H	E5887, E5907
	C. subgen. *Leprocybe* sp.	E5632
	C. subgen. *Sericeocybe* sp.	E5442
	C. subgen. *Phlegmacium* sp. 1	E5516, E5645
	C. subgen. *Phlegmacium* sp. 2	E5893
	C. subgen. *Phlegmacium* sp. 3	E5908, E5478
	C. subgen. *Phlegmacium* sp. 4	E5927, E5537
	C. subgen. *Phlegmacium* sp. 5	E5848
	C. sublargus	E5392, E5387
	C. cfr sublargus 'conic cap'	E5853
	C. cfr sublargus 'short stem'	E5393
	C. cfr sublargus group A	E5477, E5596, E5582
	C. cfr sublargus group B	E5386, E5388
	C. cfr sublargus group C	E5389, E5577, E5446
	C. cfr sublargus group D	E5514, E5479
	C. vinaceo-cinereus	E5701, E5882, E5993
	Dermocybe austroveneta	E5926, E5987, G9526, G9548
	D. basirubescens	E5897
	D. clelandii	E5988, E5588, G9528
	D. cramesina	E5641
	D. sp.	E5984
	D. splendida	E5639
	Descomyces albellus	H7328[a]
	Galerina unicolor[b]	E5925[a]
	Gymnopilus austrosapineus[b]	E5448[a]
	Hebeloma aminophilum	E6025, E6032, E6033, E6034, E5929
	Inocybe australiensis	E5886[a]
	I. cystidiocatenata	E5774, E5986, E5889
	I. fibrillosibrunnea	E5898[a]
	I. sp.	E5885[a]
	Protoglossum sp. nov.	H7259[a]
Crepidotaceae	*Tubaria cfr furfuracea*[b]	E5928[a]
Elasmomycetaceae	*Macowanites* sp.	H7204, H7136
	Martellia sp. A	H7401
	M. sp. B	H7203
	M. sp. C	H7503
	E. cfr sericeum[b]	E5698[a]
	Entoloma[b] sp.	E5856[a]
Fistulinaceae	*Fistulina hepatica*[c]	E5919[a]
Gautieriaceae	*Austrogautieria manjimupana*	G9549
Hydnaceae	*Hydnum rufescens*	E5744, E5892
Hygrophoraceae	*Hygrocybe pratensis*[b]	E5891, E5909
Mesophelliaceae	*Castoreum* sp.	H7301[a]
Paxillaceae	*Paxillus muelleri*	E5390[a]
Pluteaceae	*Pluteus atromarginatus*[b]	E5895[a]

Table 1 (*cont.*)

Family	Species	Herbarium nos
Entolomataceae	*Entoloma cfr chalybaeum*[b]	E5583[a]
	P. lutescens[b]	E5637[a]
Ramariaceae	*Ramaria cfr formosa*	E5443[a]
	R. holorubella	E5575[a]
	R. lorithamnus	E5541, E5854, E5522, E5595, E5906, G9516,G9518, G9540,G9547
	R. sp.	E5904[a]
	R. versatilis	E5634[a]
Richoniellaceae	*Richoniella* sp.	H7202[a]
Russulaceae	*Lactarius clarkeae*	E5841[a]
	Russula cfr adusta	E5855[a]
	R. clelandii	E5512, E5513, E5932, E5476, E5844, E5579, E5591, G9502, G9504, G9505, G9506, G9507
	R. multicolor nom. prov.	E5850[a]
	R. neerimea	E5585, E5590, E5843, E5851
	R. persanguinea	E5905[a]
	R. sp. A	E5842, E5849, E5852
	R. sp. B	E5474, E5543, E5574
	R. sp. C	E6003[a]
	R. sp. D	E5587, E6004
	R. sp. F	E5475[a]
Stephanosporaceae	*Stephanospora flava*	H7509[a]
Strophariaceae	*Pholiota cfr highlandensis*[b]	E5594[a]
	P. sp.[b]	E5521
Thelephoraceae	*Hydnellum* sp.	E5896[a]
Tricholomataceae	*Armillaria luteobubalina*[c]	E0026[a]
	Laccaria sp. B (Grgurinovic, 1997)	E5518, E6030, G9529, G9530
	Lepiota booloola[b]	E5899[a]
	Leucopaxillus lilacinus	E5573[a]
	Lyophyllum tylicolor[b]	E5797[a]
	Mycena kurramulla[b]	E5894[a]
	Mycena pura[b]	E5888[a]
	Tricholoma eucalypticum	E5998, E6027
	T. subgen. *Contextocutis* sp. 1	E5884, E5703, E5999
	T. subgen. *Contextocutis* sp. 2	E5576
	T. subgen. *Tricholoma* sp. 3	E5515, E5589, E6000
	T. subgen. *Tricholoma* sp. 4	E5449
	T. subgen. *Tricholoma* sp. 6	E5910

[a] Details for these fungi are given in Glen *et al.* (2001).
[b] This species is saprotrophic and not known to be ectomycorrhizal.
[c] This species is parasitic and not known to be ectomycorrhizal.

possible when searching the database by fragment size, however division into discrete groups was prevented by the continuum of sizes of the many specimens. For example, E5993 had ITS *Alu*I fragment sizes of 485 and 151, which is clearly different from E5386 with fragment sizes of 559 and 150, yet when interspersed with E5984 (492 and 153 bp), E5538 (500 and 159 bp), E5930 (521 and 152 bp) and 19 other species, it was not possible to divide this group into discrete categories.

Intraspecific variation

ITS variability

One hundred and twenty nine collections of 42 fungal species (2–12 collections per species) were analysed to explore the possibility of local intraspecific variation. PCR–RFLP profiles are shown for these 42 species in Table 3. Intraspecific variation in PCR–RFLP of the ITS region was seen in twelve species, four *Cortinarius*, four *Russula*, and one each of *Hygrocybe*, *Tricholoma*, *Austroboletus* and *Clavulina*. For three of the four variable *Cortinarius* species, and one of the *Russula* species, one collection came from forest block A, while another which produced a different PCR–RFLP profile, came from block P. This was also the case for *Clavulina vinaceo-cervina*, for which three colour forms were collected: pink, brown and purplish. One of the brown collections shared an ITS profile with the pink collection, both these collections were from block P. The other brown collection was from block A, as were the purplish collections with which it shared an ITS profile. In *Tricholoma* subgen. *Contextocutis* species 1, one collection from block P differed from two collections from block A. The fourth *Cortinarius* species (*C. cfr sublargus* group C) had a contrasting pattern. The two collections sharing an ITS profile were from different blocks, and a different profile also occurred on block A. Intraspecific variation also occurred on block A in three of the four variable *Russula* species and in *Austroboletus occidentalis*. No intraspecific variation was found on block P except in the saprophyte *Hygrocybe pratensis*, two collections of this species from block P gave different ITS profiles.

The degree of intraspecific difference was also variable. In *Cortinarius cfr bellus*, the variation occurred with only one of the four restriction enzymes and was suggestive of a difference

Table 2. Sizes of restriction fragments for codes given in Tables 3–6. Sizes are reproducible with an error of ±3%, for fragments between 100 and 500 bp, but some codes, e.g. CJ for *Alu*I digestion, cover a broader range of sizes (see text for discussion).

A code	ITS *Alu*I fragment sizes	T code	ITS *Taq*I fragment sizes	H code	ITS *Hae*III fragment sizes	F Code	ITS *Hin*fI fragment sizes	MS Code	ML3/ML4 *Sau*3AI fragment sizes	MH code	ML3/ML4 *Hae*III fragment sizes
AA	820	AB	548, 458	AA	737–960	AA	940, 485, 469	AA	1075	BA	2083, 460, 335, 203, 187, 105
AB	768	AC	595, 384	AB	618	BA	575, 399, 151, 145	AB	834-940	BB	1950, 499, 226
BB	620, 201, 100	BA	438-512, 348-407	BA	698, 312	CA	461, 444, 140	BE	1288, 636, 582, 224, 125	BC	1854, 462, 184
CC	541, 273-302	BC	495-564, 324-340	BB	664, 213, 127	CB	458, 426, 347, 129	CA	917, 791, 349, 219, 205	BD	1650, 667, 169
CD	556, 235	BD	471, 327	BC	679, 223	CC	456, 228, 142, 132	CB	965, 268	BE	1570, 759, 193
CE	536, 256	CA	572, 213, 184	BD	674, 206	CD	390-464, 339-377, 129	CC	896, 276	BH	1051, 663, 219, 186
CF	496, 254	CB	496, 222, 152	BE	683, 188	CE	430, 409, 135	CD	884, 187, 122	BK	943, 656
CH	501, 192	CE	480, 202, 190	BF	677, 129	CF	394, 282, 130, 101	CE	811, 372, 124, 102	CA	968, 187
CJ	474-581, 148-170	CF	472, 202, 180	CA	759, 115, 102	CG	410, 228, 130	CG	740, 580, 520, 222, 123	CD	815, 671
CK	450, 153	CG	475, 203, 173	CB	727, 113	CH	422, 224, 149, 128	CH	750, 518, 221, 120	CE	785-884, 408
DB	500, 240, 192, 100	DD	490, 278	DA	524-614, 298-321	CJ	421, 206, 187, 130	DA	662, 561	CG	834, 182
DC	538, 180, 150	DE	474, 272	DB	530-626, 241-271	CK	435, 316	DD	686, 504, 329	DC	700, 486
DD	550, 168, 145	ED	463, 225, 143	DC	628, 196	CM	425, 292, 129	DF	589, 544, 103	DD	677, 415
DF	535, 170, 136	FC	448, 306, 140, 118, 102	DD	600, 348	CN	392, 280, 130	DJ	700, 269	DH	587-671, 300
EC	487, 226	FD	429, 342, 108	EA	554, 284, 258	DA	392, 135, 108, 100	DK	634, 262, 100	DJ	687, 264
EG	433, 248	FE	411, 321, 104	EC	533, 202	DB	385, 373, 131	EC	683, 124	DK	668, 224
EH	452, 169	FF	428, 316	ED	542, 191, 128	DC	376, 226, 205, 130	FA	571, 442, 289, 141, 123	EA	613, 556
EJ	412, 251, 142	GB	404, 387	FA	465, 223, 146, 141	DE	387, 240, 214, 131	FE	559, 266, 101	ED	504-607, 222
EL	439, 163, 152, 121	GE	396, 310, 113	FB	425, 332, 210, 196	DF	383, 229, 131, 109	FH	572, 307, 101	EF	574-718, 206
EN	411, 218, 124	GF	395, 241, 205	GA	346, 309, 244	DG	391, 206	FK	565, 330, 229	EG	607-772, 185
EP	400, 155, 132	GG	400, 108	GB	340, 318, 272	DH	374, 253, 223, 142	FL	474-541, 281-322, 103	FA	640, 534, 182
FA	394, 240, 204	HA	364, 270, 194	GC	350, 335, 238	EA	368, 314, 125	FM	502, 348, 103	FB	615, 405, 318, 191
FD	388, 204, 147	HD	374, 193, 159, 118	GD	350, 235, 191	EB	369, 246, 207, 135	GA	529, 234, 124, 104	FE	601, 213, 106
FF	340, 168, 138, 128	HG	342, 301, 192	GE	348, 262, 242	EC	370, 225, 208, 129	GB	539, 197, 125, 103	FH	554, 249, 126
FK	335, 138, 110	HH	343, 266, 193	GF	307, 273, 200	EE	342, 268, 194, 128	GC	550, 182, 123, 102	FK	556, 203, 182
FL	329, 260, 210	HJ	336, 264, 210	GG	324, 319, 198	EF	357, 318, 137, 116, 101	GE	545, 144, 126	GA	600, 258
FO	316, 211, 152	JC	335, 213	GH	356, 204, 129	FA	358, 214, 126	GF	526, 132, 124	GB	585, 236
FP	305, 216, 192	JD	322, 179	GJ	332, 209, 193, 100	FB	364, 213, 198, 126	GG	559, 156, 129	GC	657, 112
FQ	310, 152	KB	288, 200, 188, 100			FC	351, 237, 204, 128	HA	485, 171, 125, 105	HA	572, 506, 333
GB	270, 205, 146, 113	KJ	210, 146, 141, 119, 106			FD	325, 215, 113	HB	527, 485, 174, 128	JB	396, 294, 183
GC	268, 224, 148, 112	KK	216, 192			FF	365, 133	HC	465, 257, 125, 104		
GD	276, 218, 206, 150, 124, 107					GA	314, 288, 140, 113	HD	490, 142, 132, 125		
						HA	242, 182, 140, 128	HE	445, 148, 136, 122		
								HF	486, 148, 128, 106		
								HG	432, 132, 124		
								HH	454, 150, 125		
								HJ	442, 162, 126		
								HK	443, 167		
								JA	361, 316, 116		
								JC	356, 144, 126, 104		
								JD	297, 265, 101		

in only one restriction site as two of the fragments in one pattern add up to the same size as the fragment in the other profile. In *Cortinarius* subgen. *Phlegmacium* sp. 3 and *C. cfr sublargus* group C, all four digestions were variable. There were three variable digestions for *Cortinarius* sp. E. In *Clavulina vinaceo-cervina*, variation indicative of a 30 bp indel was apparent in *Alu*I, *Taq*I and *Hin*fI restriction profiles, with a *Hae*III restriction site difference.

Table 3 lists 30 species for which multiple collections were made (2–9 collections) and for which no intraspecific variation was observed in digestion profiles of the ITS. Intraspecific similarities with collections from other areas were also noted. *Austrogautieria manjimupana* (G9549) gave the same ITS profile as H7157 from Walpole (Glen *et al*. 2001). *Cortinarius australiense* from Walpole (E5419) also matched the Dwellingup collection (E5391, Table 3). The four *Laccaria* sp. B collections had matching profiles with E439 (Glen *et al*. 2001), a culture of the same species isolated from near the study site in 1980.

Mitochondrial variation

Intraspecific variation in the ML3/ML4 region occurred in eight species, five of these members of the *Cortinariaceae* (Table 3). In *Russula clelandii* and *C. cfr sublargus* group C the collections with different ITS profile were also different by *Sau*3AI digestion of the ML3/ML4 fragment. Two collections of *C. sinapicolor* gave a much larger ML3/ML4 product than the other collections, with different RFLP profiles. The pink form of *Clavulina vinaceo-cervina* had a different *Sau*3AI profile to the brown and purplish forms. The three collections of *Inocybe cystidiocatenata* each had different

ML3/ML4 profiles, the profile for E5889 consisting of the same as that for E5986 with extra bands. Several collections gave more than one fragment from the ML3/ML4 PCR, which on digestion gave multiple faint fragments. For all these collections, a second fruit-body sample was tested to confirm the results. One collection each of *Hydnum rufescens*, *Cortinarius* subgen. *Phlegmacium* species 3 and *C. globuliformis* behaved thus, while another of each gave a single PCR product, with an interpretable RFLP profile.

Interspecific variation

ITS variability

Thirty-six species were not discriminated by our method for comparing PCR–RFLP of the ITS with *Alu*I and *Taq*I. These were grouped according to ITS PCR–RFLP profiles in Table 4. Twenty-two species, 17 *Cortinarius*, 4 *Dermocybe* and 1 *Inocybe* gave similar profiles, with codes CJ for *Alu*I digestion and BA for *Taq*I digestion (AT group 1, Table 4). Other interspecific AT groups include three *Cortinarius* and one *Dermocybe* species (AT group 2, Table 4), and *Cortinarius austroalbidus*, *Hebeloma aminophilum*, and *Pholiota* sp. (AT group 3, Table 4). Four further groups after *Alu*I and *Taq*I digestion each consist of two closely related species (AT groups 4–7, Table 4). These are: *Amanita* sect. *Lepidella* sp. and *A. eucalypticum*; *Dermocybe basirubescens* and *Cortinarius* sp. E (one of the two collections); *Tricholoma* subgen. *Contextocutis* sp. 1 and sp. 2; and *Tricholoma* subgen. *Tricholoma* sp. 3 and sp. 4.

Digestion with *Hin*fI and *Hae*III discriminated six of the *Cortinarius* species in AT group 1. Eight of the remaining *Cortinarius* species shared the same ITS PCR–RFLP profile with one *Dermocybe* species, and two *Cortinarius* species and one *Inocybe* species shared another profile. These are referred to as ITS group 1 and ITS group 2, respectively. Two *Dermocybe* species are in ITS group 3, one each of *Cortinarius* and *Dermocybe* in ITS group 4. The two groups of *Tricholoma* subgenera were also not distinguished by further digestions of the ITS. The remaining AT groups were all discriminated to species level after four digestions.

Those species not discriminated by digestion of the ITS consist of two groups of *Tricholoma* species, one from each of the subgenera *Contextocutis* and *Tricholoma*, and five groups of two to nine species of *Cortinariaceae*.

Mitochondrial variation

The ML3/ML4 PCR–RFLP showed interspecific similarities, particularly in the *Russulaceae* and *Amanitaceae* (Table 5). The close relationship of members of the *Elasmomycetaceae* with the *Russulaceae* is also highlighted by similarities in ML3/ML4 PCR–RFLP profiles. Five species of *Russula*, two of *Macowanites* and one out of three *Martellia* species shared identical ML3/ML4 PCR–RFLP profiles (HH/ED). Another two *Russula* species and *Lactarius clarkeae* shared very similar profiles (HE/ED) to these eight species, however there was also intraspecific variability in one of these *Russula* species, *R. clelandii*. Four *Russula* species had different profiles, though some similarities were also evident, e.g. same *Hae*III, different *Sau*3AI profiles. Two species of *Torrendia* (from a different site; Glen *et al*. 2001) shared an ML3/ML4 profile with one species of *Amanita*, while a third *Torrendia* species shared a very similar profile with two *Amanita* species. Three species of *Boletus* shared an ML3/ML4 profile though two others had different profiles. Three species of *Tricholoma* were also grouped, as were two species of *Entoloma*. *Sau*3AI digestion resulted in much greater variability than *Hae*III digestion. Twenty-four species (20 *Cortinarius*, 1 *Inocybe* and three *Dermocybe* spp.) shared the same *Hae*III digestion profile (EG, Tables 3, 4 and 6), though there were ten different *Sau*3AI profiles in this group.

There was greater variation in ML3/ML4 profiles among members of the *Cortinariaceae*. Amplification and digestion of the ML3/ML4 fragment was able to resolve some of the ITS groups in this family thereby distinguishing more species. ITS group 1 (Table 4) was separated into three individual species, two with variable ML3/ML4 profiles, and two subgroups each of three species. However in ITS group 2, an ML3/ML4 product was not obtained from one of the two *Cortinarius* species, the other had a profile consisting of many faint fragments, and the three *Inocybe cystidiocatenata* collections each gave different ML3/ML4 profiles, resulting in inconclusive separation. ITS groups 3, 4 and 5 were resolved into individual species by digestion of the ML3/ML4 fragment. The species of *Tricholoma* that were not distinguished by the ITS were also not separated by the mitochondrial region.

Species discrimination

Of the 109 species from the study site tested, 96 were identified to species level by PCR–RFLP of the ITS and ML3/ML4 regions. PCR–RFLP of the ITS alone discriminated 86 of these species. Digestion with only two restriction enzymes, *Alu*I and *Taq*I, was sufficient to resolve 72 of these species. The other 14 also required digestion by *Hae*III and/or *Hin*fI for resolution. Data for 41 of these species were given in Glen *et al*. (2001), as indicated in Table 1. PCR–RFLP fragment size codes are given for another ten species in Table 6. For these 51 species, only one collection of each was analysed.

For the remaining 13 species, three PCR–RFLP groups each contain three species of *Cortinariaceae*, and two further groups each contain two *Tricholoma* species. The unresolved groups are: (1) *Cortinarius* sp. B, *Cortinarius* subgen. *Phlegmacium* sp. 3, and *Cortinarius cfr sublargus* group A; (2) *Cortinarius* sp. H, *Cortinarius* subgen. *Leprocybe* sp. and *Dermocybe austroveneta*; (3) *Inocybe cystidiocatenata*, *Cortinarius* sp. E and *Cortinarius* subgen. *Phlegmacium* sp. 5; (4) *Tricholoma* subgen. *Contextocutis* sp. 1 and sp. 2; and (5) *Tricholoma* subgen. *Tricholoma* sp. 3 and sp. 4.

DISCUSSION

Intraspecific variation

High degree of intraspecific variation

We found ITS PCR–RFLP intraspecific variability in 12 of the 42 species (i.e. 29%), for which more than one

Table 3. Fungal species for which more than one collection per species was tested. Columns A, T, H and F represent *Alu*I, *Taq*I, *Hae*III and *Hin*fI digestions,respectively, of the ITS fragment, and columns MS and MH are *Sau*3AI and *Hae*III digestions of the ML3/ML4 fragment. Fragment sizes for the codes are given in Table 2.

Species	A	T	H	F	MS	MH	Loc'n[e]	Herbarium no.
No intraspecific variation in ITS or ML profiles								
Amanitaceae								
Amanita eucalypti[a]	AB	BA	AA	CH	HD	EF	A	E5451, E5385
A. xanthocephala[a]	EJ	BA	AA	HA	HE	EF	A&P	E5445, G9509, G9510, G9511
Bolbitiaceae								
Conocybe subgen. *Pholiotina* sp.[a]	EN	BA	BE	CD	EC	EF	A	E6028, E5920, E5773
Boletaceae								
Boletus prolinius cfr sect. *luridi*[a]	DC	KJ	FA	DA	CB	CE	A&P	E5642, E5480, G9531
Cortinariaceae								
Cortinarius australiensis[a]	BB	AC	BA	CA	FE	EG	A&W	E5391, E5419
C. austroalbidus[a]	CD	BA	DB	CD	FL	EG	A	E5997, E5933
C. lavendulensis[a]	CJ	DD	AB	CD	FL	EG	A	E5517, E5985
C. microarcheri[a]	FO	BA	DB	CD	GB	EG	P	E5995, E5994, G9527
C. sp. C[a]	CJ	CB	DA	CD	GC	EG	A	E6026, E6029
C. sp. H	CJ	BA	DB	CD	GC	EG	P	E5887, E5907
C. subgen. *Phlegmacium* sp. 1[a]	CJ	BA	AA	CD	HA	EG	A	E5516, E5645
C. subgen. *Phlegmacium* sp. 4	CJ	BA	DA	CD	FL	JB	A&P	E5927, E5537
C. cfr sublargus group A	CJ	BA	DB	CD	FL	EG	A&P	E5477, E5596, E5582
C. cfr sublargus group B[a]	CJ	BA	GE	CD	HA	EG	A	E5386, E5388
C. cfr sublargus group D[a]	FA	GB	GC	EE	GB	CG	P	E5514, E5479
C. sublargus[a]	CJ	BA	GD	FC	HF	EG	A	E5392, E5387
C. vinaceo-cinereus	CJ	BA	DB	CD	FM	FK	P	E5701, E5882, E5993
Dermocybe austroveneta	CJ	BA	DB	CD	GC	EG	A&P	E5926, E5987, G9526, G9548
D. clelandii	CJ	BA	DB	FC	BE	BE	A	E5988, E5588
D. clelandii	CJ	BA	DB	FC	np[c]	np	A or P	G9528
Hebeloma aminophilum[a]	CD	BA	ED	CD	DJ	DJ	T	E6025, E6032, E6033, E6034, E5929
Elasmomycetaceae								
Macowanites sp.[a]	DD	BC	BD	DE	HH	ED	A	H7136, H7204
Ramariaceae								
Ramaria lorithamnus[a]	CD	JD	GH	FF	AB	FH	A&P	E5541, E5522, E5595, E5854, E5906, G9516, G9518, G9540, G9547
Russulaceae								
Russula sp. D[a]	CC	FD	BC	DG	HJ	GB	A	E5587, E6004
Tricholomataceae								
Laccaria sp. B[a]	EP	CF	AA	CD	HC	EG	A&P	E5518, E6030, G9529, G9530
Tricholoma subgen. *Tricholoma* sp. 3	AA	CA	BC	CE	DK	DH	A	E5589, E6000
T. subgen. *Tricholoma* sp. 3	AA	CA	BC	CE	np	np	A	E5515
T. eucalypticum[a]	CJ	CE	BF	CD	DK	DH	A	E5998, E6027
Intraspecific variation in ITS or ML profiles								
Boletaceae								
Austroboletus occidentalis[b]	EH	HD	AA	CK	AA	DC	A	E5450
A. occidentalis	EG	DE	AA	FD	AA	DC	A	G9567
Clavulinaceae								
Clavulina vinaceo-cervina (pink)[b]	GC	HA	GB	CM	CB	GA	P	E5911
C. vinaceo-cervina (brown)	GC	HA	GB	CM	JA	GA	P	E5777
C. vinaceo-cervina (brown)	GB	HH	GF	CN	JA	GA	A	E5776
C. vinaceo-cervina (purplish)	GB	HH	GF	CN	JA	GA	A	E5539, E5775
Cortinariaceae								
Cortinarius cfr bellus[b]	CJ	BA	GE	EC	FL	GC	A	E5921
C. cfr bellus	FQ	BA	GE	EC	FL	GC	P	E5989
C. globuliformis	CJ	BA	DB	CD	mfp[d]	mfp	A	H7327
C. globuliformis	CJ	BA	DB	CD	CD	FB	A	H7260
C. sp. E	CJ	BA	DA	CG	mfp	mfp	A	E5930
C. sp. E	CJ	ED	AA	CD	mfp	mfp	P	E5883
C. sinapicolor	CJ	BA	DB	CD	HA	EG	A	E5538, G9521, G9522
C. sinapicolor	CJ	BA	DB	CD	HB	CA	A&P	G9523, G9524
C. subgen. *Phlegmacium* sp. 3	CJ	BA	DB	CD	FL	EG	P	E5908
C. subgen. *Phlegmacium* sp. 3	CD	BD	GE	CC	mfp	mfp	A	E5478
C. cfr sublargus group C[b]	CK	FE	DC	FF	FL	EF	A	E5389
*C. cfr sublargus*group C	FD	GB	DA	CD	GB	EF	A&P	E5446, E5577
Inocybe cystidiocatenata[a]	CJ	BA	DA	CG	GA	EG	A	E5774
I. cystidiocatenata	CJ	BA	DA	CG	CH	BA	P	E5889
I. cystidiocatenata	CJ	BA	DA	CG	CG	BC	A	E5986
Hydnum rufescens[b]	CE	HJ	DB	GA	GE	FE	P	E5744
H. rufescens	CE	HJ	DB	GA	mfp	mfp	A	E5892
Hygrophoraceae								
Hygrocybe pratensis[b]	FF	BA	AA	DH	CC	EA	P	E5909
H. pratensis	EL	AB	DD	BA	np	np	P	E5891
Russulaceae								

Table 3 (*cont.*)

Species	A	T	H	F	MS	MH	Loc'n[e]	Herbarium no.
Hydnaceae								
Russula clelandii[b]	DF	BC	BD	DE	JC	ED	A&C	E5591, E5579
R. clelandii	DF	BC	AA	DC	HE	ED	A&T	E5476, E5512, E5513, E5932, G9502, G9504, G9505, G9506, G9507
R. neerimea[b]	CE	HG	AA	FA	GE	ED	A&T	E5843, E5585, E5590
R. neerimea	CE	HG	AA	FB	GE	ED	A	E5851
R. sp. A[b]	CH	BD	CB	FF	HE	ED	A	E5849
R. sp. A	FP	GE	CB	FF	HE	ED	A&T	E5852, E5842
R. sp. B[b]	CE	JC	FB	CF	HH	ED	P	E5543, E5474
R. sp. B	CE	JC	GJ	CF	HH	ED	A	E5574
Tricholomataceae								
Tricholoma subgen. *Contextocutis* sp. 1	CJ	CG	AA	CD	HK	BD	A	E5703, E5999
T. subgen. *Contextocutis* sp. 1	CJ	CG	GH	FF	HK	BD	P	E5884

[a] These species have unique ITS PCR–RFLP profiles.

[b] These species each have two unique ITS PCR–RFLP profiles.

np[c], No PCR product after repeated testing.

mfp[d], Many faint products.

Loc'n[e], Location key: A, Forest block A, unburnt 66 yr; C, Forest block C, approx 80 km from blocks A & P; P, Forest block P, regularly burnt, adjacent to block A; T, Torrens Rd, approx 20 km from blocks A & P; and W, Walpole, approx 300 km south of blocks A & P.

collection was tested. This is greater than the level of intraspecific variability found in previous studies covering larger geographical areas. Kårén *et al*. (1997) found intraspecific variability in 16% of their 44 fungal species after sampling two to four collections for each species from across Fennoscandia. Farmer & Sylvia (1998) investigated 28 species, however they examined only one specimen for 20 of those species, two each from six species and more than two collections from only two species. They found intraspecific variation in only the last two species, *Cenococcum geophilum* and *Pisolithus arhizus*, of which they tested 19 and 12 isolates, respectively. Pritsch *et al*. (1997) found no intraspecific variation in seven species of *Russula*, *Lactarius*, *Naucoria* and *Cortinarius* but variation in *Paxillus rubicundulus* over a 300 km distance. All of these studies looked for variation across a large geographic area, here it was found in a small (3 ha) study site. More work is needed to determine whether the intraspecific variability that was found here is more common than previously assumed. Sequence data will also give a better understanding of the level of variation.

Significance for interpreting the ecology of Eucalyptus *ecosystems*

The three previously mentioned studies (Kårén *et al*. 1997, Pritsch *et al*. 1997, Farmer & Sylvia 1998) were located in regions of the Northern Hemisphere where genetic diversity of the biota may be low due to geological history (Taberlet 1998). This contrasts with the high diversity of biota in south-western Australia, which is also mostly of Gondwanan rather than Laurasian origin (Hopper *et al*. 1996). Climatic and geological changes over the past 200 Myr have created a mosaic of diverse habitats which have contributed to the maintenance of high levels of plant, vertebrate and invertebrate biodiversity with a patchy distribution, unlike the large areas of relatively species-poor communities prevalent in the Northern Hemisphere (Hopper *et al*. 1996). Some groups of biota have been driven to extinction by the increasing aridity of the continent, others have survived since ancient times in relict populations creating ecological mosaics across the continent. Recent data suggests that the larger basidiomycete ectomycorrhizal populations may have been changed over time in similar ways to their hosts (Bougher & Tommerup 1996, 1999, Tommerup & Bougher 2000). Some plant genera in south-west Australia have undergone massive speciation resulting in a high degree of endemism in these genera (Hopper *et al*. 1996). Results presented in this paper strengthen previous views that similar proliferation of species and subspecies could be mirrored in the fungi which have evolved alongside their plant hosts (Bougher & Tommerup, 1996, 1999, Glen *et al*., 1998a).

This intraspecific variability in the ITS region may be a phenomenon local to the south-west of Australia, or it may be more widespread and unrecognised due to many studies using only one or a few collections from one or a few sites. Intraspecific variability in the ITS has been assumed to be low over small geographic areas (e.g. Gardes & Bruns 1996), however this may be an indication of the limitations of current detailed knowledge of populations of many larger basidiomycetes. Except for a handful of studies on particular genera (Gardes, *et al*. 1990, Agerer, Kraigher & Javornik 1996, Hibbett & Donoghue 1996, Farnet, Roux & Le Petit 1999, Gomes *et al*. 1999, Vasiliauskas, Johanneson & Stenlid 1999) there has been little work across most broad groups of larger basidomycete fungi to test this assumption. Likewise localised variation has been examined in very few ascomycete ectomycorrhizal fungi (Guillemaud *et al*. 1996, Kagan-zur 1998). While the situation in the Northern Hemisphere, and possibly even the eastern part of Australia (Chambers *et al*. 1999), may be quite different, it is apparent that intraspecific variation in the ITS is sufficiently common in Western Australian fungi to necessitate the examination of more than one collection of a species when conducting molecular investigations, whether for community studies or phylogenetic analysis.

Table 4. Fungal species which were not distinguished by *Alu*I and *Taq*I digestion of the ITS fragment. Fungi are grouped by PCR–RFLP profile. Columns A, T, H and F represent *Alu*I, *Taq*I, *Hae*III and *Hin*fI digestions, respectively, of the ITS fragment, and columns MS and MH are *Sau*3AI and *Hae*III digestions of the ML3/ML4 fragment. Fragment sizes for the codes are given in Table 2.

	A	T	H	F	MS	MH	Species	Herbarium nos
AT Group 1								
ITS Group 1								
	CJ	BA	DB	CD	CD	FB	*Cortinarius globuliformis*	H7260
	CJ	BA	DB	CD	mfp[a]	mfp	*C. globuliformis*	H7327
	CJ	BA	DB	CD	FM	FK	*C. vinaceo-cinereus*	E5701, E5882, E5993
	CJ	BA	DB	CD	FL	EG	*C.* sp. B	E5593
	CJ	BA	DB	CD	FL	EG	*C.* subgen. *Phlegmacium* sp. 3[c]	E5908
	CJ	BA	DB	CD	FL	EG	*C. cfr sublargus* group A	E5477, E5596, E5582
	CJ	BA	DB	CD	GC	EG	*C.* sp. H	E5887, E5907
	CJ	BA	DB	CD	GC	EG	*C.* subgen. *Leprocybe* sp.	E5632
	CJ	BA	DB	CD	GC	EG	*Dermocybe austroveneta*	E5926, E5987, G9526, G9548
	CJ	BA	DB	CD	HA	EG	*C. sinapicolor*	E5538, G9521,G9522
	CJ	BA	DB	CD	HB	CA	*C. sinapicolor*	G9523, G9524
ITS Group 2								
	CJ	BA	DA	CG	CG	BC	*Inocybe cystidiocatenata*	E5986
	CJ	BA	DA	CG	CH	BA	*I. cystidiocatenata*	E5889
	CJ	BA	DA	CG	GA	EG	*I. cystidiocatenata*	E5774
	CJ	BA	DA	CG	mfp	mfp	*C.* sp. E[c]	E5930
	CJ	BA	DA	CG	np[b]	np	*C.* subgen. *Phlegmacium* sp. 5	E5848
ITS Group 3								
	CJ	BA	DB	FC	BE	BE	*Dermocybe clelandii*	E5988, E5588
	CJ	BA	DB	FC	np	np	*D. clelandii*	G9528
	CJ	BA	DB	FC	GC	EG	*D. cramesina*	E5641
ITS Group 4								
	CJ	BA	DA	CD	FL	JB	*Cortinarius* subgen. *Phlegmacium* sp. 4	E5927, E5537
	CJ	BA	DA	CD	FA	BH	*Dermocybe* sp.	E5984
Others in AT Group 1								
	CJ	BA	AA	CD	HA	EG	*C.* subgen. *Phlegmacium* sp. 1	E5516, E5645
	CJ	BA	GA	FF	FL	EG	*C. rotundisporus*	E5540
	CJ	BA	GE	EC	FL	GC	*C. cfr bellus*[c]	E5921
	CJ	BA	GE	CD	HA	EG	*C. cfr sublargus* group B	E5386, E5388
	CJ	BA	EA	CB	mfp	mfp	*C.* sp D	E5441
	CJ	BA	GD	FC	HF	EG	*C. sublargus*	E5392, E5387
AT Group 2								
	CJ	GB	DA	CD	FK	EG	*C.* sp. A	E5903
ITS Group 5								
	CJ	GB	DB	CD	FL	EG	*C. fiveashianus*	E5636
	CJ	GB	DB	CD	mfp	mfp	*C.* subgen. *Sericeocybe* sp.	E5442
	CJ	GB	DB	CD	CE	FA	*Dermocybe splendida*	E5639
AT Group 3								
	CD	BA	DB	CD	FL	EG	*C. austroalbidus*	E5997, E5933
	CD	BA	ED	CD	DJ	DJ	*Hebeloma aminophilum*	E6025, E6032, E6033, E6034, E5929
	CD	BA	DB	FC	CC	CD	*Pholiota* sp.	E5521
AT Group 4								
ITS Group 6								
	CJ	CG	AA	CD	HK	BD	*Tricholoma* subgen. *Contextocutis* sp. 1[c]	E5703, E5999
	CJ	CG	AA	CD	np	np	*T.* subgen. *Contextocutis* sp. 2	E5576
AT Group 5								
ITS Group 7								
	AA	CA	BC	CE	DK	DH	*Tricholoma* subgen. *Tricholoma* sp. 3	E5589, E6000
	AA	CA	BC	CE	np	np	*Tricholoma* subgen. *Tricholoma* sp. 3	E5515
	AA	CA	BC	CE	DK	DH	*Tricholoma* subgen. *Tricholoma* sp. 4	E5449
AT Group 6								
	AB	BA	AA	CH	HD	EF	*Amanita eucalypti*	E5451, E5385
	AB	BA	BB	CJ	GF	EF	*Amanita* sect. *Lepidella* sp.	E5584
AT Group 7								
	CJ	ED	DB	CD	GC	EG	*Dermocybe basirubescens*	E5897
	CJ	ED	AA	CD	mfp	mfp	*Cortinarius* sp. E[c]	E5883

mfp[a], Many faint products.

np[b], No PCR product after repeated testing.

[c] One of two ITS PCR–RFLP profiles for this species.

Implications for community analysis

We hypothesise that there are more species of fungi present at the study site than have been collected, since basidiomata of other species have been found nearby. The numerous hypogeous species are difficult to detect without severe disturbance to a site. A similar situation was found for pine (Gardes & Bruns 1996) and black alder (Pritsch *et al.* 1997) forests. In a patch of North American pine forest basidiomata of ten different species had been collected and root-tip analysis revealed a further ten PCR–RFLP profiles (two matching sporocarps from a nearby area) and in the

Table 5. ML3/ML4 PCR–RFLP profiles of selected fungi to demonstrate the interspecific similarities of this genomic region. Some fungi from areas other than the study site are included.

ML3/4 *Sau*3AI digestions		ML3/4 *Hae*III digestions			
Code	Fragment sizes	Code	Fragment sizes	Species	Herbarium nos
Amanitaceae					
GF	502, 131, 124	EF	622, 210	*Amanita* sp. sect. *Lepidella*	E5584
HD	491, 145, 135, 130	EF	649, 199	*A. eucalypti*	E5451, E5385
HD	484, 144, 134, 126	EF	602, 207	*A. umbrinella*	E5845
HD	485, 139, 128, 122	EF	609, 214	*Torrendia arenaria*[a]	H7501
HE	442, 144, 132, 124	EF	612, 195	*A. xanthocephala*	E5445, G9509, G9510, G9511
HE	458, 141, 131, 123	EF	600, 205	*T. grandis*[a]	E5495
HE	456, 139, 129, 121	EF	585, 207	*T. inculta*[a]	H7346
Boletaceae					
AA	1025	DC	712, 474	*Austroboletus occidentalis*	E5450, G9567
AB	922	DD	677, 415	*Boletellus obscurecoccineus*	E6001
CB	936, 262	CE	785, 392	Boletoid hypogeous	H7399
CB	998, 268	CE	884, 405	*Boletus prolinius cfr* sect. *luridi*	E5480
CB	940, 271	CE	815, 423	*B. sinapecruentus*	E5444
Elasmomycetaceae					
DA	662, 561	ED	600, 232	*Martellia* sp. C	H7503
HG	432, 132, 124	EF	577, 198	*Martellia* sp. B	H7203
HH	449, 146, 126	ED	597, 219	*Macowanites luteiroseus*[a]	H7142, H7151
HH	440, 153, 125	ED	592, 223	*Macowanites* sp.	H7204, H7136
HH	448, 155, 122	ED	578, 222	*Martellia* sp. A	H7401
Entolomataceae					
FH	575, 305, 101	DH	639, 291	*Entoloma cfr chalybaeum*	E5583
FH	583, 309, 101	DH	587, 291	*E.* sp.	E5856
Russulaceae					
DF	589, 544, 103	EG	737, 184	*Lactarius eucalypti*	E4644
GG	559, 156, 129	ED	590, 229	*Russula cfr adusta*	E5855
GE	552, 142, 124	ED	590, 214	*R. neerimea*	E5585, E5590, E5843, E5851
HE	443, 156, 139, 122	ED	571, 224	*L. clarkeae*	E5841
HE	445, 146, 131, 123	ED	562, 216	*R. clelandii*	E5512, E5513, E5932, E5476, E5844
HE	446, 156, 138, 122	ED	573, 223	*R.* sp. A	E5842, E5849, E5852
HH	462, 150, 125	ED	590, 229	*R. persanguinea*	E5905
HH	446, 147, 124	ED	571, 221	*R.* sp. B	E5474, E5543, E5574
HH	471, 149, 128	ED	583, 218	*R.* sp. C	E6003
HH	450, 144, 125	ED	599, 215	*R.* sp. E	E5185
HH	438, 154, 126	ED	571, 227	*R.* sp. F	E5475
HJ	440, 162, 128	GB	594, 235	*R. multicolor*	E5850
HJ	443, 163, 124	GB	576, 238	*R.* sp. D	E5587, E6004
JC	356, 144, 126, 104	ED	592, 215	*R. clelandii*	E5591, E5579
Tricholomataceaae					
DK	662, 257, 100	DH	671, 298	*Tricholoma* subgen. *Tricholoma* sp. 3	E5589, E6000
DK	690, 267, 102	DH	660, 309	*T.* subgen. *Tricholoma* sp. 4	E5449
DK	604, 260, 100	DH	624, 299	*T. eucalypticum*	E6027
HK	440, 167	BD	1650, 666, 169	*T.* subgen. *Tricholoma* sp. 1	E5884, E5703, E5999
np[b]		np		*T.* subgen. *Tricholoma* sp. 2	E5576
CB	982, 262	BK	943, 656	*T.* subgen. *Tricholoma* sp. 6	E5910

[a] These collections came from a different area and further details are in Glen *et al.* (2001).

np[b], No PCR product after repeated testing.

alder, eight of 16 PCR–RFLP profiles from ectomycorrhiza were matched to those of basidiomata, leaving eight unidentified. Gehring *et al.* (1998) detected 51 RFLP types on mycorrhiza of pinyon pines, only seven of these matched sporocarp profiles. Jonsson *et al.* (1999) found 80 PCR–RFLP profiles on root-tips in a Swedish mixed forest but could only match 11 of these to sporocarps, despite having a database of profiles for 66 fungal species, though most of these were not collected from the study sites. It is expected, therefore, that unrecognisable PCR–RFLP profiles will also be produced from root-tips in the jarrah forest, even though a large number of fungal species has been studied there. Knowledge of the degree of intraspecific variation that can be expected is necessary for a greater understanding of what each unmatched profile might represent.

Intraspecific variation may confound measures of species diversity based on different PCR–RFLP profiles where there is no match to a described fungus. It is nevertheless an indication of genetic variability within a community. Measurement of variation in bacterial 16S rRNA gene fragments, either by sequencing or SSCP gels, has been used as a measure of richness in bacterial communities (Schwieger & Tebbe, 1998) where it has been shown that standard culturing techniques only sample a small proportion of the bacteria present. A similar sampling problem may occur in ectomycorrhizal community studies based on sporocarp surveys. Even in regions with low diversity of ectomycorrhizal fungi and with long-term sporocarp sampling (Gardes & Bruns 1996, Pritsch *et al.* 1997, Mahmood, Finlay & Erland 1999) PCR–RFLP profiles unmatched to identifiable fungal species have been produced from root-tips, raising the possibility that some ectomycorrhizal fungi may never, or only extremely rarely, produce sporocarps. Mapping the distribution of these unidentified fungi by PCR–RFLP profiles may provide

Table 6. Fungal species distinguished by *Alu*I and *Taq*I digestion of the ITS, additional to those in Glen *et al*. (2001) and Tables 3–5, and for which only one collection of each species was tested. Columns A, T, H and F represent *Alu*I, *Taq*I, *Hae*III and *Hin*fI digestions, respectively, of the ITS fragment, and columns MS and MH are *Sau*3AI and *Hae*III digestions of the ML3/ML4 fragment. Fragment sizes for the codes are given in Table 2.

Herb. no.	Species	A	T	H	F	MS	MH
Cortinariaceae							
E6031	*Cortinarius* sp. F	CJ	GG	DB	CD	GC	EG
E5592	*C*. sp. G	DB	BA	DD	FC	FH	EG
E5893	*C*. subgen. *Phlegmacium* sp. 2	CJ	BD	GE	CC	JD	HA
E5853	*C. cfr sublargus* "conic cap"	EC	FF	EC	EA	HA	EG
E5393	*C. cfr sublargus* "short stem"	FD	GB	DA	AA	GB	EF
Elasmomycetaceae							
H7401	*Martellia* sp. A	CC	KK	GG	DB	HH	ED
H7203	*M*. sp. B	CC	BC	AA	DB	HG	EF
H7503	*M*. sp. C	FL	BD	AA	DB	DA	ED
Gautieriaceae							
G9549	*Austrogautieria manjimupana*	FK	FC	CA	EF	CA	BB
Tricholomataceae							
E5910	*Tricholoma* subgen. *Tricholoma* sp. 6	GD	KB	AA	CD	CB	BK

previously unobtainable information about their occurrence, abundance and ecology. The availability of this new information reinforces the need to gain a better understanding of species and intraspecific variation, both morphological and molecular, and how this affects community analyses, ecological interactions and conservation. What this genetic variation at a single locus means in terms of overall genetic variability needs to be determined for each genetic locus and group of organisms.

Taxonomic implications

Species concepts in fungi are constantly undergoing revision, with some currently accepted species shown to consist of a complex of biological species (Brasier, 1997). Previous morphological criteria have been insufficient to resolve these taxonomic groups. This, combined with the acknowledged shortfall of Australian fungal taxonomy, means that the number of ITS PCR–RFLP profiles may be as valid a measure of taxon richness and diversity as the number of currently accepted species. The collections referred to as *Cortinarius cfr sublargus*, of which there are six morphological groups, are distinguished by PCR–RFLP of the ITS and ML3/ML4 regions into seven groups, five of which match a morphological grouping, the last two matching a single morphological group. Morphological (and microscopic) classification was conducted independently of molecular typing. It is possible that the last morphological group contains two biological species without distinguishing morphological features, yet lacking the ability to interbreed. Biological species which have arisen through clonal propagation and/or geological or ecological separation of ancestral lineages have been shown to exist in *Armillaria* and *Heterobasidion* (Brasier 1997). This may also be the case for some of the fungi discussed here. The difficulty of *in vitro* culturing of many ectomycorrhizal species makes the demonstration of such species complexes unlikely at present for many species. However, Aanen & Kuyper (1999) have demonstrated the presence of 22 sexual incompatibility groups in the *Hebeloma crustuliniforme*/*H. alpinum* species group which is divided into four morphospecies. More evidence correlating this phenomenon with the degree of intraspecific variation in genomic DNA sequences is needed before ITS sequence variation without strongly supporting morphological and biological data is sufficient evidence on which to propose the existence of separate species.

Site and population structure implications

The pattern of distribution of variable species across the two forest blocks suggests that variability at these two loci may reflect a real genetic difference between the strains occurring at each site. A different species composition on the two adjacent blocks, which have contrasting fire histories, has been observed by basidiome collection over several years (Bougher, Tommerup & Bolsenbroek, unpublished). It is possible that the differing environmental factors have resulted in strain differences within species as well as species differences in the ectomycorrhizal fungi colonising each of the blocks. Regular fire disturbance may have provided opportunities for colonisation by new genets of ectomycorrhizal species. Such an issue is an example of the questions which may be addressed by proper application of molecular identification of field material such as fungal mycelium colonising root-tips.

In the *Russulaceae* some degree of intraspecific variation was found in four of the five species for which more than one collection was tested, though in other work (Glen *et al*., 1998*a*) collections of *R. persanguinea* from across Australia and *R. multicolor* from across south-western Australia were invariable in ITS PCR–RFLP profiles. Species of *Russula* are generally regarded as being late-stage colonisers (Visser 1995), suggesting that individuals are long-lived and do not readily establish from spores. At the study site, species of *Russula* are found much more frequently on block A than on block P, both as basidiomes and ectomycorrhiza (Glen *et al*. 1998b). The occurrence of intraspecific variation may be a result of long-term clonal propagation with sequence variation arising through mutation and becoming fixed in hyphal individuals. There was no indication, such as a fragment sum larger than

expected, of the presence of more than one ITS sequence in a single basidiome, as was found in the ML3/ML4 profiles for some fungi. The lack of ITS heterozygosity combined with the co-existence of ITS variants is consistent with an asexual lifestyle.

In our study, the *Cortinariaceae* account for almost half of the variable species (four of the twelve species with intraspecific ITS variation and five of the eight with variation in the ML3/ML4 region), but they account for 60% of the species for which more than one collection was tested, so there does not appear to be a greater level of intraspecific variability in this family than in others. No more than two ITS PCR–RFLP profiles were found for any species, though up to twelve collections of a species were analysed. Three ML3/ML4 PCR–RFLP profiles were found in three collections of *Inocybe cystidiocatenata*.

Interspecific variation

Confusion in the Cortinariaceae

There appears to be a greater degree of interspecific similarity within the ITS of species of *Cortinariaceae* compared to other families. Seventeen species of *Cortinarius* from three subgenera, four *Dermocybe*, and one *Inocybe* species produced similar profiles when the ITS fragment was digested with *Alu*I and *Taq*I. Digestion of this fragment with a further two restriction enzymes yielded ten profiles. Though digestion of the ML3/ML4 fragment provides some further resolution, even after six digestions of two genomic regions, only 13 of the 24 species were distinguished by the groupings used here. Most of the large groups are caused by the definition of PCR–RFLP profiles with broad size ranges. Another way to present these groups would have been to define a larger number of profiles with a narrower range of sizes and to include each species in two or more groups to allow for sizing error. As we intended to highlight any uncertainties that could occur with identification of field material using this method, we have retained a single grouping for all the species which overlap. Even greater discrimination may be obtained by adjacent electrophoresis of similar species, but this was not done for the same reason.

The possibility that specimens had been mixed, either when collected or when dried specimens were studied and returned to the packet, was checked by re-sampling and PCR–RFLP testing of a further two basidiomata from each collection which showed either intraspecific variability or interspecific similarity. In each case, these additional results verified the original profile, however the possibility of such misidentification illustrates the need for collections to contain the full range of developmental stages and identification to include microscopic, macroscopic and biochemical information and accurate photographs for future cross-referencing. It also highlights the need for voucher specimens to be retained for all molecular data so that inappropriate morphological identifications can be corrected in the future.

A similar combination of interspecific similarity and intraspecific variation was also noted by Kårén *et al*. (1997), who found ten species of *Cortinarius* were separated into only three PCR–RFLP types after digestion of the amplified ITS with three endonucleases. One species (*C. camphoratus*) produced two of these profiles; each was shared with two other species. Jonsson *et al*. (1999) had five groups of *Cortinarius* species containing 2–15 species which could not be distinguished from each other. The large number of species tested in this genus possibly makes discrimination more difficult then in other genera with fewer representatives. However, in pairwise comparisons, Pritsch *et al*. (1997) also found fewer differentiating restriction enzymes for their five *Cortinarius* and putative *Cortinarius* species than for species in other genera such as *Russula* and *Lactarius*.

By contrast, Chambers *et al*. (1999) distinguished ten species in the *Cortinariaceae*, by PCR–RFLP of the ITS using *Hin*fI and *Hae*III. We also found *Hin*fI and *Hae*III digestion of the ITS useful in distinguishing some groups of *Cortinariaceae*, but not adequate to give species level identification of all the *Cortinariaceae* present at our study site. Our fragment sizes for *C. sinapicolor* and *D. austroveneta* are compatible with their sequences of these species, though there is some disparity with their given restriction fragment sizes. This shows the need to determine reproducibility and accuracy of fragment sizes if these are to be used to distinguish species. In addition, they report no intraspecific variation in ten species including *C. rotundisporis*, however the same group (Sawyer, Chambers & Cairney 1999) reported intraspecific variation in ITS PCR–RFLP profiles and DNA sequences in *C. rotundisporis* from the same site. Some of our variable species (e.g. *Cortinarius* sp. E) have yet to be named, but detailed macroscopic and microscopic morphological examinations revealed no clear distinguishing features consistent with species-level taxa (Bougher 1995–99, pers. comm.).

The mitochondrial profiles provide additional but not complete resolution. The occurrence of what appears to be more than one mitochondrial sequence (digestions resulting in many faint fragments) amplified by these primers is also a disadvantage, as it reduces the reliability of the mitochondrial fragment for identification of some species. We have included these profiles as examples of intra-specific variation, though it may be necessary to clone these amplicons to properly assess their origins. It may be possible to address the problem of identifying members of the *Cortinariaceae* from ectomycorrhizas by resorting to sequencing of one or both of the above regions or by PCR–RFLP of a more variable region such as the IGS.

It is possible that ITS sequence variation among the *Cortinariaceae* is not detected by the restriction enzymes used. Kårén *et al*. (1997) sequenced the ITS regions of two *Cortinarius* species which gave identical PCR–RFLP profiles with three endonucleases and found 6% variation between the two sequences. An examination of the sequences revealed that digestion by *Mae*I (CTAG) would produce different fragment sizes for the two species. As sequence databases amass greater numbers of fungal ITS sequences, it will be more efficient to study sequences to determine which endonucleases will distinguish hard-to-separate taxa than to perform additional digestions until the correct one is reached by trial and error. However, a low level of sequence variation may not be detectable by any restriction enzyme. Two closely related

species may have ITS sequence variation of as little as one or two percent (Liu *et al*. 1997, Johannesson *et al*. 1999, Høiland & Holst-Jensen 2000).

The other possible explanation for this intra-family similarity is that this region is more conserved within the *Cortinariaceae*, in comparison to other basidiomycete families such as the *Russulaceae*. Given the large number of species and the extreme morphological heterogeneity of the family, it is the more likely explanation. Kårén *et al*. (1997) propose a recent evolutionary radiation within the genus *Cortinarius* to explain this. Our data would raise the possibility that this was a world-wide event and not so recent a radiation given the geological history of ancient continental separation and flora evolution discussed above (Hopper *et al*. 1996, Taberlet 1998, Bougher & Tommerup 1996, Specht 1996, Tommerup & Bougher 2000). It is possible that there is sequence conservation in the ITS, despite it generally being regarded as a non-coding and therefore unconserved region. Shinohara, LoBuglio & Rogers (1999) suggest that the ITS2 in *Cenococcum geophilum* is under evolutionary pressure to maintain RNA secondary structure involved in post-transcriptional processing of rRNA. A comparison of relative variability of the ITS and other genomic regions in a large number of *Cortinarius* species will be necessary to resolve this question.

Variable versus conserved regions

The variability in the mitochondrial region among the *Cortinariaceae* provides additional support for the idea of a mechanism for ITS sequence conservation in this group. In many species, the level of variation in the ITS, as determined by PCR–RFLP, is greater than in mitochondrial regions, whereas in the *Cortinariaceae* the reverse appears to be the case, at least for many Australian species examined so far. Sequencing and comparison of variability with other regions would be necessary to confirm such a hypothesis. Bruns *et al*. (1998) sequenced an adjacent mitochondrial region in a large number of ectomycorrhizal basidiomycetes and analysed the data phylogenetically. They concluded that short branch lengths in five groups may be indicative of recent radiations, including in the *Russulaceae* and *Amanitaceae*, but not the *Cortinariaceae*, in contrast to Kårén *et al*. (1997). This reinforces our findings of putatively different relative rates of variability in different fungal families, and suggests that more than one genomic region needs to be analysed before we can reach any conclusions about radiations.

Conservation of ITS DNA sequence would have repercussions for phylogenetic analyses based on ITS sequences. Misleading relationships could be erroneously supported, especially where rate-dependent analyses such as UPGMA are employed (Nei 1991). This would be particularly problematical if this conservation occurs in some groups and not others. This may be the case in the *Cortinariaceae*, as some species are readily distinguished by two digestions of the ITS.

Whatever the cause, this interspecific similarity emphasises the insufficiency of PCR–RFLP of one DNA region to give confident species-level identification across a broad spectrum of fungal families.

PCR–RFLP consistency and reliability for fungal identification

Intraspecific similarities

Sixty-five per cent of the species tested showed no intraspecific variation, even after testing of up to nine collections. Two species from the study site also matched ITS PCR–RFLP profiles of the same species from a site 300 km further south. In addition, the restriction fragment sizes of our Western Australian collections of *Dermocybe austroveneta*, *Cortinarius sinapicolor* and *C. rotundisporus* are consistent with the sequences for those species as identified by Chambers *et al*. (1999) from the eastern side of the continent. The *Taq*I, *Hae*III and *Hin*fI restriction fragment sizes of *C. cfr sublargus* group A and the *Alu*I, *Hae*III and *Hin*fI restriction fragment sizes of two of the collections of *C. cfr sublargus* group C are consistent with the *C. radicatus* (a synonym of *C. sublargus*) sequences of Chambers *et al*. (1999). Our collections identified as *C. sublargus* agree only in the *Taq*I restriction fragment sizes. None of our 14 collections identified as *C. sublargus* or *C. cfr sublargus* match all the restriction enzyme sites in the given sequence. This further highlights the taxonomic difficulties encountered in this family and the need for retention of voucher specimens and thorough morphological and biochemical descriptions of collections.

Mitochondrial patterns

Overall, the availability of a mitochondrial region in population studies should prove valuable, despite the lack of amplification or ambiguous products from some collections. In addition, the use of a second region provides an extra level of confidence in identification as well as species discrimination among some groups of fungi which cannot be differentiated using PCR–RFLP of the ITS region. The lack of amplification of individual collections or species may be caused by the presence of introns (White *et al*. 1990) or by a single nucleotide change in one of the primer sites. The occurrence of such variation even in conserved regions can be expected. This explains the need for degenerate primers to amplify the ITS of a broad range of larger basidiomycetes. The ML primers are not degenerate so a greater failure rate is to be expected.

In three of the eight species with variable ML3/ML4 PCR–RFLP profiles and in three other species, many faint fragments were observed after digestion, indicating the presence of more than one amplified fragment. It is unknown whether the two or more products are amplified from a single type of mitochondria or whether more than one mitochondrial type is present in a single fungal cell. The inheritance of mitochondria in most fungi is itself an unresolved question and this technique may help provide some of the answers. In some species, variation in the ITS appears to coincide with variation in the ML3/ML4 profile, e.g. *Russula clelandii*, in others, the two regions vary independently, e.g. *Clavulina vinaceo-cervina*.

Tools for fungal detection

The sizes of all restriction fragments from the ITSF/ITSR

and ML3/ML4 PCR products have been stored in a database to facilitate the matching of profiles for the identification of fungi growing on ectomycorrhizal root-tips, including large community population studies requiring the processing of very large numbers of samples. The data presented here and in Glen *et al*. (2001) show that PCR–RFLP of the ITS and ML3/ML4 regions is able to distinguish the majority of species of ectomycorrhizal fungi found fruiting in a highly complex community, such as that occurring in eucalyptus forest of Western Australia, with some equivocation among the *Cortinariaceae*.

The need to amplify and digest two genomic regions with more than the usual two enzymes illustrates the demands placed on molecular tools by highly diverse fungal communities. Under optimal conditions one genomic region may be sufficient to distinguish certain fungal species, however, variability or uniformity at a single locus cannot be assumed to be consistent across broader groups of fungi. This is exemplified by the ML3/ML4 region which is uniform (as far as PCR–RFLP analysis shows) across six species of *Russula* and the related hypogeous species *Macowanites* sp., *M. luteiroseus* and *Martellia* sp. (Table 5, and Glen *et al*. 2001), yet shows intraspecific variation in *Inocybe cystidiocatenata* and *Hydnum rufescens* from specimens collected in a small (3 ha) area.

There is also a clear necessity to analyse more than one collection of a species to determine the degree of intraspecific variability, even for studies that concentrate on a small geographic area. The correspondence of PCR–RFLP profiles with individual species is not exact, as some species may be represented by more than one profile, whereas another profile may represent several species. This emphasises the mutual dependence of classical taxonomy and molecular analyses.

The database of PCR–RFLP profiles of basidiomycete fungi from a single geographic region provides a valuable resource for the assessment of below-ground communities and populations in the jarrah forest as well as giving information of significance to taxonomy and fungal lifestyles in *Eucalyptus* ecosystems. The high diversity and subspecific variation found in a small area emphasises the taxonomic development needed to gain an understanding of the Australian, and indeed the whole Gondwanan, ectomycorrhizal biodiversity.

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