Specificity, sensitivity and discrimination of primers for PCR–RFLP of larger basidiomycetes and their applicability to identification of ectomycorrhizal fungi in *Eucalyptus* forests and plantations

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Techniques to rapidly identify the basidiomycete fungal partner of ectomycorrhizal associations would be a major advantage for ecological, fungal population dynamics and life history studies of epigeous and hypogeous forms in plantations, forests, wild lands and other native or natural vegetation. PCR–RFLP (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) identification of DNA regions is an available technique; however, primers which have a high probability of amplifying only the basidiomycete DNA are needed. Here we have assessed the specificity, sensitivity and discrimination of six different primer pairs, three targeting nuclear and three mitochondrial regions, for use in identification of Australian basidiomycete fungi from *Eucalyptus* forests by matching PCR–RFLP patterns to morphologically defined species. Two sets of primers, one newly designed and targeting the nuclear ribosomal DNA internal transcribed spacers (ITS) and the other amplifying a fragment of mitochondrial large subunit ribosomal DNA met the requirements of high specificity and sensitivity, amplifying DNA from a broad range of larger basidiomycetes, with no amplification of plant, bacterial or ascomycete DNA. The specificity of the ITS primer pair was compared with that of ITS1-F/ITS4-B. PCR–RFLP of the two regions discriminated fungi to species level for 91 fungal species from 28 families. Hence these two DNA regions and the specific primers are a potential practical PCR–RFLP tool for identifying basidiomycetes associated with plants from field samples.

INTRODUCTION

Ectomycorrhizal fungi are crucial to nutrient cycling and may be keystone organisms in many ecosystems (Smith & Read 1997, Tommerup & Bougher 1999). Australian forests, and particularly eucalypt forests are strongly ectomycorrhizal (Bougher 1995, Bougher & Tommerup 1996, Bougher & Syme 1998, Tommerup & Bougher 1999). However as ectomycorrhizal fungi are cryptic organisms which only become morphologically taxonomically distinguishable during a short sexual reproductive season, if at all, there is a dearth of information on the ecological behaviour and community population dynamics of most species. Many produce basidiomata rarely and only under certain conditions or have hypogeous basidiomata, presenting sampling problems (Luoma & Frenkel 1991, Johnson 1994, Bougher 1995, Bougher & Tommerup 1996, Tommerup & Bougher 1999). Additionally, many ectomycorrhizal species are unculturable (Bougher & Tommerup 1996, Smith & Read 1997, Trappe 1977). At present, the distribution, especially the fine-scale distribution, of particular ectomycorrhizal fungal species is practically unknown in either Laurasian or Gondwanan vegetation or in plantations. The relationship between mycorrhiza and basidiomata abundance has been shown to be low for some fungal species in pine forests (Gardes & Bruns 1996, Gehring *et al*. 1998, Jonsson *et al*. 1999) and is unknown for eucalypt forests. Morphotyping of ectomycorrhiza has been used in some studies; however, it has been demonstrated that morphotypes do not correspond to fungal species – some morphotypes are produced by more than one fungal species, while one fungal species may produce more than one morphotype (Timonen, Tammi & Sen 1997). Methods to accurately identify fungi on root tips or confirm identity of basidiomata, particularly when single or few of them are produced, are needed for ecological and biodiversity investigations of these and related saprotrophic and pathogenic fungi and for developing ecologically based management strategies for ecosystems.

PCR–RFLP (polymerase chain reaction–restriction fragment length polymorphism) is a technique that has been used in the detection and identification of fungi infecting humans, animals, plants and even wine cultures (Henson & French 1993, Matsumoto *et al*. 1997, Guillamon *et al*. 1998, Xu, Mitchell & Vilgalys 1999). Interspecific variation in the base sequence of a PCR-amplified DNA fragment is detected by one or more restriction enzymes which each cleave the DNA

strand at a characteristic recognition site of (usually) 4–6 base pairs. A pattern produced by electrophoresis of the fragments can be matched to reference patterns from previously identified species. The advantages of this technique include: (1) speed and the capacity for automation; (2) sensitivity; (3) ability to discriminate among several fungi from one set of results; and (4) absence of the need to obtain sporulation phases required for morphological identification or a pure culture of the fungus. Whereas the first characteristic may be of major importance in human, animal or plant disease diagnostics, the last three make this technique particularly suitable for research into the ecology of ectomycorrhizal fungi.

PCR–RFLP of the rDNA ITS (internal transcribed spacer of the ribosomal DNA) has been used by several researchers to track one or a few fungal isolates under field conditions in natural forests or plantations (Henrion *et al*. 1994, Erland 1995), to investigate species associated with a host (Kraiger, Agerer & Javornik 1995, Pritsch *et al*. 1997); or intraspecific variation in a genus (Gardes *et al*. 1990, 1991). Yet there is potential for this technique to be applied to more comprehensive surveys of ectomycorrhizal communities as done in a patch of pine forest containing 10–20 species of ectomycorrhizal fungi (Gardes & Bruns 1996) and alder forest with up to 16 species (Pritsch *et al*. 1997). Also, to discriminate fungi in communities, 44 species of ectomycorrhizal fungi from Fennoscandia were examined using three restriction enzymes and 77 % of the species could be distinguished from each other (Kårén *et al*. 1997). They found species in the genus *Cortinarius* to be the most difficult to separate. The genera *Cortinarius* and *Dermocybe* are well represented in Australia with many species, both named and as yet unnamed (Bougher & Hilton 1989, Horak & Wood 1990, Grgurinovic 1997, Chambers, Sawyer & Cairney 1999). Farmer & Sylvia (1998) conducted a survey using seven restriction enzymes among 28 species of North American ectomycorrhizal fungi. They distinguished all of these except for two *Rhizopogon* species which gave identical patterns with the seven restriction enzymes. No *Cortinarius* or *Dermocybe* species were included in this study.

The rDNA ITS is the most popular target region for PCR–RFLP identification. This region is amenable to PCR because of the high copy number and conserved flanking regions and discriminates well among taxa because of the variability in the spacer region. The spacers are removed during post-transcriptional processing so there is less selective pressure to prevent the accumulation of mutations in the DNA sequence. Other regions have also been identified as potentially useful, including the glycerinaldehyde-3-phosphate dehydrogenase gene (Kreuzinger *et al*. 1996), chitin synthase genes (Mehmann, Brunner & Braus 1994) and mitochondrial rRNA genes (Bruns *et al*. 1998). The use of more than one genomic region would provide an extra level of confidence in fungal identification and the inclusion of mitochondrial regions could provide further information as there are different inheritance patterns for nuclear and mitochondrial genomes.

The high diversity of Australian ectomycorrhizal species places great demands on the molecular and morphological tools used in fungal identification. We doubted whether one fragment of DNA would be sufficient to differentiate the 200 species expected at one site. We also expected a significant number of unrecognisable PCR–RFLP profiles to be generated from root-tips, as these have been found even in well-studied ecosystems of relatively low diversity (Gardes & Bruns 1996, Gehring *et al*. 1998, Jonsson *et al*. 1999) and Australia has many hypogeous species which are not frequently sampled as basidiomes (Claridge, Cork & Trappe 2000, Bougher & Lebel 2000). We therefore required a high degree of certainty that the ectomycorrhizal fungal DNA would be preferentially amplified. In addition to the high diversity, *Eucalyptus*, *Acacia* and *Allocasuarina* ectomycorrhiza, in contrast to those of pines, beeches and oaks, are superficial, with a Hartig net only in the outermost cortical cell layer and variable mantle, which may be only a few cells deep (Malajczuk, Dell & Bougher 1987, Tommerup & Bougher 1999). Thus the ratio of fungal to plant DNA may be quite low compared to ectomycorrhiza of many Northern Hemisphere associations and the ratio of ectomycorrhizal to DNA of other soil fungi may also be low. Preliminary work with ascomycete-specific primers resulted in amplification of a product from all field and glass-house grown plant material, though not from tissue-cultured plant material. This emphasised the need for careful selection of primers and PCR conditions to avoid possible amplification of non-target DNA, as discussed by Zhang, Wendel & Clark (1997) and Schulze & Bahnweg (1998).

Basidiomycete species are found to predominate in Australian ecosystems and there is a high degree of endemism (Castellano & Bougher 1994, Bougher & Tommerup 1996, Bougher & Lebel 2000, Claridge *et al*. 2000). One study covering 136 sites reported 10 species of *Ascomycota*, three *Zygomycota* and 79 *Basidiomycota* (Claridge *et al*. 2000). We therefore elected to concentrate on basidiomycete ectomycorrhizal fungi and to optimise or improve upon the specificity of currently used fungal and basidiomycete-specific primers. As the PCR–RFLP databases for identification of ectomycorrhizal fungi are based mainly on northern hemisphere species, and very little has been published on molecular identification of Australian ectomycorrhizal fungi, compatibility with current databases was not the prime consideration. The advantages of greater specificity were considered to outweigh the potential disadvantage of undesirable amplification of non-target DNA. However, as ectomycorrhizal fungi belong to a broad taxonomic spectrum of basidiomycetes, primers must be able to amplify DNA from fungi in diverse orders. To determine the suitability of primer pairs for use in PCR–RFLP identification of ectomycorrhizal fungi from root-tips in eucalyptus forests, woodlands, woody heathlands and plantations we tested six primer pairs targeting three nuclear and three mitochondrial regions for specificity, sensitivity and species discrimination on identified collections of 92 fungal species from 28 families.

MATERIALS AND METHODS

Basidiomycete fungi

Both fresh collections and dried material from the CSIRO Forestry and Forest Products Herbarium, Perth, W.A. were used as sources of DNA from identified basidiomata. Fresh

Table 1. Fungal collections used to assess RFLP of nuclear rDNA ITS and mt LSU PCR products. Herb. no. is the herbarium accession code for collections in the CSIRO Forestry and Forest Products Mycology Herbarium. For each digestion, the fragment sizes and a letter code for ease of comparison are given. Sizes between 100 and 500 bp are reproducible with an error of 3 %, though some codes, e.g. code CJ for *Alu*I and BA for *Taq*I, cover a broad range of sizes. This is because of the large number of fungi, mainly in the genus *Cortinarius*, with similar patterns, producing a continuum of fragment sizes without clear demarcation of groups. The ITS products were amplified using the primers ITSF/ITSR, except for *Hydnum rufescens* and *Austrogautieria manjimupana*, for which the specific primers HRITSF and AMITSR were used. The mitochondrial products were amplified using the primers ML3/ML4.

Herb. no.	Species	ITS *Alu*I digestions		ITS *Taq*I digestions		ML3/4 *Sau*3AI digestions		ML3/4 *Hae*III digestions	
		Code	Fragment sizes	Code	Fragment sizes	Code	Fragment sizes	Code	Fragment sizes
Agaricaceae									
E884	*Macrolepiota molybdites*	CE	539, 247	EG	476, 195, 132, 122	DH	693, 295	DF	699, 305
Amanitaceae									
E5385	*Amanita eucalypti*	AB	786	BA	490, 369	HD	491, 145, 135, 130	EF	649, 199
E5845	*A. umbrinella*	DA	512, 492, 243	FG	413, 315, 299, 196	HD	484, 144, 134, 126	EF	602, 207
E5445	*A. xanthocephala*	EJ	412, 251, 142	BA	471, 353	HE	442, 144, 132, 124	EF	612, 195
H7501	*Torrendia arenaria*	EK	435, 242, 126	BA	472, 354	HD	485, 139, 128, 122	EF	609, 214
E5495	*T. grandis*	EB	491, 250, 100	DC	480, 305	HE	468, 141, 131, 123	EF	600, 205
H7346	*T. inculta*	EB	476, 243, 100	FF	420, 314	HE	456, 139, 129, 121	EF	585, 207
Bankeraceae									
E5778	*Phellodon* sp.	CM	540, 130	HK	336, 254, 140, 112, 108	JB	356, 253, 226	AB	766
Bolbitiaceae									
E6028	*Conocybe*[a] subgen. *Pholiotina* sp.	EN	409, 219, 124	BA	489, 374	EC	655, 123	EF	590, 194
Boletaceae									
E6001	*Boletellus obscurecoccineus*	BC	596, 203	GD	436, 136, 100	AB	922	DD	677, 415
H7399	Boletoid hypogeous sp.	CE	522, 266	LA	156, 146, 115, 111	CB	936, 262	CE	785, 392
E5480	*B. prolinus* (cfr sect. *Luridi*)	DC	537, 183, 153	KJ	210, 146, 141, 119, 106	CB	998, 268	CE	884, 405
E5444	*B. sinapecruentus*	CB	529, 308	KG	225, 211, 164, 147, 110	CB	940, 271	CE	815, 423
Ceratobasidiaceae									
	Rhizoctonia solani[b] AG 8	FB	392, 243, 124	BD	477, 326	np[c]		np	
	Waitea[b] sp.	CG	511, 214	KD	273, 195, 146, 139, 136	np		np	
Clavulinaceae									
E5775	*Clavulina vinaceo-cervina*	GB	271, 206, 147, 110	HH	343, 268, 195	JA	364, 317, 115	GA	615, 259
Coniophoracea									
E5542	*Podoserpula pusio*	GG	249, 221, 170, 134	GB	415, 375	DG	701, 338, 291	CF	843, 382
Cortinariaceae									
E5419	*Cortinarius australiensis*	BB	643, 200, 103	AC	595, 384	FE	535, 270, 105	EG	633, 183
E5517	*C. lavendulensis*	CJ	498, 164	DD	494, 280	FL	496, 316, 104	EG	675, 185
E4063	*C. phalarus*	np	np	np	np	FL	474, 281, 102	EG	688, 184
E4447	*C. phalarus*	np	np	np	np	FL	474, 281, 102	EG	688, 184
E5593	*C.* sp.	CJ	562, 156	BA	480, 381	FL	487, 279, 102	EG	630, 184
E5632	*C.* subgen. *Leprocybe* sp.	CJ	518, 154	BA	492, 385	GC	552, 186, 123, 102	EG	734, 191
E5636	*C.* (*Myxacium*) *fiveashianus*	CJ	512, 152	GB	400, 374	FL	486, 288, 104	EG	686, 184
E5994	*C.* (*Myxacium*) *microarcheri*	FO	317, 212, 153	BA	499, 348	GB	531, 197, 125, 102	EG	717, 181
E5540	*C.* (*Myxacium*) *rotundisporus*	CJ	516, 158	BA	482, 376	FJ	541, 308, 104	EG	682, 184
E5538	*C.* (*Myxacium*) *sinapicolor*	CJ	500, 159	BA	482, 375	HA	485, 166, 125, 104	EG	615, 183
E5386	*C.* cf. *sublargus*	CJ	559, 150	BA	468, 381	HA	485, 185, 124, 103	EG	654, 184
E5993	*C. vinaceo-cinereus*	CJ	485, 151	BA	478, 367	FM	503, 348, 102	FK	550, 202, 181
E4996	*C. vinaceolamellatus*	CF	496, 254	BA	482, 369	FJ	535, 293, 103	EG	649, 183
E5639	*Dermocybe splendida*	CJ	523, 153	GB	411, 388	CE	811, 372, 124, 102	FA	640, 534, 182
E5417	*Descolea maculata*	FL	332, 267, 208	AD	526, 395	FD	574, 265	EF	574, 207
H7328	*Descomyces albellus*	CL	542, 120	BA	471, 378	FC	576, 314	ED	626, 222
E5925	*Galerina unicolor*[a]	BE	647, 123	EA	474, 372, 246, 138	GF	525, 133, 125, 102	EF	651, 213
E5448	*Gymnopilus austrosapineus*[a]	FN	319, 249, 128, 112	EB	478, 238, 137	GD	548, 126, 102	EG	642, 186
E6025	*Hebeloma aminophilum*	CD	550, 236	BA	490, 362	DJ	680, 268	DJ	674, 264
E5886	*Inocybe australiensis*	CG	521, 213	BA	476, 349	ED	638, 134, 122	ED	654, 227
E5898	*I. fibrillosbrunnea*	CE	532, 246	BA	462, 378	EF	604, 124	EF	630, 212
E5885	*I.* sp.	AB	777	BB	498, 429	EF	658, 128	ED	646, 224
H7259	*Protoglossum* sp. nov.	EA	482, 397, 202, 183, 158	GB	398, 380	FL	510, 308, 106	EG	657, 183
E5420	*Rozites symeae*	FH	342, 186, 154	BA	488, 394	CF	797, 376, 102	HB	571, 442, 184
H4317	*Setchelliogaster australiensis*	AC	646	BA	509, 390	np		np	
H6684	*Thaxterogaster basipurpureum*	CJ	497, 150	BA	483, 383	FL	491, 321, 104	GC	658, 111
H7265	*T.* sp. nov	CJ	536, 154	BA	485, 367	FJ	541, 314, 103	EG	693, 190
H6214	*Timgrovea* sp. nov.	DG	554, 135, 120	BA	481, 376	FB	571, 329	EC	640, 218
Crepidotaceae									
E5928	*Tubaria* cfr *furfuracea*[a]	GF	271, 125	BA	513, 380	FE	575, 259, 100	ED	636, 228
Elasmomycetaceae									
H7142	*Macowanites luteiroseus*	CB	523, 302	BC	545, 329	HH	449, 146, 126	ED	597, 219
H7503	*Martellia* sp.	FL	326, 254, 212	BD	483, 336	DA	662, 561	ED	600, 232
Entolomataceae									
E5583	*Entoloma* cfr *chalybaeum*[a]	GA	276, 258	FH	407, 318, 205	FH	575, 305, 101	DH	639, 291
E5698	*E.* cfr *sericeum*[a]	GE	274, 244, 234	CF	468, 198, 171	FG	547, 295, 193, 100	FC	615, 263, 189
E5856	*E.*[a] sp.	FG	343, 248	HL	341, 195, 185	FH	583, 309, 101	DH	587, 291
Fistulinaceae									
E5919	*Fistulina hepatica*[b]	BA	622, 233, 200, 100	CF	470, 206, 183	BD	1281, 729, 525, 337, 258	DA	721, 618, 296
Gautieriaceae									
H7157	*Austrogautieria manjimupana*	FK	335, 138, 110	FC	450, 308, 140, 118, 101	AB	834	EC	620, 228
Hydnaceae									
E5744	*Hydnum rufescens*	CE	541, 260	HJ	336, 264, 210	GE	540, 144, 127	FE	601, 213, 106
Hygrophoraceae									
E5909	*Hygrocybe pratensis*[a]	FF	340, 168, 138, 128	BA	501, 407	CC	895, 275	EA	613, 556
Mesophelliaceae									
H7301	*Castoreum* sp.	AC	606	AA	558, 507, 129	EB	722, 162	JA	440, 258, 206

Table 1 (*cont.*).

Herb. no.	Species	ITS *Alu*I digestions		ITS *Taq*I digestions		ML3/4 *Sau*3AI digestions		ML3/4 *Hae*III digestions	
		Code	Fragment sizes	Code	Fragment sizes	Code	Fragment sizes	Code	Fragment sizes
Paxillaceae									
E5390	*Paxillus muelleri*	CC	517, 273	CD	491, 221, 138	BG	1129, 366	CC	900, 615
Pluteaceae									
E5895	*Pluteus atromarginatus*[a]	CD	540, 240	HB	388, 235, 226	GH	512, 244, 144	EC	624, 216
E5637	*P. lutescens*	DH	498, 150, 117	BD	458, 338	BF	1206, 254, 105	BG	1237, 778, 531, 337, 202
Ramariaceae									
E5443	*Ramaria* cfr *formosa*	AC	659	HE	390, 170, 130, 120	AB	868	FJ	616, 244, 124
E5575	*R. holorubella*	CD	544, 225	FA	459, 437, 339	AB	836	HC	577, 126, 116, 103
E5906	*R. lorithamnus*	CD	532, 236	JD	316, 176	AB	890	FH	558, 246, 125
E5904	*R.* sp.	AC	681	EF	474, 190, 148	AB	889	HD	578, 152, 115, 108
E5634	*R. versatilis*	EC	494, 229	KH	226, 215, 188	np		np	
Richoniellaceae									
H7202	*Richoniella* sp.	AC	654	KE	224, 165, 122, 102	AB	934	DG	702, 285
Russulaceae									
E5841	*Lactarius clarkeae*	CH	506, 196	JA	338, 322	HE	443, 156, 139, 122	ED	571, 224
E4644	*L. eucalypti*	CJ	575, 154	BA	471, 370	DF	589, 544, 103	EG	737, 184
E5855	*Russula* cfr *adusta*	CE	533, 266	KA	295, 186, 166	GG	559, 156, 129	ED	590, 229
E5512	*R. clelandii*	DF	528, 170, 134	BC	526, 328	HE	445, 146, 131, 123	ED	562, 216
E5476	*R. clelandii*	DF	531, 174, 135	BC	543, 334	HE	440, 147, 130, 125	ED	594, 216
E5850	*R. multicolor*[d]	CC	547, 292	FB	440, 348, 114, 100	HJ	440, 162, 128	ED	594, 235
E5585	*R. neerimea*	CE	544, 251	HG	346, 298, 189	GE	552, 142, 124	ED	590, 214
E5905	*R. persanguinea*	CC	517, 282	JB	334, 272	HH	462, 150, 125	ED	590, 229
E6003	*R.* sp. C	CE	541, 269	JC	347, 215	HH	471, 149, 128	ED	583, 218
E5185	*R.* sp. E	CC	560, 290	BC	546, 334	HH	450, 144, 125	ED	599, 215
E5475	*R.* sp. F	EQ	404, 148, 120	HC	384, 147, 141, 109	HH	438, 154, 126	ED	571, 227
Sclerodermataceae									
H7530	*Pisolithus albus*[d]	BD	602, 146	KF	222, 192, 123, 103	BB	1550, 169	BF	1500, 172
H7529	*P. microcarpus*	EM	434, 151, 100	KC	283, 122, 108	BB	1550, 169	BF	1500, 172
H601	*Scleroderma cepa*	ED	429, 298	LB	129, 118, 106	BC	1448, 152	CB	935, 460, 145
H604	*S. areolatum*	EF	437, 268	LB	129, 118, 106	BC	1448, 152	CB	935, 460, 145
Stephanosporaceae									
H7509	*Stephanospora flava*	CC	566, 283	DB	492, 322, 102	DB	629, 496, 234	DB	700, 619
Stereaceae									
E5272	*Stereum*[a] sp.	BE	654, 123	DA	472, 138, 125	EA	618, 271	EB	613, 283
Strophariaceae									
E5594	*Pholiota highlandensis*[a]	FC	383, 228, 122	BA	490, 368	FE	582, 271, 100	GA	620, 257
E5521	*Pholiota* sp.[a]	CD	551, 234	BA	490, 368	CC	896, 276	CD	815, 671
Thelephoraceae									
E5896	*Hydnellum* sp.	FE	366, 254, 115, 112	EC	474, 246, 240, 138	AB	851	FD	577, 207, 160
E4817	*Thelephora congesta*	BE	632, 116	BE	485, 224	DC	675, 323, 124	DE	658, 432
Tricholomataceae									
E0026	*Armillaria luteobubalina*[b]	FM	326, 159, 149, 121, 108	GC	429, 322, 229	BA	1404, 284	BJ	1000, 725, 195
E0439	*Laccaria* sp. (Grgurinovic 1997: 304)	EP	396, 155, 132	CF	479, 200, 180	HC	468, 260, 128, 105	EG	711, 190
E5899	*Lepiota booloola*[a]	FJ	348, 148, 120	HF	342, 300, 141	np		np	
E5573	*Leucopaxillus lilacinus*	DE	558, 165, 137	BA	497, 402	KA	216	KA	216
E5797	*Lyophyllum tylicolor*[a]	CD	538, 238	CF	467, 205, 174	np		np	
E5894	*Mycena kurramulla*[a]	BF	666, 118, 104	BA	506, 348	DE	693, 509, 293	AA	1206
E5888	*M. pura*[a]	BF	642, 120, 104	BA	460, 358	DD	686, 504, 329	AA	1135
E6027	*Tricholoma eucalypticum*	CJ	537, 170	CE	478, 203, 192	DK	604, 260, 100	DH	624, 299

[a] This species is saprotrophic and not known to be ectomycorrhizal.
[b] This species is parasitic and not known to be ectomycorrhizal.
[c] np, no product (repeated testing).
[d] Provisional names not yet validly published.

basidiomata were collected from 1995 to 1998. Only specimens including the full range of development of basidiomata were used to ensure positive identification from a complete set of taxonomic characters, including microscopic characters. 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 putatively have mycorrhizal and saprotrophic species, and to provide a more complete taxonomic framework for developing specific and sensitive primers than would be obtained with mycorrhizal taxa alone. Samples for DNA extraction were stored on ice and freeze-dried as soon as possible after collection. After optimisation of PCR conditions using DNA extracted from freshly collected specimens, older air-dried specimens from the herbarium were also used. In particular, *Cortinarius phalarus* and *Rozites symeae* were included as they are reported to be of Gondwanan origin (Bougher & Hilton 1989, Bougher, Fuhrer & Horak 1994). Gondwanan fungi are not well represented in DNA sequence databases, raising the possibility of them having mismatches with the primers, which were designed from sequences of Northern hemisphere fungi. 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 fungi used is given in Table 1.

Negative controls

Negative controls, to eliminate primers which amplified non-basidiomycete DNA, were seven plant species: *Acacia pulchella*, *Eucalyptus marginata*, *Banksia grandis*, *Persoonia longifolia*, *Dryandra nivea*, *Macrozamia reidlei*, and *Pteridium esculentum*; eight ascomycete fungi, *Aleuria rhenana*, *Gyromitra infula*,

Helvella lacunosa, *Labyrinthomyces* sp., *Morchella* sp., *Neurospora intermedia*, *Peziza whitei*, and *Trichoglossum hirsutum*; a rust, *Puccinia helianthi*; *Escherichia coli*; and three mixed bacterial and yeast cultures, designated C, G and H-initiated by inoculating Oxoid no. 2 broth with small fragments of basidiomata of, respectively, *Cortinarius* sp., *Gymnopilus* sp. and *Hydnum rufescens* and incubating at 37 °C for 16 h with shaking.

DNA extraction

DNA was extracted by the method of Raeder & Broda (1985) with the following modifications. Ground, freeze-dried samples were incubated in extraction buffer for 1 h at 65 °. After phenol–chloroform extraction and ribonuclease A digestion, DNA was precipitated overnight at −20 ° in 1 volume of isopropanol. Plant DNA was extracted from leaves by the method of Byrne *et al*. (1994).

PCR amplification

Amplification was performed in 10–100 µl volumes containing, unless otherwise stated, 0.1 ng μl^{-1} template DNA, 0.08 U μl^{-1} Tth+ polymerase (Biotech International), in 67 mM Tris–HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45 % Triton X-100, 0.2 mg ml^{-1} gelatine, and 0.2 mM each of dATP, dTTP, dCTP and dGTP (Biotech International). Primer and $MgCl_2$ concentration and thermocycler programs were varied for individual experiments, as detailed in the results section. The reaction mixture was covered with a drop of paraffin oil and amplification was carried out in a Hybaid Omnigene thermocycler. Conditions for amplification with ITS1-F and ITS4-B were as outlined in Gardes & Bruns (1993). A 5 µl aliquot was electrophoresed on a 4.5 % polyacrylamide gel for 16 h at 2 V cm^{-1} or a 1 % agarose gel at 5 V cm^{-1} for 1 h. All of the fungi in Table 2 were tested at least twice.

Primers

The six primer pairs tested were MYK1/MYK2 for conserved regions of the chitin synthase gene (Bowen *et al*. 1992), ML3/ML4, ML5/ML6 and ML7/ML8 for the mitochondrial large sub-unit of ribosomal DNA (White *et al*. 1990), newly designed ITS primers specific for hymenomycetes (ITSF/ITSR) and CNL12/5SA for the IGS region of the ribosomal DNA (Henrion, Le Tacon & Martin 1992). The specificity of the basidiomycete ITS primers (ITS1-F/ITS4-B) of Gardes & Bruns (1993), using their thermocycler conditions, was also compared to the ITSF/ITSR primer pair.

Sensitivity

Sensitivity of the PCR reactions was tested by amplifying dilutions of DNA from three species of basidiomycetes, with template concentrations ranging from 10 to 0.1 pg μl^{-1}. Re-amplification of faint products was also tested using the same primers, and for the ITS region, internal primers ITS1 and ITS4 (White *et al*. 1990).

DNA sequencing

DNA sequencing was carried out with an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit on an ABI 373 XL sequencer with stretch upgrade. Fragments of 25S or 18S rDNA were amplified using the primer pairs NL5mun/NL6Bmun (Egger 1995) and NS7/NS8 (White *et al*. 1990), cut from a 1 % agarose gel, purified with a Bresaclean kit and used as sequencing template with the same primers.

Restriction digestion and PAGE

A 2–5 µl aliquot of PCR product was digested for 2–3 h in a final volume of 10 µl with 1 U of the restriction enzymes *Alu*I, *Taq*I, *Hin*fI, *Hae*III, *Sau*3AI, *Msp*I, *Rsa*I, *Hin*6I, *Bsp*LI, *Tai*I, *Pvu*II, *Sma*I and *Eco*RI (Promega, Biotech International and Boehringer–Mannheim) according to the manufacturer's instructions. The entire reaction was electrophoresed on a 6 % polyarcylamide gel for 3 h at 10 V cm^{-1}. Gels were stained for 5 min in 1 µg ml^{-1} ethidium bromide and photographed under uv light. Fragments were sized by reference to three equidistant lanes of size standards on each gel (*Eco*RI and *Hin*dIII digested bacteriophage lambda DNA and *Hpa*II digested pUC19 DNA, Biotech International) with Molecular Analyst software (Bio-Rad). In the interests of speed and practicality for the analysis of large populations of mycorrhiza or mycelium from field sites the PCR reaction was not purified before restriction digestion. Due to the lower visibility and resolution of smaller fragments, only those fragments larger than 100 bp have been considered significant for species discrimination. For reproducibility of fragment sizing, the average size from two or three separate gels was taken. The range of sizes for a single fragment varied by 3 %.

RESULTS

ITS primer design

Primers were designed from 18S and 25S rDNA sequences obtained from Genbank through the Australian National Genome Information Service (ANGIS) and aligned using Clustalv (Higgins, Bleasby & Fuchs 1992). The aim was to amplify fungi of the class *Basidiomycetes* (approximating to the 'hymenomycete lineage' of Swann & Taylor 1993) and therefore to capture the basidiomycetes which form well-developed basidiomata and exclude the simple septate basidiomycetes and smuts (the other two lineages of Swann & Taylor 1993). Regions were chosen which were conserved within the class *Basidiomycetes*, yet differed from rust fungi, yeasts (basidiomycetous and ascomycetous) and ascomycetes (Figs 1, 2). After optimisation of the PCR conditions, the DNA sequences of the relevant 18S and 26S rDNA regions were obtained from those target fungi which did not amplify, and the primers redesigned, or additional primers made. The alignments and primer sequences are shown in Figs 1 and 2. The ITSR primer site overlaps the site of the ITS4-B primer designed by Gardes & Bruns (1993), however degenerate bases were incorporated in ITSR to accommodate the low level of variation among target species. ITSF is located 25 bp upstream of ITS1-F.

Group 1	CCCTGTTGCTGAGAAGCTGATC
Group 2	....A..................
Group 3	..............CT.....
3 *Lentinus* and 3 *Polyporus* species	......C........CT.....
Athelia bombacina and *Lepiota procera*	..T.A..................
Auricularia auricula	..T...................
Auricularia polytricha	T.T...................
Basidiomycete sp.	....A...........T.....
Cortinarius phalarus	TG..A...A.......T.....
Heterotextus alpinus	T..................G..
Lentinula edodes	.T....C...............
Lentinus suavissimus	..T............C......
Leucosporidium lari-marini	..T...C.........T.ACC.
Neolentinus lepidus, N. adhaerens	..............T.....
Pleurotus ostreatus, Dacrymyces chrysospermus	....A...........T.....
Russula compacta	...C.....CA.N...T..G..
Schizophyllum commune	..TCA...........T.....
Thanatephorus praticola	.T..A..................
Filobasidiella neoformans	...CT.G...............
Mrakia frigida	..T.A.......A......C..
Cystofilobasidium capitatum	..T...C.........T.ACC.
Erythrobasidium hasegawianum	..TA..C.........T...A.
Cronartium ribicola and *Peridermium harknesseii*	.TT.AC..G.TCA...T.CTA.
ITSF primer sequence	CCCTRTTGCTGAGAAXYTGRTC
Hydnum rufescens	.T..A.A.T...A...T.....
HRITSF primer sequence	CTCTATAGTTGAAAAXTTGATC

Fig. 1. Alignment of 18S rDNA sequences for design of the ITSF primer. The sequences for *C. phalarus* and *H. rufescens* were determined in this paper, the rest were obtained through ANGIS. This region occurs 25 bp upstream of the ITS1-F primer of Gardes & Bruns (1993) and 63 bases upstream of the ITS1 primer (White *et al.* 1990). Group 1 is comprised of *Xerocomus chrysenteron* and *Boletus santanus*; group 2 of *Agaricus bisporus, Coprinus cinereus, Phyllotopsis nidulans* and *Pleurotus tuberregium*; group 3 *Panus fulvus, P. lecomtei, P. johorensis* and *Spongipellis unicolor*. *F. neoformans, M. frigida, C. capitatum* and *E. hasegawianum* are basidiomycetous yeasts, *C. ribicola* and *P. harknesseii* are pine stem rusts. R represents purines, A and G; Y represents pyrimidines, C and T; and X represents G and C.

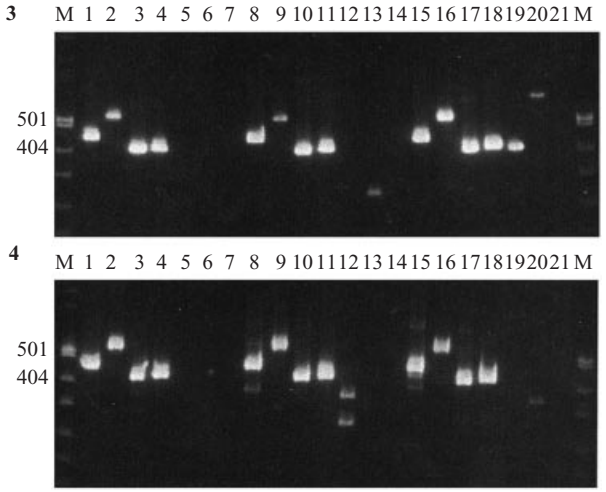
Group 4	TTCCAGGAGACTTGTACACGGTCC
Group 5	.......G..............
Group 6	............A..........
Group 7	.....A.......A..........
Group 8	.....A.................
Collybia earliae and *Stereum complicatum*	........A..............
Lactarius corrugis	..............G........
Laetiporus sulphureus	.....A.G..............
Lentinus dactyloides	.......G..-..A..........
Russula mairei	..............G......T.
Candida (30 species)	.....A.GA..A.AG...A.AG..
ITSR primer sequence	TTCCAGGAGACTTRTRCACGGTYC
Austrogautieria manjimupana	.....A.G..........T....T
AMITSR primer sequence	TTCCAGGAGACTTRTRCAYGGT
ITS4-B (Gardes & Bruns 1993)	CAGGAGACTTGTACACGGTCCAG

Fig. 2. Sequences from 26S rDNA (non-sense strand is shown) aligned for the design of the ITSR primer. The sequence for *A. manjimupana* was determined here, the rest were obtained through ANGIS. The selected region overlaps that used by Gardes & Bruns (1993) for the primer ITS4-B, though ITSR incorporates degenerate nucleotides at three sites. R represents purines, A and G, Y represents pyrimidines, C and T, and – represents a gap introduced for alignment. Group 4 is comprised of *Boletus retipes, Cortinarius stuntzii, C. phalarus, Hebeloma crustuliniforme, Pleurotus djanor, Phylloporus rhodoxanthus, Chlorophyllum molybdites, Leucoagaricus naucinus, Leucocoprinus luteus, Crinipellis campanella, Macrolepiota rachodes, Lentinellus montanus, L. omphallodes, Lentinula edodes, Panaeolina foenisecii* and several unidentified *Agaricales* isolated from fungus gardens of various ant species; group 5 of *Pleurotus pulmonarius, P. eryngii, P. populinus, P. fossulatus, P. cornucopiae, P. dryinus* and *P. ostreatus*; group 6 *Polyporus arcularius, P. squamosus, Lentinus lepidus, L. ponderosus, L. strigosus, L. tigrinus, L. sulcatus, L. kauffmannii. L. crinitus* and *Panellus stipticus*; group 7 *Lentinus lepideus, L. strigosus* and *Ganoderma lucidum*; group 8 *Trametes versicolor, Lenzites betulina, Grifola fondosa, Lentinus torulosis* and *L. velutinus*. All are basidiomycetes except the 30 species of *Candida*, which are ascomycetous yeasts. R represents purines, A and G; Y represents pyrimidines, C and T.

Primer screening

The six primer pairs were initially screened with DNA from four different genera of ectomycorrhizal basidiomycete fungi (E439, *Laccaria* sp. B culture; E5386, *Cortinarius sublargus* cf.; E5476, *Russula clelandii*; and E5480, *Boletus prolinus* cf.), one plant (*Eucalyptus marginata*) and one mixed bacterial and yeast sample, under a range of conditions (annealing temperature, $MgCl_2$ concentration and primer concentration). $MgCl_2$ concentrations of 1.6, 1.8 and 2.0 mM were tested with primer concentrations of 0.25 and 1.0 μM across a range of annealing temperatures which differed for each primer pair. Figs 3 and 4 give an example of the results obtained for the ML5/ML6 primers. The basic thermocycler programme consisted of an initial denaturation of 95 °C for 2 min followed by 40 cycles of 30 s at 95 °, 1 min at the annealing temperature, 2 min at 72 °, with a final extension of 8 min at 72 °.

Three primer pairs, ITSF/ITSR, ML3/ML4 and ML5/ML6



Figs 3–4. 5 % acrylamide gels of ML5/ML6 PCR for optimisation of conditions. Annealing temperature was 60 °C (**Fig. 3**) or 62 ° (**Fig. 4**). $MgCl_2$ concentration was 1.6 mM (lanes 1–7), 1.8 mM (lanes 8–14) or 2.0 mM (lanes 15–21). Templates are E439 (*Laccaria* sp. B culture) in lanes 1, 8 & 15; E5386 (*Cortinarius cf. sublargus*) in lanes 2, 9 & 16; E5476 (*Russula* sp.) in lanes 3, 10 & 17; E5480 (*Boletus cf. prolinus*) in lanes 4, 11 & 18; a mixed bacterial culture in lanes 5, 12 & 19; *Eucalyptus marginata* in lanes 6, 13 & 20; no DNA control in lanes 7, 14 & 21. M is molecular weight markers, *Hin*dIII and *Eco*RI digested λ DNA and *Hpa*II digested pUC18 DNA.

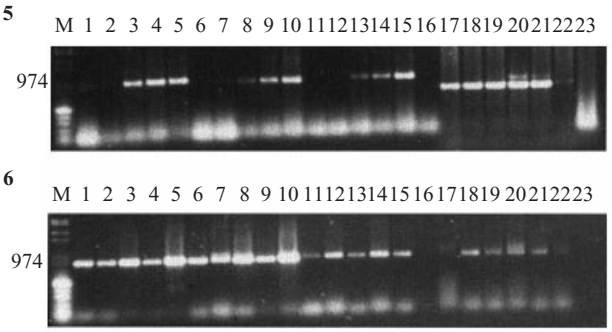
yielded fungal-specific PCR products under these conditions. ITSF/ITSR amplified all four fungal templates at an annealing temperature of 56 °, 2 mM $MgCl_2$ and 1 μM primers. The ML3/ML4 primer pair gave a fungal specific product with an annealing temperature of 59 °, 1.8 mM $MgCl_2$ and 0.25 μM primers. Optimised conditions for ML5/ML6 were an annealing temperature of 60 °, 1.6 mM $MgCl_2$ and 0.25 μM primers. An annealing temperature of 62 ° was also fungal specific at this stage, though we chose the lower temperature as more likely to amplify a wider range of basidiomycetes. We discontinued using primers for which conditions could not be optimised to yield PCR products from the four fungal species with no product from the plant or bacterial DNA. The chitin synthase primer pair, in addition to poor specificity for basidiomycetes, required at least 2–10 ng of template DNA per μl. The product for those basidiomycetes which did amplify was a series of fragments rather than a discrete band. The ML7/ML8 primers were tested with annealing temperatures of 50 °, 52 ° and 54 ° and amplified a strong product from the *E. marginata* DNA under most conditions tested, with poor or no amplification of the *C. sublargus* cf. and *R. clelandii* samples (E5386 and E5476). The IGS primers (CNL12/5SA) were tested at annealing temperatures of 55 °, 60 ° and 63 ° with 2 mM $MgCl_2$, then at 57 ° with 1.6, 1.8 and 2.0 mM $MgCl_2$. No amplification was obtained from *R. clelandii* DNA with a poor product from *C. sublargus* cf. and a faint product from *E. marginata* DNA. Further fungal templates were tested, showing that while *Laccaria* and *Boletus* amplified well, *Cortinarius* and *Ramaria* were unreliable and *Amanita* and *Russula* were not amplified at the higher annealing temperatures which prevented amplification of the *E. marginata* DNA.

The three primer pairs which gave a fungal-specific product from the four fungal DNA templates were then tested against a larger range of basidiomycetes (Table 1), eight ascomycetes, six plant and three multiple bacterial and yeast DNA templates. After lowering the annealing temperature for ITSF/ITSR to 54 ° to enable amplification of some additional basidiomycetes which were not amplified with an annealing temperature of 56 °, ITSF/ITSR and ML3/ML4 primers maintained high specificity. To ensure clarity of pattern from *Amanita* species, the number of cycles in the ITS programme was reduced from 40 to 35. ML5/ML6 failed to amplify about 25 % of the basidiomycetes tested so were not used further. Apart from old specimens which probably had degraded DNA (e.g. the two *C. phalarus* specimens), only two of the species were not amplified with ITSF/ITSR, these were *Hydnum rufescens* and *Austrogautieria manjimupana*. Seven species were also not amplified by ML3/ML4 (Table 1). The optimised conditions for the two primer pairs were thus set as: 1 μM each primer, 2 mM $MgCl_2$, 54 ° annealing temp. and 35 cycles of PCR for the ITSF/ITSR primers, and 0.25 μM primers, 1.8 mM $MgCl_2$, 59 ° annealing temp. and 40 cycles for the ML3/ML4 primers.

PCR amplification

After optimisation of the reaction conditions, two primer pairs, ITSF/ITSR and ML3/ML4, amplified DNA from the basidiomata of a wide range of basidiomycete fungi (Table 1), with no amplification from the DNA of a rust (*Puccinia helianthi*), seven ascomycetes (*Aleuria rhenana*, *Gyromitra infula*, *Helvella lacunosa*, *Labyrinthomyces* sp., *Peziza whitei*, *Trichoglossum hirsutum* and *Morchella* sp.), seven plants (*Acacia pulchella*, *Banksia menziesii*, *Dryandra nivea*, *E. marginata*, *Macrozamia* sp., *Persoonia* sp. and *Pteridium esculentum*), unidentified yeasts (in the mixed bacterial cultures), or bacteria (*E. coli* and DNA extracted from three mixed cultures of bacteria). DNA was amplified from fungi belonging to 28 families (Table 1). The product sizes mostly ranged from 850 to 950 bp for the ITS fragment, and from 790 to 1640 bp for the ML3/ML4 fragment (Table 1), with a few larger and one smaller product of 216 bp for *Leucopaxillus lilacinus*. An ITS PCR product was not obtained from some of the older herbarium specimens, including the two *C. phalarus* collections, another *L. lilacinus* collection (E5083) and another *Descolea maculata* collection (E789).

By comparison, a PCR product was not obtained for nine of the collections in Table 1 (E4063 & E4447, *C. phalarus*; E5778, *Phellodon* sp.; E5634, *Ramaria versatilis*; E5906, *R. lorithamnus*; E5841, *Lactarius clarkeae*; E5797, *Lyophyllum tylicolor*; H7157, *Austrogautieria manjimupana* and H4317, *Setchelliogaster australiensis*) when amplification was attempted with ITS1-F and ITS4-B using the conditions specified in Gardes & Bruns (1993). These fungi were tested at least twice. In addition, ITS1-F/ITS4-B amplified DNA of an ascomycete, *Aleuria rhenana*, and gave faint products for *Neurospora intermedia* and *E. marginata*. ITS1-F/ITS4-B did, however, give a stronger product from some of the dried herbarium material, where the DNA may have been partly degraded.



Figs 5–6. 1 % agarose gels of 5 µl aliquots of PCR with (**Fig. 5**) ITSF/ITSR primers and (**Fig. 6**) ML3/ML4 primers. Template for reactions in lanes 1–5 was E5512 (*Russula clelandii*) DNA, lanes 6–10 E5445 (*Amanita xanthocephala*) DNA, lanes 11–15 E5386 (*Cortinarius sublargus* cf.) DNA. Template concentrations were 1 pg/10 µl reaction for lanes 1, 6 & 10; 3 pg for lanes 2, 7, 11; 10 pg for lanes 3, 8, 12; 30 pg for lanes 4, 9, 14 and 100 pg for lanes 5, 10, 15. Lanes 16 & 23 are no DNA controls. Lanes 17–22 contain a re-amplification of the PCR products in lanes 11–16, using primers (**Fig. 5**) ITS1/ITS4 or (**Fig. 6**) ML3/ML4, and a thermocycler programme reduced to 20 cycles. Lanes M are molecular size standards, *Hin*dIII and *Eco*RI digested λ DNA and *Hpa*II digested pUC18 DNA.

Sequencing and primer modifications

After testing of the full range of fungi revealed that *Austrogautieria manjimupana*, *C. phalarus* and *Hydnum rufescens* were not amplified with ITSF/ITSR, fragments of the 18S and 26S rDNA were amplified and sequenced. The sequences were aligned with those used to design ITSF and ITSR and two new primers, HRITSF and AMITSR (Figs 1 and 2), were designed. HRITSF/ITSR amplified *H. rufescens* DNA, ITSF/AMITSR amplified DNA of *Austrogautieria manjimupana* and the majority of fungi in Table 1, but failed to amplify six of these which were amplified by ITSF/ITSR (H7346, *Torrendia inculta*; E5778, *Phellodon* sp.; E5517, *Cortinarius lavendulensis*; E5417, *Descolea maculata*; H4317, *S. australiensis* and E5443, *Ramaria formosa*). The two additional primers were therefore used only for the two species that were not amplified with ITSF/ITSR, which were used for all other species. Sequencing of fragments of the 18S and 26S rDNA of *C. phalarus* revealed 100 % match with ITS1-F, ITS4-B and ITSR, with mismatches to ITSF in the 5′ region, so non-amplification was ascribed to DNA degradation.

Primer Sensitivity

Using 1 ng of template DNA, the yield was sufficient to enable visualisation of fragments after digestion of 2–10 µl of PCR product. The sensitivity of the primers was tested by amplifying dilutions of DNA template from 1 to 100 pg in a 10 µl reaction (Figs 5–6). ITSF/ITSR amplified from 10 pg of template DNA in a 10 µl reaction, while ML3/ML4 amplified as little as 1 pg. The ML3/ML4 primer pair could be used to re-amplify the PCR product with the number of cycles in the second amplification reduced to 20. The ITSF/ITSR primer pair produced a smear on re-amplification with the same primers, however the internal primer pair ITS1/ITS4 could be used for re-amplification of the ITSF/ITSR product (Fig. 5). After re-amplification with ITS1/ITS4, an ITS product was obtained from the reaction which had 1 pg of template DNA, even though a visible product had not been obtained for the original ITSF/ITSR-primed reaction.

Restriction enzyme digestion and PAGE

The restriction enzymes with 4 bp recognition sites that were used (with the exception of *Msp*I, which cleaves at CCGG) digested the ITS PCR product from most of the fungi tested. The enzymes with 6 bp recognition sites each cut a smaller group, e.g. *Pvu*II cut the ITS fragment from some of the *Ramaria* species, but none of the other genera. As most species were distinguished by digestion of the ITS PCR product with *Alu*I and *Taq*I, the sizes of these fragments are given in Table 1. In addition to fragment sizes, a code for each digestion is given that indicates those patterns which are significantly different to each other. Two patterns are regarded as significantly different if at least one fragment varies in size by at least 10 %, as variation of ± 3 % may result from sizing inaccuracy. Exceptions occur where a large number of fungi give similar patterns, which cannot easily be separated into discrete groups. For example code CJ for *Alu*I digestion covers a range of sizes between 474 and 581 bp for one of the two fragments. Codes with the same initial letter have the largest fragment in the same size range, but may not otherwise be similar.

Species discrimination

Of the 92 species analysed, 78 produced unique PCR–RFLP patterns when the ITS product was separately digested with *Alu*I and *Taq*I (Table 1). Three groups of 2–9 species were not distinguishable to species level after *Alu*I and *Taq*I digestion. The large group contained *Cortinarius* sp., *C.* subgen. *Leprocybe* sp., *C. rotundisporus*, *C. sinapicolor*, *C. vinaceo-cervina*, *C. sublargus*, two *Thaxterogaster* species, and *Lactarius eucalypti*. All except *L. eucalypti* were members of the *Cortinariaceae*. The two other groups were *C. fiveashianus* and *Dermocybe splendida*; and *Hebeloma aminophilum* and *Pholiota* sp. *Mycena pura* and *M. kurramulla* both had codes BF/BA for *Alu*I and *Taq*I patterns, however the sizes of the *Taq*I fragments were sufficiently different to discriminate these two species when comparisons were made on fragment sizes. Similarly, *Cortinarius* sp. and *C. vinaceo-cinereus* were at opposite ends of the range of sizes of *Alu*I restriction fragments for code CJ, though here discrimination was confused by the presence of several other fungi that fit into the CJ/BA pattern. *C. phalarus* was not distinguished as the ITS was not amplified, due to probable DNA degradation in the two available collections.

Digestion with *Hin*fI and *Hae*III discriminated *C. rotundisporus*, *C. sublargus cf.*, the two *Thaxterogaster* species and *L. eucalypti* but not the other four species in the large group (Table 2). *H. aminophilum* and *Pholiota* sp. were also discriminated, but not *C. fiveashianus* from *D. splendida*. However, amplification and digestion of the ML3/ML4 fragment was able to resolve these groups of fungi. For the 92 species of fungi studied, except for *C. phalarus*, amplification

Table 2. *Hin*fI and *Hae*III ITS restriction fragments for those fungi which were not discriminated by *Alu*I and *Taq*I digestion of the ITS. Herb. no. is the accession code for the CSIRO Forestry and Forest Products Mycology Herbarium. For each digestion, the fragment sizes and a letter code for ease of comparison are given.

Herb.		ITS *Hin*fI digestions		ITS *Hae*III digestions	
No.	Species	Code	Fragment sizes	Code	Fragment sizes
E5592	*Cortinarius* sp.	CD	429, 353, 128	DB	567, 265
E5632	*C.* subgen. *Leprocybe* sp.	CD	441, 365, 128	DB	580, 256
E5540	*C.* (*Myxacium*) *rotundisporus*	FF	365, 128	GA	346, 309, 244
E5538	*C.* (*Myxacium*) *sinapicolor*	CD	423, 353, 129	DB	600, 259
E5386	*C.* cfr *sublargus*	CD	427, 343, 128	GE	343, 267, 249
E5993	*C. vinaceo-cinereus*	CD	425, 369, 128	DB	567, 265
H6684	*Thaxterogaster basipurpureum*	EB	369, 246, 207, 135	GE	353, 260, 246
H7265	*T.* sp. nov	CG	418, 228, 130	DB	610, 251
E4644	*Lactarius eucalypti*	CD	428, 345, 127	EB	498, 305
E6025	*Hebeloma aminophilum*	CD	415, 372, 128	ED	530, 193, 129
E5521	*Pholiota* sp.	EC	362, 235, 201, 128	DB	614, 256
E5636	*Cortinarius* (*Myxacium*) *fiveashianus*	CD	427, 370, 130	DB	589, 255
E5639	*Dermocybe splendida*	CD	447, 357, 128	DB	551, 255

and digestion of the ITS and ML3/ML4 fragments has discriminated them to species level.

Identification of an unknown fungus

The most efficient protocol to identify an unknown fungus is to amplify the ITS region and digest the product with *Alu*I and *Taq*I. If the pattern matches a group rather than an individual species, digestion of the ITS with *Hin*fI and *Hae*III or amplification and digestion of the ML3/ML4 fragment is the next step, depending on which group the pattern matches. In some cases, all of these may be necessary. If no ITS product is obtained, or if the ITS PCR–RFLP pattern is unrecognisable, then some information about relationships may be obtained from PCR–RFLP of the ML3/ML4 fragment, as these patterns are more conserved within some families (e.g. Amanitaceae and Russulaceae), despite the higher variation in the Cortinariaceae.

DISCUSSION

The ITS primer pair described here exhibits a greater level of specificity for the hymenomycetes in the sense of Swann & Taylor (1993) than any pair of ITS primers previously described. Primer specificity is of considerable importance when amplifying DNA from a mixed source (Gardes & Bruns 1993). The specificity of these primers was excellent for *Agaricales*, good for *Boletales*, intermediate for the *Aphyllophorales* and poor for *Hydnales*. This probably reflects a degree of genetic divergence in the 18S and 26S rDNA among these higher taxonomic groups, but also the availability of rDNA sequences for these classes of fungi. Amplification of *Russula* DNA was also somewhat erratic until a published sequence of 18S rDNA for *R. compacta* was deposited (Genbank, Accession no. AF026582). It showed that the third base from the 3′ end of the ITSF primer was G rather than A. The primer was modified accordingly and resulted in a far greater efficiency in amplification of *Russula* DNA templates. The ITSR primer was also modified to enable amplification of *A. manjimupana* DNA after sequencing a fragment of the 26S rDNA of that fungus. However the modified primer failed to amplify six other species from Table 1, so the original primer was used for most fungi. Inability to amplify the collections of *C. phalarus* was most likely due to degradation of the DNA of these specimens as sequencing of the relevant regions indicated a complete match with ITSR and three mismatches with ITSF, the two bases at the 5′ end and one base 14 bases from the 3′ end of the primer. At an annealing temperature of 54 ° C, this is unlikely to prevent primer annealing and extension. Amplification with ITS1-F/ITS4-B was also unsuccessful despite a 100 % match to these primers. Shorter PCR products were amplified from the 18S and 26S regions to enable the sequencing to be carried out, though the full-length ITS PCR product was not amplified using ITSF/ITSR or ITS1-F/ITS4-B.

Sequence variation in the primer regions was sufficient to prevent amplification of two species of fungi, *A. manjimupana* and *Hydnum rufescens*. *A. manjimupana* had three mismatches to the ITSR primer, including one at the 3′ end. This species was also not amplified by ITS1-F/ITS4-B, probably for the same reason. *Austrogautieria* is an Australian genus, though the Gautieriacea are considered to be underground relatives of the Boletes, and four species of *Boletus* amplified well. *H. rufescens* had four mismatches to the ITSF primer, though a published sequence for *H. repandum* (Genbank Acc. No. AF026641) was an exact match. This intrageneric heterogeneity within a highly conserved DNA region may be explained by the work of Hibbett & Thorn (2000) who maintain that the currently accepted families and orders of larger basidiomycete taxonomy do not reflect the rDNA gene phylogeny of basidiomycete taxa or may simply be an expression of the stochastic nature of DNA sequence evolution.

A comparison of the specificities of the ITSF/ITSR, ITSF/AMITSR and ITS1-F/ITS4-B primer pairs reveals that none were capable of amplifying the DNA of all the larger basidiomycete fungi tested here. Hence any of the primer combinations would result in non-amplification of DNA of root-tips colonised by these and possibly other unknown fungi. The advantages of improved specificity outweigh the disadvantages of non-amplification of a small number of

species and the availability of specific primers (HRITSF and AMITSR) allows the presence of these fungi to be tested on root-tip DNA samples which fail to amplify with the basidiomycete primers.

The ML3/ML4 primer pair was specific for larger basidiomycetes under optimised conditions, and sensitive, but these primers did not meet the third criterion, the ability to distinguish a large range of species. They may prove useful as a backup, however, in cases where no product is obtained from the ITS primers (e.g. *Hydnum rufescens*) or where the product is unable to satisfactorily distinguish among species (e.g. some species in the *Cortinariaceae*). The ITS region was also found by Kårén *et al*. (1997) to be insufficient to distinguish among all *Cortinarius* species. Chambers *et al*. (1999) were able to distinguish ten species of *Cortinariaceae* by ITS PCR–RFLP, however a large number of *Cortinarius* species are known to occur in eucalypt forests (Bougher 1995, CSIRO Forestry and Forest Products Mycological Research Herbarium, Bougher & Tommerup, pers. comm.). A mitochondrial region for population and community studies is useful because mitochondria have different inheritance patterns to nuclear genes. Additionally, a mitochondrial region may provide discrimination at a higher taxonomic level (genus or family) for the identification of fungi which give an unrecognisable ITS pattern, as we have shown the ML3/ML4 pattern is more conserved within most genera and families. Bruns *et al*. (1998) have demonstrated the usefulness of sequencing of the ML5/ML6 region, but PCR–RFLP of the ML3/ML4 region may be a practical alternative in some families or groups of genera.

Other primers, for example ML7/ML8 and ITS1-F/ITS4-B, produced amplicons from plant or ascomycete DNA samples. The plant DNA was extracted from non-axenic leaf tissue. The PCR products could therefore have been amplified from DNA of surface contaminating or endophytic fungi, or plant DNA, or both (Zhang *et al*. 1997). Because of amplification of this unidentified but non-target DNA, work was not continued with these primers. For ecological or population studies, the root-tip samples from forest soil will in all likelihood be contaminated with many non-target fungi (Summerbell 1990), so it was crucial to keep the specificity of the primers as narrow as possible while still amplifying as many as possible of the larger basidiomycetes.

PCR of the chitin synthase gene was much less sensitive compared to the nuclear and mitochondrial rDNA genes, probably because of lower copy number. Kreuzinger *et al*. (1996) also found lower sensitivity in amplifying glyceraldehyde-3-phosphate dehydrogenase genes of ectomycorrhizal fungi, having to resort to nested PCR and Southern blotting to visualise products. The availability of alternative genomic regions may be desirable for some studies, but for studies requiring species identification of a large number of samples, a target fragment which is easily amplifiable from small quantities of template is preferable.

Apart from ecological and biodiversity investigations, basidiomycete specific primers have a wide range of other applied and research applications, including verification of culture collection isolates, the tracking of inoculated strains of ectomycorrhizal fungi in plantation, field and microcosm experiments and taxonomic studies on herbarium specimens. The nuclear rDNA ITS and mitochondrial rDNA are useful genomic regions for taxonomic and phylogenetic studies. The DNA of collections as young as ten years old may be degraded to a sufficient extent to prevent reliable amplification by PCR, as the *Cortinarius phalarus* specimens which gave no PCR product using ITSF/ITSR or ITS1-F/ITS4-B. Shorter fragments of 26S and 18S rDNA were amplifiable using NL5mun/NL6Bmun (Egger 1995) and NS7/NS8 (White *et al*. 1990). Sequencing of these shorter fragments revealed a close match to all four primers, ITSF, ITSR, ITS1-F and ITS4-B. We concluded that the nuclear DNA was degraded to fragments shorter than 900 bp. A mitochondrial product of approx. 900 bp was amplified, however this was probably because of a higher copy number of the mitochondrial genome or preferential survival of mtDNA because of different organelle genome packaging. In cases where amplification from older collections was poor, the ITS1-F/ITS4-B primers sometimes gave a greater yield. This could be because ITSF and ITSR primers contain three degenerate nucleotide sites, resulting in a complex mixture of primers with a lower effective concentration of each primer. This must be balanced against the lack of amplification of a larger number of species from ITS1-F/ITS4-B. Even freshly dried fungi may suffer some DNA degradation depending on the drying method. In such cases, the use of non-specific primers may result in amplification of DNA from contaminating bacteria or yeasts (O'Donnell *et al*. 1997), causing confusion, delays, or the expensive sequencing of non-target DNA fragments (Zhang *et al*. 1997).

The PCR reactions developed here are sufficiently sensitive to amplify from one to ten pg of target DNA, which enhances the value of the technique for ecological studies such as determining the fungi associated with rare and endangered flora or flora in harsh environments (Massicotte *et al*. 1998, Bougher & Tommerup 1999). Consistency of results has also been demonstrated on up to ten collections of the same species (Glen *et al*. 2001). Thus the described primers meet the requirements of tools for the detection and identification of ectomycorrhizal fungi.

The primers may be equally efficient for discriminating Northern Hemisphere fungi as they were designed mainly using sequences from them. In addition, the ITSF/ITSR primer pair are compatible with existing databases of RFLP patterns created by digestion of fragments amplified by the commonly used primers ITS1, ITS1-F, ITS4 and ITS4-B. These are all internal to ITSF/ITSR and re-amplification has been demonstrated using ITS1/ITS4. While this involves an additional step, several papers have discussed the need to try several dilutions of DNA template to obtain a PCR product from root samples (Henrion *et al*. 1994, Erland, 1995). Where this is necessary, it may prove more efficient to amplify several template concentrations in small reaction volumes, then re-amplify the product for PCR–RFLP, than to routinely amplify in larger volumes. Re-amplification is also useful to obtain a sufficient quantity of PCR product for sequencing template.

Expansion of existing databases to incorporate ML3/ML4 RFLP patterns may also prove illuminating where several species of *Cortinariaceae* occur, as several groups of these are not distinguished by PCR–RFLP of the ITS (Kårén *et al*. 1997,

Jonsson *et al*. 1999). The two DNA regions and the specific primers have the potential to be a practical PCR–RFLP tool for ecological, community population and life history studies, biodiversity assessments of higher basidiomycetes and ecosystem management of a crucial component of the soil nutrient cycling processes in *Eucalyptus* forests and plantations.

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