A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis

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Abstract

**We have assembled a sequence database for 80 genera of Basidiomycota from the
Hymenomycete lineage (*sensu* Swann & Taylor 1993) for a small region of the mitochon-
drial large subunit rRNA gene. Our taxonomic sample is highly biased toward known
ectomycorrhizal (EM) taxa, but also includes some related saprobic species. This gene
fragment can be amplified directly from mycorrhizae, sequenced, and used to determine
the family or subfamily of many unknown mycorrhizal basidiomycetes. The method is
robust to minor sequencing errors, minor misalignments, and method of phylogenetic
analysis. Evolutionary inferences are limited by the small size and conservative nature of
the gene fragment. Nevertheless two interesting patterns emerge: (i) the switch between
ectomycorrhizae and saprobic lifestyles appears to have happened convergently several
and perhaps many times; and (ii) at least five independent lineages of ectomycorrhizal
fungi are characterized by very short branch lengths. We estimate that two of these groups
radiated in the mid-Tertiary, and we speculate that these radiations may have been caused
by the expanding geographical range of their host trees during this period. The aligned
database, which will continue to be updated, can be obtained from the following site on
the WorldWide Web: http://mendel.berkeley.edu/boletus.html.**

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clock

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Introduction

The diversity of ectomycorrhizal (EM) fungi is huge.
Thousands of species are known on worldwide or regional
scales and tens of species are frequently encountered even
within monoculture forests of 0.1 ha (Bruns 1995). This
diversity alone would represent an intimidating factor for
many ecological studies, but the difficulty in dealing with
EM fungi is compounded by the fact that most species are
identifiable only by their fruiting structures.

Much effort has been made to remedy this problem, but
all of the existing methods still leave significant numbers
of unknowns. Morphological approaches have resulted in

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beautifully illustrated manuals (Agerer 1987; Ingleby *et al.*
1990), but the number of species described in this way are
relatively few and many common types are essentially
described as imperfect states with unknown affinities
(Agerer 1987; Ingleby *et al.* 1990; Agerer 1994). Molecular
methods currently available enable one to match restric-
tion fragment length polymorphisms (RFLP) patterns of
unknown mycorrhizae to known fungi (Gardes *et al.* 1991;
Henrion *et al.* 1992; Gardes & Bruns 1993; Kårén *et al.* 1997),
or to use DNA probes to test for specifically characterized
taxa or genotypes (Marmeisse *et al.* 1992; Bruns & Gardes
1993). These methods offer three main advantages over
morphological methods: (i) some can be directed at strain-
level or at least subspecific-level identification; (ii) they
require less time to learn than morphological methods;

and (iii) large numbers of samples can be dealt with more easily because the initial visual sorting process is relatively fast and mycorrhizal tips can then be freeze-dried and stored indefinitely. Unfortunately, RFLP or probe-based methods have the same limitation as morphological approaches: unmatched types remain unknown.

In this study we present a sequence database that helps to place unknowns into smaller, essentially family or subfamily sized, monophyletic groups. The region chosen for this database is a small ($\approx$ 400 bp) fragment of the mitochondrial large subunit rRNA gene. The fungal sequences for this region can be amplified directly from individually extracted mycorrhizal root tips using fungal-specific primers, and the resulting products can be sequenced and compared to known sequences in the database. This method, in combination with the other available molecular methods, enables us to identify virtually all mycorrhizal samples we have encountered to some meaningful taxonomic level (Cullings *et al.* 1996; Gardes & Bruns 1996; Taylor & Bruns 1997).

Materials and methods

Extractions of DNA were made from herbarium samples, cultures of identified basidiomycetes and freeze-dried mycorrhizae collected in nature (Table 1) by methods described previously (Bruns *et al.* 1990; Gardes & Bruns 1993). The unknown mycorrhizae were derived from *Pinus muricata*, members of the Monotropoideae, and orchids; details of these studies have been reported elsewhere (Cullings *et al.* 1996; Gardes & Bruns 1996; Taylor & Bruns 1997). Diluted crude extracts were used as templates for 35 amplification cycles using the ML5 and ML6 primers (White *et al.* 1990), an annealing temperature of 53 °C or 55 °C, and other cycling parameters as previously described (Bruns *et al.* 1990).

Sequences of the PCR products were determined manually with S35 labelling using single-stranded templates generated from asymmetric reactions as described (Bruns *et al.* 1990), or by cycle sequencing of double-stranded products using fluorescent dideoxy-terminators and an ABI 377 automated sequencer according to the manufacturer's instructions (Applied Biosystems, Prism kit). The primers ML5 and ML6 were used as sequencing primers. Sequences were determined for both strands, compared, and corrected for the 67 taxa indicated (Table 1). All other sequences were determined in only a single direction but error correction in these was facilitated by comparison to closely related sequences and the original data for all variant positions were re-examined and confirmed or corrected.

An initial alignment of $\approx$ 60 taxa was made with the

Table 1 Taxa and specimens in the database

Tree location*	Taxon†	Isolate‡	ds/ss§	Accession no.**
14–15	*Agaricus brunnescens*	SAR88/411	ds	S:1333156
9	*Albatrellus ellisii*	TDB-1493	ss	S:1333174
7	*Albatrellus flettii*	TRH264	ds	S:1333189
9	*Albatrellus peckianus*	DAOM-216310 *	ds	S:1333202
7	*Albatrellus skamanius*	JL 92–89	ds	S:1333243
9	*Albatrellus syringae*	DAOM-216918	ds	S:1333261
6	*Alpova olivaceotinctus*	JMT-5376	ds	S:1333271
12	*Amanita calyptrata*	TDB-1498	ds	S:1333278
12	*Amanita francheti*	TDB-928	ds	S:1333291
12	*Amanita gemmata*	TDB-1523	ds	S:1333302
12	*Amanita magniverrucata*	TDB-1514	ds	S:1333348
12	*Amanita muscaria*	TDB-1513	ds	S:1333370
12	*Amanita pachycolea*	TDB-1508	ds	S:1333371
12	*Amanita pantherina*	BRECKON306	ds	S:1333372
12	*Amanita phalloides*	TDB-1639	ds	S:1333373
12	*Amanita silvicola*	TDB-1506	ds	S:1333374
11	*Armillaria albolanaripes*	TDB-1404	ss	S:1333375
11	*Asterophora lycoperdoides*	TDB-1227	ss	S:1333376
1	*Austroboletus betula*	RV-9.2*	ss	S:1333377
13–14	*Bolbitius vitellinus*	SAR 84–100	ds	S:1333378
1	*Boletellus ananas*	HDT-6597	ds	S:1333379
1	*Boletellus chrysenteroides*	TDB-513	ss	S:1333380
1	*Boletellus russellii*	TDB-800	ss	S:1333381
1	*Boletus affinis*	TDB-538	ss	S:1333382
1	*Boletus edulis*	TDB-1002	ss	S:1333383

Table 1 *Continued*

Tree location*	Taxon†	Isolate‡	ds/ss§	Accession no.**
1	*Boletus flaviporus*	TDB-1008	ss	S:1333384
1	*Boletus mirabilis*	TDB-1306	ss	S:1333385
1	*Boletus pallidus*	TDB-1231	ss	S:1333386
1	*Boletus satanas*	TDB-1000	ds	S:1333387
1	*Boletus smithii*	TDB-970	ss	S:1333388
1	*Boletus subglabripes*	TDB-634	ss	S:1333389
1	*Boletus viridiflavus*	TDB-1236	ss	S:1333390
8–9	*Bondarzewia montana*	TDB-1471	ss	S:1333391
6	*Brauniellula albipes*	F-2431	ds	S:1333392
7	*Byssoporia terrestris*	Z-14*	ss	S:1333393
17	*Cantharellus cibarius*	TDB-1427	ds	S:1333394
17	*Cantharellus cinnabarinus*	TDB-389	ss	S:1333395
17	*Cantharellus tubaeformis*	TDB-1434	ss	S:1333396
2	*Chalciporus piperatoides*	TDB-973	ss	S:1333397
1	*Chamonixia ambigua*	HS-2021	ds	S:1333398
6	*Chroogomphus vinicolour*	TDB-1010	ds	S:1333399
4	*Coniophora arida*	FP-104367-SP*	ss	S:1333400
4	*Coniophora puteana*	FP-102011*	ss	S:1333401
4	*Coniophora puteana*	MAD515*	ss	S:1333402
13	*Cortinarius ponderosus*	HDT-53966	ds	S:1333403
13	*Cortinarius vanduzerensis*	TRH281	ds	S:1333404
13	*Cortinarius violaceus*	TDB-1320	ds	S:1333405
1	*Gastroboletus citrinibrunneus*	HDT-40189	ds	S:1333406
16	*Gautieria monticola*	SNF-115	ds	S:1333407
6	*Gomphidius glutinosus*	TDB-957	ds	S:1333408
16	*Gomphus clavatus*	TDB-1583	ss	S:1333409
16	*Gomphus floccosus*	TDB-1310	ss	S:1333411
5	*Gyrodon merulioides*	TDB-532*	ds	S:1333414
5	*Gyroporus cyanescens*	TDB-1214	ds	S:1333416
13	*Hebeloma crustuliniforme*	TRH277	ds	S:1333418
8–9	*Heterobasidion annosum*	KV-340	ds	S:1333421
15	*Hygrocybe cantharellus*	TDB-334	ds	S:1333423
3	*Hygrophoropsis aurantiaca*	TDB-585	ds	S:1333425
15	*Hygrophorus pudorinus*	TDB-1557	ds	S:1333428
15	*Hygrophorus sordidus*	TDB-727	ds	S:1333437
15	*Hygrophorus speciosus*	TDB-650	ds	S:1333488
6	*Hymenogaster sublilacinus*	F2250	ds	S:1333489
13	*Inocybe sororia*	TDB-1427	ss	S:1333490
16	*Kavinia alboviridis*	SNF-284	ds	S:1333491
14	*Laccaria laccata*	HDT 53791	ds	S:1333492
8	*Lactarius piperatus*	TDB-1223	ss	S:1333493
8	*Lactarius volemus*	TDB-1225	ss	S:1333494
1	*Leccinum holopus*	DJM-592	ds	S:1333495
1	*Leccinum manzanitae*	TDB-969	ss	S:1333496
1	*Leccinum rubropunctum*	TDB-1203	ss	S:1333497
11	*Leucopaxillus amarus*	TDB-1336	ss	S:1333498
6	*Melanogaster tuberiformis*	TDB-1042, JMT-26	ss	S:1333499
13	*Naematoloma aurantiaca*	TDB-585*	ds	S:1333500
13	*Nolanea sericea*	SAR 88–415	ss	S:1333501
8–9	*Panus conchatus*	TDB-1049	ss	S:1333502
2	*Paragyrodon sphaerosporus*	TDB-420*	ds	S:1333503
3	*Paxillus atrotomentosus*	TDB-782*	ss	S:1333504
2	*Paxillus involutus*	TDB-642*	ds	S:1333505
3	*Paxillus statuum*	REH-5904	ds	S:1333506
5	*Phaeogyroporus portentosus*	HDT-42534	ds	S:1333507
1	*Phylloporus rhodoxanthus*	TDB-540*	ds	S:1333508
14–15	*Piloderma croceum*	CBS 294.77	ds	S:1333509

Table 1 *Continued*

Tree location*	Taxon†	Isolate‡	ds/ss§	Accession no.**
5	*Pisolithus arrhizus*	TDB-1051,1052	ss	S:1333510
9	*Polyporoletus sublividus*	DAOM 194363	ds	S:1333511
10	*Pseudotomentella tristis*	LT-60	ds	S:1333512
1	*Pulveroboletus ravenelii*	TDB-1307	ds	S:1333513
16	*Ramaria araiospora*	TDB-1414	ss	S:1333514
16	*Ramaria conjunctipes*	TDB-1479	ss	S:1333515
6	*Rhizopogon truncatus*	AHS-68359	ss	S:1333516
6	*Rhizopogon evadens*	TDB-1303	ss	S:1333517
6	*Rhizopogon ochraceorubens*	TDB-1015	ss	S:1333518
6	*Rhizopogon subcaerulescens*	F-2882	ds	S:1333519
6	*Rhizopogon villosulus*	AHS-65445	ss	S:1333520
6	*Rhizopogon vinicolour*	AHS-68595	ss	S:1333521
8	*Russula laurocerasi*	TDB-1222	ss	S:1333522
8	*Russula rosacea*	TDB-895	ds	S:1333523
10	*Sarcodon imbricatum*	LT-2	ds	S:1333524
18	*Sebacina sp.*	UAMH6444*	ds	S:1333525
3	*Serpula himantioides*	Bud-205-A*	ds	S:1333526
3	*Serpula himantioides*	FP-94342-R*	ss	S:1333527
3	*Serpula incrassata*	L-11504-SP*	ss	S:1333528
	Serpula incrassata	MAD563	ss	S:1333529
1	*Strobilomyces floccopus*	TDB-1213	ss	S:1333530
6	*Suillus cavipes*	TDB-645	ds	S:1333531
6	*Suillus ochraceoroseus*	SAR-84–137*	ds	S:1333532
6	*Suillus sinuspaulianus*	DAOM-66996*	ds	S:1333533
6	*Suillus tomentosus*	TDB-661*	ss	S:1333534
3	*Tapinella panuoides*	RLG-12933-SP	ds	S:1333535
10	*Thelephora sp.*	TDB-1504	ds	S:1333536
10	*Thelephora terrestris*	S-142*,1542	ss	S:1333537
10	*Tomentella atrorubra*	LT64	ds	S:1333538
10	*Tomentella cinerascens*	LT66	ds	S:1333539
10	*Tomentella lateritia*	LT56	ds	S:1333540
10	*Tomentella sublilacina*	TDB-2015	ds	S:1333541
11	*Tricholoma flavovirens*	TDB-1395	ss	S:1333542
11	*Tricholoma manzanitae*	KMS 194	ds	S:1333543
11	*Tricholoma pardinum*	TDB-1032	ss	S:1333544
6	*Truncocolumella citrina*	AHS-30164	ds	S:1333545
18	*Tulasnella irregularis*	UAMH-574*	ds	S:1333546
1	*Tylopilus alboater*	TDB-1206	ss	S:1333547
14–15	*Waitea circinata*	GA-846*	ds	S:1333645
1	*Xerocomus chrysenteron*	TDB-365*	ds	S:1333649
1	*Xerocomus subtomentosus*	TDB-991	ss	S:1333651
12	1MR (*Pinus*) *Amanita gemmata*	935F2 ᴍL5	ss	L46376
12	2MR (*Pinus*) *Amanita francheti*	995AA ML5	ss	L46377
10	3MR (*Pinus*) *Tomentella sublilicina*	935E2 ᴍL5	ss	L46378
10	4MR (*Pinus*) *Tomentella sublilicina*	935BR ML5	ss	L46379
10	5MR (*Pinus*) *Tomentella sublilicina*	SEEDLING19	ss	S:1333657
10	6MR (*Pinus*) *Tomentella sublilicina*	'930C-ML6'	ss	L46380
10	7MR (*Pinus*)	939B ML5	ss	L46381
10	8MR (*Pinus*)	942C2R ML5	ss	L46382
1	9MR (*Pinus*)	945 A2 ᴍL5	ss	L46383
1	10MR (*Pinus*)	936F2R ML5	ss	L46384
6	11MR (*Pinus*) *Rhizopogon subcaerulescens*	995AB ML5	ss	L46385
8	12MR (*Pinus*) *Russula xerampelina*	944B ML5	ss	L46386
8	13MR (*Pinus*) *Russula xerampelina*	942B2 ᴍL5	ss	L46387
17	14MR (*Pinus*)	936AR ML5	ss	L46388
17	15MR (*Pinus*)	935E ML5	ss	L46389
1	16MR (*Pinus*) *Xerocomus chrysenteron*	996 ʙc2R ML	ss	L46390

Table 1 *Continued*

Tree location*	Taxon†	Isolate‡	ds/ss§	Accession no.**
1	17MR (*Pinus*)	930 B PATT	ss	L46391
1	18MR (*Pinus*)	945 B PATT	ss	L46392
6	19MR (*Pinus*)	927 B PATT	ss	L46393
14	20MR (*Pinus*) *Laccaria amethysteo-occidentalis*	996 BA ML5	ss	L46394
10	21MR (*Pinus*)	915 R^2 ML5	ss	S:1333667
8	22MR (*Pinus*) *Russula brevipes*	SEEDLING B	ss	L46395
10	23MR (*Pinus*)	SD 41 FALL	ss	S:1386427
10	24MR (*Pinus*)	SD 49 FALL	ss	S:1386428
6	25MR (*Monotropa hypopithys*)	4M 3 12 92	ss	S:1386429
6	26MR (*Monotropa hypopithys*)	C1 & Lake grant2	ss	S:1386430
12	27MR (*Hemitomes*)	spoint	ss	S:1386431
8	28MR (*Monotropa uniflora*)	mich1	ss	S:1386432
6	29MR (*Monotropa hypopithys*)	lake wtII	ss	S:1386433
8	30MR (*Monotropa uniflora*)	23	ss	S:1386434
8	31MR (*Monotropa uniflora*)	22 28 32	ss	S:1386435

*Position on tree in Fig. 2, two numbers are given for taxa located between numbered groups.

†MR taxa are field-collected ectomycorrhizae.

‡Cultures are indicated*; all others are from either dried fruit body collections or field collected mycorrhizae for MR collections.

§ds, sequence determined in both directions; ss, sequence determined in a single direction.

**S, accesssion numbers are for the Genome Sequence Database; others are GenBank.

CLUSTAL V multiple alignment program (Higgins *et al.* 1992) on a Sun Sparc station.

This alignment was examined and adjusted manually using Microsoft Word on a large-screen Macintosh computer. Manual alignment was facilitated by the use of a colour font. Sample names were temporarily removed during manual alignment to avoid bias. Additional taxa were aligned in small groups and added into the large alignment manually by the same method. After viewing the results of initial phylogenetic analyses, the alignment within well-supported monophyletic groups was re-examined and adjusted. This was done to ensure that all identical or nearly identical sequences were aligned in the same way.

The final alignment, which excludes the unalignable 5′ portion of the ML5/ML6 fragment (Fig. 1), was formatted as a PAUP file (Swofford 1993). All base positions included in the analysis were written in upper case, a small internal region that could not be aligned was written in lower case, and these latter bases were then ignored by equating them to missing data. Most gaps that were introduced for alignment purposes were also treated as missing data, but a few were coded as fifth character states 'X'. The criteria used for these codings are the same as those described previously (Bruns *et al.* 1992). Exclusion sets of taxa were setup for convenient analysis of subsets of the data. PHYLIP formatted files, used for distance analysis, were derived from the PAUP file. Other than format they also differed from the latter in that all gaps were treated as missing data.

Neighbour-joining analysis was initially conducted on all known and unknown taxa using Kimura 2-parameter

distances; the programs DNADIST and NEIGHBOR from PHYLIP 3.4 (Felsenstein 1995) were used to generate the distance matrix and to produce the tree. Confidence in the branches of the neighbour-joining tree was assessed by bootstrap analysis (Felsenstein 1985), using 500 replicates. The programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE in the PHYLIP package (Felsenstein 1995) were used for this purpose. All PHYLIP programs were run on a Sun Sparc station.

Parsimony analyses were conducted with PAUP 3.1.1 on subsets of taxa to assess the effects of method of analysis. The taxa used were selected based on results from the neighbour-joining analysis such that multiple representatives from all major clades and divergent taxa not clearly placed in such groups were included. The representatives selected were chosen to maximize sequence differences within major clades. A total of 10 random addition sequences were run using the heuristic search option in PAUP 3.1.1 (Swofford 1993) on a Macintosh Quadra 800. Small batches of unknown taxa were analysed with this subset of knowns to compare placements between parsimony and neighbour-joining methods.

Later analyses were conducted with a beta version of PAUP (4.0d52) written by David Swofford using neighbour-joining of the patristic distance matrix on a Power Macintosh 7500/100. Confidence in branches was assessed with 1000 bootstrap replicates.

Molecular clock estimates were made for 1137 aligned positions from the 5′ and central portions of the nuclear small subunit (Nu-SSU) rRNA gene in the following way.

Loc.	Primer	Sequence	Position relative to introns
	ML5	ctcggcaaattatcctcataag	interrupted by intron a
	ML6	cagtagaagctgcatagggtc	3' to intron e
1	MLIN3	cgacacaggttcgtaggtag	5' to target fragment
2	ML5.5	gatcctcatagttaagaccgc	3' to intron a, 5' to others
3	CML5.5	tagcggtcttaactatgagg	5' to intron b
4	MLIN5R	ttattgataccggaccac	3' to intron c, 5' to d
5	MLIN4	ggtagcaaaataaattggcc	5' to intron c, 3' to b
6	MLIN1R	agttgtagagacagtgaagc	3' to intron d, 5' to e
7	MLIN2	ggccattaaatgtggtccgg	interrupted by intron c
8	CML7.5	ccgccccagtcaaactgcc	3' to target fragment
9	MLIN1F	gcttcactgtctctacaact	5' to intron e, 3' to others

Fig. 1. A. Diagrammatic representation of sequenced portions of the ML5–ML6 region. Total size of the region inclusive of primers is 416 bp in *Suillus sinuspaulianus*. Most other taxa have very similar-sized fragments. The cross-hatched regions indicate size-variable portions of the fragment. The 5′-most region, which contains the ML5 primer sequence, was excluded from the analysis because it varies as much as 100 bp in size and the sequences are too different to be aligned. The database starts immediately after this region with the conserved sequence indicated. Most taxa have a sequence that is nearly identical to the top line. The lower line shows the variant positions that are typical of members of the Russulaceae. The shaded region contains small inserts of less than 15 bp in some agarics; it is included in the database, but portions of it are excluded from the analysis as described in the Materials and methods. The 3′-most portion of the region is not determined for most taxa in the database, because of technical difficulties associated with its proximity to the ML6 primer. B. Five introns encountered infrequently in the taxa sampled. Approximate locations of know introns is shown (vertical arrows, a–e). Primers that work around these are indicated (horizontal arrows) and their names and sequences are given. Flanking regions of the gene that are outside of the target fragment are shown with dashed lines.

The shortest parsimony tree was found for the following 13 selected taxa (GenBank accession number): *Tremella moriformis* (U00977), *T. globospora* (U00976), *Spongipellis unicolour* (M59760), *Boletus satanas* (M94337), *Xerocomus chrysenteron* (M94340), *Phylloporus rhodoxanthus* (M90825), *Paragyrodon sphaerosporus* (M90826), *Paxillus atrotomentosus* (M90824), *Chroogomphus vinicolour* (M90822), *Gomphidius glutinosus* (M90823), *Rhizopogon subcaerulescens* (M90827), *Suillus cavipes* (M90828), *Suillus sinuspaulianus* (M90829). All 24 trees that were three steps longer or less were compared with maximum likelihood

(DNAML, no clock assumption, Felsenstein 1995), and those that were significantly worse than the best tree were rejected. The remaining trees were examined visually and those that included *Paragyrodon* within, rather than as the sister group to, the boletoid group were rejected; this criterion was used because four rRNA genes examined to date (Mt-LSU, Mt-SSU Nu-SSU, and Nu-LSU) all depict *Paragyrodon* as outside the boletoid group and collectively show strong support for this relationship even though the Nu-SSU gene does not specify this relationship strongly (Bruns & Szaro 1992; T. D. Bruns, unpublished data). The remaining 12 trees, which differed only in the branching orders within the boletoid group and in the placement of *Paxillus* relative the suilloid and boletoid groups, were each submitted to maximum likelihood with a clock constraint (DNAMLK, Felsenstein 1995); this program forces the constraint that each terminal branch is equidistant from the root and thus corrects for the rate differences on a given topology. Calibration for the root of the tree was based on Berbee and Taylor's estimate of 220 Ma for the divergence of *Tremella* from polypores, agarics and boletes (Berbee & Taylor 1993). A 100 Ma error was allowed by also using estimates of 270 and 170 Ma.

Results and Discussion

Phylogenetic analyses and placement of unknowns

A total of 152 sequences were determined for the ML5–ML6 region of the mitochondrial large subunit. Of these sequences, 121 were derived from identified samples and 31 from initially unidentified mycorrhizae (Table 1). The known samples include representatives of 80 genera from 17 families. We were able to determine sequences for virtually all samples. The only consistent exceptions can be attributed to taxa that contained introns in this region. We know that at least five introns can be present and these can dramatically increase the size of the region and in some cases disrupt the ML5 primer site (Li 1995); this can make it difficult to amplify or sequence the region from DNA templates. Among the taxa we have sampled, however, introns were rarely encountered and were only found within a subset of species of *Albatrellus, Byssoporia, Coniophora, Heterobasidion, Hydnellum, Hygrophorus, Kavinia, Macrolepiota, Rhizopogon,* and *Suillus*. Because these introns were fairly rare and variably present even within species, we did not try to use them for identification purposes, but instead tried to work around them. Obtaining full-length sequence in species containing multiple introns was often difficult and not always achieved. Primers that avoid the introns and amplify the flanking pieces of the structural gene (Fig. 1) helped us to obtain at least partial sequences in all but two species (*Albatrellus ovinus* and *Hydnellum peckii*).

Alternatively, rRNA templates and reverse transcriptase PCR could be used, but this approach may necessitate different preservation and extraction methods to ensure that RNA templates are not degraded.

We were able to determine sequences directly from field-collected mycorrhizae in virtually all cases. Although contaminating soil fungi must have been present on most or all of these samples, they did not appear to contribute to the sequences determined. We say this because the sequence clarity was usually very good and the unknown sequences were placed into well-defined lineages of EM fungi (Fig. 2). Furthermore, many of these placements have been confirmed by internal transcribed spacer region (ITS) RFLP matches or oligonucleotide probing (Cullings *et al*. 1996; Gardes & Bruns 1996). These two other methods use different primers and target different regions and thus are independent of the ML5/ML6 sequence analyses (Bruns & Gardes 1993; Gardes & Bruns 1993; Bruns 1996)

After exclusion of the unalignable 5′ portion of the fragment and 15 bp of internal positions (Fig. 1), the remaining sequence was represented by 339 aligned positions within the database; ≈ 40 bp of the 3′ end was not determined in the majority of the taxa. In total, the number of variable positions is 181, and 143 of these are cladistically informative (i.e. they contain variant states that are shared by two or more taxa).

A neighbour-joining tree based on patristic distances generated from PAUP is shown and branches supported by more than 50% of the bootstrap replicates are indicated (Fig. 2). A tree based on Kimura 2-parameter distances generated by PHYLIP contained all but one of the major groupings shown in Fig. 2a. The only exception was that the position of the Hygrophoraceae (group 15, Fig. 2) was shifted and was no longer monophyletic. In both trees all of the unknowns were placed within the same groups indicated.
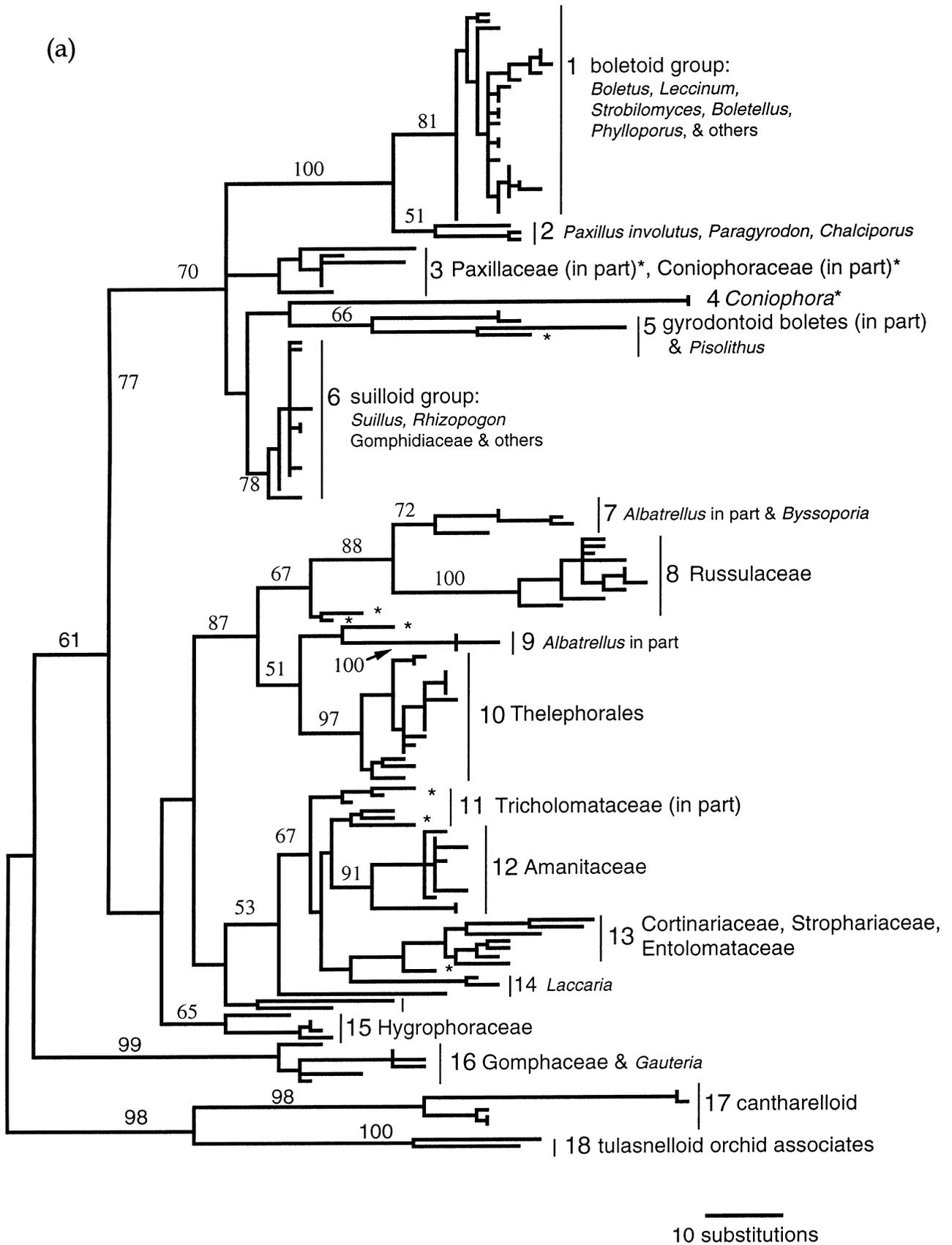
The large number of taxa, the relatively low number of informative characters, and the many near-zero branch lengths made the number of equally parsimonious trees very high and the computational time too long to allow for a complete analysis of the entire dataset with parsimony. However, even very short (< 10 min) and incomplete parsimony runs using the whole dataset resulted in the same placement of all of the unknown taxa into the same numbered family or subfamily groups indicated (Fig. 2). Longer runs with subsets of taxa resulted in very similar trees to the one shown in Fig. 2; all the branches that were supported by more than 60% of the neighbour-joining bootstrap replicates and also many of the lesser supported branches were also found with the partial parsimony analyses.

Even partial sequences resulted in fairly confident and apparently accurate placements at the family or subfamily level. The placements of partial sequences of *Gomphus clavatus*, *Ramaria conjunctipes*, and *Kavinia alboviridis* are good examples, as all were placed with other known members of the Gomphaceae (group 16, Fig. 2d) with high confidence (99% bootstrap). Thelephoroid unknowns MR-1, 4 and 7 are also good examples; these have recently been confirmed to be closely related to *Thelephora* and *Tomentella* by sequence analysis of the internal transcribed spacer region (D. L. Taylor, unpublished results). Partial sequences, however, often yielded artifactually long-terminal branches especially if the missing data were in highly conserved regions. This was true if neighbour-joining distances were displayed, and it is the reason that we chose to display character changes (i.e. parsimony distances) on the tree shown.

Sequence error and minor misalignments also appear to have little effect on placement of unknowns. The major effect was that the terminal branch lengths were exaggerated. We did not test this in a rigorous way, but we have observed this effect from preliminary analysis of many unknowns in which the sequences were initially incomplete, poor in quality, or misaligned. Yet all such sequences were placed correctly by the phylogenetic analysis as judged by later analysis of completed and accurate sequences, or by ITS-RFLP matches to species within the groups. The phylogenetic resolution was low in many parts of the tree as judged by bootstrap analysis (i.e. those < 70% in Fig. 2). Fortunately the low phylogenetic resolution had almost no effect on the family or subfamily placement of the unknown mycorrhizal fungi we encountered. This apparent contradiction is true because the unknowns we encountered and tested turned out to be members of groups that were well sampled and strongly supported by phylogenetic analyses. The strong support is due to the fact that very few sequence differences occur within most major mycorrhizal lineages sampled, while sequence variation between these groups and other taxa is moderate to large (Table 2). Indeed, many closely related species and genera have identical or nearly identical sequences in this region. For example, among the nine species of *Amanita* sampled, six have identical sequences and only *A. francheti* differs by more than 2%. Similarly, within the suilloid group, some species of *Suillus*, *Rhizopogon* and Gomphidiaceae have identical sequences, and all others placed within this group differ at only a few positions.

Placements within the boletoid (Fig. 2, group 1) and suilloid groups (group 6), the Russulaceae (group 8), the Thelephorales (group 10), and *Amanita* (group 12) were typically unequivocal because these groups are both well sampled in our database and have very distinct and relatively uniform ML5/ML6 sequences. High bootstrap values define all of these lineages except the suilloid group (group 6), and even in this case the bootstrap value was moderately high (78%) and within the range that can be considered as strong (Hillis & Bull 1993). Furthermore,

(a)

1 boletoid group:
*Boletus, Leccinum,
Strobilomyces, Boletellus,
Phylloporus,* & others

81

100

51

2 *Paxillus involutus, Paragyrodon, Chalciporus*

70

3 Paxillaceae (in part)*, Coniophoraceae (in part)*

4 *Coniophora**

66

5 gyrodontoid boletes (in part)
& *Pisolithus*

77

6 suilloid group:
Suillus, Rhizopogon
Gomphidiaceae & others

78

72

7 *Albatrellus* in part & *Byssoporia*

88

67

100

8 Russulaceae

87

51

9 *Albatrellus* in part

100

97

10 Thelephorales

61

11 Tricholomataceae (in part)

67

12 Amanitaceae

91

53

13 Cortinariaceae, Strophariaceae,
Entolomataceae

14 *Laccaria*

65

15 Hygrophoraceae

99

16 Gomphaceae & *Gauteria*

98

17 cantharelloid

98

100

18 tulasnelloid orchid associates

10 substitutions

(b)

Boletus affinis
Phylloporus rhodoxanthus
Boletus satanas
Boletus pallidus
Xerocomus subtomentosus
Leccinum manzanitae
Leccinum holopus
Leccinum rubropunctum
Chamonixia ambigua
Strobilomyces floccopus
Boletus flaviporus
Boletus subglabripes
Boletus smithii
Austroboletus betula
Boletellus chrysenteroides
Boletus viridiflavus
Boletellus ananas
Boletus edulis
17MR (Pinus)
Boletellus russellii
Tylopilus alboater
Pulveroboletus ravenelii
9-MR (Pinus)
10MR (Pinus) PS
16MR (Pinus) X. chrysenteron
18MR (Pinus)
Xerocomus chrysenteron
Boletus mirabilis
Gastroboletus citrinibrunneus

1

81

100

51

Chalciporus piperatoides
Paxillus involutus
Paragyrodon sphaerosporus

99

2

Paxillus atrotomentosus *
Serpula incrassata *
Paxillus statuum ?
Serpula himantioides *
Tapinella panuoides *
Hygrophoropsis aurantiaca *

3

70

100

Coniophora arida *
Coniophora puteana *

4

66

100

Gyroporus cyanescens
Pisolithus arrhizus

100

Phaeogyroporus portentosus *?
Gyrodon merulioides *

5

Melanogaster tuberiformis
Rhizopogon evadens
11MR (Pinus) Rhizopogon subcaerulescens
25MR (Monotropa hypopithys) OP PS
Hymenogaster sublilacinus
Rhizopogon vinicolor
Gomphidius glutinosus
Truncocolumella citrina
Rhizopogon truncatus
26MR (Monotropa hypopithys) OP
Suillus ochraceoroseus
Alpova olivaceotinctus
Rhizopogon ochraceorubens
Rhizopogon subcaerulescens
Chroogomphus vinicolor
Suillus sinuspaulianus
Suillus cavipes
29MR (Monotropa hypopithys) OP PS
Rhizopogon villosulus
Suillus tomentosus
Brauniellula albidipes
19MR (Pinus) OP

6

78

10 substitutions

(c)


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                                                    71  Albatrellus-ellisii
                                              72 ┌─────── Albatrellus skamanius          ┐ 7
                                                 └─────── Albatrellus flettii            ┘
                                              ──── Byssoporia terrestris
                                    88                 Lactarius piperatus              ┐
                                                       30MR (Monotropa uniflora)        │
                                                       31MR (Monotropa uniflora)        │
                                                       Lactarius volemus                │
                                                       12MR (Pinus) R. xerampelina      │
                          67                           13MR (Pinus) R. xerampelina      │ 8
                                              100      Russula rosacea                  │
                                                       28MR (Monotropa uniflora )       │
                                                       Russula laurocerasi              │
                                                       22MR Russula brevipes            ┘
                           91  ┌── Bondarzewia montana *
                              └─ Heterobasidion annosum *
                     87              Panus conchatus *
                                100  Albatrellus peckianus                               ┐ 9
                                     Polyporoletus sublividus ?                          │
                         51          Albatrellus syringae                                ┘
                                     Thelephora terrestris
                                     Thelephora sp
                                        3-MR (Pinus) Tomentella sublilicina PS
                                        4-MR (Pinus) Tomentella sublilicina PS
                                        8-MR thelephoroid sp2
                                 60     7-MR thelephoroid sp2 PS
                                        Tomentella lateritia
                                     Tomentella sublilicina
                                     Tomentella atrorubra                                │ 10
                                        6-MR (Pinus) Tomentella sublilicina
                                        5-MR (Pinus) Tomentella sublilicina
                            97          23MR (Pinus)
                                        24MR (Pinus)
                                     Tomentella cinerascens
                                        21MR (Pinus)
                                     Pseudotomentella tristis
                                     Sarcodon imbricatum
                                           Leucopaxillus amarus *?                        ┐
                                          Tricholoma manzanitae                          │ 11
                                          Tricholoma flavovirens                         ┘
                                       Tricholoma pardinum
                                       Asterophora lycoperdoides *
                                       Armillaria albolanaripes *?
                    67                    Amanita phalloides                             ┐
                                          Amanita calyptrata                            │
                                          Amanita silvicola                             │
                                          Amanita gemmata                               │
                                          1MR (Pinus) A. gemmata                        │
                                          Amanita muscaria                              │ 12
                                          Amanita pantherina                            │
                                          Amanita magniverrucata                        │
                             91           27MR (Hemitomes)                              │
                                          Amanita pachycolea                            │
                                          2MR (Pinus) A. francheti                      │
                                          Amanita francheti                             ┘
                                             81    Nolanea sericea *?                   ┐
                                       57      Naematoloma aurantiaca *                 │
                                                Hebeloma crustuliniforme               │ 13
                                             84  Cortinarius violaceus                  │
                      53                         Cortinarius vanduzerensis              │
                                                Cortinarius ponderosus                 │
                                                Inocybe sororia                        ┘
                                             Bolbitius vitellinus *
                                        100   20MR (Pinus) L. amethysteo-occidentalis   │ 14
          56                                 Laccaria laccata
                                     Agaricus brunnescens *
                              Piloderma croceum
                              Waitea circinata
```

10 substitutions

Fig. 2 a, b, c and d. Phylogenetic placement of unknowns. The phylogram is based on a neighbour-joining analysis of patristic distances. Horizontal distance is based on number of inferred substitutions (i.e. parsimony criteria). Vertical distance is arbitrary. Numbers indicate percentage of bootstrap replicates from a sample of 1000 that support the indicated branches; unlabelled branches have values less than 50% or are in parts of the tree where the branch lengths are too small to label. All sequences other than unknown mycorrhizae were derived from identified herbarium collections or cultures (Table 1). 'Sebacina sp.' may not conform to the current circumscription of that genus, but we give the name that was originally reported for it (Currah et al. 1990). Groups named in lower-case letters are not currently recognized as formal taxa; we use them here for convenient reference to apparently monophyletic lineages. OMR, cultured from orchid mycorrhizae. PS, partial sequence. OP, confirmed as suilloid (group 6) by oligonucleotide probing (Bruns & Gardes 1993); MR, unknown mycorrhiza, preceded by a unique number, followed by plant host in parentheses and, if the type has been matched by ITS-RFLP, the fungal species name is given; *, taxa that are nonmycorrhizal; *?, suspected to be nonmycorrhizal; ?, unknown ecology.

virtually all unknowns that we initially identified as suilloid by phylogenetic analysis were also confirmed with oligonucleotide probes, ITS-RFLP matches, or ITS-sequence analysis (Bruns & Gardes 1993; Gardes & Bruns 1993, 1996; Cullings et al. 1996).

Cantharellus, Hygrophorus, and the Gomphaceae also have very distinct sequences that should allow unknowns from these groups to be recognized easily, even though our sample remains fairly small. Interestingly, there is one group of unknown mycorrhizal sequences (14MR and

Table 2 Kimura 2-parameter distances expressed as percentage base substitutions for selected parts of the dataset

Taxon	Distances range within group*	Distance to next closest known taxon*
Amanitaceae 8 species, 6 sections	0–4.3	3.2
Boletoid group 24 spp., 11–12 genera	0–2.0	3.1 (8.6)†
Cantharellaceae 3 spp., 1 genus	0–0.04	25
Cantharelloid group *Cantharellus* plus unknowns 14, 26	0–12	25
Gomphaceae 3 spp., 3 genera	0–4.3	12.3
Hygrophoraceae 3 spp., 2 genera	0.4–5.9	8.0
Russulaceae 4 spp. 2 genera	1.9–3.2	7.8
Suilloid group 17 spp., 9–11 genera	0–0.5	2.5
Thelephorales 5–6 species, 5 genera, and unknowns	0–3.7	5.4

*taxa represented by partial sequences are excluded from these distance measures, because their values are inflated by the missing data; see text.

†3.1 is the minimum distance from members of the boletoid clad to *Chalciporus*, *Paxillus involutus* or *Paragyrodon*. The next-closest relatives are the other members of the Paxillaceae and *Serpula* at 8.6%.

15MR) that is part of a strongly supported monophyletic group with *Cantharellus*, but the sequences are quite distinct from the three known sequences of *Cantharellus* that we sampled (Fig. 2). These unknowns were associated with *Pinus muricata* in coastal California. We have also found nearly identical sequences associated with *Sarcodes sanguinea* in the Sierras, but in neither case were we able to match these unknowns to any fruiting species (Cullings *et al.* 1996; Gardes & Bruns 1996). We know that they are closely related to *Clavulina cristata*.

Use of this database enables one to identify the fungal component of many unknown mycorrhizae to the level of family or subfamily. This is an improvement over the current state of affairs in which many mycorrhizal fungi are not assignable to any meaningful taxonomic group. It is particularly useful when the morphology of the interaction is atypical due to the influence of the plant host. The best examples are the monotropoid and some orchid mycorrhizae, both of which were found to have specific EM associations that were previously unknown (Cullings *et al.* 1996; Taylor & Bruns 1997). Another advantage of this database is that it could potentially be used for identification of EM fungi in nonmycorrhizal states such as mycelial mats and rhizomorphs.

Sequence-based family identifications can also be used to narrow the search for species-level identification by other molecular methods such as ITS-RFLP matching. Indeed, many of our successful RFLP matches in previous studies were facilitated by phylogenetic placement of unknown ML5/ML6 sequences (Cullings *et al.* 1996; Gardes & Bruns 1996; Taylor & Bruns 1997). One previously unmatched type, 20MR (Gardes & Bruns 1996), has now been identified as *Laccaria amethysteo-occidentalis*. It was initially missed because its ITS-RFLP differed from the isolate of the species with which it was compared, but after adding *Laccaria laccata* to the database it became clear that this unknown was likely to be a *Laccaria*. This encouraged us to try more extensive ITS-RFLP comparisons, and these revealed that some isolates of *Laccaria amethysteo-occidentalis* were perfect ITS-RFLP matches to the unknown.

Assessing placement of unknowns

To use this database for identification it is important to realize that confidence of the placement within the tree is an important criterion. Two types of evidence can be used: (i) internal confidence, and (ii) external independent evidence. To access internal confidence we have used bootstrap analysis (Felsenstein 1985), but this may not be necessary to use each time a new unknown is analysed. From the analyses we report here it seems safe to say that placements of unknowns will be strongly supported within any of the groups listed in Table 2 if their sequence differences from other members of the group fall within the range listed. The suilloid group is a minor exception; placements into this group are likely to be only moderately supported by bootstrap but, as discussed above, are very likely to be correct. Placements within Cortinariaceae, Tricholomataceae, and related families (lineages 11, 13, and 14) are more difficult to interpret, because relationships among these taxa are not resolved well by these data.

Independent evidence is the strongest confirmation. We have used ITS-RFLP and oligonucleotide probe analysis where feasible. The large number of candidate species that one may need to compare in order to find an ITS-RFLP match will remain a problem, but extensive ITS-RFLP databases should help to solve this (Kårén *et al.* 1997). Oligonucleotide probes will probably have a limited value in the foreseeable future because few are currently available and they require significant effort to test

thoroughly. Confirmation by careful morphological characterization is also feasible for distinctive and well described types (Agerer 1987; Ingleby *et al*. 1990).

Evolutionary implications

The limited phylogenetic resolution of this region results in low confidence in many of the major branches of the tree shown, and the highly biased selection of taxa toward EM species would be another problem if phylogenetic estimation were the main goal. Nevertheless, two interesting evolutionary patterns transcend these problems and are worth noting: (i) saprobic and EM taxa are intermixed throughout the tree, and (ii) all of the EM groups for which we have large samples exhibit very short within-group branch lengths relative to other branches in the tree.

The first pattern can be seen in several parts of the tree. In the Boletales (Fig. 2a, all groups) the wood-decaying species of the Coniophoraceae (groups 3 and 4) and Paxillaceae (group 3) appear to be the close relatives of the boletoid and suilloid groups, the two largest samples of mycorrhizal fungi in our database. This connection of the Coniophoraceae and Paxillaceae to the Boletaceae is also supported by secondary chemistry (Gill & Steglich 1987). At the base of the Russulaceae and the Thelephorales are three wood-decaying taxa: *Bondarzewia*, *Heterobasidion* and *Panus*. *Bondarzewia* has been hypothesized to be related to the Russulaceae, based on morphological characters (Singer 1986) and this hypothesis is also suggested by independent sequence data from the mitochondrial small subunit rRNA gene (Hibbett & Donoghue 1995). The latter work by Hibbet and Donoghue also placed other wood-decaying taxa (*Auriscalpium*, *Lentinellus*, *Echinodontium* and *Gloeocystidiellum*) into the clade that includes the Russulaceae. Finally, within the central area of the tree (Fig. 2c), saprobic and mycoparasitic taxa such as *Agaricus*, *Asterophora*, *Bolbitius*, and *Nematoloma* are intermixed with EM taxa such as *Tricholoma*, *Inocybe*, and *Cortinarius*. The exact relationships within this loose group are not clear from these data, as judged by multiple equally parsimonious trees, short internodal branches, and weak bootstrap values; nevertheless, it is clear that the sequences of both nonmycorrhizal and EM taxa are very similar to each other within this cluster. Collectively these examples show that the switch between saprobic and EM lifestyles probably happened convergently several and perhaps many times. These examples suggest that different lineages of EM basidiomycetes may well have different biochemical capacities which in turn may relate to their ability to degrade litter and extract mineral nutrients (Bruns 1995).

The second pattern, that of the short branches, can be best seen in the boletoid (Fig. 2, group 1) and suilloid

groups (group 6), the Russulaceae (group 8), the Thelephorales (group 10) and the Amanitaceae (group 12). In these lineages the samples are large and diverse enough that taxon selection is unlikely to be the reason for the short branch lengths. If read from a molecular clock perspective, the short branches suggest that these five groups, and perhaps several others, represent relatively recent radiations. We can not directly address the time scale of the radiation with the ML5/ML6 data because this molecule has not previously been used for molecular clock estimates. Thus, no estimated rate of change nor any dated branches have been determined. Furthermore, the small size of the molecule would limit the resolution of time estimates. For these reasons we turned to the nuc SSU rRNA gene, which has been previously used for molecular clock estimates in the fungi (Berbee & Taylor 1993; Simon *et al*. 1993), and for which data are available for representatives of two of these five raditions: the suilloid and boletoid groups. We estimated divergence times for these two groups by submitting 12 possible topologies to a maximum likelihood molecular clock model (DNAMLK), using Berbee and Taylor's estimate of 220 Ma, and allowing an error of ± 50 Ma on this estimate (Fig. 3). This approach yielded estimates ranging from 60 to 35 Ma for the suilloid group and 94–31 Ma for the boletoid group. The larger range and greater age of estimates for the boletoid group is probably caused by the inclusion of *Phylloporus*, which is know to have an accelerated rate of change in the nuc SSU rRNA gene (Bruns & Szaro 1992). In any case both sets of estimates essentially fall in the early to mid Tertiary period and coincide with the only fossil ectomycorrhiza found to date – a 50 Ma old middle Eocene ectomycorrhiza that appears to be suilloid (Lepage *et al*. 1997). The upper end of our range of estimates also overlaps the Eocene-Oligocene transition. During this time the earth's climate became cooler and more temperate, and trees in the Pinaceae and Fagales, both obligate ectomycorrhizal taxa, came to dominate the temperate forests (Berggren & Prothero 1992). Combining these facts and estimates with our original observation of short branch lengths in at least five lineages of ectomycorrhizal basidiomycetes, we speculate there has been a convergent radiation of several groups of EM fungi in response to the expanding geographical ranges of their shared plant hosts. This hypothesis may later be rejected if many of the nonectomycorrhizal fungal groups also appear to radiate during a similar time period, but our current EM-biased taxomonic sample does not allow us to address this issue.

This database in relation to current and future needs

We view this database as a working version that we will continue to develop over the next several years. Updated

Fig. 3 Estimated divergence times for the Boletales (1), boletoid group (2), and suilloid group (3) based on maximum likelihood analysis of nuclear small subunit rRNA gene sequences. Berbee & Taylor's (1993) estimated divergence of 220 Ma (b) for the node indicated (*) is assumed. Estimated times are given graphically for the Boletales and suilloid group and in tabular form for all three lineages. Range of estimates is derived by allowing ± 50 Ma (a & c) variation from the Berbee and Taylor date and through analysis of 12 other topologies that differ slightly from the one shown. All 12 trees shared the internal branches indicated in bold and were not significantly different from each other based on Kishino & Hasegawa (1989) tests. The tree is drawn to the geological time scale shown. Epochs of the Tertiary: P, Palaeocene; E, Eocene; O, Oligocene; M, Miocene; unmarked, Pliocene.

versions will be posted on our website as they become available. In its current state it is a useful supplement to existing methods such as ITS-RFLP analysis and detailed morphotyping. It fills a need for identification of types that cannot be determined by other methods. The main advantages of the ML5-ML6 fragment are: its small size, its alignability, and the availability of fungal-specific primers to amplify it.

Although most major EM groups are included in the database, there are several significant omissions. The most obvious is the lack of any ascomycetes. To date we have not been able to amplify these well with the ML5/ML6 primers. This is not too surprising given that known ascomycetous sequences (e.g. *Saccharomyces*, *Aspergillus*, *Neurospora* and *Podospora*) are very divergent and essentially unalignable relative to the basidiomycetous sequences in the database. Within the Basidiomycota there are also some important omissions. These include non-thelephoroid resupinate taxa (e.g. *Amphinema*), gasteroid fungi of uncertain taxonomic placement (e.g. *Leucogaster*, *Leucophleps*, and others), *Coltricia* (Hymenochaetaceae) and additional taxa within the Tricholomataceae and Cortinariaceae.

Other than such omissions, which can be corrected over time, this database still has one major disadvantage: its lack of resolution among closely related genera. For this reason we expect that this database will probably be replaced by one based on sequences from the ITS region. The main advantages of ITS sequences are their much greater resolution among closely related species and genera (Bruns 1996). ITS is such a popular target for phylogenetic analysis that sequences of it are rapidly accumulating; these include sequences from some EM groups such as *Cortinarius*, *Dermocybe*, *Suillus*, *Tricharina*, and *Wilcoxina* (Liu *et al.* 1995; Egger 1996; Kretzer *et al.* 1996; Kretzer & Bruns 1997; Liu *et al.* 1997). Currently,

many other important EM groups remain unsampled. Thus, until many more ITS sequences are available the database presented here will remain a useful tool for the identification of EM fungi.

Acknowledgements

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The authors are interested in the ecology of ectomycorrhizal fungi. The development of molecular identification methods has been a necessary step in exploring this interest.