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Nucleotide Sequence Variation of Chitin Synthase Genes among Ectomycorrhizal Fungi and Its Potential Use in Taxonomy

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DNA sequences of single-copy genes coding for chitin synthases (UDP-*N*-acetyl-D-glucosamine:chitin 4-β-*N*-acetylglucosaminyltransferase; EC 2.4.1.16) were used to characterize ectomycorrhizal fungi. Degenerate primers deduced from short, completely conserved amino acid stretches flanking a region of about 200 amino acids of zymogenic chitin synthases allowed the amplification of DNA fragments of several members of this gene family. Different DNA band patterns were obtained from basidiomycetes because of variation in the number and length of amplified fragments. Cloning and sequencing of the most prominent DNA fragments revealed that these differences were due to various introns at conserved positions. The presence of introns in basidiomycetous fungi therefore has a potential use in identification of genera by analyzing PCR-generated DNA fragment patterns. Analyses of the nucleotide sequences of cloned fragments revealed variations in nucleotide sequences from 4 to 45%. By comparison of the deduced amino acid sequences, the majority of the DNA fragments were identified as members of genes for chitin synthase class II. The deduced amino acid sequences from species of the same genus differed only in one amino acid residue, whereas identity between the amino acid sequences of ascomycetous and basidiomycetous fungi within the same taxonomic class was found to be approximately 43 to 66%. Phylogenetic analysis of the amino acid sequence of class II chitin synthase-encoding gene fragments by using parsimony confirmed the current taxonomic groupings. In addition, our data revealed a fourth class of putative zymogenic chitin synthases.

Many ascomycete and basidiomycete genera form ectomycorrhizal symbioses with gymnosperm and angiosperm trees. The mutualistic association enhances the ability of the host plant to obtain nutrients and water from the soil, and the plant provides the fungus with its primary source of carbohydrates (21). The identification of the fungal symbiont has always been extremely difficult because (i) morphological and structural characteristics of ectomycorrhizae do not vary greatly, (ii) several species of ectomycorrhizal symbionts cannot be isolated in pure culture, and (iii) very few ectomycorrhizal fungi form fruiting bodies in pure culture (13, 22, 36).

Use of the PCR (33) allows characterization of the fungal symbionts by amplification of specific sequences. Identification can thus be achieved by analysis of PCR-amplified products by restriction fragment length polymorphisms, sequencing, or oligonucleotide probing (19). Most studies aiming at the identification of ecto- or endomycorrhizal fungi have been based on ribosomal DNA, specifically by using the internal transcribed spacer (ITS) region. Several features make the ITS a convenient target region: (i) in fungi the entire ITS region is often between 600 and 800 bp and can readily be amplified with universal primers that are complementary to sequences within the rRNA genes (43); (ii) the multicopy nature of the rDNA repeat makes the ITS region easy to amplify from small DNA samples; and (iii) several studies have demonstrated that the ITS region is often highly variable among morphologically distinct fungal species (1, 3, 12, 17, 19, 29).

To identify ecto- or endomycorrhizae, it is desirable that specific or preferential amplification of fungal DNA and not of plant DNA can be achieved, since DNA must often be amplified from mixtures of plant and fungal DNAs. To design specific primers that amplify fungal ribosomal genes or the ITS region, a large number of fungal sequences should be available for comparison. Amplification and direct sequencing of ribosomal genes by universal primers made it possible to design endomycorrhiza-specific primers from the 18S rDNA. PCR amplification with these primers and further analysis of the PCR fragments allowed the characterization of endomycorrhizal fungi (40, 43). In other studies ectomycorrhizae were identified by restriction fragment length polymorphism and sequence analysis of PCR fragments amplified from fungus-specific primers of the ITS region (18, 19), and the suilloid group of ectomycorrhizae could be identified by oligonucleotide hybridization to PCR-amplified products of mitochondrial rRNA genes (8).

Another approach to identification of the fungal symbiont of ecto- or endomycorrhizae would involve the analysis of sequences of genes specific for fungi, e.g., chitin synthase-encoding genes. Chitin, the β-1-4-linked polymer of *N*-acetylglucosamine, is an important component of fungal cell walls and is not known to occur in plants or bacteria (11, 37). Comparison of derived amino acid sequences of the two chitin synthase zymogens (CHS1 and CHS2) of the ascomycete *Saccharomyces cerevisiae* with the derived sequence of a closely related *Candida albicans* gene (UDP-*N*-acetyl-D-glucosamine: chitin 4-β-*N*-acetylglucosaminyltransferase; EC 2.4.1.16) revealed a highly conserved region of about 200 amino acids (5). Short stretches of identical amino acid sequences flanking this region are suitable for designing degenerate PCR primers. Taking advantage of this fact, homologous DNA fragments derived from zymogenic chitin synthase-coding genes of approximately 600 bp were amplified in a study involving primarily ascomycetous fungi (5). Fragments homologous to the

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TABLE 1. Taxonomic affinities of fungal species and chitin synthase-encoding gene designations

Class[a]	Order[b]	Family	Species	Strain	Species code[c]	Gene designation	No.[d]
A	Elaphomycetales (15)	Elaphomycetaceae	*Elaphomyces muricatus*	FK28	Elm	*ElmCHS1*	14
						ElmCHS2	1
	Tuberales (45)	Tuberaceae	*Tuber uncinatum*	FK65	Tuu	*TuuCHS1*	2
B	Agaricales (920)	Cortinariaceae	*Hebeloma crustuliniforme*	FK5	Hec	*HecCHS1*	3
			Hebeloma mesophaeum	FK3	Hem	*HemCHS1*	4
			Cortinarius odorifer	FK73	Coo	*CooCHS1*	5
						CooCHS2	15
		Tricholomataceae	*Laccaria laccata*	FK40	Lal	*LalCHS1*	6
						LalCHS2	16
	Boletales (100)	Boletaceae	*Boletus edulis*	FK13	Boe	*BoeCHS1*	7
			Xerocomus badius	FK66	Xeb	*XebCHS1*	8
	Hymenogastrales (65)	Rhizopogonaceae	*Rhizopogon vulgaris*	FK71	Rhv	*RhvCHS1*	9
						RhvCHS2	13
	Russulales (250)	Russulaceae	*Russula adulterina*	FK47	Rua	*RuaCHS1*	10
			Lactarius deterrimus	FK41	Lcd	*LcdCHS1*	11
	Aphyllophorales (40)	Cantharellaceae	*Cantharellus cibarius*	FK69	Cac	*CacCHS1*	12

[a] A, ascomycetes; B, basidiomycetes.
[b] The approximate number of known ectomycorrhizal species in central Europe within an order is given in parentheses.
[c] A code consisting of three characters is indicated for each fungal species and is used in the gene designation.
[d] Numbers are used to identify DNA fragments in the figures.

nonzymogenic chitin synthase gene *CSD2* of *S. cerevisiae* (10) and *CACHS3* of *C. albicans* (41) could not be amplified because they lack the sequences used for primer design. Amino acid sequence analyses revealed up to three chitin synthase-encoding fragments per fungal species, falling into three distinct classes, presumably representing separate functional groups (5). Further analysis of these DNA sequences allows a classification of fungi within the individual classes (5).

We report here the amplification of chromosomal DNA derived from ectomycorrhizal fungi by using fungus-specific primers, describe their analysis, and suggest their utility for the identification of different genera. The fungal taxa targeted by these primers belonged preferentially to different families of basidiomycetes and in addition to two families of ascomycetes. They were chosen to reflect the distribution of ectomycorrhizal species within fungal families in Europe.

MATERIALS AND METHODS

Selection of taxa and DNA extraction. Fungal taxa were chosen corresponding to the distribution of ectomycorrhizal species within fungal families in Central Europe according to Moser (30, 31) and Jülich (27) and are given in Table 1. Confirmatory specimens of the fungi used are deposited at the Swiss Federal Institute for Forest, Snow and Landscape Research fungal collection, Birmensdorf, Switzerland. DNAs were isolated from all fungi from dried fruiting bodies by a modification of the method of Bruns et al. (6). The extraction was performed with phenol-methylenechloride and methylenechloride instead of phenol-chloroform, and in some cases an RNase digestion step as described previously (45) was added.

PCR conditions. A DNA region within the open reading frame of chitin synthase genes was amplified. The sequences of the oligonucleotides MYK1 and MYK2 were published by Bowen et al. (5). The reaction mixture consisted of a template solution of genomic DNA of various species analyzed and a master mix (4 nmol of each nucleotide, the appropriate amount of the 10× Super-*Taq* reaction buffer, 100 mM Tris-HCl [pH 9.0], 500 mM KCl, 0.1% (wt/vol) gelatin, 15 mM $MgCl_2$, 1% Triton X-100) containing 0.5 U of Super-*Taq* DNA polymerase (Staehelin, Basel, Switzerland) and 50 pmol of each primer in a 20-μl total reaction volume, overlaid with mineral oil. Temperature cycling was carried out with a thermocycler (Biometra, Göttingen, Germany). An initial denaturation step of 94°C for 3 min was followed by 40 cycles consisting of a 1-min denaturing step at 94°C, a 1-min annealing step at 42°C, and a 1-min extension step at 72°C, followed by a final extension step of 10 min at 72°C.

Cloning and sequencing. PCR products were separated on low-melting-point agarose gels, isolated, digested by restriction nucleases *Xho*I and *Hin*dIII, rerun on and isolated from low-melting-point agarose gels, and inserted into the *Hin*dIII and *Xho*I sites of plasmid pGEMZf+ (Promega Biotec, Madison, Wis.). DNA manipulations and transformation of *Escherichia coli* were performed by standard methods (38). The chitin synthase gene fragments were sequenced by the method of Sanger et al. (39), using universal sequencing primers. PCR primers and sequence-specific primers for DNA sequencing were synthesized by Microsynth, Windisch, Switzerland.

Computer analysis. Translation and alignments of amino acid sequences were performed with a software package (Genetics Computer Group, Madison, Wis.), using the programs MAP, TRANSLATE, PILEUP, and CLUSTAL (14, 26). The neighbor-joining analysis was performed with the PHYLIP software package version 3.5 (16). The phylogenetic tree and the percent identity values were derived by PAUP 3.1 (42).

Nucleotide sequence accession numbers. Reported sequences are deposited in the EMBL data bank. Accession numbers are X78087 (*Boletus edulis*), X78088 (*Cantharellus cibarius*), X78089-90 (*Cortinarius odorifer*), X78091-92 (*Elaphomyces muricatus*), X78093 (*Hebeloma crustuliniforme*), X78094 (*Hebeloma mesophaeum*), X78095-96 (*Laccaria laccata*), X78097 (*Lactarius deterrimus*), X78098-99 (*Rhizopogon vulgaris*), X78100 (*Russula adulterina*), X78101 (*Tuber uncinatum*), and X78102 (*Xerocomus badius*).

RESULTS

Different PCR fragment patterns of several species of ectomycorrhizal fungi. PCRs were performed with chromosomal DNA extracted from fruiting bodies of several ectomy-

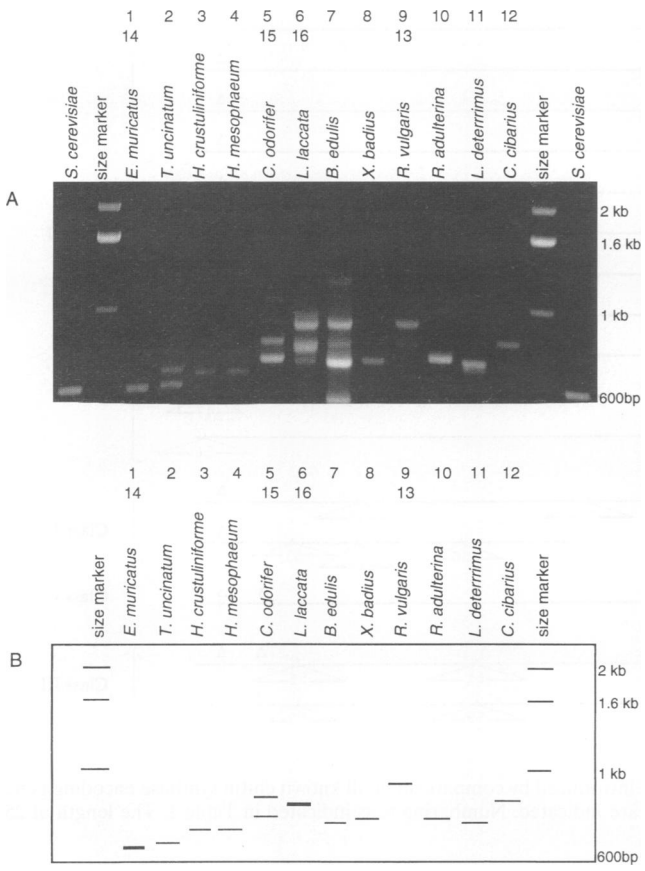

FIG. 1. (A) Agarose gel showing preferential amplification from genomic DNA of various species of ectomycorrhizal fungi and of *S. cerevisiae* by using MYK1 and MYK2 as DNA primers in a PCR. A kilobase ladder (Life Technologies Inc., Basel, Switzerland) was used as the size marker. Numbering is as indicated in Table 1. (B) Schematic diagram of cloned PCR fragments. The most prominent bands derived by both primers which were cloned into plasmid pGEM7Zf+ for further analysis by DNA sequencing are shown. Numbering is as indicated in Table 1.

corrhizal fungi (listed in Table 1) and from *S. cerevisiae* cells as a control. Fragments were directly assessed by viewing the amplified DNA after gel electrophoresis (Fig. 1A). PCRs performed with bacterial DNA (*Propionigenium modestum*) and plant DNA (*Picea abies*) did not result in any detectable amplified products (data not shown). The amplified products of 12 ectomycorrhizal fungi derived from the two primers varied from one to four fragments of lengths from 600 to 900 bp. The two corresponding fragments amplified from *S. cerevisiae* were both approximately 600 bp long and resulted in a single band on these gels. The DNAs of the two ascomycete species analyzed gave fragments of sizes comparable to those of *S. cerevisiae*, in contrast to the DNA of all basidiomycete species, which gave fragments larger than 600 bp. Fragment patterns of the two *Hebeloma* species were identical. This corresponded to the results of Gardes et al. (19) that length polymorphism between ectomycorrhizal genera and relative uniformity within the genus *Laccaria* was detected in PCR amplicons of the ITS region.

Analysis of PCR fragments by sequencing. The most prominent PCR fragments from each fungal species (Fig. 1B) were cloned, and the DNA sequences were determined. Multiple alignments of these nucleotide sequences with known chitin synthase-encoding gene fragments (5) suggested the presence of various introns (Fig. 2). All introns were confirmed by identifying the consensus exon/intron splice junction sequences seen in other chitin synthase gene fragments and in other fungal genes (2). None of the sequences of the analyzed ascomycetous fungi contained any introns. This was in agreement with the only other study performed with PCR-amplified gene fragments encoding parts of primarily ascomycetous chitin synthase proteins (5). These authors found only a single chitin synthase-encoding gene fragment derived from an ascomycete which contained an intron. This gene, *HcCHS3*, originated from *Histoplasma capsulatum*. However, two introns upstream and downstream of the amplification product *NcChs3* have been reported in the *chs-1* gene of *Neurospora crassa* (44). All introns reported in the current study were of the characteristically short length of 45 to 60 bp reported for fungal genes (20). Thus, the differences in the sizes of the PCR fragments were due to a different number and length of introns within the amplified DNA region.

Comparison of nucleotide sequences and deduced amino acid sequences. The nucleotide sequence analysis of all cloned chitin synthase-encoding gene fragments showed about 4% interspecific variation. Sequences of the same family showed variations of about 12 to 18%, and sequences of the same order or class showed variations of up to 45% (data not shown). These data were in agreement with the interspecific variation of about 3 to 5% found in the ITS region of several *Laccaria* species (19).

A comparison of all analyzed amino acid sequences yielded identity values varying from 42 to 99.5% (Fig. 3). The only difference between the two amino acid sequences of the two *Hebeloma* species was a single amino acid substitution at position 65 from glycine to aspartate. The corresponding nucleotide sequences showed more differences (4%) because of the use of different codons for the same amino acids (data not shown).

There are three known classes of zymogenic chitin synthases (5). An alignment of the amino acid sequences found in this work with known sequences of the three chitin synthase classes revealed that most of the analyzed fragments belonged to class II (Fig. 4). One of the two amino acid sequences from *Laccaria laccata* and *Cortinarius odorifer* belonged to class III and one of two from *Elaphomyces muricatus* to class I. Each class is characterized by particular conserved residues or gaps. In class II, two sequences (one from CacChs1 of *Cantharellus cibarius* and one from UmChs2 of *Ustilago maydis* [5]) were characterized by four missing amino acids (positions 102 to 105). The two partial deduced amino acid sequences of *Rhizopogon vulgaris* were assigned to the same class because of many conserved residues typical of class II chitin synthases. However, comparison of the nucleotide and derived amino acid sequences of both fragments in the overlapping part revealed that they cannot be derived from the same chromosomal chitin synthase gene. The deduced amino acid sequence of the chitin synthase-encoding gene fragment of RhvChs1 was more similar to the sequences of *Cantharellus cibarius* and *Ustilago maydis* (5) than to all other class II fragments. In a neighbor-joining analysis the three partial deduced amino acid sequences RhvChs1, CacChs1, and UmChs2 (5) were grouped together (data not shown). These data suggest that a further class of zymogenic chitin synthases exists, which we name class IV.

Positioning of introns. The relative positions and length variations of the introns of the different classes of chitin synthase-encoding gene fragments were analyzed (Fig. 2).

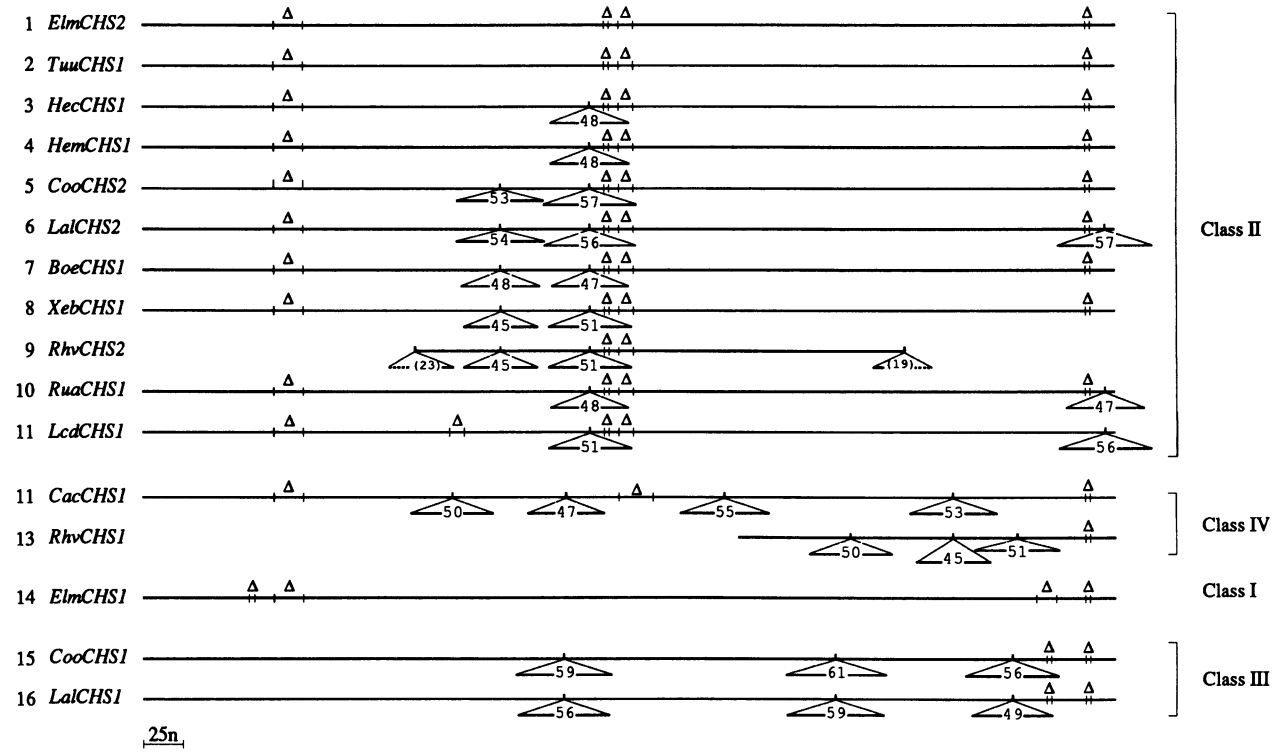

FIG. 2. Gene structure of partial chitin synthase genes. Δ represents gaps introduced by comparison of all known chitin synthase encoding gene fragments. Triangles represent intron sequences. The lengths of the introns are indicated. Numbering is as indicated in Table 1. The length of 25 nucleotides (25n) is indicated as a size standard.

Although the number of introns differed within the basidiomycetes, their positions within the classes were usually conserved. The only exceptions were *CacCHS1* from *Cantharellus cibarius* and *RhvCHS1* from *Rhizopogon vulgaris*, which showed three introns at different positions, and *UmCHS2* from *Ustilago maydis*, which showed no introns at all (5). Both *Hebeloma* species showed a single intron at the same position in the amplified region (amino acid 92). The position of this intron was conserved in all class II sequences of basidiomycetes except in the above-mentioned sequences. An intron at amino acid 73/74 was found in the orders Boletales and Agaricales, and a third intron at amino acid position 198 was present only in the families Tricholomataceae and Russulaceae. Intron positions known for the ascomycetous *Histoplasma capsulatum HcCHS3* did not match intron positions of class III fragments of the two basidiomycetous fungi *Laccaria laccata* and *Cortinarius odorifer* (5).

	1 ElmChs2	2 TuuChs1	3 HecChs1	4 HemChs1	5 CooChs2	6 LalChs2	8 XebChs1	7 BoeChs1	9 RuaChs1	11 LcdChs1	12 CacChs1	14 ElmChs1	16 LalChs1	15 CooChs1
1 ElmChs2	-	77.2	64.1	65.6	66.1	65.1	64.6	65.6	66.1	53.2	52.7	54.3	45.2	43.4
2 TuuChs1		-	63.0	63.5	64.0	62.4	62.4	63.0	64.6	53.2	51.6	53.3	46.8	44.7
3 HecChs1			-	99.5	95.8	92.6	87.8	88.4	82.0	64.1	60.3	58.7	48.4	49.5
4 HemChs1				-	96.3	93.1	88.4	88.9	82.5	64.1	60.9	59.2	48.9	50.0
5 CooChs2					-	89.9	86.8	87.8	81.5	64.6	61.4	59.8	49.5	50.5
6 LalChs2						-	91.5	89.9	85.2	68.8	60.9	60.3	50.5	49.5
8 XebChs1							-	96.3	85.7	69.4	63.0	59.2	51.6	49.5
7 BoeChs1								-	86.8	68.7	63.0	59.2	50.5	49.5
9 RuaChs1									-	72.0	57.1	57.1	51.6	50.0
11 LcdChs1										-	48.1	47.5	43.2	41.6
12 CacChs1											-	51.7	42.4	42.6
14 ElmChs1												-	49.7	49.2
16 LalChs1													-	91.4
15 CooChs1														-

FIG. 3. Percent identity of conserved amino acid residues between chitin synthase sequences. Numbering is as indicated in Table 1.

Phylogenetic analysis of the deduced amino acid sequences of class II fragments. Bootstrap analysis of the deduced amino acid sequences of class II fragments was performed as a means of validating relationships shown by the neighbor-joining analysis. Bootstrapping is a statistical method used to evaluate the confidence level of the phylogenetic estimate by random resampling of the data. The bootstrap value indicates the percentage of the bootstrap replications that support the group's descent from a given branch. It was performed with the heuristic algorithm by using a step matrix specifying the cost of changing from one amino acid to another. A total of 1,000 bootstrap replicates were performed on putative class II sequences. The deduced protein sequences CacChs1, RhvChs1, and UmChs2 were used as the hypothetical outgroup to construct a parsimonious tree (Fig. 5). The protein sequence of ScChs2 of the basidiomycetous *Schizophyllum commune* was used as reference for class II. The consistency index, measuring the deviation of a tree from a perfect fit to data (28), obtained for the consensus tree derived from the branch and bound tree search was 0.977 (data not shown). The demarcation of the class IV amino acid sequences, CacChs1, RhvChs1, and UmChs2, which were proposed to belong to a different class from class II, was confirmed with a bootstrap value of 98% (Fig. 5). Within the remaining class II protein fragments, taxonomic relationships were confirmed.

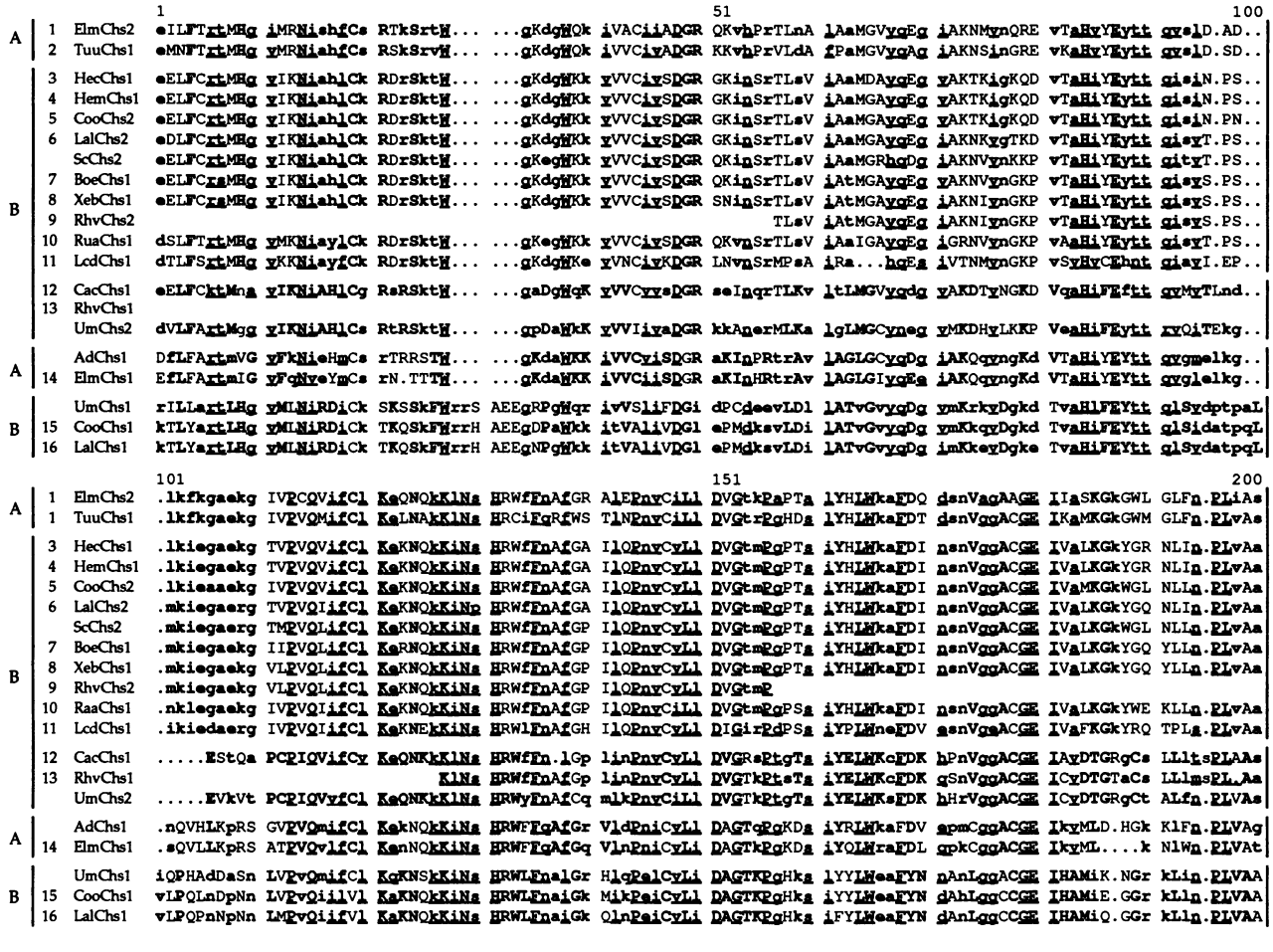

FIG. 4. Multiple sequence alignment of deduced amino acid sequences of 16 fungal chitin synthase-encoding gene fragments. The alignment was derived by PILEUP and CLUSTAL (14, 26). As a standard for each class, we used different known sequences for chitin synthases: ScChs2 from *Schizophyllum commune* and UmChs2 from *Ustilago maydis* for class II, UmChs1 from *Ustilago maydis* for class III, and AdChs1 from the ascomycete *Aspergillus nidulans* for class I (5). Boldface capital letters indicate complete identity, whereas boldface lowercase letters indicate conservative substitutions. Underlined boldface capital and lowercase letters are for identity and conserved substitutions, respectively, over all four classes of chitin synthases. Boldface letters which are not underlined are for identity or conserved substitutions within each class. Identities and conserved substitutions are determined by alignment with chitin synthase fragments found so far in the gene bank. Numbering is as indicated in Table 1. A, ascomycetous; B, basidiomycetous.

DISCUSSION

Methods previously used to identify mycorrhizal fungal species by PCR depended on the amplification of ribosomal DNA (8, 18, 19, 24, 25, 40). The method presented here, however, applied to ectomycorrhiza-forming basidiomycetous species, is based on the amplification of a DNA region from a small, fungus-specific gene family. Primers designed by Bowen et al. (5) to amplify a conserved region common to a gene family produced not just one but several fragments from chromosomal DNA. In contrast to ribosomal DNA, in which only length polymorphism could be found, the use of the chitin synthase gene family allows detection of both length and number polymorphisms. The combination of these two features and the uniformity of the amplified products within the two *Hebeloma* representatives might allow the identification of basidiomycetous genera directly by PCR fragment patterns without any further analysis of the fragments. Such variation in number and length of DNA fragments is due mainly to various introns which are to some extent conserved in their positions. In ascomycetes, however, such distinctions are not possible (5).

Most of the prominent DNA fragments analysed in this study encode class II chitin synthases. The preference for class II DNA fragments in basidiomycetes was also observed in an earlier study on chitin synthase (5). A phylogenetic analysis performed with the derived amino acid sequences of chitin synthase class II-encoding DNA fragments confirmed the current taxonomic grouping. The *Rhizopogon* species was assigned to the order Boletales, a transfer already proposed after studies of the ribosomal DNA (7–9).

Our results suggest an extension of the classification of the present chitin synthase family. Each of the three previous known classes of the zymogenic members of chitin synthases is characterized by its own set of conserved amino acid residues within this region and by specific gaps which have to be introduced into the amino acid sequences when comparing all three classes. Representatives CacChs1, RhvChs1, and

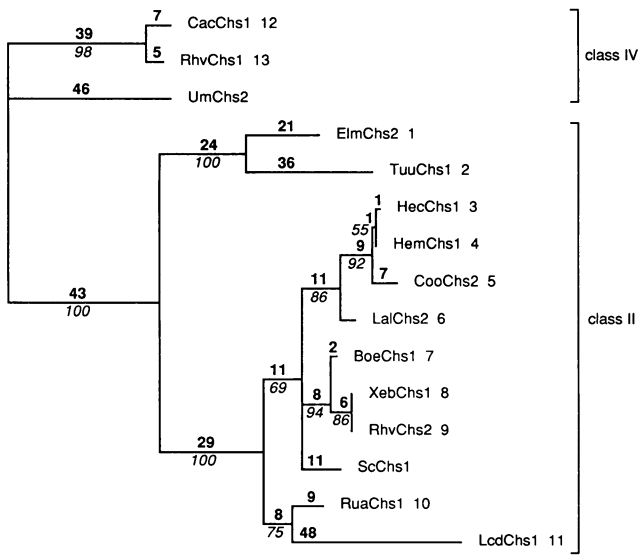

FIG. 5. Phylogenetic analysis of the deduced amino acid sequences of putative class II and IV chitin synthase fragments. The two basidiomycetous amino acid sequences already known—ScChs2 from *Schizophyllum commune* and UmChs2 from *Ustilago maydis* (5)—are used as standards for class II and the putative class IV, respectively. The amino acid sequences were compared by parsimony using PAUP 3.1 (42). Italic numbers below the lines are the bootstrap results from 1,000 samplings performed with the heuristic algorithm. Boldface numbers above the lines show the tree lengths. Numbering is as indicated in Table 1.

UmChs2 (5) of a new fourth class are characterized by the expansion of a gap and their own set of conserved amino acid residues. The neighbor-joining analysis clusters the three deduced amino acid sequences, and the phylogenetic analysis of representatives of class II and putative members of class IV confirms the demarcation of class IV with a bootstrap value of 98%, thus supporting the existence of a fourth zymogenic chitin synthase class. Further evidence is given by the different set of conserved relative positions of introns found in these sequences.

Conservation of intron positions has also been found in other fungal genes, such as the glyceraldehyde-3-phosphate dehydrogenase-encoding genes (23), acetyl coenzyme A synthetase-encoding genes (4), and lignin peroxidase-encoding genes (34). It has been suggested that comparison of intron presence and position within related genes from different genera might shed light on the evolution of individual genes. In a chitin synthase-encoding gene of the zygomycete *Phycomyces blakesleeanus*, best matching the motifs found in class II (32), an intron could be detected at a position completely different from that reported for the basidiomycetous class II sequences. Even the positions of the introns in the chitin synthase class III-encoding gene fragment of *Histoplasma capsulatum*—the only known chitin synthase-encoding ascomycetous fragment sequence containing an intron—do not match the positions in class III representatives of the basidiomycetous fungi (5). Therefore, we conclude that distinct sets of intron positions are conserved not only in different classes but also in different orders of fungi. From these observations we assume that none of the events giving rise to the introns observed within this gene family has occurred in a putative ancestral gene and before the divergence of basidiomycetes, zygomycetes, and ascomycetes, irrespective of whether that event involved a net loss (15) or gain (35) of introns.

In summary, this approach seems to have a potential use for identifying ectomycorrhizal fungi, assuming that fragment pattern uniformity within genera will be confirmed. Using DNA extracted from fruiting bodies, in the step following this pilot project we will test whether this method proves good with DNA extracted from fungal mycelia or ectomycorrhizae.

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REFERENCES

1. **Anderson, J. B., and E. Stasovski.** 1992. Molecular phylogeny of northern hemisphere species of *Armillaria*. Mycologia **84:**505–516.
2. **Ballance, D. J.** 1986. Sequences important for gene expression in filamentous fungi. Yeast **2:**224–236.
3. **Baura, G., T. M. Szaro, and T. D. Bruns.** 1992. *Gastrosuillus laricinus* is a recent derivative of *Suillus grevillei*: molecular evidence. Mycologia **84:**592–597.
4. **Birch, P. R. J., P. F. G. Sims, and P. Broda.** 1992. Nucleotide sequence of a gene from *Phanerochaete chrysosporium* that shows homology to the *facA* gene of *Aspergillus nidulans*. DNA Sequence **2:**319–323.
5. **Bowen, A. R., J. L. Chen-Wu, M. Momany, R. Young, P. J. Szaniszlo, and P. W. Robbins.** 1992. Classification of fungal chitin synthases. Proc. Natl. Acad. Sci. USA **89:**519–523.
6. **Bruns, T. D., R. Fogel, and J. W. Taylor.** 1990. Amplification and sequencing of DNA from fungal herbarium species. Mycologia **82:**175–184.
7. **Bruns, T. D., R. Fogel, T. J. White, and J. D. Palmer.** 1989. Evolution of mushroom of a false-truffle from a mushroom ancestor. Nature (London) **339:**140–142.
8. **Bruns, T. D., and M. Gardes.** 1993. Molecular tools for identification of ectomycorrhizal fungi: taxon-specific oligonucleotide probes for suilloid fungi. Mol. Ecol. **2:**233–242.
9. **Bruns, T. D., and T. M. Szaro.** 1992. Rate and mode differences between nuclear and mitochondrial small-subunit rRNA genes in mushrooms. Mol. Biol. Evol. **9:**836–855.
10. **Bulawa, C. E.** 1992. *CSD2*, *CSD3*, and *CSD4*, genes required for chitin synthesis in *Saccharomyces cerevisiae*: the *CSD2* gene product is related to chitin synthases and to developmentally regulated proteins in *Rhizobium* species and *Xenopus laevis*. Mol. Cell. Biol. **12:**1764–1776.
11. **Bulawa, C. E.** 1993. Genetics and molecular biology of chitin synthesis in fungi. Annu. Rev. Microbiol. **47:**505–534.
12. **Chen, W., J. W. Hoy, and R. W. Schneider.** 1992. Species-specific polymorphisms in transcribed ribosomal DNA of five *Pythium* species. Exp. Mycol. **16:**22–34.
13. **Danielson, R. M.** 1984. Ectomycorrhizal associations in jack pine stands in northeastern Canada. Can. J. Bot. **62:**932–939.
14. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
15. **Doolittle, W. F.** 1978. Genes in pieces: were they ever together? Nature (London) **272:**581–582.
16. **Felsenstein, J.** 1989. PHYLIP—Phylogeny Inference Package (version 3.2). Cladistics **5:**164–166.
17. **Gardes, M., and T. D. Bruns.** 1991. Rapid characterization of

ectomycorrhizae using RFLP pattern of their PCR amplified-ITS. Mycol. Soc. Newsl. **41**:14.
18. **Gardes, M., and T. D. Bruns.** 1993. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol. Evol. **2**:113–118.
19. **Gardes, M., T. H. White, J. A. Fortin, T. D. Bruns, and J. W. Taylor.** 1991. Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. Can. J. Bot. **69**:180–190.
20. **Gurr, S. J., S. E. Unkles, and J. R. Kinghorn.** 1988. The structure and organisation of nuclear genes in filamentous fungi, p. 93–139. *In* J. R. Kinghorn (ed.), Gene structure in eukaryotic microbes. IRL Press, Oxford.
21. **Harley, J. L.** 1989. The significance of mycorrhiza. Mycol. Res. **92**:129–139.
22. **Harley, J. L., and S. E. Smith.** 1983. Mycorrhizal symbiosis. Academic Press, Ltd., London.
23. **Harmsen, M. C., F. H. J. Schuren, S. M. Mouhka, C. M. van Zuilen, P. J. Punt, and J. G. H. Wessels.** 1992. Sequence analysis of the glyceraldehyde-3-phosphate dehydrogenase genes from the basidiomycetes *Schizophyllum commune*, *Phanerochaete chrysosporium* and *Agaricus bisporus*. Curr. Genet. **22**:447–454.
24. **Henrion, B., G. Chevalier, and F. Martin.** 1994. Typing truffle species by PCR amplification of the ribosomal DNA spacers. Mycol. Res. **98**:37–43.
25. **Henrion, B., F. Le Tacon, and F. Martin.** 1992. Rapid identification of genetic variation of ectomycorrhizal fungi by amplification of ribosomal RNA genes. New Phytol. **122**:289–298.
26. **Higgins, D. G., and P. M. Sharp.** 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene **73**:237–244.
27. **Jülich, W.** 1984. Die Nichtblätterpilze, Gallertpilze und Bauchpilze. Kleine Kryptogamenflora IIb/1. Gustav Fischer Verlag, Stuttgart, Germany.
28. **Kluge, A. G., and J. S. Farris.** 1969. Quantitative phyletics and the evolution of anurans. Syst. Zool. **18**:1–32.
29. **Lee, S. B., and J. W. Taylor.** 1992. Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. Mol. Biol. Evol. **9**:636–653.
30. **Moser, M.** 1963. Ascomyceten. Kleine Kryptogamenflora IIa. Gustav Fischer Verlag, Stuttgart, Germany.
31. **Moser, M.** 1984. Die Röhrlinge und Blätterpilze. Kleine Kryptogamenflora IIb/2. Gustav Fischer Verlag, Stuttgart, Germany.
32. **Miyazaki, A., M. Momany, P. J. Szaniszlo, M. Jayaram, and T. Ootaki.** 1993. Chitin synthase-encoding gene(s) of the zygomycete fungus *Phycomyces blakesleeanus*. Gene **134**:129–134.
33. **Mullis, K. B., and F. A. Faloona.** 1987. Specific synthesis of DNA in vitro via a polymerase-catalysed chain reaction. Methods Enzymol. **155**:335–350.
34. **Naidu, P. S., Y. Z. Zhang, and C. A. Reddy.** 1990. Characterization of a new lignin peroxidase gene (*GLG6*) from *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **173**:994–1000.
35. **Rogers, J. H.** 1989. How were introns inserted into nuclear genes? Trends Genet. **5**:213–216.
36. **Rogers, S. O., S. Rehner, C. Bledsoe, G. J. Mueller, and J. F. Ammirati.** 1989. Extraction of DNA from basidiomycetes for ribosomal DNA hybridizations. Can. J. Bot. **67**:1235–1243.
37. **Ruiz-Herrera, J., R. S. Ramon, and J. P. Martinez.** 1992. Chitin biosynthesis in fungi, p. 281–312. *In* D. K. Arora et al. (ed.), Handbook of applied mycology, vol. 4. Fungal biotechnology. Marcel Dekker, Inc., New York.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
40. **Simon, L., M. Lalonde, and T. D. Bruns.** 1992. Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Appl. Environ. Microbiol. **58**:291–295.
41. **Sudoh, M., X. Nagahashi, M. Doi, A. Ohta, M. Takagi, and M. Arisawa.** 1993. Cloning of the chitin synthase 3 gene from *Candida albicans* and its expression during yeast-hyphal transition. Mol. Gen. Genet. **241**:351–358.
42. **Swofford, D. L.** 1993. PAUP 3.1.1: phylogenetic analysis using parsimony, version 3.1. Computer program distributed by the Illinois Natural History Survey, Champaign, Ill.
43. **White, T. J., T. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal genes for phylogenetics, p. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc. San Diego, Calif.
44. **Yarden, O., and C. Yanofsky.** 1991. Chitin synthase I plays a major role in cell-wall biogenesis in *Neurospora crassa*. Genes Dev. **5**:2420–2430.
45. **Zolan, M. E., and P. J. Pukkila.** 1986. Inheritance of DNA methylation in *Coprinus cinereus*. Mol. Cell. Biol. **6**:195–200.