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

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Received 22 March 1994/Accepted 19 June 1994

DNA sequences of single-copy genes coding for chitin synthases (UDP-N-acetyl-D-glucosamin:chitin 4-β-N-acetylglucosaminyltransferase; EC 2.4.1.16) were used to characterize ectomycorrhizal fungi. Degenerate primers derived from short, completely conserved amino acid stretches flanking a region of about 200 amino acids of zymogenic chitin synthase 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 by 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 ascomycetous and basidiomycetous 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 endomycorrhizae 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 suillloid 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 synthasezymogens (CHS1 and CHS2) of the ascomycete Saccharomyces cerevisiae with the derived sequence of a closely related Candida albicans gene (UDP-N-acetyl-d-glucosaminc: 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
The approximate number of known ectomycorrhizal species in central Europe within an order is given in parentheses.

4 Numbers are used to identify DNA fragments in the figures.
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, HeCHS3, 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 ranging 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 neighboring 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).
Although the number of introns differed within the basidiomycetes, their positions within the classes were usually conserved. The only exceptions were CacChs1 from *Cantarellaellus 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. Introns 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 *Cor- narius odorifer* (5).

**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 basidiomycetes *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. 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 positions. 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.

**FIG. 3.** Percent identity of conserved amino acid residues between chitin synthase sequences. Numbering is as indicated in Table 1.
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 Schizotalea 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 conservations substitutions, respectively, over four classes of chitin synthases. Boldface letters which are not underlined are for identity or conservation 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, ascomycetes; B, basidiomycetes.

**DISCUSSION**

Methods previously used to identify mycorrhizal fungal species by PCR depend on the amplification of ribosomal DNA (18, 19, 24, 25, 40). The method presented here, however, applied to ectomycorrhiza-forming basidiomycete 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 Heloderma representatives might allow the identification of basidiomycete 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 *Rhizogongen* 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.

ACKNOWLEDGMENTS

We thank Orlando Petri for helpful discussion and assistance in the use of phylogenetic computer programs, and we thank Simon Egli for assisting in selection of fungal species and placing the WSL collection at our disposal. We thank Uschi Gerike and Urs Büchler for DNA samples of bacteria and plants. We also thank Michal Kertesz, Markus Künzler, and Roney Graf for critical reading of the manuscript and all other members of the yeast research group for helpful discussions. We are especially grateful to Ralf Hütt for generous support.

This work was supported by Swiss National Foundation grant 31.30778.91, by the Swiss Federal Institute of Technology, and by the Swiss Federal Institute for Forest, Snow, and Landscape Research.

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