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The Nuclear Ribosomal DNA Intergenic Spacer as a Target Sequence To Study Intraspecific Diversity of the Ectomycorrhizal Basidiomycete *Hebeloma cylindrosporum* Directly on *Pinus* Root Systems

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Polymorphism of the nuclear ribosomal DNA intergenic spacer (IGS) of the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* was studied to evaluate whether this sequence could be used in field studies to estimate the diversity of strains forming mycorrhizas on individual *Pinus pinaster* root systems. This sequence was amplified by PCR from 125 haploid homokaryotic strains collected in 14 *P. pinaster* stands along the Atlantic coast of France by using conserved oligonucleotide primers. Restriction enzyme digestion of the amplified 3.4-kbp-long IGS allowed us to characterize 24 alleles whose frequencies differed. Nine of these alleles were found only once, whereas about 60% of the strains contained four of the alleles. Local populations could be almost as diverse as the entire population along a 150-km stretch of coastline that was examined; for example, 13 alleles were found in a single forest stand. The IGS from one strain was partially sequenced, and the sequence data were used to design oligonucleotides which allowed separate PCR amplification of three different segments of the IGS. Most polymorphisms observed among the full-length IGS regions resulted from polymorphisms in an internal ca. 1,500-bp-long sequence characterized by length variations that may have resulted from variable numbers of a T_2AG_3 motif. This internal polymorphic sequence could not be amplified from the genomes of nine other *Hebeloma* species. Analysis of this internal sequence amplified from the haploid progenies of 10 fruiting bodies collected in a 70-m^2 area resulted in identification of six allelic forms and seven distinct diplotypes out of the 21 possible different combinations. Moreover, optimization of the PCR conditions resulted in amplification of this sequence from more than 80% of the DNA samples extracted from individual *H. cylindrosporum* infected *P. pinaster* mycorrhizal root tips, thus demonstrating the usefulness of this sequence for studying the below-ground diversity of mycorrhizas formed by genets belonging to the same fungal species.

In most terrestrial ecosystems, the root system of a single plant may host a variety of symbionts. Thus, different fungal ectomycorrhizal (ECM) species may contribute, according to their relative abundance on the root system and to their physiological properties, to improving the growth and fitness of a common host tree. These multipartner symbioses are not stable but evolve during the life of the host plant, as illustrated by results based on samples of basidiocarps of different ECM species found in the vicinity of individual trees (23). It was recently found that results of ECM community studies based on basidiocarp records (above-ground view) may be misleading and do not necessarily reflect the frequency and abundance of individual ECM species on root systems (below-ground view) (13, 24).

At the intraspecific level, genetically distinct mycelia of the same ECM species can also coexist on the root system of a single tree. This was demonstrated after coinoculation of *Pinus banksiana* seedlings with different strains of *Laccaria bicolor* (9) and was deduced from the results of field studies which showed that up to nine different genets of the agaric *Hebeloma cylindrosporum* can be found in a 1-m^2 patch of ground under a *Pinus pinaster* tree (14). Population studies of the latter ECM species were conducted after basidiocarp samples were obtained in the same forest stands in successive fruiting seasons, and the results revealed that there were high levels of genetic diversity within local populations. At two of the three sites sampled, there were never more than two basidiocarps that had the same genotype (i.e., emerged from the same below-ground mycelium). Moreover, none of the genets identified during the first year of the study was found 3 years later. Such observations made at the intraspecific level pose questions similar to the questions already addressed at the community level concerning the below-ground distribution of individual genets.

To answer such questions, a powerful method is needed to discriminate between different below-ground individuals. One of the most accurate methods for identifying the fungal symbiont present in a single, field-collected, mycorrhizal root tip is PCR-restriction fragment length polymorphism (RFLP) analysis of polymorphic DNA sequences. In ECM community studies, one of the most-studied DNA sequences is the PCR-amplified nuclear ribosomal internal transcribed spacer (ITS) sequence. ITS sequences can easily be amplified from minute amounts of DNA with PCR primers (34) and are conserved at the species level; however, there are enough differences be-

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tween related fungal taxa so that RFLPs generated with several endonucleases are a fairly good way to distinguish between different fungal species (10, 12, 18, 22, 32). An alternative to the ITS is a portion of the large mitochondrial ribosomal DNA (rDNA) gene which can also be amplified easily; this region has been sequenced in more than 100 ECM taxa (4).

When a single ECM species is examined, it is advisable to design species-specific primers in order to amplify the sequence only from mycorrhizas formed by the ECM species being studied. For such a survey of intraspecific diversity, a single DNA sequence could eventually be studied if it is highly polymorphic with many different allelic forms. Such a sequence must also reveal polymorphisms in potentially related strains collected in very small areas (i.e., strains colonizing the same root system). The sequence targeted in this study was the nuclear rDNA intergenic spacer (IGS) sequence of *H. cylindrosporum*. This sequence, which spans from the 3′ end of the 26S rRNA gene to the 5′ end of the 18S rRNA gene, may contain the 5S rRNA gene. Previous studies of *H. cylindrosporum* have shown that the entire IGS sequence can be amplified easily by using DNA extracted from pure mycelial cultures (15). IGS amplified from dikaryotic strains produce different RFLP patterns, which in some strains result from heterozygoty at the rDNA locus (14). Intraspecific polymorphisms in this sequence are not limited to *H. cylindrosporum* and have been shown to occur in the IGS of other homobasidiomycete species (1, 17, 21, 29).

The aim of this study was to evaluate the amount and distribution of polymorphism in the IGS in populations of *H. cylindrosporum* occurring along the Atlantic coast of France, where this fungal species is one of the dominant ECM species associated with *P. pinaster* growing on the backs of the coastal sand dunes. We also identified within the IGS a highly polymorphic region which can be directly amplified from DNA extracted from mycorrhizas by using species-specific PCR primers.

MATERIALS AND METHODS

Fungal strains. In the fall of 1995, 68 *H. cylindrosporum* sporulating basidiocarps were collected in 12 *P. pinaster* forest stands along the Atlantic coast of Les Landes (south western France) and two stands on the coast of Brittany in western France (Fig. 1). At each site except Le Porge, the minimal distance between two basidiocarp samples was 5 m in order to prevent sampling of basidiocarps emerging from the same below-ground mycelium (14). At Le Porge, basidiocarp samples were collected in two different areas. One area was along a footpath through the sand dunes leading to the seashore; in this area the sampling procedure was the same as the sampling procedure used at the other sites, and the fungal strains collected were designated the LPDu strains. The second Le Porge area was the 70-m^2 area studied by Gryta et al. (14), and the fungal strains collected were designated the LP strains. Basidiospore germination was obtained for all of the basidiocarps sampled (8). For each basidiocarp except the basidiocarps collected in Brittany and at Le Porge, a single haploid progeny was analyzed. For each of the basidiocarps collected in Brittany and at Le Porge, between two and four haploid progenies were analyzed. This analysis resulted in a total of 125 different homokaryotic strains. In addition, laboratory homokaryotic strain h1 (8) was used to clone and sequence the IGS. Furthermore, two dikaryotic mycelia, GCA6 and GCC2, which were collected in 1993 at the Grand Crohot site (14) and harbored different IGS types, were used for in vitro synthesis of mycorrhizas.

The specificity of the PCR primers was tested by using one strain of each of the following nine *Hebeloma* species: one species belonging to subgenus *Myxocybe* (*Hebeloma radicosum*) and eight species belonging to subgenus *Hebeloma*, including members of section Porphyrospora (*Hebeloma sarcophyllum*), section Hebeloma (*Hebeloma mesophaeum*), and section Denudata (the section to which *H. cylindrosporum* belongs) (*Hebeloma spoliatum*, *Hebeloma vaccinum*, *Hebeloma hiemale*, *Hebeloma cavipes*, *Hebeloma bulbiferum*, and *Hebeloma crustuliniforme*). Strains of these species are kept in the culture collection of our laboratory. In addition, strains of *Thelephora terrestris*, *Pisolithus tinctorius*, *Suillus bovinus*, and *Lycoperdon* sp., four basidiomycete ECM species frequently encountered in *P. pinaster* stands, were also used to test primer specificity.

Media and culture conditions. Basidiospores were germinated and mycelia were grown on solid YMG medium (25) supplemented with 30 mg of chloramphenicol per liter at 22°C in the dark. For DNA extraction, the YMG plates were covered with a cellophane membrane to allow recovery of the mycelium. Single germinating spores were isolated with a binocular microscope, and the resulting mycelia were checked with the microscope for the absence of the clamp connections which characterize homokaryons of *H. cylindrosporum*.

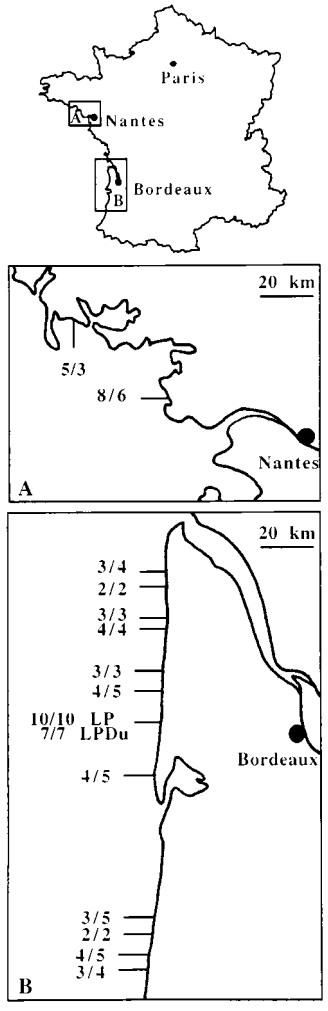

FIG. 1. Locations of the different sites along the Atlantic coast of France where basidiocarps of *H. cylindrosporum* were obtained. (A) South Brittany (two sites). (B) Coast of Les Landes (12 sites). For each site the number of IGS alleles/number of basidiocarps collected is given; this ratio could be greater than 1 when several homokaryons of one basidiocarp were analyzed (the two sites in Brittany).

Mycorrhizas were produced in vitro in 14-cm petri dishes that were half-filled with autoclaved soil collected at the Grand Crohot and Le Porge sites (14). A suspension of hyphal fragments from dikaryotic GCA6 or GCC2 mycelia, obtained by maceration in water, was added to the soil. One-month-old sterile seedlings of *P. pinaster* were placed flat in the petri dishes, and their roots were covered with soil. The plates were incubated in a culture room at 22°C with a cycle consisting of 16 h of light (energy fluence rate, 1.2 W m^{-2}) and 8 h of darkness. Three months after inoculation the root systems were washed, and the short dichotomous mycorrhizas were collected and stored at −20°C.

PCR and RFLP analyses. DNA was extracted from freeze-dried mycelia by the method of Van Kan et al. (33) and then resuspended in water. DNA was extracted from individual mycorrhizas as described by Henrion et al. (16), except that 1% (wt/vol) insoluble polyvinylpolypyrrolidone was added to remove humic acids (19). The DNA extracted from one mycorrhiza was resuspended in 10 μl of water. The sequences of the different PCR primers used in this study are shown in Table 1, and the positions of these sequences on the IGS restriction map are shown in Fig. 2. PCR were carried out in 50-μl reaction mixtures containing 80 ng of genomic DNA, each primer at a concentration of 100 nM, each deoxynucleoside triphosphate at a concentration of 200 μM, 1.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase, and the appropriate buffer supplied by the manufacturer

TABLE 1. PCR primers used in this study

Primer	Sequence	Reference
126	5′-ACCACCTAGGACGGTCATCA-3′	14
127	5′-GTAGAGTAGCCTTGTTGTTACGATC-3′	14
5SA	5′-CAGAGTCCTATGGCCGTGGAT-3′	18
5SA′	5′-ATCCACGGCCATAGGACTCTG-3′	34
IGS2A	5′-TTAACTAGCCCAACCCCACTTT-3′	This study
IGS2AI	5′-AAAGTGGGGTTGGGCTAGTTAAG-3′	This study

(GIBCO-BRL). When DNA extracted from mycorrhizas were used, 4 μl of a DNA solution was added, the concentration of each primer was increased to 400 nM, and 0.5% (wt/vol) bovine serum albumin (BSA) was added (35). For primers 126 and 127, primers 126 and IGS2A, and primers 127 and 5SA, the amplification conditions were as follows: initial denaturation at 95°C for 3 min and then 37 cycles consisting of 95°C for 2 min, 50°C for 1 min, and 72°C for 3 min. The reaction was completed by a 10-min extension step at 72°C. For primers IGS2AI and 5SA′, the annealing temperature was increased to 55°C. Control reaction mixtures without DNA were always run in parallel to ensure that there was no contaminating DNA in the solutions.

Appropriate amounts of the amplified sequences were digested with *Hae*III and *Nde*II, and the restriction fragments were separated by electrophoresis on 2.5% agarose gels (1.66% multipurpose agarose and 0.83% standard agarose; Appligene Oncor) in 0.5× TBE buffer (0.04 M Tris HCl, 0.04 M boric acid, 1 mM Na_2EDTA; pH 8.0). The gels were stained with ethidium bromide and photographed.

Cloning and sequencing. The two internal *Bcl*I-*Bcl*I fragments of IGS2 amplified from *H. cylindrosporum* h1 (Fig. 2) were cloned into the *Bam*HI site of plasmid pBluescript SK (Stratagene) and propagated in *Escherichia coli* DH5α (26). The shortest fragment (600 bp) was sequenced from both extremities by using primers T3 and T7 (Fig. 2, sequence b). A 594-bp sequence was obtained from the 5′ end of the longest *Bcl*I-*Bcl*I fragment (Fig. 2, sequence a). Sequences were generated by the chain termination method (27) by using an Applied Biosystems automatic sequencer (Génome Express Co., Grenoble, France). The sequences obtained were used to design oligonucleotide primers for PCR by avoiding repeated sequences and internal secondary structures and choosing an annealing temperature greater than 50°C.

Nucleotide sequence accession numbers. The nucleotide sequences of sequences a and b have been deposited in the EMBL database under accession no. AJ006148 and AJ006147, respectively.

RESULTS

Characterization and distribution of the different alleles. PCR amplification of the IGS from 124 of the 125 homokaryons with primers 126 and 127 yielded a single DNA fragment that was about 3.4 kb long; the only exception was one strain for which two DNA fragments which differed from each other by less than 150 bp were amplified. Prolonged electrophoretic migration was necessary in order to reveal slight size polymorphisms; the maximum difference between the smallest and largest amplified fragments was 300 bp. RFLPs were generated by using the four-cutter endonucleases *Hae*III and *Nde*II, which have been shown to reveal polymorphisms in this sequence amplified from dikaryotic mycelia of *H. cylindrosporum* (15). *Hae*III generated 12 different restriction profiles (profiles H1 to H12), and *Nde*II generated nine different restriction profiles (profiles N1 to N9) (Fig. 3A). Some of the IGS which produced the same restriction pattern could be distinguished from each other by slight differences in size which specifically affected the largest restriction fragment (Fig. 3A, lanes N1). These size differences could be distinguished only when DNA samples were separated in contiguous lanes on the same agarose gel. One *Hae*III (or *Nde*II) profile could be associated with one or several *Nde*II (or *Hae*III) profiles, so 24 different combinations (24 IGS alleles) were identified (Fig. 4). Allele frequencies were calculated for the whole Les Landes coastline except the Le Porge sites (both LP and LPDu strains), which were analyzed separately in order to take into account the different sampling strategies used for the homokaryotic strains (Fig. 1). Some alleles occurred very frequently. Four (N2H1, N1H5, N1H2, and N1H4) accounted for 60% of the homokaryons collected along the Les Landes coastline excluding the Le Porge site, and two of these alleles were also well represented at the Le Porge site (Fig. 4). In contrast, nine alleles were each observed in the homokaryotic progenies of only one basidiocarp. When the different sites were examined, at each of them the number of alleles identified was either higher than, equal to, or less than by only one the number of basidiocarps sampled (Fig. 1). When we examined the Le Porge site, which was sampled more extensively than the other sites, we found that 10 of the 14 alleles occurred at at least one other site, either along the Les Landes coast or in Brittany. In 60% of the 26 basidiocarps, for which several homokaryotic progenies were analyzed, two alleles were identified, suggesting that these basidiocarps were heterozygous at this locus; this was, for example, the case for 7 of the 10 basidiocarps collected in the small, 70-m^2 LP strain area.

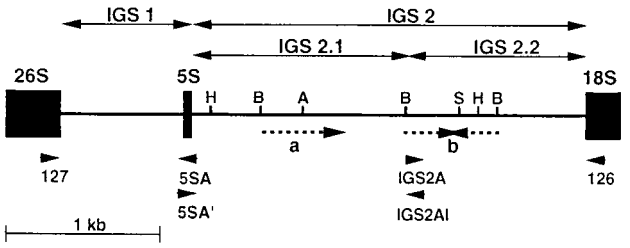

FIG. 2. Restriction map of the IGS sequence of *H. cylindrosporum* h1, showing the positions of the different PCR primers (arrowheads) and the designations of the segments studied. The dashed lines indicate the positions of the two regions sequenced; Abbreviations: A, *Apa*I; B, *Bcl*I; H, *Hin*cII; S, *Sac*I.

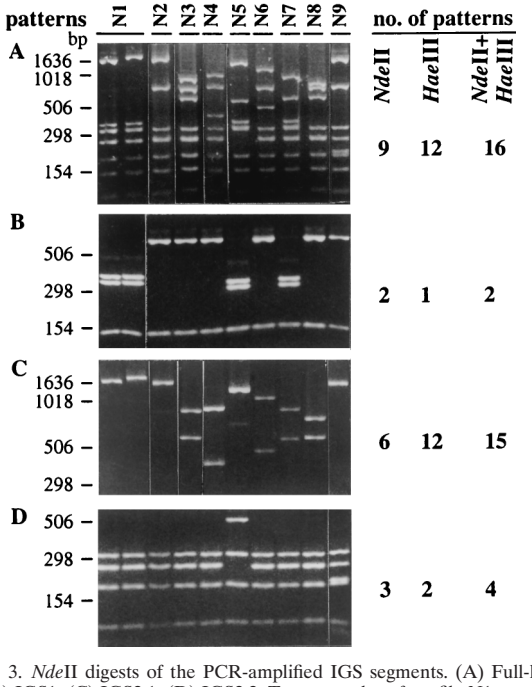

FIG. 3. *Nde*II digests of the PCR-amplified IGS segments. (A) Full-length IGS. (B) IGS1. (C) IGS2.1. (D) IGS2.2. Two examples of profile N1 are shown to illustrate the small size variations which affected the largest restriction fragment in the full-length IGS digests. For the different IGS segments the total numbers of *Nde*II, *Hae*III, and *Nde*II-*Hae*III restriction patterns are shown; for IGS2.1 (C) undigested sequences were considered to be identical.

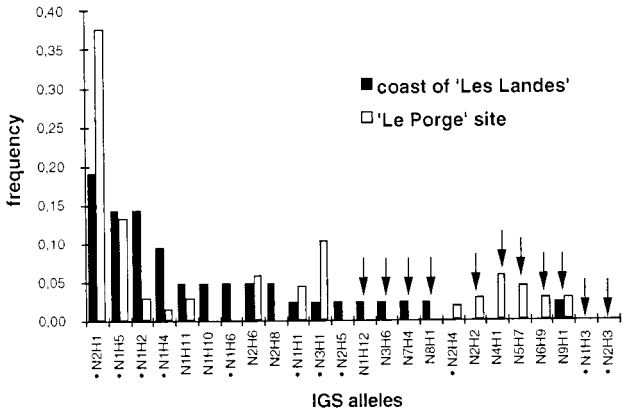

FIG. 4. Frequencies of the different IGS alleles in the *H. cylindrosporum* homokaryons collected along the coast of Les Landes excluding the Le Porge site and frequencies at the Le Porge site. At the Le Porge site, in order to take into account the variable number of homokaryotic progenies analyzed for each basidiocarp, the frequency of allele x (f_x) was: $f_x = (\Sigma f_{xi})/17$, where f_{xi} is the frequency of allele x in the progenies of the ith basidiocarp. Seventeen was the number of basidiocarps collected at this site. Arrows indicate alleles found only in the progenies of one basidiocarp. Dots indicate alleles identified in Brittany populations.

IGS sequence analysis and design and specificity of PCR primers. A restriction map was constructed for the IGS amplified from laboratory strain h1 (Fig. 2). PCR performed with different combinations of primer 5SA or 5SA′ with primers 126 and 127, which anneal to conserved regions of the 5S, 18S, and 26S ribosomal genes (Table 1 and Fig. 2), allowed us to localize the 5S gene on the restriction map. This gene is closer to the 26S gene (850-bp IGS1) than to the 18S gene (2.6-kbp IGS2) (Fig. 2), and all three genes are transcribed in the same orientation (data not shown). The two internal *Bcl*I-*Bcl*I fragments of IGS2 were cloned and partially sequenced (Fig. 2, sequences a and b). None of the partial sequences obtained for a single strand of DNA exhibited a significant level of homology to any database DNA sequence. The 594-bp sequence a was characterized by the occurrence of perfect or truncated T_2AG_3 or G_3T_2A repeats either in tandem or separated by short unique sequences (Fig. 5). These repeats were not found in the other IGS2 segment sequenced (Fig. 2, sequence b). The 5′ end of IGS2.2 was chosen for two almost complementary PCR primers, IGS2A and IGS2AI (Table 1), which, when used with primers 5SA′ and 126, respectively, allowed amplification of two nonoverlapping segments of IGS2 (Fig. 2).

Using the IGS2AI-5SA′ primer combination did not result in amplification from the genomic DNA of the nine other *Hebeloma* species tested, whereas using conserved primers 126 and 5SA′ resulted in amplification of the IGS2 sequence of all these species (data not shown). Using primers IGS2AI and 5SA′ also did not result in amplification of the genomic DNA extracted from the four other ECM species frequently found in the dune ecosystem.

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   1 TGATCAGTAG GTGTTTAGGG TAGGTAGTTT GTGAGTTGGG TGTAGTTAGT  50
  51 TGGATAGTTG GATATGAATT GATAAAGTTT GGCAGTTGAA AAGGCCTTGG 100
 101 ATGAGCAGAA AGTTGGTTTT AGAGATTCAC TGGGAACGCG TGTCTCGGAA 150
 151 GCCCGCCGCA GGTGGAAAAA TTTTGAGAAT AACCTTTCTC CGGGACGCGT 200
 201 GTCTCGGAAG CCCGCCGCAG GTGGAAAAAT TTTAGGGATA ACATTTCTCT 250

 251 GGGACGCGTG TCTCGGGGCC CGCCGCAGGT GAATTTTTTT AGGGAAGAGT 300
 301 GTTCAGATTT AGGGTAGGTT ACGCAAGGTT TATAGTGATA AGTTTAGTTA 350
 351 GGGTTATCAA GTTTGGTTTG GTCAGAAGGT TAGATTTAGA TGTGGGTTAG 400
 401 GAATTAGGGT TAGCGAGTGT CAGAGGTTAG AGAGTTAGGT ANGGGTTAGG 450
 451 GTTAGGGGTT AGGGTTAGTG TTAGGGAATT AGTGTTAGGG AGTTGAAGTT 500

 501 AGGGAGTTGT GCAGGTTAGG GTGTTAGGGT TAGGGGTTAG GGTTAGTGTT 550
 551 AGGGTTAGAA TTAGAGAGTT AGGGTTAGGG AATTAGAATC AGGT       594
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FIG. 5. Nucleotide sequence of segment a of IGS2.1 from *H. cylindrosporum* h1 (Fig. 2). The T_2AG_3 motifs are underlined. The *Apa*I site shown in Fig. 2 is enclosed in a box.

Distribution of polymorphisms within the IGS. The IGS was divided into three segments which were amplified separately; segment IGS1 was amplified with primers 5SA and 127, segment IGS2.1 was amplified with primers 5SA′ and IGS2AI, and segment IGS2.2 was amplified with primers IGS2A and 126 (Fig. 2). These three sequences were amplified from 20 different homokaryotic strains, each representing one of the different IGS restriction profiles generated with *Hae*III and *Nde*II. For the outermost IGS1 and IGS2.2 segments, all PCR yielded a single DNA fragment when a primer annealing temperature of 50°C was used. No length polymorphism was observed when we examined the different IGS1 and IGS2.2 sequences, which were 850 and 1,230 bp long, respectively. For the IGS2.1 sequence, increasing the primer annealing temperature to 55°C was necessary in order to amplify a single DNA fragment for most strains; two fragments were always obtained for one strain. The latter strain was the only strain for which amplification of the entire IGS sequence also yielded two fragments, thus suggesting that its rDNA gene cluster may contain two IGS sequences that differ in length. In contrast to segments IGS1 and IGS2.2, significant length variations were observed for segment IGS2.1 fragments from different strains. These size variations were similar in magnitude to the size variations observed for the entire IGS sequence, thus demonstrating that the size variations observed for the different IGS resulted exclusively from size variations in IGS2.1.

All PCR products were digested separately with *Hae*III and *Nde*II (Fig. 3). IGS1 and IGS2.2 were poorly polymorphic. IGS1 was monomorphic with *Hae*III, and only two restriction patterns were generated with *Nde*II, compared to the 12 and 9 IGS RFLP patterns generated with *Hae*III and *Nde*II, respectively (Fig. 3). For IGS2.2, two and three RFLP patterns were obtained with *Hae*III and *Nde*II, respectively (Fig. 3). IGS2.1 was the most polymorphic sequence; 12 and 6 RFLP patterns were obtained with *Hae*III and *Nde*II, respectively. As shown in Fig. 3 for *Nde*II, which either did not cut or cut only once in IGS2.1, restriction polymorphisms in IGS2.1 not only reflected a variable number of restriction sites but also reflected the length polymorphisms which were accentuated after restriction enzyme digestion. In order to calculate the number of restriction patterns, all nondigested IGS2.1 sequences were considered identical even if they differed in size. Despite this approximation, when the IGS2.1 *Hae*III and *Nde*II restriction patterns were combined, 15 different IGS2.1 patterns were obtained, compared to the 16 IGS alleles which were originally identified.

For each of the 10 basidiocarps collected at the LP site, whose IGS alleles were identified previously, the IGS2.1 sequence was amplified either from one homokaryotic progeny (when the progenies were homozygous) or from two progenies (when the progenies were heterozygous) so that all of the IGS alleles occurring in this population were represented. Digestion of the PCR products resulted in identification of six IGS2.1 types, and the six basidiocarps appeared to be heterozygous. An analysis of this selected region revealed that there were seven different basidiocarps, compared to the nine basidiocarps which were discriminated by the analysis of the full-length IGS.

Amplification from mycorrhizas. Mycorrhizas were synthesized in vitro in two different sandy soils characteristic of the

dune ecosystem. The Grand Crohot soil was almost devoid of organic matter, in contrast to the Le Porge soil, which contained up to 4% organic matter (14). The PCR mixture used to amplify IGS2.1 from DNA extracted from mycelia did not allow reproducible amplification of this sequence when DNA extracted from mycorrhizas were used. To improve both the reproducibility and the PCR yield, we studied the influence of different factors; among these factors were the concentrations of different components of the PCR mixture (primers, Mg^{2+}, template DNA) and the presence of different molecules (dimethyl sulfoxide, Tween, BSA, gelatin). The best results were obtained by simultaneously using a high oligonucleotide concentration and adding BSA, which allowed amplification from 78% of the DNA samples tested irrespective of the fungal strain used for inoculation or of the origin of the soil (108 and 150 mycorrhizas obtained from Grand Crohot and Le Porge soils, respectively, were analyzed). The BSA-containing reaction mixture did not allow reproducible amplification of the entire IGS sequence with primers 126 and 127 and DNA extracted from mycorrhizas. Digestion of IGS2.1 amplified from mycorrhizas with either *Hae*III or *Nde*II resulted in restriction profiles identical to the restriction profiles of IGS2.1 amplified from the DNA of the original strain grown in pure culture; thus, the strains forming the mycorrhizas could be identified unambiguously (data not shown).

DISCUSSION

IGS allelic diversity. Our study of *H. cylindrosporum* isolates collected under *Pinus* trees along the west coast of France revealed a high level of diversity in the IGS of the nuclear rDNA genes of this species; a minimum of 24 alleles were identified in 125 haploid strains. IGS alleles were defined on the basis of their *Nde*II and *Hae*III restriction patterns. The number of alleles identified increased with the number of basidiocarps analyzed but did not increase proportionately. The ratio between these two values was $1 \pm x$ for each of the sample sites where less than six basidiocarps were collected; it decreased to 0.76 at the Le Porge site, where 17 basidiocarps were analyzed, and to 0.35 for the complete survey (24 alleles for 68 basidiocarps). This tendency indicates that most of the IGS alleles present in the geographic area examined have been identified. Moreover, the frequency of each individual allele suggests that probably only rare alleles (frequency, less than 0.05) remain to be identified. This tendency also indicates that although the most abundant alleles have been identified, not all of the alleles present have been characterized even in the most extensively sampled sites and that local sites can be almost as diverse as the whole geographic area, as exemplified by the Le Porge site. Furthermore, up to 60% of the genets are heterozygous at the rDNA locus, which confirms the data of Gryta et al. (14), who found a high degree of genetic diversity within local populations of *H. cylindrosporum* which reflected a high level of outbreeding in this heterothallic species. Allele distribution seems to be homogeneous along the coastline examined, as no significant difference or gradient in allele occurrence could be identified from south to north. Two of the most frequent alleles detected along the coastline are also the two most frequent alleles in the local population at the Le Porge site. Altogether, these observations suggest that there is a low degree of spatial subdivision over the area sampled, a situation which can be compared to the absence of a clear subdivision of the population of the agaric root pathogen *Armillaria gallica* in eastern North America (28). For the latter species, Saville et al. suggested that in the area considered, all available forest sites were occupied and there were frequent genetic exchanges between sites, which prevented local differentiation. Genetic differentiation between populations of *H. cylindrosporum* may occur on a different scale within the geographic range of this species, which is a characteristic organism of European sand dune forest ecosystems. This hypothesis could be tested by using strains collected along the coast of the Italian peninsula and the coast of Sardinia (7) in the Mediterranean basin, an area which is both distant and isolated from the Atlantic coast dune ecosystem which we studied.

Molecular organization of the IGS. The structure of the fungal IGS is not known for many species. In the homobasidiomycete group, IGS1 sequences have been determined for closely related Northern Hemisphere *Armillaria* species (2, 31) and for *L. bicolor* (29). Total IGS1 and IGS2 sequences are available only for the pathogen *Filobasidiella neoformans* (11), and partial IGS1 and IGS2 sequences are also available for the agaric *Tricholoma matsutake* (20). Restriction maps and the location of the 5S gene have been determined for some other organisms, such as *Coprinus* sp. (5, 36), *Laccaria* sp. (1, 29), *Pleurotus cornucopiae* (21), and *Schizophyllum commune* (30). The overall structure of the IGS of *H. cylindrosporum* is similar in a number of ways to the structures of the other homobasidiomycete IGS that have been studied. One common feature is the presence of the 5S gene, which is transcribed in the same orientation as the other rRNA genes; the only reported exception is the 5S gene of *Coprinus comatus*, which is transcribed in the opposite direction (6). Another common feature is the fact that the IGS1 sequence is shorter than the IGS2 sequence; the only exception to this occurs in the IGS of *P. cornucopiae* (21). The polymorphisms in the IGS of *H. cylindrosporum* were found to be greater in the half of IGS2 next to the 3′ end of the 5S gene than in the other half. Polymorphisms in this region resulted from both size variations and restriction enzyme site polymorphisms, whereas polymorphisms in other regions resulted exclusively from restriction enzyme site polymorphisms. Partial sequencing of this region revealed the presence of several perfect or imperfect TA_2C_3 repeats which were not found in the sequenced portion of the less polymorphic IGS2.2 sequence. The presence of short reiterated motifs in rDNA IGS sequences is well documented in higher plants (3) but has not been reported previously in fungi. An examination of more than 10 ascomycete and basidiomycete IGS sequences found in GenBank failed to reveal similar motifs in IGS2. However, it is too early to conclude that *H. cylindrosporum* is an exception, since only one other partial IGS2 sequence from an agaric species (*T. matsutake*) has been published and three or four copies of a similar T_2AG_3 motif have been shown to be present in IGS1 of the ECM basidiomycete *L. bicolor* (29). Length differences between IGS2 sequences may result from variable numbers of T_2AG_3 repeats similar to what is observed for microsatellite loci in other organisms.

In all but one of the homokaryotic mycelia of *H. cylindrosporum* studied, amplification of the IGS yielded a single DNA product, and for each DNA product the sum of the sizes of the restriction fragments obtained with either *Nde*II or *Hae*III was identical to the size of the uncut fragment. This suggests that in a haploid nucleus, all of the repeat units of the rDNA cluster have the same IGS sequence. Amplification of two fragments of different sizes was observed with only one strain, which suggests that in this rare case there are two different IGS in the rDNA cluster. Until recently, it was thought that meiotic recombination was inhibited within fungal rDNA clusters and that sequence heterogeneities in repeat units did not exist or existed very transiently (5). However, sequence heterogeneities resulting from meiotic crossovers have been found in a

few homokaryotic progenies of a heterozygous dikaryon of the agaric *L. bicolor* (29).

Application to field studies. The hypervariable IGS2.1 sequence was amplified for all of the IGS alleles by combining a universal primer (5SA′) and a specific primer (IGS2AI), which indicated that the sequence identified by the specific primer was present in all of the IGS alleles. As this primer set failed to amplify any DNA fragment from the genomes of nine other *Hebeloma* species, we concluded that the IGS2AI oligonucleotide sequence is probably specific to *H. cylindrosporum*. Furthermore, optimization of the PCR conditions with DNA extracted from mycorrhizas obtained from soil resulted in reproducible amplification of IGS2.1. These two characteristics, species specificity of the primer and capacity to amplify IGS2.1 from limited amounts of fungal material, should lead to identification of mycorrhizas formed by this species in the field.

For application to studies on the correlation between aboveground diversity (based on basidiocarp sampling) and belowground diversity (based on mycorrhiza sampling), this sequence must fulfill a second prerequisite; it must be highly polymorphic among genets collected in very limited areas (a few square meters and even a few square decimeters). This characteristic was tested by analyzing homokaryotic strains derived from 10 basidiocarps collected at the 70-m^2 LP site. Diversity was analyzed at two levels. First, we analyzed the allelic diversity, which corresponded to the six different IGS2.1 sequences detected in this sample of strains; and second, we analyzed the genotypic diversity, which for heterothallic basidiomycete species such as *H. cylindrosporum* may be different from the allelic diversity, as the mycelia which form the mycorrhizas and differentiate fruiting bodies in the field are dikaryotic. In this study, six basidiocarps appeared to be heterozygous at the rDNA locus, and seven different diplotypes were distinguished. At such a limited sampling site, the basidiocarps that have identical IGS2.1 types either emerge from the same below-ground mycelium or emerge from distinct mycelia which happen to have identical rDNA alleles. With six different alleles, the maximum number of diplotype combinations is 21, but each of these combinations should not occur at the same frequency, as allele frequencies are not identical. As not all allele combinations have been detected at the basidiocarp level, it may be possible to detect additional genotypes on the host plant root systems at the LP site.

Conclusions. The rDNA IGS sequence of *H. cylindrosporum* appears to be a very good candidate sequence for evaluating the diversity in local populations of this species. This sequence is highly variable even within local populations, and many allelic forms of this molecule can be identified by using only two restriction enzymes. The variability within this sequence is not evenly distributed and is concentrated in an internal portion which can be amplified efficiently from DNA extracted from mycorrhizas by using a species-specific oligonucleotide. Previously published data for different fungal species indicate that intraspecific variability within the IGS is commonplace, so an approach similar to the one which we used with *H. cylindrosporum* could be used with other species. Moreover, the strategy of concentrating on a single DNA sequence to study intraspecific diversity seems to be particularly appropriate for heterothallic basidiomycete species whose genotypic diversity is likely to be higher than the allelic diversity.

Such a method should allow characterization of the different genotypes that form mycorrhizas on host plant root systems and the genotypes which occasionally or regularly form fruiting bodies. The data from such studies should increase our knowledge of the structure and dynamics of below-ground and above-ground populations of ECM species.

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REFERENCES

1. **Albee, S. R., G. M. Muller, and B. R. Kropp.** 1996. Polymorphisms in the large intergenic spacer of the nuclear ribosomal repeat identify *Laccaria proxima* strains. Mycologia **88:**970–976.
2. **Anderson, J. B., and E. Stasovski.** 1992. Molecular phylogeny of Northern Hemisphere species of *Armillaria*. Mycologia **84:**505–516.
3. **Bhatia, S., M. S. Negi, and M. Lakshmikumaran.** 1996. Structural analysis of the rDNA intergenic spacer of *Brassica nigra*: evolutionary divergence of the spacers of the three diploid *Brassica* species. J. Mol. Evol. **43:**460–468.
4. **Bruns, T. D., T. M. Szaro, M. Gardes, K. W. Cullings, J. J. Pan, D. L. Taylor, T. R. Horton, A. Kratzer, M. Garbelotto, and Y. Liss.** 1998. A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis. Mol. Ecol. **7:**257–272.
5. **Cassidy, J. R., D. Moore, B. C. Lu, and P. J. Pukkila.** 1984. Unusual organization and lack of recombination in the ribosomal RNA genes of *Coprinus cinereus*. Curr. Genet. **8:**607–613.
6. **Cassidy, J. R., and P. J. Pukkila.** 1987. Inversion of 5S ribosomal RNA genes within the genus *Coprinus*. Curr. Genet. **12:**33–36.
7. **Contu, M.** 1991. Contributo allo studio del genere *Hebeloma* (Basidiomycetes, Cortinariaceae) in Sardegna (Italia). I. Rev. Iberoam. Micol. **8:**38–42.
8. **Debaud, J. C., and G. Gay.** 1987. *In vitro* fruiting under controlled conditions of the ectomycorrhizal fungus *Hebeloma cylindrosporum* associated with *Pinus pinaster*. New Phytol. **105:**429–435.
9. **De La Bastide, P., B. R. Kropp, and Y. Piché.** 1995. Population structure and mycelial phenotypic variability of the ectomycorrhizal basidiomycete *Laccaria bicolor* (Maire) Orton. Mycorrhiza **5:**371–379.
10. **Erland, S., B. Henrion, F. Martin, L. A. Glover, and I. J. Alexander.** 1994. Identification of the ectomycorrhizal basidiomycete *Tylospora fibrillosa* Donk by RFLP analysis of the PCR-amplified ITS and IGS regions of the ribosomal DNA. New Phytol. **126:**525–532.
11. **Fan, M., L. C. Chen, M. A. Ragan, R. R. Gutell, J. R. Warner, B. P. Currie, and A. Casadevall.** 1995. The 5S rRNA and the rRNA intergenic spacer of the two varieties of *Cryptococcus neoformans*. J. Med. Vet. Mycol. **33:**215–221.
12. **Gardes, M., and T. D. Bruns.** 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol. Ecol. **2:**113–118.
13. **Gardes, M., and T. D. Bruns.** 1996. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. Can. J. Bot. **74:**1572–1583.
14. **Gryta, H., J. C. Debaud, A. Effosse, G. Gay, and R. Marmeisse.** 1997. Fine-scale structure of populations of the ectomycorrhizal fungus *Hebeloma cylindrosporum* in coastal sand dune forest ecosystems. Mol. Ecol. **6:**353–364.
15. **Gryta, H., R. Marmeisse, G. Gay, J. Guinberteau, and J. C. Debaud.** 1996. Molecular characterization of *Hebeloma cylindrosporum*: an ectomycorrhizal basidiomycete associated with *Pinus pinaster* in coastal sand dunes, p. 35–38. *In* C. Azcon-Aguilar and J. M. Barea (ed.), Proceedings of the Fourth European Symposium on Mycorrhizas. Office for Official Publications of the European Communities, Luxembourg, Luxembourg.
16. **Henrion, B., G. Chevalier, and F. Martin.** 1993. Typing truffle species by PCR amplification of the ribosomal DNA spacers. Mycol. Res. **98:**37–43.
17. **Henrion, B., C. Di Battista, D. Bouchard, D. Vairelles, B. D. Thompson, F. Le Tacon, and F. Martin.** 1994. Monitoring the persistence of *Laccaria bicolor* as an ectomycorrhizal symbiont of nursery-grown Douglas fir by PCR of the rDNA intergenic spacer. Mol. Ecol. **3:**571–580.
18. **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.
19. **Holden, W. E., J. K. Janson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54:**703–711.
20. **Hwang, S., and J. Kim.** 1998. Small-subunit ribosomal DNA of an ectomycorrhizal fungus *Tricholoma matsutake*: sequence, structure and phylogenetic analysis. Mol. Cell. **8:**251–258.
21. **Iraçabal, B., and J. Labarère.** 1994. Restriction site and length polymorphism of the rDNA unit in the cultivated basidiomycete *Pleurotus cornucopiae*. Theor. Appl. Genet. **88:**824–830.
22. **Karén, O., N. Högberg, A. Dahlberg, L. Jonsson, and J. E. Nylund.** 1997. Inter- and intraspecific variation in the ITS region of rDNA of ectomycor-

rhizal fungi in Fennoscandia as detected by endonuclease analysis. New Phytol. **136:**313–325.
23. **Last, F. T., J. Dighton, and P. A. Mason.** 1987. Successions of the sheating mycorrhizal fungi. Trends Ecol. Evol. **58:**3553–3560.
24. **Mehmann, B., S. Egli, G. H. Braus, and I. Brunner.** 1995. Coincidence between molecularly or morphologically classified ectomycorrhizal morphotypes and fruitbodies in a spruce forest, p. 41–52. *In* V. Stocchi, P. Bonfante, and M. Nuti (ed.), Biotechnology of ectomycorrhizae. Plenum Press, New York, N.Y.
25. **Rao, P. S., and D. J. Niederpruem.** 1969. Carbohydrate metabolism during morphogenesis of *Coprinus lagopus* (sensu Buller). J. Bacteriol. **100:**1222–1228.
26. **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.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
28. **Saville, B. J., H. Yoell, and B. Anderson.** 1996. Genetic exchange and recombination in populations of the root-infecting fungus *Armillaria gallica*. Mol. Ecol. **5:**485–497.
29. **Selosse, M. A., G. Costa, C. Di Battista, F. Le Tacon, and F. Martin.** 1996. Meiotic segregation and recombination of the intergenic spacer of the ribosomal DNA in the ectomycorrhizal basidiomycete *Laccaria bicolor*. Curr. Genet. **30:**332–337.
30. **Specht, C. A., C. P. Novotny, and R. C. Ulrich.** 1984. Strain specific differences in ribosomal DNA from the fungus *Schizophyllum commune*. Curr. Genet. **8:**219–222.
31. **Terashima, K., J. Y. Chae, T. Yajima, T. Igarashi, and K. Miura.** 1998. Phylogenetic analysis of Japanese *Armillaria* based on the intergenic spacer (IGS) sequences of their ribosomal DNA. Eur. J. For. Pathol. **28:**11–19.
32. **Timonen, S., H. Tammi, and R. Sen.** 1997. Characterization of the host genotype and fungal diversity in Scots pine ectomycorrhiza from natural humus microcosms using isozyme and PCR-RFLP analyses. New Phytol. **135:**313–323.
33. **Van Kan, J. A. L., G. F. J. M. Van den Ackerveken, and P. J. G. M. De Wit.** 1991. Cloning and characterization of the cDNA of avirulence *avr*9 of the fungal pathogen *Cladosporium fulvum*, the causal agent of tomato leaf mold. Mol. Plant Microbe Interact. **4:**52–59.
34. **White, T. J., T. D. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA 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 amplifications. Academic Press, San Diego, Calif.
35. **Wilson, I. G.** 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. **63:**3741–3751.
36. **Wu, M. M. J., J. R. Cassidy, and P. J. Pukkila.** 1983. Polymorphisms in DNA of *Coprinus cinereus*. Curr. Genet. **7:**385–392.