A Diverse Population of Introns in the Nuclear Ribosomal Genes of Ericoid Mycorrhizal Fungi Includes Elements with Sequence Similarity to Endonuclease-Coding Genes

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Ericoid mycorrhizal fungi form symbioses with the roots of members of the Ericales. Although only two genera have been identified in culture, the taxonomic diversity of ericoid symbionts is certainly wider. Genetic variation among 40 ericoid fungal isolates was investigated in this study. PCR amplification of the nuclear small-subunit ribosomal DNA (SSU rDNA) and of the internal transcribed spacer (ITS), followed by sequencing, led to the discovery of DNA insertions of various sizes in the SSU rDNA of most isolates. They reached sizes of almost 1,800 bp and occurred in up to five different insertion sites. Their positions and sizes were generally correlated with morphological and ITS-RFLP grouping of the isolates, although some insertions were found to be optional among isolates of the same species, and insertions were not always present in all SSU rDNA repeats within an isolate. Most insertions were identified as typical group I introns, possessing the conserved motifs characteristic of this group. However, other insertions lack these motifs and form a distinct group that includes other fungal ribosomal introns. Alignments with almost 70 additional sequences from fungal nuclear SSU rDNA introns indicate that introns inserted at the same site along the rDNA gene are generally homologous, but they also suggest the possibility of some horizontal transfers. Two of the ericoid fungal introns showed strong homology with a conserved motif found in endonuclease genes from nuclear rDNA introns.

Introduction

Ericoid mycorrhizal fungi are a diverse group of fungi that form symbiotic associations with plants in the Ericales (Perotto et al. 1995; Read 1996; Straker 1996). The fine roots of these plants are colonized in nature by both Ascomycetes and Basidiomycetes (Bonfante 1980; Peterson, Mueller, and Englander 1980), but taxonomic identification is confined to the Ascomycetes, as Basidiomycetes symbionts have not yet been isolated in pure culture. Species from two genera are reported as mycorrhizal partners of ericaceous plants: *Hymenoscyphus ericae* (Read) Korf and Kernan (Read 1974), with its anamorph *Scytalidium vaccinii* Dalpé, Litten, and Siegler (Egger and Siegler 1993), and *Oidiodendron* spp., as well as representatives of 16 groups of ascomycete fungi (Peterson, Mueller, and Englander 1980), but taxonomic identification is confined to the Ascomycetes, as Basidiomycetes symbionts have not yet been isolated in pure culture. Species from two genera are reported as mycorrhizal partners of ericaceous plants: *Hymenoscyphus ericae* (Read) Korf and Kernan (Read 1974), with its anamorph *Scytalidium vaccinii* Dalpé, Litten, and Siegler (Egger and Siegler 1993), and *Oidiodendron* spp. (Couture, Fortin, and Dalpé 1983; Dalpé 1986), with their teleomorphs in the Gymnoascaceae and Mixothri- caceae (Dalpé 1989; Hambleton et al. 1998).

Sterile mycelia with different colony morphologies have also been described as common symbionts of ericoid roots in North America (Stoyke, Egger, and Currah 1992; Hambleton and Currah 1997), Europe (Duclos and Fortin 1983; Perotto et al. 1990, 1996), South Africa (Straker and Mitchell 1985), and Australia (Hutton, Dixon, and Sivathamparam 1994; Liu, Chambers, and Cairney 1998), where they often form the majority of mycorrhizal isolates. Their taxonomic position, however, is unknown because they lack the morphological structures that could be used for identification.

The nuclear ribosomal genes have been extensively used for taxonomic purposes in fungi (e.g., Berbee and Taylor 1993; Gargas et al. 1995). Therefore, we have begun to sequence these genes to determine the genetic diversity of ericoid mycelia collected worldwide, focusing on the nuclear small subunit (SSU) rDNA genes and the internal transcribed sequences (ITSs). While sequencing the SSU rDNA, we have discovered introns in most of the ericoid isolates. The presence and sequence of an intron in the SSU rDNA of one *H. ericae* isolate has already been reported by Egger, Osmond, and Goodier (1995). In this paper, we analyzed the entire SSU rDNA of several isolates of *H. ericae* and of *Oidiodendron* spp., as well as representatives of 16 groups of sterile mycelia. The sites of intron insertion have been mapped in all isolates and shown to occur at five different positions, including sites rarely described in fungi. Sequence analysis demonstrates that many of these introns belong to group I.

Group I introns are a structural and functional group with a widespread but irregular distribution (Dujon 1989) and are frequently found in lower eukaryotes, especially algae and fungi (Dujon 1989; Johansen, Muscarella, and Vogt 1996). They occur at several locations along the chloroplast and mitochondrial genome, including protein coding genes, but in the nuclear genome they seem to be restricted to the rDNA genes. Several have been shown to splice both in vitro and in vivo due to the autocatalytic properties of the intron RNA. Insertion in intronless copies of the same gene, a process called homing (Cech 1990), is usually catalyzed by an intron-encoded DNA endonuclease (Belfort and Roberts 1997), although alternative mechanisms have been suggested (Roman and Woodson 1998). In fungi, nuclear group I introns have been found both in the Ascomycetes and the Basidiomycetes. In...
Sterile mycelia

As the databases on ribosomal fungal sequences are becoming larger, reports on ribosomal insertions are also increasing rapidly. We used a large set of intron sequences to investigate the relationships existing among ericoid introns occurring at the different insertion sites and those of other fungi.

Most cases described in the literature, one or two introns have been found in the SSU rDNA of the same organism (see Gargas, DePriest, and Taylor [1995] and Johansen, Muscarella, and Vogt [1996] for references). An exception is the group of lichen-forming fungi, one of the most complex systems, where relatively small insertions have been described in as many as eight different sites in Lecanora dispersa (Gargas, DePriest, and Taylor 1995).

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Materials and Methods

ERICOID MYCELIA AND CULTURE CONDITIONS

The isolates investigated are listed in table 1. Most sterile fungal isolates from northern Italy were previously grouped on the basis of their ITS–restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) profiles (Perotto et al. 1996). Mycelia were grown on 2% malt agar medium and subcultured into liquid 2% malt medium for DNA extraction.

DNA Extractions

Whole-cell DNA was extracted in Eppendorf tubes from 30–50 mg fresh weight of fungal material follow-

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Table 1

<table>
<thead>
<tr>
<th>Fungal Isolates</th>
<th>Origin</th>
<th>Strain Number*</th>
<th>Reference</th>
<th>SSU rDNA Insertions</th>
</tr>
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<tbody>
<tr>
<td><em>H. ericae</em> ...</td>
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<td>CLM1340.98</td>
<td>Pearson and Read (1973)</td>
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<td>Yes</td>
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<tr>
<td><em>H. ericae</em> 100</td>
<td>Great Britain</td>
<td>CLM1342.98</td>
<td>Pearson and Read (1973)</td>
<td>Yes</td>
</tr>
<tr>
<td><em>H. ericae</em> 101</td>
<td>Great Britain</td>
<td>CLM1343.98</td>
<td>Leake and Read (1991)</td>
<td>Yes</td>
</tr>
<tr>
<td><em>H. ericae</em> CV3</td>
<td>Great Britain</td>
<td>CLM1344.98</td>
<td>D. J. Read (unpublished)</td>
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<td><em>H. ericae</em> CV4</td>
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<td>CLM1345.98</td>
<td>D. J. Read (unpublished)</td>
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<td><em>H. ericae</em> CV5</td>
<td>Great Britain</td>
<td>CLM1346.98</td>
<td>D. J. Read (unpublished)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Oidiodendron isolates

| O. maius 89 | Canada | CLM1347.98 | Dalpé (1986) | No |
| O. maius 90 | Canada | CLM1348.98 | Dalpé (1986) | Yes |
| O. maius 91 | Canada | CLM1349.98 | Dalpé (1986) | Yes |
| O. sp. 150 | Slovenia | CLM1350.98 | N. Gogala (unpublished) | No |
| O. sp. 151 | Slovenia | CLM1351.98 | N. Gogala (unpublished) | No |
| O. maius (L.Iba/6) | Italy | CLM1352.98 | Perotto et al. (1996) | Yes |
| O. maius (L.Ibb/1) | Italy | CLM1353.98 | Perotto et al. (1996) | Yes |
| O. maius (L.Ibb/8) | Italy | CLM1354.98 | Perotto et al. (1996) | Yes |
| O. maius (L.Ibb/12) | Italy | CLM1355.98 | Perotto et al. (1996) | Yes |
| O. maius (L.Ibb/5) | Italy | CLM1356.98 | Perotto et al. (1996) | Yes |
| O. maius (L.Ibb/20) | Italy | CLM1357.98 | Perotto et al. (1996) | Yes |
| O. maius (L.Ibb/7) | Italy | CLM1358.98 | Perotto et al. (1996) | Yes |
| O. maius (L.Ibb/6) | Italy | CLM1359.98 | Perotto et al. (1996) | Yes |
| O. citrinum | Italy | CLM1360.98 | C. Varese (unpublished) | No |

Sterile mycelia

G1 (III.Ib/1) | Italy | CLM1361.98 | Perotto et al. (1996) | Yes |
| G1 (III.Ib/9) | Italy | CLM1362.98 | Perotto et al. (1996) | Yes |
| G1 (L.Iba/11) | Italy | CLM1363.98 | Perotto et al. (1996) | Yes |
| G2 (III.Ib/8) | Italy | CLM1364.98 | Perotto et al. (1996) | Yes |
| G2 (III.Ia/3) | Italy | CLM1365.98 | Perotto et al. (1996) | Yes |
| H (L.Iib/4) | Italy | CLM1366.98 | Perotto et al. (1996) | No |
| H (L.Iib/22) | Italy | CLM1367.98 | Perotto et al. (1996) | No |
| 1 (III.ib/3) | Italy | CLM1368.98 | Perotto et al. (1996) | Yes |
| I2 (III.ib/9) | Italy | CLM1369.98 | Perotto et al. (1996) | Yes |
| J2 (III.Iib/4) | Italy | CLM1370.98 | Perotto et al. (1996) | Yes |
| PSIV | Italy | CLM1371.98 | Perotto et al. (1990) | Yes |
| C5 | Italy | CLM1372.98 | Perotto et al. (1997) | Yes |
| S26. | Italy | CLM1373.98 | Perotto et al. (1997) | No |
| Duclos I | France | CLM1374.98 | Duclos and Fortin (1983) | Yes |
| Duclos VI | France | CLM1375.98 | Duclos and Fortin (1983) | Yes |
| Duclos VII | France | CLM1376.98 | Duclos and Fortin (1983) | Yes |
| Duclos IX | France | CLM1377.98 | Duclos and Fortin (1983) | Yes |
| Duclos XIX | France | CLM1378.98 | Duclos and Fortin (1983) | No |
| Isolate from Erica hispidula South Africa | CLM1379.98 | Straker and Mitchell (1985) | No |
| Isolate from Erica mauroitana South Africa | CLM1380.98 | Straker and Mitchell (1985) | No |

* Mycological Collection of the Department of Plant Biology, University of Torino, Italy.
ing a protocol modified by Lee and Taylor (1990). Briefly, samples were frozen in liquid nitrogen and homogenized with a small glass pestle. After thawing in 600 ml lysis buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% v/v 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl [pH 8.0]), samples were incubated at 65°C for 1 h, followed by centrifugation in an Eppendorf centrifuge to eliminate debris. The supernatant was then extracted once with an equal volume of phenol : chloroform : isooctyl alcohol and re-extracted with chloroform : isooctyl alcohol (24:1 v/v). After precipitation of DNA with isopropanol and rinsing of the pellet with 70% ethanol, DNA was resuspended in 100–200 ml of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA.

PCR Amplification

The reaction components for the PCR reaction and the thermal cycling conditions were those described by Gardes and Bruns (1993). Taq polymerase from Dynazyme (Celbio) was used, and the amplification products were analyzed by electrophoresis on agarose gel after staining with ethidium bromide. Primers to conserved regions of the rDNA corresponded to those designed by White et al. (1990). Additional primers were designed for primer to synthesize the second strand (primer combinations are described in more detail in the legend to fig. 3), and 2.5 U of RedTaq polymerase (Sigma). The conditions for DNA amplification were the same as those described by Gardes and Bruns (1993). As a control, total DNA was amplified for each sample using the same primer combinations. Samples were separated on 1.2% agarose gels in 0.5 × TAE.

Sequence Analysis

DNA fragments, either as PCR products or as cloned inserts, were sequenced from both ends using an ABI 373A Stretch DNA sequencer (Service de Sequençage, University of Laval, Canada). Accession numbers for the ericoid intron sequences are provided in table 3. When present, the conserved P, Q, R, and S regions were identified by comparing them with published sequences either visually or through BLAST searches against gene banks.

About 70 sequences of fungal introns identified in the SSU rDNA gene were obtained from gene banks through published references or BLAST searches using all the ericoid intron sequences as queries (table 2). The insertion sites for each retrieved intron sequence were checked either on the gene bank data sheet or, when missing, directly with the authors. The excised and the retrieved intron sequences were aligned with the CLUSTAL X program (Thompson et al. 1997), and the alignment was manually adjusted with GeneDoc (Nicholas, Nicholas, and Deerfield 1997). Phylogenetic clusters were identified by the neighbor-joining method with 1,000 bootstrap replicates. Distances were corrected for multiple substitutions using the Kimura two-parameter model. Alignments can be viewed at the EBI server (url ftp://ftp.ebi.ac.uk/pub/databases/embl/align/). A search for protein homologs in data banks was carried out with the BlastX option at the NCBI web site by using the intron nucleotide sequences as queries.

Results

Variable Sizes of the Nuclear SSU rDNA Genes

Total DNA extracted from the mycelia listed in table 1 was PCR-amplified with primers NS5/ITS4 for subsequent sequencing (fig. 1). However, bands larger than expected were often observed when the PCR fragments were separated by agarose gel electrophoresis (fig. 2). Occasionally (see lane 2 in fig. 2), DNA fragments of different sizes were found for individual isolates after PCR amplification, with one corresponding to the expected size and the other being larger. This situation, also shown in subsequent experiments, indicates a heterogeneous size of the rDNA repeats. A size in-
### Table 2
List of the Fungal Intron Sequences (All Occurring in the Nuclear SSU rDNA) Retrieved from GenBank and Aligned to the Introns of Ericoid Fungi

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Organism</th>
<th>Site</th>
<th>Length (bp)</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stego287</td>
<td>Stegobium paniceum endosymbiont</td>
<td>287</td>
<td>210</td>
<td>D49657</td>
</tr>
<tr>
<td>M.inve516</td>
<td>Mycoarachis inversa (Ascomycota)</td>
<td>516</td>
<td>289</td>
<td>AB012953</td>
</tr>
<tr>
<td>T para516</td>
<td>Trichocoma paradoxus (Ascomycota, Plectomycetes, Eurotiales)</td>
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<td>417</td>
<td>AB013743</td>
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<td>R.dacr516</td>
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<td>404</td>
<td>D13459</td>
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<td>T.flav516</td>
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<td>Myrioclerotinia scirpicola (Ascomycota, Discomycetes, Leotiales)</td>
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### Table 2
Continued

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Retrieved fungal intron sequences that could not be aligned to ericoid introns

- *Lecanora dispersa* (Ascomycota, Discomycetes, Lecanorales) 516 295 L37734
- *L. dispersa* (Ascomycota, Discomycetes, Lecanorales) 789 181 L37734
- *L. dispersa* (Ascomycota, Discomycetes, Lecanorales) 943 112 L37734
crease was never observed for PCR fragments obtained with primers ITS1/ITS4, which amplify a region comprising the ITS and the 5.8S rDNA (data not shown).

A large set of oligonucleotide primers designed to match conserved regions was then used to analyze the complete SSU rDNA gene (fig. 1) and map the occurrences and positions of DNA insertions. Several PCR amplifications indicated that the rDNA of most, but not all, isolates contains repeats larger than expected (table 1). Subsequent sequencing of the NS5/ITS4 fragments confirmed that the size increase was due to DNA insertions which occurred exclusively in the SSU rDNA region.

An RT-PCR experiment carried out on representatives of the different ericoid isolates showed that these insertions are spliced during rRNA maturation independent of their sizes and positions along the ribosomal gene (fig. 3).

Characterization of DNA Insertions

PCR-amplified fragments containing DNA insertions were sequenced in representative strains of the different ericoid groups (table 1). The exact positions and sequences of all rDNA insertions were established (fig. 4), with the exception of the optional insertion of *H. ericae* in position 1506, whose sequence was already published by Egger, Osmond, and Goodier (1995). Insertion sites were numbered in accordance with the *Escherichia coli* SSU rDNA (Gargas, DePriest, and Taylor 1995). No insertions were found in the 5′ half of the SSU rDNA, whereas five possible insertion sites were identified in the 3′ half. Comparison of isolates with DNA insertions in the same positions revealed fragments of variable size (fig. 4). Only a partial sequence of about 1,300 bp was obtained for the larger insertion of sterile mycelium C5, and this was not considered in the subsequent alignments. Insertion sizes ranged from almost 1,800 bp for sterile isolate PSIV down to 185 bp for sterile mycelia I2 and Duclos VI (fig. 4).

Most DNA insertions contained four conserved regions (termed P, Q, R, and S; table 3) that have been described as a characteristic trait of group I introns (Cech 1988; Dujon 1989). None of these typical regions were found in the DNA insertions in position 1199. Similarly, no conserved group I intron sequences were found in the optional intron identified at position 989 in some of the *O. maius* isolates (fig. 5 and table 3).

Intraspecific Variability in the Occurrence of Introns in the SSU rDNA

Investigation of intraspecific variability in the occurrence of introns in several *O. maius* and *H. ericae* isolates detected introns at specific insertion sites in some isolates but not in others (figs. 5 and 6). Eight *O. maius* strains were chosen as representative of about 40 isolates derived from the same sampling site and previously grouped according to their RAPD profiles (Perotto et al. 1996). *Oidiodendron maius* 89, an isolate originally received as *Oidiodendron griseum* but recently reclassified on the basis of morphological and molecular features (Hambleton, Egger, and Currah 1998), was also included in the analysis.

After PCR amplification with primers 18SB/NS4, only *O. maius* 89 showed a DNA fragment of the expected size, whereas all other isolates gave DNA fragments of the same size but larger than expected. When a region of the SSU rDNA farther downstream was amplified, about half of these isolates showed an additional insertion (fig. 5). Sequencing of this optional insertion revealed that it did not contain any of the short sequences characteristic of group I introns. Its position (nucleotide 989, relative to *E. coli*) corresponds to an insertion site not found in other ericoid fungi (fig. 4) and rarely described for the nuclear SSU rDNA (see table 2).

Each of the seven *H. ericae* isolates had one intron either in position 943 (revealed by primers NS5/NS6 in fig. 6A) or in position 1506 (revealed by primers NS7/NS8 in fig. 6B), but none had introns at both sites.

---

**Table 2**

Continued

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**Fig. 2.** Result of the RT-PCR experiment on total RNA extracted from ericoid fungal isolates. The PCR amplification was designed to outline the presence/absence of insertions in different regions of the SSU rDNA and of the corresponding rRNA with a combination of oligonucleotide primers (see Fig. 1). A, Gel showing the results of RT-PCR using the 18SE oligonucleotide to prime the RT reaction. The same primer was used for the PCR amplification of genomic DNA. In samples 1–4, the NS5 primer was added to the PCR reaction to amplify a fragment of the expected size of about 670 bp. In samples 5 and 6, the NS7 primer was used to generate a fragment of the expected length of about 390 bp. B, RT-PCR amplification that used the 18SD oligonucleotide to prime the RT reaction. The NS5 primer was added to the PCR reaction to amplify a fragment of the expected size of about 200 bp. The same primers (18SD/NS5) were used to amplify genomic DNA. In all DNA samples, amplification products show the presence of insertions. In contrast, all samples have corresponding rRNA segments of the correct size, indicating splicing of the insertions. Sample 1, Duclos I; sample 2, Duclos VI; sample 3, I1 (III.Iib/3); sample 4, I2 (III.Iib/4); sample 5, G1 (I.IIib/9); sample 6, PSIV; sample 7, Oidiodendron maius (III.Iib/7); sample 8, PSIV; sample 9, Duclos VIII; sample 10, G1 (I.IIib/9). M, control reactions for the DNA and cDNA amplifications, respectively. M, PCR digested with Hae III.
Variability in the Occurrence of Ribosomal Introns Within the Genome

Ribosomal genes occur in the genome as tandem repeats. The results shown for some of the isolates in figure 2 (lane 2) and figure 5 (lane 4) suggest that not all rDNA repeats have intron insertions. The same was more clearly demonstrated for *H. ericae* CV4 (fig. 6B), as two DNA fragments were equally amplified with the same pair of primers, their sizes corresponding to an rDNA copy lacking an intron and an rDNA copy harboring an intron, respectively.

An apparently contradictory result, later explained by sequencing, demonstrated the same phenomenon for a number of sterile mycelia that showed single bands after PCR amplification. Amplifications with NS5/NS8 primers revealed insertions in this DNA segment, but when genomic DNA was amplified with internal primers to map these insertions more precisely (NS5/NS6 and NS7/NS8; see fig. 1), these isolates gave amplified fragments corresponding in size to intronless DNA copies (data not shown). DNA sequencing, however, revealed that all introns that failed to appear during PCR mapping were inserted at position 1199, which interrupts the annealing site of NS6 and NS7 primers.

Intron Sequence Analysis

The ericoid intron sequences were excised from the flanking DNA, and a first alignment of the complete sequence was attempted with CLUSTAL X. Insertions at positions 989 and 1199, which did not contain the conserved sequences of group I introns, were too divergent to be aligned with the others and were considered

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**Fig. 4.**—Diagram showing the positions of DNA insertions in the SSU rDNA of ericoid isolates. The sizes of DNA insertions and their exact positions (relative to *Escherichia coli* rDNA) were established on the basis of sequence information.
perotto et al.

Fig. 6.—Intron variability among isolates of *Hymenoscyphus ericae*. A, Gel showing a band shift corresponding to the intron at position 943, as revealed by amplification with primers NS5/NS6. G. *Hymenoscyphus ericae* strains CV3 and CV4 do not contain the intron at this site. B, The same isolates after amplification with primers NS7/18SE. The band shift reveals an intron at position 1506 for *H. ericae* strains CV3 and CV4, but not for the other isolates. A possible explanation for the double band observed in lane 6 is given in the text. Below is a diagram showing the relative positions of the primer and the amplified fragments. The two lanes contain PCR products obtained with different dilutions of starting DNA. Isolate 1, *H. ericae*; isolate 2, *H. ericae* A; isolate 3, *H. ericae* 100; isolate 4, *H. ericae* 101; isolate 5, *H. ericae* CV3; isolate 6, *H. ericae* CV4; isolate 7, *H. ericae* CV5. M, lambda DNA digested with *Eco*RI and *Hind*III; M’, pUC18 digested with *Hae*III.

Because the sequences were different in size and contained very variable regions, two different alignments were carried out. A first alignment considered both variable and conserved regions, and manual modifications of the CLUSTAL X alignment involved only the exclusion of the most peripheral sequence regions and the realignment of the S region in some isolates (intron sequences H.eri100 943; *H. ericae* CV5 943; DVIII 943; PSIV 943, and P.lign 943) due to the presence of long intervening sequences between the R and S regions (alignment accession number DS39188). Even though the alignment of the variable regions is uncertain for the most distant comparisons, we used this alignment to generate the unrooted tree shown in figure 7 because this gives a clear picture of the overall similarity of the sequences. When the analysis was confined to the most conserved regions (alignment accession number DS39188), the tree had a similar topology except that the introns at site 789 were nested within the clade of introns at site 1506 (see fig. 8). This indicates that introns at site 789 are related to those at site 1506 in the more conserved regions, but their more variable regions identify them as a separate, well-supported group. Intron at site 789 seem to be quite rare in fungi. In ericoid fungi, they were found exclusively in *O. maius* isolates and in the sterile mycelium Duclos IX. The lichen-forming species *Lecanora dispersa* and *Myriosclerotinia scirpicola* (table 2) were the only other fungi (both Ascomycetes) in which introns at this position were reported. However, the intron from *L. dispersa* could not be aligned with the ericoid introns.

In the alignment corresponding to figure 8, we also included the conserved regions of nuclear SSU rDNA introns found in some green algae and occurring at the same insertion sites as the ericoid fungal introns. The positions of these introns on the resulting unrooted tree indicate that they cluster according to insertion site rather than according to the different phylogenetic position of their host (fig. 8).

Some insertions identified in ericoid isolates are not typical group I introns because they lack the short conserved regions (alignment accession number DS39188), the tree had a similar topology except that the introns at site 789 were nested within the clade of introns at site 1506 (see fig. 8). This indicates that introns at site 789 are related to those at site 1506 in the more conserved regions, but their more variable regions identify them as a separate, well-supported group. Intron at site 789 seem to be quite rare in fungi. In ericoid fungi, they were found exclusively in *O. maius* isolates and in the sterile mycelium Duclos IX. The lichen-forming species *Lecanora dispersa* and *Myriosclerotinia scirpicola* (table 2) were the only other fungi (both Ascomycetes) in which introns at this position were reported. However, the intron from *L. dispersa* could not be aligned with the ericoid introns.

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Introns in the rDNA of Ericoid Fungi

Fig. 7.—Unrooted neighbor-joining tree obtained from the alignment of conserved and variable regions of fungal group I introns inserted in the nuclear SSU rDNA at sites 789, 943, and 1506. Separate branches that are well supported by bootstrap values contain introns found at the different sites. The alignment is available from the EBI server (alignment accession number DS39187). Percentage bootstrap support (out of 1,000 trials) is shown when it exceeds 70%. The abbreviation for each fungal species or isolate is listed in table 2.

Fig. 7.—Unrooted neighbor-joining tree obtained from the alignment of conserved and variable regions of fungal group I introns inserted in the nuclear SSU rDNA at sites 789, 943, and 1506. Separate branches that are well supported by bootstrap values contain introns found at the different sites. The alignment is available from the EBI server (alignment accession number DS39187). Percentage bootstrap support (out of 1,000 trials) is shown when it exceeds 70%. The abbreviation for each fungal species or isolate is listed in table 2.

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...and this alignment was used to generate the unrooted tree shown in figure 10. Introns at position 516 formed a well-supported group, those in position 989 formed a poorly supported cluster (53%), and those at positions 287 and 1199 formed a single mixed group. When the alignment considered only the most conserved regions, indicated in boxes in figure 9, the corresponding unrooted tree showed that the cluster of introns at position 516 was still highly supported, whereas all the others formed a mixed group (data not shown).
In this case, the coding sequence was located on the antisense strand compared with that encoding the rRNA. For both ericoid introns, no homologies were found with any other proteins. The sequence of other long rDNA introns (e.g., H. ericae, Pseudohalonectria lignicola) was also used as query in BlastX searches, but no significant homology with any protein could be found.

Discussion

Introns Are a Common Feature of the Nuclear SSU rDNA of Most Ericoid Isolates

Our analysis of the nuclear rDNA of a wide range of ericoid mycorrhizal fungi, initiated to investigate their genetic diversity, has revealed that the SSU rDNA varies significantly in size. Most isolates gave DNA fragments larger than expected for eukaryotes when tested in PCR experiments with universal rDNA primers. Sequence analysis revealed that this increase was caused by additional DNA insertions that occurred at five possible locations on the nuclear SSU rDNA and often corresponded to subgroup IC1 group I introns (Michel and Westhof 1990), featuring typical P, Q, R, and S regions.

Sizes of ericoid fungal introns are very variable and correspond to those most commonly observed for other fungal introns (about 200–500 bp). In some groups, however, they tend to be quite large. Five fungal groups contained insertions longer than 800 bp, with a maximum length of about 1,800 bp in sterile mycelium PSIV, which is to our knowledge the largest group I intron identified so far in the nuclear SSU rDNA of eukaryotes. Concerning the sites of insertion identified in the SSU rDNA of ericoid fungi, some, such as sites 943 and 1506, are conserved and very commonly found in lower eukaryotes whereas others, like sites 789 and 989, are rare and have been identified only sporadically.

Intragenomic Variability in the Presence of Group I Introns

In filamentous fungi, nuclear rDNA genes are present in tandem repeats ranging from about 60 copies in Coprinus (Cassidy et al. 1984) to 220 in Neurospora crassa (Russell et al. 1984). The homing ability of group I introns allows them to insert at specific sites of intronless rDNA genes and spread over gene repeats (Cech 1990; Lambowitz and Belfort 1993; Belfort and Roberts 1997). However, data on Ascomycetes (DePriest 1993) and Basidiomycetes (Hibbett 1996) indicate that some constraints on intron transposition may exist, since introns are not necessarily found on all rDNA repeats within the genome. The same observation was made in this study for several ericoid fungal isolates, for which the relative number of intronless and intron-containing rDNA repeats seems to be quite variable. For many sterile isolates, intronless rDNA repeats probably made up a minor percentage in the genome, as they could be amplified and generate a DNA fragment only with primers that excluded intron-containing copies by annealing directly to the intron insertion site. In other cases, two bands of equal intensity were amplified, suggesting a
Introns in the rDNA of Ericoid Fungi

**Fig. 9.**—Aligned sequences of fungal insertions lacking the typical P, Q, R, and S regions. Because the introns are of very different sizes, the peripheral sequence regions were not considered in the alignment. Four conserved regions were identified during the alignment and are indicated in boxes. A consensus sequence is indicated above the alignment. Nucleotides in uppercase letters are 90% conserved, nucleotides in lowercase letters are 70%-90% conserved, and an “n” indicates that no nucleotides are conserved. A pair of lowercase letters indicates that the two nucleotides frequently occupy a position and together account for 90% of the sequences. This alignment was used to originate the unrooted tree shown in Figure 10.

It remains an open question whether the heterogeneity observed in the rDNA repeats occurs within a single haploid nucleus, or whether rDNA copies respectively lacking and containing introns are separated into different nuclei of a heterokaryotic mycelium. Heterogeneity in ribosomal gene sequences has been shown within a single coenocytic spore of arbuscular mycorrhizal fungi (Lloyd-MacGilp et al. 1996; Lanfranco, Delpero, and Bonfante 1996; Trouvelot et al. 1999). In the case of *Scutellospora castanea*, in situ hybridization has revealed that these different sequences are found within the same nucleus (Trouvelot et al. 1999).

### Mobility of Group I Introns

Horizontal transmission of group I introns among taxa has been discussed since their discovery on account of their scattered distribution and presence/absence in related taxa. In fungi, this hypothesis has been supported by phylogenetic analysis of the Homobasidiomycetes (Hibbett 1996) and the archaeascomycetous *Protomycys* (Nishida, Tajiri, and Sugiyama 1998). Its investigation in ericoid fungi, however, will first require determination of the phylogenetic relationship of the sterile ericoid mycelia with each other and with *H. ericae* and the genus *Oidiodendron*.

Sequence analysis of the fungal introns indicates that insertions sharing the same site on the SSU rDNA gene form distinct lineages and are likely related. This hypothesis was already suggested by Hibbett (1996) and Bhattacharya, Friedl, and Damberger (1996) and is now supported in fungi by the analysis of a much larger number of intron sequences. In particular, introns in position 943 (relative to *E. coli*) are clearly distinct from those at site 1506. These two sites are certainly very ancient, as they are found in several protists (see Gargas, De-Priest, and Taylor 1995). Site 789 is an uncommon and probably more recent insertion site, and introns in this position also form a well-supported group.

It remains an open question whether the clusters gathering together insertions found at the same position in different taxa result from their occurrence in a common progenitor, with a loss in all the intermediate lineages, or from horizontal transfer across different phyla.
Fig. 10.—Unrooted tree based on the alignment shown in figure 9. The introns found at site 516 form a well-supported branch, those at site 989 form a group with relatively low bootstrap support, and those at sites 1199 and 287 form a mixed group. Percentage bootstrap support (out of 1,000 trials) is shown when it exceeds 50%. The abbreviation for each fungal species or isolate is listed in table 2.

Although the cost of intron gain and intron loss is not known, the wide phylogenetic distance between some of the organisms in the tree (both Basidiomycetes and Ascomycetes) would suggest the second hypothesis as the most parsimonious. If this is the case, the mechanisms that allow introns to home in a predefined position must be very efficient, since no exceptions were found for the introns at position 943, for example. Lateral movement along the rDNA gene may nevertheless be possible in some cases and has been suggested by several authors (e.g., Bhattacharya, Friedl, and Damberger 1996; Hibbett 1996). If introns in position 789 are a more recent acquisition, their closer similarities with introns at position 1506 suggest that they may have originated from the transposition of introns originally in position 1506. The close relatedness between the intron at position 1199 of the ericoid isolate II and that found at site 287 in the yeastlike endosymbiont of an anobiid beetle, Stegobium paniceum, also suggests intron transposition. Close similarity between introns at positions 287 and 1199 was also found by Bhattacharya, Friedl, and Damberger (1996) in the lichen-forming fungus Lecanora dispersa, although these sequences could not be aligned with those of ericoid introns.

Phylogenetic analysis has also indicated that horizontal transmission of group I introns may have occurred between organelles (Turmel et al. 1995) and also between fungi and plants during the intimate contacts established during pathogenic or symbiotic interactions (Nishida and Sugiyama 1995; Adams, Clements, and Vaughn 1998). In ericoid symbiosis, the fungal and plant cytoplasms are separated only by a thin interface (Perotto et al. 1995), so a possibility exists that some introns may thus have been transmitted from the fungus to the host, although this aspect has not been investigated.

Isolates of the Same Species Contain Optional Introns

Screening of ericoid isolates also raised the question of how these introns are inherited/transmitted at a lower taxonomic rank. This aspect could be investigated in O. maius and H. ericae, for which the taxonomic

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**Fig. 11.**—Organization of two introns found in ericoid sterile isolates PSIV and II. These introns are inserted, respectively, at positions 943 and 1199 on the SSU rDNA. A. Diagram showing the relative positions of the conserved intron sequences and of the nucleotide region corresponding to the His-Cys box. Arrows indicate the expected direction of transcription. B, the His-Cys box motif of sterile ericoid isolates PSIV and II aligned to the corresponding motifs present in nuclear rDNA introns of Nectria galligena, Physarum polycephalum, and Naegleria jamiesoni (Johansen and Haugen 1999). Only the most conserved amino acids are indicated in bold, although other residues are also conserved in most sequences.
identification of isolates is certain. Heterogeneity among isolates of the same species, expressed by the presence/absence of specific introns, was found in both species. For *H. ericae*, our results are supported by recent findings on genetically related isolates from ectomycorrhizal roots, showing the variable presence of the intron at position 1506 (T. Vrålstad, personal communication). Heterogeneity has often been reported in eukaryotes (see Johansen, Muscarella, and Vogt 1996). In fungi, it has been described in natural populations of the lichen complex *Cladonia merochlorophaeae* (DePriest and Been 1992; DePriest 1993), in the ectomycorrhizal Ascomycetes *Cenococcum geophilum* (Shinohara, Lobuglio, and Rogers 1996), and in *Sclerotinia sclerotiorum* isolates (Carbone, Anderson, and Kohn 1995), in which an optional intron was found in the mitochondrial SSU rDNA.

**Mechanisms of Intron Mobility**

Mobile group I introns generally encode a site-specific endonuclease that cleaves near the site of intron insertion (see Lambowitz and Belfort 1993). Genes coding for endonucleases have been reported for several introns occurring in the cell organelles, where they share either the LAGLI-DAGLI or the GIY-YIG motifs (Johansen, Embley, and Willassen 1993; Lambowitz and Belfort 1993; Belfort and Roberts 1997). In contrast, very few intron-encoded endonucleases are known for nuclear rDNA. They were first described in extrachromosomal rDNA of the slime molds *Physarum polycephalum* and *Didymium iridis* and the amoeboid flagellate *Naegleria* (Einvik, Elde, and Johansen 1998). In fungi, intron-encoded endonucleases have recently been reported in the nuclear SSU rDNA of *Nectria galligena* (Johansen and Haugen 1999). In this species, a polymorphic insertion element either containing or lacking an endonuclease-encoding open reading frame (ORF) was observed in different isolates (Crockard et al. 1998). All endonucleases encoded by nuclear rDNA introns seem to be members of a distinct family defined by a conserved 30-amino-acid segment including a His-Cys motif (Johansen, Embley, and Willassen 1993).

Two of the longest introns identified in the nuclear SSU rDNA of ericoid fungi may also code for endonucleases, as one of the possible reading frames revealed strong homologies with the conserved 30-amino-acid region of *P. polycephalum* and *Naegleria*. Similar to *N. galligena*, the region encoding for the His-Cys box in the 1199 intron of ericoid isolate I1 was found on the antisense rDNA strand, thus indicating that a separate transcription event would be needed to generate an mRNA for the endonuclease.

Alternative mechanisms based on the reversal of the splicing reaction at the RNA level have been proposed to explain mobility of introns in the absence of endonuclease-coding sequences (Lambowitz and Belfort 1993; Roman and Woodson 1998). Since analysis of most ericoid—and, in general, fungal—introns excludes the presence of possible coding sequences, mechanisms based on reverse splicing and loss may explain the scattered distribution of nuclear rDNA introns. However, it cannot be excluded that endonuclease-coding sequences were once present in a larger number of fungal species but were lost over time. It is well documented that ORFs coding for endonucleases can be mobile themselves (see Lambowitz and Belfort 1993).

**Some Insertion Elements Lack the Consensus Sequences of Typical Group I Introns**

Several fungal intron sequences deposited in gene banks do not contain the consensus sequences of group I introns. Sequence studies of lichen-forming fungi led to the suggestion that some of the smaller DNA insertions lacking the *P*, *Q*, *R*, and *S* regions may be remnants of larger group I introns that went through incorrect splicing (Grube, Gargas, and DePriest 1996; Stenroos and DePriest 1998). Ductus VI and Sterile mycelium I2, which feature the smallest insertions (185 bp) in position 1199, are closely related by ITS sequencing to sterile mycelium I1 (unpublished data), which displays a large intron (1,330 bp) in the same position. However, alignment to this larger insertion did not support the hypothesis of incorrect splicing of a previously larger intron related to the I1 intron.

The large sizes of some of these introns (up to 1,330 bp), their occurrence at specific sites on the SSU rDNA distinct from those colonized by the subgroup IC1 group I introns, and the presence of four well-conserved sequence regions indicate that these less abundant nuclear ribosomal introns are not relics but represent a distinct population of insertional elements in fungi. These conserved regions had not been previously identified, although they seem to be involved in the formation of a secondary structure similar to other group I introns, as recently suggested by Johansen and Haugen (1999) for the *N. galligena* intron at position 1199. Analysis of the mature rRNA of ericoid isolates shows that all introns are correctly spliced during rRNA processing, indicating that proper folding is obtained for members of this intron subgroup.

In conclusion, we have identified a high level of polymorphism in the nuclear SSU rDNA genes due to insertion, at distinct positions, of a heterogeneous population of introns that are different in size and sequence. Two distinct subgroups of introns colonize the rDNA of ericoid fungi, one showing the conserved regions of subgroup IC1 group I introns and the other featuring conserved (but distinct) nucleotide sequences. Comparisons with other fungal sequences have allowed us to strengthen the hypothesis that, if transferred horizontally across taxa as suggested by phylogenetic evidence (Hibbett 1996), group I introns usually home very specifically into the same insertion site. Homing may be less specific, or transposition events may occur more frequently, for the second group of introns, as we found high similarity for insertions in at least three different sites. The finding of sequences related to endonuclease genes in some of the ericoid introns may provide an additional clue to lead to an understanding of the mechanisms of intron mobility in nuclear introns.

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of the ribosomal internal transcribed spacers within and among isolates of *Glomus mosseae* and related mycorrhizal fungi. New Phytol. 133:103–111.


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