The nuclear rDNA intergenic spacer of the ectomycorrhizal basidiomycete Laccaria bicolor: structural analysis and allelic polymorphism

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The nuclear rDNA intergenic spacer (IGS) of the ectomycorrhizal basidiomycete Laccaria bicolor was amplified and sequenced to identify the source of its intraspecific polymorphism. The IGS was amplified by PCR in several L. bicolor strains and shown to exhibit multiple bands and length polymorphism. The IGS loci were shown to segregate in a 1:1 ratio within haploid progenies in three dikaryotic strains, suggesting that divergent IGS haplotypes were present in the two nuclei of these strains. The two haplotypes of L. bicolor 5238N were sequenced: the β-haplotype was 4160 bp in length, whereas the size of the α-haplotype was estimated to be about 4700 bp. These represent the largest published fungal IGS sequences to date. These sequences can be subdivided into three main regions, IGSY, 5S rDNA and IGSZ. The IGS sequences are AT-rich and contain numerous occurrences of three types of subrepeats (e.g. T,AC). The length polymorphism, observed between the IGS sequence of the α- and β-haplotypes, results from the insertion of various numbers of a 71 bp subrepeat, called B, occurring in IGSZ. This variation in subrepeat number suggests that the two haplotypes resulted from unequal cross-overs. The L. bicolor IGS was aligned with IGS sequences of two other Tricholomataceae (i.e. Tricholoma matsutake and Callya fusipes). No sequence similarity was observed between these IGSs, but homologous subrepeats were found in L. bicolor and T. matsutake. Analysis of IGS length polymorphism is therefore an efficient tool for investigating genetic relationships between genets and within progenies in natural fungal populations.

**Keywords:** Laccaria bicolor, basidiomycete, intergenic spacers, rDNA, repeated DNA

**INTRODUCTION**

Nuclear rDNA provides useful inter- and intraspecific polymorphisms to type higher fungi. There are multiple copies of 18S, 5·8S, 25S and 5S rRNA genes, which are arranged as head-to-tail repeats separated by non-coding spacers. They are located at one or several loci and have identical sequences due to concerted evolution (Garber et al., 1988). The coding sequences of rRNA are highly conserved between species of ectomycorrhizal fungi, but the internal transcribed spacers (ITS) between the 18S, 5·8S and 25S RNA sequences, which are removed during processing, and also the intergenic spacers (IGS), residing between each transcription unit, exhibit a high inter- and intraspecific variability (Henrion et al., 1992; Albee et al., 1996; Selosse et al., 1996; Gryta et al., 1997; Martin et al., 1998). The IGS region, which includes the 5S rRNA gene in most fungal species, is worthy of particular attention as it contains a variety of transcriptional regulatory elements and tandem arrays of subrepeating sequence motifs (Baldridge et al., 1992). IGS sequences often contain sufficient variation to allow examination of genetic relationships between fungal populations and individuals (Anderson et al., 1990; Albee et al., 1996; Gryta et al., 1997; Selosse et al., 1998; Guidot et al., 1999). The IGS varies greatly in length among different fungal species, ranging from

**Abbreviation:** IGS, intergenic spacer.

The NCBI accession number for the sequence reported in this paper is AF116534.
about 1 to over 4 kb (Garber et al., 1988). Such length heterogeneity is likely to be a result of the presence of many tandem or dispersed subrepeat domains. However, the long length of fungal IGS sequences may explain the paucity of sequence data available in databases to date. The entire sequence of this region is known only for a few fungal rDNAs, e.g. heterogeneity is likely to be a result of the presence of *Laccaria bicolor* databases to date. The entire sequence of this region is for *Crassa neofoymans* communication) and partial sequences are also available for *Tricholoma matsutake* (Hwang & Kim, 1995, 1998) and *Hebeloma cylindrosporum* (Guidot et al., 1999).

*Laccaria bicolor* ([Maire] Orton) is an ectomycorrhizal basidiomycete (Tricholomataceae) having a haplodikaryotic life cycle. The dikaryotic mycelium forms an ectomycorrhizal mutualistic association on tree roots. This symbiosis plays a fundamental role in the biology and ecology of forest trees, affecting growth, water and nutrient absorption, and providing protection from root diseases (Smith & Read, 1997). Questions about the temporal and spatial distributions of populations of this ectomycorrhizal fungus, together with the origin and maintenance of its genetic variation, are therefore critical to understand how populations evolve and disappear at different stages of development of forest ecosystems (Martin et al., 1998; Selosse et al., 1998). Each genotype arises in a unique mating event and then vegetatively expands as a host root-connected mycelium throughout the humus layer of forest soils. Molecular markers have been designed to identify *L. bicolor* and allow the study of its populations in nurseries and plantations (Selosse et al., 1998). Since several different strains are currently used for nursery inoculation of Douglas fir (Villeneuve et al., 1991; Le Tacon et al., 1992), this species provides an ideal model for strain tracking and analysis of population effects of outplanting of inoculated trees.

PCR/RFLP analysis of IGS in *L. bicolor* revealed variation indicative of sequence polymorphisms among isolates in this species (Henrion et al., 1992; Selosse et al., 1996). IGS polymorphisms allowed us to discriminate most genets present in natural populations (Selosse et al., 1998) and haplotypes within progenies (Selosse et al., 1998). Each polymorphic sequence is assumed to be due to the presence of unique IGS sequences. IGS polymorphism became useful for monitoring population structure (Selosse et al., 1998). The previously published IGS sequence of *L. bicolor* S238N (GenBank accession no. L25898) was assembled to the IGS sequence using Sequencher. Searches for sequence homologues in the non-redundant DNA database (March 1999) of the National Center for Biotechnology Information (NCBI) was carried out using BLAST 2.0 (Altschul et al., 1997). Multiple sequence alignments were performed with the Multalin

### METHODS

#### Strains, growth and breeding

A detailed description of the origin of the American strain S238N of *Laccaria bicolor* ([Maire] Orton) (Hymenomycetes, Agaricales, Tricholomataceae) used in this analysis is provided by Di Battista et al. (1996). The two other *L. bicolor* strains used are from the Collection of Ectomycorrhizal Fungi (INRA, Nancy, France);

DNA extraction, PCR amplification and enzymic digestion. Total DNA was extracted by the hexadecyltrimethylammonium bromide (CTAB)/proteinase K method as described by Henrion et al. (1994). The IGS sequence, containing the 3' end of the 25S rDNA and the 25S/5S spacer, was amplified as described by Selosse et al. (1996) using primers CNL12/SSA. The IGS sequence, containing the 5S rDNA plus the 5S/18S spacer, was amplified with the primers revSSA (5'-ATCCACGGCCATAGGACTCTG-3') and revNS1 (5'-GAGACAAGCATATGACTAC-3') with priming sites at the 5' end of the 5S rDNA and at the 5' end of the 18S rDNA, respectively. Primers 11 (5'-TCCAGGTGTACCATATATGCC-3') and 12 (5'-GGTTTGTCCGGAGATATGG-3') were used to amplify the central region VII of IGS. Primers were supplied by Bioprobe Systems and Taq DNA polymerase by Appligene-Oncor. Reactions were performed in a Perkin-Elmer GeneAmp 9600 thermocycler under the following conditions: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. Digestion of PCR products was carried out as described by Henrion et al. (1994).

#### Sequencing

Sequencing of IGS was carried out on two monokaryons from *L. bicolor* S238N, namely HC3 and HC4, carrying different rDNA haplotypes (respectively the x- and y-haplotypes; Selosse et al., 1996; Fig. 1a). PCR products were purified using a Qiaguid Spin PCR Purification Kit (Qiagen). The sequencing reactions were performed using the PRISM Ready Reaction Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems). Sequencing reactions were initially primed using the revSSA or revNS1 primers and then 12 internal primers were designed for primer walking along the y-type IGS. Sequencing redundancy was between 17 and 3. The sequencing reaction products were analysed using an ABI model 373S DNA sequencer (Perkin-Elmer Applied Biosystems). Raw sequence data were edited and contigs were assembled using the AutoAssembler (Perkin-Elmer Applied Biosystems) and Sequencer (Gene Codes C) programs. Regions V, VI and VII of IGS were also sequenced from the x-type IGS of *L. bicolor* S238N. The previously published IGS sequence of *L. bicolor* S238N (GenBank accession no. L25898) was assembled to the IGS sequence using Sequencher. Searches for sequence homologues in the non-redundant DNA database (March 1999) of the National Center for Biotechnology Information (NCBI) was carried out using BLAST 2.0 (Altschul et al., 1997). Multiple sequence alignments were performed with the Multalin
program (Corpet, 1988) on the IBCP server (Institut de Biologie et Chimie des Protéines, Lyon, France; http://pbil.ibcp.fr/NPSA/npsa-multalin.html).

RESULTS

Heterozygosity of L. bicolor rDNA revealed by amplification of IGS

The 5S rDNA and the intergenic spacer between the 5S and 18S rDNAs (so-called IGS) were amplified by PCR in three dikaryotic strains of L. bicolor, S238N, 81306 and N203. Two (strains S238N and N203) or three (strain 81306) PCR products were observed after electrophoretic separation (e.g. strain S238N in Fig. 1a, lane 5), suggesting that IGS length heterogeneity existed within individuals. To study further the origin of this length heterogeneity and the inheritance of the amplified fragments, monokaryons germinated from spores of these three L. bicolor strains were analysed (Table 1). For S238N (Fig. 1a, lane 5), 47 monokaryons showed a pattern, referred to as the α-haplotype, with only the 3.9 kb fragment (Fig. 1a, lane 3) and 39 monokaryons exhibited a single 3-4 kb fragment, the β-haplotype (Fig. 1a, lane 4). Five monokaryons produced the α + β parental pattern of strain S238N (Table 1). This latter pattern probably resulted from meiotic recombination within the rDNA repeats (Selosse et al., 1996). For strain N203, which exhibited two fragments after PCR amplification, two haplotypes were also recovered in the haploid progeny, each allowing the amplification of a single fragment (Table 1). In strain 81306, a small 0.5 kb fragment was amplified (data not shown) in addition to two 3-5 and 3-3 kb fragments, as already observed in some Laccaria proxima strains (Albee et al., 1996). Monokaryons from this strain showed either the 3.5 kb fragment or the 3-3 and the 0-5 kb fragments (Table 1). These data confirmed and extended our previous results (Selosse et al., 1996), suggesting a meiotic segregation of two IGS haplotypes, α and β, with the fragments amplified in dikaryons corresponding to two allelic IGS haplotypes.

Features of the L. bicolor IGS

The sequencing data obtained on the β-type IGS of L. bicolor S238N correlated very well with (i) the overall length of IGS spacer and its RFLP pattern (Fig. 1a, lane 2), as determined by agarose gel electrophoresis. Previous PCR amplification of this IGS (Selosse et al., 1996) suggested a longer length, but this was probably due to size overestimation on acrylamide gels of amplified DNA fragments following separation in acrylamide gels (Stellwagen, 1983). The IGS sequence was assembled to the previously sequenced IGS (GenBank accession no. L25898). The total IGS was 4160 bp in length. The junctions between the spacers and coding regions were inferred from sequences reported for other fungi. The sequence includes a small portion of the flanking 25S and 18S coding regions, IGS (Selosse et al., 1996), 5S rDNA and IGS (present study), and its G+C content is 41.8 mol%. The general organization of the L. bicolor IGS (Fig. 2) is typical of most fungal, plant and animal ribosomal intergenic spacers (Cordesse et al., 1993). It consists of several repeated sequences and unique regions. Nine different regions with respect to sequence repetition and rRNA gene identity (I–IX; Fig. 2) can be distinguished in the sequence. Region I contains 288 bp of the 3’ end of 25S rDNA (46.2% G+C). Region II corresponds to 505 bp of IGS (37% G+C) and contains a series of subrepeats, T₂A₂, called A. Region III (117 bp in length, 60.7% G+C) corresponds to the 5S rRNA coding sequence. IGS covers regions IV–VIII (3215 bp, 41% G+C) and consists of two central repeated regions (V and VII) and unique regions flanking these repeated sequences. The central repetitive regions extend from nt 1450 to 1592 (subrepeats A, region V) and from nt 1718 to 2602 (subrepeats B, region VII) (Fig. 2). Region IX contains 35 bp of 18S rRNA sequence. The length of region VII

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**Fig. 1.** Agarose gels showing the uncut and digested rDNA IGS (a) or amplified region VII of IGS (b) from L. bicolor strain S238N. (a) Lanes: 1, amplified IGS of monokaryon HC3 (α-type) digested by RsaI; 2, amplified IGS of monokaryon HC4 (β-type) digested by RsaI; 3, uncut amplified IGS of monokaryon HC3 (α-type); 4, uncut amplified IGS of monokaryon HC4 (β-type); 5, uncut amplified IGS of the parental strain S238N. (b) Region VII, containing the B subrepeats, was amplified using primers II and I2 flanking this central region (see Fig. 2). Amplification and restriction products were separated on 1.5% (w/v) agarose gels. Size markers are shown on the left (φX-174 DNA digested by Haelll, and the 3500 and 4250 bp markers are from λ DNA cut by EcoRI and HindIII).
Table 1. Mendelian segregation of rDNA IGS types (α and β) among monokaryons derived from the Laccaria bicolor dikaryotic strains 5238N, N203 and 81306

<table>
<thead>
<tr>
<th>Strain</th>
<th>Size of IGS, haplotypes (kb)</th>
<th>Segregation in the progeny*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>α</td>
<td>β</td>
</tr>
<tr>
<td>S238N</td>
<td>3.9</td>
<td>3.4</td>
</tr>
<tr>
<td>N203</td>
<td>3.8</td>
<td>3.5</td>
</tr>
<tr>
<td>81306</td>
<td>3.5</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* (NS), x² not significantly different from 1:1 segregation at the 0.05 level.
† Monokaryons recombinant for the rDNA.
‡ An additional 0.5 kb fragment was also amplified from this haplotype (see text).

Fig. 2. Diagrammatic representation of the organization of IGS regions of L. bicolor S238N (β-haplotype) with indication of subrepeats A, B and C. Regions I-IX have been defined based on the occurrence of rDNA coding sequences and subrepeats A, B and C. In region VII, B subrepeats identical in the α- and β-haplotypes are shown as black arrowheads, whereas the grey box containing black arrowheads indicates the B subrepeats of divergent sequence (see Fig. 3a). The diagonally shaded areas indicate regions containing A subrepeats.

for the β-haplotype correlates well with the approximately 960 bp length of the PCR fragment amplified with primers I1 and I2 flanking this region (Fig. 1b).

Fourteen copies of subrepeat A, TA₃C₃, occurred in region V of IGS₂, whereas three were detected in IGS₁ (region II) (Selosse et al., 1996). It may be worth mentioning that this subrepeat was also found in IGS₃ of the ectomycorrhizal Hebeloma cylindrosorum (accession no. A006148; Guidot et al., 1999) and the telomere hexamer array repeat region of the human chromosome (accession no. U32384). The second subrepeat family, named B, is present 100 bp downstream of the subrepeat A region. This subrepeat block consists of 13 copies of the element B which occur in tandem (Fig. 3a). All units of element B are 70–71 bp in length and consist of three degenerated subrepeats of 20, 19 and 32 bp, except B13 which is truncated. The consensus sequences of the B subrepeats are (identities underlined):

- Ba, CGGAATAGACTACAAAGTC; Bb, ATAGATAGACTACAAACGC; and Bc, TATAAAAGACTACAAAGTCACTACAAACAT. The Bc element may be a result of incomplete duplication of a Ba- or Bb-like element, as it contains two ACTACA₃ subrepeats. Alignment of IGS₁ and IGS₂ sequences revealed that the B subrepeat can also be interpreted as a repetition of an inverted sequence occurring in IGS₁ (Fig. 3b), from nt 657 to 725.

A third subrepeat, named C, occurred three times in IGS₂ (Fig. 3c). This 88 bp element (restricted to a 36 bp element in repetition C1) is highly homologous to a repeat sequence of Tricholoma matsutake IGS₂ (the DR1 repeat; Hwang & Kim, 1998): 32 of the 34 bp of the central part of motif C are identical to one of the DR1 repeats (TmDR1a, Fig. 3c). This motif may correspond to a conserved regulatory sequence for the transcription of 25S rRNA.

With the addition of L. bicolor, full-length IGS sequences currently exist for two other members of the Tricholomataceae, C. fusipes (B. Marçais, INRA-Nancy, personal communication) and T. matsutake (Hwang & Kim, 1998). These sequences ranged in size from 2.2 to over 4.7 kb. The sequence variation prevented unambiguous alignment of these IGSs. As described above, sequence conservation exists only in the region TmDR1a.

Comparison of the two IGS haplotypes of L. bicolor S238N

RFLP analyses using various enzymes (Alul, HinfI, MboI, MspI, SauIla) showed that the length differences between the amplified α- and β-type IGS₂ of L. bicolor S238N always mapped to a single fragment (e.g. the 0.9 or 1.4 kb fragment produced after digestion by RsaI; Fig. 1a, lanes 1 and 2). This suggests an almost identical sequence for both haplotypes, with a single insertion responsible for the observed length difference. The variable region, which was not digested by the enzymes tested, corresponds to the central repeat-rich region VII.
as shown by PCR amplification using the internal primers designed for sequencing the \( \beta \)-type. The PCR fragment, amplified using primers I1 and I2 flanking the repeated region VII, exhibited the expected \( \beta \)-type, flanking a series of 71 bp subrepeats (region VII) (Fig. 3a). The subrepeats occurring at the 5' and 3' ends of region VII were identical in the two haplotypes, with subrepeats B1, B2 and B3 at the 5' end, and B8 and B9 at the 3' end. The sequence of the inner repeats in the \( \alpha \)-haplotype (grey box in Fig. 2) differed from the \( \beta \)-haplotype by point mutations. The sequence of the most central repeats for the \( \beta \)-haplotype could not be exactly established since a sequencing number of subrepeat B in region VII of this sequence. The presence of very similar, short (20, 19 and 20 bp) subrepeats are given according to the GenBank IGS, subrepeats from (RevlGSl, accession no. U62538; Hwang et al., 1998) and the corresponding consensus sequence. Nucleotide positions of subrepeats are given according to the GenBank L. bicolor IGS sequence (see also Fig. 2 for localization of subrepeats).

**DISCUSSION**

The utility of the IGS region in population genetics of ectomycorrhizal fungi will depend on a thorough understanding of its inheritance and mechanisms by which this region evolved. Length variation in IGS has been reported in many fungi, including the basidiomycetes Coprinus cinereus (Wu et al., 1983), Laccaria proxima (Albee et al., 1996), Pleurotus cornucopiae (Iraga et al., 1994) and H. cylindrosporum (Gryta et al., 1997), as well as other fungi such as Cochliobolus heterostrophus (Garber et al., 1988) and Fusarium oxysporum (Appel & Gordon, 1996). The differences are attributed to insertions or deletions in the arrays of subrepeats within the IGS region. We found inter- and intraspecific length variations in IGS, (this study) was reported in IGS, of the ectomycorrhizal fungi, as shown by PCR amplification using the internal primers designed for sequencing the \( \beta \)-type. The PCR fragment, amplified using primers I1 and I2 flanking the repeated region VII, exhibited the expected 0.5 kb length difference between the two haplotypes (Fig. 1b). This inner fragment of the \( \alpha \)-type was sequenced and was found to have non-repetitive regions identical to regions VI and VIII of the \( \beta \)-type, flanking a series of 71 bp B subrepeats (region VII) (Fig. 3a). The subrepeats occurring at the 5' and 3' ends of region VII were identical in the two haplotypes, with subrepeats B1, B2 and B3 at the 5' end, and B8 and B9 at the 3' end. The sequence of the inner repeats in the \( \alpha \)-haplotype (grey box in Fig. 2) differed from the \( \beta \)-haplotype by point mutations. The sequence of the most central repeats for the \( \beta \)-haplotype could not be exactly established since a sequencing number of subrepeat B in region VII of this sequence. The presence of very similar, short (20, 19 and 32 bp, respectively) internally repeated sequences within the
71 bp B subrepeat suggests that this subrepeat evolved by amplification of segments of a shorter ancestral subrepeat, then subsequent amplification of the resulting larger units. The amplification of the segments corresponding to the subrepeats may have resulted from recombination, since crossing-over occurs during meiosis (Selosse et al., 1996), or between sister chromatids during the vegetative life cycle (Ganley & Scott, 1998) of L. bicolor. Polymerase slippage during replication (Tautz et al., 1986) may have contributed to their formation. The length differences in region VII of the two haplotypes of L. bicolor S238N are evidence of unequal crossing-over events, probably due to misalignment of the repeats. The concerted evolution of the repeats probably contributed to the homogenization of the rDNA repeats within each nucleus, leading to fixation of the newly formed IGS [see Ganley & Scott (1998) for a discussion].

The repeated regions found in IGS are probably involved in the regulation of the transcription of rRNA, as described for other eukaryotic IGS (Baldrige et al., 1991). Subrepeat C, which strikingly resembles a sequence from IGS2 of the agaric species T. matsutake (Fig. 3c), may be involved in the transcription of 25S rRNA, although it is lacking in IGS2 of another Tricholomataceae, C. fusipes (B. Marçais, INRA-Nancy, personal communication). This suggests that functional domains within IGSs may be variable even in closely related species. The lack of homology with other sequences from DNA databases is due to the high IGS divergence among higher fungi, but also to the paucity of IGS2 sequence data for homobasidiomycetes.

This study was undertaken to explain the length variations between IGSs found in genets of L. bicolor (Selosse et al., 1998). The insertions and deletions of subrepeats A and B in IGS (Figs 1b and 3a) explain the high level of polymorphisms of this sequence in natural populations (Selosse et al., 1998). Variation of the subrepeat number, as well as the frequent formation of heteroduplexes during amplification of IGS1 (Selosse et al., 1996), allowed us to use IGS as a mendelian marker for population genetics (Selosse et al., 1998). Together with SCARs (Bonello et al., 1998), IGS amplification will allow us to study fungal populations using DNA extracted from mycorrhizas. Variation in this sequence is mainly located in the central repeated region and specific primers, such as I1 and I2, could therefore be designed for amplifying the different alleles found in local populations (Guidot et al., 1999). IGS therefore appears to be an excellent target sequence for studying intraspecific genetic relationships in ectomycorrhizal basidiomycetes (Selosse et al., 1998; Gryta et al., 1997).

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