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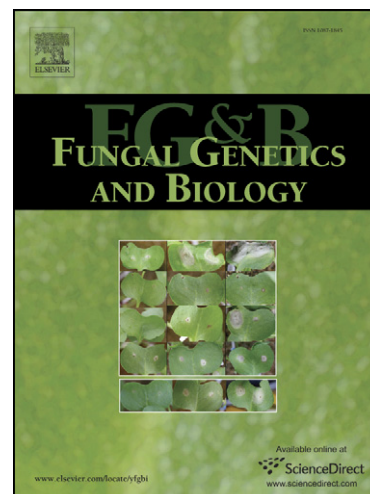
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**Molecular evolution of mitochondrial ribosomal DNA in the  
fungal genus *Tricholoma*: Barcoding implications**

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**Abstract**

The molecular evolution of the V6 and V9 domains of the mitochondrial SSU-rDNA was investigated to evaluate the use of these sequences for DNA barcodes in the Basidiomycota division. The PCR products from 27 isolates belonging to 11 *Tricholoma* species were sequenced. Both domains in the isolates belonging to the same species had identical sequences. All the species possess distinctive V9 sequences due to point mutations and insertion/deletion events. Secondary structures revealed that the insertion-deletion events occurred in regions not directly involved in the maintenance of the standard SSU-rRNA structure. The inserted sequences possess conserved motifs that enable their alignment among phylogenetically distant species. Hence, the V9 domain by displaying identical sequences within species, an adequate divergence level, easy amplification, and alignment represents an alternative molecular marker for the Basidiomycota division and opens the way for this sequence to be used as specific molecular markers of the fungal kingdom.

*Keywords:* Basidiomycota, *Tricholoma*, Mitochondrial SSU rDNA, DNA barcodes

## 1. Introduction

In fungi, studies on the evolution of mitochondrial genomes and genes are of great interest to understand the evolutionary history of fungal populations through key characteristics such as high copy number, lack of recombination and high substitution rate. Several complete mitochondrial genomes from fungi are available in the GenBank. Most of these sequences concern Ascomycota, either yeast taxa (more than 17 sequences) or filamentous fungi (more than 10 sequences). Although the Basidiomycota division gathers most of the edible and cultivated mushrooms (Chang and Miles, 1991), several highly damaging phytopathogenic fungi (Lievens et al., 1998) and symbiotic fungi (Smith and Read 1997) molecular studies on their mitochondrial genomes are scarce and only five mitochondrial complete genomes: *Schizophyllum commune* (GenBank accession no. **AF402141**), *Cryptococcus neoformans* (GenBank accession no. **NC 004336**), *Moniliophthora perniciosa* (GenBank accession no. **NC 005927**), *Ustilago maydis* (GenBank accession no. **NC 008368**) and *Pleurotus ostreatus* (GenBank accession no. **EF204913**) are available. The Basidiomycota division constitutes crucial and attractive models to study the evolution of the fungal mitochondrial genomes and genes because of the great variation in size of their mitochondrial genomes, from 36 kbp to 176 kbp (Hintz et al., 1985; Bruns et al., 1988) and because of the scarcity of available complete mitochondrial sequences as compared to the Ascomycota division.

Among the mitochondrial genes, the *cox1* gene with encodes a sub-unit of complex III of the respiratory chain, is universally carried by the eukaryotic mitochondrial genomes and encodes a highly conserved protein. As the result, this gene has been largely used for studying phylogenetics relationship of organisms belonging to the Plant (Bowe et al., 1999) or Animal Kingdoms (Garcia-Varela et al., 2006). In the later, the small region (600-700 pb) of the *cox1*

gene has been demonstrated to be a highly efficient tool for identification of numerous species (Hebert et al., 2004; Hajibabaei et al., 2006) because it is short enough to be quickly and easily sequenced and displays significant level genetic variability and divergence between species.

Moreover, in animal Kingdom, the *coxI* gene does not contain any introns contrasting with the abundance of group I introns reported in this gene in the basidiomycetous fungi. (Table 1). Six species among the eight available in Genbank database have at least two introns. Unfortunately, the amplification of the expected small portion of the *coxI* gene by PCR using universal primers could generate PCR products with a large variation in size resulting from the presence of numerous introns, thus preventing its use as an appropriate genetic locus for the DNA barcodes in the Basidiomycota division.

In this context, we searched others regions of the mitochondrial genome possessing significant interspecific variability and usable for taxonomic identification of the basidiomycetous fungi.

Beside the *coxI* gene, the small sub-unit rDNA gene (SSU-rDNA) constitutes interesting molecules by his important evolutionary dimension and structural complexity (Okamoto et al. 1996). This gene consists of regions with varying degrees of sequence divergence, divided into the highly conserved core interrupted by nine domains with large length variations (Neefs et al., 1993). Because of these features, SSU rDNA has been used extensively in systematic and phylogenetic studies of Basidiomycota (Hibbett et al., 1997; Ko and Jung, 1999; Binder and Hibbett; 2002; Uhart et al., 2007). Three of the nine variable domains (V4, V6 and V9) commonly described from the SSU-rDNA were reported to be species-specific and used as an efficient taxonomic marker in several Basidiomycota genera (Gonzalez and Labarere, 1999; 2000).

In this paper, we focused the work on the two hyper variable regions (V6 and V9) of the mitochondrial SSU-rDNA. We determined the sequences of both regions from 27 isolates of 11 species belonging to the genus *Tricholoma*. Alignment of the resulting sequences and comparison of their secondary structures allowed us to deduce nucleotide sequence variations and the localisation of the insertions/deletions events occurring in each mitochondrial domain. Finally their use as appropriate loci for DNA barcoding is discussed. The *Tricholoma* genus was chosen because it is monophyletic and gathers a hundred indexed species placed in the largest family of the Agaricales (Moncalvo et al., 2000).

## 2. Materials and methods

### 2.1. Isolates, media and culture conditions

The isolates were obtained from basidiocarps collected in France and different European countries (Table 2). All these isolates are preserved in the Laboratory Evolution et Diversité Biologique in a CTAB 2X Buffer (100 mM Tris HCl (pH 8), 1.4 M NaCl, 20 mM Na<sub>2</sub> EDTA, 2% CTAB), or in a dry state. The media and culture conditions of the fungal isolates were as previously described (Mouhamadou et al., 2006).

### 2.2. DNA extraction and PCR

For each isolate, fungal total DNA was extracted using the Wizard genomic DNA purification kit (Promega) according to the manufacturer's recommendation. The PCRs were carried out according to conventional protocols using GoTaq DNA polymerase in 5x colorless

Go Taq reaction Buffer (Promega Corp., Madison, WI, USA) and primers are synthesized by Operon (Cologne, Germany). The couple of primers V6U/V6R (V6U: TTAGTCGGTCTCGGAGCA; V6R: TGACGACAGCCATGCAAC) and V9U/V9R (V9U: CCGTGATGAACTAACCGT; V9R: TTCCAGTACAAGCTACCT) (Gonzalez and Labarere, 1998) were used to amplify the regions containing variable domains V6 and V9, respectively. The PCRs were performed in a Programmable Thermal Cycler PTC 100 (MJ Research, MA). Amplifications were carried out in 50  $\mu$ L reaction mixtures containing 10–30 ng of fungal DNA, 4 mM of both primers, 200 mM of each dNTP, 1U of GoTaq DNA polymerase, 50mM KCl, 10mM Tris-HCl (pH = 8.3), 2mM MgCl<sub>2</sub> and Triton X- 100 0.1% (v/v). Reactions were run for 40 cycles at 95°C for 30 s (denaturation step), 2°C below the T<sub>m</sub> of both primers for 30 s (annealing step) and 72°C for 1 min (elongation step). A final elongation for 10 min at 72°C was included at the end of the 40th cycle. The PCR products were analyzed by electrophoresis in agarose gels (1%, w/v) containing 200 ng/ml of ethidium bromide, in TAE buffer 1x (Tris base 0.4M, acetic acid 1 % (v/v), EDTA 0.5 M pH 8.0).

### 2.3. Sequencing and sequence analysis

PCR products were sequenced on both strands by Genoscreen (Lille, France). Comparisons with sequences of the GenBank and EMBL databases were made with the BLAST search algorithm (Altschul et al., 1990). Alignments of nucleotide sequences were carried out with Clustal W software (Thompson et al., 1994). The secondary structures of the SSU-rRNAs were obtained with the help of the RNA folding (Zuker and Jacobson, 1995) and were checked manually to improve the accuracy with the model for archae, bacteria, plastids, and mitochondria SSU-rRNA (Neefs et al., 1993 and Wuyts et al., 2002). Phylogenetic analyses were carried out with the entire nucleotide sequences of V9 domain.

The tree was constructed without imposing a species as an outgroup and obtained with the neighbor-joining method (Saitou and Nei, 1987), deriving from matrices of distances based on the distance model proposed by Kimura (1983). Tree topology was evaluated by performing bootstrap analysis of 1000 data sets using MEGA 3.1 (Molecular Evolutionary Genetics Analysis).

**GenBank accession nos.** For the V6 domain (this work): *T. cingulatum*, [EU639426](#); *T. scalpturatum* group1, [EU639427](#); *T. inocybeoides*, [EU639428](#); *T. scalpturatum* group2, [EU639429](#); *T. triste*, [EU639430](#); *T. gausapatum*, [EU639431](#); *T. basirubens*, [EU639432](#); *T. fulvum*, [EU639433](#); *T. populinum*, [EU639434](#); *T. imbricatum*, [EU639435](#); *T. atosquamosum*, [EU639436](#). For the V9 domain (this work): *T. atosquamosum*, [EU639437](#); *T. cingulatum*, [EU639438](#); *T. fulvum*, [EU639439](#); *T. populinum*, [EU639440](#); *T. scalpturatum* group1, [EU639441](#); *T. imbricatum*, [EU639442](#); *T. inocybeoides*, [EU639443](#); *T. triste*, [EU639444](#); *T. basirubens*, [EU639445](#); *T. gausapatum*, [EU639446](#); *T. sclapturatum* group2, [EU639447](#). For the genus *Pleurotus*: [AF091924](#), [AF091925](#), [AF091926](#), [AF091927](#), [AF091928](#), [AF091929](#), [AF091930](#), [AF091931](#), [AF091932](#), [AF091933](#), [AF091934](#), [AF091935](#), [AF091936](#), [AF091937](#), [AF091938](#). For the genus *Agrocybe*: [AF080420](#), [AF080421](#), [AF080422](#), [AF080423](#), [AF080424](#), [AF080425](#), [AF080426](#), [AF080427](#), [AF080428](#), [AF080429](#).

### 3. Results

#### 3.1. PCR Amplification and length variation of the V6 and V9 domains

To study the V6 and V9 domains of mitochondrial SSU rDNA in the genus *Tricholoma*, we first analyzed 18 isolates from different geographical origins (Table 2) of two genetic groups of the taxon *T. scalpturatum* (9 for *T. scalpturatum* group 1 and 9 for *T. scalpturatum* group 2) considered as two cryptic species (Gryta et al., 2006, Carriconde et al., 2008). From each genetic group, total DNA were extracted and then subjected to PCR amplification using the couple of primers V6U/V6R and V9U/V9R, previously described (Gonzalez and Labarere, 1998). For each species, PCR products were sequenced in order to

determine precisely the size of both domains. All isolates from the same cryptic species possessed exactly the same domain length: 64 nt or 78 nt for the V6 domain, 194 nt or 197 nt for the V9 domain of *T. scalpturatum* group 1 and group 2, respectively. However, variations in size were observed among the 11 species of the genus *Tricholoma* (Table 3). The V6 domain varies from 64 nt in *T. scalpturatum* group1 and *T. cingulatum* to 120 nt in *T. basirubens* and the V9 domain from 194 nt in *T. scalpturatum* group1 and *T. cingulatum* to 270 nt in *T. gausapatum*. Some species possessed the same length for one domain (Table 3), but only *T. cingulatum* and *T. scalpturatum* group1 shared the same V6 and V9 size (Table 3).

### 3.2. Sequence analysis of the V6 and V9 domains within the genus *Tricholoma*

The comparison of nucleotide sequence of each variable domain showed that all isolates from the same species had identical sequences. In contrast, the alignment of the 11 *Tricholoma* species (Fig. 1) revealed (i) conserved sequences in 5' and 3' part of both variable domains and (ii) the regions affected by large variations in length and sequence leading to the determination of two kinds of event. The point mutations distributed throughout each entire domain sequence which the percentage varies for 0 to 37 % for the V6 domain and 1 to 24 % for the V9 domain (Table 3). Moreover, *T. cingulatum* and *T. scalpturatum* group 1 whose V6 or V9 domains were the same length (64 nt and 194 nt for the V6 and V9 domains, respectively), shared identical V6 sequences, while the V9 domain displayed one point mutation (Fig 1B). In the same way, *T. scalpturatum* group 2 and *T. inocybeoides* possess identical V6 sequences, but differ by their V9 sequences. So despite the identical V6 sequence of some species, all the species studied could be discriminated on the basis of their V9 nucleotide sequences.

In addition to the interspecific variations due to the point mutations, insertion / deletion events were observed in the central part of each variable domain. The size of each variable domain varied up to a factor 2 for the V6 or 1.4 for the V9 domain according to the species. The largest domains were not specific to one single species, for example, *T. basirubens* possessed the largest V6 domain whereas, the largest V9 domain was found in *T. gausapatum*. However the shortest domains always occurred in *T. cingulatum* and *T. scalpturatum* group 1.

To determine precisely the sites where the insertion / deletion events had occurred, the secondary structures of V6 and V9 domains for each species were determined. Using the V6 secondary structure (Fig 3A), despite the sequence variations due to the insertion/deletion events, all the species studied possess the same topology characterized by the presence of P37-1 and P37-2 helices. However, in four species (*T. cingulatum*, *T. inocybeoides*, *T. scalpturatum* group 1 and group 2) the absence of the V6-1 motif in the central part of the V6 domain leads to a shortening of the loop located at the end of P37-1 helix. It appeared that sequence variations in the V6 domain were essentially due to the differences in the number of nucleotides in the loops varying from 5 nt to 45 nt.

To determine the origin of the inserted sequence, the search for homologous sequences with the *T. basirubens* V6-1 motif that constitutes the longest inserted motif was carried out, and failed to reveal any significant similarity with the sequences of the GenBank and EMBL databases. However, this sequence is AT-rich (75% A + T) showing its mitochondrial origin.

For the V9 domain, four groups of secondary structures were observed (Fig. 3B), characterized essentially by the presence or absence of additional helices. In all species, the V9 domain possessed a canonical topology with the P49-3 helix (Neefs et al., 1993). However, one or two additional helices were observed due to the insertion / deletion events. Two groups of species, *T. populinum* and *T. fulvum* (group A) on the one hand and *T.*

*gausapatum*, *T. basirubens*, *T. atosquamosum* and *T. triste* (group B) on the other hand posses an additional P49-1 helix due to the insertion of V9-1 motif. This motif varying from 25 nt to 46 nt was not strictly conserved among these species, but was absent in the V9 of the six other *Tricholoma* studied. Both groups are differentiated by the absence of P49-2 helix and the elongation of P49-3 helix in *T. populinum* and *T. fulvum* V9 domain. The third group: *T. inocybeoides*, *T. scalpturatum* group 1, group 2 and *T. cingulatum* (group C) is characterized only by the additional P49-2 helix due to the presence of V9-2 motif. It is to be noticed that similar V9-2 motif recovered in the *T. populinum* and *T. fulvum* V9 domains, does not form any stable secondary structure, but allows the extension of the P49-3 helix. The fourth group (group D) only constituted by *T. imbricatum*, is characterized by the absence of the P49-1 helix and the elongation of the P49-2 helix. For this comparison, sequence variations of V9 domains were due essentially to the presence of P49-1, P49-2, or both helices and the variation of length of P49-2 or P49-3 helices.

To determine whether the grouping of the species studied based on their V9 secondary structures is in accordance with the phylogenetic relationship classically described for these species, a phylogenetic analysis was carried out with the V9 sequences using the neighbour-joining method. Four groups of species were clearly recovered (Fig. 4) corresponding to the four groups evidenced by the secondary structures. The first group (group C) is constituted by both *T. cingulatum* highly related to *T. scalpturatum* 1 supported by a highly bootstrap value (100) and *T. inocybeoides* related to *T. scalpturatum* 2 (Bootstrap value = 75). The second group (group B) also contained four species: *T. basirubens* highly related to *T. atosquamosum* (Bootstrap value = 100) and *T. triste* related to *T. gausapatum* (Bootstrap value = 98). Both groups are separated from a more distant group A joining *T. populinum* and *T. fulvum* (Bootstrap value = 97). *T. imbricatum* (group D) ranges in an outgroup position on the tree.

This phylogenetic analysis gave additional information about the two cryptic species *T. scalpturatum* group 1 and group 2. Indeed, on the phylogram of figure 4, within the group C, both species, regarded as a single species, are clearly separated in two subgroups (Bootstrap value = 100). Surprisingly, *T. scalpturatum* group 1 is more closely related to *T. cingulatum* than to *T. scalpturatum* group 2. In the same way, *T. scalpturatum* group 2 is more closely related to *T. inocybeoides* than to *T. scalpturatum* group 1.

#### 4. Discussion

Ours results show that the size and sequences of each variable domain (V6 and V9) of several isolates from different geographic origins of *T. scalpturatum* group 1 and group 2 were strictly identical. This conservation of nucleotide sequences is in agreement with the functional role of these sequences. The lack of variation within species suggests that both domains are under the selection pressure, which prevents any mutation events. The conservation of both variable domains has been reported in various genera of polypores (Soon et al, 2002), *Agrocybe* (Gonzalez et al., 1999) and *Pleurotus* (Gonzalez et al., 2000) and this conservation offers the advantage of using a single isolate of each species to study the taxonomy of the *Tricholoma* genus. Therefore the use of mitochondrial V6 and V9 sequences for the identification of species does not require the verification by other sources of data in comparison with nuclear ITS rDNA in which intraspecific sequence variations have been reported (O'Donnell and Cigelnik 1997). As a matter of fact, the ITS sequences of two strains of *T. mustake* available in GenBank and EMBL databases (GenBank Accession nos. **AB286068** and **AB368513**) were compared and we found 3% of nucleotide divergences between both strains. In the same way, 10 nucleotide variations have been described in the ITS1 sequences and four in the ITS2 sequences from two isolates (D383 and D1136) of *Pleurotus*

*cornucopiae* (Vilgalys and Sun, 1994).

In contrast to the highly intraspecific nucleotide conservation, both the V6 and V9 domains revealed interspecific variations due to the point mutations and insertion or deletion events. The secondary structures of each domain were determined and showed that insertions /deletions of polynucleotide occurred in the same section of variable domains. The inserted motifs occurring in certain species led mainly to the elongation of the loop in the V6 domain or to the presence of additional helices in the V9 domain suggesting that they are not directly involved in the interaction with ribosomal proteins and therefore not essential for the mitochondrion.

In order to determine whether the inserted sequences contain some phylogenetic information, these sequences were aligned using the clustalW software. Except in two groups of species (*T. scalpturatum* group 2 and *T. inocybeoides*, *T. cingulatum* and *T. scalpturatum* group1) with the V6 domain, all the inserted sequences are different in size but possess conserved motif sequences. Hence, it is speculated that these inserted sequences may be derived from the same sequence, followed by the divergence of the inserted copy mainly mediated by large deletions. This result is strongly confirmed by the phylogenetic analysis carried out with the entire nucleotide sequences of the V9 domain which group the *Tricholoma* species studied in four major clades corresponding to the four groups evidenced with the secondary structures.

Moreover two cryptic species *T. scalpturatum* group 1 and group 2 considered by morphological characters as a unique species were clearly separated in two distinct clades. This result is in agreement with the analysis carried out with ITS (Carriconde et al., 2008) and several genes such as  $\beta$ -tubulin, glyceraldehyde-3-phosphate deshydrogenase (Jargeat, personal communication), which clearly distinguish both cryptic species.

Because of the presence of conserved motifs in the inserted sequences, the entire V6 or V9 sequences were accurately aligned. A limited number of *Tricholoma* species (*T. scalpturatum* group 2 and *T. inocybeoides* or *T. cingulatum* and *T. scalpturatum* group1) displayed identical V6 sequences. For these species, further studies are required for their taxonomic identification. Interestingly, these species as well as all the other species studied were well separated by their V9 nucleotide sequences.

To demonstrate the efficiency of the V9 domain in the discrimination of fungal species, complete V9 sequences were searched in Genbank database. Although sequences were available for several species of *Ganoderma*, they were excluded from the final analysis because they had partial sequences and did not contain the informative sites of the V9 domain. Only the two genera *Agrocybe* and *Pleurotus* had complete sequences for several species. Secondary structures were investigated for 10 and 15 species of *Agrocybe* and *Pleurotus*, respectively, and the sequences compared with those of *Tricholoma* species (Table 4). Overall, the V9 domain shows a relatively high degree of polymorphism (22%, 20% and 14% in *Agrocybe*, *Pleurotus* and *Tricholoma*, respectively), The level of interspecific variation was sufficient to discriminate all species except *P. pulmonarius* and *P. sajor-caju*. Of interest, the latter name is often used for cultivated strains of *P. pulmonarius* (R. Petersen and K. Hughes, unpublished data), suggesting a classic case of name misuse for the cultivated taxon. In addition to the three genera that contain multiple V9 sequences in Genbank database, we also analyzed the complete V9 domain of *Ustilago maydis*, *Moniliophthora perniciosa* and *Schizophyllum commune*. Significant interspecific variations (45-30%) were found among the three distant phylogenetic species.

Interestingly, the relatively high level of divergence observed does not prevent the alignment of these sequences across the fungal genera despite their phylogenetic remoteness. Moreover, no intraspecific variations were observed demonstrating that the V9 domain

represents a powerful molecular marker for species identification of Basidiomycota division and thus for the DNA barcodes.

Up to now, the mitochondrial *cox1* gene encoding the subunit 1 of cytochrome oxidase has been largely used for identification of species belonging to animal kingdom in which the sequences of about 700 nt had shown to possess a high level of nucleotide divergences and yielded species-level resolution in more than 95% of the studied taxa (Hebert et al., 2004).

The use of the *cox 1* gene on a large scale was proposed as a molecular marker for the identification of varied species belonging to different kingdoms. However, numerous works on plants (Kress et al., 2005) showed that the rate of interspecific variability of this gene did not allow species level resolution because of the slow evolution of the *cox1* gene. Therefore, the portion of the plastid *matK* gene possessing a high level of interspecific variation have been proposed for the plant DNA barcodes (Lahaye et al., 2008).

In fungi, the potential efficiency of species identification using the *cox 1* gene has been scarcely studied. Seifert et al. (2007) have recently proposed the widespread use of this gene in the fungal kingdom, but their study was based only on species belonging to the *Penicillium* genus, in which most species are devoid of any introns contrasting with the wide distribution of group 1 introns described on this gene among the Basidiomycota division (Gonzalez et al., 1998; GenBank Accession No. NC 005927; NC\_008368). We explored the potentiality of the *cox1* gene as a molecular taxonomic marker in the European population of *T. sculpturatum* group 1 and we have detected the presence of introns in two isolates from diverse geographical origin (data not shown). We were unable to obtain PCR amplification in other isolates probably due to the presence of introns, preventing the use of this gene as a molecular marker for DNA barcodes for basidiomycetous fungi.

In this article, we studied the evolution of the SSU-rDNA, and the V9 domain showed a high level of divergence between *Tricholoma* species due to point mutations and insertions /

deletions of nucleotide sequences. These insertions / deletions do not occur randomly during the evolution of the species and they possess conserved motifs that enable their alignment among phylogenetically distant species. Hence, the V9 domain by displaying an adequate divergence level, easy amplification, and alignment represents an alternative powerful molecular marker for the taxonomic identification of the basidiomycotous fungi. However, future investigations are required on other genera to use this molecular marker on a large-scale for DNA barcode in Basidiomycota division.

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**References**

- Altschul, S.F., Gish, W., Miller W., Myers E.W., Lipman, D.J., 1990. Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.
- Binder, M., Hibbett, D.S., 2002. Higher-level phylogenetic relationships of Homobasidiomycetes (mushroom-forming fungi) inferred from four rDNA regions. *Mol Phylogenet. Evol.* 22, 76-90.
- Bowe, L.M., Coat, G., dePamphilis, C.W., 1999. Phylogeny of seed plants based on all three genomic compartments: Extant gymnosperms are monophyletic and Gnetales' closest relatives are conifers. *PNAS* 97, 4092-4097.
- Bruns, T.D., Palmer, J.H., Shumard, D.S., Grossman, G.I., Hudspeth, M.E.S., 1988. Mitochondrial DNA of *Suillus*: Three-fold size change in molecules that share a common gene order. *Curr. Genet.* 13, 49-56.
- Carriconde, F., Gardes, M., Jargeat, P., Heilmann-Clausen, J., Mouhamadou, B., Gryta, H 2008. Population Evidence of Cryptic Species and Geographical Structure in the Cosmopolitan Ectomycorrhizal Fungus, *Tricholoma scalpturatum*. *Microb. Ecol. In Press*
- Chang, S.T., Miles, P.G., 1991. Recent trends in world production of cultivated edible mushrooms. *Mushroom Journal* 504, 15–18.
- Garcia-Varela, M., Nadler, S.A., 2006. Phylogenetic relationships among Syndermata inferred from nuclear and mitochondrial gene sequences. *Mol. Phylogenet. Evol.* 40, 61-72.
- Gryta, H., Carriconde, F., Charcosset, J.Y., Jargeat, P., Gardes, M., 2006. Population dynamics of the ectomycorrhizal fungal species *Tricholoma populinum* and *Tricholoma scalpturatum* associated with black poplar under differing environmental conditions. *Environ. Microbiol.* 8, 773-86.
- Gonzalez, P., Barroso, G., Labarere, J., 1998. Molecular analysis of the split *cox 1* gene from the basidiomycota *Agrocybe aegerita* : relationship of its introns with homologous Ascomycota introns and divergence levels from common ancestral copies. *Gene* 220, 45-53.
- Gonzalez, P., Labarere, J., 1999. Sequence and secondary structure of the mitochondrial small-subunit rRNA V4, V6, and V9 domains reveal highly species-specific variations within the genus *Agrocybe*. *Appl. Environ. Microbiol.* 64, 4149–4160.
- Gonzalez, P., Labarere, J., 2000. Phylogenetic relationships of *Pleurotus* species according to the sequence and secondary structure of the mitochondrial SSU-rRNA V4, V6 and V9 domains. *Microbiology* 146, 209–221.
- Hajibabaei, M., Janzen, D.H., Burns, J.M., Hallwachs, W., Hebert, P.D., 2006. DNA barcodes distinguish species of tropical Lepidoptera. *PNAS* 103, 968-971.

- Hebert, P.D., Stoeckle, M.Y., Zemplak, T.S., Francis, C.M., 2004. Free in PMC Identification of Birds through DNA Barcodes. *PloS. Biol.* 2, e312.
- Hibbett, D.S., Pine, E.M., Langer, E., Langer, G., Donoghue, M.J., 1997. Evolution of gilled mushrooms and puffballs inferred from ribosomal DNA sequences. *PNAS* 94, 1202–1206.
- Hintz, W.E.A., Mohan, M., Anderson, J.B., Horgen, P.A., 1985. The mitochondrial DANN of *Agaricus*; Heterogeneity in *A. bitirquis* and homogeneity in *A. brunnescens*. *Curr. Genet.* 9, 127-132.
- Horton, T.R., 2002. Molecular approaches to ectomycorrhizal diversity studies: variation in ITS at a local scale. *Plant and soil* 244, 29-39.
- Kimura, M., 1983. *The Neutral Theory of Molecular Evolution*. Cambridge University Press, Cambridge, MA.
- Ko, K.S., Jung, H.S., 1999. Molecular phylogeny of *Trametes* and related genera. *Antonie Van Leeuwenhoek.* 75, 191-199.
- Kress, J.W., Wurdack, K.J., Zimmer, E.A.C., Weigt, L.A., Janzen, D.H., 2005. Use of DNA barcodes to identify flowering plants *PNAS* 102, 8369–8374.
- Lahaye, R., van der Bank, M., Bogarin, D., Warner, J., Pupulin, F., Gigot, G., Maurin, O., Duthoit, S., Barraclough, T.G., Savolainen, V., 2008. DNA barcoding the floras of biodiversity hotspots. *PNAS* 105, 2923-2928.
- Lievens, B., Brouwer, M., Vanachter, A.C., Levesque, C.A., Cammue, B.P., Thomma, B.P., 2005. Quantitative assessment of phytopathogenic fungi in various substrates using a DNA microarray. *Environ. Microbiol.* 7, 1698-1710.
- Moncalvo, J.M., Lutzoni, F.M., Rehner, S.A., Jonson, J., Vilgalys, T., 2000. Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Syst. Biol.* 49, 278-305.
- Mouhamadou, B., Ferandon, C., Barroso, G., Labarere, J., 2006. The mitochondrial apocytochrome b genes of two *Agrocybe* species suggest lateral transfers of group I homing introns among phylogenetically distant fungi. *Fungal Genet. Biol.* 43, 135-145.
- Neefs, J.M., Van de Peer, Y., De Rijk, P., Chapelle, S., De Wachter, R., 1993. Compilation of small ribosomal subunit RNA structures. *Nuc. Acids. Res.* 21, 3025–3049.
- O'Donnell, K., Cigelnik, E., 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol. Phylogenet. Evol.* 7, 103-106.
- Okamoto, K., Sekito, T., Yoshida, K., 1996. The secondary structure and phylogenetic relationship deduced from complete nucleotide sequence of mitochondrial small subunit rRNA in yeast *Hansenula wingei*. *Genes. Genet. Syst.* 71, 69–74.

- Saitou, N., Nei, M. 1987. The Neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.
- Seifert, A.K., Samson, R.A., deWaard, R.J., Houbraken, J., Levesque, C.A., Moncalvo, J.M., Louis-Seize, G., Hebert, P., 2007. Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case. *PNAS* 104, 3901-3906.
- Smith, S.E., Read, D.J., 1997. *Mycorrhizal Symbiosis*. London: 2nd edn. UK: Academic Press.
- Soon, G.H., Wonjin, J., 2002. Amplification of mitochondrial small subunit ribosomal DNA of polypores and its potential for phylogenetic analysis. *Mycologia* 94, 823-833.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic. Acids. Res.* 22, 4673–4680.
- Uhart, M., Sirand-Pugnet, P., Labarere, J., 2007. Evolution of mitochondrial SSU-rDNA variable domain sequences and rRNA secondary structures, and phylogeny of the *Agrocybe aegerita* multispecies complex. *Research in Microbiology* 158, 203-212.
- Vilgalys, R., Sun, B., 1994. Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of the ribosomal DNA sequence. *PNAS* 91, 4599-4603.
- Wuyts, J., Van de Peer, Y., Winkelmans, T., De Wachter, R., 2002. The European database on small subunit ribosomal RNA. *Nucl. Acids Res.* 30, 183–185.
- Zuker, M., Jacobson, A.B., 1995. “Well-determined” regions in RNA secondary structure prediction: analysis of small subunit ribosomal RNA. *Nucl. Acids Res.* 23, 2791–2798.

Fig. 1 : Alignment of the V6 domain sequences of 11 species of the genus *Tricholoma*. Sequences strictly conserved among the 11 species are indicated by asterisks. The inserted sequences are indicated in grey. *Timb*, *T. imbricatum*; *Tc*, *T. cingulatum*; *Tsg1*, *T. scalpturatum group1*; *Ti*, *T. inocybeoides*; *Tsg2*, *T. scalpturatum group2*; *Tb*, *T. basirubens*; *Tr*, *T. triste*; *Tg*, *T. gausapatum*; *Ta*, *T. atosquamosum*; *Tf*, *T. fulvum*; *Tp*, *T. populinum*.

Fig. 2: Alignment of the V9 sequences of 11 species of the genus *Tricholoma*. Sequences strictly conserved among the 16 species are indicated by asterisks. V9-1 and V9-2 boxes represent putative insertions/deletions events.

Fig. 3: Putative secondary structure of the variable domains V6 (A) and V9 (B) in the *Tricholoma* genus. The secondary structures B1 (group B), B2 (group C), B3 (group A) and B4 (group D), represent each of the four groups of structures evidenced in this study.

Fig. 4 : Consensus tree of 1000 trees obtained by the neighbour-joining method, based of the entire sequences of the V9 variable domain. Groups defined by comparison of the secondary structures are indicated.

Table 1. Number of introns in *cox1* gene of the basidiomycetous fungi available in Genbank database.

Species	GenBank accession n°	Number of intron
<i>Pleurotus ostreatus</i>	<u>NC 009905</u>	9
<i>Ustilago maydis</i>	<u>NC 008368</u>	8
<i>Moniliophthora perniciosa</i>	<u>NC 005927</u>	6
<i>Agrocybe aegerita</i>	<u>AF010257</u>	4
<i>Tilletia walkeri</i>	<u>NC 010651</u>	4
<i>Rhodotorula glutinis</i>	<u>AB248915</u>	2
<i>Schizophyllum commune</i>	<u>NC 003049</u>	0
<i>Cryptococcus neoformans</i>	<u>AY560610</u>	0

Table 2. *Tricholoma* isolates used in this study

Strain	Origin
<i>T. scalpturatum</i> group 1	
FC053	Denmark
FC059	Denmark
125572A	England
FC086	Sweden
AV04-242	France
Rim05-75	France
Rim05-49	France
AV05-2	France
M098-68	France
<i>T. scalpturatum</i> group 2	
FC068	Sweden
FC037	Denmark
F135443A	England
Cooo14c	France
Cooo13a	France
AV04-78	France
AV05-42	France
AV04-14	France
Rim05-15	France
<i>T. inocybeoides</i> TSV2	France
<i>T. populinum</i> PAF02	France
<i>T. fulvum</i> C0-02	France
<i>T. imbricatum</i> CD02	France
<i>T. cingulatum</i> TC1	France
<i>T. gausapatum</i> AM03	France
<i>T. triste</i> Bes06.2	France
<i>T. atrosquamosum</i> SVC 03.2	France
<i>T. basirubens</i> SCV03	France





Table 4. Length variations and percentage of nucleotide divergences of the V9 domain at different taxonomic levels of three basidiomycetous genera.

Genus	Number of strain	Number of species	Mean domain size (nt)	Intraspecific variations (%)	Means of interspecific variations (%)	Range of means (%)
<i>Agrocybe</i>	42	10	328	0 <sup>+</sup>	22	5-30
<i>Pleurotus</i>	48	15	260	0 <sup>+</sup>	20	0-35
<i>Tricholoma</i>	27	11	254	0*	14	1-24

\* The intraspecific variations in the genus *Tricholoma* were investigated in two cryptic species (*T. scalpturatum* group 1 and group 2). <sup>+</sup>The intraspecific variations in the *Agrocybe* and *Pleurotus* genera are those reported by Gonzalez and Labarere, 1999; 2000, respectively.

V6 - 1

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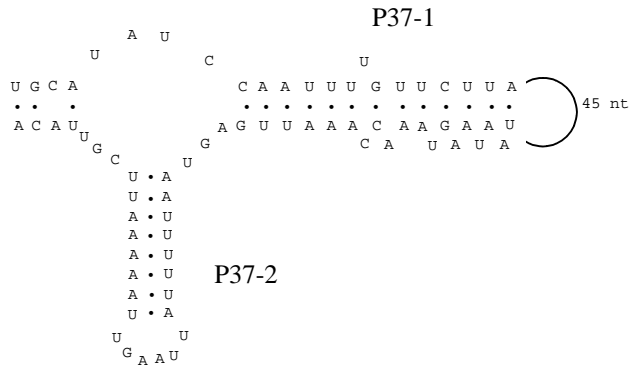
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Ts g1   TTGCATATCCAAT-----TA--TATTT--ATTA-----TAA----TTGAGTAATTTTATAATATTTAAAAATTCGTTTACA
Ti      TTGCATATCCAAT-----TAGGTTATTTGAATAAGAAGAA-CTAA----TTGAGTAATTTTATAATATTTAAAAATTCGTTTACA
Ts g2   TTGCATATCCAAT-----TAGGTTATTTGAATAAGAAGAA-CTAA----TTGAGTAATTTTATAATATTTAAAAATTCGTTTACA
Ttr     TTGCATATCCAATTAT-GTTCTTAGTTAATTC-----TAAGTTCTTTTAAATAAGAA-AA-CTAA----TTGAGTAATTTTATAATATTTAAAAATTCGTTTACA
Tg      TTGCATATCCAATGAT-GTTCTTAGTTAATTC-----TAAGTTCTTTTAAATAAGAA-AA-CTAA----TTGAGTAATTTTATAATATTTAAAAATTCGTTTACA
Ta      TTGCATATCCAATTTT-GGTCTTAGTTTATTC-----TAAGTTCTTTTAAATAAGTA-AA-CCAAA----TTGAGTAATTTTATT-TAATTAATAATTCGTTTACA
Tb      TTGCATATCCAATTTT-GTTCTTAGTTTATCTGAGTTCCTTTGTTCTTAGTTTATTCCTAAATTCCTTTTAAATAAGTA-AA-CCAAA----TTGAGTAATTTTATTA-AGTTAAAAATTCGTTTACA
Tf      TTGCATAAACAATTTT-GTTATTAGAGTATTTT-----ATTCTAATGTCCTTTTAAATAAGAAAAACACAAA----TTGAGTAATTTTATAATTTTAAAAATTCGTTTACA
Tp      TTGCATAACCAATTTTGTATTAGAAAAATTT-----TTTCTAATATCTTTTAAAGTAAGAAAAA-CATAA----TTGAGTAATTTTATAGTTTTTAAAAAGTTCGGTTACA
Timb    TTGCATATCCAATCCTTATTTTAAATTTACT-----TAAATTTATTTACGTGCAAA-AATATTAAGAAAGATTGAGTAGTTCTTTTAAATTAAGAAACTCGTTTACA
*****  ****                                **  *  **  *                                *****  ***  *  *  *  *  *  *  *  *  *  *  *  *  *

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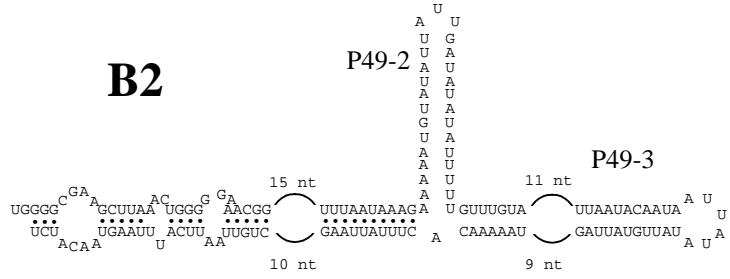
Figure 1



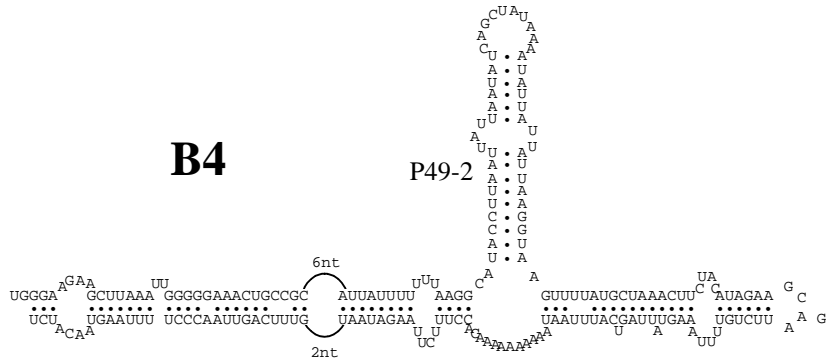
**A**



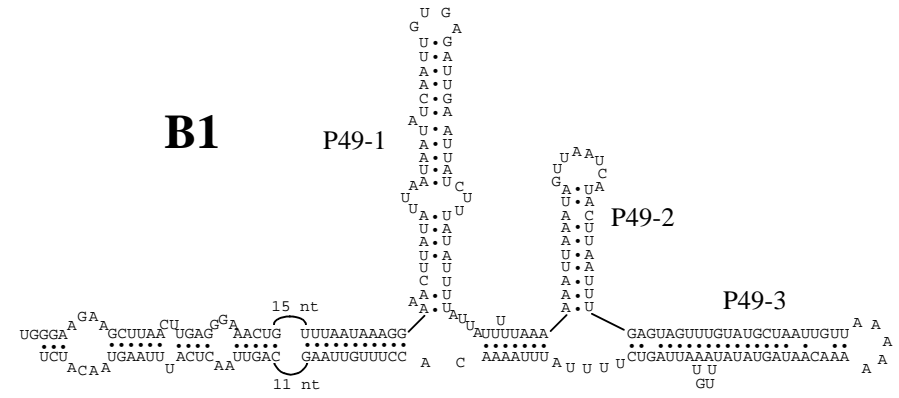
**B2**



**B4**



**B1**



**B3**

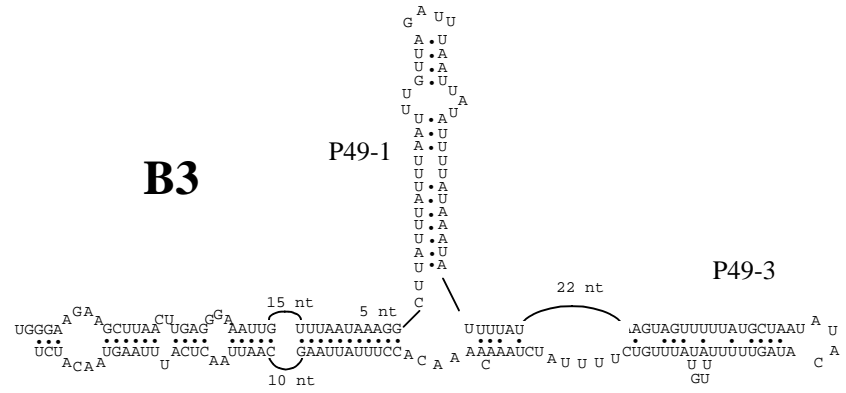


Figure 3

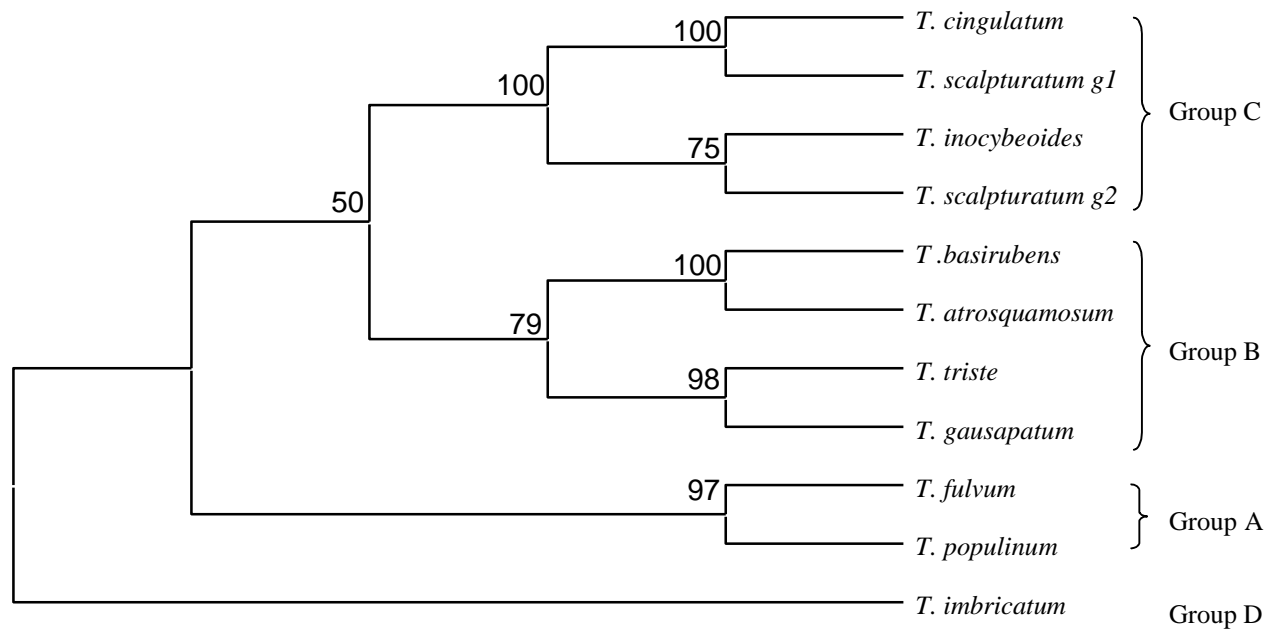


Figure 4