FROM THE COMPARATIVE ANALYSIS OF FUNGAL MITOCHONDRIAL GENES TO THE DEVELOPMENT OF TAXONOMIC AND PHYLOGENETIC TOOLS

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ABSTRACT

The complete sequence of the mitochondrial *cox1* gene, encoding the largest subunit of the cytochrome oxidase of the Basidiomycota *Agaricus bisporus* has been achieved. It has the longest *cox1* gene (29,902 nt) with the largest number of group I introns (18 group I introns) reported to date in any eukaryote. The group I introns in the *A. bisporus cox1* gene are similar to those reported in other Basidiomycetes includeing: 3 of the 4 introns in *Agrocybe aegerita*, 7 of the 9 introns in *Pleurotus ostreatus*, 3 of the 6 introns in *Moniliophthora perniciosa*, and 10 of the 15 introns in *Trametes cingulata*. constituting 18 of the 23 introns described in all Basidiomycota available genes, Moreover, the *A. bisporus cox1* gene possesses two introns specifically reported in this gene (*iAbi1* and *iAbi14*) and one intron (*iAbi 18*) possessing orthologous sequences only in the Ascomycota phylum but unknown to date in the Basidiomycota. Hence, *A. bisporus cox1* gene contains three-quarters (18/24) of the fungal introns described in all the fungal *cox1* genes from Dikarya (Ascomycota and Basidiomycota). From the *A. bisporus* sequence, primers were designed to evaluate the potential of rare and widely distributed introns to act as taxonomic and/or phylogenetic markers of species belonging to the genus *Agaricus*. Indeed, we found that the rare introns could be specifically recovered in some strains and/or species. Sequences of the widely distributed fungal introns provide information on the phylogeny and spread of introns among distant as well as closely related species.

Keywords: *Agaricus* genus; mitochondria; *cox1* gene; group I intron; phylogeny

INTRODUCTION

Although the fungal Dikarya subkingdom (including the Ascomycota and Basidiomycota phyla) contains a broad range of taxa with a great variety of morphologies, ecologies and life cycles, it is sometimes difficult, even for specialized mycologists, to define distinct and unambiguous species boundaries based on their phenotypic differences. In this context, there is an urgent need to obtain improved and cost-effective molecular markers to help discriminate closely related species, especially for species belonging to the same genus and with commercial interests.

To date, molecular identification of fungi relies mostly on nuclear DNA markers, such as the conserved LSU-rDNA (18S), SSU-rDNA (28S) or the frequently studied variable spacers (ITS1 and ITS2) within the nuclear ribosomal gene cluster. Currently, the 18S and 28S are used to discriminate high taxonomic levels such as family and genera while the internal transcribed spacers (ITS) allow the characterization of organisms at the species level [1]. However, in recent

years, it has been necessary to develop several additional nuclear markers to resolve the inefficiency of ITS to discriminate some well-characterized species or to identify cryptic species in some fungal species complexes, as well as to clarify phylogenetic relationship between related as well as distant species. The β-tubulin gene (BenA) [2], the elongation-factor EF-1-α [3], the second largest subunit of RNA polymerase II (RPB2) [4] have often been used for these purposes.

Besides these nuclear DNA sequences, the potential use of mitochondrial DNA specific markers has become increasingly common. Indeed, mitochondrial DNA markers possess several interesting features such as their high copy number allowing an easy recovering of the sequences and the paucity of repetitive regions often involved in misleading results. Furthermore, mitochondrial DNA (mtDNA) has been reported to be less affected by genetic recombination, mainly due to a predominantly uniparental heredity, and to show a higher rate of evolution than the nuclear genome. These features make mitochondrial DNA a potentially powerful source of molecular markers to identify species [5]. Hence, a 648 nt sequence located at the 5' end of the *cox1* gene encoding the subunit I of the "cytochrome c oxydase" (complex IV of the respiratory chain) has been widely used in a successful "DNA barcoding" method in several animal groups such as insects or birds [6]. With a taxonomic resolution higher than 95% in most of the Metazoa group, this mitochondrial region was proposed as the core of global bio-identification systems for eukaryotes [7].

In this way, in the Metazoa related kingdom of fungi, the *cox1* gene was recently shown suitable for discriminating fungal species in the taxonomically challenging genus *Penicillium* [8]. However, in most genera of the fungal kingdom, this approach is hampered by the presence of several large group I introns, frequently occurring in mitochondrial coding sequences and, especially in the *cox1* gene which is the mitochondrial gene showing the highest number of introns [9]. Owing to this wealth of introns, few fungal complete *cox1* gene sequences are available in database, especially for species belonging to the Basidiomycetes where only six sequences have been reported and correctly annotated to date (*Moniliophtora perniciosa, Pleurotus ostreatus, Agrocybe aegerita, Schyzophyllum commune, Agaricus bisporus* and *Trametes cingulata)* [9].

Here, we report the analysis of three types of mitochondrial sequences with the aim to define molecular markers suitable as taxonomic and/or phylogenetic tools at different taxonomic ranks and/or for strain fingerprinting: (i) Variables domains of the SSU-rDNA of the mitoribosome, (ii) the sequences of a" rare" group I intron, and (iii) a widely distributed intron carried by the *cox1* gene. The taxonomic and phylogenetic potentials of these mitochondrial markers will be discussed by comparing with the sequences of the conventional nuclear ribosomal cluster.

The comparative analysis was carried out with six strains representing five *Agaricus* species (*A. boisseletti, A. gennadii, A. arvensis, A. subrufescens* and *A. bisporus*) belonging to four different taxonomic sections of this genus. Two *A. subrufescens* strains from two geographical origins (France and Brazil) were included in the analysis for comparison.

MATERIALS AND METHODS

***Agaricus* species sampling and determination.** Sporophores representing six *Agaricus* strains (Table 1) were collected and morphologically identified. Sequences of the nuclear ribosomal unit obtained from these stains were established and compared with sequences available in the GenBank (data not shown).

Table 1: *Agaricus* strains and sequences

Section	*Agaricus* species	strain N°	ITS Acc N°	V6 domain	V9 domain	intron i7	intron i18
Sanguinolenti							
	A. boisseletti	CA369		CA369	nd	CA369	0
	A. boisseletti	CA123	DQ182531				
Chitonioides							
	A. gennadii	CA387		CA387	CA387	CA387	CA387
	A. gennadii	Gn17	AF432881				
Arvenses							
	A. arvensis	CA640		CA640	CA640	CA640	CA640
	A. arvensis	strain 15	AJ887993				
	A. subrufescens	CA516		CA516	CA516	CA516	CA516
	A. subrufescens	CA454		CA454	CA454	CA454	CA454
	A. subrufescens	I_101_S1	AY818660				
Bivelares							
	A. bisporus	BS518		BS518	BS518	BS518	BS518
	A. bisporus	ATCC MYA-4626	GU327642				

***In vitro* DNA manipulation and sequencing.** Sequences used as molecular markers were obtained by conventional procedures from cloned PCR products.

Total DNA of fungal strains were extracted from 0.1 g of dried carpophores after grinding in liquid nitrogen to generate a fine powder. Nucleic acids were extracted according to the *N*-cethyl-*NNN*-trimethyl ammonium bromide (CTAB) procedure adapted to small quantities of basidiomycete mycelia by [10]. DNA (OD_{260}) was quantified using a NanoDrop spectrophotometer (NanoDrop ND-1000, Nanodrop technologie, DE, USA), diluted in deionized sterilized Milli-Q water (Milli-Q water system production, Millipore, Saint-Quentin en Yveline, France) and stored at -20°C.

PCR amplifications were carried out using the Go *Taq* polymerase from Promega Corp. (Madison, Wis, USA) and with corresponding primer pairs synthesized by Eurofins MWG Operon (Germany). PCR were performed in a Programmable Thermal Cycler PTC 200 (MJ Research Inc., Watertown, Mass., USA). Each reaction contained 10 to 100 ng of fungal genomic DNA, 1 µM of both primers, 200 µM of each dNTP, 1 unit of *Taq* DNA polymerase, in a final volume of 50 µl of the appropriate buffer. Reactions were run for 30 cycles at 95 ° C for 30s, then two degrees below the lowest Tm of both oligonucleotides for 30s, 72° C for 1 to 2 min, and one final cycle at 72° C for 5 min. An aliquot of 10 µl of each PCR product was analysed by agarose (1%, w/v) gel electrophoresis containing 200ng/ml of ethidium bromide, in TEB buffer [11].

DNA Sequencing. PCR products were purified with the Wizard SV gel and PCR Clean-Up System (Promega Corp.Madison, WI, USA) before they were sequenced by the primer walking methods using the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Courtaboeuf, France) and corresponding primers used for the initial PCR amplifications. Sequence reactions were carried out, according to the supplier recommendations, in a final volume of 10 µl containing 100 ng of PCR product and 0.5 µM of the specific primer. Sequence reactions were conducted in a thermocycler by applying an initial denaturation step at 95°C for 1 minute; 27 cycles each composed of the three following steps: 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. The reaction products were ethanol precipitated, dried then separated by capillary electrophoresis (on an automated sequencer ABI 3130x1, ABI Prism Corp., France) at the genomic platform of the University Bordeaux Segalen (France). Sequencing profiles were edited and corrected using the BioEdit sequence alignment editor v7.0.9 free software (Ibis Biosciences Carlsbad, CA, USA).

Sequence analyses. Comparisons with sequences of the GenBank and EMBL databases were performed using the search algorithm BLAST [12]. Multi-alignments of nucleic acid and/or proteins were performed using Clustal W algorithm [13] or Muscle [14] for multiple alignment and Gblocks for automatic alignment curation [15]. For phylogenic analyses, the sequence data were aligned and checked for accuracy manually. Pairwise evolutionary distances based on unambiguous nucleotides were computed using the dnadist (Jukes and Cantor option) and neighbor-joining programs in the PHYLIP suite. Seqboot was used for Bootstrap analysis [18], using algorithm version 3.572c. One thousand Bootstrap replicates were employed to determine confidence in the branches order. (The phylogenetic softwares used were part of the PHYLIP package version 3.572 Mac executables [19].

The alignments were also submitted to the PhyML tree building program [16] and TreeDyn for tree drawing [19]. PhyML was run with the aLRT statistical test of branch support. These programs were obtained on line at: http://www.phylogeny.fr/ [20, 21]. In this case, confidence in the branches order was measured by the ratio test developed by [22] working with the PhyML tree building program at the phylogeny site (http://www.phylogeny.fr/) [20, 21].

RESULTS AND DISCUSSION

Comparison of the molecular organization of the *cox1* gene in *A. bisporus* (section Bivelares) and in other Basidiomycete species

The complete sequence of the mitochondrial *cox1* gene of *Agaricus bisporus* was achieved. This gene is both the longest mitochondrial gene (29,902 nt) and the largest intron reservoir reported to date in an eukaryote [9]. It possesses 18 group I and one group II introns. An exhaustive analysis of the group I introns available in *cox1* genes shows that they are ancestral mobile genetic elements, whose frequent events of loss (according to the "late theory") and gain by lateral transfer ("early theory") would combine to obtain the observed wide and patchy distribution extending on several kingdoms [23]. Its distributions are consistent with both the "early" and "late" paradigms, which are still matters of debate [24, 25]. However, the overview of the intron distribution in eukaryotes indicates that they are mainly evolving towards elimination and, in such a landscape of eroded and lost intron sequences, the *A. bisporus* largest intron reservoir, by its singular dynamics of intron keeping and catching, would constitute the most fitted relic of an early split gene [9]. However, the analysis was carried out on phylogenetically distant organisms extending on several kingdoms.

When the analysis was limited to the Dikarya, results show that the *Abi cox1* gene possesses most of the group I introns available in other Basidiomycete *cox1* genes: it contains 3 of the 4 introns in *Agrocybe aegerita*, 7 of the 9 introns in *Pleurotus ostreatus*, 3 of the 6 introns in *Moniliophthora perniciosa*, 10 of the 15 introns in *Trametes cingulata, and* 18 of the 23 introns described in all basidiomycota available genes (Fig. 1). Moreover, the *A. bisporus cox1* gene possesses two introns only reported in this organism (*iAbi1* and *iAbi14*) and one intron (*iAbi 18*) possessing orthologous sequences found only in the Ascomycota phylum so far but unknown to date in the Basidiomycota. Hence, *A. bisporus cox1* gene contains three quarters(18/24) of the fungal introns described in all the fungal *cox1* genes from Dikarya, i. e. from all the available Ascomycota and Basidiomycota *cox1* genic sequences [9].

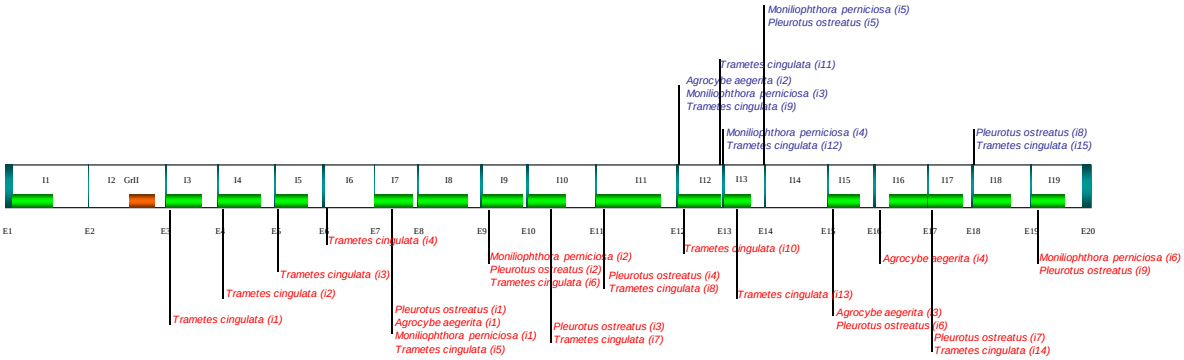

Figure 1: Molecular organization of the *A. bisporus* strain BS518 *cox1* gene.
The position of orthologous (red) and not orthologous (blue) introns reported in other Basidiomycota are indicated below and above the *A. bisporus cox1* gene, respectively. Blue boxes represent exons E1 to E20. The names of the introns are indicated in the empty boxes. The putative functional *heg* in group I introns are shown by green boxes; the eroded reverse transcriptase gene carried by the *iAbi 2* group II intron by an orange box.

In other to investigate the evolution (loss and/or gain), the ancestral feature and the correlated potentiality of *cox1* group I introns to act as phylogenetic markers, we have studied the occurrence and phylogenetic relationships of two group I introns reported in the *Abi cox1* gene (strain BS518) in five additional *Agaricus* species belonging to four different sections. The evolution of these intronic sequences was compared with that of the nuclear ribosomal unit and with that of variable domains of another mitochondrial gene, namely the SSU-rDNA, encoding the small rRNA (16S) of the mitoribosome.

Occurrence and phylogenetic analysis of the widely distributed *iAbi7* group I intron. The first intron studied was *iAbi7* (1207 nt) which is the most widely distributed group I intron in eukaryotes (present in all the divisions of the fungal kingdom and also in the Viridiplantae kingdom). All the available Basidiomycete *cox1* genes reported to date possess orthologous sequences of the *iAbi7* intron, with the exception of *Schizophyllum commune* whose mitochondrial genome does not possess any intron.

From the *A. bisporus cox1* gene, a couple of specific primers located in the upstream (primer U7: 5'ACAGGGTGGACGGTA3') and downstream (primer R7: 5'GATTCCTGATAAAGGAGG3') exon regions flanking *iAbi7* were defined and used in PCR to amplify six *Agaricus* strains as matrix. As shown in Table 1, all the studied strains generated a PCR product of a large size around 1,200 nt. For each strain, this PCR product was purified and sequenced. The resulting sequences confirmed the presence in the *cox1* gene of each strain of an *iAbi7* orthologous sequence. Moreover, all these orthologous introns possess a large ORF corresponding to the *heg* encoding a putatively functional Homing Endonuclease (HE) involved in the transfer and site-specific integration (homing) of the mobile intron. These results suggest that the *iAbi7* intron is a mobile genetic element with likely conserved functions in the *Agaricus* genus.

A phylogenetic tree was constructed by two methods: a distance (Neighbor-Joining) method (Fig. 2C) and the maximum likelihood method. The trees were obtained from the MUSCLE alignment of a 754 nt sequence read on both strands and located in the central part of the intron sequence and in the central part of the *heg* (Homing Endonuclease Gene) carried by these orthologous introns.

These trees confirm the close relationship between the six *iAbi7* orthologs. The deduced relationship among the strains are in agreement with those deduced from the trees based either on the nuclear ribosomal cluster (figure 2A) or on the compiled sequences of two variable domains

(V6 and V9) of the mitochondrial SSU-rDNA (figure 2B). Particularly, sequences of the intron 7 of both strains of *A. subrufescens* (CA454 from Brazil and CA516 from France) were identical to each other and to the *A. Arvensis* strain CA640 which belongs to the same section Arvenses. When these sequences were compared with *iAbi7* sequence of *A. bisporus* which belongs to the second clade of the tree, they showed 82,6 % of nt identity.

The agreement between the *iAbi7* phylogeny and those based on the nuclear ribosomal gene cluster (figure 2A) or based on the compiled sequences of two variable domains (V6 and V9) of the mitochondrial SSU-rDNA (figure 2B) argues for an ancestral feature of the *iAbi7* intron. This result, along with the high conservation of the *i7* intron within the *cox1* genes, and the wide distribution of *heg* within the *i7* intron represent a powerful tool for phylogenetic studies of the genus *Agaricus*, and more particularly at the section level.

Occurrence and phylogenetic analysis of the rare *iAbi18* group I intron. The second studied intron is *iAbi18* (1148 nt), a rare intron only reported in *A. bisporus* in the Basidiomycota and two species in the Ascomycota, *Gibberella zea* and *Penicillium marneffei*.

From the *A. bisporus cox1* gene, a couple of specific primers located in the upstream (primer U18: 5'TGCAGGTTTCTATTATTGG3') and downstream (primer R18: 5'AAGTGTTGAGGGAAAAATG3') exon regions flanking *iAbi18* were defined and used in PCR to amplify the six *Agaricus* strains. As shown in table 1, only one of the six strains, *A. boisseletti* CA123 did not possess orthologous sequence to *iAbi18*. In this case, the PCR product with a size of 120 nt corresponded to the size of the COX1 CDS located between both primers. The exon nature of the PCR product was verified by sequencing. This confirms that the presence of a group I intron in a strain or species is optional. However, the study of several *A. boisseletti* strains will be needed to determine if the observed intron loss concerns the strain level or has to be extended to the species level.

The large size PCR product (around 1,300 nt) obtained with the five other strains was sequenced. The resulting sequences confirmed the presence in the *cox1* gene of each strain of an *iAbi18* orthologous sequence, carrying a putative functional *heg*.

Moreover, the two orthologs carried by both *A. subrufescens* strains from Brazil and France were identical, with a 100% of nt sequence identity.

Similar to the analyses for iAbi7 sequences above, trees were constructed by the Distance and PhyML programs. The trees (figure 2D) were obtained from the MUSCLE alignment of a 896 nt sequence read on both strands, and located in the central part of the intron sequence and in the central part of the *heg* (Homing Endonuclease Gene) carried by the intron. These trees confirmed the close relationships between the five *iAbi18* orthologs. It is to be noted that the *iAbi18* ortholog harboured by the *A. Arvensis* strain *(iAarv18*) follows the phylogenetic relationships deduced from the trees based on the nuclear ribosomal unit and on the mitochondrial variable domains as well as on the *iAbi7* orthologous sequences. However, in the Arvenses section, the sequence of *A. arvensis* was highly diverged from the two sequences of *A. subrufescens*. Indeed, *iAarv18* possesses 91.4 % nt identity with *iAbi18* and 92.3% with *iAsub18*, although *A. arvensis* and *A. subrufescens* are two phylogenetically closely related species (belonging to the same Arvenses section).

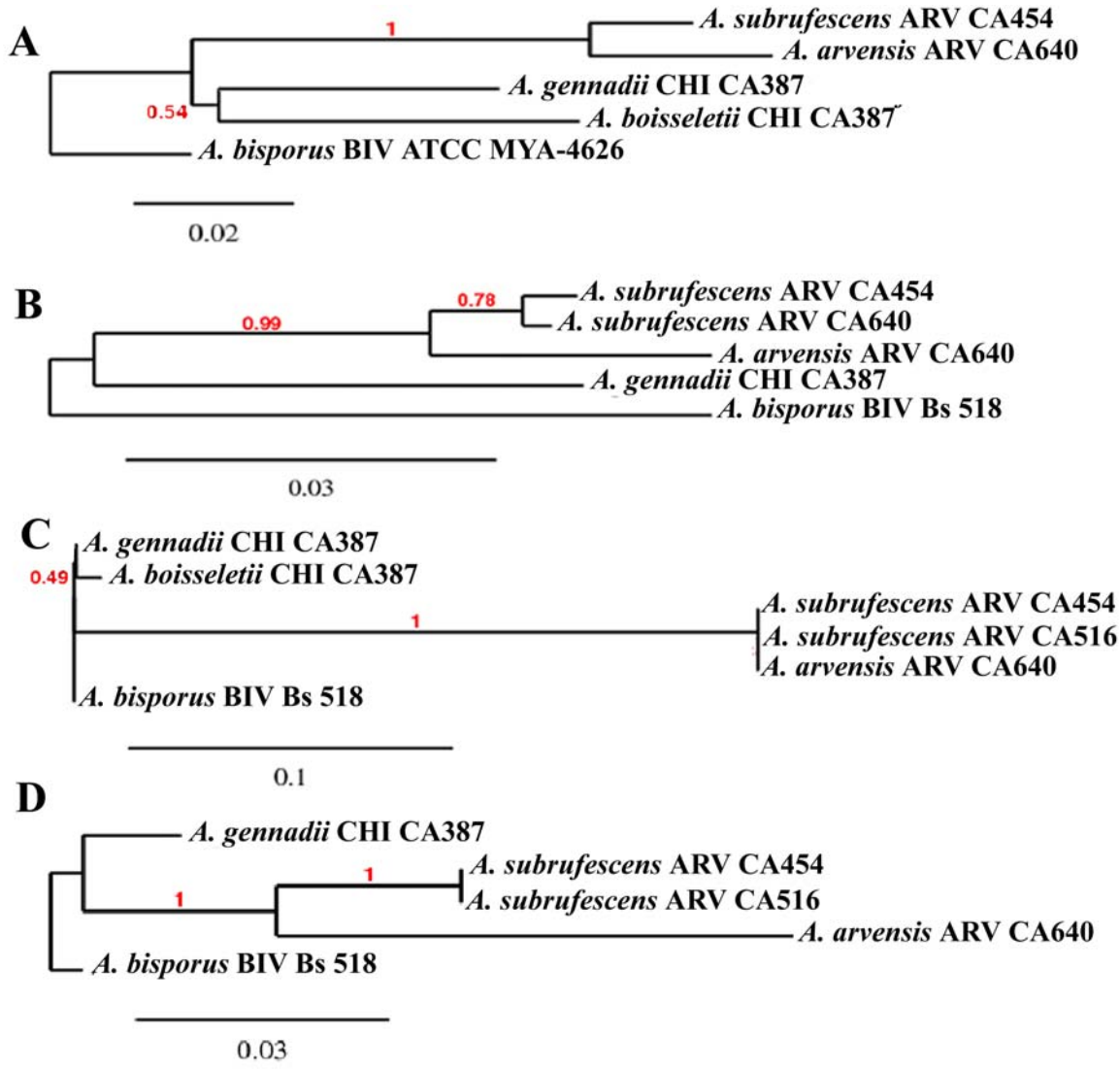

Figure 2: Distance (Neighbor) cladograms based on the nuclear ribosomal unit (A), a compilation of the V6 and V9 variable domains (B), the orthologs of the *iAbi7* intron (C) and *iAbi18* intron (D).
The Bootstraps values indicated above branches were obtained with 1,000 replicates. The cladograms based PhyML program (maximum likelihood) led to similar trees with branches supported by comparable SH-like Branch supports.

CONCLUSION

This report focused on three different mitochondrial molecular markers and six strains representing five species and four sections of the *Agaricus* genus. From the preliminary results, two types of mitochondrial sequences appear as potentially suitable tools to add phylogenetic and/or taxonomic information to the well-established nuclear ribosomal units.

The first one is the compiled sequences of two variable domains (V6 and V9) of the SSU-rDNA, encoding the 16S RNA of the small-subunit of the mito-ribosome. Indeed, these domains mainly evolve by length mutations involving indel (insertion/deletion) sequences and, consequently, can easily lead to CAPS markers for species determination.

The second kind of sequences is constituted by the orthologs of the *iAbi7* intron. Indeed, this group I intron appears widely distributed in the eukaryote kingdom, but also in the *Agaricus* genus. Moreover, this mobile genetic element carries a structural gene, encoding a Homing Endonuclease (HE) which seems to have maintained its function during evolution, and consequently can constitute a permanent phylogenetic marker, to replace the “barcoding region ”

of the *cox1* gene which is split too much by several large group I introns in the fungi, and especially in the *Agaricus* genus (data not shown).

The third studied sequence, the "rare" group I intron *iAbi18*, was shown to be frequent but not universally distributed in the *Agaricus* genus. Additionally, one of its orthologous sequences described in an *A. arvensis* strain reveals an unexpected phylogenetic behavior, suggesting that its evolution might not strictly follow evolution by descent but could involve lateral gene transfer between a distantly related species. This behavior does not allow to consider it as an easy phylogenetic marker but opens the way to the discovery of still unknown transfers of mitochondrial sequences.

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