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Molecular gene organisation and secondary structure of the mitochondrial large subunit ribosomal RNA from the cultivated Basidiomycota *Agrocybe aegerita*: a 13 kb gene possessing six unusual nucleotide extensions and eight introns

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ABSTRACT

The complete gene sequence and secondary structure of the mitochondrial LSU rRNA from the cultivated Basidiomycota *Agrocybe aegerita* was derived by chromosome walking. The *A.aegerita* LSU rRNA gene (13 526 nt) represents, to date, the longest described, due to the highest number of introns (eight) and the occurrence of six long nucleotidic extensions. Seven introns belong to group I, while the intronic sequence i5 constitutes the first typical group II intron reported in a fungal mitochondrial LSU rDNA. As with most fungal LSU rDNA introns reported to date, four introns (i5–i8) are distributed in domain V associated with the peptidyl-transferase activity. One intron (i1) is located in domain I, and three (i2–i4) in domain II. The introns i2–i8 possess homologies with other fungal, algal or protozoan introns located at the same position in LSU rDNAs. One of them (i6) is located at the same insertion site as most Ascomycota or algae LSU introns, suggesting a possible inheritance from a common ancestor. On the contrary, intron i1 is located at a so-far unreported insertion site. Among the six unusual nucleotide extensions, five are located in domain I and one in domain V. This is the first report of a mitochondrial LSU rRNA gene sequence and secondary structure for the whole Basidiomycota division.

INTRODUCTION

Although the Basidiomycota division includes most cultivated mushrooms and several highly damaging phytopathogenic fungi, molecular studies on the Basidiomycota mitochondrial gene organisation have recently begun to develop, leading to the report of four complete mitochondrial gene sequences for the whole division: the *cox3* (1) and *nad5* (2) genes of *Schizophyllum commune*, and the SSU rRNA (3) and the *cox1* (4) genes of the edible mushroom *Agrocybe aegerita*.

Agrocybe aegerita is a cultivated Basidiomycota species belonging to the Agaricales order whose mtDNA, 80 kb in size, has been previously cloned and mapped (5). Restriction fragment length polymorphism (RFLP) analysis of the mitochondrial genome (6) has indicated four different haplotypes widely distributed in a European wild-type strain population (7). Moreover, subgenomic molecules present at a low level and resulting from rearrangements between distant mitochondrial fragments have been demonstrated (8), among which one rearrangement involved mitochondrial regions encoding and surrounding the LSU rRNA and SSU rRNA.

This rearrangement prompted us to investigate the molecular organisation of both rRNA genes. Indeed, rRNAs by their structural complexity and evolutionary dimension are the most investigated molecules in eukaryotes, prokaryotes and organelles. The *A.aegerita* SSU rRNA gene sequence revealed the unusual occurrence of a group I intron within its sequence (3). Moreover, previous studies on *A.aegerita* mitochondrial *cox1* gene showed that it is split by four large intronic sequences. In this way, in Ascomycota, algae and protozoa, most mitochondrial LSU rDNAs have been reported to possess introns (9). To date, no complete sequence of a mitochondrial LSU rDNA has been available for the Basidiomycota division. Thus, determination of the mitochondrial LSU rDNA sequence of *A.aegerita* is of great interest; to improve our knowledge of the organisation of Basidiomycota genes, and to provide more information on the occurrence of intronic sequences within the mitochondrial genes of *A.aegerita*.

We report here the complete sequence of the *A.aegerita* mitochondrial LSU rRNA gene. The secondary structures of this rRNA and of its eight introns were derived, and the molecular organisation of the gene compared with previously described fungal mitochondrial LSU rRNA genes. The location of each intron has been compared with those of previously reported mitochondrial LSU rDNA introns in fungi, algae and protozoa. The unusually long nucleotide extensions found in domains I and

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V were compared with those present in the LSU rRNA of other organisms. The occurrence of so many intronic sequences and the putative origin of some of them are discussed.

MATERIALS AND METHODS

Strain and culture

The wild-type *A.aegerita* strain SM 47 used in this study is conserved in the collection of the Laboratory of Molecular Genetics and Breeding of Cultivated Mushrooms, and was obtained by sub-culture on CYM solid medium (10) of a fragment of a wild basidiocarp collected from Agen (France). The *Escherichia coli* strain JM83 (11) used for cloning was cultured in LB medium (12).

Cloning of the mitochondrial fragments in pGEM7 Z f(+) plasmid vector

The 5′ part of the mitochondrial LSU rDNA was previously located on a cloned *Hin*dIII fragment by heterologous hybridisation with a part of the *E.coli* rrnB operon (5). For the first step of the chromosome walking, the *A.aegerita* SM 47 strain mtDNA was purified on a CsCl gradient in the presence of bisbenzimide, according to Fukumasa-Nakai *et al*. (13). *Sca*I, *Hae*III, *Kpn*I, *Bam*HI and *Xba*I libraries representatives of the *A.aegerita* mtDNA were constructed in *E.coli* JM83 at appropriate sites of the pGEM7 Z f(+) vector using a conventional cloning procedure (12). From these libraries, mitochondrial fragments carrying the LSU rRNA gene were isolated by hybridisations on colonies using previously cloned overlapping fragments as probes.

DNA labelling and hybridisations

The recombinant plasmids (100 ng) used as probes were digested by the appropriate restriction endonucleases, then labelled with 925 kBq of [α-^{32}P]dCTP (110 TBq/mmol, Amersham, UK) using the Random Primers DNA Labelling Kit (Promega Corp., Madison, WI). All the probes had a specific radioactivity >10^8 c.p.m./µg DNA.

Southern hybridisations of *A.aegerita* total DNA with cloned probes were carried out as previously described (6).

Colony hybridisations

Colony hybridisations were performed on clones of each mtDNA library according to the method described by Salvado and Labarère (14). The mitochondrial fragments used as probes were recovered after digestion of the appropriate recombinant plasmids and electrophoresis in a 0.8% (w/v) Nusieve GTG agarose gel (FMC BioProduct, Rockland, ME), using a Geneclean Kit (Bio 101 Inc., CA), before being radio-labelled.

DNA sequencing

The mitochondrial inserts were sub-cloned in both orientations in the pGEM7 Z f(+) plasmid vector, then processed to generate nested deletions using the Erase-a-base system, according to the manufacturer's recommendations (Promega Corp., Madison, WI). Recombinant plasmids were purified from *E.coli* JM83 clones by a conventional miniprep method (12). Sequences were determined on both strands by using the M13-40 primer, the M13 reverse primer or specific 18mer oligonucleotides (Eurogentec, Belgium). The sequencing reactions were performed using the dideoxy-chain termination method (15), with the Sequenase II (United States Biochemical Corp., Cleveland, OH) and 185 kBq [α-^{35}S]dATP (>37 TBq/mM, Amersham, UK). The labelled fragments were denatured, separated by electrophoresis on 6% (w/v) polyacrylamide gels and revealed by autoradiography. Sequence analyses were performed with the DNA Strider 1.2 software. Comparisons with sequences of the GenBank and EMBL databases were performed using the search algorithm BLAST (16). Alignments of nucleotidic and proteic sequences were carried out with CLUSTAL W package (17) and PILEUP software from GCG Wisconsing Package.

RESULTS

Molecular cloning and sequencing of the *A.aegerita* mitochondrial LSU rDNA

Heterologous hybridisations with restriction fragments from the *E.coli* rrnB operon (5) have shown that the *A.aegerita* mitochondrial LSU rRNA gene was carried by a cloned *Hin*dIII fragment named H_1-LSU. Comparison of the complete sequence (3413 nt) of this fragment with GenBank and EMBL databases revealed that it possessed only the central part of the *A.aegerita* mitochondrial LSU rDNA. To determine the complete sequence of this gene, a chromosome walking approach was then used from both boundaries of the H_1-LSU fragment.

The size of overlapping fragments was determined by Southern hybridisation of the *A.aegerita* total DNA with probes constituted by Exonuclease III-generated 500 nt long sequences located on both sides of the H_1-LSU fragment. The overlapping restriction fragments, of a size allowing their molecular cloning, were selected and isolated from corresponding mitochondrial *A.aegerita* libraries by colony hybridisations.

The complete mitochondrial LSU rDNA was carried by seven overlapping restriction fragments obtained after two or four steps of chromosome walking, in the 3′ and 5′ sides of the H_1-LSU sequence, respectively (Fig. 1).

The seven mitochondrial inserts were sequenced on both strands. The resulting sequence (accession no. AF087656), from the *Ava*II site of the AS-LSU fragment to the *Hin*dIII site of the H_2-LSU fragment, had a total size of 14 061 nt representing about one-fifth of the total *A.aegerita* mitochondrial genome.

Characterisation of eight intronic sequences within the mitochondrial LSU rRNA gene

Comparison of the complete LSU rDNA sequence of *A.aegerita* with GenBank and EMBL databases indicated two types of sequences (Fig. 1): (i) sequences (E1–E9) possessing >50% identity with previously described partial exonic sequences of Basidiomycota mitochondrial LSU rDNAs (18) or with bacterial LSU rDNAs such as *Rhospirillum rubrum* and *Zoogloea ramigera* (19); (ii) sequences (i1–i8) possessing >60% identity with group I or group II introns from Ascomycota, plant, algae, protozoa and bacteria. This sequence comparison suggested that the mitochondrial LSU rDNA of *A.aegerita* possessed eight intronic sequences. The intron boundaries were deduced from the intron secondary structures. Seven introns belonged to group I (i1, i2, i3, i4, i6, i7 and i8) and possessed canonical conserved sequences P, Q, R and S of the subgroup IA or IB intron cores (20). The exact 5′ and 3′ ends of these introns were determined

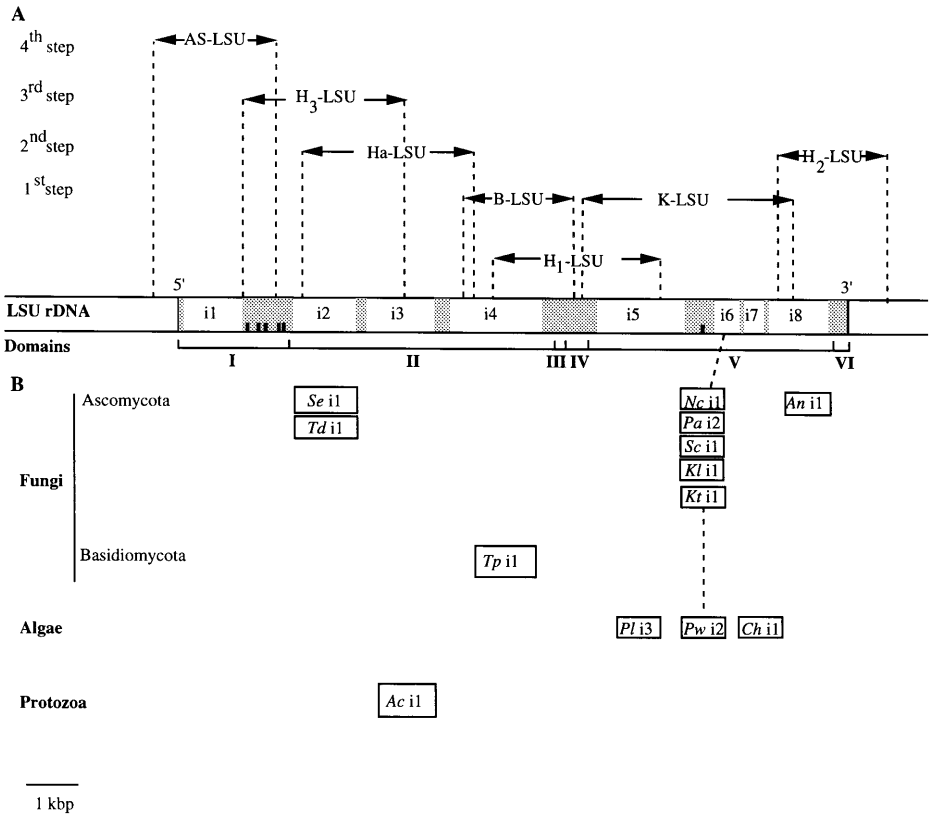


Figure 1. (**A**) Molecular organisation of the *A.aegerita* mtDNA encoding the LSU rRNA. Inserts and cloned fragments used for sequencing are indicated. Exonic sequences (E1–E9) are indicated in grey. The locations within the gene of the six nucleotide extensions are represented by small black boxes. (**B**) Introns possessing the same location as *A.aegerita* LSU rDNA introns. *An*, *Aspergillus nidulans* (38); *Ac*, *Acanthamoeba castellanii* (39); *Ch*, *Chlamydomonas humicola* (40); *Kl*, *Kluyveromyces lactis* (31); *Kt*, *Kluyveromyces thermotolerans* (31); *Nc*, *N.crassa* (9); *Pa*, *P.anserina* (9); *Pl*, *P.littoralis* (36); *Pw*, *P.whickeramii* (9); *Sc*, *S.cerevisiae* (9); *Se*, *Saccharomyces exiguus* (accession no. AJ229047); *Td*, *Torulaspora delbrueckii* (accession no. AJ229052); *Tp*, *Trimorphomyces papiliomaceus* (30). All these introns are mitochondrial LSU rDNA introns except that of *C.humicola*, which is a chloroplast intron.

from the establishment of the Internal Guide Sequence (IGS) constituted by an alternative base-pairing between a sequence located on the 3′ side of the P1 helix and the sequence immediately downstream of the P9 structure (Fig. 2).

The seven group I introns possessed sizes of 1155 bp (nt 32–1186), 1258 bp (nt 2242–3499), 1463 bp (nt 3675–5137), 1931 bp (nt 5479–7409), 543 bp (nt 10 980–11 522), 369 bp (nt 11 574–11 942) and 1186 bp (nt 12 027–13 212) for i1, i2, i3, i4, i6, i7 and i8, respectively.

The presence of the P2 helix and of an additional helix between the conserved sequence S and the 3′ part of the P3 helix showed that i6 and i8 belong to subgroup IA1. The presence of the P2 helix and of two additional helices between the conserved sequence S and the 3′ part of the P3 helix, in i2 and i3, were characteristic of subgroup IA3 introns. The lack of the P2 helix and an extensive P5 loop showed that i1 and i4 belonged to subgroup IB2. Owing to the presence of the P2 helix and of a large P5 loop, i7 was a subgroup IB4 intron (Fig. 2).

Searching for open reading frames (ORFs) in these seven group I introns showed that three of them (i3, i4 and i8) could encode for a putative protein. Translation of the ORFs was performed using the *Neurospora crassa* mitochondrial genetic code. Indeed, previous studies of the mitochondrial genome of *A.aegerita* have shown that in this species the UGA codon is not a stop codon and could putatively encode for a tryptophan amino acid (3). The *A.aegerita* introns i3, i4 and i8 encoded for a protein of 326, 298 and 137 amino acids, respectively. All these proteins possessed two LAGLI-DADG motifs characteristic of the endonucleases involved in intron I mobility (21). Each of the three coding sequences were contained in a loop of the intron secondary structure: i3 in the loop of the P8 helix, i4 in those of the P9 helix and i8 in the loop of the P1 helix.

Beside these seven group I introns, the *A.aegerita* i5 was found to belong to group II introns, as confirmed by its secondary structure (Fig. 3) corresponding to the group II intron model (22). The presence of the conserved motif AARC in the $C1^{(ii)}$–$C1^{(i)}$ internal loop, the bulging A on the 3′ side of the hairpin VI and the usual RAY intron end showed that i5 belonged to subgroup IIB1 and had a total size of 1784 bp (nt 10 230–12 013). Searching for an ORF revealed two short sequences encoding for putative proteins of 120 and 137 amino acids, respectively. Comparison using the search algorithm BLAST revealed no homology with reverse transcriptase-like proteins usually encoded by group II introns (21), suggesting that the two ORFs evidenced in *A.aegerita* i5 could simply result from a random distribution of codons.

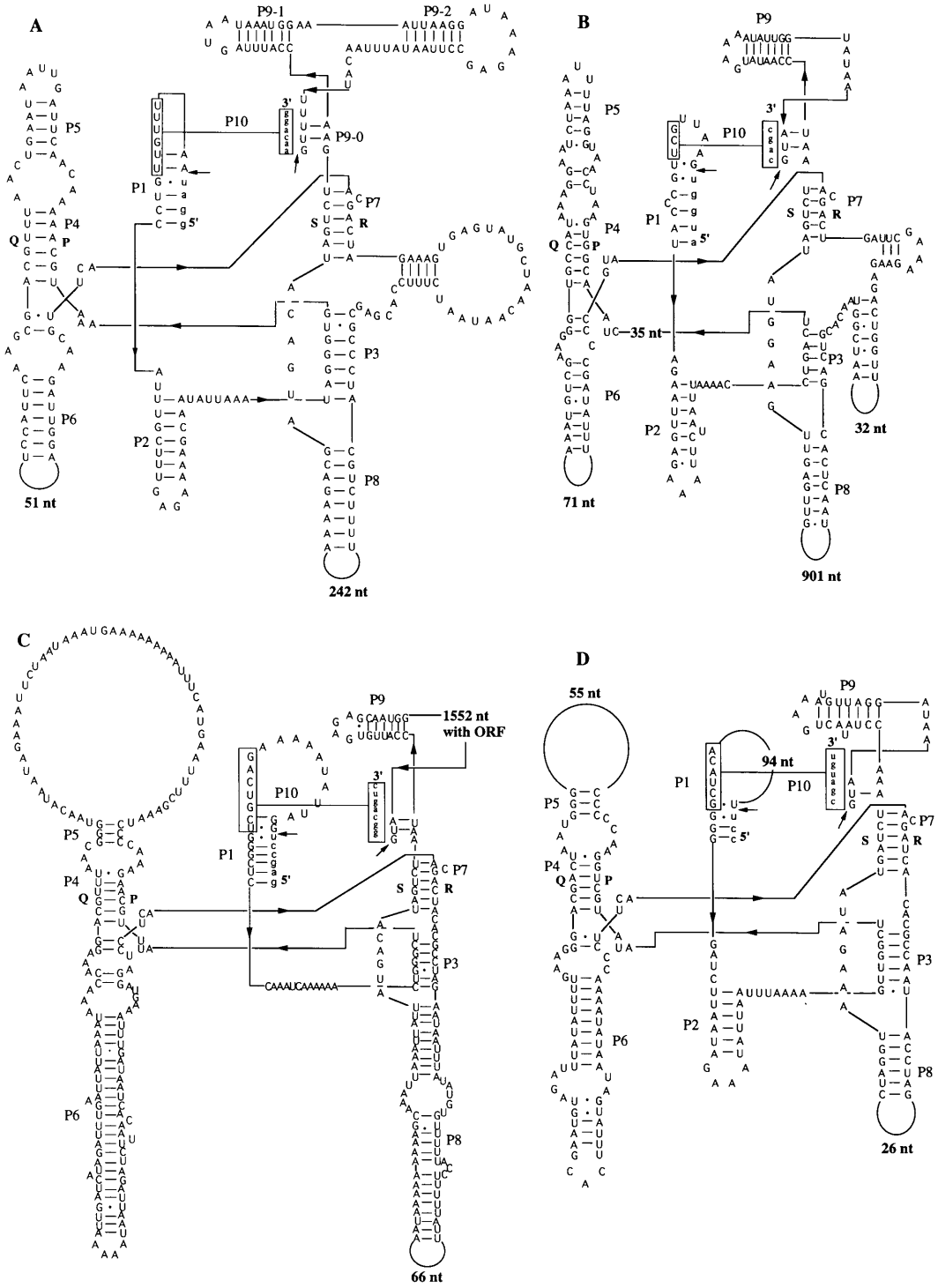


Figure 2. An example of each type of group I secondary structures found in the LSU rDNA of *A.aegerita*: (**A**) subgroup IA1 (i6), (**B**) subgroup IA3 (i2), (**C**) subgroup IB2 (i4) and (**D**) subgroup IB4 (i7). The conserved sequences (P, Q, R and S) of the group I intron core and the helices noted P1–P9 are indicated (41). The IGS sequences constituting helix P10 are framed. Putative splice sites at 5′ and 3′ boundaries are shown by arrows.

Molecular gene organisation and secondary structure of the mitochondrial LSU rRNA

The sequence of the mitochondrial LSU rRNA of *A.aegerita* was obtained after removing the eight intronic sequences described above. The secondary structure of the LSU rRNA was determined by comparison with the previously described secondary structures of *E.coli* (23) and of the Ascomycota *Saccharomyces cerevisiae* (24). All the characteristic helices and loops found in these two models were recovered in the mitochondrial LSU rRNA of

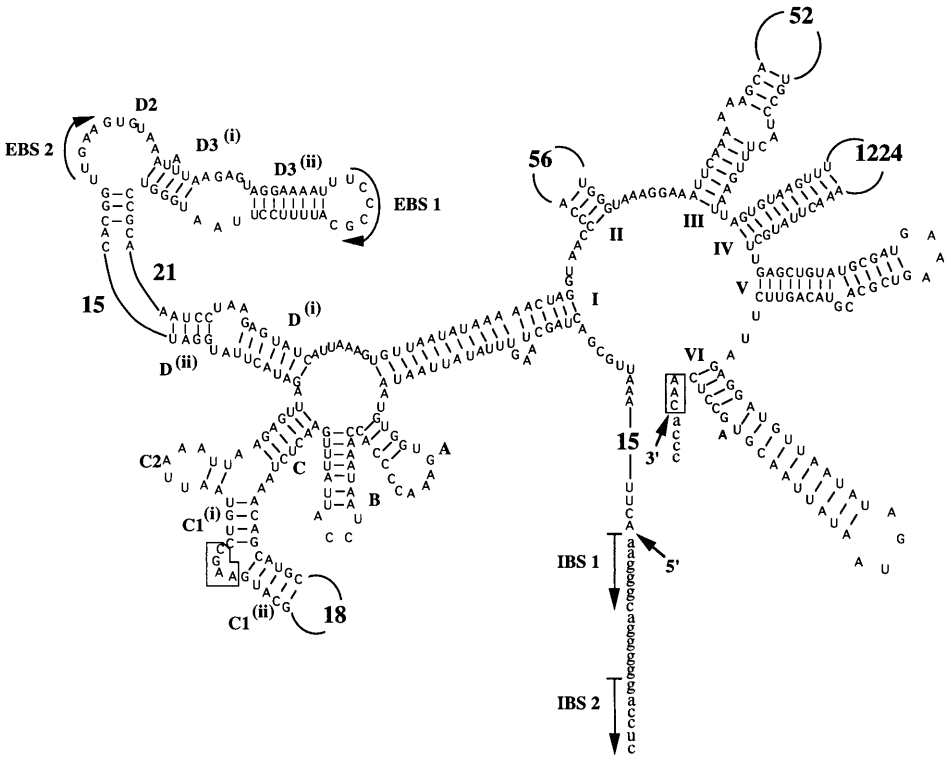


Figure 3. Secondary structure of the group II intron (i5) of the *A.aegerita* mitochondrial LSU rDNA. Domains and subdomains are numbered according to the conventional nomenclature (22). Intron binding sites (IBS) and exon binding sites (EBS) are indicated by arrows. Conserved motifs AARC in subdomain C and RAY at the 3′ end are framed. The bulged A in the helix VI is in bold.

A.aegerita (Fig. 4A and B), and constituted the six classical domains numbered according to the conventional nomenclature (25). The secondary structure allowed the precise determination of the 5′ and 3′ ends and of the complete rRNA size, i.e. 3837 nt.

Interestingly, the secondary structure of the *A.aegerita* LSU rRNA revealed some specific characteristics. Indeed, six long and unusual nucleotidic extensions were found within two domains: domain I for the first five sequences, and domain V for the last one (Fig. 4A and B). These sequences had a size of 40 bases (nt 1272–1311), 193 bases (nt 1463–1665), 115 bases (nt 1707–1821), 108 bases (nt 1927–2034), 109 bases (nt 2039–2147) and 241 bases (nt 10556–10796), respectively.

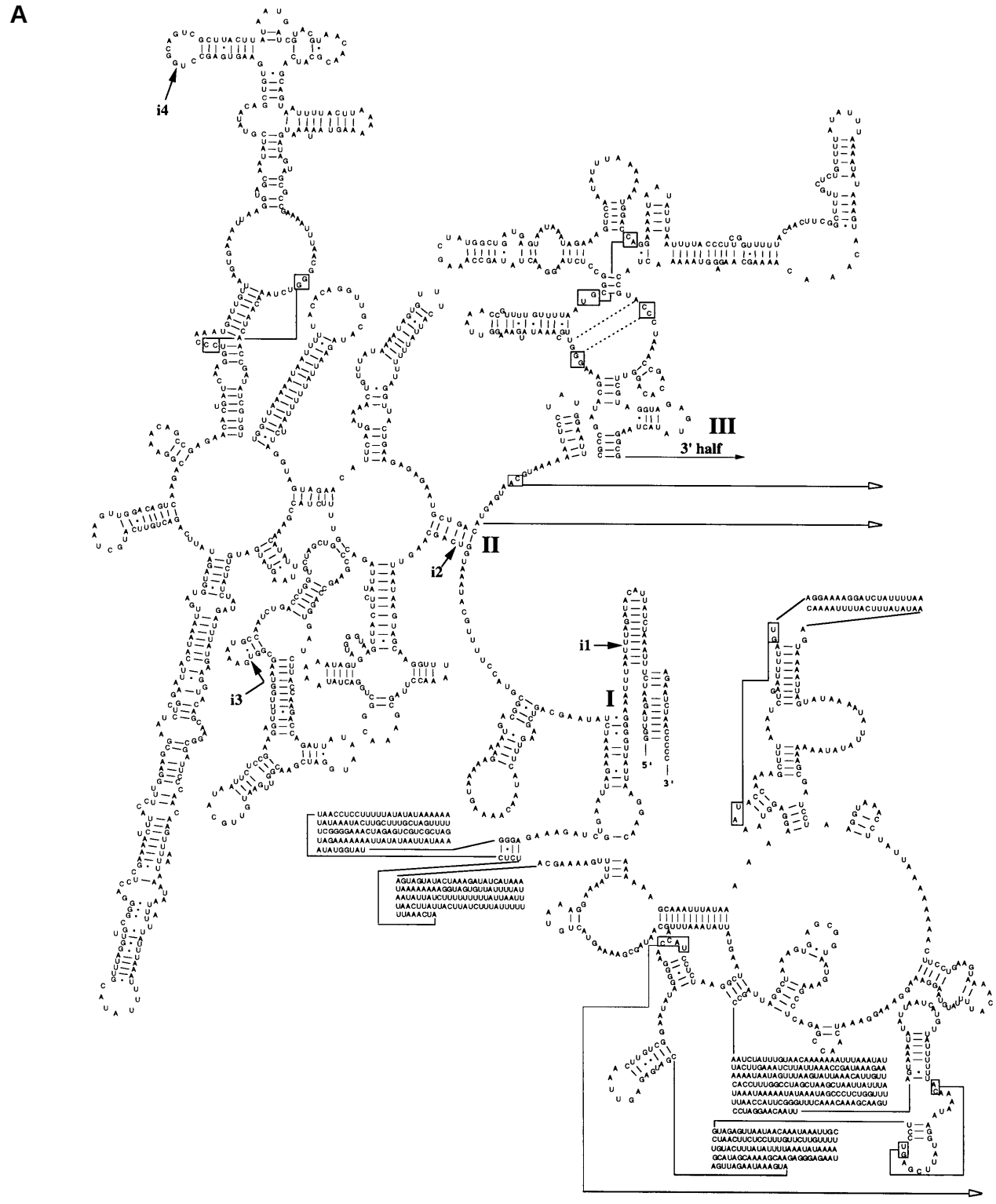
Comparison with the GenBank and EMBL databases showed that the *A.aegerita* mitochondrial LSU rRNA was homologous to partial sequences from other Basidiomycota species overlapping the domains IV and V. Alignment of these partial sequences with the corresponding sequence of *A.aegerita* (763 bases; nt 8259–8554 joined to nt 10 339–10 615), indicated high percentages of identity ranging from 68% with the Boletale *Tapinella panuoides* (18) to 94% with the Agaricale *Laccaria laccata*. Moreover, the nucleotide extension found in the domain V of *A.aegerita* (241 nt) was homologous to the extension (141 nt) located at the same site in the *L.laccata* LSU rRNA (accession no. AF006486).

Establishment of the secondary structure of the LSU rRNA allowed the accurate location of each intron within this molecule. The location of the seven group I introns was compared with the location of previously reported LSU rDNA introns. From this comparison, the i1 intron of *A.aegerita* occupied a position in domain I, not reported so far in other organisms, while the other introns were inserted at sites previously described for fungal, algal or protozoan mitochondrial LSU rDNA introns (Fig. 1).

DISCUSSION

The complete sequence of the mitochondrial LSU rDNA from the Basidiomycota *A.aegerita* constitutes the first report of such a gene in this fungal division. This gene extends for 13 526 bp and is, to our knowledge, the longest mitochondrial LSU rDNA reported so far. This unusual length is due mainly to the presence of six nucleotide extensions (adding 806 nt to the usual 2900 nt of LSU rRNA), as well as to the occurrence of eight intronic sequences within the gene.

The six nucleotide extensions were located in two domains: five in domain I and one in domain V. It should be noted that such extensions were previously reported in the LSU rRNAs of the Ascomycota *S.cerevisiae* (24) and of the algae *Prothoteca whickeramii* (26). In these two species, the additional sequences are also located at the same sites in the domains I and V, suggesting that length variations occurring in these two domains would have no effect on the mitochondrial ribosome activity. It should be noted that the nucleotide extensions recovered in different organisms were highly different in size and sequence. For example, in the mitochondrial LSU rRNA of *P.whickeramii*, the two nucleotide extensions of 75 and 163 nt found in domain I were located at the same site as those of 193 and 115 nt of *A.aegerita*, respectively. In this way, previous studies of the *E.coli*



23S rRNA showed that LSU rRNAs with variable stem lengths are able to enter freely into polysomes (27).

Beside these extensions, the large size of the LSU rDNA of *A.aegerita* is mainly due to the occurrence of seven large group I introns and one group II intron, representing ~73% of the total gene size. In contrast, most mitochondrial LSU rDNAs of other fungi possessed no introns or only a few (up to two) (9). Hence, *A.aegerita* appears to be the most intron-rich LSU rDNA reported to date. Four introns (i5–i8) are located in the domain V associated with the peptidyl transferase activity (28). Interestingly, it should be noted that most fungal introns reported so far in LSU rDNAs are located in this domain (9). In most fungal species, the single intron of the LSU rDNA possesses the same location and homologies with the *A.aegerita* i6. The presence of this intron in

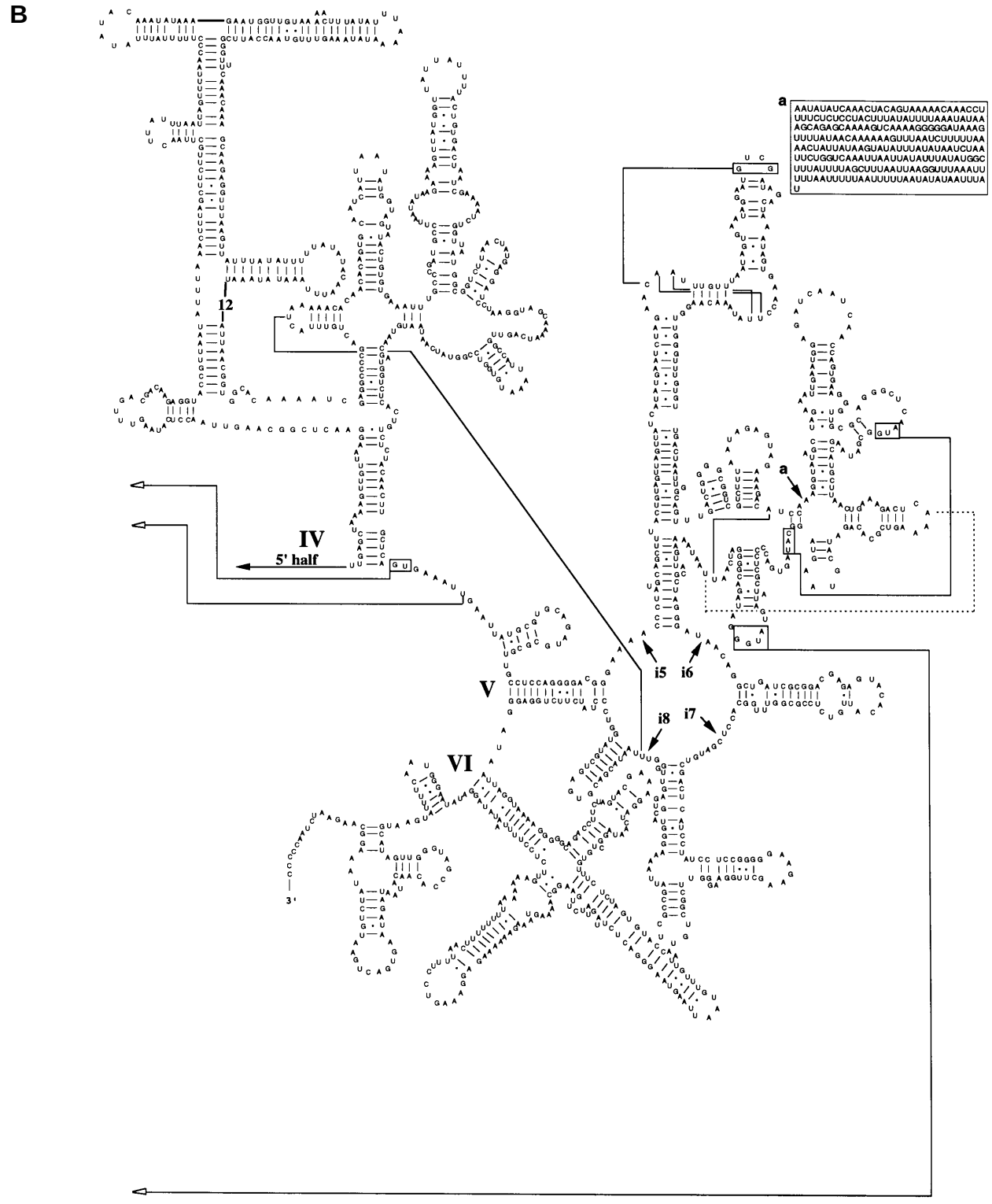


Figure 4. Secondary structure of the mitochondrial LSU rRNA of the cultivated Basidiomycota *A.aegerita*. (**A**) (Previous page) 5′ part; (**B**) 3′ part. The name of each intron is indicated and their locations within the gene shown by arrows.

numerous fungal species could reveal an intron lateral transfer between Ascomycota and Basidiomycota species. Another possibility is that this intron is a remnant of an intron already present in the common ancestor of both Basidiomycota and Ascomycota. Lateral transfers of group I introns have been previously reported between species of the same kingdom and between species belonging to two different kingdoms. Indeed, a lateral transfer involving a group I intron of the *cox1* gene was reported between the

land plant *Peperomia polybotrya* and an Ascomycota fungal donor (29). Lateral transfers between Ascomycota and Basidiomycota species have been previously suggested between the *A.aegerita* and the *Sclerotinia sclerotiorum* SSU rDNA introns (3) or between the *A.aegerita* i4 and *Podospora anserina* i14 *cox1* introns (4). However, the lack in *A.aegerita* i6 of an ORF encoding for an endonuclease classically involved in the spreading and homing of group I introns (21) is rather in favour of the inheritance from a common ancestor than of an intron transfer. In this case, the intron-less species would result from an intron loss.

Contrasting with the wide distribution of the i6 intron, introns homologous to the other *A.aegerita* introns were rarely found in previously reported mitochondrial LSU rDNAs (9,30,31). Hence, i3 location was only encountered in protozoa, and i5 and i7 only present in algae mitochondrial or chloroplastic LSU rDNAs. In the same way, i4 was recovered in only one Basidiomycota species, and i2 and i8 in only one or two Ascomycota species. In contrast, the *A.aegerita* i1 location constitutes a new insertion site not yet described.

Beside these widespread group I introns, the occurrence of group II introns is quite unusual within mitochondrial genes. In fungi, such introns have been identified in the *cox1* and *cytb* genes of *S.cerevisiae* (32,33), the *cox1* and *nd5* genes of *P.anserina* (34) or in the *cytb* of *Schizosaccharomyces pombe* (35). Therefore, to our knowledge, the *A.aegerita* i5 represents the first report of a group II intron in a fungal mitochondrial LSU rDNA. However, intronic sequences of this type have already been described in the mitochondrial LSU rDNA of the brown algae *Pilayella littoralis* (36), which possesses four subgroup IIB introns.

The location specificity of group I and group II introns suggests that introns are not randomly scattered within the LSU rDNAs among the different kingdoms, and that only a few sites can be occupied by these mobile genetic elements. It should be noted that domain V possessed the highest number of introns putative locations.

Interestingly, studies of the mitochondrial genes of the Basidiomycota *A.aegerita* reported so far have shown that these genes were intron-rich. Indeed, one group I intron has been found in the SSU rDNA (3) and four in the *cox1* gene (4). Recently, it has been shown that the number and the location of introns within the nuclear *ABH1* gene of *Agaricus bisporus* seemed to have an effect on the level of gene expression (37). Therefore, by analogy, it could be hypothesised that mitochondrial introns could influence the transcription level of some *A.aegerita* mitochondrial genes.

In conclusion, our results show that the mitochondrial LSU rDNA from the basidiomycota *A.aegerita* is of an uncommonly large size due to the presence of a high number of introns and the occurrence of six nucleotide extensions. Future prospects would have to investigate if these characteristics are specific of *A.aegerita* or widespread in the Basidiomycota division. This raises the question of the putative role of intronic sequences. In this way, it would be of great interest to study if the transcript level of the mitochondrial genes is correlated with the number and the location of these mobile genetic elements.

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