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Mitochondrial tRNAs in the lower fungus *Spizellomyces punctatus*: tRNA editing and UAG 'stop' codons recognized as leucine

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ABSTRACT

The mitochondrial DNA of the chytridiomycete fungus *Spizellomyces punctatus* encodes only eight tRNAs, although a minimal set of 24–25 tRNAs is normally found in fungi. One of these tRNAs has a CAU anticodon and is structurally related to leucine tRNAs, which would permit the translation of the UAG 'stop' codons that occur in most of its protein genes. The predicted structures of all *S.punctatus* tRNAs have the common feature of containing one to three mis-pairings in the first three positions of their acceptor stems. Such mis-pairing is expected to impair proper folding and processing of tRNAs from their precursors. Five of these eight RNAs were shown to be edited at the RNA level, in the 5′ portion of the molecules. These changes include both pyrimidine to purine and A to G substitutions that restore normal pairing in the acceptor stem. Editing was not found at other positions of the tRNAs, or in the mitochondrial mRNAs of *S.punctatus*. While tRNA editing has not been observed in other fungi, the editing pattern in *S.punctatus* is virtually identical to that described in the amoeboid protozoan *Acanthamoeba castellanii*. If this type of mitochondrial tRNA editing has originated from their common ancestor, one has to assume that it was independently lost in plants, animals and in most fungi. Alternatively, editing might have evolved independently, or the genes coding for the components of the editing machinery were laterally transferred.

INTRODUCTION

Recently, an increasing number of very different RNA editing mechanisms has been discovered in eukaryotes, mostly in mitochondrial systems. Post-transcriptional insertions and deletions of uridine residues were the first to be detected in the mitochondrial RNAs of kinetoplastid protozoa (1,2). Editing in kinetoplastids has provided examples of the most extensive RNA editing, to the point that the corresponding genes can no longer be recognized by comparative sequence analysis (3–5). The editing machinery of kinetoplastid protozoa consists of a ribonucleoprotein complex, and the information that permits specific insertion or deletion of nucleotides is provided by short guide RNA (gRNA) molecules (6,7). The RNA editing that occurs in the mitochondria of the slime mold *Physarum polycephalum* appears mechanistically unrelated to editing in kinetoplastids, consisting of preferential insertion of single nucleotides and (less frequently) of dinucleotides (8,9). The specificity of the process directing these insertions remains unexplained because gRNAs have not been identified in the mitochondria of *Physarum*, and neither conserved sequence motifs nor structural features of the RNAs in proximity to the editing sites have been noted. Plant mitochondrial and chloroplast RNA editing consists of pyrimidine (mainly C-to-U) conversions (10,11), which seem to occur within a particular sequence context. It remains unclear how sequence context determines the specificity of editing and if it is the only source of information for this process (12–14). Recently, two distinct forms of tRNA editing were found in the mitochondria of Metazoa. In a marsupial, $tRNA^{Asp}$ is edited at the second anticodon position by a C-to-U conversion which creates the expected asparagine anticodon (15). In land snails, several tRNAs are edited in the 3′ half of the acceptor stems, possibly by polyadenylation following the removal of the 3′ nucleotides of the mismatched regions (16).

RNA editing is not confined to organelles of eukaryotes, but has been also discovered in several transcripts of nuclear genes. The editing of the glutamate ion channel receptor mRNA in mammalian brain consists of an A to I conversion, most probably catalyzed by a double-stranded RNA-dependent adenosine deaminase. The base pairings between the sequence surrounding an editing site and adjacent intron sequences are essential for the specificity and efficiency of editing at this site (17–19). Hepatitis delta virus (HDV) RNA is also edited by an A to I conversion mechanism which is provided by the host cells and requires a double stranded region centered at the editing site in addition to some primary sequence specificity (19,20). The mammalian apolipoprotein B mRNA is an example of RNA editing at a single position which changes a C to a U. The editing reaction is a site-specific cytidine deamination, which creates an in-frame termination codon and allows the production of two functionally different proteins, Apo100 and Apo48, from the unedited and edited mRNA, respectively (21,22). The specificity and efficiency of *apoB*

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mRNA editing is provided by a sequence of 22 nucleotides (nt) surrounding the editing site (23). The human NF-1 mRNA is edited by a single C-to-U conversion, which creates a stop codon, and which seems to be directed by a sequence motif similar to the one in *apoB* mRNA (24). The Wilm's tumour susceptibility (WT-1) mRNA (25) is edited by U-to-C conversion mechanism, the reverse of *apoB* mRNA editing.

Recently, a novel editing mechanism was found in mitochondria of the amoeboid protozoan, *Acanthamoeba castellanii* (26). This post-transcriptional editing consists of both pyrimidine-to-purine transversions (U-to-A or U-to-G) and A-to-G transitions, which occurs at the first 3 bp of tRNA acceptor stems. In this article we describe a tRNA editing mechanism in the mitochondria of the chytridiomycete fungus *S.punctatus* that is seemingly identical to that originally described in the case of *A.castellanii* mitochondria.

MATERIALS AND METHODS

Strains, cultures, preparation and cloning of mtDNA from *Spizellomyces punctatus, Rhizophlyctis rosea* and '*Rhizophydium* sp.'

The strains *Spizellomyces punctatus* BR117 (27) and *Rhizophlyctis rosea* BR186 were kindly provided by Dr D. Barr (Agriculture Canada, Ottawa). The strain *Rhizophydium sp.* was obtained from the Carolina Biological Supply Company (strain 15-6220). An analysis of its morphological characters showed that this isolate is likely not a Chytridiales but a member of the Spizellomycetales, probably in the genera *Spizellomyces* (J. Longcore, Maine University, personal communication). Therefore, its name appears in quotation marks ('*Rhizophydium sp.*') in this article. *S.punctatus* and '*Rhizophydium sp.*' were grown on a medium containing 0.5% yeast extract, 3% dextrose or glycerol (pH 5.8 adjusted with KH_2PO_4). *R.rosea* was grown on the same medium, supplemented with 1.7% yeast nitrogen base w/o amino acids and ammonium sulphate (Difco). Cell culture, purification of mtDNA, by cesium chloride/bisbenzimide density gradient centrifugation of total cellular DNA, and cloning procedures were performed according to previously published protocols (28).

Sequencing of mtDNA

The *S.punctatus cox1* and tRNA genes were sequenced from a random clone library (http://megasun.bch.umontreal.ca/ogmp/ssdna.html) in a Bluescript KS+ vector (Stratagene). Sequencing of single-stranded DNA templates was performed by the dideoxy chain termination method (29). To permit extended reading of sequences, high-resolution polyacrylamide-gel electrophoresis was performed (30). Both DNA strands were sequenced. The partial *cox1* sequences of *R.rosea* and '*Rhizophydium sp.*' were amplified with specific oligonucleotides that match highly conserved domains of the *cox1* gene. The PCR fragments were cloned and several independent clones were sequenced on both strands. No sequence polymorphisms caused by potential DNA amplification errors were detected.

Phylogenetic analysis

The COX1 sequences of *Marchantia polymorpha* (Acc. number M68929), *Prototheca wickerhamii* (Acc. number U02970), *Allomyces macrogynus* (Acc. number U41288), *Aspergillus nidulans* (Acc. number X00790) and *Schizosaccharomyces pombe* (Acc. number X54421) were obtained from GenBank. A collection of all protein sequences used in the phylogenetic analysis can be obtained via the WWW at http://megasun.bch.umontreal.ca/people/lang/FMGP/proteins.html. All amino acid positions of the various COX1 sequences could be aligned without ambiguities, and were used for the analysis. The data were analyzed by a distance approach (PROTDIST, FITCH or NEIGHBOR) (31,32) and submitted to bootstrap analysis (31). Alternative trees (not shown) were constructed with a maximum likelihood algorithm (PROTML) (33), using an exhaustive tree optimisation procedure with the -jf parameters. No difference was observed between the trees constructed either by the two distance approaches (using either FITCH or NEIGHBOR), or by the maximum likelihood algorithm.

RNA purification

Cells were disrupted with glass beads (34), and mitochondria were isolated by differential centrifugation, after which they were lysed with 0.5% SDS in the presence of 100 µg/ml proteinase K. Total mitochondrial RNAs were purified from this lysate by repeated phenol–chloroform extractions, followed by two ethanol precipitations. A mitochondrial tRNA fraction was enriched by centrifugation (Beckman SW50 TI rotor, 4 h at 40 000 r.p.m.) of total mitochondrial RNAs through a CsCl cushion (0.3 g CsCl added/ml TE buffer). The upper tRNA-containing fractions of the gradient were identified by agarose gel electrophoresis and were then pooled. The RNA was desalted and concentrated by two ethanol precipitations.

Oligonucleotides

The oligonucleotides used for reverse transcriptase sequencing were either obtained from GSD (Toronto, Canada) or from the oligonucleotide synthesis service in the Department of Biochemistry at the University of Montreal:

(Lys) GSD 940-311:	5′-TAGGAGTGACAGGACTTGAACC-3′
(Leu) GSD 940-312:	5′-TAGACCTGGTGGGACTTGAACC-3′
(Gln) #2766:	5′-GACTCGAACCTGCACATCATGGT-3′
(Met) #2702:	5′-AAGTCCGGGCAGGAGTTGAACC-3′
(Tyr) #2704:	5′-TGTGGGCGGTAGGATCTGAACC-3′

Reverse transcriptase sequencing

The reverse transcriptase (RT) RNA sequencing protocol was adapted from a published procedure (35), with the following modifications: no actinomycin D was added to the sequencing reaction, the final hybridization temperature was lowered to 25°C, and AMV reverse transcriptase (Boehringer) was used for the reaction. DNA was removed from the samples by digestion with DNAse I (Pharmacia, FPLC pure). Sequence ambiguities, caused by the pausing of reverse transcriptase during cDNA synthesis, were resolved by treating the cDNAs with terminal deoxynucleotidyl transferase. The terminal transferase (Promega) reaction was performed as published elsewhere (26). The sequencing reaction products were precipitated before loading on 6, 8 or 10% polyacrylamide gels that were prepared and processed as for DNA sequencing (see above). Gels were run for 2.5–3 h at 37 W (3000 V max). The dried gels were exposed to X-ray film with intensifying screens, for 1–8 days at –70°C.

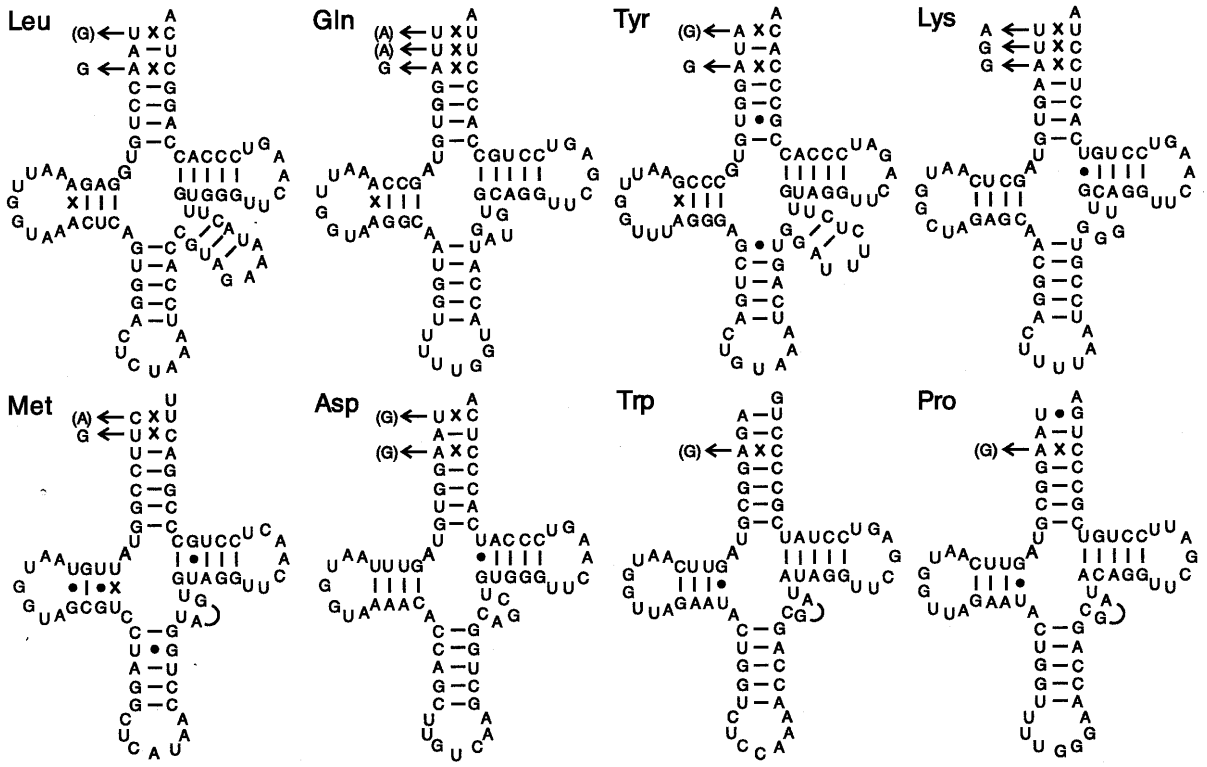

Figure 1. Secondary structures of *S.punctatus* mitochondrial tRNAs, derived from gene sequences. The tRNAs are identified by their assumed amino acid specificity, in three-letter code. The mismatches in the acceptor stems of the tRNAs and the changes necessary to restore a conventional acceptor stem pairing of tRNAs are indicated by arrows. Nucleotide changes that were expected but were not confirmed experimentally are given in parentheses to distinguish them from editing events that were verified by sequencing of the mature tRNAs.

RESULTS

A reduced set of unusual tRNA genes, and UAG codons assigned to leucine in *Spizellomyces* mtDNA

We have sequenced the mtDNA of the chytridiomycete *Spizellomyces punctatus* and have identified eight putative tRNA genes (Fig. 1). On the basis of their anticodon sequences, seven of the predicted tRNAs are expected to recognize lysine, aspartic acid, tryptophan, methionine, tyrosine, glutamine and proline codons. The remaining tRNA (labelled $tRNA^{Leu}$ in Fig. 1) has an anticodon 5′-CUA-3′ which, if unmodified, would recognize UAG stop codons. UAG codons have indeed been found within many reading frames of conserved mitochondrial protein genes (such as *cox1*, *cox2*, *cox3* and *cob)* of *S.punctatus*, as well as in intronic open reading frames (ORFs).

In Figure 2, an alignment of selected regions of the *S.punctatus* mitochondrial COX1 sequence with several mitochondrial homologs shows that the UAG codons found in this gene occur predominantly at positions corresponding to conserved leucine codons. We also sequenced part of the *cox1* genes of two organisms related to *S.punctatus*, *Rhizophlyctis rosea* and '*Rhizophydium sp.*' to determine if they had the same type of codon reassignment. In *R.rosea*, we found five UAG codons within the known stretch of 323 codons which are located in positions different from the *S.punctatus* UAG codons. Also these codons are predominantly located at positions with conserved leucines. In the known *cox1* sequence of '*Rhizophydium sp.*' (153 codons), a single UAG codon is present at a position also found in *S.punctatus.*

A phylogenetic analysis with the COX1 protein sequences shows that *S.punctatus*, '*Rhizophydium sp.*' and *R.rosea* are members of one lineage and that another chytridiomycete, *Allomyces macrogynus*, groups together with several Ascomycetes in a sister lineage (Fig. 3). This phylogenetic grouping is consistent with the hypothesis that the use of UAG (leucine) codons evolved once, prior to the divergence of the two *Spizellomyces* species and *R.rosea*.

The eight tRNA genes of *S.punctatus* are all encoded in the largest of the three circular DNA molecules (58.8, 1.4 and 1.1 kb in size) that constitute its mitochondrial genome (http://megasun.bch.umontreal.ca/People/lang/species/spunc/spunc.html). Only two of these genes, $tRNA^{Pro}$(TGG) and $tRNA^{Trp}$(CCA), could be identified by searching with a specific tRNA search program (TRNASCAN; ref. 36). The other tRNAs do not fold into classical 'cloverleaf' secondary structures (Fig. 1) and were recognized by searching for sequence patterns diagnostic of the highly conserved TψC domain. In common with other mitochondrial tRNA sequences, *S.punctatus* mitochondrial tRNA sequences have changes in otherwise invariant and semi-invariant nucleotide positions (37). A few minor deviations from the structural model were noted in the D-loop regions of $tRNA^{Leu}$, $tRNA^{Met}$ and $tRNA^{Tyr}$, but the most important anomaly is the presence of one to three mismatches at the first three bases pairs of the acceptor stem in all eight tRNAs. The pattern of mis-pairings and the changes necessary to permit the formation of a conserved

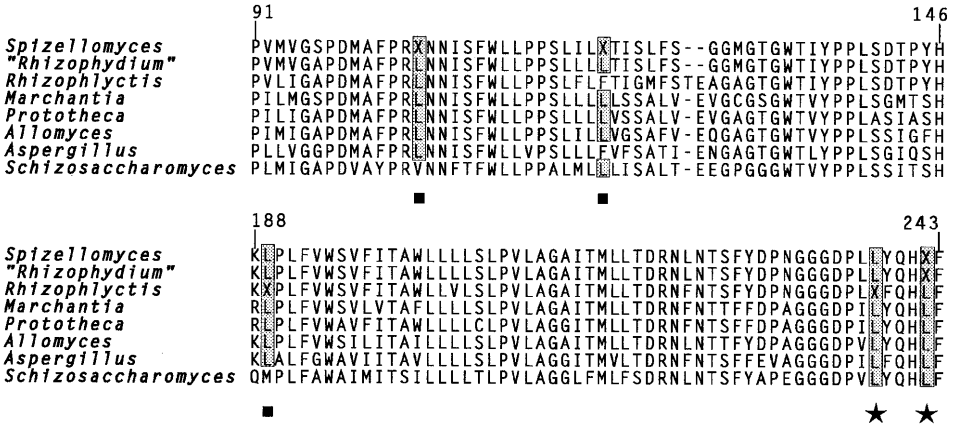

Figure 2. Alignment of *S.punctatus*, '*Rhizophydium sp.*' and *R.rosea* COX1 protein sequences. The numbering of the amino acids follows that of the *S.pombe* sequence. The letter (**X**) within the aligned COX1 sequences indicates the presence of UAG codons. The symbols (★) and (■) in the consensus sequence indicate that a UAG codon in *S.punctatus*, '*Rhizophydium sp.*' or *R.rosea* is located at a position corresponding to leucines in all or most other species respectively.

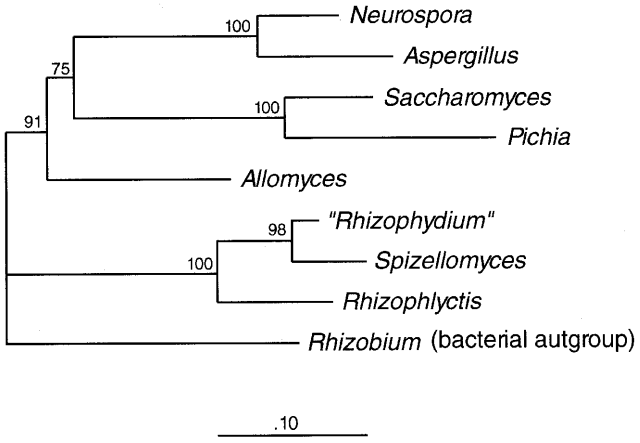

Figure 3. Phylogenetic tree of COX1 protein sequences. This tree was inferred using PROTDIST and FITCH, with its robustness tested by bootstrap analysis (31,32). The percentage of bootstrap support is indicated for each node.

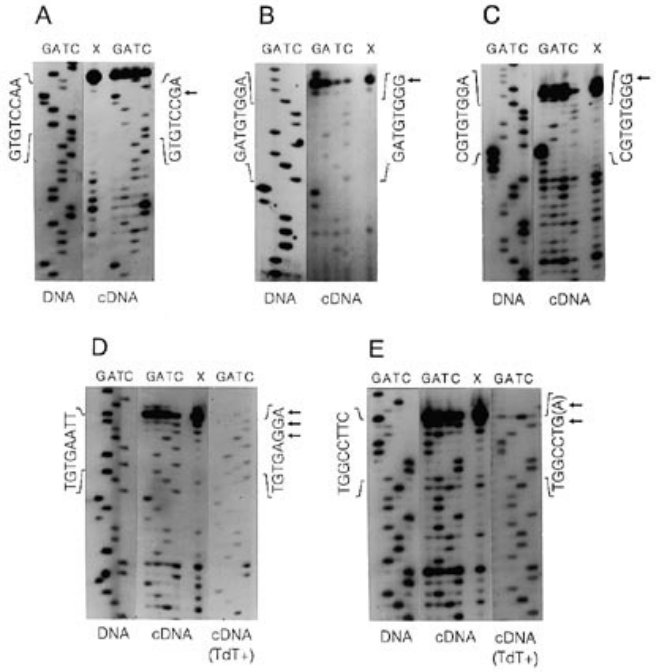

Figure 4. Comparison of tRNA gene and mature tRNA sequences. Sequences are given in the following order: (**A**) $tRNA^{Leu}$, (**B**) $tRNA^{Gln}$, (**C**) $tRNA^{Tyr}$, (**D**) $tRNA^{Lys}$, (**E**) $tRNA^{Met}$. The complements of the sequences actually determined are indicated, reading 5′ to 3′ from top to bottom. The arrows indicate differences between the tRNA and its corresponding gene sequences. In (D) and (E), also the sequencing reactions processed with terminal deoxynucleotidyl transferase are shown (TdT+).

secondary structure is the same as that previously observed in the amoeboid protozoan, *Acanthamoeba castellanii*, whose mitochondrial tRNAs undergo a novel type of tRNA editing (26).

Pattern of tRNA editing in *Spizellomyces* mitochondria

To determine if *S.punctatus* mitochondrial tRNAs are edited as predicted by sequence analysis, we purified the bulk of tRNAs from total mitochondrial RNA, and sequenced five tRNAs directly from this pool using a reverse transcriptase sequencing protocol and gene-specific primers. Primers were chosen for sequencing such that they would bind specifically towards the 3′-end of individual tRNA species. All five gene sequences differ from the corresponding tRNA sequences in at least one of the first three nucleotides at the 5′-end (Fig. 4). We have determined that $tRNA^{Lys}$ is processed from a mitochondrial RNA precursor. Because the RNA precursor is present at a low level in total mitochondrial RNAs, its sequence was obtained by exposing the autoradiographic film for a longer period (results not shown). The sequence of the RNA precursor molecule, extending upstream of the tRNA coding region, was identical to the corresponding mtDNA sequence.

The editing changes in *S.punctatus* mitochondria involve the replacement of pyrimidines or purines by purines, preferentially from A to G (8×), followed by U to G (4×), U to A (3×) and C to A (1×). At the position of a mis-pairing, the nucleotide in the 3′ half of the acceptor stem is always a pyrimidine, predominantly a C, and editing completely restores the conserved secondary structure of the acceptor stems by generating a standard base pair (G·C or A·U; Fig. 1). The mitochondrial tRNA editing in

S.punctatus shows striking similarities to that of *A.castellanii* (26). In both species, purine to pyrimidine as well as G-to-A changes never occur, and the substituting nucleotide is always a purine that is complementary to a pyrimidine in the 3′ half of the acceptor stem. Most importantly, editing in both species is restricted to the first three positions of the acceptor stem. Only minor differences are observed at the level of exchange frequencies. In *S.punctatus*, the second position is the least frequently edited, and seven out of eight A-to-G changes are observed at the third position. In *A.castellanii* the third position is the least frequently edited, and A to G changes occur predominantly at the first position (six out of seven) (26,38,39).

To determine whether other editing sites are present in the tRNAs, we determined their sequences up to nucleotide position 36, by further analyzing the sequencing reaction products on high-percentage polyacrylamide gels. We found that A/A, G/A and U/U mis-pairings in the DU region of the $tRNA^{Leu}$, $tRNA^{Gln}$ and $tRNA^{Tyr}$ genes, and the U·G pair in the acceptor stem of $tRNA^{Tyr}$ are not edited, an observation also made for *A.castellanii* tRNAs. Also the anticodon of $tRNA^{Leu}$ remains unchanged. Partial sequencing of the *cox1* and *cob* mRNAs shows that *S.punctatus* mitochondrial mRNAs contain the UAG codons found in the gene sequences (all four UAG codons in a total of 1035 nucleotides of mRNA sequence; results not shown). Thus, editing of transcripts in *S.punctatus* mitochondria appears to be restricted to the first three positions in the tRNAs.

DISCUSSION

A reduced number of tRNA genes and a tRNA that recognizes UAG 'stop' codons

Usually, mtDNAs of fungi code for 24 or more tRNAs, a set that is sufficient to read all codons in mitochondrial protein genes, assuming that the mitochondrion-specific codon recognition rules are followed (for a review, see 37,40). The lower fungus *S.punctatus* is an exception in that its mtDNA encodes only eight tRNAs. Consequently, the majority of its mitochondrial tRNAs have to be imported into the mitochondria (other examples that provide precedents are the green alga *Chlamydomonas reinhardtii*, the ciliate *Tetrahymena pyriformis*, plants and kinetoplastid protozoa; 41,42).

A special requirement of the *S.punctatus* mitochondrial translation system is the recognition of UAG 'stop' as leucine codons. We have shown that these codons are not changed by mRNA editing (data not shown). Consequently, UAG codons have to be recognized by a tRNA with a CUA anticodon, which, as expected, is one of the tRNAs encoded in the *S.punctatus* mtDNA. This tRNA has a long extra arm (Fig. 1) characteristic of leucine tRNAs, corroborating both its assignment as a leucine tRNA as well as its possible evolutionary origin from a leucine tRNA. This modification of the genetic code is found in the *cox1* gene of '*Rhizophydium* sp.' and *R.rosea*, which are phylogenetically related to *S.punctatus* (Figs 2 and 3). This codon reassignment does not exist in other fungal mitochondria. Very recently, UAG sense codons were also found in mitochondria of several chlorophyceae (43), which likely code for leucines in some instances and alanine in others. The nature of the corresponding two types of tRNAs that would recognize UAG codons remains unknown, in these cases.

The very close branching of '*Rhizophydium sp.*' and *Spizellomyces punctatus* (Fig. 3) is further evidence for an incorrect taxonomic classification of the '*Rhizophydium sp.*' isolate, which should rather be reclassified as *Spizellomyces sp.* (see also the comment in the first paragraph of the Materials and Methods section).

Features and biological significance of tRNA editing in *S.punctatus*

Mitochondrial tRNAs, many of which are notable for their deviation from conventional structure, may in the most extreme cases completely lack the D- or TψC loops (39,40), but they almost always contain a conserved acceptor stem, occasionally with minor mis-pairings. The acceptor stem is indispensable for the definition of the tRNA three-dimensional structure (44–47), and the first three base pairs of some tRNAs have been shown to undergo specific interactions with their respective aminoacyl-tRNA synthetases (48,49). In addition, the recognition and the kinetics of 5′-end processing of tRNA precursors by RNAse P depends, among other factors, on the presence of a conventional acceptor stem (50–52). Because of the mis-pairings in the first three positions of their acceptor stem, the structures of *S.punctatus* mitochondrial tRNAs, as predicted from the gene sequences, would most likely not be processed from RNA precursors by RNAse P and a 3′-end processing enzyme (53). Even if processing did occur, it is unlikely that the resulting tRNA structures would be recognized by the aminoacyl-tRNA synthetases and charged with the respective amino acid.

The absence of even a low percentage of non-edited nucleotides in the mature tRNA sequences both of *S.punctatus* (Fig. 4) and *A.castellanii* (26) could be explained by a very efficient editing process, but it most likely reflects a requirement of RNAse P for conventional acceptor stem pairings. Therefore, the complete editing of the precursor RNAs should either precede the processing of the tRNAs, or could be also intimately coupled with processing.

Only five of eight tRNAs were shown to be edited at the RNA level, resulting in the restoration of the conserved acceptor stem pairings. From the remaining three structures, $tRNA^{Asp}$ will certainly have to be edited, because it contains two mis-pairings that would prevent the formation of a conventional acceptor stem. On the other hand, $tRNA^{Trp}$ and $tRNA^{Pro}$ have single A·C pairs at the third position of the acceptor stem, which might not be sufficient to disturb their proper folding. However, it is questionable whether these two unedited tRNA structures will be recognized by the mitochondrial RNAse P as well as by their respective aminoacyl-tRNA synthetases, and so it would also be interesting to sequence these two tRNAs.

As discussed by Lonergan and Gray (26), the editing mechanism could be considered as a form of 'directed mismatch repair' in which the 3′ half of the tRNA acceptor stem defines the position and the nature of the edited nucleotides. This mechanism would involve a sequential or total removal of the first three nucleotides followed by a replacement synthesis. Following this hypothesis, any nucleotide editing change should in principle be possible. In contrast, a compilation of potential and actual editing sites in *A.castellanii* and *S.punctatus* shows that the nucleotide replaced through editing is always a purine (because the 3′ partner is always a pyrimidine). A nucleotide-biased replacement mechanism is reminiscent of the transglycosylation reaction that

is involved in tRNA modification in nuclear, bacterial and organellar systems (54), where the hypermodified base quenine (Q base) is incorporated at the wobble position of anticodons. Because the enzyme that catalyzes this post-transcriptional base exchange is a tRNA-guanine transglycosylase (55), this reaction has to be regarded as editing rather than tRNA modification. A similar enzyme could be involved in this type of tRNA editing, however, as the known transglycosylation reactions act at single-stranded RNA positions, a transglycosylase with a different specificity has to be postulated.

A common evolutionary origin of mitochondrial tRNA editing in *S.punctatus* and *A.castellanii* ?

The tRNA editing systems in *S.punctatus* and *A.castellanii* share the following features: (i) changes occur exclusively in the first three nucleotides of the tRNA, and editing is not observed in rRNAs or mRNAs; and (ii) edited nucleotides are always replaced with purines. Only minor differences are observed in the nucleotide-specific exchange frequencies at the three editing positions, and it is likely that tRNA editing in both organisms is mediated by a similar enzymatic process. The existence of virtually identical editing mechanisms in *S.punctatus* and *A.castellanii* could be explained by its presence in a common ancestor. However, this scenario is unlikely, as *S.punctatus* is a member of the Chytridiomycota that have been clearly associated with the fungi (Eumycota), both on the basis of ultrastructural information (56,57) and nuclear sequence data (58,59). This type of tRNA editing has not been found in mitochondria of any other fungus, not even in the most closely related chytridiomycete, *Allomyces macrogynus* (60). *A.castellanii*, on the other hand, is an amoeboid protist that has no known evolutionary connection to the fungi, either on the ultrastructural or on the sequence level. In molecular phylogenies, *A.castellanii* appears on a branch basal to the divergence points of plants, animals and fungi (61). Consequently, a single, common origin of tRNA editing would have had to be followed by several independent losses in the three major lineages plants, animals and fungi.

Alternatively, a similar editing system might have either evolved independently in these two distant lineages or was acquired by lateral transfer. These hypotheses can be tested by sequence comparison of components of the editing machinery, in *A.castellanii*, *S.punctatus* and species related to them. We are currently investigating the distribution of tRNA editing in lower fungi in order to further test these hypotheses.

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