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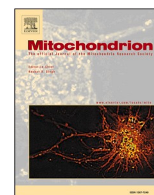

Comparative mitogenomics of Agaricomycetes: Diversity, abundance, impact and coding potential of putative open-reading frames

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A B S T R A C T

The mitochondrion is an organelle found in eukaryote organisms, and it is vital for different cellular pathways. The mitochondrion has its own DNA molecule and, because its genetic content is relatively conserved, despite the variation of size and structure, mitogenome sequences have been widely used as a promising molecular biomarker for taxonomy and evolution in fungi. In this study, the mitogenomes of two fungal species of Agaricomycetes class, *Phellinotus piptadeniae* and *Trametes villosa*, were assembled and annotated for the first time. We used these newly sequenced mitogenomes for comparative analyses with other 55 mitogenomes of Agaricomycetes available in public databases. Mitochondrial DNA (mtDNA) size and content are highly variable and non-coding and intronic regions, homing endonucleases (HEGs), and unidentified ORFs (uORFs) significantly contribute to the total size of the mitogenome. Furthermore, accessory genes (most of them as HEGs) are shared between distantly related species, most likely as a consequence of horizontal gene transfer events. Conversely, uORFs are only shared between taxonomically related species, most probably as a result of vertical evolutionary inheritance. Additionally, codon usage varies among mitogenomes and the GC content of mitochondrial features may be used to distinguish coding from non-coding sequences. Our results also indicated that transposition events of mitochondrial genes to the nuclear genome are not common. Despite the variation of size and content of the mitogenomes, mitochondrial genes seemed to be reliable molecular markers in our time-divergence analysis, even though the nucleotide substitution rates of mitochondrial and nuclear genomes of fungi are quite different. We also showed that many events of mitochondrial gene shuffling probably happened amongst the Agaricomycetes during evolution, which created differences in the gene order among species, even those of the same genus. Altogether, our study revealed new information regarding evolutionary dynamics in Agaricomycetes.

1. Introduction

The mitochondrion is a bi-membranous organelle vital for a wide range of cellular pathways that, unlike most organelles, has an exclusive genome (mitochondrial DNA - mtDNA) that works rather independently from the nuclear genome, although they still interact with each other

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(Chatre and Ricchetti, 2014; Lechuga-Vieco et al., 2020). Nonetheless, during evolution, many of the mitochondrial genes have been transferred to the nuclear genome, and, currently, only the so-called 'mitochondrial core-genes' — those encoding for proteins associated with the oxidative phosphorylation pathway and for the ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) — are present (Adams and Palmer, 2003). Nevertheless, even though the genetic content of animal, fungal, and plant mitochondria is relatively conserved, in fungal mitochondrial genomes there is a great variation of size and structure. For instance, the mitochondrial genome of *Lactarius volemus* is 38 kbp long (Li et al., 2019a) while the fungus *Rhizoctonia solani* mitogenome size is 236 kbp long (Losada et al., 2014). This variation has been widely corroborated in other studies and can be attributed to the number and size of accessory elements, such as introns, homing endonucleases (HEs), and unidentified ORFs (uORFs) (Burger et al., 2003; Zhang et al., 2015).

Introns are sequences inserted in genes that are removed during the pre-mRNA processing. In fungal mitochondria, two types of introns are found, named group I and group II, which differ from each other in sequence, structure, and splicing mechanism (Belfort et al., 2002). Different studies have reported that introns may contain genes encoding for proteins called homing endonucleases (Chevalier and Stoddard, 2001; Pellenz et al., 2002). Homing endonuclease genes (HEGs) can propagate through the genome because their enzymatic product is capable of cleaving the DNA strand in specific loci and activate the homologous recombination repair pathway, consequently generating gene conversion events (Stoddard, 2011).

HEGs are classified into six different families based on their protein primary structure and occur in organisms of every biological kingdom (Stoddard, 2014). In fungi, HEGs of three families are found; however, only the LAGLIDADG and GIY-YIG families have been identified in mitochondrial genomes (Hafez and Hausner, 2012). Although HEGs can be found as free-standing genes, they are most commonly contained within introns (Hafez and Hausner, 2012). Furthermore, even though the main function of HEGs is to propagate themselves, some may also play additional biological roles such as maturase activity and transcriptional repression (Stoddard, 2014). Mobile elements such as HEGs can alter ORFs, and, thus, create uORFs or introns (Al-Reedy et al., 2012). Unidentified ORFs (uORFs) in fungal mitogenomes have been pointed out as features capable of producing unknown RNAs or peptides, or already known molecules that have not been correctly identified yet (Burger et al., 2012; Flot and Tillier, 2007; Luban et al., 2005). Similarly to HEGs, uORFs can be associated with intronic regions or be free-standing genes (Sellem et al., 1996).

Alteration of mitochondrial gene products can affect mitochondrial integrity and function, promoting migration and integration of mtDNA sequences into the nuclear genome (Hazkani-Covo et al., 2010). Different studies have reported the presence of mtDNA sequences in the nuclear genome of different organisms, including humans (Mourier et al., 2001; Richly and Leister, 2004), and the presence of nuclear mtDNA sequences (NUMTs) in fungi has been known for several years, being firstly detected in *Neurospora crassa* (van den Boogaart et al., 1982) and, years later, in different yeast species (Hazkani-Covo et al., 2010; Sacerdot et al., 2008; Thorsness and Fox, 1990). NUMTs are almost identical to their correspondent mitochondrial sequences; however, they are frequently considered pseudogenes, since the mitochondrial and nuclear genetic codes are different and consequently they would not generate the same product (Lopez et al., 1994).

Mitochondrial genomes have been widely used as molecular markers in phylogenetic studies because of their low recombination rate, usually conserved gene content, maternal inheritance mechanism, and simplicity and ease of isolation and sequencing when compared to the nuclear genome (Rubinoff and Holland, 2005; Smith, 2016). Thus, many studies have used the mtDNA to determine the phylogenetic and evolutionary relationships among fungal species (Chen et al., 2019; Fonseca et al., 2020; Li et al., 2019b; Stone et al., 2010). Nonetheless, despite their recognized importance, fungal mitogenomes are still largely underrepresented in public databases (Smith, 2016), which has impacted the advance of studies regarding fungal biology, classification, and evolution (Chatre and Ricchetti, 2014).

In our study, the mitogenomes of two fungal species of Agaricomycetes, *Phellinotus piptadeniae* (Teixeira) Drechsler-Santos & Robledo and *Trametes villosa* (Sw.) Kreisel were sequenced, assembled, and annotated for the first time. *Phellinotus* was recently described as a new genus of the Hymenochaetales order (Drechsler-Santos et al., 2016) while *T. villosa* belongs to a widely known and studied genus of Polyporales. *T. villosa* has high biotechnological potential as a consequence of its capacity to synthesize ligninolytic enzymes (de Oliveira Carneiro et al., 2017; Silva et al., 2014); on the other hand, *P. piptadeniae* has not yet been explored regarding its enzymatic or metabolic capacity. These newly sequenced mitogenomes were used for comparative analyses with other 55 mitogenomes of Agaricomycete fungi available in public databases. Our results indicated that mitogenomes of Agaricomycete fungi vary in size and content, even within the same species, and that transposition of mitochondrial genes to the nuclear genome are not common. We also showed that non-core genes sequences are shared among distantly related species, most likely as a consequence of horizontal gene transfer events. On the other hand, uORF sequences, whose products do not have any known protein domain, are only shared at the family or genus levels, probably as a result of vertical inheritance. Additionally, we reported that codon usage can vary among the mitogenomes and that the GC content of mitochondrial features may be used to distinguish coding from non-coding sequences. Moreover, we showed that time-divergence analysis using mitochondrial genes can be as reliable as estimates based on nuclear genes.

2. Material and methods

2.1. Fungal isolates origin, DNA extraction, and sequencing

The *Phellinotus piptadeniae* isolate was collected in the Parque Municipal do Córrego Grande (Florianópolis, SC, Brazil) and identified using morphological characters as described by (Drechsler-Santos et al., 2016). *Trametes villosa* CCMB561 was isolated from the semiarid region of Brazil and identified using morphological characters and molecular markers (de Oliveira Carneiro et al., 2017). We extracted the total DNA of each isolate using FastDNA Spin Kit (MP Biomedicals) following the manufacturer's instructions, and the genomic DNA was qualitatively and quantitatively analyzed by agarose gel electrophoresis (1%) and measurement in Nanodrop 1000ND spectrophotometer (Thermo Scientific). The sequencing library was prepared from genomic DNA with the NEBNext Fast DNA Fragmentation and Library Preparation Kit (New England Biolabs). We evaluated the quality of the library using Agilent 2100 Bioanalyzer (Agilent Technologies) and performed paired-end sequencing using Illumina HiSeq 2500 platform (Illumina Inc.). The mitochondrial DNA sequencing was carried out simultaneously with nuclear DNA.

2.2. New mitogenomes assembly and annotation

Raw DNA reads gathered from the total genome sequencing of the fungi *P. piptadeniae* and *T. villosa* went through a quality control step in which we removed adapters and nucleotides with Phred score lower than 20 using BBDuk, a software in BBTools v. 36.86 package (Bushnell, 2014). The overall quality and length of the reads were analyzed using FastQC v. 0.11.5 software (Andrews, 2010). Both genomes were assembled using SPAdes v. 3.11.1 (Bankevich et al., 2012), and the contigs referring to the mitochondrial genomes identified through similarity searches against fungal mtDNA database using BLASTn (Altschul et al., 1990). After identification, we annotated the mitogenomes using three different programs: MITOS2 (Bernt et al., 2013) to identify the mitochondrial genes, using "NCBI fungi RefSeq 81" database and genetic code number four as parameters; RNAweasel (Beck and Lang, 2009) to

identify introns; and MFannot v. 1.33 (Beck and Lang, 2010) to identify uORFs. The events of gene duplication or fragmentation were confirmed and the theoretical products of uORFs identified using the NCBI Conserved Domain Search tool (Marchler-Bauer et al., 2015). Graphic representations of both mitogenomes were created using Geneious v. 6.0.6 (https://www.geneious.com/).

All the mitochondrial genes, rRNAs, and tRNAs were named according to their usual nomenclature; HEGs were identified using "lagli" if they belonged to the LAGLIDADG family or "giy" if their family was GIY-YIG. Introns were named according to the nomenclature suggested by (Zhang and Zhang, 2019). Lastly, uORFs were named using "orf" followed by the size of their potential protein product. All the information regarding feature position, size, and strand location in both fungal genomes are available in Supplementary data 1.

2.3. Comparative analyses of mitogenomes

For the comparative analyses, we retrieved mitogenomes belonging to fungi from Agaricomycetes class from two different public databases: Organelle Genome Resources (https://www.ncbi.nlm.nih.gov/genome/organelle/) and Joint Genome Institute (https://genome.jgi.doe.gov/portal/). The access codes for all the mitogenomes used in this study are available in Supplementary data 2. We reannotated all the mitogenomes retrieved from public databases using the same pipeline utilized for the two fungal isolates originally sequenced in this study (item 2.2). Annotation files can be found in Supplementary data 3. Graphics showing the length and relative composition of the mitogenomes were built using R v. 4.0.2 (R Core Team, 2015) through *ggplot2* package v. 3.3.2 (Wickham, 2011). If a HEG or an uORF was inside an intron, the size of the intronic sequence was determined subtracting the length of the elements. We estimated the correlation between mtDNA total length and the intronic, coding, and non-coding regions using Pearson's correlation analyses performed with the *ggplot2* package. For such analyses the coding regions were subdivided into three different groups: i) mitochondrial core-genes, rRNAs and tRNAs; ii) HEG sequences; and iii) uORF sequences.

2.4. Sequence sharing among Agaricomycetes

We extracted and pairwise aligned nucleotide sequences identified in the fungal mitogenomes to search for sequence similarity using BLASTn. We built a matrix based on the pairs of homologous sequences with a minimum percentage of 60% for coverage and 70% for identity. The data was later considered as the proportion of sequences that two mitogenomes are sharing between themselves. Sequence sharing between the mitogenomes was showed as color matrixes that we built using *ggplot2* and *reshape2* v. 1.4.4 (Wickham, 2012) packages. The sharing of sequences between fungal species of the Hymenochaetales and Polyporales orders was showed using chord diagrams that we built using *circlize* package v. 0.4.10 in R (Gu et al., 2014). We aligned homing endonuclease genes using MAFFT v. 7 (Katoh et al., 2019) and built maximum-likelihood phylogenetic trees with bootstrap value of 1,000 using RAxML-HPC2 in XSEDE via CIPRES Science Gateway (Miller et al., 2010).

2.5. Codon usage and GC content analyses

For the codon usage analysis, we calculated the relative synonymous codon usage (RSCU) values of the concatenated sequences of the mitochondrial protein-coding core genes, non-core genes (uORFs which we managed to predict their product), and uORF sequences present in the mitogenomes using MEGA X (Kumar et al., 2018). We then performed a T-SNE analysis based on the RSCU values using the R package *Rtsne* v. 0.15 (Krijthe, 2015) and calculated the GC content of mitochondrial protein-coding core genes, non-core genes, uORFs, and introns using an in-house Perl script (Supplementary data 4). We produced correlation of codon usage profiles of the different features among different mitogenomes using the *corrplot* R package v. 0.84 (Wei and Simko, 2017), color matrixes in R using the *ggplot2* and *reshape2* packages, and plotted the GC content data using *ggplot2* R package.

2.6. Presence of mitochondrial genes in the nuclear genomes

We evaluated the presence of mitochondrial genes in the nuclear genomes using all but four fungal species, for which the nuclear genomes were not available in the public databases (see Supplementary data 2 for access codes). The sequences corresponding to the genes related to the oxidative phosphorylation pathway (*atp6*, *atp8*, *atp9*, *cob*, *cox1*, *cox2*, *cox3*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5,* and *nad6*), those encoding for the ribosomal RNAs (*rns* and *rnl*), and the one that encodes for the ribosomal protein 3 (*rps3*) were used in the searches. Except for *rrns*, *rrnl,* and *rps3* genes, we translated all the other sequences into amino acids using ExPASy's online translation tool (https://web.expasy.org/translate/) under the genetic code number four. We then aligned the sequences against the nuclear genomes using BLASTn and tBLASTn, and only hits with identity $\geq$ 85%, coverage $\geq$ 50% and E-value $\leq 1E^{-10}$ were considered as copies of the mitochondrial genes (method adapted from (Fonseca et al., 2020).

2.7. Phylogenetic inference and gene order

We used annotations provided by MITOS2 to determine the gene order of the mitogenomes considering *cox1* gene as the starting point. We selected to determine the phylogenetic relationships among the fungi using *atp8*, *cob*, *cox2*, *cox3*, *nad2*, *nad3*, *nad4*, *nad5,* and *nad6* genes concatenated sequences, since we did not detect copies of these genes in the nuclear genomes. We aligned the sequences using MAFFT v. 7 (Katoh et al., 2019) and used its output to build a maximum-likelihood phylogenetic tree with bootstrap value of 1,000 using RAxML-HPC2 in XSEDE via CIPRES Science Gateway (Miller et al., 2010). We used the mitochondrial genomes of the fungi *Puccinia triticina* (Subphylum Pucciniomycotina, NCBI accession code NC_044103.1) and *Ustilago bromivora* (Subphylum Ustilaginomycotina, NCBI accession code LT558140.1) as the external group. We estimated the divergence time among the species using MEGA-X (Kumar et al., 2018) under the "General Time Reversible" substitution model using four different calibration points obtained from TimeTree (Kumar et al., 2017). The calibration points were: 187 – 236 MYA between Hymenochaetales and Polyporales as well as Russulales and Polyporales orders (Da Lage et al., 2013; Wisitrassameewong et al., 2016); 229 – 266 MYA between Agaricales and Boletales orders (Nagy et al., 2011; Riess et al., 2016) and 230 – 312 MYA between Sebacinales and Hymenochaetales orders (Da Lage et al., 2013; Riess et al., 2016).

3. Results and discussion

3.1. Assembly and annotation of P. Piptadeniae and T. Villosa mitogenomes

The assembly of the reads provided a single mitochondrial contig for both *P. piptadeniae* and *T. villosa*. The mitogenomes were deposited on GenBank under the accession numbers MW255595 and MW255594, respectively.

P. piptadeniae and *T. villosa* mitogenomes are circular molecules of 137,390 and 56,885 bp and GC content of 24.1% and 25.5%, respectively, which is in agreement with a previous study conducted by (Franco et al., 2017) that pointed out that fungal mitogenomes GC content are estimated to be of 24.4 ± 7.3%. We identified the 14 genes related to the oxidative phosphorylation pathway, the two rRNAs subunits, a set of tRNAs encoding for all the 20 amino acids, and the ribosomal protein subunit 3 (*rps3*) gene in the two mitogenomes studied (Fig. 1). Furthermore, we noticed that three tRNAs and eight uORFs were annotated in the reverse strand of *P. piptadeniae* mitogenome,

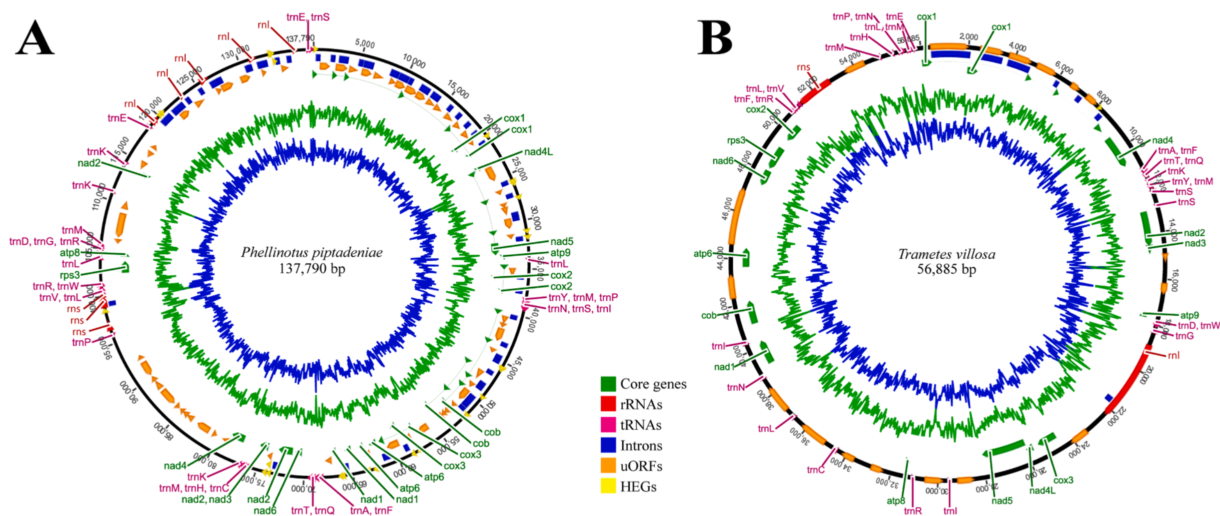

Fig. 1. Graphic representation of the mitogenomes of *Phellinotus piptadeniae* (A, left) and *Trametes villosa* (B, right). The inner blue and green circles represent, respectively, the GC and AT contents. The features were either colored in green (genes), red (rRNAs), pink (tRNAs), blue (introns), orange (uORFs), or yellow (HEGs). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

whereas the genes *atp8*, *atp9*, *cox2*, *nad3*, *rps3,* and *rns*, ten tRNAs and four uORFs were annotated in the reverse strand of *T. villosa* mitogenome. Such findings were expected because in Basidiomycota both strands of the mitochondrial genome can harbor genes, whereas in Ascomycota usually only one strand contains the mitochondrial genes (Aguileta et al., 2014).

The annotation of the mitogenomes revealed a higher number of introns, HEGs, and uORF sequences for *P. piptadeniae* than for *T. villosa*. We identified a total of 47 introns (7.8% of the total mitogenome size), 27 HEGs (24 LAGLIDADG and three GIY-YIG; 4.7%), and 64 uORFs (40.1%) in *P. piptadeniae* mitogenome, in contrast with seven introns (1.7%), one HEG (GIY-YIG; 0.1%) and 18 uORFs (30.2%) in the mitogenome of *T. villosa*. Furthermore, we detected protein domains related to HEGs in 32 uORFs of *P. piptadeniae* and in five of *T. villosa.* Thus, the total number of HEGs found in the mtDNA of *P. piptadeniae* was 59 (55 LAGLIDADG and 4 GIY-YIG) and 6 for *T. villosa* (4 LAGLIDADG and 2 GIY-YIG).

Differences in the mtDNA size and distribution of accessory elements, such as introns, HEGs, and uORFs, have been observed in fungi of other groups, such as the Hypocreales order (Al-Reedy et al., 2012; Zhang et al., 2015) and Leotiomycetes class (Chen et al., 2019). In all of these studies, the number of accessory elements was positively correlated with the mtDNA size, which suggests that the size difference observed for *P. piptadeniae* and *T. villosa* may be influenced by the presence and size of these elements.

As expected, most of the introns found in the mitogenome of *P. piptadeniae* (93.6%) and all the *T. villosa* introns were classified as group I (Supplementary data 1). It is widely known that mitochondrial group I introns are the most commonly found in fungi, and similar results have been reported for other species of the Agaricomycetes class, such as *Agaricus bisporus* (Férandon et al., 2013) and *Phlebia radiata* (Salavirta et al., 2014), both with approximately 93.5% of introns classified as group I.

Additionally, we identified some uORFs in the mitogenomes of both *P. piptadeniae* and *T. villosa* as a non-core mitochondrial gene, the one encoding for DNA polymerase (*dpo*) (Supplementary data 4). Although the DNA polymerase enzyme is crucial for DNA replication, the mtDNA polymerase is encoded in the nuclear genome (Sazer and Sherwood, 1990), and the *dpo* genes in mitochondria are associated with replication of plasmids (Griffiths and Yang, 1995). Some studies have established a relationship between the presence of this gene with plasmid integration in the mitochondrial DNA, an event linked to the increase of the mitogenome size (Férandon et al., 2013; Formighieri et al., 2008; Mardanov et al., 2014). Nevertheless, since we found *dpo* genes in both mitogenomes, plasmid integration events alone cannot fully explain the size difference observed in the mtDNA of *P. piptadeniae* and *T. villosa*, suggesting the existence of other factors, such as the amount of introns annotated and the size of intergenic regions, as previously reported for the genera *Fusarium* (Al-Reedy et al., 2012) and *Lyophyllum* (Li et al., 2019b).

3.2. Mitogenomes of Agaricomycetes fungi are highly heterogeneous

Comparative mitogenome analyses included 52 different fungal species distributed across seven orders (Agaricales, Boletales, Cantharellales, Hymenochaetales, Polyporales, Russulales, and Sebacinales) besides the two newly characterized mitogenomes. Our results show that mitogenome relative content and size varied significantly among the different species evaluated. The sum of the core-genes, rRNAs, and tRNAs made up approximately half of the total size of some mitogenomes (such as *Lactarius hatsudake*, *Lyophyllum decastes,* and *Russula virescens*), while for others (*Agaricus bisporus*, *Ganoderma calidophilum* and *Phlebia radiata*) such genes contributed for one quarter or less of the size. The contribution of the non-coding intergenic region for the total mitogenomes size also varied; the proportions were, for instance, 10% and 60% for *Russula abietina* and *Rhizoctonia solani* AG3-Rhs1AP, respectively. (Fig. 2A).

Variation in the mitogenome size ranged from 35,445 (*Lactarius volemus*) to 235,849 bp (*Rhizoctonia solani*) and was observed at both inter and intraspecific levels. We detected striking differences of 70 Kbp between two *Rhizoctonia solani* isolates until subtle differences of 100 bp between two isolates of *Flammulina velutipes*. Conversely, the two *Ganoderma lucidum* isolates had very similar mitogenome sizes, which varied only 5 bp (Fig. 2B).

A recent mitogenomic comparative study of the Leotiomycetes class (Ascomycota) described similar results, with mitogenome size ranging from 27 kbp (*Pseudogymnoascus pannorum)* to 203 kbp (*Sclerotinia borealis*). The same study reported high variability in the relative content of the mitogenomes, with the core-genes, rRNAs, and tRNAs making up from 10% (in *Sclerotinia borealis)* to 75% of the content (in *Cairneyella variabilis*). The contribution of non-coding intergenic regions to the mitogenomes range from 15% (in *Cairneyella variabilis)* to 33% (in *Sclerotinia sclerotiorum*,) (Chen et al., 2019).

Our correlation analyses revealed a positive correlation between the mitogenome sizes and the lengths of the non-coding regions ($R = 0.88$, $p < 2.2e\text{-}16$), the intronic regions ($R = 0.87$, $p < 2.2e\text{-}16$), the sum of HEGs

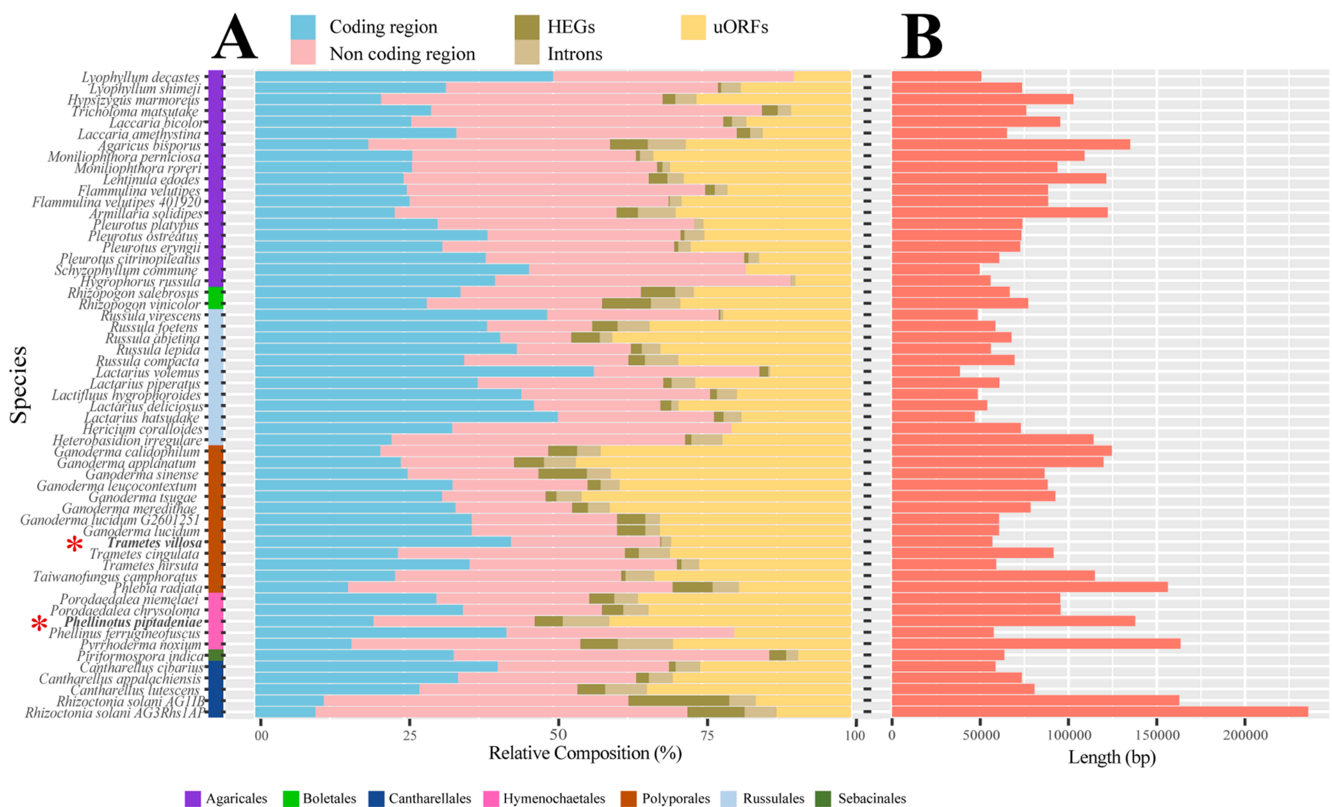

Fig. 2. Mitogenome relative composition (A, left) and absolute size (B, right). The relative composition is shown in percentage, while the mitogenome total size is displayed in base pairs. The names of *P. piptadeniae* and *T. villosa* are shown in bold and with a red asterisk on the side. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sizes ($R = 0.79$, $p = 3.4\text{e-}13$) and the uORFs sequences ($R = 0.69$, $p = 4\text{e-}09$) (Fig. 3). The size of the coding regions (core-genes, rRNAs, and tRNAs), however, had little influence on the total size of the mitogenomes ($R = 0.27$, $p < 0.045$). The positive influence of non-coding and intronic regions in the mtDNA sizes was also reported for Ascomycetes like species from Hypocreales order (Fonseca et al., 2020) and species of the genera *Aspergillus* and *Penicillium* (Joardar et al., 2012), *Lachancea* (Friedrich et al., 2012), *Rhynchosporium* (Torriani et al., 2014), and *Pichia* (Jung et al., 2010).

The described mitogenome sizes and content variations among different fungi corroborate with the idea that mitochondrial genome characterization studies can provide important data to help delimitating species or even fungal populations (Rubinoff and Holland, 2005; Smith, 2016). The observed structural differences are most likely caused by the insertion of repetitive elements, gain or loss of introns, and/or the transposition of mitochondrial genes to the nuclear genome, which are often the cause of the variations detected in the mitogenome sizes (Burger et al., 2003).

3.3. Possible horizontal transfer events in mitogenome sequences

In the studied fungal mitogenomes, we characterized through sequence similarity the protein product of 901 out of 1605 uORFs (56.1%). These protein products corresponded to non-core mitochondrial genes (and will be referenced as 'non-core genes' from now on), and most of them (742 or 82.4%) was identified as HEGs, from which 688 (92.7%) belonged to the LAGLIDADG family (Supplementary data 5). Other studies have already reported the presence of predicted uORFs classified as HEGs (Deng et al., 2016; Salavirta et al., 2014), as well as the predominance of the LAGLIDADG over the GIY-YIG family (Chen et al., 2019; Fonseca et al., 2020). In our analysis, the sharing of non-core mitochondrial genes was observed for both closely and distantly related species (Fig. 4A), while the sharing of uORF with no similarity with known protein-encoding genes was more common amongst fungi of the same genus or family (Fig. 4B).

We also analyzed the sequence sharing pattern within Hymenochaetales and Polyporales that encompass the two fungal isolates sequenced in this study. Our chord graphic (Supplementary data 6) shows that the sharing is more intense within the species belonging to Polyporales than Hymenochaetales. This can be a consequence of the higher representativeness of mitogenomes of Polyporales (13 distinct mitogenomes, in comparison with 5 of Hymenochaetales) in the dataset, a consequence of the uneven representation in mitogenomic databases (Smith, 2016), or could indicate that some taxonomical groups are more prone to share sequences. Nevertheless, we acknowledge that additional studies are necessary to better understand this data pattern.

Most of the mitochondrial sequences of *P. piptadeniae* and *T. villosa* shared with other fungi were classified as HEGs, as observed for all the metagenomes we evaluated in this study. From the 64 uORFs found in the mitogenome of *P. piptadeniae*, 14 (21.9%) were shared with other fungi and all of them were identified as HEGs. In *T. villosa*, five out of 18 uORFs identified (27.8%) were shared with other fungi, and four out of the five were classified as HEGs (Supplementary data 7).

Our results suggest that two different mechanisms could be involved in the sharing of mitochondrial sequences among the mitogenomes studied. Since many non-core mitochondrial genes shared between distant phylogenetic fungi are HEGs, our first hypothesis is that they are product of horizontal transfer events. HEGs have been detected in all the domains of life and their ability to cleave the DNA strand in specific target-sites and promoting gene conversion events is widely reported (Hausner, 2003; Stoddard, 2011). Using such a mechanism, HEGs are capable of transposing to different regions of the same genome or even to different genomes (Koufopanou et al., 2002).

In our study, we detected HEGs of the LAGLIDADG and GIY-YIG

Fig. 3. Pearson's correlation between the mitochondrial genome sizes and the lengths of coding regions (A), non-coding regions (B), intronic regions (C), HEGs sizes (D), and uORFs sequences (E).

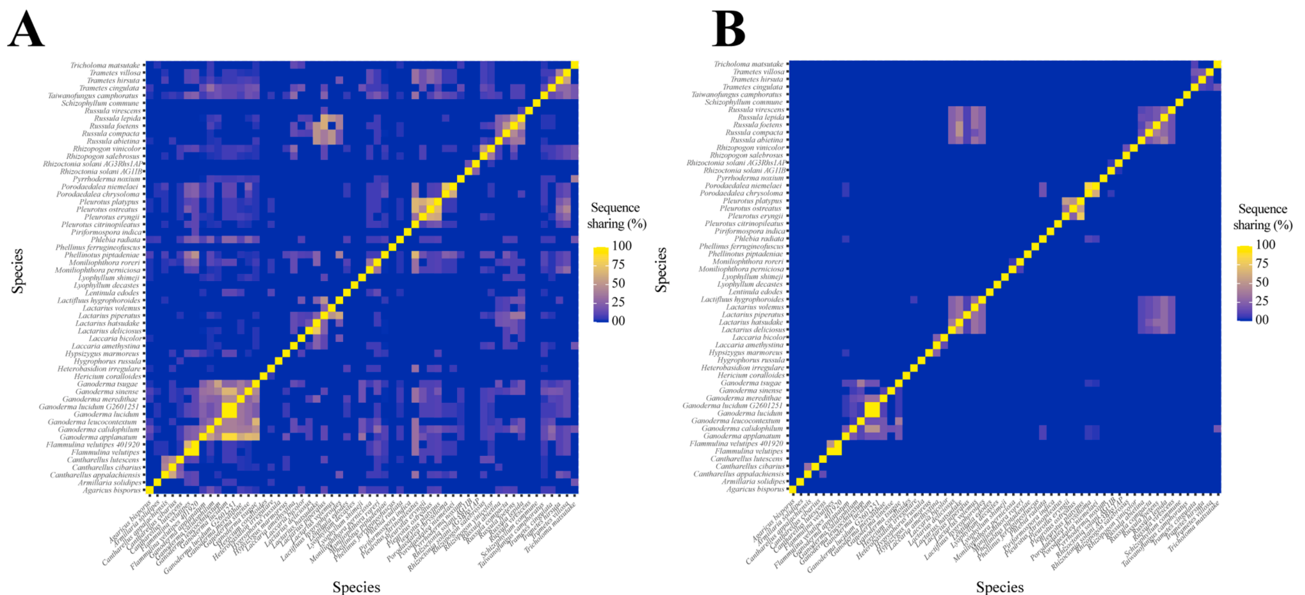

Fig. 4. Percentage of sequence sharing among fungi of the Agaricomycetes class from 0% (purple) to 100% (yellow). (A) Non-core mitochondrial genes and (B) unidentified ORFs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

families, which are frequently identified within group I introns. In agreement to that, only 137 HEGs (20.8%) identified in our analysis were found outside introns (free-standing), whereas 521 (79.2%) were classified as intronic (Supplementary data 4). Furthermore, the genes found harboring HEGs were *cox1* (328), *cob* (78), *nad5* (68), *cox3* (15), *cox2* (14), *nad4* (12), *nad1* (4), and *nad2* (2). A similar result was obtained in a study in which the authors analyzed the coevolution of fungal mitochondrial introns and HEGs, and found out that *cox1* and *cob* are the genes that most commonly harbor HEGs (Megarioti and Kouvelis, 2020).

Many studies indicated that homologous sequences of group I introns containing HEGs are common among distantly related species, which suggests the occurrence of horizontal transfer events (Chevalier and Stoddard, 2001; Gimble, 2000; Hafez and Hausner, 2012; Turmel et al., 1995). In fungi, HEGs located inside mitochondrial introns have been linked to the sharing of sequences between distantly related species of *Armillaria* (Kolesnikova et al., 2019), *Aspergillus*, and *Penicillium* (Joardar et al., 2012). To further investigate the possibility that these sequences are shared as a consequence of horizontal gene transfer, we built maximum-likelihood phylogenetic trees of GIY-YIG and LAGLIDADG HEGs taking into account their insertion sites (Supplementary data 8 and 9, respectively). Our results show that HEGs inserted in the same gene tend to cluster together, even the ones of phylogenetically distant species, with few exceptions. According to a recently published study by (Megarioti and Kouvelis, 2020), introns and HEGs are under a dynamic coevolution, with introns tending to have conserved localization and HEGs usually having conserved site recognition, suggesting that the presence of similar intronic HEGs inserted in the same genes in distantly related species may be due to horizontal gene transfer events.

Moreover, we also detected a sharing of uORFs that have no similarity with known protein-encoding genes between closely related species, and, therefore, our second hypothesis is that mitochondrial sequences may be shared between species as a consequence of an inheritance mechanism from a common ancestor. Although intraspecific horizontal transfer events between fungi can occur, they have been associated with mitochondrial plasmids and endonucleases (Hamari et al., 2003; Rosewich and Kistler, 2000).

3.4. Assessment of uORFs coding potential based on molecular signatures

It has been shown that codon usage pattern has a direct impact on the time and energy spent during mRNA translation, and mitochondrial genes are believed to have a preference for the use of certain codons, especially in organisms that have rapid growth rates, thus, saving time and energy for cell development (Sharp et al., 2005). We calculated and compared their respective relative synonymous codon usage (RSCU) values, an index often used in codon usage bias studies, and we detected that it is not homogenous among Agaricomycetes mitochondrial genomes (Supplementary data 10 and 11). As seen in Supplementary data 11A-C, the fungi *Cantharellus* sp., *Moniliopththora* sp., *Lentinula edodes*, *Flammulina velutipes*, *Armillaria solidipes,* and all the species in Polyporales order have a preference for the TGA (or UGA) codon, whereas the other species analyzed have a preference for the TGG (or UGG) codon, both coding for tryptophan residues. Similar results were reported in a study conducted by (LaBella et al., 2019), in which the authors identified considerable variation in genome-wide codon usage for budding yeasts at subphylum level. In addition, our t-SNE analysis and correlograms also showed that the codon usage of core genes is different from of those for non-core mitochondrial genes and uORFs (Fig. 5A and Supplementary data 12).

In our analyses, we found that 10% of the protein-encoding core genes, 12.3% of the non-core genes, and 33.2% of uORFs are located on the reverse strand (Supplementary data 13), which may be one of the reasons for the difference in codon usage between the groups evaluated. As shown in Fig. 5A, even the mitochondrial core genes did not form a single cluster, implying the existence of codon usage variations among features of the same group. Variation in mitochondrial protein composition due to gene location (either forward or reverse strand) has been reported in mammalian mitogenomes (Min and Hickey, 2007) and may occur in fungal mitogenomes. Furthermore, as a consequence of the asymmetric replication process of the mitochondrial genome, mutation rates in the two strands are not the same, and, thus, influencing codon usage (Jia and Higgs, 2008).

Another factor that seems to be linked to codon usage is the GC content. Although GC-rich mitogenomes have been described in animals, fungi, green algae, and land plants, the majority of mitogenomes already sequenced are rich in adenosine and thymine (Smith, 2012), and in a study conducted by (Singer and Hickey, 2000), the authors showed that AT-rich genomes have a higher usage of AT-rich codons in their genes. Therefore, we compared the GC content of the mitochondrial core genes, non-core genes, uORFs, and intronic sequences. Our results indicated that the GC content of intronic sequences is very different from the same content of the others features analyzed (all *p-values* are$<$2.22e-

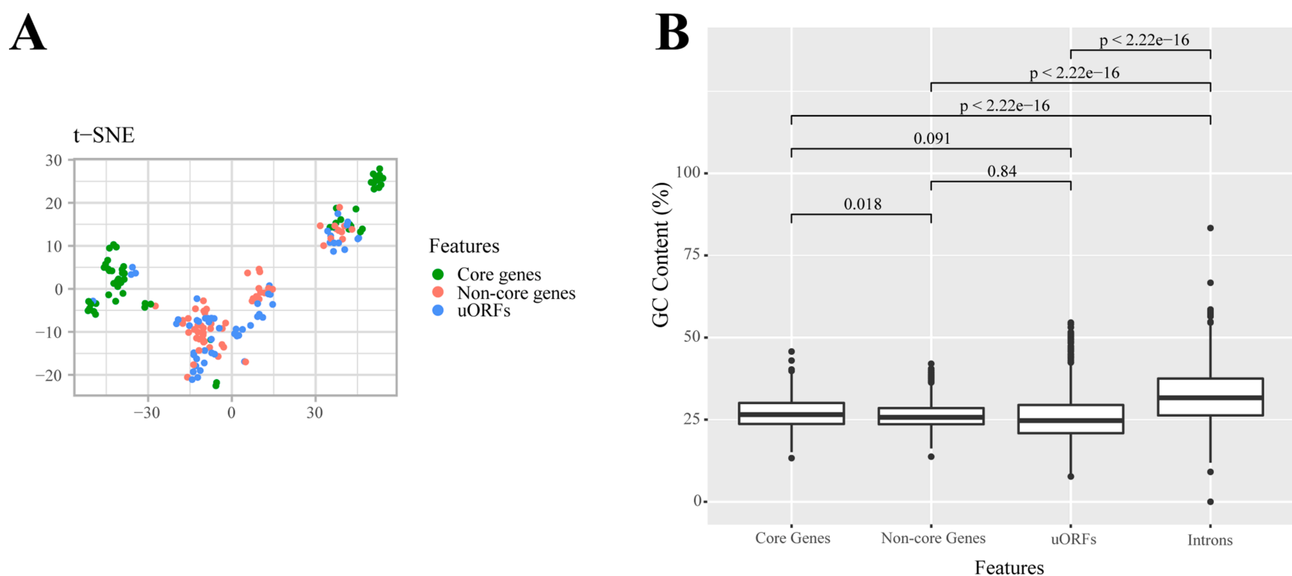

Fig. 5. Clustering analysis of the codon usage (RSCU) in mitochondrial core genes, non-core genes, and uORFs (A, left). GC content of mitochondrial core genes, non-core genes, uORFs, and introns (B, right).

16) (Fig. 5B), which may be an indicative that the GC content can be used as a coding-potential marker. It has been reported that in humans and other animals the GC content can be used as a splicing marker during transcription in virtue of the difference between the GC contents of exons and their flanking introns (Amit et al., 2012). Therefore, since in our analysis we observed that the GC content of introns (32.45 $\pm$ 9.01%) is higher than in core genes (26.82 $\pm$ 5.18%), non-core genes (26.27 $\pm$ 4.05%), and uORFs (26.21 $\pm$ 8.25%), our results may indicate that the GC content of intronic sequences acts as a splicing marker in fungal mitochondrial genes.

Moreover, our results showed that the GC content of uORFs is similar to that of both core and non-core genes (*p-values* equal to 0.091 and 0.84, respectively) (Fig. 5B), suggesting that uORFs may be under the same selective pressures that act on known protein-coding genes in mitogenomes. Codon optimization has been linked to increased mRNA stability and translational speed (Presnyak et al., 2015), which is an important factor in mitochondria because mitogenomes usually harbor few copies of tRNAs genes for each amino acid, most commonly only one. Despite of the existence of codon optimization, we acknowledge the necessity of further confirming our hypothesis that uORFs are functional genes through experimental methods, such as RNA sequencing and proteomic analyses. Furthermore, we noticed that the GC contents of core and non-core genes are slightly different ($p = 0.018$) (Fig. 5B) and we hypothesize that the small difference observed may be due to recent events of plasmid integration into the mtDNA. Out of the 901 non-core mitochondrial genes identified in this study, 111 (12.3%) were homologous to the plasmid genes *dpo* or *rpo* (Supplementary data 5). Previous studies have shown that mitochondrial plasmids have different GC content when compared to the main mitochondrial genome (Handa et al., 2002; McDermott et al., 2008), and that it is possible to detect recent plasmid integration events by analyzing the GC content profile of the mitogenome (Formighieri et al., 2008).

3.5. NUMT is an uncommon phenomenon among Agaricomycete fungi

We detected the presence of copies of mitochondrial genes in the nuclear genomes in only nine out of the 54 (16.7%) species evaluated, and for most of them (six out of nine, or 66.7%) only one gene was detected. Additionally, out of the 17 mitochondrial genes searched, we found only eight in the nuclear genomes, and *nad4L* was the most commonly found (detected in four different fungal species), followed by the genes *atp9* and *rnl* (both detected in two different fungal species), and *atp6*, *cox1*, *nad1*, *rns,* and *rps3,* which appeared only once each (Table 1).

Previous studies classified the presence of NUMTs in fungal genomes as a common phenomenon, with species with more than 200 NUMTs detected (Hazkani-Covo et al., 2010; Sacerdot et al., 2008). However, unlike our study, the authors of the previous studies were not specifically searching for copies of mitochondrial genes in the nuclear genome, but rather they considered any mitogenome sequence. Thus, the number of NUMTs that are copies of mitochondrial genes may be lower. Moreover, the quantity of NUMTs detected can also vary depending on the method and the completeness of the genome (Hazkani-Covo et al., 2010), which may become data comparison difficult.

In a recent study of the Hypocreales order (Fonseca et al., 2020), the authors found that 11 out of 18 (61.1%) fungal species have at least one copy of a mitochondrial gene in the nuclear genome. Moreover, out of the 17 genes evaluated, only three were not found in the nuclear genomes. Those findings are in contrast with ours, since a significantly lower number of fungi in our study had NUMTs, and the number of genes with nuclear copies is also different. Such divergences suggest that different fungal groups (Ascomycota $\times$ Basidiomycota) may have distinct rates of transposing events of mitochondrial genes to the nuclear genome. In yeasts, for instance, the rate at which mtDNA sequences are exported to the nucleus can vary as a consequence of environmental conditions (Hazkani-Covo et al., 2010). Therefore, it seems there is no consensus regarding the frequency of NUMTs in fungi or agreement on which mitochondrial genes are most commonly found in the nuclear genomes, suggesting the need for more studies on this theme.

NUMTs are found in most eukaryotic organisms, but their functions are not fully understood. Some authors suggest that most NUMTs are nonfunctional because the genetic code of the mitochondrial and nuclear genomes are different, and, thus, nuclear copies would not produce the same protein as their mitochondrial counterpart (Lopez et al., 1994; Pozzi and Dowling, 2019). However, in *Saccharomyces cerevisiae*, nuclear insertions of mtDNA acquired new functions and now encode for different proteins (Noutsos et al., 2007), or became important to the replication of chromosomal DNA (Chatre and Ricchetti, 2011). Additionally, the NUMT content seems to be strongly correlated with the nuclear genome size, while no correlation was found between the presence of NUMTs and the size of mtDNA (Hazkani-Covo et al., 2010).

3.6. Phylogenetic divergence time inference and gene ordering

Our phylogenetic inference analysis revealed that, except for the order Cantharellales, all the orders formed monophyletic groups with high bootstrap values, which is in agreement with the applicability of the mtDNA as a phylogenetic tool (Fig. 6). It was also possible to detect

Table 1
Mitochondrial genes also found in the nuclear genome.

Mitochondrial genes	*Agaricus bisporus*	*Ganoderma lucidum*	*Laccaria bicolor*	*Pleurotus ostreatus*	*Pleurotus platypus*	*Porodaedalea niemelaei*	*Rhizopogon vinicolor*	*Taiwanofungus camphoratus*	*Tricholoma matsutake*	TOTAL
atp6		1								1
atp8						X				0
atp9					1		1			2
cob										0
cox1				1						1
cox2										0
cox3										0
nad1					1					1
nad2										0
nad3										0
nad4										0
nad4L	1		1		1		1			4
nad5										0
nad6										0
rns									1	1
rnl				1		1				2
rps3								1		1

Table 1 Species evaluated in this study for which mitochondrial genes were also detected in the nuclear genomes. The box corresponding to the *atp8* gene for *Porodaedalea niemelaei* contains a “X” because the mitogenome lacks this gene, so it was not possible to evaluate whether it is also present in the nuclear genome or not.

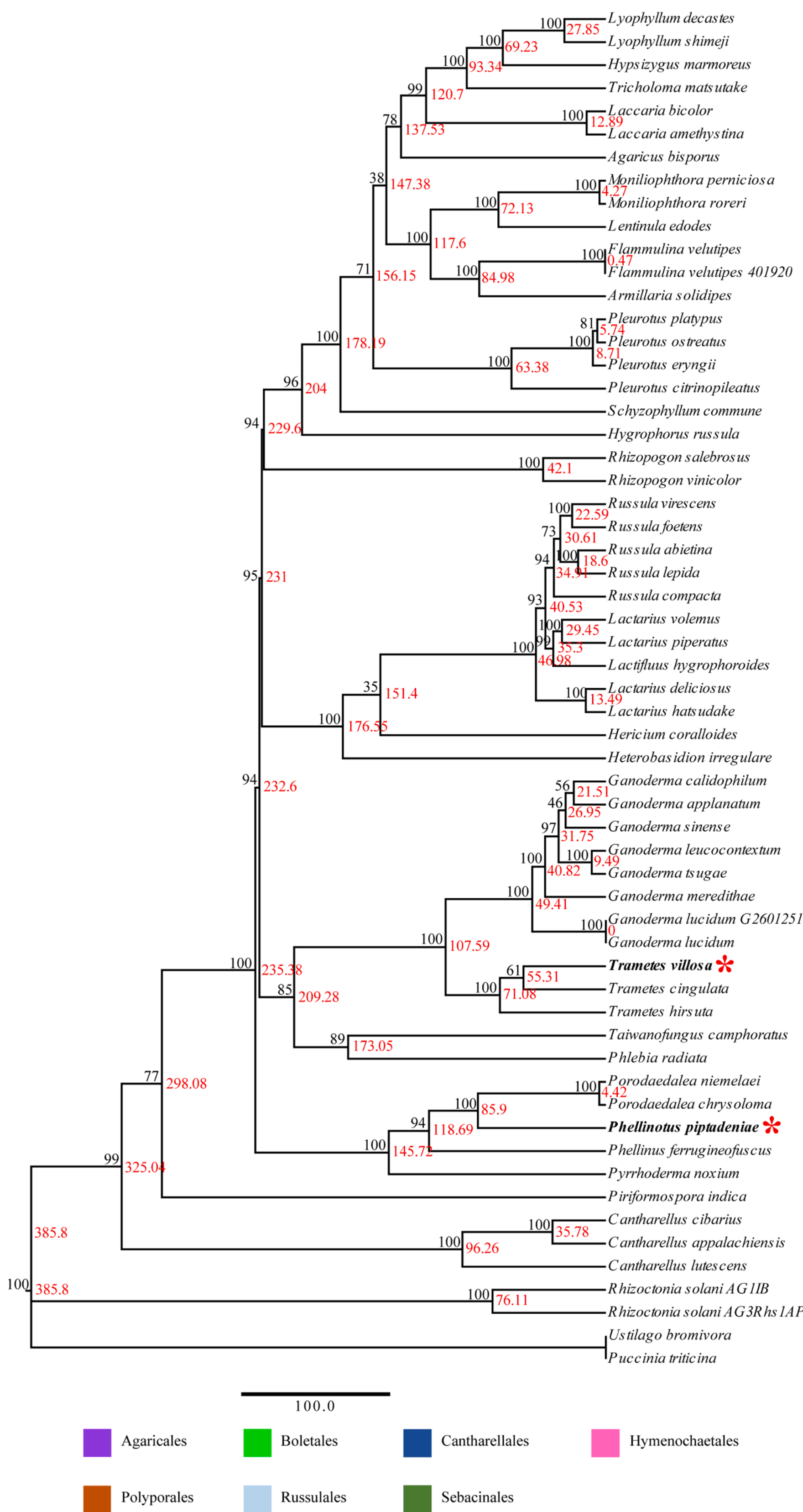

Fig. 6. Maximum-likelihood phylogenetic reconstruction of the fungal species (Agaricomycetes) evaluated in this study. Red values in the nodes represent divergence time in millions of years, and black values indicate the bootstrap values. The names of *P. piptadeniae* and *T. villosa* are marked by a red asterisk. Divergence time was not computed between species of the outgroup. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

monophyletic genera, such as *Ganoderma*, *Pleurotus*, *Russula,* and *Trametes*.

Our time-divergence analysis suggested that the Cantharelalles order diverged approximately 386 million years ago (MYA) and is the oldest in the Agaricomycetes class. This result is in agreement with studies based on nuclear genes that have established the Cantharelalles order as the most basal group of the Agaricomycotina subphylum, which diverged 406 MYA (He et al., 2019; Zhao et al., 2017). Our analysis also suggested that the divergence events of the orders Hymenochaetales, Polyporales, Russulales, and Agaricales happened in a short time – 235, 233, 231, and 230 MYA, respectively. This result is similar to an estimate based on the nuclear genome, which suggests that the Hymenochaetales order diverged 236.6 MYA (Feng et al., 2012), but is in disagreement with another study that established the divergence time of Agaricales order at 123.6 MYA (Zhu et al., 2019). Besides, our data suggested that *Phellinotus piptadeniae* may have diverged 86 MYA, making it older than *Trametes villosa*, whose divergence time is 55 MYA.

The divergence time is an estimative based on the differences of aligned sequences that are accumulated throughout evolution (Kishino et al., 2001). Therefore, the differences found in our time-divergence analysis and those reported in other studies may be related to the different nucleotide substitution rates among fungal groups. Moreover, mitogenomic nucleotide substitution rate is not the same as the nuclear one, which can cause divergences in results (Berbee and Taylor, 2010; Sandor et al., 2018).

Furthermore, according to our analysis, the two *Flamullina velutipes* and *Rhizoctonia solani* strains diverged 0.47 and 76.1 MYA, respectively. In comparison with other species, the divergence time between the two *R. solani* strains is higher than those for the species of *Ganoderma, Laccaria*, *Lactarius*, *Lyophyllum*, *Moniliophthora*, *Pleurotus*, *Porodaedalea*, *Rhizopogon*, *Russula,* and *Trametes*. Outstanding genetic differences among *R. solani* isolates have already been identified, which led different authors to conclude that this taxon is a species complex of different groups with different evolutionary histories (Cubeta and Vilgalys, 1997), many of which are genetically incompatible (Ajayi-Oyetunde and Bradley, 2018). On the other hand, the two *Ganoderma lucidum* strains showed divergence time equal to zero, which is in agreement with our data that showed high similarity in size and content for their mtDNAs.

Except for *Porodaedalea niemelaei*, which lacks *atp8* gene, the mitogenome of all studied fungi is comprised of 14 mitochondrial protein-encoding genes, the two rRNAs subunits, *rps3* gene, and sets of tRNAs. This is not the first report of a missing gene in a fungal mitogenome. (Cummings et al., 1990) have already reported the lack of *atp9* gene in the mitogenome of the fungus *Podospora anserina*; even so, in comparison with other fungi, the genetic content of Agaricomycetes mitogenomes is well conserved (Sandor et al., 2018).

Besides the conservation of coding sequences, we noticed that the gene order varied considerably among the mitogenomes evaluated (Supplementary data 14). In the genera *Cantharellus*, *Ganoderma,* and *Trametes*, as well as in the Hymenochaetales order and Russulaceae family, the core-gene ordering is quite conserved. However, by analyzing the disposition of tRNAs, we can detect possible genetic rearrangements, such as deletion of a copy of *trnW* before *atp8* in *C. appalachiensis*, insertion of two copies of *trnR* in *G. sinense* (one between *nad5* and *rns* and the other one after *cob*), and inversion of the segment *atp9-trnD-trnW-trnG* in *T. villosa*. These observations are in agreement with a recently published study, in which the authors showed that, in the Hypocreales order, genetic rearrangement events involving tRNAs occur with a higher rate than events involving core-genes (Zhang et al., 2020). On the other hand, within species of the genera *Laccaria* and *Lyophyllum*, the gene ordering was not maintained. Moreover, for some species, only one core-gene appears to be out of order, such as *atp6* in *Russula compacta*, *rnl* in *Phellinotus piptadeniae,* and *nad3* in *Ganoderma meredithae*.

Differences in the gene ordering of mtDNAs have been described as consequences of non-homologous recombination events, that are undertaken by repetitive DNA sequences, plasmid integration, and mobile elements, such as HEGs (Aguileta et al., 2014; Li et al., 2019b). Fungal mitogenomes are dynamic and frequently undergo structural changes (Sandor et al., 2018). It is interesting to notice that some species with conserved gene ordering displayed higher divergence time than species without conserved gene ordering, such as *Rhizopogon* sp. (divergence time of 42.1 millions of years ago and conserved gene order) and *Lyophyllum* sp. (divergence time of 27.85 millions of years ago and non-conserved gene ordering). These findings suggest that divergence time may not be a factor (or the only factor) for the mitogenome gene shuffling. (Aguileta et al., 2014), investigating the relationship of mitochondrial gene ordering and the divergence time in Dikarya, also concluded that divergence time alone does not explain the changes in gene ordering.

In comparison to studies focused on fungi of Leotiomycetes class (Chen et al., 2019) and Hypocreales order (Sordariomycetes class) (Fonseca et al., 2020), our data revealed higher mitochondrial gene ordering variation. Nonetheless, high gene ordering variability was described by (Wang et al., 2019), who investigated the mitochondrial gene ordering of 13 Agaricomycetes fungi. These findings are in agreement with conclusions made by (Aguileta et al., 2014) that, although gene rearrangements are reported in all fungal groups, Basidiomycota seems to have the most variable gene ordering within the Kingdom Fungi.

The uneven sampling of groups is an important factor that may impact the results of different studies using mitochondrial genomes (Smith, 2016). In our study, the Agaricales order presented low gene ordering conservation, while in Russulales, the order of genes was quite conserved. The Agaricales order was comprised of 18 different species distributed in 12 genera, while the Russulales order was represented by 12 species of only 5 genera. The sampling bias could influence the conclusions drawn; however, this is not a technique-related artifact, but a consequence of the differential representation of the taxa in the current databases. Therefore, for more conclusive results regarding the conservation of mitochondrial gene order among Agaricomycete fungi, continuous studies including more mitogenomes are necessary.

4. Conclusions

In this study a comparative analysis of 57 different mitochondrial genomes of fungal species of the Agaricomycetes class, a group containing many economical and biotechnological relevant fungi, was performed. Alongside the mitochondrial genome sequences available at online public databases, our study also contributed with two newly sequenced mitogenomes of the species *Phellinotus piptadeniae* and *Trametes villosa*. The high variation in mtDNA size and content of the fungi evaluated can be mainly attributed to the presence of non-coding and intronic regions, HEGs, and uORFs. The observed sharing of homologous sequences from different mitogenomes revealed the possible existence of horizontal gene transfer events mediated by the activity of HEGs. Our analyses also revealed that codon usage varies among mitogenomes and that the GC content of mitochondrial features may be used to distinguish coding from non-coding sequences. Moreover, transposition events of mitochondrial genes to the nuclear genome are not common in Agaricomycetes. Despite the variation of size and content of the mitogenomes, mitogenomes seem to be reliable molecular markers according to our time-divergence analysis.

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CRediT authorship contribution statement

Daniel S. Araújo: Conceptualization, Investigation, Formal analysis, Writing - original draft, Visualization. **Ruth B. De-Paula:** Investigation. **Luiz M.R. Tomé:** Investigation. **Gabriel Quintanilha-Peixoto:** Investigation. **Carlos A. Salvador-Montoya:** Investigation. **Luiz-Eduardo Del-Bem:** Conceptualization, Investigation, Writing - review & editing. **Fernanda Badotti:** Investigation, Writing - review & editing. **Vasco A. C. Azevedo:** Investigation, Resources. **Bertram Brenig:** Investigation, Resources. **Eric R.G.R. Aguiar:** Conceptualization, Investigation, Formal analysis, Writing - review & editing. **Elisandro R. Drechsler-Santos:** Investigation, Resources. **Paula L.C. Fonseca:** Conceptualization, Investigation, Formal analysis, Writing - review & editing, Supervision. **Aristóteles Góes-Neto:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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