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The mitochondrial genome of *Morchella importuna* (272.2 kb) is the largest among fungi and contains numerous introns, mitochondrial non-conserved open reading frames and repetitive sequences

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ABSTRACT

The complete mitochondrial genome of *Morchella importuna*, the famous edible and medicinal mushroom, was assembled as a 272,238 bp single circular dsDNA. As the largest mitogenome among fungi, it exhibits several distinct characteristics. The mitogenome of *M. importuna* encoded 14 core conserved mitochondrial protein-coding genes and 151 mitochondrial non-conserved open reading frames (ncORFs) were predicted, of which 61 were annotated as homing endonuclease genes, and 108 were confirmed to be expressed during the vegetative growth stages of *M. importuna*. In addition, 34 introns were identified in seven core genes (*cob*, *cox*1, *cox*2, *cox*3, *nad*1, *nad*4 and *nad*5) and two rRNA genes (*rrnS* and *rrnL*) with a length from 383 bp to 7453 bp, and eight large introns with a length range of 2340 bp to 7453 bp contained multiple intronic mtORFs. Moreover, 34 group I (IA, IB, IC1, IC2, ID and derived group I introns) and four group II intron domains were identified for the 34 introns, including five hybrid ones. Furthermore, the *M. importuna* mitogenome showed the presence of about 18.7% mitogenomic interspersed repeats. These and the aforementioned ncORFs and introns, contributed to the enlarged size of the mitogenome.

1. Introduction

Mitochondria, the morphologically distinctive bimembrane organelles, are typically referred to as the power factories of cells due to their contribution to energy supply, but they also participate in several other important processes, including ion homeostasis, intermediary metabolism and cell senescence and apoptosis [1,2]. Mitochondria have their own genetic system as a vestigial genome originating from an endosymbiotic α-proteobacterial ancestor [3]. Most of the genomic material of the progenitor was rapidly lost or transferred to the nuclear genome during evolution. Depending on the species, the DNA that remains in the mitochondria ranges from approximately 11 kb (*Hanseniaspora uvarum*) to 235.8 kb (*Rhizoctonia solani*) in length, and it usually contains 14 conserved genes in fungi [4]. The remaining conserved genes are mainly involved in respiration and/or oxidative phosphorylation and translation, as well as transcription, RNA maturation and protein import [5]. Besides the core set of genes, a varying number of introns and some mitochondrial non-conserved open reading frames (ncORFs) encoding proteins of unknown function have also been reported in fungal mitogenomes [5,6].

The large size variations of mitogenomes between different fungal species mainly result from intergenic regions and introns, such as the *Ascomycota* fungus *Ophiocordyceps sinensis*, with introns accounting for 68.5% of the whole mitogenome; *Phlebia radiata*, with 80% of the 156 kb mitogenome composed of intronic and intergenic regions; *Schizophyllum commune*, with no introns present in the 49.7 kb mitogenome; *H. uvarum*, which has an extremely compact linear mitogenome, with only 5.1% (571 bp) intergenic regions, making it the smallest mitochondrion in fungi [4,5,7,8]. The number and size of introns are highly variable in the mitogenome of the fungal kingdom and are the major sources of variation in fungal mitogenomes [9].

Mitochondrial introns can be classified into group I and II domains according to their conserved secondary RNA structure, which often carries the motifs GIY-YIG or LAGLIDADG of homing endonuclease genes (*hges*) [9]. Group I introns are predominantly present in fungal mitogenomes, whereas group II introns dominate plant mitogenomes [10]. Both group I and II introns are able to move and proliferate in the mitochondrial genomes through a mechanism called intron homing, which is mediated by HEGs of intron-encoded (maturases) or host genome-encoded factors [9,11].

In addition to the conserved mitochondrial ORFs, the mitochondrial genome usually contains non-conserved ORFs (ncORFs) of different quantity, which exist either in the intronic regions as an intronic ORF

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or in intergenic regions as a free-standing ORF. The ncORFs are poorly conserved even among closely related species and their roles remain a mystery [5,6]. The quantity of ncORFs varies obviously in fungal mitochondria, especially in species with a large mitogenome size [5,12,13]. Some of the free-standing ORFs may also be functional because they contain HEG domains. In *Hypomyces aurantius* (72 kb), five out of the eight free-standing ORFs were found to encode proteins similar to homing endonucleases [14]. Sequence similarity analysis of free-standing ORFs showed lateral gene transfer rather than intragenomic proliferation, but their exact function in the mitogenome remains unknown [13].

Horizontal DNA transfer and repetitive sequences have played a significant role in the evolution and size expansion of mitogenome [12,15]. The mitochondrial gene horizontal transfer in yeast provided evidence for the emergence of introns, exons, and intron-lacking protein genes across the mitogenome, implying a propensity common in the entire fungal kingdom and other eukaryotic lineages [16]. Recent mitogenome analysis of fungi also suggests mitogenomic duplication as a potential reason for their size expansion [5,12].

In recent years, the number of complete filamentous fungal mitogenomes has significantly increased, and >500 complete fungal mitochondrial genomes are available in the NCBI Organelle Genome database; however, only the *Pyronema confluens* mitogenome in the Pezizomycetes of *Ascomycetes* has been simply described with a length of 191 kb [17]. Pezizomycetes is a basal branch of Pezizomycotina, which comprises one order, Pezizales, with the fruiting body being usually named as discoid or cup-shaped by shape except for the hypogeous truffle [18]. Some of them have important economic and medicinal values, such as *Tuber* spp., *Morchella* spp. and *Helvella* spp. *Morchella* is a highly desired edible mushroom with a worldwide distribution, and as long been hunted by mycophiles and gourmets for its delicate taste and unique appearance [19]. In recent years, the domestication and cultivation of *Morchella* have achieved great success in China, but the instability of cultivation caused by a weak understanding of the biological basis of this species seriously restricts the development of morel's industry. To help solve this problem, this work attempted to *de novo* assemble the complete mitogenome of *M. importuna*, with a focus on the possible reasons for the size expansion of the mitogenome and bioinformatics functional analysis of the ncORFs.

2. Materials/methods

2.1. Samples collection, sequencing and mitogenome assembly

The strains, culture conditions, and DNA sequencing protocol used in this paper have been reported previously [20]. The complete mitogenome could not be assembled in the nuclear *de novo* genome project, which meant that the mitogenome assembly had to be carried out in a gradual extension strategy. In short, the core conserved mitochondrial proteins were extracted from the mitochondrial genome of species with close phylogenetic relationships for database construction, with the purpose of screening the possible mitochondrial seed fragments from the final assembled genome and PacBio long sequencing data. Then, the mitochondrial genome was obtained by a new *de novo* assembly using long screened reads. First, by comparing homologous mitochondrial proteins with the final assembled genome, the Morimp01GS063 and Morimp01GS082 contigs were obtained. Alignment of short sequence reads showed that the median base coverage of the whole genome was 82×, but the median/mean base coverage was 783×/750× for Morimp01GS063 and 140×/201× for Morimp01GS082. This suggested that Morimp01GS063 is most possibly a mitogenome fragment, due to the higher copy number of the mitochondrial genome, and thus Morimp01GS082 was maintained as a candidate. Then, the Morimp01GS063 sequence was used as a seed for comparison with the intermediate assembly results of different software during genome assembly (Edena, AllPathsLG, DBG2OLC and FALCON), with the purpose to obtain a maximal number of prolonged candidate mitochondrial sequences. However, no effective results were obtained from the AllPaths-LG, FALCON or DBG2OLC genome assembly. In the results assembled by Edena, lots of contigs were matched, and those with a base coverage >400× were filtered as new candidate mitochondrial fragments, which were further compared with the sequencing data of PacBio to extract candidate long sequencing reads for the final mitogenome assembly. Finally, the extracted long reads were assembled by CANU software and seven new scaffolds were formed [21]. One of them with a sequence of 286,949 bp was identified as the complete mitogenome sequence by TBLASTP analysis of the mitogenome homologous protein database, and an overlap of its beginning and ending was determined by self-alignment. For the detailed technical parameters, please refer to the Supplementary Detailed_Parameters.doc.

2.2. Mitogenome annotation and bioinformatics analysis

The mitochondrial ORFs were identified automatically with MFannot and RNAweasel using genetic code 4 (http://megasun.bch.umontreal.ca/RNAweasel/). Functional assignments were performed based on sequence similarity to characterize fungal mitochondrial proteins using BLASTP searches against Nr and InterPro databases. The tRNAs were identified by tRNAscan-SE [22]. The mitochondrial physical map was drawn using OGDraw (version 1.4) software [23]. The transcriptional regulation of mitochondrial genes was evaluated using the transcriptome sequencing data of the vegetative growth of *M. importuna* at three stages [24]. The read coverage of the mapping sites was calculated by hisat2 software (https://ccb.jhu.edu/software/hisat2/index.shtml), and refer to the Supplementary (Detailed_Parameters.doc) for detailed calculation parameters. Repetitive sequences were identified and analyzed by local BLASTN searches of mtDNA against itself or nuclear genome at a cut-off e-value of 10^{-5}.

2.3. DNA and RNA extraction and PCR amplification

The DNA and cDNA were prepared as reported by Liu et al. and Zhang et al. [20,25], and were used to verify the coding genes or to determine the intron boundaries or the accuracy of sequence splicing. Primers were designed by the NCBI primer-BLAST design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast). Detailed information about the primers used in this paper is shown in Supplemental Table S1. The amplification protocol was the same as reported by Zhang et al. [25]. The amplified products were detected by 1.5% agarose gel electrophoresis and sequenced by Sanger sequencing.

2.4. Phylogenetic inference

The gene order of fourteen conserved protein-coding genes and the large and small ribosomal RNA genes in *M. importuna* were compared with those of 30 other known mitogenomes retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/genome/organelle/). The fourteen conserved proteins used for phylogenetic analysis included three ATPase subunits (*atp*6, *atp*8 and *atp*9), four subunits of the respiratory chain complexes (*cob*, *cox*1, *cox*2 and *cox*3), and seven NADH dehydrogenase subunits (*nad*1, *nad*2, *nad*3, *nad*4, *nad*4L, *nad*5 and *nad*6). A phylogenetic tree was constructed using the mitogenome of *Rhizopus oryzae* (*Mucoromycotina*) as outgroup. The multiple sequence alignment was performed by MAFFT v7.158b software [26], the Prottest v3.4 software was used to calculate the best amino-acid replacement models [27], and the RAxML version 8.1.24 software was used to calculate the maximum-likelihood species trees with 1000 bootstrap-replications [28]. The final trees were drawn using the TreeView software [29].

3. Results and discussion

3.1. Mitogenome assembly of M. importuna in a gradual extension strategy

The mitogenome could not be identified by the normal genome *de novo* assembly [20]. Therefore, a gradual extension strategy was adopted in this paper, as mentioned above, and finally, the 272,238 bp circular mitochondrial physical map of *M. importuna* was obtained (Fig. 1) (GenBank Acc. No. MK527108). This is the first time that we have accomplished the complete assembly of the mitogenome of *M. importuna*. The mitogenome assembly was mainly dependent on the PacBio long sequencing read approach, with an average read length of about 10 k to span the repetitive regions (refer to the following section for details).

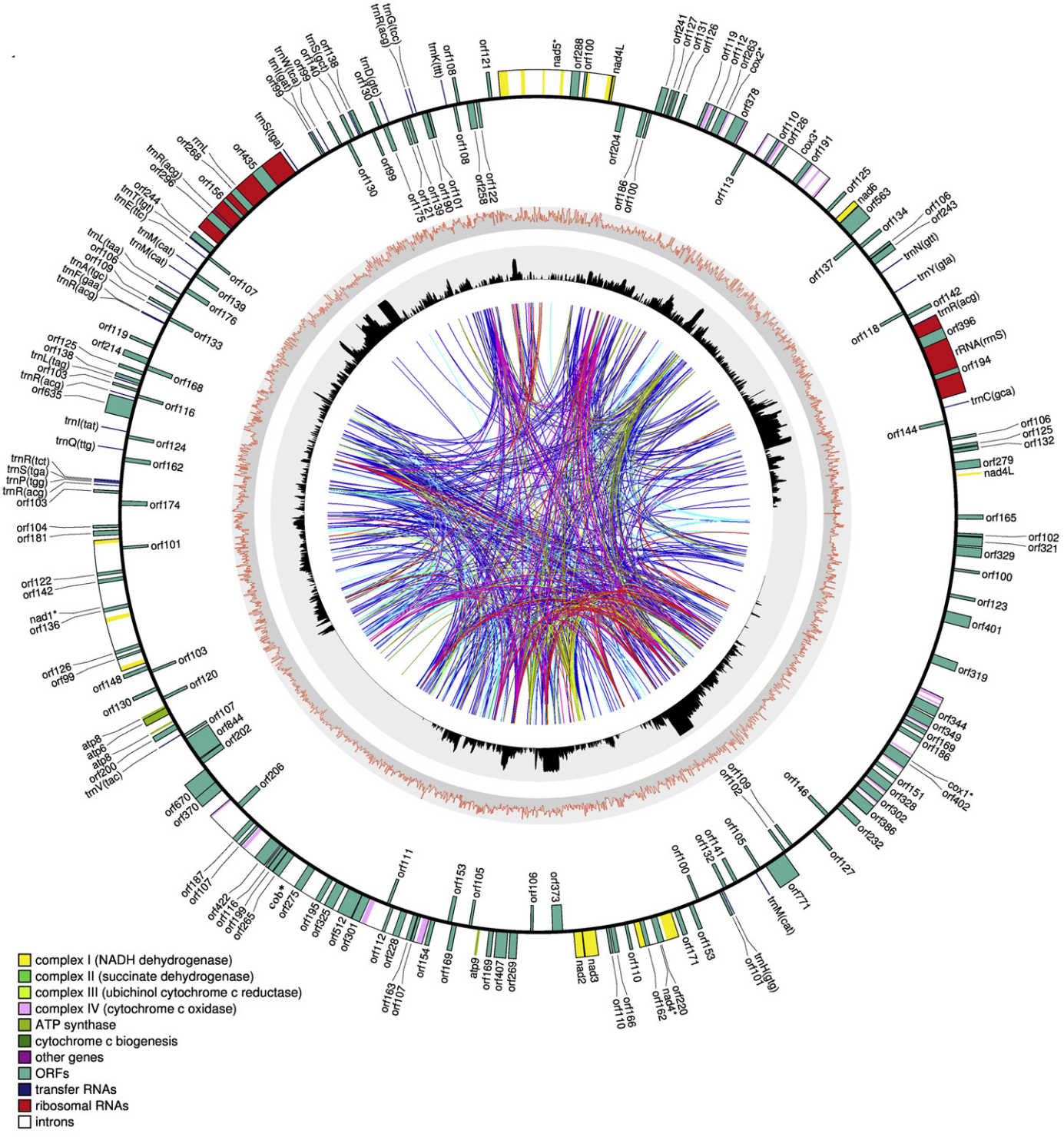

Fig. 1. The 272.2 kb physical map of the mitogenome of *Morchella importuna*. The first ring from the outside represents the core *M. importuna* mitochondrial protein coding genes, rRNAs, tRNAs, *rps*3 and ncORFs, inside the circle are the genes of the antisense strand; outside the circle are the genes of the sense strand. The second ring represents the GC percentage content. The next inner circle represents the RNA-seq coverage (TPM, log2) of the vegetative stages. The innermost circle shows the repetitive sequences within the mitogenome, and the colors of the six links represent different lengths of repeat sequences, <50 bp, 50–100 bp, 100–150 bp, 200–250 bp, 250–300 bp and >300 bp indicated by blue, wathet, light brown, light green, purplish and red, respectively.

3.2. Characteristics of the mitogenome of M. importuna

To our knowledge, the 272 kb mitogenome of *M. importuna* is the largest in size to date in the kingdom of fungi, which is far larger than the reported 203 kb-size mitogenome of *Sclerotinia borealis* of *Ascomycetes*, and the 235 kb-size mitogenome of another soil fungus *Rhizoctonia solani* of the *Basidiomycota* phylum [12,13]. The total GC content of the *M. importuna* mitogenome was 39.94%, and it has almost the same percentage content in coding regions and intron regions, which is much higher than the average value of the other fungi (24.52%, n = 537, Eukaryota, Fungi group; 18/8/2018, https://www.ncbi.nlm.nih.gov/genome/browse#!/organelles/).

The data of the *M. importuna* mitogenome (SCYDJ1-A1 v1.0: Project: 1047733) was released in the JGI database (https://genome.jgi.doe.gov/Morimp1/Morimp1.home.html), and has 30 contigs, two scaffolds and 5.5% gap, with a mitogenome contig size of 259,232 bp. The lengths of the two scaffolds were 272,253 bp and 1953 bp. The SCYDJ1-A1 mitogenome was blasted against our mitogenome, and the results showed a high similarity between them. The scaffold_1 (272,253 bp) of the SCYDJ1-A1 mitogenome was aligned to our mitogenome with a query coverage of 93% and an identity of 99% (NCBI blastn online web server). Another small mitogenome fragment (scaffold_2 1953 bp) of SCYDJ1-A1was aligned with 99% identity as well. On this point, the mitogenome of *M. importuna* was indeed relatively large in size.

The prediction and annotation of protein-coding genes revealed a total of 171 hypothetical genes in the *M. importuna* mitogenome, which was also the largest in fungi, far exceeding the 127 of *R. solani*, 126 of *P. radiate*, 95 of *S. borealis* and 94 of *Chrysoporthe austroafricana* [8,12,13,30]. Fourteen of these genes were identified as typical mitochondrial genes. Interestingly, two copies of *atp*8, one truncated *nad*4L (273 aa) and two predicted *rnp*B genes encoding RNAse P (mtP-RNA) were also identified. All of the 14 typical mitochondrial genes and the repetitive genes were on the same strand (Fig. 1). As commonly found in the fungal mitogenome, the *nad2*/*nad3* genes were joined together, and the *nad4L*/*nad5* genes were only four bases from each other in the *M. importuna* mitogenome [5,12,31]. Furthermore, 151 ncORFs were identified to be uniformly dispersed in the *M. importuna* mitogenome, including 39 intronic ORFs and 112 free-standing ORFs, with 45 of them on the antisense strand and their length ranging from 300 to 2535 bp.

Additionally, 34 introns were identified to range from 383 bp (*rrnS* gene intron i5) to 7453 bp (*nad*1 gene intron i1). Except for the *nad*4i1 intron, which was not annotated to the known intron domains, 34 and 4 of the other introns were annotated to group I and group II intron domains, respectively. Five hybrid introns were identified with two different domains, and all of them were in large introns (*rrnLi*3 3819 bp, *nad*1i2 4724 bp, *cob*i1 4173 bp, *cob*i3 6840 bp and *cob*i5 5369 bp) (Fig. 1, Table 1).

Based on the Nr and InterPro annotation, 61 genes in the 151 ncORFs associated with self-splicing mobile genetic elements were identified as *hegs*, and they all contained the LAGLIDADG or GIY-YIG conservative domain. The mitogenome was found to contain 22 GIY-YIG and 39 LAGLIDADG HEG genes, with 29 *hegs* being annotated in intronic ORFs, and the remaining 32 *hegs* present in free-standing ORFs. Meanwhile, 118 ORFs could be annotated by Nr and Interpro, and whether the remaining 33 ORFs have any specific functions or are pseudogenes remains to be confirmed in further studies.

A set of 32 different tRNAs for the 20 amino acids was identified to be widely distributed in the *M. importuna* mitogenome (Supplemental Table S2). All tRNAs were on the same strand, as typically occurs in conserved genes, and ranged from 68 to 88 bp in length. Among them, four tRNAs (trnR, trnS_2, trnP and trnR_6) were clustered from 132,623 to 133,215 between ORF103 and ORF162, and the other 28 tRNAs were scattered throughout the whole mitogenome (Fig. 1). This was different from other fungi, as tRNAs usually exist in clusters [32–34].

The protein coding sequences totaled 112,940 bp, including 14 core conserved protein-coding genes, 151 ncORFs, 32 tRNA genes, two rRNA genes (*rrnS* and *rrnL*), two *rpn*B genes and one *rps*3 gene, accounting for 41.49% of the genome size. The 14 mitochondrial conserved genes, tRNA and rRNA regions occupied only 8.52% of the mitogenome; the intergenic sequences, 99,920 bp (36.70% of the total); the intronic sequences, 84,131 bp (30.90% of the total); the free-standing ORFs regions, 64,989 bp (23.87% of the total), which dominated the mitogenome and were responsible for the huge mitogenome of *M. importuna* (Table 2).

3.3. Interspersed repeats of M. importuna

The most salient feature of the *M. importuna* mitogenome was the presence of a large number of interspersed repeats. At the threshold of 1e-5, 2216 repetitive units were identified within the mitogenome by global BLASTN alignments, with a total cumulative length of 230,860 bp. After global identity alignments at 90% cut-off threshold by CD-HIT [35], the 2216 repetitive elements were classified into 447 unique sequence units with a total length of 50,922 bp, accounting for 18.7% of the mitogenome. Such a large number of repetitive sequences in the mitochondria genome had been reported in *R. solani*, covering about 34% of the whole mitogenome and once topping the list of mitogenomes in fungi [12]. Besides these repetitive sequences, there were few large-scale internal repeats in the mitochondrial genome of fungi. The length of the repeat sequences of *M. importuna* ranged from 27 to 564 bp. Some long sequence units had multiple repetitions, such as the 50 bp fragment at the position of 177,510 to 177,559 with the highest repetition (22 times), and five repetitive fragments over 200 bp that are repeated >10 times each (Fig. 1).

Sequence alignment of the mitogenome with the nuclear genome of *M. importuna* showed that at the threshold of 1e-5, one end of the genome scaffold Morimp01GS025 and one end of Morimp01GS039 aligned to the mitogenome. However, it was confirmed by PCR amplification and sequencing with specific primers that the specific regions of the nuclear genome had been incorrectly assembled (Supplemental Table S1). To further verify whether the horizontal transfer of foreign DNA sequences resulted in the large mitochondrial genome of *M. importuna*, we compared the mitogenome with the nr/nt, refseq_genomes, wgs, HTGS and gss databases in NCBI (default parameters). The results showed no alignment of other sequences in the *M. importuna* mitogenome, implying that the expansion of the mitogenome could have been mainly intrinsic.

3.4. Mitochondrial gene expression of M. importuna

The numerous ncORFs are the most unusual feature of the *M. importuna* mitogenome. To evaluate whether these ncORFs are functional, the transcriptome data of *M. importuna* at the vegetative growth stage were aligned [24]. The results showed that except for the conserved genes, 108 out of the 151 ncORFs were expressed with a total TPM (Transcripts Per Million) in the range of 454 to 2,100,078, and the expression of the majority of ncORFs was below the average level (102/108, with an average and median TPM of 50,929.39 and 1794.60, respectively) (Supplemental Table S3). Both small and large ribosomal RNA genes were highly expressed with a total TPM of 655,176.39 and 904,672, respectively.

Interestingly, some ncORFs had extremely high expression levels. For instance, orf105 and orf771 (Fig. 1, Supplemental Table S3) had a total TPM of 2,100,078, which exceeded the expression level of the large ribosomal RNA subunits. The orf105 could not be annotated by any of the methods used. The orf771 functional annotation showed that it contains some conservative domains, such as RT_G2_intron (reverse transcriptase with group II intron origin, e-value: 7.45e-80), RVT_1 (reverse transcriptase: RNA-dependent DNA polymerase, e-value: 1.38e-20) and intron_maturas2 (Group II intron reverse transcriptase, maturase and DNA endonuclease activities, e-value: 1.00e-17). The predicted gene orf793 was also annotated as an RNA-

Table 1
Location and annotation of introns and intronic ORFs in the mitogenome of *Morchella importuna*.

Gene name	Intron								Intronic ORF[c]			
	No.	Start	Stop	Size (bp)	Type RNAweasel[b]	E-value	Start	Stop	Gene name	Start	Stop	Homing endonuclease[d]
	i1	12885	14910	2026	II (domainV)	6.08E-14	14839	14881	G-orf194	14103	14687	LAGLIDADG
	i2	15066	17229	2164	II (domainV)	1.66E-06	17157	17199				
rrnS	i3	17495	18928	1434	IC2	2.34E-35	17530	17758	G-orf396	17633	18823	LAGLIDADG
	i4	19249	19642	394	IC1	1.26E-27	19308	19587				
	i5	19669	20052	384	I (derived, A)	6.84E-03	19768	19932				
	i1	37245	38342	1098	IB (complete)	3.95E-17	38115	38313				
cox3	i2	38412	39169	758	II (domainV)	2.11E-12	39090	39130				
	i3	39284	42952	3669	IB (complete)	4.12E-17	40654	41498	G-orf191	39284	39859	LAGLIDADG
	i4	43191	44430	1240	I (derived, A)	4.79E-03	44209	44374	G-orf110_1	43193	43525	
cox2	i1	47013	48780	1768	IB (complete)	2.32E-18	48362	48749	G-orf378	47013	48149	GIY-YIG
	i2	48910	50641	1732	ID	2.52E-29	50470	50623	G-orf263	48910	49701	LAGLIDADG
	i1	61157	63069	1913	IB (complete)	5.63E-21	61195	61682				
	i2	63223	65616	2394	IB (complete)	9.66E-22	65024	65533	G-orf100_2	63223	63525	LAGLIDADG
nad5	i3	65764	67492	1729	IB (complete)	1.82E-25	65786	67432				
	i4	67534	69450	1917	IB (complete)	6.70E-24	67572	69248				
	i5	69693	71126	1434	IB (complete)	8.45E-20	70157	70352				
	i1	96568	98827	2260	IC2	5.92E-30	98494	98727	G-orf435	97053	98360	GIY-YIG
	i2	99686	101261	1576	IA	6.16E-22	99735	101204	G-orf268	100339	101145	LAGLIDADG
rrnL	i3[a]	101311	105129	3819	IB (complete)	3.98E-17	102980	103164	G-orf156	101733	102203	LAGLIDADG
					I (derived, A)	2.17E-05	104723	104894	G-orf296	103646	104536	GIY-YIG
	i1	138954	146407	7454	IA (5′ partial)	7.27E-06	141833	142016	G-orf101_2	139746	139441	
									G-orf122_2	141811	142179	
									G-orf142_2	142504	142932	GIY-YIG
nad1									G-orf136	145360	145770	
	i2[a]	146666	151389	4724	IB (5′, partial)	8.71E-04	146746	147242	G-orf126_3	149646	150026	
					I (derived, B1)	2.65E-06	148487	148745	G-orf99_4	150290	150589	
	i1[a]	168648	172820	4173	IB (complete)	3.03E-20	171139	171350	G-orf206	169936	169316	
					II (domainV)	2.11E-12	170972	171012	G-orf187	171401	171964	LAGLIDADG
									G-orf107_3	172247	172570	LAGLIDADG
	i2	173028	175558	2531	ID	4.95E-35	173999	174118	G-orf422	174234	175502	LAGLIDADG
	i3[a]	175592	182431	6840	IB (complete)	8.44E-28	177723	177995	G-orf199	175902	176501	LAGLIDADG
					ID	2.27E-31	177413	177534	G-orf265	176494	177291	LAGLIDADG
									G-orf275	179036	179863	LAGLIDADG
cob									G-orf195	181439	182026	GIY-YIG
	i4	182485	186480	3996	IA	3.63E-18	183543	183862	G-orf512	183883	185421	LAGLIDADG
									G-orf301	185414	186319	LAGLIDADG
	i5[a]	186788	192156	5369	IB (complete)	1.58E-18	189520	189737	G-orf111	188445	188110	
					IA	9.52E-18	189184	189433	G-orf112_2	188605	188943	
									G-orf228	189722	190408	LAGLIDADG
									G-orf163	191076	191567	GIY-YIG
									G-orf107_4	191575	191898	GIY-YIG
nad4	i1	214653	216992	2340					G-orf162_2	214728	215216	LAGLIDADG
									G-orf220	215971	216633	
	i1	240109	241693	1585	IB (complete)	1.33E-21	241318	241664	G-orf386	240109	241269	LAGLIDADG
	i2	241735	242982	1248	IB (complete)	1.18E-15	242714	242953	G-orf302	241736	242644	LAGLIDADG
	i3	243088	245846	2759	IB (3′, partial)	3.39E-08	244293	245619	G-orf328	243089	244075	LAGLIDADG
cox1	i4	245954	247414	1461	IB (complete)	4.30E-19	247198	247370	G-orf402	245956	247164	GIY-YIG
	i5	247462	250512	3051	IC2	6.91E-39	247499	247787				
	i6	250588	253078	2491	IB (complete)	1.09E-23	250615	251654	G-orf349	250588	251637	LAGLIDADG
	i7	253093	253526	434	I (derived, A)	7.48E-04	253260	253357				

[a] Different intron domains are in one intron.
[b] Annotations for intron types were performed using RNAweasel online tools (http://megasun.bch.umontreal.ca/cgi-bin/RNAweasel/RNAweaselInterface.pl).
[c] Intronic ORF was obtained by MFannot prediction.
[d] Homing endonuclease annotations were obtained by Nr or InterPro, which identifies the conservative.

dependent DNA polymerase in the *cob* intron of *P. radiata*, which was considered to have been horizontally transferred recently from a distant gene with a potential genetic origin of the virus [8]. Orf373, another interesting mitochondrial ORF, also had a high transcription level with a total TPM of 115,652 on the antisense strand in the region about 22 kb upstream of the orf771 gene, and it was annotated as containing the conservative domain of reverse transcriptase RT_G2_intron (e-value: 3.48e-91).

Highly transcribed RTs (reverse transcriptase) were reported in the 157 kb-sized mitochondrial genome of *O. sinensis*, which, just like the *M. importuna* mitogenome, also contains a number of repetitive sequences, with both RTs and HEGs having the ability to move around the genome and increase the number of copies, resulting in sequence repetition and the increase of genome size [5]. Increased reverse transcriptase activity was observed when plasmids are integrated in the mitochondrial genome [36]. Such template-switching reverse transcriptase could explain both the loss and gain of introns [36,37]. In plants, these reverse transcriptase genes are thought to be associated with group II intron splicing function and/or other cellular functions [37]. In the isolated organelle, the sustained high expression during vegetative growth implied that, apart from reverse transcription, insertion and maturation enzyme activity, the RT_G2_intronic ORFs (orf771 and orf373) may have other unknown important functions, but this is beyond the scope of this study.

3.5. Phylogenetic analysis of *M. importuna* and other fungi

Considering the limited information about the mitochondrial genome of Pezizomycetes, we performed a comprehensive phylogenetic analysis of the novel *M. importuna* mitogenome in combination with

Table 2
Characteristics of the mitogenome of *Morchella importuna*.

Size of mitochondrial genome (bp)	272,238
Rate of GC (%)	39.94
Number of standard PCGs (n)	14
Introns (n)	34
ORFs (n)	151
rRNAs (n)	2
tRNAs (n)	32
Genic regions (bp)	15,839
Intergenic regions (bp)	75,167
Intronic regions (bp)	84,131
Intronic ORF regions (bp)	24,753
Free-standing ORF regions (bp)	64,989
tRNA genes (bp)	2378
rRNA genes (bp)	4981
Total ORF regions (bp)	89,742

the mitogenomes of other higher taxonomic unit species, including two species of *Mucoromycota*, six species of *Basidiomycota* and the other 22 species of *Ascomycota*. The mitogenome phylogenetic tree was constructed using the maximum-likelihood method and the LG+I+G+F model with 14 core concatenated mtDNA-encoded proteins. The phylogenetic tree based on the mitochondrial genome has the same topological structure as previously reported [20]. Consistent with the expectation, the *Mucoromycotina*, *Glomeromycotina* and *Basidiomycota* were clustered at the basal position. Meanwhile, 23 species of *Ascomycota* were clustered under a large clade, with *M. importuna* and *P. omphalodes* on the closest branch, indicating the same class and order (Pezizomycetes and Pezizales) but a different family (Morchaceae and Pyronemataceae). The other 21 *Ascomycota* species were clustered in four independent clades, representing the four classes of Eurotiomycetes, Sordariomycetes, Leotiomycetes and Dothideomycetes (Fig. 2A).

As *P. omphalodes* is the closest species to *M. importuna* in the current public database, their mitochondrial genome collinearity was analyzed by Mauve [38]. The alignment of the mitogenomes revealed a remarkable difference without obvious synteny between each other (Fig. 2B). This result is understandable as they are nevertheless genetically distant and belong to two different families.

4. Discussion

Mitogenomes are assumed to be highly reduced in the protein gene content of the descent from their α-proteobacterial ancestor and almost only retain the genes involved in respiration and protein synthesis [5]. The gene contents of mitochondria are quite conserved. Typical fungal mitogenomes usually encode 30–40 genes, and their sizes vary greatly from 11 kb to the current 272 kb in *M. importuna* [2,4]. The variation in the fungal mitogenome length is closely related to the number and sequence length of introns as well as ncORFs. Additionally, the direct introduction of exogenous DNA such as plasmid-related DNA and sequence duplication also leads to the expansion of the mitochondrial genome.

The mitogenomes of fungi often possess a different number of introns, from 0.15 to 4 kb in size, and the overall intron content ranges from the intron-free mitogenomes of both *Aspergillus terreus* and *Phialocephala fortinii* to 68% in *O. sinensis* [1,5,39,40]. In the *Rhynchosporium* clade, the intron content ranges from 8.1% in *R. orthosporum* to 39.8% in both *R. agropyri* and *R. secalis* [6]. *P. anserina* has a relatively large mitochondrial genome of approximately 100 kb, including 36 introns that account for about 60% of the mitogenome [41], resulting in a large size difference from 87 kb to 101 kb in ten different *P. anserina* strains due to optional introns in mitochondrial DNA [42]. Mitogenome size differences are also reported to be primarily related to the number of introns, such as in *Fusarium* sp. and *S. borealis* [13,33]. The increase in the mitogenome size of *S. borealis* was mainly ascribed to the insertion of 61 introns, which was far greater than that of other fungi [13]. Here, a total of 34 introns were identified in seven conserved typical mitochondrial genes and two ribosomal RNAs of *M. importuna*. Interestingly, among the large introns identified, *nad*1i1 is the largest with a length of 7453 bp, including four intronic ORFs, followed by *cob*i3 and *cob*i5, which are also >5 kb and contain multiple intronic ORFs. This phenomenon of large introns containing multiple ORFs showed that the mitochondrial introns could be tolerant to the insertion of DNA fragments, which should be the reason for the enlargement of the *M. importuna* mitochondrial size of intrinsic origin, although the amplified sequence source was unknown.

Mitochondrial ncORFs are often present in a large fungal mitogenome, such as in *R. solani* (119 ncORFs in a 235 kb mitogenome) [12], *P. radiata* (108 ncORFs in a 156 kb mitogenome) [8], *S. borealis* (80 ncORFs in a 203 kb mitogenome) [13] and *O. sinensis* (58 ncORFs in a 157 kb mitogenome) [5], accounting for 18%–26% of the corresponding mitogenome size. The origins and functions of the ncORFs have not yet been elucidated, and some mechanisms were proposed to explain it, such as the introduction of novel point mutations and small indels in the pre-existing mitochondrial gene, the gene transfer from the nuclear genome to mitogenome or the horizontal transfer from another species [6]. There are 151 ncORFs in the mitogenome of *M. importuna*, including 39 intronic ORFs, and 108 of them were expressed, but their exact function remains unknown. The real answer to this question probably should start with the comparison of the mitogenomes of more closely related fungal species [43]. The total length of these ncORFs is 89 kb, accounting for 34% of the whole mitogenome, and these ncORFs may help to explain the increased size of the mitogenome of *M. importuna*.

Some large-size fungal mitochondrial genomes were reported to contain a certain proportion of repetitive sequences [8,12,13]. The most obvious example was *R. solani* (235 kb), with one-third of the genome being occupied by interspersed repeats, which provided evidence for mitochondrial size expansion and highlighted the role of interspersed repeat elements in the evolution of fungal mitochondria [12]. Based on the numerous occurrences of nuclear DNA recombination between the heterokaryon parent strains, Losada et al. speculated that the heterokaryotic structure may also be responsible for the increase of fine mitochondrial DNA, but there was no evidence of horizontal gene transfer or recombination in *R. solani* [12]. Numerous short inverted repeats were reported to be hidden in the glomeromycete fungus *Funneliformis mosseae* (134.9 kb), suggesting their involvement in the dispersal of numerous mobile elements, contributing to mitogenome expansion [10]. In *R. solani*, some repetitive elements appeared to be clustered on islands in the mitogenome, indicating that these regions might be hotspots for insertion and many elements could jump consecutively into these locations, or a single large element could be introduced to the genome, then copied and become degenerated in some cases [12].

Plasmid-related sequences were able to integrate into the mitogenome and an increasing number of reports have described plasmid-related sequences in plant and fungal mtDNAs [44], such as in *A. bitorquis* [45], *Pleurotus ostreatus* [31], *Moniliophthora perniciosa* [46] and *Fusarium* species [33]. The linear plasmid pMC3-2 with a length of 6044 bp, was found to be localized in the mitochondria of *M. conica* [47], and 26% of its sequence (gb|X63909.1, pos: 1064 bp, 2087..2512;2512..3151) was aligned to the *M. importuna* mitogenome with 84% sequence identity (e-value = 0) at the antisense strand (pos:57825..57396;57296..56651). One ORF (orf186_1) was annotated in this region and probably encoded a B-type DNA-polymerase, corresponding to the ORF2 of pMC3-2(e-value = 5.61992e-62) [47]. At least eight strains of *Morchella* sp. have been reported to have plasmids [48], while no reports are available about the presence of plasmids in *M. importuna* at present. Therefore, it is still not clear whether the mitochondrial genes divide into plasmids, the linear plasmids integrate into the mitochondrial genome, or both scenarios occur.

Homing endonucleases genes, which are common in fungi and other eukaryotic mitogenomes, are assumed to represent 'semi-selfish

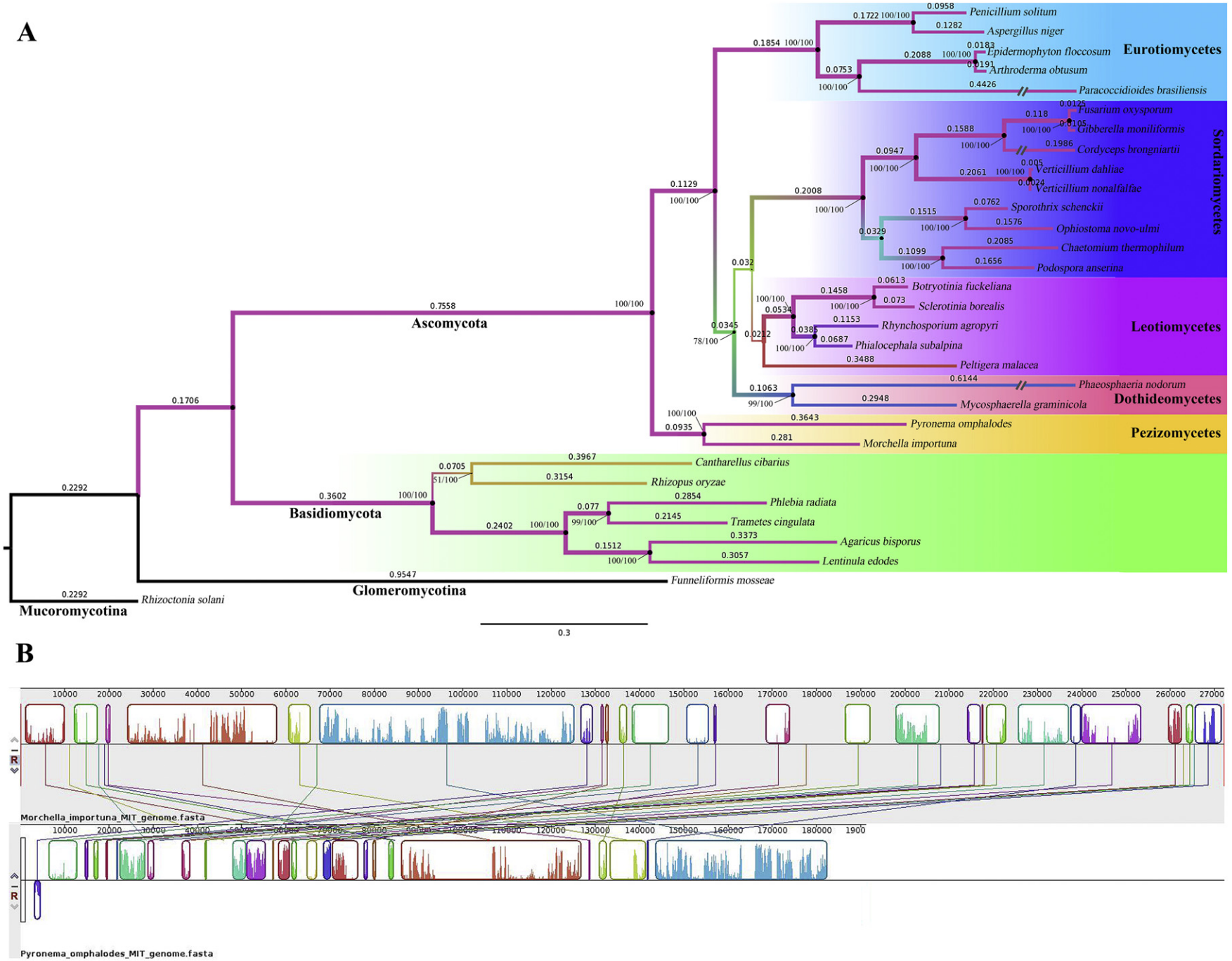

Fig. 2. Phylogenetic and comparative analysis of *Morchella importuna*. (A) The phylogenetic tree was constructed using the 14 core concatenated mtDNA-encoded proteins of 31 fungal species (detailed mitogenome information used in panel A can be found in the Supplement Table S4). (B) Mitogenome comparative collinearity analysis between *Morchella importuna* and *Pyronema omphalodes*.

elements' that promote the homing of introns by cleaving DNA at a specific site and can also function as intron-specific maturases by facilitating RNA splicing [49,50]. Thus, they have been proposed as the driving force for the wide distribution of group I introns through horizontal DNA transfer, even between phylogenetically distant species from different kingdoms [51]. Homing endonucleases can promote site-specific homologous recombination events, leading to insertion, deletion, mutation, or repair of DNA double-strand breaks, which have been well studied in yeast and mammalian cells [52,53]. Mitochondria play a key role in eukaryotic aging and many mechanisms are involved in keeping the mitochondria functioning under cumulative age-related damage. One of the mechanisms of DNA repair is (homologous) recombination, a process that can also lead to new combinations of undamaged genes and thus may have a role in maintaining well-functional mitochondria [54]. Mitochondrial homologous recombination occurred between natural populations of *P. anserine*, which increased their life span, especially in late life stages [54,55]. Just like *P. anserine*, a large number of morel species (*M. elata*, *M. importuna* and *M. sextelata*, among others) have undergone rapid and systematic aging during natural culture conditions [56,57]. The new evidence showed that this aging was related to oxidative damage [58]. Thus, the relationship between the rapid aging of morel and the mitogenome needs to be further studied. The expansion of the *R. solani* mitogenome was presumed to be related to *hges* [12]. As a considerable number of expressed *hegs* and reverse transcriptases were annotated in the mitogenome of *M. importuna*, they are supposed to contribute to the increase of the mitogenome size.

5. Conclusion

In this study, the 272,238 bp single circular mitochondrial genome of *M. importuna* was assembled for the first time. In addition to 14 core conserved mitochondrial protein-coding genes, 151 ncORFs were predicted. Sixty-one of the ncORFs were annotated as *hges* and 108 of were shown to be transcribed. Furthermore, 34 introns were identified with a length from 383 bp to 7453 bp, and eight large introns contain multiple intronic ORFs. Moreover, 34 group I (IA, IB, IC1, IC2, ID and derived group I introns) and four group II domains were determined in the 34 introns, including five hybrid introns. As the largest among fungi thus far, this mitogenome exhibits several distinct characteristics, including ~18.7% of mitogenomic interspersed repeats as well as a fairly large number of introns and free-standing ORFs. The insertion of introns, numerous ncORFs and repetitive sequences, possibly contributed jointly as evolutionary events to the increase in the size of the *M. importuna* mitogenome.

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CRediT authorship contribution statement

Wei Liu: Conceptualization, Methodology, Supervision, Resources, Data curation, Software, Writing - original draft, Writing - review & editing. **Yingli Cai:** Methodology, Resources, Data curation. **Qianqian Zhang:** Methodology, Resources, Data curation. **Lianfu Chen:** Software, Methodology. **Fang Shu:** Methodology, Resources, Data curation. **Xiaolong Ma:** Methodology, Resources, Data curation. **Yinbing Bian:** Conceptualization, Methodology, Supervision, Funding acquisition, Writing - review & editing.

Declaration of competing interest

We have no conflict of interest to declare.

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