The mitochondrial genome of the Basidiomycete fungus

*Pleurotus ostreatus* (oyster mushroom)

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**Abstract**

In this study, the full mitochondrial genome of a basidiomycete fungus, *Pleurotus ostreatus*, was sequenced and analyzed. It is a circular DNA molecule of 73 242 bp and contains 44 known genes encoding 18 proteins and 26 RNA genes. The protein-coding genes include 14 common mitochondrial genes, one ribosomal subunit protein 3 gene, one RNA polymerase gene and two DNA polymerase genes. In addition, one RNA and one DNA polymerase genes were identified in a mitochondrial plasmid. These two genes show relatively low similarities to their homologs in the mitochondrial genome but they are nearly identical to the known mitochondrial plasmid genes from another *Pleurotus ostreatus* strain. This suggests that the plasmid may mediate the horizontal gene transfer of the DNA and RNA polymerase genes into mitochondrial genome, and such a transfer may be an ancient event. Phylogenetic analysis based on the *cox1* ORFs verified the traditional classification of *Pleurotus ostreatus* among fungi. However, the discordances were observed in the phylogenetic trees based on the six *cox1* intronic ORFs of *Pleurotus ostreatus* and their homologs in other species, suggesting that these intronic ORFs are foreign DNA sequences obtained through HGT. In summary, this analysis provides valuable information towards the understanding of the evolution of fungal mtDNA.

**Introduction**

*Pleurotus* species (*Pleurotaceae*, Basidiomycetes) are worldwide distributed macrofungi and usually grow on hardwood in terrestrial ecosystems (Vilgalys & Sun, 1994). Similar to other white-rot fungi, they are important agents of biodegradation, due to their ability to break down plant materials, especially cellulose and lignin (Carlile et al., 1994). Most *Pleurotus* fungi are edible mushrooms with high commercial value, and thus they have been widely cultivated (Cohen et al., 2002). *Pleurotus ostreatus* (i.e. oyster mushroom) is a complex species including a number of varieties, subspecies and cultivated strains. Previous research on *Pleurotus ostreatus* has been mainly focused on taxonomy, nutrition, health-stimulating materials and waste biodegradation (Cohen et al., 2002; Park et al., 2006; Saavedra et al., 2006; Sainos et al., 2006; Sarangi et al., 2006). However, the knowledge on genomics and evolutionary history of *Pleurotus ostreatus* is far from being profound.

In fungi, mitochondrial genomes (mtDNAs) were widely used to address the issues such as classification, mitochondrial origination, and horizontal gene transfer (HGT) (Walker & Doolittle, 1982; Bullerwell et al., 2003a, b; Seif et al., 2005; Seifert et al., 2007). Several reports addressed the classification of the *Pleurotus* species based on phylogenetic analyses using mitochondrial small subunit ribosomal (rns) gene. For example, using V4, V6 and V9 domains of mitochondrial *rns*, 49 *Pleurotus* species were grouped into 14 clusters according to sequence similarity (Gonzalez & Labarere, 1998, 2000). These results clarified ambiguities in earlier classification, and nominated *Pleurotus ostreatus* and *Pleurotus floridus* as the same species (Gonzalez & Labarere, 2000). A similar clustering analysis was applied to distinguish *Pleurotus ostreatus* from two close species showing identical restriction fragment length polymorphism (RFLP) types (Bao et al., 2005).

An interesting topic on fungal mtDNA is the diversity of genomic size and gene content among different species and strains. DNA polymerase gene, for instance, is usually present in basidiomycete mtDNAs that have a relatively large size, such as in *Agaricus bisporus* (Robinson et al., 1991), *Crinipellis perniciosa* (NC_005927) and *Agrocybe*...
were reported. The zonation, gene content, intergenic repeats and intronic ORFs was determined and its genome organization, as well as for the comparative genomics of fungal mitochondria.

In the present study, the complete mtDNA sequence of *P. ostreatus* was determined and its genome organization, gene content, intergenic repeats and intronic ORFs were reported. The *cox1* genes and the intronic ORFs were then used to reconstruct the phylogenetic relationship of fungi and lower eukaryotes, and the possible occurrence of HGT was discussed.

Materials and methods

Preparation of mtDNA

The fungus strain, *Pleurotus ostreatus* P51, was obtained from the Edible Cultivated Mushroom Collection of Chengdu, Southwest China. It is a representative strain of several cultivated strains isolated from southwest of China on the basis of consistent RFLP pattern. The mycelium was grown statically on a shallow layer of potato yeast (1%) exudate glucose (5%) medium for 8–14 days at room temperature and was harvested for preparation of mtDNA. Briefly, 50–100 g of wet mycelium was used to isolate crude DNA and the AT-rich mtDNA band was recovered as described elsewhere (Garber & Yoder, 1983).

Cloning and sequencing of mtDNA

The mtDNA was digested with single or combined restriction enzymes (RE) according to its RE map and the digested DNA fragments were cloned into the corresponding RE-sites of the Bluescript KS+ vector (Invitrogen). Recombinant plasmids were identified by colony hybridization using P32-labeled probe mtDNA. An ordered library for the circular mitochondrial genomic map was constructed by RE mapping or cloned fragment walking. The whole-genome sequence was assembled using SeqMan of the Lasergene Package (DNASTAR, Madison, WI) and was further confirmed by PCR amplification and sequencing of the overlapped fragments. The mtDNA sequence has been deposited in GenBank (accession number: EF204913). The DNA and RNA polymerase genes on the mitochondrial plasmid were determined by directly sequencing the PCR amplicons. The primers covering the whole plasmid sequences of pMLP1 (NC_002135) and pMLP2 (AF355103) in another *Pleurotus ostreatus* strain NEFA2 (Kim et al., 2000) were designed. All the DNA fragments based on the pMLP1 were successfully amplified and sequenced. The ORF sequences of plasmid DNA polymerase (*pdpo*) and plasmid RNA polymerase (*prop*) were deposited in GenBank.

Genome annotation

Hypothetical ORFs longer than 70 amino acids were retrieved by a tool for annotation, ARTEMIS (www.sanger.ac.uk/Software/Artemis). The products of these ORFs were annotated by blasting against the NCBI protein database (http://www.ncbi.nlm.nih.gov/). The RNA genes and *atp8* gene were located in reference to their homologs on *C. neoformans* mtDNA (NC_005927). After extraction of the hypothetical ORFs, the remaining fragments were again subjected to BLAST search in the NCBI, aiming to identify more known genes or motifs that could not be identified in the above procedures.

Analyses of codon usage bias and repeat sequences

Codon usage was assessed by the Relative Synonymous Codon Usage (RSCU) value (Sharp et al., 1986). The RSCU for a particular codon (i) is given by RSCUi = \( \frac{X_i/n}{X_i/\sum X_i} \)

where \( X_i \) is the number of times that the ith codon has been used for a given amino acid, \( X_i \) is the total number of times that all the synonymous codons have been used for the same
amino acid, and \( n \) is the number of synonymous codons for that amino acid. The tandem repeats were searched with TRFfinder (Benson, 1999), and a minimum alignment score was set to 70. DNA secondary structures of intergenic motifs were predicted by Mfold (bioinfo.hku.hk/Pise/mfold.html). The algorithm for inverted repeat identification is as described in a previous report (Wang & Leung, 2006). The inverted repeats were searched using a sliding window of 2 kb across the mtDNA, and one copy of the inverted repeats was set to be >15 bp.

Construction of phylogenetic tree

The amino acid sequences of cox1 and its intronic ORFs of Pleurotus ostreatus, other fungi, as well as some lower eukaryotes, were used to construct a phylogeny. Homologs of the nine cox1 intronic ORFs of Pleurotus ostreatus were searched by blasting their amino acid sequences against the database in the NCBI, with a threshold alignment score of 40. If more than one ORF in a species showed high similarity to a given Pleurotus ostreatus intronic cox1 ORF, the one with the highest alignment score was retained as a homolog. If both cox1 gene and homologous ORF were found in a species, they were all used in the phylogenetic analyses. Thus the data were successfully collected from 28 species of fungi and lower eukaryotes. Schizopyllum commune cox1 gene was also included in the data set even though it does not have any intronic ORFs. The amino acid sequence of the homologous ORFs of the 28 species were used to constitute six datasets, each consisting of a Pleurotus ostreatus intronic ORF and at least 12 homologous ORFs from the other species. The remaining Pleurotus ostreatus intronic ORFs were not used for the analysis because of the insufficient number of homologs identified in database.

The amino acid sequences of the cox1 and its intronic ORFs \((n = 6, \text{designated as i1–i6})\) were independently aligned using CLUSTALW, followed by manual adjustment. Phylogenetic trees were reconstructed for each of the alignments using the Bayesian Markov Chain Monte Carlo method (BMCMC) implemented in MrBayes (Ronquist & Huelsenbeck, 2003) based on the JTT amino acid substitution model. The BMCMC trees are majority consensus trees summarized from two sets of four tempered MCMC chains of \( 10^7 \) states sampled every 1000th generation, with the initial 10% states discarded.

Results

Gene content and gene order

The mitochondrial genome of Pleurotus ostreatus is a circular DNA molecule of 73 242 bp with 26.4% GC content. According to the BLAST results, 44 known genes were identified, including 18 protein-coding genes and 26 RNA genes (Fig. 1). Among the protein-coding genes, there are 14 common mitochondrial genes encoding cytochrome oxidase subunits 1–3 (cox1, cox2, cox3), apocytochrome b of bc1 complex (cob), NADH subunits 1–6 of complex I (nad1, nad2, nad3, nad4, nad4L, nad5, nad6), ATP synthase subunits 6, 8 and 9 (atp6, atp8, atp9). The remaining four protein-coding genes encode one ribosomal small subunit protein 3 (rps3), one RNA polymerase gene (rpo) and two putative B-type DNA polymerase genes (dpo1 and dpo2). The 26 RNA genes include small and large subunit RNA genes (rns and rnl) and 24 tRNA genes. The isoacceptors produced by the 24 tRNA genes are carriers for the 20 common amino acids. Besides these, five hypothetical ORFs were found (>100 aa) that lacked homology to any known genes in the GenBank (Fig. 1).

Group I introns were found in cox1, cox2, nad2 and nad4. The splicing sites of cox1 exons were identified by multiple alignments with cox1 genes from other species. Within the nine introns of cox1, there are correspondingly nine intronic ORFs homologous to known endonuclease genes. In contrast, the introns of cox2, nad2 and nad4 are relatively short and do not have any intronic ORFs.

The gene order among the mtDNAs of Pleurotus ostreatus, C. perniciosa and S. commune was compared. The results indicate frequent occurrences of gene shuffling events among these mtDNAs. Pleurotus ostreatus and C. perniciosa exhibit a more similar gene order, and in accordance with that, they are classified under the same taxonomical order: Agaricales. Interestingly, the linkage between nad genes

![Fig. 1](physical-map-of-pleurotus-ostreatus-mitochondrial-genome.png)
(nad2-nad3, nad4L-nad5 and nad4-nad6) remains intact among these three mtDNAs. Between *Pleurotus ostreatus* nad4L and nad5, there is no intergenic sequence and in fact the stop codon of nad4L is immediately followed by the initiation codon of nad5. This is also the case for nad2-nad3 in *S. commune*. Assuming insertions of intergenic sequences are later events in mitochondrial evolution, the absence of intergenic sequences in the nad genes of *Pleurotus ostreatus* and *S. commune* possibly stands for an ancient feature of mtDNA in fungi.

Fourteen common mitochondrial genes were used to calculate the frequency of codon usage, and a strong bias of using A or T at the first and third codon positions was observed (data not shown). For example, synonymous codons for Leu exhibit a strong usage bias between TTA and CTG (RSCU_TTA = 4.9 vs. RSCU_CTA = 0.35). The only exception is the codon for tryptophan. The tryptophan codon TGA in mold translation is only used for three times, which is much less frequent than its synonyms TGG for 62 times. This observed codon bias is completely contrary to the tryptophan codon usage in the mitochondrial genome, in which *TryTGG* is a rarely used codon (Saccone *et al.*, 2006). At present, the biological significance of *TryTGG* bias in *Pleurotus ostreatus* mitochondrial genome remains unsolved.

### DNA and RNA polymerases

One RNA polymerase (*rpo*) and two DNA polymerase (*dpo1* and *dpo2*) genes were identified in *Pleurotus ostreatus* mtDNA and they showed, at the amino acid level, a significant homology to their conserved protein family (Pfam00940 and Pfam 03175, respectively). In the aligned part, sequence identity is 32% between *rpo* and Pfam00940, and 30% between the *dpo*s and Pfam 03175. In fact, both the bacteriophage-type single-chain RNA polymerase (Pfam00940) and the B-type DNA polymerase (Pfam 03175) genes are widely reported in fungal plasmids, mtDNAs, chloroplast DNAs and viruses. In addition, these RNA and DNA polymerases are also commonly found in most of the reported mitochondrial plasmids (Griffiths, 1995; Cahan & Kennell, 2005). Interestingly, the organization of *rpo* and *dpo* in *Pleurotus ostreatus* mtDNA is very similar to the organization of these genes in the mitochondrial plasmids, pMLP1 and pMLP2 of *Pleurotus ostreatus*, with *rpo* and *dpo* located nearby but in opposite orientations (Kim *et al.*, 2000).

In order to compare the RNA and DNA polymerases encoded by mtDNA and those encoded in the mitochondrial plasmid, both genes on the mitochondrial plasmid of *Pleurotus ostreatus* P51 were sequenced. The *rpo* gene on mtDNA shows only 28% amino acid identity to the plasmid RNA polymerase (*prpo*); *dpo1* and *dpo2* genes on mtDNA show the same amino acid identity (also 28%) to their homologs in the plasmid (*pdpo*). A multiple alignment among *dpo1*, *dpo2* and *pdpo* shows that both *dpo1* and *dpo2* on mtDNA are a part of *pdpo* with a more conserved overlapping region. About 60 aa in the overlapping shows 53% identity between *dpo1* and *dpo2* (Fig. 2). It was therefore speculated that the two genes were shortened from two inversely duplicated copies during evolution.

Next the plasmid in the strain was compared with the pMLP1. At the nucleotide level, both RNA and DNA polymerase genes on the plasmid showed >98% identity to the homologs on the pMLP1. However, a big difference was observed in the intergenic region between *prop* and *pdpo*, including several large deletions and nucleotide mutations (data not shown).

### Phylogenetic analysis and horizontal gene transfer

The phylogenetic relationship of *Pleurotus ostreatus* to 24 other fungi species was reconstructed using the ORFs of *cox1*, with five species of ichthyosporeans, green algae and liverwort as the outgroup. *Pleurotus ostreatus* and the other three basidiomycete species were clustered in the same clade associated with posterior probability support (>95%). Among the three mushroom species, *Pleurotus ostreatus* and *A. aegerita* are phylogenetically closer than the pathogenic mushroom *C. perniciosa* (Fig. 3). Overall, the phylogenetic relationship is in agreement with the classification of basidiomycetes in fungi (Bullerwell *et al.*, 2003a; Bullerwell & Lang, 2005; Seif *et al.*, 2005).

Phylogenetic trees were also reconstructed with six of the nine *cox1* intronic ORFs. In contrast, these six phylogenetic trees are generally discordant from each other and are not consistent with the current fungal classification (Fig. 4). The intronic ORFs of *Pleurotus ostreatus*, for example, are clustered with the homologs of completely different species in i2, i3 and i4 phylogenetic trees with high posterior probability support. These phylogenetic discordances...
among the intronic ORFs can also be observed in *Podospora anserina*. *Podospora anserina* shares a monophyletic relationship with *Smittium culisetae*, *C. pemiciosa* and *S. culisetae* in i1, i3 and i4 trees, respectively, but clusters with *C. perniciosa*/*Pleurotus ostreatus* and *Kluyveromyces thermotolerans*/*Pichia canadensis* in i5 and i6 trees, respectively (Fig. 4). These observed phylogenetic discordances implicate the HGT of these intronic ORFs among these species.

It is noted that a number of homologs of *Pleurotus ostreatus* cox1 intronic ORFs were also identified in non-cox1 genes in mtDNA of other fungal species, including *nad1*, *nad5*, *atp1* and *rnl*, and a number of chloroplast genes. The cox1 intronic ORF i3 has the widest spectrum of non-cox1 intronic homologs, implying its high mobility through HGT.

Repetitive fragments and secondary structures in intergenic regions

Two fragments that are able to form large secondary structures were identified. Firstly, a 299-bp fragment, which is located within a tRNA gene-rich region at 19.6 kb, was predicted to form a stable secondary structure with dG = −67 using Mfold. Secondly, a 466-bp fragment, which is located between *rpo* and *dpo1*, was predicted to form a secondary structure as stable as the first one (dG = −68). The potential function of these secondary structures in the mitochondrial genome is unclear at present. Tandem repeats were searched within the full mtDNA and the most frequently identified motif was CT(G/C)CTA(T/C)G. Among the CT(G/C)CTA(T/C)G repeats, (CTGCTATG)n and (CTGCTACG)n form long inverted repeats with (CATAG CAG)n and (CGTAGCAG)n, respectively. Twenty inverted repeats were identified in the genome. Occasionally, the inverted repeats were found within coding regions.

The inverted repeats in mtDNA of *Podospora anserina* were also investigated, which is a model species for studying mitochondrial instability and aging (Albert & Sellem, 2002). Under the same criteria, 45 inverted repeats were found in the mtDNA of *Podospora anserina*, despite the fact that the genome size of these two species is comparable (73 kb in *Pleurotus ostreatus* vs 94 kb in *Podospora anserina*). Thus, the presence of such a large number of inverted repeats might explain, at least in part, a reasonable instability of *Podospora anserina* mtDNA because inverted repeats are able to induce recombination and introduce instability to a genome by forming stem-loop structure and Holiday junction (Lobachev et al., 1998; Shlyakhtenko et al., 2000). The influence of such repeats to the stability of *Pleurotus ostreatus* mitochondrial genome is an object for investigation because the issue was not well documented in this species.

Discussion

In this study, the gene content and genomic features of the mtDNA from a basidiomycete fungus *Pleurotus ostreatus* strain P51, as well as its phylogenetic relationship with other fungal species, were characterized. It is interesting that a relatively large number of introns (n = 9) were identified in the cox1 gene of *Pleurotus ostreatus*. A summary in a recent report indicates that the cox1 gene of *Podospora anserina* represents the most complicated gene structure by owning seven introns (Seifert et al., 2007), while the cox1 gene of *Pleurotus ostreatus* has two extra introns. This special feature of cox1 gene in *Pleurotus ostreatus* deserves further
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Fig. 4. HGT evidence from phylogenies of cox1 intronic ORFs. (a) to (f) represent phylogenies of i1–i6, respectively. The numbers following the species names are as suggested in Fig. 3. In addition, the number 5 refers to nonfungal species, including ichthyosporeans, green algae and liverwort. The scale bars denote the 0.2 amino acid substitutions per site. The numbers at the nodes are posterior probability support of the clade. All the phylogenies are mid-point rooted.

investigation. The size of the intergenic and intronic regions of known basidiomycete mtDNAs varies greatly, accounting largely for their genome size variation. For example, both intron and intronic ORFs can be found in the mtDNA of C. perniciosa but are absent in S. commune. The absence of introns in the S. commune mtDNA is accompanied with a relatively small size of its genic regions (exons plus introns). Interestingly, although the number of introns in Pleurotus ostreatus and S. commune is substantially different, their intergenic regions both account for the almost same
proportion (39%) in their mtDNAs, suggesting that the expansion of intergenic regions precisely correlates with that of genic regions in these fungal mitochondrial genomes.

In this study, dpo and rpo were both identified in the mitochondrial plasmid and mtDNA of Pleurotus ostreatus. Although gene sharing between mitochondrial plasmids and mtDNAs has been widely reported (Cahan & Kennell, 2005), the mechanism explaining the duplication remains elusive.

The discordances of phylogenetic signal of intronic ORFs of cox1 among different fungal special suggests the possibility of HGT of these ORFs. However, the mechanism responsible for HGT among mtDNAs is far from being understood. Even though mitochondrial plasmids have been proposed as possible mediators, their mobility is in doubt at present. A early study proposed a mechanism of the insertion of the mobile intronic ORFs (mostly endonuclease) (Eddy & Gold, 1992). Nonetheless, it is still enigmatic as to how these mobile intronic ORFs can be transferred across two barriers (cellular member and mitochondrial member) and spread in a wide spectrum of hosts and genes.

In summary, this analysis provides valuable information towards the understanding of the evolution of fungal mtDNA. With more mtDNAs from higher basidiomycetes become available, the biological and evolutionary significance of the fungal genome expansion will be investigated more thoroughly. Moreover, the common mitochondrial genes identified in this study, e.g. cox1, may provide a reference for the classification of basidiomycetes in the future.

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Authors’ contribution
Y.W. and F.Z. contributed equally to this study.

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