RESEARCH LETTER

The mitochondrial genome of the wood-degrading basidiomycete *Trametes cingulata*

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Abstract

We present the 91 500 bp mitochondrial genome of the wood-degrading basidiomycete *Trametes cingulata* and compare it with the mitochondrial genomes of five additional *Basidiomycota* species. The *Trametes* mitochondrial genome encodes 15 proteins, 25 tRNAs and the small and large rRNAs. All of the genes, except one tRNA, are found on the same DNA strand. Several additional ORFs have also been identified; however, their sequences have not been conserved across the species we compared and they show no similarity to any known gene, suggesting that they may not correspond to authentic genes. The presence of endonuclease-like sequences in introns suggests a mechanism that explains the diversity of mitochondrial genome sizes that are unrelated to the gene content.

Introduction

It is generally accepted that mitochondria have a monophyletic origin and represent an ancient symbiosis between a free-living *Alphaproteobacterium* and an autotrophic archebacterium (Gray & Doolittle, 1982; Martin & Muller, 1998). While most of the ancestral alphaproteobacterial genes have been lost or transferred to the nucleus, mitochondria usually maintain about 30–40 transcribed genes, although the number varies from 3 to 67 (Adams & Palmer, 2003). Mitochondrial genomes vary in size from about 20 kb in protozoa, fungi and animals to more than 200 kb in plants (Lang *et al.*, 1999).

Of the 70 fungal mitochondrial genomes available at NCBI (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=4751&opt=organelle), the higher fungi are represented by 51 sequences of *Ascomycota* and seven of *Basidiomycota*. These genomes vary in size from 18 844 nucleotides (nt) in *Hanseniaspora uvarum* to 109 103 nt in *Moniliophthora perniciosa*. There is no apparent correlation of genome size and gene content: size differences can be attributed to the size of introns and intergenic regions and the presence of integrated plasmids. Of the two major types of mitochondrial introns, type I is the norm in fungal mitochondrial genomes, while type II are usually present only in plant mitochondrial genomes (Lang *et al.*, 2007).

Trametes cingulata (Bakshi *et al.*, 1970) is a heterothallic dikaryon originally isolated from rotting *Shorea robusta* lumber. We present the sequence of the *T. cingulata* mitochondrial genome and compare it with the mitochondrial genomes of five basidiomycete species. *Trametes* is a representative genus of the polypore clade in the subphylum Agaricomycotina (Ko & Jung, 1999; Hibbett *et al.*, 2007). The available mitochondrial genomes of *Basidiomycota* at NCBI are represented by four species of Agaricomycotina including *Pleurotus ostreatus* (Wang *et al.*, 2008), *M. perniciosa* (Formighieri *et al.*, 2008), *Schizophyllum commune* and *Cryptococcus neoformans* var. *grubii*. The Ustilaginomycotina is a sister clade of the Agaricomycotina and we have selected *Ustilago maydis* as a representative for this group.

Materials and methods

Preparation of DNA

Trametes cingulata was obtained from the American Type Culture Collection (http://www.atcc.org accession number 26747) and maintained and grown on 2% malt extract agar

plates at room temperature. DNA was isolated from hyphae essentially as described by Raeder & Broda (1985). Hyphae were collected by filtration or centrifugation, washed with 20 mM EDTA, pH 8.0, and freeze-dried for 24–48 h. Samples were crushed at room temperature in a mortar and resuspended in extraction buffer (200 mM Tris-Cl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) using about 2 mL per 0.1 g of dried tissue. Phenol (~0.7 vol.) was added to the slurry, which was then mixed for 2 min. Following the addition of ~0.3 vol. chloroform, mixing and centrifugation at 10 000 ***g*** for 1 h, the aqueous layer was transferred to a new tube and 1/20 vol. of 20 mg mL^{-1} RNAse A was added and incubated 37 °C for 20 min. The RNAse was extracted with 1 vol. chloroform and the tube was centrifuged at 10 000 ***g*** for 10 min. DNA was precipitated from the aqueous layer by the slow addition of isopropanol (~1 vol.). The precipitated mass of DNA was sequentially washed with 50% isopropanol and 70% ethanol, dried briefly and resuspended in TE buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA). This preparation included genomic and mtDNA.

Sequencing and assembly

DNA was sequenced using a GLS FLX sequencer (http://www.454.com) and assembled using a GS DE NOVO ASSEMBLER (version 1.1.03). The final mitochondrial genome assembly was performed using bioinformatic procedures developed at the Computational Genetics Laboratory at the Minnesota Supercomputing Institute. The raw end reads of the assembled contigs were compared using BLAST (Altschul *et al.*, 1990) and reassembled using CAP3 (Huang & Madan, 1999). The assembled sequence was manually examined for errors.

Genome annotation

Potential genes were identified using BLAST and the annotation of significant hits was accepted. ORFs larger than 100 codons were identified using ORFfinder (http://www.ncbi.nlm.nih.gov/projects/gorf/) and codon usage table 4 (Mold, Protozoan, and Coelenterate Mitochondrial Code). The predicted exon–intron boundaries for three selected genes, cytochrome oxidase subunits 1 (*cox1*) and 2 (*cox2*) and the small ribosomal subunit (*rns*) gene were confirmed by sequencing reverse transcriptase (RT)-PCR products. Total RNA from fungal hyphae growing in 2% malt extract was obtained using TRIzol reagent (Invitrogen Corp., CA) and RT-PCR was performed using the Omniscript RT Kit (Qiagen Inc., CA) following the manufacturer's recommended protocol. The primers used are shown in Table 1. Intronic sequences were analyzed using RNAweasel (Lang *et al.*, 2007).

The mitochondrial genomes and annotation of *P. ostreatus*, *M. perniciosa*, *S. commune*, *C. neoformans* and *U. maydis* are available at GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide) under accession numbers EF204913, AY376688, AF402141, AY101381 and DQ157700, respectively. The accession number for the *T. cingulata* mitochondrial genome is GU723273.

Table 1. Primers used in RT-PCR and sequencing of the *cox1*, *cox2* and *rns* genes from RNA of *Trametes cingulata*

Primer description	Primer sequence (5′–3′)
RT primer for *rns*	ATCCAGCTGCACTTTCCAGT
Forward primer for *rns* PCR and sequencing	ATTTTGGTTCCGATTGAACG
Reverse primer for *rns* PCR and sequencing	CCCTGCTATGACTTTTGAGATG
RT primer for *cox1*	CATATTAAAGGCATGGAATGGA
Forward primer for *cox1* PCR and sequencing	TTCTTGGCTATCTTCTACTAATGCT
Reverse primer for *cox1* PCR and sequencing	AAAGTAAGATGGTACTAACCAAGGAT
RT primer for *cox2*	TGAATCTATCCATGCTAAATAATCTTG
Forward primer for *cox2* PCR and sequencing	ATGATGCTCCACAACCTTGG
Reverse primer for *cox2* PCR and sequencing	GCTATCGGCATAAATCCATGA

Results

Genome assembly and annotation

The *T. cingulata* mitochondrial genome was assembled into a single 91 500 bp circular molecule with a coverage depth of about 140-fold. BLAST comparison with other fungal mitochondrial genomes identified genes encoding 15 proteins and the small and large rRNAs (Fig. 1). tRNAscan-SE (Lowe & Eddy, 1997) identified 25 tRNAs in the genome corresponding to all 20 amino acids. We also found five ORFs not overlapping any other gene on either strand and larger than 100 codons (Fig. 1). However, these ORFs showed little similarity to sequences found in the mitochondrial genomes of *P. ostreatus*, *M. perniciosa*, *S. commune*, *C. neoformans* and *U. maydis* (Fig. 1, rings v–ix). Additionally, TBLASTX and BLASTN comparison of these five ORFs with the nonredundant database did not identify any sequence with an expected value of < 0.1, further indicating that they may not be authentic. GC skew analysis has been used to identify the origin of replication in bacterial genomes (Grigoriev, 1998) and a similar technique has been proposed in fungal mitochondrial genomes (Formighieri *et al.*, 2008). We were unable to detect any obvious origin of replication based on the GC content or GC skew analysis.

Gene structure and gene order

Like the mitochondrial genes of the other Agaricomycotina, most of the *T. cingulata* mitochondrial genes are located on

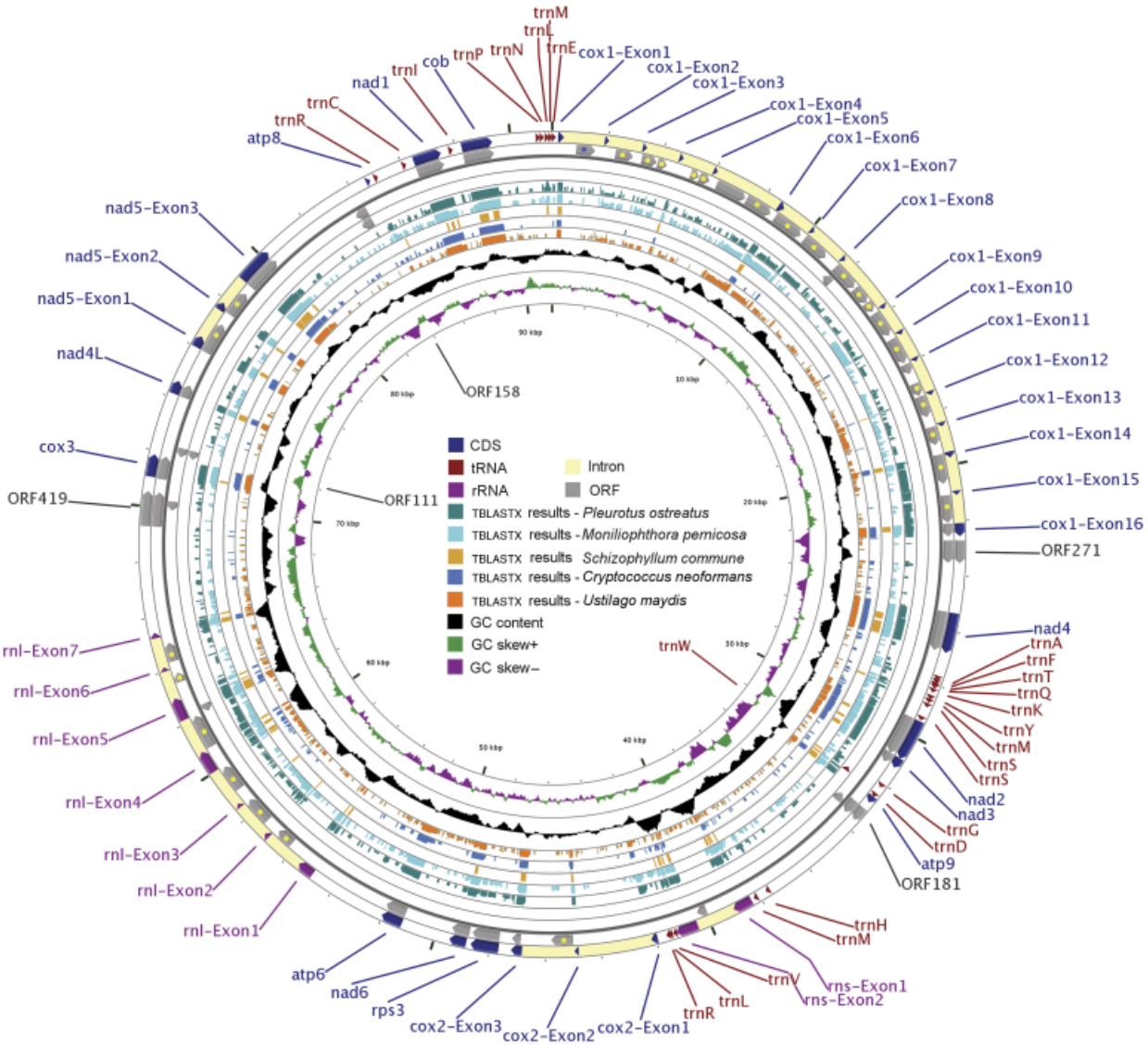

Fig. 1. Physical map of the *Trametes cingulata* mitochondrial genome and a comparison with mitochondrial genomes of five additional basidiomycete species. From the outside in, the various rings represent (i) *Trametes* genes identified in the clockwise strand; (ii) ORFs in the clockwise strand; (iii) ORFs in the anticlockwise strand; and (iv) genes identified in the anticlockwise strand. Rings v through ix represent the TBLASTX results with a maximum expected value of 10^{-10} when the *Trametes* mitochondrial genome was used to query the mitochondrial genomes of *Pleurotus ostreatus*, *Moniliophthora perniciosa*, *Schizophyllum commune*, *Cryptococcus neoformans* and *Ustilago maydis*, respectively. (x) GC content as the deviation from the average over the entire genome and; (xi) GC skew as the deviation from the average over the entire genome. ORFs depicted are at least 100 codons long. The number following the ORF label represents the length of the corresponding polypeptide. The image was generated using CGVIEW (Grant & Stothard, 2008). The numbering of the bases has been performed from an arbitrary point between genes trnM and trnE. Blue or yellow dots in ring (ii) show the presence of GIY-YIG or LAGLIDADG-like endonuclease sequences, respectively.

one strand. The only identifiable gene on the anticlockwise strand is the one encoding $tRNA^{Trp}$, which is found nowhere else in this genome. While gene order is not conserved among the mitochondrial genomes of *T. cingulata*, *P. ostreatus*, *M. perniciosa*, *S. commune*, *C. neoformans* and *U. maydis*, they share a similar set of genes (Fig. 2). The position, size and number of introns have not been conserved among these species (Table 2).

Introns were detected in the *cox1*, *cox2*, *nad5*, *rns* and *rnl* genes. All of these are type I introns, except for the single type II intron in *rns*. All type I introns contained endonuclease-like gene sequences with the conserved LAGLIDADG motif, except for the first intron in the *cox1* gene, which had the GIY-YIG motif. The endonuclease in *cox1* intron-12 appears to be truncated and does not have the full LAGLIDADG domain.

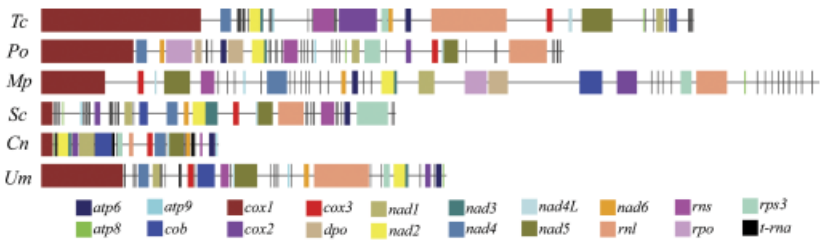

Fig. 2. Gene order in the mitochondrial genomes of *Trametes cingulata* (*Tc*), *Pleurotus ostreatus* (*Po*), *Moniliophthora perniciosa* (*Mp*), *Schizophyllum commune* (*Sc*), *Cryptococcus neoformans* (*Cn*) and *Ustilago maydis* (*Um*). Actual genome sizes are (*Tc*) 91 500 bp, (*Po*) 73 242 bp, (*Mp*) 109 103 bp, (*Sc*) 49 704 bp, (*Cn*) 24 874 bp and (*Um*) 56 814 bp. Unidentified ORFs and introns are not shown.

Table 2. The gene content and the number of introns found in the mitochondrial genomes of the basidiomycetes *Trametes cingulata* (*Tc*), *Pleurotus ostreatus* (*Po*), *Moniliophthora perniciosa* (*Mp*), *Schizophyllum commune* (*Sc*), *Cryptococcus neoformans* (*Cn*), *Ustilago maydis* (*Um*) and the ascomycete *Aspergillus niger* (*An*)

	Number of introns in						
Genes	*Tc*	*Po*	*Mp*	*Sc*	*Cn*	*Um*	*An*
cox1	15	9	6	0	0	8	1
cox2	2	1	2	0	0	0	0
cox3	0	0	0	0	0	0	0
atp6	0	0	0	0	0	0	0
atp8	0	0	0	0	0	0	0
atp9	0	0	0	0	0	0	0
nad1	0	0	1	0	1	0	0
nad2	0	1	0	0	0	0	0
nad3	0	0	0	0	0	0	0
nad4	0	1	1	0	0	0	0
nad4L	0	0	0	0	0	0	1
nad5	2	0	1	0	0	1	0
nad6	0	0	0	0	0	0	0
rnl	6	0	0	0	0	2	1
rns	1	0	0	0	0	0	0
rps3	0	0	0	0	0	0	–
cob	0	0	2	0	0	1	0
dpo	–	0	0	–	–	–	–
rpo	–	0	0	–	–	–	–

The absence of a gene is indicated by a dash (–).

Of the genes found in the mitochondrial genome of *T. cingulata*, the structure of *cox1* is the most complex. Of the 16 exons that make up the *cox1* gene, five are smaller than 20 nt long, with the smallest two being only 11 nt. All 15 introns have at least one ORF larger than 100 codons. ORFs encoding endonuclease-like sequences were also seen in all other introns, except for intron-1 of *cox2*. The reading frames of the exons 1 and 2 of *nad5* continue well beyond the predicted splice sites into the respective introns. These extended reading frames also encode endonuclease-like sequences within an ORF (Fig. 1).

While the coding regions have been well conserved among the Agaricomycotina and to a lesser extent with *U. maydis*, the introns show less similarities (Fig. 1). *Trametes cingulata* intronic ORFs show greater sequence similarity to *P. ostreatus* and *M. perniciosa* than to the more distantly related *U. maydis*. *Schizophyllum commune* and *C. neoformans* do not have introns in the same genes as *T. cingulata*. The DNA and RNA polymerases *dpo* and *rpo*, which have been reported in *P. ostreatus* and *M. perniciosa*, are not present in the *T. cingulata* mitochondrial genome nor were they annotated or obvious in the *S. commune*, *C. neoformans* or *U. maydis* mitochondrial genomes.

tRNAs and codon bias

The 25 identified tRNAs genes represent all 20 amino acids and include three copies encoding $tRNA^{Met}$ and two each of $tRNA^{Arg}$, $tRNA^{Ser}$ and $tRNA^{Leu}$. Single genes encode the other 16 tRNAs. We analyzed codon usage for the 15 protein-encoding annotated genes (Supporting Information, Table S1) and found that all of these genes use TAA as the stop codon, except for *nad5*, which uses TAG. *nad5* is also the only gene that uses GAG as a codon for glutamic acid and AGG for arginine, which is otherwise encoded exclusively by AGA. Other codons for arginine, CGG, CGT and CGC, are not used. The glycine codon GGC is also not used. At least one of these four otherwise unused codons is used one or more times in all five of the unidentified ORFs, lending additional support to the hypothesis that these ORFs are not expressed genes. The alternate codon for tryptophan TGA that differs from the standard codon table is not used either as a codon for tryptophan or to represent a stop codon in the 15 protein-encoding annotated genes. However, it is present twice in ORF111 and once in ORF158. *Nad4L* is the only gene that uses AAG to code for lysine and does it only at one position.

Only 13% of the codons for tyrosine use TAC, with the rest using TAT. A similar situation is seen with the asparagine codon AAC, which is used by only 9% of the codons, while the majority use AAT. Where more than one codon is used for an amino acid, codons with A or T in the third position are used more than twice as often as those with G or C. There is a significant bias toward A and T, which compose 75.5% of this genome.

Discussion

A significant proportion of the *T. cingulata* genome is made up of the *cox1* gene that is punctuated by large type I introns.

Type I introns are usually characterized by the presence of long ORFs encoding endonucleases that are involved in intron mobility and self-splicing. The endonucleases, often referred to as homing endonucleases, have rare recognition sites and cleave the target gene, which activates the cell's DNA repair mechanism. This leads to precise insertion of the intron into the target gene (Lang *et al.*, 2007). All of the type I introns in the *T. cingulata* mitochondrial genome have an ORF with either a LAGLIDADG or a GIY-YIG endonuclease-like sequence. These endonucleases could be responsible for intron homing, whereby introns move into previously intronless genes, a mechanism that could account for the large differences in the size of the mitochondrial genomes that are unrelated to the gene content. The variability in the size of *cox1* is apparent and can be directly attributed to the number of introns in the gene (Fig. 2, Table 2).

The gene structure and content of the *T. cingulata* mitochondrial genome is very similar to the genomes of the recently published genomes of *P. ostreatus* and *M. perniciosa*. The same subset of genes is also seen in the other basidiomycetes we used in this study and the ascomycete *Aspergillus niger* (Juhasz *et al.*, 2008), with one or more minor changes such as the apparent absence of *rps3* in *A. niger* (Table 2), although this gene is usually present in other ascomycetes. The DNA and RNA polymerases reported in the mitochondrial genomes of *P. ostreatus* and *M. perniciosa* are thought to be from integrated plasmids (Formighieri *et al.*, 2008; Wang *et al.*, 2008), a feature absent in the *T. cingulata* mitochondrial genome.

The phylogeny of *Trametes* species and related genera has proven difficult using morphological characteristics (Ko & Jung, 1999) and rDNA studies (Matheny *et al.*, 2007). The number of *Trametes* species is unknown and ranges from a conservative 50 in the Catalogue of Life (Bisby *et al.*, 2009) to 335 in the Index Fungorum database (http://www.indexfungorum.org). The polypore clade includes many wood-degrading species that are ecologically and industrially important including the widely studied *Phanerochaete chrysosporium* (Tien & Kirk, 1983; Wariishi *et al.*, 1991; Vanden Wymelenberg *et al.*, 2006). The mitochondrial genome sequence of *T. cingulata* provides another tool for evolutionary biologists to clarify the evolutionary relationships among this group.

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Authors' contribution

S.H. and J.S.G. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Cumulative codon use in the *cox1*, *cox2*, *cox3*, *cob*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*, *rps3*, *atp6*, *atp8* and *atp9* mitochondrial genes of *Trametes cingulata*.

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