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SHORT COMMUNICATION

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Organization of the mitochondrial genome of *Fusarium oxysporum* (anamorphic Hypocreales)

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Abstract A 33.4-kb fragment of the mitochondrial genome of *Fusarium oxysporum* has been sequenced. The fragment contains the complete gene sequences for 13 of the 14 proteins typically encoded by the mitochondrial genome of filamentous ascomycetes. Similarity searching revealed all encoded proteins to be most similar to those from other members of the Hypocreales. The fragment contains the complete small subunit rRNA gene, partial large rRNA subunit gene, and 12 tRNAs. Two introns were present, one in the *nad5* gene and one in the large rRNA subunit gene, the latter containing a ribosomal protein gene.

Key words Long PCR · Mitochondria · Organelle genomics

Fusarium oxysporum Schlecht.: Fr. (anamorphic Hypocreales: Ascomycota) is a ubiquitous soilborne fungus comprising numerous saprophytic and plant pathogenic forms (Gordon and Martyn 1997). It is a major wilt pathogen, affecting many economically important crops, and has been traditionally divided into host-specific formae speciales. Since the mid-1980s, strains have further been divided into vegetative-compatibility groups (VCGs) (Katan 1999), which appear to be natural groups, in contrast to traditional formae speciales (Baayen et al. 2000).

Variation in the mitochondrial genome has been used to characterize many groups of fungi (Bruns et al. 1991), including various formae speciales and VCGs of *F. oxysporum*. The mitochondrial genome of *F. oxysporum* is a circular molecule that varies in size between approximately 45 and 52 kb (Kistler and Benny 1989; Marriott et al. 1984). Restriction fragment length polymorphisms (RFLPs) of the mitochondrial genome of *F. oxysporum* have been used to characterize strains from hosts such as chickpea (Perez-Artes et al. 1995), muskmelon (Jacobson and Gordon 1990), and watermelon (Kim et al. 1992). Some of these studies also determined the positions of several rRNA and protein-coding genes. Baayen et al. (2000) later conducted a phylogenetic analysis of various formae speciales and VCGs using sequences from several nuclear and mitochondrial genes including part of the mitochondrial rRNA small subunit. Cunnington (2006) sequenced several mitochondrial intergenic regions from five isolates of *F. oxysporum* and found that one of these regions appeared to be more variable than introns in the elongation factor 1-α gene that has commonly been used for phylogenetic studies within *F. oxysporum*. Within the Hypocreales, the mitochondrial genomes of *Hypocrea jecorina* Berk. & Broome (Chambergo et al. 2002), *Lecanicillium lecanii* (Zimm.) Zare & W. Gams (Kouvelis et al. 2004), and *Metarhizium anisopliae* (Metschn.) Sorokīn (Ghikas et al. 2006) have been sequenced. The aim of this work was to sequence the mitochondrial genome of *F. oxysporum* to allow further investigation into its use to identify formae speciales and VCGs.

Fusarium oxysporum collection VPRI 19292, isolated from carnation, was selected for sequencing. A culture was grown on potato dextrose agar, the mycelium was scraped from the agar surface, and total DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Doncaster, Victoria, Australia) according to the manufacturer's instructions. A range of polymerase chain reaction (PCR) primers were designed from conserved regions of the mitochondrial genomes of the hypocrealean fungi *H. jecorina* (GenBank AF447590) and *L. lecanii* (AF487277). Seven sets of primers were designed in an attempt to use long PCR to amplify the entire mitochondrial genome in 3- to 7-kb fragments (Table 1). Polymerase chain reactions were performed in 25- or 50-μL volumes using the TaqPlus Long PCR System (Stratagene, Sydney, Australia) with the low-salt buffer according to the manufacturer's instructions. Annealing temperatures of 50°–60°C were used, with 6- to 10-min extension periods. Products were cleaned with a QIAquick PCR purification

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Table 1. PCR primers used to amplify the *F. oxysporum* mitochondrial genome

Region	Forward primer (5′–3′)	Reverse primer (5′–3′)	PCR product size (kb)
nad4-rns	GATCTCCTCCACCTGCTACTTC	CAACTTCCACTACGCGAACCG	5.0
rns-rnl	CAGCAGTGAGGAATATTGGTCAATG	CCCATTATGCAAAAGGTACGTTCTT	5.3
rnl-rnl	GTATATACGCAGGCCAATGCTTA	CCTTAACCAACTTAGCTTTCCTGC	4.9
nad2-cox2	TATAGAAATTCAGAATTATCTAC	TTCATATTAATTCTATTAGTGTACC	4.0
cox2-cytb	CCAAGACAGTGCTACTCCACAAATG	CATTAGGCATAAAGAATACAAAG	7.4
cytb-cox1	ATAGTTGAGTTCATTTGAGGAGGTT	ACACTAGGTCCACTATGACTTTGTA	2.7
cox1-nad4	TACAAAGTCATAGTGGACCTAGTGT	GCTAAAACTATACTTCCACCTAAAGG	5.2

Table 2. Partial mitochondrial genome sequence of *Fusarium oxysporum*

Genetic element	Location (nt)	Size		Codon	
		bp	aa	Start	Stop
nad2 (partial)	<1–1159	–	–	–	TAA
nad3	1160–1573	414	137	ATG	TAG
atp9	2429–2653	225	74	ATG	TAA
cox2	3731–4480	750	249	ATG	TAA
trna-arg1	4613–4683	71			
nad4L	5333–5602	270	89	ATG	TAA
nad5	Join: (5602–6318, 7329–8600)	1989	662	ATG	TAG
nad5 intron ORF[a]	6319–7233	915	304	–	TAA
trna-arg2	8998–9068	71			
cob	10442–11614	1173	390	ATG	TAA
trna-cys	11816–11888	73			
cox1	13244–14836	1593	530	ATG	TAA
trna-arg3	15104–15174	71			
nad1	16812–17921	1110	369	ATG	TAG
nad4	18143–19666	1524	507	ATG	TAA
atp8	19802–19948	147	48	ATG	TAA
atp6	20442–21242	801	266	ATG	TAA
rns	21858–23540	1683			
trna-tyr	23555–23638	84			
trna-asp	23639–23712	74			
trna-ser1	23942–24023	82			
trna-asn	24424–24494	71			
cox3	24540–25349	810	269	ATG	TAA
nad6	26247–26918	672	223	ATG	TAA
trna-val	27221–27292	72			
trna-ile	27418–27489	72			
trna-ser2	27491–27578	88			
trna-pro	27651–27723	73			
rnl (partial)	Join: (28241–30777, 33001–>33396)	–			
rps	31207–32634	1428	475	ATG	TAA

[a] In frame with upstream exon

kit (Qiagen) and sequenced using Applied Biosystems (Scoresby, Victoria, Australia) BigDye technology. A primer walking strategy was used to sequence the products in both directions. If products were not amplified, one or both primers were redesigned based on sequence data obtained from other amplified fragments. Sequences were joined manually using a text editor, or by using the multiple sequence alignment program ClustalX (Thompson et al. 1997). Open reading frames (ORFs) and genes were located using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Ribosomal RNA genes were identified using Blast2 (Altschul et al. 1997) searches of GenBank, and tRNAs were located using tRNAscan-SE 1.21 (Lowe and Eddy 1997). Codon usage was determined using CodonW (Peden 1997).

Of the mitochondrial genome, 33396 bases were amplified in seven segments and sequenced. Despite numerous attempts, the remaining portion could not be amplified by long PCR. The sequenced region contained 15 protein coding genes, 12 tRNAs, and 2 rRNAs (Table 2). Partial sequences were obtained for the *nad2* and rRNA large subunit genes. All protein and RNA genes were encoded on the same strand. Overall AT content was 69%, rising to 72.8% in protein-coding genes, but considerably lower in

tRNAs (60.8%) and rRNAs (64.6%). The sequence has been deposited on GenBank (AY874423).

The 15 protein-coding genes identified comprise the ATP synthase subunits (*atp6*, *atp8*, and *atp9*), cytochrome oxidase subunits (*cox1*, *cox2*, and *cox3*), apocytochrome b (*cob*), the reduced nicotinamide adenine dinucleotide ubiquinone oxidoreductase subunits (*nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, and *nad6*), and the 5S ribosomal protein (*rps3*). Their order is identical to that of *H. jecorina* (AF447590), *L. lecanii* (AF487277), and *M. anisopliae* (AY884128). In the sequence from *F. oxysporum* the ORFs for *nad4L* and *nad5* overlap by one base, such that the terminal A in the stop codon for *nad4L* is also the first A in the start (methionine) codon for *nad5* (see Table 2).

The typical fungal mitochondrial genetic code (code 4) was found in *F. oxysporum*, i.e., the universal code but with TGA coding for tryptophan, rather than a termination. Codons were particularly biased away from G and C at the third codon position, which had a GC content of 14.6%. Codon CGA was not used at all; codons CTC, TCG, TGC, TGG, CGC, CGG, AGG, and GGC were used no more than five times. The use of these rare codons was biased toward the *rps3* gene and *nad5* intron ORF. Codons used most frequently were AT rich, i.e., TTA (540), ATA (395), TTT (257), and AAT (235). Stop codons were also biased, with 13 genes terminating with TAA but only 3 with TAG. The most commonly used amino acids were leucine (687), isoleucine (581), serine (417), and phenylalanine (383). Cysteine (29), tryptophan (70), and histidine (94) were the least used.

Twelve tRNA genes were found in the sequenced fragment. The arrangement of each of these, and general tRNA arrangement on the mitochondrial genome, was quite similar to those for *H. jecorina* and *L. lecanii*. Surprisingly, three of the tRNAs code for arginine (*trna-arg 1–3*), which is one of the least used amino acids in the genes. The 16S rRNA gene (*rns*) was sequenced, as was the 5′-end of the 23S rRNA gene (*rnl*). The latter contains a 2224-bp intron, in which resides the 1427-bp intronic ribosomal small subunit 3 protein (*rps*3) ORF.

In addition to the intron in the 23S rRNA gene (*rps3*), an intron was found in a protein-coding gene. The *nad5* gene contains a group I intron with an ORF fused in frame with the gene. The ORF shows significant sequence homology to an ORF in the same position in the *nad5* gene of the mitochondrial genome of *Neurospora crassa* Shear & B.O. Dodge. The ORF contains two dodecapeptide (LAGLIDADG) regions, typical of group I intron ORFs. Intergenic regions were generally large, with six at least 1 kb in size. Intergenic regions accounted for approximately 31% of the fragment. Repetitive elements were not found.

The mitochondrial genome of *F. oxysporum* is very similar in structure and content to other ascomycetes, particularly filamentous ascomycetes within the Pezizomycotina. As expected, genes and proteins were most similar to those of other members of the Hypocreales, and gene order was identical, with the exception of the position of a few tRNA genes. The overlapping of the *nad4L* and *nad5* genes is common in filamentous ascomycetes (Woo et al. 2003); however, the *nad2-nad3* overlap in *L. lecanii* was not found here, nor was it found in *H. jecorina* or *M. anisopliae*. Transfer RNA gene distribution was more similar to *H. jecorina* and *M. anisopliae* than *L. lecanii*, but *F. oxysporum* has an extra arginine (*arg2*) tRNA gene between the *nad5* and *cob* genes. The intergenic spacer regions (31% of the fragment) are considerably larger than the 13.7% in *L. lecanii* (Kouvelis et al. 2004). However, variation in the size of the intergenic regions is known to vary considerably between similar fungi. For example, in *Schizosaccharomyces pombe* Lindner the intergenic regions comprise only 11% of the mitochondrial genome, but these constitute 49% in *S. octosporus* Beij. and 76% in *S. japonicus* Yukawa & Maki (Bullerwell et al. 2003).

It is unclear what genetic elements are in the region that was not sequenced. The unsequenced region should be between 12 and 19 kb in length, based on previous reports of the entire mitochondrial genome ranging in size from 45 to 52 kb (Kistler and Benny 1989). This region only occupies approximately 2 kb in *L. lecanii*, where it contains the remaining parts of the *nad2* and *rnl* genes and 12 tRNA genes. It is possible that there are a number of large introns in the *nad2* or *rnl* genes, but introns have not been previously recorded in the *nad2* gene in fungi, and only one or two introns have been reported in the *rnl* gene of ascomycetes (Woo et al. 2003).

The reason for the failure to amplify the remaining portion of the mitochondrial genome is not known. The other fragments were amplified relatively easily, especially when secondary primers were designed from sequences obtained using the initial primer sets. Multiple primer sets failed to amplify the remaining fragment. With the exception of *L. muscarium* (Kouvelis et al. 2004), all other fungal mitochondrial genomes sequenced in the last few years have been obtained by shotgun sequencing, e.g., *H. jecorina* (Chambergo et al. 2002), *P. marneffei* Segretain (Woo et al. 2003), *M. anisopliae* (Ghikas et al. 2006), *Verticillium dahliae* Kleb. (Pantou et al. 2006), and three fission yeasts (Bullerwell et al. 2003).

During the preparation of this article, a complete mitochondrial genome for an isolate of *F. oxysporum* appeared on GenBank (accession AY945289). This sequence has not yet been published, but a phylogenetic paper comparing this mitochondrial genome with those of other ascomycetes is in preparation (M.A. Typas, University of Athens, Greece, personal communication). This sequence is 34.5 kb in length, only 1.1 kb larger than the sequence obtained in this study. The gene order is identical. Intergenic space sizes are also very similar. The small size of this mitochondrial genome is surprising. Previous studies have mapped the *F. oxysporum* mitochondrial genome and found it to be closer to 50 kb in length. For example, the map produced by Jacobsen and Gordon (1990) showed that the region (*rnl-nad*2) that could not be sequenced in this study is about 12 kb long, whereas this region in GenBank AY945289 is only 1.5 kb long. This highly variable region should be the focus of future studies, as there appears to be very significant variation. It is possible that all the length variation reported in the *F.*

oxysporum mitochondrial genome could be occurring in the *rnl-nad*2 region.

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