The distribution of optional mitochondrial introns encoding putative homing endonuclease genes in the *Fusarium oxysporum* complex

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Cunnington J. H. (2009) The distribution of optional mitochondrial introns encoding putative homing endonuclease genes in the Fusarium oxysporum complex. – Sydowia 61 (1): 1–9.

To determine if optional introns are responsible for size variation in the Fusarium oxysporum mitochondrial genome, the distribution of introns in five genes was determined for 16 isolates. Introns were found in two genes. These introns contained open reading frames coding for putative homing endonuclease genes. A homing endonuclease gene containing two LAGLIDADG motifs was present in an intron in the NADH ubiquinone oxidoreductase subunit 5 gene of all isolates. Nine isolates had a homing endonuclease gene with a GIY-YIG motif in an intron in the apocytochrome b (*cob*) gene. A second intron containing a LAGLIDADG homing endonuclease gene was found in the cob gene of two isolates. Phylogenetic analysis of the isolates based on partial translation elongation factor 1-alpha gene intron sequences revealed a relationship between evolutionary history and homing endonuclease gene distribution in the cob gene. The absence, or presence of one or two homing endonuclease genes in the *cob* gene, correlated with previously reported *F. oxysporum* clades 2, 3, and 1, respectively. This information could be used to help delimit subspecific taxa in the *F. oxysporum* complex.

Keywords: mitochondrial genomics, Hypocreales, phylogeny

Fusarium oxysporum Schlecht:Fr. (anamorphic Hypocreales: Ascomycota) is a major plant pathogen, affecting many economically important crops. It is a species complex that has been divided into host specific *formae speciales* and into vegetative-compatibility groups (VCGs) (Katan 1999, Baayen *et al.* 2000). Variation in the mitochondrial DNA has been used to characterize many groups of fungi (Bruns *et al.* 1991), including various *formae speciales* and VCGs of *F. oxysporum* from hosts such as chickpea (Perez-Artes *et al.* 1995), muskmelon (Jacobson & Gordon 1990), and watermelon (Kim *et al.* 1992). The mitochondrial genome of *F. oxysporum* is a circular molecule that was reported to vary in size between approximately 45 kb and 52 kb (Kistler & Benny 1989, Marriott *et al.* 1984). But, the mitochondrial genome of a *F. oxysporum* isolate was recently sequenced and found to be only 34.5 kb (GenBank accession

AY945289). A second, almost complete, mitochondrial genome sequence from a different isolate of *F. oxysporum* (Cunnington 2007) was found to be only 33.4 kb. These sizes are significantly smaller than earlier reports, i.e. 45–52 kb.

A possible reason for these differences is the presence of optional introns. Litter *et al.* (2005) found that the mitochondrial genomes of *Cryptococcus neoformans* var. *neoformans* (San Felice) Vuill. and *C. neoformans* var. *grubii* Franzot, Salkin & Casadevall differed by about 8 kb due to the distribution of optional introns. *Cryptococcus neoformans* var. *neoformans* had nine more introns than *C. neoformans* var. *grubii*. Five of these were in the cytochrome oxidase 1 (*cox*1) gene. Subspecies of *Ophiostoma novo-ulmi* Braiser also differ in the distribution of optional mitochondrial introns (Gibb & Hausner 2005). *Ophiostoma novo-ulmi* subsp. *ulmi* has introns in the mitochondrial ribosomal RNA large subunit genes, rRNA small subunit gene and *cox*1 gene. All these introns are absent from *O. novo-ulmi* subsp. *americana* Braiser & S.A. Kirk (Gibb & Hausner 2005).

These optional introns often contain genes coding for homing endonucleases. Homing endonuclease genes (HEG's) are a class of highly invasive mobile genetic element that are common in bacteria and eukaryotes. In most of the examples listed above, the optional introns were Group I introns containing HEG's belonging to the LAGLIDADG and GIY-YIG groups. These are two of the most common groups of HEG's and are often found in the mitochondrial genomes of fungi (Gimble *et al.* 2000).

The aim of this study was to determine the presence of optional mitochondrial introns in a range of *Fusarium oxysporum* isolates to see if they are responsible for the variation in mitochondrial genome size reported in the literature.

Materials and Methods

Isolates and DNA extraction

Sixteen *Fusarium oxysporum* isolates were chosen at random from the fungal culture collection in herbarium VPRI at the Victorian Department of Primary Industries (Table 1). They were all collected in southeastern Australia over the last 30 years. Cultures were grown on potato dextrose agar, the mycelium was scraped from the agar surface, and total DNA extracted using a DNeasy™ Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

Intron distribution

Five protein coding genes were selected based on the reported frequency of introns in ascomycete mitochondrial genes (Woo *et al.*

Tab. 1. – Introns type and occurrence in five mitochondrial genes of eighteen strains of *F. oxysporum*.

Strain	Host	Gene					Translation elongation factor clade and GenBank no.
		cob	*cox1*	*cox2*	*nad1*	*nad5*	
AY945289[a]	–	–	–	–	–	LAGLIDADG	N/A
AY874423[a]	*Dianthus caryophyllus*	–	–	–	–	LAGLIDADG	N/A
VPRI 12300	*Solanum tuberosum*	–	–	–	–	LAGLIDADG	2 (DQ487322)
VPRI 17577	*Linum usitatissimum*	–	–	–	–	LAGLIDADG	2 (EU035591)
VPRI 11639	*Daucus carota*	–	–	–	–	LAGLIDADG	2 (EU035592)
VPRI 16963	*Solanum tuberosum*	–	–	–	–	LAGLIDADG	2 (EU035593)
VPRI 10358	*Dianthus caryophyllus*	–	–	–	–	LAGLIDADG	2 (EU035594)
VPRI 16234	*Solanum tuberosum*	–	–	–	–	LAGLIDADG	2 (EU035595)
VPRI 10403	*Triticum aestivum*	–	–	–	–	LAGLIDADG	2 (EU035596)
VPRI 10408	*Solanum tuberosum*	GIY-YIG	–	–	–	LAGLIDADG	N/A[b](EU035597)
VPRI 10405	*Triticum aestivum*	GIY-YIG	–	–	–	LAGLIDADG	N/A[b] (DQ487325)
VPRI 11681	*Lycopersicon esculentum*	GIY-YIG	–	–	–	LAGLIDADG	N/A[b] (DQ487323)
VPRI 13039	*Capsicum annum*	GIY-YIG (GenBank EU035603)	–	–	–	LAGLIDADG	3 (DQ487326)
VPRI 10605	*Hyacinthus orientalis*	GIY-YIG	–	–	–	LAGLIDADG	3 (DQ487324)
VPRI 32134	*Allium cepa*	GIY-YIG	–	–	–	LAGLIDADG	3 (EU035598)
VPRI 32289	*Phoenix canariensis*	GIY-YIG	–	–	–	LAGLIDADG	3 (EU035599)
VPRI 32287	*Phoenix canariensis*	GIY-YIG, LAGLIDADG (GenBank EU035604)	–	–	–	LAGLIDADG	1 (EU035600)
VPRI 32288	*Phoenix canariensis*	GIY-YIG, LAGLIDADG	–	–	–	LAGLIDADG	1 (EU035601)

[a] GenBank sequences [b] Clade not defined.

2003). These were the genes encoding apocytochrome b (*cob*), cytochrome oxidase c subunit 1 (*cox*1), cytochrome oxidase c subunit 2 (*cox*2), NADH ubiquinone oxidoreductase subunit 1 (*nad*1) and the NADH ubiquinone oxidoreductase subunit 5 (*nad*5). PCR primers (Table 2) were designed for these genes based on the *F. oxysporum* mitochondrial genome sequence (GenBank AY945289). Polymerase chain reactions were performed in 25 or 50 µL volumes using the TaqPlus® Long PCR System (Stratagene) with the low salt buffer according to the manufacturer's instructions. Cycling times were 2 min 94 °C, then 35 cycles of 10 sec 94 °C, 30 sec 50 °C, and 6 min 68 °C. The size of the PCR products was determined on a 1 % agarose gel in TBE.

Where introns were found, PCR products were cleaned with a QIAquick PCR Purification Kit (Qiagen) and sequenced by primer walking using Applied Biosystems BigDye technology. Open reading frames (ORFs) and were located and identified using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and Blast2 (Altschul *et al.* 1997) searches of GenBank. In most cases only partial unidirectional sequencing from each end of the PCR product was conducted to confirm the intron type and position. But, for each different intron found, one representative was completely sequenced and deposited on GenBank.

Phylogenetic analysis

To determine the phylogenetic relationships between isolates, translation elongation factor 1-alpha (EF 1-α) gene intron sequences were obtained. Polymerase chain reactions were performed in 25 µL volumes containing 200 µM of each dNTP, 1.5 mM $MgCl_2$, 2.5 µL 10x buffer, 4 ng each of primers EF-1 and EF-2 (O'Donnell *et al.* 1998) and 0.5 units of Taq polymerase. Cycling times were 2 min 94 °C, then 35 cycles of 30 sec 94 °C, 30 sec 55 °C and 1 min 72 °C. Products were cleaned with a QIAquick PCR purification kit (Qiagen) and sequenced using Applied Biosystems BigDye technology. Sequences were aligned using ClustalX (Thompson *et al.* 1997). A minimum evolution tree was constructed with MEGA2 (Kumar *et al.* 2001) using the Kimura-2-parameter method and a complete deletion of gapped sites. *Fusarium foetens* (AY320084) was used as the outgroup.

Results

Introns were found in the *nad*5 and cob genes. The occurrence of these introns is shown in Table 1. Partial sequencing demonstrated that all isolates have the same intron inserted in the same position in

Tab. 2. – Primers used in study to amplify five protein-coding genes in the *F. oxysporum* mitochondrial genome.

Region	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size of PCR product (no introns)
cob	TGGTTATTTAATAGACGCTTCACAA	AAACGGGCTTTCTACGTGTTT	900bp
*cox*1	TGAACTTAGTGGGCCAGGAG	TGAGGTTTAGGTGGGCTTGA	900bp
*cox*2	TATTTCCAAGACAGTGCTACTCC	ATTCCACATATTTCTGAACATTG	500bp
*nad*1	CAAGTATGCAAAGAAGACTAGGTC	GGGAAAGAGGCTCTTACTCAG	600bp
*nad*5	TTTTTGGTAGAAAAGTCGGTGTTA	CCACGTCATCTACTACCTGAACAA	2900bp[a]

[a] All isolates had an intron in the *nad*5 gene.

the *nad*5 gene. This intron is also present in the two *F. oxysporum* mitochondrial genome sequences on GenBank (Table 1). The intron codes for a putative homing endonuclease that is fused in frame with the *nad*5 gene. The homing endonuclease is of the LAGLIDADG type that is common in the mitochondrial genomes of fungi. No differences were found between the partial sequences of *nad*5 gene and intron regions of the isolates.

Fig. 1. – Structure of the middle of the cob gene in members of *F. oxysporum* Clade 1. The *cob* gene is represented in black, the putative homing endonuclease genes (fused in frame with the gene) are grey and the introns are white. Size of depicted region is 3453 bases, GenBank accession EU035604.

There was a variable distribution of introns in the *cob* gene (Table 1). Seven isolates had no introns, while seven produced a PCR product approximately 2150 bases in length. Complete sequencing of one of these long PCR products (EU035603) revealed that a single intron, 1238 bases long coding for a putative GIY-YIG homing endonuclease, was fused in frame with the gene. Partial sequencing of the other isolates showed that they also contained the same intron inserted in the same position in the *cob* gene. The partial sequences showed no variation in the 5′ cob coding region, and only 1–2 bases variation in the first 200 bases of the intron.

Two isolates produced a *cob* gene PCR product of approximately 3500 bases. Complete sequencing of one of these isolates (EU035604) showed that the same GIY-YIG homing endonuclease encoding intron was present in the same position as in the other isolates, but that a second intron, 1233 bases long, was also present. This intron contained a putative LAGLIDADG homing endonuclease gene, and was also fused in frame with the *cob* gene. Figure 1 shows the arrangement of the *cob* gene containing these two introns. Partial sequencing of the second isolate confirmed that it had the same two introns inserted in the same positions. No sequence variation was detected, but the 5′ 200 bases of the GIY-YIG endonuclease encoding intron differed by 7–8 bases from the GIY-YIG endonuclease encoding introns in the isolates without the LAGLIDADG endonuclease encoding intron.

All the introns were Group I. Although the complete secondary structure for these introns was not determined, each intron was found to have the typical Group I P7 stem comprising the R (GACUA) and S (UAGUC) elements (data not shown).

Phylogenetic analysis using translation elongation factor 1-alpha (EF 1-α) gene intron sequences showed a correlation between

evolutionary history and the distribution of introns in the *cob* gene (Figure 2). Most isolates could be placed in Clades 1–3 of the *F. oxysporum* complex that were proposed by Baayen *et al.* (2000). Isolates with no *cob* introns were in Clade 2, isolates with only a GIY-YIG endonuclease encoding intron were in Clade 3, and isolates with both the GIY-YIG and LAGLIDADG endonuclease encoding introns were in Clade 1. Isolates that did not belong to any of the three clades had only the GIY-YIG endonuclease encoding intron.

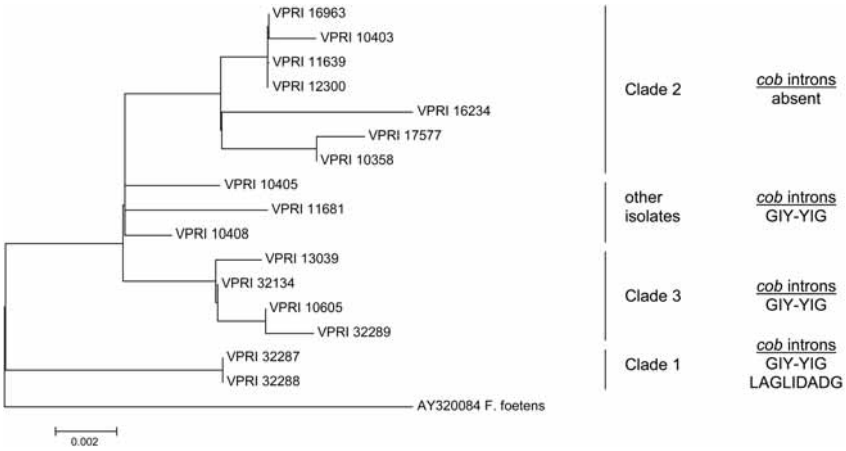

Fig. 2. – Minimum evolution tree from partial translation elongation factor 1-alpha gene sequences, showing the evolutionary relationship between *F. oxysporum* isolates and the distributions of homing endonuclease genes in the mitochondrial *cob* gene. Clades refer to those clades proposed by Baayen *et al.* (2000). Scale is 2 changes per 1000 bases.

Discussion

Although optional mitochondrial introns were found in this study, there were too few to account for the published size variation in the *F. oxysporum* mitochondrial genome. But, the correlation between *cob* intron distribution and evolutionary history is interesting. It appears that Jacobson and Gordon (1990) encountered these optional introns in the *cob* gene of *F. oxysporum* f. sp. *melonis*. They found an optional 1.4 kb insert in a *Pst*I mitochondrial genomic DNA restriction fragment that contained the cob gene. It is possible that this was one of the homing endonuclease encoding introns found in this study. Their isolates that contained the insert belonged in rare VCG's that were found to be genetically divergent from the other VCG's of f. sp. *melonis* that lacked the insert.

There appears to be different levels of variability in the distribution of optional mitochondrial introns in different groups of

ascomycetes. Belcour *et al.* (1997) found a range of optional Group I and Group II introns in strains of *Podospora anserina* (Rabenh.) Niessl. Collins and Lambowitz (1983) found significant variation in the distribution of introns in the cox1 gene of wild-type *Neurospora crassa* Shear & B.O. Dodge isolates. In contrast, there are several reports of a more conserved mitochondrial intron distribution that correlates with closely related taxa. The examples of subspecific taxa of *C. neoformans* and *O. novo-ulmi* were given in the introduction. Another good example is *Aspergillus niger* Tiegh. and the closely related *A. tubingensis* Mosseray (1934). These two species have different cox1 intron distributions that appear to be so stable that it was suggested that they could be used as diagnostic markers to differentiate the two species (Juhasz *et al.* 2007).

It is possible that the distribution of optional mitochondrial introns in the *F. oxysporum* complex could play a role in breaking up the complex into well defined subspecific taxa. Most isolates of *F. oxysporum* characterized by Baayen *et al.* (2000) could be placed into three evolutionary clades. But, several isolates did not fall into any of these, and neither did several isolates used in this study. The distribution of optional mitochondrial introns in these isolates was the same as those isolates in Clade 3. Therefore, lumping these isolates with Clade 3 becomes an option if the *F. oxysporum* complex were split into three subspecific taxa. Although this would mean that the Clade 3 taxon would not be monophyletic. There also exists the possibility of developing a simple diagnostic PCR test to differentiate the three clades of the *F. oxysporum* using these optional mitochondrial introns.

Acknowledgments

I thank Dr Rodney Jones and Dr Motiul Quader for reviewing the manuscript. This work was funded by the Victorian Government initiative, Our Rural Landscape.

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(Manuscript accepted 15 April 2009; Corresponding Editor: R. Pöder)