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Short Communication

Novel primers developed from mitochondrial intergenic spacers are useful for multi-locus sequence typing of *Fusarium oxysporum* strains

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Abstract

The *Fusarium oxysporum* species complex causes serious vascular diseases of a wide range of crops. In an effort to locate new highly variable regions for multi-locus sequence typing (MLST) of *Fusarium oxysporum* populations, mitochondrial intergenic regions were targeted. Five regions were amplified and sequenced from five randomly selected isolates. For these isolates, all regions contained at least as many variable sites as the mitochondrial ribosomal RNA small subunit region commonly used in MLST. Most regions contained short indels; however, 80–120 base indels were present in the *nad*5-*arg*2 intergenic region. Only the *nad*5-a*rg*2 region was found to be more variable than the single copy nuclear translation elongation factor (1α) gene introns. The *nad*5-*arg*2 region is likely to be a useful inclusion in MLST of *Fusarium oxysporum* populations.

Fusarium oxysporum is a cosmopolitan species complex that occupies several ecological niches as a saprophyte, plant endophyte or plant pathogen (Kistler, 2001). It is in this last role that *F. oxysporum* has received most attention, as it is responsible for many serious vascular diseases of plants (Gordon and Martyn, 1997). Early attempts to genetically characterise strains were based on restriction endonuclease digestion of the mitochondrial genome (e.g., Jacobson and Gordon, 1990). But, amplified fragment length polymorphisms (AFLPs) and multi-locus sequence typing (MLST) are now more commonly used methods (O'Donnell et al., 2004). In regards to the latter, the commonly targeted regions are the nuclear translation elongation factor 1-alpha (TEF 1-α) gene introns, nuclear ribosomal DNA intergenic spacer (IGS) regions, and the mitochondrial ribosomal DNA small subunit (SSU) gene (O'Donnell et al., 1998; Baayen et al., 2000; O'Donnell et al., 2004). Of these, the mtDNA SSU region is the least informative, but given that this is the only region that codes for a functional product, it is not surprising that it does not contain as many variable sites as an intron in a single copy nuclear gene (O'Donnell et al., 2004). Given that mtDNA is considered an appropriate target for population level genetic variation studies (Bruns et al., 1991), it is likely that non-coding (intergenic) regions would be more suitable than the SSU region for use in MLST.

As a first step to understanding variation within the mitochondrial genome of *F. oxysporum*, a 33.4 kb portion (GenBank AY874423, to be presented elsewhere) containing all the protein coding genes has been sequenced from isolate VPRI 19292, ex *Dianthus*. The organisation of the genome is very similar to that reported for *Hypocrea jecorina* (Chambergo et al., 2002) and *Lecanicillium muscarium* (Kouvelis et al., 2004), other members of the Hypocreales. The most notable features of the *F. oxysporum* mitochondrial genome are the large intergenic regions (approx.

31% of the sequence) and the presence of only a single intron in a protein coding gene. In these respects, the mitochondrial genome differs significantly from *H. jecorina* (many introns with short intergenic regions) and *L. muscarium* (no introns and short intergenic regions). The aim of this work was to examine sequence variability in the intergenic regions of the mitochondrial genome of *F. oxysporum* to determine if any could be useful additions to MLST studies.

Fusarium oxysporum isolates VPRI 10405 (ex *Triticum*), 10605 (ex *Hyacinthus*), 11681 (ex *Lycopersicon*), 12300 (ex *Solanum*) and 13039 (ex *Capsicum*) were chosen at random from the fungal culture collection at the Victorian Department of Primary Industries (VPRI). Cultures were grown on potato dextrose agar (Oxoid, Thebarton, SA, Australia), the mycelium was scraped from the agar surface, and total DNA extracted using a DNeasy™ Plant Mini Kit (Qiagen, Doncaster, VIC, Australia) according to the manufacturer's instructions. PCR primers were designed for five intergenic regions (Table 1) using the partial *F. oxysporum* mitochondrial genome sequence (GenBank AY874423). Regions were chosen based on size (300–600 bases), such that they could be sequenced in a single reaction. Polymerase chain reactions were performed in 25 μl volumes containing 200 μM of each dNTP, 1.5 mM $MgCl_2$, 2.5 μl 10× buffer, 4 ng of each primer and 0.5 units of *Taq* polymerase (Roche, Castle Hill, NSW, Australia). Cycling times were 2 min at 94 °C, then 35 cycles of 30 sec at 94 °C, 30 sec at 50 °C and 1 min at 72 °C. To compare the variation within these regions to other gene regions often used in *F. oxysporum* MLST studies, the mtDNA SSU region was amplified using primers MS1 and MS2 (White et al., 1990), and TEF 1-α gene was amplified using primers EF-1 and EF-2 (O'Donnell et al., 1998). An annealing temperature of 55 °C was used for the TEF 1-α gene. Products were cleaned with a QIAquick PCR purification kit (Qiagen) and sequenced using Applied Biosystems (Scoresby, VIC, Australia) BigDye technology. Both strands were sequenced.

The five intergenic regions were successfully amplified and sequenced, as were the mitochondrial rDNA SSU gene and the TEF 1-α gene. These sequences have been deposited in GenBank as accessions DQ487292-DQ487326. The sequences were aligned and gapped regions greater than four bases in length were removed. The distribution of variable sites within these final alignments is shown in Tables 2, 3 and 4. The SSU, *arg*1-*nad*4L, *cox*1-*arg*2, *ser-asn* and *pro-lsu* regions contained similar levels of variation, i.e., between 9 and 13 variable sites. Of these, only the *ser-asn* region contained indels of significant length. These consisted of 16 base and 29 base insertions in isolates VPRI 10605, 13039 and 11681, but there were no variable sites within these insertions.

The remaining intergenic region, *nad*5-*arg*2, contained 34 variable sites, approximately twice the number as the TEF 1-α gene introns. There were also significant length polymorphisms in this region due to the presence of 80–120 base insertions at its beginning in isolates VPRI 10605, 13039 and 11681. These insertions contained a small number of additional variable sites. Of the five intergenic regions examined in this study, the *nad*5-*arg*2 provides the most variability. Another two intergenic regions of appropriate size were initially targeted, but the primers designed failed to amplify these regions consistently. The remaining intergenic regions in the sequenced portion of the *F. oxysporum* mitochondrial genome are either very small (< 300 bases) or are so large that multiple sequencing reaction would be required to sequence the region. It is interesting to note that the *nad*5-*arg*2 is one of the smaller regions examined in this study. Future studies should examine the variation in the other small intergenic regions. Examination of the TEF 1-α gene introns sequences revealed that isolates VPRI 10605 and

Table 1. Primers developed in this study

Region	Forward primer (5′–3′)	Reverse primer (5′–3′)	Approximate size
*arg*1-*nad*4L	MIS1F (GGTAGAACAAGATGGTACGGTC)	MIS1R (CTATTAAATACAAACCCTAAGATTCC)	650
*nad*5-*arg*2	MIS2F (GTAGTAGATGACGTGGTAAATTC)	MIS2R (ATCTCATCTCAGGGTCGAACT)	450–570
*cox*1-*arg*3	MIS3F (GGATTCTACACAGACGTACTGC)	MIS3R (CTCATCTCAGGGTCGAACTAAG)	380
ser-asn	MIS4F (GGTAGGGTAAGATATTTGCTAAG)	MIS4R (AGCCCCTAAGATGAATCGAAC)	670
pro-lsu	MIS5F (GTAGGCACTTCGTTTGGGACG)	MIS5R (ATATTTAGCCTTTGAGGAAGGT)	450

Table 2. Sequence variation in the mitochondrial rDNA SSU gene and translation elongation factor 1-alpha gene from isolates of *F. oxysporum*

Isolate	SSU									Translation elongation factor1-alpha																
	522	524	533	560	565	568	575	577	578	78	112	189	234	263	288	322	334	335	386	396	406	412	601	602	636	637
AY874423[A]	T	A	A	G	A	A	T	A	A	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#
VPRI 12300	.	.	.	.	.	.	.	.	.	A	C	C	A	C	A	C	-	-	-	G	G	G	G	C	A	T
VPRI 10405	.	.	G	.	G	C	.	.	.	T	.	.	G	.	G	.	C	-	-	C	.	.	.	T	.	.
VPRI 10605	.	G	.	A	.	C	-	C	T	T	T	T	G	.	.	T	T	-	A	C	.	.	.	.	-	-
VPRI 13039	.	G	.	A	.	C	-	C	T	T	T	T	G	.	.	.	T	-	A	C	.	C	.	.	.	.
VPRI 11681	G	G	.	A	.	C	.	C	T	T	.	.	G	A	.	.	T	T	A	C	C	.	T	.	.	.

Numbers, written vertically, indicate variable base positions in modified alignment (discussed in text). Dots indicate identity with reference sequence, dashes indicate gaps.

[#]Sequence not available for this isolate.

[A]VPRI 19292.

Table 3. Sequence variation in the mitochondrial *arg*1-nad*4L, cox*1-*arg*2 and *ser-asn* intergenic regions of *F. oxysporum*

Isolate	*arg*1-*nad*4L									*cox*1-*arg*2														*ser-asn*												
	91	266	317	320	361	372	488	489	616	42	83	91	92	95	96	97	107	152	153	165	182	216	255	7	126	135	143	210	250	293	294	349	387	421	441	578
AY874423[A]	G	A	-	T	A	A	T	-	G	C	T	G	G	-	-	T	A	G	-	T	G	T	G	G	G	T	A	C	G	-	-	T	G	A	T	A
VPRI 12300	.	.	-	.	.	.	.	-	.	.	.	.	.	-	-	.	.	-	-	.	.	.	.	T	.	.	.	.	.	-	-	.	.	.	.	.
VPRI 10405	A	.	-	.	.	.	-	-	.	T	C	.	.	-	-	A	.	-	-	C	A	.	.	.	T	C	.	T	A	A	-	.	.	.	.	.
VPRI 10605	A	G	-	C	.	.	-	-	T	.	.	A	A	A	T	A	.	.	G	C	A	.	A	.	T	C	G	T	.	A	-	A	A	G	A	T
VPRI 13039	A	G	-	C	.	.	.	-	T	.	.	A	A	A	T	A	.	.	G	C	.	C	.	.	.	C	G	T	.	A	A	A	A	G	A	T
VPRI 11681	A	G	A	C	G	G	.	T	.	.	.	A	A	A	T	A	-	.	G	C	.	.	.	.	T	C	G	.	.	-	-	A	A	G	A	T

[A]VPRI 19292.

Table 4. Sequence variation in the mitochondrial *pro-lsu* and *nad*5-*arg*2 intergenic regions of *F. oxysporum*

Isolate	*pro-lsu*												*nad*5-*arg*2																																	
	48	73	74	78	135	136	137	138	201	393	455	500	1	12	42	54	56	57	59	60	61	62	64	65	67	140	150	176	184	198	207	208	212	231	232	244	245	273	280	281	282	340	341	342	343	399
AY874423[A]	C	G	C	T	-	-	-	-	A	G	A	G	C	C	C	C	A	G	T	T	A	A	C	T	G	T	G	G	-	C	A	T	T	-	-	G	A	T	C	T	-	T	G	C	T	T
VPRI 12300	.	.	.	.	-	-	-	-	.	.	.	.	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	-	-	.	.	.	T	.	-	.	.	.	.	.
VPRI 10405	.	-	-	C	-	-	-	-	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	G	.	A	T	T	A	G	C	.	.	G	-	-	-	-	.
VPRI 10605	.	.	.	.	T	T	T	-	G	A	G	C	G	.	.	G	G	A	G	A	.	-	T	C	C	-	T	A	A	.	G	C	.	T	-	A	G	C	.	A	G	.	.	.	.	A
VPRI 13039	T	.	.	.	T	T	T	T	G	A	G	C	G	.	.	G	G	A	G	A	.	-	T	C	C	-	T	A	A	.	G	C	.	-	-	A	G	C	.	A	G	.	.	.	.	A
VPRI 11681	T	.	.	.	T	T	T	-	G	.	G	.	G	T	T	G	G	A	.	.	T	-	T	.	C	.	T	A	-	T	G	C	.	-	-	A	G	C	.	.	G	.	.	.	.	A

[A]VPRI 19292.

13039 belong in *F. oxysporum* Clade 3 (Baayen et al., 2000), and VPRI 12300 belongs in Clade 2. The other isolates could not be placed in any clade.

There have been few reports of mitochondrial IGS for population genetic studies in fungi. This is probably due to few complete fungal mitochondrial genome sequences, at least until recently. Mitochondrial intergenic regions were examined in *Phytophthora infestans* populations, although much lower levels of variation were encountered

(Wattier et al., 2003); only nine variable sites were found across five regions. Although *F. oxysporum* is a species complex, given the high level of variation found here it is possible that these regions will be useful for other groups of fungi. The primers designed here have been successfully used with *F. verticillioides* (data not shown) and may be appropriate for a range of other *Fusarium* species. However, based on a comparison with the *H. jecorina* mitochondrial genome, these primers will not be applicable throughout the Hypocreales.

O'Donnell et al. (2004) stated that single copy nuclear genes interrupted by large or numerous introns are essential for MLST of Fusaria. This study has shown that mitochondrial IGS have the potential to provide more information than such nuclear regions, and thus must also be seriously considered for future MLST studies.

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