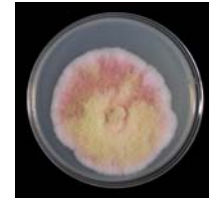


Multiple Copies of *Cox1* in Species of the Fungal Genus *Fusarium* (Ascomycota, order Hypocreales, family Nectriaceae)

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Introduction

Fungal barcoding with *Cox1* is in its infancy. Most published sequences are derived from mitochondrial genomes and there are few published sets of primers. The frequent reports of introns in fungal *Cox1* have led to some reservations among mycologists about using this gene for barcoding. If introns are frequent and of significant length, they may interfere with PCR by disrupting priming sites or increasing the length of the amplicon. However, in the first comprehensive study of *Cox1* in a fungal genus, of *Penicillium* subgenus *Penicillium* (Seifert et al., PNAS 104: 3901-3906, 2007), very few introns were found.

Fusarium is a mould genus of about 125 known species, classified in the Ascomycete order Hypocreales. The species are widely distributed on plants and in the soil and are typically characterized by the production of banana-shaped macroconidia. The genus includes several economically important plant pathogenic species, such as *F. graminearum*, *F. oxysporum*, and *F. solani*. Several *Fusarium* spp. also produce mycotoxins (moniliformin, fumosins, trichothecenes) that are subject to international regulations. Crops and other food commodities contaminated with these toxins affect human and animal health by chronic or acute toxicity, or long term carcinogenic effects.

A successful DNA barcoding system for identifying species of this genus would have potential commercial value. There is a growing body of literature on using genes such as *Translation Elongation Factor 1- α* (*TEF*) for identification of *Fusarium* species, and this technology is being used with increasing frequency in research on these fungi. This study was a preliminary survey of *Cox1* in *Fusarium*, using newly designed primers.

Materials and methods

Samples. Cultures were obtained from the Canadian Collection of Fungal Cultures (DAOM). DNA was isolated using a variety of methods, and most extracts were derived from ongoing projects on the taxonomy of *Fusarium* and other genera of the Hypocreales.

Primer design. Initial primer design for amplification of the *Cox1* barcode in Hypocreales was done from the few sequences available in GenBank; *Fusarium oxysporum*, *Metarhizium anisopliae*, *Hypocrea jecorina*, and *Lecanicillium muscarium*. One reverse primer and three different forward primers were designed (Table 1) and tested for PCR and sequencing.

Primer name	Sequence	Pairs with	Description
AHyFu-F	5'-CTTAGTGGCCAGGAGTTC AATA-3'	AHyFu-R	Barcode, forward, from <i>F. oxysporum</i> AY874423
AHyFu-R	ACCTCAGGGTGTCCGAAGAAT	AHyFu-F, F14b-F	Barcode, reverse, from <i>F. oxysporum</i> AY874423
F13-F	TTAAAAGTATCGAAAATCAAAAAGGTGT	F13-R	Intron 3, forward, for <i>Fusarium</i>
F13-R	ATCTATCTCTTATTTCTTGGCTCATTGGTT	F13-F	Intron 3, reverse, for <i>Fusarium</i>
F14b-F	CCTTAAAACATAGTACCCGAC	AHyFu-R	Intron 4b, forward to end of barcode, for <i>Fusarium</i>

Table 1: Primer sequences used to amplify and sequence the *Cox1* region of *Fusarium* species and other members of the Hypocreales.

Intron number	1	2	3	4a	4b	5	6	7	8	9	10	11a	11b	Total bp
Barcode position (bp)	8	55	68	97	97	138	170	253	256	350	472	566	566	567
<i>F. oxysporum</i> AY874423														
<i>F. dimerum</i> DAOM 235647														567
<i>G. moniliformis</i> genome														1498
<i>F. lateritium</i> BBA 62244				512+										692+
<i>F. solani</i> DAOM 235651														861+
<i>F. babinda</i> DAOM 235678														633+
<i>F. flocciferum</i> BBA 64535				712+	229+									2000
<i>Hypocrea jecorina</i> NC03388								1282						2987
<i>Podospira anserina</i> X55026.1	2539			1238		1260	2500	1507		1456	2251	1407	1474	14724
<i>Aspergillus japonicus</i> AF315722		842+		1239										
<i>Neurospora crassa</i> X14669		2631			1345							1222		5765

Table 2: Intron positions in the barcode region of the *Peizomycotina*. The barcode of *Fusarium oxysporum* (AY874423) was used as the reference. The letters a and b distinguish between two nonorthologous introns (unalignable) found at the same position. Examples are shown of different strains where introns were recovered, with the intron length (in bp) and position in the barcode where the intron was found. A + sign indicates that only a partial sequence was obtained and the final length of the intron is greater than the indicated number. The rows in yellow are in the Hypocreales; the remaining rows are other members of the *Peizomycotina* for which mitochondrial genome sequences are available.

Acknowledgements

We are grateful to the Canadian Collection of Fungal Cultures (DAOM, Ottawa) and the Institut für Mikrobiologie culture collection (BBA, Berlin) for providing cultures and DNA preps. This research was funded by the Genome Canada grant to the Canadian BarCode of Life Network.

Results

Primers AHyFu-F and AHyFu-R, designed from genome sequences of *F. oxysporum* to amplify a 567 bp fragment of *Cox1*, yielded PCR fragments of three sizes: 570 bp (no introns), ~2000 bp and ~3000 bp (introns present). Subsequent sequencing confirmed introns in some species and not in others (Figs. 1-3).

A survey of intron location and length using sequences from GenBank, revealed 11 potential intron sites in the 567 bp barcode region (Table 2) of the *Peizomycotina* (including the Hypocreales). At two sites, two unalignable introns occurred in the same location, labeled as a or b in Table 2. Three intron positions (3, 4, 11) were found in *Fusarium*; at two positions, two different sequences types were found (4a, 4b; 11a, 11b). In our initial survey, most species had either no introns or introns 3 and 4b. *Fusarium circinatum* and *F. sacchari* had heterozygous bases in the barcode sequences, indicating more than one *Cox1* copy in these haploid strains (Fig. 1, side panels).

We designed intron specific primers to test for the presence of introns in cultures where they were not initially seen (Table 1). Primers were for a 337 bp fragment of intron 3 (F13-F and F13-R) and a second primer (F14b-F) paired with the barcode reverse primer (AHyFu-R) to give a 724 bp fragment starting in intron 4b. When these primers were tested on *Fusarium* samples that had previously yielded no introns, intron-containing sequences were amplified, confirming the presence of multiple copies of *Cox1*. Some samples, such as *F. torulosum* (BBA 64465) and *F. circinatum* (DAOM 235758), had heterozygous bases in intron 3 suggesting multiple copies of the intron (side panels in Fig. 2).

Multiple copies of *Cox1* confound the interpretation of the barcode tree of *Fusarium*, with several strains and/or species showing up in multiple locations. These are not misidentified strains. The identity of all strains was confirmed with *TEF* sequencing (data not shown). Perhaps with comprehensive sampling, each main cluster within the barcode tree will reiterate the species phylogeny. However, there appears to be a lack of differentiation among the species, at least within some *Cox1* copies, with species from different sections of the genus having identical sequences (green arrows in Fig. 1).

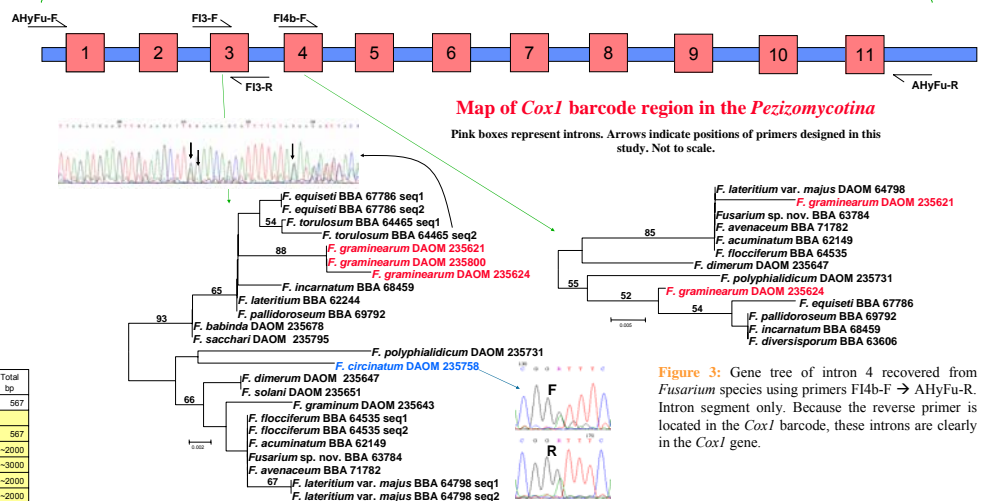


Figure 2: Gene tree of intron 3 recovered from *Fusarium*. Primers F13-F → F13-R. Although this intron was recovered in some barcode sequences, it is unproven that all of these introns actually occur in the *Cox1* gene; they could represent mobile mitochondrial introns. The side panel shows a heterozygous base pair detected in the intron of one strain.



Micrograph of a macroconidium and spore producing cells of *Fusarium graminearum*.

Figure 1: *Cox1* barcode region of *Fusarium*, gene tree excluding introns. Blue labels and red labels indicate two of the species with two copies. Side panels show heterozygous bases detected in barcode sequences of two species. Primers AHyFu-F → AHyFu-R. ** indicates sequences with introns removed.

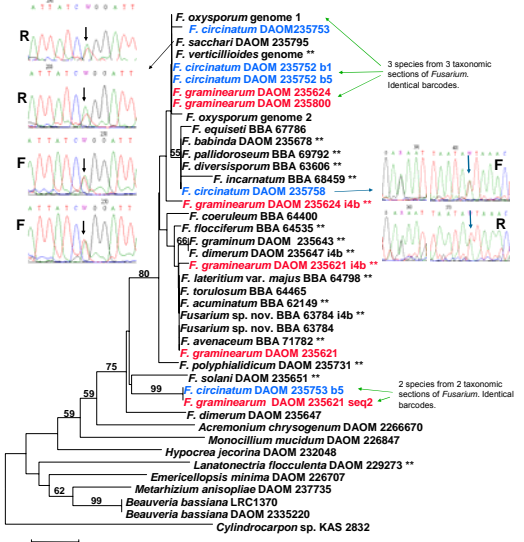


Figure 3: Gene tree of intron 4 recovered from *Fusarium* species using primers F14b-F → AHyFu-R. Intron segment only. Because the reverse primer is located in the *Cox1* barcode, these introns are clearly in the *Cox1* gene.

Conclusions

- Introns occur frequently in the *Cox1* gene of the *Fusarium* species surveyed.
- Multiple copies of *Cox1* were found in individual strains of *Fusarium* species, and different copies were found among different cultures of the same species.
- Using intron-specific primers, introns could be amplified from some DNA preps that did not reveal them using the barcode primers.
- With introns excised, the barcode tree of *Fusarium* shows many species occurring in multiple locations because of their multiple *Cox1* copies; this makes comparison with established phylogenies based on nuclear genes difficult.
- Homologous copies of *Cox1* from *Fusarium* species seem to exhibit insufficient variation to function as an effective identification species.
- This must be confirmed with a broader survey of *Fusarium* species and strains, using a method that will focus on transcribed copies of the gene, such as reverse transcriptase PCR.