

ALTERNATIVE OXIDASE GENE (AOX I): A GOOD DNA BARCODING CANDIDATE FOR THE GENUS *FUSARIUM*

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EL GEN DE LA OXIDASA ALTERNATIVA (AOXI): UN BUEN CANDIDATO PARA "CÓDIGO DE BARRAS DE ADN" DEL GÉNERO *FUSARIUM*

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RESUMEN

"DNA barcoding" es una herramienta de análisis taxonómico que usa una secuencia estándar corta presente en todos los taxa de interés, con suficiente variación de secuencia para discriminar entre especies. Los miembros del género *Fusarium* son conocidos por ser saprófitos y patógenos importantes de plantas y humanos, y su identificación taxonómica comúnmente depende de características macro y microscópicas, y métodos moleculares. Sin embargo, la identificación puede ser difícil debido a la falta de algunas estructuras en cultivo o poco polimorfismo en el DNA de secuencias ribosomales. "DNA barcoding" podría proveer un método confiable y rápido para superar estos problemas. Este estudio preliminar evaluó secuencias de *Cox* (mitochondrial cytochrome oxidase subunidad 1) y *Aox* (alternative oxidase) como potenciales "DNA barcodes" para la identificación de especies de *Fusarium*. Se extrajo ADN de 12 aislados de *Fusarium* previamente identificados por métodos tradicionales en complejos diferentes: *F. solani*, *F. oxysporum* y *Gibbelleria fujikuroi*. Para la amplificación por PCR del gen *Cox I* se usaron cebadores diseñados en un estudio previo y se observaron amplicones de aproximadamente 600 pb en todos los aislados. Para el gen *Aox*, nuestro grupo diseñó cebadores obteniendo amplificación de una región de aproximadamente 800 bp en todas las especies de *Fusarium*. Las secuencias fueron agrupadas en árboles, adicionalmente la divergencia intra e interespecífica fue estimada con el modelo K2P. Los resultados mostraron que la secuencia de *Aox* agrupó los aislados en los complejos de especies de *Fusarium* apropiados de acuerdo a la caracterización morfológica previa, mostrando su potencial para diferenciar aislados de *Fusarium* a nivel de especies. El análisis de similitud de *Cox I* no mostró variación suficiente para discriminar entre aislados. En conclusión, la secuencia de 800 bp de AOX es candidato a convertirse en una secuencia "barcode", pero se necesita un mayor número de especies y aislados para probar su habilidad discriminante.

Palabras clave: *Fusarium*, Barcode, *Aox*, *Cox1*, variación intra e inter específica

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ABSTRACT

DNA barcoding is a tool for taxonomy analysis that uses a short standard genomic sequence present in all the taxa of interest, which has enough sequence variation to discriminate among species. Members of the genus *Fusarium* are recognized as saprophytes and important plant and human pathogens, and their taxonomic identification commonly relies on macro and microscopical characteristics and molecular methods. However, identification can be difficult due to the lack of some structures in culture or poor polymorphism in ribosomal DNA sequences. Barcoding could provide an easy and reliable method to overcome these problems. This study is a preliminary study to evaluate sequences of *Cox* (mitochondrial cytochrome oxidase subunit I) and *Aox* (alternative oxidase) as potential DNA barcodes for identification of *Fusarium* species. DNA was extracted from 12 *Fusarium* isolates previously identified by traditional methods into different complexes: *F. solani*, *F. oxysporum* and *Gibbellera fujikuroi*. For PCR amplification of *Cox 1* gene, primers designed in a previous study were used and amplicons of approximately 600 pb were observed in all the isolates. For the amplification of *Aox*, primers were designed by our group showing amplification of a region of 800 bp approximately in all *Fusarium* species. The sequences were clustered using phylogenetic trees, additionally, intra- and interspecific divergence were estimated with the K2P model. These results showed that the *Aox* sequence clustered the isolates into the appropriate *Fusarium* species complex, according to previous morphological characterization, indicating its potential to differentiate *Fusarium* isolates to the species level. Similarity analysis of *Cox1* sequences showed insufficient variation to discriminate among isolates. In conclusion, the 800 bp sequence of *AOX* is a candidate to become a DNA barcode sequence, but more species and isolates are needed to further test its discriminating ability.

Key words: *Fusarium*, Barcode, *Aox*, *Cox1*, intra and inter-specific variation

INTRODUCTION

Members of genus *Fusarium* are recognized as important saprophytes and important plant and animal pathogens. Their routine taxonomic identification relies on macro and microscopical characteristics and molecular methods. However, identification can be difficult due to the lack of some structures in culture and poor polymorphism in ribosomal sequences (Geiser et al., 2004; Leslie y Summerell, 2006). "DNA barcoding" is a tool that aims to become a taxonomic method using a short standard genomic sequence, present in all the taxa of interest and showing enough sequence variation to discriminate among species (Hajibabaei

et al., 2007). The project "Barcode of Life" arises as a tool in the identification of species and has generated much attention and controversy (Ebach and Holdrege, 2005; Hebert et al., 2003) The concept that a short sequence of DNA can be used in the diagnosis of taxa with relative speed, efficiency and accuracy has become a reality for mammals, birds, fish and some groups of insects, using as a sequence of marker gene encoding subunit I of cytochrome c oxidase *CoxI* (Hebert et al., 2004; Barrett et al., 2005). For other taxa like fungi, a conserved sequence with enough resolution to identify species has not been found.

In the case of *Penicillium*, sequences of *Cox I* ITS and β -tubulin (BenA) genes have been evaluated by Seifert et al. (2007) for the identification of molecular marker DNA barcode type. They suggested the use of *Cox I* as possible marker and proposed that it should be tested for other groups of fungi. In regards to *Fusarium*, in a preliminary study by Gilmore et al. (2009) was reported the presence of introns and multiple copies of the gene *Cox I*, which produced PCR amplicons of different sizes and their sequences generated non stable trees.

Our working hypothesis is that the gene *Aox* (alternative oxidase) could provide a reliable barcoding method to overcome identification problems in the genus *Fusarium*. This study evaluated sequences of *Cox I* and *Aox* as potential DNA barcodes for identification of *Fusarium* species. Sequences of *Cox I* have been used as DNA barcodes for several groups including Oomycete genera (Seifert, 2009). As far as we know, *Aox* has not been used as barcode, but we consider it a potential candidate due to its role in an alternative metabolic pathway when oxidative phosphorylation is affected. It is a nuclear gene but its protein product is active at the mitochondrial membrane (Atteia et al., 2004; Lorin et al., 2006).

MATERIALS AND METHODS

Samples. Twelve fungal isolates from human and plant hosts were used in this study. These isolates were obtained from LAMFU-Universidad de Los Andes (Bogotá, Colombia) culture collection. Monosporic cultures were grown on Potato Dextrose Agar (PDA) and then they were identified by morphological analysis and using the taxonomic keys from Leslie and Summerell, (2006) and Nelson et al., (1983). Also, molecular characterization was done by sequencing the ITS standard region using the primer ITS4 and TS5. The isolates included different complexes of *F. solani*, *F. oxysporum* and *Gibberella fujikuroi*. For comparison, DNA from *Alternaria sp.* and *Pestalotia sp.* was used.

Species	Source	Code
<i>Fusarium oxysporum</i>	LAMFU*	FO1157
<i>Fusarium oxysporum</i>	LAMFU	FO1232
<i>Fusarium oxysporum</i>	LAMFU	FO2055
<i>Fusarium solani</i>	LAMFU	FS558
<i>Fusarium solani</i>	LAMFU	FS1394
<i>Fusarium solani</i>	LAMFU	FS2110
<i>Fusarium moniliforme</i>	LAMFU	FPSC497
<i>Fusarium moniliforme</i>	LAMFU	FV2001
<i>Fusarium moniliforme</i>	LAMFU	FPSC1229
<i>Fusarium proliferatum</i>	LAMFU	FP561
<i>Fusarium proliferatum</i>	LAMFU	FP695
<i>Fusarium proliferatum</i>	LAMFU	FP2103
<i>Pestalotia sp.</i>	UMNG**	PS200
<i>Alternaria sp.</i>	UMNG	AL100

Table 1. List of *Fusarium* species tested and species used as controls

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Primer design.

Primers AHyFu-F and AHyFu-R for *Cox1* were obtained from Gilmore *et al.* (2009) (Table 2). Primers for *Aox* were designed using sequences of *F. oxysporum* (Transcript:FOXG_00039T0), *F. verticillioides* (Transcript:FVEG_01477T0) and *F. graminearum* (Transcript:FGSG_01342T0) freely available in BROAD database (<http://www.broadinstitute.org>) (Table 2).

Gene	Name	Sequence (5'-3')	Reference
Cox1	AHyFu-F	5'-CTTAGTGGGCCAGGAGTTCAATA-3'	Gilmore <i>et al.</i> (2009)
	AHyFu-R	5'-ACCTCAGGGTGTCCGAAGAAT-3'	Gilmore <i>et al.</i> (2009)
Aox	Fw1F.EG	5'-GTGACTTCTTTCCGGTCAAAGAGAC-3'	This Work
	Re2F.EG	5'-GGATCAAGTCCTTCATCGTACGGTGC-3'	This Work

Table 2: Primer sequences used to amplify and sequence the *Cox1* and *Aox* regions from *Fusarium* isolates

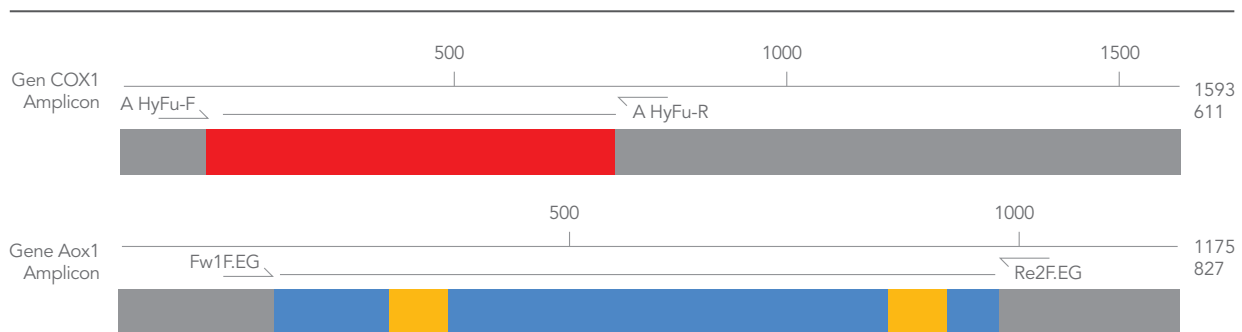


Figure 1. Maps of the amplified regions of *Cox1* and *Aox*. The red box indicates *Cox 1* and blue boxes for *Aox*, represent the amplified regions, yellow boxes represent possible introns and arrows indicate position of primers.

DNA extraction. *Fusarium* isolates were activated in PDA (potato dextrose agar) 5% and incubated 8 days at 24°C after which monospore cultures were grown. For DNA extractions 100 mg of lyophilized mycelium from monospore cultures was used. Ground lyophilized micellium was treated with (2% CTAB, 3% SDS, 250 mM NaCl, 200mM Tris-HCl pH 8.5 y 25mM EDTA) with 2-mercaptoetanol 1% and incubated at 65°C for 2h, after which

phenol:chloroform:isoamyl alcohol extraction was performed. DNA was washed and precipitated with ethanol 95%, dissolved in TE buffer 1X (Tris-HCl 0.1mM y EDTA 1mM) and stored at -20°C.

PCR amplification. Standard PCR amplifications were performed for both pairs of primers using Accuzyme High Fidelity Polymerase (BIOLINE, London, UK), to generate amplicons for sequencing. Amplifications were done in a 25-µL reaction volume

containing 1 μ l (10 ng) of DNA, 0.5 μ M of each primer AHyFu-F and AHyFu-R, 1 U of Accuzyme (BIOLINE, London, Uk), 1 \times Accuzyme reaction buffer, 0.2 mM dNTPs. Thermocycling program was as follows: 2 min at 95°C, 30 cycles consisting of denaturation for 1 min at 94°C, annealing temperature of 54°C, extension of 68°C for 1 min and a final extension of 68°C for 8 min. For Aox Fw1.FEG and Re2.FEG primers were used. The PCR was performed with the same conditions of the Cox PCR, except for the annealing temperature (56°C). DNA was sequenced by Macogen, Korea. Sequenced amplicons were assembled and edited with CLC Main Workbench 5.0. The consensus sequence obtained for each species was used for BLAST analysis (parameters by defect) against *Fusarium* Comparative Genome Platform data base (<http://fcgp.fusariumdb.org/> 2009).

Clustering analysis and barcode gap estimation. DNA sequences were aligned with MUSCLE (Edgar, 2004) and evolutionary model GTR+G was determined using jModelTest software (default options) (Posada, 2008). Trees were inferred using Rax ML software (Stamatakis, 2006) using maximum likelihood analysis with 1000 bootstrap replicates, and the appropriate evolutionary model. Intra- and interspecific divergence was estimated in MEGA 4 (Tamura et al. 2007) according to K2P (Kimura 2-Parameter) model. To establish the presence of a barcode gap, identity values were used to build graphics of the maximum interspecific variation percent in relation to the minimum percent of interspecific variation (Hebert et al. 2003).

RESULTS

Amplicons of approximately 600 pb for Cox1 and 800 pb for Aox were observed with the primers tested. An isolate of *F. verticillioides* generated two amplicons, one of the expected size and other of approximately 2300 pb (Figure 2). All the *Fusarium* species could be amplified but not *Alternaria* sp. or *Pestalotia* sp. with any primers.

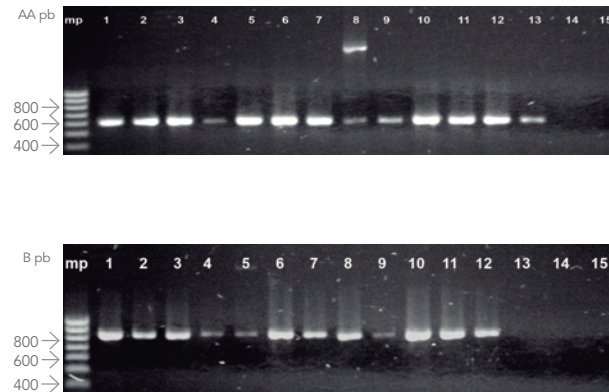


Figure 2. Agarose gel electrophoresis of PCR products. A): Cox1 PCR products; B): Aox PCR products. Lane mp molecular weight marker Hyperladder IV Bioline; lane 1, *F. oxysporum*1167; lane 2, *F. oxysporum*1232; lane 3, *F. oxysporum*2055; Lane 4, *F. solani*558; lane 5, *F. solani*1394; lane 6, *F. solani*2110; lane 7, *F. pseudocircinatum* 497; lane 8, *F. verticillioides* 2001; lane 9, *F. pseudocircinatum* 1229, lane 10, *F. proliferatum*561; lane 11, *F. proliferatum*695; lane 12, *F. proliferatum*2103; lane 13, *Pestalotia*100; lane 14, *Alternaria*sp300; lane 15, Control H₂O.

To confirm the identity of the sequences obtained by PCR, they were compared by BLAST-n (Altschul et al., 1990) analysis with the *Fusarium* Comparative Genome Platform data base (Park et al., 2011) (<http://fcgp.fusariumdb.org/> 2008) (Table 3). All sequences obtained presented similarity with sequences of the genus *Fusarium*. Regardless of isolate, all sequences obtained with the Cox 1 gene primers, showed similarity with Cox 1 sequences of *F. oxysporum* (similarity between 99.9 and 90.0, E value - 0 in all cases). Sequences of Aox gene from *F. oxysporum*, *F. pseudocircinatum* and *F. proliferatum* showed similarity with sequences reported for *F. oxysporum* (similarities between 99.9% and 89.5% E value - 0 in all cases) and sequences of *F. solani* and *F. verticillioides* isolates coincided with sequences of their species (similarities between 96.7% and 94.6% for *F. solani* and for *F. verticillioides* 97.4%, E value - 0 in all cases).

Query sequences	"BLASTn" RESULTS (FCGP)					
	COX			AOX		
	Organismo	% Similarity	Expect value	Organismo	% Similarity	Expect value
F. oxysporum 1157	<i>F. oxysporum</i>	99.8	0.0	<i>F. oxysporum</i>	99.9	0.0
F. oxysporum 1232	<i>F. oxysporum</i>	99.8	0.0	<i>F. oxysporum</i>	99.9	0.0
F. oxysporum 2055	<i>F. oxysporum</i>	99.8	0.0	<i>F. oxysporum</i>	99.9	0.0
F. solani 558	<i>F. oxysporum</i>	97.5	0.0	<i>F. solani</i>	94,6	0.0
F. solani 1394	<i>F. verticillioides</i>	91.1	0.0	<i>F. solani</i>	96,71	0.0
F. solani 2110	<i>F. oxysporum</i>	97	0.0	<i>F. solani</i>	96,5	0.0
F. pseudocircinatum 497	<i>F. oxysporum</i>	93.8	0.0	<i>F. oxysporum</i>	89.5	0.0
F. verticillioides 2001	<i>F. oxysporum</i>	97,4	0.0	<i>F. verticillioides</i>	99.9	0.0
F. pseudocircinatum 1229	<i>F. oxysporum</i>	93.8	0.0	<i>F. oxysporum</i>	89.5	0.0
F. proliferatum 561	<i>F. oxysporum</i>	97,1	0.0	<i>F. oxysporum</i>	96.4	0.0
F. proliferatum 695	<i>F. oxysporum</i>	97,1	0.0	<i>F. oxysporum</i>	96.7	0.0
F. proliferatum 2103	<i>F. oxysporum</i>	97,1	0.0	<i>F. oxysporum</i>	96,7	0.0
Pestalotia 200 (En NCBI)	<i>Verticillium dahliae</i>	90,0	0.0			

Table 3. Results of BLASTn analysis of sequences obtained from PCR analysis of Cox1 and Aox against *Fusarium* Comparative Genome Platform data base.

Sequences obtained in this work were submitted to Genbank but accession numbers have not been assigned yet. However sequences are available upon request. These sequences and some downloaded from *Fusarium* Comparative Genome Platform data base were used to build alignments of sequences for each species. In Figures 4 and 5,

intraspecific polymorphism can be observed in orange and yellow letters for Cox1 and Aox sequences respectively and interspecific variation is represented by green and purple columns Cox1 and Aox sequences respectively. Sequences of Aox gene showed larger intra and interspecific variation than those of Cox1 gene.



Figure 3. Alignments of sequences of Cox 1 gene obtained with AHyFu-F/AHyFu-R primers

Letters in orange show intra specific polymorphism. Green bars represent percent of inter specific similarity. Numbers to the right represent the length of the sequence. (*) sequences downloaded Fusarium Comparative Genome Platform data base. Images obtained with CLC main workbook 5.0.

Using tools for phylogenetic analysis, sequences of both genes were clustered using the Maximum Likelihood (ML) (Stamatakis, 2006) algorithm. Clustering analysis of *Aox* sequences showed enough polymorphism to differentiate among species and grouped isolates into the appropriate *Fusarium*

species complexes, according to previous taxonomical determination (Figure 5). Sequences derived from *Cox 1* gene produced clusters containing sequences from different species and was unable to cluster sequences belonging to the same species, for example in the case of *F. verticillioides* and *F. solani* (Figure 5).

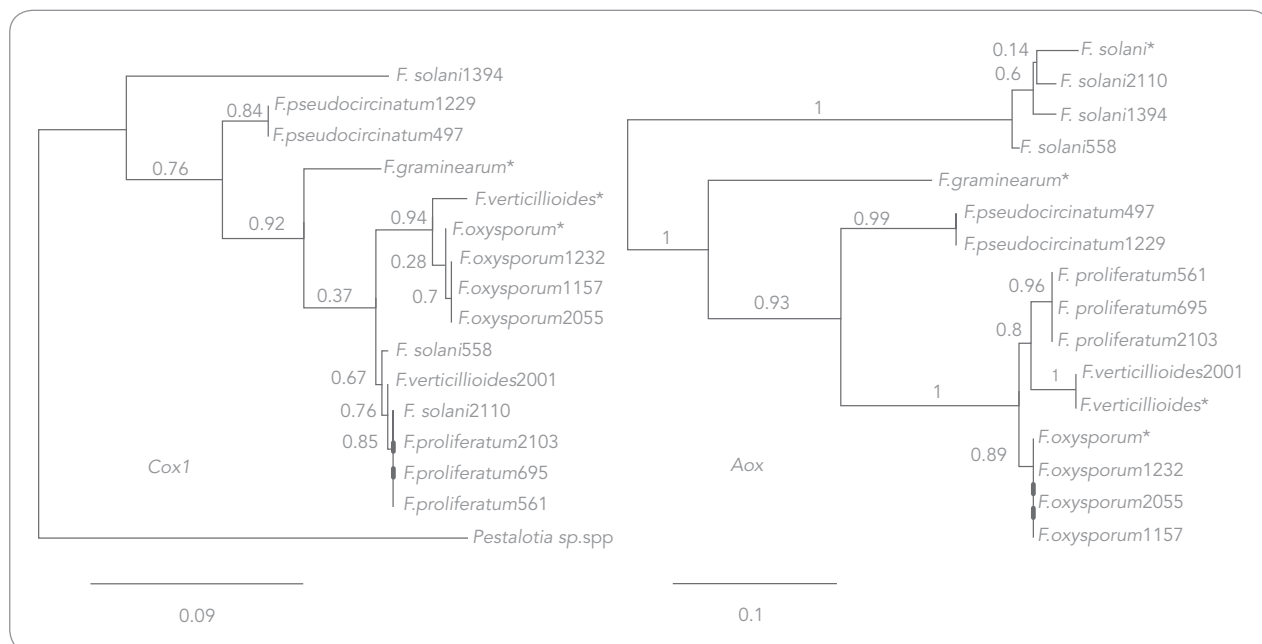


Figure 5. Clustering analysis for *Cox1* gene sequences (left) and *Aox* gene sequences (right). Numbers represent branch support. Trees were inferred using maximum likelihood analysis with 1000 bootstrap replicates, using RaxML software (Stamatakis, 2006).

* Sequences downloaded from *Fusarium* comparative genome platform data base (www.fusariumdb.org).

Barcode gap analysis was performed with sequences of *Cox 1* and *Aox* genes using the K2P model, to compare the interspecific variation against the intraspecific variation. Figure 6 shows that sequences derived from *Aox* gene presented a barcode gap for the species evaluated in this work (*F. oxysporum*, *F. verticillioides*, *F. proliferatum*, *F. pseudocircinatum* and *F. solani*), making this sequence a suitable region for DNA barcoding. On the other hand, barcode gap was absent in the *Cox 1* gene sequences.

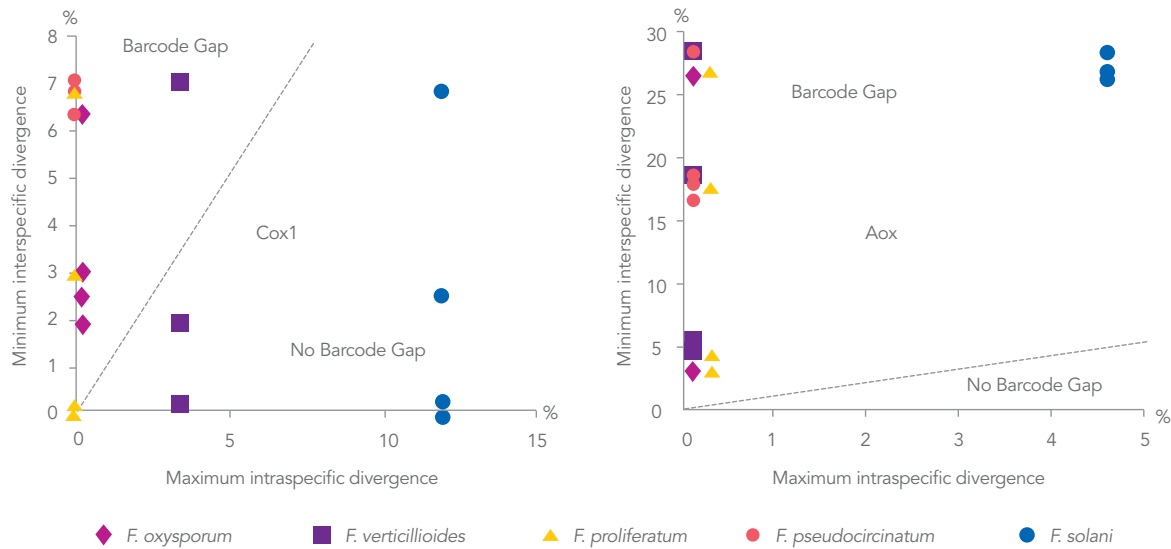


Figure 6. DNA barcode gap plot. Sequence divergence in five *Fusarium* species. Shapes indicate species pair comparisons, dotted blue line represent the threshold where intra and interspecific variation are equal.

DISCUSSION

This is a preliminary study in the search of a sequence that meets the requirements of a DNA Barcode for the taxonomic identification of species in the genus *Fusarium*. We evaluated 12 *Fusarium* isolates belonging to species of the *F. oxysporum*, *F. solani* and *Gibberella fujikuroi* complexes and two possible candidate genes *Cox 1* and *Aox*. A DNA Barcode is a tool that allows identification of samples to species level with relative velocity, high efficiency and precision (Ebach and Holdrege, 2005; Hebert et al., 2003) (<http://www.boldsystems.org>). Although some controversy has been raised, DNA barcodes have become a reality for species of mammals, fishes, birds and some insects among others, using as marker the *Cox1* and (Hebert et al., 2004; Barrett et al., 2005). In the case of fungi, *Cox1*, ITS and β -tubulin (*BenA*) gene sequences had been proposed as possible DNA barcode sequences for the genus *Penicillium* (Schoch et al., 2012; Seifert et al., 2007). These

authors evaluated *Cox1* gene as a possible DNA barcode in other groups of fungi. In the genus *Fusarium* Gilmore et al., (2009), reported in a preliminary study the presence of introns and multiple copies in the *Cox1* gene, producing amplicons of different sizes unsuitable for DNA barcode markers. Similar results were obtained by Schoch et al., (2012) evaluating *Cox1* in several fungal lineages, but proposed that ITS region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation.

The results obtained in this work with primers AH y Fu-F/AHyFu-R previously designed by Gilmore et al., (2009) produced amplicons of the expected size (611 bp) in 11 of 12 evaluated isolates of the complex *F. oxysporum*, *F. solani* and *Gibberella fujikuroi* complexes. One isolate of *F. verticillioides* generated two amplicons, one of the expected size and one

of approximately 2300 bp (Figure 2). The 2300 pb band was sequenced and it aligned with sequences of 611pb band, suggesting that the larger band corresponded to a copy of the gene that contained introns lacking in the shorter band. This result is first objection to the use of marker *Cox1* type "DNA Barcode" (Gilmore et al., 2009; Schoch et al., 2012). Additionally, *Cox 1* gene showed insufficient sequence variation to discriminate between *Fusarium* species (Figures 3 and 6), supporting previously published results (Gilmore et al., 2009; Schoch et al., 2012).

On the other hand, the evaluation of *Aox* gene showed that this sequence was a potential DNA Barcode for identification of *Fusarium* species, at least for the species tested. Using freely available sequences, we designed primers Fw1F.EG and Re2F.EG which amplified isolates of all the *Fusarium* species tested producing amplicons of approximately 800 pb (Figure 2). No amplification was observed for isolates of *Alternaria* sp. and *pestalotia* sp, in contrast to amplification obtained with *Cox 1* primers for the from *Pestalotia* sp. sample (Figure 2).

According to Kress and Erickson (2007) a locus must meet three basic criteria to be useful as a DNA Barcode: (i) show significant variability and genetic divergence between species (ii) present conserved regions for the development of universal primers directed at a broad taxonomic group and, (iii) the designed

primers must amplify a short sequence that facilitate the DNA extraction and sequencing by the current available methods. Results of the *Aox* gene sequence obtained in this work showed all three characteristics. First, sequence variability at the intraspecific level was smaller than the interspecific divergence observed among the evaluated species (Figure 6). Second, in respect to the design of primers, we identified conserved regions for the *Fusarium* genus that allowed the design of primers and finally the amplicons obtained with these primers generated bands of about 800 pb that were easily purified and sequenced. Kress and Erickson (2008) proposed that a DNA Barcode must have a sequence with a length between 400 and 800 bp, while Hebert et al., (2003) and Seifert et al., (2007) ideal lengths of 600 bp. In the sequence that we obtained, most of the variation was observed in the first 500 nucleotides suggesting that a shorter region that meets all the criteria including length should be evaluated in future work.

Multiple alignments of the sequences of *Fusarium* *Aox* gene generated two gaps of about 60 bp in regions of the alignment (Figure 4), which correspond to sites where introns were predicted. The data showed that the length of introns did not vary among isolates of the same species, except in *F. solani*, but to determine if this trend is maintained a larger sample should be evaluated. According to Seifert et al. (2007), the

Aox sequence clustered the isolates into the appropriate *Fusarium* species complex, according to previous morphological characterization, indicating its potential to differentiate *Fusarium* isolates to the species level

presence of introns of significant length could interfere with PCR by disrupting the priming sites or increasing the length of the amplicon alignments complicated by the fact that these introns do not occur regularly in all isolates of *Fusarium*. However, Seifert et al. (2007), also discussed that intronic regions could provide useful sequences to distinguish among closely related species.

To identify whether *Aox* and *Cox1* sequences had useful Barcode gaps, the divergence was estimated through the K2P model. Additionally, plots of the maximum values of intraspecific variation versus the minimum values of interspecific divergence were performed (Figure 6). The graph obtained for *Cox1* showed overlaps of the divergence range of the sequences of the *F. solani* complex isolates with samples of the *G. fujikuroi* complex and that species of the complex *F. oxysporum* had a gap respect to the other complexes. This observation supports the conclusion that *Cox1* is not a good candidate for DNA Barcode for *Fusarium*. In contrast, the region

of 800 bp (obtained with the Fw1F.EG/Re2F.EG primers) of the *Aox* gene presented lower intraspecific variability compared to the interspecific divergence, creating gaps between all the tested complexes. According to a large gap between the range of intra and interspecific variation is one of the key features of a Barcode molecular marker. In conclusion, *Aox* is potential DNA Barcoding marker for the genus *Fusarium* but this preliminary result must be confirmed with evaluation of a larger number of samples per species and more species of the genus. Our group is working in this subject at the moment.

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