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NADH Dehydrogenase Subunit 6: A Suitable Secondary Barcode for Speciation of Genus Fusarium

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Ultimately for some groups of fungi there may need to be two barcodes to meet the requirements of identifying very closely related fungal species. In order to develop a suitable barcode system for *Fusarium* species complexes, universally accepted fungal barcode, Internal Transcribed Spacer (ITS, 600bp) was examined for the barcoding performance with other potential barcode loci *viz.,* Cytochrome Oxidase subunit I (COI, 567bp) and Translation elongation factor 1 (tef-I, 700 bp). One novel genomic region (NADH dehydrogenase subunit 6 (ND6), 900bp) was also be evaluated. Multilocus sequence Typing (MLST) of 22 *Fusarium* isolates representing eleven different species (*F. acuminatum, F. chlamydosporum, F. graminearum, F. oxysporum, F. pallidoroseum F. poae, F. solani, F. sporotrichioides, F. subglutinans, F. udum and F. verticillioides*), were compared. The ITS regions did not work well within the *Fusarium* species complex, and its near relatives due to less barcode gap (0.12). Comparative sequence analyses suggested COI to be a better marker for intraspecific and interspecific variability because of greater barcode gap (0.50), Transition/Transversion ratio (1.48) and evolutionary divergence (0.49). However, the presence of mobile introns and low PCR success in CO1 region poses a serious difficulty in the PCR and bioinformatic surveys. Therefore, ND6 could be considered as a suitable secondary barcode candidate for *Fusarium* species complex due to the absence of introns, justified barcode gap (0.48), higher Transition/Transversion ratio (1.42) and evolutionary divergence (0.44).

Key words: Barcode, Cytochrome Oxidase subunit I, Fusarium species, ITS and NADH dehydrogenase subunit 6 gene.

Fusarium species is known a devastating plant pathogens which cause disease in a number of agriculturally, medicinally and industrially important crops. Symptomology of the diseases caused by different *Fusarium* species has also high range of variability from wilts, scabs, root rots, cankers upto foolish elongation in hosts[1].

Fusarium taxonomy is a confusion catalogue due to different species complexes *viz., Giberella fujikori, Fusarium chlamydosporum, F. incarnatum, F. equiseti, F.solani, F. oxysporum* species complexes. Many times the classification of *Fusarium* has been revised[8] but morphological identification upto species level is very complication. Some species have not any morphological differences called as Sibling species but at the other hand, cultural variation is too high within isolates of different *Fusarium* species varieties. This poorly standardized morphological characterization always resulted in misidentification of *Fusarium* species which is very essential to propose an adequate management practices.

Molecular phylogenetic analysis was also explored extensively to examine the taxonomy of *Fusarium* species and have proposed new taxonomic systems based on the phylogenetic species concept. However, many phylogenetic relationships remain unclear as only few

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comprehensive phylogenetic analyses of the *Fusarium* genus have been performed[21].

Recently, DNA barcode concept has came in existence to identify different organism based on short DNA segment. In order to search a potential barcode for *Fusarium* species complex, the sequences most commonly used to distinguish *Fusarium* spp. are portions of the genomic sequences encoding the translocation elongation factor 1-á (EF-1)[23], â-tubulin (tub2)[10], calmodulin[12], internally transcribed spacer regions in the ribosomal repeat region (ITS1 and ITS2)[9, 20], and the intergenic spacer region (IGS)[24]. Not all sequences work equally well for all species, with tef1 gene being the most widely accepted across the genus. Also, â-tub2 was reported not to work well within the *Fusarium* solani species complex[7, 15]. Therefore, since *Fusarium* is having low ITS interspecific variability which has been proposed as universal barcode[17], secondary markers must be used to accurately report genetic diversity[3].

Cytochrome oxidase subunit 1 (COI) was a default region to be a barcode marker for fungi because it is universal barcode marker for animals and insects. But presence of introns was a major concern to use this maker in fungal DNA barcoding[14]. According to Gilmore et. al[4] introns in *Fusarium* species are at three of the known intron positions (3, 4, 11); at two positions two different sequences types are present (4a, 4b and 11a,11b). In this survey, the COI of most *Fusarium* strains had either no introns present, or introns 3 and 4b.

A mitochondrial region, NADH dehydrogenase subunit 6 (ND6) gene, can be explored as barcode candidates for *Fusarium* due to their paucity of introns and to their length[16].

A successful DNA barcoding system for identifying species of *Fusarium* would have great research value, and potential commercial value. Quarantine plant pathologists and regulators guarding the food system routinely deal with species of this genus. Private seed testing laboratories identify *Fusarium* species present in thousands of grain samples every year. *Fusarium* species are also increasingly isolated from human infections, or from medical products intended for human use[11].

This study was designed to evaluate EF-1, ITS, COI and ND6 for a short potential barcode for identifying species of *Fusarium* species.

MATERIALS AND METHODS

Fusarium isolates

Twenty two *Fusarium* isolates representing eleven different species (*F. acuminatum, F. chlamydosporum, F. graminearum, F. oxysporum, F. pallidoroseum F. poae, F. solani, F. sporotrichioides, F. subglutinans, F. udum and F. verticillioides*) were obtained from the Indian Type culture collection (ITCC), New Delhi, India (Table 1). The isolates were cultured in potato dextrose broth (PDB) to obtain mycelia mat for DNA extraction. Agar discs were cut out from an actively growing fungal colony and placed into 250 ml conical flask containing 100 ml of potato dextrose broth. These cultures were incubated at 24±1ºC in a incubator. The mycelial mats were harvested by filtration through a double layered muslin cloth / Whatman filter paper No.1, washed repeatedly with distilled water. The obtained mycelia mat of each isolate was stored at -20°C for genomic DNA extraction.

DNA Extraction

Genomic DNA was extracted using a C-TAB method[2] with a slight modification. Harvested mycelial mats of isolates were weighed (200mg) and crushed with 600µl of DNA extraction buffer (3% CTAB buffer) separately using sterilized pestle and mortar. The suspensions were collected in 1.5ml eppendorf tubes and incubated at 65 ºC for 30 minutes over water bath with occasional stirring of suspension to get cleared suspension. After incubation the solutions were transferred to new tubes and add equal volume of Chloroform: Phenol (1:1) to each tube and were centrifuged at 14000 rpm for 30 minutes at 4ºC. Subsequently the supernatants were transferred to a new marked 1.5 ml eppendorf tubes and repeat the same for twice.

The upper aqueous phase containing DNA were transferred to the new tubes and add 2.5 volumes of absolute ethanol then stored at -20ºC for overnight. The samples were taken out and centrifuged at 12000 rpm for 20 minutes to get DNA pellet. The pellet collected was washed with ice cold 70 % ethanol. Finally air dried for 15-20 minutes, resuspended in 100 ìl of Tris-EDTA (TE) buffer having 10 mM Tris-HCl (pH 8.0) 1 mM EDTA and stored at -20 ºC in small aliquots for further use.

Primer Designing

To select secondary barcode for *Fusarium*, ND6 gene sequence (1788 bp) was taken from *Fusarium oxysporum* full genome provided by *Fusarium* Comparative Database (http://www.broadinstitute.org/annotation/genome/*Fusarium* group/MultiHome.html). Primer was designed by primer 3 software. FOD F- (5'-TGAGGGGTCTGGCTACTGTC-3') 20-Nucleotide and FOD R- (5'-GGGAGACACCAACTTGCCT-3') 19-Nucleotide. The FOD region was amplified with the primer FOD F and FOD R. Amplification was performed in 50 ìl of reaction mixture containing 5ìl of 10X PCR buffer, 0.5ìl of 25 mMMgCl2, 1 ìl of 10 mM dNTP's, 1ìl 0f 10 pmol/µl of each primers (FOD1 and FOD4), 1ìl of 10 ng of template DNA, 0.5ìl of (3U/ìl) Taq polymerase (Bangalore Genei,India) and 39 ìl nuclease free water and PCR was performed using AB, (BIOER GenePro) Thermocycler.

Samples were subjected to initial denaturation for 5 min at 95 ºC and followed by 35 cycles containing 1 min denaturation at 95 ºC; 45 s annealing at 61 ºC; 1 min extension at 72 ºC and the final extension was set up at 72°C for 10 min. Amplified products were electrophoresed on a 1.2% gel in TAE buffer and visualized by staining with ethidium bromide and photographed using a Genius Gel Documentation System (Syngene Inc, Cambridge,UK). The sizes of fragments were analyzed by comparison with 100-bp ladder DNA.

Amplification and sequencing genes

The ribosomal RNA gene (rDNA) cluster region, including ITS1 anITS2 region, Cytochrome Oxidase subunit I (COI) and Translation elongation factor 1 (tef-1) were selected as the regions for analysis. The ITS region and tef-1 region of 16 isolates of five different species of *Fusarium* were amplified using universal primer Table 1;[18, 22] respectively. COI region was amplified for 22 isolates of 11 different *Fusarium* species using primer designed by Gilmore et. al[4]. The PCR products were sequenced by the in an automated ABI 3100 Genetic Analyser (Applied Biosystems, USA) by Bangalore Genei (Bangalore, India). The sequences were assembled using Clustal X version 2.0. The sequences determined in this study have been deposited in GenBank (Table 2.)

Success rates of test barcode sequence acquisition

The success rates of PCR amplification and sequencing were evaluated. PCR amplification was considered as successful when there was a single and clear band of the expected size on agarose gels. A high quality chromatogram counted as successful sequencing. The success rate of PCR amplification multiplied by that of sequencing determined the success rate of PCR amplification and sequencing.

Transition/Transversion ratio and Average Evolutionary Divergence

The basic sequence statistics including nucleotide frequencies, transition/transversion (ns/nv) ratio and maximum evolutionary divergence of sequences were computed by Molecular Evolutionary Genetics Analysis (MEGA)[19].

Barcode Gap

The intra- and inter-specific pairwise distances were calculated using the K2P model in MEGA 5[19].

Bracode gap= Maximum Intraspecific distance – Minimum Interspecific distance

Intron Mapping of COI region

Intron 3 and Inron 4 b was amplified based on designed primer by Gilomore et. al. [4] (Table 3).

RESULTS

The potentiality of all four targeted loci amplified and sequences from 22 isolates of eleven different species of *Fusarium* were analysed by comparing their PCR and sequence success rate, Inter-and inter-specific divergence and barcode gap. The results obtained are concised below.

Development of species primer for novel loci NADH dehydrogenase subunit 6 (ND6)

The specific primer sets were designed based on the annotated gene of full genome sequence of *Fusarium oxysporum*. These specific primer sets gave amplicons of 1788 bp different *Fusarium* species. The sequence of the specific primer pairs were FOD F- 'TGAGGGGTCTGGCT ACTGTC' and FOD R- 'GGGAGACACCAACTTG CCT'. These primers were given amplification in all tested isolates belonging to 11 different species of *Fusarium* (Fig.1).

PCR amplification and sequencing success rate

PCR amplification was generally 100% with ITS, tef-1 and ND6. But only 73% PCR success was recorded with COI region. The sequencing success rates for the four loci, *viz.*, ITS, tef-1, COI

and ND6 were 90%, 95%, 85% and 96%, respectively.

Transition/Transversion ratio and Average Evolutionary Divergence

Fusarium of ITS region, tef 1gene, cox 1 gene and ND 6 gene were amplified and sequenced. Sequences were aligned by clustal w. Mean base pairs of ITS, COI, tef-1 and ND6 loci respectively 526 bp, 586 bp, 694 bp and 918 bp. According to the base pair size ND6 gene sequence having maximum 918 bp comparatively ITS, COI, tef-1 region sequences. Transition / Transversion ratio of ND6 region 1.42 whereas COI region having 1.96. But the problem in COI region is having introns due to that amplification of the gene problematic depends upon the species of *Fusarium*. Transistion / Transversion ratio of ITS region (0.564) and tef 1 gene (1.153) is very less comparatively ND6 and COI region. Evolutionary divergence of the ND6 gene is 0.44. This was highest among ITS region (0.173), COI region (0.427) and tef-1 gene (0.380).

Barcode gap

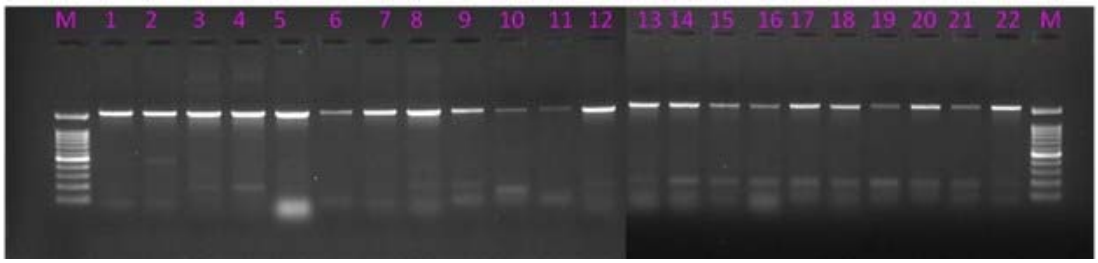
Based on Inter and intraspecific divergence, barcode gap was found highest in COI (0.50), followed by for ND6 (0.49) Barcode gap was found minimum in ITS (0.16) and tef-1 (0.38).

Intron Mapping for COI region

Twenty isolates of 16 different species was also subjected to the Intron mapping of COI region. Intron 3 and 4b was amplified but amplification was recorded in 5 isolates (11, 12 .13, 15 and 19) only in Intron 4b.

Table 1. Primer used for the study

S. No.	Genomic region	Primers	References
1	ITS	ITS1(5'-CTTGGTCATTTAGAGGAAGTAA-3') ITS4 (5'-TCCTCCGCTTATTGATATGC-3')	White *et al.* (1990)
2	tef-1	EF-1(5'-ATGGGTAAGGAAGACAAGAC-3') EF-2 (5'- GGAGGTACCAGTGATCATGTT-3')	Skovgaard *et al.* (2001)
3	COI	AHyFU-F (5'-CTTAGTGGGCCAGGAGTTCAATA-3') AHyFU-R (5'-CCTCAGGGTGTCCGAAGAAT-3')	Gilmore *et al.* (2009)
4	COIIntron 3	Fus-I3-F (52 -TTAAAAGTATCGAAAAATCAAAAAGG TGT-32)Fus-I3-R (52 -ATCTATCTCTTATTTCTTGGCT CATTGGTT-32).	Gilmore *et al.* (2009)
5	COIIntron 4b	Fus-I4b-F (52 -CCTTTAAAACTAGTACCGCAGAC-32) ,AHyFU-R (5'-CCTCAGGGTGTCCGAAGAAT-3')	Gilmore *et al.* (2009)



1: (6386) *Fusarium oxysporum;* 2: (6392) *Fusarium oxysporum;* 3: (6398) *Fusarium oxysporum;* 4: (5175) *Fusarium oxysporum;* 5: (6393) *Fusarium verticilloides;* 6: (6385) *Fusarium verticilloides;* 7: (6335) *Fusarium verticilloides;* 8: (3555) *Fusarium subglutinans;* 9: (6387) *Fusarium pallidoroseum;* 10: (6342) *Fusarium solani;* 11: (6334) *Fusarium solani;* 12: (5320) *Fusarium graminearum;* 13: (5240) *Fusarium graminearum;* 14: (5508) *Fusarium chlamydosporum;* 15: (5245) *Fusarium chlamydosporum;* 16: (5241) *Fusarium udum;* 17: (4873) *Fusarium udum;* 18: (555) *Fusarium oxysporum;* 19: (4362) *Fusarium sporotrichioides;* 20: (3970) *Fusarium subglutinans;* 21: (1528) *Fusarium poae;* 22: (3354) *Fusarium acuminatum.*

Fig. 1. Amplification of ND6 region of different *Fusarium* species

DISCUSSION

DNA barcoding is aimed at developing a 'biological barcode' to enable identification of any organism at the species level. DNA barcoding has been promoted as a potentially powerful method for the efficient, accurate and high throughput assignment of unknown specimens to known species. Reference barcodes must be derived from expertly identified vouchers deposited in biological collections. Interspecific variation should exceed intraspecific variation (the barcode gap), and barcoding is optimal when a sequence is constant and unique to one species[5-6].

Ideally, the barcode locus would be the same for all kingdoms. The cytochrome c oxidase subunit 1 (CO1; Universal barcode for animals) is more reliable in a few clades of closely related fungal species[13] but not all. Due to presence of large database and universal primers, ITS is proposed as the standard barcode[17]. This proposal will be applicable for many fungal genera but not for fungi which are having species complexes *viz.,* *Fusarium*. For the fungi having low ITS interspecific variability, secondary markers must be used to accurately report genetic diversity. Therefore, this study was design to search a potential secondary barcode for Genus *Fusarium*.

Three well established genomic loci were explored to develop a suitable barcode system for *Fusarium* species complex *viz.,* Cytochrome Oxidase subunit I (COI, 567bp) and Translation elongation factor 1 (tef-I, 700 bp) and universally accepted fungal barcode, Internal Transcribed Spacer (ITS, 600bp). One novel genomic region (NADH dehydrogenase subunit 6 (ND6), 900bp) was also be evaluated. This is the first time that ND6 fragments have been selected for *Fusarium* identification and DNA barcoding.

A primer pair was design to amplify ND6 region of mitochondrial DNA. The selected primer was validated with 22 different isolates of 11 different *Fusarium* species. Targeted amplicon of about 1500 bp was amplified in all tested 22 isolates. According to Ascomycota intron map[16], full NADH dehydrogenase subunit 6 (ND6) genes could be considered as barcode candidates for Ascomycota due to their paucity of introns and to their length,

Table 2. Different Fusarium species used for sequence analysis

S.No	Name of the species	ITCC No.	ITS	*EF-1*	COI	ND6
1	*Fusarium oxysporum*	6386	HQ384392	JQ065359	JQ065344	KC488628
2	*Fusarium oxysporum*	6392	HQ384393	JQ065361	JQ065346	KC488629
3	*Fusarium oxysporum*	6398	HQ384400	JQ065370	JQ065347	KC488630
4	*Fusarium oxysporum*	5175	HQ384394	JQ065360	JQ065352	KC488631
5	*Fusarium oxysporum*	55	HQ384395	JQ065362	JQ065353	KC488638
6	*Fusarium verticilloides*	6396	HQ384401	JQ065358	JQ065349	NS*
7	*Fusarium verticilloides*	6385	HQ384402	JQ065363	JQ065350	KC488644
8	*Fusarium verticilloides*	6335	HQ384403	JQ065369	JQ065351	KC488645
9	*Fusarium subglutinans*	3555	HQ995669*	JF270302*	SE#	KC488637
10	*Fusarium pallidoroseum*	6387	HQ384388	JQ065364	JQ065341	KC488633
11	*Fusarium solani*	6334	HQ384390	JQ065355	JQ065339	NS*
12	*Fusarium solani*	3594	HQ384396	JQ065356	JQ065340	NS*
13	*Fusarium graminearum*	5320	GU183128*	HQ008669*	SE#	KC488635
14	*Fusarium graminearum*	5240	GQ221858*	HQ008667*	SE#	KC488642
15	*Fusarium chlamydosporum*	5508	HQ905456*	GQ505410*	SE#	KC488636
16	*Fusarium chlamydosporum*	5245	HQ905451*	GQ505412*	SE#	KC488648
17	*Fusarium udum*	5241	JQ436560*	AF160275*	SE#	KC488647
18	*Fusarium udum*	4873	U34575*	AF160276*	SE#	KC488640
19	*Fusarium sporotrichioides*	4362	HQ845043*	EF521146*	SE#	KC488639
20	*Fusarium subglutinans*	3970	FJ158133*	JF270287*	GQ503090*	KC488641
21	*Fusarium poae*	1528	JN942836*	EF512024*	SE#	KC488643
22	*Fusarium acuminatum*	3354	GQ922916*	EF521138*	FJ590531*	KC488649

Sequence taken from NCBI, SE # Sequence error - sequence data is interfered with Introns, NS Not submitted in NCBI

Table 3. Different Fusarium isolates used for Intron mapping of COI region

S. No.	*Fusarium* species	ITCC No.
1	*F. concolar*	5149
2	*F. culmorum*	146, 147
3	*F. graminearum*	5320, 5334
4	*F. sambucinum*	4031
5	*F. heterosporum*	1525
6	*F. trichothecoides*	3657
7	*F. udum*	4873
8	*F. dimereum*	3864
9	*F. merismoides*	4900
10	*F. acuminatum*	3354
11	*F. equeseti*	6118, 6237
12	*F. solani*	6334, 6338
13	*F. chlamydosporum*	5508
14	*F. avenaceum*	5116
15	*F. poae*	1528
16	*F. tricinctum*	6244

above 400 bp, comparable to the lower end size of the length range of barcodes successfully used in animals.

Multilocus Sequence Typing (MLST) was compared. The ITS regions was not showed potentiality due to less barcode gap (0.12). Comparative sequence analyses suggested COI to be a better barcode region because of greater barcode gap (0.50), Transition/Transversion ratio (1.48) and evolutionary divergence (0.49). However, the presence of mobile introns and low PCR success in CO1 region poses a serious difficulty to select this region as barcode marker. Therefore, ND6 could be considered as a suitable secondary barcode candidate for *Fusarium* species complex due to the absence of introns, justified barcode gap (0.48), higher Transition/Transversion ratio (1.42) and evolutionary divergence (0.44). In order to search a potential barcode for *Fusarium* species complex, the sequences most commonly used to distinguish *Fusarium* spp. are portions of the genomic sequences encoding the translocation elongation factor 1-á (*EF-1*)[23], â-tubulin (tub2)[10] calmodulin[12], Cytochrome oxidase subunit 1 (COI)[4], internally transcribed spacer regions in the ribosomal repeat region (ITS1 and ITS2)[9,20] and the intergenic spacer region (IGS)[24]. Not all sequences work equally well for all species, with tef1 gene being the most widely accepted across the genus.

Therefore, ND6 can be used as potential barcode loci for Genus *Fusarium*. It enable DNA barcoding of *Fusarium* species complex easy and authentic.

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