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FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*

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Abstract

One of the greatest impediments to the study of *Fusarium* has been the incorrect and confused application of species names to toxigenic and pathogenic isolates, owing in large part to intrinsic limitations of morphological species recognition and its application. To address this problem, we have created FUSARIUM-ID v. 1.0, a publicly available database of partial translation elongation factor 1-alpha (TEF) DNA sequences, presently representing a selected sample of the diversity of the genus diversity, with excellent representation of Type-B trichothecene toxin producers, and the *Gibberella fujikuroi, Fusarium oxysporum* and *F. solani* species complexes. Users can generate sequences using primers that are conserved across the genus, and use the sequence as a query to BLAST the database, which can be accessed at http://fusarium.cbio.psu.edu, or in a phylogenetic analysis. Correct identification of a known species in these groups often can be performed using this gene region alone. This growing database will contain only vouchered sequences attached to publicly available cultures. In the future, FUSARIUM-ID will be expanded to include additional sequences, including multiple sequences from the same species, sequences from new and revised species, and information from additional genes.

Introduction

Fusarium, the single most important genus of toxigenic fungi, has had a confusing and unstable taxonomic history. A number of factors, including a lack of clear morphological characters separating species, leading to species concepts that are too broad, together with variation and mutation in culture, have conspired to create taxonomic systems that poorly reflect species diversity. The result of this confusion is the rampant misapplication and inconsistent application of species names to toxigenic and pathogenic isolates. With the recent advent of multilocus phylogenetic methods which allow for the objective identification of species boundaries in the Fungi (Taylor et al., 2000), relationships among well-defined *Fusarium* species have been inferred, showing a great deal of species diversity that was vastly under-estimated by all previous morphological treatments (Aoki and O'Donnell, 1999; Geiser et al., 2001; O'Donnell, 2000; O'Donnell et al., 1998a, b, c; Ward et al., 2002). Most *Fusarium* isolates studied by mycotoxicologists and plant pathologists in the first three quarters of the 20th century were initially identified incorrectly using one of several oversimplified morphological systems. For example, the name of the toxin nivalenol was based on the misidentification of an isolate producing it as *Fusarium nivale*. Since then, *F. nivale* has been moved to a new genus, *Microdochium*, and the nivalenol producer was re-identified first as *F.*

tricinctum and *F. sporotrichioides* by two different researchers using morphology, and finally as a new species, *F. kyushuense*, using molecular phylogenetics (Aoki and O'Donnell, 1998). Marasas et al. (1984) undertook a huge effort to correctly identify hundreds of toxigenic isolates associated with the production of various mycotoxins using the then-current system. These authors debunked and confirmed many reports of mycotoxin production associated with particular *Fusarium* species, but their efforts could only reflect the current state of the art of morphological species recognition. They recognized 30 *Fusarium* species, and while it served as an advance over the previous nine species system of described by Snyder and Hansen (1940, 1941, 1945), it too vastly under-estimated the underlying phylogenetic diversity and grouped species into sections that do not reflect evolution. Nearly 20 years later, we find ourselves again needing to correlate previous reports of mycotoxin production with phylogenetically defined species, such that identifying toxigenic isolates still remains an enormous challenge. Species newly identified using phylogenetic methods are generally difficult or impossible to identify using conventional morphological traits (Aoki et al., 2003).

Much of the motivation for the streamlined morphology-based *Fusarium* taxonomic systems of the mid- to late-20th century came from a desire to make identification simple and reliable, and in hindsight at the cost of over-simplification. Now that phylogenetics is providing an excellent framework for objective species recognition, there is a need to apply the same tools to provide reliable species identification in the laboratory. Phylogenetic species recognition in *Fusarium* has relied mostly on Genealogical Concordance Phylogenetic Species Recognition (GCPSR; Taylor et al., 2000), a method which identifies shared partitions among multiple gene genealogies as landmarks for species boundaries. While two or more gene genealogies are required for this method of recognizing species, in many instances species may be identified accurately using a single DNA sequence marker, as long as background phylogenetic analyses have been performed using the marker along with others, thereby validating its diagnostic utility.

The markers of choice for species-level phylogenetics in fungi are intron-rich portions of protein-coding genes (Geiser, 2003). These gene regions tend to evolve at a rate higher than that which is observed at the species level in more commonly applied markers such as the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene repeat (O'Donnell, 2000; O'Donnell et al., 1998a). Moreover, many fusaria within the *Gibberella* clade possess non-orthologous copies of the ITS2, which can lead to incorrect phylogenetic inferences (O'Donnell and Cigelnik, 1997; O'Donnell et al., 1998a).

The translation elongation factor 1-α (TEF) gene, which encodes an essential part of the protein translation machinery, has high phylogenetic utility because it is (i) highly informative at the species level in *Fusarium*; (ii) non-orthologous copies of the gene have not been detected in the genus; and (iii) universal primers have been designed that work across the phylogenetic breadth of the genus. This gene was first used as a phylogenetic marker to infer species- and generic-level relationships among Lepidoptera (Cho et al., 1995; Mitchell et al., 1997). Primers were first developed in the fungi to investigate lineages within the *F. oxysporum* complex (O'Donnell et al., 1998c). The ef1 and ef2 primers were designed based on sites shared in exons between *Trichoderma reesei* (Hypocreales/Sordariomycetes/Pezizomycotina/Ascomycota) and *Histoplasma capsulatum* (Eurotiales/Eurotiomycetes/Pezizomycotina/Ascomycota), and they can be applied to a wide variety of filamentous ascomycetes. These primers amplify an ~700 bp region of TEF, flanking three introns that total over half of the amplicon's length, in all known fusaria (Figure 1). This gene appears to be consistently single-copy in *Fusarium*, and it shows a high level of sequence polymorphism among closely related species, even in comparison to the intron-rich portions of protein-coding genes such as calmodulin, beta-tubulin and histone H3. For these reasons, TEF has become the marker of choice as a single-locus identification tool in *Fusarium*.

As of September 2003, the GenBank database contained TEF sequences from 463 isolates of *Fusarium*. From these and other sequences, we assembled the first generation of FUSARIUM-ID v. 1.0, a database currently consisting of 175 sequences representing a phylogenetically diverse selection of TEF sequences from the genus, and placed it on a local BLAST server, which can be accessed at http://fusarium.cbio.psu.edu.

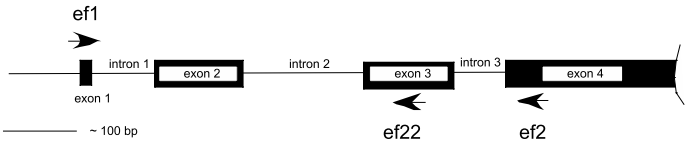

Figure 1. Map of the TEF gene region in *Fusarium* used in FUSARIUM-ID, with primer locations.

Current phylogenetic subgroups within the genus represented in FUSARIUM-ID v. 1.0 are summarized in Table 1. Certain groups are well-covered in FUSARIUM-ID v. 1.0, particularly the *F. oxysporum*, *F. solani*, and *Gibberella fujikuroi* species complexes and the Type-B trichothecene toxin-producing clade (i.e., *Gibberella zeae* complex: *F. graminearum* and its eight sister species together with *F. culmorum, F. cerealis, F. lunulosporum* and *F. pseudograminearum*) (Geiser et al., 2001; O'Donnell, 2000; O'Donnell et al., 1998a, c; O'Donnell et al., 2000a, b, Ward et al., 2002), with considerably less complete sampling of other groups, including the A-type trichothecene toxin-producing clade, *F. lateritium* and relatives, and poorly defined species such as *F. equiseti* and *F. longipes*. Such poorly characterized species are listed in the database as '*Fusarium* sp. cf', because we are currently unsure of their correct identification and cannot be sure until rigorous morphological and multilocus phylogenetic studies are completed. Likewise, dozens of phylogenetic species that have been recognized within the *F. solani* and *F. oxysporum* complexes await taxonomic treatment. Also, many species that have *Cosmospora* and *Nectria*-like teleomorphs, including *F.*

Table 1. Summary of species represented in the FUSARIUM-ID v. 1.0 database

Species complex	Species and names represented in FUSARIUM-ID[1]
Gibberella fujikuroi species complex: Excellent representation	*Fusarium acutatum*, *F. andiyazi*, *F. anthophilum*, *F. bactridioides*, *F. begoniae*, *F. brevicatenulatum*, *F. bulbicola*, *F. circinatum*, *F. circinatum*, *F.* sp. cf. *concentricum*, *F. denticulatum*, *F. dlaminii*, *F. fractiflexum*, *F. fujikuroi*, *F. globosum*, *F. guttiforme*, *F. konzum*, *F. lactis*, *F. mangiferae*, *F. napiforme*, *F. nygamai*, *F. phyllophilum*, *F. proliferatum*, *F. pseudoanthophilum*, *F. pseudocircinatum*, *F. pseudonygamai*, *F. ramigenum*, *F. sacchari*, *F. sterilihyphosum*, *F. subglutinans*, *F. succisae*, *F. thapsinum*, *F. udum*, *F. verticillioides* and 20 undescribed spp.
Type A trichothecene producing species and relatives: Fair representation	*Fusarium* sp. cf. *armeniacum*, *F.* sp. cf. *brachygibbosum*, *F. kyushuense*, *F. langsethae*, *F.* sp. cf. *longipes*, *F. musarum*, *F.* sp. cf. *poae*, *F. robustum*, *F.* sp. cf. *sambucinum*, *F.* sp. cf. *sporotrichioides*, *F.* sp. cf. *tumidum*, *F. venenatum*
Type-B trichothecene producers and relatives: Excellent representation	*Fusarium cerealis* (= *F. crookwellense*), *F. culmorum*, *F. graminearum* and 8 related spp., *F. lunulosporum*, *F. pseudograminearum*
F. oxysporum and relatives: Good representation	*Fusarium commune*, *F. hostae*, *F. miscanthi*, 45 lineages of cf. '*F. oxysporum*', *F. redolens*
F. solani species complex: Good representation	*Fusarium phaseoli*, 25 lineages of cf. '*F. solani*', *F. tucumaniae*, *F. virguliforme*
Other lineages: Fair to poor representation	*Fusarium* sp. cf. *acuminatum*, *F. anguioides*, *F. avenaceum*, *F. aywerti*, *F. beomiforme*, *F.* sp. cf. *bullatum*, *F.* sp.cf. *camptoceras*, *F. dimerum*, *F.* sp.cf. *equiseti*, *F.* sp. cf. *heterosporum*, *F. lateritium*, *F. merismoides*, *F.* sp. cf. *negundis*, *F. nelsonii*, *F. nisikadoi*, *F. nurragi*, *F.* sp. cf. *pallidoroseum*, *F.* sp. cf. *polyphialidicum*, *F.* sp. cf. *pyriforme*, *F.* sp. cf. *tricinctum*, *F.* sp. cf. *xylarioides*

[1] Species that are currently based on tenuous or non-existent phylogenetic support are listed as 'sp. cf.'. Any identification of these species should be considered critically.

merismoides, *F. dimerum* and species associated with insects such as *F. coccophilum*, are currently not represented in the database. Representatives of these groups will be added in later versions of the database.

Materials and methods

The approach for identifying an isolate of *Fusarium* using the FUSARIUM-ID database is outlined in Figure 2. It involves obtaining a pure culture of an isolate, extracting genomic DNA, amplification of the TEF gene region, and sequencing. BLAST is then used to identify the closest matches between the unknown sequence and those contained in the FUSARIUM-ID sequence database.

Obtaining a pure culture

Mixed cultures or cultures contaminated with bacteria and other fungi are a problem for all types of identification. Care should be taken to obtain a pure culture, particularly one derived from a single conidium or ascospore to ensure that the culture represents a clone. Methods for obtaining pure cultures are outlined by Summerell et al. (2003).

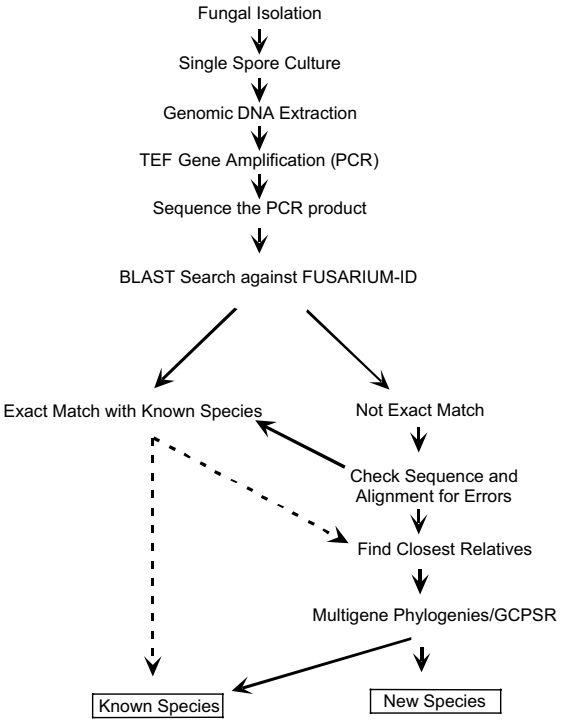

Figure 2. Flowchart describing the process of using TEF DNA sequences to identify *Fusarium* species.

DNA extraction

DNA extraction from *Fusarium* is very simple and a variety of methods can be used. We recommend either a simple and inexpensive CTAB extraction procedure (O'Donnell et al., 1997), or a commercial kit (i.e., Dneasy, Qiagen, Valencia, CA). Both methods work on fresh, frozen or lyophilized mycelium, grown in a liquid culture on a weak nutrient medium such as half-strength potato dextrose broth.

Polymerase chain reaction

A standard polymerase chain reaction (PCR) protocol is used to amplify the TEF gene region. ef1 (forward primer; 5′-ATGGGTAAGGA(A/G)GACAAGAC-3) and ef2 (reverse primer; 5′-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3′ (O'Donnell et al., 1998c) primers are used in a PCR reaction, with an annealing temperature of 53 °C. An ~700 bp product is amplified, as illustrated in Figure 1.

DNA sequencing

The TEF PCR product generated using the ef1 and ef2 primers is then used as a template for DNA sequencing, once the unincorporated nucleotides and primers are removed by filtration over Montage PCR96 cleanup plates (Millipore Corp., Billerica, MA) or via Sephadex G50 (Pharmacia; Pistcataway, NJ) or Qiaquick (Qiagen, Valencia, CA) spin columns. Using the latest capillary technology, a DNA sequence generated from the ef2 primer alone usually produces high enough quality data to produce an unambiguous identification. Sequencing from both directions using the ef1 and ef2 primers, however, provides an internal check for sequence quality. Whether one or two primers are used, a careful check of the raw sequence data for accurate base calls is a necessity. Replacement of ambiguous sites with 'N' will not lead to a mistaken identification. As an alternative

to the ef2 primer for sequencing, the internal reverse ef22 primer (5′-AGGAACCCTTACCGAGCTC-3′) can be used to generate an ~450 bp sequence that is generally highly diagnostic at the species level because it covers the most informative, intron-rich 5′ portion of the ef1/ef2 PCR product.

Identification by comparison with databases

The FUSARIUM-ID server at http://fusarium.cbio.psu.edu contains a BLAST search tool that allows users to query unknown sequences against the database. Alternatively, the GenBank database is publicly available for identification purposes, and can be accessed via the Entrez website at the US National Center for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov/Entrez/. However, because there is no quality control regarding correct identification of sequences in the GenBank database, and there is potential for misidentified accessions, we strongly recommend using FUSARIUM-ID because it contains vouchered and well-characterized sequences that correspond to publicly available cultures that can be used for confirmation. Of course, FUSARIUM-ID can be used in conjunction with GenBank.

Once edited, the TEF sequence from an isolate of interest can be copied and pasted into a web browser connected to the BLAST server and used as a query for comparison to the database. Nucleotide BLAST will retrieve the closest matches to the query sequence, and present the matches as a series of DNA alignments with corresponding percentage match information. These alignments must be checked carefully, however, because BLAST will view stretches of the same base as N's if the default 'mask simple sequence repeats' option is used. This is not the default option on the FUSARIUM-ID BLAST server, but it is on the NCBI BLAST server. Because the TEF region often contains a long stretch of thymidine (T) residues, a perfect match of a 650 bp sequence with 10 consecutive T's in the middle may be misreported as only a 640/650 (98%) match. These mistakes are easily spotted from the alignments provided in the BLAST output, if the masking option is mistakenly left on. The BLAST alignment output also provides an opportunity to go back and re-check every polymorphic site for basecall errors.

Discussion

What do matches mean?

As is the case with any sort of fungal identification, DNA sequence-based identification is a reiterative process that may require further investigation and consideration of other factors, including morphology and ecology. In the end, it will almost always provide a much stronger inference than an analysis that does not utilize sequence information, but the strength of the inference will depend on the amount of context information—sequences available in the databases—just as is the case for other methodologies.

An exact sequence match with a known isolate in the database can be considered very close to an unambiguous species identification, but even this is not always the case. It is possible that phylogenetically distinct species may have identical TEF sequences even if they are recognized by GCPSR based on other genes. What does it mean when the query sequence is slightly different, say by one or a few bases, from all of the sequences in the database?

There are a number of possible explanations for a sequence not perfectly matching anything in the database, and the following steps are recommended under these scenarios:

(1) The query sequence may be an allelic variant that is not present in FUSARIUM-ID, from a known species (i.e., described or undescribed and phylogenetically distinct). In this case, it is expected that there would be close matches (within one or a few nucleotide polymorphisms) within the database, and the list of 'best hits' in the BLAST result would be known related species in a particular species complex. In some cases, TEF sequences that differ by one or a few differences may come from the same species, and other clues (host origin, morphology) may confirm the species ID suggested by the closest DNA sequence match. To confirm the species identification in such a case, a phylogenetic analysis of the TEF sequence along with other intron-rich protein-coding genes is recommended. For species complexes that have already been characterized well (e.g., *Gibberella fujikuroi*, *F. solani*, *F. oxysporum*, *Gibberella zeae*), good published data are currently available, including sequences from other genes (e.g., beta-tubulin, mitochondrial small subunit ribosomal

RNA gene). There is no set number of sequence differences that indicate a species boundary, and it is up to the researcher at that point to take additional steps if necessary, including generating DNA sequences from other loci to confirm genealogical concordance through phylogenetic analyses. Variation among TEF alleles within a species can be extremely variable; all known isolates of the soybean sudden death syndrome pathogen within the United States, *F. virguliforme* (*F. solani* species complex), have identical TEF sequences, whereas TEF alleles within *F. proliferatum* may vary by as much as 1.5%. In later versions of the FUSARIUM-ID, multiple sequences will be added from species to represent known allelic variation.

(2) The query sequence is from a new species that is not represented in FUSARIUM-ID (i.e., undescribed and phylogenetically distinct). In this case, which may be difficult to distinguish from the previous case, there may be a number of equally good matches, each different from the query sequence by several or more nucleotides. The same thing is recommended as in the previous case: generating more DNA sequences from more genes, and testing for GCPSR.

(3) The query sequence is from a known (i.e., described) species, but there is no sequence for it in FUSARIUM-ID. This is less likely to occur within the well-characterized species complexes listed above, but may occur in a number of known toxigenic lineages, until high-resolution phylogenetic analyses are completed. Such a failure to match is currently most likely to occur in the Type-A trichothecene toxin-producing clade that is currently under intensive study using GCPSR (Ward et al., unpubl.), as well as in other groups of *Fusarium* with *Gibberella* teleomorphs, and species associated with *Cosmospora* and *Nectria*-like teleomorphs. However, some representatives of these groups are present in FUSARIUM-ID, and the closest matches identified in a BLAST search should provide clues as to the possible species identification or clade to which it belongs.

(4) The query sequence corresponds to a species that is currently poorly defined. Many of the *Fusarium* species that are commonly encountered, including important toxigenic species such as *F. sporotrichioides*, *F. poae* and *F. longipes*, as well as commonly encountered species such as *F. equiseti*, appear to be non-monophyletic as traditionally circumscribed, and they are in need of critical study. Any *Fusarium* species that has not yet been subjected to the scrutiny of GCPSR should be viewed with caution.

Updating the FUSARIUM-ID sequence database

Any TEF sequence from any *Fusarium* species can be added to FUSARIUM-ID, as long as it corresponds to a culture that is available through one or several major international culture collections where it will be openly distributed. In future versions, we will be adding more TEF sequences to the database, and adding databases from other genes of interest as well so that multilocus identifications can be performed. Eventually, these databases can be used as a basis for developing rapid and reliable hybridization- and chip-based methods for identification, circumventing the need for DNA sequencing (Fessehaie et al., 2003).

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