Phylogenetic evidence indicates the important mycotoxigenic strains Fn-2, Fn-3, Fn-2B and Fn-M represent a new species of Fusarium

K. O'DONNELL*

K・オドネル*：マイコトキシン産生株Fn-2，Fn-3， Fn-2BがFusariumの新種であることを示す系統発生学的根拠

Summary

Phylogenetic relationships of important trichothecene-producing strains originally identified as Fusarium nivale Fn-2, Fn-3 and Fn-2B and F. episphaeria Fn-M were investigated within the Gibberella lineage of Fusarium using nucleotide characters obtained by sequencing polymerase chain reaction (PCR) DNA amplified from the following 4 loci: nuclear 28S ribosomal DNA, nuclear ribosomal internal transcribed spacer (ITS) region, mitochondrial small subunit (mtSSU) ribosomal DNA, and β-tubulin gene exons and introns. Parsimony analysis of the individual and combined data sets indicate that Fn-2, Fn-3, Fn-2B, and Fn-M represent an undescribed species of Fusarium. Bootstrap and decay analysis identified a clade containing F. sambucinum and several other diacetoxysclerone (DAS) and/or T-2 trichothecene-producing species as a sister to Fusarium sp. n. The outgroup species used to root the tree, by contrast, produce a different set of mycotoxins that include deoxynivalenol and zearalenone. These results emphasize the importance of investigating the evolution of Fusarium toxins the context of a robust species-level phylogeny.

Introduction

The genus Fusarium Link comprises a large number of cosmopolitan, pleoanamorphic species (Hypocreales: Hypocreaceae), many of which are mycotoxigenic. Mycotoxins produced by fusaria are implicated in the disease of many crops, farm animals, and humans. Fusaria are some of the most challenging fungi for morphological systematists as illustrated by the discordant species concepts adopted in the following taxonomic treatments of the genus: Snyder and Hansen accepted 9 species, Booth 51 species and varieties, Gerlach and Nirenberg 101 species and varieties, and Nelson et al. 30 species. These four diverse taxonomic schemes are based exclusively on morphological species concepts that are often conflicting. As a result, identification of Fusarium species remains problematical for specialists and nonspecialists alike. Correct identification of important toxigenic strains using morphology alone is further complicated when atypical or degenerate cultures are encountered. For this reason, a molecular phylogenetic approach using parsimony analysis of DNA sequence data from multiple loci offers enormous potential for the identification of key mycotoxigenic strains, that is independent of

* Microbial Properties Research, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture (Peoria, Illinois 61604)
Mycotoxins

cultural morphology, and these molecular systematic data provide an objective avenue for investigating species boundaries using a phylogenetic species concept.7

In this study, DNA sequences from one mitochondrial and three nuclear loci were used to investigate the phylogenetic relationships and identity of trichothecene-producing strains originally published as Fusarium nivale Ces. ex Sacc. Fn-2, Fn-3 and Fn-2B from wheat in Kyushu, Japan8–12, and F. episphaeria (Tode ex Fr.) Snyder & Hansen Fn-M from vinyl plates on a farm in Shikoku, Japan13. These strains occupy an important place in the Fusarium mycotoxigenic literature because it was from these strains that the trichothecenes nivalenol14–16, fusarenon-X (= fusarenon or nivalenol monoacetate)17,18, and deacetylnivalenol (= nivalenol diacetate)19 were first isolated and characterized.

Fn-2 and Fn-3 were sent to the ARS Culture Collection (NRRL) by Prof. Hiroshii Tsunoda in 1966 and Fn-2B and Fn-M by Prof. Yoshio Ueno in 1971 for identification. Subsequently, these strains were identified at NRRL by John J. Ellis as F. tricinctum (Corda) Sacc. and at the Medical Research Council Collection (MRC; Tygerberg, South Africa) by Marasas et al.21 as F. sporotrichioides Sherb. Molecular systematic results reported here, however, indicate that Fn-2, Fn-3, Fn-2B, and Fn-M represent an undescribed, phylogenetically distinct species which is most closely related to a clade containing F. sambucinum Fuckel and other type A trichothecene-producing species20,21 of the species sampled. By contrast, species within a separate lineage of fusaria used to root the tree produce a different spectrum of mycotoxins, including zearalenone and deoxynivalenol (DON = vomitoxin)22. These results suggest that molecular phylogenetics should prove to be a valuable tool to investigate the evolution of toxins within lineages and to predict toxin production for the many new species of Fusarium that will be discovered over the next decade.

Materials and Methods

Fungal strains sequenced and analyzed phylogenetically (Table 1) are stored cryogenically at −175°C or by lyophilization or in the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL. Mycelium for DNA extraction was grown in yeast malt broth, harvested and freeze-dried overnight as described previously22. Genomic DNA was extracted from approximately 50 mg of lyophilized mycelium using a CTAB (hexadecyltrimethylammonium bromide; Sigma Chemical Co.; St Louis, MO) miniprep protocol23. Following extraction, the DNA pellet was resuspended in 100 μL of double-distilled H2O and stored at −20°C.

Dilute samples for the polymerase chain reaction (PCR) were prepared by adding 4 μL of the genomic DNA stocks to 1 mL of double-distilled H2O. Locations and sequences of the PCR and sequencing primers have been published24–26. PCR products were amplified in a Perkin-Elmer 9600 thermal cycler using the fastest ramp times as follows: 40 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C, followed by a 4°C soak. Following purification of the amplified DNA with GeneClean II (Bio 101, La Jolla, CA), both strands were sequenced with the Applied Biosystems dye terminator cycle sequencing kit (Foster City, CA) in a Perkin-Elmer 9600 thermal cycler programmed using the fastest ramp times as follows: 25 cycles of 15 s at 96°C and 4 min at 55°C,
Table 1 Strains sequenced and analyzed phylogenetically.

<table>
<thead>
<tr>
<th>Species</th>
<th>NRRL strain number</th>
<th>Equivalent number</th>
<th>Received from</th>
<th>GenBank accession numbers(^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. accumatum Ellis &amp; Everh.</td>
<td>6227</td>
<td>—</td>
<td>NRRL isolate</td>
<td>28S: U85516, ITS: U85533, mtSSU: U85550, B-tubulin: U85567</td>
</tr>
<tr>
<td>F. cerealis Cooke(^{d})</td>
<td>13721</td>
<td>KF-748</td>
<td>P. Golinski</td>
<td>28S: U85517, ITS: U85534, mtSSU: U85551, B-tubulin: U85568</td>
</tr>
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<td>F. graminearum Schwabe</td>
<td>5883</td>
<td>GZ-17</td>
<td>NRRL isolate</td>
<td>28S: U85520, ITS: U85537, mtSSU: U85554, B-tubulin: U85571</td>
</tr>
<tr>
<td>F. lanusporum Gerlach</td>
<td>13393</td>
<td>R-5822</td>
<td>FRC</td>
<td>28S: U85521, ITS: U85538, mtSSU: U85555, B-tubulin: U85572</td>
</tr>
<tr>
<td>F. tumidum Sherb.</td>
<td>13394</td>
<td>R-5823</td>
<td>FRC</td>
<td>28S: U85526, ITS: U85543, mtSSU: U85560, B-tubulin: U85577</td>
</tr>
<tr>
<td></td>
<td>25348</td>
<td>Fn-2B</td>
<td>Y. Ueno</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>26204 = A-14731</td>
<td>Fn-3</td>
<td>H. Tsunoda</td>
<td>—</td>
</tr>
<tr>
<td>Fusarium sp.</td>
<td>22189</td>
<td>BBA 64262</td>
<td>H. I. Nirenberg</td>
<td>28S: U85531, ITS: U85548, mtSSU: U85565, B-tubulin: U85582</td>
</tr>
<tr>
<td>Fusarium sp.</td>
<td>22192</td>
<td>BBA 64918</td>
<td>H. I. Nirenberg</td>
<td>28S: U85532, ITS: U85549, mtSSU: U85566, B-tubulin: U85583</td>
</tr>
</tbody>
</table>

\(^{a}\) BBA = Biologische Bundesanstalt für Land-und Forstwirtschaft, Berlin, Germany; Fn-2, Fn-3 = H. Tsunoda, Food Research Institute, Ministry of Agriculture and Forestry, Tokyo, Japan; Fn-2B, Fn-M = Y. Ueno, Department of Toxicology and Microbial Chemistry, Science University of Tokyo; KF = Department of Plant Pathology, Agricultural University of Warsaw, Pozan, Poland; Prentice = N. Prentice, ARS, Madison, WI from W. C. Gordon, Winnipeg, Canada.

\(^{b}\) FRC = Fusarium Research Center, Department of Plant Pathology, Pennsylvania State University, University Park, PA.

\(^{c}\) http://www.ncbi.nlm.nih.gov; sequences obtained for NRRL 26204 were not deposited in GenBank but they indicate that this strain is conspecific with the other Fusarium sp. n. strains from Japan.

\(^{d}\) F. cerealis [syn = F. crookwellense Burgess et al.\(^{e}\)].

\(^{e}\) Strains accessioned under the A numbers and subsequently given NRRL numbers.
followed by a 4°C soak. An Applied Biosystems 373A DNA sequencer was used to analyze the sequencing reaction mixtures. GenBank accession numbers are listed in Table 1. Aligned sequences are available from the author upon request.

DNA sequences were visually aligned with TSE, a DOS text editor software program (SemWare; Marietta, GA) and analyzed with PAUP 3.1.1. Maximum parsimony trees were inferred from the aligned sequences for each data set using the heuristic search option with 1000 random addition sequences. The outgroup method was used to root the trees. Support for the phylogenetic groupings was assessed with 1000 bootstrap replications, and by decay indices (also known as Bremer support) calculated up to 15 steps. MacClade was used to determine the base pair composition and the approximate transition/transversion pattern of the combined data. Nixon and Wheeler's phylogenetic species concept was adopted in this study in which species are defined as the smallest set of populations or lineages diagnosable by a unique combination of fixed derived characters (i.e., apomorphies).

Results

Complete sequences for the four regions compared (Fig. 1A) were obtained for all strains listed in Table 1. The β-tubulin gene accounts for the greatest number of substitutions within the combined data set (Fig. 1B, 55%) followed by the mitochondrial small subunit (mtSSU) rDNA (22.7%), the nuclear internal transcribed spacer (ITS) rDNA region (17.5%), and the 5′-most portion of the nuclear 28S rDNA (4.8%). The average transition/transversion ratio approximated with MacClade for the combined data set is 2.5 (150/60). Chi-square goodness of fit techniques were used to test substitutional mutation values. The sequences exhibit a significant ($P<0.01$) bias towards transitions (150 of 210 unambiguous substitutions = 71.4%), and among transitions, base substitutions are strongly biased toward C-T transition mutations ($P<0.01$; 100 of 150

![Fig. 1](image-url)  
(A) Map of the four regions sequenced that comprise the combined data set (28S rDNA = 535 bp, ITS rDNA = 562 bp, mtSSU rDNA = 776 bp, β-tubulin gene = 622 bp). (B) MacClade chart of the single most parsimonious tree found by PAUP, using a 25 bp interval, showing the distribution of steps (=substitutions) within each of the loci sequenced that comprise the combined data set (see phylogram in Fig. 6).
Fig. 2 One of 2 equally most-parsimonious phylogenograms for nuclear 28S rDNA sequences rooted with sequences of *Fusarium graminearum* and related species. Edge length (= steps) is indicated together with bootstrap percentages above 50%. CI = consistency index, RI = retention index.

Fig. 3 One of 18 equally most-parsimonious outgroup rooted trees for nuclear rDNA ITS sequences. Bootstrap intervals above 50% and edge length are indicated. CI = consistency index, RI = retention index.

Fig. 4 One of 32,600 equally most-parsimonious outgroup rooted trees for mtSSU rDNA sequences. Edge length and bootstrap replication frequencies above 50% are indicated. CI = consistency index, RI = retention index.
unambiguous transitions = 66.7%). Highly conserved regions were identified within the central portion of the 28S and ITS (=5.8S rDNA) rDNA sequences and the 5' half of the mtSSU rDNA (Fig. 1B). Boundaries of the ITS1, 5.8S rDNA gene, ITS2 and 28S rDNA were identified by comparison with published sequences. The *Fusarium* β-tubulin is orthologous with *benA* of *Aspergillus nidulans* (Eidam) Wint. and *tub2* of *Epichloë typhina* (Pers.) Tul.

Parsimony analysis of gene trees inferred from sequences of the 5'-most portion of the nuclear 28S rDNA yielded two equally most-parsimonious trees that are largely unresolved (Fig. 2; the outgroup species contributed 7 of the 15 steps). Monophyly of the ingroup taxa, which include *Fusarium* sp. n. (Fn-2, Fn-3, Fn-2B, and Fn-M), received modest bootstrap support (82%). The ingroup taxa were strongly supported as monophyletic (100% bootstrap) within the rDNA ITS gene tree (Fig. 3). *Fusarium* sp. n. (Fn-2, Fn-3, Fn-2B, and Fn-M) occupies a phylogenetically distinct position within the ITS gene tree but its sister is unresolved. Support for the monophyly of the ingroup received a 99% bootstrap interval within the mtSSU rDNA tree (Fig. 4); however, relationships within the ingroup are unresolved. The outgroup species within the mtSSU rDNA
tree contribute slightly over half of the tree length (37-38 steps). When the analysis was extended to the β-tubulin gene (Fig. 5), considerable phylogenetic structure was resolved within the ingroup which was strongly supported as monophyletic (100% bootstrap). The outgroup species contribute only 33 (19.7%) of the 170 step tree length. Of the species sampled, a *F. sporotrichioides* (sect. *Sporotrichiella* Wollenw.)*-F. acuminatum* Ellis & Everhart (sect. *Gibbosum* Wollenw.) clade (98% bootstrap) was identified as a sister of the remaining ingroup taxa. Within this latter clade, *F. poae* (Peck) Wollenw. (sect. *Sporotrichiella*) was strongly supported (96% bootstrap) as a sister to a modestly supported clade (78% bootstrap) containing the following two subgroups: *Fusarium* sp. n. (Fn-2, Fn-3, Fn-2B, and Fn-M) (sect. *Sporotrichiella sensu* Marasas *et al.*), and a lineage containing *F. sambucinum* and related species (sect. *Discolor* Wollenw., pro parte). Because the individual data sets yielded trees that were generally corroborating topologically, parsimony analysis was conducted on the combined nuclear 28S rDNA, ribosomal ITS region, mtSSU rDNA, and β-tubulin gene data sets. A heuristic search implemented by PAUP 3.1.1 with 1000 random addition sequences yielded a single most parsimonious outgroup-rooted tree (Fig. 6; length = 319 steps, CI = 0.862, RI = 0.893). The phylogram shown in Fig. 6 is concordant in most details with gene trees inferred from each of the individual data sets, especially the β-tubulin gene tree (Fig. 5). Bootstrap intervals and decay indices calculated for the tree inferred from the combined data were generally concordant (Fig. 6).

**Discussion**

This study reports the results of a molecular phylogenetic analysis of several mycotoxigenic strains from Japan referred to here as *Fusarium* sp. n. (Fn-2, Fn-3, Fn-2B, and Fn-M) and their close relatives within the Gibberella clade of *Fusarium*. The β-tubulin gene and data from the combined loci each produced fully resolved cladograms that were largely concordant. These cladograms are taken as the best available estimate of the species phylogeny because most of the species have been recognized in one or more morphologically-based taxonomic treatment of the genus, and concordance of gene trees inferred from the unlinked loci suggests that each reflects the same underlying phylogeny. Phylogenetic analysis of the β-tubulin gene data alone provided measures of clade stability comparable to that produced from analysis of the combined 28S rDNA, ribosomal ITS region, mtSSU rDNA, and β-tubulin gene data, a result consistent with the discovery that most of the phylogenetic signal in the combined data set is contributed by the β-tubulin locus. Similar findings were reported by O'Donnell and Cigelnik for an investigation of the Gibberella fujikuroi and *Fusarium oxysporum* species complexes. Poor resolution of species within the 28S and ITS rDNA gene trees is consistent with previous findings and emphasizes the point that these loci lack sufficient resolution for distinguishing closely related species within *Fusarium* and probably many other toxigenic fungi as well. Because many nodes and terminal taxa were poorly supported in the present analysis of the combined data set, the species phylogeny requires further testing through the analysis of additional rapidly evolving nuclear genes that possess large introns as in β-tubulin.

Contrary to the findings of Guadet *et al.* results of the present and previous studies indicate that the sectional classification of *Fusarium* is artificial, and as such, it lacks robust
power to accurately predict biological properties such as toxin production of these agronomically-important fungi. Pending a complete phylogenetically-based revision of the genus, the available data indicate that the sections should be viewed as non-monophyletic species groupings.

Previously, strains of *Fusarium* sp. n. (Fn-2, Fn-3, Fn-2B, and Fn-M) were identified as *F. nivale* [= *Microdochium nivale* (Fries) Samuels & Hallett], *F. episphaeria*, *F. tricinctum*, and most recently as *F. sporotrichioides*. *Fusarium* sp. n. NRRL 3509 (=Fn-2) was identified as *F. sporotrichioides* primarily because both species were thought to produce the same two types of microconidia on polyphialides in the aerial mycelium. However, microscopic examination of NRRL 3509 revealed that this strain produces fusoid-to-ovoid microconidia, not fusoid and pyriform as in *F. sporotrichioides*. Other morphological differences between these two species include the absence of chlamydospores and sclerotia in *Fusarium* sp. n. (Fn-2, Fn-3, Fn-2B, and Fn-M), but their presence is typical of *F. sporotrichioides*. Marasas et al. noted two additional differences between *Fusarium* sp. n. (Fn-2, Fn-3, Fn-2B, and Fn-M) and *F. sporotrichioides*: geographic origin (warm for *Fusarium* sp. n. in Kyushu and Shikoku, Japan while *F. sporotrichioides* is more common in cooler regions), and different toxin production (nivalenol and fusarenon-X in *Fusarium* sp. n. and diacetoxyscripenol (DAS), T-2, and HT-2 in *F. sporotrichioides*). Collectively, these differences are fully concordant with the molecular data which strongly indicate that the Japanese strains (Fn-2, Fn-3, Fn-2B, and Fn-M) represent an undescribed species. Whether this species is endemic to Japan, or introduced, is an open question since its only known host is the cosmopolitan grain wheat.

An important implication of the present study is that the number of *Fusarium* species will increase substantially through ongoing morphological and molecular systematic studies as is evidenced by the discovery of three undescribed, phylogenetically distinct species in the present study: *Fusarium* sp. n. (Fn-2) from Japan, *Fusarium* sp. 22189 reported as *F. sambucinum* on *Glycine max* (L.) Merr. from Brazil and *Fusarium* sp. 22192 published as *Gibberella pulicaris* (Fr.) Sacc. (= *Fusarium sambucinum*) on a palm tree from Indonesia. These latter two new species are distinguished from *F. sambucinum* by the production of T-2 toxin instead of DAS, and from each other and *F. sambucinum* via the random amplified polymorphic DNA (RAPD) technique. In addition, mating studies have demonstrated that NRRL 22189 and 22192 are infertile with *F. sambucinum* testers. These results demonstrate a perfect concordance between the phylogenetic and biological species concepts for *G. pulicaris*, indicating that both species concepts identify the same taxon. That both concepts show similar concordance in phylogenetically diverse lineages of *Fusarium* suggests that either concept accurately predicts the boundaries of the other. However, because teleomorphs are unknown from most of the species included in this study, identification of strains by mating experiments for all but a few species is currently impossible. Given the close relationship of *F. tumidum* Sherb. and *F. sambucinum* in the phylogram inferred from the combined data, there is a reasonable possibility that mating experiments may demonstrate that these two taxa are members of the same biological species.

One of the more important implications of the present study is the importance of investigating the evolution of *Fusarium* toxins within the context of a robust species-level phylogeny.
Within the relatively small data set analyzed in this study, the following three toxin lineages were resolved: nivalenol/nivalenol mono-and diacetate (\textit{Fusarium} sp. n. Fn-2)\textsuperscript{31}, DAS/T-2 toxin (= \textit{F. sambucinum} clade)\textsuperscript{20}, and DON/zearalenone (= \textit{F. graminearum} clade)\textsuperscript{2,20}. It is important to note that toxin production has not been characterized for all of the species within the \textit{F. sambucinum} and \textit{F. graminearum} clades but explicit, testable predictions can be made for these species based on the available data. In addition to their potential for elucidating the evolution of toxins within \textit{Fusarium}, molecular characters also provide a wealth of discrete nucleotide data that can be used to diagnose species and to design molecular diagnostics for the rapid and accurate identification of unknown strains.

During the course of this investigation, Fn-2B (= A-18476 = NRRL 6490 = MAFF 237646) was identified as \textit{Fusarium} sp. n. after it had been stored by lyophilization ever since it was sent to the ARS Culture Collection (NRRL) by Prof. Yoshio Ueno in 1971. Of the strains of \textit{Fusarium} sp. available for study, NRRL 6490 is in the best condition morphologically and the formal diagnosis being prepared for this interesting toxigenic species from Japan is based largely on this strain\textsuperscript{44,45}.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

\textbf{References}

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