Toxicity, Pathogenicity, and Genetic Differentiation of Five Species of *Fusarium* from Sorghum and Millet

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ABSTRACT

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Fusarium isolates recovered from sorghum and millet are commonly identified as *F. moniliforme*, but with the recognition of new species in this group, the strains given this name are being re-evaluated. We analyzed five strains each from five *Fusarium* species (*F. andiyazi*, *F. nygamai*, *F. pseudonygamai*, *F. thapsinum*, and *F. verticillioides*) often associated with sorghum and millet for their ability to produce fumonisin and moniliformin, their toxicity to ducklings, and their ability to cause disease on sorghum seedlings in vitro. These species can be distinguished with isozymes (fumarase, NADP-dependent isocitrate dehydrogenase, and malate dehydrogenase) and with banding patterns resulting from ampli-

fied fragment length polymorphisms. Two species, *F. pseudonygamai* and *F. thapsinum*, produced high levels of moniliformin, but little or no fumonisins, and were consistently highly toxigenic in the duckling tests. Two species, *F. verticillioides* and *F. nygamai*, produced high levels of fumonisins, but little or no moniliformin, and also were toxigenic in the duckling tests. *F. andiyazi* produced little or no toxin and was the least toxigenic in the duckling tests. In sorghum seedling pathogenicity tests, *F. thapsinum* was the most virulent followed by *F. andiyazi*, then *F. verticillioides*, and finally *F. nygamai* and *F. pseudonygamai*, which were similar to each other. Thus, these five species, which would once have all been called *F. moniliforme*, differ sufficiently in terms of plant pathogenicity and toxin production profile, that their previous misidentification could explain inconsistencies in the literature and differences observed by researchers who thought they were all working with the same fungal species.

Fusarium spp. can cause stalk rots, ear rots, and grain mold, resulting in serious production losses in both maize and sorghum (12,72), and produce mycotoxins that are harmful to both humans and domesticated animals. In high incidence areas of human esophageal cancer in Africa, e.g., portions of the Eastern Cape Province of South Africa, traditional indigenous African staple crops such as sorghum (*Sorghum bicolor* (L.) Moench) and pearl millet (*Pennisetum glaucum* (L.) R.Br) have been replaced by maize (*Zea mays* L.) during the past 50 to 100 years (59). This change in staple diet may have affected the nutritional status of the populations at risk for esophageal cancer and may have altered the kinds and amounts of *Fusarium* mycotoxins to which these populations are exposed.

Risks associated with *Fusarium* toxins usually are assessed based on the *Fusarium* species present, as not all species produce all toxins. Important toxins produced by these fungi include the fumonisins (14) and moniliformin (45). Based on duckling toxicity tests (32) and brine shrimp assays (34), additional mycotoxigenic compounds produced by these fungi probably remain to be identified.

Fusarium spp. from sorghum and millet have only recently become the subject of in depth research and often were included only as isolates of peripheral interest in earlier studies. Modern work on the taxonomy of the genus *Fusarium* began with Wollenweber and Reinking (74), who established the section *Liseola* within the genus *Fusarium* for species that produce microconidia

in chains and/or false heads and macroconidia, but that do not produce chlamydospores. A chain-forming *Fusarium* sp. isolated from moldy maize in Nebraska and described as *F. moniliforme* Sheldon (65) was included in section *Liseola* by Wollenweber and Reinking (74), with two other species and three varieties, for a total of six taxa. Doidge (9) recognized only two representatives of section *Liseola* in South Africa, i.e., *F. moniliforme* with microconidia in chains and *F. moniliforme* var. *subglutinans* Wollenw. & Reinking with microconidia not in chains. All the above taxa were later reduced to a single species, *F. moniliforme* Sheldon emend. Snyder & Hansen by Snyder and Hansen (66).

Booth (3) initiated the re-evaluation of the species in section *Liseola* that is still in progress by separating *F. moniliforme* var. *subglutinans* from *F. moniliforme* due to the absence of microconidial chains and the presence of polyphialides. Gerlach and Nirenberg (15) expanded the number of taxa in section *Liseola* to 10 and pointed out that, based on priority, the correct name for *F. moniliforme* is *F. verticillioides* (Sacc.) Nirenberg (50), and the name of the teleomorph is *Gibberella moniliformis* Wineland (73); a position now generally accepted in the *Fusarium* research community (64). Nelson et al. (49) accepted four taxa in section *Liseola*, including *F. moniliforme* and another chain-forming species, *F. proliferatum* (Matsushima) Nirenberg, which was separated because of the presence of polyphialides. They also elevated *F. moniliforme* var. *subglutinans* to species rank as *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas. In the last 20 years, five additional chain-forming *Fusarium* species in section *Liseola* have been described; *F. andiyazi* Marasas, Rheeder, Lamprecht, Zeller & Leslie (44); *F. globosum* Rheeder, Marasas & Nelson (60); *F. miscanthi* W. Gams et al. (13); *F. nisekadoi* Aoki & Nirenberg (51); and *F. thapsinum* Klittich, Leslie,

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Nelson & Marasas (26). These taxonomic treatments differed somewhat from each other, but only two of them (26,44) differentiated between *F. moniliforme* (or *F. verticillioide*s) from maize and sorghum, whereas some stated explicitly that *F. moniliforme* occurs on both maize and sorghum (3,9,33,74).

These taxonomic difficulties are further complicated by chain-forming *Fusarium* species that resemble those seen in section *Liseola*, but that also produce chlamydospores (e.g., *F. nygamai* Burgess & Trimboli [6] and *F. napiforme* Marasas, Nelson & Rabie [42]), which along with *F. dlamini* Marasas, Nelson & Toussoun (40) were placed in the new section *Dlamini* by Kwasna et al. (27). This new section has not been widely used, and instead many members of both sections *Liseola* and *Elegans* are now referred to as part of the “*Gibberella fujikuroi* species complex” (28,30,31). DNA sequence data indicate that section *Liseola* is paraphyletic and that *F. nygamai*, *F. napiforme*, and *F. dlamini* are “phylogenetically nested within section *Liseola*” (54). The “*G. fujikuroi* species complex” now contains at least 46 phylogenetically distinct lineages (54,55), including 10 new species described by Nirenberg and O'Donnell (52), two species described by Nirenberg et al. (53), and most recently, *F. andiyazi* (44) and *F. konzum* Zeller, Summerell & Leslie (76). At present there are at least 15 described *Fusarium* species in the “*G. fujikuroi* complex” that produce microconidia in chains: *F. andiyazi*; *F. annulatum* Bugnicourt; *F. brevicatenulatum* Nirenberg, O'Donnell, Kroschel & Andrianavo; *F. fujikuroi*; *F. globosum*; *F. lactis* Pirota & Riboni; *F. napiforme*; *F. nygamai*; *F. phyllophilum* Nirenberg & O'Donnell; *F. proliferatum*; *F. pseudoanthophilum* Nirenberg, O'Donnell & Mubutanhema; *F. pseudocircinatum* O'Donnell & Nirenberg; *F. pseudonygamai* O'Donnell & Nirenberg; *F. thapsinum*; and *F. verticillioide*s, but not all of these have been recovered from sorghum and millets.

In addition to maize and sorghum, Booth (3) stated that *F. moniliforme* also occurs on rice and sugarcane, is a “major para-

site” of several members of the family Gramineae, and occurs on at least 31 other families of plants. This purported host range of *F. moniliforme* was extended even further by Bacon et al. (1), who calculated that 11,000+ species of plants could serve as a host for this fungus. In this context, it is not surprising that the fungus that causes “head blight” or “grain mold” of sorghum has been, and often still is, referred to as *F. moniliforme* and considered to be conspecific with the fungus by the same name associated with ear and stalk rot of maize (2,11,12,33,47,56–58).

Our objective in this study was to evaluate in detail a limited number of strains (five) from each of five taxa (*F. andiyazi*, *F. nygamai*, *F. pseudonygamai*, *F. thapsinum*, and *F. verticillioide*s) commonly recovered from sorghum, millet, and maize in South Africa. Three of these taxa, *F. verticillioide*s, *F. thapsinum*, and *F. nygamai*, are relatively well described and understood. The other two, *F. pseudonygamai* and *F. andiyazi*, are more recently described and have not been as extensively characterized. Our working hypothesis is that these five species have commonly been confounded in the past, probably all identified as *F. moniliforme* from sorghum and millet, resulting in the confusingly different toxicological and phytopathogenic properties that are simultaneously attributed to *F. moniliforme*. To distinguish strains and species, we used various genetic and molecular techniques (e.g., sexual crosses [28,30,31], vegetative compatibility [29], isozymes [16], and amplified fragment length polymorphisms [AFLPs] [42,68,75,76]) to develop a composite picture of the differences between species that is consistent with differences in economically important traits such as mycotoxin profiles, duckling toxicity, and virulence toward sorghum seedlings.

MATERIALS AND METHODS

Strains and culture conditions. Twenty-five strains (Table 1) were used in these studies. Strain numbers from collections are

TABLE 1. Strains used in this study

MRC strain ^y	KSU strain ^y	Mating type ^z	Host	Geographic origin	Reference
<i>Fusarium andiyazi</i>					
5993	3860	<i>MAT-1</i>	Sorghum grain	Greytown, South Africa	44
5995	3862	<i>MAT-1</i>	Sorghum grain	Greytown, South Africa	44
<u>6122</u>	<u>4804</u>	<i>MAT-2</i>	Sorghum soil debris	Greytown, South Africa	44
6123	4812	<i>MAT-2</i>	Sorghum soil debris	Greytown, South Africa	44
6126	4807	<i>MAT-1</i>	Sorghum soil debris	Greytown, South Africa	44
<i>F. nygamai</i>					
<u>3997</u>	<u>1782</u>	<i>MAT-1</i>	Sorghum root	Narrabri, Australia	6
3998	1783	<i>MAT-1</i>	Root debris	Rockhampton, Australia	6
4010	1795	<i>MAT-2</i>	Sorghum field soil	Springbok Flats, South Africa	6
4011	1796	<i>MAT-1</i>	Sorghum field soil	Springbok Flats, South Africa	6
4012	1797	<i>MAT-1</i>	Sorghum field soil	Butterworth, South Africa	6
<i>F. pseudonygamai</i>					
1240	1762	<i>MAT-1</i>	Pearl millet grain	Ovambo, Namibia	43
1247	1765	<i>MAT-2</i>	Pearl millet grain	Ovambo, Namibia	43
2203	1767	<i>MAT-1</i>	Pearl millet grain	Ovambo, Namibia	43
4149	1801	<i>MAT-1</i>	Pearl millet grain	Kavango, Namibia	43
4150	1802	<i>MAT-1</i>	Pearl millet grain	Kavango, Namibia	43
<i>F. thapsinum</i>					
6001	3868	<i>MAT-2</i>	Sorghum root	Kwazulu-Natal, South Africa	26
6002	3869	<i>MAT-2</i>	Sorghum root	Kwazulu-Natal, South Africa	26
6003	3870	<i>MAT-2</i>	Sorghum root	Kwazulu-Natal, South Africa	26
6004	3871	<i>MAT-2</i>	Sorghum root	Kwazulu-Natal, South Africa	26
6148	3884	<i>MAT-1</i>	Sorghum seed	Northwest Province, South Africa	26
<i>F. verticillioide</i> s					
826	488	<i>MAT-1</i>	Maize grain	Transkei, South Africa	14,32
1065	4796	<i>MAT-2</i>	Maize grain	Transkei, South Africa	32
4315	4801	<i>MAT-2</i>	Maize grain	Transkei, South Africa	32
4317	4802	<i>MAT-1</i>	Maize grain	Transkei, South Africa	32
4321	4803	<i>MAT-1</i>	Maize grain	Transkei, South Africa	32

^y MRC strain number (Medical Research Council, Tygerberg, South Africa in the collection of PROMEC); KSU strain number (Kansas State University, Department of Plant Pathology). Ex-holotype cultures are underlined.

^z Mating type was determined by crosses with standard tester strains or by polymerase chain reaction amplification of a fragment from the *MAT-2* HMG box using primers Gfmat2a and Gfmat2b of Kerényi et al. (21) or primers Gfmat1a and Gfmat1b of Steenkamp et al. (67).

denoted as follows: KSU (Kansas State University, Manhattan), MRC (Medical Research Council, Tygerberg), FGSC (Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City), BBA (Biologische Bundesanstalt für Land-und Forstwirtschaft, Berlin, Germany), and FRC (*Fusarium* Research Center, Pennsylvania State University, University Park). Two of the *F. pseudonygamai* strains, MRC 1240 and MRC 2203, are vitamin auxotrophs that required the vitamin mixture of Correll et al. (7) for growth in minimal medium. For AFLP studies, we included the ex-holotype strain of *F. pseudonygamai* (MRC 7537, KSU X-10762, NRRL 13592, BBA 69552, and FRC M-1166). This strain has a pedigree that indicates it was first collected by one of us as MRC 515 from pearl millet in Namibia (39,41, 43,48), and assigned to either *F. moniliforme* or *F. nygamai*, but with atypical morphological characters, and does not originate from Nigeria as claimed in the original species description (52). For isozyme studies we also included established mating-type tester strains as comparison standards. We used the following standard *G. fujikuroi* mating population mating-type tester strains (22,28,30,36) to test for sexual cross-fertility: FGSC 7600 (*MATA-1*), FGSC 7603 (*MATA-2*), FGSC 7611 (*MATB-1*), FGSC 7610 (*MATB-2*), FGSC 8931 (*MATC-1*), FGSC 8932 (*MATC-2*), FGSC 7615 (*MATD-1*), FGSC 7614 (*MATD-2*), FGSC 7616 (*MATE-1*), FGSC 7617 (*MATE-2*), FGSC 7057 (*MATF-1*), FGSC 7056 (*MATF-2*), FGSC 8934 (*MATG-1*), and FGSC 8933 (*MATG-2*).

Strains were routinely cultured on carnation leaf agar (10) or on modified Czapek's complete medium (7). Strains were maintained as lyophilized cultures (MRC) or as spore suspensions in 15% glycerol frozen at -70°C (KSU).

Radial growth rates of all 25 strains were determined by measuring colony diameters of single conidial cultures on potato dextrose agar (Difco Laboratories, Detroit) in 90-mm-diameter petri dishes incubated for 72 h in total darkness at 25 or 30°C . Measurements were made on three replicate cultures that each originated from single conidia per temperature for each strain.

Sexual crosses and diagnosis of mating type. We determined the mating type of all 25 strains following polymerase chain reaction (PCR) amplification with the primers of Kerényi et al. (21) or Steenkamp et al. (67). Strains also were crossed with standard mating-type tester strains on carrot agar according to the techniques of Klittich and Leslie (24) for *G. thapsina* and *G. fujikuroi* mating populations A to E. Crosses with testers of *G. nygamai* Klaasen & Nelson were made by using the tester strains and protocols of Klaasen and Nelson (22). Within *F. andiyazi*, *F. nygamai*, and *F. pseudonygamai*, strains were intercrossed in all possible pairwise combinations, with each strain serving as both the male and the female parent in crosses with other members of the group. All crosses were repeated at least twice. Fertile crosses produced perithecia that exuded a cirrus of ascospores.

DNA manipulations. DNA was isolated with a CTAB protocol, as described by Kerényi et al. (21), and stored in $1\times$ Tris-EDTA (61) at 4°C until used. AFLPs were generated essentially as described by Vos et al. (71), with the modifications of Marasas et al. (44), and analyzed as described by Marasas et al. (44) and Zeller et al. (75). In brief, we used two primer pair combinations (*EcoRI*+TT/*MseI*+AC and *EcoRI*+GG/*MseI*+TG) and analyzed up to 150 bands in intraspecific comparisons and up to 360 bands in the interspecific pairs. The *EcoRI* primers in the final PCR amplifications were labeled with ^{32}P . Labeled DNA fragments were detected by autoradiography following separation on 6% polyacrylamide gels. AFLP profiles were scored manually for the presence or absence of bands, and genetic similarity was assessed with the Dice coefficient.

Vegetative compatibility tests. Nitrate nonutilizing (*nit*) mutants were generated from all 25 strains and used in complementation tests to identify strains that belonged to the same vegetative compatibility group (VCG) (7,24,25). The *nit* mutants

were generated on a minimal medium containing 2% KClO_3 and assigned to one of three classes—*nit1*, *nit3*, or NitM—based on their ability to grow on media containing differential nitrogen sources (7). *F. nygamai* strains MRC 4010 and MRC 4012 and *F. andiyazi* strain MRC 6123 were resistant to 2% KClO_3 but sensitive to 3% KClO_3 . Complementary mutants in the same VCG form a prototrophic heterokaryon when cultured together on a minimal medium containing KNO_3 as the sole nitrogen source. Whenever possible, a *nit1* and a NitM mutant pair from each strain was used in the pairing procedure. If phenotypically distinct mutants from the same parental strain could not form a heterokaryon, then the parental strain was designated as heterokaryon self-incompatible (8). Pairings were made in 24-well hybridoma plates as described by Klittich and Leslie (25). All pairings were tested at least twice.

Isozyme phenotype. Isozymes were resolved and isolated on the basis of protein banding patterns in starch gels as described by Huss et al. (16). We tested the isozymes triose phosphate isomerase (TPI), EC 5.3.1.1; NADP-dependent isocitrate dehydrogenase (IDH), EC 1.1.1.42; fumarase (FUM), EC 4.2.1.2; malate dehydrogenase (MDH), EC 1.1.1.37; mannitol dehydrogenase (MADH), EC 1.1.1.67; glucose-6-phosphate dehydrogenase (G6PDH), EC 1.1.1.49; and phosphogluconate dehydrogenase (PGDH), EC 1.1.1.44.

Secondary metabolite production. Fumonisin B₁ and B₂ contents of cultures grown on patties of ground maize were determined by the method of Sydenham et al. (69) as modified by Leslie et al. (32); detection limit 1 $\mu\text{g/g}$. Moniliformin levels were initially determined by the method of Thiel (70); detection limit 60 $\mu\text{g/g}$. Strains that produced little or no moniliformin in this assay were re-examined using the more sensitive method of Scott and Lawrence (63); detection limit 1 $\mu\text{g/g}$.

Duckling toxicity protocol. All 25 strains were tested for toxicity to ducklings according to the methods of Marasas et al. (38,46) as modified by Leslie et al. (32). The ducklings and their feed were weighed at the beginning of each experiment, and the total feed intake value for each group of four ducklings was calculated from the amount of feed remaining at the end of the test. Mortality was recorded daily, and the mean day of death was calculated from the number of ducklings that died on different days. Cultures were considered toxic if they caused the death of three or four of the four ducklings in a single group. A toxicity index was calculated by multiplying the amount of feed intake, in grams, by the mean day of death to obtain an inverse measure of toxicity for the cultures (19). This test was conducted once with the different strains serving as replicates by which the general toxicity of a species was estimated.

Sorghum pathogenicity assays. Pathogenicity tests were conducted in an agar test tube assay. Strains were assayed for pathogenicity by inoculating seedlings of sorghum cvs. PAN 8560 and PAN 8472. Seed was treated in a water bath at 55°C for 30 min to eliminate seedborne fungi, but we had to increase the length of the heat treatment from 5 min, as described previously (17,18), to 30 min to ensure that all of the endogenous *Fusarium* propagules were dead. Disinfested seeds were germinated on 0.6% water agar. After incubation for 14 days, sterile germinated seeds were transferred to test tubes (200 by 30 mm) containing 20 ml of solidified sterile Hoagland's no. 2 medium (one seed per tube). The tubes were plugged with sterile nonabsorbent cotton and incubated for 10 days with a 14-h photoperiod (Sylvania Gro-Lux, WFS96T12/Gro/QHO WS 215W, GTE Products Corp., Danvers, MA) at 25°C by day and 18°C by night. Uniform seedlings were selected for inoculation. For each of the 25 *Fusarium* strains, inoculum was prepared and seedling tubes were inoculated with 0.5 ml of a spore suspension containing 1×10^6 spores per ml and incubated for 30 additional days under the same conditions as those used for the initial growth of the seedlings. Controls were inoculated with 0.5 ml of sterile potato dextrose broth

(Difco Laboratories), the medium in which the cultures had been grown. Roots were rated visually for disease severity on a 1 to 5 scale reflecting the proportion of the root system with visual lesions as described by Schreuder et al. (62). The foliage of the seedlings was excised, and shoot and root length was measured. Root and shoot dry mass were determined by weighing after drying at 60°C for 3 days.

The experiment was done twice. There were three replicates (blocks) in the first experiment and 12 in the second. The treatment design was a 2 × 26 factorial with two cultivars and 26 fungal treatments (one control and five strains from each of the five species). An experimental unit was a single plant. Factorial analysis of variance was performed on the root and shoot length dry mass data. Disease severity ratings were ranked before the data were subjected to analysis of variance (version 6; SAS Institute, Cary, NC) according to the manufacturer's instructions. Student's *t* test and least significant differences at the 5% significance level were calculated to compare means.

RESULTS

Vegetative growth. Radial growth rates for all five taxa (Table 2) were similar at both 25 and 30°C, ranging from a mean of 25 mm in 72 h at either 25 or 30°C for *F. nygamai* to 32 mm in 72 h at 30°C for *F. andiyazi*. Pigmentation of the reverse side of the colony was purple for *F. andiyazi*; white, peach, or purple for *F. nygamai*, *F. pseudonygamai*, and *F. verticillioideis*, respectively; and white or yellow for *F. thapsinum*.

Mating type and sexual cross-fertility. Both mating types were observed in all five species (Table 1). The *F. verticillioideis* and *F. thapsinum* strains were all fertile as males with one of the two standard tester strains for those species. Two of the *F. verticil-*

lioides strains, MRC 826 and MRC 4317, also were fertile as the female parent in a reciprocal cross with the standard tester strains. Strains assigned to *F. nygamai*, *F. pseudonygamai*, or *F. andiyazi* were not cross-fertile with tester strains from *G. fujikuroi* mating populations A to E, *G. thapsina*, or *G. nygamai*, and mating-type diagnoses were based on PCR amplification with primers specific for either *MAT-1* or *MAT-2* (21,67).

Within *F. nygamai*, *F. pseudonygamai*, and *F. andiyazi*, the five strains were crossed in all possible pairwise combinations, but no fertile crosses were observed. We were unable to repeat the cross reported by Klaasen and Nelson (22) in which strain MRC 4011 was reported to be cross-fertile with one of the *G. nygamai* tester strains; however, we also were unable to obtain a positive control cross between the two *G. nygamai* tester strains. In crosses with testers from *G. fujikuroi* mating population B, we found very few fertile perithecia in some crosses with *F. nygamai* strain MRC 4012 and *F. andiyazi* strains MRC 5993 and MRC 6126, and with all of the *F. pseudonygamai* strains. These rare, apparently fertile perithecia were assumed to have resulted from self-fertilization by the tester strains for mating population B, as previously reported (5).

Vegetative compatibility. We tested all five strains from each species (Table 1) for vegetative compatibility with the other strains in that species. We used four independently derived *nit* mutants from each strain, two *nit1* and two *NitM*, in the pairings. One of the 25 strains tested, *F. nygamai* MRC 4011, was heterokaryon self-incompatible. All of the other strains were heterokaryon self-compatible. Within each species we found no strains that were in the same VCG, and thus concluded that none of the 25 strains were vegetative clones.

Isozyme polymorphisms. Four isozymes (FUM, IDH, MDH, and TPI) have collectively been used to distinguish *G. fujikuroi*

TABLE 2. Radial growth rates, toxins produced, and toxicities of strains of *Fusarium verticillioideis*, *F. thapsinum*, *F. nygamai*, *F. pseudonygamai*, and *F. andiyazi*

Strain ^w	Mycotoxin (µg/g)			Duckling toxicity index ^x	Radial growth (mm/72 h ± SD)	
	Fumonisin B ₁	Fumonisin B ₂	Moniliformin		25°C	30°C
<i>F. andiyazi</i>						
5993	Tr ^y	ND	ND	690	30 ± 1.5	31 ± 1.5
5995	Tr	ND	ND	7,600 ²	33 ± 0.0	34 ± 2.6
6122	Tr	ND	ND	440	32 ± 0.6	33 ± 1.2
6123	Tr	ND	ND	590	29 ± 3.2	31 ± 0.6
6126	ND ^z	ND	ND	420	28 ± 1.7	29 ± 1.0
<i>F. nygamai</i>						
3997	3,200	1,600	2,000	35	27 ± 3.1	28 ± 2.3
3998	3,800	3,200	140	45	26 ± 0.6	24 ± 2.0
4010	2,300	700	Tr	74	24 ± 1.5	24 ± 1.5
4011	Tr	ND	49	5,500 ²	26 ± 2.0	28 ± 1.2
4012	1,500	5,700	380	68	24 ± 0.6	22 ± 1.0
<i>F. pseudonygamai</i>						
1240	Tr	Tr	2,400	20	30 ± 0.6	29 ± 1.5
1247	ND	ND	270	18	27 ± 0.6	26 ± 1.5
2203	Tr	Tr	1,100	170	25 ± 0.6	24 ± 0.0
4149	Tr	Tr	4,800	20	27 ± 0.6	28 ± 0.6
4150	Tr	Tr	1,900	48	24 ± 0.6	28 ± 0.6
<i>F. thapsinum</i>						
6001	Tr	Tr	698	35	22 ± 0.6	19 ± 1.5
6002	Tr	Tr	1,198	18	24 ± 1.0	20 ± 0.6
6003	20	9	1,742	18	28 ± 1.2	26 ± 0.6
6004	Tr	Tr	898	30	24 ± 0.6	27 ± 0.6
6148	ND	ND	296	43	19 ± 2.1	14 ± 1.0
<i>F. verticillioideis</i>						
826	3,700	800	ND	95	21 ± 1.0	27 ± 1.0
1065	400	80	ND	120	30 ± 0.6	35 ± 1.5
4315	3,000	650	ND	43	27 ± 0.1	25 ± 0.6
4317	120	20	ND	60	24 ± 1.0	26 ± 0.6
4321	2,000	160	ND	40	23 ± 0.6	22 ± 3.8

^w Strain number from the MRC strain collection.

^x Toxicity index = number of grams of feed consumed × mean day of death. Thus, the smaller the number, the more toxic the strain. A numerical superscript indicates the number of ducklings that died during the 14-day examination period if that number was less than the total of four.

^y Tr = trace (for fumonisin, 1 to 4 µg/g, and for moniliformin, 1 to 5 µg/g).

^z ND = not detected (for fumonisin, <1 µg/g, and for moniliformin, <1 µg/g).

mating populations A to E, *F. thapsinum*, and *F. nygamai* (16). The profiles for MADH, PGDH, and G6PDH were monomorphic, as previously observed.

For three of the diagnostic isozymes, only previously identified forms were observed. For FUM (Table 3), only the previously described α and γ forms were observed, although three of the five *F. nygamai* strains (MRC 3997, MRC 3998, and MRC 4010) had the γ form, which was not detected in the two previously tested strains of this species. For MDH (Table 3), we observed only the previously described α and γ forms, with all of the *F. andiyazi* and *F. pseudonygamai* strains having the γ form. The five *F. nygamai* strains all had the α form, as reported previously (16). Members of *G. fujikuroi* mating population A have a unique form of TPI (β), with the remaining mating populations, including *F. nygamai*, having a faster running (α) form. Both *F. pseudonygamai* and *F. andiyazi* have the α form of TPI.

IDH was the only isozyme for which new forms were observed (Fig. 1; Table 3). For *F. andiyazi*, only the previously described β form was observed. Four of the five *F. pseudonygamai* strains have the previously described γ form of IDH, and one strain (MRC 1240) has a new form (α/β) that appears to represent a composite of the α and β forms previously observed (Table 3). There also are two new forms of IDH not previously observed in *F. nygamai*. One is the previously existing β form seen in *F. verticillioide*s, *F. sacchari*, *F. fujikuroi*, and *F. proliferatum*, and occasionally in *F. thapsinum* (Table 3), which we observed in strain MRC 4012. The other is a new form (δ) not previously observed in strains from any of the *G. fujikuroi* mating populations, which is intermediate between the previously described β and ω forms, and observed in strain MRC 4011 (Fig. 1).

AFLP polymorphism. We compared banding patterns from the five strains from each species with those from representatives of the other four species and with the ex-holotype strain (MRC

7537) of *F. pseudonygamai*. Within each of *F. andiyazi*, *F. pseudo-nygamai*, *F. thapsinum*, and *F. verticillioide*s, the tested strains were $\geq 73\%$ similar in AFLP profile (Table 4). The diversity among the *F. nygamai* strains was greater than that observed within the other groups. Strains MRC 3997, 3998, 4010, and 4011 were all $\geq 71\%$ similar in AFLP fingerprint profile. Strain MRC 4012 was considerably different from the other four *F. nygamai* strains, with similarity ranging from 21 to 26%. These values are comparable to those observed between strains belonging to different species (Table 4) (34,36,44,68,76). Thus, MRC 4012 probably belongs to a species that has not yet been differentiated from *F. nygamai*. The greatest similarity observed between strains in different species was 33% for MRC 6148 (*F. thapsinum*) and MRC 3997 (*F. nygamai*). Our *F. pseudonygamai* strains shared $>76\%$ of their bands with the ex-holotype strain and were $>73\%$ similar to one another (Table 4). This similarity is the primary reason for our identification of these strains as *F. pseudonygamai* rather than *F. nygamai*, as had been done previously (46).

Duckling toxicity. With two exceptions (MRC 4011 and MRC 5995), maize cultures of all the *Fusarium* strains caused the death of four out of four ducklings within 14 days (Table 2). The toxicity index is the number of grams of feed consumed multiplied by the mean day of death. Thus, the smaller the number for the toxicity index, the more toxic the strain. Strains of *F. pseudo-nygamai*, *F. thapsinum*, and *F. verticillioide*s were the most toxic, because all of the strains had duckling toxicity indices <170 , i.e., they caused the death of four out of four ducklings in the shortest time and after consumption of the smallest amount of feed. *F. nygamai* probably should be grouped with these three species, but one strain, MRC 4011, caused the death of only two of the four ducklings and consequently had a very high duckling toxicity index (5,500). The least toxic species was *F. andiyazi* with relatively high duckling toxicity indices (420 to 690) for four strains, while one strain, MRC 5995, caused the death of only two of the four ducklings and had the highest duckling toxicity index (7,600) of all the strains tested.

Secondary metabolite production. The mycotoxin profiles of the five *Fusarium* species with respect to the production of fumonisins (FB₁ and FB₂) and moniliformin agreed relatively well

TABLE 3. Isozyme alleles for strains from *Fusarium nygamai*, *F. andiyazi*, *F. pseudonygamai*, and the known mating populations of *Gibberella fujikuroi*

Strain ^x	Isozyme ^y			
	FUM-M	IDH-M	MDH-M	TPI-S6
<i>F. andiyazi</i>	γ	β	γ	α
<i>F. nygamai</i> (3997, 3998, 4010, 4012)	γ	ω	α	α
<i>F. nygamai</i> (4011)	α	δ (new)	α	α
<i>F. pseudonygamai</i> (1247, 2203, 4149, 4150)	γ	γ	γ	α
<i>F. pseudonygamai</i> (1240)	γ	α/β (new)	γ	α
<i>G. fujikuroi</i> mating populations ^z				
A (<i>G. moniliformis</i>)	α	β	α	β
B (<i>G. sacchari</i>)	α or β	β	α	α
C (<i>G. fujikuroi</i>)	α	β	β	α
D (<i>G. intermedia</i>)	α or β	β	β	α
E (<i>G. subglutinans</i>)	δ	γ	γ	α
F (<i>G. thapsina</i>)	γ	α or β	α	α
G (<i>G. nygamai</i>)	α (or γ)	ω	α	α
		(β or δ or)		

^x Strain number from the MRC strain collection, for *F. nygamai*, *F. pseudonygamai* and *F. andiyazi*, or the letter designating the mating population of *G. fujikuroi*.

^y Isozymes identified from separations in starch gels. The three letters before the dash indicate the isozyme, while the letters and numbers following the dash indicate the buffer. For procedural details, see Huss et al. (16). Isozymes: FUM, fumarase (EC 4.2.1.2); IDH, NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42); MDH, malate dehydrogenase (EC 1.1.1.37); and TPI, triose phosphate isomerase (EC 5.3.1.1). Buffers: M, morpholine and S6, Soltis no. 6. Isozyme forms are the same as those given in Huss et al. (16), except for the IDH forms designated "new", which are reported here for the first time and illustrated in Figure 1.

^z Isozyme data for *G. fujikuroi* mating populations A to G is a composite of the data of Huss et al. (16). The less-common alleles are in parentheses. MP-A = *F. verticillioide*s, MP-B = *F. sacchari*, MP-C = *F. fujikuroi*, MP-D = *F. proliferatum*, MP-E = *F. subglutinans*, MP-F = *F. thapsinum*, and MP-G = *F. nygamai*.

Fig. 1. Resolution of NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42) on starch gels showing new forms identified in this study. **A**, Lane 1 (*Gibberella nygamai* FGSC 8934, ω), lane 2 (*G. nygamai* MRC 3997, ω), lane 3 (*G. nygamai* MRC 3998, ω), lane 4 (*G. nygamai* MRC 4010, ω), lane 5 (*G. nygamai* MRC 4011, δ), lane 6 (*G. nygamai* MRC 4012, β), lane 7 (*Fusarium sacchari* FGSC 7610, β), lane 8 (*F. sacchari* FGSC 7611, β), lane 9 (*G. nygamai* FGSC 8933, ω), and lane 10 (*G. thapsina* FGSC 7056, α). **B**, Lane 1 (*G. intermedia* FGSC 7615, β), lane 2 (*G. nygamai* FGSC 8934, ω), lane 3 (*G. thapsina* FGSC 7056, α), lane 4 (missing), lane 5 (*F. pseudonygamai* MRC 1240, α/β), lane 6 (*F. pseudonygamai* MRC 1247, γ), lane 7 (*F. pseudonygamai* MRC 2203, γ), lane 8 (*F. pseudonygamai* MRC 4149, γ), lane 9 (*F. pseudonygamai* MRC 4152, γ), and lane 10 (*G. subglutinans* FGSC 7616, γ).

with their respective toxicity indices (Table 2). Two of the most toxic species (*F. thapsinum* and *F. pseudonygamai*) also produced the most moniliformin. The high levels of moniliformin (270 to 4,800 µg/g) produced by all of the strains of these two species probably were responsible for their low toxicity indices and correspondingly high levels of toxicity to ducklings. Some strains of *F. nygamai* also produced relatively high levels of moniliformin (up to 2,000 µg/g). None of the strains in the least toxic species (*F. andiyazi*) produced any moniliformin.

The highest levels of FB₁ were produced by *F. verticillioide*s, ranging from 120 to 3,700 µg/g, and *F. nygamai*, ranging from 1,500 to 3,800 µg/g. The highest FB₂ producer was *F. nygamai* (ND-5,700 µg/g) followed by *F. verticillioide*s (20 to 800 µg/g). The ratios of FB₁/FB₂ produced by *F. verticillioide*s were 4.6 to 12.5/1 compared with 0.3 to 3.3/1 for *F. nygamai*. One strain of *F. nygamai* (MRC 4012) produced more FB₂ than FB₁. Strains of *F. thapsinum* produced, at most, traces of FB₁ or FB₂, and none of the strains of *F. andiyazi* and *F. pseudonygamai* produced any detectable fumonisins.

Sorghum pathogenicity. Strains of all five *Fusarium* taxa were pathogenic to sorghum seedlings, as indicated by disease severity rank means that were significantly higher ($P < 0.05$) than the uninoculated control (Table 5). Based on mean values, *F. andiyazi* was significantly more virulent to the seedlings than were *F. verticillioide*s, *F. nygamai*, and *F. pseudonygamai* with respect to disease severity, root length, shoot length, and root dry mass. However, *F. andiyazi* was significantly less virulent to sorghum than *F. thapsinum*, with respect to all of these parameters as well as root dry mass.

DISCUSSION

Differentiating *Fusarium* spp. in the *G. fujikuroi* species complex is difficult without relying on DNA-based techniques (35,

68). The five taxa examined in this study were first separated on the basis of morphology with the molecular data collected later to support the tentative differentiation of these groups. We have proposed (34,36,44,68,76) that strains sharing ≥65% of their AFLP bands are conspecific, and that those sharing ≤40% definitely are not. In at least two cases, i.e., the C and D mating populations of *G. fujikuroi* (36) and lineages 6 and 7 of *G. zeae* (20), in which the AFLP similarities are >40% but <65%, members of the different groups retain some cross-fertility. We used the 40 and 65% similarity cutoffs to identify our strains of *F. pseudonygamai* to species through a comparison with the ex-holotype strain. Based on AFLPs, all of the groups of strains we worked with are single species, except for *F. nygamai*. Our five *F. nygamai* strains probably represent two species, because MRC 4012 is <26% similar to the other four *F. nygamai* strains. Our inability to obtain fertile crosses even with the *G. nygamai* mating-type tester strains made it impossible for us to test whether this strain belonged to the same biological species, and we suggest that *F. nygamai* as described by Burgess and Trimboli (6) is heterogeneous, i.e., MRC 4012 probably is a member of a cryptic species that has yet to be clearly separated from *F. nygamai*.

Methods other than AFLPs also could be used to distinguish these species. DNA sequences suffice to distinguish these species (44,54,55,76), with portions of the genes encoding β-tubulin (*tub*) and translocation elongation factor 1-α (*tef*) the most commonly analyzed sequences. Isozymes were used previously to separate *F. verticillioide*s, *F. nygamai*, and *F. thapsinum* (16). Both *F. andiyazi* and *F. pseudonygamai* have γ alleles for FUM and MDH, a pattern that is thus far unique to them. These two species can be distinguished based on their IDH alleles, for which *F. andiyazi* has the β allele and *F. pseudonygamai* has either the γ allele or a unique α/β allele. Until female-fertile strains are identified, probably in a larger population-based study of these species, and mating-type tester strains are synthesized, sexual crosses for

TABLE 4. Amplified fragment length polymorphism (AFLP) similarities between the strains of *Fusarium andiyazi*, *F. nygamai*, *F. pseudonygamai*, *F. thapsinum*, and *F. verticillioide*s^w

Strain ^x	6122 ^y	3997 ^y	6001	1247	7537 ^y	826				
<i>F. andiyazi</i>							5993	5995	6123	6126
5993	0.926 ^z	0.255	0.207	0.289	0.265	0.198	1.000	...	...	...
5995	0.947	0.236	0.207	0.289	0.245	0.198	0.936	1.000	...	...
6123	0.957	0.236	0.224	0.289	0.245	0.198	0.915	0.968	1.000	...
6126	0.957	0.239	0.206	0.292	0.247	0.178	0.968	0.946	0.968	1.000
<i>F. nygamai</i>							3998	4010	4011	4012
3998	0.286	0.724	0.246	0.281	0.209	0.278	1.000	...	...	...
4010	0.252	0.763	0.305	0.305	0.264	0.283	0.756	1.000	...	...
4011	0.250	0.721	0.306	0.245	0.242	0.283	0.714	0.796	1.000	...
4012	0.170	0.231	0.202	0.185	0.183	0.255	0.262	0.212	0.208	1.000
<i>F. pseudonygamai</i>							1240	2203	4149	4152
1240	0.262	0.265	0.207	0.900	0.889	0.272	1.000	...	...	...
2203	0.235	0.222	0.212	0.827	0.819	0.265	0.881	1.000	...	...
4149	0.304	0.262	0.234	0.766	0.779	0.227	0.921	0.735	1.000	...
4150	0.247	0.214	0.222	0.848	0.740	0.280	0.902	0.816	0.753	1.000
<i>F. thapsinum</i>							6002	6003	6004	6148
6002	0.220	0.704	0.980	0.216	0.214	0.271	1.000	...	...	...
6003	0.220	0.704	0.980	0.216	0.214	0.271	1.000	1.000	...	...
6004	0.220	0.704	0.980	0.216	0.214	0.271	1.000	1.000	1.000	...
6148	0.252	0.676	0.736	0.190	0.170	0.263	0.748	0.748	0.748	1.000
<i>F. verticillioide</i> s							1065	4315	4317	4321
1065	0.189	0.255	0.265	0.309	0.286	0.857	1.000	...	...	...
4315	0.196	0.243	0.253	0.277	0.253	0.932	0.813	1.000	...	...
4317	0.196	0.224	0.274	0.255	0.232	0.909	0.835	0.909	1.000	...
4321	0.182	0.233	0.286	0.289	0.264	0.952	0.828	0.905	0.905	1.000

^w Comparisons in which the two strains share <40% of the bands in the AFLP fingerprints are shown with a shaded background.

^x MRC strain number.

^y MRC 6122, MRC 7537, and MRC 3997 are ex-holotype strains of *F. andiyazi*, *F. pseudonygamai*, and *F. nygamai*, respectively.

^z Similarity was calculated as the Dice coefficient of pairwise distances between each strain.

identifying these species will not be possible. Until then, we anticipate that most workers will encounter difficulty if using only the described morphological features to distinguish these species.

Strains from seven of the known mating populations in the *G. fujikuroi* species complex have been recovered from sorghum, although the F mating population (*F. thapsinum*) usually dominates (26,30,37). Thus far, *F. andiyazi* has been recovered from sorghum in Ethiopia, Nigeria, South Africa, and the United States (44). Some of the “sterile” isolates recovered by Mansuetus et al. (37) from Tanzania and the unusual isolates of *F. nygamai* from Nigeria, Lesotho, and Zimbabwe (56–58), which were not cross-fertile with the *G. moniliformis*, *G. thapsinum*, or *G. nygamai* testers (23), also could be *F. andiyazi*. We know less about the geographic distribution of *F. pseudonygamai*, but the five strains used in this study originated from pearl millet grain in Namibia (43), and we have identified isolates of this species from maize in the United States and from sorghum and pearl millet from Mali and Nigeria (J. F. Leslie and K. A. Zeller, unpublished data).

All five of the species we examined occur in Africa, with *F. verticillioides* common on maize, *F. thapsinum* and *F. andiyazi* on sorghum, and *F. pseudonygamai* on pearl millet. *F. proliferatum*, which can make both fumonisins and moniliformin, also occurs in Africa and can be recovered from maize, sorghum, and millet. Much of the previous literature referring to *F. moniliforme*

from sorghum and millet probably refers to *F. thapsinum*, *F. andiyazi*, and/or *F. pseudonygamai*, and not to the sibling species *F. verticillioides*. Of these three sorghum and millet fungi, none produce high levels of fumonisins, two (*F. thapsinum* and *F. pseudonygamai*) produce high levels of moniliformin, and one (*F. andiyazi*) produces no more than a trace of either of these toxins. Thus, subsistence level farmers relying on sorghum or millet as their primary food are much more likely to encounter high levels of moniliformin in their diet, while those that rely on maize are more likely to encounter high levels of fumonisins (because *F. verticillioides* is the dominant *Fusarium* species on maize). Significant human health problems have not been convincingly associated with moniliformin, but this compound is toxic to animals such as ducklings, rats, mice, chickens, and swine (38,39).

In contrast with the animal toxicity data, the *F. andiyazi* strains caused significantly more disease on sorghum than did the *F. nygamai* and *F. pseudonygamai* strains. *F. nygamai* is not commonly regarded as an economically important plant pathogen, although it has been reported to cause reduction in levels of the parasitic weed *Striga hermonthica* in sorghum (4). Strains of *F. nygamai* parasitic to *Striga asiatica* were described as *F. brevicatenulatum* (53), and these strains of *F. nygamai* could belong to *F. brevicatenulatum* and not to *F. nygamai*. Our pathogenicity

TABLE 5. Disease severity and root and shoot length and dry mass of sorghum seedlings inoculated with strains of *Fusarium thapsinum*, *F. andiyazi*, *F. verticillioides*, *F. nygamai*, and *F. pseudonygamai*

Strain ^x	Disease severity ^{yz}	Shoot		Root	
		Dry mass (mg) ^z	Length (mm) ^z	Dry mass (mg) ^z	Length (mm) ^z
<i>F. thapsinum</i>					
6001	440 a	39 h	210 gh	14 fgh	66 i
6002	430 a	43 gh	220 gh	16 defg	66 i
6003	450 a	40 h	190 i	13 gh	68 hi
6004	440 a	39 h	190 i	13 gh	66 i
6148	460 a	39 h	210 hi	12 h	67 hi
Mean	440 E	40 E	210 D	13 B	67 D
<i>F. andiyazi</i>					
5993	380 ab	46 fgh	250 ef	17 bcdef	66 i
5995	330 bc	52 bcdef	270 cd	18 abcd	80 h
6122	380 ab	43 gh	240 f	18 abcde	76 hi
6123	460 a	43 gh	230 fg	16 cdefg	71 hi
6126	310 bc	50 efg	260 de	19 abc	76 hi
Mean	370 D	47 D	250 C	17 A	74 C
<i>F. verticillioides</i>					
826	150 efg	59 ab	300 a	18 abcde	120 ab
1065	240 cde	50 defg	280 bc	15 efg	100 efg
4315	210 def	59 abc	290 ab	19 ab	120 abcde
4317	260 cd	57 abcde	290 ab	18 abcde	110 defg
4321	210 def	56 abcde	290 ab	18 abcde	110 bcdef
Mean	210 C	56 B	290 A	18 A	110 B
<i>F. nygamai</i>					
3997	170 defg	55 abcdef	290 ab	18 abcde	110 cdefg
3998	260 cd	52 cdef	260 de	16 bcdef	98 g
4010	200 defg	61 a	290 ab	20 a	120 abcde
4011	100 gh	52 bcdef	280 bc	18 abcde	120 abcde
4012	130 fgh	54 abcde	280 bc	17 abcdef	100 fg
Mean	170B	55 BC	280 B	18 A	110 B
<i>F. pseudonygamai</i>					
1240	110 gh	51 def	280 bc	17 abcde	120 ab
1247	210 def	51 def	280 bcd	16 bcdef	100 fg
2203	140 fg	57 abcd	290 ab	19 abc	130 a
4149	160 defg	50 efg	280 bcd	16 bcdef	110 defg
4152	190 defg	51 def	280 bc	16 cdefg	100 fg
Mean	160 B	52 C	280 B	17 A	110 B
Control	43 h A	61 a A	290 ab A	17 abcde A	120 abc A

^x MRC strain number.

^y Rank mean.

^z Values within a column followed by the same letter are not significantly different ($P = 0.05$). Lowercase letters are used to compare individual strains. Uppercase letters are used to compare means of species.

tests showed that *F. thapsinum* > *F. andiyazi* > *F. verticillioides* > *F. nygamai* = *F. pseudonygamai* with respect to disease on sorghum seedlings. These results are somewhat different from those of Jardine and Leslie (17), who used 11 strains of *F. thapsinum* and two strains of *F. verticillioides* but found no significant differences in the ability of these strains to induce disease following stalk rot inoculation of mature sorghum plants. *F. thapsinum* probably is the major *Fusarium* pathogen of sorghum, but *F. andiyazi* and *F. verticillioides* also may be important when they colonize sorghum. *F. andiyazi* might be associated with other *Fusarium*-related diseases of sorghum, e.g., grain mold, in which *F. thapsinum* perhaps plays a minor role. *F. nygamai* and *F. pseudonygamai* also caused more disease than that observed in the control, but until more distribution data is published, the importance of these species under field conditions will be difficult to critically evaluate. Further studies, preferably with both greenhouse and field tests, however, will be needed to confirm these results.

Jardine and Leslie (17,18) had problems with cross-contamination when conducting pathogenicity tests with *F. thapsinum* and *F. verticillioides* on mature maize and sorghum plants. This cross-contamination is likely the result of the normal endophytic colonization of these plants by various *Fusarium* spp. One of the biggest problems encountered when screening maize and sorghum for resistance/sensitivity to *Fusarium* spp. is ensuring that the disease observed is due to the strain(s) that was inoculated and not to those already asymptotically present in the plant. The test tube assay may be one means of circumventing this problem, if the ratings obtained in this study are predictive of those obtained from studies with mature plants, and could allow comparisons between strains and a more rapid assessment of sensitivity/resistance to the diseases caused by these fungi.

In conclusion, we think that the recognition of different species within the *G. fujikuroi* species complex from sorghum and millet will make evaluation of the plant pathological and human health risks posed by these fungi, both individually and collectively, much easier to discern. Additional work is needed, especially with *F. andiyazi* and *F. pseudonygamai*, to confirm the breadth of their distribution, to determine their host preferences and mycotoxin profiles, to develop good mating-type tester strains and identify the teleomorph, and to provide breeders with the strains they need to rigorously challenge their material.

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