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Multilocus genotyping and molecular phylogenetics resolve a novel head blight pathogen within the *Fusarium graminearum* species complex from Ethiopia

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ABSTRACT

A survey of *Fusarium* head blight (FHB)-contaminated wheat in Ethiopia recovered 31 isolates resembling members of the *Fusarium graminearum* species complex. Results of a multilocus genotyping (MLGT) assay for FHB species and trichothecene chemotype determination suggested that 22 of these isolates might represent a new species within the *Fg* complex. Phylogenetic analyses of multilocus DNA sequence data resolved the 22 Ethiopian isolates as a novel, phylogenetically distinct species. The new species also appears to be novel in that MLGT probe data and sequence analysis of both ends of the TRI-cluster identified 15ADON and NIV recombination blocks, documenting inter-chemotype recombination involving the chemotype-determining genes near the ends of the TRI-cluster. Results of pathogenicity experiments and analyses of trichothecene mycotoxins demonstrated that this novel *Fg* complex species could induce FHB on wheat and elaborate 15ADON *in planta*. Herein the FHB pathogen from Ethiopia is formally described as a novel species.

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1. Introduction

Outbreaks and epidemics of *Fusarium* head blight (FHB) over the past two decades have had a devastating impact on the world's agricultural economy (reviewed in Goswami and Kistler, 2004). FHB attributed losses within North America during the 1990s were estimated to exceed $3 billion (Windels, 2000), due to significant reduction in cereal yield and quality, and price discounts associated with toxin-contaminated grain. Preventing trichothecenes from entering the food chain is an enormous challenge to animal health and food safety worldwide in that these sesquiterpenoids alter immune function (Pestka and Smolinski, 2005), inhibit eukaryotic protein synthesis (McLaughlin et al., 1977; Ueno et al., 1973), and have been linked to various mycotoxicoses of humans and other animals (Peraica et al., 1999; Ueno and Ishii, 1985). In addition, trichothecene mycotoxins are acutely phytotoxic and function as virulence factors on some cereals (Jansen et al., 2005; Maier et al., 2006). Trichothecene toxin differences also appear to be adaptive in that three strain-specific profiles, or chemotypes (Miller et al., 1991), have been maintained by balancing selection over multiple speciation events (Ward et al., 2002).

Members of the *Fusarium graminearum* species complex (*Fg* complex) are the primary etiological agents of FHB worldwide (O'Donnell et al., 2000, 2004; Starkey et al., 2007), and every species within the *Fg* complex is capable of producing B-trichothecenes *in planta*. Until recently, members of the *Fg* complex were thought to represent a single cosmopolitan species (*F. graminearum*; teleomorph *Gibberella zeae*) based on morphological species recognition (Booth, 1971; Gerlach and Nirenberg, 1982; Leslie and Summerell, 2006; Nelson et al., 1983). Robust hypotheses of species limits, employing phylogenetic species recognition based on genealogical concordance (GCPSR; Taylor et al., 2000), however, strongly indicated that *F. graminearum sensu lato* comprises at least 11 phylogenetically distinct species with marked biogeographic

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structure (O'Donnell et al. 2000, 2004; Starkey et al., 2007), a hallmark of allopatric speciation (Giraud et al., 2008). Fortunately, *F. graminearum sensu stricto* and *G. zeae* appear to be conspecific as virtually all FHB at their respective type localities in Europe and North America is caused by this species. To facilitate accurate communication of their geographic distribution, host range and mycotoxin potential within the scientific community, the ten previously unnamed species within the *Fg* complex were all formally described according to the international code of botanical nomenclature (O'Donnell et al., 2004; Starkey et al., 2007).

The primary objective of the present study was to expand our knowledge of FHB species and mycotoxin diversity by utilizing a high-throughput multilocus genotyping (MLGT) assay (Ward et al., 2008) to screen for novel FHB pathogen and trichothecene toxin diversity among fusaria recovered from wheat seed in Ethiopia. Results of the MLGT assay and multilocus molecular phylogenetics identified a novel FHB pathogen with trichothecene gene cluster termini reflective of inter-chemotype recombination. Isolates of this novel pathogen were characterized further by determining their morphological phenotypes, their ability to induce FHB on wheat and produce trichothecene mycotoxins *in planta*. Based on the results of these integrated analyses, employing the criterion of genealogical exclusivity to delimit species boundaries (GCPSR, Taylor et al., 2000), herein the newly discovered FHB pathogen from Ethiopia is formally described as a novel species within the *Fg* complex.

2. Materials and methods

2.1. Strains and multilocus genotyping

Thirty-one fusaria identified as *Fusarium* cf. *graminearum* were isolated from wheat seed variety HAR-1685 produced during the 2003 growing season in the Gugsa womberma ($N = 10$) and Bure districts ($N = 12$) in the Amhara region, and in the Arsi-robe district ($N = 9$) in the Oromia region of Ethiopia. Because morphological species recognition fails to differentiate seven of the species within the *Fg* complex (O'Donnell et al., 2004; Starkey et al., 2007), these isolates were accessioned into the ARS Culture Collection (NRRL) for species and trichothecene chemotype determination, using a multilocus genotyping (MLGT) assay (Ward et al., 2008).

2.2. Phylogenetic analysis of species limits

Based on the results of the MLGT assay, partial translation elongation factor (*EF-1α*, 725 bp), 3-*O*-acetyltransferase (*Tri101*, 1329 bp; Kimura et al., 1998), and ammonia ligase (*URA*, 1388 bp) gene sequences from the 31 Ethiopian isolates were generated. These sequences were combined with 58 sequences representing all known B-trichothecene clade species (Starkey et al., 2007), and then were analyzed as individual partitions by maximum parsimony to assess species identity. Results of these analyses indicated that 22 of the isolates might represent a new *Fg* clade species. Five of the putatively novel Ethiopian isolates (NRRL 46710 = et-2689, NRRL 46718 = et-26851 and NRRL 46722 = et-2681 from the Gugsa womberma district, and NRRL 46726 = et-23210 and NRRL 46738 = et-2325 from the Bure district) were selected for multilocus DNA sequencing (13 genes totaling 16.3 kb) to assess their evolutionary relationships and species status employing genealogical concordance phylogenetic species recognition (GCPSR; Taylor et al., 2000; Dettman et al., 2003a,b). To produce a multilocus alignment that accurately reflected positional homology, three regions within the intergenic B region of the mating type locus (MAT) totaling 275 bp were excluded as ambiguously aligned from all subsequent analyses.

Unweighted maximum parsimony (MP) analyses of individual and combined data partitions were conducted in PAUP* v. 4.0b10 (Swofford, 2002), using the heuristic search option with 1000 random sequence addition replicates, MULPARS on, and the tree bisection–reconnection (TBR) branch-swapping algorithm. Results of a hierarchical likelihood ratio test using MrModeltest ver. 2.2 (Posada and Crandall, 1998), evaluated in PAUP*, indicated that the general-time-reversible model with a proportion of invariant sites and gamma distributed rate heterogeneity (GTR + I + Γ) was the best-fit model of nucleotide substitution for maximum likelihood (ML) analysis of the combined dataset. The best ML tree received a negative log likelihood (−lnL) score of −40205.67700 based on the results of 10 independent ML heuristic phylogenetic analyses, using the GTR + I + Γ model of nucleotide substitution in GARLI (Zwickl, 2006). Clade support was assessed via nonparametric bootstrapping of the combined dataset under MP and ML criteria, employing 1000 pseudo-replicates of the data. MP bootstrap analyses were conducted with PAUPRat (Sikes and Lewis, 2001) in PAUP*, using 10 random addition sequences per replicate and TBR branch swapping. ML bootstrapping was conducted with GARLI (Zwickl, 2006), after halving the default generations without improving the topology parameter to 5000. MP and ML bootstrapping of the individual partitions employed, respectively, 1000 and 200 pseudo-replicates of the data.

DNA sequence data generated for the five isolates of the new *Fg* complex species from Ethiopia have been deposited in GenBank under accession numbers FJ240230-FJ240349. GenBank accession numbers for the other isolates included this study have been reported previously (Starkey et al., 2007). The name *Fusarium aethiopicum* has been registered in MycoBank (www.mycobank.org).

2.3. Pathogenicity experiments, mycotoxin and phenotypic analyses

The highly susceptible hard red spring wheat cultivar Norm was used for all pathogenicity experiments as reported previously (Goswami and Kistler, 2005; Starkey et al., 2007). Pathogenicity of four isolates of the new *Fg* complex species was compared with the moderately pathogenic *F. graminearum* strain NRRL 31084 (=PH-1), which was used as a positive control, and the non-host pathogen, *F. verticillioides* NRRL 20956, which was included as a negative control. Analyses of trichothecene mycotoxin production *in planta* followed published protocols (O'Donnell et al., 2004; Ward et al., 2002). Trichothecene metabolite profiles were used to assign the four Ethiopian isolates analyzed for mycotoxins to a 15-acetyldeoxynivalenol (15ADON) chemotype (Rodrigues-Fo et al., 2002). Phenotypic analyses followed protocols described previously (Aoki and O'Donnell, 1999; O'Donnell et al., 2004). The holotype of the new species described in the present study (NRRL 46726 = et-23210) has been deposited in the U.S. National Fungus Collection (BPI 878409), USDA/ARS, Beltsville, MD, USA, as a dried culture. Cultures are available from the ARS Culture Collection (NRRL), the Centraalbureau voor Schimmelcultures (CBS) Fungal Diversity Center, and the Fusarium Research Center (FRC).

2.4. Trichothecene gene cluster evolution

DNA sequences of the *TRI3*, *TRI7*, *TRI8*, *TRI12* and *TRI13* genes from four *F. aethiopicum* isolates and 40 additional isolates used in a previous analysis of trichothecene gene cluster evolution were determined or obtained from public databases (Ward et al., 2002). DNA sequences were edited and aligned with Sequencher (version 4.1.2, Gene Codes). Phylogenetic analyses were conducted under a distance framework using the neighbor-joining algorithm and the Kimura two-parameter model as implemented in MEGA (version 4.0). Relative support for individual nodes was assessed by bootstrap analysis with 2000 replications. DNA sequences have been

deposited in the GenBank database (Accession numbers FJ152462-FJ152481).

3. Results and discussion

3.1. Species limits, phylogeny and biogeography

A high-throughput multilocus genotyping (MLGT) assay (Ward et al., 2008) was used to screen 31 *Fusarium* cf. *graminearum* isolates from Ethiopia for FHB pathogen diversity and trichothecene toxin potential. All nine isolates from the Arsi-robe district produced positive results with both of the MLGT probes specific to *F. boothii*. However, the MLGT results for isolates from the Gugsa womberma ($N = 10$) and Bure districts ($N = 12$) indicated that these 22 isolates might represent a new B-trichothecene clade species within the *Fg* complex. These isolates produced positive results with clade probes for the B-trichothecene lineage and the *Fg* complex, but produced negative results with probes specific to the 11 previously described species within the *Fg* complex (O'Donnell et al., 2000, 2004; Starkey et al., 2007).

The species identity of the 31 isolates was assessed further via unweighted maximum parsimony analyses of partial translation elongation factor (*EF-1α*, 725 bp), 3-*O*-acetyltransferase (*Tri101*, 1329 bp), and ammonia ligase (*URA*, 1388 bp) gene sequences. The results of the parsimony analyses were consistent with the MLGT data, confirming that all nine isolates from the Arsi-robe district were *Fusarium boothii*, and indicating that the remaining 22 isolates might represent a novel species within the *Fg* complex (not shown). In order to rigorously assess evolutionary relationships and the species status of the latter group, a 13-gene multilocus DNA sequence dataset was constructed for five of these novel isolates and analyzed phylogenetically together with sequences of 58 isolates chosen to represent all known B-FHB species (Fig. 1). The results of parsimony analyses for the eight individual data-partitions and the combined 13-gene dataset (16.3 kb/strain) are summarized in Table 1. The genealogical exclusivity (monophyly) of the novel isolates from Ethiopia was strongly supported by MP and ML bootstrap values for six of the eight individual partitions (>80%) and the combined 13-gene dataset (100%; Fig. 1; Table 1). Evolutionary relationships for this group were unresolved by the *EF-1α* and ITS-28S rDNA partitions. However, analyses of these partitions were uninformative and did not contradict the monophyly of the novel Ethiopian isolates relative to other species within the *Fg* complex. Using reciprocal monophyly as the exclusivity criterion under GCPSR, the results of the phylogenetic analyses strongly support the recognition of these isolates as a novel species within the *Fg* complex. Herein this novel species is formally described as *Fusarium aethiopicum* (see Appendix A).

Morphological species recognition failed to distinguish *F. aethiopicum* from three other species within the *Fg* complex (i.e., *F. graminearum*, *F. vorosii and F. asiaticum*), all of which produce 5-septate macroconidia of similar length and width (Figs. 2 and 3; also see Table 5 in Starkey et al., 2007). This result is not surprising given the well-documented limitations of morphological species recognition in delimiting agriculturally (Couch and Kohn, 2002; Fournier et al., 2005; Le Gac et al., 2007; Geiser et al., 1998; Schardl and Craven, 2003; Starkey et al., 2007; Zhang et al., 2006) and medically important species (Balajee et al., 2005; Bovers et al., 2008; Cruse et al., 2002; Fisher et al., 2002; Koufopanou et al., 1997; Matute et al., 2006; Pringle et al., 2005; Zhang et al., 2006). In contrast, the use of reciprocal monophyly as an exclusivity criterion for defining species limits in Fungi (reviewed in Taylor et al., 2000; Taylor et al., 2006a,b), plants (Baum and Donoghue, 1995) and animals (Avise and Wollenberg, 1997) has gained widespread acceptance within the systematic community over the past decade, due to its utilitarian approach and foundation in population and molecular evolutionary genetics. Recent coalescent-based approaches suggest the genealogical non-discordance criterion (*sensu* Dettman et al., 2003a,b) employed in the present study is overly conservative, particularly given the likelihood of encountering incomplete lineage sorting in recently evolved species (Knowles and Carstens, 2007; Maddison and Knowles, 2007) . However, this underscores the fact that species recognized under this operational criterion, such as *F. aethiopicum*, likely have an extended history of genetic isolation.

In combined analysis of the 13-gene dataset, *F. aethiopicum* formed a strongly supported clade with *F. acaciae-mearnsii* and a phylogenetically distinct species represented by a single strain from South African soil (NRRL 34461 = FRC R-8601 *Fusarium* sp.). In an earlier study, NRRL 34461 was provisionally included in a phylogenetically diverse *F. acaciae-mearnsii* (Starkey et al., 2007). However, inclusion of the *F. aethiopicum* isolates in the present study strongly indicated that NRRL 34461 *Fusarium* sp. and *F. acaciae-mearnsii* are phylogenetically distinct. Of further note, NRRL 34461 *Fusarium* sp. did not form a clade with *F. aethiopicum* or *F. acaciae-mearnsii* in bootstrap analyses of the individual partitions, indicating that it is not an interspecies hybrid. It is noteworthy that NRRL 34461 *Fusarium* sp. and *F. acaciae-mearnsii* also are morphologically distinct in that they form asymmetric conidia that are widest above and below the mid-region, respectively (Starkey et al., 2007). However, we have refrained from formally describing NRRL 34461 *Fusarium* sp. in anticipation that additional isolates will become available for study, thereby allowing its species identity and exclusivity to be rigorously assessed.

The phylogenetic relationships between *F. aethiopicum*, *F. acaciae-mearnsii* and NRRL 34461 *Fusarium* sp. indicate that this clade may be endemic to the Southern Hemisphere (eg., Africa or Australia). All of the *F. aethiopicum* were isolated in Ethiopia, while NRRL 34461 was isolated from South African soil. *F. acaciae-mearnsii* was initially identified from South Africa, but is most likely native to Australia, given the Australian origin of its host and two genetically diverse isolates of this species (i.e., NRRL 34207 and 34197). Under this scenario, this stem canker and collar rot pathogen of *Acacia mearnsii* (Roux et al., 2001) may have been introduced to South Africa from Australia along with the host.

3.2. Pathogenicity, toxin production and trichothecene gene cluster evolution

Two multilocus haplotypes were identified among the 22 *F. aethiopicum* isolates examined, and two representatives of each of these haplotypes were selected for analyses of pathogenicity, toxin production and trichothecene gene cluster evolution. Consistent with observations of FHB in Ethiopian wheat fields and stored seed lots, results of the pathogenicity experiment conducted on red hard spring wheat cultivar Norm demonstrated that all four strains of *F. aethiopicum* tested could induce head blight in an environmentally controlled greenhouse experiment. As noted for other species within the *Fg* complex (Goswami and Kistler, 2005), the four isolates tested varied in their aggressiveness on wheat. Unlike controls inoculated with the non-host pathogen *F. verticillioides*, all *F. aethiopicum* strains produced typical FHB disease symptoms on wheat even though three of the four strains caused significantly less disease than the moderately aggressive *F. graminearum* strain 31084 ($p < 0.05$; Supplementary Table).

Trichothecene mycotoxin analyses of *F. aethiopicum* infected wheat spikelets demonstrated that all isolates were highly toxigenic. Each strain accumulated significantly more DON in inoculated wheat than *F. graminearum* strain 31084 ($p < 0.04$), and levels of DON were comparable to some of the most toxigenic strains of *F. graminearum* found in North America (Goswami and

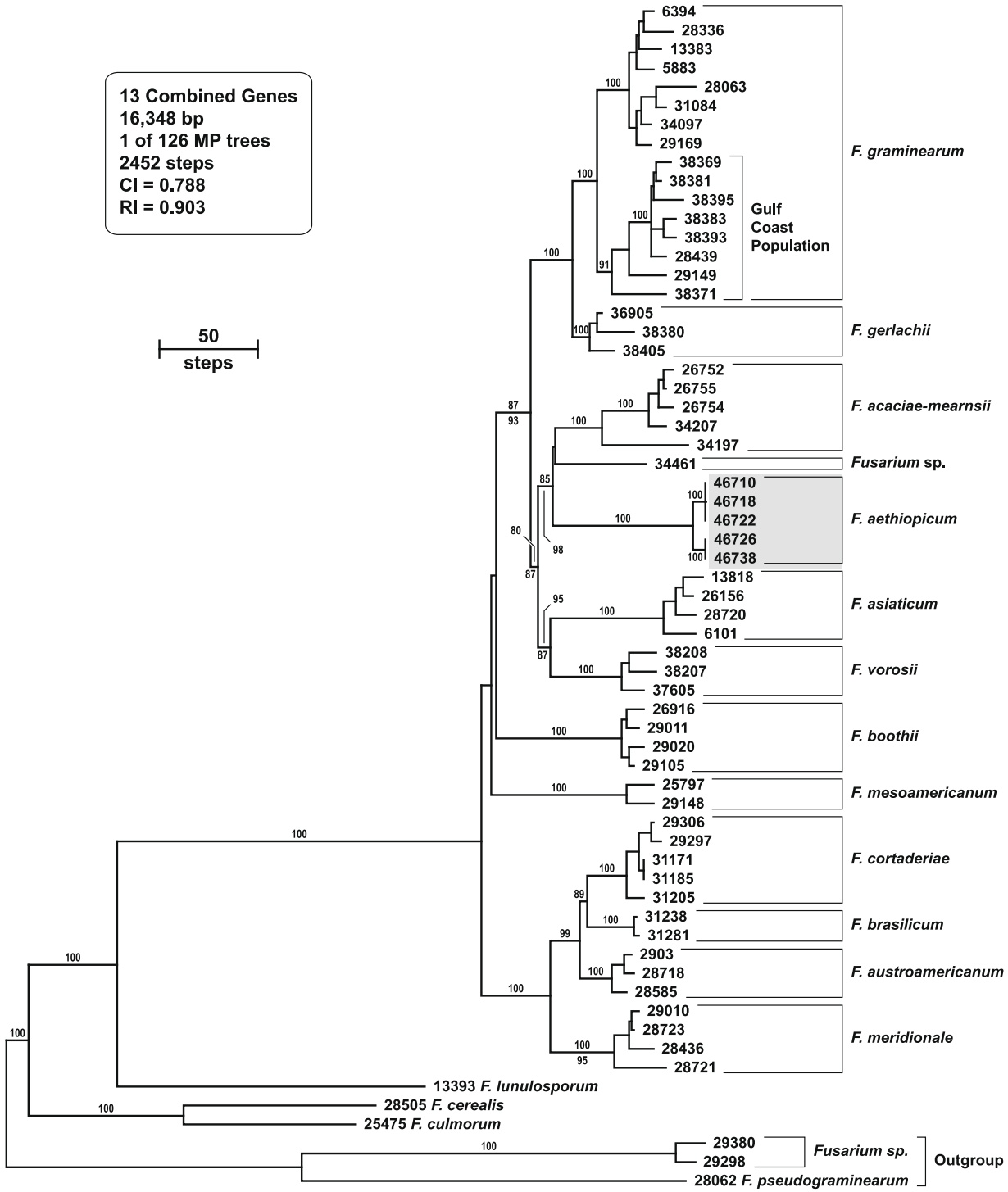

Fig. 1. One of 126 most-parsimonious phylograms inferred from the combined dataset of 16.3 kb from eight loci comprising portions of 13 nuclear genes rooted with sequences of *Fusarium pseudograminearum* NRRL 28062 and *Fusarium* sp. NRRL 29380 and 29298. Branches that received ⩾70% maximum parsimony (MP) bootstrap values are indicated above branches. A maximum likelihood bootstrap value of ⩾70% is indicated below a branch only if it differed by ⩾5% from that obtained by MP bootstrapping. Note that MP and ML bootstrap values for 27 of the 31 nodes differed by <5%. *F. aethiopicum* (shaded) forms a strongly supported clade with *F. acaciae-mearnsii* isolates from Australian soil (NRRL 34207 and 34197) and *Acacia mearnsii* stem canker from South African forestry plantations (NRRL 26752, 26754 and 26755; Roux et al., 2001) and NRRL 34461 *Fusarium* sp. from South African soil. A second bracket indicates the genetically divergent U.S. Gulf Coast population of *F. graminearum*.

Kistler, 2005; Supplementary Table). These experiments also demonstrated that these strains have a 15ADON chemotype and produce 15ADON as the major acetyl-ester deoxynivalenol derivative *in planta*. In MLGT analysis of the 31 Ethiopian isolates, the nine *F. boothii* isolates from the Arsi-robe district were positive for both of the probes targeting genetic variation specific to isolates with a 15ADON chemotype. However, the 22 *F. aethiopicum* isolates produced mixed results with the trichothecene chemotype probes, which target chemotype-specific variation at either end of the trichothecene biosynthetic and regulatory gene cluster (TRI-cluster; Ward et al., 2002, 2008). These isolates produced positive results with the 15ADON-specific probe from *TRI12*, which is con-

Table 1
Tree statistics and summary of phylogenetic analyses[a]

Locus[b]	Sequence length (bp)	# Haplotypes[c]	# MPTs	MPT length	CI	RI	Syn	Aut	Bootstrap support (%) *F. aethiopicum*[d]	# *Fg* species supported as monophyletic[c]
α-Tubulin (1)	1686	32	32	114	0.94	0.97	66	41	86	7
β-Tubulin (4)	1257	38	90	108	0.78	0.85	50	31	93, 92	3
EF-1α (2)	648	34	>200,000	86	0.91	0.96	55	20	<70	5
Histone H3 (2)	449	26	18	70	0.86	0.95	39	18	87, 80	5
MAT (2)	5910	58	48	958	0.84	0.94	514	245	100	11
URA-Tri101-PHO (4)	4124	50	336	691	0.8	0.91	368	161	100	10
ITS-28S rDNA (4)	1133	17	2	23	0.96	0.97	8	14	<70	0
Reductase (2)	1141	43	1948	261	0.87	0.91	138	75	81, 82	9
Combined	16,348	59	126	2452	0.79	0.9	1238	605	100	11

[a] *Abbreviations used:* bp, base pairs; MPTs, most-parsimonous trees; CI, consistency index; RI, retention index; Syn, synapomorphy or parsimony informative character; Aut, autapomorphy, uniquely derived character or parsimony uninformative character.
[b] The number in parenthesis indicates the chromosome location in the genetic and physical map (Gale et al., 2005) and whole genome sequence of NRRL 31084 = PH-1 *F. graminearum* (Cuomo et al., 2007).
[c] Haplotypes were identified using DnaSP Ver. 4.20.2 (Rozas et al., 2003), including gapped sites.
[d] MP and ML bootstrap values supporting *F. aethiopicum* monophyly were identical for five of the individual partitions and the combined dataset, and were comparable for the other three partitions where MP and ML values are listed left-to-right.

sistent with the toxin production data, but were also positive for the nivalenol (NIV)-specific probe from *TRI3*.

In order to determine the basis for the disagreement between *TRI3* and *TRI12* chemotype probes, we performed phylogenetic analyses of DNA sequences from *TRI3*, *TRI12*, and three additional genes located at either end of the TRI-cluster (Fig. 4) that are directly responsible for chemotype differences. The phylogenetic placement of the *F. aethiopicum* sequences in gene genealogies from all of the examined TRI-cluster genes except *TRI3* was consistent with chemical analyses demonstrating that these isolates had a 15ADON chemotype (Fig. 5). In addition, the *F. aethiopicum* isolates harbored mutations in *TRI7* and *TRI13* that interrupted the open reading frames of these chemotype-determining genes, and which are typical of isolates with a 15ADON chemotype (Lee et al., 2002; Kim et al., 2003). However, the *TRI3* sequences from these isolates were clearly nested within the NIV chemotype clade (Fig. 5), indicating they were the result of recombination between chemotype lineages, and demonstrating that MLGT results for these isolates accurately reflected the evolutionary history of their TRI-cluster genes. While TRI-cluster haplotypes are mosaics of multiple evolutionary histories resulting from the joint effects of recombination and balancing selection, recombination between chemotype lineages appears to be limited at either end of the TRI-cluster by the need to maintain co-adapted allelic combinations for genes that directly contribute to chemotype differences (Ward et al., 2002). The *F. aethiopicum* isolates are unusual in this regard (Ward et al., 2002, 2008). However, *TRI3* genes from isolates with a NIV or 15ADON chemotype are predicted to encode fully functional 15-*O*-acetyltransferases, and the rarity of recombination between NIV and 15ADON chemotype lineages on the *TRI3* side of the TRI-cluster is likely due to the chemotype-specific pseudogene polymorphism in the adjacent *TRI7* gene (Kimura et al. 2003), which is required for production of 4-acetylnivalenol, but not DON or its acetyl-derivatives (Lee et al., 2002). All isolates with a predicted NIV chemotype possessed uninterrupted *TRI7* open reading frames, while 15ADON isolates possessed *TRI7* pseudogene sequences, and *TRI7* was deleted from the TRI-cluster of all 3ADON isolates examined.

3.3. Incorporation of *F. aethiopicum* probes into the MLGT assay

The previously published MLGT assay for species identification and trichothecene chemotype prediction (Ward et al., 2008) was updated by incorporating probes for identification of *F. aethiopicum* strains. DNA sequence data from the *F. aethiopicum* isolates were compared with DNA sequences from the 53 isolates used to design the original MLGT assay, and probes were designed to match substitutions that were specific to the *F. aethiopicum* isolates. Two probes, ATae(9), 5′-TAATCTTCTATATCAACATCTTAC GGCCAGCTAC CAGGCCTG-3′ and REDae(66), 5′-TAACATTACAACTATACTATCTAC CGGAAGAAACATGATGGGT-3′ were developed from variation identified in the *TRI101* and reductase genes respectively. Each probe was appended with a unique sequence tag (underlined) used to sort extension products via hybridization with fluorescent microspheres (Luminex Corporation) as described previously (Ward et al., 2008). The expanded MLGT assay was run as previously described (Ward et al., 2008) against an isolate panel consisting of the five *F. aethiopicum* isolates that were sequenced for the multilocus phylogenetic analyses and 53 isolates that were used in the design and validation of the original MLGT assay. Index of discrimination values (Ward et al., 2008) for ATae(9) and REDae(66) were 4.3 and 10.6 respectively, meaning that fluorescence intensity values for these probes were at least 4-fold higher among *F. aethiopicum* isolates than the highest values observed among the 53 isolates tested from the other species within the B-trichothecene lineage of FHB pathogens. Incorporation of the two new probes did not interfere

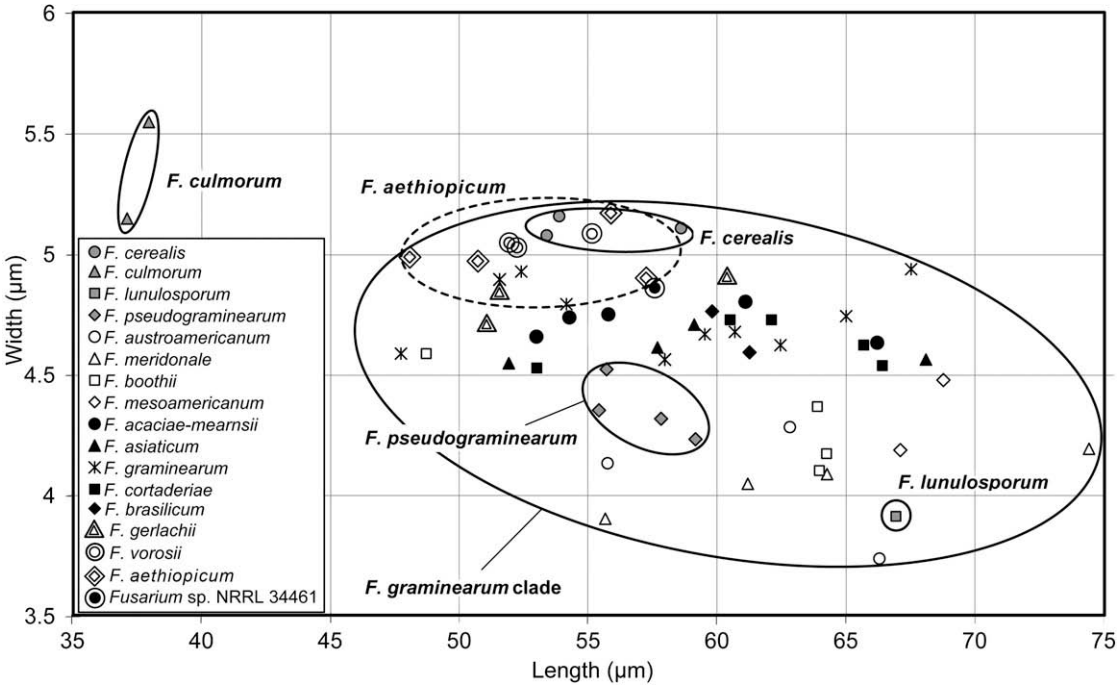

Fig. 2. Length and width of 5-septate conidia of B-trichothecene clade species cultured under continuous black light. The two newly delimited species include NRRL 34461 *Fusarium* sp. indicated by a white circle enclosing a black dot and *F. aethiopicum* represented by a white double-lined diamond. 5-Septate conidia formed by *F. aethiopicum* and three species within the *F. graminearum* species complex (i.e., *F. graminearum*, *F. vorosii* and *F. asiaticum*) are identical, indicating that these species cannot be distinguished using morphological species recognition.

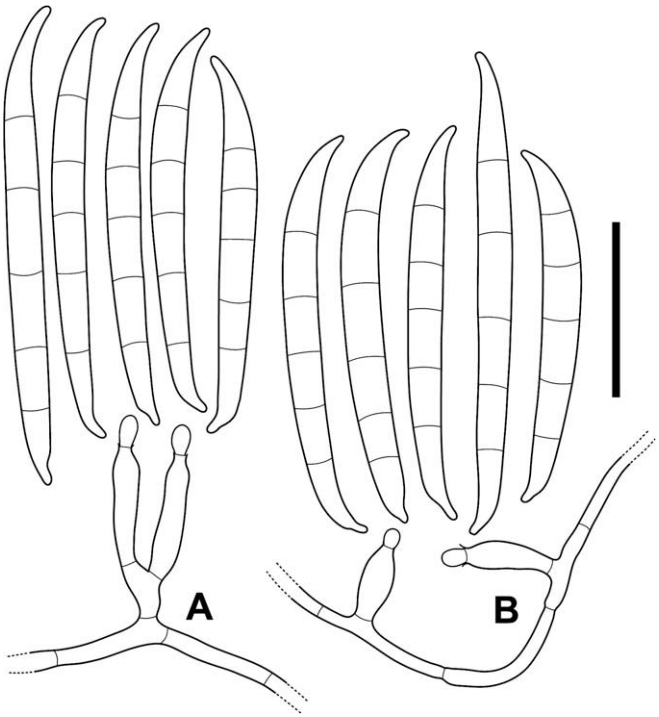

Fig. 3. Phialidic conidiophores and 5-septate sporodochial conidia formed by *Fusarium aethiopicum* on SNA under black light. Fusiform conidia are slightly curved and typically widest above the mid-region; they possess a prominent foot cell, but the distal most cells lack a narrow beak.

with the ability of the previously developed probes to provide for the identification of previously described species and trichothecene chemotypes. These results demonstrate that the expanded MLGT assay, consisting of 43 probes, provides for the identification of all 17 described B-FHB species and the three B-trichothecene chemotypes, and highlight the ability to easily expand the MLGT assay to accommodate novel variation.

4. Conclusions

One of the primary advances in our understanding of FHB pathogen evolution and diversity is the demonstration that morphological species recognition fails to resolve seven of the *Fg* complex species discovered by multilocus phylogenetics (O'Donnell et al., 2000, 2004; Starkey et al., 2007; Ward et al., 2002). Recognizing that the morphospecies *F. graminearum* comprises at least 13 phylogenetically distinct, biogeographically structured species, most of which appear to be foreign to North America, highlights the challenge FHB poses to plant disease specialists, plant breeders, and quarantine officials in a world interconnected by the global trade of agricultural and horticultural products. Considering that many *Fg* complex species appear to have evolved allopatrically (Giraud et al., 2008), it seems likely that several FHB pathogens have been introduced into non-indigenous areas relatively recently, especially in countries that rely extensively on agricultural imports (Lee et al., 2004; Monds et al., 2005; O'Donnell et al., 2004; Roux et al., 2001; Starkey et al., 2007). By analogy, the low FHB species diversity reported within North America (Gale et al., 2007; Ward et al., 2008; Zeller et al.,

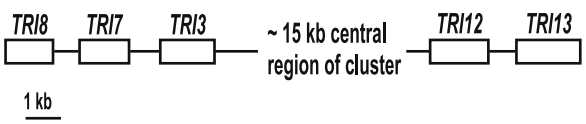

Fig. 4. Trichothecene (TRI) toxin biosynthesis and regulatory gene cluster organization in *Fusarium*. The five TRI genes sequenced are identified. Luminex probes for B-clade species and toxin chemotype determination target *TRI3* and *TRI12* near either end of the TRI cluster.

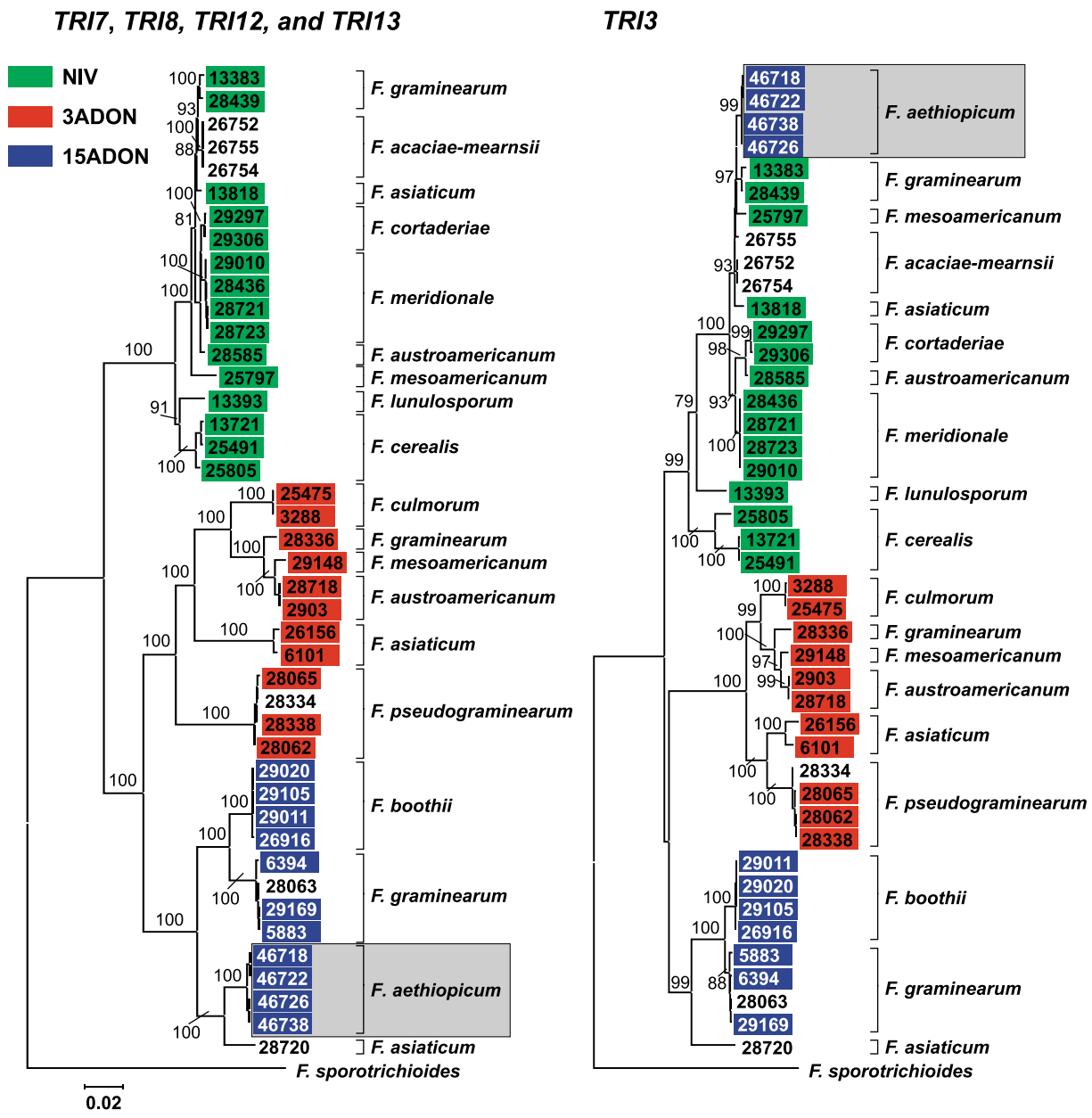

Fig. 5. Neighbor-joining bootstrapped phylograms inferred from a combined analysis of *TRI7*, *TRI8*, *TRI12* and *TRI13* and individual analysis of *TRI3* which nest isolates of *Fusarium aethiopicum*, respectively, within 15ADON and NIV chemotype clades. Because *TRI13* is a pseudogene in the *F. aethiopicum* isolates, they cannot produce NIV (Lee et al., 2002). Colored blocks indicate the chemotype of each strain (see legend). Numbers above nodes indicate the frequency (%) with which branches were recovered from 2000 bootstrap replicates of the data. Each phylogram is rooted with sequences of *F. sporotrichioides*.

2004), Europe (Gagkaeva and Yli-Mattila, 2004; Láday et al., 2004; Tóth et al., 2005; Waalwijk et al., 2003; Qu et al., 2008b), Japan and China (Suga et al., 2008; Qu et al., 2008a,b) may be attributed to a combination of factors, including stringent inspections of and/or less dependence on agricultural imports, together with biogeographic data suggesting that the majority of species within the *Fg* complex have evolutionary origins in the Southern Hemisphere (O'Donnell et al., 2004; Starkey et al., 2007). As FHB is a significant threat to cereal production worldwide, information on the global distribution of FHB pathogen diversity will be critical to identifying and implementing pathogen control strategies, and developing plant germplasm that offers broad resistance to a diverse complex of FHB pathogens. In the present study, a previously described MLGT assay was used to identify a novel FHB species from Ethiopia, demonstrating the utility of this assay as a high-throughput platform for B-clade species and trichothecene mycotoxin determination. As such, it provides active global molecular surveillance programs and quarantine officials worldwide with a requisite tool for detecting foreign FHB pathogens so that proactive steps can be taken to prevent them from being introduced into non-indigenous areas by anthropomorphic activities. Moreover, the increasing database of global molecular surveillance information is providing a detailed understanding of the geographic distribution, host range and evolutionary dynamics of the FHB pathogens and their toxin potential, all of which are critical for monitoring changes in and combating this economically devastating disease worldwide.

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Appendix A

Species description.

Fusarium aethiopicum O'Donnell, Aberra, Kistler et T. Aoki sp. nov. (Figs. 2 and 3)

Anamorphe morphologia idem ac *Fusarium graminearum, F. asiaticum et F. vorosii*. Conidia sporodochialia in SNA tantum sub illuminatione nigra formata, (1-)3-5(-7)-septata; 5-septata 37.5-48.1-57.3-71.5 × 4-4.90-5.17-6 μm, ex uno latere modice curvata, in dimidio superiore plerumque Paulo laëtiora. Positio systematicum per sequentiis α-tubulini, β-tubulini, HIS, MAT, reductasi, *URA, Tri101* et *PHO* a speciebus similibus differens.

Fusarium aethiopicum is indistinguishable morphologically from *F. graminearum, F. asiaticum* and *F. vorosii* based on colony and conidial characters on PDA and SNA under black light (Figs. 2 and 3). Five-septate sporodochial conidia formed by *F. aethiopicum* on SNA under continuous black light are ~5 μm wide on average (size ranges of total and average values: 5-septate: 37.5-48.1-57.3-71.5 × 4-4.9-5.2-6; ex Type = NRRL 46726; 43-55.9-70 × 4.5-5.2-6) μm, one side moderately curved and often widest above the mid-region, and lacking a narrow beak at the apex (Aoki and O'Donnell, 1999; O'Donnell et al., 2004). Delimitation of this species is strongly supported using the exclusivity criterion under GCPSR in which reciprocal monophyly received strong MP and ML bootstrap support from analyses of α-tubulin, β-tubulin, HIS, MAT, reductase, *URA-Tri101-PHO* and the combined dataset (Table 1). *F. aethiopicum* formed a robust clade with *F. acaciae-mearnsii* and NRRL 34461 *Fusarium* sp. in the multilocus phylogeny (Fig. 1), however, evolutionary relationships among these three species remain unresolved.

Distribution: Ethiopia.

HOLOTYPUS: BPI 878409, a dried culture, isolated from stored wheat grain, variety HAR-1685 *Triticum aestivum* L., in the Bure district in the west Gojam zone of the Amhara region of Ethiopia, Dereje Aberra 26 Feb., 2007, deposited in the herbarium of BPI, U.S.A. Ex-holotype culture: NRRL 46726 = et-23210 = CBS 122858. Other strains examined: NRRL 46710 = et-2689, NRRL 46718 = et-26851 = CBS 122856 and NRRL 46722 = et-2681 = CBS 122857 from the Gugsa womberma district, and NRRL 46738 = et-2325 = CBS 122859 from the Bure district, both located in the west Gojam zone of the Amhara region.

Etymology: *aethiopicum*; after the country where it was discovered.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2008.09.002.

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