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ABSTRACT

The ability to produce fumonisin mycotoxins varies among members of the black aspergilli. Previously, analyses of selected genes in the fumonisin biosynthetic gene (*fum*) cluster in black aspergilli from California grapes indicated that fumonisin-nonproducing isolates of *Aspergillus welwitschiae* lack six *fum* genes, but nonproducing isolates of *Aspergillus niger* do not. In the current study, analyses of black aspergilli from grapes from the Mediterranean Basin indicate that the genomic context of the *fum* cluster is the same in isolates of *A. niger* and *A. welwitschiae* regardless of fumonisin-production ability and that full-length clusters occur in producing isolates of both species and nonproducing isolates of *A. niger*. In contrast, the cluster has undergone an eight-gene deletion in fumonisin-nonproducing isolates of *A. welwitschiae*. Phylogenetic analyses suggest each species consists of a mixed population of fumonisin-producing and nonproducing individuals, and that existence of both production phenotypes may provide a selective advantage to these species. Differences in gene content of *fum* cluster homologues and phylogenetic relationships of *fum* genes suggest that the mutation(s) responsible for the nonproduction phenotype differs, and therefore arose independently, in the two species. Partial *fum* cluster homologues were also identified in genome sequences of four other black *Aspergillus* species. Gene content of these partial clusters and phylogenetic relationships of *fum* sequences indicate that non-random partial deletion of the cluster has occurred multiple times among the species. This in turn suggests that an intact cluster and fumonisin production were once more widespread among black aspergilli.

1. Introduction

The black aspergilli, also known as *Aspergillus* section *Nigri* (Gams et al., 1985), can cause food spoilage and occur worldwide on a wide variety of substrates, including soil, grains, forage crops, fruits, vegetables, nuts, cotton, and meat and dairy products (Raper and Fennell, 1965; Pitt and Hocking, 2007, 2009). Some black *Aspergillus* species are also important for commercial production of organic acids, hydrolytic enzymes, and fermented beverages (Hong et al., 2013; Raper and Fennell, 1965; Varga et al., 2000). Although multiple species of black aspergilli are difficult to distinguish from one another by morphology, differentiation between species has been facilitated by DNA sequence-based methods (Hong et al., 2013; Perrone et al., 2011; Varga et al., 2011). For example, a recent DNA-based phylogenetic analysis resolved *Aspergillus awamori* from a collection of *A. niger* isolates (Perrone et al., 2011). *A. awamori* was renamed *A. welwitschiae* because it is distinct from black aspergilli used for fermentation of the Okinawan beverage awamori, and it is identical to a previously described black *Aspergillus* isolates from the plant *Welwitschiae mirabilis* (Hong et al., 2013).

Some black aspergilli can produce the mycotoxins ochratoxins (Abarca et al., 2003, 2004; Wicklow et al., 1996; Varga et al., 2000; Cabanes et al., 2002; Sage et al., 2004; Samson et al., 2004) and fumonisins (Frisvad et al., 2007, 2011; Perrone et al., 2011). Reports of these mycotoxins in raisins, grape must, and wine have raised food safety concerns about the occurrence of black aspergilli on grapes (Logrieco et al., 2009, 2010). Fumonisins are a health concern because they can cause multiple diseases in experimental rodents and some livestock, and they are epidemiologically associated with several human diseases, including oesophageal cancer (Marasas et al., 2004). Fumonisins were first identified in cultures

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of the fungus *Fusarium* ~25 years ago, and there is an extensive literature on production by different species as well as the genetics and biochemistry of fumonisin biosynthesis in *Fusarium* (Alexander et al., 2009; Rheeder et al., 1992; Uhlig et al., 2012). In contrast, fumonisin production in black aspergilli was first reported less than a decade ago (Frisvad et al., 2007), and as a result, the literature on production in *Aspergillus* is relatively small.

Fumonisins consist of a linear polyketide-derived backbone with two methyl, one amine, one to four hydroxyl, and two tricarboxyl ester groups located at various positions along the backbone. Genetic and biochemical analyses of *Fusarium* identified a 16 to 17-gene fumonisin biosynthetic gene (abbreviated *FUM* in *Fusarium*, *fum* in *Aspergillus*) cluster that encodes biosynthetic enzymes necessary for formation of fumonisins as well as two transport proteins and a transcription factor (Alexander et al., 2009). A homologue of the *Fusarium FUM* cluster is present in the *A. niger* genome (Baker, 2006; Pel et al., 2007). The latter cluster includes homologues of 11 of the *Fusarium* genes: the polyketide synthase (*fum1*), hydroxylase (*fum3*, *fum6*, *fum15*), dehydrogenase (*fum7*), aminotransferase (*fum8*), acyl-CoA synthase (*fum10*), carbonyl reductase (*fum13*), condensation-domain protein (*fum14*), ABC transporter (*fum19*), and transcription factor (*fum21*) genes. The *A. niger* cluster also includes a short-chain dehydrogenase gene (designated here as *sdr1*) that is not present in the *Fusarium* cluster and whose function, if any, in fumonisin biosynthesis has not been reported (Baker, 2006; Pel et al., 2007). Notably absent from the *A. niger fum* cluster is a homologue of the *Fusarium FUM2* gene, which is responsible for hydroxylation of the fumonisin backbone at carbon atom 10 (Proctor et al., 2006). The lack of a *FUM2* homologue in the *A. niger* cluster is consistent with the results of a majority of studies showing that this species produces only fumonisins that lack a hydroxyl at carbon atom 10, e.g. fumonisins B_2, B_4, and B_6 (FB_2, FB_4, FB_6 respectively) (Frisvad et al., 2007, 2011; Mansson et al., 2010; Noonim et al., 2009).

With the recent taxonomic revision of the black aspergilli, fumonisin production has been confirmed in only two species: *A. niger* and *A. welwitschiae* (formerly *A. awamori*) (Perrone et al., 2011; Palumbo et al., 2013; Varga et al., 2010, 2011). However, the ability to produce fumonisins is not uniform among isolates of these two species. In six independent studies 28–81% of the *A. niger* and/or *A. welwitschiae* isolates examined produced fumonisins (Abrunhosa et al., 2011; Frisvad et al., 2011; Mogensen et al., 2010; Susca et al., 2010; Palumbo et al., 2011; Varga et al., 2010). This variation in ability begs the question: what is the genetic basis for the difference in fumonisin production and nonproduction in *A. niger* and *A. welwitschiae*? In one study that addressed this question in black aspergilli isolated from grapes grown in the Mediterranean Basin, the *fum8* gene was not detected in most fumonisin-nonproducing isolates of *A. niger* that were examined. However, this study was done before DNA sequence analyses resolved *A. niger* into *A. niger sensu stricto* and *A. welwitschiae* (Perrone et al., 2011; Varga et al., 2011; Hong et al., 2013). Because both of these species can occur on grapes (Palumbo et al., 2013), it is possible that the collection of *A. niger* examined by Susca et al. (2010) included isolates of *A. welwitschiae* as well as *A. niger*. A subsequent study examined the presence of eight *fum* genes (*fum1*, *fum3* [also designated as *fum9*], *fum6* – *fum8*, *fum13*, *fum14*, and *fum19*) in *A. niger* and *A. welwitschiae* isolates from grapes grown in California (Palumbo et al., 2013). All eight genes were detected in FB_2-nonproducing isolates of *A. niger*, but only *fum1* and *fum19* were detected in nonproducing isolates of *A. welwitschiae*. Thus, the results of two studies indicate that in *A. niger* and *A. welwitschiae* collectively, the absence of one or more *fum* genes could account for the FB_2-nonproduction phenotype in some isolates but not others.

The objective of the current study was to clarify the gene content and organization of the *fum* cluster in FB_2-nonproducing isolates of *A. niger* and *A. welwitschiae* recovered from grapes. PCR, Southern blot, and sequence data of the *fum* cluster region provide evidence that the *fum* cluster in nonproducing isolates of *A. niger* includes all 11 *fum* genes as well as *sdr1*, whereas the cluster in nonproducing isolates of *A. welwitschiae* has undergone a large deletion. These findings combined with the results of phylogenetic analyses suggest the mixtures of fumonisin-producing and nonproducing individuals arose independently in the two species. In addition, genome sequence data suggest that the *fum* cluster and perhaps fumonisin production were once more widespread among members of the black aspergilli than they currently are.

2. Materials and methods

2.1. Fungal cultures

In this study, we employ the species nomenclature for black aspergilli used by Hong et al. (2013). The 58 black *Aspergillus* isolates analyzed in this study were obtained from the agro-food important toxigenic fungi – ITEM (Istituto Tossine e Micotossine da Parassiti Vegetali) Culture Collection at the Institute of Sciences of Food Production (ISPA), National Research Council, Italy. Further information about the isolates (year of isolation, depositor, toxin production, etc.) are available at the ISPA Website (server.ispa.cnr.it/ITEM/Collection/). Fifty-one isolates were recovered from grapes grown in the Mediterranean Basin: nine from Greece (ITEM 5218, 5219, 5253, 5266, 5267, 5272, 5276, 5277, 5283), 15 from Italy (ITEM 4717, 4853, 4858, 4859, 4863, 6122, 6123, 6126, 6127, 6128, 7090, 7091, 7097, 7468, 10355), 16 from Turkey (ITEM 11945, 12918, 14266, 14270, 14273, 14275, 14281, 14282, 14286, 14297, 14303, 14305, 14307, 14308, 14309, 14310), three from Spain (ITEM 4947, 4951, 9720), six from Portugal (ITEM 4545, 4547, 4552, 6140, 6142, 6144), and two from Israel (ITEM 6292 and 6293). In addition, three isolates were recovered from cashew nuts, ITEM 11448 from Brazil and ITEM 11552 and ITEM 11553 from India; and two isolates were recovered from raisins, ITEM 10927 from Turkey and ITEM 10954 from Chile. The study also included the *A. niger* type strain ITEM 4501, which was recovered from a tannin-gallic acid fermentation in the USA, and the *A. welwitschiae* reference strain ITEM 4502 (Perrone et al., 2011), the origin of which is unknown.

2.2. Fumonisin analysis

All isolates were grown in darkness for 7 days on Czapek yeast autolysate medium with 20% sucrose (CY20S) solidified with agar according to the method published by Frisvad et al. (2007). Fumonisins were analyzed according to the method of Frisvad et al. (2007) with slight modifications (De Girolamo et al., 2011). One g of CY20S culture was extracted with 5 mL of methanol/water (70:30, v/v) on an orbital shaker for 60 min. One hundred μL of the extract were diluted with 900 μL of acetonitrile/water (30:70, v/v) and then filtered using RC 0.2 μm filters (Phenomenex, U.S.A.). Fifty μL of diluted extract was derivatized by mixing with 50 μL of o-phtaldialdehyde (OPA) for 50 s. using a high performance liquid chromatograph (HPLC) autosampler (Agilent, Waldbronn, Germany). Three min. after the start of derivatization, 100 μL of the extract was injected by full loop into an Agilent 1100 series HPLC (Agilent, Waldbronn, Germany). The analytical column was a Symmetry Shield RP18 15 cm × 4.6 mm, 5 μm with a guard column inlet filter (0.5 μm × 3 mm diameter, Rheodyne Inc. CA, USA), and the mobile phase consisting of a binary gradient was applied as follows: the initial composition of the mobile phase consisted of 57% solvent A (water–acetic acid, 99/1, v/v)/43% solvent B (acetonitrile–acetic acid, 99/1, v/v), and was kept constant

for 5 min, then solvent B was linearly increased to 54% over 21 min, then to 58% at 25 min, after which the resulting solvent combination (42% A/58% B) was held constant for 5 min. The column temperature was 30 °C. The flow rate of the mobile phase was 0.8 mL/min. The excitation and emission wavelengths of the fluorometric detector were set at 335 and 440 nm respectively. Fumonisins were quantified by comparing chromatographic peak areas to those of standards. The detection limits for FB_1, FB_2 and FB_3 were 0.1 μg/g based on a signal-to-noise ratio of 3:1.

2.3. DNA isolation

Fungal isolates were grown in liquid Wikerham's medium (40 g of glucose, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract, and water up to 1 L) on a rotary shaker (120 rpm). Mycelia were harvested by filtration, lyophilized, and then ground using 5-mm iron beads in a Mixer Mill MM 400 (Retsch). Genomic DNA was extracted from 10 mg of ground mycelia with the Wizard® Magnetic DNA Purification System for Food (Promega). The quality and quantity of the resulting DNA were assessed by agarose gel electrophoresis and by spectrometry with a Spectophotometer ND-1000 (Nano Drop).

2.4. PCR amplifications

PCR primers (Table 1) were used to screen for the presence of the *fum* cluster genes *fum1*, *fum3*, *fum6*, *fum7*, *fum8*, *fum10*, *fum13*, *fum14*, *fum15*, and *fum21* (2 regions designated I and II), as well as the *fum19-fum15* intergenic region and a region downstream of *fum6*. Primers were designed based on *fum* cluster sequences in *A. niger* strains ATCC 1015 (in the Joint Genome Institute (JGI) database) and CBS513.88 *A. niger* strains (in the GenBank database: accession AM269971.1) (Supplemental Table S1). When designing primers, we avoided regions where there was variability in the nucleotide sequences in *A. niger* strains ATCC 1015 and CBS513.88 whenever possible (Andersen et al., 2011). Primers CL1 and CL2A (O'Donnell et al., 2000) were used to amplify and sequence a ~650-bp fragment of the calmodulin gene (*caM*); primers BT2a and BT2b (Glass and Donaldson, 1995) were used to amplify sequence a 450-bp fragment of the β-tubulin gene (*benA*); and primers A-EF_F/A-EF_R (Perrone et al., 2011) were used to amplify and sequence a 700-bp fragment of the translation elongation factor 1α gene (*ef-1α*).

Amplification reactions were performed in a 25-μL volume: 0.6 U of HotMaster Taq DNA Polymerase (5 Prime, Hamburg, Germany), 1x HotMaster Taq DNA Polymerase buffer with 25 mM Mg^{2+}, 300 mM of each primer, 200 μM of each deoxynucleoside triphosphate, and approximately 20 ng of fungal DNA as template. Cycling conditions were as follows: denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 50 s, annealing at appropriate temperature for each primer pair for 30 s, extension at 72 °C for 50 s; final extension at 72 °C for 5 min, followed by cooling at 4 °C until recovery of the samples. All PCR amplicons were examined by electrophoresis in a 1.2% (w/v) ethidium bromide-stained agarose gel.

2.5. DNA sequencing and phylogenetic analysis

Prior to sequence analysis, amplicons were purified by the enzymatic mixture *E. coli* Exonuclease I/Shrimp Alkaline Phosphatase. The nucleotide sequence of both strands was determined with the Big-Dye Terminator Cycle Sequencing Ready Reaction Kit. Sequence reactions were purified by filtration through Sephadex G-50 (Pharmacia, Uppsala, Sweden) equilibrated in double-distilled water and then analyzed with a 3730 DNA Analyzer (Applied Biosystems). Sequence reads were viewed and when necessary edited with the BioNumerics 5.1 software (Applied Maths). To determine the nucleotide sequence of the *fum* cluster in *A. niger* strain ITEM 10355 and *A. welwitschiae* strains ITEM 7468 and ITEM 11945, the entire genome sequence of each strain was obtained from BaseClear, a private company in Leiden, The Netherlands. Sequence data were generated by the Illumina Casava pipeline version 1.8.2 sequencing system and included paired reads. The number of sequence reads was 22.3, 12.3 to 22.7 million and average

Table 1
PCR primer used for screen *A. niger/A.welwitschiae* strains.

Primer name	Sequence	Tm primer	Primer coordinates on FUM CLUSTER–Scaffold 1 of *A. niger* v1.0 (ATCC1015-JGI portal)	Amplicon size (bp)	Gene	Reference
fum3r-234F	TACCATGGACCACTTTCCCG	59	2,013,504–2,013,523	651	FUM3	This work
fum3-884R	AAGTTCCTCAAGCGGCAGTC	58	2,014,135–2,014,154			
fum13-200F	ATGCTCTTCACCTCCTCCGG	60	2,008,576–2,008,595	651	FUM13	This work
fum13-850R	CACTCAACGAGGAGCCTTCG	60	2,009,207–2,009,226			
fum14-102F	TTGGGCTGATGTGCTCTGTC	58	2,006,129–2,006,148	730	FUM14	This work
fum14-831R	ACCTCGTAGACGTAATTGAGTAGTCCT	58	2,006,832–2,006,858			
fum10-756F	GTCATTATTCCTCCGGCCCT	59	2,016,981–2,017,000	651	FUM10	This work
fum10-1406	TGGGATTCGAAAGCATACCG	59	2,017,612–2,017,631			
fum7-600F	CAACAGCCCGAATCCCAGTA	60	2,015,113–2,015,132	681	FUM7	This work
fum7-1280R	GCTCAGTCTTGCCCATCGTG	60	2,015,774–2,015,793			
fum1-4583F	GGGTTCCAGGCAGAATCGTAC	59	1,990,159–1,990,179	701	FUM1	This work
fum1-5283R	GAACTCACATCCTTTTGGGCC	59	1,990,839–1,990,859			
fum15-299F	CGATTGGTAGCCCGAGGAA	60	1,999,811–1,999,829	701	FUM15	This work
fum15-999R	CTTGATATTGCGGAGTGGTCC	58	2,000,491–2,000,511			
fum15-14-1395F	CATTTCATGGGACCTCAGCC	59	2,002,525–2,002,544	703	FUM21 I	This work
fum15-14-2097R	AAGCACAGGTTCCGAATTTGA	58	2,003,207–2,003,227			
fum15-14-2997F	GGGTCCCATTGCCTCAATT	58	2,004,127–2,004,145	705	FUM21 II	This work
fum15-14-3701R	CAATGGAGTCGACGGTGTCAC	60	2,004,811–2,004,831			
fum19-15-62F	ACACCGCGAGAATTCCATG	58	1,998,657–1,998,675	868	FUM19-FUM15	This work
fum19-15-929R	GCAGGCTGGTAGTAGCGACAT	58	1,999,504–1,999,524			
fum>6-11F	CAAAAGACACCGCCCGTCT	60	2,023,641–2,023,659	667	Down-stream fum6	This work
fum>6-677R	TTGACGCCCTGTACAAGGC	58	2,024,289–2,024,307			
fum6-1701F	CTGTGAGGCCCTGGCACTT	60	2,021,591–2,021,609	849	FUM6	This work
fum6-2549R	TCTGCCGGAGCTCAACGTA	59	2,022,421–2,022,439			
vnF1	TTCGTTTGAGTGGTGGCA	56	2,011,611–2,011,628	862	FUM8	Susca et al. (2010)
vnR3	CAACTCCATASTTCWWGRRAGCCT	57	2,010,766–2,010,789			

read length was 50, 99 and 98 for strains ITEM 10355, 7468 and 11945 respectively. Sequence reads were trimmed and assembled using the program CLC Genomics Workbench version 5.5.1 (CLC-bio). The sequence coverage for the genomes was 32X for ITEM 10355 and ITEM 7468 and 60X for ITEM 11945. The *fum* cluster region was identified in the resulting assembled genome sequences with the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) function in CLC Genomics Workbench. Query sequences used in the BLAST analysis were from *A. niger fum* gene homologues recovered from the *A. niger* ATCC 1015 genome sequence database at the JGI website (http://genome.jgi-psf.org). Genes within the *fum* cluster region of the ITEM 10355, 7468 and 11945 genome sequences were then manually annotated.

Initial species identification of field isolates was determined by assessing the nucleotide sequence identity of *caM* amplicons with references sequences. BLAST (Altschul et al., 1990) at the National Center for Biotechnology Information (NCBI), *caM* sequences of the isolates were queried against NCBI accessions AJ964872 and AJ964874, the *caM* sequences of the *A. niger* type strain (ITEM 4501T = CBS 554.65T) and *A. welwitschiae* type strain (ITEM 4509T = CBS 557.65T) respectively.

To infer phylogenetic relationships, nucleotide sequences of housekeeping and *fum* genes were aligned with ClustalW and then subjected to maximum likelihood and maximum parsimony analyses as implemented in MEGA5 (Tamura et al., 2011). All positions containing gaps and missing data were eliminated from the analyses. Statistical significance of branches in the resulting trees was assessed by bootstrap analysis using 500 or 1000 pseudoreplications (Felsenstein, 1985). Selection within orthologs of *fum1* was assessed by estimating differences in rates of nonsynonymous and synonymous substitutions at each codon of *fum1*. This was accomplished using the program Selecton version 2.4, which estimates the ratio of nonsynonymous to synonymous substitution rates using a Bayesian approach (Stern et al., 2007).

2.6. Southern blot analysis

A subset of 11 *Aspergillus* isolates was examined for the presence of *fum* genes and *sdr1* by Southern blot analysis. Genomic DNA was isolated from lyophilized mycelia that was ground to a powder and then suspended in extraction buffer (200 mM Tris–Cl, pH 8, 250 mM NaCl, 25 mM EDTA, pH 8, and 0.5% SDS) at 50 mg per 250 μL of buffer. Genomic DNA was purified from the suspension with the DNeasy Plant Mini Kit (Qiagen) following the protocol recommended by the manufacturer. Approximately 5 μg of genomic DNA was digested with restriction enzyme *Hind*III, electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and then subjected to a standard Southern hybridization protocol (Sambrook et al., 1989). Southern hybridization probes were generated by standard PCR with either the same primers used for the PCR screen (Table 2) or primers that amplified a fragment that spanned but was larger than the amplicon from the PCR screen. Following amplification, DNA fragments were subjected to agarose gel electrophoresis, purified with the UltraClean (MoBio Laboratories) method, and then labelled with ^{32}P using the Ready-to-Go DNA labelling kit (Amersham Biosciences).

3. Results

3.1. Species identification

Initial species identities of 56 *Aspergillus* isolates employed in this study were determined by comparisons of the nucleotide sequence of the *caM* sequence from each isolate with reference *caM* sequences from the type strains of *A. niger* (ITEM 4501) and *A. welwitschiae* (ITEM 4509). The *caM* sequences from 19 isolates were >99% identical to that of type strain ITEM 4501, indicating that they were *A. niger*. The *caM* sequences from 34 other isolates were >99% identical to the *caM* sequence of type strain 4509, indicating that they were *A. welwitschiae*. The identities of three other isolates, ITEM 11448, ITEM 11552 and ITEM 11553, were ambiguous because their *caM* sequences exhibited 99.0% and 98.8% identity to the reference sequences for *A. niger* and *A. welwitschiae* respectively. These three isolates differ from all other isolates examined in that they were isolated from dried fruit or cashew nuts from Brazil (ITEM 11448) or India (ITEM 11552 and ITEM 11553) rather than from grapes or raisins in the Mediterranean Basin.

To further resolve relationships of the *Aspergillus* strains, nucleotide sequences of fragments of three genes, *benA*, *caM* and *ef-1α*, from a subset of 51 isolates were subjected to maximum likelihood (ML) and maximum parsimony (MP) analysis. The *caM* sequences resolved isolates identified as either *A. niger* or *A. welwitschiae* into separate well-supported clades in the ML and MP analyses, but analyses with either *benA* or *ef-1α* sequences did not consistently resolve isolates of these two species into monophyletic clades (Supplemental Fig. S1). Because there were no conflicting branches that were strongly supported in trees generated with individual genes, we conducted ML and MP analyses with the concatenated *benA*, *caM* and *ef-1α* sequences. These analyses resolved isolates of the two species into separate and well-supported clades (Fig. 1). However, bootstrap values (84–96) for the clades corresponding to each species were not as high as obtained with the *caM* sequence alone. Isolates ITEM 11552 and ITEM 11553, which had ambiguous identities based on *caM* sequence comparisons, were resolved as a distinct well-supported clade that was basal to the *A. welwitschiae* clade (Fig. 1). The analyses did not provide strong support for the placement of isolate ITEM 11448. Thus, the phylogenetic analyses did not provide definitive species identities for isolates ITEM 11448, ITEM 11552 and ITEM 11553.

3.2. Fumonisins production

Because *A. niger* produces FB_2 at levels that are 8 and 200 times as high as FB_4 and FB_6 respectively (Frisvad et al., 2011), we employed FB_2 as a marker for fumonisin production. To assess FB_2 production, we grew isolates on CY20S, a medium that supports some of the highest levels of fumonisin production reported for *A. niger* (Frisvad et al., 2007). HPLC analysis of CY20S cultures of 58 *Aspergillus* isolates revealed that 14 *A. niger* isolates, including the *A. niger* type strains ITEM 4501, produced 0.5–3.3 μg FB_2 per g culture, and 10 *A. welwitschiae* isolates, including the *A. welwitschiae* reference strains ITEM 4502, produced 1.0–25.3 μg FB_2 per g culture. Six *A. niger* strains and 25 *A. welwitschiae* strains did not produce detectable levels of FB_2 under the conditions examined, nor did strains ITEM 11448, ITEM 11552 and ITEM 11553. The analytical method employed in this study was able to detect FB_1 and FB_3 in addition to FB_2. However, neither FB_1 nor FB_3 was detected in any of *A. niger* or *A. welwitschiae* cultures examined.

In order to gain insight into the relationships of FB_2-producing and nonproducing isolates of *Aspergillus*, we examined the production phenotypes in the context of the phylogeny generated with the concatenated sequences of three housekeeping genes: *benA*, *caM* and *ef-1α*. Although phylogenetic analysis resolved *A. welwitschiae* and *A. niger* into separate well-supported monophyletic clades, the analysis did not provide strong support for multiple clades within either species. Within *A. welwitschiae*, however, there was one well-supported sub-specific clade (ML bootstrap value = 85) that consisted of the majority of *A. welwitschiae* isolates analyzed, including both FB_2-producing and nonproducing isolates (Fig. 1). This sub-specific clade provides evidence that some

Table 2
PCR screening of *A. niger* and *A. welwitschiae* strains versus fum genes and intergenic regions.

ITEM	*Aspergillus* species	*FB_2 Production	*fum1* (701 bp)	*fum19-15* (868 bp)	*fum15* (701 bp)	*fum21* I (703 bp)	*fum21* II (705 bp)	*fum14* (730 bp)	*fum13* (651 bp)	*fum8* (800 bp)	*fum3* (651 bp)	*fum7* (681 bp)	*fum10* (651 bp)	*fum6* (849 bp)	Dowstream *fum6* (667 bp)
5266	***A. welwitschiae***	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7097	***A. welwitschiae***	+	+[1]	+	+[1]	+	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+
14303	***A. welwitschiae***	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14305	***A. welwitschiae***	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14308	***A. welwitschiae***	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11945	***A. welwitschiae***	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14297	***A. welwitschiae***	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4502	***A. welwitschiae***	+	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+
4951	*A. welwitschiae*	+	+	+	+	n.t.	+	+	+	+	+	+	+	n.t	n.t
5277	*A. welwitschiae*	+	+	+	+	n.t.	+	+	+	+	+	+	+	+	n.t
7468	***A. welwitschiae***	-	+	+	+	-	-	-	-	-	-	-	-	-	+
5253	***A. welwitschiae***	-	+	+	+	-	-	-	-	-	-	-	-	-	+
6122	***A. welwitschiae***	-	+	+	+	-	-	-	-	-	-	-	-	-	+
6127	***A. welwitschiae***	-	+[1]	+	+[1]	-	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	+
6293	***A. welwitschiae***	-	+[1]	+	+[1]	-	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	+
14310	***A. welwitschiae***	-	+[1]	+	+[1]	-	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	+
4552	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	-	n.t
4545	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	-	+
4717	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	-	+
4853	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	n.t	n.t
4858	*A. welwitschiae*	-	+[1]	+	+[1]	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	-[1]	+
4859	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	-	+
4863	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	-	+
4947	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	-	+
5267	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	-	+
5272	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	-	+
5283	*A. welwitschiae*	-	+	+	+	n.t.	n.t.	-	-	-	-	-	-	n.t	n.t
6123	*A. welwitschiae*	-	+	+	+	n.t.	n.t.	-	-	-	-	-	-	n.t	n.t
6126	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	-	n.t
6128	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	n.t	n.t
6140	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	n.t	n.t
6142	*A. welwitschiae*	-	+	n.t.	n.t.	-	-	-	-	-	-	-	-	n.t	n.t
6144	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	-	n.t
14307	*A. welwitschiae*	-	+	+	+	-	-	-	-	-	-	-	-	n.t	n.t
14309	*A. welwitschiae*	-	+	-	+	n.t.	n.t.	-	-	-	-	-	-	n.t	n.t
11448	***Aspergillus sp.***	-	+	+	+	-	-	-	-	-	-	-	-	-	+
11552	***Aspergillus sp.***	-	+	+	+	-	-	-	-	-	-	-	-	-	+
11553	***Aspergillus sp.***	-	+	+	+	-	-	-	-	-	-	-	-	-	+
4501[T]	***A. niger***	+	+[1]	+	+[1]	+	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+
7091	***A. niger***	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5276	***A. niger***	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6292	***A. niger***	+	+[1]	+	+[1]	+	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+
14266	***A. niger***	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14286	***A. niger***	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4547	*A. niger*	+	+[1]	+	+[1]	+	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+
10927	*A. niger*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14270	*A. niger*	+	+	+	+	n.t.	+	+	+	+	+	+	+	n.t	n.t
14273	*A. niger*	+	+	+	+	n.t.	+	+	+	+	+	+	+	n.t	n.t
14275	*A. niger*	+	+	+	+	n.t.	+	+	+	+	+	+	+	n.t	n.t
14281	*A. niger*	+	+	+	+	n.t.	+	+	+	+	+	+	+	n.t	n.t
14282	*A. niger*	+	+	+	+	n.t.	+	+	+	+	+	+	+	n.t	n.t
5218	***A. niger***	-	+	+	+	+	+	+	+	+	+	+	+	+	+
5219	***A. niger***	-	+[1]	+	+[1]	+	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+[1]	+
7090	***A. niger***	-	+	+	+	+	+	+	+	+	+	+	+	+	+
12918	***A. niger***	-	+	+	+	+	+	+	+	+	+	+	+	+	+
9720	***A. niger***	-	+	+	+	+	+	+	+	+	+	+	+	+	+
10355	*A. niger*	-	+	+	+	+	+	+	+	+	+	+	+	+	+
10954	*A. niger*	-	+	n.t	n.t	+	+	+	+	n.t	+	+	+	+	+

Bold character: Strains with all amplicons sequenced.
n.t. = Not tested.
[1] Data confirmed by Southern blot analysis.
*LOD (FB_2) = 0.1 μg/g.
[T] Type strain.

FB_2-nonproducing isolates of *A. welwitschiae* are more closely related to producing isolates than they are to other nonproducing isolates. Resolution of sub-specific clades within *A. niger* was not sufficient to draw conclusions about the relatedness of producing and nonproducing isolates in this species.

3.3. *fum* Cluster analysis

All experimental isolates were screened by PCR to assess the presence and/or absence of the *fum* cluster. The PCR assay employed primer pairs designed to amplify 13 different regions within the cluster (Fig. 2). Eleven fragments corresponded to segments within the coding regions of *fum1*, *fum15*, *fum21* (two segments designated I and II), *fum14*, *fum13*, *fum8*, *fum3*, *fum7*, *fum10* and *fum6*; one fragment corresponded to the *fum19-fum15* intergenic region; and one fragment corresponded to sequence downstream of the *fum6* coding region (Fig. 2). Fragments of the expected size were amplified for all 13 regions from FB_2-producing isolates of *A. niger* and *A. welwitschiae* as well as from all FB_2-nonproducing isolates of *A. niger* (Table 2 and Fig. 2). In contrast, only fragments corresponding to *fum1*, *fum15*, the *fum19-fum15* intergenic region, and the region downstream of *fum6* were amplified from FB_2-nonproducing isolates of *A. welwitschiae* and isolates ITEM 11448, ITEM 11552 and ITEM 11553 (Table 2).

Southern blot analysis was done to obtain additional evidence for the presence and absence of *fum* genes in a subset of 11 isolates. This analysis employed hybridization probes for all 12 genes in the *A. niger fum* cluster: *fum1*, *fum19*, *fum15*, *fum21*, *fum14*, *fum13*, *fum8*, *fum3*, *fum7*, *fum10*, *fum6*, and *sdr1*. The results of the

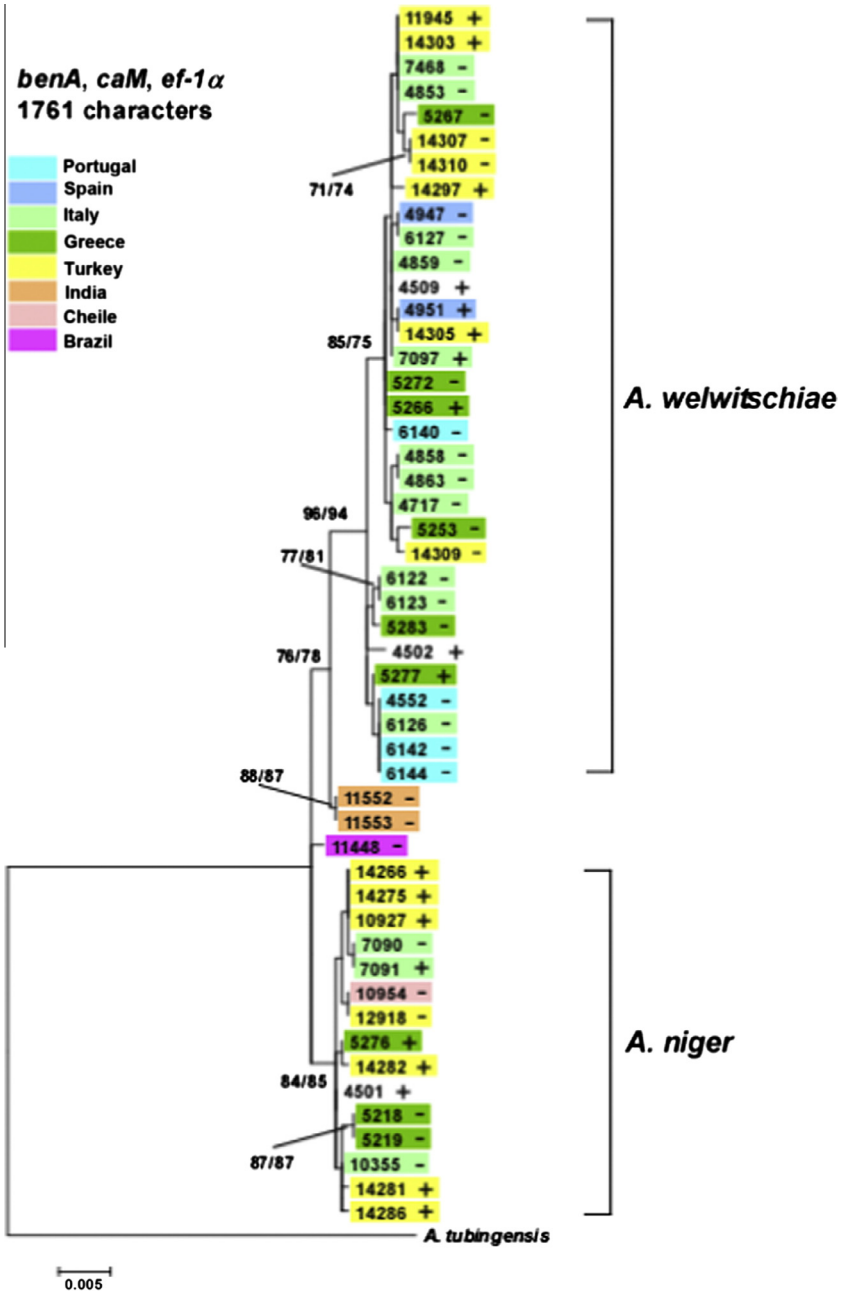

Fig. 1. Phylogenetic tree generated from concatenated nucleotide sequences of *benA*, *caM* and *ef-1α* fragments from a subset of 51 *Aspergillus* strains. Nucleotide sequences were aligned by ClustalW and the resulting alignment was analyzed by maximum likelihood and maximum parsimony analysis as implemented in MEGA5. Numbers near branches are bootstrap values; values to the left of slash are from maximum likelihood analysis, and values to the right of slash are from maximum parsimony analysis. All positions containing gaps and missing data were eliminated from the analyses. The + and – symbols next to strain designations indicate that strains produced or did not produce FB_2, respectively, in the fumonisin production assay.

Southern analysis were consistent with the PCR data; hybridization signals were detected for all 12 genes in FB_2-producing isolates of *A. niger* and *A. welwitschiae* as well as for nonproducing isolates of *A. niger*, but hybridization signals were detected for only *fum1*, *fum19* and *fum15* in FB_2-nonproducing isolates of *A. welwitschiae* (Table 3; Supplemental Fig. S2).

Nucleotide sequence data for the entire *fum* cluster in two FB_2-producing strains (CBS 513.88 and ATCC 1015) of *A. niger* have been reported previously (Pel et al., 2007; Baker, 2006). To further assess nucleotide sequence, content and arrangement of genes in the *fum* cluster among isolates of *Aspergillus*, we obtained sequence data for the *fum* region in FB_2-nonproducing isolates of *A. niger* (ITEM 10355) and *A. welwitschiae* (ITEM 7468) as well as in an FB_2-producing isolate of *A. welwitschiae* (ITEM 11945). In *A. niger*, the content and arrangement of *fum* cluster genes is the same for the FB_2-producing and nonproducing isolates examined (Fig. 3). The sequence data also indicate that the *fum* genes in the FB_2-nonproducing isolate (ITEM 10355) of *A. niger* are functional; i.e. the genes do not include insertions or deletions that would introduce frame shifts or premature stop codons relative to the orthologues in the two producing strains. However, the *fum1* coding region in the nonproducing isolate of *A. niger* includes a 12-nucleotide deletion that is not present in the *fum1* homologues of producing strains CBS 513.88 and ATCC 1015. The *fum1*-encoded polyketide synthase catalyzes synthesis of the fumonisin backbone (Alexander et al., 2009; Du et al., 2008), therefore mutations that

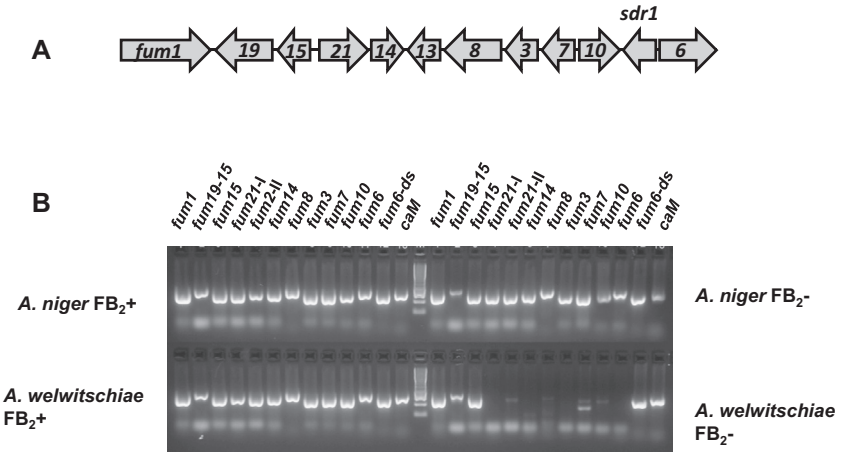

Fig. 2. (A) Organization of genes in the *A. niger fum* cluster. (B) PCR analysis of 12 regions within the *fum* cluster and the *caM* gene in four representative isolates of *Aspergillus*: *A. niger* isolates ITEM 7091 (FB_2 producer = FB_2+) and ITEM 10355 (nonproducer = FB_2−), and *A. welwitschiae* isolates ITEM 11945 (FB_2+) and ITEM 7468 (FB_2−). The lanes labeled *fum19-15* correspond to PCR products for the *fum19-fum15* intergenic region. The lanes labeled *fum6-ds* correspond to the PCR products for the region downstream of the *fum6* coding region. Sequence analysis revealed that the products amplified from the FB_2-nonproducing strain of *A. welwitschiae* with the primer pairs for *fum21*, *fum7*, *fum8* and *fum10* were nonspecific amplicons.

Table 3
Southern blot analysis of *fum* genes[a] in selected strains of *A. niger* and *A. welwitschiae*.

Species	ITEM No.	*fum1*	*fum19*	*fum15*	*fum21*	*fum14*	*fum13*	*fum8*	*fum3*	*fum7*	*fum10*	*sdr1*	*fum6*
A. niger	4501	+	+	+	+	+	+	+	+	+	+	+	+
A. niger	4547	+	+	+	+	+	+	+	+	+	+	+	+
A. niger	5219[b]	+	+	+	+	+	+	+	+	+	+	+	+
A. niger	6292	+	+	+	+	+	+	+	+	+	+	+	+
A. welwitschiae	4502	+	+	+	+	+	+	+	+	+	+	+	+
A. welwitschiae	4858[b]	+	+	+	–	–	–	–	–	–	–	–	–
A. welwitschiae	6127[b]	+	+	+	–	–	–	–	–	–	–	–	–
A. welwitschiae	6293[b]	+	+	+	–	–	–	–	–	–	–	–	–
A. welwitschiae	7097	+	+	+	+	+	+	+	+	+	+	+	+
A. welwitschiae	14310[b]	+	+	+	–	–	–	–	–	–	–	–	–

[a] The + and − symbols indicate that a signal was or was not detected, respectively, in the Southern analysis of *fum* genes and *sdr1*.
[b] Strains that did not produce fumonisins under the experimental conditions.

render the gene nonfunctional would result in a nonproduction phenotype. We used PCR and sequence analysis to determine whether the 12-nucleotide deletion was present in five other nonproducing and three producing isolates of *A. niger*. The deletion was not present in any of these isolates. Thus, if the deletion is responsible for fumonisin nonproduction in isolate ITEM 10355, it is not responsible for nonproduction in some other nonproducing isolates of *A. niger*.

In an attempt to identify other mutations that could be responsible for the FB_2-nonproduction phenotype in *A. niger*, we utilized *fum*-gene fragments amplified in the PCR screen (Table 2) from a subset of six FB_2-producing and six nonproducing isolates of *A. niger*. Sequence analysis revealed that, with one exception, fragments from nonproducing isolates did not include nucleotide substitutions, insertions or deletions that would introduce premature stop codons into *fum*-gene coding sequences. The exception was a substitution that introduced a premature stop codon into the *fum10* coding region of nonproducing isolate ITEM 12918. Functional analysis of the *F. verticillioides fum10* homologue indicates that it is required for formation of FB_2 (Butchko et al., 2006; Zaleta-Rivera et al., 2006). Thus, the premature stop codon *fum10* could account for the lack of FB_2 production in ITEM 12918. Although we did not observe nucleotide changes that would have an obvious effect on *fum* gene function in other nonproducing isolates, it should be kept in mind that the sequences of the PCR products represented only ~30% of coding regions in the *fum* cluster, and mutations could exist in gene segments that were not sequenced.

The content and arrangement of genes in the *fum* cluster of the FB_2-producing isolate (ITEM 11945) of *A. welwitschiae* was the same as in the *A. niger* isolates examined. That is, the ITEM 11945 cluster includes homologues of the 11 known *fum* genes as well as *sdr1* (Fig. 3). In contrast, the *fum* cluster in the FB_2-nonproducing isolate of *A. welwitschiae* (ITEM 7468) includes full-length homologues of *fum1*, *fum19* and *fum15* as well as a truncated homologue of *fum6* but no other *fum* genes (Fig. 3); there is no evidence for *fum21*, *fum14*, *fum13*, *fum8*, *fum3*, *fum7*, *fum10*, and *sdr1* in this region or elsewhere in the genome of ITEM 7468. The sequence data are consistent with the PCR and Southern analyses described above (Tables 2 and 3). Failure to detect a truncated *fum6* in nonproducing isolates of *A. welwitschiae* in the PCR and Southern analyses can be explained by the fact that the probe used in each analysis did not correspond to the region of *fum6* present in ITEM 7468.

3.4. Partial fum cluster in black aspergilli

Analysis of genome sequence databases available at the JGI and NCBI revealed the presence of remnants of *fum* cluster homologues in three other black aspergilli species: *A. brasiliensis*, *A. tubingensis*, and *A. luchuensis* strains CBS 106.47 (synonym *A. acidus*) and IFO 4308 (synonym *A. kawachii*, Futagami et al., 2011), (Supplemental

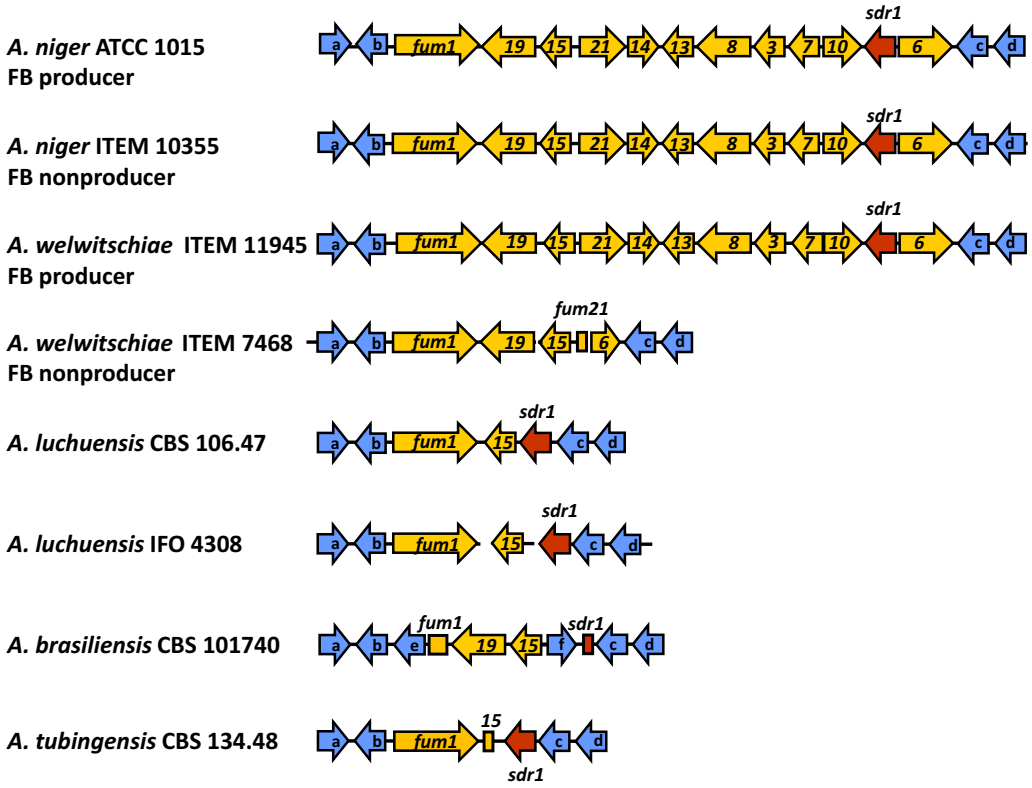

Fig. 3. Content and organization of genes in the *fum* cluster region of FB_2-producing and nonproducing strains of black aspergilli species: *A. niger* strains ATCC 1015 (FB_2-producer) and ITEM 10355 (nonproducer); *A. welwitschiae* strains ITEM 11945 (FB_2-producer) and ITEM 7468 (nonproducer); *A. acidus* strain CBS 106.47; *A. brasiliensis* CBS strain 101740; *A. kawachii* strain IFO 4308; and *A. tubingensis* strain CBS 134.48. Sequence data for strains ITEM 7468, 10355, and 11945 were generated during the course of this study; data for *A. kawachii* were downloaded from the NCBI database; and data for all other strains were downloaded from the JGI database. The *A. kawachii* genes shown here were present on three short contigs, and based on the data it is not possible to determine whether there are additional genes between the contigs. However, according to phylogenetic analysis of Hong et al. (2013), *A. acidus* and *A. kawachii* are the same species, *A. luchuensis*. Yellow arrows labeled with numbers represent known *fum* genes; numbers correspond to *fum* designations. According to BLAST analysis against the NCBI database, Genes a – f (blue arrows) are predicted to encode: a – polyketide synthase; b – major facilitator superfamily transport protein; c – 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase/methionine synthase; d – alkaline phytoceramidase; e – unknown hypothetical protein; f – heterokaryon incompatibility protein. Genes represented by boxes rather than arrows do not have full-length coding regions, and therefore are likely to be pseudogenes. Sequence data for the *fum* cluster region in *A. niger* strain 10355 and *A. welwitschiae* strains ITEM 7468 and 11945 have been submitted to NCBI/GenBank as accessions KJ934797, KJ934796 and KJ934798 respectively.

Table S1). These remnants are similar to the partially deleted cluster in *A. welwitschiae* in that they have retained all or part of *fum1* and *fum15*, and in the case of *A. brasiliensis* all of *fum19* (Fig. 3). In addition, genes immediately flanking the intact and partial clusters are conserved (Fig. 3), indicating that the genomic context, and therefore most likely the chromosomal location, of the intact and partial clusters is the same. The partial clusters in *A. brasiliensis*, *A. luchuensis*, and *A. tubingensis* differed from the partial cluster in *A. welwitschiae* in that they included all or part of *sdr1*.

3.5. Phylogenetic analysis of fum cluster sequences

A subset of 13 *A. welwitschiae* and 11 *A. niger* isolates were selected to assess the phylogenetic relationships of *fum* genes in FB_2-producing and nonproducing isolates. For this analysis, we employed DNA sequence data generated from fragments amplified in the PCR screen (Table 2) or from genome sequence data when available. The sequence analysis confirmed that the PCR amplicons were the target *fum* gene fragments. Because of the large deletion in the *fum* cluster and our PCR strategy, we had limited sequence data (only *fum1* and *fum15* fragments) for phylogenetic analyses that included FB_2-nonproducing isolates of *A. welwitschiae*. Analyses of *fum1* and *fum15* alone or combined were consistent; internodes with significant support (i.e. bootstrap values >70) did not conflict between trees generated with the *fum1* and/or *fum15* datasets (Fig. 4, Supplemental Fig. S3). These datasets did not resolve either *A. niger* or *A. welwitschiae* isolates into a well-supported monophyletic clade; nor did they resolve FB_2-producing and nonproducing isolates of *A. niger* into distinct clades (Fig. 4). However, the combined *fum1-fum15* dataset resolved FB_2-producing isolates of *A. welwitschiae* into a well-supported monophyletic clade that did not include nonproducing isolates. FB_2-nonproducing isolates of *A. welwitschiae* were not resolved into a distinct clade. In addition, in an unrooted tree generated from the combined *fum1-fum15* dataset, the three isolates (ITEM 11448, ITEM 11552 and ITEM 11553) for which the species identity could not be determined were resolved as a well-supported clade (bootstrap value 99%).

When FB_2-nonproducing isolates of *A. welwitschiae* were excluded, we were able to conduct phylogenetic analyses with fragments of nine *fum* genes: *fum1*, *fum15*, *fum21*, *fum14*, *fum13*, *fum8*, *fum3*, *fum7* and *fum10*. Well-supported internodes of individual unrooted trees were largely consistent with trees based on concatenated sequences of the nine genes (Fig. 5, Supplemental Fig. S3), but there were several exceptions to this. For example, the five *A. niger* isolates ITEM 5218, ITEM 5219, ITEM 7090, ITEM 7091 and ITEM 10355 were resolved into a single well-supported clade in some trees, but into different well-supported clades in others (Supplemental Fig. S3). In the analysis using concatenated *fum* sequences, all fumonisin-producing *A. welwitschiae* isolates were resolved into a well-supported monophyletic clade (Fig. 5). In trees generated with concatenated sequences [*fum1*, *fum15*,

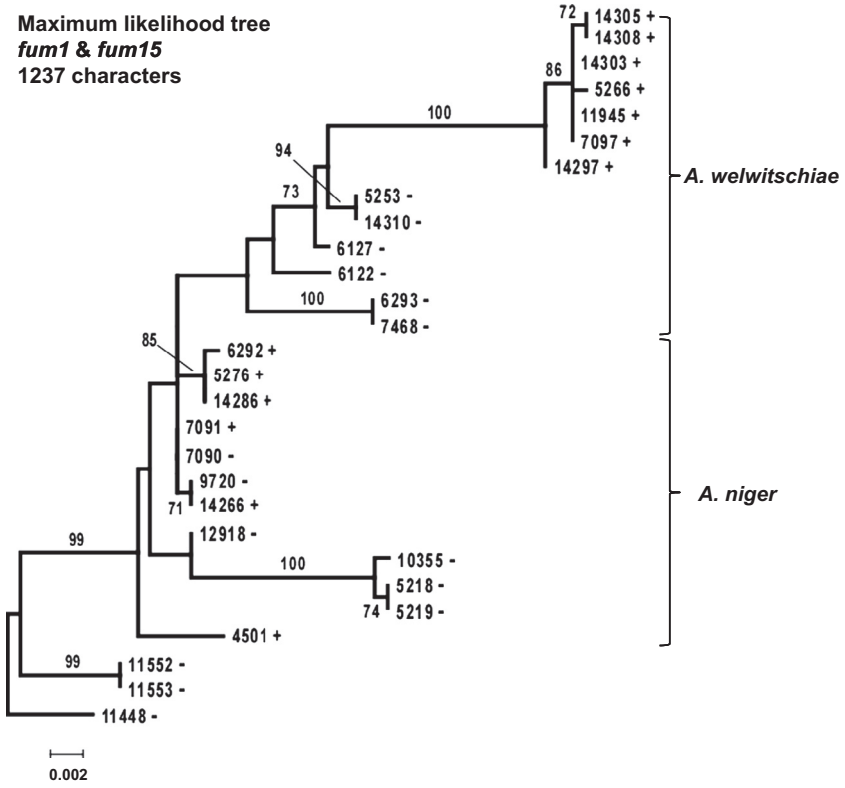

Fig. 4. Phylogenetic tree generated from concatenated nucleotide sequences of *fum1* and *fum15* amplified from selected strains of *A. niger* and *A. welwitschiae*. Numbers near branches are bootstrap values. For this data set, the Kimura 2-parameter model with gamma distribution was the optimal nucleotide substitution model and was used in maximum likelihood analysis of the aligned data. The + and − symbols next to isolate designations indicate that isolates produced or did not produce FB_2, respectively, in the fumonisin production assay. Nucleotide sequences for *fum* gene fragments have been submitted to the European Bioinformatics Institute with accession numbers LK931880-LK931897 (*fum1*) and LK931998-LK932015 (*fum15*).

fum21 (segments I and II), *fum14*, *fum13*, *fum8*, *fum3*, *fum7* and *fum10*] and rooted with *A. welwitschiae* sequences, all *A. niger* isolates were also resolved as a well-supported clade (Supplemental Fig. S4). In both unrooted (Fig. 5) and rooted (Supplemental Fig. S4) trees generated with the concatenated data set, *A. niger* isolates were resolved into two well-supported clades, both of which included at least one FB_2-producing and multiple nonproducing isolates. In fact, some isolates of *A. niger* (e.g. isolates ITEM 7091 and ITEM 7090) with different fumonisin production phenotypes were more closely related to one another than to isolates with the same phenotype (Fig. 5). Thus, in *A. niger* there is evidence that *fum* sequences from FB_2-producing and nonproducing isolates do not constitute two distinct clades.

We also conducted a phylogenetic analysis of full-length *fum1* sequences from black *Aspergillus* species from which sequence data for an intact or partial *fum* cluster was available. The truncated *fum1* sequence in *A. brasiliensis* was excluded from this analysis, because it corresponded to only 5% of the full-length *fum1* sequence. The results of this phylogenetic analysis indicate that *fum1* sequences from *A. niger* and *A. welwitschiae* are more closely related to one another than either is to sequences from *A. luchuensis* or *A. tubingensis* (Fig. 6). These results are consistent with results of phylogenetic analysis of combined sequences of *benA*, *caM*, and the rDNA internal spacer sequences (ITS) of black aspergilli that indicated *A. niger* and *A. welwitschiae* are more closely related to one another than either is to the three other species (Hong et al., 2013). The four *fum1* homologues from partial *fum* clusters used for the analysis shown in Fig. 6 appear to be functional, because their nucleotide sequences do not include frame shifts or internal stop codons. The exception is a single-nucleotide deletion near the 3′ end of the *fum1* homologue in *A. welwitschiae* strain ITEM 7468. This deletion is predicted to both truncate the corresponding protein by three amino acids and change the resulting five carboxy-terminal amino acids such that they are completely different from the amino acids at the equivalent position in other homologues.

When selection for a functional protein-encoding gene is maintained, nonsynonymous substitutions (i.e. mutations that alter amino acid sequence) are retained less frequently than synonymous substitutions (i.e. mutations that do not alter amino acid sequence) (Nei and Kumar, 2000). To assess whether selection for a functional *fum1* was relaxed or change following partial deletion of the cluster, we used the Selecton program (Stern et al., 2007) to estimate differences in the rates of synonymous and nonsynonymous substitutions along the entire length of the *fum1* coding region of the eight aspergilli isolates/species shown in Fig. 6. The results of the analysis provide evidence for purifying selection (i.e. selection against nucleotide changes that alter the amino acid sequence) at positions along the length of the *fum1*-encoded polyketide synthase and, therefore, selection against changes with the potential to negatively affect or change enzyme function.

4. Discussion

Production of fumonisin mycotoxins by black aspergilli is of concern because collectively these fungi can occur on a diversity of food and feed crops, can cause food spoilage, and are important for industrial fermentation, including production of compounds (e.g. citric acid) that are used in food. The results of the current study provide evidence that the predominant black aspergilli species on grapes in the Mediterranean Basin are *A. niger* and *A. welwitschiae*, two species that also predominate on grapes in California (Palumbo et al., 2013). The previous analysis of black

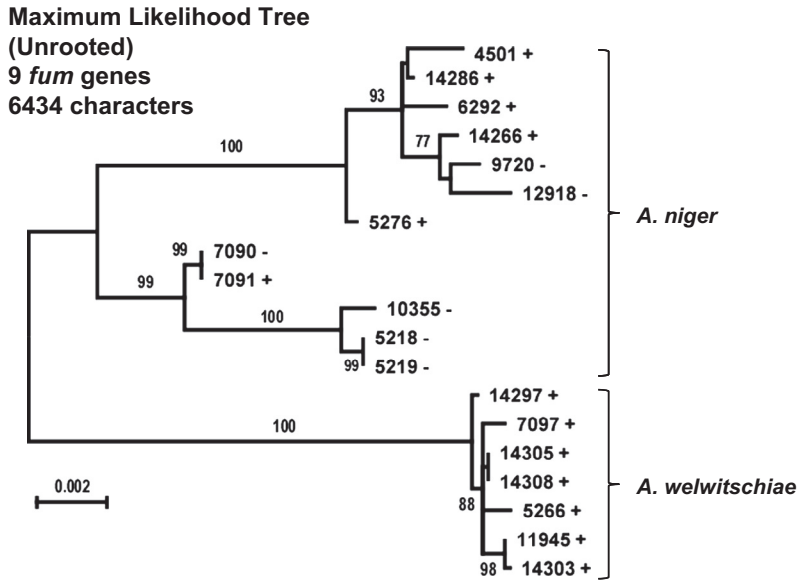

Fig. 5. Unrooted phylogenetic tree generated from concatenated nucleotide sequences of *fum1*, *fum15*, *fum21* (segments I and II), *fum14*, *fum13*, *fum8*, *fum3*, *fum7* and *fum10* from selected strains of *A. niger* and *A. welwitschiae*. Numbers near branches are bootstrap values. For this data set, the Kimura 2-parameter model with gamma distribution was the optimal nucleotide substitution model and was used in maximum likelihood analysis of the aligned data. The + and − symbols next to isolate designations indicate that isolates produced or did not produce FB_2, respectively, in the fumonisin production assay. Nucleotide sequences for *fum* gene fragments have been submitted to the European Bioinformatics Institute with accession numbers LK931880-LK931897 (*fum1*), LK931998-LK932015 (*fum15*), LK931923-LK931937 (*fum21-I*), LK931938-LK931952 (*fum21-II*), LK931908-LK931922 (*fum14*), LK931983-LK931997 (*fum13*), LK931898-LK931907 (*fum8*), LK931953-LK931967 (*fum3*), LK931865-LK931880 (*fum7*), LK931968-LK931982 (*fum10*).

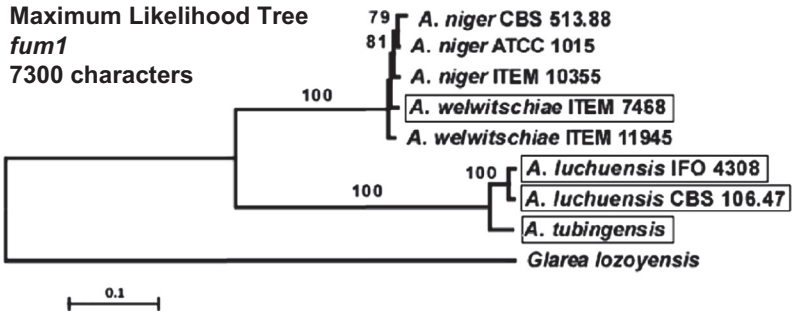

Fig. 6. Phylogenetic tree generated from full-length nucleotide *fum1* coding region sequences from intact and partial *fum* clusters in members of the *A. niger* clade. Numbers near branches are bootstrap values. For this data set, the Kimura 2-parameter model with gamma distribution was the optimal nucleotide substitution model and was used in the maximum likelihood analysis of the aligned data. Boxes around names indicate species/strains with a partial *fum* cluster.

aspergilli from California grapes indicated that fumonisin-nonproduction was associated with the absence of six *fum* genes in *A. welwitschiae* but not in *A. niger* (Palumbo et al., 2013). Results of the current study further clarify the structure of the *fum* cluster in fumonisin-producing and nonproducing isolates of these species. The results of our analyses indicate that gene content and organization within the *fum* cluster is the same in fumonisin-producing isolates of both species and in nonproducing isolates of *A. niger*, but that the cluster has undergone an eight-gene deletion in nonproducing isolates of *A. welwitschiae* (Fig. 3, Tables 1 and 2).

The lack of FB_2 nonproduction in some *A. niger* isolates that was observed in the current study is consistent with previous reports of fumonisin nonproduction in other isolates of this species (Frisvad et al., 2007; Palumbo et al., 2013; Varga et al., 2010). Because we examined production under only one culture condition (i.e. on CY20S medium), and because fumonisin production can vary under different conditions (Frisvad et al., 2007, 2011), our results do not exclude the possibility that *A. niger* isolates that did not produce FB_2 on CY20S medium could produce FB_2 on other media. Nevertheless, production of 0.5–3.3 μg FB_2 per g CY20S culture in some isolates of *A. niger* that we examined and the lack of production in other isolates indicate a fundamental difference(s) between these two groups of isolates. Comparisons of *fum* cluster sequences in the FB_2-producing and nonproducing isolates of *A. niger* do not provide definitive evidence for the genetic basis for the difference and raise the possibility that the cause of nonproduction may differ among isolates. The data suggest that nonproduction could result from four types of genetic modifications: (i) a small deletion within a *fum* gene coding region that renders the corresponding protein nonfunctional; (ii) a base substitution in a *fum* gene coding region that introduces a premature stop codon or causes an amino acid substitution that renders the corresponding protein nonfunctional; (iii) a base substitution in a *fum* cluster regulatory sequence that disrupts *fum* gene expression; or (iv) a mutation outside the *fum* cluster that negatively affects fumonisin production, e.g. by blocking expression of cluster genes. Evidence for nonproduction resulting from a small deletion within a *fum* gene is the 12-base deletion in the *fum1* homologue of *A. niger* isolate ITEM 10355. This deletion is located between the predicted β-ketoacyl synthase and acyl transferase domains of the *fum1*-encoded polyketide synthase (Proctor et al., 1999), and although these two domains are essential for function of the polyketide synthase, it is unclear whether a

small in-frame deletion between them could affect function. In addition, the *fum1* deletion occurs in a region that includes multiple in-frame deletions/insertions of >20 bases in *fum1* homologues in other fungi, e.g. *Aspergillus*, *Fusarium*, *Glarea*, and *Tolypocladium* (data not shown). Thus the deletion in ITEM 10355 would not necessarily affect the functionality of the polyketide synthase, and therefore, the ability of ITEM 10355 to produce fumonisins. The only evidence that a base substitution within a *fum* gene coding region could contribute to FB_2 nonproduction is the substitution and resulting premature stop codon in *fum10* of isolate ITEM 12918. In *Fusarium*, the *fum10* homologue is required for formation of the tricarboxyl esters of fumonisins and, therefore, for production of FB_2 or FB_4. However, *fum10* is not required for synthesis of fumonisin biosynthetic intermediates known as hydrolyzed fumonisins, because they lack the tricarboxyl esters (Butchko et al., 2006). Assuming *fum10* has the same function in *Aspergillus* and *Fusarium*, it is possible that isolate ITEM 12918 produces hydrolyzed fumonisins. Examination of ITEM 12918 for production of hydrolyzed fumonisins could help resolve the role of the premature stop codon in *fum10* in the FB_2-nonproduction phenotype in this strain.

Data from the current study do not directly address the possibility that fumonisin nonproduction in *A. niger* results from a mutation of a regulatory element within or outside the *fum* cluster. However, a previous study of *A. niger* revealed that the level of expression of eight cluster genes was relatively high in a fumonisin-producing isolate, whereas expression of some *fum* genes was markedly reduced in nonproducing isolates (Palumbo et al., 2013). In some isolates, expression of genes (e.g. *fum1* and *fum8*) that are essential for formation of the fumonisin backbone was not detected. Although environmental conditions can have differing effects on fumonisin production in *Aspergillus* and *Fusarium* (Mogensen et al., 2009), common themes in the genetic mechanisms that regulate fungal secondary metabolism (Keller et al., 2005) indicate that regulation of *fum* gene expression in *Aspergillus* and *Fusarium* likely includes some of the same genetic and physiological components. In *Fusarium*, the *FUM* cluster gene *FUM21* encodes a transcription factor that induces expression of other cluster genes (Brown et al., 2007). Examination of the full-length *fum21* sequence in *A. niger* nonproducing isolate ITEM 10355 did not reveal any deletions, insertions or base substitutions that would have an obvious negative affect on its activity. *Fusarium fum* gene homologues are also regulated by proteins encoded by genes located outside the cluster. These proteins include transcription factors that regulate carbon and nitrogen metabolism and proteins involved in methylation and acetylation of chromatin and thereby modification of the accessibility of DNA to transcriptional machinery (Woloshuk and Shim, 2013). Homologues of such regulatory genes are present in *Aspergillus* and could contribute to regulation of fumonisin production in *A. niger*. Based on current data, however, it is not possible to draw any conclusions as to whether such regulators contribute to the fumonisin-nonproduction phenotype in isolates of *A. niger*.

The close phylogenetic relationships of *fum* genes in some fumonisin-producing and nonproducing isolates of *A. niger* (Fig. 5) is consistent with nonproduction resulting from a mutation in a regulatory gene located outside the cluster or with the hypothesis that nonproduction has arisen multiple times in *A. niger* by small mutations within the *fum* cluster. The 12-base deletion in *fum1* of ITEM 10355 and the premature stop codon in *fum10* of ITEM 12918 indicate that different nonproducing isolates of *A. niger* can have different mutations that potentially affect fumonisin production. Thus, the genetic basis for fumonisin nonproduction in *A. niger* remains unclear. Functional analyses could help to resolve this issue. For example, if nonproduction is the result of the 12-base deletion in the *fum1* homologue of ITEM 10355, transformation-mediated introduction of a *fum1* homologue from a producing isolate into this isolate should restore fumonisin production. However, if nonproduction is the result of a mutation outside the cluster, it should be possible to restore production by inducing expression of *fum* cluster genes, e.g. by constitutive expression of the cluster regulatory gene, *fum21*.

The eight-gene deletion in fumonisin-nonproducing isolates of *A. welwitschiae* is consistent with the hypothesis that the absence of *fum* genes is the genetic basis for nonproduction in this species (Palumbo et al., 2013), because *Fusarium* orthologues of some of the deleted genes are required for synthesis of FB_2 (Alexander et al., 2009; Uhlig et al., 2012; Du et al., 2008). While partial deletion of the cluster would prevent fumonisin biosynthesis from occurring and provides a straightforward explanation for nonproduction in *A. welwitschiae*, the presence of the deletion does not rule out the possibility that the original genetic cause of nonproduction was a mutation(s) in a regulatory gene located outside the *fum* cluster, and that deletion of *fum* genes occurred after mutation of the regulatory gene. As with *A. niger*, functional analyses could provide further insight into the genetic basis of fumonisin nonproduction in *A. welwitschiae*. For example, if nonproduction is due solely to the partial deletion, transformation-mediated introduction of a functional *fum* cluster from a producing isolate into a nonproducing isolate should restore fumonisin production.

The phylogenetic analysis of *benA*, *caM* and *ef-1α* resolved a clade within *A. welwitschiae* that consisted of both fumonisin-producing and nonproducing isolates (Fig. 1). This indicates that isolates with different production abilities can be more closely related to one another than isolates with the same production ability, and therefore, that this species is not divided into two diverging lineages, one consisting of only producing isolates and the other of only nonproducing isolates. In contrast to the housekeeping genes, phylogenetic analysis of *fum1* and *fum15* resolved all of the fumonisin-producing isolates of *A. welwitschiae* into a well-supported clade that did not include nonproducing isolates (Fig. 4). Together, the resolution of producing isolates into a single clade in the *fum1*/*fum15* phylogeny, the identical pattern of *fum* gene loss in nonproducing isolates, and the identical genomic context of the *fum* cluster in producing and nonproducing isolates suggest that the intact and partial clusters constitute alternate alleles of a *fum* cluster locus. However, although the partial *fum* cluster homologues in *A. welwitschiae* isolates appear to constitute the same allele based on gene content and organization, the *fum1*-*fum15* sequences from partial clusters were not resolved into a well-supported monophyletic clade in the gene tree (Fig. 4). Thus, there may be multiple partial *fum* clusters alleles in *A. welwitschiae*.

The phylogenetic trees inferred from housekeeping genes (Fig. 1) suggest that relatively closely related isolates of *A. welwitschiae* can have different *fum* cluster alleles and that relatively distantly related isolates can have the same allele. The presence of different alleles of a locus in closely related individuals of a species is indicative of genetic recombination. A likely mechanism for such recombination is heterothallic sexual reproduction, i.e. when two distantly related individuals with different alleles undergo sexual reproduction they can give rise to closely related individuals (i.e. progeny) with the different alleles. Although, a sexual cycle has not been reported in *A. welwitschiae*, one has been reported in the black *Aspergillus* species *A. tubingensis* (Horn et al., 2013) as well as several other aspergilli that were once considered to be asexual (Horn et al., 2009). In addition, formation of sclerotia, a prerequisite for sexual reproduction in multiple aspergilli, have been induced in *A. niger* and some other black aspergilli, although not yet in *A. welwitschiae* (Frisvad et al., 2014). Furthermore, a full complement of early sexual development genes, including the *mat-1* mating-type gene, are present in genome sequences of *A. niger* (Pel et al., 2007). Thus, it is possible that an as yet unreported

sexual cycle has contributed the presence of the different *fum* cluster alleles in closely related isolates of *A. welwitschiae*. If this is the case, it would be less likely that the original genetic cause of nonproduction in *A. welwitschiae* is a mutation in a regulatory gene located outside the cluster, because genetic recombination during meiosis could give rise to fumonisin-nonproducing progeny with the mutated regulatory gene and an intact *fum* cluster. Such a scenario is not consistent with the finding that all fumonisin nonproducing isolates of *A. welwitschiae* examined to date lack some *fum* genes (Palumbo et al., 2013; and Table 2 of current study).

The results of the current and previous studies indicate that on grape *A. niger* and *A. welwitschiae* consist of mixed populations of fumonisin-producing and nonproducing individuals. Based on current data, we propose the following hypothesis for how the mixed population arose in *A. welwitschiae*: after *A. welwitschiae* diverged from *A.* niger, the *fum* cluster underwent an eight-gene deletion in one or more strains; subsequently, both partially deleted and intact forms of the cluster were retained and exchanged through genetic recombination, possibly by meiosis. More extensive analyses of housekeeping genes and the *fum* cluster from more isolates of *A. welwitschiae* should provide additional support for or against this hypothesis. Although existing data do not point to a clear hypothesis for how the mixed population of fumonisin-producing and nonproducing isolates arose in *A. niger*, the data indicate two fundamental differences from how such a mixture arose in *A. welwitschiae*. First, the mixed population in *A. niger* was not facilitated by a large deletion within the *fum* cluster. Second, the close phylogenetic relationships of *fum* sequences in some producing and nonproducing isolates of *A. niger* (Fig. 5) suggest recent exchange of *fum* sequences between fumonisin-producing and nonproducing individuals and possibly that the *fum* gene mutation(s) responsible for nonproduction occurred relatively recently. In *A. welwitschiae*, by contrast, *fum* sequences from producing strains are relatively distantly related to those of nonproducing strains. There is evidence for variation in secondary metabolite production phenotypes (chemotypes) among individuals in other fungal species. For example, the ability to produce aflatoxins varies among isolates of *A. flavus*, and likely results from deletion of genes or point mutations within the aflatoxin biosynthetic gene (*afl*) cluster (Chang et al., 2005; Mauro et al., 2013; Moore et al., 2009). In addition, isolates of some *Fusarium* species can differ in the kinds of trichothecene mycotoxins (e.g. nivalenol, 3-acetyl deoxynivalenol, or 15-acetyl deoxynivalenol) they produce as a result of loss or variation in function of trichothecene biosynthetic genes (Kimura et al., 2003; Lee et al., 2002; Ward et al., 2002). Thus, the variation in fumonisin production ability in *A. niger* and *A. welwitschiae* provides a further example of intra-species variation in mycotoxin chemotypes among fungi.

The *A. niger* clade of black aspergilli consists of at least 12 phylogenetically distinct species, including *A. brasiliensis*, *A. luchuensis*, *A. niger*, *A. tubingensis*, and *A. welwitschiae* (Hong et al., 2013). The similarities in gene content of the partial *fum* clusters in these species (Fig. 3) suggests that the partial cluster could have resulted from a deletion event in their common ancestor followed by vertical inheritance of the resulting partial cluster through multiple speciation events. In this scenario, partial deletion would have occurred prior to speciation, and as a result, *fum* sequences from intact versus partial clusters in the same species should be more distantly related to one another than *fum* sequences from partial clusters from different species. But, this is not the case; the *fum1* homologue from the partial *A. welwitschiae* cluster is more closely related to the homologue from the intact *A. welwitschiae* cluster than to homologues from partial clusters in other species (Fig. 6). This suggests that the deletion(s) resulting in the partial *fum* cluster in *A. welwitschiae* occurred after this species diverged from other species with partial clusters. An alternative explanation for the similarity in gene content of partial *fum* cluster orthologues is non-random loss of genes in different species. There is evidence for non-random gene loss in the *afl* cluster among isolates of *A. flavus* (Chang et al., 2005; Mauro et al., 2013; Moore et al., 2009) and in the gibberellic acid (GA) biosynthetic gene cluster within and among species of *Fusarium* (Bömke and Tudzynski, 2009; Wiemann et al., 2013). For both the *afl* and GA clusters, some of the deleted genes are responsible for early steps in the biosynthetic pathways such that the pathways are blocked before metabolites with aflatoxin- or GA-like structures are formed. This suggests that the non-random loss could reflect selection for isolates that do not produce aflatoxin- and GA-like metabolites. The apparent non-random gene loss within the *Aspergillus fum* cluster differs in that a full-length *fum1* has been retained in the partial clusters of the species examined except for *A. brasiliensis*. The *fum1*-encoded polyketide synthase is responsible for the first step in fumonisin biosynthesis, namely synthesis of the linear polyketide that forms the backbone of the fumonisin structure (Alexander et al., 2009; Du et al., 2008). Even though other genes (e.g. *fum6*, *fum8*, and *fum21*) that are absent in the partial clusters are also necessary for formation of fumonisin-like metabolites (Alexander et al., 2009; Uhlig et al., 2012; Du et al., 2008), if non-random gene loss were due only to selection for loss of production of fumonisin-like molecules, loss of the *fum1* side of the cluster would be expected to occur in some species. Thus, non-random gene loss in *fum* cluster orthologs among black aspergilli could result from selection for a functional *fum1* and production of the corresponding polyketide. This hypothesis is consistent with the absence of frame shift mutations and of nucleotide changes that introduce premature stop codons in the coding region of *fum1* homologues from all partial clusters except for the *A. brasiliensis fum1* homologue. In addition, the hypothesis is consistent with results indicating selection against nucleotide changes that alter the amino acid sequence, and potentially function, of the *fum1*-encoded enzyme (i.e. purifying selection) regardless of whether *fum1* was in an intact or partial cluster (Supplemental Table S2). However, the latter results (Supplemental Fig. S5) should be interpreted with caution, because if partial deletion of the cluster and relaxation of selection had occurred relatively recently, most of the evolutionary history of *fum1* would have occurred in the presence of selection, and as a result the signature of purifying selection in *fum1* would likely still persist. Given that *A. brasiliensis*, *A. luchuensis*, *A. niger*, *A. tubingensis*, and *A. welwitschiae* represent a large proportion of the phylogenetic diversity of the *A. niger* clade (Hong et al., 2013), it is possible that other species in this clade also have an intact or partial *fum* cluster. Furthermore, given the evidence that in at least some cases partial deletion of the cluster occurred after speciation, it is also possible that the cluster and the ability to produce fumonisins were once more widespread in this group of fungi than they currently are.

The results of the current study provide further insight into the evolutionary history and the genetic bases for variation in secondary metabolite production within fungal species. This insight includes evidence suggesting that a sexual cycle has contributed to variation in fumonisin production in the black *Aspergillus* species *A. welwitschiae*, evidence that is consistent with reports of sexual reproduction in other *Aspergillus* species once considered to be asexual. Analysis of mating type genes, sexual reproduction, and functional analyses of *fum* clusters should provide further insight into the genetic basis for variation in fumonisin production in black aspergilli and further contribute to diagnostic methods to distinguish between producing and nonproducing isolates.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2014.09.009.

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