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Birth, death and horizontal transfer of the fumonisin biosynthetic gene cluster during the evolutionary diversification of *Fusarium*

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Summary

Fumonisins are a family of carcinogenic secondary metabolites produced by members of the *Fusarium fujikuroi* species complex (FFSC) and rare strains of *Fusarium oxysporum*. In *Fusarium*, fumonisin biosynthetic genes (*FUM*) are clustered, and the cluster is uniform in gene organization. Here, sequence analyses indicated that the cluster exists in five different genomic contexts, defining five cluster types. In *FUM* gene genealogies, evolutionary relationships between fusaria with different cluster types were largely incongruent with species relationships inferred from primary-metabolism (PM) gene genealogies, and *FUM* cluster types are not trans-specific. In addition, synonymous site divergence analyses indicated that three *FUM* cluster types predate diversification of FFSC. The data are not consistent with balancing selection or interspecific hybridization, but they are consistent with two competing hypotheses: (i) multiple horizontal transfers of the cluster from unknown donors to FFSC recipients and (ii) cluster duplication and loss (birth and death). Furthermore, low levels of *FUM* gene divergence in *F. bulbicola*, an FFSC species, and *F. oxysporum* provide evidence for horizontal transfer of the cluster from the former, or a closely related species, to the latter. Thus, uniform gene organization within the *FUM* cluster belies a complex evolutionary history that has not always paralleled the evolution of *Fusarium*.

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Introduction

The filamentous fungus *Fusarium* produces toxic secondary metabolites, also known as mycotoxins, that can accumulate in food and feed crops and thereby pose health risks to humans and livestock. Fumonisins are a family of mycotoxins that can accumulate in maize and some other crops (Munkvold and Desjardins, 1997; Moretti *et al.*, 2010; Proctor *et al.*, 2010), cause multiple diseases in animals, and are epidemiologically associated with esophageal cancer as well as neural tube defects in some human populations (Gelderblom *et al.*, 1988; Marasas *et al.*, 2004). Fumonisins are linear, polyketide-derived molecules that can be classified as B or C fumonisins (FBs or FCs respectively) based on presence (FB) or absence (FC) of a terminal methyl group derived from an amino acid (Fig. 1) (Musser and Plattner, 1997; Sewram *et al.*, 2005). Most known fumonisin-producing fusaria are members of the *Fusarium* (*Gibberella*) *fujikuroi* species complex (FFSC) and produce predominantly FBs (Nelson *et al.*, 1992; Rheeder *et al.*, 2002; Proctor *et al.*, 2004). Although, isolates of *Fusarium oxysporum*, which is closely related to but not part of the FFSC, do not generally produce fumonisins, FC production has been confirmed in two isolates of this species (Seo *et al.*, 1996; Sewram *et al.*, 2005).

In filamentous fungi, secondary metabolite biosynthetic genes are typically located in gene clusters (Keller *et al.*, 2005). A fumonisin biosynthetic gene (*FUM*) cluster has been described in the FFSC species *Fusarium proliferatum* (Waalwijk *et al.*, 2004), *Fusarium verticillioides* (Proctor *et al.*, 2003), one of the rare fumonisin-producing strains (FRC O-1890) of *F. oxysporum* (Proctor *et al.*,

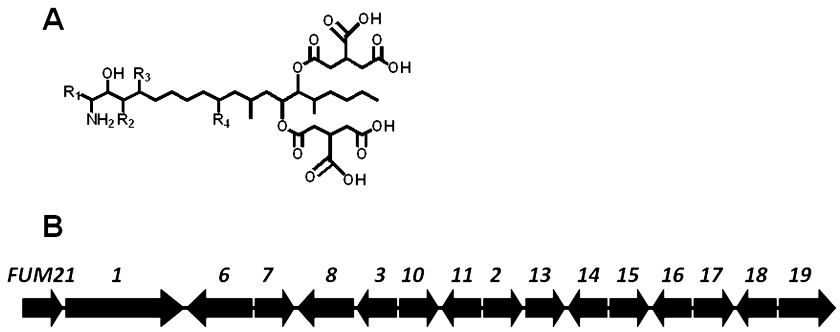

Fig. 1. A. Structure of B and C fumonisins (FBs and FCs). R_1 is a methyl group (CH_3) in FBs and a hydrogen atom in FCs. Within FBs and FCs, R_2, R_3 and R_4 are either a hydroxyl group (OH) or a hydrogen atom (H) depending on the fumonisin. For example, in FC_1 and FB_1 R_2 = H, R_3 = OH and R_4 = OH; in hydroxy-FC_1 R_2 = OH, R_3 = OH and R_4 = OH.
B. Organization of the 16-gene *FUM* cluster described in *F. oxysporum*, *F. proliferatum* and *F. verticillioides*. A single expressed sequence tag from *F. verticillioides* and genomic DNA sequence data from *F. verticillioides* and *F. oxysporum* FRC O-1890 provide evidence for an additional small gene (*FUM20*) located between *FUM2* and *FUM13*. *FUM20* is unusual in that its transcript overlaps with that of *FUM2* and it is predicted to encode a small (44-amino-acid) protein (Brown *et al.*, 2005).

2008), and in the more distantly related fungus *Aspergillus niger* (Khaldi and Wolfe, 2011). The *Fusarium* cluster includes 16 genes that encode biosynthetic enzymes as well as regulatory and transport proteins (Fig. 1). Functions of most of the genes in fumonisin biosynthesis in *F. verticillioides* have been determined by gene inactivation and heterologous expression analyses (Butchko *et al.*, 2003; 2006; Ding *et al.*, 2004; Yi *et al.*, 2005; Zaleta-Rivera *et al.*, 2006). Within the cluster, the *FUM1* gene encodes a polyketide synthase that catalyses synthesis of a linear polyketide that forms the backbone structure of fumonisins. In addition, the *FUM8* gene encodes an α-oxoamine synthase that mediates whether fusaria produce FBs or FCs by catalysing the condensation of the linear polyketide with alanine to produce FBs or with glycine to produce FCs (Branham and Plattner, 1993; Sewram *et al.*, 2005; Proctor *et al.*, 2008). The number, order and orientation of genes within *FUM* clusters in *F. verticillioides*, *F. oxysporum* and *F. proliferatum* are the same (Proctor *et al.*, 2003; 2008; Waalwijk *et al.*, 2004). However, the sequences flanking the clusters differ, indicating that the cluster is in a different genomic location in these three species.

The FFSC consists of more than 50 phylogenetically distinct species, many of which are pathogens and/or endophytes of economically important crops (O'Donnell *et al.*, 1998; Kvas *et al.*, 2009). For example, *F. proliferatum* and *F. verticillioides* are maize ear rot pathogens. The latter species is also considered a maize endophyte and is among the fungi most frequently recovered from healthy maize kernels (Munkvold and Desjardins, 1997). The FFSC has been delineated from other fusaria by DNA-based phylogenetic analyses that have also resolved the complex into three major lineages, designated as the African, American and Asian clades (O'Donnell *et al.*, 1998; 2000b; Summerell *et al.*, 2010). Although fumonisin production has been reported in one or more species in each FFSC clade (Nelson *et al.*, 1992; Rheeder *et al.*, 2002), production is discontinuous in that it is not correlated with phylogenetic relationships of species (O'Donnell *et al.*, 2000b; Rheeder *et al.*, 2002; Proctor *et al.*, 2004). Furthermore, Southern blot, PCR and sequence analyses indicate that *FUM* genes are absent from at least some FFSC species and *F. oxysporum* isolates that do not produce fumonisins (Mirete *et al.*, 2004; Proctor *et al.*, 2004; Glenn *et al.*, 2008; Stepien *et al.*, 2010; Van Hove *et al.*, 2011). Thus, the cluster can be present in one species, absent in a second closely related species, and present in a third distantly related species (Mirete *et al.*, 2004; Proctor *et al.*, 2004; Waalwijk *et al.*, 2004; Glenn *et al.*, 2008; Stepien *et al.*, 2010; Van Hove *et al.*, 2011).

Previous phylogenetic analyses of *FUM1* and *FUM8* in seven fumonisin-producing fusaria (Proctor *et al.*, 2004) indicated differences between *FUM* gene genealogies and those of primary-metabolism (PM) genes, i.e. species phylogenies, reported in another study (O'Donnell *et al.*, 1998). This finding indicates that the evolutionary history of *FUM* genes has not mirrored that of the fusaria in which the genes occur. The objectives of the current study were to (i) further evaluate the evolutionary relationships between *FUM* clusters in *Fusarium*, (ii) assess variation in the genomic context of the *FUM* cluster among species, and (iii) characterize the evolutionary processes that have shaped *FUM* cluster diversity in *Fusarium*. The results demonstrate that the phylogenetic discord of *FUM* and PM genes coincides with differences in *FUM* cluster genomic context; that *FUM* cluster diversity in the FFSC predates the diversification of this species complex; and that phylogenetic discord observed between *FUM* and PM genes is consistent with horizontal transfer of the cluster as well as cluster duplication, sorting and loss (birth and death).

Results

Phylogenetic analyses

To evaluate potential discord between *FUM* cluster evolution and the phylogenetic relationships of fumonisin-producing *Fusarium* species, we conducted phylogenetic analyses of nine *FUM* genes and 12 PM genes from representative isolates of 10 fumonisin-producing species (Table 1). Each selected *FUM* gene (*FUM1*, *FUM3*, *FUM6*, *FUM7*, *FUM8*, *FUM10*, *FUM13*, *FUM14* and *FUM19*) is required for wild-type fumonisin production in *F. verticillioides*, and together they span almost the entire length of the *FUM* cluster (Alexander *et al*., 2009). The PM genes included eight genes (*CAL1*, *CPR1*, *HIS3*, *RED1*, *RPB1*, *RPB2*, *TEF1* and *TUB2*) that have been used previously to infer phylogenetic relationships within *Fusarium* (O'Donnell *et al*., 1998; 2004; Proctor *et al*., 2009); two global regulatory genes, *LAE1* and *FLB2* (or *FlbB*), that also affect fungal secondary metabolism (Lafon *et al*., 2006; Wiemann *et al*., 2010); and a dehygrogenase gene, *ZBD1*, that flanks the *FUM* cluster in *F. verticillioides* (Proctor *et al*., 2003).

Species trees inferred from maximum likelihood (ML) analysis of the combined nucleotide sequences of the 12 PM genes strongly supported *F. oxysporum* FRC O-1890 as distinct from a monophyletic FFSC, and resolved members of the FFSC into three well supported clades (Fig. 2) corresponding to the previously described African, American and Asian clades (O'Donnell *et al*., 1998; 2000b). Phylogenetic reconstructions derived from neighbour-joining (NJ), maximum parsimony (MP) and Bayesian analyses of the combined PM genes were identical to the ML tree (Fig. 2). Bootstrap analyses under ML, MP and NJ produced 100% support for every internode except the one defining a sister-group relationship between the Asian and African clades, which was supported by at least 83% of bootstrap replicates. Bayesian posterior probability (BPP) values of 1 were obtained for every internode in the PM phylogeny. Well-supported (> 70% of bootstrap replicates) internodes in individual PM gene trees (Supplemental Fig. S1) were congruent with the combined PM topology except for the placement of *Fusarium phyllophilum* in ML trees derived from *CAL1* and *RPB2* (bootstrap scores = 74% and 71% respectively).

Phylogenetic relationships inferred from combined analyses of the nine *FUM* genes were similar to the species phylogeny in support for monophyletic African and Asian clades (Fig. 2), but monophyly of the FFSC and the American clade were strongly contradicted in the combined and individual analyses of *FUM* genes (Fig. 2; Supplemental Fig. S1). Specifically, *F. oxysporum* was nested within the FFSC as a sister-species to *Fusarium bulbicola* (American clade species) in the combined (ML, MP and NJ bootstrap = 100%; BPP = 1) and individual analyses of *FUM* gene sequences. These two species were resolved as a sister-group to a monophyletic African clade in the combined *FUM* gene analyses (ML, MP and NJ bootstrap > 83%; BPP = 0.99) and in six of the nine individual *FUM* gene trees. The American-clade species *Fusarium anthophilum* was most closely related to a monophyletic Asian clade in the combined *FUM* gene phylogeny (ML, MP and NJ bootstrap = 100%; BPP = 1) and in eight of the nine individual *FUM* gene trees.

Shimodaira–Hasagawa (SH) tests were used to evaluate alternative phylogenetic hypotheses and to assess the statistical significance of the topological differences between *FUM* and PM trees. Constraint analyses that forced the PM topology onto the *FUM* data set provided a significantly worse explanation of the data than the unconstrained *FUM* tree ($P < 0.01$). Trees recovered from analyses that enforced limited constraints requiring only American clade monophyly or FFSC monophyly also provided significantly worse explanations of the *FUM* data than the unconstrained *FUM* tree ($P < 0.01$). Reciprocal analyses conducted on the PM data demonstrated that the optimal PM tree (species tree) offered a significantly better explanation of the PM data than trees that were consistent with (i) the *FUM* tree topology, (ii) a sister-species relationship between *F. bulbicola* and *F. oxysporum*, or (iii) sister relationships between *F. anthophilum* and the Asian clade and between *F. bulbicola* and the African clade ($P < 0.01$).

Given the incongruence between *FUM* cluster evolution and species relationships, we analysed intraspecific variation using a subset of four *FUM* genes (*FUM1*, *FUM8*, *FUM14* and *FUM19*) from two to eight isolates of each fumonisin-producing FFSC species included in this study. Individual and combined analyses with this larger set of strains produced a topology (Fig. 3, Supplemental Fig. S2) that was completely consistent with the well-supported relationships among *FUM* sequences in Fig. 2. In addition, each of the FFSC species were resolved as monophyletic groups in the analyses of four *FUM* genes (Fig. 3) indicating that *FUM* cluster polymorphism was not trans-specific and that the *FUM* gene sequences from strains included in the nine gene analyses were representative of the *FUM* sequence diversity within their respective species.

Genomic context of FUM cluster

We also examined the genomic context of the *FUM* cluster by determining the sequence of ~ 3–20 kb of DNA flanking each side of the cluster. The results indicate that the cluster can occur in five genomic contexts (Fig. 4, Table 2), designated here as GC1, GC2, GC3a, GC3b and GC4. GC1 is the same context previously described for the *FUM* cluster in *F. verticillioides* (Proctor *et al*., 2003) and the *FUM* cluster remnant in *Fusarium musae* (Glenn *et al*.,

Table 1. Strains of *Fusarium* examined in this study.

Species	Strain[a]	Other designations	Fumonisin[b]	Reference
***Fusarium* (*Gibberella*) *fujikuroi* species complex**				
F. anthophilum	NRRL 25214[c]	BBA 62266	FC	O'Donnell *et al.* (1998)
	NRRL 13293	FRC M-1211	None	O'Donnell *et al.* (1998)
	NRRL 13602	CBS 737.97, FRC M-1355	FC	O'Donnell *et al.* (1998)
	NRRL 25217	BBA 64252	None	O'Donnell *et al.* (1998)
	Fant-1		nd	Current study
F. bulbicola	NRRL 13618[c]	BBA 63628, CBS 220.76	NONE	O'Donnell *et al.* (1998)
	NRRL 13600	BBA 63620, FRC M-1354	nd	O'Donnell *et al.* (1998)
	NRRL 22947	BBA 63620, CBS 245.59	FC	O'Donnell *et al.* (1998)
	NRRL 25176	BBA 63622	None	O'Donnell *et al.* (1998)
F. fujikuroi	HKM 41[c]	FGSC 8382	FB	Desjardins *et al.* (2000)
	41-79		FB	Desjardins *et al.* (2007)
	41-84		FB	Desjardins *et al.* (2007)
	Fp0		nd	
	GGM 118		nd	Desjardins *et al.* (2007)
	GL 24		None	Proctor *et al.* (2004)
	GL 28		None	Proctor *et al.* (2004)
F. globosum	NRRL 26131[c]	CBS 428.97, FRC M-8014, MRC 6647	FB	O'Donnell *et al.* (1998); Moses *et al.* (2010)
	NRRL 26133	CBS 430.97, MRC 6657	FB	O'Donnell *et al.* (1998); Moses *et al.* (2010)
F. nygamai	FRC M-7492[c]	MUCL 47026, FGSC 8934	FB	Klaasen and Nelson (1996)
	FRC M-7491	FGSC 8933	FB	Klaasen and Nelson (1996)
	NRRL 13448	BBA 69862, CBS 749.97, FRC M-1375	nd	O'Donnell *et al.* (1998)
	NRRL 25449	BBA 63175, CBS 413.97	nd	O'Donnell *et al.* (1998)
	NRRL 25596		nd	O'Donnell *et al.* (1998)
F. phyllophilum	NRRL 13617[c]	MUCL 43905, CBS 216.76	FB	O'Donnell *et al.* (1998)
	NRRL 25053	BBA 7983, CBS 246.61	nd	O'Donnell *et al.* (1998)
	NRRL 25178	BBA 63625,	nd	O'Donnell *et al.* (1998)
	NRRL 25341	MUCL 46369	nd	O'Donnell *et al.* (1998)
F. proliferatum	ITEM 2287		FB	Láday *et al.* (2004)
	GGM 185		FB	Desjardins *et al.* (2007)
	HKM 10		FB	Desjardins *et al.* (2000)
	ITEM 1301		nd	
	ITEM 1456		nd	Láday *et al.* (2004)
	ITEM 1799		nd	Láday *et al.* (2004)
	ITEM 2829		nd	
	ITEM 4292		nd	Láday *et al.* (2004)
F. ramigenum	NRRL 25208[c]	MUCL 46367, CBS 418.97	FB	O'Donnell *et al.* (1998)
	NRRL 25305		nd	O'Donnell *et al.* (1998)
	ITEM 2837		FB	Moretti *et al.* (2010)
	ITEM 2846		FB	Moretti *et al.* (2010)
	ITEM 2848		None	Moretti *et al.* (2010)
F. verticillioides	FRC M-3125	NRRL 20956, FGSC 7600	FB	Leslie *et al.* (1992)
	41-109		FB	Desjardins *et al.* (2007)
	AMRF-1		FB	Current study
	ISU-3		nd	
	ISU-94040		nd	
	FRC M-3120		FB	Leslie *et al.* (1992)
	FRC M-5500		None	Proctor *et al.* (2006)
Fusarium oxysporum				
F. oxysporum	FRC O-1890	CAR, NRRL 39464	FC	Proctor *et al.* (2008); Seo *et al.* (1996)

a. BBA indicates the Biologische Bundesanstalt fur Land- und Forstwirtschaft collection, Berlin, Germany; CBS indicates the Centraal Bureau voor Schimmelcultures collection, Utrecht, the Netherlands; FGSC indicates the Fungal Genetics Stock Center culture collection at the University of Missouri, Kansas City (Missouri), USA; FRC indicates the *Fusarium* Research Center collection at Pennsylvania State University, University Park, USA; ITEM indicates the collection at the Institute of Sciences of Food Production, National Research Council, Bari, Italy; MRC indicates the Medical Research Council collection, Tygerberg, South Africa; MUCL indicates the collection at the Mycothèque de l'Université catholique de Louvain, Louvain-la-Neuve, Belgium; NRRL indicates collection at the US Department of Agriculture, Agriculture Research Service, National Center for Agricultural Utilization Research (USDA ARS NCAUR), Peoria (Illinois), USA. Strain HKM 41 was obtained from A.E. Desjardins at USDA ARS NCAUR. *F. oxysporum* strain FRC O-1890 (= CAR, NRRL 39464) was obtained from Y.-W. Lee, Seoul National University. Strains GL 24 and GL 28 were provided by Thomas Gordon, University of California, Davis, California. Strains ISU-3 and ISU-94040 were provided by Gary Munkvold, Iowa State University, Ames, Iowa.

b. FB and FC indicate that B or C fumonisins, respectively, were the predominant fumonisins produced by the strain indicated. Production by most species listed has been reported previously (Nelson *et al.*, 1992; Fotso *et al.*, 2002; Rheeder *et al.*, 2002; Proctor *et al.*, 2004; Glenn *et al.*, 2008; Moretti *et al.*, 2010). Data on production by strains of *F. anthophilum* and *F. bulbicola* were determined during the course of the current study. *F. anthophilum* strains NRRL 13602 and NRRL 25214 produced FC_1, hydroxy-FC_1 and FC_2 at 3–11, 12–45 and 7–45 μg per g cracked maize kernel culture respectively. *F. bulbicola* strain NRRL 22947 produced FC_1, FC_2 and FC_3 at 117, 2 and 19 μg per g culture respectively.

c. Strains used for construction of GenomeWalker library for analysis of *FUM* cluster flanking regions.

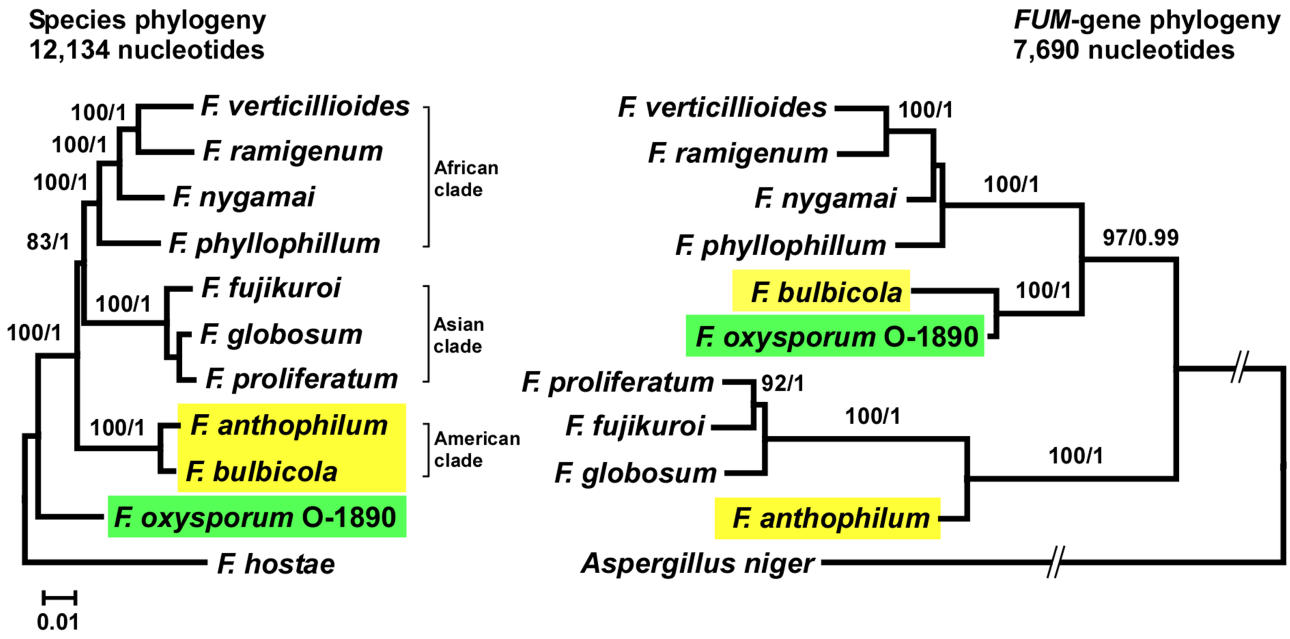

Fig. 2. Species and *FUM* gene phylogenies based on maximum likelihood analysis of concatenated nucleotide sequences of 12 PM and nine *FUM* genes amplified from fumonisin-producing species of *Fusarium*. Numbers near branches indicate bootstrap values based on 1000 pseudoreplicates from maximum likelihood analysis (left of slash mark) and posterior probabilities from Bayesian analysis (right of slash mark). GenBank accession numbers for sequences generated and employed in this analysis are KF416128–KF415191 (*FUM* genes) and KF466326–KF466456 (PM genes).

2008; Van Hove *et al.*, 2011). GC2 was observed in all African-clade species examined (Fig. 4, Table 2). The organization of genes in GC2 was confirmed by examination of the two recently released genome sequences of *F. fujikuroi* (Jeong *et al.*, 2013; Wiemann *et al.*, 2013). GC3a and GC3b were observed in American-clade species *F. anthophilum* and *F. bulbicola* respectively. GC3a and GC3b are similar in that the three genes (*CPM1*, *MFS2* and *DOX1*) flanking the *FUM19* side of the cluster are homologous (Fig. 4, Table 2). In contrast, GC3a and GC3b differ in the *FUM21*-flanking region. In GC3a (*F. anthophilum*), *FUM21* is flanked by genes *CPM2* and *TSP1* (Fig. 4, Table 2). We obtained only ~1500 bp of sequence data upstream of *FUM21* in GC3b (*F. bulbicola*), and within this region there was no evidence for genes. Within the ~ 300 bp immediately upstream of *FUM21*, the DNA sequences of GC3a and GC3b shared multiple 20- to 87-bp-long regions with up to 80% identity. More than 300 bp upstream of *FUM21*, there was no significant sequence identity. We identified a *F. bulbicola* homologue of *CPM2* with 85% identity to the *F. anthophilum CPM2*. However, analysis of the *CPM2* flanking region in *F. bulbicola* revealed that *CPM2* and *FUM21* are not located within 17 kb of one another in this species. BLASTX analysis indicated there are five genes located within the 15.5 kb of sequence immediately upstream of the *F. bulbicola CPM2* homologue. In *F. anthophilum*, in contrast, the intergenic region between *CPM2* and *FUM21* is 4 kb, and there is no evidence for other genes between them. Thus, although the DNA sequences of *F. anthophilum* and *F. bulbicola* exhibit significant similarity within the first 300 bp upstream of *FUM21*, the sequences are completely different further upstream. This indicates that the *FUM21* flanking regions in these species are not the same and, therefore, constitute different genomic contexts. One attribute common to the *FUM21* flanking region in these two species is AT-rich sequences. *F. anthophilum* has a 1 kb AT-rich segment beginning 600 bp upstream of the *FUM21* start codon, and *F. bulbicola* has two shorter segments (≤ 350 bp) within the 1500 nucleotides upstream of the *FUM21* start codon.

In GC4, the genomic context of the *FUM* cluster in *F. oxysporum* strain FRC O-1890 (Proctor *et al.*, 2008), there is no evidence for a full-length gene within the ~ 2800 bp region upstream of *FUM21*. However, this region includes three apparent gene fragments that are 140–377 bp in length and are located adjacent to one another. The deduced amino acid sequence of one of these fragments (377 bp) exhibits 65% identity to putative transposase genes from other fungi, including other strains of *F. oxysporum*. The region upstream of *FUM21* in GC4 also includes two AT-rich regions of ~ 340 and ~ 400 bp. GC4 is like GC3a (*F. anthophilum*) and GC3b (*F. bulbicola*) in that the *FUM19* side of the cluster is flanked by a homologue of *CPM1*. The 770 bp of sequence available upstream of *CPM1* in GC4 (*F. oxysporum*) does not include sequences

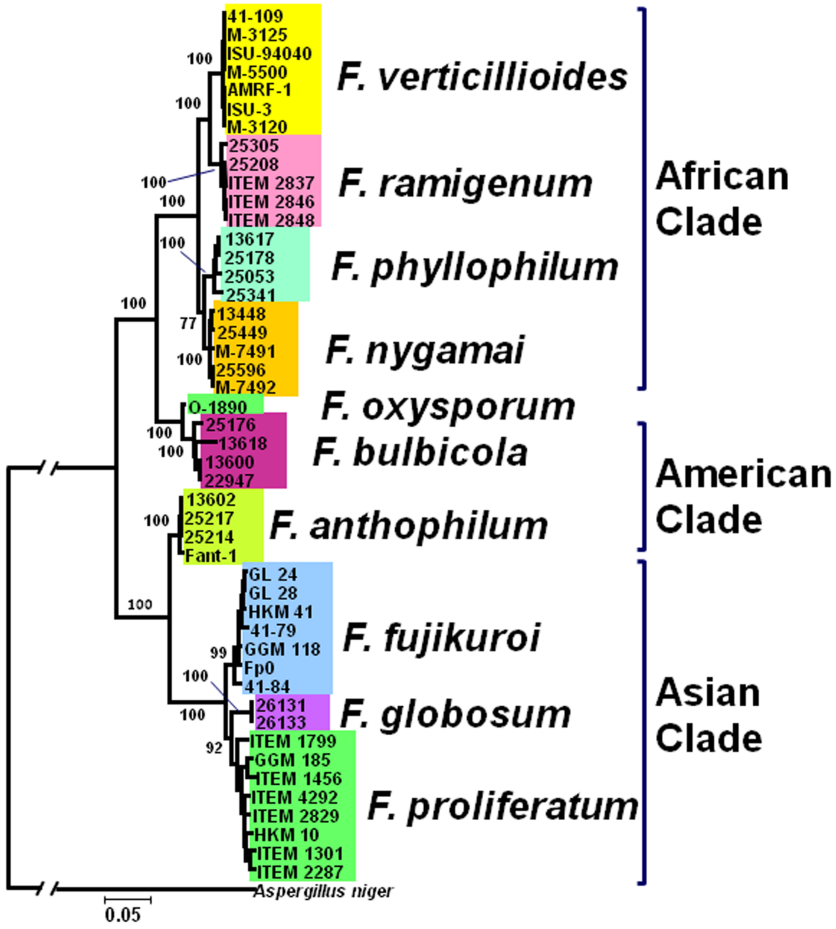

Fig. 3. Maximum likelihood tree generated from concatenated nucleotide sequences (2802 characters) of *FUM1*, *FUM8*, *FUM14* and *FUM19* from multiple isolates of nine FFSC species. Numbers near branches are bootstrap values based on 500 pseudoreplicates. The analysis employed the GTR+G substitution model. Strain designations consisting of only five numbers (e.g. 13448) are from the ARS (NRRL) culture collection (Table 1).

with similarity to any previously described genes and lacks significant identity to the sequence upstream of *CPM1* in GC3a and GC3b.

Examination of sequence data at the National Center for Biotechnology Information (NCBI) and the Broad Institute indicate that the genes flanking the two ends of the *FUM* cluster in GC1 or GC2 are adjacent to or near one another in other *Fusarium* genomes. For example, the genome sequences of *F. fujikuroi* (Jeong *et al.*, 2013; Wiemann *et al.*, 2013) and *F. oxysporum* strain FGSC 4287 (Ma *et al.*, 2010) has homologues of the *ZNF1*, *ZBD1*, *ORF20* and *ORF21* arranged in the same order as in GC1. However, unlike GC1, there is no *FUM* cluster located between the *F. oxysporum* FGSC 4287 homologues of *ZBD1* and *ORF20*. Instead, these two genes are located only 1550 bp apart. Likewise, the *F. oxysporum* FGSC 4287 and *F. verticillioides* (Ma *et al.*, 2010) genome sequences have homologues of *MFS1*, *ZCB1* and *GAT1* arranged in the same order as in GC2, but without a *FUM* cluster between *ZCB1* and *GAT1*. A similar arrangement of *FUM* cluster-flanking genes from GC3a was not evident in other *Fusarium* genome sequences. In fact, there is no evidence for closely related homologues of the GC3a/GC3b *FUM19* flanking genes *CPM1*, *MFS2* and *DOX1* in any of the *Fusarium* genome sequences examined. However, there is a ~ 220 bp remnant of *CPM1* located ~ 10 bp downstream of *FUM19* in GC1 (Fig. 4).

Together, these results provide evidence that the *FUM* cluster can exist in five different genomic contexts in the fusaria examined. The differences in genomic contexts are concordant with genealogies inferred from PM genes (Fig. 4), but discordant with genealogies inferred from *FUM* genes (Fig. 2), indicating an association between differences in genomic context of the cluster and the phylogenetic discord.

Analysis of synonymous site divergence

The timing of *FUM* cluster divergence relative to divergence of species and clades in which the cluster occurs was assessed by comparing the number of synonymous differences per synonymous site (d_S) in *FUM* versus PM genes. Non-synonymous substitutions were excluded from the analyses in order to minimize the impact of variation in substitution rates resulting from gene-specific differences in selective constraint. Within the FFSC, d_S

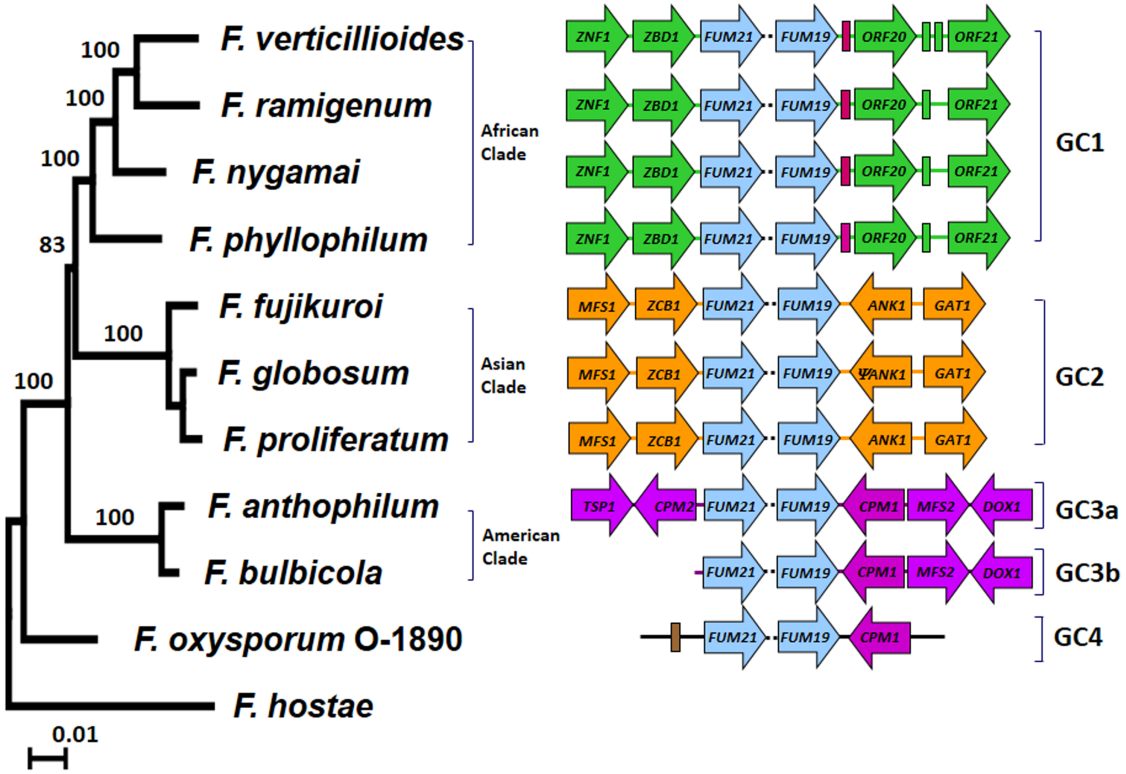

Fig. 4. Species phylogeny (left) and organization of genes flanking the *FUM* cluster (right) in 10 species of *Fusarium*. The species phylogeny is the same as that shown in Fig. 2. Numbers near branches indicate bootstrap values based on 1000 pseudoreplications. For the *FUM* cluster flanking regions, coloured arrows indicate relative positions and orientations of genes. Only the terminal genes (*FUM19* and *FUM21*) of the *FUM* cluster are shown; internal portions of the cluster are represented by a dashed line. GC1, GC2, GC3a, GC3b and GC4 are designations for the different genomic contexts. The sizes of and distances between genes are not to scale. For the African clade, the rust-coloured box between *FUM19* and *ORF20* represents a ~ 200 bp remnant of *CPM1*, and the green box(es) between *ORF20* and *ORF21* represents a remnant of a gene most similar to *F. graminearum* gene FGSG_00274. The brown box upstream *FUM21* in *F. oxysporum* (GC4) represents remnants of three different genes: one most closely related to the *F. graminearum* hypothetical protein-encoding gene FGSG_12015, another to a transposase gene, and the third to an oxidoreductase gene. In *F. globosum*, the *ANK1* homologue is a pseudo-gene and is therefore labelled as *ΨANK1*. GenBank accessions for the *FUM* cluster flanking regions in the species examined in this study are KF482460–KF482467, with the exception of *F. oxysporum* (EU477239) and *F. verticillioides* (AF155773), which were previously described in detail (Proctor *et al.*, 2003; 2008).

values for *FUM* genes were significantly greater ($P < 0.001$) than for PM genes in five of the six pairwise comparisons involving species or clades that differ in genomic context of the *FUM* cluster (Table 3). For example, in a comparison of the American clade species *F. anthophilum* (GC3a) and *F. bulbicola* (GC3b), divergence of *FUM* genes ($d_S = 0.365$) was more than 10-fold greater than PM genes ($d_S = 0.034$). These differences suggest that divergence of some *FUM* cluster types (defined by genomic context) predate divergence of the species or clades being compared. The comparison of the Asian clade (GC2) and *F. anthophilum* (GC3a) was an exception, in that divergence of *FUM* ($d_S = 0.189$) and PM genes ($d_S = 0.197$) was not significantly different, which is consistent with the expected pattern for orthologous *FUM* clusters. *FUM* gene divergence values were equal to or lower than values for PM gene in seven of the nine pairwise comparisons of species in which the *FUM* cluster is in the same genomic context (i.e. within the Asian or African clades) (Supplemental Table S1). These comparisons and the similar levels of divergence of *FUM* and PM genes in comparisons of Asian clade species and *F. anthophilum* indicate the evidence for an ancient divergence of some *FUM* cluster types is likely not due to elevated rates of synonymous substitution in *FUM* cluster genes. Thus, differences in d_S values of *FUM* and PM genes likely reflect different lengths of time that the genes have been diverging rather than different rates of synonymous substitution.

Interestingly, divergence of *FUM* genes ($d_S = 0.028$) was significantly less ($P < 0.001$) than PM genes ($d_S = 0.179$) when comparing *F. oxysporum* FRC O-1890 (GC4) and *F. bulbicola* (GC3b). Combined with the phylogenetic results demonstrating that *FUM* cluster sequences from *F. oxysporum* are nested within the FFSC, the very recent divergence of the *FUM* clusters in GC3b and GC4 provides

Table 2. Descriptions of genes in *FUM* cluster flanking regions.

Genomic context[a]	Region flanking	Tentative designation[b]	*Fusarium* homologues[c]	Predicted functions
GC1	*FUM21*	*ZNF1*	FVEG_00313 FOXG_01188 FGSG_00272	Zinc (RING) finger protein – transcription factor
		ZBD1	FVEG_00314 FOXG_01187 FGSG_00273	Zinc-binding dehydrogenase
	FUM19	*ORF20*	FVEG_00330 FOXG_01186	No known protein domains
		ORF21	FVEG_00331 FOXG_01185 FGSG_00275	No known protein domains
GC2	*FUM21*	*MFS1*	FVEG_10012 FOXG_11815 FGSG_03891	Major facilitator superfamily transport protein
		ZCB1	FVEG_10011 FOXG_11816	Zinc(2)-Cysteine(6) transcription factor
	FUM19	*ANK1*	None	Ankyrin repeat protein
		GAT1	FVEG_10010 FOXG_11820 FGSG_03892	Glutamine amidotransferase-like domain
GC3a	*FUM21*	*CPM2*	FVEG_10523 FOXG_11944	Cytochrome P450 monooxygenase
		TSP1 (P)	None	Transposase
GC3a & GC3b	*FUM19*	*CPM1*	None	Cytochrome P450 monooxygenase
		MFS2	FGSG_03360	Major facilitator superfamily transport protein
		DOX1	None	Dioxygenase
GC4	*FUM21*	None	None	None
	FUM19	*CPM1*	None	Cytochrome P450 monooxygenase

a. Genomic contexts of the *FUM* cluster as indicated in the text.
b. Tentative designations for genes are based on predicted functions, which are in turn based on similarity/identity to gene/proteins reported to have functions in the NCBI/GenBank database. Similarity was determined by BLASTX and/or BLASTP analysis against the NCBI database. (P) indicates that sequence for only a part of a coding region was obtained.
c. Homologues in the *Fusarium* Comparative Database for which the per cent identity of the predicted amino acid sequence is > 55% based on BLAST analysis. FGSG, FOXG and FVEG numbers indicate sequences from *F. graminearum*, *F. oxysporum* and *F. verticillioides* respectively.

strong evidence for horizontal transfer of the *FUM* cluster between *F. oxysporum* and *F. bulbicola* or a closely related member of the FFSC. This transfer event also appears to have included the *CPM1* gene, which is adjacent to *FUM19* in American clade species and *F. oxysporum* FRC O-1890 (Fig. 4). Genetic divergence of *CPM1* sequences in *F. bulbicola* and *F. oxysporum* FRC O-1890 ($d_S = 0.037$) was not significantly different from that of *FUM* gene sequences ($d_S = 0.028$), and was significantly less ($P < 0.001$) than the divergence of PM gene sequences used to infer species relationships ($d_S = 0.174$). Similarly, the *CPM1, MFS2* and *DOX1* genes, which are adjacent to *FUM19* in both *F. bulbicola* and *F. anthophilum*, displayed levels of synonymous divergence ($d_S = 0.365$) consistent with those observed for *FUM* gene sequences in these species ($d_S = 0.305$), and significantly greater ($P < 0.001$) than was

Table 3. Synonymous site divergence estimates (d_S) from concatenated sequences of 12 PM genes (above diagonal) and nine *FUM* genes (below diagonal) for species/clades with the *FUM* cluster in different genomic contexts.

	F. anthophilum	*F. bulbicola*	Asian clade	African clade	*F. oxysporum* O-1890
F. anthophilum (GC3a)[a]		0.034 ± 0.004	0.197 ± 0.010	0.179 ± 0.009	0.179 ± 0.009
F. bulbicola (GC3b)	0.365 ± 0.017		0.192 ± 0.010	0.173 ± 0.009	0.174 ± 0.009
Asian clade (GC2)	0.189 ± 0.011	0.534 ± 0.023		0.173 ± 0.009	0.181 ± 0.009
African clade (GC1)	0.429 ± 0.019	0.242 ± 0.012	0.589 ± 0.026		0.173 ± 0.009
F. oxysporum O-1890 (GC4)	0.341 ± 0.016	0.028 ± 0.004	0.509 ± 0.022	0.221 ± 0.012	

a. Designations in parentheses indicate genomic contexts of the *FUM* cluster as described in the text and shown in Fig. 4.
d_S values were estimated by two methods, Codeml-Maximum likelihood and modified Nie-Gojobori. The two methods yielded highly similar results, and therefore, only the results of Codeml-Maximum likelihood are presented.

Table 4. Comparisons of two residues in the deduced amino acid sequence of Fum8 that exhibit a consistent difference in FB- versus FC-producing *Fusarium* species.

Species	Fumonisin production[a]	Genomic context	Deduced amino acid sequence[b]	
			Residue 552	Residue 580
F. nygamai	FB	GC1	LEAKAPLNL	GPSSARWFY
F. phyllophilum	FB	GC1	LEAKAPLNL	GPSSARWFY
F. ramigenum	FB	GC1	LEAKAPLNL	GPSSARWFY
F. verticillioides	FB	GC1	LEAKAPLNL	GPSSARWFY
F. fujikuroi	FB	GC2	LEGIAPLSL	GPSSARWFY
F. globosum	FB	GC2	LEGIAPLSL	GPSSARWFY
F. proliferatum	FB	GC2	LEGIAPLSL	GPSSARWFY
F. anthophilum	FC	GC3a	LEAKPPLNL	GPSSVRWYY
F. bulbicola	FC	GC3b	LEAKPPLNL	GPSSVRWFY
F. oxysporum	FC	GC4	LEAKPPLNL	GPSSVRWFY
A. niger	FB		MPSQDLFPA	GPCSARWFY

a. Except for *F. anthophilum* and *F. bulbicola*, fumonisin production was determined previously (Rheeder *et al.*, 2002; Proctor *et al.*, 2004;Sewram *et al.*, 2005). Despite two attempts using our standard crack maize kernel assay and liquid chromatography-mass spectroscopy analysis, we were not able to confirm the previous report (Nelson *et al.*, 1992) of low-level FB production in *F. anthophilum* strains FRC M-854 and FRC M-1238; the strains did not produce detectable levels of fumonisin in the assay. In addition, we found that the previous report (Nelson *et al.*, 1992) of high FB production in a third *F. anthophilum* strain (FRC M-1139) was most likely the result of species misidentification; FRC M-1139 produced high levels of FBs in our standard assay, but analysis of its *TEF1* sequence at the Fusarium-ID database (Geiser *et al.*, 2004) revealed that the strain was most likely *F. proliferatum* (data not shown).
b. The letters are standard abbreviations for amino acids (e.g. A = alanine, V = valine).
The residues correspond to amino acids 552 and 580 (highlighted in black) in the *F. verticillioides* Fum8 homologue (NCBI GenBank accession AAG27130.3).

observed for PM genes ($d_S = 0.034$). As such, it appears that divergence of the *CPM1, MFS2* and *DOX1* homologues in *F. anthophilum* and *F. bulbicola* predates divergence of the FFSC and that these three genes have evolved in concert with the *FUM* cluster in the two species.

Fumonisin production and variation in FUM8

With the exception of *F. anthophilum* and *F. bulbicola*, fumonisin production has been confirmed in multiple reports in the literature for the FFSC species shown in Table 1. To clarify the fumonisin production phenotypes of *F. anthophilum* and *F. bulbicola*, we subjected three to four strains of each species to a fumonisin production assay by growing them on cracked maize kernel medium for 10 days and analysing culture extracts by liquid chromatography-mass spectroscopy. *F. anthophilum* strains NRRL 13602 and NRRL 25214 produced fumonisins FC_1, hydroxy-FC_1 and FC_2, but no or only trace amounts of FBs; whereas *F. anthophilum* strains NRRL 13293 and NRRL 25217 produced no detectable fumonisins (Table 1). *F. bulbicola* strain NRRL 22947 produced FC_1, FC_2 and FC_3, but no FBs; and *F. bulbicola* strains NRRL 13618 or NRRL 25176 produced no detectable fumonisins (Table 1).

Of the fumonisin-producing species examined in this study, the majority produce predominantly FBs, and three (*F. anthophilum*, *F. bulbicola* and *F. oxysporum*) produce predominantly FCs (Table 1). Because the *FUM8*-encoded enzyme, Fum8, determines FB versus FC production (Proctor *et al.*, 2008), we compared the predicted amino acid sequence of Fum8, corresponding to the region of *FUM8* used in the phylogenetic analysis, in order to identify differences in the sequences that might contribute to production of FBs versus FCs. The Fum8 homologue in the distantly related FB-producing fungus *A. niger* was also included in this analysis. Within the Fum8 region examined, only two residues differed consistently between FB and FC-producing fusaria. The first residue corresponded to amino acid 552 in the *F. verticillioides* Fum8 homologue and was an alanine in all FB producers and a proline in all FC producers (Table 4). However, this difference was not conserved in *A. niger*. In fact, the *A. niger* Fum8 sequence exhibits no identity to the *Fusarium* Fum8 sequence over a 17-amino-acid region that includes residue 552. The second residue corresponded to amino acid 580 in the *F. verticillioides* Fum8 homologue and was an alanine in the FB-producing fusaria as well as in *A. niger*. In contrast, the corresponding residue was a valine in the Fum8 homologues of FC-producing fusaria (Table 4).

Discussion

The results of this study extend previous evidence of discord between the evolutionary histories of two *FUM* cluster genes (Proctor *et al.*, 2004) and species relationships within the FFSC (O'Donnell *et al.*, 1998) by providing direct comparisons of phylogenies inferred from nine *FUM* genes and 12 PM genes. Discord between *FUM* cluster evolution and the phylogenetic relationships of fumonisin-producing fusaria was strongly supported by

bootstrap analyses and Bayesian posterior probabilities (Fig. 2), and by direct tests of alternative phylogenetic hypotheses via constraint analyses. In addition, our analyses indicated an association between phylogenetic discord and differences in *FUM* cluster genomic context and that *FUM* cluster diversity in the FFSC predates diversification of this species complex. Cases of phylogenetic discord and unexpectedly high levels of genetic divergence were previously observed in analyses of the aflatoxin (*afl*) and trichothecene (*TRI*) biosynthetic gene clusters in *Aspergillus* and *Fusarium* respectively (Ward *et al*., 2002; Carbone *et al*., 2007). This discord was attributed to trans-species evolution of *afl* and *TRI* polymorphism caused by balancing selection to maintain diversity of metabolite production phenotypes (chemotypes). In contrast to these examples, *FUM* cluster polymorphism was not trans-specific (Fig. 3), phylogenetic incongruence and high levels of synonymous divergence were directly related to differences in *FUM* cluster genomic context (Figs 2 and 4 and Table 3), and relationships inferred from *FUM* cluster genes were not consistent with fumonisin chemotype differences (Table 1). These findings indicate that balancing selection and trans-species evolution are not likely explanations for the phylogenetic discord and unexpectedly high levels of synonymous divergence between *FUM* cluster types occupying different genomic locations in *Fusarium*.

Interspecies hybridization is another potential cause of phylogenetic discord. However, the affects of hybridization should be evident across the genome, and not restricted to a single locus. Two hallmarks for such hybridization are discord among PM genes and/or multiple copies of individual PM genes per genome (O'Donnell *et al*., 2000a; Moon *et al*., 2004). We found no evidence for either of these phenomena among species with different *FUM* cluster types. Therefore, hybridization between the *Fusarium* species examined, or their close relatives, is not a likely explanation for the observed phylogenetic discord.

Horizontal transfer and FUM cluster evolution

Recent documentation of horizontal transfer (HT) of secondary metabolite gene clusters between fungi (Khaldi *et al*., 2008; Slot and Rokas, 2011; Campbell *et al*., 2012) indicates that HT is another potential cause of the phylogenetic discord of *FUM* and PM genes (Fig. 2). The observation that *FUM* cluster sequences from *F. oxysporum* strain FRC O-1890 were deeply nested within the FFSC in individual and combined *FUM* gene trees (Figs 2 and 3, and Supplemental Figs S1 and S2) is consistent with HT of the *FUM* cluster between *F. bulbicola* or a closely related member of the FFSC and *F. oxysporum*. The relative magnitude of synonymous divergence (Table 3) suggests the transfer occurred relatively recently, probably following the split between the American clade species *F. bulbicola* and *F. anthophilum*. Multiple lines of evidence suggest that the transfer occurred from the American clade to *F. oxysporum* rather than the reverse. First, if transfer had occurred from *F. oxysporum* to *F. bulbicola*, *FUM* sequences from these two species should be resolved on a branch that formed an outgroup relative to all other members of the FFSC, in the same manner that *F. oxysporum* is resolved as an outgroup to the FFSC in PM gene genealogies. Transfer from the FFSC American clade to *F. oxysporum* is also supported by the fact that fumonisin production and the *FUM* cluster occur in multiple species of the FFSC, but production has been confirmed in only two isolates of *F. oxysporum* (Seo *et al*., 1996; Sewram *et al*., 2005) and the cluster has been reported only in strain FRC O-1890 (Proctor *et al*., 2008). HT-mediated acquisition of the *FUM* cluster by *F. oxysporum* is also consistent with evidence that ~ 25% of the *F. oxysporum* genome is composed of DNA acquired from other organisms (Ma *et al*., 2010).

Phylogenetic relationships among *FUM* cluster sequences (Figs 2 and 3) were also suggestive of two HT events among members of the FFSC; one between the African clade and *F. bulbicola*, and a second between the Asian clade and *F. anthophilum*. To test the hypotheses that such HT events occurred, we assessed the relative levels of synonymous divergence of *FUM* and PM genes between these species or clades. If the HT events had occurred, *FUM* cluster genes would be expected to have lower levels of sequence divergence than PM genes in comparisons of putative donor and recipient species, as was the case for *F. bulbicola* and *F. oxysporum* (Table 3) and in recently reported instances of HT involving secondary metabolite biosynthetic (SMB) gene clusters in fungi (Slot and Rokas, 2011; Campbell *et al*., 2012). These lower levels of divergence of transferred SMB clusters occur because the split between donor and recipient species necessarily predates HT of the clusters. However, branch lengths (Fig. 2) and synonymous divergence values (Table 3) strongly contradicted hypotheses of HT between members of the FFSC. In fact, these analyses indicated that *FUM* cluster diversity among some FFSC species predates the diversification of the FFSC, which is the opposite of what would be expected if HT of the *FUM* cluster had occurred among members of this species complex.

A model based on HT can still be used to explain the phylogenetic incongruence (Fig. 2) and deep divergence of *FUM* cluster sequences (Table 3), but it requires multiple independent transfers of the *FUM* cluster into multiple lineages of the FFSC from unidentified donors outside of the FFSC (Fig. 5A). In this model, the *FUM* cluster was acquired by an ancestor of the FFSC via HT from an unknown donor, followed by vertical inheritance and retention of the cluster in the Asian clade and *F. anthophilum* as

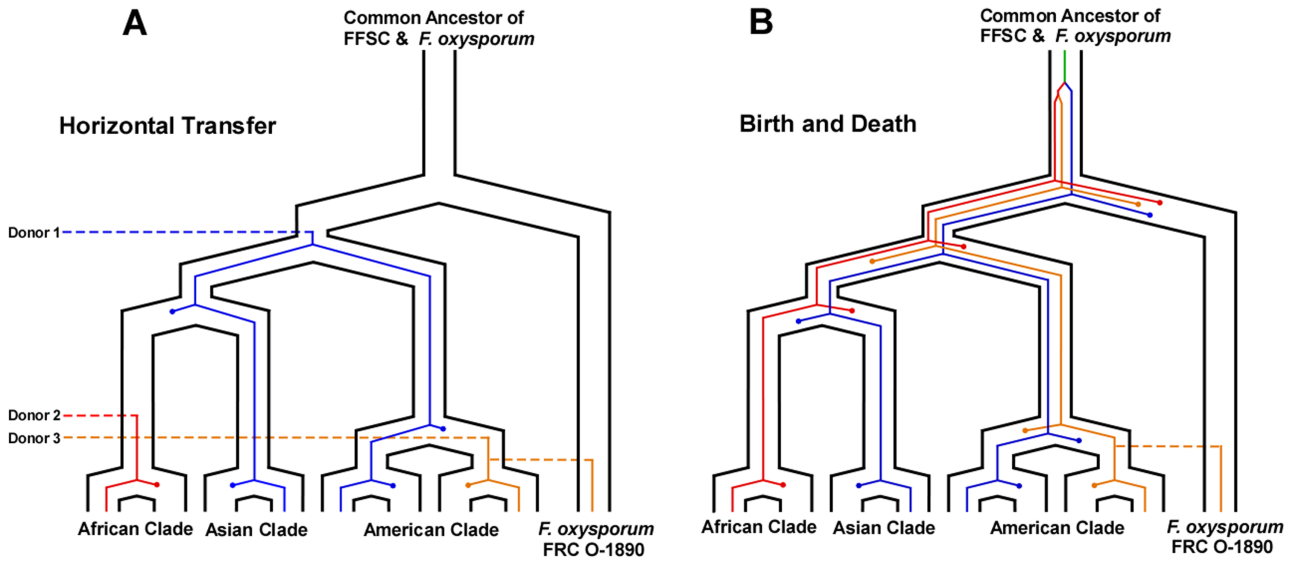

Fig. 5. Models for the evolutionary history of the *FUM* cluster during divergence of the FFSC and *F. oxysporum* O-1890.
A. Model based on independent horizontal transfer of the *FUM* cluster from three unknown but related donors to three lineages within the FFSC.
B. Model based on duplication followed by retention or loss (birth and death) of the *FUM* cluster. In both models, thick black lines depict the divergence of *Fusarium* lineages from the common ancestor of the FFSC and *F. oxysporum*; solid coloured lines represent evolution of the *FUM* cluster during divergence of the *Fusarium* lineages; closed circle indicate loss of a cluster homologue; and the dashed coloured line indicates horizontal transfer. In the birth-and-death model, bifurcations of coloured lines prior to divergence of the FFSC and *F. oxysporum* indicate cluster duplication events.

well as its loss from *F. bulbicola* and the ancestor of the African clade. This model also requires a second transfer of the *FUM* cluster from a second unknown donor to an ancestor of the African clade, and a third transfer from a third unknown donor to an ancestor of *F. bulbicola*. Because of the distant relationship of *FUM* genes in *A. niger* and *Fusarium* (Fig. 2), *A. niger* could not have been one of the donors. Alternative HT scenarios are possible but are not less complicated than the one presented here. Thus, an HT model that explains the discord between *FUM* gene and species phylogenies within the FFSC requires a minimum of three HGT events from unknown donors to multiple lineages within the FFSC. There are an increasing number of studies documenting HT of genes and entire biosynthetic gene clusters between fungi (Khaldi *et al.*, 2008; Mehrabi *et al.*, 2011; Slot and Rokas, 2011; Campbell *et al.*, 2012; Gardiner *et al.*, 2012). However, the number of independent HT events depicted in Fig. 5A involving closely related species of fungi would be unprecedented and suggestive of a strong selective advantage associated with the acquisition of the *FUM* cluster by species of *Fusarium*. The little and conflicting information available on the role of fumonisins in the ecology of *Fusarium* makes it difficult to speculate about the adaptive value of the cluster. Furthermore, fumonisin production appears to have been lost in multiple species or lineages of the FFSC (Proctor *et al.*, 2004; Glenn *et al.*, 2008; Van Hove *et al.*, 2011), suggesting frequent loss of selection. Given this, we contend that a model of *FUM* cluster evolution within the FFSC that relies solely on horizontal transfers is improbable. Nevertheless, such a model cannot be ruled out given existing information.

Birth, death and FUM cluster evolution

The birth-and-death model of evolution that has been applied to several multigene families (Nei and Rooney, 2005; Rooney and Ward, 2005) offers an alternative explanation for the observed phylogenetic incongruence (Fig. 2) and deep divergence of *FUM* cluster sequences (Table 3). Birth-and-death evolution involves duplication and subsequent maintenance or loss of gene copies to describe expansion, diversification and contraction of multigene families (Nei and Rooney, 2005). When this model is used to explain *FUM* cluster evolution, the three highly divergent *FUM* cluster types found in the African clade (GC1), *F. bulbicola* (GC3b), and the Asian clade plus *F. anthophilum* (GC2/GC3a) represent paralogues derived from ancient duplication events rather than homologues of the cluster derived from speciation (orthologous clusters) or HT (xenologous clusters) events (Fig. 5B). Cluster duplication and paralogy could explain the incongruence between *FUM* gene trees and species trees within the FFSC because phylogenetic relationships and levels of divergence among paralogues would reflect the pattern and timing of cluster duplication rather than

speciation events. However, *FUM* cluster types in the Asian clade and *F. anthophilum* are interpreted as orthologous because the level of divergence observed between their *FUM* gene sequences was not significantly different than levels of divergence between PM genes. This suggests that divergence of the *FUM* cluster in the Asian-clade species and *F. anthophilum* coincided with the split between the Asian and American clades. Furthermore, the presence of the *F. anthophilum* and Asian clade *FUM* cluster orthologues in different genomic contexts suggests that relocation of the *FUM* cluster within a genome can occur in the absence of duplication or HT.

Synonymous divergence between the three putative *FUM* cluster paralogues was significantly greater than between PM genes in comparisons of *F. oxysporum* and the FFSC (Table 3), indicating that all three paralogues were present in the common ancestor of the *F. oxysporum* and FFSC lineages. However, PCR and Southern data from multiple species (Proctor *et al.*, 2004; Stepien *et al.*, 2010) and analyses of the *F. fujikuroi* and *F. verticillioides* genomes (Ma *et al.*, 2010; Jeong *et al.*, 2013) indicate that there is only one copy of the *FUM* cluster in extant, fumonisin-producing species of *Fusarium* that have been examined. Therefore, acceptance of the birth-and-death model requires us to postulate that following duplication events of the *FUM* cluster in an ancestral *Fusarium* genome, the evolutionary history of the FFSC has been marked by reductions in *FUM* cluster copy number (Fig. 5B). The discontinuous distribution of fumonisin production within the FFSC combined with PCR and Southern data indicating that fumonisin-nonproducing FFSC species lack an intact *FUM* cluster (Proctor *et al.*, 2004; Glenn *et al.*, 2008; Van Hove *et al.*, 2011) provides indirect evidence of *FUM* cluster loss. When evidence for the presence and absence of *FUM* genes is mapped onto FFSC species phylogenies (O'Donnell *et al.*, 1998; Kvas *et al.*, 2009) it appears that the *FUM* cluster has been lost at least 15 times during the evolution of the FFSC (Supplemental Fig. S3). Some of these losses map to relatively short branches such that closely related or sibling species (e.g. *F. musae–F. verticillioides*, *F. napiforme–F. ramigenum* and *F. phyllophilum–F. udum*) now differ in the presence and absence of the *FUM* cluster. In addition, there is direct evidence for *FUM* cluster loss in *F. musae*, which lacks a cluster but has remnants of *FUM21* and *FUM19* in the same genomic context (GC1) as the intact clusters in other African clade species (Glenn *et al.*, 2008; Van Hove *et al.*, 2011). Although our analysis did not identify any fusaria with multiple paralogues of the *FUM* cluster, there are examples of two or more copies of SMB clusters in other fungal species: two paralogues of a putative polyketide biosynthetic cluster in *F. oxysporum* (Brown *et al.*, 2012); three paralogues of a putative non-ribosomal peptide biosynthetic cluster in *Pyrenophora tritici-repentis* (Manning *et al.*, 2013); and two paralogues of the *afl* cluster in *Aspergillus parasiticus* (Chang and Yu, 2002).

Under a birth-and-death model, the discontinuous distribution of fumonisin production and the phylogenetic discord between *FUM* gene and species phylogenies would have resulted from differential loss of *FUM* cluster paralogues in different lineages through a process known as paralogue sorting (Rooney and Ward, 2008). In such a scenario, some relatively distantly related lineages (e.g. *F. bulbicola* and the African clade) retained more closely related paralogues, whereas some closely related lineages (e.g. *F. anthophilum* and *F. bulbicola*) retained more distantly related paralogues, and some lineages or species lost all copies of the *FUM* cluster. Paralogue sorting is similar to the well-characterized process of lineage sorting (Avise *et al.*, 1983), which describes the non-phylogenetic transmittal of alleles at a single locus during speciation, and failure to consider evidence of paralogue sorting has resulted in false inferences of HT (Rooney and Ward, 2008).

Loss of cluster paralogues could also account for absence of the *FUM* cluster in most strains of *F. oxysporum* despite *FUM* gene divergence data indicating that multiple *FUM* cluster paralogues were present in the common ancestor of *F. oxysporum* and the FFSC. The absence of the cluster is evident from an inability to detect *FUM* genes in 11 *F. oxysporum* genome sequences at the Broad Institute's Comparative Fusarium Database or by Southern blot or PCR of 15 strains of this species (Proctor *et al.*, 2004; R.H. Proctor and A. Moretti, unpublished). The exception to this is strain FRC O-1890, which our data indicate acquired the *FUM* cluster via HT from the American clade. Thus, it appears that HT reintroduced the *FUM* cluster and fumonisin production into a lineage of *Fusarium* that had previously lost this metabolic capability. A similar mechanism was responsible for the reacquisition of the biotin biosynthetic pathway in *Saccharomyces cerevisiae* (Hall and Dietrich, 2007). In this context, the results of the current study suggest a mixed model of *FUM* cluster evolution that incorporates HT as well as cluster duplication and loss (birth and death) with paralogue sorting. A similar pattern of evolution was described for the virulence-associated internalin gene family in *Listeria*, where a relatively recent HT event was overlaid on a background of gene duplication and loss events consistent with birth-and-death evolution (Rooney and Ward, 2008). To our knowledge, the current study is the first to explicitly apply the birth-and-death model with paralogue sorting to the evolution of an SMB gene cluster, and in fact, the first application of the model to a biosynthetic pathway. However, the model is very similar to that used to describe the evolution of the avirulence gene (*ACE1*) cluster, in which *ACE1* cluster dupli-

cation occurred in the ancestor of the Pezizomycotina and was followed by additional duplications, horizontal transfers and loss of some or all paralogues in many lineages (Khaldi *et al*., 2008).

Evolution of FC production

Comparison of FB and FC production among fusaria indicates a correlation between fumonisin chemotype and the three major clades of the FFSC. All fumonisin-producing African and Asian-clade species that have been examined produce predominantly FBs, whereas all fumonisin-producing American-clade species that have been examined produce predominantly FCs (Table 1) (Proctor *et al*., 2004; Sewram *et al*., 2005). FB but not FC production has also been reported in two other fungal genera: *A. niger* (Frisvad *et al*., 2007) and three species of *Tolypocladium* (Mogensen *et al*., 2010). Based on the occurrence of FB production in *Aspergillus*, *Fusarium* and *Tolypocladium* but FC production only in *Fusarium*, we propose that FB production is ancestral and that a change(s) in *FUM8* sequence in the American clade of the FFSC resulted in a switch from FB to FC production. Because American clade species *F. anthophilum* and *F. bulbicola* have non-orthologous *FUM* clusters (e.g. different paralogues in the birth-and-death model or xenologues in the HT model) the *FUM8* mutation(s) leading to the change from FB to FC production would have had to occur twice, once in each homologue type, to account for the current distribution of FC production in the American clade and its absence in the African and Asian clades.

Comparison of deduced amino acid sequences of *FUM8* homologues revealed a perfect correlation between differences at residue 580 and FB versus FC production (Table 4). Residue 580 was previously proposed (Proctor *et al*., 2008) to play a role in determining FB versus FC production in *Fusarium* because mutation of the corresponding residue in another α-oxoamine synthase changed the amino acid substrate specificity of the enzyme (Shoolingin-Jordan *et al*., 2003). Thus, the current data provide further correlative evidence for the role of this variation in determining whether fungi produce FBs or FCs. In an extension of the hypothesis for evolution of FC production from FB production, residue 580 would have changed independently from an alanine to a valine in the Fum8 encoded by the *FUM*-cluster homologues in *F. anthophilum* and *F. bulbicola*. Such a change in amino acid sequence would require a change in only one nucleotide in the corresponding codon of the *FUM8* coding region: GCN (alanine) to GTN (valine). Mutation analysis of residue 580 as well as other Fum8 residues that are consistently different in FC and FB-producing fungi should aid in determining the role of the residues in FB versus FC production.

Conclusion

The results presented here demonstrate that *FUM* cluster evolution in *Fusarium* has been complex and is not adequately represented by the species phylogeny. Current data from the *FUM* cluster do not permit definitive rejection of the multiple HT model of *FUM* cluster evolution or the mixed model of birth and death with HT. Further evaluation of these models will be facilitated by the availability of an increasing number of genome sequences from *Fusarium* and other filamentous fungi, which will enable us to search for potential *FUM* cluster donors (in support of the HT model) or the presence of multiple *FUM* cluster paralogues or their remnants within a single genome (in support of the birth-and-death model). Regardless of which model is correct, this study adds to a number of previous studies in collectively demonstrating that SMB gene cluster diversity in filamentous fungi has been influenced by a variety of dynamic processes, including cluster duplication and loss (birth and death), balancing selection, shifts in functional constraint, translocation and HT (Ward *et al*., 2002; Carbone *et al*., 2007; Khaldi *et al*., 2008; Khaldi and Wolfe, 2011; Slot and Rokas, 2011). Furthermore, it appears that a combination of these processes has shaped the evolution and current distribution of some SMB clusters and contributed to metabolic diversity in fungi.

Experimental procedures

Strains and media

Fusarium strains used in this study are listed in Table 1. Species selected for the analysis either were previously reported to produce fumonisins or were identified as fumonisin producers during the course of this study. Strains were stored as 15% glycerol stocks at –80°C and grown on V-8 juice agar medium (Tuite, 1969) to prepare spores and mycelia to inoculate other media. For isolation of genomic DNA, *Fusarium* strains were grown in liquid GYEP medium (2% glucose, 0.1% yeast extract, 0.1% peptone) for 2–4 days at 200 r.p.m. and 28°C. For fumonisin production, strains were grown in cracked maize kernel medium at 25°C as described previously (Seo *et al*., 2001).

Nucleic acid manipulations

To isolate genomic DNA, cultures grown in liquid GYEP medium were harvested by vacuum filtration, lyophilized, ground to a powder and 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 buffer. DNA used for PCR or to prepare GenomeWalker libraries was purified from this suspension with the DNeasy Plant Mini Kit as described by the manufacturer (Qiagen). Some DNA used only for PCR was prepared with a modified UltraClean protocol (Mo Bio Laboratories). Briefly, the mycelia suspension described above was extracted with an equal volume of a 1:1 (v/v) mixture of

TRIS-equilibrated phenol and chloroform:isoamyl alcohol (24:1). Genomic DNA was purified from the resulting aqueous phase with the UltraClean DNA Purification kit as specified by the manufacturer except that the aqueous phase was mixed with two volumes of sodium iodide solution and 5 μl of Ultra-Bind solution. For genome sequence analysis of *F. anthophilum* and *F. bulbicola*, genomic DNA was obtained by growing the fungi in liquid GYEP medium and extracting DNA with the ZR Soil Microbe DNA MidiPrep kit (Zymo Research Corporation) following the manufacturer's specifications.

Oligonucleotide primers used to amplify fragments of *FUM* and PM genes are listed in Supplemental Table S2. Primers were synthesized by Integrated DNA Technologies or by Sigma Life Science-Genosys. Standard PCR amplifications employed *Taq* DNA polymerase (Qiagen) or Platinum PCR SuperMix polymerase (Invitrogen Life Technologies) and conditions recommended by the manufacturers. PCR amplification employed DNA polymerase contained in the Platinum SuperMix High Fidelity reagents (Invitrogen) using conditions recommended by the manufacturer. PCR products were analysed by agarose gel electrophoresis and were purified from the agarose with the UltraClean DNA Purification protocol (Mo Bio Laboratories) prior to their being used in nucleotide sequencing reactions.

For *F. anthophilum*, *F. bulbicola*, *F. fujikuroi*, *F. globosum*, *F. nygamai*, *F. phyllophilum* and *F. ramigenum*, DNA sequences of regions flanking the *FUM* cluster were obtained using the Universal GenomeWalker Kit (Clontech Laboratories) as previously described (Proctor *et al*., 2008; 2009). The initial gene-specific primers for this protocol were designed based on the sequences of fragments of *FUM19* and *FUM21*. For most species, several rounds of GenomeWalker PCR were performed. When the protocol did not yield a PCR product, we obtained sequence data for a given species by standard PCR methods using primers complementary to genes flanking the *FUM* cluster in one or more closely related species. Nucleotide sequence analysis employed BigDye Terminator version 3.1 (Applied Biosystems) reagents and UltraClean-purified PCR products (Mo Bio Laboratories) as DNA templates. Sequence reactions were purified with the BigDye Xterminator Purification protocol and analysed with a 3730 DNA analyser at the USDA-ARS-NCAUR DNA Sequence Facility. Sequence data were viewed and edited with Sequencher version 5.0 (Gene Codes Corporation). Additional sequence data for the *FUM* cluster flanking regions in *F. anthophilum* NRRL 25214 and *F. bulbicola* NRRL 13618 were obtained from genome sequences of these strains. The genome sequence data were acquired with an Ion Torrent system (Life Technologies). Sequence reads were trimmed and assembled and the resulting data were analysed with the CLC Genomics Workbench software (CLC Bio).

Phylogenetic analysis and genetic distance estimation

The 12 PM genes used to infer species phylogenies and the proteins they encode were: *CAL1* calmodulin; *CPR1*, NADPH-cytochrome P450 reductase; *FLB1*, regulator of G protein signalling; *HIS3*, histone H3; *LAE1*, velvet complex methyltransferase, *RED1*, reductase; *RPB1*, RNA polymerase II largest subunit; *RPB2*, RNA polymerase II largest subunit; *TEF1* (also *EF-1α*), translation elongation factor 1α; *TPS1*, α,α-trehalose-phosphate synthase; *TUB2*, β-tubulin; and *ZBD1*, zinc-binding dehydrogenase (Steenkamp *et al*., 2002; O'Donnell *et al*., 2004; 2007; Malonek *et al*., 2005). Nucleotide sequences of *A. niger FUM* genes were retrieved from the Joint Genome Institute (http://genome.jgi-psf.org) via BLAST analysis with the corresponding *F. verticillioides* homologues.

Nucleotide sequences were aligned by CLUSTALW+ or MUSCLE as implemented in the program MEGA5 (Tamura *et al*., 2011). Phylogenetic relationships were inferred from aligned sequences using maximum likelihood (ML), neighbour-joining (NJ) and maximum parsimony (MP) as implemented in MEGA5, as well as by Bayesian estimation using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) as implemented on the CIPRES Science Gateway web server (Miller *et al*., 2010). The best fit model of molecular evolution for use in ML, NJ and Bayesian analyses was selected based on estimation of the Bayesian Information Criterion (BIC) for each of the 24-nucleotide substitution models included in MEGA5. MP analyses were conducted with CNI branch-swapping on 10 random starting trees. In all three analyses all gaps and missing data were eliminated, and support for individual nodes was estimated via non-parametric bootstrapping with 1000 pseudoreplications of the data. Bayesian estimations of phylogeny employed the best fit model of molecular evolution for each with two independent runs of four simultaneous chains of 10 000 000 generations, sampling every 1000 generations and using a burnin period of 100 000 generations. In order to assess the significance of the discord observed between the *FUM* and PM gene trees, we conducted Shimodaira–Hasagawa (SH) tests (Shimodaira and Hasagawa, 1999) using RAxML 7.2.8 (Stamatakis, 2006) implemented on the CIPRES Science Gateway web server.

Estimates of genetic divergence were based on the number of synonymous substitutions per synonymous site (d_S) estimated from concatenated sequence alignments of *FUM* and PM genes used in the phylogenetic inference analyses described above, and from individual alignments of genes flanking the *FUM* cluster. Two methods were employed to estimate d_S values: 1) Maximum likelihood estimates of d_S were obtained using the CODEML program of PAML 4 (Yang, 2013) implemented on the Phylemon2 web server (Sánchez *et al*., 2011); and 2) estimates of d_S were also obtained using the modified Nei-Gojobori method with Jukes-Cantor correction (Zhang *et al*., 1998) implemented in MEGA5. Standard errors were estimated using the bootstrap method with 1000 pseudoreplications of the data. All positions containing gaps were eliminated from the analyses. The significance of differences in genetic divergence estimates was assessed using *Z* tests.

Fumonisin production assay

Two-week-old cultures grown on 10 g cracked maize kernel medium were extracted with 25 ml of a 50% acetonitrile-50% water solution for 3 h with shaking at 200 r.p.m. Extracts were centrifuged at 380 *g* for 5 min. The supernatant was then analysed for fumonisins by reverse-phase liquid chromatography coupled to a mass spectrometer (LC-MS) as previously described (Plattner *et al*., 1996; Proctor *et al*., 2006). Fumonisins were identified and quantified by comparison of retention

times, masses and mass spectra to those of FB and FC standards (Plattner *et al*., 1996).

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