Fungal Genetics and Biology 46 (2009) 936–948

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

A two-locus DNA sequence database for typing plant and human pathogens within the *Fusarium oxysporum* species complex

Kerry O'Donnell [a,*], Cécile Gueidan [b], Stacy Sink [a], Peter R. Johnston [c], Pedro W. Crous [b], Anthony Glenn [d], Ron Riley [d], Nicholas C. Zitomer [d], Patrick Colyer [e], Cees Waalwijk [f], Theo van der Lee [f], Antonio Moretti [g], Seogchan Kang [h], Hye-Seon Kim [h], David M. Geiser [h], Jean H. Juba [h], Robert P. Baayen [i,1], Matthew G. Cromey [j], Sean Bithell [j], Deanna A. Sutton [k], Kerstin Skovgaard [l], Randy Ploetz [m], H. Corby Kistler [n], Monica Elliott [o], Mike Davis [p], Brice A.J. Sarver [q]

[a] *Microbial Genomics Research Unit, Agricultural Research Service, US Department of Agriculture, Peoria, IL 61604, United States*
[b] *CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands*
[c] *Landcare Research, Auckland, New Zealand*
[d] *USDA-ARS, Athens, GA 30605, United States*
[e] *Louisiana State University Agricultural Center, Bossier City, LA 71040, United States*
[f] *Plant Research International, Wageningen University and Research, P.O. Box 16, 6700 Wageningen, The Netherlands*
[g] *CNR (Research National Council), ISPA Institute of Sciences of Food Production, Bari, Italy*
[h] *Department of Plant Pathology, The Pennsylvania State University, University Park, PA 16802, United States*
[i] *Ministry of Agriculture, Nature and Food Quality, P.O. Box 20401, 2500 EK The Hague, The Netherlands*
[j] *Crop and Food Research, Christchurch, New Zealand*
[k] *Department of Pathology, University of Texas Health Science Center, San Antonio, TX 78229, United States*
[l] *National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark*
[m] *University of Florida, Homestead, FL, United States*
[n] *Cereal Disease Laboratory, Agricultural Research Service, US Department of Agriculture, St. Paul, MN 55108, United States*
[o] *University of Florida, Ft. Lauderdale, FL 33301, United States*
[p] *Department of Plant Pathology, University of California, Davis, CA 95616, United States*
[q] *Department of Biological Sciences, University of Idaho, Moscow, ID 83844, United States*

ARTICLE INFO

Article history:
Received 8 June 2009
Accepted 18 August 2009
Available online 26 August 2009

Keywords:
Beauvericin
EF-1α
Enniatin
Fumonisin
Fusariosis
Genealogical discordance
IGS rDNA
Moniliformin
Mycotoxin
Phylogeny
Vascular wilt

ABSTRACT

We constructed a two-locus database, comprising partial translation elongation factor (*EF-1α*) gene sequences and nearly full-length sequences of the nuclear ribosomal intergenic spacer region (IGS rDNA) for 850 isolates spanning the phylogenetic breadth of the *Fusarium oxysporum* species complex (FOSC). Of the 850 isolates typed, 101 *EF-1α*, 203 IGS rDNA, and 256 two-locus sequence types (STs) were differentiated. Analysis of the combined dataset suggests that two-thirds of the STs might be associated with a single host plant. This analysis also revealed that the 26 STs associated with human mycoses were genetically diverse, including several which appear to be nosocomial in origin. A congruence analysis, comparing partial *EF-1α* and IGS rDNA bootstrap consensus, identified a significant number of conflicting relationships dispersed throughout the bipartitions, suggesting that some of the IGS rDNA sequences may be non-orthologous. We also evaluated enniatin, fumonisin and moniliformin mycotoxin production *in vitro* within a phylogenetic framework.

Published by Elsevier Inc.

1. Introduction

Members of the *Fusarium oxysporum* species complex (FOSC) are ubiquitous soil borne pathogens responsible for vascular wilts, rots, and damping-off diseases of a broad range of agronomically and horticulturally important crops (Baayen et al., 2000; Michielse

* Corresponding author. Fax: +1 309 681 6672.
E-mail address: kerry.odonnell@ars.usda.gov (K. O'Donnell).
URL: http://www.ars.usda.gov/sp2UserFiles/Place/36207000/MGB-ODonnell2.pdf (K. O'Donnell).
[1] Present address: European Commission, Directorate for Health and Consumers, Brussels, Belgium.

1087-1845/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.fgb.2009.08.006

and Rep, 2009). With more than 80 putatively plant host-specific formae speciales described (Kistler et al., 1998; Katan, 1999; Katan and Di Primo, 1999), members of the FOSC collectively represent the most commonly encountered and economically important species complex within *Fusarium*. A number of these fusaria are also clinically important, causing localized or deeply invasive life-threatening infections in humans and other animals (O'Donnell et al., 2004, 2007; Ortoneda et al., 2004). Mortality in patients who are persistently and severely neutropenic is typically 100% (Dignani and Anaissie, 2004).

Due to the staggering economic losses that members of the FOSC inflict on agriculture worldwide, an extensive body of literature exists on the genetic and pathogenic characterization of individual formae speciales into vegetative compatibility groups (VCGs). Formae speciales are defined based on pathogenicity to one or more plant hosts, whereas VCGs are defined based on their ability to anastomose and form heterokaryons (Gordon and Martyn, 1997). VCGs have proven to be excellent predictors of evolutionary origin (Elias et al., 1993), and they appear to be predominantly or exclusively clonal lineages. Some formae speciales are further divided into races based on virulence to a differential set of cultivars of one or more plant species. VCGs may harbor several races, which in such cases seem to differ only in cultivar-specific virulence genes (Baayen et al., 2000). Molecular phylogenetic analyses have shown that formae speciales with two or more VCGs may in some cases be polyphyletic (O'Donnell et al., 1998b; Baayen et al., 2000; Skovgaard et al., 2001; Mbofung et al., 2007; Fourie et al., 2009), calling into question the taxonomic value of the forma specialis naming system. Although putatively non-pathogenic strains have been described, and some have been employed successfully as biocontrol agents to suppress soil borne pathogens (Larkin et al., 1996; Fuchs et al., 1997; Olivain et al., 2006), the null hypothesis that some isolates may be non-pathogenic is virtually impossible to test given the huge number of potential vascular plant hosts. This problem is exacerbated because the phylogenetic history of the FOSC appears to be characterized by numerous host jumps based on geographic proximity rather than taxonomic relatedness (O'Donnell et al., 1998b; Baayen et al., 2000; Roy, 2001), and by the horizontal transfer of genes contributing to host specificity (van der Does et al., 2008).

Although diverse molecular markers have been developed to identify various formae speciales (Baayen et al., 2000; Groenewald et al., 2006; Lievens et al., 2007, 2008), time-consuming pathogenicity assays are currently the gold standard for identifying host-specific pathogens within the FOSC. Given the high level of phylogenetic diversity and large number of formae speciales, multilocus DNA sequence typing (MLST) currently represents the most robust approach for characterizing the genetic diversity of the FOSC. Moreover, DNA sequence data are ideally suited for sharing and developing web-accessible databases for the purpose of pathogen identification and strain typing via the Internet (Taylor and Fisher, 2003; Geiser et al., 2004; Park et al., 2008). Towards this end, the primary objective of the present study was focused on assessing the utility of partial translation elongation factor (*EF-1α*, 634 bp alignment) and nearly full-length nuclear ribosomal DNA intergenic spacer (IGS rDNA, 2220 bp alignment) region sequences for developing a two-locus database for the identification of formae speciales, opportunistic pathogens of humans and other animals, and environmental contaminants of hospital plumbing systems or food and beverage processing facilities. In addition, experiments were conducted to assess the potential of genetically diverse members of the FOSC to produce moniliformin, fumonisin, and enniatin mycotoxins *in vitro*. The FOSC database, including the electropherograms, will be incorporated into the next version of the web-accessible FUSARIUM-ID database (Geiser et al., 2004).

2. Materials and methods

2.1. Strains

To provide dense sampling of formae speciales genetic diversity, FOSC isolates were obtained from diverse sources (see Supplementary Table 1), with the majority obtained from the following three internationally accessible culture collections: CBS-KNAW Fungal Biodiversity Center (CBS, *N* = 157), Utrecht, The Netherlands; Fusarium Research Center (FRC, *N* = 119), Pennsylvania State University; and The International Collection of Microorganisms from Plants (ICMP, *N* = 246), Landcare Research, Auckland, New Zealand. We also included isolates characterized genetically in previous studies of the FOSC (O'Donnell et al., 1998b, 2004; Baayen et al., 2000; Skovgaard et al., 2001). In addition, some isolates included in this study were initially identified as members of the FOSC after conducting BLAST searches of the FUSARIUM-ID database (Geiser et al., 2004) (http://www.fusariumdb.org/), using partial translation elongation factor sequences as the query. Sequences of the sister taxon of the FOSC, *F. foetens*, were used to root the phylogeny (Schroers et al., 2004). We also included isolates associated with mycotic infections of humans and other animals, contaminants of food and beverage production facilities, putative non-pathogens, and biocontrol agents to assess their relationship to one another and to isolates of 68 described formae speciales, which we were able to obtain from internationally accessible culture collections (Table 1). All isolates are stored cryogenetically in liquid nitrogen vapors at ~175 °C in the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL (http://nrrl.ncaur.usda.gov/).

2.2. DNA manipulations

Genomic DNA was extracted from freeze-dried mycelium using a CTAB protocol as described previously (O'Donnell and Cigelnik, 1997). The two-locus typing scheme consisted of sequences of the nuclear ribosomal DNA intergenic spacer region (IGS rDNA) and the 5′ intron-rich portion of the *EF-1α* gene. PCR and sequencing primers for the IGS rDNA are listed in Fig. 1. Of the seven internal sequencing primers designed in this study, only four were typically needed (i.e., iNL11, NLa, CNSa and iCNS1) to span the region analyzed (2220 bp alignment). PCR primers EF-1 and EF-2 for the *EF-1α* gene are reported in O'Donnell et al. (1998b). A partial sequence of the *EF-1α* gene was obtained using EF-3 (GTAA GGAGGASAAGACTCACC) and EF-22 (AGGAACCCTTACCGAGCTC) as sequencing primers. PCR amplifications employed Platinum® *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) in an Applied Biosystems (ABI) 9700 thermocycler (Emeryville, CA) using the following program: 1 cycle of 90 s at 94 °C; 40 cycles of 30 s at 94 °C, 90 s at 55 °C, and 3 min at 68 °C; followed by 1 cycle of 5 min at 68 °C and a 4 °C soak. All PCR products were sized via electrophoresis in 1.5% agarose gels run in 1× TAE buffer, stained with ethidium bromide, and then photographed over a UV trans-illuminator. Prior to sequencing on an ABI 3730 capillary sequencer, amplicons were purified using Montage PCR_{96} filter plates (Millipore Corp. Billerica, MA). DNA sequencing reactions were conducted in a 10 μl volume with 0.5–2 μl of ABI BigDye version 3.1 terminator reaction mix, 2–4 pmol of each sequencing primer and approximately 50 ng of cleaned amplicon. Sequence data was edited and then aligned with Sequencher version 4.1.2 (Gene Codes, Ann Arbor, MI), after which they were exported as nexus files. The *EF-1α* and IGS rDNA sequence files were imported separately into DnaSP (Rozas et al., 2007) so that they could be exported as FASTA files which were then aligned using CLUSTAL-X (Larkin et al., 2007). Due to the number of length-variable indels,

Table 1
Summary of formae speciales included in study.

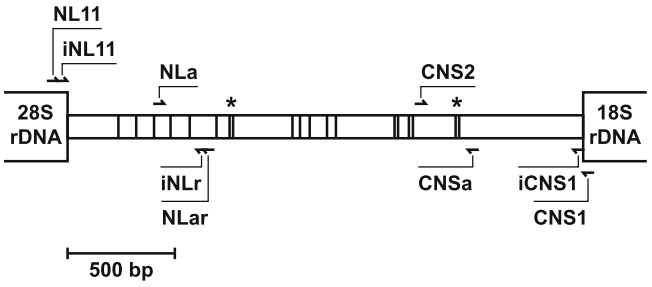
Forma specialis[a]	# Isolates[b]	# Haplotypes[c]	VCGs[d]	Two-locus haplotype # [=ST]
aechmeae	1	1		5
albedinis	3	2	1	30, 80
allii	1	1	⩾1	64
apii	5	4	3	2, 6, 145, 181
arctii	1	1		65
asparagi	8	5	⩾1	7, 51, 93, 106, 112
basilica	5	2	1	66, 193
batatas	6	6	⩾2	7, 34, 142, 162, 163, 178
betae	1	1	⩾7	182
bouvardiae	1	1		44
callistephi	3	2		2, 8
canariensis	3	2	⩾1	41, 200
cannabis	1	1		66
cassiae	1	1		70
cattleyae	1	1		9
cepae	3	3	1	7, 12, 212
chrysanthemi	4	2	2	10, 44
conglutinans	17	8	⩾3	2, 27, 160, 174, 252, 253, 255, 256
cubense	25	13	⩾24	7, 25, 36–39, 115–116, 135–141
cucumerinum	2	2	⩾6	71, 91
cucurbitacearum	2	2		3, 45
cyclaminis	1	1	3	149
dianthi	34	9	6	7, 42, 46, 87–90, 101, 158
elaeidis	6	2	⩾5	11, 256
erythroxyli	1	1	2	79
fabae	2	2		2 (=*F. inflexum*), 30
fatshederae	1	1		43
fragariae	1	1	⩾4	72
gladioli	28	8	6	7, 22, 23, 91–94
glycines	1	1		35
heliotropae	1	1		22
koae	1	1		231
lactucum	1	1	1	22
lagenariae	1	1	1	35
lentis	1	1		171
lilii	4	2	1	73, 107
lini	15	6	1	2, 90, 110, 111, 154, 159
loti	1	1	1	2
lupine	3	2	⩾2	47, 164
lycopersici	37	8	⩾4	43, 56, 63, 108, 150, 165, 166, 208
matthioli	5	3	1	13, 22, 198
medicaginis	5	3	3	14, 82, 93
melongenae	3	2	1	74, 194
melonis	18	5	⩾8	3, 4, 63, 165, 167
meniscoideum (var)	2	1		69
momordicae	2	2		67, 84
narcissi	3	1		8
nicotianae	1	1	⩾2	93
niveum	5	4	3	150, 156, 166, 223
opuntiarum	22	6	7	15, 30, 95–97, 103
palmarum	5	2		250, 251
passiflorae	2	2		16, 172
perniciosum	7	4		17, 94, 225, 226
phaseoli	4	2	8	51, 93
pini	3	1		18
pisi	26	10	⩾5	2, 27, 51, 90, 93, 145, 151, 155, 161, 169
radicis-lycopersici	5	4	⩾9	40, 55, 165, 168
raphani	5	2	1	19, 254
rauvolfiae	1	1		75
sesame	1	1		76
spinaciae	3	2	3	85, 86
tracheiphilum	4	4	1	20, 81, 153, 174
tuberose	1	1	6	21
tulipae	3	1	1	22
vanillae	1	1	⩾2	77
vasinfectum	135	24	⩾12	7, 23, 28–32, 63, 81, 113, 115–125, 133, 134, 220
voandzeiae	1	1		68
zingiberi	3	2		78, 119

[a] After the 850 isolate dataset was completed and analyzed, we typed the biocontrol strain Fo47 as ST 283 (Fuchs et al., 1997) and four isolates of *F. oxysporum* f. sp *radicis-cucumerinum* (NRRL 53513–53516) as ST 286 (Lievens et al., 2007).

[b] Of the 850 isolates typed, a total of 510 were assigned to one of 68 formae speciales. In addition, two isolates of the outgroup species, *Fusarium foetens*, represented STs 59 and 186 (Schroers et al., 2004).

[c] DNA sequences from the combined *EF-1α* gene and nuclear ribosomal intergenic spacer (IGS rDNA) region were used to identify 256 unique two-locus haplotypes (=STs).

[d] Based on Katan (1999) and Katan and Di Primo (1999).



NL11 5'-CTGAACGCCTCTAAGTCAG
iNL11 5'-AGGCTTCGGCTTAGCGTCTTAG
NLa 5'-TCTAGGGTAGGCKRGTTTGTC
iNLr 5'-AATTCTACTTACCCTAGAGC
NLar 5'-GGTAAGTAGAATTCGAGTTTC
CNS2 5'-AACTTCAATCGCCTCTCACG
CNSa 5'-TCTCATRTACCCTCCGAGACC
iCNS1 5'-TTTCGCAGTGAGGTCGGCAG
CNS1 5'-GAGACAAGCATATGACTAC

Fig. 1. Map of the nuclear ribosomal intergenic spacer region (IGS rDNA). PCR and sequencing primers are indicated by numbered half-arrows. PCR primers NL11 and iNL11 are nested within the 3′ end of the nuclear large subunit 28S rDNA whereas CNS1 is nested within the 5′ end of the nuclear small subunit 18S rDNA gene. Vertical lines indicate 19 repeated elements identified by DNA Strider version 1.4f18. Fifteen of these elements are represented by a 12 bp imperfect repeat with the consensus sequence GRTVYAGGGTAG. The remaining four elements, indicated by asterisks, consist of two 20 bp and 15 bp perfect repeats.

CLUSTAL-X (Larkin et al., 2007) was employed to obtain an initial alignment of the *EF-1α* and IGS rDNA partitions followed by manual improvement to establish positional homology. Phylogenetically informative indels were coded as a fifth character state, using TextPad for Windows (http://www.textpad.com/). No ambiguously aligned indel-containing positions were detected within the two-locus dataset. This finding is consistent with studies indicating a relatively shallow phylogenetic divergence within the FOSC. Nine indels within the *EF-1α* gene and 40 indels within the IGS rDNA partition were coded, irrespective of length, as a single phylogenetically informative (synapomorphic) fifth character state for the parsimony analyses. Gaps were considered to be homologous only if their 5′ and 3′ termini were identical (Simmons and Ochoterena, 2000). In other words, indels of different lengths that occupied overlapping nucleotide positions were not considered to be homologous. Indel coding only introduced a slight amount of homoplasy (CI = 0.4954 for combined dataset compared with a CI = 0.4865 for the 49 coded nucleotide positions), suggesting that phylogenetic signal from indels within the FOSC is generally concordant with that of nucleotide base substitutions at the low taxonomic level sampled. Although the results of recent studies support indel coding at lower taxonomic levels as a reliable source of phylogenetically informative characters (Kawakita et al., 2003; González et al., 2006), no consensus strategy on how to code coding them has been reached thus far. The DNA sequence data reported in this study have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/) under accession numbers FJ985264 – FJ985680 (Supplementary Table 2).

Southern analysis was conducted on ten diverse *F. oxysporum* isolates (Table 2), suspected of harboring divergent, intragenomic paralogous or xenologous copies of the IGS rDNA region, using a [α-^{32}P] labeled IGS rDNA amplicon from isolate NRRL 26220 (CBS 186.53 = FRC O-2063) *F. oxysporum* f. sp. *raphani* representing ST 2 as the probe. Genomic DNA isolated using the CTAB method was further purified using DNeasy columns (Qiagen, Valencia, CA), and restriction enzyme digestions were performed using 25 U of HaeIII and Sau3AI in separate reactions according to the manufacturer's instructions (New England BioLabs, Beverly, MA). Approximately 5 μg of restricted genomic DNA was loaded into each lane and DNA was blotted onto nitrocellulose membranes and probed using standard methods (Sambrook et al., 1989). The IGS rDNA amplicons from these strains, which were PCR amplified using primers NL11 and CNS1, were also digested with the same restriction enzymes and were separated using a 2% agarose gel. To assess whether the same 10 *F. oxysporum* isolates included in the Southern experiment (Table 2) possessed divergent, non-orthologous IGS rDNA sequences, an allele-specific PCR was conducted using two ITS2 type-specific reverse primers (e.g., IR and IIR; see Fig. 3 in O'Donnell and Cigelnik, 1997) in separate reactions with the forward primer NL11. PCR conditions used for this assay, which was conducted in an Applied Biosystems 9700 thermocycler, included 1 cycle of 30 s at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 58 °C, and 6 min at 68 °C; followed by 1 cycle of 5 min at 68 °C and a 4 °C soak.

2.3. Phylogenetic analyses and compatibility of datasets

Analyses using COLLAPSE version 1.1 (http://inbio.byu.edu/Faculty/kac/crandall_lab/Computer.html) identified 101 *EF-1α*, 203 IGS rDNA and 256 two-locus haplotypes. The collapsed 256 isolate dataset, which contained all of the unique two-locus haplotypes, was used for all subsequent phylogenetic analyses employing maximum parsimony (MP; Swofford, 2002) and maximum likelihood (ML; Zwickl, 2006). MP analyses of the combined partitions for the 256 isolate dataset were conducted using the parsimony ratchet (Nixon, 1999) implemented in PAUPRat (Sikes and Lewis, 2001) and PAUP* version 4.0 beta (Swofford, 2002). Searches for the shortest trees employed five independent parsimony ratchet runs

Table 2
Strains included in genomic Southern and allele-specific PCR experiments.

Isolates[a]	EF1		IGS rDNA				
	BS[b] (%)	# Differences	# Differences	# Substitutions	TS[c]	TV[d]	Indels (size of each in bp)[e]
36389 × 38289	93	0	72	68	52	16	1, 1, 2
26990 × 28369	98	0	36	30	23	7	1, 2, 3
26406 × 32885	86	2	30	25	16	9	5
36356 × 36472	88	0	109	27	20	7	1, 26, 55
32558 × 36102	95	2	107	78	61	17	1, 1, 1, 1, 1, 9, 15

[a] The five pairs of isolates were selected because they possessed identical or nearly identical *EF-1α* alleles but highly divergent, putatively non-orthologous IGS rDNA sequences. NRRL 26406 shares the same two-locus ST with NRRL 34936 *F. oxysporum* f. sp. *lycopersici*, the strain whose whole genome was sequenced (http://www.broad.mit.edu/).
[b] BS, bootstrap support. Clade support for each pair of isolates was between 86–95% based on MP bootstrapping of the *EF-1α* partition.
[c] TS, transitions.
[d] TV, transversions.
[e] Alignment of the IGS rDNA of each pair of isolates required the insertion of one to seven gaps 1–55 bp in length. The number of differences between each pair of IGS sequences represents the sum of the # of substitutions and total indel length.

of 200 iterations, using tree-bisection and reconnection branch swapping (TBR), and 1000 random sequence addition replicates. Nonparametric bootstrapping was used to assess clade support with PAUPRat in PAUP*, employing 1000 pseudo-replicates of the data, 10 random addition sequences per replicate, and TBR branch swapping. The general-time-reversible model with a proportion of invariant sites and gamma distributed rate heterogeneity (GTR + I + Γ) was selected as the best-fit model of nucleotide substitution for the combined dataset by the hierarchical likelihood ratio tests in MrModeltest version 2.2 (Posada, 2003), implemented in PAUP*. The best ML tree received a negative log likelihood ($-\ln L$) score of −18318.888549 based on the results of ten independent ML heuristic phylogenetic analyses, using the GTR + I + Γ model of nucleotide substitution in GARLI version 0.96 (Zwickl, 2006). Nonparametric bootstrap analysis of the 256 isolate dataset was conducted in GARLI, using a dual 2 GHz Power Mac G5. The ML bootstrap employed 200 ML pseudo-replicates of the data, in which the generations without improving the topology parameter was set at 5000. DNA Strider version 1.4f18 (obtained from Christian Marck, SBGM, CEA/Saclay, 91191 Gif-Sur-Yvette Cedex, France) was used to search for repetitive sub-repeat sequence motifs within the IGS rDNA, using a repeat size of $\geqslant$12 bp with no mismatched base pairs and no repeat sequence overlap.

Compatibility between the *EF-1α* and IGS rDNA datasets was assessed using the partition-homogeneity test (PHT; Farris et al., 1994) as implemented in PAUP*, and Compat.py version 3.0 (Kauff and Lutzoni, 2002a,b). For the Compat analysis, the two datasets (*EF-1α*, 634 characters, 101 synapomorphies; IGS rDNA, 2220 characters, 583 synapomorphies) were initially analyzed separately using MP as implemented in PAUP* (Swofford, 2002). Bootstrap analyses of 1000 replicates were conducted with the default settings, except for the following: gaps were treated as missing data, using one random addition sequence, and only one tree was saved per replicate. The 50% majority rule consensus trees were computed using the *EF-1α* and IGS rDNA bootstrap tree samples, and bootstrap support values were saved as branch length. Two strains of *F. foetens* were used as outgroup taxa to root the phylogenies. The bootstrap consensus trees were then used as input files in Compat, using a bootstrap support threshold of 70% to assess topological conflict.

2.4. Mycotoxin production

Genetically diverse members of the FOSC were assessed for their potential to produce moniliformin, fumonisin, beauvericin and other enniatin mycotoxins *in vitro*. To sample the genetic diversity of the FOSC, strains included in the mycotoxin analyses were specifically selected from the three major clades previously identified via molecular phylogenetics (O'Donnell et al., 1998b) and by including $\geqslant$10 different formae speciales. To assess fumonisin mycotoxin production, autoclaved cracked corn kernels (5 g hydrated with 50% water (v/w) in a 20 mL scintillation vial) were inoculated with a suspension of 1×10^7 conidia. Three vials were inoculated for each strain. After 14 days incubation at 27 °C in the dark, 10 mL of acetonitrile:water (1:1) containing 5% formic acid were added to each vial. After the vials were vigorously shaken to disrupt the cultures, they were placed on a rocker shaker for 3 h. Extracts were diluted 1000-fold in acetonitrile:water (3:7) containing 1% formic acid. After filtration using a Spin-X 0.45 μm nylon centrifugation filter (Corning Inc., Corning, NY), the diluted samples were analyzed by LC–MS as previously described (Williams et al., 2006). In addition to fumonisin B_1, B_2, and B_3 (FB1, FB2, and FB3), scans were added to detect fumonisin C_1, C_2, C_3, and C_4 (FC_1, FC_2, FC_3, and FC_4) based on their known masses and fragmentation patterns (Bartok et al., 2006). Analyses of the fumonisin C analogs included a qualitative standard of FC1 (generously supplied by Jennifer Tonos; USDA, ARS, Crop Genetics and Production Research Unit, Stoneville, MS). The fumonisin C analogs were quantified based on FB1 equivalents because pure standards were not available.

Moniliformin production was assessed by thin layer chromatography (TLC) by spotting 10 μL of each of the undiluted acidified acetonitrile:water extracts on a TLC sheet along a line of origin (Whatman Ltd., Maidstone, Kent, England; silica gel coating, 254-nm UV indicator, aluminum backing). TLC sheets were developed in a saturated chamber containing acetonitrile:toluene:water:formic acid (80:10:10:1) which gave good separation of moniliformin (R_f = 0.42) from the fungal pigments. Developed sheets were dried to evaporate the solvents and then they were photographed under UV light (254 nm) using an Alpha Innotech FluorChem 8000 digital imaging system (San Leandro, CA). Spot densitometry and linear log analysis of integrated density values, relative to a gradient of known standard, were used to calculate the amount of moniliformin in the extracts (Sigma–Aldrich, St. Louis, MO). The detection limit for moniliformin was approximately 30 μg/g of corn culture material.

The cyclohexadepsipeptides beauvericin and enniatin A_1, B and B_1 were extracted from autoclaved rice kernels (10 g hydrated with 45% water in 50 mL vials), which had been inoculated in triplicate with 1×10^7 conidia and incubated 4 weeks at 25 °C. Preparation and processing of samples and analyses by HPLC were performed as described previously (Moretti et al., 2002).

3. Results and discussion

3.1. Molecular data and genealogical discordance

We constructed a two-locus DNA sequence database, comprising partial *EF-1α* and nearly full-length IGS rDNA sequences, to support the molecular identification of host-specific plant pathogens (i.e., formae speciales), clinically and environmentally relevant isolates, and putative non-pathogens within the *F. oxysporum* species complex (FOSC). These loci were chosen because they possessed the greatest nucleotide diversity compared with the small number of loci tested to date within this complex (O'Donnell et al., 1998b; Baayen et al., 2000; Mbofung et al., 2007). Two isolates of the sister taxon of the FOSC, *F. foetens*, were included as outgroup taxa for rooting the phylogenies based on more inclusive analyses (Schroers et al., 2004). Amplicons were approximately 680 bp for the *EF-1α* gene and ranged from 2.4–2.6 kb in length for the IGS rDNA. Due to the number of length-variable indels, CLUSTAL-X (Larkin et al., 2007) was employed to obtain an initial alignment of the *EF-1α* and IGS rDNA partitions followed by manual improvement to establish positional homology. Manual alignment of the *EF-1α* gene dataset required the insertion of nine parsimony informative indels 1-to-4 bp in length within the intronic regions, yielding a total of 101 phylogenetically informative and 39 autapomorphic characters. In addition to coding nine indels within the *EF-1α* gene as a phylogenetically informative fifth character state, the 40 indels within the IGS rDNA region, which ranged from 1-to-99 bp in length, were coded similarly for the MP analysis, yielding 583 synapomorphic and 188 autapomorphic nucleotide characters. To obtain alignments of the bipartitions, which did not contain any missing sequence data, approximately 40 bp were trimmed from the conserved ends of the *EF-1α* and 250 bp IGS rDNA sequence alignments. By trimming the ends to exclude all missing sequence data, we were able to collapse the dataset with the software program COLLAPSE version 1.1 to identify all of the unique haplotypes in the individual and combined datasets. The *EF-1α* gene and nuclear IGS rDNA alignments contained 634 and 2220 nucleotide characters, respectively, totaling 2854 bp of

aligned DNA sequence per isolate. The aligned *EF-1α* sequences, excluding indels, ranged from 626 to 632 bp in length. By way of contrast, as much as 9.8% length variation was observed within the IGS rDNA, with NRRL 38290 *F. oxysporum* f. sp. *pini* (1932 bp) and NRRL 36408 *F. oxysporum* f. sp. *lupini* (2121 bp) possessing, respectively, the shortest and longest IGS rDNA sequences in the 2220 bp alignment.

To identify whether there was any potential incongruence among the bipartitions, a 69 h test run of the partition-homogeneity test was conducted in PAUP* based on 100 replicates yielded a *P* value of 0.01, indicating that the datasets harbored significant heterogeneity. Moreover, because the sum of lengths in the original partition (i.e., 1691 steps) was 233–270 steps shorter than the 99 shuffled partitions, we feel justified in assuming the P value after 1000 replicates would approximate 0.001. In addition, the program Compat (Kauff and Lutzoni, 2002a) was used to identify topological conflict between supported clades (support value $\geqslant$70%) in the *EF-1α* gene and IGS rDNA bootstrapped phylogenies. Such conflicts are assumed if a group of taxa is monophyletic in one tree and non-monophyletic in the other. In this dataset, 42 conflicting nodes were detected between these two partitions (Fig. 2). Some of these conflicts were overlapping, meaning they resulted from the conflicting placements of the same taxon (or taxa), but involved nested well-supported clades. Conflicts affected both terminal and deeper nodes, and they were distributed in all parts of the trees where well-supported nodes were present. Unlike the PHT, which is a global measure of homogeneity between datasets, Compat provides much more useful information by identifying incongruent nodes.

Based on the Compat congruence analysis, which identified conflicting relationships between the placement of isolates in the *EF-1α* gene and IGS rDNA phylogenies (Fig. 2), coupled with the results of previous studies of the FOSC which indicated that *EF-1α* phylogenies are concordant with those inferred from nuclear genes and the mitochondrial small subunit (mtSSU) ribosomal rDNA (O'Donnell et al., 1998b; Baayen et al., 2000; Skovgaard et al., 2001; Mbofung et al., 2007), we attempted to identify the source(s) of the discordance within the IGS rDNA. Initially we suspected that variable numbers of sub-repeats within the IGS rDNA might play a role in the topological discordance. These elements have been reported in a diverse range of fungi (Martin et al., 1999; Pramateftaki et al., 2000; Pantou et al., 2003), in which they have contributed to IGS length heterogeneity even within the same isolate (Chang et al., 2008). Mbofung et al. (2007) recently documented these elements in the FOSC IGS rDNA. To evaluate whether variable numbers of sub-repeats were responsible for the discordance, we identified five pairs of isolates that were nested in well-supported but topologically discordant clades in the bipartitions (Table 2). The *EF-1α* alleles were identical in three of the five pairs and differed by only two nucleotide substitutions in the other two. By contrast, divergence within the IGS rDNA in these five pairs ranged from 30 to 109 differences, including one to seven indels ranging between 1 and 55 bp in size (Table 2). Nineteen sub-repeats were identified within the IGS rDNA of the five pairs, using a repeat size of $\geqslant$12 bp with no repeat sequence overlap and no mismatched base pairs search parameters within DNA Strider version 1.4f18. In contrast to reports of head-to-tail tandemly repeated elements found in other fungi (Pramateftaki et al., 2000), none of the 19 elements were contiguous in the FOSC IGS rDNA (Fig. 1). Instead, 15 of these elements were represented by a 12 bp imperfect repeat with the consensus sequence GRTVYAGGGTAG dispersed over approximately 1.5 kb in the central portion of the IGS rDNA (Fig. 1). As first reported in *F. oxysporum* f. sp. *lactucae* by Mbofung et al. (2007), we also identified the GGTGTAGGGTAG motif in the majority of the sub-repeats in the present study (i.e., 5 of 15). The four other repeated sequences we identified included two 20 bp (GTGAGTCGATTTTTTTGTTT; nucleotide positions 683–702 and 1795–1814) and two 15 bp perfect repeats (TGAATTTTGCGGAAA; nucleotide positions 714–728 and 1823–1840) which were separated by a divergent region of 11 bp in the IGS alignment (identified by asterisks in Fig. 1). The same four elements were also identified in the same position in *F. oxysporum* f. sp. *lactucae* where they correspond to sub-repeats 22 and 15A (Fig. 1B in Mbofung et al., 2007).

Taken together, these analyses demonstrate that the discordance within the IGS rDNA is due to nucleotide polymorphism residing outside the sub-repeats, rather than variable numbers of or divergence within the sub-repeats. Some of these highly conserved elements are thought to play a role in the regulation of rRNA synthesis (Ganley et al., 2005). As first noted by Mbofung et al. (2007) in *F. oxysporum* f. sp. *lactucae*, our phylogenetic footprinting revealed that the ends of the IGS rDNA sequence were highly conserved across the FOSC (Fig. 1). The 0.6 kb region immediately 5′ to the nuclear small subunit (18S) rRNA-encoding gene, also known as the external transcribed spacer (Eickbush and Eickbush, 2007), contains essential coding and noncoding elements, including a 35S rRNA promoter. Given the high conservation of this promoter region in *Saccharomyces* spp. (Ganley et al., 2005), and in members of the FOSC, it appears to be under significant functional constraint.

Because members of the FOSC possess highly divergent non-orthologous ITS2 rDNA sequences (O'Donnell and Cigelnik, 1997; O'Donnell et al., 1998a), and rDNA loci have been identified on multiple chromosomes in some isolates within the FOSC (Boehm et al., 1994), we suspected that their genomes might harbor paralogous (e.g., via gene duplication and subsequent divergence) or xenologous (e.g., via horizontal gene transfer) copies of the IGS rDNA region. To evaluate these hypotheses, we digested genomic DNA of 10 strains with HaeIII and Sau3AI (Table 2), both of which recognize four bp target sequences, and probed the resulting Southern blot with an amplified IGS fragment (same as the one sequenced). The blot showed 3–4 bands hybridizing to the probe, but some of the bands appeared to consist of more than one fragment, which was expected from the predicted RFLP patterns based on their IGS sequences. We also amplified the IGS region from all 10 strains and digested the resulting products with HaeIII and Sau3AI, which resulted in 4–5 distinct fragments. In both analyses, we did not see any signs of the presence of polymorphic IGS repeats within these strains. Because these methods are crude, the negative results do not confirm the lack of heterogeneous copies of IGS. However, the clean sequence data obtained from these amplicons also support the supposition that if heterogeneous copies of the IGS are present they are likely to be in low copy number, as a mixture of polymorphic IGS rDNA templates would likely yield unreadable electropherograms.

Although members of the FOSC sampled to date possess highly divergent ITS2 types (O'Donnell and Cigelnik, 1997; O'Donnell et al., 1998a), all members of this complex appear to be fixed for a Type I ITS2 as the major type (i.e., the type detected by PCR and direct sequencing). In other words, the FOSC ITS2 sequences amplified by the conserved ITS4 and ITS5 primers represent an orthologous set. To assess whether divergent, non-orthologous IGS rDNA sequences were linked to the divergent ITS2 rDNA Type I and Type II sequences, the 10 isolates were subjected to an allele-specific PCR, using two reverse primers nested within the ITS2 rDNA (IR and IIR; see Fig. 3 in O'Donnell and Cigelnik, 1997) combined in separate reactions with forward primer NL11. Sequence analysis of the amplicons, however, indicated that the same IGS rDNA sequence type was linked to each non-orthologous ITS2 rDNA sequence type. Lastly, BLAST searches of the whole genome sequence of *F. oxysporum* f. sp. *lycopersici* strain NRRL 34936 (http://www.broad.mit.edu/annotation/genome/fusarium), using the IGS rDNA sequence of this isolate as the query, only identified one sequence type, even though the placement of the sequenced

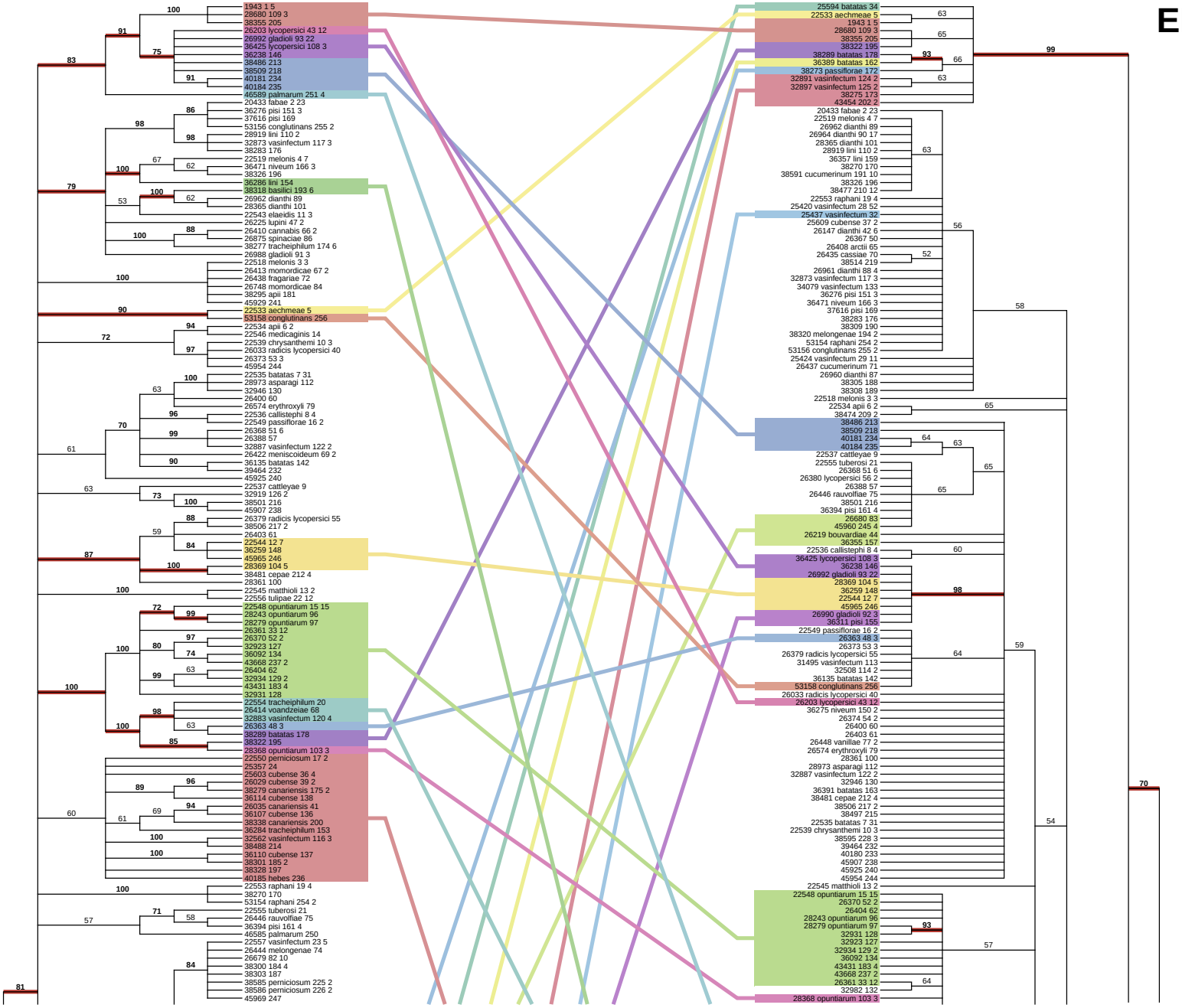


Fig. 2. Topological conflicts between IGS rDNA and *EF-1α*. Topological incongruence, as detected by Compat (Kauff and Lutzoni, 2002a), is indicated by colored boxes and lines linking the same isolates in the two trees. Bold red branches identify nodes involved in conflicting relationships. MP bootstrap support is indicated above the branches, with values ⩾70% shown in bold.

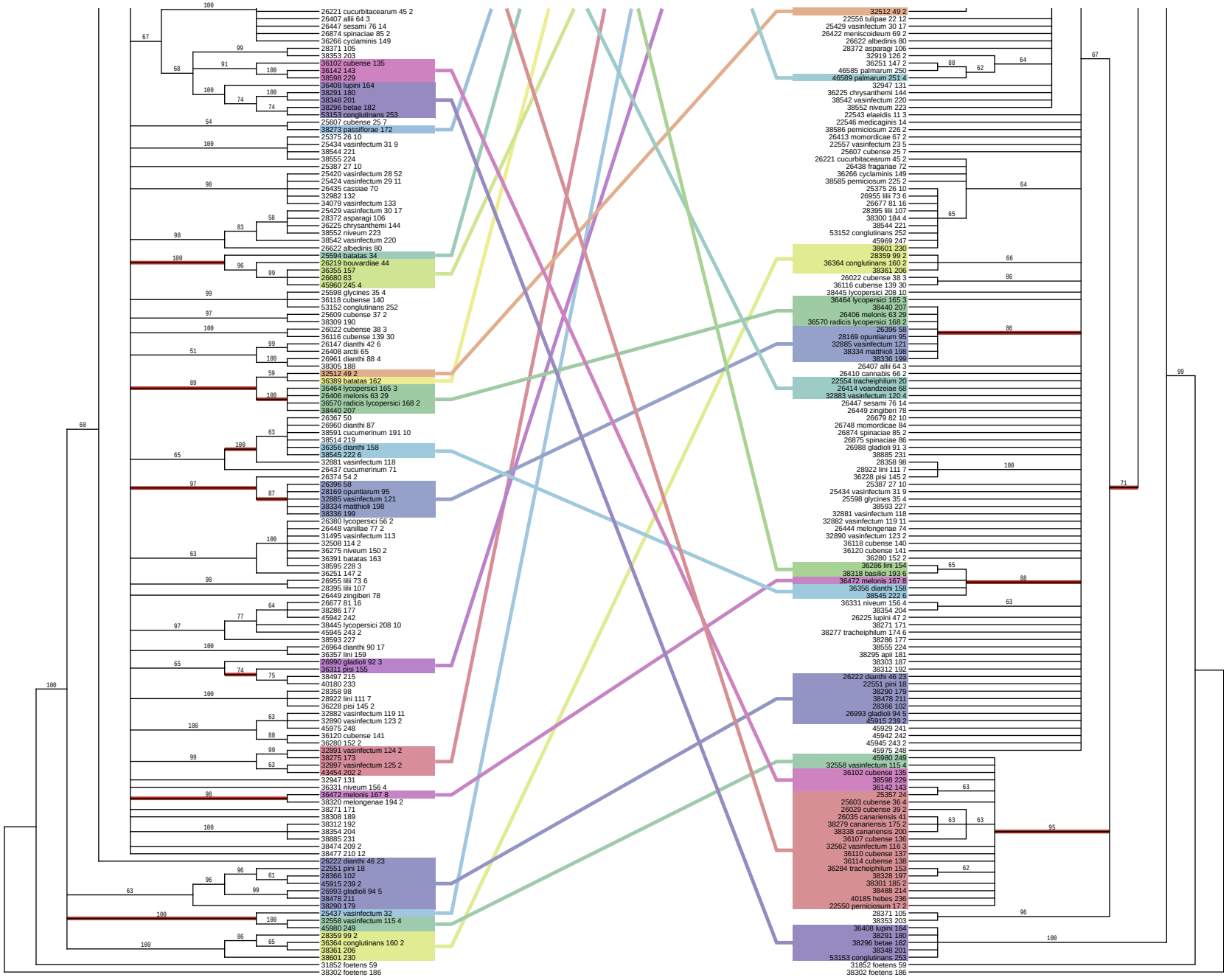


Fig. 2 (*continued*)

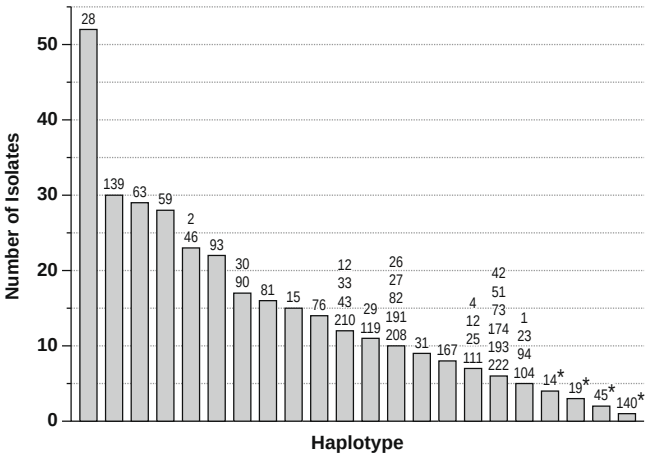


Fig. 3. Histogram showing the tailed distribution of isolates in each of the 22 haplotype classes defined by the number of isolates per two-locus haplotype. The number(s) above each bar identifies the number of ST(s) within each haplotype class, except for the four asterisked-numbers to the right which identify the number of STs within each haplotype class.

strain in the *EF-1α* and IGS rDNA bootstrapped phylogenies was topologically discordant (Fig. 2, Table 2; note that NRRL 34936 and 26406 both are ST 63). In summary, if divergent paralogous and/or xenologous IGS rDNA sequences are present in members of the FOSC, our results indicate that they must be in such low copy number as to have gone undetected.

Even though the underlying genetic mechanism(s) that are responsible for the homoplasy we detected at the IGS rDNA locus are unknown, results from other studies provide some possible explanations. Horizontal gene transfer (HGT) of a virulence locus has been reported within *F. oxysporum* f. sp. *lycopersici* (van der Does et al., 2008), and parasexual recombination has been demonstrated experimentally between vegetatively incompatible FOSC isolates under laboratory conditions (Molnár et al., 1990). Thus, the 42 genealogical discordant clades we detected in the *EF-1α* and IGS rDNA bootstrapped partitions may have resulted from multiple independent HGT and/or introgressive hybridization events. In support of this interpretation, HGT of virulence genes (He et al., 1998; Liu et al., 2003; Friesen et al., 2006; van der Does et al., 2008), and hybridization-mediated introgression of rRNA genes (Hughes and Petersen, 2001; Xie et al., 2008) have been reported in phylogenetically diverse fungi. In addition, HGT has been theorized to account for the discontinuous distribution of the *Fot1* transposon within the FOSC (Daboussi et al., 2002). Lastly, the genetic duplication and aneuploidy reported in the FOSC (Kistler et al., 1995) shows parallels with the parasexual cycle in *Candida ablicans* (Forche et al., 2008). Future studies are needed to elucidate whether HGT, possibly mediated by parasexuality, has played a major role in the reproductive success of the clonal or near clonal members of the FOSC, by generating adaptive mitotic recombinants (Schoustra et al., 2007).

3.2. DNA sequence-based typing scheme

Given the apparent underlying discordant evolutionary histories of the *EF-1α* and IGS rDNA, analyses of the combined dataset were restricted to determining the utility of the two-locus typing scheme for distinguishing individual formae speciales, indoor contaminants of hospitals or food and beverage production facilities, opportunistic pathogens of humans and other animals, and putative non-pathogens, including strains used for the biocontrol of soil borne pathogens. Towards this end, the 254 unique two-locus haplotypes within the ingroup were parsed into seven haplotype groups (Supplementary Fig. S1) based primarily on ST-host association. This subdivision revealed that approximately two-thirds of the STs ($N = 164$) were associated with a single host (i.e., haplotype groups 1 and 2). However, it should be noted that pathogenicity has not been determined for the 58 STs within haplotype group 2, except for those isolates considered as non-pathogenic to a particular host. Although three haplotype groups were associated with two or more hosts (i.e., haplotype groups 3–5), comprising 55 of the STs, only the 21 STs within haplotype group 3 were shared by two or more formae speciales. It is noteworthy that five STs (i.e., 2, 7, 22, 30 and 93) within haplotype group 3 were linked to four or more formae speciales, with ST 2 being shared by seven host-specific pathogens (i.e., f. spp. *apii*, *callistephi*, *conglutinans*, *fabae*, *lini*, *loti* and *pisi*) and ST 7 by eight (i.e., f. spp. *asparagi*, *batatas*, *cepae*, *cubense*, *dianthi*, *gladioli*, *phaseoli* and *vasinfectum*). Several of the formae speciales involved (f. spp. *apii*, *asparagi*, *batatas pisi*, *vasinfectum*, and others) have been shown to cross-infect the hosts of other formae speciales; isolates received as a specific forma specialis might in such cases also be classifiable as another forma specialis, in particular in the case of *F. oxysporum* f. sp. *vasinfectum* (Armstrong and Armstrong, 1975; Davis et al., 2006). Isolates belonging to different formae speciales and sharing their STs should be tested systematically for cross-pathogenicity in future investigations.

Haplotype group 6, with 10 novel STs and seven STs which are also shared by haplotype group 7, includes indoor contaminants, notably of hospital water systems and food and beverage production facilities, together with soil isolates or isolates whose host/substrate data is incomplete. Haplotype groups 6 and 7 also contain ST 33, previously reported as the widespread FOSC 3-a clonal lineage (O'Donnell et al., 2004, 2007; Chang et al., 2006), and STs 49 and 54. These three STs have been recovered as hospital contaminants and from mycotic infection of humans, suggesting nosocomiality. Lastly, haplotype group 7 contains 26 STs whose membership is based on at least one isolate in each ST being recovered from an opportunistic infection of humans or other animals. Opportunistic human pathogens within the FOSC are genetically diverse not only in terms of numbers of distinct STs ($N = 26$), but because they are nested within the three major clades that comprise the phylogenetic breadth of this complex (O'Donnell et al., 1998b, 2004). Half of the STs within this group exclusively contained clinically relevant isolates, and these included 10 singleton STs and STs in which all three (ST 48 = FOSC 3-c and ST 109 = FOSC 4-c) or all five isolates (ST 1 = FOSC 4-b) were recovered from opportunistic infections of humans. The remaining 13 STs within haplotype group 7 contained isolates that were also assigned to one other haplotype group. Of these, ST 33 (=FOSC 3-a; Chang et al., 2006) appears to be the most important member of haplotype groups 6 and 7, given how common it has been recovered from infections of humans, and hospital plumbing and food and beverage production facilities (O'Donnell et al., 2004, 2007). In addition to the human pathogenic FOSC STs mentioned above, the ability of clinically important fusaria to colonize plumbing systems via biofilms appears to be widespread within *Fusarium* (Imamura et al., 2007; O'Donnell et al., 2008; Schroers et al., 2009), and may help explain why fusaria were almost exclusively responsible for the 2005 and 2006 contact lens solution-associated keratitis outbreaks in Asia and the United States (Chang et al., 2006; O'Donnell et al., 2007).

Of the 848 ingroup taxa, 504 (59.4%) were received as one of 68 described formae speciales (Table 1), while the remaining 344 isolates were received without a formae speciales designation. ST 28, with 52 isolates, is the most common ST represented in the database (Fig. 3, Table 1). All of the isolates within this ST were received as *F. oxysporum* f. sp. *vasinfectum* except for NRRL 38307 (=FRC O-1259). The two-locus typing scheme we employed failed to

identify more than one ST within a VCG. This finding is consistent with previous studies indicating VCGs typically possess a highly clonal population structure (Kistler, 1997), but it could also reflect the low resolution of the current typing scheme based on two genetic loci. Indeed, the clonal nature of VCGs does not exclude further evolution. VCGs may contain several races which are closely related genetically (Baayen et al., 1997, 2000), suggesting gene-for-gene evolution of virulence; examples are to be found in *F. oxysporum* f. sp. *dianthi* VCG 0022 (ST 90; races 1 and 8), *F. oxysporum* f. sp. *gladioli* VCG 0340 (ST 93; races 1, 2i, 2n, 3 and 4), and *F. oxysporum* f.sp. *melonis* VCGs 0135 and 0138-II (ST 167; races 0, 1, 2 and 1.2). In cases where a specific race is reported for more than a single VCG (or ST), this may be due to lack a differential host varieties for resolving differences in virulence (Armstrong and Armstrong, 1975) as has been repeatedly demonstrated (Baayen et al., 1997; Mes et al., 1994).

We also populated the database with 27 putatively non-pathogenic isolates, including strains that have been used to control fusarium wilts of strawberry (NRRL 45881 = Fus-IMJF), tomato, cucumber and other crops (NRRL 53506 = Fo47; Fuchs et al., 1997; Benhamou et al., 2002). STs of 13 of the 27 putative non-pathogens matched a ST of a known forma specialis (Supplementary Table 3), Non-pathogenic isolates generally did not match the ST of the known forma specialis from the same host and typically appear to be unrelated. An exception is isolate NRRL 26442 from lily which was originally isolated and stored as f. sp. *lilii*, but is no longer pathogenic. Of the two biocontrol strains we typed, only the strain used for suppressing wilt of strawberry in Sweden (NRRL 45881 = Fus-IMJF) shared a two-locus ST with a known forma specialis (i.e., *F. oxysporum* f. sp. *raphani*, ST 19), suggesting that the former isolate should be tested for pathogenicity to *Raphanus sativus*, before being released in an area where radish and other cruciferous crops are grown.

Consistent with reports that soilborne members of FOSC are genetically diverse (Edel et al., 1997, 2001), 85% of the putatively non-pathogenic isolates were resolved as a unique ST (i.e., 23 of 27; Supplementary Table 3), and these were nested within the three major clades previously identified within the FOSC (O'Donnell et al., 1998b). Nonetheless, future developments in the database are needed to accurately assess evolutionary relationships among biocontrol and plant pathogenic fusaria within a robust phylogenetic framework. Even in instances where HGT of virulence genes has been reported (van der Does et al., 2008), by extensive sampling of known formae speciales, it should be possible in the near future to develop a high-resolution MLST scheme comparable to those currently available for typing human pathogenic *Candida* species (Odds and Jacobsen, 2008). Such a scheme is critical to advancing our ability to assess the risk of releasing putatively non-pathogenic FOSC biocontrol isolates into the environment (Cook et al., 1996). In addition, this database should provide a robust phylogenetic framework to evaluate whether different modes of biocontrol activity have evolved in these isolates (Fravel et al., 2003).

The primary objective of the present study was to develop a comprehensive DNA sequence database for identifying pathogens within the FOSC that could minimize and ultimately eliminate the need for time-consuming bioassays. Toward this end, the FOSC two-locus database, including the DNA sequence alignments and electropherograms, will be incorporated into the internet-accessible FUSARIUM-ID database (http://fusarium.cbio.psu.edu/). FUSARIUM-ID will evolve to Cyber-infrastructure for *Fusarium* (CiF; http://www.fusariumdb.org/), together with a similarly comprehensive MLST database for members of the *F. solani* species complex (O'Donnell et al., 2008). Inclusion of the downloadable alignments will facilitate identification of new and existing STs, help predict the forma specialis of unknowns, and assist researchers in validating the accuracy of new sequence data by taking advantage of their comprehensive phylogenetic footprint.

We emphasize the importance of depositing representative isolates of all newly described formae speciales, races and VCGs of *F. oxysporum* in publically accessible culture collections (Kang et al., 2006). Host and substrate of origin should be identified, and assumptions of pathogenicity should be verified (Koch's postulates); isolates taken from a host associated with a known forma specialis need not belong to that forma specialis, even in the case of typical symptomatology. Well-preserved and well-characterized isolates are essential for any study on pathogenic diversity or host-pathogen interactions, in absence of which the value of such studies is at stake. In addition, data used to generate MLSTs or otherwise characterize a taxon should be archived in a centralized database that can be edited by the scientific community. For example, the ability to correct ambiguous DNA sequence data would be most useful (Bruns et al., 1998). To illustrate the value of transparent and verifiable data, we did not incorporate GenBank data in the FOSC database due to problems we encountered with the reliability of these data (Pennisi, 2008).

The primary strength of the current version of the FOSC database is the dense sampling provided by 848 isolates representing 68 formae speciales and 256 unique STs. This database will continue to be updated as new FOSC pathogens and formae speciales are typed. Unlike web-accessible databases for the identification of *Trichoderma* (Druzhinina et al., 2005), ectomycorrhizal basidiomycetes (Bruns et al., 1998), *Fusarium* (Geiser et al., 2004), and *Phytophthora* (Park et al., 2008), where BLAST searches and phylogenetic analyses are employed to identify species, the homoplastic evolutionary history of the IGS rDNA precludes the use of molecular phylogenetics to accurately identify relationships within the FOSC. At present, we recommend identifying unknowns as new or known STs by collapsing each two-locus dataset to a minimal set of novel haplotypes.

3.3. Mycotoxin production

Based on published reports of mycotoxin production within the FOSC (Rabie et al., 1982; Seo et al., 1996), we investigated moniliformin and fumonisin B and C series toxin production *in vitro* on cracked corn cultures. The 19 phylogenetically diverse strains included in the mycotoxin analyses were specifically selected to sample from all of the FOSC clades previously identified (O'Donnell et al., 1998b, 2004) and included 18 unique STs, 11 formae speciales and one isolate of *Fusarium foetens*. NRRL 29056 *F. verticillioides* (FRC M-3125), NRRL 39464 *F. oxysporum* (FRC O-1890; Seo et al., 1996) and NRRL 22037 *F. proliferatum* were used as positive controls for fumonisin production (Supplementary Table 4). The latter isolate was also used as a positive control for moniliformin production. In comparison to the positive control, TLC assessment of moniliformin production demonstrated that of the other strains tested, only NRRL 26029 *F. oxysporum* f. sp. *cubense* (VCG 01210) was able to produce moniliformin, but at very low levels (34 ± 6.0 ppm). Similarly, other than the positive controls, only NRRL 39464 *F. oxysporum* (FRC O-1890 = strain CAR, ex *Dianthus caryophyllus* from Korea), representing the singleton ST 232, was able to elaborate fumonisin B and C series analogues. As originally reported (Seo et al., 1996), NRRL 39464 produced mostly FC_1 and little FB_1 or FB_2, in contrast to NRRL 22037 *F. proliferatum* and NRRL 29056 *F. verticillioides*, which elaborated high levels of FB_1, moderate levels of FB_2, and minor amounts of FC_1, FC_2, or FC_3. Fumonisins and moniliformin were not detected in cultures of NRRL 31852 *F. foetens*, the sister species of the FOSC. Our results are consistent with the findings of Proctor et al. (2004) who found that of the four FOSC isolates tested; only NRRL 39464 possessed fumonisin biosynthetic genes and produced fumonisins. Results of the present

study suggest that production of this toxin by members of the FOSC may be limited (Seo et al., 1996). By way of contrast, our results that suggest limited moniliformin production within the FOSC differ from those of Rabie et al. (1982) who reported that 14 of 24 toxic isolates produced this toxin. However, because the strains included in Rabie et al. (1982) were not characterized genetically, it is possible that they isolated a widespread moniliformin-producing clone within the FOSC, or more likely not even members of the FOSC as currently circumscribed phylogenetically. At the time of their publication (Rabie et al., 1982), over 10 species recognized today outside the FOSC would have been identified by them as *F. oxysporum* using most morphological treatments (i.e., by the production of chlamydospores, monophialides but no polyphialides, and microconidia in false heads rather than chains; Nirenberg and O'Donnell, 1998; O'Donnell et al., 1998a).

In addition, based on a report of cyclohexadepsipeptide mycotoxin production within the FOSC (Moretti et al., 2002), we investigated the potential of 40 genetically diverse isolates representing 30 STs and nine formae speciales selected from three major clades of the FOSC (O'Donnell et al., 1998b) to produce the beauvericin and enniatin A_1, B and B_1 *in vitro*. Beauvericin production was observed in the majority (30 out of 40) of isolates analyzed (Supplementary Table 5), ranging from minute amounts in some isolates to 240 ppm in NRRL 26991 *F. oxysporum* f. sp. *gladioli*, 245 ppm in NRRL 28384 *F. oxysporum* f. sp. *asparagi* and 300 ppm in NRRL 26961 *F. oxysporum* f. sp. *dianthi*. The potential to produce enniatin A_1, B and B_1 appeared to be slightly less frequent, with 23 out of 40 isolates producing enniatin B, while production of enniatin A and B_1 was observed in only five and four isolates, respectively. These results generally parallel those obtained from isolates of *F. oxysporum* f. sp. *melonis* by Moretti et al. (2002) who reported that 37 of 44 isolates produced beauvericin, whereas only 11 isolates produced enniatin B, and none produced A and B_1 enniatins. In contrast to the apparent limited potential to produce moniliformin and fumonisins, our results indicate beauvericin and enniatin B cyclohexadepsipeptide mycotoxin production appears to be widespread among members of the FOSC. Two competing hypotheses can be proposed to explain the discontinuous phylogenetic distribution of moniliformin and fumonisin production within the FOSC (Patron et al., 2007): (1) horizontal gene cluster transfer, possibly mediated by dispensable chromosomes as reported for *F. solani* f. sp. *pisi* (Temporini and VanEtten, 2004), followed by subsequent inactivation or loss, and (2) phylogenetic inheritance followed by vertical transmission and subsequent inactivation or loss (Kroken et al., 2003). Evidence for the latter hypothesis has been documented in genetically divergent isolates of *F. verticillioides* from banana where the fumonisin gene cluster has been completely deleted (Glenn et al., 2008).

3.4. Conclusions

The widespread genealogical discordance between the IGS rDNA and *EF-1α* bipartitions reported herein, together with the discovery of non-orthologous ITS2 types within the FOSC and *Gibberella fujikuroi* species complexes (O'Donnell and Cigelnik, 1997; O'Donnell et al., 1998a), provides a compelling argument against using single-locus data, especially from nuclear ribosomal RNA genes, for phylogenetic reconstruction and for inferring species limits within the Fungi (Taylor et al., 2000). Although the IGS rDNA has become one of the most popular loci for investigating genetic diversity within the FOSC (Fujinaga et al., 2005; Kawabe et al., 2005; Mbofung et al., 2007; Enya et al., 2008), and its high levels of nucleotide diversity provide a high degree of discriminatory power useful for isolate identification, results of the present study strongly indicate that the homoplastic evolutionary history of this locus obscures accurate phylogenetic relationships within this complex. Of the relatively small number of genes that have been sampled to date within the FOSC (ex., IGS rDNA, *EF-1α*, polygalacturonases, mitochondrial small subunit ribosomal RNA, phosphate permease, β-tubulin, nitrate reductase, *MAT 1* and *MAT2*), only the *EF-1α* and IGS rDNA genes appear to have much phylogenetic signal. However, genealogical discordance involving the IGS rDNA (Mbofung et al., 2007; Fourie et al., 2009; present study) precludes the use of this locus for molecular phylogenetics within the FOSC. Similarly, discordant phylogenies were recovered employing four polygalacturonase genes (Hirano and Arie, 2009) and a mitochondrial repeat region (Fourie et al., 2009), which suggests that identifying an orthologous set of loci for phylogenetic inference within the FOSC will be a challenging endeavor.

The major limitation of the current version of the FOSC database is that the resolution is too poor to distinguish all of the formae speciales and VCGs. Future improvements in the database will take advantage of the genomes of 10 phylogenetically diverse fusaria that are being sequenced at the Broad Institute of MIT and Harvard (H. C. Kistler, personal communication). Once these genomes become available online, it should be possible to profile phylogenetically informative genes (Townsend, 2007; Aguileta et al., 2008) to identify an orthologous set of loci for a high-resolution MLST typing schemes for epidemiological investigations (O'Donnell et al., 2004; Chang et al., 2006) and molecular phylogenetics. In addition, these genomes should harbor VNTR loci, which might prove to be ideal for high throughput strain typing (Taylor and Fisher, 2003; Ward et al., 2008), and these genomic resources should also facilitate identification of virulence genes for specific formae speciales (races) if required (Recorbet et al., 2003; van der Does et al., 2008).

Moreover, a highly resolved phylogeny would provide the necessary framework to address critical questions concerning the population biology and reproductive mode of these fungi including the degree of clonality within the FOSC (Taylor et al., 1999; Halkett et al., 2005), and whether host specificity has evolved vertically and/or horizontally within the FOSC (van der Does et al., 2008). Given their importance to agriculture and medicine, knowledge gained from these basic studies should promote more informed disease control strategies and quarantine regulations, and assist development of cultivars with broad resistance to the plethora of host-specific pathogens within the FOSC.

Acknowledgments

Special thanks are due the individuals and culture collections listed in Supplementary Table 1 for generously sharing strains, Alison Strom and Jean Juba for excellent technical assistance, and Don Fraser for preparing the publication figures. The mention of firm names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture over other firms or similar products not mentioned.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2009.08.006.

References

Aguileta, G., Marthey, S., Chiapello, H., Lebrun, M.-H., Rodolphe, F., Fournier, E., Gendrault-Jacquemard, A., Giraud, T., 2008. Assessing the performance of single-copy genes for recovering robust phylogenies. Syst. Biol. 57, 613–627.

Armstrong, G.M., Armstrong, J.K., 1975. Reflections on the wilt fusaria. Ann. Rev. Phytopathol. 13, 95–103.

Baayen, R.P., Van Dreven, F., Krijger, M.C., Waalwijk, C., 1997. Genetic diversity in *Fusarium oxysporum* f. sp. *dianthi* and *Fusarium redolens* f.sp. *dianthi*. Eur. J. Plant Pathol. 103, 395–408.

Baayen, R.P., O'Donnell, K., Bonants, P.J.M., Cigelnik, E., Kroon, L.P.N.M., Roebroeck, E.J.A., Waalwijk, C., 2000. Gene genealogies and AFLP analyses in the *Fusarium*

oxysporum complex identify monophyletic and nonmonophyletic formae speciales causing wilt and rot diseases. Phytopathology 90, 891–900.
Bartók, T., Szécsi, Á., Szekeres, Á., Mesterhazy, Á., Bartók, M., 2006. Detection of new fumonisin mycotoxins and fumonisin-like compounds by reversed-phase high-performance liquid chromatography/electrospray ionization ion trap mass spectrometry. Rapid Commun. Mass Spectrom. 20, 2447–2462.
Benhamou, N., Garand, C., Goulet, A., 2002. Ability of nonpathogenic *Fusarium oxysporum* strain Fo47 to induce resistance against *Pythium ultimum* infection in cucumber. Appl. Environ. Microbiol. 68, 4044–4060.
Boehm, E.W.A., Ploetz, R.C., Kistler, H.C., 1994. Statistical analysis of electrophoretic karyotype variation among vegetative compatibility groups of *Fusarium oxysporum* f. sp. *cubense*. Mol. Plant Microbe Interact. 7, 196–207.
Bruns, T.D., Szaro, T.M., Gardes, M., Cullings, K.W., Pan, J.J., Taylor, D.L., Horton, T.R., Kretzer, A., Garbelotto, M., Li, Y., 1998. A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis. Mol. Ecol. 7, 257–272.
Chang, D.C., Grant, G.B., O'Donnell, K., Wannemuehler, K.A., Noble-Wang, J., Rao, C.Y., Jacobson, L.M., Crowell, C.S., Sneed, R.S., Lewis, F.M.T., Schaffzin, J.K., Kainer, M.A., Genese, C.A., Alfonso, E.C., Jones, D.B., Srinivasan, A., Fridkin, S.K., Park, B.J., 2006. A multistate outbreak of *Fusarium* keratitis associated with use of a contact lens solution. JAMA 296, 953–963.
Chang, J.-C., Hsu, M.M.-L., Barton, R.C., Jackson, C.J., 2008. High-frequency intragenomic heterogeneity of the ribosomal DNA intergenic spacer region in *Trichophyton violaceum*. Eukar. Cell 7, 721–726.
Cook, J.M., Bruckart, W.L., Coulson, J.R., Goettel, M.S., Humber, R.A., Lumsden, R.D., Maddox, J.V., McManus, M.L., Moore, L., Meyer, S.F., Quimby Jr., P.C., Stack, J.P., Vaughn, J.L., 1996. Safety of microorganisms intended for pest and plant disease control: a framework for scientific evaluation. Biol. Control 7, 333–351.
Daboussi, M.-J., Davière, J.-M., Graziani, S., Langin, T., 2002. Evolution of the *Fot1* transposons in the genus *Fusarium*: discontinuous distribution and epigenetic inactivation. Mol. Biol. Evol. 19, 510–520.
Davis, R.M., Colyer, P.D., Rothrock, C.S., Kochman, J.K., 2006. Fusarium wilt of cotton: population diversity and implications for management. Plant Dis. 90, 692–703.
Dignani, M.C., Anaissie, E.J., 2004. Human fusariosis. Clin. Microbiol. Infect. 10, 67–75.
Druzhinina, I.S., Kopchinskiy, A.G., Komoń, M., Bissett, J., Szakacs, G., Kubicek, C.P., 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genet. Biol. 42, 813–828.
Edel, V., Steinberg, C., Gautheron, N., Alabouvette, C., 1997. Populations of nonpathogenic *Fusarium oxysporum* associated with roots of four plant species compared to soilborne populations. Phytopathology 87, 693–697.
Edel, V., Steinberg, C., Gautheron, N., Recorbet, G., Alabouvette, C., 2001. Genetic diversity of *Fusarium oxysporum* populations isolated from different soils in France. FEMS Microbiol. Ecol. 36, 61–71.
Eickbush, T.H., Eickbush, D.G., 2007. Finely orchestrated movements: evolution of the ribosomal RNA genes. Genetics 175, 477–485.
Elias, K.S., Zamir, D., Lichtman-Pleban, T., Katan, T., 1993. Population structure of *Fusarium oxysporum* f. sp. *lycopersici*: restriction fragment length polymorphisms provide genetic evidence that vegetative compatibility group is an indicator of evolutionary origin. Mol. Plant Microbe Interact. 6, 565–572.
Enya, J., Togawa, M., Takeuchi, T., Yoshida, S., Tsushima, S., Arie, T., Sakai, T., 2008. Biological and phylogenetic characterization of *Fusarium oxysporum* complex, which causes yellows on *Brassica* spp., and proposal of *F. oxysporum* f. sp. *rapae*, a novel forma specialis pathogenic on *B. rapa* in Japan. Phytopathology 98, 475–483.
Farris, J.S., Kallersjö, M., Kluge, A.G., Bult, C., 1994. Testing significance of incongruence. Cladistics 10, 315–319.
Forche, A., Alby, K., Schaefer, D., Johnson, A.D., Berman, J., Bennett, R.J., 2008. The parasexual cycle in *Candida albicans* provides an alternative pathway to meiosis for the formation of recombinant strains. PLoS Biol. 6 (5), e110. doi:10.1371/journal. pbio. 0060110.
Fourie, G., Steenkamp, E.T., Gordon, T.R., Viljoen, A., 2009. Evolutionary relationships among the *Fusarium oxysporum* f. sp. *cubense* vegetative compatibility groups. Appl. Environ. Microbiol. 75, 4770–4781.
Fravel, D., Olivain, C., Alabouvette, C., 2003. *Fusarium oxysporum* and its biocontrol. New Phytol. 157, 493–502.
Friesen, T.L., Stukenbrock, E.H., Liu, Z., Meinhardt, S., Ling, H., Faris, J.D., Rasmussen, J.B., Solomon, P.S., McDonald, B.A., Oliver, R.P., 2006. Emergence of a new disease as a result of interspecific virulence gene transfer. Nat. Genet. 38, 953–956.
Fuchs, J.-G., Moënne-Loccoz, Y., Défago, G., 1997. Nonpathogenic *Fusarium oxysporum* strain Fo47 induces resistance to Fusarium wilt in tomato. Plant Dis. 81, 492–496.
Fujinaga, M., Ogiso, H., Shinohara, H., Tsushima, S., Nishimura, N., Togawa, M., Saito, H., Nozue, M., 2005. Phylogenetic relationships between the lettuce root rot pathogen *Fusarium oxysporum* f. sp. *lactucae* race 1, 2, and 3 based on the sequence of the intergenic spacer region of its ribosomal DNA. J. Gen. Plant Pathol. 71, 402–407.
Ganley, A.R.D., Hayashi, K., Horiuchi, T., Kobayashi, T., 2005. Identifying gene-independent noncoding functional elements in the yeast ribosomal DNA by phylogenetic footprinting. Proc. Natl. Acad. Sci. USA 102, 11787–11792.
Geiser, D.M., Jiménez-Gasco, M.delM., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T.J., Zhang, N., Kuldau, G.A., O'Donnell, K., 2004. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. Eur. J. Plant Pathol. 110, 473–479.
Glenn, A.E., Zitomer, N.C., Zimeri, A.M., Williams, L.D., Riley, R.T., Proctor, R.H., 2008. Transformation-mediated complementation of a *FUM* gene cluster deletion in *Fusarium verticillioides* restores both fumonisin production and pathogenicity on maize seedlings. Mol. Plant Microbe Interact. 21, 87–97.
González, D., Cubeta, M.A., Vilgalys, R., 2006. Phylogenetic utility of indels within ribosomal DNA and β-tubulin sequences from fungi in the *Rhizoctonia solani* species complex. Mol. Phylogenet. Evol. 40, 459–470.
Gordon, T.R., Martyn, R.D., 1997. The evolutionary biology of *Fusarium oxysporum*. Annu. Rev. Phytopathol. 35, 111–128.
Groenewald, S., van den Berg, N., Marasas, W.F.O., Viljoen, A., 2006. The application of high-throughput AFLP's in assessing genetic diversity in *Fusarium oxysporum* f. sp. *cubense*. Mycol. Res. 110, 297–305.
Halkett, F., Simon, J.-C., Balloux, F., 2005. Tackling the population genetics of clonal and partially clonal organisms. TREE 20, 194–201.
He, C., Rusu, A.G., Poplawski, A.M., Irwin, J.A.G., Manners, J.M., 1998. Transfer of a supernumerary chromosome between vegetatively incompatible biotypes of the fungus *Colletotrichum gloeosporioides*. Genetics 150, 1459–1466.
Hirano, Y., Arie, T., 2009. Variation and phylogeny of *Fusarium oxysporum* isolates based on nucleotide sequences of polygalacturonase genes. Microbes Environ. 24, 113–120.
Hughes, K.W., Petersen, R.H., 2001. Apparent recombination or gene conversion in the ribosomal ITS region of a *Flammulina* (Fungi, Agaricales) hybrid. Mol. Biol. Evol. 18, 94–96.
Imamura, Y., Chandra, J., Mukherjee, P.K., Lattif, A.A., Szczotka-Flynn, L.B., Pearlman, E., Lass, J.H., O'Donnell, K., Ghannoum, M.A., 2007. *Fusarium* and *Candida albicans* biofilms on soft contact lenses: model development, influence of lens type and susceptibility to lens care solutions. Antimicrob. Agents Chemother. 52, 171–182.
Kang, S., Blair, J.E., Geiser, D.M., Khang, C.-H., Park, S.-Y., Gahegan, M., O'Donnell, K., Luster, D.G., Kim, S.-H., Ivors, K.L., Lee, Y.-H., Lee, Y.-W., Grünwald, N.J., Martin, F.N., Coffey, M.D., Veeraraghavan, N., Makalowska, I., 2006. Plant pathogen culture collections: it takes a village to preserve these resources vital to the advancement of agricultural security and plant pathology. Phytopathology 96, 920–925.
Katan, T., 1999. Current status of vegetability groups in *Fusarium oxysporum*. Phytoparasitica 27, 51–64.
Katan, T., Di Primo, P., 1999. Current status of vegetability groups in Fusarium oxysporum. Phytoparasitica 27 (Suppl. 1999), 1–5.
Kauff, F., Lutzoni, F., 2002a. Compat.py – a program to detect topological conflict between supported clades in phylogenetic trees. <http://www.lutzonilab.net/pages/download.shtml>.
Kauff, F., Lutzoni, F., 2002b. Phylogeny of the Gyalectales and Ostropales (Ascomycota, Fungi): among and within order relationships based on nuclear ribosomal RNA small and large subunits. Mol. Phylogenet. Evol. 25, 138–156.
Kawabe, M., Kobayashi, Y., Okada, G., Yamaguchi, I., Teraoka, T., Arie, T., 2005. Three evolutionary lineages of tomato wilt pathogen, *Fusarium oxysporum* f. sp. *lycopersici*, based on sequences of IGS, *MAT1*, and *pg1*, are each composed of isolates of a single mating type and a single or closely related vegetative compatibility group. J. Gen. Pl. Pathol. 71, 263–272.
Kawakita, A., Sota, T., Ascher, J.S., Ito, M., Tanaka, H., Kato, M., 2003. Evolution and phylogenetic utility of alignment gaps within intron sequences of three nuclear genes in bumble bees (*Bombus*). Mol. Biol. Evol. 20, 87–92.
Kistler, H.C., 1997. Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum*. Phytopathology 27, 474–479.
Kistler, H.C., Benny, U., Boehm, E.W.A., Katan, T., 1995. Genetic duplication in *Fusarium oxysporum*. Curr. Genet. 28, 173–176.
Kistler, H.C., Alabouvette, C., Baayen, R.P., Bentley, S., Brayford, D., Coddington, A., Correll, J., Daboussi, M.-J., Elias, K., Fernandez, D., Gordon, T.R., Katan, T., Kim, H.G., Leslie, J.F., Martyn, R.D., Migheli, Q., Moore, N.Y., O'Donnell, K., Ploetz, R.C., Rutherford, M.A., Summerell, B., Waalwijk, C., Woo, S., 1998. Systematic numbering of vegetative compatibility groups in the plant pathogenic fungus *Fusarium oxysporum*. Phytopathology 88, 30–32.
Kroken, S., Glass, N.L., Taylor, J.W., Yoder, O.C., Turgeon, B.C., 2003. Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. Proc. Natl. Acad. Sci. USA 100, 15670–15675.
Larkin, R.P., Hopkins, D.L., Martin, F.N., 1996. Suppression of *Fusarium* wilt of watermelon by nonpathogenic *Fusarium oxysporum* and other microorganisms recovered from a disease-suppressive soil. Phytopathology 86, 812–819.
Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X, version 2.0.. Bioinformatics 23, 2947–2948.
Lievens, B., Claes, L., Vakalounakis, D.J., Vanachter, A.C.R.C., Thomma, B.P.H.J., 2007. A robust identification and detection assay to discriminate the cucumber pathogens *Fusarum oxysporum* f. sp. *cucumerinum* and f. sp. *radicis-cucumerinum*. Envir. Microbiol. 9, 2145–2161.
Lievens, B., Rep, M., Thomma, P.H.J., 2008. Recent developments in the molecular discrimination of *formae speciales* of *Fusarium oxysporum*. Pest Manage. Sci. 64, 781–788.
Liu, X., Inlow, M., VanEtten, H.D., 2003. Expression profiles of pea pathogenicity (*PEP*) genes in vivo and in vitro, characterization of the flanking regions of the *PEP* cluster and evidence that the *PEP* cluster region resulted from horizontal gene transfer in the fungal pathogen *Nectria haematococca*. Curr. Genet. 44, 95–103.

Martin, F., Selosse, M.-A., Le Tacon, F., 1999. The nuclear rDNA intergenic spacer of the ectomycorrhizal basidiomycete *Laccaria bicolor*: structural analysis and allelic polymorphism. Microbiology 145, 1605–1611.

Mbofung, G.Y., Hong, S.G., Pryor, B.M., 2007. Phylogeny of *Fusarium oxysporum* f. sp. *lactucae* inferred from mitochondrial small subunit, elongation factor 1-α, and nuclear ribosomal intergenic spacer sequence data. Phytopathology 97, 87–98.

Mes, J.J., Van Doorn, J., Roebroeck, E.J.A., Van Egmond, E., Van Aartrijk, J., Boonekamp, P.M., 1994. Restriction fragment length polymorphisms, races and vegetative compatibility groups within a worldwide collection of *Fusarium oxysporum* f. sp. *gladioli*. Plant Pathol. 43, 362–370.

Michielse, C.B., Rep, M., 2009. Pathogen profile update: *Fusarium oxysporum*. Mol. Plant Pathol. 10, 311–324.

Molnár, A., Sulyok, L., Hornok, L., 1990. Parasexual recombination between vegetatively incompatible strains in *Fusarium oxysporum*. Mycol. Res. 94, 393–398.

Moretti, A., Belisario, A., Tafuri, A., Ritieni, A., Corazza, L., Logrieco, A., 2002. Production of beauvericin by different races of *Fusarium oxysporum* f. sp. *melonis*, the Fusarium wilt agent of muskmelon. Eur. J. Plant Pathol. 108, 661–666.

Nirenberg, H.I., O'Donnell, K., 1998. New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. Mycologia 90, 434–458.

Nixon, J.C., 1999. The parsimony ratchet, a new method for rapid parsimony analysis. Cladistics 15, 407–414.

Odds, F.C., Jacobsen, M.D., 2008. Multilocus sequence typing of pathogenic *Candida* species. Eukar. Cell 7, 1075–1084.

O'Donnell, K., Cigelnik, E., 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylogenet. Evol. 7, 103–116.

O'Donnell, K., Cigelnik, E., Nirenberg, H.I., 1998a. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90, 465–493.

O'Donnell, K., Kistler, H.C., Cigelnik, E., Ploetz, R.C., 1998b. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci. USA 95, 2044–2049.

O'Donnell, K., Sutton, D.A., Rinaldi, M.G., Magnon, K.C., Cox, P.A., Revankar, S.G., Sanche, S., Geiser, D.M., Juba, J.H., van Burik, J.-A.H., Padhye, A.A., Anaissie, E.J., Francesconi, A., Walsh, T.J., Robinson, J.S., 2004. Genetic diversity of human pathogenic members of the *Fusarium oxysporum* complex inferred from multilocus DNA sequence data and amplified fragment length polymorphism analyses: evidence for the recent dispersion of a geographically widespread clonal lineage and nosocomial origin. J. Clin. Microbiol. 42, 5109–5120.

O'Donnell, K., Sarver, B.A.J., Brandt, M., Chang, D.C., Noble-Wang, J., Park, B.J., Sutton, D.A., Benjamin, L., Lindsley, M., Padhye, A., Geiser, D.M., Ward, T.J., 2007. Phylogenetic diversity and microsphere array-based genotyping of human pathogenic fusaria, including isolates from the multistate contact lens-associated US keratitis outbreaks of 2005 and 2006. J. Clin. Microbiol. 45, 2235–2248.

O'Donnell, K., Sutton, D.A., Fothergill, A., McCarthy, D., Rinaldi, M.G., Brandt, M.E., Zhang, N., Geiser, D.M., 2008. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. J. Clin. Microbiol. 46, 2477–2490.

Olivain, C., Humbert, C., Nahalkova, J., Fatehi, J., L'Haridon, F.L., Alabouvette, C., 2006. Colonization of tomato root by pathogenic and nonpathogenic *Fusarium oxysporum* strains inoculated together and separately into the soil. Appl. Environ. Microbiol. 72, 1523–1531.

Ortoneda, M., Guarro, J., Madrid, M.P., Caracuel, Z., Roncero, M.I.G., Mayayo, E., Di Pietro, A., 2004. *Fusarium oxysporum* as multihost model for the genetic dissection of fungal virulence in plants and animals. Infect. Immun. 72, 1760–1766.

Pantou, M., Mavridou, A., Typas, M.A., 2003. IGS sequence variation, group-I introns and the complete nuclear ribosomal DNA of the entomopathogenic fungus *Metarhizium*: excellent tools for isolate detection and phylogenetic analysis. Fungal Genet. Biol. 38, 159–174.

Park, J., Park, B., Veeraraghavan, N., Jung, K., Lee, Y.-H., Blair, J.E., Geiser, D.M., Isard, S., Mansfield, M.A., Nikolaeva, E., Park, S.-Y., Russo, J., Kim, S.H., Greene, M., Ivors, K.L., Balci, Y., Peiman, M., Erwin, D.C., Coffey, M.D., Rossman, A., Farr, D., Cline, E., Grünwald, N.J., Luster, D.G., Schrandt, J., Martin, F., Ribeiro, O.K., Makalowska, I., Kang, S., 2008. *Phytophthora* database: a forensic database supporting the identification and monitoring of *Phytophthora*. Plant Dis. 92, 966–972.

Patron, N.J., Waller, R.F., Cozijnsen, A.J., Straney, D.C., Gardiner, D.M., Nierman, W.C., Howlett, B.J., 2007. Origin and distribution of epipolythiodioxopiperazine (ETP) gene clusters in filamentous ascomycetes. BMC Evol. Biol. 7, 174. doi:10.1186/1471-2148-7-174.

Pennisi, E., 2008. DNA data: proposal to "Wikify" GenBank meets with stiff resistance. Science 319, 1598–1599.

Posada, D., 2003. Using Modeltest and PAUP* to select a model of nucleotide substitution. In: Baxevanis, A.D., Petsko, G.A., Stein, L.D., Yates, G.D., Davison, D.B., Page, R.D.M. (Eds.), Current Protocols in Bioinformatics. John Wiley and Sons, Inc., New York (Sections 6.5.1–6.5.14).

Pramateftaki, P.V., Antoniou, P.P., Typas, M.A., 2000. The complete DNA sequence of the nuclear ribosomal RNA gene complex of *Verticillium dahliae*: intraspecific heterogeneity within the intergenic spacer region. Fungal Genet. Biol. 29, 19–27.

Proctor, R.H., Plattner, R.D., Brown, D.W., Seo, J.-A., Lee, Y.-W., 2004. Discontinuous distribution of fumonisin biosynthetic genes in the *Gibberella fujikuroi* species complex. Mycol. Res. 108, 815–822.

Rabie, C.J., Marasas, W.F.O., Thiel, P.G., Lübben, A., Vleggaar, R., 1982. Moniliformin production and toxicity of different *Fusarium* species from Southern Africa. Appl. Environ. Microbiol. 43, 517–521.

Recorbet, G., Steinberg, C., Olivain, C., Edel, V., Trouvelot, S., Dumas-Gaudot, E., Gianinazzi, S., Alabouvette, C., 2003. Wanted: pathogenesis-related marker molecules for *Fusarium oxysporum*. New Phytol. 159, 73–92.

Roy, B.A., 2001. Patterns of association between crucifers and their flower-mimic pathogens: host jumps are more common than coevolution or cospeciation. Evolution 55, 41–53.

Rozas, J., Sánchez-DelBarrio, J.C., Messeguer, X., Rozas, R., 2007. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19, 2496–2497.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Schoustra, S.E., Debets, A.J.M., Slakhorst, M., Hoekstra, R.F., 2007. Mitotic recombination accelerates adaptation in the fungus *Aspergillus nidulans*. PLoS Genet. 3 (4), :e68.

Schroers, H.-J., Baayen, R.P., Meffert, J.P., Hooftman, M., O'Donnell, K., 2004. *Fusarium foetens*, a new species pathogenic to begonia elatior hybrids (*Begonia* x *hiemalis*) and the sister taxon of the *Fusarium oxysporum* species complex. Mycologia 95, 393–406.

Schroers, H.-J., O'Donnell, K., Lamprecht, S.C., Kammeyer, P.L., Johnson, S., Sutton, D.A., Rinaldi, M.G., Summerbell, R.C., 2009. Taxonomy and phylogeny of the *Fusarium dimerum* species group. Mycologia 101, 44–70.

Seo, J.-A., Kim, J.-C., Lee, Y.-W., 1996. Isolation and characterization of two new type C fumonisins produced by *Fusarium oxysporum*. J. Nat. Prod. 59, 1003–1005.

Sikes, D.S., Lewis, P.O., 2001. Software manual for PAUPRat: a tool to implement parsimony ratchet searches using PAUP*. <http://users.iab.uaf.edu/~derek sikes/software2.htm>.

Simmons, M.P., Ochoterena, H., 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49, 369–381.

Skovgaard, K., Nirenberg, H.I., O'Donnell, K., Rosendahl, S., 2001. Evolution of *Fusarium oxysporum* f. sp. *vasinfectum* races inferred from multigene genealogies. Phytopathology 91, 1231–1237.

Swofford, D.L., 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods), version 4. Sinauer Associates, Sunderland, MA.

Taylor, J.W., Fisher, M.C., 2003. Fungal multilocus sequence typing – it's not just for bacteria. Curr. Opinion Microbiol. 6, 351–356.

Taylor, J.W., Jacobson, D.J., Fisher, M.C., 1999. The evolution of asexual fungi: Reproduction, speciation and classification. Annu. Rev. Phytopathol. 37, 197–246.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., Fisher, M.C., 2000. Phylogenetic species recognition and species concepts in Fungi. Fungal Genet. Biol. 31, 21–32.

Temporini, E.D., VanEtten, H.D., 2004. An analysis of the phylogenetic distribution of the pea pathogenicity genes of *Nectria haematococca* MPVI supports the hypothesis of their origin by horizontal transfer and uncovers a potentially new pathogen of garden pea: *Neocosmospora boniensis*. Curr. Genet. 46, 29–36.

Townsend, J.P., 2007. Profiling phylogenetic informativeness. Syst. Biol. 56, 222–231.

van der Does, H.C., Lievens, B., Claes, L., Houterman, P.M., Cornelissen, B.J.C., Rep, M., 2008. The presence of a virulence locus discriminates *Fusarium oxysporum* isolates causing tomato wilt from other isolates. Environ. Microbiol. 10, 1475–1485.

Ward, T.J., Clear, R.M., Rooney, A.P., O'Donnell, K., Gaba, D., Patrick, S., Starkey, D.E., Gilbert, J., Geiser, D.M., Nowicki, T.W., 2008. An adaptive evolutionary shift in Fusarium head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America. Fungal Genet. Biol. 45, 473–484.

Williams, L.D., Glenn, A.E., Bacon, C.W., Smith, M.A., Riley, R.T., 2006. Fumonisin production and bioavailability to maize seedlings grown from seeds inoculated with *Fusarium verticillioides* and grown in natural soils. J. Agric. Food Chem. 54, 5694–5700.

Xie, J., Fu, Y., Jiang, D., Li, G., Huang, J., Li, B., Hsiang, T., Peng, Y., 2008. Intergeneric transfer of ribosomal genes between two fungi. BMC Evol. Biol. 8. doi:10.1186/1471-2148/8/87.

Zwickl, D.J., 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation, The University of Texas at Austin.