

# Genome Sequencing of Multiple Isolates Highlights Subtelomeric Genomic Diversity within *Fusarium fujikuroi*

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**Data deposition:** This project has been deposited at GenBank under the accessions: JRVH00000000 (*Fusarium fujikuroi* KSU 3368), JRVG00000000 (*F. fujikuroi* KSU X-10626), and JRVF00000000 (*F. fujikuroi* FGSC 8932).

## Abstract

Comparisons of draft genome sequences of three geographically distinct isolates of *Fusarium fujikuroi* with two recently published genome sequences from the same species suggest diverse profiles of secondary metabolite production within *F. fujikuroi*. Species- and lineage-specific genes, many of which appear to exhibit expression profiles that are consistent with roles in host–pathogen interactions and adaptation to environmental changes, are concentrated in subtelomeric regions. These genomic compartments also exhibit distinct gene densities and compositional characteristics with respect to other genomic partitions, and likely play a role in the generation of molecular diversity. Our data provide additional evidence that gene duplication, divergence, and differential loss play important roles in *F. fujikuroi* genome evolution and suggest that hundreds of lineage-specific genes might have been acquired through horizontal gene transfer.

**Key words:** mycotoxin, comparative genomics, secondary metabolites.

## Introduction

*Fusarium fujikuroi* ([Hsieh et al. 1977] synonym, *Gibberella fujikuroi* mating population C) is the causal agent of the rice disease bakanae (Leslie and Summerell 2006) which, due to fungal production and secretion of gibberellic acids, is characterized by the formation of excessively elongated seedlings with chlorotic stems and leaves (Sunder 1998).

In addition to plant hormones (Rademacher 1997; Tudzynski 1999, 2005), *F. fujikuroi* can potentially produce a broad spectrum of secondary metabolites (SMs), including pigments (Linnemannstons et al. 2002; Prado et al. 2004; Rodriguez-Ortiz et al. 2013), mycotoxins, such as fumonisins (Proctor et al. 2004), moniliformin (Harvey et al. 1997), fusaric

acid, beauvericin and fusarin C (Marasas et al. 1988; Barrero et al. 1991; Desjardins et al. 2000; Leslie, Zeller, Logrieco, et al. 2004), as well as other compounds of biological interest (Avalos et al. 2007).

Here, we present draft genome sequences of three geographically distinct *F. fujikuroi* isolates and summarize the results of comparative genomic analyses incorporating two other recently published complete *F. fujikuroi* genome sequences (Jeong et al. 2013; Wiemann et al. 2013) and that of the closely related species *Fusarium verticillioides* ITEM 7600 (Ma et al. 2013) (table 1) focusing on genome-wide evolutionary patterns as well as characterized and candidate SM biosynthesis genes and gene clusters.

**Table 1**

Strains Used in This Study

Strain Number	Species	Origin	Reference
KSU 3368	<i>F. fujikuroi</i>	Thailand, rice (1990)	This Study
KSU X-10626	<i>F. fujikuroi</i>	Konza Prairie (USA), <i>Schizachyrium scoparium</i> (1997)	This Study, Leslie, Zeller, Logrieco, et al. 2004; Leslie, Zeller, Wohler, et al. 2004
FGSC 8932	<i>F. fujikuroi</i>	Taiwan, rice	This Study, Kuhlman 1982; Leslie, Zeller, Wohler, et al. 2004
B14	<i>F. fujikuroi</i>	South Korea, rice	Jeong et al. 2013
IMI58289	<i>F. fujikuroi</i>	Taiwan, rice	Wiemann et al. 2013
ITEM 7600	<i>F. verticillioides</i>	California, maize	Leslie and Dickman 1991

## Materials and Methods

### Genome Sequencing, Assembly, and Annotation

For *F. fujikuroi* FGSC 8932 and KSU X-10626 strains both paired-end and large insert mate pair libraries were prepared and sequenced on an Illumina HiSeq2000 machine at the University of Missouri's DNA Core Facility. For (KSU 3668), a single paired-end library was sequenced on an Illumina MiSeq machine. Reads were subjected to quality trimming using the Trimmomatic program (Bolger et al. 2014) with default parameters.

Assembly was performed using the Velvet software (Zerbino and Birney 2008) with a comprehensive grid-search employed to optimize assembly parameters. Scaffolding was performed, where mate-pair data were available, using SSPACE (Boetzer and Pirovano 2014).

Gene annotation was performed using the Augustus software (Stanke et al. 2006) with a model trained on *Fusarium graminearum* (Cuomo et al. 2007) and predicted transcript sequences from *F. fujikuroi* IMI58289 (Wiemann et al. 2013).

tRNAs were annotated using tRNAscan-SE (Lowe and Eddy 1997) with default parameters. TransposonPsi (available at <http://transposonpsi.sourceforge.net/>, last accessed February 2015) was used to predict transposons. RepeatMasker (available at <http://www.repeatmasker.org>, last accessed February 2015) and RepeatScout (Price et al. 2005) were used with standard parameters to predict repeats.

### Clusters of Orthologous Genes

All against all BLASTp (Altschul et al. 1990) searches were performed using the BLOSUM80 matrix. Orthology was defined where pairs of genes were each others best reciprocal hits with  $P$  value  $\leq 1E-5$  and where "second-best" hits produce bit scores less than 90% of that associated with the best match.

### Genome Alignment, Single Nucleotide Polymorphisms, and Structural Variation

Genome alignment and single nucleotide polymorphism (SNP) calling were performed using MAUVE (Darling et al. 2004) with block size = 50,000, using the IMI58289 (Wiemann et al. 2013) assembly as reference. SNP densities and identity

rates were estimated using custom scripts. For analyses of chr IV, MUMmer (Delcher et al. 2002) was used with default parameters other than the minimal match length (50 bp). Inversion frequencies were estimated using MegaBLAST and ad hoc scripts.

### Recombination and Phylogenetic Analyses

Hierarchical clustering was performed using the R implementation of the neighbor joining algorithm. The Phi test for recombination was performed on 100 kb windows (sliding by 50 kb) using the profile program from the PhiPack package (Bruen et al. 2006).

### Functional Enrichment Analyses

Pfam domains were annotated using the PfamScan script (<ftp://ftp.sanger.ac.uk/pub/databases/Pfam/Tools/>, last accessed February 2015) with default parameters. Gene Ontology (GO) enrichment analyses were performed using a custom script implementing a Fisher exact test with the Benjamini–Hochberg False Discovery Rate (FDR) correction.

### Codon Usage

Codon usage analyses were performed using the CodonW suite (Peden 1999).

## Results

### Genome Sequencing, Assembly, and Annotation

Standard paired-end and mate-pair libraries prepared from strains FGSC 8932 and KSU X-10626 were sequenced on an Illumina HiSeq 2000 instrument. For KSU 3368, a single paired-end library was sequenced on the Illumina MiSeq platform. In all cases, reads were subjected to quality trimming. Contig assembly and scaffolding were performed using Velvet (Zerbino and Birney 2008) and SSPACE (Boetzer and Pirovano 2014) respectively. Assembly, scaffolding, and annotation statistics are presented in table 2, and the final theoretical coverages (number of bases submitted to the assembler divided by published assembly length) were 169 $\times$  for KSU 3368, 176 $\times$  for KSU X-10626, and 27 $\times$  for KSU 3368. The genome sequences are available from

**Table 2**  
Genome Assembly Statistics

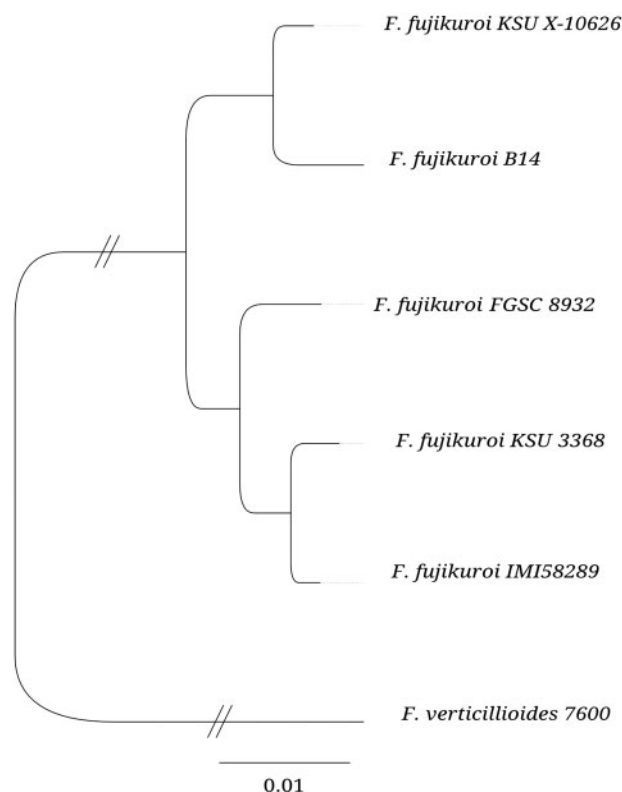
	FGSC 8932	KSU X-10626	KSU 3368
No. of PE reads	52,460,000	55,010,000	6,343,126
PE insert size (bp)	389	586	410
No. of MP reads	61,332,000	60,803,000	na
MP insert size (kb)	3.09	3.21	na
No. of contigs	1,286	636	2,964
N50 contigs (kb)	27	75	4.5
No. of scaffolds	291	167	2,309
N50 scaffolds (kb)	180	1,020	5.2
Total assembly size (Mb)	43.096	43.11	43.199
No. of predicted protein-coding genes	14,832	14,801	15,188
No. of intact TE	35	38	25
Recently duplicated (Mb)	1.89	1.92	1.91
Alignable with IMI58289 (Mb)	40.9	40.5	41.2
% identity with IMI58289	98.94	98.08	99.4
No. of genes shared with IMI58289	14,566	14,245	14,557
No. of genes shared with B14	14,386	14,574	14,406

GenBank under the accession numbers JRVH000000000 (KSU 3368), JRVG000000000 (KSU X-10626), and JRVF000000000 (FGSC 8932).

### Recombination and Sequence Similarity in the Genealogy of *F. fujikuroi* Genomes

*Fusarium fujikuroi* is capable of sexual reproduction, and consequently meiotic recombination. However, some isolated *F. fujikuroi* populations have been reported to undergo predominantly asexual reproduction (Carter et al. 2008). Accordingly, the IMI58289 anchored multiple genome alignment was subjected to the Phi test for recombination, as implemented in the PhiPack software (Bruen et al. 2006). These analyses (supplementary fig. S1, Supplementary Material online) strongly suggest that extensive recombination has occurred since the divergence of the isolates under study.

Character-based phylogenetic reconstruction methods are unsuitable for the analysis of data sets where extensive recombination is expected, although hierarchical clustering based on average identity of aligned genome regions (fig. 1) indicates KSU X-10626 is most similar to B14, whereas the Thai isolate KSU 3368 and the FGSC 8932 isolate are more similar to IMI58289. Although both KSU X-10626 and FGSC 8932 are cross-fertile with isolates from both *F. fujikuroi* and *Fusarium proliferatum* (Leslie, Zeller, Logrieco, et al. 2004; Leslie, Zeller, Wohler, et al. 2004), our analyses provide no direct support for the hypothesis that these strains are hybrids between these two species. Further resolution of this issue awaits the availability of whole-genome sequences from *F. proliferatum*.

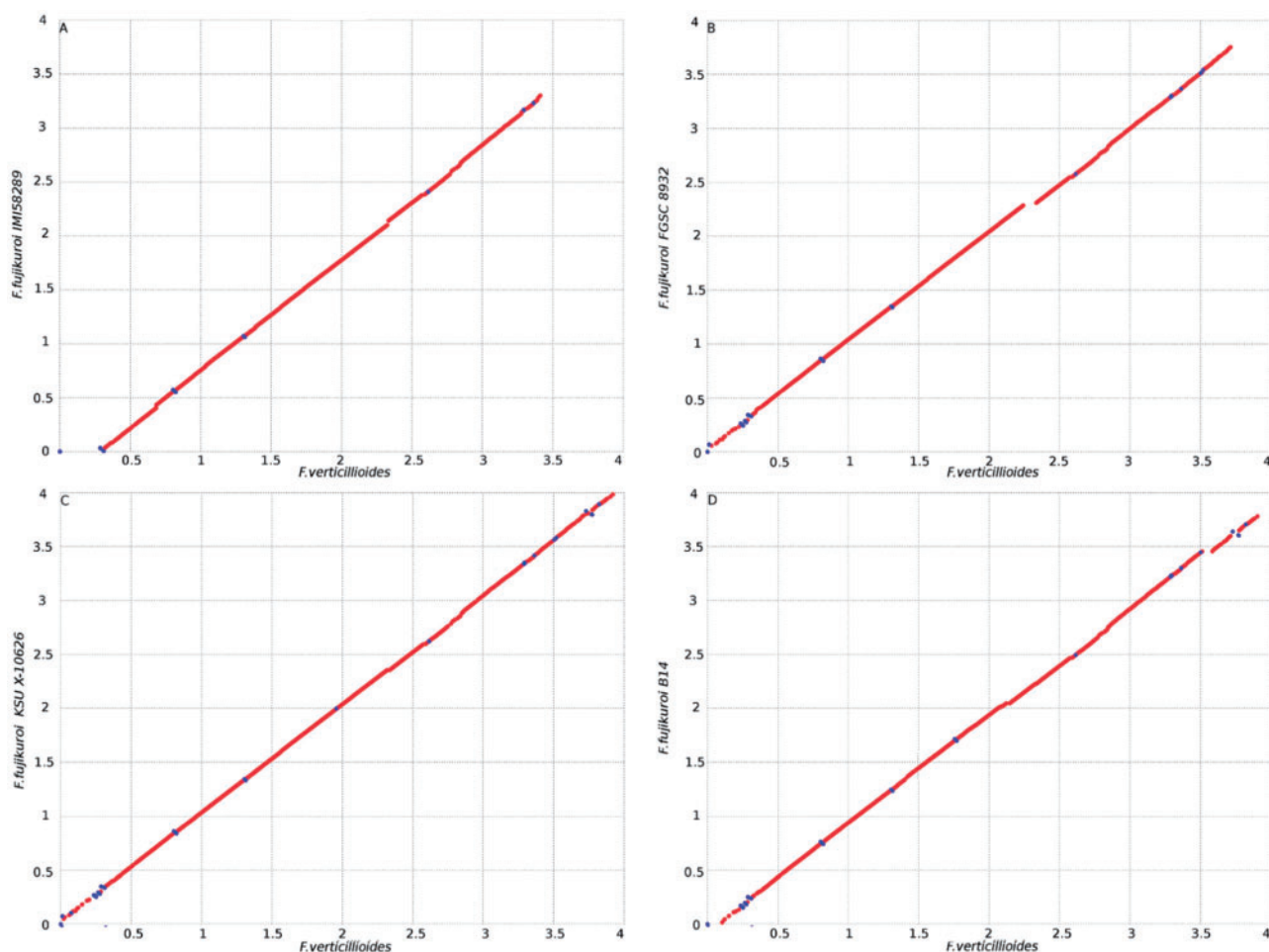


**Fig. 1.**—Hierarchical clustering based on average identity of aligned genome regions. Neighbor Joining tree based on raw genetic distances between alignable regions of *F. fujikuroi* and *F. verticillioides* genomes. All internal branches received a 100% bootstrap support.

### Synteny and Structural Variation among *F. fujikuroi* Genomes

Synteny between *F. fujikuroi* isolates is high, indeed for FGSC 8932 and KSU X-10626, less than 0.30% and 0.43% respectively of the aligned sequence displays inversions or translocations longer than 1 kb with respect to IMI58289. Similar statistics were not generated for KSU 3368 given the fragmented nature of the assembly. Aligned regions show 98.1% (KSU X-10626) to 99.4% (KSU 3368) identity with IMI58289 (table 2).

Although *F. fujikuroi* IMI58289 is highly syntenous with *F. verticillioides*, it lacks both extremities of chr IV (for a total of around 1 Mb of DNA encoding around 400 genes) with respect to the latter (Wiemann et al. 2013). While the *F. fujikuroi* genomes sequenced here, and the B14 strain (Jeong et al. 2013) all have different subtelomeric deletions in chr IV (fig. 2), 80 genes present in these regions in *F. verticillioides* but absent in IMI58289 are found widely dispersed in the subtelomeric regions of the other available *F. fujikuroi* genomes. Additional smaller deletions in chr III and VII of IMI58289 (Wiemann et al. 2013) with respect to *F. verticillioides* are conserved in all *F. fujikuroi* isolates. A chr V



**Fig. 2.**—Dot plot representation of alignments between chr IV of *F. verticillioides* ITEM 7600 and homologous supercontigs from *F. fujikuroi* isolates. (A) *F. fujikuroi* IMI58289, (B) *F. fujikuroi* FGSC 8932, (C) *F. fujikuroi* KSU X-10626, and (D) *F. fujikuroi* B14. The plots illustrate the variability of deletions at the termini of chr IV of various *F. fujikuroi* isolates relative to *F. verticillioides*. *Fusarium fujikuroi* KSU 3368 is not represented due to the fragmented nature of the genome assembly.

telomeric deletion in IMI58289 has a more complex and interesting pattern of conservation between isolates (see below). Supernumerary chr XII (Wiemann et al. 2013) is present in all of the strains sequenced in this study.

The mating type genes are conserved in order and orientation in all the isolates on chromosome VI. B14, FGSC 8932, KSU 3368, and IMI58289 are identified as *MAT-2* while X-10626 carries the *MAT-1* idiomorph.

### Duplications and Compositional Patterns

Subtelomeric regions (within 350 kb of the termini of each IMI58289 chromosome) constitute about 18% of the genomes and yet contain 32% of the intraspecific SNPs (0.0625 SNPs per site vs. 0.0225 for the rest of the genome,  $P$  (hypergeometric)  $\leq 10E-308$ ). They exhibit a marginal (53% vs. 51%), but highly significant ( $P$  (hypergeometric)  $\leq 10E-308$ ) increase in AT content with respect to the rest of the genome

as well as a reduced gene density (0.25 genes per kb vs. 0.38 genes per kb,  $P$  (hypergeometric)  $\leq 1E-140$ ); 36.7% of subtelomeric DNA is “genic,” while the corresponding figure for the rest of the genome is 47.72%.

Potentially recently duplicated sequences (regions showing greater than 89% identity to other sequences in the same genome) were identified in a sliding window BLASTn analysis (400 bp windows) and constituted up to 2 Mb (5.9%) per genome (table 2). Approximately 42% of this genetic material was associated with genomic loci identified as Transposable Element (TE)-derived.

A distinctly nonuniform frequency distribution of sequence identity levels between duplicated loci (supplementary fig. S2, Supplementary Material online) is consistent with past bursts of gene duplication. However, a strong negative correlation between AT content and percentage identity of copies (R-Pearson = 0.5,  $P$  value  $\leq 1E-16$ )—with

**Table 3**Variation of Putative SM Biosynthetic Gene Clusters in *F. fujikuroi* Isolates

Core Gene ID	Gene Symbol <sup>a</sup>	SM Product	B14	KSU X-10626	FGSC 8932	KSU 3368	IMI58289	Acidic		Basic	
								UP	DW	UP	DW
FFUJ_03506	<i>NRPS10</i>	Unknown	0 <sup>b</sup>	0 <sup>b</sup>	1	1	1	0	0	0	1
FFUJ_00003	<i>NRPS31</i>	Apicidin-like	2 <sup>b</sup>	2 <sup>b</sup>	11	11	11	0	11	3	3
FFUJ_02105	<i>PKS6</i>	Fusaric acid	5	5	5 <sup>c</sup>	5	5	0	5	0	5
FFUJ_12239	<i>PKS19</i>	Unknown	6	6	0 <sup>b</sup>	6	6	0	3	0	2
FFUJ_09241	<i>PKS11</i>	Fumonisin	15	15	8 <sup>b</sup>	15	15	11	0	7	0
FFUJ_08113	<i>NRPS4</i>	Unknown	3	3	3	2 <sup>d</sup>	3	0	0	0	0
FFUJ_11199	<i>PKS16</i>	Unknown	4	4	3 <sup>d</sup>	4	4	3	0	3	0
LW93_15044	new PKS	Unknown	7	7	4	0 <sup>b</sup>	0 <sup>b</sup>	NA	NA	NA	NA

NOTE.—For each cluster, the number of genes present in each isolate is reported. The number of genes up- (UP) or down- (DW) regulated in IMI 58289 when nitrogen-limiting conditions were applied in acidic or basic media is reported (data from Wiemann et al. [2013]).

<sup>a</sup>Defined by Weimann et al. (2013) except for the new PKS (LW93\_15044), the current work.

<sup>b</sup>Core gene is absent.

<sup>c</sup>Cluster subject to genomic rearrangement.

<sup>d</sup>Core gene present, loss of single accessory gene.

regions rich in TE-derived sequences often showing AT content  $\geq 70\%$ —might also be consistent with the action of a Repeat Induced Point mutation (RIP)-like mechanism (Galagan and Selker 2004) in *F. fujikuroi* genomes. Inactivation of duplicated genes, following meiosis, is known to occur in *F. verticillioides* (Leslie and Dickman 1991).

### Lineage-Specific Genes

*Fusarium verticillioides* and all *F. fujikuroi* genomes share 8,199 genes. Another 1,017 genes are species specific (SS), or specific to, and universally present in, the five *F. fujikuroi* isolates with respect to other available *Fusarium* genomes. An additional 1,210 genes are lineage specific (LS)—specific to a subset of *F. fujikuroi* genomes or unique to a single genome, with a maximum of 96 genes being unique to a single *F. fujikuroi* isolate (FGSC 8932). Both SS and LS genes are enriched in transcription factors, transporters, and genes associated with SM production. Subtelomeric regions are significantly enriched in putative LS and SS genes for all *F. fujikuroi* isolates ( $P$  value (hypergeometric test)  $\leq 1.0E-170$ ). This observation is consistent with previous reports of a subtelomeric bias in LS genes in other fungal species including *Aspergillus fumigatus* (Nierman et al. 2005), *Saccharomyces cerevisiae* (Zakian 1996), and *Pichia stipitis* (Jeffries et al. 2007). Codon usage indices suggest that subtelomeric genes use a lower frequency of optimal codons and display a lower codon adaptation index with a higher effective number of codons.

For 176 SS and 216 LS genes, best near full-length matches (covering  $>50\%$  of the protein sequence) were obtained outside the genus *Fusarium*, with affinities to *Colletotrichum* spp. (81 genes), *Trichoderma* spp. (37 genes) and *Aspergillus* spp. (32 genes) being the most frequent. These findings are consistent either with widespread independent loss of ancestral

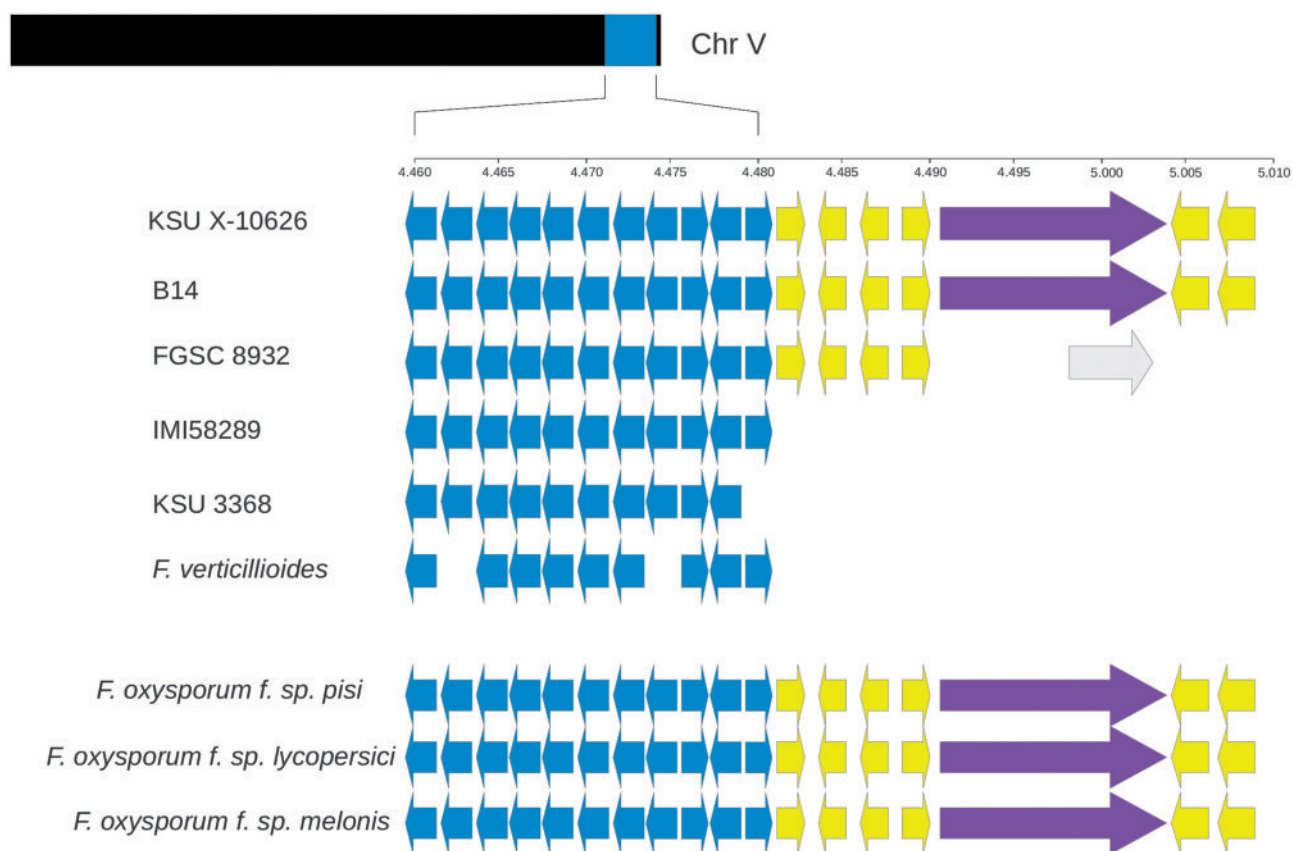
genes in many fungal lineages or with recent SS and LS gene acquisition through horizontal gene transfer.

We also identified 496 SS and 547 LS genes with best Blast hits against *F. fujikuroi* genes previously assigned to other clusters of orthologous genes. Of these genes, 82% retain the same number of introns as their best match, suggesting that they originate from gene duplication rather than from retrotranscription-based mechanisms.

Wiemann et al. (2013) used a custom microarray to profile *F. fujikuroi* IMI58289 gene expression under varying pH and nitrogen concentrations and conditions designed to simulate plant infection. These data (GEO series accession: GSE43745) indicate that SS genes, with respect to core *F. fujikuroi* genes, tend to be upregulated under nitrogen-limiting conditions (both acidic and basic) (supplementary fig. S3, Supplementary Material online), which is consistent with roles in infection-related processes (Coleman et al. 1997; Snoeijers et al. 2000).

### Comparative Analysis of PKS, NRPS, and Putative Novel SM Biosynthetic Gene Clusters

Wiemann et al. (2013) recently identified gene clusters likely involved in SM production in *F. fujikuroi* IMI58289. The composition and arrangement of most of these clusters is conserved across all of the *F. fujikuroi* genomes compared here. All clusters predicted to be complete are potentially active, as they contain no deletions or premature stop codons. Table 3 provides a summary of within *F. fujikuroi* polymorphism in gene content or arrangement in putative SM gene clusters. The *F. fujikuroi* FGSC 8932 genome assembly lacks the entire *PKS19* cluster. It also lacks seven genes from the fumonisin (*PKS11*) biosynthetic cluster, including *FUM19*, the ABC transporter required for toxin synthesis/export. This observation is consistent with the apparent lack of fumonisin production by



**Fig. 3.**—Putative novel SM gene cluster present on the distal subtelomeric region of chr V of *F. fujikuroi* KSU X-10626 and B14. Conservation of a putative novel SM biosynthetic cluster between *F. fujikuroi* and *F. oxysporum* isolates, with lack of conservation apparent in *F. verticillioides*. Genes in blue are present at the subtelomere of all species; the PKS gene is shown in violet with yellow genes representing the presumed accessory components of the cluster. The gray gene in FGSC 8932 is a predicted partial PKS gene recovered as orthologous to the PKS of the novel cluster. The inferred telomeric location of this cluster was derived from synteny of flanking genes with the IMI58289 assembly.

this isolate (Studt et al. 2012). *Fusarium fujikuroi* FGSC 8932 also exhibits a rearrangement of the *PKS6* (fusaric acid) cluster, whereby the PKS gene is inverted and moved 30 kb away from the rest of the cluster relative to other *Fusarium* species.

Orthologs of the *NRPS10* (functionally uncharacterized) and *NRPS31* genes of *F. fujikuroi* IMI58289 (responsible for the production of an apicidin-like compound [Wiemann et al. 2013]) are absent from the *F. fujikuroi* KSU X-10626 and B14 genomes. The *NRPS31* cluster genes, situated close to a telomere on chr I, show high identity with homologs from *F. semitectum* (Wiemann et al. 2013).

A deletion at the proximal subtelomeric region of chr V of IMI58289 noted by Wiemann et al. (2013), and not involving SM genes is conserved in all isolates studied here. Interestingly, in strains X-10626 and B14 the distal subtelomeric region of the same chromosome contains an additional PKS gene with an associated cluster of six genes, absent from the genome of *F. verticillioides*, but closely related to a homologous cluster in *Fusarium oxysporum* (fig. 3). FGSC 8932 retains only four of the genes from this PKS cluster.

The *NRPS4* and *PKS16* clusters exhibit apparent deletions of one gene in single isolates (although the NRPS and PKS genes, respectively, are present). Subtelomeric regions are enriched for SM clusters ( $P$  value  $\leq 1E-22$ ) and contain 23 clusters, (including five of the eight variable clusters displayed in table 3). Among these clusters, only *PKS11* (fumonisins) and *PKS16* (uncharacterized compound) show consistent upregulation under nitrogen-limiting conditions (table 3, data from Wiemann et al. [2013]). Fumonisin production by *F. verticillioides* is required for development of foliar disease symptoms on maize seedlings (Glenn et al. 2008). Wiemann et al. (2013) showed that *F. fujikuroi* can produce only very limited amounts of the toxin compared with *F. verticillioides*, suggesting that the production of fumonisins by *F. fujikuroi* may not be essential for its pathogenesis of rice. The *PKS16* cluster contains only four genes and is poorly characterized with respect to its function and the SM it produces. FFUJ\_11198, the gene which is absent from this cluster in FGSC 8932, contains a serine hydrolase domain (PFAM03959) and is upregulated in nitrogen-limiting conditions. Expression data from Wiemann

et al. (2013) for the 37 conserved SM gene clusters are reported in [supplementary table S1, Supplementary Material online](#).

## Discussion

Comparative genomic analyses of geographically distinct *F. fujikuroi* isolates reveal, as proposed for other phylogenetically distant fungi (Zakian 1996; Nierman et al. 2005; Jeffries et al. 2007) that both sequence variability and the presence of LS and SS genes are concentrated in subtelomeric regions. LS and SS gene sets are enriched in transcription factors, transporters, and genes associated with SM production. Furthermore, publicly available expression data indicate that many of these genes are upregulated under nitrogen-limiting conditions. Focused generation of genetic diversity in subtelomeric regions may thus be particularly important for allowing the fungus to rapidly adapt to changing environments and host colonization. Indeed, our analyses suggest that gene duplication events are concentrated in these genomic regions although rapid divergence of recently duplicated sequences is likely driven by a RIP-like mechanism. These observations notwithstanding, the identification of nearly 400 LS and SS genes that recover best matches outside the genus *Fusarium* may suggest a role for HGT in the evolution of *F. fujikuroi*.

Although SM clusters are for the most part conserved within the species, X-10626 and B14 strains possess a PKS cluster absent from both *F. verticillioides* and other *F. fujikuroi* strains but present in *F. oxysporum*. This and several other differences in SM cluster gene content are most parsimoniously explained by gene loss events. Our data confirm recent comparative genome analyses indicating that *Fusarium* and other filamentous fungi have the genetic potential to produce many more SMs than previously thought (Ma et al. 2013).

The novel *F. fujikuroi* genome sequences presented here will provide an important resource for comparative and gene expression studies in this economically important pathogen. More generally, our findings underline the likely importance of distinctive evolutionary mechanisms in the generation of diversity among pathogenic fungi. Additional investigations into these mechanisms represent high priorities from both basic and applied standpoints.

## Supplementary Material

[Supplementary table S1](#) and [figures S1–S3](#) are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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