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Variability of chromosome structure in pathogenic fungi – of "ends and odds"

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Abstract

Chromatin structure can affect the organization and maintenance of chromosomes. Recent discoveries in several filamentous fungi suggest mechanisms for the clustering and co-regulation of secondary metabolite genes or pathogenicity islands. An extreme case of this may be fungal "accessory", "conditionally dispensable", or "supernumerary" chromosomes that often confer beneficial traits. Fungal supernumerary chromosomes may be derived by similar mechanisms as animal or plant B chromosomes, and we thus propose that this term should be reconsidered to capture the wide variety of fungal accessory chromosomes. In some fungi, both the "ends" of chromosomes and these "odd" B chromosomes are enriched with a silencing histone modification, H3 lysine 27 trimethylation (H3K27me3), suggesting parallel mechanisms in evolving subtelomeric or B-chromosomal pathogenicity islands and secondary metabolite clusters (SMCs).

Keywords

chromatin; histone modification; B chromosome; supernumerary chromosome; accessory chromosome; H3K27 methylation; H3K9 methylation; fungi; secondary metabolite

Introduction

Genome organization of fungal plant and animal pathogens plays important roles in their pathogenicity. Here we discuss two aspects of this organization in filamentous ascomycetes, first reviewing recent work on the physical linkage of secondary metabolite pathways into gene clusters, their distribution across the genome, and their co-regulation as affected by chromatin structure (the "ends"). We then turn to B chromosomes ("supernumerary", "accessory" or "lineage-specific" chromosomes - the "odds") and their importance for pathogenicity. Both features have important consequences for the lifecycle of fungal pathogens and both have been under intense scrutiny in the past few years, driven largely by the development of new technologies. We focus on these phenomena because we feel that

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they offer unique opportunities for molecular mycologists to contribute to answering fundamental questions in chromosome biology.

Secondary metabolite clusters (SMCs)

Fungi produce a large variety of secondary metabolites – compounds not essential for normal growth but nonetheless important in certain environments or developmental stages [1]. Most fungal secondary metabolites fall into four classes defined by the keystone enzyme in their biosynthetic pathway: isoprenoids by prenyltransferases (PTs), nonribosomal peptides by non-ribosomal peptide synthetases (NRPSs), terpenoids by terpene cyclases (TCs) and polyketides by polyketide synthases (PKSs) [2]. Compounds from SMCs play key roles in fungal pathogenicity of humans, animals and crops. The genus *Fusarium*, for example, harbors major pathogens of numerous crops, especially cereals, causing diseases that reduce crop yields and may render the remaining harvest unusable as food or feed due to the presence of mycotoxins. The terpenoid deoxynivalenol (DON) inhibits protein synthesis, causing cellular stress and has both acute and chronic effects on humans and animals [3,4]. DON is produced from farnesyl pyrophosphate by the action of 12 enzymes. In *Fusarium graminearum*, nine of these are clustered at one locus along with two regulatory genes while the remaining three biosynthetic enzymes are at two separate loci [5]. Such clustering of genes within a secondary metabolite pathway is a general rule in fungi [6], and other well-known examples include the 54 kb, 23 gene sterigmatocystin cluster in *Aspergillus nidulans* [7], the 68 kb, 23 gene sirodesmin cluster in *Leptosphaeria maculans* [8], the 64 kb, 17 gene lovastatin cluster of *Aspergillus terreus* [9] and the 75 kb, 15 gene fumonisin cluster of the *Gibberella moniliformis* species complex [10]. Along with biosynthesis genes, SMCs commonly contain transporter genes that can confer resistance to toxic secondary metabolites. Transcription factors that control expression of the cluster are often, but not always present in or near the SMC. Only about a quarter of all easily predictable SMCs have been assigned final products, and developing methods to coordinately control their expression to enable compound identification is a major goal of current studies.

Regulation of secondary metabolite clusters

Analyses of the many almost complete fungal genome sequences have shed light on the location of SMCs on chromosomes. Overwhelmingly, SMCs are localized closer to the ends of chromosomes, in what can be broadly defined as “subtelomeric regions”, and they are often flanked by repetitive elements [2,11–13]. These findings suggested potential mechanisms of coordinated regulation of multiple clusters by shared transcription factors or chromatin modifications [2,14]. Genes within fungal SMCs are often coordinately regulated by a hierarchy of control systems. Many SMCs encode $Zn(II)_2Cys_6$ transcription factors (TFs) that activate the cluster. The best studied example is *Aspergillus* AflR, which activates sterigmatocystin biosynthesis genes and production of aflatoxin by binding to a preferred consensus sequence, TCG(G/C)(A/T)NN(G/C)CG(A/G), present in the promoters of these genes [15,16]. Positive global regulators, like the fungal-specific putative protein methyltransferase LaeA [17], may control larger regions that are activated by several TFs,

for example FapR for fumagillin and pseurotin and an unknown non-SMC TF for the neighboring fumitremorgin cluster [18].

Expression of activating TFs is often not sufficient for cluster expression, as SMCs can be embedded within transcriptionally silent heterochromatin that must be remodeled before expression is possible. It has been proposed that nucleosomes of the *Aspergillus* aflatoxin cluster are trimethylated on lysine 9 of histone H3 (H3K9me3), which is bound by the chromo domain of Heterochromatin Protein-1 (HP1, in *Aspergillus* HepA), though genome-wide histone modification maps have not been produced yet. H3K9me3 and HP1 binding results in gene silencing in other systems, which in *Aspergillus* may somehow be relieved by the action of LaeA [19]. In *L. maculans*, both HP1 and the H3K9 methyltransferase KMT1 (DIM-5/ClrD/SUVAR39) affect the expression of pathogenicity factors [20] and we predict that SMCs will be shown to be affected once genome-wide ChIP-seq is conducted.

In contrast, in *Fusarium graminearum* most SMCs are associated with a different repressive chromatin mark, H3K27me3, especially when grown in rich medium with high nitrogen levels (Fig. 1A). Upon deletion of the H3K27 methyltransferase gene, *kmt6*, more than half of these SMCs are expressed under normally repressive conditions [13]. In *Neurospora crassa*, deletion of the *kmt6* homologue, *set-7*, also resulted in complete loss of H3K27me3 [21] but in this genus SMCs are mostly not associated with H3K27me3 or H3K9me3 (Fig. 1B). Two SMCs (*eas* and *ltm*) in the alkaloid-producing fungus *Epichloe festucae* show enrichment of H3K9me3 and H3K27me3, in a life stage-dependent manner; enrichment was increased in axenic cultures when compared to symbiotic growth in plant tissue [22]. This study also revealed interesting interactions between H3K9 and H3K27 methylation, as there appears to be cooperativity between the two histone marks in gene silencing. In accord with the currently available data, our unpublished results suggest that similar patterns hold within a given genus (e.g. *Fusarium* and *Zymoseptoria*), but that different fungal clades use slightly different versions of chromatin silencing. Overall there does not seem to be a single, universal solution to coordinated regulation of SMCs. Future work will refine the emerging picture and likely also contribute to a basic understanding of silencing pathways in all eukaryotes that make use of H3K27 methylation.

Cluster maintenance in fungal lineages

The pressures resulting in mechanisms for clustering of secondary metabolite genes remain unclear. In some cases clustering may be a byproduct from horizontal transfer of entire clusters from a fungus or bacterium into a naïve species. For example, a high degree of sequence and syntenic conservation supports the wholesale transfer of the *Aspergillus* sterigmatocystin cluster to *Podospora anserina* [23], and comparative phylogenetics supports the interkingdom transfer of a 6-methylsalicylic acid PKS from actinobacteria to the progenitor of the Ascomycetes [24]. Alternatively, and not mutually exclusive, the need to efficiently regulate SMCs may drive gene clustering. Evidence for this exists in the trichothecene pathway gene distributions within the genus *Fusarium*. In *F. graminearum* and *F. sporotrichioides* the pathway is fragmented across three loci, whereas in *F. equiseti* it is condensed into two. The ancestral pathway most likely existed across three loci as in *F. graminearum* and *F. sporotrichioides* and became consolidated within *F. equiseti*[10].

Furthermore, the *F. equiseti* cluster contains a $Zn(II)_2Cys_6$ transcription factor that is absent from *F. graminearum* and *F. sporotrichioides.* This is consistent with a distinct –if not more efficient– regulatory pathway. Strict coordination of gene expression may be particularly important during the biosynthesis of secondary metabolites as intermediate compounds are potentially toxic. Indeed, a recent analysis of the simplest possible gene clusters, gene pairs, uncovered a strong bias for the pairing of enzymes that share a toxic intermediate [25]. Moreover, many of these gene pairs are divergently oriented around a single promoter, an arrangement that favors tight co-regulation.

Unlinked secondary metabolite pathways genes are at great risk of "disassembly" during meiotic recombination unless mechanisms exist to reduce recombination rates within these regions. Recombination is not evenly distributed across fungal genomes. Notably, the centromeres of *Schizosaccharomyces pombe* seem to have very low recombination rates in part because they are heterochromatic [26,27]. Reduced recombination rates within heterochromatin may relate to its condensed nature, which makes it refractory to programmed double-strand break formation during meiosis [28]. As double strand breaks are central to meiotic recombination and correlated to high recombination rates [29,30] heterochromatic regions have lower recombination rates than highly expressed regions. Thus secondary metabolite pathways may be clustered within large domains of facultative heterochromatin to limit recombination and thus retain intact clusters. However, recombination profiles of *F. graminerarum* [31,32] suggest that the facultative heterochromatin marked by H3K27 methylation [13] carries the most variable DNA sequence. Solving this paradox will be a focus of investigations in coming years.

B chromosomes (Bs)

Many eukaryotes, in fact perhaps as many as 15% of all plant species, carry additional (B) chromosomes in excess of the normal haploid or diploid set of A chromosomes (for a review see a chapter in [33]. By definition, Bs are not strictly necessary chromosomes, they are "conditionally dispensable" (CD), "supernumerary" or "accessory" to the core genome contained on the A chromosomes [34–36]. In many plant species and in some fungi, Bs are found only in specific lineages of closely related species, so they are sometimes referred to as "lineage-specific" chromosomes [37]. This is a somewhat biased term as it depends on the current state of knowledge covering specific taxa – what is currently "lineage-specific" may become "genus-specific" by discovery of the proper line or strain. Over the past 20 years, mycologists have roundly rejected "B chromosome" to describe the more specialized supernumerary chromosomes in fungi, though we propose that this term still applies to fungi. Here we outline why.

What do B chromosomes do? Mostly they are harmful to the host plant or animal - at best they are neutral elements. In fungi, however, we find an overwhelming majority of beneficial Bs, perhaps one reason why mycologists prefer specific terms rather than "B chromosome". Often the benefit to the fungus lies in the ability to use genes encoded on Bs to colonize plant material or detoxify plant defense compounds, perhaps another reason to separate them from the overall gene-poor Bs of plants and animals, explaining why they

have been called “pathogenicity” chromosomes [37,38]. Selective advantage via pathogenicity determinants does not seem to hold for all fungal Bs, however [2,39].

Overall, there are more commonalities than differences among Bs from different kingdoms, as revealed by the voluminous literature [33], even though there are differences in *sensu stricto* definitions [40,41]. In most cases B chromosomes use various forms of meiotic drive and self-accumulation mechanisms to propagate – many of the inheritance patterns are non-Mendelian. Of course this is difficult to study in fungi without extant sexual stage [41]. Nevertheless, general correlations can be derived: (a) higher numbers of Bs in one nucleus, and growth under harsher environmental conditions make negative effects of Bs more pronounced; (b) the behavior of B chromosomes can affect recombination of A chromosomes; (c) in plants, inbreeding promotes the accumulation and spread of beneficial Bs; (d) presence of Bs is positively correlated with low ploidy, low chromosome numbers and large genomes (i.e. larger domains of repetitive DNA); (e) Bs are more prevalent in genomes with acrocentric chromosomes, and smaller Bs are mitotically less stable than large Bs [33,40,42]. Some of these correlations apply to fungal Bs, thus it seems advantageous to mycologists to use the same term as researchers who work with animal and plant species. The other correlations need to be investigated.

How big are B chromosomes? The simple rule in fungi is “rarely larger than the smallest A chromosome”. In most eukaryotes, Bs are indeed small, containing sometimes only the elements required for propagation, i.e. telomeres and some centromeric DNA for kinetochore and spindle attachment. The variety is staggering, however! There are so many different types of Bs in plants and animals that there are no phylogenomic rules discernable for true classification by size and DNA content. This begs the question: What classes of genes or elements are located on Bs? Overall Bs are depleted for genes and enriched for repeated elements, either satellite repeats or active and mutated transposons, often of different types than on the As [35,37]. Few genes have been found on Bs in plants or animals; the most gene-rich Bs are those of various filamentous fungi. Thus, it appears that increase of gene density on Bs is positively correlated with benefit to the host organism. A special case can be made for Bs that contain rDNA clusters. These are much more frequently found among plant and animal Bs and this finding, combined with data showing that rDNA segregates late during division, suggest that rDNA is causally involved in increased non-disjunction and thus mitotic or meiotic drive of Bs [33].

Matching the finding of few active genes, cytological data suggest that Bs are largely heterochromatic. This idea requires further study in fungi, however. Even in plants only half of all Bs are composed of truly constitutive heterochromatin. Long stretches of euchromatin and facultative heterochromatin can be found, though these are matching mostly transposable elements. In preliminary studies, we determined some chromatin features of B chromosomes in several genera of fungi, and found that while they are heterochromatic, they are mostly associated with H3K27me3, a hallmark of facultative heterochromatin that can be activated under the appropriate conditions (Fig. 2). We feel that the genetically tractable fungi, for which many biochemical and cytological methods exist, will become important models to unravel the varied chromatin structures of B chromosomes.

Two of the most obvious and vexing questions remain unanswered. Where do Bs originate? And how are they maintained or lost? Clearly Bs are either generated within the host by aberrant chromosome segregation during division, or they are acquired from closely or distantly related taxa. Horizontal Chromosome Transfer (HCT) should be inefficient and rare, as otherwise there would be a large collection of fungal Bs available for study, all actively degenerating from the previous donor A chromosomes to avoid pairing with As during meiosis. Of course, donors may provide already degenerated Bs to the new host, which may explain the very different transposon or repeat content and codon bias observed on some fungal Bs [35,37,43]. While some interspecific hybridization may occur within many genera, particularly in the fungi, endogenous sources seem more obvious. One mechanism would be wholesale duplication of A chromosomes followed by degeneration and accumulation of transposons to generate large Bs that shrink only over time. Alternatively, missegregation or Robertsonian chromosome fusions may result in duplication of short regions from As, presumably containing centromeric or “proto-centromeric” DNA to generate very small Bs that “grow from the centromere out” [33]. Based on what we know about the behavior of centromeric chromatin, the generation and maintenance of heterochromatin, and the inheritance of minichromosomes this latter scenario seems much more likely. There is evidence for both pathways from different organisms [33], but no clear and decisive answers have emerged. Fungi offer opportunities to test both possibilities by experiment, rather than deduction from the age-old evidence generated by natural evolution.

Recent studies in fungi showed that the two pathways are not exclusive, perhaps even for a single species. Horizontal chromosomes transfer (HCT) can occur in *F. oxysporum* f. sp. *lycopersici*, at least under strong selection pressure [37]. The Bs of this taxon are enriched for transposable elements and contain genes with distinct evolutionary profiles that are required for pathogenicity on specific host plants. Genes on the Bs are fungal in origin but appear only poorly related to the host’s genes [37]. This study demonstrated transfer of B chromosomes experimentally, converting a non-pathogenic strain into a pathogenic strain. The authors favor the idea that HCT between isolated strains “explain the polyphyletic origin of host specificity and the emergence of new pathogenic lineages”. Interestingly, this study also showed that mixed A and B chromosomes exist in *F. oxysporum* f. sp. *lycopersici*, as chromosomes 1 and 2 show repeat content and other sequence features that revealed As with translocated B sequences. B chromosomes 3 and 6 are also larger than the smallest A. This suggests that either translocations after HCT can occur or that endogenous mechanisms may be involved that shuffle chromosomes after acquisition.

The endogenous generation of Bs by chromosome fusion followed by degenerative breakage, as proposed by Barbara McClintock’s model of “breakage-fusion-bridge” (BFB) cycles [44,45], may be the origin of at least one of the eight Bs in the reference strain of *Zymoseptoria tritici* [36]. This model suggests that repetitive DNA or perhaps specific chromatin structure may be required for non-allelic homologous recombination between repeats to generate a dicentric and acentric chromosome. The dicentric chromosome (here Chromosome 17) may have undergone BFB cycles [36], while the acentric chromosome was simply lost in subsequent divisions. This testable hypothesis will result in renewed efforts to

observe how exactly one extra centromere is deleted or degenerated and how acentric, potentially small chromosomes are either lost or may acquire a new centric region, or "neocentromere". *Zymoseptoria* is quickly developing into a model genus, combining excellent genome resources [46–48] with opportunities for biochemical and cytological analyses. What is currently lacking is a genetic system under laboratory conditions, though controlled crosses have been carried out in the field [39,49]. Making genetics a routine feature in species with Bs will also aid in addressing how these chromosomes are maintained (or quite frequently lost) during meiosis [39]. We predict that *Zymoseptoria* and certain *Fusarium* species will become facile models to answer these very basic questions about the biology of B chromosomes. That these questions are of general interest was illustrated in a recent review on mammalian and yeast genome destabilization [50].

Concluding remarks

The intriguing connection between the "ends", large subtelomeric blocks of mostly non-syntenic DNA, and the "odds", supernumerary or B chromosomes, seems to be chromatin structure that is characterized by the presence of nucleosomes with trimethylated H3K27. The *F. graminearum* species complex presents an interesting case. Many determinants for pathogenicity and most SMCs reside not only in the subtelomeric regions but also within the four chromosomes, which were predicted to result from chromosome fusions of the ancestral 11 or 12 chromosomes [31]. Recombination profiles [31,32] and chromatin structure analyses [13] suggest that the epigenetically defined and usually silent or "cryptic" regions of this genome maintain diversity but paradoxically also have been maintained in this form over millennia. Thus, future work will address how H3K27me3 and other chromatin marks are involved in the formation and maintenance of facultative heterochromatic domains on A *versus* B chromosomes, with special emphasis on DNA recombination and repair.

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* of special interest

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Highlights

- Histone H3K27me3 generates facultative heterochromatin in some fungi.
- Fungal core genomes are contained on “A chromosomes”.
- Supernumerary chromosomes of fungi are specialized “B chromosomes”.
- Some fungal B chromosomes are “pathogenicity” chromosomes.
- Subtelomeric regions and B chromosomes share widespread H3K27me3.

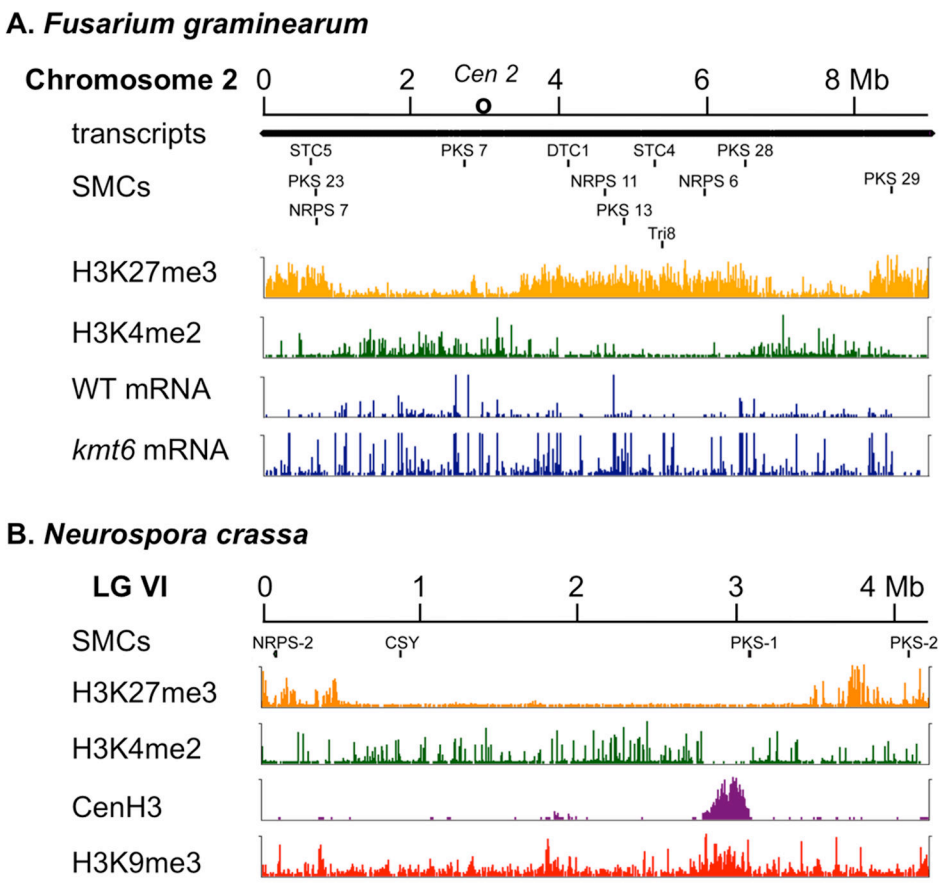

Figure 1. Comparison of chromatin features of *Fusarium* and *Neurospora* chromosomes with SMCs

A. *Fusarium graminearum* Chromosome 2 contains twelve SMCs that can be identified by keystone enzymes (from left to right: STC5, FGSG_08181; PKS23, FGSG_08208; NRPS7, FGSG_08209; PKS7, FGSG_08795; DTC1, FGSG_03066; NRPS11, FGSG_03245; PKS13, FGSG_03340; STC4, FGSG_03494; Tri8, FGSG_03532; NRPS6, FGSG_03747; PKS28, FGSG_03964; PKS29, FGSG_04588; nomenclature for SMCs according to [2]. Except for PKS7, all SMCs are in silent regions enriched with H3K27me3 (orange). None are in regions enriched with H3K4me2 (green), a modification most often found in the promoters of active genes. This suggested that none of these SMCs are expressed in wild type (WT), which was validated by RNA-seq (WT mRNA). In contrast, all SMCs are expressed or overexpressed in an H3K27me3 mutant, *kmt6* (*kmt6* mRNA), except for STC5 and PKS7 that either require activating factors (STC5) or are not subject to gene silencing by H3K27me3 (PKS7). The thousands of transcripts generated or predicted from Chromosome 2 are indicated, at this resolution as solid bar (transcripts). The centromeric region (*Cen2*) is indicated by a circle. B. *Neurospora crassa* Linkage Group (LG) VI has four SMCs (from left to right: NRPS-2, NCU07119; CSY, NCU04801; PKS-1, NCU06013, PKS-2, NCU05011). Of the four clusters, only NRPS-2 is enriched for H3K27me3 (orange). CSY and PKS-1 are enriched in H3K4me2 (green) and expressed at low levels under standard growth conditions (Vogel's minimal medium at 32C), while PKS-2 is not enriched for any of the tested histone modifications and not expressed under standard laboratory

conditions. The *pks-1* gene resides near the right edge of *Cen VI* (CenH3 localization in purple), directly next to a tract of H3K9me3 (red). Histones and their modifications were assayed byCh IP-seq of chromatin precipitated with antibodies against GFP-tagged CenH3, H3K4me2, H3K9me3 and H3K27me3 as described [13].

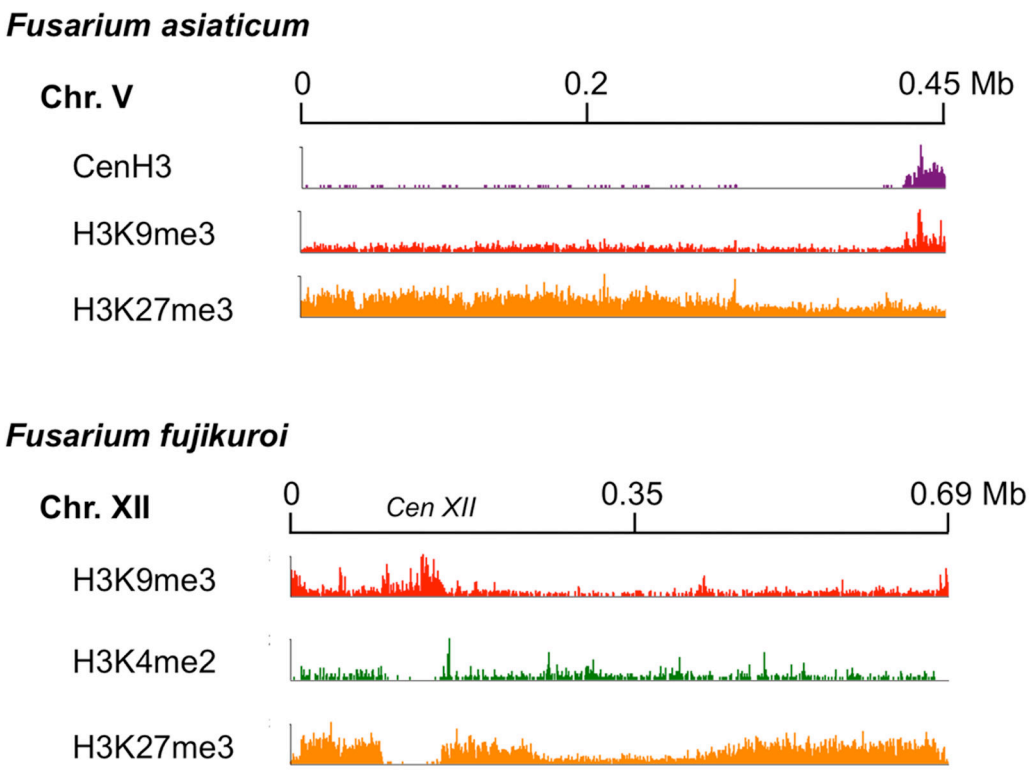

Figure 2. Putative B chromosome of *F. asiaticum* and *F. fujikuroi*

ChIP-seq of chromatin precipitated with antibodies against GFP-tagged CenH3 (purple), H3K4me2 (green), H3K9me3 (red) and H3K27me3 (orange) suggests that H3K27me3 acts as the major gene silencing modification on *Fusarium* B chromosomes (Sung-Hwan Yun, Lena Studt, Bettina Tudzynski, Lanelle Connolly, Kristina Smith and Michael Freitag, preliminary data). Note the predicted acrocentric centromeres in both species, either determind by Cen H3 ChIP (purple in *F. fujikuroi*) or predicted by large region of H3K9me3 (red, *F. fujikuroi*).