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The genomic organization of plant pathogenicity in *Fusarium* species

Martijn Rep[1] and H Corby Kistler[2]

Comparative genomics is a powerful tool to infer the molecular basis of fungal pathogenicity and its evolution by identifying differences in gene content and genomic organization between fungi with different hosts or modes of infection. Through comparative analysis, pathogenicity-related chromosomes have been identified in *Fusarium oxysporum* and *Fusarium solani* that contain genes for host-specific virulence. Lateral transfer of pathogenicity chromosomes, inferred from genomic data, now has been experimentally confirmed. Likewise, comparative genomics reveals the evolutionary relationships among toxin gene clusters whereby the loss and gain of genes from the cluster may be understood in an evolutionary context of toxin diversification. The genomic milieu of effector genes, encoding small secreted proteins, also suggests mechanisms that promote genetic diversification for the benefit of the pathogen.

Addresses
[1] Plant Pathology, Swammerdam Institute for Life Sciences, Faculty of Science, University of Amsterdam, P.O. Box 94215, 1090 GE Amsterdam, The Netherlands
[2] USDA ARS Cereal Disease Laboratory, 1551 Lindig St., University of Minnesota, St. Paul, MN 55108, USA

Corresponding authors: Rep, Martijn (m.rep@uva.nl) and Kistler, H Corby (hckist@umn.edu)

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Introduction

Genomic sequencing of fungal phytopathogens has revolutionized the study of plant pathogenesis. Whole genome sequence (WGS) data for individual fungal genomes accelerated classical forward and reverse genetic approaches for identifying pathogenicity genes. More recently, the availability of several WGS assemblies for comparative genomic analysis has enabled unprecedented opportunities for tracing the evolutionary origin (and demise) of genes and molecules that influence the outcome of fungal–plant interactions. Moreover, the overall genomic organization of fungal pathogenicity-related genes has suggested novel modes of molecular diversification and genetic transmission. We here highlight recent insights into the evolution of disease-causing ability among plant pathogenic fungi, focusing on the comparative genomic analysis of *Fusarium* species with additional reference to other fungi.

Comparative genomics

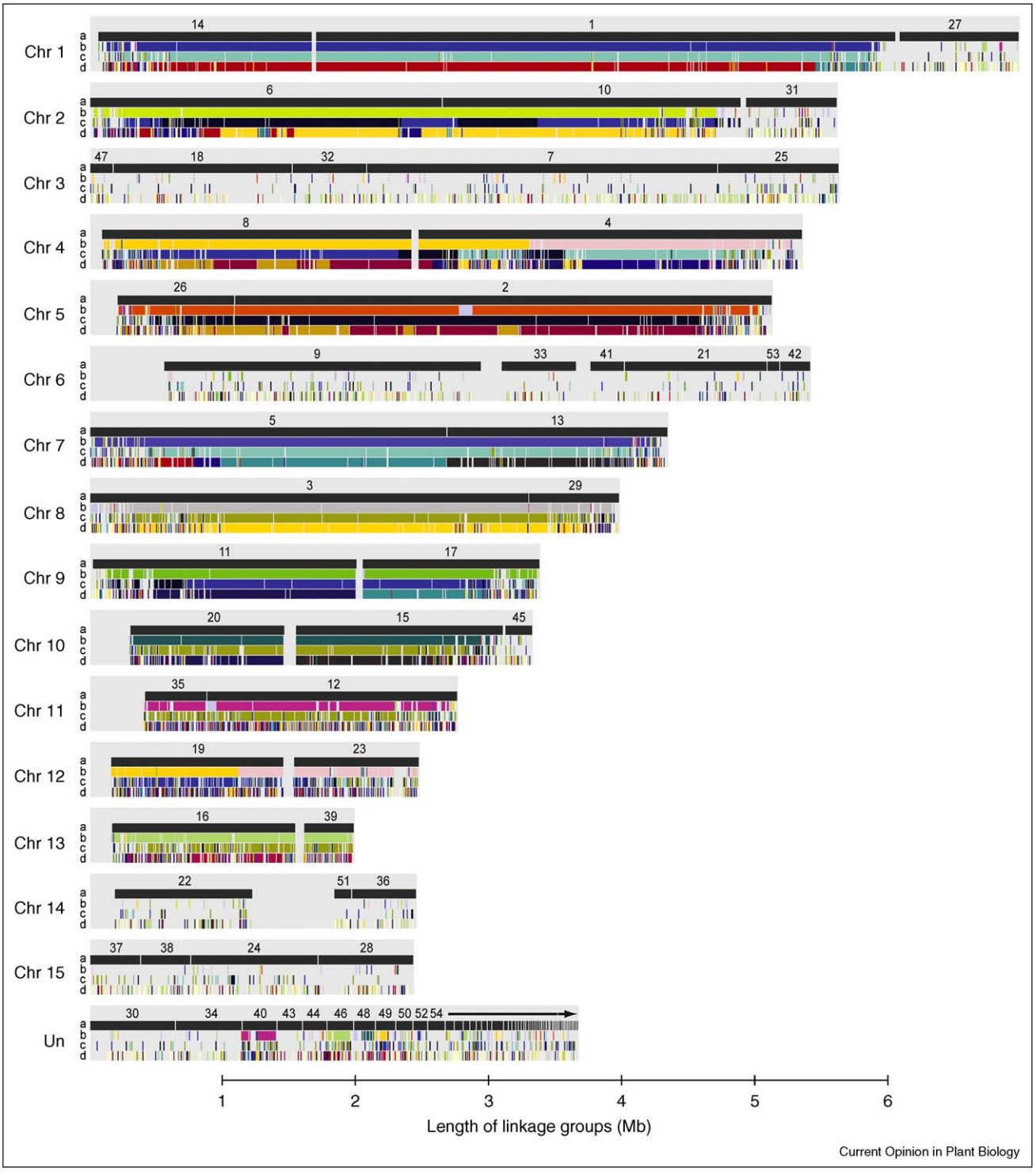
In 2007 the Broad Institute released its first *Fusarium* comparative genomics web site (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html), which brought together high quality sequence assemblies of the plant pathogenic fungus *Fusarium graminearum*, sequenced previously [1•] and two new WGS for the species *Fusarium verticillioides* and *Fusarium oxysporum*. At the same time, the Joint Genome Institute (JGI) released a WGS for *Fusarium solani* (*Nectria haematococca*) (http://genome.jgi-psf.org/Necha2/Necha2.home.html). The four genomes share considerable sequence similarity as well as extensive synteny (Figure 1) [2•,3••].

The *Fusarium* genomes consist of a core region with approximately 9000 genes considered to be orthologous due to high sequence similarity and conserved gene order [3••]. Each species also contains thousands of genes that are unique to each genome, many of which are found near the ends of chromosomes. In *F. graminearum*, these telomere proximal regions are rich in gene diversity as measured by SNP density [1•] and are regions of elevated recombination [4]. Distinctively, *F. graminearum* also contains interstitial chromosomal regions of high diversity and recombination that appear to have been created by an ancestral telomeric fusion of chromosomes [1•,3••]. How these interstitial regions of *F. graminearum* chromosomes have maintained high genetic diversity, normally associated with telomeres, remains a mystery.

Comparative genomics with closely related *Aspergillus* species also has revealed large species-specific gene sets, which are concentrated in subgenomic regions called chromosomal islands [5••]. Interestingly, these regions have a subtelomeric bias too. This could be due to the inherent recombinogenic nature of chromosome ends but seems to have functional implications as well; subtelomeric gene expression is associated with invasive growth of *Aspergillus fumigatus* in mammals and *ex vivo* neutrophil exposure [6].

Instead of genomic islands, both *F. oxysporum* and *F. solani* contain supernumerary chromosomes that largely consist of lineage-specific sequences and make up a

Figure 1



Comparison of Fusarium Genomes. Synteny of the *F. oxysporum*, *F. verticillioides*, *F. graminearum*, and *F. solani* genomes determined by BLASTN alignment (cutoff 1e-10). Chromosomes (Chr) of *F. oxysporum* are used as a reference and the scale (bottom) measures chromosome size in megabases (Mb). Grey boxes indicate chromosome size determined by optical mapping, upon which are superimposed corresponding WGS assembly scaffolds displayed as numbered black bars. Unassembled scaffolds (Un) are concatenated for ease of display. Regions of shared gene order with chromosomes of *F. verticillioides*, *F. graminearum* and *F. solani*, are shown beneath each *F. oxysporum* chromosome. Sequences from individual chromosomes in each species are color-coded so that blocks of solid color represent regions of synteny with the *F. oxysporum* genome. Little homology or synteny is apparent for the lineage specific regions of *F. oxysporum* chromosomes 3, 6, 14 and 15 and for telomere-proximal portions of chromosomes 1 and 2. Figure modified from supplemental material of [3••].

substantial fraction of the entire genome [2•,3••]. Lineage-specific portions of *F. oxysporum* and *F. solani* are highly enriched in transposable elements. Regions of high genome diversity in *Fusarium*, whether at chromosome ends, special interstitial sites or on supernumerary chromosomes, also have been shown to be rich in genes predicted to be involved in fungal–host interactions. High diversity regions are enriched for predicted secreted proteins, carbohydrate-active enzymes and genes specifically expressed during plant infection [1•,3••,7].

Effector genes, pathogenicity chromosomes and horizontal transfer

Effectors are proteins secreted by pathogens that promote virulence, commonly by interacting with plant host proteins [8]. Because of these interactions, effector genes are frequently involved in molecular arms races between pathogen and plant and subject to accelerated evolution [9]. The location of effector genes in a genome may affect the rate at which they evolve through mutation or recombination. In *Leptosphaeria maculans*, for instance, the effector (*Avr*) genes that have been identified through positional cloning all reside in AT-rich genomic subregions of up to several hundred kb consisting mostly of remnants of retrotransposons [10,11•,12]. These long chromosomal segments of uniform AT/GC content are similar to isochores found in other eukaryotes.

Although these highly particular, isochore-like genomic subregions presently have been found only in *L. maculans*, the genomic location of effector genes is also non-random in several other fungi. In the corn pathogen *Ustilago maydis*, a significant fraction (18%) of the genes for secreted proteins are organized in 12 clusters (3–26 genes per cluster) dispersed in the genome [13]. Most clusters contain tandem arrays of 2–5 related genes. This is remarkable since overall, the genome of *U. maydis* is largely devoid of repetitive DNA. Expression of most genes in the *U. maydis* effector gene clusters is co-regulated and induced during plant infection [13].

In the rice blast fungus, *Magnaporthe oryzae*, effector genes are not clustered but are frequently found near telomeres, where gene duplication and gene conversion may be more frequent [14,15,16••]. One example of an apparent recent telomeric duplication in *Fusarium* is the effector gene *SIX8* in *F. oxysporum*, which is present in identical copies close to at least eight telomeres in the genome of the tomato-specific pathogenic strain (M Rep, LJ Ma, unpublished observations).

Interestingly, all other known effector genes in the tomato-pathogenic strain of *F. oxysporum* are localized on a single, transposon-rich 'pathogenicity chromosome' of ~2 Mb [3••]. This chromosome and another smaller strain-specific chromosome can undergo transfer between pathogenic and non-pathogenic strains during co-cultivation, resulting in a new pathogenic lineage. Lateral chromosome transfer, first observed in *Colletotrichum gloeosporioides* [17•], likely explains the existence of host-specific groups of strains (*formae speciales*) of *F. oxysporum* composed of several independent lineages. This is supported by the observation that the sequences of the effector genes on the pathogenicity chromosome are, with few exceptions, identical between disparate lineages and highly specific to lineages which are pathogenic to tomato [18].

In the pea-pathogenic form of *F. solani*, supernumerary chromosomes also harbor genes for host-specific virulence, including detoxification mechanisms and host nutrient utilization [19,20]. Three supernumerary chromosomes with sizes between 0.5 and 1.6 Mb have characteristics that distinguish them from the rest of the genome including higher levels of repeat sequences (mostly transposons), more duplicated and unique genes, smaller average gene size and a lower G+C content. Like in *F. oxysporum*, sequences of the genes on these chromosomes suggest activities consistent with habitat specialization but no involvement in essential cellular functions [2•]. For *Alternaria alternata*, too, host plant-specific pathogenicity can be attributed to supernumerary chromosomes, of 1.0–1.7 Mb [21•]. Genes for synthesis of host-selective toxins that are crucial for host-specificity are located on these chromosomes [22].

Transposons

Could the proximity of effector genes to repeats or transposons accelerate their evolution? While *Leptosphaeria* represents an extreme case of repetitive genomic context of effector genes, the context of effector genes in other fungi is also often transposon rich [16••,23]. In *Leptosphaeria*, both transposons and effector genes appear to undergo mutation through Repeat Induced Point Mutation (RIP) in the AT-rich genomic subregions [24]. In *F. solani*, 72% of the repetitive sequences but only 4% of the unique sequences have been subjected to RIP [2•]. It will be interesting to determine whether a repetitive genomic environment promotes mutation of non-repetitive effector genes residing within it. Accelerated DNA sequence divergence coupled with positive selection for effector diversification might link transposons to the evolution of nearby effector genes. A repeat-rich genomic environment might also promote homologous recombination among different repeats leading to duplication, chimeric gene formation or gene loss. In line with this, transposons are associated with gene gain/loss events in *M. oryzae* [16••]. Also, transposons are known to mediate chromosomal rearrangements [25] and to affect chromatin structure [26], which may influence effector gene expression and possibly also mutation rates.

The pathogenicity-related and other supernumerary chromosomes that have been analyzed are all enriched

for transposable elements. Interestingly, in a clear case of horizontal gene transfer between fungi, a single gene encoding a host-selective toxin was transferred together with an hAT-family DNA transposon [27]. It remains to be determined whether association of transposons with mobile virulence-related genes or whole chromosomes is coincidental, resulting from 'tolerance' of non-essential genomic regions to transposon insertion, or whether transposons are selected for function in the transfer process. For now this remains mostly speculative; mechanisms through which transposons may promote horizontal transfer of genes or chromosomes are currently unknown.

Evolution of toxin diversity at biosynthetic gene clusters

Fungal genes for biosynthesis of secondary metabolites, including toxic compounds produced in plants, often are clustered at a single locus and are co-expressed [28]. Two major themes in toxin cluster evolution have been revealed by comparative genomics: trans-species polymorphism and linkage disequilibrium within blocks of genes, each correlated to 'chemotype' [29–31]. Chemotypes are distinct spectra of metabolites/toxins produced by related strains or species. Examples of toxin chemotypes are the nivalenol (NIV)- *versus* deoxynivalenol (DON)-producing strains of *Fusarium*. Polymorphisms in multiple genes in the toxin pathway lead to separate chemotypes. As selection is likely occurring on the toxic pathway end-product(s), recombination between genes that define different chemotypes might result in combinations that produce no toxins or a level or spectrum of toxin with reduced biological activity. Negative selection against recombination of genes defining different chemotypes might, therefore, underlie the observed patterns of linkage disequilibrium across the gene cluster [29,30].

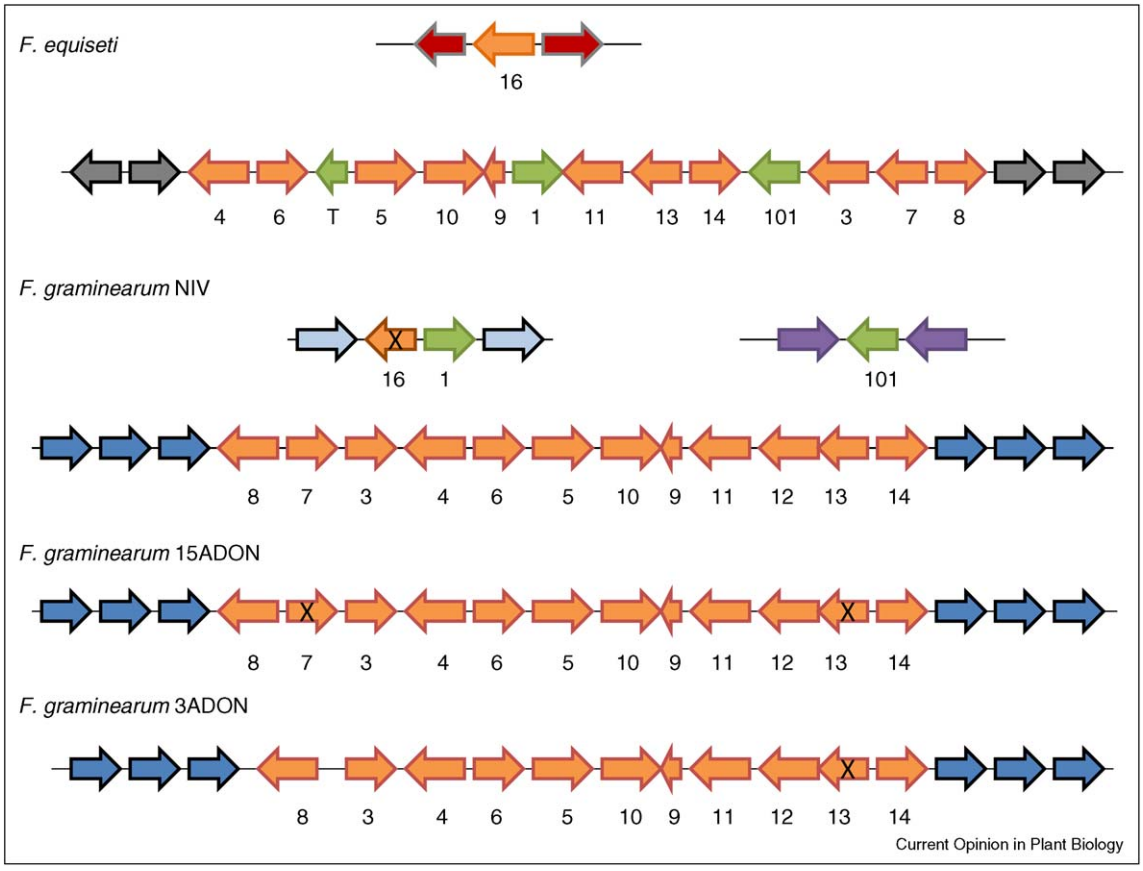
Trans-species polymorphisms correlated with chemotype suggest that cluster diversification arose prior to species divergence. This interpretation is supported by coalescence analysis of toxin cluster and non-cluster genes in *Aspergillus* whereby cluster genes display a twofold greater difference in the estimated time to the most common recent ancestor [31]. Trans-species polymorphism may be due to balancing selection, and so it is tempting to speculate how balancing selection may explain the persistence of toxin chemotypes. One possibility is that chemotypes may confer differential fitness in different hosts. Trichothecenes produced by *F. graminearum* are known to alter the pathogenicity of the fungus in complex and host-specific ways. For example, the trichothecene NIV, but not DON, is required for maximum aggressiveness on maize but not on wheat [32]. If differences in disease level reflect fitness differences, then chemotype diversity may be maintained due to differential fitness of chemotypes in the various hosts of *Fusarium*.

Recent comparative genomics of the trichothecene gene cluster among *Fusarium* species has captured the process of how genetic elements are added and subtracted from clusters resulting in different chemotypes (Figure 2). In *F. graminearum* the trichothecene gene cluster consists of 10–12 contiguous genes as well as two other genes, *Tri1* and *Tri101*, which are at separate loci outside the main cluster [33]. While the intact gene cluster in *F. graminearum* results in strains that produce the toxic compound NIV, naturally occurring alternative forms of the gene cluster exist for different chemotypes [29,34] that have pseudogenes or deletions for *Tri7* and *Tri13*. Tri13 is a cytochrome P-450 monooxygenase responsible for the 4-position hydroxyl that distinguishes NIV and DON chemotypes. Targeted mutation of the *Tri13* gene in the NIV cluster therefore results in strains that produce DON [35].

Further diversification occurs for trichothecene chemotypes in species distant to *F. graminearum*. *F. sporotrichioides* and related species produce A-type trichothecenes, such as T-2 toxin, which differs from NIV and DON by having an isovalerate ester at the 8 position oxygen, rather than a carbonyl. These differences result from catalytic divergence of the cytochrome P-450 enzymes encoded by *Tri1*. In *F. graminearum* Tri1p oxygenates both C-7 and C-8 (which results in a hydroxyl at C-7 and a carbonyl at C-8) whereas in *F. sporotrichioides*, only C-8 is hydroxylated by Tri1p [36,37]. In *F. graminearum*, *Tri1* is separate from the main trichothecene gene cluster at a telomere-proximal, high diversity region of the genome, whereas the main cluster is at a low diversity genomic position. We speculate that the diversification for chemotype made possible by the divergence of *Tri1* was facilitated by its position near the telomere. Like the cluster itself, *Tri1* also exhibits trans-species polymorphism that appears to track with chemotype [33]. However, in *Fusarium equiseti* and related species, *Tri1* has relocated to the main trichothecene cluster along with *Tri101* and a gene for a predicted transcription factor [33]. The variation generated at a high diversity genomic locus may be 'locked in' by migration to the cluster where diversification and recombination may be limited.

The sporadic phylogenetic distribution of secondary metabolite gene clusters among fungal species initially led to the hypothesis that many gene clusters could have been horizontally transferred among species as they have been in bacteria (reviewed in [38]). The phylogeny of genes within clusters, however, often is congruent with the phylogeny inferred from housekeeping genes. Most cluster evolution therefore seems to occur by way of vertical transmission, with cluster duplication and diversification combined with frequent cluster loss resulting in a pattern resembling horizontal transfer [39]. Distinguishing between vertical and horizontal transmission has been facilitated by DNA sequence information from a wide range of filamentous fungi, which has enabled

Figure 2



Comparison of trichothecene biosynthetic gene clusters from different *Fusarium* species and chemotypes. Clusters are shown for *F. equiseti*, and (top to bottom) *F. graminearum* NIV, 15 ADON and 3 ADON chemotypes. Arrows in orange represent trichothecene biosynthetic (*Tri*) genes with gene numbers given below. Arrows in green are *Tri* genes that have moved to the *Tri* cluster in *F. equiseti*. *T* is a predicted transcription factor. Arrows with an 'X' indicate pseudogenes. Arrows with the same color adjacent to *Tri* genes are homologous genes based on between-species comparisons. Comparisons are derived from figures presented in Lee *et al.* [35] and Proctor *et al.* [33].

identification of ancestral duplications and the ability to distinguish orthologous sequences from paralogous sequences.

Using this approach, a few fungal gene clusters responsible for the synthesis of plant-associated, bioactive compounds have been shown to have likely evolved by way of horizontal transfer. The sirodesmin/gliotoxin gene clusters that encode cyclic peptide derived toxins in several fungal species [40] and the ACE1 and ACE1-like gene clusters that encode enzymes apparently capable of synthesizing small molecules that can act as plant effectors [39,41] each show evidence for horiziontal transfer. Additionally, as mentioned above, toxin gene clusters in *A. alternata* may have been horizontally transferred by virtue of their position on mobile supernumerary chromosomes [21•].

Conclusions

Comparative genomics greatly enhances the rate of discovery of genes that form the basis of fungal pathogenicity. An important finding is that the most highly diverse genes found among strains of the same genus or even the same species, appear to be enriched for those involved in niche adaptation, including the colonization of living plant tissue. These genes or clusters often are not randomly dispersed in the genome, but rather tend to concentrate in genomic islands, telomere-proximal regions or even entire lineage-specific chromosomes. This organization and their association with mobile genetic elements make them functionally analogous to genomic islands and plasmids in bacteria.

What evolutionary processes organize genes involved in pathogenicity and habitat adaptation into clusters and subgenomic regions? Answering this question will require explanations for both mechanism and function. Do rates of mutation, recombination and duplication differ between conserved genomic regions and those associated with plant interactions? Does chromatin modification play a role in differences in the regulation of gene expression between these regions? Is an increased level of organiz-

ation, from pathogenicity gene cluster to pathogenicity chromosome, advantageous for the fungus? Are pathogenicity-related subgenomic regions more prone to repositioning within a genome or to mobility between genomes? Exactly what role do transposons play in gene expression, mutation, recombination and mobility? The answers to these questions, raised by comparative genomic analysis, will bring us closer to a unified understanding of how fungal genomes adapt to a pathogenic existence.

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