Drivers of genetic diversity in secondary metabolic gene clusters in a fungal population

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Summary

Filamentous fungi produce a diverse array of secondary metabolites (SMs) critical for defense, virulence, and communication. The metabolic pathways that produce SMs are found in contiguous gene clusters in fungal genomes, an atypical arrangement for metabolic pathways in other eukaryotes. Comparative studies of filamentous fungal species have shown that SM gene clusters are often either highly divergent or uniquely present in one or a handful of species, hampering efforts to determine the genetic basis and evolutionary drivers of SM gene cluster divergence. Here we examined SM variation in 66 cosmopolitan strains of a single species, the opportunistic human pathogen *Aspergillus fumigatus.* Investigation of genome-wide population-level variation showed that *A. fumigatus* strains contained five general types of variation in SM gene clusters: non-functional gene polymorphisms, gene gain and loss polymorphisms, whole cluster gain and loss polymorphisms, allelic polymorphisms where different alleles corresponded to distinct, non-homologous clusters, and location polymorphisms in which a cluster was found to differ in its genomic location across strains. These polymorphisms affect the function of representative *A. fumigatus* SM gene clusters, such as those involved in the production of gliotoxin, fumigaclavine, and helvolic acid, as well as the function of clusters with undefined products. In addition to enabling the identification of polymorphisms whose detection requires extensive genome-wide synteny conservation (e.g., mobile gene clusters and non-homologous cluster alleles), our population genomics approach also implicated multiple underlying genetic drivers, including point mutations, recombination, genomic deletion and insertion events, as well as horizontal gene transfer from distant fungi. Finally, most of the population variants that we uncover have been previously hypothesized to contribute to SM gene cluster diversity across entire fungal classes and phyla. We suggest that the drivers of genetic diversity operating within a fungal population shown here are sufficient to explain SM cluster macroevolutionary patterns.

Keywords: chemodiversity, specialized metabolism, genome evolution, genome architecture, gene loss, genomic rearrangement

Introduction

Filamentous fungi produce a diverse array of small molecules that function as toxins, antibiotics, and pigments [1]. Though by definition these so-called specialized or secondary metabolites (SMs) are not strictly necessary for growth and development, they are critical to the lifestyle of filamentous fungi [2]. For example, antibiotic SMs gain give their fungal producers a competitive edge in environments crowded with other microbes [3]. SMs can additionally mediate communication between and within species, as well as contribute to virulence on animal and plant hosts in pathogenic fungi [4,5].

A genomic hallmark of SMs in filamentous fungi is that the biosynthetic pathways that produce them are typically organized into contiguous gene clusters in the genome [6]. These gene clusters contain the chemical backbone synthesis genes whose enzymatic products produce a core metabolite, such as non-ribosomal peptide synthases (NRPS) and polyketide synthases (PKS), tailoring enzymes that chemically modify the metabolite, transporters involved in product export, and transcription factors that control the expression of the clustered genes [6]. Filamentous fungal genomes, particularly those in the phylum Ascomycota [6], typically contain dozens of SM gene clusters. However, most individual SM gene clusters appear to be either species-specific or narrowly taxonomically distributed in only a handful of species [6,7]. SM gene clusters that are more broadly distributed show discontinuous taxonomic distributions and are often highly divergent between species. Consequently, the identity and total number of SM gene clusters can vary widely even between very closely related species whose genomes exhibit very high sequence and synteny conservation [8,9].

In the last decade, several comparative studies have described macroevolutionary patterns of SM gene cluster diversity. For example, studies centered on genomic comparisons of closely related species, such as members of the same genus, have identified several different types of inter-species divergence, from single nucleotide substitutions (e.g., differences in fumonisins produced by *Fusarium* species are caused by variants in one gene [10]), to gene gain / loss events (e.g., the trichothecene gene clusters in *Fusarium* species and the aflatoxin family SM gene clusters in *Aspergillus* species) [11–16], and genomic rearrangements (e.g., the

86 trichothecene gene clusters in *Fusarium*) [11]. Additionally, genetic and genomic comparisons
87 across fungal orders and classes have identified several instances of gene gain or loss [17–19]
88 and horizontal gene transfer [13,20–23] acting on individual genes or on entire gene clusters,
89 providing explanations for the diversity and discontinuity of the taxonomic distribution of
90 certain SM gene clusters across fungal species.
91
92 Although inter-species comparative studies have substantially contributed to our
93 understanding of SM diversity, the high levels of evolutionary divergence of SM clusters make
94 inference of the genetic drivers of SM gene cluster evolution challenging; simply put, it has
95 been difficult to "catch" the mechanisms that generate SM gene cluster variation "in the act".
96 Several previous studies have examined intra-species or population-level differences in
97 individual SM gene clusters, typically focusing on the presence and frequency of non-functional
98 alleles of clusters involved in production of mycotoxins. Examples of clusters exhibiting such
99 polymorphisms include the gibberellin gene cluster in *Fusarium oxysporum* [24], the fumonisin
100 gene cluster in *Fusarium fujikuroi* [25], the aflatoxin and cyclopiazonic gene clusters in
101 *Aspergillus flavus* [26], and the bikaverin gene cluster in *Botrytis cinerea* [27]. While these
102 studies have greatly advanced our understanding of SM gene cluster genetic variation and
103 highlighted the importance of population-level analyses, studies examining the entirety of SM
104 gene cluster polymorphisms in fungal populations are so far lacking. We currently do not
105 understand the types and frequency of SM gene cluster polymorphisms in populations,
106 whether these polymorphisms affect all types of SM gene clusters, as well as the genetic drivers
107 of SM gene cluster evolution.
108
109 To address these questions, we investigated the genetic diversity of all 36 known and
110 predicted SM gene clusters in whole genome sequence data from 66 strains, 8 of which were
111 sequenced in this study, of the opportunistic human pathogen *Aspergillus fumigatus*, a species
112 with cosmopolitan distribution and panmictic population structure [28]. We found that 13 SM
113 gene clusters were generally conserved and harbored low amounts of variation. In contrast, the
114 remaining 23 SM gene clusters were highly variable and contained one or more of five different

115 types of genetic variation: single-nucleotide polymorphisms including nonsense and frameshift
116 variants, individual gene gain and loss polymorphisms, entire cluster gain and loss
117 polymorphisms, polymorphisms associated with changes in cluster genomic location, and
118 clusters with non-homologous alleles resembling the idiomorphs of fungal mating loci. Many
119 clusters contained interesting combinations of these different polymorphisms, such as
120 pseudogenization in some strains and entire cluster loss in others. The types of variants we find
121 are likely generated by a combination of DNA replication and repair errors, recombination,
122 genomic insertions and deletions, and horizontal transfer. We additionally find an enrichment
123 for transposable elements (TEs) around horizontally transferred clusters, clusters that change in
124 genomic locations, and idiomorphic clusters. Taken together, our results provide a guide to
125 both the types of polymorphisms and the genetic drivers of SM gene cluster diversification in
126 filamentous fungi. As most of the genetic variants that we observe have been previously
127 associated with SM gene cluster diversity across much larger evolutionary distances and
128 timescales, we argue that population-level processes influencing SM gene cluster diversity are
129 sufficient to explain SM cluster macroevolutionary patterns.
130

131

Results

We analyzed the genomes of 66 globally distributed strains of *Aspergillus fumigatus* for polymorphisms in SM gene clusters. We performed whole-genome sequencing on 8 strains, and collected the remaining 58 strains from publicly available databases including NCBI Genome and the NCBI Short Read Archive (Figure 1, Table S1) [28–32]. We analyzed all strains for polymorphisms in 33 curated SM gene clusters present in the reference Af293 genome and additionally searched for novel SM gene clusters (see Methods). These examinations revealed five distinct types of polymorphisms which influence SM gene cluster variation (Table 1):

a) Single nucleotide and short indel polymorphisms. 33 / 33 SM gene clusters (present in the reference Af293 strain) contained multiple genes with missense SNPs and short indel variants in at least one strain in the population. 23 / 33 SM gene clusters contained one or more genes with frameshift or nonsense variants in the population.
b) Gene content polymorphisms involving loss or gain of one or more genes. 6 / 33 SM gene clusters contained a gene content polymorphism in the population.
c) Whole SM gene cluster gain and loss polymorphisms. 3 / 33 SM gene clusters present in the genome of the reference Af293 strain were absent in at least one strain in the population and an additional 3 previously unknown SM gene clusters were present in the population.
d) Idiomorphic polymorphisms. One locus contained multiple non-homologous SM gene cluster alleles in different strains of the population.
e) Genomic location polymorphisms. 2 / 33 SM gene clusters were found in different genomic locations (e.g., different chromosomes) between strains.

Single-nucleotide and indel polymorphisms

It is well established that single nucleotide polymorphisms (SNPs) and short indel polymorphisms are caused by errors in DNA replication and repair, and are a major source of genomic variation [33]. Non-synonymous SNPs and indels with missense, frameshift, and nonsense effects were widespread across the 33 SM reference gene clusters (Table S2). Every

160 strain contained numerous missense mutations and at least one nonsense or frameshift
161 mutation in its SM gene clusters. Although missense mutations are likely to influence SM
162 production, the functional effects of nonsense and frameshift mutations are comparatively
163 easier to infer from genomic sequence data because they often lead to truncated proteins
164 lacking a significant portion of their amino acid sequence. For example, a frameshift mutation in
165 the polyketide synthase (PKS) of the trypacidin gene cluster in the A1163 strain results in loss of
166 trypacidin production [34]. Interestingly, we identified a premature stop codon (Gln273*) in a
167 transcription factor required for trypacidin production, *tpcD,* in a strain sequenced in this study
168 (MO79587EXP). These data suggest that function of this SM gene cluster has been lost through
169 at least two independent genetic events in *A. fumigatus*.
170
171 Individual nonsense or frameshift variants ranged from very common in the population
172 to rarer variants present in one or a handful of strains. For example, the non-ribosomal peptide
173 synthase (NRPS) *pes3* gene (Afu5g12730) in SM gene cluster 21 harbors 16 nonsense or
174 frameshift polymorphisms in 55 strains. Seven of these polymorphisms are common and
175 present in 10 or more strains, while seven are rarer and found in 5 or fewer strains. Strains with
176 lab-mutated null alleles of the *pes3* gene are more virulent than strains with functional copies
177 [35], which may explain the widespread occurrence of null *pes3* alleles in the *Aspergillus*
178 population.
179

180 **Gene content polymorphisms**

181 We additionally identified several SM gene clusters that gained or lost genes in some
182 strains. These gene content polymorphisms were most likely generated through genomic
183 deletion or insertion events and were often present in high frequencies in the population (Table
184 1). In three cases, these polymorphisms impact backbone synthesis genes, rendering the SM
185 gene cluster non-functional. One example involves SM gene cluster 14, whose standard
186 composition includes a pyoverdine synthase gene, an NRPS-like gene, an NRPS backbone gene,
187 and several additional modification genes (Figure 2A). We discovered that 4 / 66 strains lack an
188 11-kb region on the 3' end of the cluster which normally contains an NRPS gene and two

189 additional cluster genes, and the first non-SM genes on the 3' end flanking the cluster. All *A.*
190 *fumigatus* strains contain a *copia* family transposable element [36,37] at the 3' end of the
191 cluster, suggesting that transposable elements may have been involved in the generation of this
192 polymorphism. Three of the strains lacking the 11-Kb region form a clade in the SNP-based
193 phylogeny (the fourth, RP-2014 was not included in this phylogeny as short read data were not
194 available). While this polymorphism could have arisen through a deletion event, a homologous
195 cluster lacking the 11-kb region is also present in the reference genomes of *Aspergillus lentulus*
196 and *Aspergillus fischerianus*, close relatives of *A. fumigatus* (Figure 2). The most parsimonious
197 explanation is that the genome of the *A. fumigatus* ancestor contained an SM gene cluster that
198 lacked the 11-kb region, and that this genomic region was subsequently gained and increased in
199 frequency in the *A. fumigatus* population.
200
201 Two additional gene content polymorphisms affecting SM backbone genes were
202 restricted to one strain each and appear to have arisen through genomic deletion events.
203 Specifically, strain IF1SWF4 lacks an 8-Kb region near the helvolic acid SM gene cluster,
204 resulting in the loss of the backbone oxidosqualene cyclase gene as well an upstream region
205 containing two non-SM genes (Figure S1). Strain LMB35Aa lacks a 54-kb region on the end of
206 chromosome 2, which includes five genes from the telomere-proximal fumigaclavine C cluster
207 (Figure S1).
208
209 In three other cases, gene content polymorphisms involved gene loss or truncation
210 events of non-backbone structural genes. We found that the second half of the ORF of the *gliM*
211 *O*-methyltransferase gene in the gliotoxin gene cluster has been lost in 2 / 66 strains (Figure
212 S1), and that the first half of the permease *fmqE* in the fumiquinazoline gene cluster has been
213 lost in 4 / 66 strains (Figure S1). Finally, an ABC transporter gene in SM cluster 21 has been
214 almost entirely lost in 21 / 66 strains (Figure S2). This deletion event is found in strains that are
215 related in the SNP phylogeny but does not perfectly mirror population structure (Figure S2).
216

217 **Whole gene cluster gain and loss polymorphisms**

218 Several SM gene clusters were gained or lost entirely in 13 / 66 strains. We observed
219 instances where a cluster present in the genome of the reference Af293 strain was absent or
220 pseudogenized in other strains as well as cases in which SM clusters present in other strains
221 were absent from the reference Af293 strain.

222

223 The most notable example of an SM gene cluster that was present in the Af293
224 reference genome but absent or pseudogenized in others was SM cluster 4. This cluster
225 contains 5 genes on the tip of the Af293 chromosome 1 and contains orthologs to five of the six
226 genes in the fusarielin gene cluster in *Fusarium graminearum* [38]. This cluster is also present in
227 several other *Aspergillus* species, including *A. clavatus* and *A. niger* [38]. Phylogenetic analysis
228 of the genes in this SM gene cluster is consistent with horizontal gene transfer between fungi in
229 the class Sordariomycetes and fungi in the class Eurotiomycetes, or alternatively with extensive
230 gene loss in both Sordariomycetes and Eurotiomycetes (Figure S3). This gene cluster is entirely
231 absent in 4 / 66 strains, and its genes are undergoing pseudogenization in an additional 43
232 strains via multiple independent mutational events (Figure 3A). These four strains form a single
233 clade on the SNP-based phylogeny, suggesting that this cluster was lost in a single deletion
234 event (Figure 3B). Further, 19 strains shared a single frameshift variant in the polyketide
235 synthase gene (4380_4381insAATGGGCT; frameshift at Glu1461 in Afu1g17740) and an
236 additional 13 strains shared a single frameshift variant (242delG; frameshift at Gly81) in an
237 aldose 1-epimerase gene (Afu1g17723). Eleven other strains each contained one to several
238 frameshift or nonsense polymorphisms involving nine unique mutational sites, suggesting that
239 this pathway is undergoing multiple independent pseudogenization events. Five of these strains
240 contained multiple distinct frameshifts and premature stop codons in more than one gene in
241 the cluster, indicating that the entire pathway is pseudogenized in these strains. The
242 distributions of pseudogenizing variants only in the aldose 1-epimerase gene and of
243 pseudogenizing variants in multiple genes in the cluster roughly correlate with population
244 structure, while the distribution of pseudogenizing variants in the PKS backbone gene does not
245 (Figure 3B).

246

247 By searching for novel SM gene clusters in the genomes of the other 65 *A. fumigatus*
248 strains, we found three SM gene clusters that were absent from the genome of the Af293
249 reference strain. As SM gene clusters are often present in repeat-rich and subtelomeric regions
250 that are challenging to assemble [39,40], these strains might harbor additional novel SM gene
251 clusters.
252
253 One of the novel SM gene clusters that we identified, cluster 34, was present in all but
254 two of the strains (Af293 and F7763). This cluster contains a PKS backbone gene, one PKS-like
255 gene with a single PKS associated domain, nine genes with putative biosynthetic functions
256 involved in secondary metabolism, and six hypothetical proteins (Figure 3C). The two strains
257 that lack this cluster contain a likely non-functional cluster fragment that includes the PKS-like
258 gene, two biosynthetic genes, and three hypothetical proteins. Interestingly, the 3' region
259 flanking this cluster is syntenic across all 66 strains but the 5' region is not, suggesting that a
260 recombination or deletion event may have resulted in the loss of this cluster in the Af293 and
261 F7763 strains.
262
263 The other SM gene clusters that were absent from the Af293 genome are present at
264 lower frequencies in the population; cluster 35 is present in 2 / 66 strains and cluster 36 in 4 /
265 66 strains. Cluster 35 is located in a region syntenic with an Af293 chromosome 4 region and is
266 flanked on both sides by transposable elements (Figure S4). The two strains that contain this
267 novel cluster are not sister to each other in the SNP-based phylogeny (Figure S4). Eight of the
268 14 genes in this SM gene cluster are homologous to genes in an SM gene cluster in the genome
269 of the insect pathogenic fungus *Metarhizium anisopliae* (Figure S4). Phylogenetic analysis of
270 these 8 genes is consistent with a horizontal transfer event (Figure S5). Cluster 36 is an NRPS
271 containing cluster located on genomic scaffolds that lack homology to either the Af293 or
272 A1163 genomes, making it impossible to determine on which chromosome this cluster is
273 located (Figure S3). Two of the strains containing this novel cluster are sister to each other in
274 the SNP-based phylogeny, while the third is distant to these two (Figure S4). The evolutionary

histories of the genes in the cluster are consistent with vertical inheritance and are present in multiple *Aspergillus* species.

Idiomorph polymorphisms

One of the most peculiar types of polymorphisms that we identified is a locus containing different unrelated alleles of SM gene clusters, reminiscent of the idiomorph alleles at the fungal mating loci [41]. This locus, which resides on chromosome 3 and corresponds to cluster 10 in the Af293 genome (Figure 4), was previously described as being strain-specific in a comparison between Af293 and A1163 [30] and is thought to reside in a recombination hot spot [28]. Our analysis showed that there are at least 6 different alleles of this cluster in the population, containing 4 different types of key enzymes involved in natural product biosynthesis: a polyketide synthase (PKS) non-ribosomal peptide synthetase (NRPS) hybrid, a highly reducing (HR) PKS, a non-reducing (NR) PKS and an NRPS-like enzyme (Figure 4). Two additional alleles were present in only one strain each (Figure S6).

In the Af293 reference genome, the cluster present at this locus contains one NR-PKS along with an NRPS-like gene (Allele B). In the A1163 reference genome and 17 other strains, there is a PKS-NRPS and an HR-NRPS (Allele E). These alleles show an almost complete lack of sequence similarity except for a conserved hypothetical protein and a fragment of the HR-PKS in the Af293 allele; in contrast, the upstream and downstream flanking regions of the two alleles, which do not contain any backbone genes, are syntenic. Remarkably, another allele, present in 12 strains, contains all of the genes from both the Af293 and A1163 clusters (Allele D). The remaining three alleles contain various combinations of these genes. One allele found in 22 strains contains some A1163-specific genes including the HR-PKS and no Af293-specific genes (Allele F), while another allele found in 3 strains contains some Af293-specific genes including the NRPS-like gene but no A1163 genes (Allele A). The final allele, present in 8 strains, contains the entire Af293 allele as well as part of the A1163 allele containing the HR-PKS (Allele C). Every allele is littered with long terminal repeat sequence fragments from *gypsy* and *copia* TE families as well as with sequence fragments from DNA transposons from the *mariner* family

304 [36]. In some cases, these TEs correspond with breakpoints in synteny between alleles,
305 suggesting that the diverse alleles of this SM gene cluster may arise via TE-driven
306 recombination. Further, both of the alleles that are restricted to a single strain had an insertion
307 event of several genes near a TE, though the rest of the locus is highly similar to one of the
308 more common alleles (Figure S6).
309
310 Preliminary metabolite analysis comparing one of the strains (08-19-02-30) that
311 contains the allele harboring all four key enzymes (Allele D) to two strains (08-12-12-13 and 08-
312 19-02-10) that only codes for the putative cluster the HR-PKS backbone gene (Allele F) by
313 untargeted XCMS analysis [42] revealed the presence of 2 unique masses in 08-19-02-30 (Table
314 S4; Fig. 4B). Further analysis is underway to investigate whether any of these m/z can be
315 directly linked to the allele present in 08-19-02-30.
316
317 The evolutionary history of this highly diverse locus is unclear (Figure 4C). While it is
318 tempting to speculate that the largest allele containing all observed genes represents the
319 ancestral state, it does not explain the presence of a shared hypothetical protein and PKS gene
320 fragment between the Af293 locus (Allele C) and the A1163 locus (Allele B). The distributions of
321 Alleles A, B, C, and F roughly correlate with the population structure of the strains, while the
322 distribution of Alleles D and E do not (Figure 4C).
323

324 **Genomic location polymorphisms**

325 The final type of polymorphism that we observed is associated with SM gene clusters
326 that are located in different genomic locations in different strains, suggesting that these SM
327 gene clusters are behaving like mobile elements. This type of polymorphism was observed in
328 SM gene clusters 1 and 33, both of which produce as yet identified products, and are present at
329 low frequencies in the population.
330
331 SM gene cluster 1, which is present in six strains at three different genomic locations
332 (Figure 5A), consists of a PKS and four other modification genes that are always flanked by a 15

333 Kb region (upstream) and a 43 Kb region (downstream) containing TEs. In the reference Af293
334 strain and in strain F7763, this SM gene cluster and its flanking regions are located on
335 chromosome 1, while in strains dutch3, F13619, and Z5 they are located between Afu4g07320
336 and Afu4g07340 on chromosome 4. In contrast, in strain JCM_10253, the cluster and flanking
337 regions are located on chromosome 8 immediately adjacent to the 3' end of the intertwined
338 fumagillin and pseurotin SM gene supercluster [43]. The strains containing the allele on
339 Chromosome 1 are sister to each other in the SNP-based phylogeny, while the other strains are
340 scattered across the tree and do not reflect the underlying population structure (Figure 5C).
341
342 In 5 / 6 strains, the cluster appears to be functional and does not contain nonsense SNPs
343 or indels. However, the cluster found on chromosome 1 in strain F7763 contains two stop
344 codons in the oxidoreductase gene (Gln121* and Gln220*) and two premature stop codons in
345 the polyketide synthase (Gln1156* and Gln1542*), suggesting this strain contains a null allele.
346
347 This "jumping" gene cluster is not present in any other sequenced *Aspergillus* genus,
348 and phylogenetic analysis of its constituent genes is consistent with horizontal gene transfer
349 between fungi (Figure S7). Specifically, this gene cluster is also present in *Phaeosphaeria*
350 *nodorum,* a plant pathogen from the class Dothideomycetes, *Pseudogymnoascus pannorum*, a
351 fungus isolated from permafrost from the Leotiomycetes, *Escovopsis weberi*, and a fungal
352 parasite of fungus-growing ants from the Sordariomycetes (Figure 5B). One additional species,
353 the endophyte *Hypoxylon* sp. CI4A from the class Sordariomycetes, contains four of the five
354 cluster genes but is missing Afu1g00970, an MFS drug transporter. However, this species
355 contains an unrelated gene annotated as an MFS drug transporter immediately adjacent to this
356 cluster, so this species may be using a different transporter (Figure 5B). None of these fungi
357 contain the upstream or downstream TE-rich flanking regions present in *A. fumigatus*, and each
358 fungus contains additional unique genes with putative biosynthetic functions adjacent to the
359 transferred cluster. The most likely explanation for this change in flanking regions is that this
360 SM gene cluster was transferred into *A. fumigatus* once and has subsequently moved its
361 location in the genome.

362

363 The second SM gene cluster that shows variation in its genomic location across strains,
364 cluster 33, contains a terpene synthase. This cluster is present in only 5 strains at 3 distinct
365 locations (Figure S8). Similar to cluster 1, this cluster is also flanked by TEs, and in one strain the
366 clusters is located 58 Kb from another SM gene cluster. Two strains that contain the cluster in
367 the same genomic location are sister to each other in the SNP-based phylogeny, while the other
368 three strains containing the cluster do not reflect the underlying population structure (Figure
369 S8). In contrast to cluster 1, this cluster does not appear to have been horizontally transferred
370 between fungi and its genes are present in other sequenced *Aspergillus* species. As this mobile
371 cluster was not horizontally transferred, it is possible that the horizontal transfer of cluster 1
372 and its mobile nature throughout the *A. fumigatus* genome are driven by different mechanisms.
373

374 **Discussion**

375 Our examination of 66 genomes from strains of *Aspergillus fumigatus* revealed that five
376 general types of polymorphisms describe variation in SM gene clusters in a fungal population.
377 These polymorphisms include variation in single nucleotides, gene and gene cluster gains and
378 losses, non-homologous clusters at the same genomic position, and changes in genomic
379 locations of clusters (Figure 6). In several cases, the genetic mechanisms that gave rise to these
380 polymorphisms and their functional consequences are clear. Polymorphisms in single-
381 nucleotides most likely arose from errors in DNA replication or repair, while gene loss
382 polymorphisms likely arose from genomic deletion or recombination events. Others, such as SM
383 gene clusters that exist as non-homologous alleles or that entered the population through
384 horizontal transfer, could arise through multiple genetic mechanisms. Transposable elements
385 are common in polymorphic SM gene clusters and are found flanking mobile and horizontally
386 transferred clusters, as well as in regions adjacent to non-homologous alleles and where gene
387 gain has occurred, suggesting they contribute to SM gene cluster diversity. Using a population
388 genomics approach to identify SM gene cluster variants allowed us to also capture and describe
389 novel polymorphisms, including mobile gene clusters and idiomorphic clusters, which are
390 difficult to identify in comparative genomic studies between species whose conservation of
391 genome-wide synteny is low. Strikingly, the variants and genetic drivers we observe at the
392 population level are also implicated as driving SM gene cluster variation between fungal
393 species, suggesting that the observed microevolutionary processes are sufficient to explain
394 macroevolutionary patterns of SM gene cluster evolution.

395

396 The first novel type of polymorphism was observed in SM gene clusters 1 and 33, which
397 by occupying different genomic locations in different strains, appear to behave in a manner
398 similar to mobile genetic elements. Interestingly, both clusters are located near or immediately
399 adjacent to other SM gene clusters in some strains. For example, cluster 1 is located
400 immediately adjacent to the intertwined fumagillin and pseurotin supercluster [43] in one
401 strain. This supercluster is regulated by the transcriptional factor *fapR* and is located in a
402 chromosomal region controlled by the master SM regulators *laeA* and *veA* [43,44], raising the
403 hypothesis that these mobile gene clusters might be co-opting the regulatory machinery

404 already in place. Previous work has hypothesized that the fumagillin and pseurotin supercluster
405 formed through genomic rearrangement events placing the once-independent gene clusters in
406 close proximity to each other [43]. Our observation that this mobile gene cluster is located in
407 this same region not only supports this hypothesis but also implicates transposable elements as
408 one of the mechanisms by which such genomic rearrangements are formed. These
409 superclusters may also represent an intermediate stage in the formation of new SM gene
410 clusters. Supercluster formation, potentially mediated by mobile gene clusters, and followed
411 by gene loss, could explain macroevolutionary patterns of SM gene clusters have shown that
412 clustered genes in one species can be dispersed over multiple gene clusters in other species
413 [9,11].
414
415 The second novel type of polymorphism was the presence of multiple non-homologous
416 alleles at the cluster 10 locus, echoing the structure of the idiomorphic mating type locus [41].
417 This region has previously been reported to be a recombination hotspot in *A. fumigatus* [28]
418 and all alleles contain numerous transposable elements. Thus, it is possible that polymorphism
419 at this locus originated via SM gene cluster fusion or splitting events driven by transposable
420 elements. Interestingly, two other previously described instances of SM gene cluster variation
421 bear close resemblance to the *A. fumigatus* idiomorphic SM gene cluster 10 locus. The first is
422 the presence of two non-homologous *Aspergillus flavus* alleles, where some strains contain a 9-
423 gene sesquiterpene-like SM gene cluster and others contain a non-homologous 6-gene SM gene
424 cluster at the same genomic location [45]. The second is the presence of two non-homologous
425 SM gene clusters at the same, well-conserved, locus in a comparison of six species of
426 dermatophyte fungi [46]. Based on these results, we hypothesize that idiomorphic clusters may
427 be common in fungal populations and contribute to the broad diversity of SM gene clusters
428 across filamentous fungi.
429
430 The remaining types of SM polymorphism in the *A. fumigatus* population have
431 previously been described at the species level. For example, null alleles have been reported for
432 many individual SM gene clusters across diverse fungal species [10,17,26,27] and our results

433 show that it is a widespread phenomenon, affecting two-thirds of the *A. fumigatus* SM gene
434 clusters. Consistent with previous literature reporting the presence of additional SM gene
435 clusters not present in the reference strain [24,25], we also identify four low-frequency SM
436 gene clusters in the *A. fumigatus* population, two of which are horizontally transferred from
437 distantly related fungi and two that appear vertically inherited. We find numerous cases of
438 gene loss and relatively fewer cases of gene gain; previous studies have implicated these
439 processes across numerous types of SM gene clusters and reported their effects on metabolite
440 production [47]. Previous work has indicated that gene and gene cluster gain and loss tends to
441 occur near telomeres, suggesting that higher rates of genetic events like recombination lead to
442 loss [48]. While we do find several cases of gene and gene cluster loss in subtelomeric clusters,
443 including the fumigaclavine gene cluster and the helvolic acid gene cluster, we also find gene
444 loss in clusters that are not located near telomeres, such as the gliotoxin gene cluster and the
445 fumiquinazoline gene cluster. These findings suggest that while gene and gene cluster loss can
446 occur in telomeric regions, clusters in other genomic regions also experience high rates of loss.
447
448 Previous work has demonstrated that SM gene clusters, like the metabolites that they
449 produce, are highly divergent between fungal species. Our examination of genome-wide
450 variation shows that these SM gene clusters are correspondingly diverse within individual
451 strains of a single fungal species. Furthermore, the observed genetic changes in SM gene
452 clusters are widespread across different types of gene clusters and are caused by many
453 underlying genetic drivers, including gene and gene cluster gain, loss, non-homologous cluster
454 alleles, and mobile gene clusters. The net effect of these substitutions, gains, losses, and
455 rearrangements raises the hypothesis that fungal SM gene clusters are likely in a state of
456 evolutionary flux, constantly altering their SM gene cluster repertoire, and consequently
457 modifying and diversifying their chemodiversity.
458

459 **Methods**

460 **Strains analyzed**

461 Eight strains of *A. fumigatus* were isolated from four patients with recurrent cases of
462 aspergillosis in the Portuguese Oncology Institute in Porto, Portugal. Each strain was
463 determined to be *A. fumigatus* using macroscopic features of the culture and microscopic
464 morphology observed in the slide preparation from the colonies with lactophenol solution [49].
465 Based on the morphological characterization, all clinical strains were classified as *A. fumigatus*
466 *complex*-Fumigati. After whole genome sequencing, retrieval and examination of the beta
467 tubulin and calmodulin sequences of each strain confirmed that all strains belonged to *A.*
468 *fumigatus* (see Phylogenetic Analysis and Figure S9). The genomes of all eight strains were
469 sequenced using 150bp Illumina paired-end sequence reads at the Genomic Services Lab of
470 Hudson Alpha (Huntsville, Alabama, USA). Genomic libraries were constructed with the Illumina
471 TruSeq library kit and sequenced on an Illumina HiSeq 2500 sequencer. Samples of all eight
472 strains were sequenced at greater than 180X coverage or depth (Table S1). Short read
473 sequences for these 8 strains are available in the Short Read Archive under accession
474 SRP109032.

475

476 In addition to the 8 strains sequenced in this study, we retrieved 58 *A. fumigatus* strains
477 with publicly available whole genome sequencing data, resulting in a population genomics
478 dataset of 66 strains (Table S1). The strains used included both environmental and clinical
479 strains and were isolated from multiple continents. Genome assemblies for 10 of these strains,
480 including the Af293 and A1163 reference strains, were available for download from GenBank
481 [28–32,50]. For 6 of these strains, short read sequences were also available from the NCBI Short
482 Read Archive (SRA), which were used for variant discovery only (see Single nucleotide variant
483 (SNV) and indel discovery) and not for genome assembly. Short read sequences were not
484 available for the remaining 4 strains. Short read sequences were downloaded for an additional
485 48 strains from the Short Read Archive if they were sequenced with paired-end reads and at
486 greater than 30x coverage.

487

488 **Single nucleotide variant (SNV) and indel discovery**

489 All strains with available short read data (62 of 66 strains) were aligned to both the
490 Af293 and A1163 reference genomes using BWA mem version 0.7.12-r1044 [51]. Coverage of
491 genes present in the reference genome was calculated using bedtools v2.25.0 [52]. SNV and
492 indel discovery and genotyping was performed relative to the Af293 reference genome and was
493 conducted across all samples simultaneously using the Genome Analysis Toolkit version 3.5-0-
494 g36282e4 with recommended hard filtering parameters [53–55] and annotated using snpEff
495 version 4.2 [56].

496

497 ***De novo* genome assembly and gene annotation**

498 All 56 strains without publicly available genome assemblies were *de novo* assembled
499 using the iWGS pipeline [57]. Specifically, all strains were assembled using SPAdes v3.6.2 and
500 MaSuRCA v3.1.3 and resulting assemblies were evaluated using QUAST v3.2 [58–60]. The
501 average N50 of assemblies constructed with this strategy was 463 KB (Table S1). Genes were
502 annotated in these assemblies as well as in five GenBank assemblies with no predicted genes
503 using augustus v3.2.2 trained on *A. fumigatus* gene models [61]. Repetitive elements were
504 annotated in all assemblies using RepeatMasker version open-4.0.6 [62].

505

506 **Secondary metabolic gene cluster annotation and discovery**

507 Secondary metabolic gene clusters in the Af293 reference genome were taken from two
508 recent reviews, both of which considered computational and experimental data to delineate
509 cluster boundaries [63,64] (Table S3). The genomes of the other 65 strains were scanned for
510 novel SM gene clusters using identified using antiSMASH v3.0.5.1 [65]. To prevent potential
511 assembly errors from confounding the analysis, any inference about changes in genomic
512 locations of genes or gene clusters was additionally verified by manually inspecting alignments
513 and ensuring that paired end reads supported an alternative genomic location (see SNV and
514 indel discovery). Cases where paired end reads did not support the change in genomic location
515 or where mapping was ambiguous or low quality were discarded.

516

517 **Phylogenetic analysis**

518 To confirm all strains in this analysis belonged to the species *Aspergillus fumigatus*, the
519 genomic sequences of the beta tubulin and calmodulin genes were extracted from the
520 assembled genomes of all strains. Gene phylogenies were constructed using *Aspergillus*
521 *fischerianus* as an outgroup using RAxML v8.0.25 with the GTRGAMMA substitution model [66].
522 The tree was midpoint rooted and all branches with bootstrap support less than 80% were
523 collapsed (Figure S9).

524

525 To construct a SNP-based strain phylogeny, biallelic SNPs with no missing data were
526 pruned using SNPRelate v1.8.0 with a linkage disequilibrium threshold of 0.8 [67]. A total of
527 15,274 SNVs were used to create a phylogeny using RAxML v8.0.25 with the ASC_BINGAMMA
528 substitution model [66]. The tree was midpoint rooted and all branches with bootstrap support
529 less than 80% were collapsed.

530

531 To understand the evolutionary histories of specific SM gene clusters showing unusual
532 taxonomic distributions, we reconstructed the phylogenetic trees of their SM genes.
533 Specifically, SM cluster protein sequences were queried against a local copy of the NCBI non-
534 redundant protein database (downloaded May 30, 2017) using phmmer, a member of the
535 HMMER3 software suite [68] using acceleration parameters --F1 1e-5 --F2 1e-7 --F3 1e-10. A
536 custom perl script sorted the phmmer results based on the normalized bitscore (nbs), where
537 nbs was calculated as the bitscore of the single best-scoring domain in the hit sequence divided
538 by the best bitscore possible for the query sequence (i.e., the bitscore of the query aligned to
539 itself). No more than five hits were retained for each unique NCBI Taxonomy ID. Full-length
540 proteins corresponding to the top 100 hits (E-value < 1 × 10-10) to each query sequence were
541 extracted from the local database using esl-sfetch [68]. Sequences were aligned with MAFFT
542 v7.310 using the E-INS-i strategy and the BLOSUM30 amino acid scoring matrix [69] and
543 trimmed with trimAL v1.4.rev15 using its gappyout strategy [70]. The topologies were inferred
544 using maximum likelihood as implemented in RAxML v8.2.9 [66] using empirically determined
545 substitution models and rapid bootstrapping (1000 replications). The phylogenies were

546 midpoint rooted and branches with less than 80% bootstrap support were collapsed using the
547 ape and phangorn R packages [71,72]. Phylogenies were visualized using ITOL version 3.0 [73].
548

549 **Differential metabolite analysis**

550 For natural product analysis, 5 x 106 spores/mL for the indicated strains were grown in
551 50 mL liquid GMM [74] for five days at 25 °C and 250 rpm in duplicates. Supernatants were
552 extracted with equal volumes of ethyl acetate, dried down and resuspened in 20% acetonitrile
553 (ACN). Each sample was analyzed by ultra-high performance liquid chromatography (UHPLC)
554 coupled with mass spectrometry (MS). The samples were separated on a ZORBAX Eclipse XDB-
555 C18 column (Agilent, 2.1 x 150 mm with a 1.8 µM particle size using a binary gradient of 0.5 %
556 (v/v) formic acid (FA) as solvent A and 0.5 % (v/v) FA in ACN as solvent B that was delivered by a
557 VanquishTM UHPLC system (Thermo Scientific) with a flow rate of 0.2 mL/min. The binary
558 gradient started with 20% B that was increased with a linear gradient to 100% B in 15 min
559 followed by an isocratic step at 100% B for 5 min. Before every run, the system was equilibrated
560 for 5 min at 20% B. The UHPLC system was coupled to a Q Exactive hybrid quadrupole
561 OritrapTM MS (Thermo Scientific). For electrospray ionization, the ion voltage was set at +/-3.5
562 kV in positive and negative mode, respectively. Nitrogen was used as sheath gas at a flow rate
563 of 45 and as sweep gas at a flow rate of 2. Data analysis was performed using XCMS [42] and
564 Maven [75] software.
565

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Author contributions

Conceptualization, A.R., G.H.G., A.L.L.; Methodology, A.L.L., J.H.W, C.L, P.W., J.M.P.; Investigation, A.L.L.; Visualization, A.L.L., J.H.W, P.W.; Resources, G.H.G., F.R., N.P.K., C.L.; Writing, A.L.L, A.R.

583 **Table 1. Types and rates of SM gene cluster variants in *A. fumigatus* strains.**

584

Description	Phenotype	Drivers	Frequency at cluster level	Frequency at strain level	Previous reports
Single-nucleotide polymorphisms and indels	Potential for protein function change (missense); abrogation of protein function (nonsense and frameshift)	DNA replication errors; relaxation of purifying selection	100% (33/33 clusters; missense); 70% (23/33 clusters; nonsense and frameshift)	Every strain affected	Bikaverin in *Botrytis* [17,27], aflatoxin in *Aspergillus oryzae* and *Aspergillus flavus* [26], fumonisins in *Fusarium* [10], many others
Gene content polymorphisms	Loss of gene cluster function; structural changes in the metabolite; change in cluster expression or metabolite transport	Deletion and insertion events; recombination; transposable elements	6 clusters	27 / 66 strains	Trichothecene in *Fusarium*, aflatoxin and sterigmatocystin in *Aspergillus* [11–15]
Whole gene cluster polymorphisms	Loss or gain of novel metabolites	Deletion and insertion events; horizontal gene transfer; transposable elements	6 clusters	13 / 66 strains	Gibberellin and fumonisin in *Fusarium* [24,25]
fCluster idiomorphs	Changes in metabolites produced or structure of metabolites	Transposable elements; recombination; other mechanisms?	1 gene cluster	8 unique identified alleles	Putative SM gene clusters in dermatophytes; putative SM gene cluster in *Aspergillus flavus* and *Aspergillus oryzae* [45,46]
Mobile gene clusters	Potential for change in gene regulation	Transposable elements; horizontal gene transfer; other mechanisms?	2 gene clusters	8 / 66 strains	None

585

586

587
588 **Table S1. Summary of strains, sequence data, and assemblies used.**
589
590 **Table S2. All nonsynonymous variants in SM gene cluster genes.**
591
592 **Table S3. Description, locus tags, and gene annotations of all reference Af293 SM gene**
593 **clusters.**
594
595 **Table S4. XCMS analysis of extracted supernatants from 08-19-02-30, 08-12-12-13, and 08-19-**
596 **02-10.**
597
598

599

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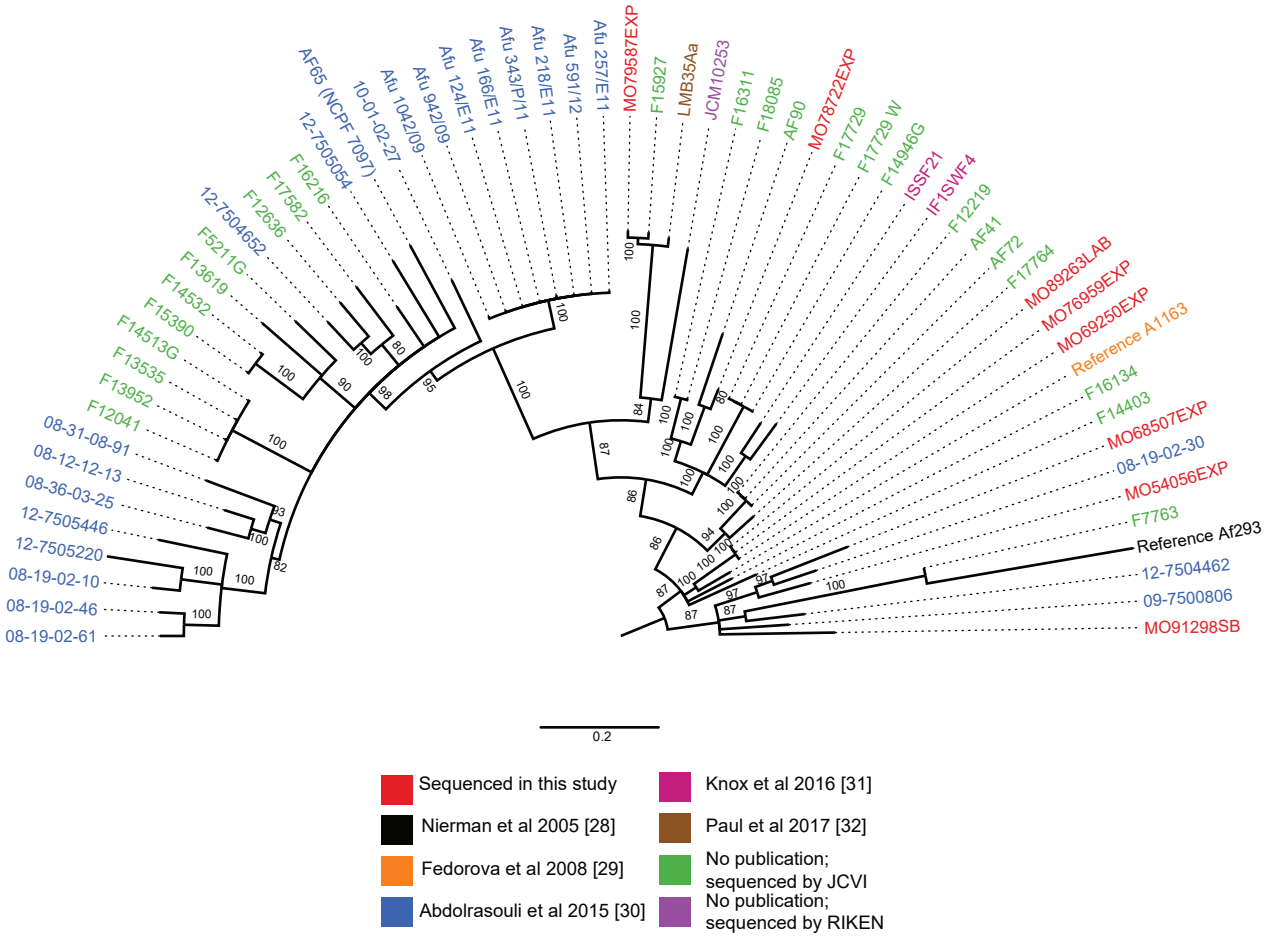


Figure 1. SNP-based phylogeny of *A. fumigatus* strains. The phylogeny was constructed using 15,274 biallelic SNPs with no missing data. The tree is midpoint rooted and all branches with bootstrap support less than 80% are collapsed. This phylogeny does not include strains Af10, Af210, Z5, or RP-2014 as short read data were not available.

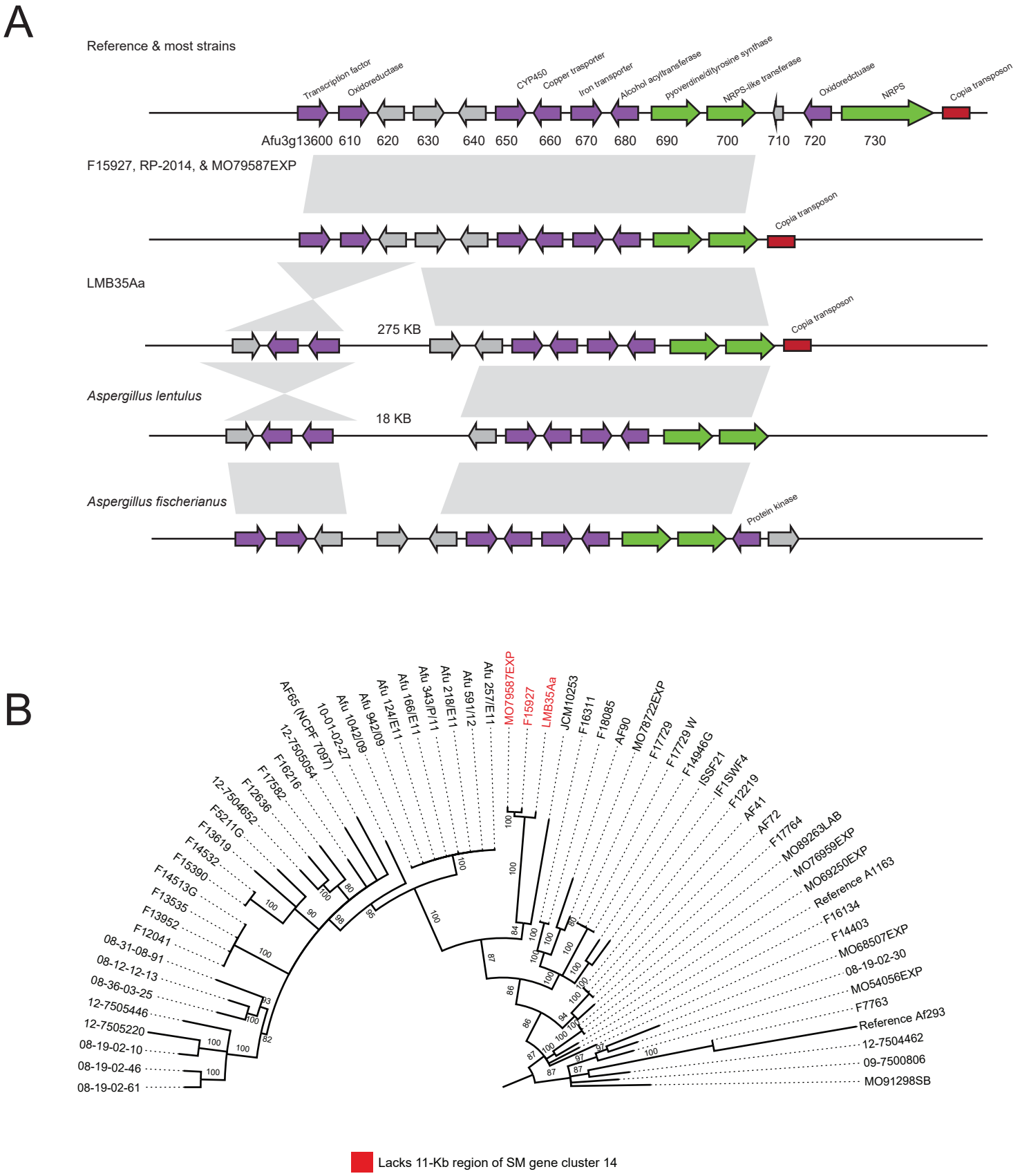


Figure 2. Differences in gene content in SM gene cluster 14 in A. fumigatus strains and closely related species. Four *A. fumigatus* strains lack an 11-Kb region in this cluster, including an NRPS backbone gene. (A) Regions upstream and downstream of this cluster are syntenic. LMB35Aa also contains a large inversion that moves a transcription factor, oxidoreductase, and hypothetical protein 275 kb away from the cluster. *Aspergillus fischerianus* and *Aspergillus lentulus*, close relatives of *A. fumigatus*, contain a cluster lacking the 11-kb region. (B) Strains without 11-Kb region highlighted in red.

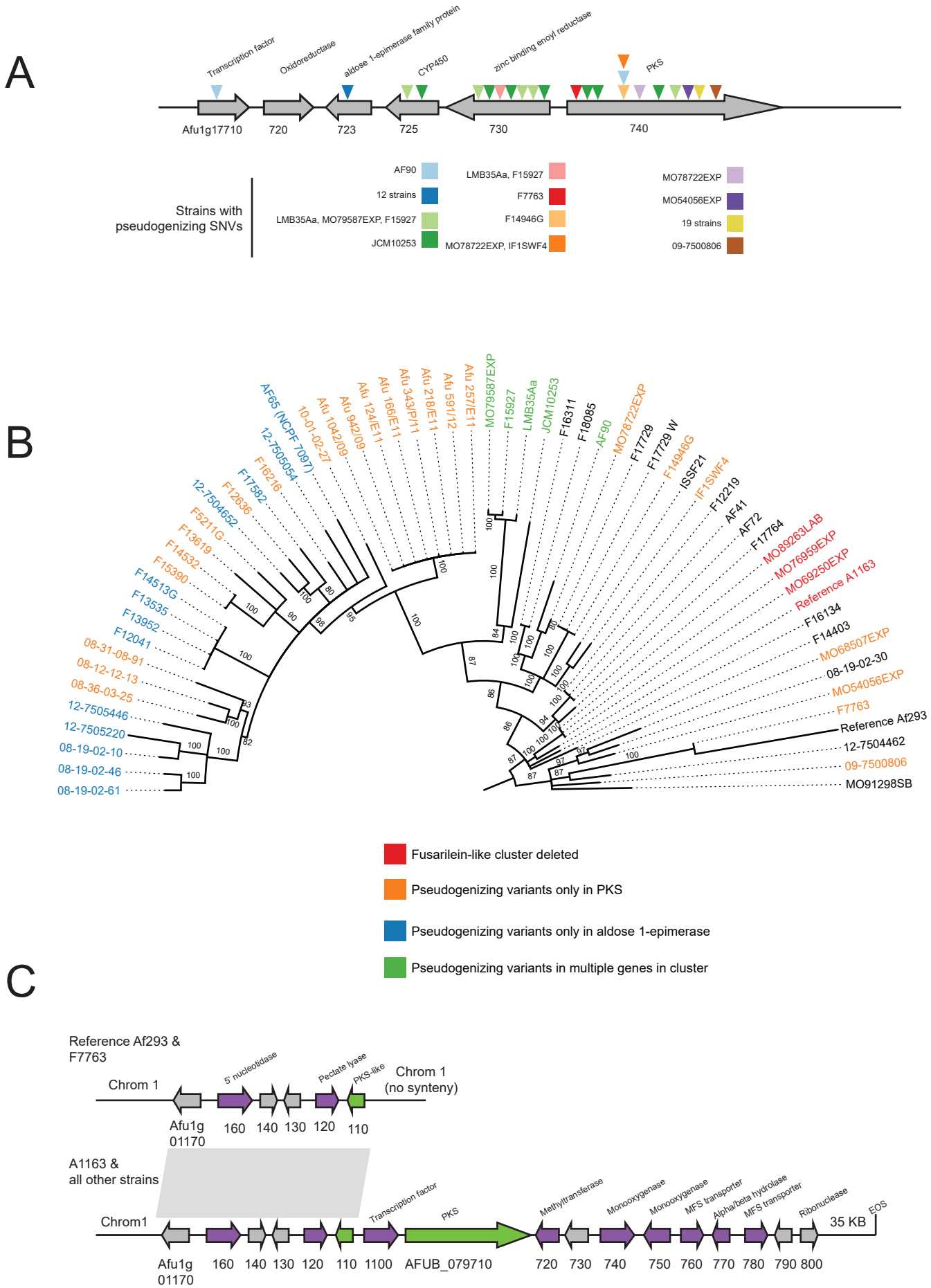


Figure 3. Pseudogenization and gene loss in SM gene clusters. (A) Positions of frameshift variants and nonsense variants in the fusarielin-like SM gene cluster 4. (B) Strains lacking the fusarielin-like cluster or containing frameshift and nonsense variants in the cluster. (C) SM gene cluster found in most *A. fumigatus* strains but absent from the Af293 reference and from the F7763 strain. EOS denotes end of scaffold.

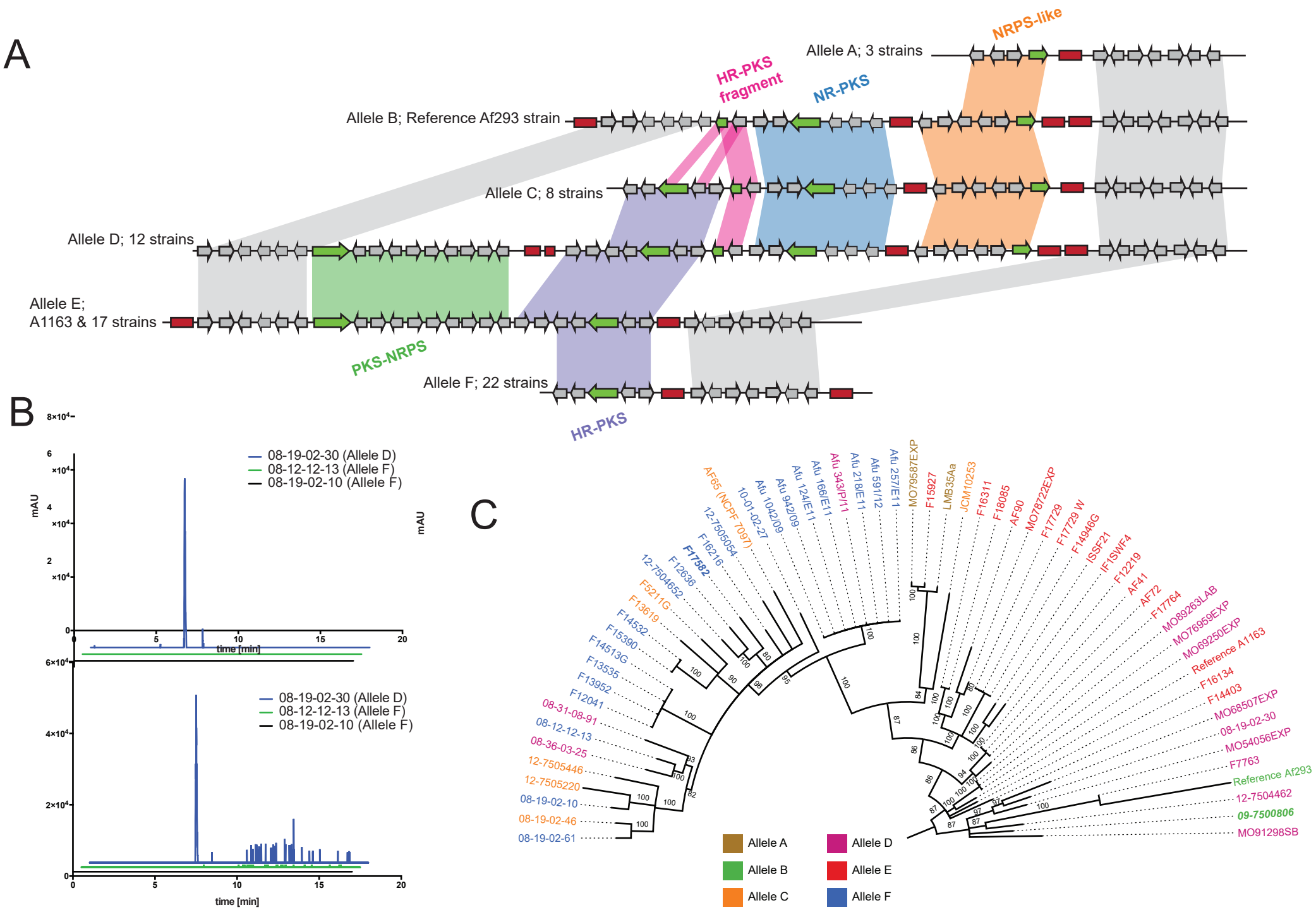


Figure 4. Six alleles of an idiomorphic SM gene cluster. (A) Alleles of SM gene cluster 10 on chromosome 3. Red boxes denote transposable elements. Green arrows denote backbone genes (PKS or NRPS). (B) Extracted ion chromatograms for the two m/z identified in negative mode from XCMS analysis comparing extracts from strains with Alleles D and F. (C) Distribution of the six cluster alleles across a SNP-based phylogeny of strains. Bolded and italicized strains have unique alleles but appear derived from another allele (see also Figure S5).

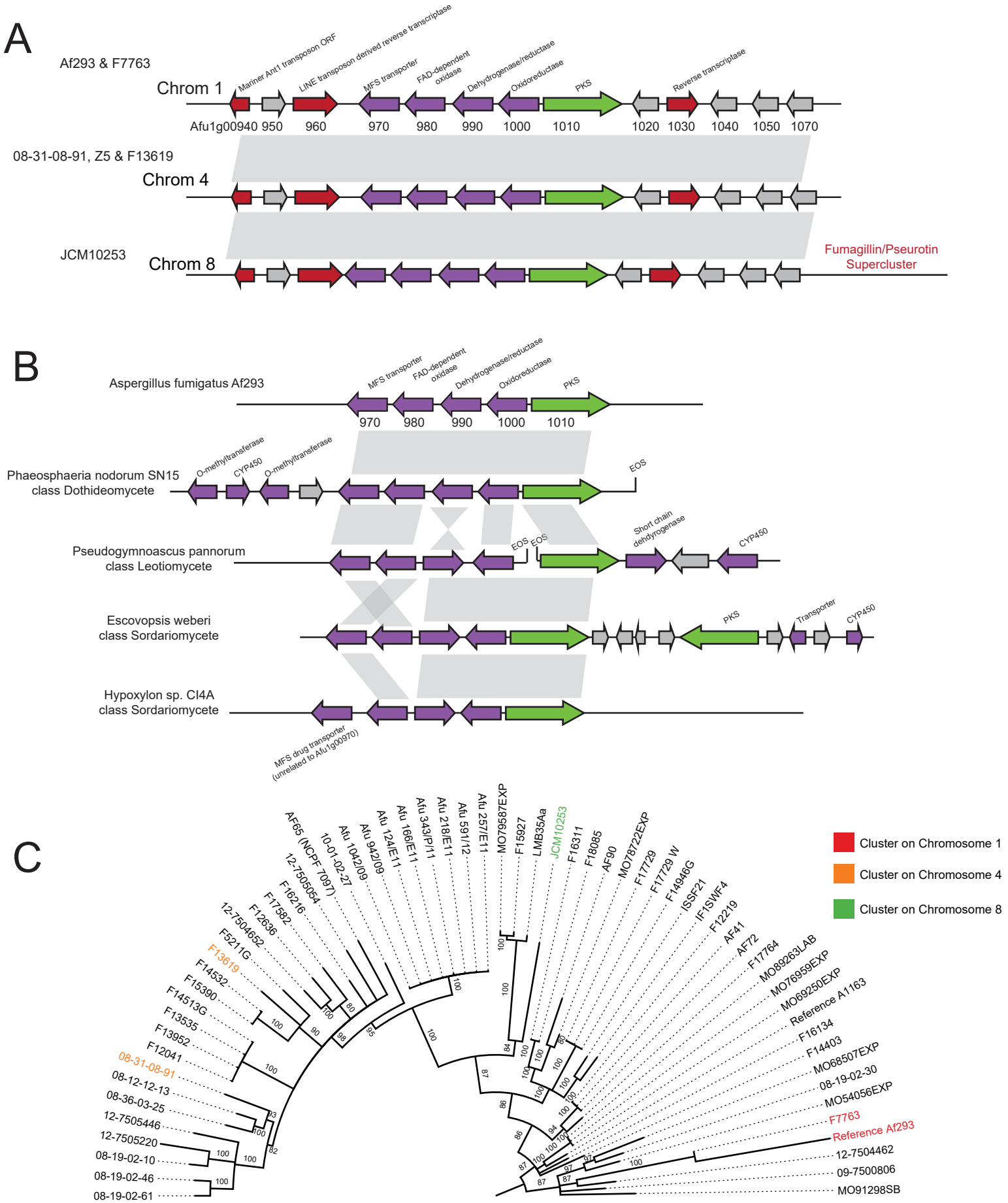


Figure 5. Multiple genomic locations of a horizontally transferred SM gene cluster. (A) SM gene cluster 1 (Afu1g00970-01010) and flanking region is found in different genomic locations.The flanking regions contain transposon-derived open reading frames including two putative reverse transcriptases. (B) Synteny of A. fumigatus SM gene cluster 1 with clusters in *Phaeosphaeria nodorum*, *Pseudogymnoascus pannorum*, *Escovopsis weberi*, and *Hypoxylon sp. CI4A.* EOS denotes end of scaffold. All species contain non-syntenic genes predicted by antiSMASH to be part of a biosynthetic gene cluster. (C) Strains containing SM gene cluster 1 in SNP-based phylogeny.

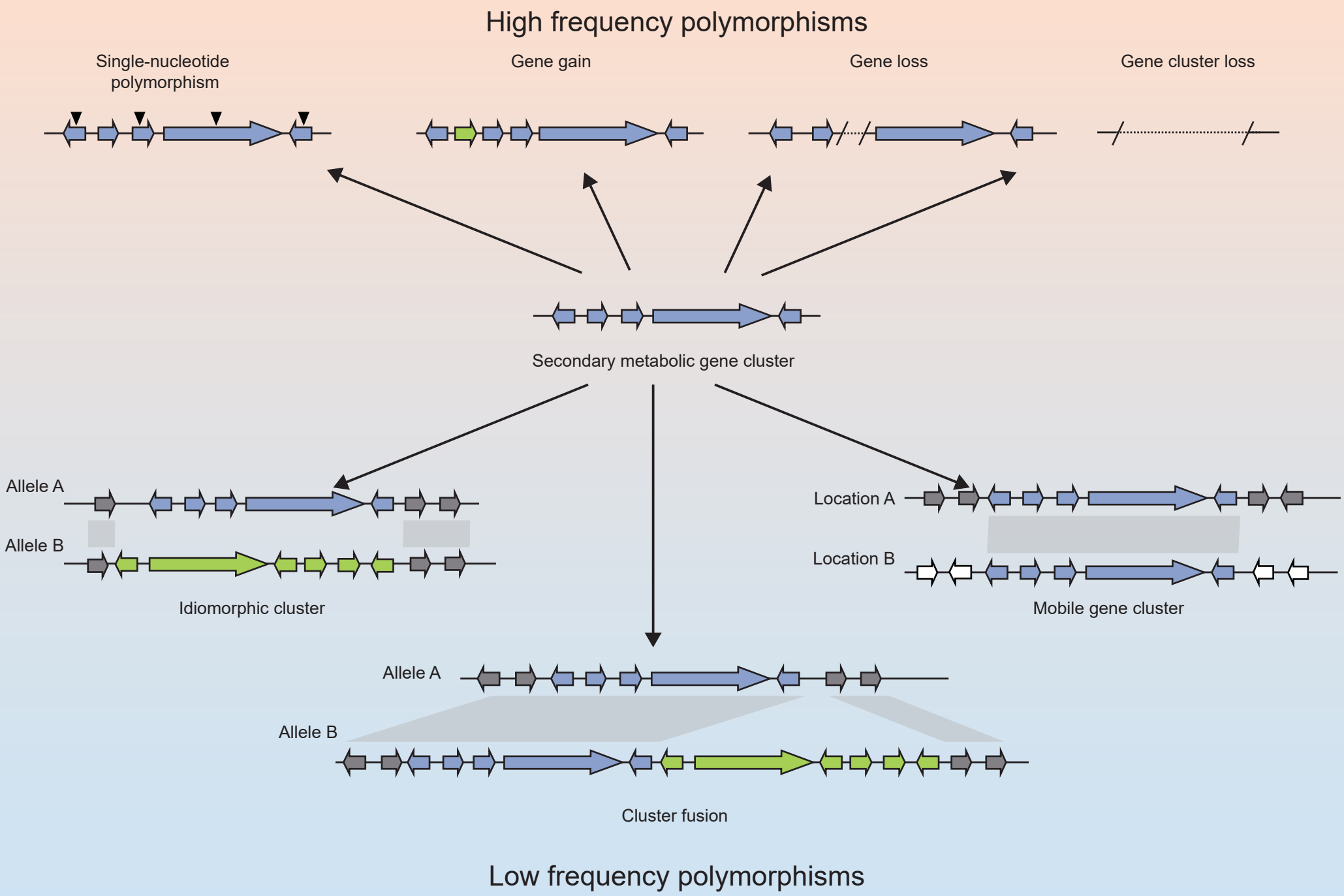


Figure 6. Types and frequencies of all SM gene cluster variants in the *A. fumigatus* population.

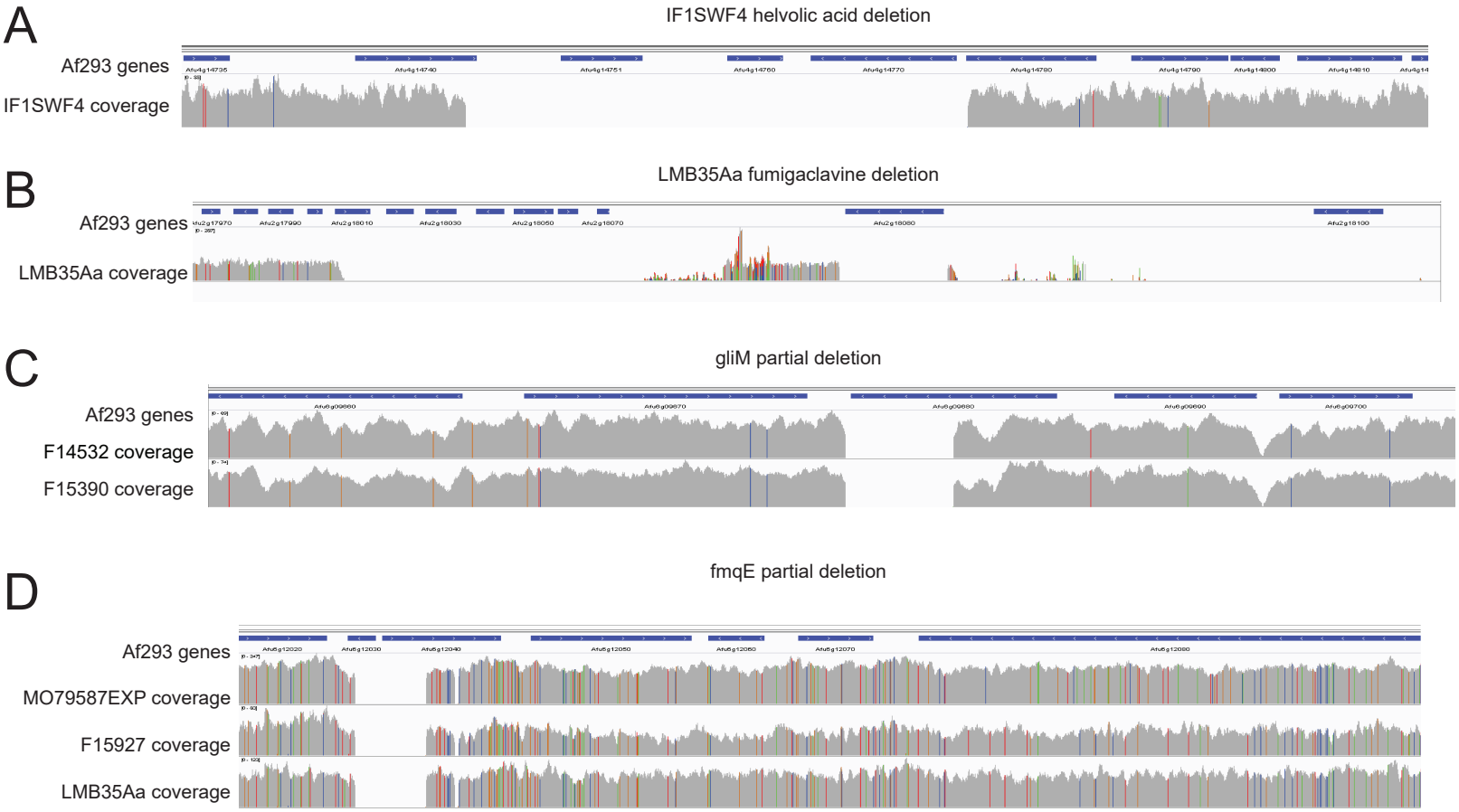


Figure S1. Alignments showing deletion of genes in SM gene clusters. A) Deletion of helvolic acid genes in IF1SWF4. B) Deletion of fumigaclavine genes in LMB35Aa. C) Partial deletion of gliM in the gliotoxin gene cluster in two strains. D) Partial deletion of fmqE in the fumiquinazoline gene cluster in 3 strains.

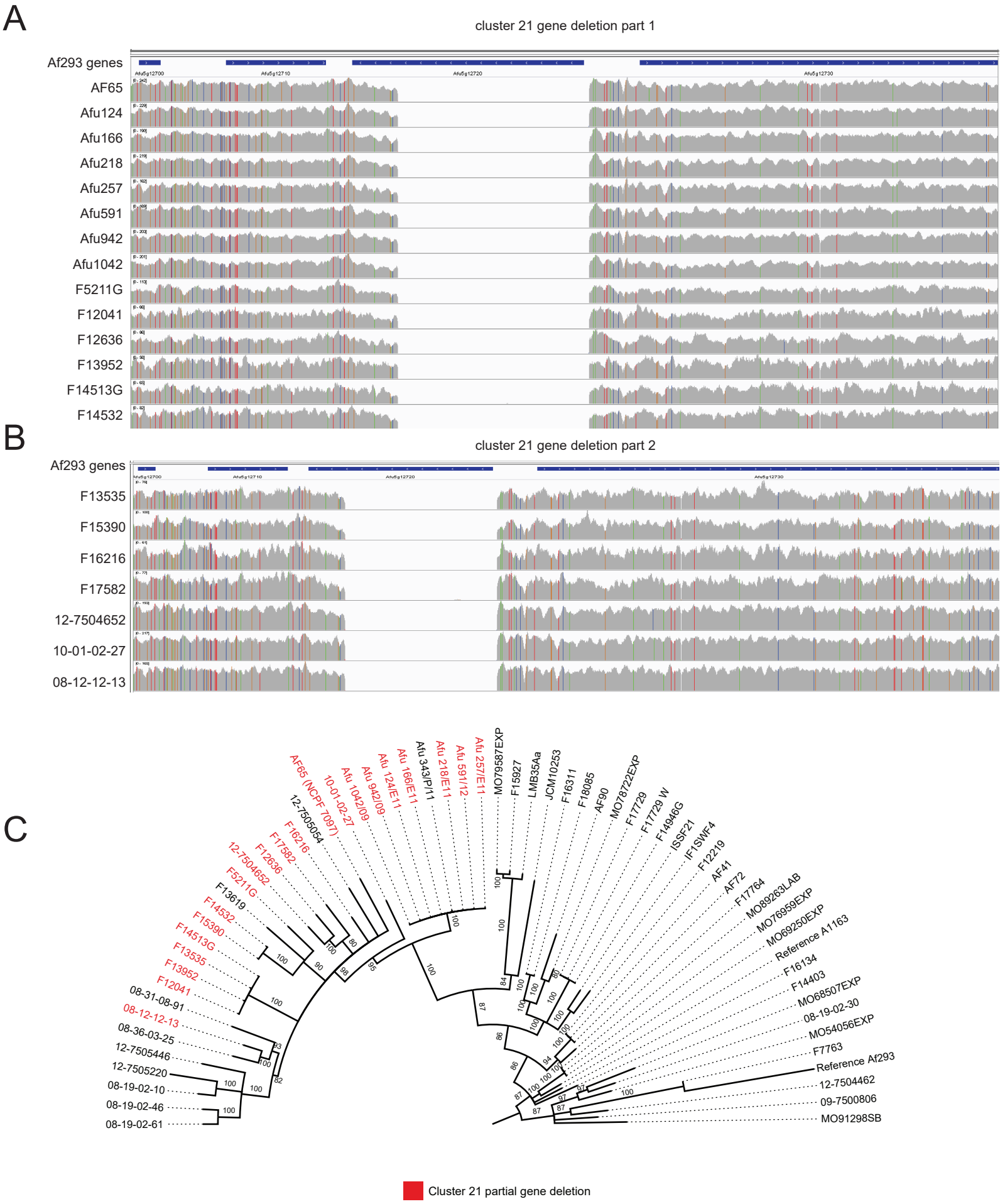
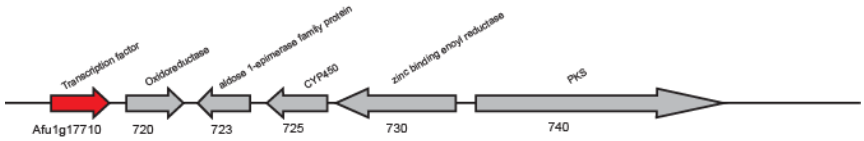


Figure S1. Partial deletion of ABC transporter gene in SM gene cluster 21. (A,B) Coverage of 21 strains with partial deletion of SM gene cluster 21. (C) Position of strains with partial gene deletion in SNP based phylogeny.

Figure S2. Gene phylogenies of the fusarielin-like SM gene cluster 4. These phylogenies are consistent with horizontal transfer between Eurotiomycete and Sordariomycete fungi or with extensive gene loss.

S2A



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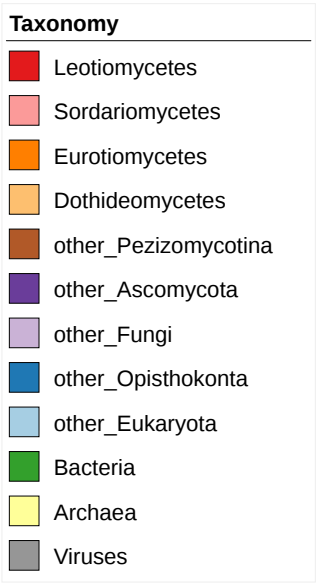
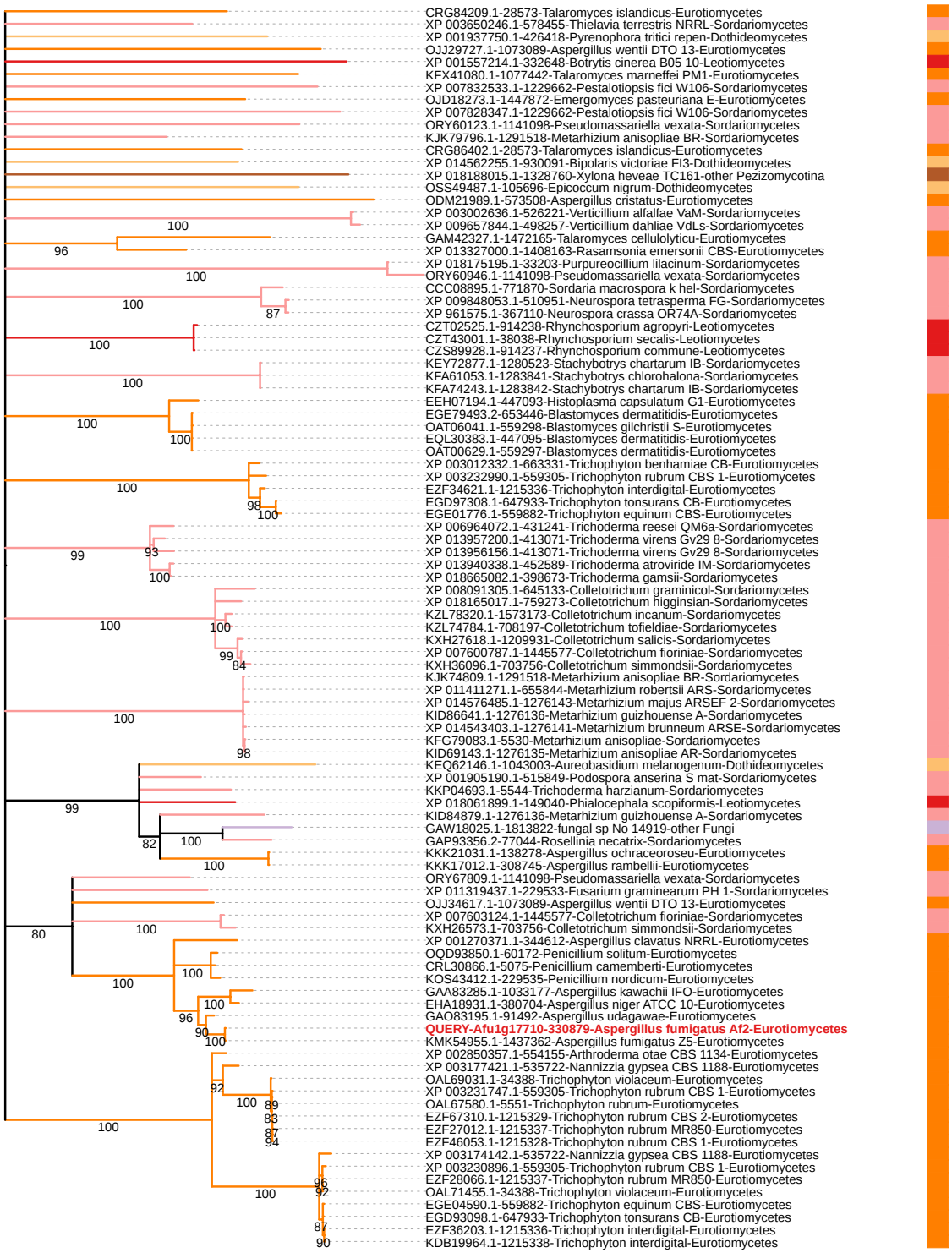
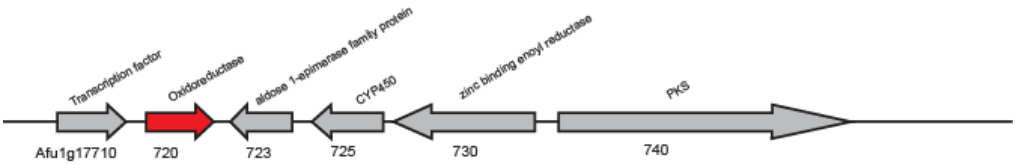




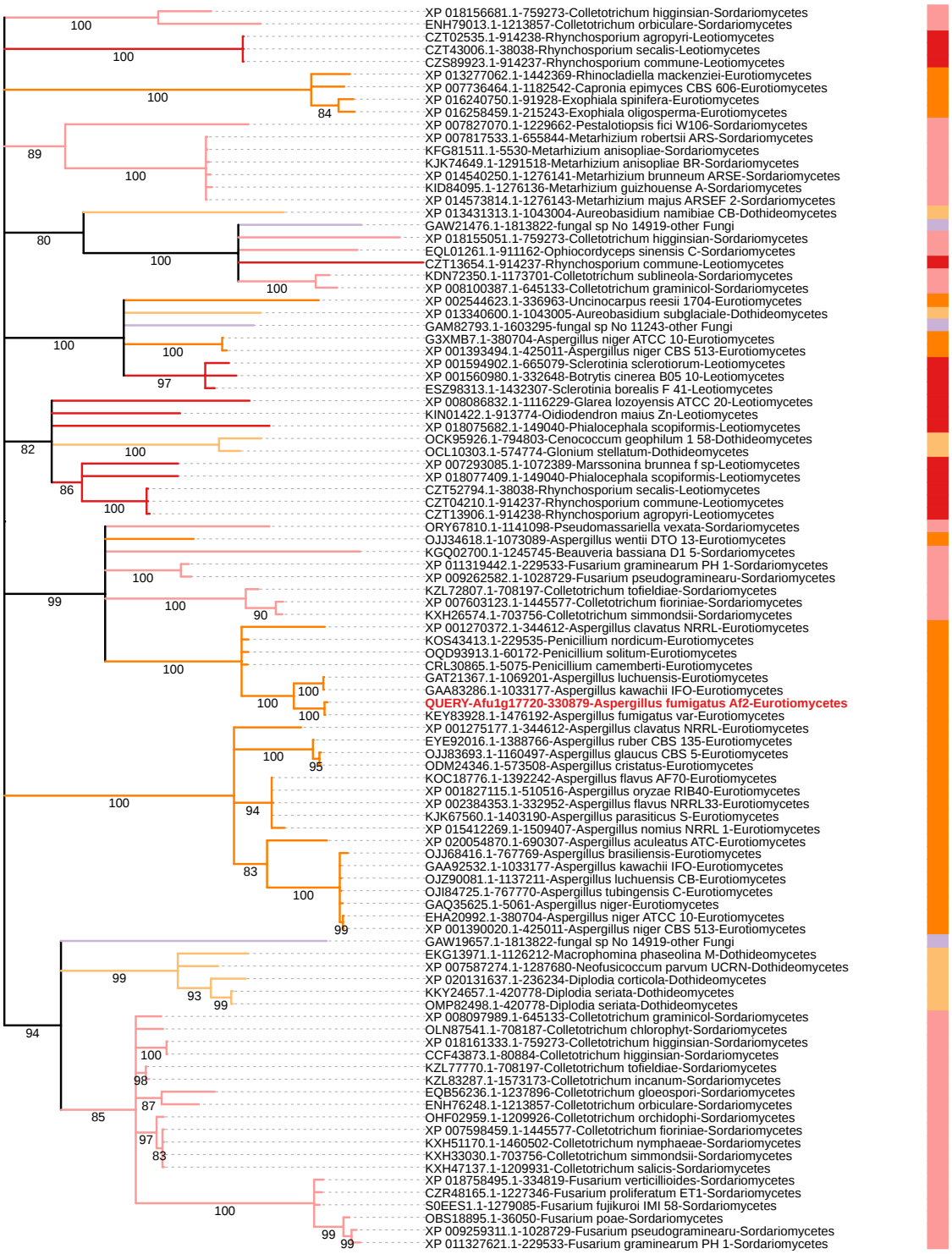
S2B



Tree scale: 0.1

Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses



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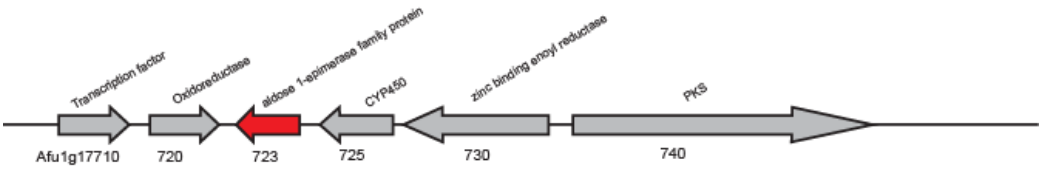

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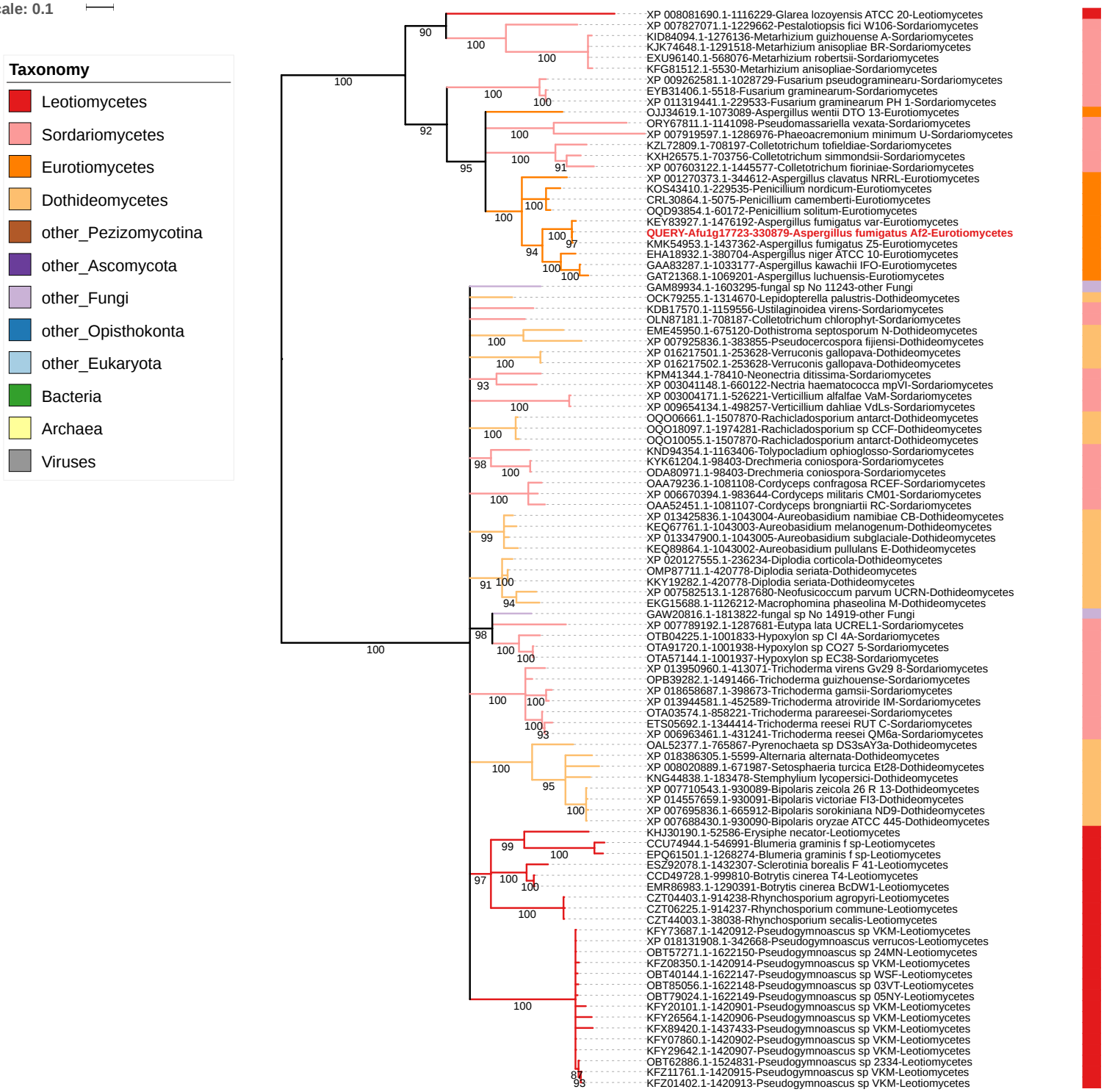
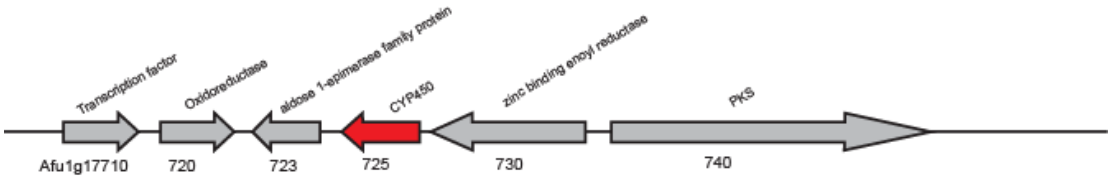


S2D



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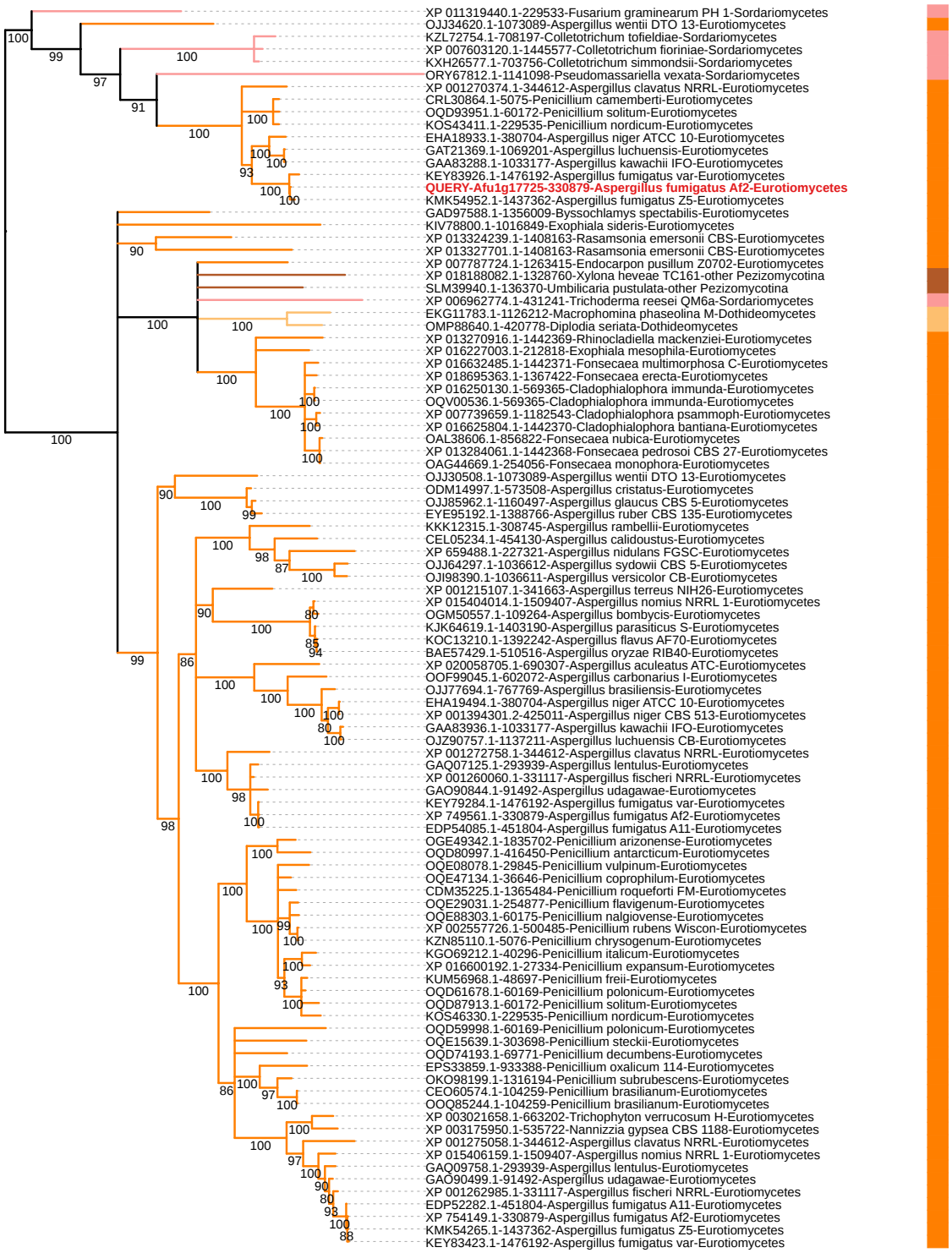

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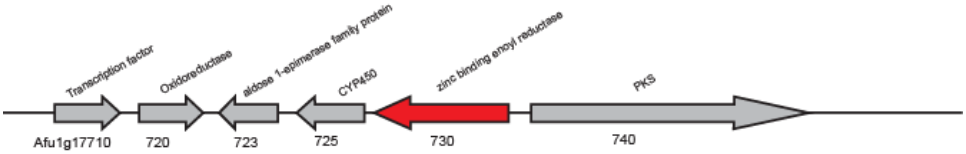
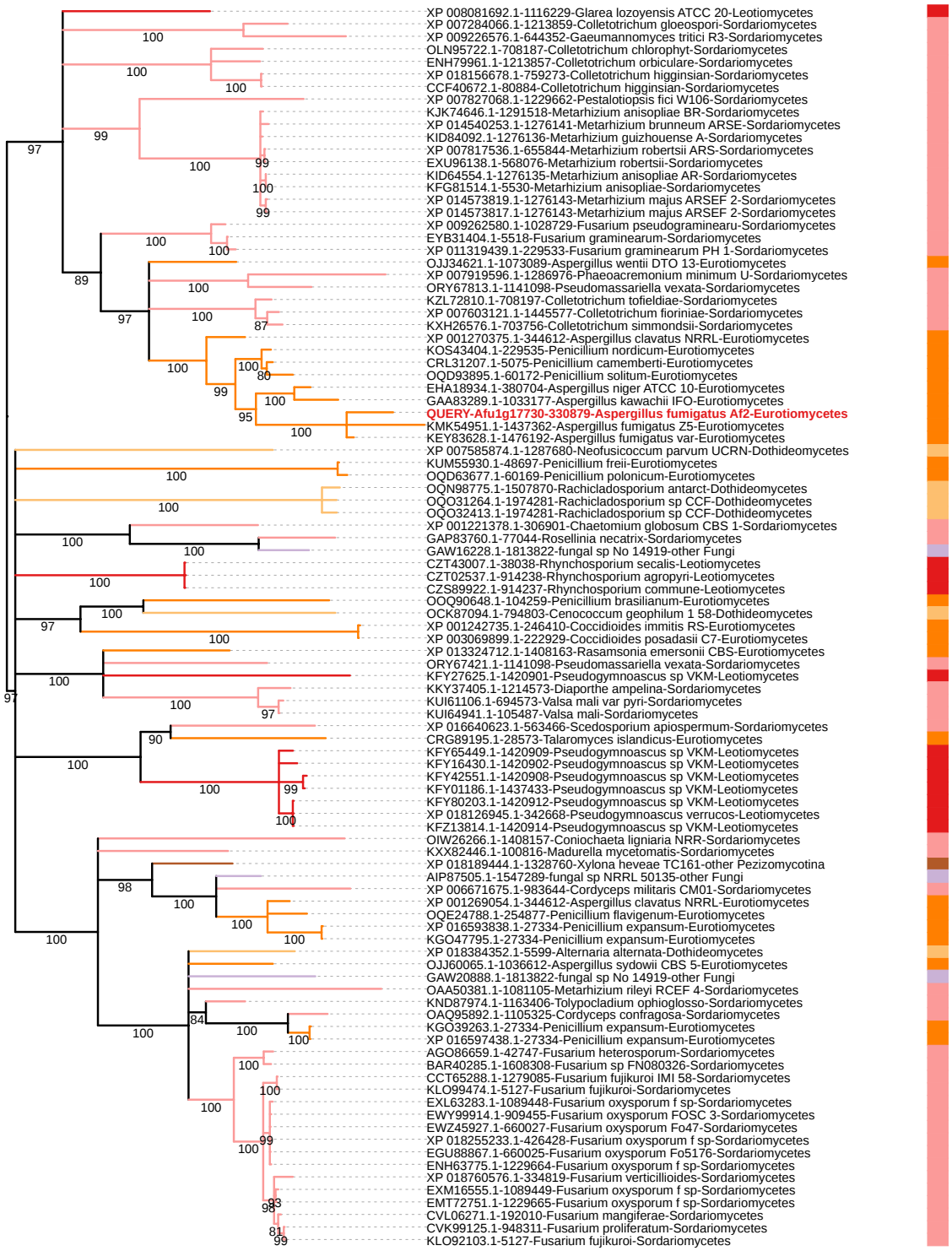


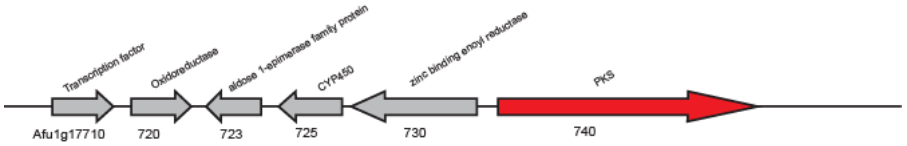
Tree scale: 0.1

Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses



S2F



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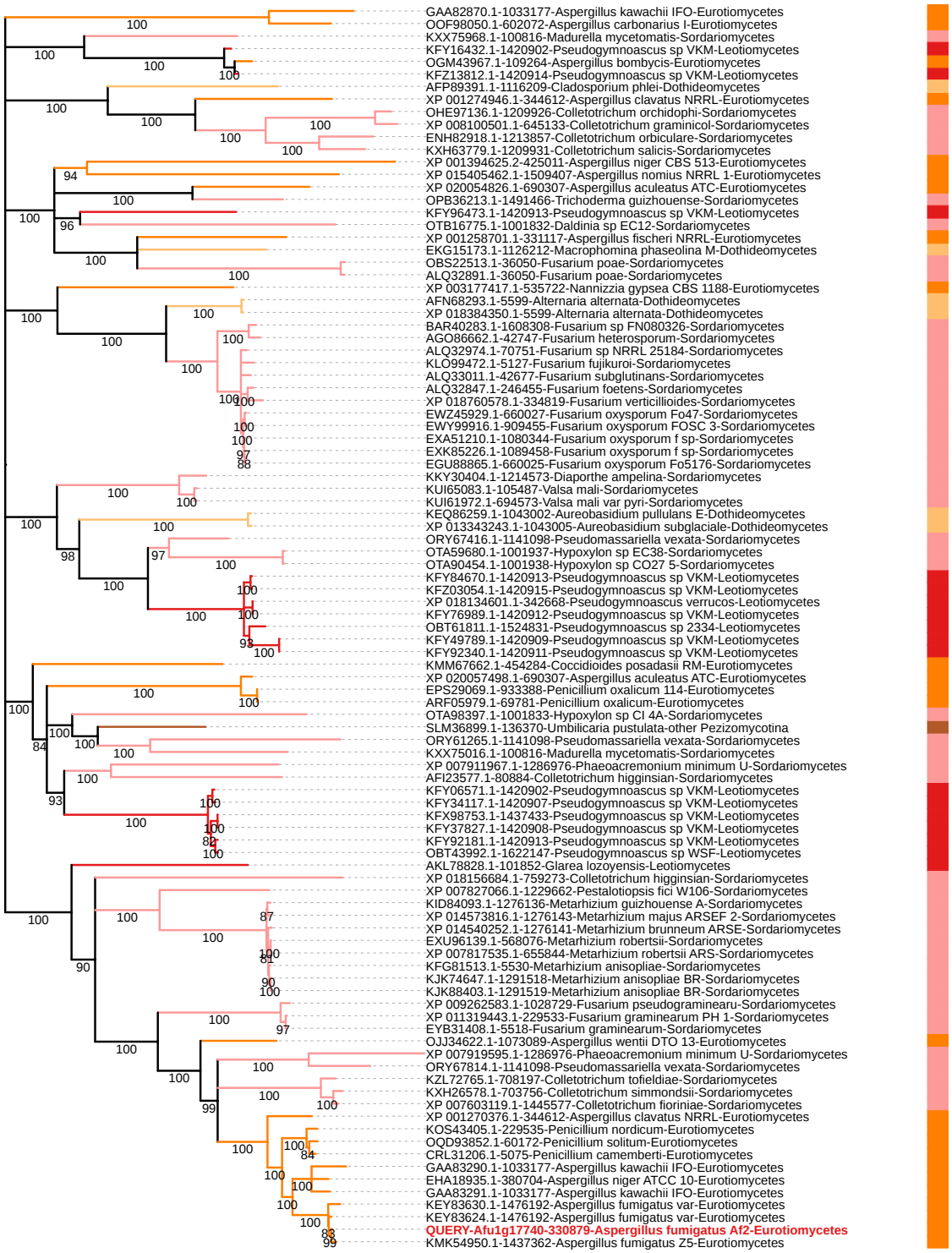

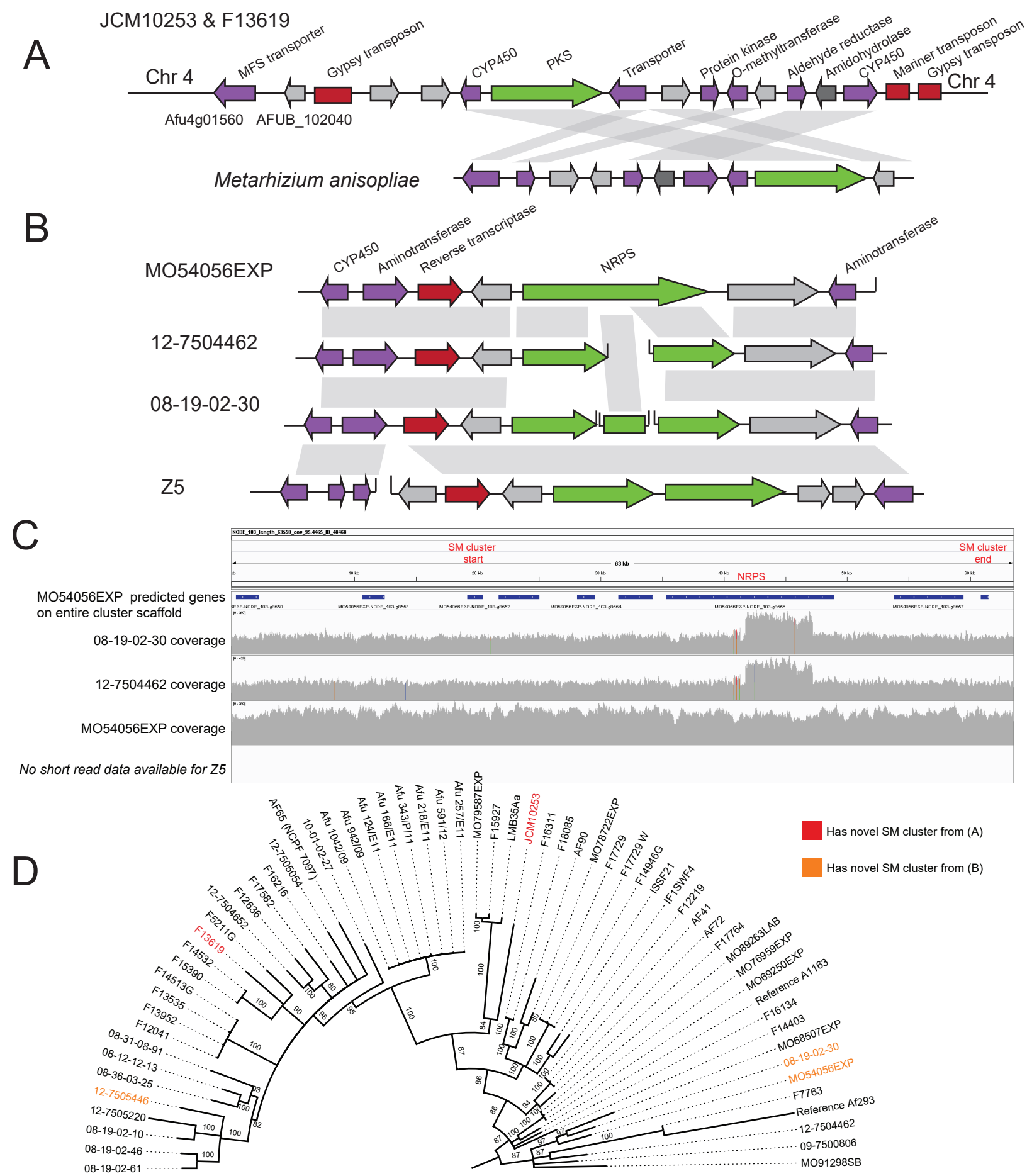

Figure S3. Novel SM gene clusters in A. fumigatus strains. (A) Synteny between a novel PKS containing cluster in two strains with an SM gene cluster in *Metarhizium anisopliae*. This novel PKS cluster is located between transposable elements in a region syntenic with the reference Af293 chromosome 4. (B) Novel SM gene cluster in MO54056EXP and 3 additional strains. This cluster is only located on one scaffold in MO540556EXP and is fragmented across the other strains (ends of scaffolds are marked). (C) Coverage data from short read alignments for MO54056EXP, 12-7504462, and 08-19-02-30 relative to the MO54056EXP scaffold containing the novel SM gene cluster.(D) Strains with novel clusters on SNP phylogeny.

Figure S4. Gene phylogenies of SM gene cluster 24. The phylogenies of several genes in this cluster are consistent with horizontal transfer between *Aspergillus fumigatus* and *Metarhizium* fungi.

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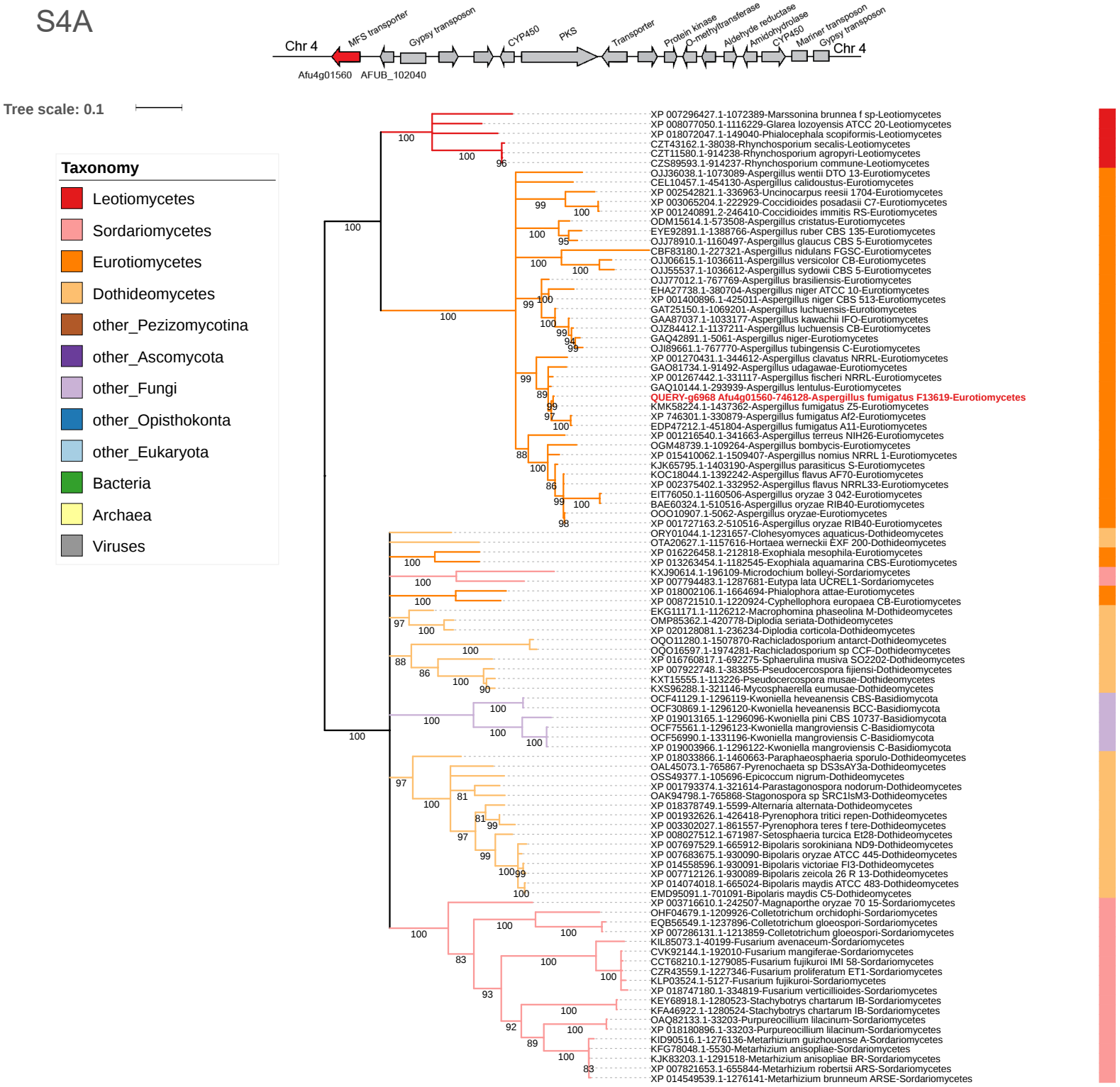

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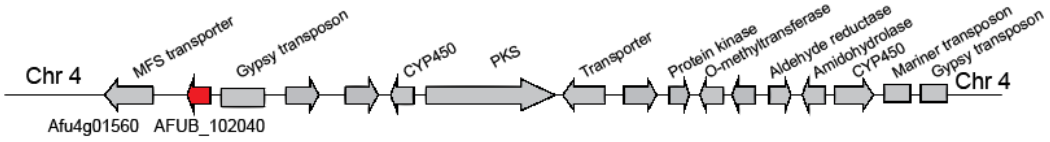

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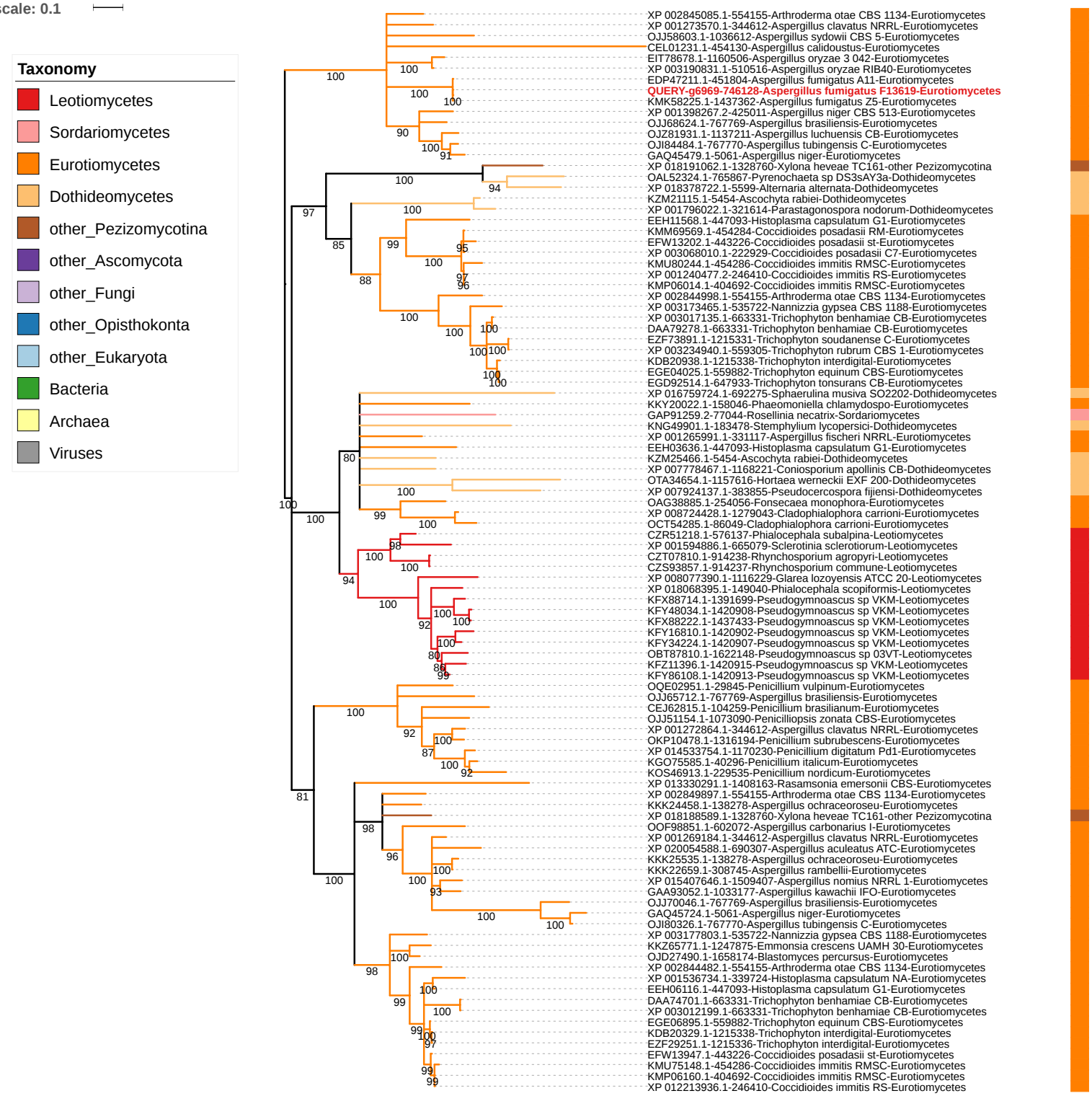

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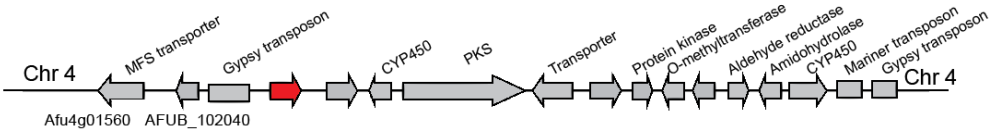
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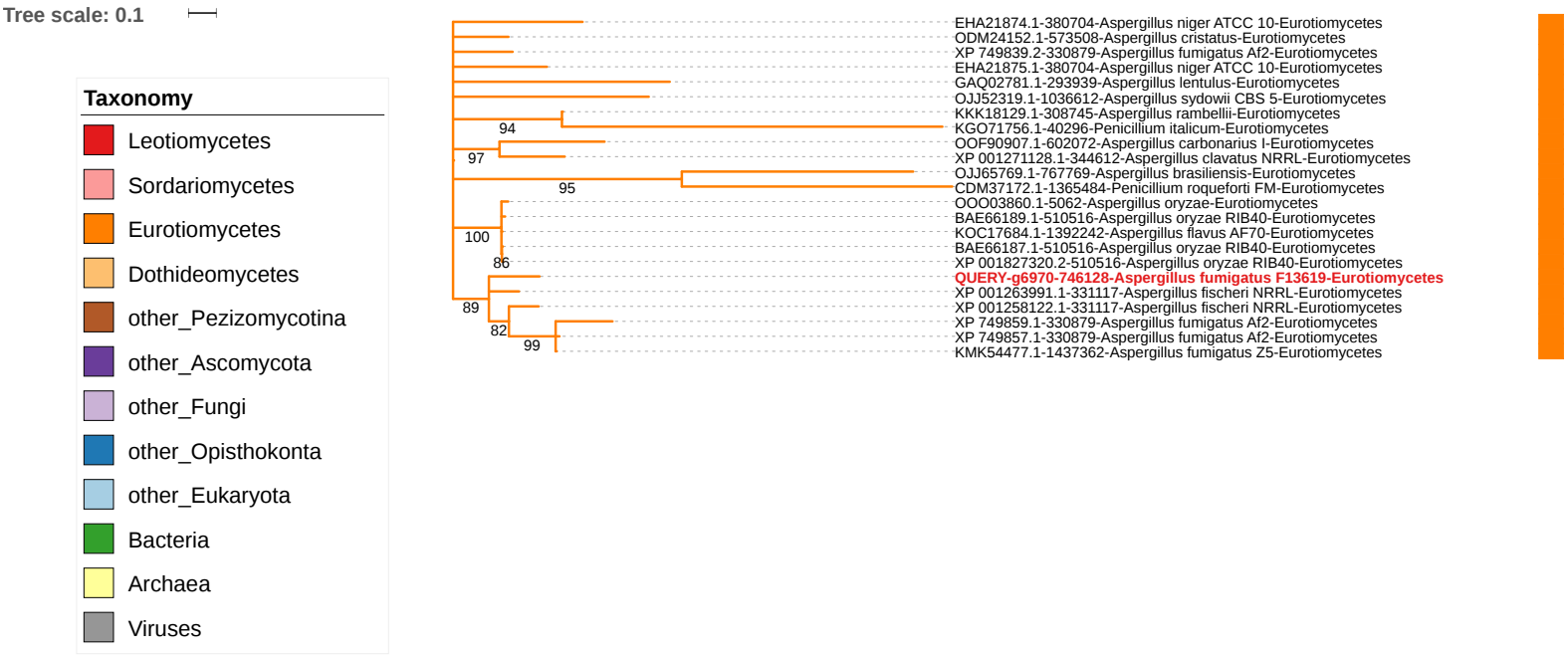
Gypsy transposon
CYP450
PKS
Transporter
Protein kinase
O-methyltransferase
Aldehyde reductase
Amidohydrolase
CYP450
Mariner transposon
Gypsy transposon
Chr 4
Afu4g01560
AFUB_102040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Sordariomycetes
Eurotiomycetes
Dothideomycetes
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other_Ascomycota
other_Fungi
other_Opisthokonta
other_Eukaryota
Bacteria
Archaea
Viruses
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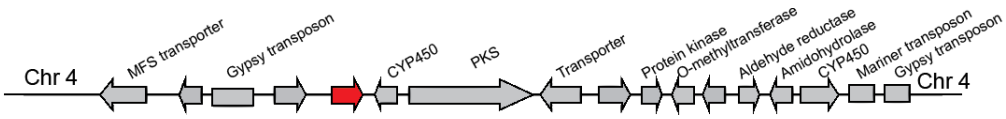
94
97
95
100
86
89
82
99

S4D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Gypsy transposon
CYP450
PKS
Transporter
Protein kinase
O-methyltransferase
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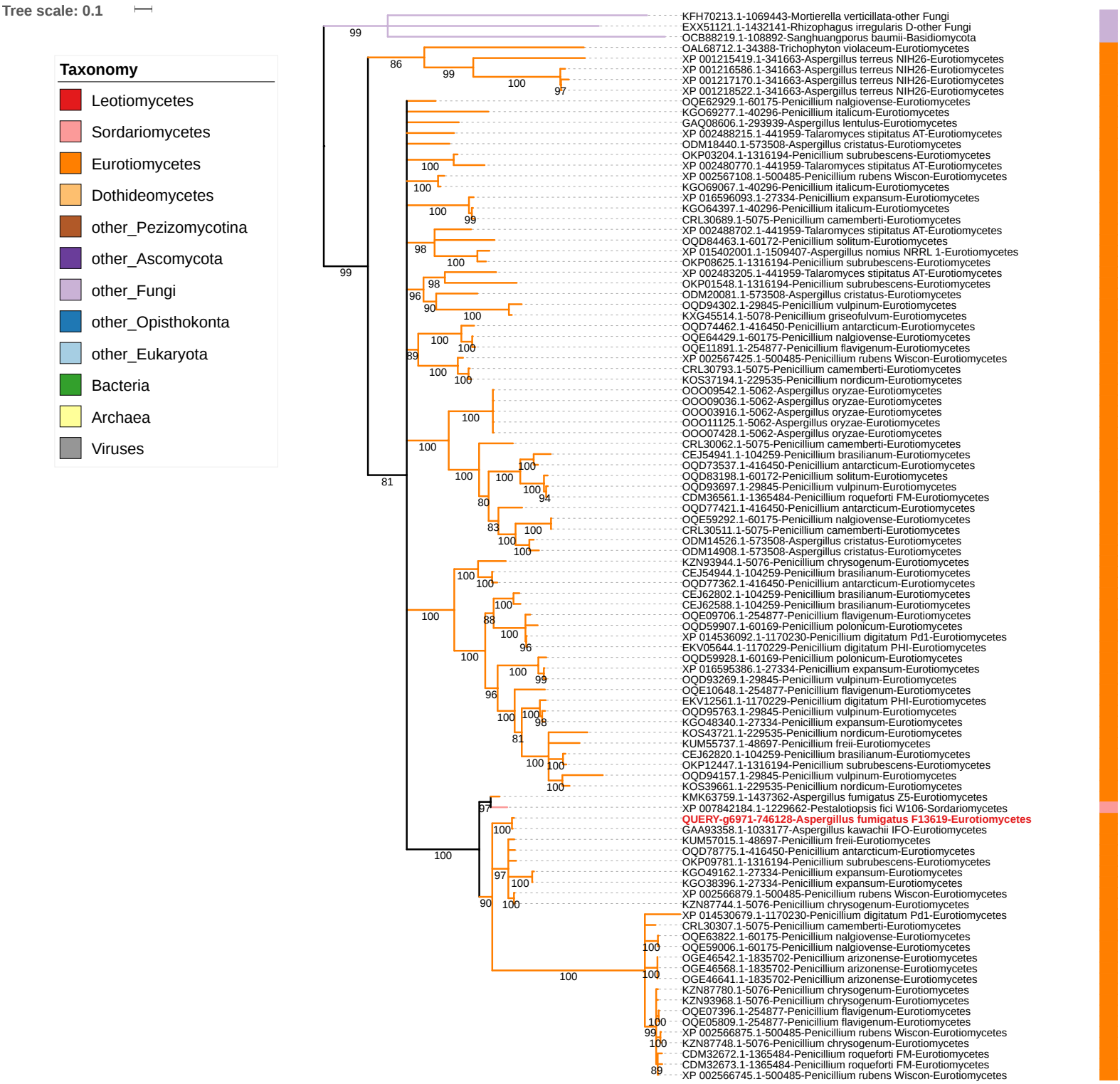
Gypsy transposon
Chr 4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Sordariomycetes
Eurotiomycetes
Dothideomycetes
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other_Fungi
other_Opisthokonta
other_Eukaryota
Bacteria
Archaea
Viruses
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OQE11891.1-254877-Penicillium flavigenum-Eurotiomycetes
XP 002567425.1-500485-Penicillium rubens Wiscon-Eurotiomycetes
CRL30793.1-5075-Penicillium camemberti-Eurotiomycetes
KOS37194.1-229535-Penicillium nordicum-Eurotiomycetes
OOO09542.1-5062-Aspergillus oryzae-Eurotiomycetes
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OQD83198.1-60172-Penicillium solitum-Eurotiomycetes
OQD93697.1-29845-Penicillium vulpinum-Eurotiomycetes
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GAA93358.1-1033177-Aspergillus kawachii IFO-Eurotiomycetes
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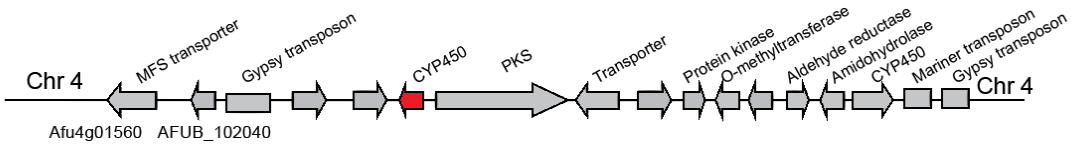
S4E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CYP450
PKS
Transporter
Protein kinase
O-methyltransferase
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Amidohydrolase
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Mariner transposon
Gypsy transposon
Chr 4
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Tree scale: 0.1
Taxonomy
Leotiomycetes
Sordariomycetes
Eurotiomycetes
Dothideomycetes
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other_Ascomycota
other_Fungi
other_Opisthokonta
other_Eukaryota
Bacteria
Archaea
Viruses
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OCW41141.1-158607-Diaporthe helianthi-Sordariomycetes
XP 016643173.1-563466-Scedosporium apiospermum-Sordariomycetes
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XP 008027816.1-671987-Setosphaeria turcica Et28-Dothideomycetes
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OCK73526.1-1314670-Lepidopterella palustris-Dothideomycetes
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KZZ96510.1-1081109-Aschersonia aleyrodis RCE-Sordariomycetes
KDN69202.1-1173701-Colletotrichum sublineola-Sordariomycetes
XP 008098964.1-645133-Colletotrichum graminicol-Sordariomycetes
XP 007286629.1-1213859-Colletotrichum gloeospori-Sordariomycetes
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KXH47916.1-1209931-Colletotrichum salicis-Sordariomycetes
OCW42213.1-158607-Diaporthe helianthi-Sordariomycetes
KXH50180.1-703756-Colletotrichum simmondsii-Sordariomycetes
OIW33137.1-1408157-Coniochaeta ligniaria NRR-Sordariomycetes
KIM94742.1-913774-Oidiodendron maius Zn-Leotiomycetes
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XP 001260852.1-331117-Aspergillus fischeri NRRL-Eurotiomycetes
OJJ40738.1-1073089-Aspergillus wentii DTO 13-Eurotiomycetes
XP 001271874.1-344612-Aspergillus clavatus NRRL-Eurotiomycetes
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XP 751828.1-330879-Aspergillus fumigatus Af2-Eurotiomycetes
EME39031.1-675120-Dothistroma septosporum N-Dothideomycetes
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XP 681087.1-227321-Aspergillus nidulans FGSC-Eurotiomycetes
KKK21817.1-308745-Aspergillus rambellii-Eurotiomycetes
XP 020053364.1-690307-Aspergillus aculeatus ATC-Eurotiomycetes
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OAG34162.1-254056-Fonsecaea monophora-Eurotiomycetes
XP 013280246.1-1442368-Fonsecaea pedrosoi CBS 27-Eurotiomycetes
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XP 018159597.1-759273-Colletotrichum higginsian-Sordariomycetes
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KZL63447.1-708197-Colletotrichum tofieldiae-Sordariomycetes
XP 008098516.1-645133-Colletotrichum graminicol-Sordariomycetes
XP 003717434.1-242507-Magnaporthe oryzae 70 15-Sordariomycetes
OOQ86043.1-104259-Penicillium brasilianum-Eurotiomycetes
OCK77653.1-1314670-Lepidopterella palustris-Dothideomycetes
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OQD73863.1-69771-Penicillium decumbens-Eurotiomycetes
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OBT43052.1-1622147-Pseudogymnoascus sp WSF-Leotiomycetes
XP 012740108.1-658429-Pseudogymnoascus destruct-Leotiomycetes
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KFY92053.1-1420913-Pseudogymnoascus sp VKM-Leotiomycetes
KFZ12582.1-1420915-Pseudogymnoascus sp VKM-Leotiomycetes
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EIT78191.1-1160506-Aspergillus oryzae 3 042-Eurotiomycetes
BAE65144.1-510516-Aspergillus oryzae RIB40-Eurotiomycetes
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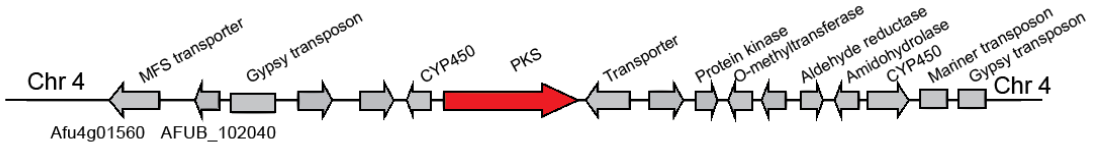
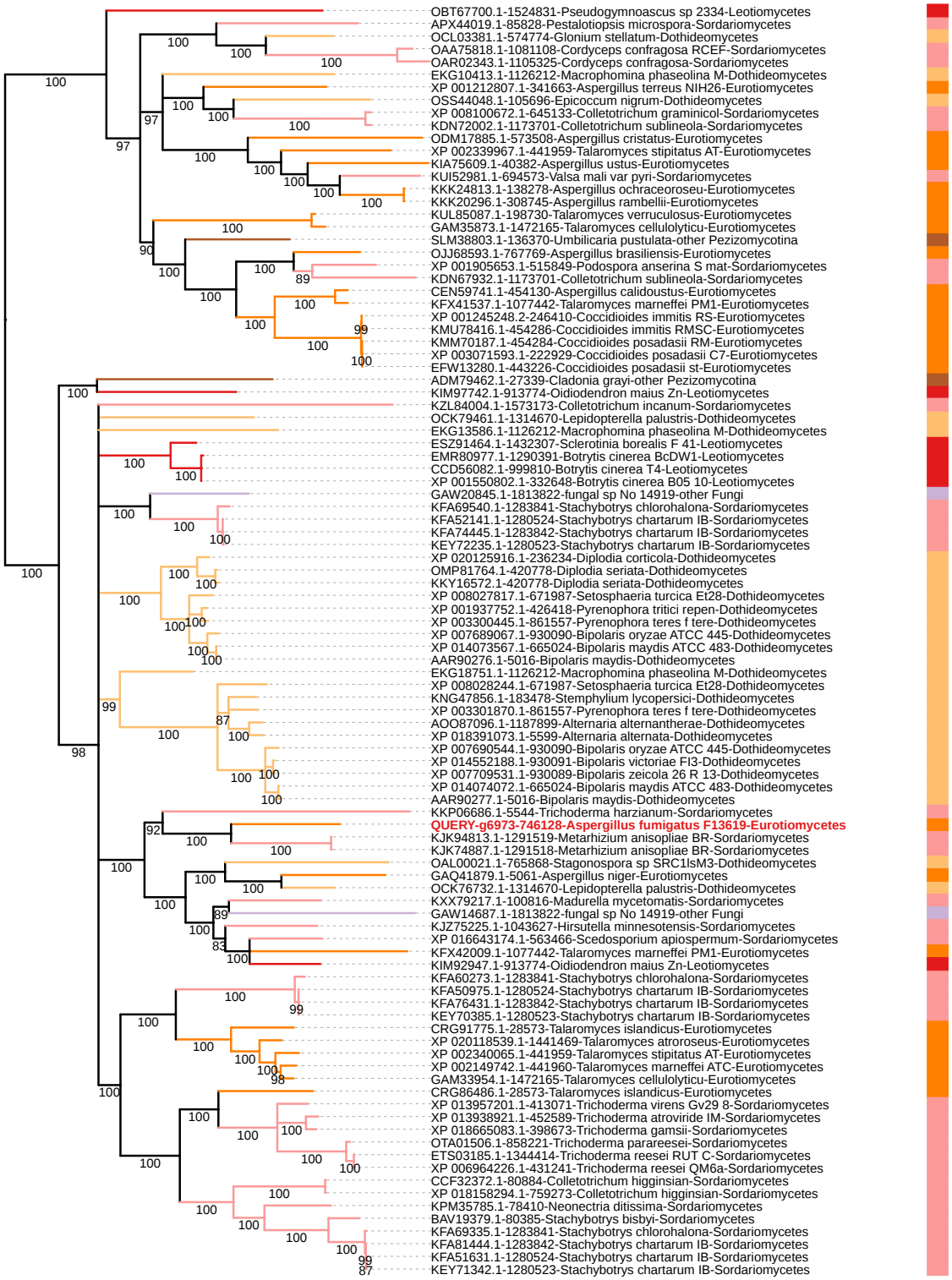
S4F



Tree scale: 0.1

Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses



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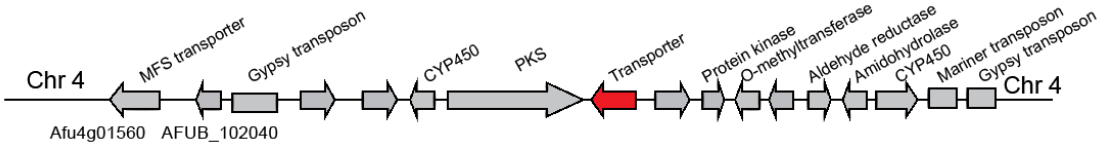
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MFS transporter
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CYP450
PKS
Transporter
Protein kinase
O-methyltransferase
Aldehyde reductase
Amidohydrolase
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Mariner transposon
Gypsy transposon
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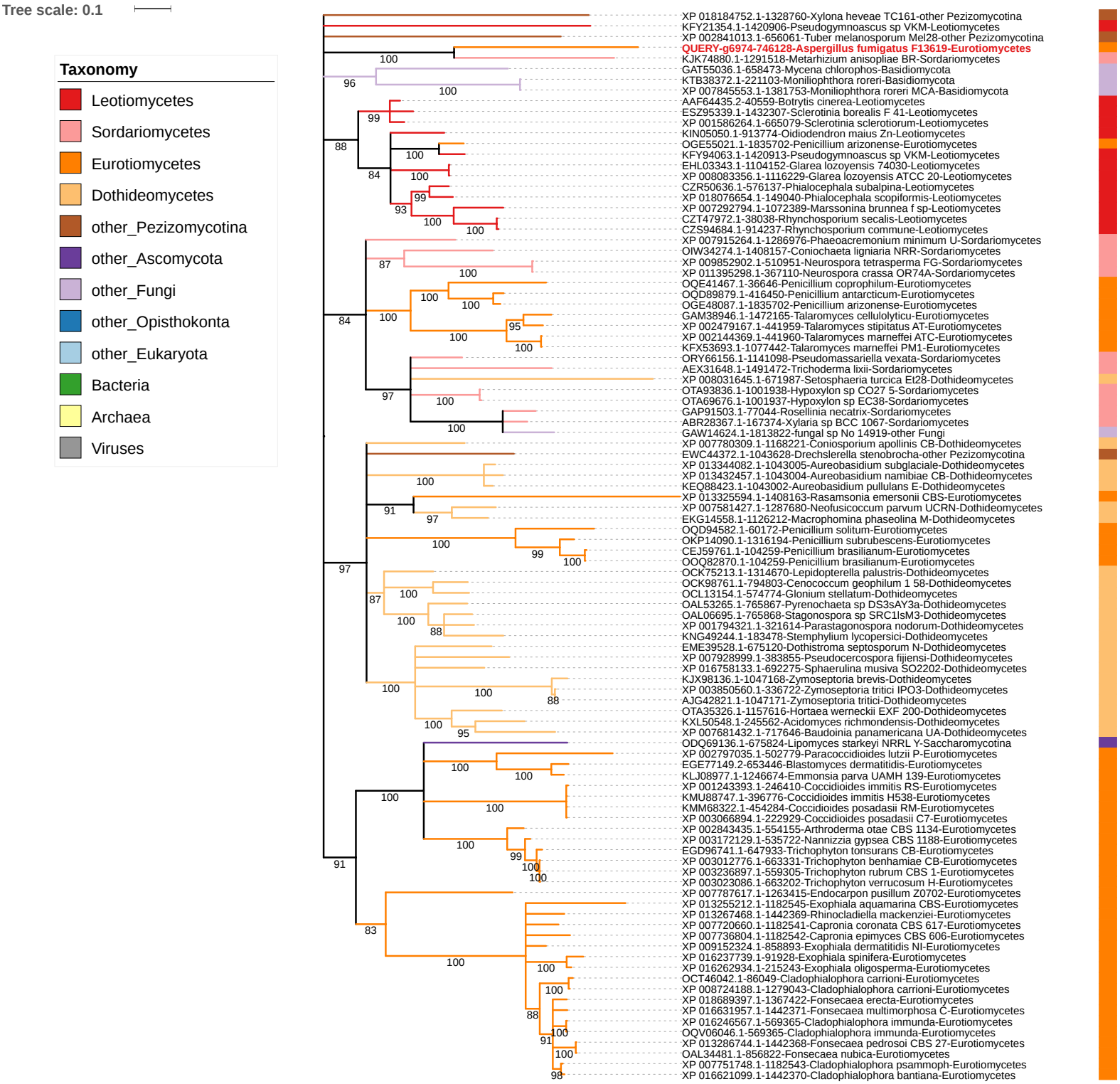
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Taxonomy
Leotiomycetes
Sordariomycetes
Eurotiomycetes
Dothideomycetes
other_Pezizomycotina
other_Ascomycota
other_Fungi
other_Opisthokonta
other_Eukaryota
Bacteria
Archaea
Viruses
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KFY21354.1-1420906-Pseudogymnoascus sp VKM-Leotiomycetes
XP 002841013.1-656061-Tuber melanosporum Mel28-other Pezizomycotina
QUERY-g6974-746128-Aspergillus fumigatus F13619-Eurotiomycetes
KJK74880.1-1291518-Metarhizium anisopliae BR-Sordariomycetes
GAT55036.1-658473-Mycena chlorophos-Basidiomycota
KTB38372.1-221103-Moniliophthora roreri-Basidiomycota
XP 007845553.1-1381753-Moniliophthora roreri MCA-Basidiomycota
AAF64435.2-40559-Botrytis cinerea-Leotiomycetes
ESZ95339.1-1432307-Sclerotinia borealis F 41-Leotiomycetes
XP 001586264.1-665079-Sclerotinia sclerotiorum-Leotiomycetes
KIN05050.1-913774-Oidiodendron maius Zn-Leotiomycetes
OGE55021.1-1835702-Penicillium arizonense-Eurotiomycetes
KFY94063.1-1420913-Pseudogymnoascus sp VKM-Leotiomycetes
EHL03343.1-1104152-Glarea lozoyensis 74030-Leotiomycetes
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XP 018076654.1-149040-Phialocephala scopiformis-Leotiomycetes
XP 007292794.1-1072389-Marssonina brunnea f sp-Leotiomycetes
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OIW34274.1-1408157-Coniochaeta ligniaria NRR-Sordariomycetes
XP 009852902.1-510951-Neurospora tetrasperma FG-Sordariomycetes
XP 011395298.1-367110-Neurospora crassa OR74A-Sordariomycetes
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OQD89879.1-416450-Penicillium antarcticum-Eurotiomycetes
OGE48087.1-1835702-Penicillium arizonense-Eurotiomycetes
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XP 002144369.1-441960-Talaromyces marneffei ATC-Eurotiomycetes
KFX53693.1-1077442-Talaromyces marneffei PM1-Eurotiomycetes
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AEX31648.1-1491472-Trichoderma lixii-Sordariomycetes
XP 008031645.1-671987-Setosphaeria turcica Et28-Dothideomycetes
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XP 013432457.1-1043004-Aureobasidium namibiae CB-Dothideomycetes
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XP 016758133.1-692275-Sphaerulina musiva SO2202-Dothideomycetes
KJX98136.1-1047168-Zymoseptoria brevis-Dothideomycetes
XP 003850560.1-336722-Zymoseptoria tritici IPO3-Dothideomycetes
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OTA35326.1-1157616-Hortaea werneckii EXF 200-Dothideomycetes
KXL50548.1-245562-Acidomyces richmondensis-Dothideomycetes
XP 007681432.1-717646-Baudoinia panamericana UA-Dothideomycetes
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XP 002797035.1-502779-Paracoccidioides lutzii P-Eurotiomycetes
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XP 002843435.1-554155-Arthroderma otae CBS 1134-Eurotiomycetes
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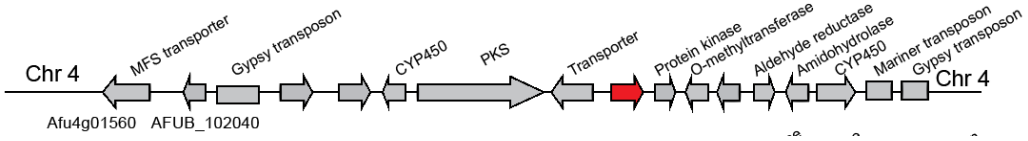

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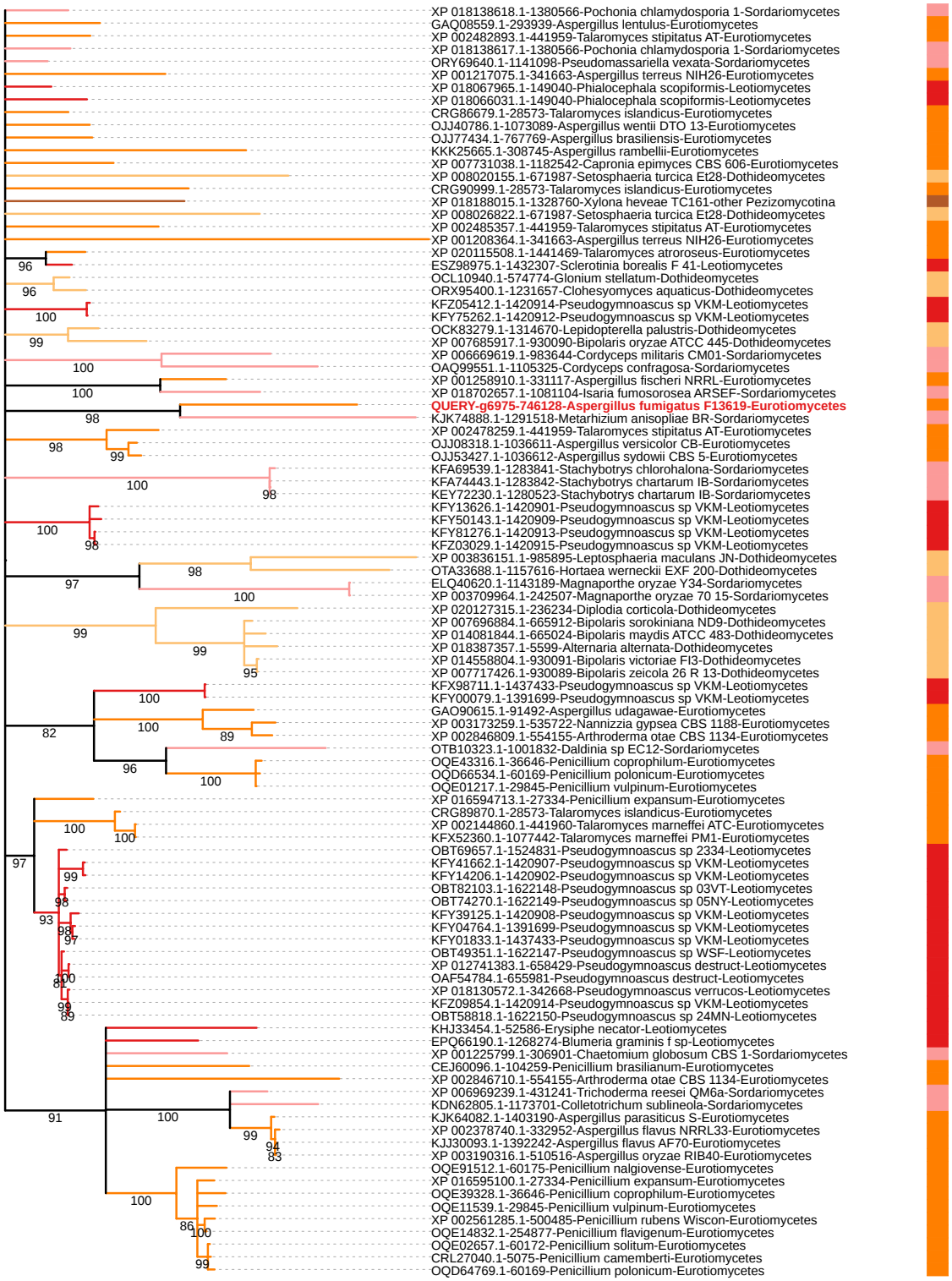
Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses



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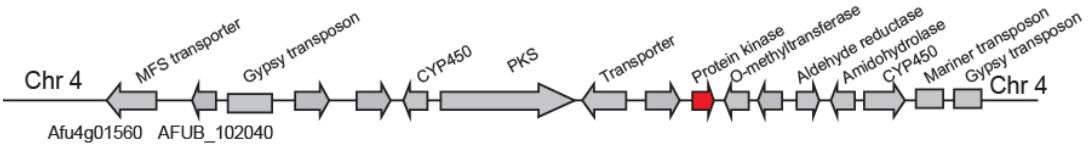
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Chr 4
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Tree scale: 0.1
Taxonomy
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Sordariomycetes
Eurotiomycetes
Dothideomycetes
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other_Fungi
other_Opisthokonta
other_Eukaryota
Bacteria
Archaea
Viruses
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XP 015699878.1-502779-Paracoccidioides lutzii P-Eurotiomycetes
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XP 002382888.1-332952-Aspergillus flavus NRRL33-Eurotiomycetes
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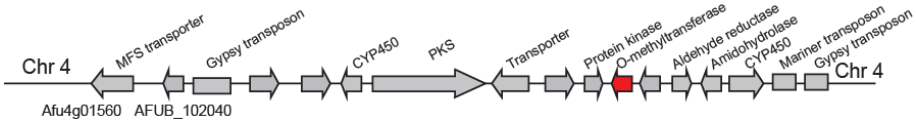
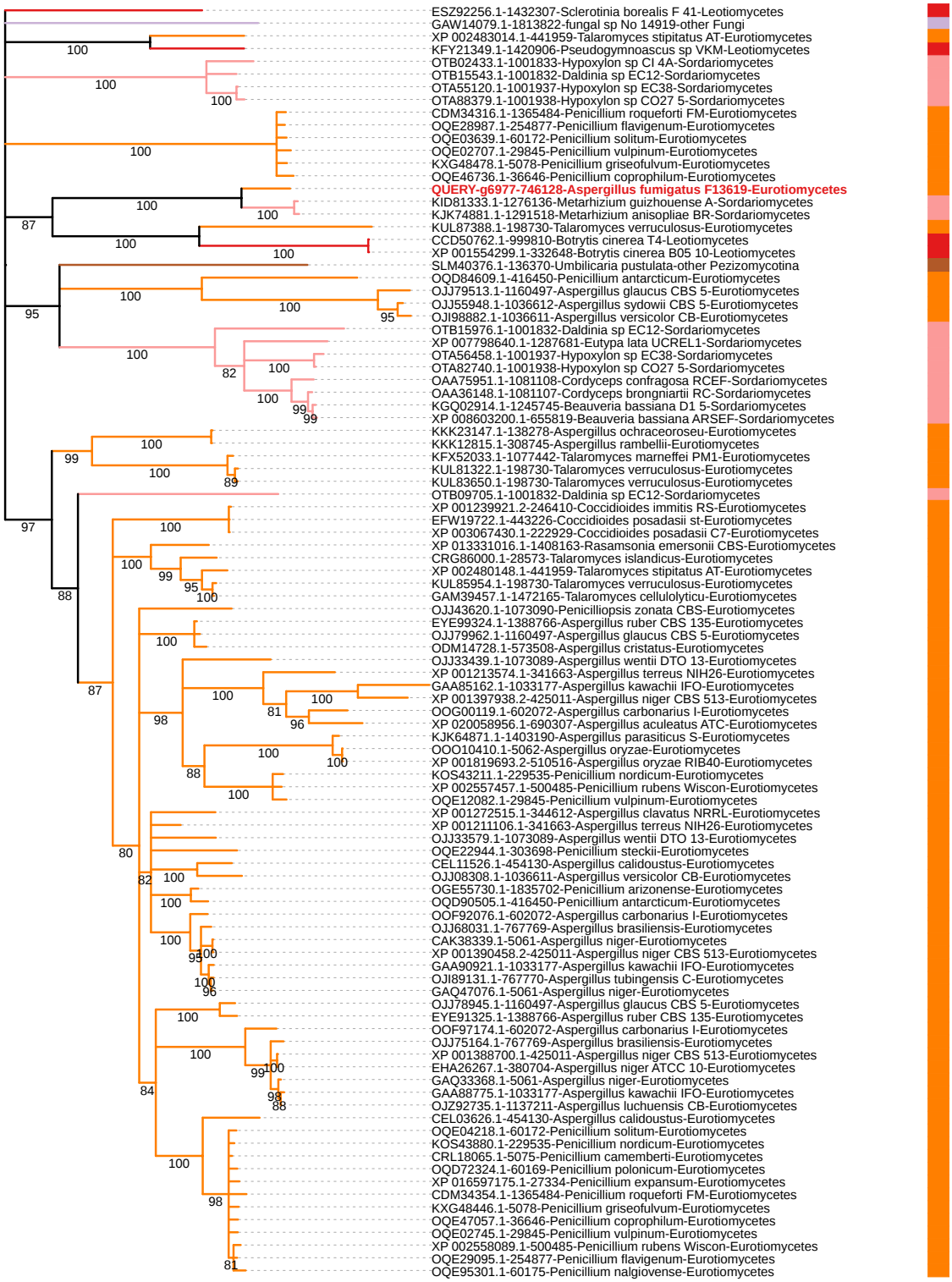
S4J



Tree scale: 0.1

Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses



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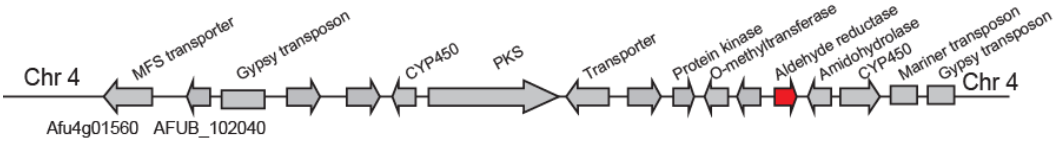

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Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
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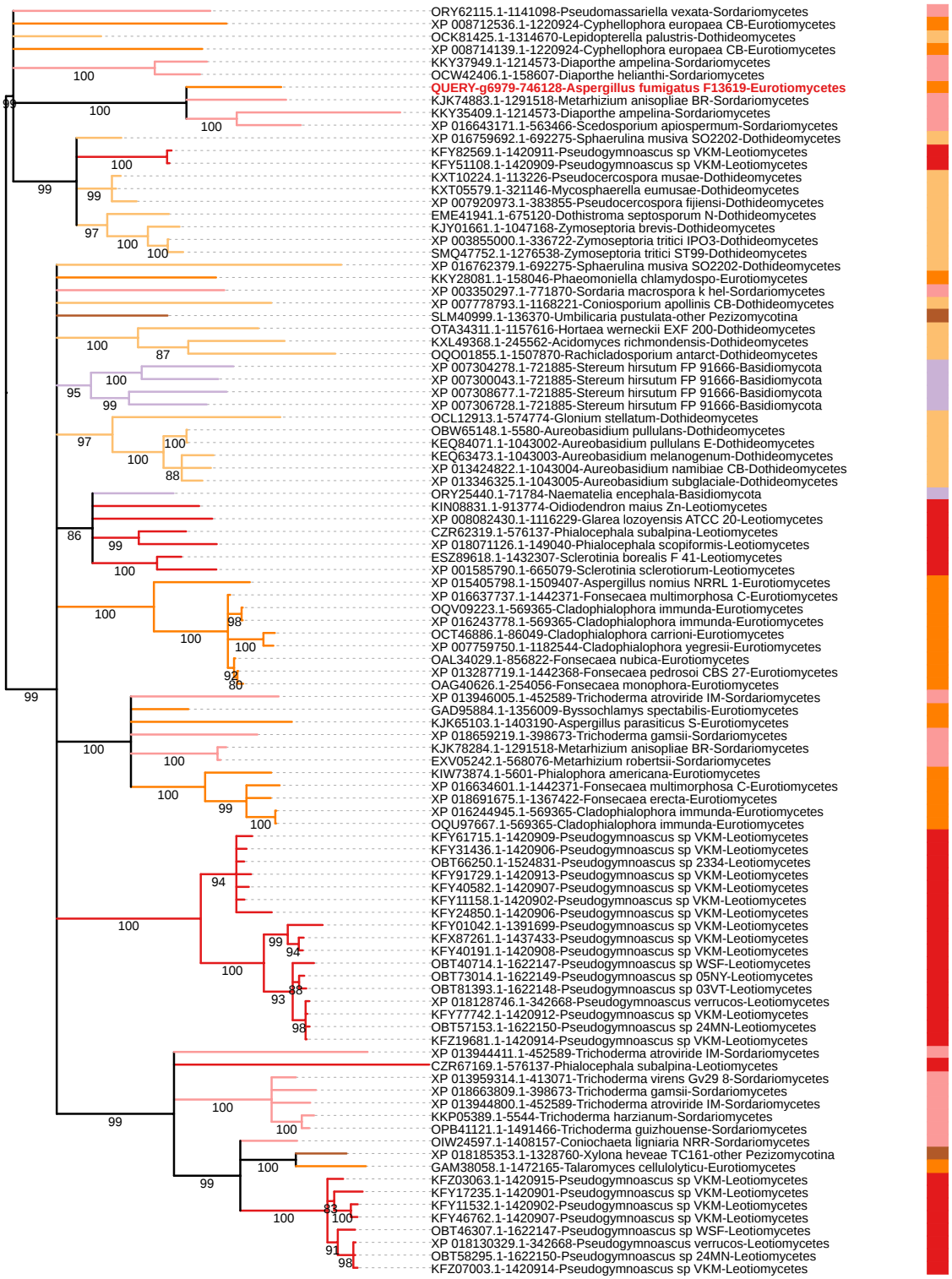
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses



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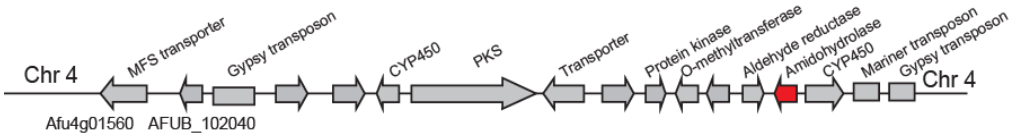

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Taxonomy

- Leotiomycetes
- Sordariomycetes
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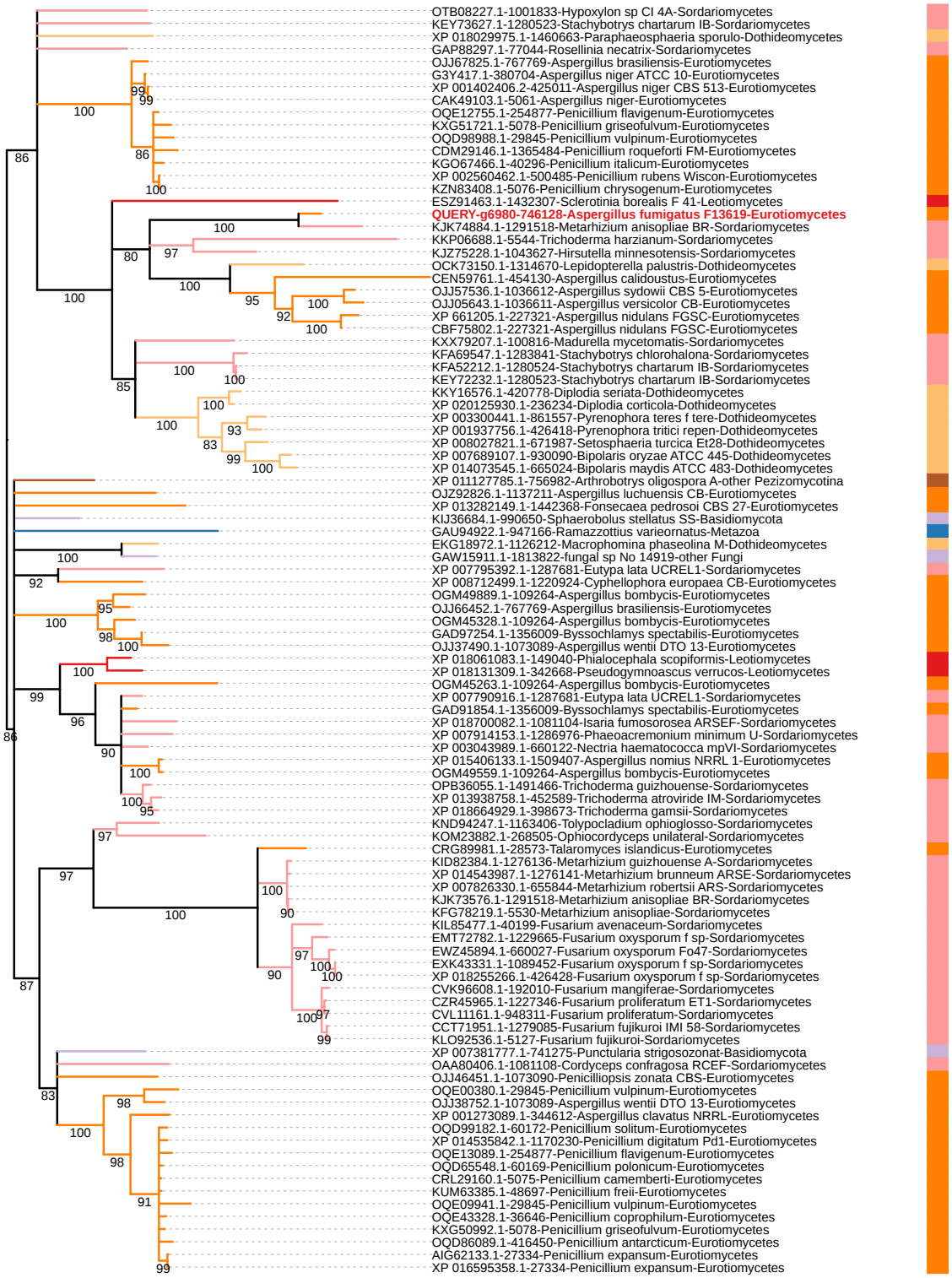
- Archaea
- Viruses



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MFS transporter
Gypsy transposon
CYP450
PKS
Transporter
Protein kinase
O-methyltransferase
Aldehyde reductase
Amidohydrolase
CYP450
Mariner transposon
Gypsy transposon
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Afu4g01560
AFUB_102040
Tree scale: 0.1
Taxonomy
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Sordariomycetes
Eurotiomycetes
Dothideomycetes
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other_Ascomycota
other_Fungi
other_Opisthokonta
other_Eukaryota
Bacteria
Archaea
Viruses
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XP 002143162.1-441960-Talaromyces marneffei ATC-Eurotiomycetes
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XP 020125921.1-236234-Diplodia corticola-Dothideomycetes
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KXX74268.1-100816-Madurella mycetomatis-Sordariomycetes
OJJ40738.1-1073089-Aspergillus wentii DTO 13-Eurotiomycetes
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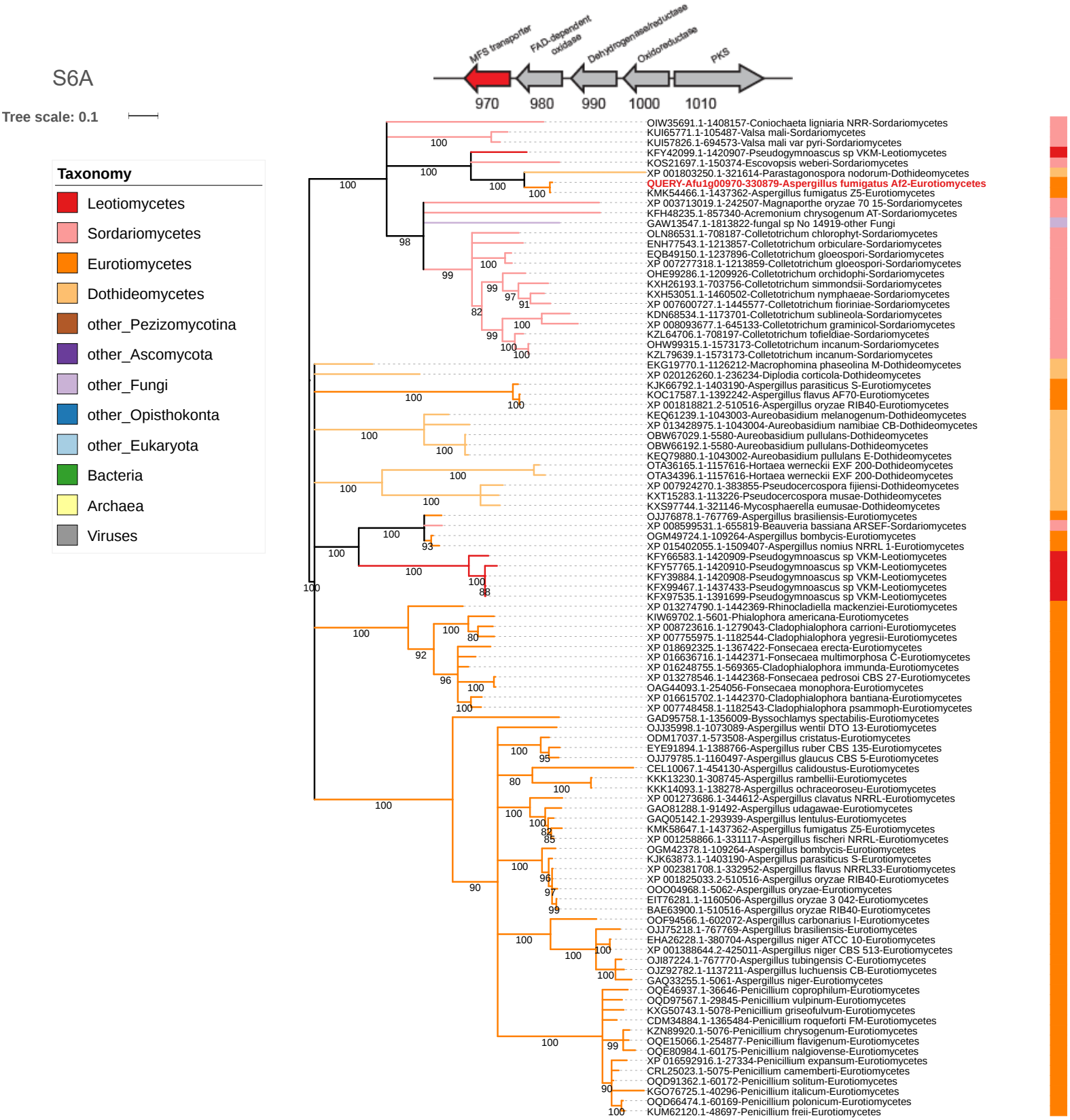
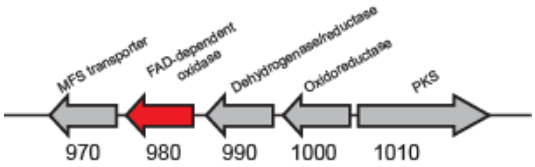
Figure S6. Gene phylogenies of the mobile SM gene cluster 1. These phylogenies are consistent with horizontal transfer between Eurotiomycete, Dothidiomycete, Leotiomycete, and Sordariomycete fungi.

S6A



S6B



Tree scale: 0.1

Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
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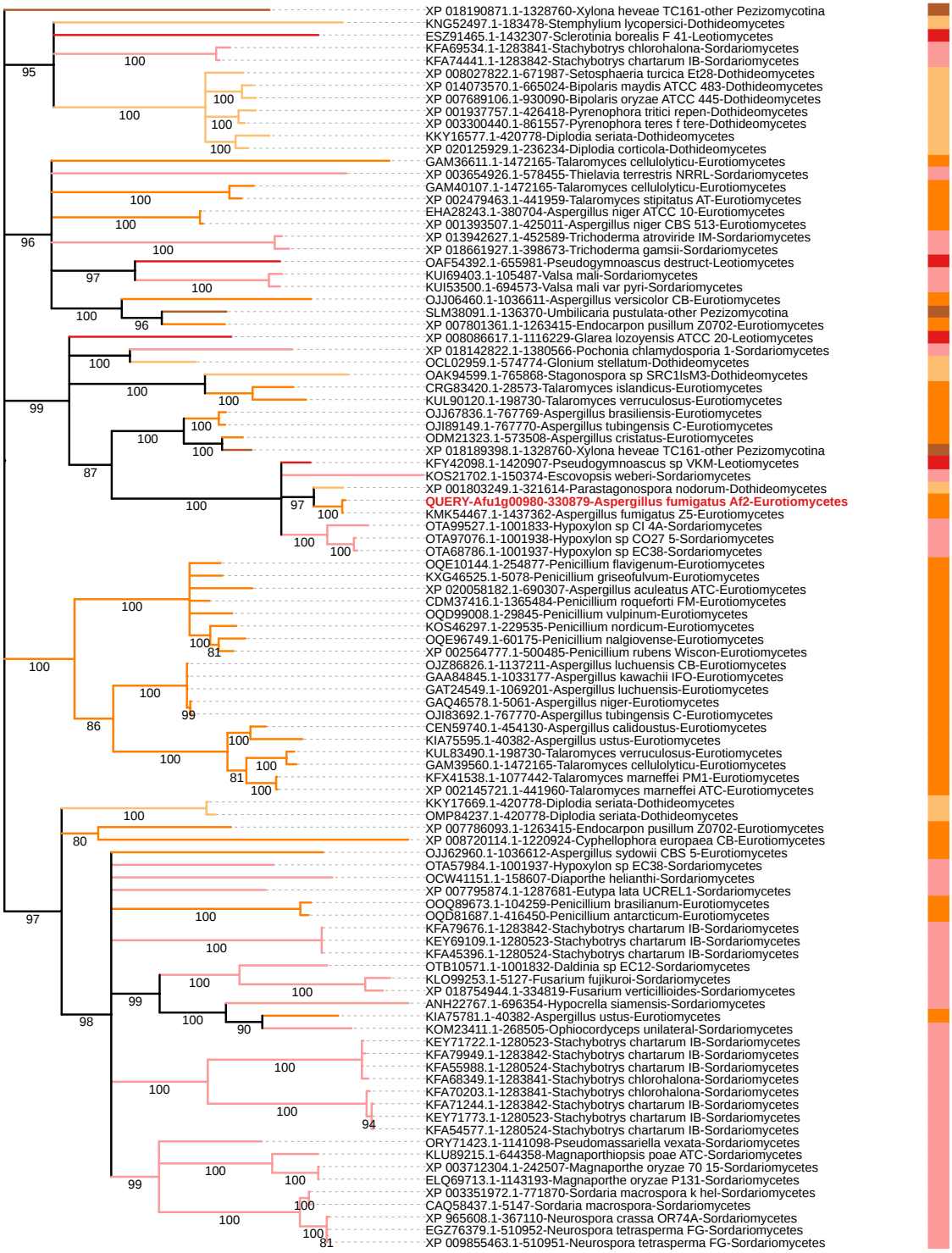
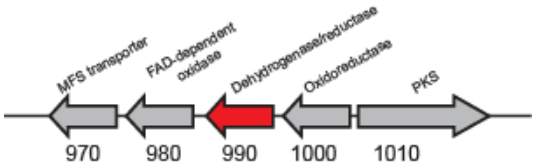
- Bacteria
- Archaea
- Viruses



S6C

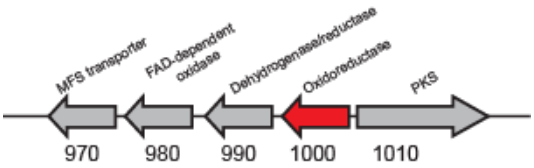


Tree scale: 0.1

Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses

S6D

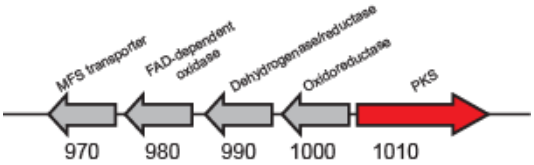


Tree scale: 0.1

Taxonomy

- Leotiomycetes
- Sordariomycetes
- Eurotiomycetes
- Dothideomycetes
- other_Pezizomycotina
- other_Ascomycota
- other_Fungi
- other_Opisthokonta
- other_Eukaryota
- Bacteria
- Archaea
- Viruses

S6E



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Taxonomy

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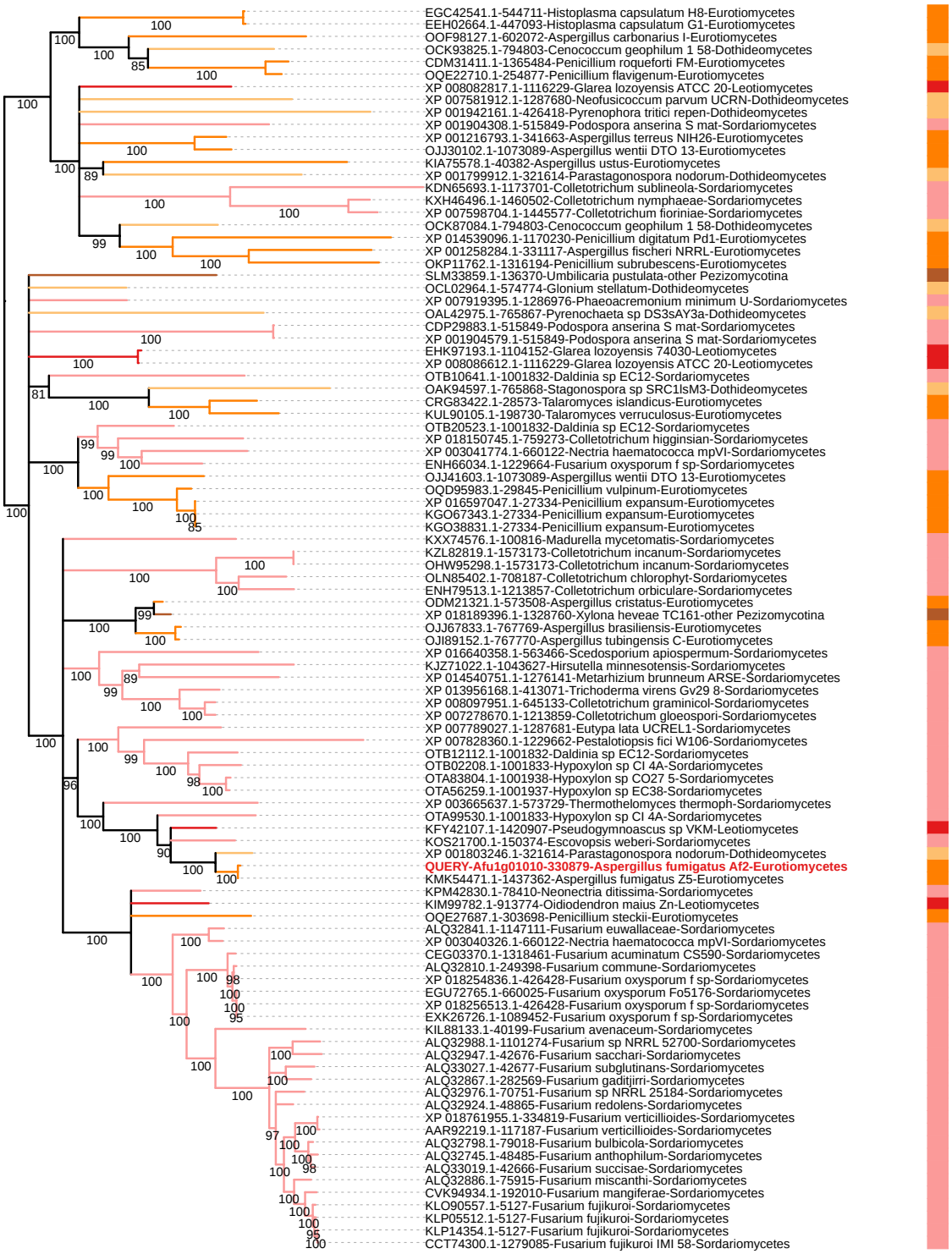


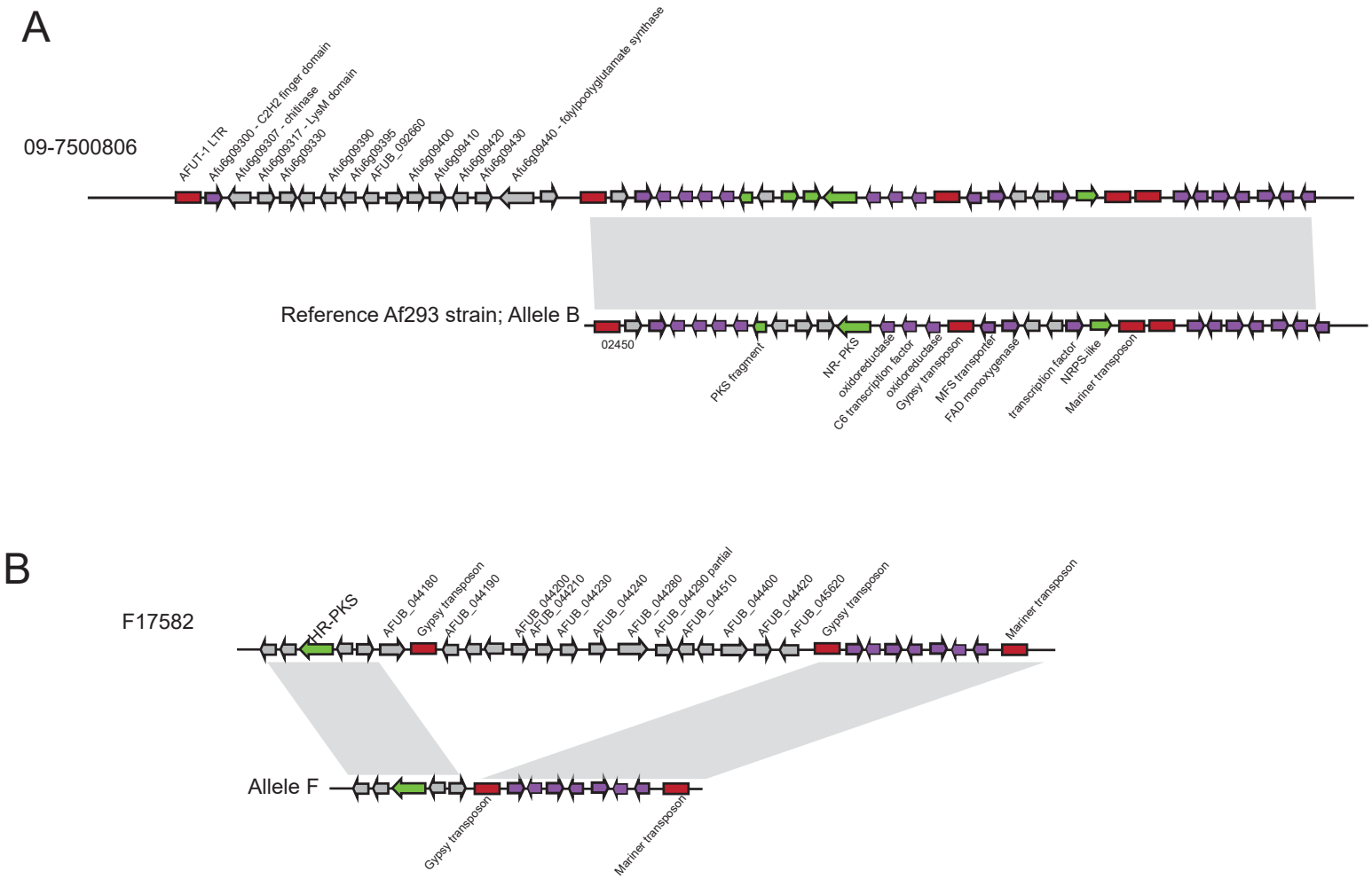
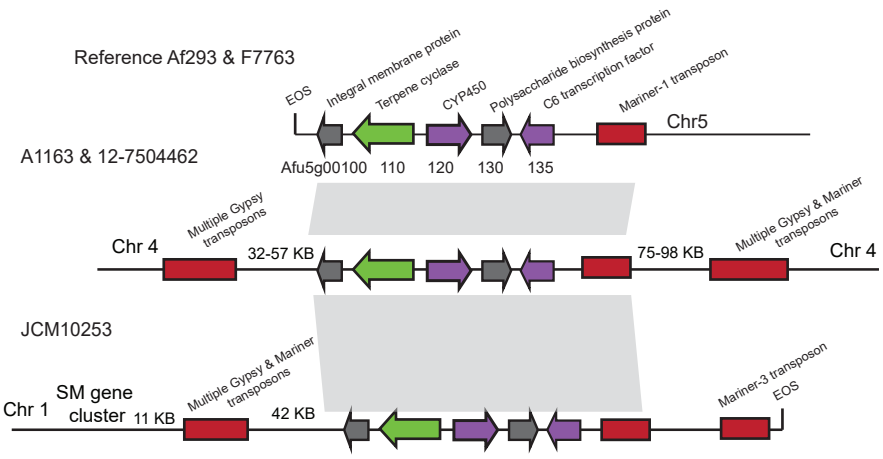


Figure S5. Two alleles of the idiomorphic SM gene cluster 10 present in one strain each.
A) This allele contains an insertion of genes from Chromosome 6 immediately upstream of Allele C (see main text Figure 4). None of these genes are likely SM gene cluster backbone genes. An additional transposable element is found flanking this insertion. B) This allele contains an insertion of genes present in the A1163 reference but not in the Af293 reference in the middle of Allele A (see main text Figure 4). None of these genes are likely SM gene cluster backbone genes. One additional transposable element is contained in this insertion.

A



B

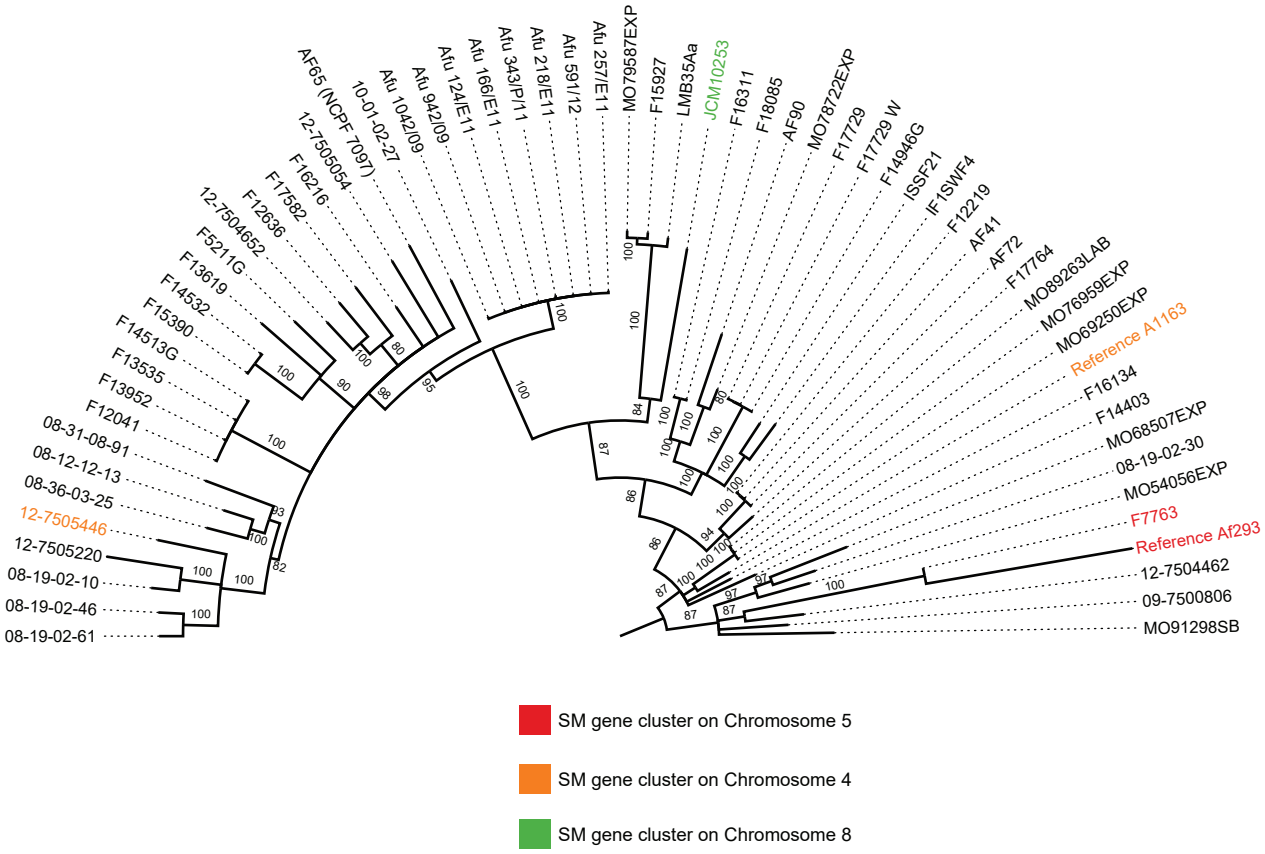


Figure S7. Genomic contexts of mobile SM gene cluster 5. (A) This gene cluster is located on Chromosome 5 in two strains, on Chromosome 4 in two strains, and on Chromosome 1 in one strain adjacent to a putative SM gene cluster. Multiple transposable elements flank the cluster in each strain. (B) Strains containing this cluster marked on SNP based phylogeny.

Figure S1. Marker gene phylogenies of all strains and *Aspergillus fischerianus*.
(A) Phylogeny of beta tubulin gene. (B) Phylogeny of calmodulin gene.