Acrophiarin (antibiotic S31794/F-1) from Penicillium arenicola shares biosynthetic features with both Aspergillus- and Leotiomycete-type echinocandins

Lan, Nan; Perlatti, Bruno; Kvitek, Daniel J.; Wiemann, Philipp; Harvey, Colin J. B.; Frisvad, Jens Christian; An, Zhiqiang; Bills, Gerald F.

Published in:
Environmental Microbiology

Link to article, DOI:
10.1111/1462-2920.15004

Publication date:
2020

Document Version
Peer reviewed version

Acrophiarin (antibiotic S31794/F-1) from *Penicillium arenicola* shares biosynthetic features with both *Aspergillus*- and *Leotiomycete*-type echinocandins

Nan Lan[1], Bruno Perlatti[1], Daniel J. Kvitek[2], Philipp Wiemann[2], Colin J.B. Harvey[2], Jens Frisvad[3], Zhiqiang An[1], Gerald F. Bills[1^]

[1]Texas Therapeutics Institute, The Brown Foundation Institute of Molecular Medicine, The University of Texas Health Science Center at Houston, Houston, Texas 77054, USA

[2]Hexagon Bio, Menlo Park, California 94025, USA

[3]Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark, DK-2800, Lyngby, Denmark

^Corresponding Author.
Gerald Bills
Professor
Kay and Ben Fortson Distinguished Chair
in Neurodegenerative Disease Research
Texas Therapeutics Institute
The Brown Foundation Institute of Molecular Medicine
The University of Texas Health Science Center at Houston
1881 East Road, 3SCR6.4676
Houston, TX 77054, USA
Office 01-713-486-2344
Mobile 01-713-505-4479
billsge@vt.edu or Gerald.F.Bills@uth.tmc.edu

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1462-2920.15004

Keywords: antifungal, Ascomycota, Aspergillaceae, fellutamides, fermentation, horizontal gene transfer, nonribosomal peptide synthetase, *Phialomyces*, secondary metabolites

Summary

The antifungal echinocandin lipopeptide, acrophiarin, was circumscribed in a patent in 1979. We confirmed that the producing strain NRRL 8095 is *Penicillium arenicola* and other strains of *P. arenicola* produced acrophiarin and acrophiarin analogues. Genome-sequencing of NRRL 8095 identified the acrophiarin gene cluster. *Penicillium arenicola* and echinocandin-producing *Aspergillus* species belong to the family Aspergillaceae of the Eurotiomycetes, but several features of acrophiarin and its gene cluster suggest a closer relationship with echinocandins from Leotiomycete fungi. These features include hydroxy-glutamine in the peptide core instead of a serine or threonine residue, the inclusion of a non-heme iron, α-ketoglutarate-dependent oxygenase for hydroxylation of the C3 of the glutamine, and a thioesterase. In addition, *Penicillium arenicola* bears similarity to Leotiomycete echinocandin-producing species because it exhibits self-resistance to exogenous echinocandins. Phylogenetic analysis of the genes of the echinocandin biosynthetic family indicated that most of the predicted proteins of acrophiarin gene cluster exhibited higher similarity to the predicted proteins of the pneumocandin gene cluster of the Leotiomycete *Glarea lozoyensis* than to those of the echinocandin B gene cluster from *A. pachycristatus*. The fellutamide gene cluster and related gene clusters are recognized as relatives of the echinocandins. Inclusion of the acrophiarin gene cluster into a comprehenisve phylogenetic analysis of echinocandin gene clusters indicates the divergent evolutionary lineages of echinocandin gene clusters are descendents from a common ancestral progenitor. The minimal ten-gene cluster may have undergone multiple gene acquisitions or losses and possibly horizontal gene transfer after the ancestral separation of the two lineages.

Introduction

The echinocandins are a family of lipohexapeptide fungal metabolites that non-competitively bind to the catalytic unit of β-1,3-glucan synthase leading to osmotic instability and fungal cell wall lysis. The antifungal drug caspofungin (CANCIDAS™) was developed from the echinocandin variant, pneumocandin B_0 (Balkovec et al., 2013). Two more echinocandin-type antifungals, micafungin (MYCAMINE™) derived from FR901370 (WF11899A), and anidulafungin (ERAXIS™) derived from echinocandin B have been brought to market (Vazquez and Sobel, 2006; Balkovec et al., 2013). A new long-acting echinocandin, rezafungin (CD101 IV), is based on the ECB natural product (Zhao et al., 2016). Because of the importance of echinocandin lipopeptides in antifungal therapy, understanding the full range of natural echinocandin variants and their underlying biosynthesis will provide options to generate and optimize new echinocandins. Furthermore, comprehensive mapping of these gene clusters across the Ascomycetes will contribute to understanding the natural functions of echinocandins in their respective producing organisms.

Our group artificially made the echinocandin acrophiarin (antibiotic S31794/F-1, Fig. 1, Table 1) (Dreyfuss and Tscherter, 1979; Dreyfuss, 1986) during mutasynthesis experiments (Chen et al., 2016b). The polyketide synthase (GLPKS4) of *Glarea lozoyensis* that is responsible for the 10,12-methyl myristate side chain of pneumocandins was inactivated by disruption of *glpks4* (Chen et al., 2016b). By exploiting the relaxed substrate specificity of the pathway's acetyl-CoA ligase (GLligase), feeding this mutant with straight-chain fatty acid precursors, e.g., myristic acid (C14) resulted in acrophiarin and other pneumocandin variants with substituted side chains (Chen et al., 2016b).

All echinocandins produced by *Aspergillus* species (Eurotiomycetes, Aspergillaceae) bear serine or threonine in the fifth position (Table 1), and have been referred to *Aspergillus*- or *Eurotiomycete*-type echinocandins (Yue et al., 2015). In contrast, Leotiomycete-type echinocandins invariably bear hydroxy-L-glutamate in the peptide's fifth position (Table 1). Based on this classification of echinocandins (Yue et al. 2015), acrophiarin from *Penicillium arenicola* appears to be a

Leotiomycete-type echinocandin from a fungus in the Aspergillaceae, albeit in a genus distinct from *Aspergillus*. Therefore, acrophiarin is unique among the echinocandins because it combines a feature of Leotiomycete-type echinocandins, hydroxy-glutamate in the cyclic peptide's fifth position with a straight-chain myristate side chain (Table 1, Fig. 1) which presumably originates from the cellular fatty acid pool.

A patent from the Swiss pharmaceutical company Sandoz (now Novartis) first disclosed acrophiarin as antibiotic S31794/F-1 (Dreyfuss and Tscherter, 1979). In the patent, infrared, ultraviolet, ^{1}H, and ^{13}C NMR spectra, and basic elemental composition data circumscribed a purified fermentation product and antifungal molecule effective against *Candida* species, however, a complete chemical structure was not proposed. The new antifungal metabolite was produced from strain NRRL 8095 from soil from British Columbia (Dreyfuss and Tscherter, 1979). In 1981, a patent from workers at Eli Lilly (Abbott and Fukuda, 1981) disclosed a chemical structure for antibiotic S31794/F-1 and proposed that it was a cyclic peptide consisting of cyclic dihydroxy-L-ornithine, L-threonine, hydroxy-L-proline, dihydroxy-L-homo-tyrosine, hydroxy-L-glutamine, hydroxy-4-methyl-L-proline that was N-acylated at the ornithine residue to myristoyl. In other words, acrophiarin was an echinocandin with a peptide core like that of the pneumocandins but with a straight myristoyl side chain rather than the dimethyl-myristoyl side chain characteristic of the pneumocandins (Figs. 1,2). The Lilly patent repeated the NMR spectra and physiochemical data from the Sandoz patent but offered no new spectral data to verify the structural assignments (Abbott and Fukuda, 1981). Antibiotic S31794/F-1 was later renamed "acrophiarin", and the producing strain was identified as *Penicillium arenicola* (Dreyfuss, 1986). *Penicillium arenicola* has been recognized as morphologically and phylogenetically distinct from the narrow phylogenetic definition of *Penicillium* and is closely related to *Phialomyces macrosporus* of the Aspergillaceae (Pitt, 1980; Houbraken and Samson, 2011; Frisvad et al., 2013). At the time of writing, *P. arenicola* has yet to be formally reclassified as a species of *Phialomyces*, therefore, we are obliged to continue to use

the name *Penicillium arenicola*. Other metabolites reported from *P. arenicola* are the γ-butyrolactone canadensolide (McCorkindale et al., 1968), and the C-gylcosylated depsides arenicolins A and B (Perlatti et al., 2020).

The biosynthetic gene clusters (BGCs) of most of the known echinocandins have been mapped and consist of ten to 14 contiguous and coregulated genes (Yue et al., 2015; Hüttel, 2017). Based on phylogenetic analysis of the protein sequences of echinocandin enzymes and comparisons structural variations among different echinocandins, they were classified into two types from divergent lineages of fungi (Yue et al., 2015). *Aspergillus*-types incorporate either serine or threonine in the core peptide's fifth position and lack a polyketide synthase (PKS) dedicated to synthesis of the side-chain. Leotiomycete-type echinocandins incorporating hydroxy-glutamate in the fifth position have either a fatty acid- or highly reducing PKS-derived side chain. Some Leotiomycete-type echinocandins undergo O-sulfation of the homotyrosine residue (e.g., FR901370, Table 1). As noted above, acrophiarin incorporates features of both echinocandin types.

To better comprehend the evolutionary processes leading to diversification of the echinocandin lipopeptides, we sequenced the genome of *P. arenicola* NRRL 8095 to identify the acrophiarin gene cluster. This newly characterized BGC is integrated into a revised evolutionary framework of echinocandins and the phylogenetically related fellutamide BCGs. The analysis indicates the acrophiarin biosynthetic and transport genes bridge the deep phylogenetic hiatus between the two types of echinocandins. These new data associate a nonribosomal peptide synthase (NRPS) adenylation domain (A domain) for the incorporation of hydroxy-L-glutamine in the echinocandin core with a species in the Aspergillaceae of the Eurotiomycetes. Based on these new findings, we offer a revised hypothesis on the origin of ancestral echinocandins and their relationship to the fellutamide gene clusters. Phylogenetic and synteny analyses of echinocandin gene clusters indicate divergent evolutionary lineages of echinocandin and fellutamide gene clusters are descendents from common ancestral progenitors. Subsequent to the ancestral separation,

echinocandin structural diversity appears to have undergone additional elaboration among the Leotiomycete fungi. Horizontal gene transfer (HGT) of a Leotiomycete-type gene cluster could plausibly explain the re-introduction of the acrophiarin gene cluster into the Aspergillaceae lineage. Furthermore, we demonstrate that acrophiarin and a series of natural acrophiarin analogs are a chemotaxonomic feature for strains of *P. arenicola*. Finally, the structural characterization of naturally occurring acrophiarin is updated with new data on its spectral properties, and its antifungal activity towards the producing strains is evaluated.

Results

Confirmation of NRRL 8095 as *Penicllium arenicola*

The patent describing antibiotic S31794/F-1 identified the producing strain as *Acrophialophora limonispora* (Dreyfuss and Tscherter, 1979). This invalid name was later corrected, and the strain was correctly identified as *Penicillium arenicola* (Dreyfuss, 1986). By microscopy, we observed the typical conidial state described in the literature (Fig. S1) (Pitt, 1980). Database searches with internal transcribed spacer and large subunit ribosomal DNA from the genome sequence of NRRL 8095 (GenBank MN512717) sequences from strains of *P. arenicola*, including the ex-type strain NRRL 3392 (Table 2) as the top BLAST hits. Furthermore, as previously noted (Pitt, 1980), strains in Table 2 were highly similar in their colony characteristics and microscopic features. We therefore concluded that NRRL 8095 is conspecific with authentic strains of *P. arenicola*.

Acrophiarin and related analogs detection and chemical structure determination

Bioassay of extracts of all four *P. arenicola* strains (Table 2, Fig. S2) in the five media tested resulted in strong inhibition of growth of *C. albicans*, but extracts were only weakly or not active towards *Cryptococcus neoformans* (Fig. 2). This pattern of antifungal activity is suggestive of echinocandins because they do not affect *C. neoformans*, possibly due to their failure to reach the site of β-glucan assembly (Thompson et al. 1999). All extracts from the two strains of the closely related *Ph.*

macrosporus were inactive towards *C. albicans* indicating echinocandin type metabolites were not produced (Fig. 2).

HPLC-MS analysis confirmed that strains NRRL 8095, 3392, 31507, and 31509 produced acrophiarin and highly related analogs (Figs. 3; 4) indicating that the acrophiarin BGC may be a consistent feature of *P. arenicola* (see results below). Additionally, we could infer that like other echinocandins, production of acrophiarin and its analogs is facile. They could be detected after a few days when significant growth accumulated (data not shown) indicating that the BGC is transcribed during exponential growth of the culture.

Acrophiarin was characterized by comparison of its MS and ^{1}H NMR spectroscopic data (Fig. S3) with those described in the literature and co-injection of purified standard made during *G. lozoyensis* mutasythesis experiments (Dreyfuss and Tscherter, 1979; Chen et al., 2016b). As the data agreed well with the published values, the principal echinocandin from *P. arenicola* was confirmed as acrophiarin. Additional peaks were observed by HPLC-MS, with *m/z* corresponding to the 15C and 16C side chain variants of pneumocandins I and K, and their presence were confirmed by co-injection with analytical standards (Chen et al., 2016b) (Fig. 3). Additional minor peaks were also observed, and analysis by MS fragmentation indicated the existence of analogs lacking some of the core peptide hydroxyls (Fig. 4), consistent with minor products observed in other pneumocandin mixtures resulting from incomplete oxidation of the core's amino acids (Masurekar et al., 1992; Li et al., 2015).

Identification of the acrophiarin biosynthetic gene cluster

Only one BGC that comprised an acetyl-CoA ligase, a NRPS with six adenylation domains, along with the *hty* genes encoding the biosynthesis of homotyrosine from tryrosine was found in the draft genome assembly for NRRL 8095. Genes in this BGC exhibited high sequence similarity to other BGCs encoding echinocandins and had a gene cluster organization and order that was intermediate

between *Aspergillus*- and Leotiomycete-types (Fig. 5; Table 3). The high level of identity and synteny with the pneumocandin BGC and other echinocandin clusters indicated with a high degree of certainty it encoded the biosynthesis of acrophiarin. Surprisingly, most of the predicted proteins of acrophiarin BGC exhibited higher similarity to the predicted proteins of the pneumocandin BGC from *G. lozoyensis* than to those of the echinocandin B BGC (Table 3). Consistent with the chemical structure of acrophiarin, the BGC lacks a PKS ortholog of GLPKS4 that assembles the pneumocandin side chain (Fig. 5; S4, Table S1). To rule out the possibility that a side-chain encoding PKS gene might lie outside the acrophiarin gene cluster, we used the predicted amino acid sequence of GLPKS4 to search a database of NRRL 8095 protein models. Two distantly related highly reducing PKS (identities, 43% and 40%, respectively) were found at other loci on other contigs, and both contained a C-methyltransferase domain, thus it seems unlikely either would be responsible for the myristoyl side chain (Fig. S5). Thus, the hypothesis that the acrophiarin side chain originates from the fatty acid pool rather from a highly reducing PKS seems parsimonious with the available data and previous mutasynthesis experiments with pneumocandins (Chen et al., 2016b).

Another unexpected feature of the acrophiarin gene cluster is the presence of an ortholog of GLHYD (Figs. 1, 5, Table 3), a gene encoding a thioesterase that apparently assists in off loading the side chain from the highly reducing PKS (Chen et al., 2016b). However, this enyzyme could also conceivably hydrolyse myristoyl-CoA/myristoryl-ACP to myristate, thus make it available to the first adenylation domin of the NRPS. Current data indicates that thioesterase-encoding genes are absent in all other *Aspergillus*-type echinocandin BGCs. Furthermore, unlike other *Aspergillus*-type, echinocandin BGCs described to date, the acrophiarin gene cluster contains an ortholog of GLOXY3 that encodes a non-heme iron, α-ketoglutarate-dependent oxygenase responsible for hydroxylation of the C3 of the glutamine (Yue et al., 2015; Hüttel, 2017). These latter two features indicate a strong affinity of the acrophiarin BGC to those of the Leotiomycete-type echinocandins.

Evolution of the acrophiarin NRPS and other pathway enzymes

In order to test whether acrophiarin NRPS modules were more similar to Eurotiomycete- or Leotiomycete-type NRPS modules, the predicted A domains were extracted from the deduced amino acid sequences for each of the echinocandin NRPSs and were aligned with our previously published data set of echinocandin-type NRPS A domains (Yue et al. 2015). The analysis also included the A domains of InpB, the first NRPS of the fellutamide BGC (Fig. 1) (Yeh et al., 2016), because in our previous work, the InpB NRPS and the InpC acetyl-CoA ligase were found to be close phylogenetic relatives of the echinocandin NRPSs and acetyl-CoA ligases, respectively. BLAST searches of public databases and bioinfomatic analysis of genes identified additional fellutamide gene clusters in species of *Aspergillus* sect. *Nidulantes* (*A. sydowii, A. versicolor, A. mulundensis. A. pachycristatus*) and fellutamide-like gene clusters in other unrelated ascomycetes, including *Spathularia flavida* (Leotiomycetes, Rhytismatales), *Bisporella* sp. (Leotiomycetes, Helotiales), and *Lobaria pulmonaria* (Lecanoromycetes, Peltigerales) indicating that the fellutamide family of lipopeptides may more widespread than previously recognized (Shigemori et al., 1991; Lee and Hong, 2011; Xu et al., 2011; Wu et al., 2014; Kjærbølling et al., 2019). Because the products of these BGCs in these latter fungi are unknown, we refer to them as fellutamide-like BGCs. These InpB and InpC orthologs were added to the analysis to explore possible relationships between the fellutamides and echinocandins, and they fell within their respective fellutamide subclades of the echinocandin linegage with significant statistical support (Figs. 6, 7).

A maximum likelihood (ML) tree of these A domains (Fig. 6) reproduced similar topological features observed in a prior analysis (Yue et al., 2015). The ML tree indicated that all the echinocandin A domains and the both A domains of the InpB NRPS (ANID_03496) of the fellutamide tripeptides from *A. nidulans* (Yeh et al., 2016), and its orthologs ASInpB from *A. sydowii*, AVInpB from *A. versicolor*, AMInpB from *A. mulundensis*, and APCInpB from *A. pachycristatus* NRRL 1140, and newly found fellutamide-like BGCs formed a distinct clade (97% support value). Therefore, the echinocandin NRPSs along with the InpB module 2 and its orthologs formed a well-supported

monophyletic lineage within the EAS (Euascomycete clade synthetase) subfamily of fungal NRPSs (Bushley and Turgeon, 2010) (Fig. 6). It should be noted that the InpB module 2 activates L-glutamine while the InpB module 1 activates L-asparagine. It is unclear whether L-glutamine-activation by the InpB module 2 and the Leotiomycete-type 5th position A domains or is indicative of a past evolutionary connection between the fellutamide and echinocandin peptide sequences, or whether incorporation of a common amino acid is conincidence.

Within the echinocandin A domain clade, the six individual A domains exhibited highly resolved intra-clade relationships (Fig. 6). The echinocandin clade was resolved into seven well supported subclades, each corresponding to one of the six amino acid positions in the echinocandin nucleus plus a clade for modules 1 and 2 of InpB and similar fellutamide-like NRPSs (Fig. 6). As is the case with other NRPS orthologs from distantly related fungi (Bushley and Turgeon, 2010), this topology indicates that the A domains for each amino acid position from each distantly related fungus are more similar to each other than the individual A domains from a given echinocandin-producing strain. As expected based on its function, the acrophiarin NRPS module 5 which activates glutamine formed a cluster (100% bootstrap value) with its corresponding Leotiomycete A domains. Although, the *Aspergillus*-type NRPS A domains were phylogenetically distinct from those of the Leotiomycete-types, the phylogenetic placement of acrophiarin A domains were in nearly all cases (except for module 4 and 6) more closely related to those of the Leotiomycetes although the fungus belongs to the Eurotiomycetes. Within each of these echinocandin A-domain clades, the branching of terminal leaves was congruent with the expected phylogenetic species trees except for the A domains from the acrophiarin NRPS. These analyses strongly supported the hypothesis that the extant echinocandin and fellutamide NRPSs were descendants from a common ancestral peptide synthetase (Fig. 6). However, the phylogenetic affinity of the acrophiarin NRPS A domains deviated from those of the *Aspergillus* species, and this phylogenetic incongruency could be interpreted as evidence for a closer relationship to echinocandin BGCs of Leotiomycete fungi.

To further explore the relationships of the acrophiarin cluster genes and the variations in their echinocandin gene orthologs, we built phylogenetic trees for individual echinocandin and related fellutamide pathway genes (Fig. 7). In trees inferred from orthologs of individual echinocandin genes, relationships among more closely related genes of the Leotiomycete- and *Aspergillus*-types were generally well resolved (bootstrap values >80%). In most single-gene trees (except for OXY4), the aculeacin genes (*A. japonicus*/*aculeatus*) formed a distinct branch from the echinocandin/mulundocandin genes (*A. pachycristatus*/*mulundensis*). Also, in the acetyl-CoA ligase tree, the fellutamide acetyl-CoA ligases (InpC) and orthologs from fellutamide-like BGCs appeared to be basal to the echinocandin ligases. InpC from *A. nidulans* fellutamide BGC exhibited about 51% identity to EcdI from NRRL 1440 echinocandin B gene cluster. This observation, along with the fact that in some *Nidulantes* species (*A. pachycristatus*/*mulundensis*) the fellutamide and echinocandin BGCs co-exist in the same genome, prompts further speculation that the fellutamide gene cluster may share common ancestry with progenitors of the echinocandin gene clusters. It should also be pointed out that fellutamides are also antifungal, albeit with a mode of action distinct from that of echinocandins (Xu et al., 2011).

The relationships among the acrophiarin cluster genes and the corresponding genes of the two lineages of echinocandins were generally unresolved (Fig. 7). In some cases, acrophiarin gene sequences fell on a distinct branch intermediate between the two echinocandin types. A few branch conflicts were observed in some gene trees, but the conflicts were statistically unsupported by bootstrap analysis. Only in the case of the acetyl-CoA ligase, did an acrophiarin gene clearly fall into the *Aspergillus*-type echinocandin clade (100% bootstrap value). Other the hand, the P450-1, HtyB, and OXY4 gene were strongly associated with the Leotiomycete-type clade with support values of 100%, 85%, and 61% respectively. Nevertheless, the overall high similarities of the acrophiarin and the Leotiomycete-type echinocandin cluster including the presence of GLHYD in the acrophiarin BCG, which is thought to assist the acetyl-CoA ligase in off-loading 10,12-methyl

myristate in *G. lozoyensis* (Chen et al., 2016b), suggested a close relationship between acrophiarin and Leotiomycete-type echinocandins, e.g., pneumocandins. On the other hand, the absence of an ortholog of GLPKS4, which provides 10,12-methyl myristate in some Leotiomycetes, suggests a hypothetical scenario where the *Aspergillus*-type echinocandins clusters were lost in most lineages of the Aspergilllaceae, inclduing the *Phialomyces* lineage. Subsequently, an acrophiarin BGC could have been reintroduced into *Phialomyces* lineage via HGT soon after the ancestral divergence of the Leotiomycete and *Aspergillus* lineages.

To compare phylogenetic relationships of echinocandin cluster orthologs to the phylogeny of the corresponding fungi in which they occur, we inferred a species tree from the concatenated sequences of six housekeeping genes (18S rRNA, 28S rRNA, ITS RNA region, β-tubulin, translation elongation factor 1-α, and RNA polymerase II subunit 2) to a tree based on the ten shared enzymes of the echinocandin pathways (NRPS, acetyl-CoA ligase, TRT, P450-2, OXY1, OXY2, OXY4, HtyA, HtyC, HtyD) (Fig. 8). We analyzed trees inferred from each housekeeping gene individually which indicated that the individual housekeeping-gene trees were congruent and consistent with current models of ascomycete species phylogeny (data not shown). The high bootstrap values for almost all branches in the species tree provided evidence for the hierarchical relationships of most of the genera examined (Fig. 8A). Even though *P. arenicola* clearly grouped with the Eurotiomycetes in the housekeeping gene tree, it fell outside the Eurotiomycetes in the echinocandin gene tree. Thus, the topologies of the species tree and the concatenated echinocandin-gene tree were conflicted, and the incongruent branch with *P. arenicola* was the cause of the conflict (Shimodaira-Hasegawa test, $P = 0$; weighted Shimodaira-Hasegawa test, $P = 0$) (Figs. 8B; S6). Such phylogenetically incongruent gene sets are classic evidence of a past HGT event. This conflict suggested a scenario where the acrophiarin BGC may have been horizonally transferred from a Leotiomycete ancestor, and this putative HGT event occurred subsequent to the divergence of Leotiomycete- and *Aspergillus*-type echinocandins.

Penicillium arenicola is self-resistant to acrophiarin and other echinocandins

Previous studies demonstrated that growth of echinocandin-producing strains of *Aspergillus* are sensitive to exogenously applied echinocandins, while the Leotiomycete-type echinocandin-producing strains are generally insensitive or have reduced sensitivity to echinocandins (Tóth et al., 2012; Yue et al., 2018). To test whether the acrophiarin-producing *P. arenicola* are susceptible to echinocandins as in Aspergilli or have innate elevated resistance to echinocandins like Leotiomycetes that produce echinocandins, we carried out a zone of inhibition (ZOI) assay with three *P*. *arenicola* strains, the pneumocandin-producing strain *G*. *lozoyensis*, the echinocandin B producing-strain *A*. *pachycristatus,* and *C*. *albicans* against pure echinocandins. Natural echinocandins (pneumocandin B_0, echinocandin B, acrophiarin) caused large ZOIs with *A*. *pachycristatus* and *C*. *albicans*, while the same compound set had little effect on the three *P*. *arenicola* strains (Fig. 9 and Fig. S7). *Glarea lozoyensis* exhibited minimal sensitivity to pneumocandin B_0 and was unaffected by acrophiarin and echinocandin B. Bioinformatic analysis of the *P. arenicola* genome sequence revealed only a single copy of FKS1. These results indicated that the acrophiarin-producing strains of *P*. *arenicola* are intrinsically resistant to echinocandins as is the case for echinocandin-producing species of the Leotiomycete lineage, e.g., *G. lozoyensis*. To date, echinocandin resistance mechanisms have been attributed to amino acid mutations in the FKS1 protein (a plasma membrane-embedded enzyme), a compensatory increase in chitin synthase, or a second auxiliary copy of FKS1 associted with an echinocandin BGC (Johnson et al., 2011; Tóth et al., 2012; Walker et al., 2015; Slot, 2017; Yue et al., 2018). Therefore, the mechanism underlying the intrinsic resistance to echinocandins in *G*. *lozoyensis* and *P*. *arenicola* remains unclear.

Discussion

The genetic mechanisms leading to the formation, persistence, and loss of complex secondary metabolite biosynthetic gene clusters remain poorly understood. A combination of genomic, phylogenetic, functional, and biochemical analyses has provided insights into the evolutionary history of echinocandin biosynthesis in species of diverse genera of Pezizomycotina (Yue et al., 2015; Hüttel, 2017; Yue et al., 2018). The above results build upon previous analyses and indicated that structural diversity of echinocandins produced by these fungi has arisen largely from gains and losses of genes and changes in specificities of adenylation sites of the core NRPS gene during the evolutionary histories of the echinocandin BGCs. A variety of mechanisms, including gene duplication, neofunctionalization, introgression, HGT, and horizontal chromosome transfer, have the potential to contribute to gain and diversification of secondary metabolic biosynthetic genes in fungi (Wisecaver et al., 2014; Koczyk et al., 2015; Florea et al., 2017; Slot, 2017; Feurtey and Stukenbrock, 2018; Thynne et al., 2019). One of the most important requisites for inferring relationships and prediction of HGT events is that all extant forms of the genes be included in the analysis (Feurtey and Stukenbrock, 2018). The inclusion of the acrophiarin BGC along with the discovery of the fellutamide BGC (Yeh et al., 2016) have filled important gaps in previous analyses of the echinocandin family. The addition of the acrophiarin BGC to our phylogenetic analyses indicates that the evolutionary histories of echinocandin-encoding genes do not always parallel the phylogenetic relationships of echinocandin-producing fungi, in contrast to previous conclusions (Yue et al., 2015). Therefore, the revised evolutionary pattern among echinocandin BGCs more resembles reticulate patterns of pathway evolution observed for other families of fungal secondary metabolites, and where conflicted gene phylogenies of some of the pathways genes have been offered as classic evidence of past HGT events (Wisecaver et al., 2014; Lind et al., 2017). One scenario supported by our phylogenetic analyses and comparative mapping of gene clusters suggests that the origin of acrophiarin BGC in *P. arenicola* of the *Phialomyces* lineage was derived from an ancestral Leotiomycete after the canonical *Aspergillus*-type echinocandin BGC had been lost during lineage sorting (Fig. 10).

A more complete picture of the extant echinocandin biosynthetic gene family allows for inference of ancestral states of the echinocandin cluster and biosynthetic boundaries of the pathway genes. Genome sequences (including draft genomes) for at least 12 strains representing eight species have enabled comparisons of the echinocandin BGC locus across representatives of nearly all types of producing strains with varying capabilities to produce echinocandins (Fig. 10). Chemotype differences among these diverse species can be attributed to the presence or absence of genes encoding key pathway steps and variations in NRPS A domains (Yue et al., 2015; Hüttel, 2017). However, some anomalous reactions remain to be discovered, e.g., the mechanism of homotyrosine O-sulfation in some Leotiomycete-type echinocandins (Table. 1). Interestingly, to date, we have not detected evidence of widespread degenerate echinocandin gene clusters and single echinocandin genes among genomes of the Pezizomycotina suggesting that these genes clusters are quickly lost from the genome when the gene cluster degenerates. Thus, the distribution of echinocandin BGCs remains relatively narrow and is based almost entirely on detection of the corresponding echinocandins in antifungal assays from limited number of species (Dreyfuss, 1986; Peláez et al., 2011; de la Cruz et al., 2012; Yue et al., 2015; Hüttel, 2017). However, the discovery of additional echinocandin and related NRPS clusters remains a possibility with more widespread genome sequencing of the Pezizomycotina and continued screening for cell-wall-active antifungal metabolites.

Hybridization and introgression may be important forces for interspecific dispersal of biosynthetic genes in fungi and in diversifying and generating novel metabolites (Olarte et al., 2015; Moore et al., 2017; Hubka et al., 2018). Although, the number of available genomes of echinocandin-producing fungi precludes a detailed analysis, interspecfic hybridization and introgression could have played a role in interspecific dispersion of the echinocandin BGCs, at least in species complexes where closely related species produce echinocandins. The greatest concentration of echinocandin-producing species recognized to date is in *Aspergillus* section *Nidulantes*. Species of

this section reported to produce echinocandins include *A. pachycristatus*, *A. spinulosporus*, *A. rugulosus*, *A. quadrilineatus*, and *A. navahoensis* which produce echinocandin B and its variants (de la Cruz et al., 2012; Chen et al., 2016a; Hüttel, 2017), and *A. mulundensis* which produces mulundocandin (Bills et al., 2016). Because of the large number of species in section *Nidulantes* (65 species, Chen et al. 2016a), additional producers of echinocandin B and its variants are likely be found with more targeted genome surveys or focused screening for cell-wall-active metabolites. Section *Nidulantes* encompasses a large complex of rapidly evolving species, with both homothallic and heterothallic species, consequently, interspecific disperial of BGCs and their chemical evolution in part may be driven by hybrization and introgression as has been observed in the *A. flavus* complex (Olarte et al., 2015; Moore et al., 2017), and section *Fumigati* (Hubka et al., 2018).

Phylogenetic analysis of ascomycete NRPS A domains also provided evidence that the echinocandin clade of NRPSs also incorporates the InpB NRPS of the fellutamide pathway and similar ortholgous NRPSs from diverse fungi (Fig. 6). Phylogenetic analyses suggested a degree of common ancestry between the two metabolite pathways (Figs. 6,7), but because the relationship appears to be ancient and predating the divergence of major ascomycete lineages, it is difficult to speculate on the relationships between the echinocandin and fellutamide gene clusters. During the first step of fellutamide B biosynthesis (Fig. 1) (Yeh et al., 2016), InpC activates 3-hydroxydodecanoic acid to form 3-hydroxydodecanoyl-AMP that is then loaded onto the T_0 domain of InpB. The 3-hydroxydodecanoyl-S-phosphopantetheinyl-T_0 is extended stepwise with L-asparagine and L-glutamine by the two condensation-adenylation-thiolation modules of InpB. The linear lipodipeptide is then transferred from InpB onto InpA for the addition of the last amino acid, L-leucine. The lipotripeptide undergoes reductive release by the thioesterase domain of InpA resulting in (2S)-fellutamide B (Yeh et al., 2016). InpF might be involved in the release and transfer of the lipodipeptide from inpB to inpA. The relatively high protein sequence similary between echinocandin acetyl-CoA ligases and InpC orthologs also suggests both these ligases might have

shared a common ancestor and their divergence in function is a result of neofunctionalization, thus resulting in paralogs.

Additionally, most echinocandin genes bear some significant similarity to non-echinocandin genes. This suggests that neofunctionalization of closely related non-echinocandin genes has contributed to the evolution of the gene clusters. For example, the four genes comprising the homo-L-tyrosine subcluster of all echinocandins BGCs and the four-gene cassette from the *Alternaria alternata* AM toxin pathway (*amt* gene cluster, Fig. 11) that is predicted to encode synthesis of the α-amino-4-phenyl-valeric acid monomer of AM toxin are examples of neofunctionalization. The genes of the Hty subpathway would need to be inherited with other pathway genes since incorporation of homotyrosine has been shown to be essential for product formation (Cacho et al., 2012).

Ten orthologs are shared in all extant echinocandin BGCs across both lineages of the echinocandin-type BGC clusters (Fig. 8B) and are necessary for the conserved core lipopeptide skeleton structure, the peripheral genes that build the nonproteinogenic amino acids, the initiation and activation of side chain variants, and ligation of the side chain with the ornithine residue (Fig. 1). Thus, the ancestral echinocandin cluster and its products could be the same as simple echinocandins produced by extant species of Aspergilli e.g., aculeacin and echinocandin B (Fig. 10, Table 1). It seems plausible the ancient echinocandin progenitor descended into both the Leotimycete and Eurotiomyctes lineages. The above phylogenetic and synteny analyses point to a Leotiomycete ancestor as the likely donor pathway for the acrophiarin BGC because of the glutamine-specific A-domain 5 of the core NRPS and the presence of the thioesterase gene (GLHYD orthologs) and GLOXY3 orthologs. During evolution of the Leotiomycete lineage, the addition of a thioesterase may have facilitated the recruitment of highly reducing PKS responsible for the branched PKS side chain of the pneumocandins, sporiofungins, and FR190239. The different species of echinocandin-producing fungi have varied habitats (soil, endophytes, litter). The dispersal by HGT to a heavily sporulating species with a broad geographic distribtution, e.g., the circumboreal *P. arenicola,*

would provide ample opportunities for intimate contact needed for HGT from a rare, host-specific or geographically restricted species of the Leotiomycetes, e.g., *G. lozoyensis*, *Venustampulla echinocandica*, or *Coleophoma cylindrospra*. Finally, *Pencillium arenicola* also appears to be more similar to the Leotiomycete echinocandin-producing species with regard to self-resistance to exogenous echinocandins. Whether this is a coincidence or there is mechanistic relationship remains to be determined.

In conclusion, the identification of the acrophiarin BGC in *P. arenicola* and the inclusion of the fellutamide gene cluster in the echinocandin biosynthetic family expands the known boundaries of echinocandin biosynthetic capabilities. The echinocandins exhibit a highly disjunct distribution among ascomycetous fungi from very different ecologies (e.g., soil, plant litter, endophytes). Therefore, a common ecological function for these metabolites remains unclear except for the fact that discovery of auxillary copies of FKS1 in some species clearly indicates their selection for interaction with fungal cell wall biosynthesis (Yue et al., 2018). Moreover, the diversity of fungi that produce echinocandins suggests their ecological roles may vary among plant-, litter-, and soil-associated species. The strong phylogenetic relationships and co-existance of genes from the fellutamide BGC cluster in some *Aspergillus* genomes suggests an origin of both pathways prior to the divergence on the major lineages of filamentous ascomycete more than 400 million years ago (Lutzoni et al., 2018) leading to independent evolutionary courses for echinocandin pathways in the Eurotiomycete and Leotiomycete fungi. The origin of the Leotiomycete-like acrophiarin BGC in *P. arenicola* could be explained by a subsequent dispersal of a Leotiomycete echinocandin gene cluster into *P. arenicola* during a HGT event. This is a significant finding for the field of fungal lipopeptide biosynthesis and expands the possibilities to identify additional antifungal compounds and biosythetic reactions encoded from the echinocandin-fellutamide family.

Experimental procedures

Strains and culture conditions.

The acrophiarin-producing strain *P. arenicola* NRRL 8095 (Dreyfuss and Tscherter, 1979) and three additional strains of *P. arenicola* were obtained from the U.S.D.A National Regional Research Laboratories (NRRL) (Table 2). Two strains of the closely related *Phialomyces macrosporus* were obtained from the IBT Culture Collection of Fungi (Table 2). The agar medium for growth and sporulation was YM agar (glucose 10 g, malt extract 3 g, yeast extract 3 g, peptone 5, agar 20 g per 1000 ml of deionized H_2O). Five different fermentation media were tested for production of acrophiarin based on their previous history for producing echinocandins. These media were: Medium I (glucose 20 g, casein peptone 5 g, $NaNO_3$ 3 g, of $KHPO_4$ 1 g, KCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 10 mg per 1000 mL of deionized H_2O) (Dreyfuss and Tscherter, 1979); Medium IV (Debono, 1980) ($ZnSO4.7H_2O$ 4.5 mg, meat peptone 30.5 g, soybean meal 15.5 g, dextrin 2.0 g, blackstrap molasses (Brer Rabbit) 10.5 g, Na_2HPO_4 4.5 g, $MgSO4.7H_2O$ 5.5 g, $FeSO4.7H_2O$ 0.10 g, cottonseed oil 40 mL per 1000 mL of deionized H_2O); Medium VI (Boeck and Kastner, 1981); Medium TG 106 (Tkacz et al., 1993) (D-mannitol 100 g, NZ amine 33 g, yeast extract 10 g, $(NH_4)_2SO_4$ 5 g, KH_2PO_4 9 g per 1000 mL of deionized H_2O), and medium SMY (Bacto neopeptone 10 g, maltose 40 g, yeast extract 10 g per 1000 ml of deionized H_2O).

For the seed cultures stage, six agar discs from 3-wk old YM agar culture were inoculated into Medium SMY (maltose 40 g, neopeptone 10 g, yeast extract 10 g, 1000 mL deionized H_2O) with 0.4% agar in 50-mL aliquots in 250-mL flasks. Seed cultures were grown at 24 °C, 220 rpm for 4 d. For the production cultures, 1-mL aliquots of the seed growth were transferred to flasks with 50 mL of Medium I, Medium IV, Medium IV, Medium TG 106, and Medium SYM respectively. For quantitative measurements of titers, five replicates of each fermentation were grown at 24 °C, 220 rpm for 12 d.

Genome sequencing and annotation

NRRL 8095 was grown in a static culture of 100 ml SMY for 14 d at 23 °C. Mycelium was filtered, pressed dry, frozen at -80 °C, and lyophilized. Genomic DNA was purified from ground mycelial powder with a Zymo Research Corporation Quick-DNA™ Fungal/Bacterial Miniprep Kit. For preparation of sequencing libraries, 500 ng of total genomic DNA were used as the template and processed using the KAPA HyperPlus Kit for PCR-free workflows (Roche, Switzerland) according to the manufacturer's instructions. Sequencing libraries were size selected for 600-800 bp fragments using a LightBench (Coastal Genomics, Canada). Whole genome sequencing was run on a HiSeq 4000 Sequencing System (Illumina, USA). The genome was assembled by SPAdes using standard parameters (Bankevich et al., 2012). *Ab initio* gene predictions from the genome assembly were made with Augustus (Stanke et al., 2004) using *A. fumigatus* as the reference genome.

The acrophiarin biosynthetic gene cluster in NRRL 8095 (GenBank MN518690) was identified by submitting the unannotated contig sequences for antiSMASH analysis (https://fungismash.secondarymetabolites.org/) and by reciprocal BLAST searches with sequences of known echinocandin biosynthetic genes from *A. pachycristatus* NRRL 11440 (JX421684, JX421685) and *Glarea lozoyensis* ATCC 20868 (PRJNA246203). The acrophiarin gene cluster occupied a single continuous locus on contig 7. Additional fellutamide BGCs from *Spathularia flavida*, *Bisporella* sp., and *Lobaria pulmonaria* were identified by BLAST searches and from annotated draft genomes available at the Department of Energy Joint Genome Institute's Mycocosm portal (https://mycocosm.jgi.doe.gov/mycocosm/home).

Screening strains for echinocandin activity

To detect echinocandin-type activity, strains (Table 2) were fermented using Medium SMY, Medium I, Medium IV, Medium VI, and TG106. Each fermentation was extracted by the addition of an equal volume of methanol (MeOH) followed by shaking for 2 hr. The H_2O-MeOH mixture was filtered and evaporated under vacuum (Fig. S2). Residues were dissolved in DMSO at 10× of the

original culture volume, and 20 µL of each DMSO extract was applied to a 4-mm well aspirated from a plate of YM agar seeded with an overnight culture of *Candida albicans* (ATCC 10231) or *Cryptococcus neoformans* H99. Plates were incubated at 25 °C and examined after 24-48 hr for zones of inhibition.

Antifungal assay for determining sensitivity of P. arenicola to echinocandins.

For zone of inhibition (ZOI) assays, fresh conidia suspensions of *P*. *arenicola* strains, pneumocandin-producing strain *G*. *lozoyensis* and echinocandin B-producing strain *A*. *pachycristatus* were added to melted YM agar at 45 °C and adjusted to a final conidial concentration to 2×10^3 conidia/ml. Twenty five-mL aliquots of the seeded agar media were poured into 9-cm Petri dishes. When the seed-agar plates are cooled and solidified, wells were made by aspirating agar with a 4-mm diam syringe tip. Acrophiarin, pneumocandin B_0 and echinocandin B were dissolved in DMSO to 250 µg/mL, and amphotericin B (250 µg/mL) was used as the control antimicrobial. Ten-µL aliquots of these compounds were added to each well. The plates of *P. arenicola*, *G. lozoyensis* and *A. pachycristatus* were incubated at 24 °C for 3, 6 and 2 days, respectively, then, ZOIs were measured and photographed.

Extraction and analysis for HPLC-MS

For each fermentation sample described in the previous step, a 5-ml aliquot of the aqueous-MeOH filtrate was evaporated to dryness, resuspended in 0.5 mL of MeOH, filtered through 0.2 µm cellulose membrane, and a 10-µL aliquot was analyzed by HPLC-MS on an Agilent 1260 HPLC equipped with a diode array detector (DAD) and coupled to an Agilent 6120 single quadrupole mass spectrometer (MS). Samples were eluted on a C18 reverse phase column (Ace Equivalence 5C18, 4.6 × 150 mm, 5 µm) with a solvent gradient of 10−100% B for 28 min (solvent A, 0.1% formic acid in H_2O; solvent B, 0.1% formic acid in acetonitrile), with flow rate of 1.0 $mL.min^{-1}$. The

chromatographic profiles were monitored by wavelength scanning from 190 to 400 nm and acquisition set at 210 nm and by positive and negative ESI-MS from *m/z* 160-1500.

Isolation and identification of acrophiarin and pneumocandin-enriched fractions

To obtain sufficient mass for purification and biological tests, the crude extracts from different fermentations were pooled, evaporated to dryness and dissolved in 300 mL of H_2O:MeOH (1:1 v/v). The extract was evaporated under vacuum until most of the MeOH was removed. The remaining aqueous sample was extracted with an equal volume of methyl ethyl ketone (MEK). After 2 hr, the organic phase was separated and evaporated under vacuum. The crude extract was dissolved in a mixture of acetonitrile (ACN) and H_2O (1:1 v/v) and fractionated with a Grace Reveleris X2 flash chromatography system using a Reveleris C18 RP 12 g cartridge (10-100% ACN over 16 min, flow rate 30 $mL.min^{-1}$), using UV and ELSD detection, resulting in 16 fractions.

Fractions 2-5 were pooled, dried, dissolved in ACN:H_2O (1:1 v/v) and further purified by semipreparative HPLC (Agilent Zorbax SB-C18 column; 5 µm; 9.4 × 250 mm; gradient of 30−60% B for 20 min (solvent A, 0.1% formic acid in H_2O; solvent B, 0.1% formic acid in acetonitrile), 4.0 $mL.min^{-1}$), yielding 2.1 mg of acrophiarin. Fractions 6 and 7 were analyzed by HPLC-MS with the same method described for crude extracts and showed the presence of other pneumocandin analogues which were not further purified. NMR data were collected on a Bruker 500 MHz NMRs equipped with a 5-mm triple resonance cryoprobe at 298 K, with tetramethylsilane used as internal standard (TMS δ_H 0).

Prediction of adenylation domain specificity

The adenylation domain (A-domain) structure determines the specific amino acids incorporated during peptide elongation and is a critical step in predicting the essential binding-pocket residues that correlate with the amino acid sequence of the NRPS product. Key positions of the A-domain

binding pockets were determined at the PKS/NRPS Analysis website (http://nrps.igs.umaryland.edu) (Bachmann and Ravel, 2009).

Phylogenetic analysis and hypothesis testing

Phylogenetic trees for all the enzymes in the echinocandin biosynthetic pathway were explored in the context of orthologous functional enzymes from across the Ascomycota. Proteins encoded by *G. lozoyensis* and *A. pachycristatus* echinocandin clusters and the *A. pachycristatus* fellutmide cluster were used as queries in BLAST searches against NCBI databases. Each set of proteins was aligned by using ClustalW implemented in MEGA 7.0 (Kumar et al., 2016), and the resulting alignment was manually adjusted. Phylogenies were inferred by the maximum likelihood (ML) method implemented in MEGA 7.0 under a JTT+G model. Bootstrap supports were calculated using the default options in MEGA 7.0 with 100 replicates per run.

To estimate the phylogenetic affinities of NRRL 8095, a combined six-gene dataset, including the DNA fragments of the small-subunit (SSU) (18S) rRNA gene, the large-subunit (28S) rRNA gene, the ITS RNA gene region, the β-tubulin gene, the EF1- gene, and the RPB2 gene were resampled from previous phylogenetic studies of the echinocandin producing fungi and other Ascomycota (Yue et al., 2015) and aligned with ClustalW implemented in MEGA 7.0. The best-fit nucleotide substitution models were determined for the alignment based on the lowest Bayesian information criterion scores. Positions containing gaps and missing data were eliminated. A GTR+G+I model was applied the alignment to construct phylogenies using the ML method with MEGA 7.0.

To test whether the phylogenies of the echinocandin pathway genes were congruent with the current classification of the Ascomycota. The concatenated phylogenetic marker gene sequences for the eight echinocandin-producing fungi were aligned with ClustalW and analyzed with MEGA 7.0 by ML method using a GTR+G+I model. Ten pathway enzymes were found to be common among the eight echinocandin-producing fungi. The amino acid sequences of the 10 enzymes

(NRPS, acyl AMP dependent ligase, ABC transporter, oxygenase 1, oxygenase 2, oxygenase 4, P450-2, isopropylmalate dehydrogenase, 2-isopropylmalate synthase, and aconitase) were combined to construct a phylogenetic tree. The concatenated amino acid sequences of these enzymes were aligned with ClustalW and analyzed with MEGA 7.0 by the ML method using a JTT+G+I+F model. Alternative hypotheses based on the tree topologies assessed under the null hypothesis that all topologies were equally good explanations of the data were tested with the Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999) and with a weighted Shimodaira-Hasegawa test as implemented in TREEFINDER (Jobb et al., 2004).

Acknowledgements

Parts of this work were supported by the Ben and Kay Fortson Endowment (to GFB) an a grant from the National Institutes of Health (R01 GM121458). The authors declare that they have no conflicts of interest. We thank Michael Dreyfuss for historical information regarding the discovery of acrophiarin.

References

Abbott, B.J., and Fukuda, D.S. 1981. S 31794/F-1 Nucleus. US Patent 4,304,716.

Bachmann, B.O., and Ravel, J. (2009) Chapter 8. Methods for in silico prediction of microbial polyketide and nonribosomal peptide biosynthetic pathways from DNA sequence data. *Meth Enzymol* **458**: 181-217.

Balkovec, J.M., Hughes, D.L., Masurekar, P., Sable, C.A., Schwartz, R.A., and Singh, S.B. (2013) Discovery and develpment of first in class antifungal caspofungin (Cancidas). A case study. *Nat Prod Rep* **31**: 15-34.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G.,

Alekseyev, M.A., and Pevzner, P.A. (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comp Biol* **19**: 455-477.

Bills, G.F., Yue, Q., Chen, L., Li, Y., An, Z., and Frisvad, J.C. (2016) *Aspergillus mulundensis sp. nov.,* a new species for the fungus producing the antifungal echinocandin lipopeptides, mulundocandins. *J Antibiotics* **69**: 141-148.

Boeck, L.D., and Kastner, R.E. 1981. Method of producing the A-30912 antibiotics. U.S. Patent 4,288,549.

Bushley, K.E., and Turgeon, B.G. (2010) Phylogenomics reveals subfamilies of fungal nonribosomal peptide synthetases and their evolutionary relationships. *BMC Evol Biol* **10**: 26.

Cacho, R.A., Jiang, W., Chooi, Y.H., Walsh, C.T., and Tang, Y. (2012) Identification and characterization of the echinocandin B biosynthetic gene cluster from *Emericella rugulosa* NRRL 11440. *J Amer Chem Soc* **134**: 16781-16790.

Chen, A.J., Frisvad, J.C., Sun, B.D., Varga, J., Kocsubé, S., Dijksterhuis, J., Kim, D.H., Hong, S.B., Houbraken, J., and Samson, R.A. (2016a) *Aspergillus* section *Nidulantes* (formerly *Emericella*): Polyphasic taxonomy, chemistry and biology. *Stud Mycol* **84**: 1-118.

Chen, L., Li, Y., Yue, Q., Loksztejn, A., Yokoyama, K., Felix, E.A., Liu, X., Zhang, N., An, Z., and Bills, G.F. (2016b) Engineering of new pneumocandin side-chain analogues from *Glarea lozoyensis* by mutasynthesis and evaluation of their antifungal activity. *ACS Chem Biol* **11**: 2724-2733.

de la Cruz, M., Martín, J., González-Menéndez, V., Pérez-Victoria, I., Moreno, C., Tormo, J.R., El Aouad, N., Guarro, J., Vicente, F., Reyes, F., and Bills, G.F. (2012) Chemical and physical modulation of antibiotic activity in *Emericella* species. *Chem Biodivers* **9**: 1095-1113.

Debono, M. 1980. Derivatives of cyclic peptide nuclei. European Patent Application 0031220 A1.

Dreyfuss, M.M. (1986) Neue Erkenntnisse aus einem pharmakologischen Pilz-screening. *Sydowia* **39**: 22-36.

Dreyfuss, M.M., and Tscherter, H. 1979. Antibiotic S31794/F-1. US Patent 4,173,629.

Feurtey, A., and Stukenbrock, E.H. (2018) Interspecific gene exchange as a driver of adaptive evolution in fungi. *Ann Rev Micobiol* **72**: 377-398.

Florea, S., Panaccione, D.G., and Schardl, C.L. (2017) Ergot alkaloids of the family Clavicipitaceae. *Phytopathology* **107**: 504-518.

Frisvad, J.C., Houbraken, J., Popma, S., and Samson, R.A. (2013) Two new *Penicillium* species *Penicillium buchwaldii* and *Penicillium spathulatum*, producing the anticancer compound asperphenamate. *FEMS Microbiol Lett* **339**: 77-92.

Houbraken, J., and Samson, R.A. (2011) Phylogeny of *Penicillium* and the segregation of Trichocomaceae into three families. *Stud Mycol* **70**: 1-51.

Hubka, V., Barrs, V., Dudová, Z., Sklenář, F., Kubátová, A., Matsuzawa, T., Yaguchi, T., Horie, Y., Nováková, A., Frisvad, J.C., Talbot, J.J., and Kolařík, M. (2018) Unravelling species boundaries in the *Aspergillus viridinutans* complex (section *Fumigati*): opportunistic human and animal pathogens capable of interspecific hybridization. *Persoonia* **41**: 142-174.

Hüttel, W. (2017) Structural diversity in echinocandin biosynthesis: the impact of oxidation steps and approaches toward an evolutionary explanation. *Z Naturforsch C* **72**: 1-20.

Jobb, G., von Haeseler, A., and Strimmer, K. (2004) TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. *BMC Evol Biol* **4**: 18.

Johnson, M.E., Katiyar, S.K., and Edlind, T.D. (2011) New FKS hot spot for acquired echinocandin resistance in *Saccharomyces cerevisiae* and its contribution to intrinsic resistance of *Scedosporium* species. *Antimicrob Agents Chemother* **55**: 3774-3781.

Kjærbølling, I., Vesth, T., and Andersen, M.R. (2019) Resistance gene-directed genome mining of 50 *Aspergillus* species. *mSystems* **4**: e00085-00019.

Koczyk, G., Dawidziuk, A., and Popiel, D. (2015) The distant siblings—A phylogenomic roadmap illuminates the origins of extant diversity in fungal aromatic polyketide biosynthesis. *Genome Biol Evol* **7**: 3132-3154.

Kumar, S., Stecher, G., and Tamura, K. (2016) MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* **33**: 1870-1874.

Lee, Y.-M., and Hong, J. (2011) A cytotoxic fellutamide analogue from the sponge-derived fungus *Aspergillus versicolor*. *Bull Korean Chem Soc* **32**: 3817-3820.

Li, Y., Chen, L., Yue, Q., Liu, X., An, Z., and Bills, G.F. (2015) Genetic manipulation of the pneumocandin biosynthetic pathway for generation of analogues and evaluation of their antifungal activity. *ACS Chem Biol* **10**: 1702-1710.

Lind, A.L., Wisecaver, J.H., Lameiras, C., Wiemann, P., Palmer, J.M., Keller, N.P., Rodrigues, F., Goldman, G.H., and Rokas, A. (2017) Drivers of genetic diversity in secondary metabolic gene clusters within a fungal species. *PLoS Biol* **15**: e2003583.

Lutzoni, F., Nowak, M.D., Alfaro, M.E., Reeb, V., Miadlikowska, J., Krug, M., Arnold, A.E., Lewis, L.A., Swofford, D.L., Hibbett, D., Hilu, K., James, T.Y., Quandt, D., and Magallón, S. (2018) Contemporaneous radiations of fungi and plants linked to symbiosis. *Nat Comm* **9**: 5451.

Masurekar, P.S., Fountoulakis, J.M., Hallada, T.C., Sosa, M.S., and Kaplan, L. (1992) Pneumocandins from *Zalerion arboricola* 2. Modification of product spectrum by mutations and medium manipulation. *J Antibiotics* **45**: 1867-1874.

McCorkindale, N.J., Wright, J.L.C., Brian, P.W., Clarke, S.M., and Hutchinson, S.A. (1968) Canadensolide - an antifungal metabolite of *Penicillium canadense*. *Tetra Lett* **9**: 727-730.

Moore, G.G., Olarte, R.A., Horn, B.W., Elliott, J.L., Singh, R., O'Neal, C.J., and Carbone, I. (2017) Global population structure and adaptive evolution of aflatoxin-producing fungi. *Ecol Evol* **7**: 9179-9191.

Olarte, R.A., Worthington, C.J., Horn, B.W., Moore, G.G., Singh, R., Monacell, J.T., Dorner, J.W., Stone, E.A., Xie, D.-Y., and Carbone, I. (2015) Enhanced diversity and aflatoxigenicity in

interspecific hybrids of *Aspergillus flavus* and *Aspergillus parasiticus*. *Mol Ecol* **24**: 1889-1909.

Peláez, F., Collado, J., Platas, G., Overy, D.P., Martín, J., Vicente, F., Gonźlez del Val, A., Basilio, A., De la Cruz, M., Tormo, J.R., Fillola, A., Arenal, F., Villareal, M., Rubio, V., Baral, H.O., Galán, R., and Bills, G.F. (2011) Phylogeny and intercontinental distribution of the pneumocandin-producing anamorphic fungus *Glarea lozoyensis*. *Mycology* **2**: 1-17.

Perlatti, B., Lan, N., Earp, C.E., AghaAmiri, S., Vargas, S.H., Azhdarinia, A., Bills, G.F., and Gloer, J.B. (2020) Arenicolins: C-glycosylated depsides from *Penicillium arenicola*. *J Nat Prod* **(in press)**.

Pitt, J.I. (1980) *The genus Penicillium and its teleomorphic states Eupenicillium and Talaromyces.* London, UK: Academic Press.

Shigemori, H., Wakuri, S., Yazawa, K., Nakamura, T., Sasaki, T., and Kobayashi, J.i. (1991) Fellutamides A and B, cytotoxic peptides from a marine fish-possessing fungus *Penicillium fellutanum*. *Tetrahedron* **47**: 8529-8534.

Shimodaira, H., and Hasegawa, M. (1999) Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol Biol Evol* **16**: 1114.

Slot, J.C. (2017) Chapter Four - Fungal Gene Cluster Diversity and Evolution. In *Advances in Genetics*. Townsend, J.P., and Wang, Z. (eds): Academic Press, pp. 141-178.

Thynne, E., Mead, O.L., Chooi, Y.-H., McDonald, M.C., and Solomon, P.S. (2019) Acquisition and loss of secondary metabolites shaped the evolutionary path of three emerging phytopathogens of wheat. *Genome Biol Evol* **11**: 890-905.

Tkacz, J.S., Giacobbe, R.A., and Monaghan, R.L. (1993) Improvement in the titer of echinocandin-type antibiotics: A magnesium-limited medium supporting the biphasic production of pneumocandins A_0 and B_0. *J Indust Microbiol* **11**: 95-103.

Tóth, V., Nagy, C.T., Pócsi, I., and Emri, T. (2012) The echinocandin B producer fungus *Aspergillus nidulans* var. *roseus* ATCC 58397 does not possess innate resistance against its lipopeptide antimycotic. *Appl Microbiol Biotech* **95**: 113-122.

Vazquez, J.A., and Sobel, J.D. (2006) Anidulafungin: A novel echinocandin. *Clin Infect Dis* **43**: 215-222.

Walker, L.A., Lee, K.K., Munro, C.A., and Gow, N.A.R. (2015) Caspofungin treatment of *Aspergillus fumigatus* results in ChsG-dependent upregulation of chitin synthesis and the formation of chitin-rich microcolonies. *Antimicrob Agents Chemother* **59**: 5932-5941.

Wisecaver, J.H., Slot, J.C., and Rokas, A. (2014) The evolution of fungal metabolic pathways. *PLoS Gen* **10**: e1004816.

Wu, C.-J., Li, C.-W., and Cui, C.-B. (2014) Seven new and two known lipopeptides as well as five known polyketides: the activated production of silent metabolites in a marine-derived fungus by chemical mutagenesis strategy using diethyl sulphate. *Marine Drugs* **12**: 1815-1838.

Xu, D., Ondeyka, J., Harris, G.H., Zink, D., Kahn, J.N., Wang, H., Bills, G., Platas, G., Wang, W., Szewczak, A.A., Liberator, P., Roemer, T., and Singh, S.B. (2011) Isolation, structure, and biological activities of fellutamides C and D from an undescribed *Metulocladosporiella* (Chaetothyriales) using the genome-wide *Candida albicans* fitness test. *J Nat Prod* **74**: 1721-1730.

Yeh, H.H., Ahuja, M., Chiang, Y.M., Oakley, C.E., Moore, S., Yoon, O., Hajoysky, H., Bok, J.W., Keller, N.P., Wang, C.C.C., and Oakley, B.R. (2016) Resistance gene-guided genome mining: Serial promoter exchanges in *Aspergillus nidulans* reveal the biosynthetic pathway for fellutamide B, a proteasome inhibitor. *ACS Chem Biol* **11**: 2275-2284.

Yue, Q., Li, Y., Chen, L., Zhang, X., Liu, X., An, Z., and Bills, G.F. (2018) Genomics-driven discovery of a novel self-resistance mechanism in the echinocandin-producing fungus *Pezicula radicicola*. *Environ Microbiol* **20**: 3154-3167.

Yue, Q., Chen, L., Zhang, X., Li, K., Sun, J., Liu, X., An, Z., and Bills, G.F. (2015) Evolution of chemical diversity in echinocandin lipopeptide antifungal metabolites. *Euk Cell* **14**: 698-718.

Zhao, Y., Perez, W.B., Jiménez-Ortigosa, C., Hough, G., Locke, J.B., Ong, V., Bartizal, K., and Perlin, D.S. (2016) CD101: a novel long-acting echinocandin. *Cell Microbiol* **18**: 1308-1316.

Table 1. Principal natural echinocandins, representative strains that produce them, acyl side chains, amino acids (AA) in positions 1-6 of peptide core, and accession numbers for genomes or gene clusters.

Naturally occurring echinocandins	**Representative strain**	**Class, Family classification**	**Acyl side chain**	**AA1**	**AA2**	**AA3**	**AA4**	**AA5**	**AA6**	**Genome or gene cluster accession**
Aculeophiarin (antibiotic S31794/F-1)	*Penicillium arenicola*	Eurotiomycetes, Aspergillaceae	myristic acid	4*R*,5*R*-dihydroxy-L-Orn	L-Thr	4*R*-hydroxy-L-Pro	3*S*,4*S*-dihydroxy-L-homoTyr	3*R*-hydroxy-L-Gln	3*S*-hydoxy-4S-methly-L-Pro	MN518690
Echinocandin B	*Aspergillus pachycristatus* NRRL 11440 (ATCC 58397)	Eurotiomycetes, Aspergillaceae	linoleic acid	4*R*,5*R*-dihydroxy-L-Orn	L-Thr	4*R*-hydroxy-L-Pro	3*S*,4*S*-dihydroxy-L-homoTyr	L-Thr	3*S*-hydoxy-4*S*-methly-L-Pro	JX421684
Mulundocandin	*Aspergillus mulundensis* DSMZ 5745	Eurotiomycetes, Aspergillaceae	12-methlymyristic acid	4*R*,5*R*-dihydroxy-L-Orn	L-Thr	4*R*-hydroxy-L-Pro	3*S*,4*S*-dihydroxy-L-homoTyr	L-Ser	3S-hydoxy-4S-methly-L-Pro	KP742486, PVWQ00000000
Aculeacin A	*Aspergillus aculeatus* ATCC 16872 (NRRL 5094)	Eurotiomycetes, Aspergillaceae	palmitic acid	4*R*,5*R*-dihydroxy-L-Orn	L-Thr	4*R*-hydroxy-L-Pro	3*S*,4*S*-dihydroxy-L-homoTyr	L-Thr	3*S*-hydoxy-4S-methly-L-Pro	JGI ATCC16872 v1.1

Pneumocandin	*Glarea lozoyensis* ATCC 20868	Leotiomycetes, Helotiaceae	10,12-dimethylmyristic acid	4*R*,5*R*-dihydroxy-L-Orn	L-Thr	4*R*-hydroxy-L-Pro	3*S*,4*S*-dihydroxy-L-homoTyr	3*R*-hydroxy-L-Gln	3*S*-hydoxy-4S-methly-L-Pro	ALVE00000000
Sporiofungin	*Pezicula radicicola* *NRRL 12192*	Leotiomycetes, Dermataceae	10,12-dimethylmyristic acid	4*R*,5*R*-dihydroxy-L-Orn	L-Ser	4*R*-hydroxy-L-Pro	3*S*-hydroxy-L-homoTyr	3*R*-hydroxy-L-Gln	3*S*-hydoxy-4S-methly-L-Pro	PDUO00000000
FR901379 (WF11899A)	*Coleophoma cylindrospora* FERM BP 6252	Leotiomycetes, Dermataceae	palmitic acid	4*R*,5*R*-dihydroxy-L-Orn	L-Thr	4*R*-hydroxy-L-Pro	3*S*, 7-dihydroxyl-L-homoTyr-7-O-sulfate	3*R*-hydroxy-L-Gln	3S-hydoxy-4S-methly-L-Pro	AB723722, AB720725, PDLN00000000
FR209602	*Coleophoma crateriformis* FERM BP 5796	Leotiomycetes, Dermataceae	palmitic acid	4*R*,5*R*-dihydroxy-L-Orn	L-Ser	4*R*-hydroxy-L-Pro	3*S*,4S,7-trihydroxyl-L-homoTyr-7-O-sulfate	3*R*-hydroxy-L-Gln	3S-hydoxy-4S-methly-L-Pro	AB720076, PDLN00000000
FR190293	*Venustampulla echinocandica* FERM BP 5553	Leotiomycetes, Pleuroascaceae	10,12-dimethylmyristic acid	4*R*,5*R*-dihydroxy-L-Orn	L-Thr	4*R*-hydroxy-L-Pro	3*S*, 7-dihydroxyl-L-homoTyr-7-O-sulfate	3*R*-hydroxy-L-Gln	4*R*,5*R*-dihydroxy-L-Orn	AB720726, NPIC00000000

TR227673[1]	*Chalara* sp.	Leotiomycetes, family unknown	12,14-dimethylpalmitic acid	4*R*,5*R*-dihydroxy-L-Orn	L-Thr	4*R*-hydroxy-L-Pro	3*S*, 7-dihydroxyl-L-homoTyr-7-O-sulfate	4*R*,5*R*-dihydroxy-L-Orn	None available

[1]Not studied. Included here for comparative purposes. See reference.

Table 2. Strains of *Penicillium arenicola* and *Phialomyces macrosporus* examined in this work and their geographic origin.

Species	**Strain number**	**Habitat**	**Geographic origin**
P. arenicola	NRRL 8095	Soil	British Columbia, Canada
P. arenicola	NRRL 3392[A]	Soil, pine forest	near Kyiv, Ukraine
P. arenicola	NRRL 31507	Mineral soil, under *Pinus resinosa*	Ontario, Canada
P. arenicola	NRRL 31509	Oil soaked soil	Norman Wells, Northwest Territories, Canada
Ph. macrosporus	IBT 31128[B]	Thermally heated soil	Rotorua, New Zealand
Ph. macrosporus	IBT 31129	Decaying needles of *Pinus luchensis*	Iriomoto-jima Island, Okinawa, Japan

[A]Ex-lectotype strain. [B]Ex-holotype strain.

Table 3. Sequence comparisons between proteins in the pneumocandin, echinocandin B, and acrophiarin gene clusters. See (Cacho et al., 2012; Li et al., 2015) for gene nomenclature.

Pneumocandin biosynthetic proteins	**Predicted function**	**Acrophiarin biosynthetic proteins**	**Coverage %; Identity %**	**Echinocandin B biosynthetic proteins**	**Coverage %; Identity %**
GLNRPS4	Non-ribosomal peptide synthetase	PANRPS4	98; 65	EcdA	98; 55
GLPKS4	Polyketide synthase	Absent	no	Absent	no
GLligase	AMP-dependent ligase	PALigase	100; 59	EcdI	98; 57
GLTRT	ABC transporter	PATRT	99; 60	EcdL	99; 51
GLHYD	Thioesterase	PAHYD	100; 57	Absent	no
GLP450-1	Cytochrome P450	PAP450-1	98; 57	HtyF	93; 52
GLP450-2	Cytochrome P450	PAP450-2	99; 55	EcdH	98; 49
GLOXY-1	Oxygenase	PAOXY-1	99; 67	EcdG	99; 56
GLOXY-2	Oxygenase	PAOXY-2	100; 69	HtyE	100; 64
GLOXY-3	Oxygenase	PAOXY-3	93; 66	Absent	No

GLOXY-4	Oxygenase	PAOXY-4	100; 71	EcdK	100; 62
GLHtyA	2-Isopropylmalate synthase	PAHtyA	82; 67	HtyA	93; 64
GLHtyB	D-Amino acid aminotransferase	PAHtyB	98; 74	HtyB	100; 64
GLHtyC	Isopropylmalate dehydrogenase	PAHtyC	98; 73	HtyC	99; 71
GLHtyD	3-Isopropylmalate dehydratase	PAHtyD	100; 65	HtyD	95; 62
GLHYP	Hypothetical protein	PAHYP	99; 38	EcdJ	56; 51

Figure Captions

Fig. 1. Biosynthetic pathways of echinocandin and fellutamide lipopeptides mentioned in this report. A. Acrophiarins. B. Fellutamides. The fellutamide pathway is redrawn from (Yeh et al., 2016).

Fig. 2. Agar diffusion assay of extracts of four strains of *P. arenicola* (NRRL 3392, 8095, 31507 and 31509) and two strains of *Ph*. *macrosporus* (IBT 31128, 31129) grown in five different fermentation media against *C. albicans* ATCC 10231 (left) and *C. neoformans* H99 (right). The extracts of each strain on each medium were arrayed in agar wells from left to right. Amphotericin B was the positive control (lower right corner). Assay wells are 4 mm in diam.

Fig. 3. A. EIC chromatograms of purified standards of acrophiarin, pneumocandin I and pneumocandin K obtained from *G. lozoyensis* (+ESI, *m/z* 900-1250). The chromatogram obtained for *P. arenicola* NRRL 3392 in medium VI was selected among crude extract chromatograms as an example of acrophiarin and related pneumocandins in crude extracts. B. MS fragments observed for: 1. Peak with retention time 16.02 minutes; 2. Purified acrophiarin from *G. lozoyensis*; 3. Peak with retention time 17.30 minutes; 4. Purified pneumocandin I from *G. lozoyensis*; 5. Peak with retention time 18.66 minutes; 6. Purified pneumocandin K from *G. lozoyensis*. See Chen et al. 2016 for production of standards.

Fig. 4. A. LC-MS analysis of fractions 6 and 7 showing the presence of several pneumocandin analogs. B. MS fragmentation observed for unidentified acrophiarin analogs 1-5.

Fig. 5. Graphic representation of the echinocandin-type gene clusters and their microsynteny. Acrophiarin gene cluster from *Penicilium arenicola* NRRL 8095, echinocandin B gene cluster from *Aspergillus pachycristatus* NRRL 11440, and pneumocandin gene cluster from *Glarea lozoyensis*. Gene functions are color coded. The intensity of the red scale bar indicates the degree of nucleotide similarity.

Fig. 6. Phylogenetic analysis of the echinocandin and fellutamide NRPS A domains. Evolutionary analyses were conducted in MEGA 7.0 by using the maximum Likelihood method based on the JTT matrix-based model. The tree is rooted with A domains of emericellamide synthetase (EasA). The corresponding amino acids that are activated by each subclade of A domains are indicated to the right. Numbers at nodes are likelihood bootstrap support values.

Fig. 7. Abbreviated trees for maximum likelihood phylogenies inferred from genes in the echinocandin biosynthetic gene clusters. Maximum likelihood phylogeny was inferred from each of the echinocandin biosynthetic-related genes. Each of the echinocandin biosynthetic-related genes forms a monophyletic lineage. Eight of thirteen *P*. *arenicola* acrophiarin biosynthetic genes group with genes *Leotiomycete*-type echinocandins. (A) Phylogeny of acyl-AMP and AMP-dependent ligases. (B, G, H and L) Phylogeny of nonheme iron, α-ketoglutarate-dependent oxygenases. (C and I) Phylogeny of cytochrome P450s. D) Phylogeny of 2-isopropylmalate synthases. E) Phylogeny of isopropylmalate dehydrogenases. F) Phylogeny of ABC transporters. J) Phylogeny of D-amino acid aminotransferases. K) Phylogeny of isopropylmalate dehydratases. M) Phylogeny of thioesterases. Numbers at nodes are likelihood support values.

Fig. 8. Maximum likelihood phylogenies of fungal species and the enzymes of the echinocandin pathway. (A) Phylogenetic reconstruction of the echinocandin producing fungi and other *Ascomycetes* using maximum likelihood analysis of a six-gene dataset consisting of DNA fragments of the 18S rRNA gene, the 28S rRNA gene, the ITS RNA region, the β-tubulin gene, the translation elongation factor 1-α gene, and the RNA polymerase II subunit 2 gene. (B) Consensus phylogenetic pattern for gene tree for 10 shared enzymes of all echinocandin pathways (left) and the established phylogenetic tree extracted from the data in panel A. Trees are rooted at the midpoint. Numbers at nodes are likelihood support values. The 10-cluster enzyme tree significantly conflicted with the species phylogeny tree (Shimodaira-Hasegawa test, $P = 0$; weighted Shimodaira-Hasegawa test, $P = 0$). Dotted lines are aids to connect branch tips to strain name.

Fig. 9. Zone of inhibition assays for evaluation of echinocandin susceptibility of *P*. *arenicola* (NRRL 8095, NRRL 31507 and NRRL 3392), *G*. *lozoyensis* (ATCC 20868), *A*. *pachycristatus* (NRRL 11440) and *C*. *albicans* (ATCC 10231). 1. Acrophiarin (250 μg/ml). 2. Pneumocandin B_0 (250 μg/ml). 3. Echinocandin B (250 μg/ml). 4. Amphotericin B (250 μg/ml). 5. DMSO.

Fig. 10. Evolutionary hypothesis for the famility of echinocandin and fellutamide biosynthetic gene clusters. Possible ancestral elements observed in the Aspergillaceae include adenylation domains on the NRPSs, an acyl-CoA ligase of the fellutamide and echinocandin pathways and their orthologs, and the Hty pathway. The origin of the Hty pathway is unknown, and it is illustrated separately from the core NRPS of the echinocandins. In the Leotiomycete lineage, a mutation in A domain 5 leads to incorporation of glutamine in position 5, and an oxygenase for glutamine hydroxylation (OXY3) and a thioesterase (HYD) were recruited. A highly reducing PKS and a presumed aryl sulfotransferase (TS) were recruited in some species of the Leotiomycetes. An arrow indicates the possible origin of the acrophiarin gene cluster in *Penicilium arenicola* by

horizontal transfer (HGT) from Leotiomycetes. Date of estimated divergence of Leotiomycete-Eurotiomycete lineages is from (Lutzoni et al., 2018).

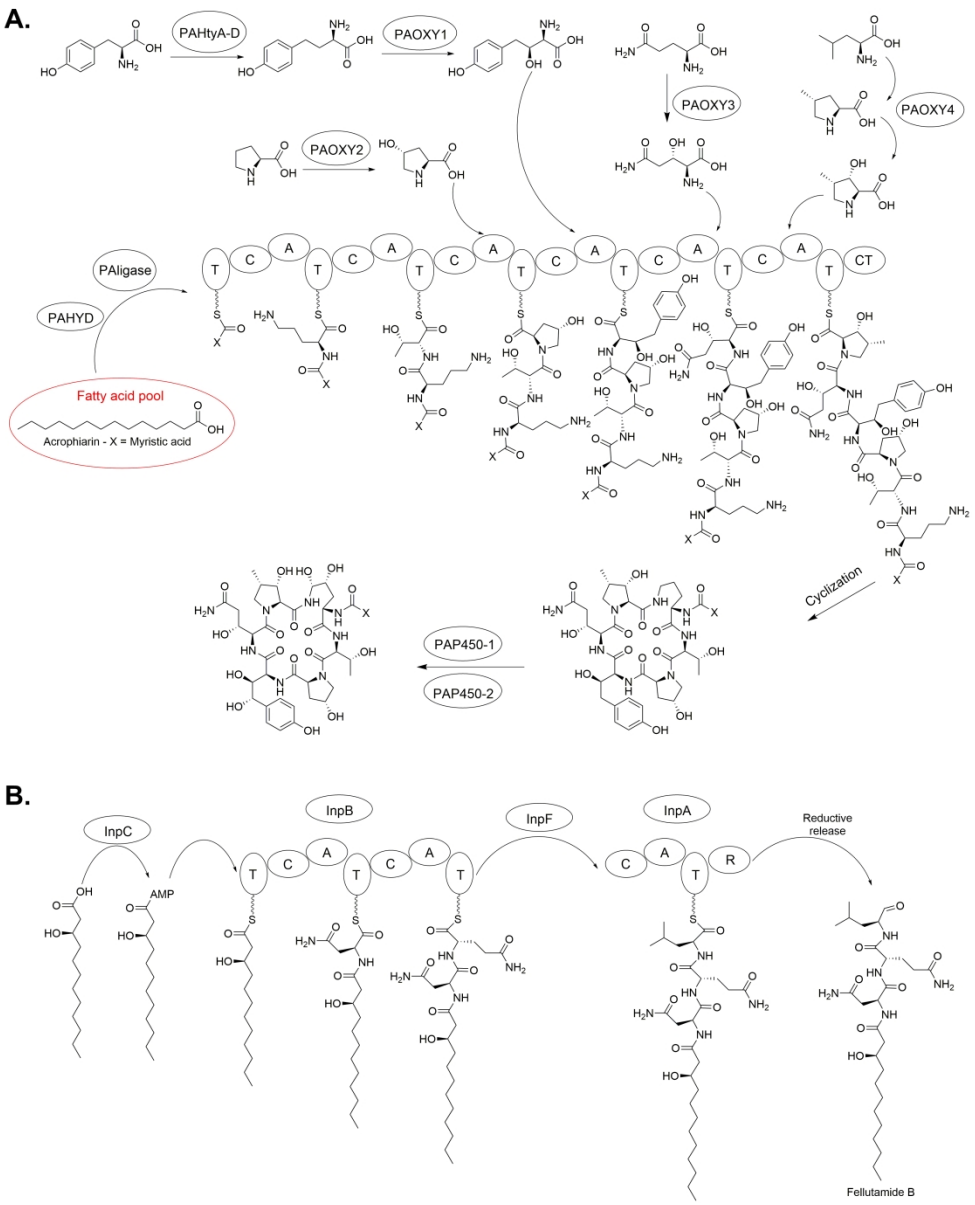

Fig. 1. Biosynthetic pathways of echinocandin and fellutamide lipopeptides mentioned in this report. A. Acrophiarins. B. Fellutamides. The fellutamide pathway is redrawn from (Yeh et al., 2016).

307x380mm (300 x 300 DPI)

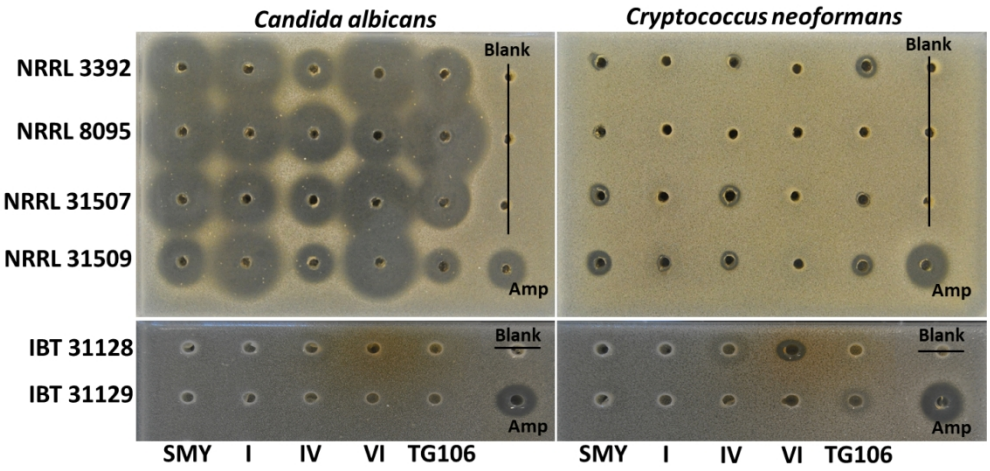

Fig. 2. Agar diffusion assay of extracts of four strains of P. arenicola (NRRL 3392, 8095, 31507 and 31509) and two strains of Ph. macrosporus (IBT 31128, 31129) grown in five different fermentation media against C. albicans ATCC 10231 (left) and C. neoformans H99 (right). The extracts of each strain on each medium were arrayed in agar wells from left to right. Amphotericin B was the positive control (lower right corner). Assay wells are 4 mm in diam.

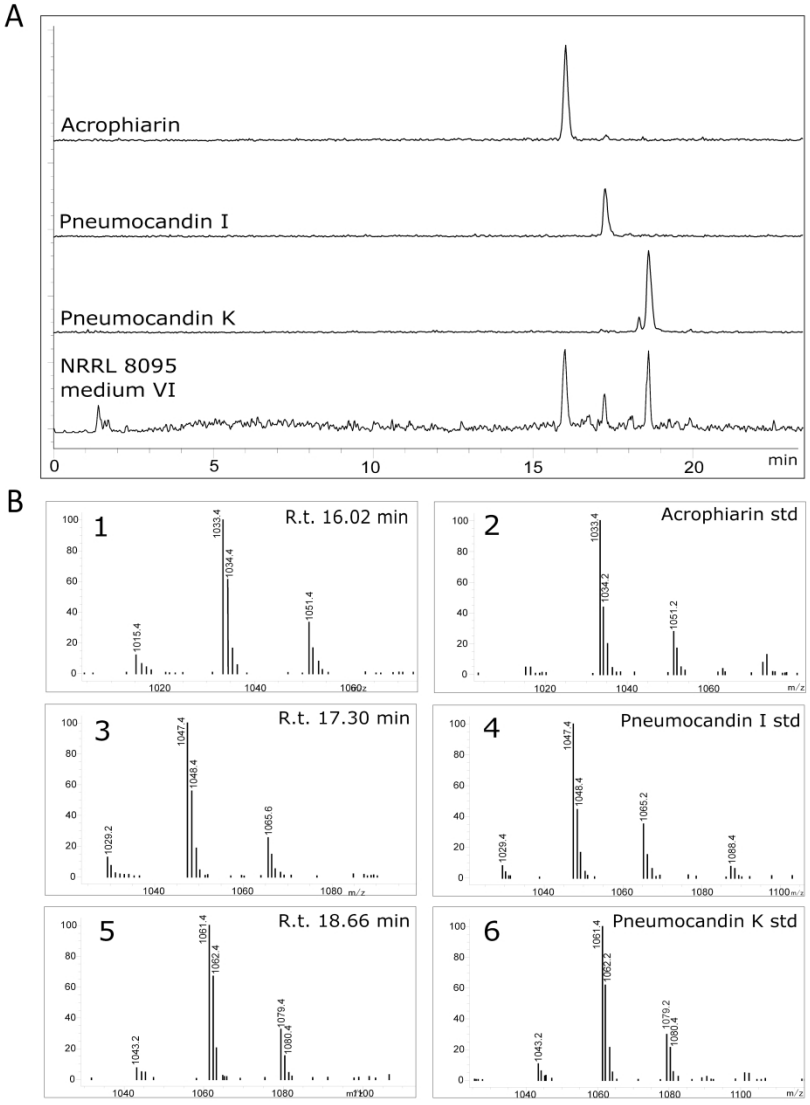

Fig. 3. A. EIC chromatograms of purified standards of acrophiarin, pneumocandin I and pneumocandin K obtained from G. lozoyensis (+ESI, m/z 900-1250). The chromatogram obtained for P. arenicola NRRL 3392 in medium VI was selected among crude extract chromatograms as an example of acrophiarin and related pneumocandins in crude extracts. B. MS fragments observed for: 1. Peak with retention time 16.02 minutes; 2. Purified acrophiarin from G. lozoyensis; 3. Peak with retention time 17.30 minutes; 4. Purified pneumocandin I from G. lozoyensis; 5. Peak with retention time 18.66 minutes; 6. Purified pneumocandin K from G. lozoyensis. See Chen et al. 2016 for production of standards.

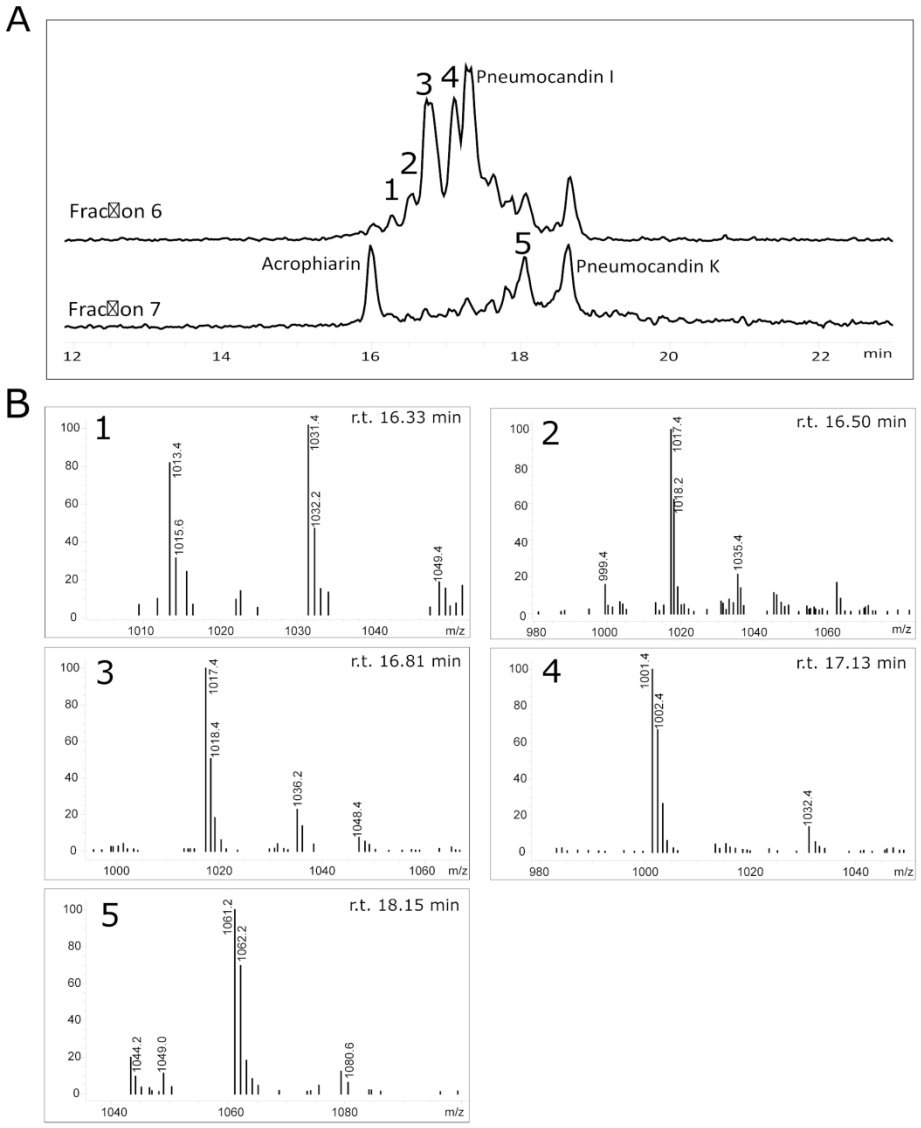

Fig. 4. A. LC-MS analysis of fractions 6 and 7 showing the presence of several pneumocandin analogs. B. MS fragmentation observed for unidentified acrophiarin analogs 1-5.

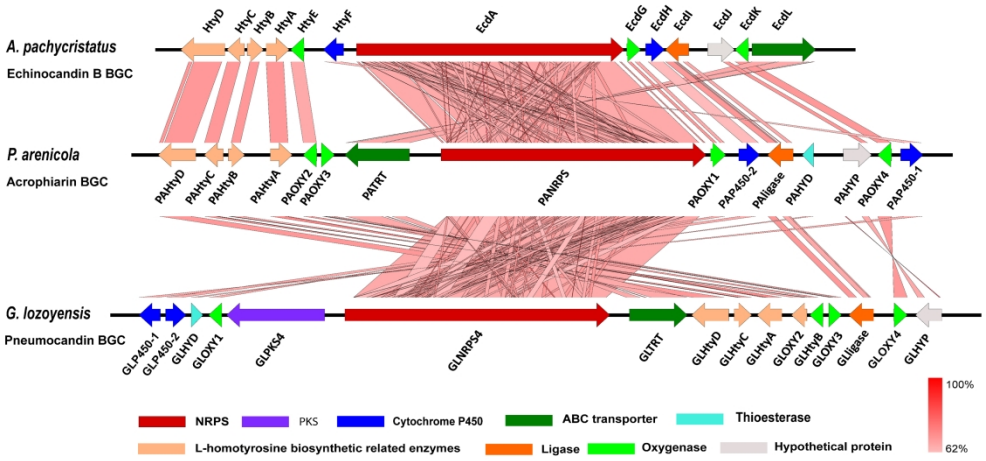

Fig. 5. Graphic representation of the echinocandin-type gene clusters and their microsynteny. Acrophiarin gene cluster from Penicilium arenicola NRRL 8095, echinocandin B gene cluster from Aspergillus pachycristatus NRRL 11440, and pneumocandin gene cluster from Glarea lozoyensis. Gene functions are color coded. The intensity of the red scale bar indicates the degree of nucleotide similarity.

338x190mm (300 x 300 DPI)

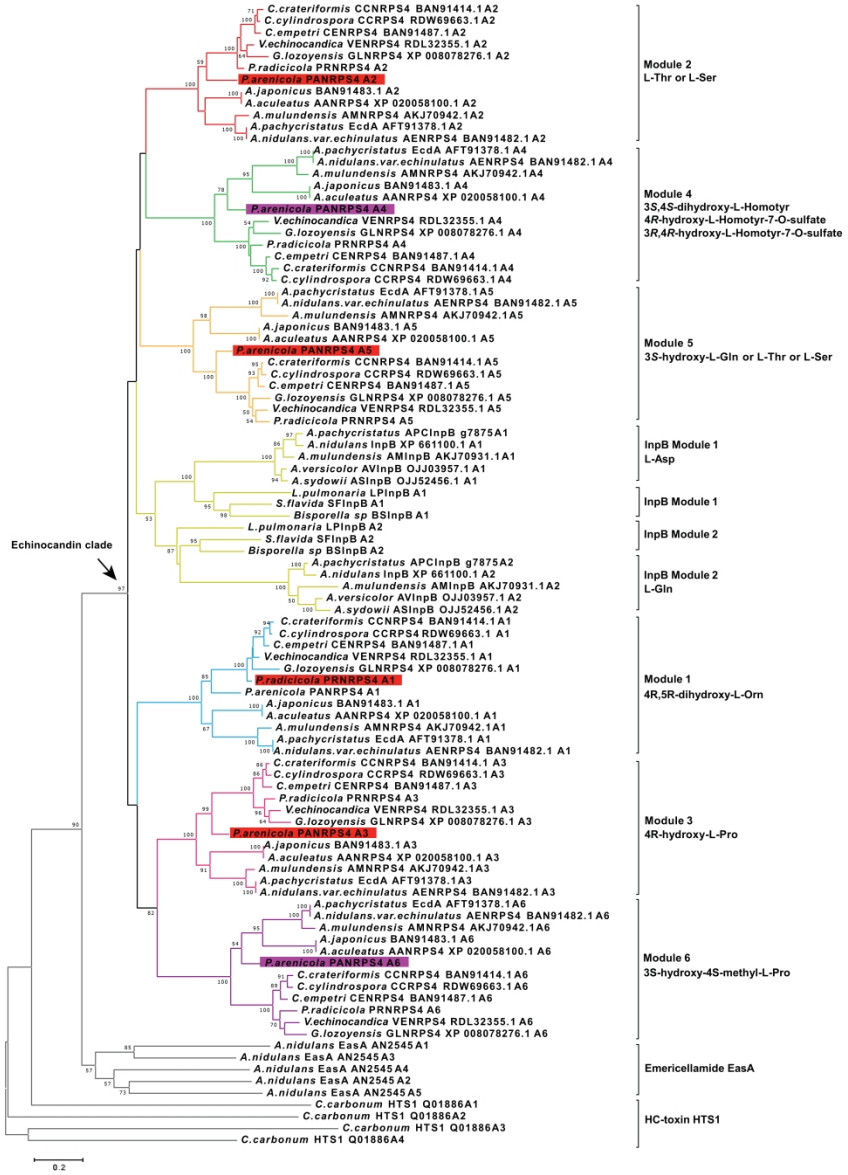

Fig. 6. Phylogenetic analysis of the echinocandin and fellutamide NRPS A domains. Evolutionary analyses were conducted in MEGA 7.0 by using the maximum Likelihood method based on the JTT matrix-based model. The tree is rooted with A domains of emericellamide synthetase (EasA). The corresponding amino acids that are activated by each subclade of A domains are indicated to the right. Numbers at nodes are likelihood bootstrap support values.

415x558mm (300 x 300 DPI)

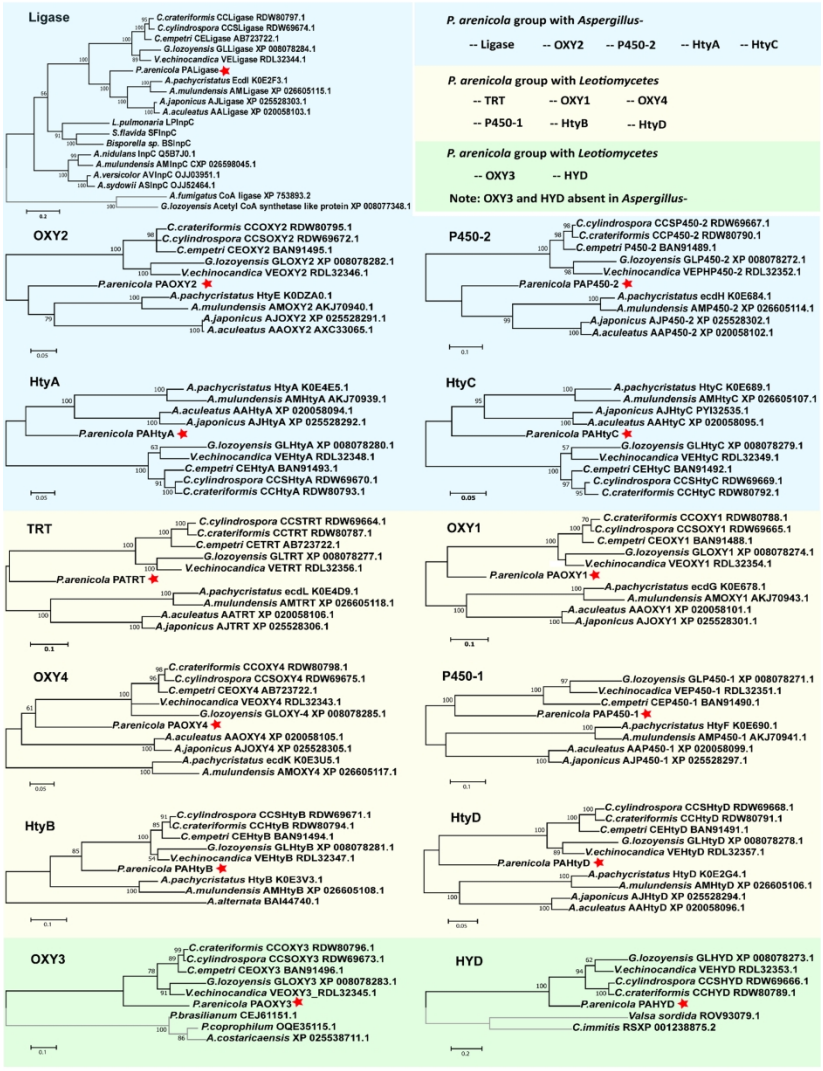

Fig. 7. Abbreviated trees for maximum likelihood phylogenies inferred from genes in the echinocandin biosynthetic gene clusters. Maximum likelihood phylogeny was inferred from each of the echinocandin biosynthetic-related genes. Each of the echinocandin biosynthetic-related genes forms a monophyletic lineage. Eight of thirteen P. arenicola acrophiarin biosynthetic genes group with genes Leotiomycete-type echinocandins. (A) Phylogeny of acyl-AMP and AMP-dependent ligases. (B, G, H and L) Phylogeny of nonheme iron, α-ketoglutarate-dependent oxygenases. (C and I) Phylogeny of cytochrome P450s. D) Phylogeny of 2-isopropylmalate synthases. E) Phylogeny of isopropylmalate dehydrogenases. F) Phylogeny of ABC transporters. J) Phylogeny of D-amino acid aminotransferases. K) Phylogeny of isopropylmalate dehydratases. M) Phylogeny of thioesterases. Numbers at nodes are likelihood support values.

190x279mm (300 x 300 DPI)

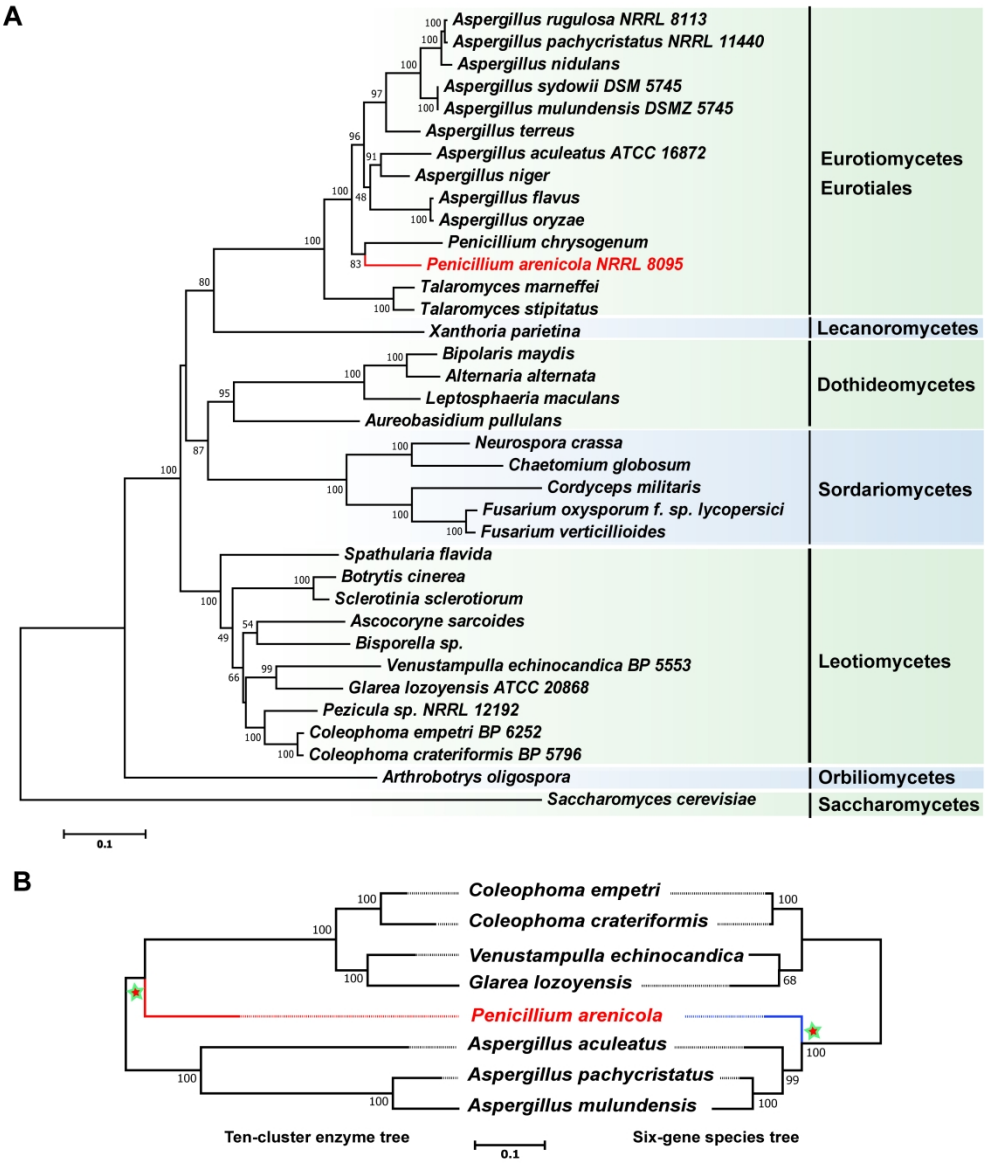

Fig. 8. Maximum likelihood phylogenies of fungal species and the enzymes of the echinocandin pathway. (A) Phylogenetic reconstruction of the echinocandin producing fungi and other Ascomycetes using maximum likelihood analysis of a six-gene dataset consisting of DNA fragments of the 18S rRNA gene, the 28S rRNA gene, the ITS RNA region, the β-tubulin gene, the translation elongation factor 1-α gene, and the RNA polymerase II subunit 2 gene. (B) Consensus phylogenetic pattern for gene tree for 10 shared enzymes of all echinocandin pathways (left) and the established phylogenetic tree extracted from the data in panel A. Trees are rooted at the midpoint. Numbers at nodes are likelihood support values. The 10-cluster enzyme tree significantly conflicted with the species phylogeny tree (Shimodaira-Hasegawa test, P = 0; weighted Shimodaira-Hasegawa test, P = 0). Dotted lines are aids to connect branch tips to strain name.

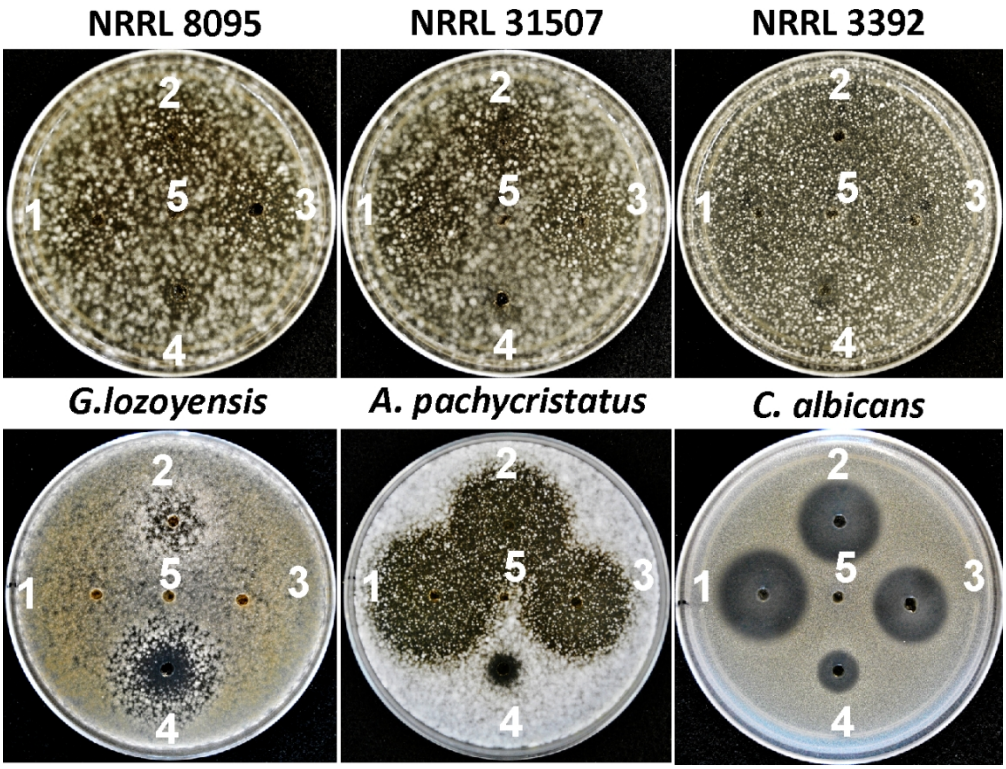

Fig. 9. Zone of inhibition assays for evaluation of echinocandin susceptibility of P. arenicola (NRRL 8095, NRRL 31507 and NRRL 3392), G. lozoyensis (ATCC 20868), A. pachycristatus (NRRL 11440) and C. albicans (ATCC 10231). 1. Acrophiarin (250 µg/ml). 2. Pneumocandin B0 (250 µg/ml). 3. Echinocandin B (250 µg/ml). 4. Amphotericin B (250 µg/ml). 5. DMSO.

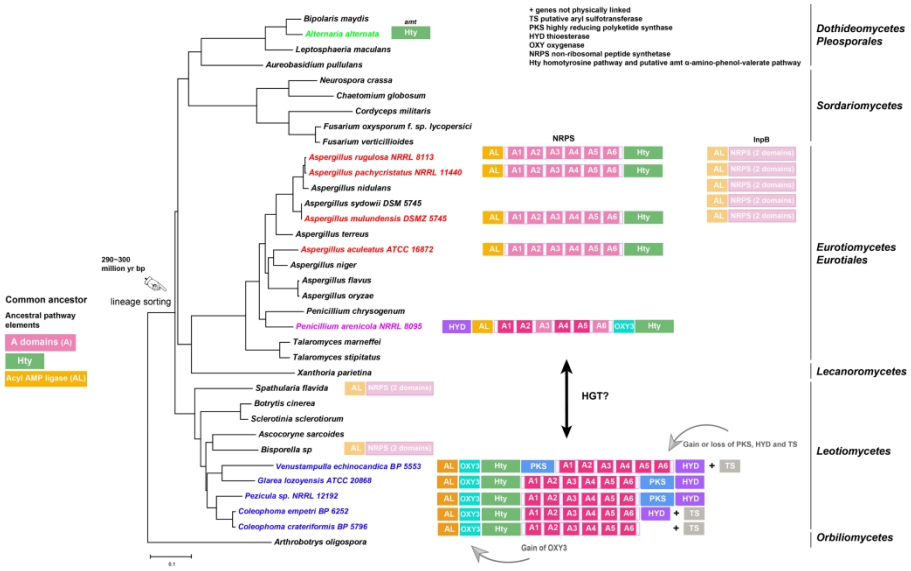

Fig. 10. Evolutionary hypothesis for the famility of echinocandin and fellutamide biosynthetic gene clusters. Possible ancestral elements observed in the Aspergillaceae include adenylation domains on the NRPSs, an acyl-CoA ligase of the fellutamide and echinocandin pathways and their orthologs, and the Hty pathway. The origin of the Hty pathway is unknown, and it is illustrated separately from the core NRPS of the echinocandins. In the Leotiomycete lineage, a mutation in A domain 5 leads to incorporation of glutamine in position 5, and an oxygenase for glutamine hydroxylation (OXY3) and a thioesterase (HYD) were recruited. A highly reducing PKS and a presumed aryl sulfotransferase (TS) were recruited in some species of the Leotiomycetes. An arrow indicates the possible origin of the acrophiarin gene cluster in Penicilium arenicola by horizontal transfer (HGT) from Leotiomycetes. Date of estimated divergence of Leotiomycete-Eurotiomycete lineages is from (Lutzoni et al., 2018).

338x190mm (300 x 300 DPI)