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Pore-forming aegerolysin and MACPF proteins in extremotolerant or extremophilic fungi

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Abstract

Aegerolysin proteins are involved in various interactions by recognising a molecular receptor in the target organism. The formation of pores in combination with larger, non-aegerolysin-like protein partners (such as membrane attack complex/perforin proteins [MACPFs]) is one of the possible responses in the presumed competitive exclusion of other organisms from the ecological niche. Bicomponent pairs are already observed at the gene level. Fungi growing under extreme conditions can be divided into ubiquitous and extremotolerant generalists which can compete with mesophilic species and rare, isolated extremophilic and extremotolerant specialists with narrow ecological amplitude that cannot compete. Under extreme conditions, there are fewer competitors, so fungal specialists generally produce less diverse and complicated profiles of specialised molecules. Since extremotolerant and extremophilic fungi have evolved in numerous branches of the fungal tree of life and aegerolysins are unevenly distributed across fungal genomes, we investigated whether aegerolysins, together with their partner proteins, contribute to the extreme survival ecology of generalists and specialists. We compiled a list of 109 thermo-, psihro-, acido-, alkali-, halo-, metallo- and polyextremo-tolerant/-philic fungal species. Several challenges were identified that affected the outcome: renaming fungal species, defining extremotolerant/extremophilic traits, identifying extremotolerant/extremophilic traits as metadata in databases and linking fungal isolates to fungal genomes. The yield of genomes coding aegerolysins or MACPFs appears to be lower in extremotolerant/extremophilic fungi compared to all fungal genomes. No candidates for pore-forming gene pairs were identified in the genomes of extremophilic fungi. Aegerolysin and MACPFs partner pairs were identified in only two of 69 species with sequenced genomes, namely in the ubiquitous metallotolerant generalists *Aspergillus niger* and *A. foetidus*. These results support the hypothesised role of these pore-forming proteins in competitive exclusion.

KEYWORDS
aegerolysins, competitive exclusion, extremophilic, extremotolerant, fungi, membrane attack complex/perforin (MACPF) domain, pore-forming proteins

1 | INTRODUCTION

Aegerolysins share some common features: low molecular weights (15–20 kDa), low identity protein sequences, low isoelectric points, stability in a wide pH range, the same protein domain (PF06355) and β-sandwich folded protein structure. Despite their occurrence in cells of certain developmental stages and their presence in secretomes, only a few aegerolysins have been studied in detail. These remarkable properties were revealed by a limited published (experimental) dataset of 23 different aegerolysins and their variants identified in 18 different species.[1] Among fungi, these 12 species belong to the Agaricales (*Pleurotus ostreatus*, *P. eryngii*, *Agrocybe aegerita* and *Moniliophthora perniciosa*) and the Polyporales (*Lignosus rhinocerotis*) in Agaricomycotina; to the Eurotiales (*Aspergillus fumigatus*, *A. niger*, *A. terreus* and *A. oryzae*) in Eurotimycetes; to the Hypocreales (*Beauveria bassiana* and *Trichoderma atroviride*) in Sordariomycetes, and to the Pleosporales (*Alternaria gaisen*) in Dothideomycetes. The other six species belong to Bacteria, Insecta or Viria.

Perhaps more is known about the use of aegerolysins than about their biological function. Most commonly, some fungal aegerolysins serve as probes for the detection, labelling and imaging of specific membrane lipids, lipid rafts, cancer cells, invertebrates or parasites. Their genes and their expression or antibodies produced against aegerolysins can serve as biomarkers or immunodiagnostic tools for the progression of fruiting body differentiation, exposure to fungal pathogens or the progression of infectious diseases. In combination with larger protein partners, some of them can form pore-forming complexes that can be used to selectively eliminate insect pests or to treat certain types of cancer cells.[2,3]

However, the biological function of aegerolysins is intriguing. They are involved in various interactions by recognising a molecular receptor (mostly lipids) in the target organism. The formation of pores in combination with larger, non-aegerolysin-like protein partners is one of the possible responses in the presumed competitive exclusion of other organisms from the ecological niche. Unexpectedly, structural models showed that aegerolysins dock five groups of larger partner proteins.[1] The two groups of larger partner proteins identified in fungal species are in the focus of this study: membrane attack complex/perforin proteins (MACPF)(i) and MACPF(ii). Proteins containing a MACPF domain are transmembrane pore-forming proteins that are important for both human immunity and virulence of pathogens. In fungi, MACPF domain-containing proteins probably contribute to several specific processes. The number of MACPF domain proteins can range from 0 to 10 or more. However, the identification and annotation of putative MACPF domain genes is generally quite error-prone due to a higher number of introns.[4]

MACPF(i) partner proteins such as pleurotolysin PlyB and erylysin EryB have a MACPF domain (PF01823) confirmed by the 13-amino acid signature Y/F-G-X_2-F/Y-X_6-G-G. The membrane-embedded pleurotolysin PlyA/PlyB pore with 13-fold symmetry from the saprotrophic and nematocidal white-rot fungus *P. ostreatus* is the only pore-forming complex that has been elucidated besides the two monomers. The heteromeric structure of the pore (PDB ID: 4V2T) consists of 26 PlyA and 13 PlyB molecules.[5] The highly identical proteins ostreolysin OlyA6 and pleurotolysin PlyA2 from *P. ostreatus* and from saprotrophic grassland-litter decomposer, facultatively biotrophic and nematocidal *P. eryngii* are the best studied aegerolysins.[6,7] *Pleurotus* aegerolysins interact with membrane domains enriched with sphingomyelin (SM) in combination with cholesterol (Chol). However, their high-affinity lipid receptor is also ceramide phosphoethanolamine (CPE), an analogue of SM and the major membrane sphingolipid of invertebrates. The pore-forming protein complexes, which consist of the *Pleurotus* aegerolysins ostreolysin OlyA6, pleurotolysin PlyA2 or erylysin EryA in combination with the MACPF(i) partner pleurotolysin PlyB, can kill plant pests such as the western corn rootworm (*Diabrotica virgifera virgifera*) and the Colorado potato beetle (*Leptinotarsa decemlineata*) by targeting CPE in the midgut of the pest's.[6,7]

However, the sequences of MACPF(ii), such as nigerolysin NigB1, Asp-hemolysin partner protein Asp-HSB, beauveriolysin BlyB and *Alternaria geisen* aegerolysin partner protein L152B, where the MACPF domain is not recognised by the algorithm, also contain the same MACPF/CDC signature.[1,8] The structural models of the MACPF(ii) proteins from the saprotrophic and human pathogen *A. fumigatus* (Asp-HSB), the entomopathogen and endophyte *B. bassiana* (BlyB), the plant pathogen *A. gaisen* (L152B) and the saprotrophic and human opportunistic pathogen *A. niger* (NigB1) show the best alignments to the structure of the insecticidal protein

GNIP1Aa.[1] This protein from the Proteobacteria *Chromobacterium piscinae* is specifically toxic to the larvae of *D. virgifera virgifera* when ingested.[9]

Fungi develop a variety of ecological strategies based on three main factors, stress, disturbance and the presence of competitors, or combination of these factors.[10] Ruderal fungi compete by being pioneers and having a fast growth rate; stress-tolerant fungi have adapted to life under more extreme ecological conditions. Combative fungi compete and cooperate by producing specialised molecules. Antagonistic mechanisms utilised by combative fungi to attack or defend against competitors include morphological changes, production of enzymes and toxins, detoxification of competitor toxins and alteration of metabolic rate.[10] Some molecules are only biosynthesised by a selected subset of organisms and/or are specific to certain ecological niches, which are described by the terms secondary or specialised metabolites. Secondary metabolites, in contrast to primary metabolites, are not essential for growth and development but are often important for the survival of organisms in their environment.[11] Specialised metabolites have a limited clade-specific or niche-specific distribution and play a particular role in ecology or physiology.[11] As stress-selected extremophilic and extremotolerant fungal species that thrive under extreme conditions have few competitors, they generally produce less diverse and complicated profiles of specialised metabolites, specialised proteins and specialised exopolysaccharides.[12]

Extremophilic and extremotolerant fungi have evolved multiple times, they are found in different branches of the fungal tree of life and their distribution is not uniform. In fact, extremotolerant and extremophilic fungi share some common adaptations, despite the phylogenetic distance between these groups and the different challenges in the various extreme environments.[13] Since aegerolysins and MACPFs are also unevenly distributed among fungal species, it is of interest to investigate whether aegerolysins and their partners contribute to the extreme survival ecology of generalists or specialists. If aegerolysins and their partners belong to the specialised proteins involved in the process of competitive exclusion and they *do not* contribute to the extreme survival ecology of extremophilic fungi and extremotolerant fungal specialists; the amount of these proteins will be lower in extremotolerant and extremophilic fungi than in average fungal species.

2 | METHODS

After identifying the extremotolerant and extremophilic fungi from the relevant literature and from a database, the current names of the species were determined together with the taxonomic lineage (Section 2.1). The fungal genome database was searched for available genome sequences (Section 2.2), and genes coding for aegerolysins (PF6355) and MACPFs (PF1823) were identified (Section 2.3). The aegerolysin gene loci were analysed for the presence of MACPFs (Section 2.4). Phylogenetic trees were inferred in comparison to previously described proteins (Section 2.5). Protein structure models were predicted and compared with existing protein structures and models (Section 2.6).

2.1 | Identification of extremotolerant and extremophilic fungal species

We searched for fungal species adapted to extreme conditions based on pH tolerance, temperature, solute concentration, heavy metal concentration or a combination of extreme conditions. To identify extremotolerant and extremophilic fungal species, a none exhaustive literature search was combined with the prefix of the species name thermo-, psychro-, acido-, alkali-, halo-, metallo-, extemo- or the metadata from the fungal genome database JGI MycoCosm.[13–22] A species was defined as polyextremophilous or extremotolerant if it was already described as such in the literature or if it exhibited two (or more) different extremes. The identified extremotolerant and extremophilic fungal species were named and taxonomically classified according to the NCBI Taxonomy website.[23]

2.2 | Determination of the available genome sequences

The fungal genome database JGI MycoCosm was searched for available genomes for the identified species; sometimes several strains were sequenced.[22]

2.3 | Determination of aegerolysins and MACPFs in the fungal genome database

To identify aegerolysins, the fungal genome database JGI MycoCosm was searched for the protein domain PF06355 using the PFAM terms search.[22] To identify MACPFs in these fungal species, the same genome database was searched for the protein domain PF01823 using the PFAM terms search. A cumulative table was generated containing the following: current names of the fungal species, other names for the same species if available, taxonomic lineages, extremotolerant or extremophilic types,

identified aegerolysins (PF6355) and MACPFs (PF1823) and sequenced genomes of fungal species with references (Table S1).

2.4 | Analysis of gene loci of strains encoding both proteins

The presence of MACPF(i) or MACPF(ii) gene pairs was identified by manual inspection of aegerolysin-containing contigs for larger high intron genes at a distance of ±10 kb from the start or stop codon of the aegerolysins, which have bi-directional 5′–5′ orientation.[24,25] The identified MACPF(i) or MACPF(ii) genes were confirmed by the presence of the typical MACPF signature.

2.5 | Construction of the phylogenetic tree for aegerolysins or aegerolysin partner proteins and comparison with known ones

Phylogenetic analysis of the aegerolysins and MACPFs aligned using the Muscle algorithm and inferred using the Maximum Likelihood method was performed using Molecular Evolutionary Genetics Analysis, version 11 (MEGA11).[26,27] The logo for the characteristic protein signature was created using the WebLogo web tool.[28] To determine identity, protein sequences were aligned using the ClustalW web tool.[29]

2.6 | Protein structure models and their comparison

The deep learning algorithm AlphaFold2 was used to model protein structures.[30] UCSF ChimeraX version 1.7 (22 February 2024) was used to run AlphaFold2 in conjunction with a free and accessible platform for protein folding ColabFold.[31,32] Some of the AlphaFold2 models were predicted on the HPC cluster ARC (National Institute of Chemistry, Slovenia) running ColabFold suite by using custom open-source scripts available at https://github.com/ajasja/af2slurm.[32,33] For structure models visualisation, cartoon representation of the structures and models was performed using PyMOL, version 2.2.0.[34]

3 | RESULTS

3.1 | Uneven distribution of aegerolysins in fungal genomes

Of the total of 2527 genomes available in the fungal genome database JGI MycoCosm (as of August 2023), the highest number of aegerolysin-containing genes was encoded by four fungal taxonomic categories, which also contain the highest number of genomes: Eurotiomycetes (345 aegerolysins), Sordariomycetes (157 aegerolysins), Agaricomycetes (99 aegerolysins) and Dothideomycetes (43 aegerolysins), which were identified in 76%, 28% and twice 19% of the sequenced genomes for each of listed taxonomic category, respectively (Figure 1). On average, there were 2.4 aegerolysins per Agaricomycetes species encoding aegerolysins, 1.7 aegerolysins per Eurotiomycetes species, 1.2 aegerolysins per Sordariomycetes species and 1 aegerolysin per Dothideomycetes species encoding aegerolysins.

3.2 | Uneven distribution of MACPF(i)s in fungal genomes

The highest number of MACPF(i)s belongs to the same four taxonomic categories, which also contain the highest number of aegerolysins and the highest number of genomes: Eurotiomycetes (219 MACPF[i]s), Sordariomycetes (207 MACPF[i]s), Agaricomycetes (105 MACPF[i]s) and Dothideomycetes (26 MACPF[i]s), but they were encoded in different proportions of the sequenced genomes for each of the listed taxonomic categories: 46%, 37%, 20% and 12%, respectively (Figure 2). On average, there was the highest number of MACPF(i) per Agaricomycetes species encoding MACPF(i)s (4.2 MACPF[i]s), followed by 2 MACPF(i)s per Eurotiomycetes species, 1.4 per Dothideomycetes species and 1.2 per Sordariomycetes species encoding MACPF(i)s.

3.3 | Fungal extremotolerant and extremophilic species

The final list comprised 109 identified extremotolerant and extremophilic species (Table S1). Most of these species were thermotolerant and thermophilic (35 species, 32%), followed by halotolerant and halophilic (21 species, 19%), metallotolerant and metallophilic as well as polyextremotolerant and polyextremophilic (13 species, 12%), alkalitolerant and alkaliphilic (12 species, 11%), acidotolerant and acidophilic (8 species, 7%) and psychrotolerant and psychrophilic species (7 species, 6%) (Figure 3). Most of them, belong to thermophilic species,[31] none of psychrotolerant species were identified. There was no obvious correlation between extreme environmental conditions and the distribution of aegerolysins or MACPFs. Most of the aegerolysins were found in metallotolerant species (11 proteins), 4 in alkalitolerant, 3 in thermophilic or halotolerant and 1 in acidophilic or polyextremophilic species. Most MACPFs were found in alkalitolerant species (21 proteins) and 2 also in thermophilic, metallotolerant or polyextremotolerant and 1 in thermotolerant species.

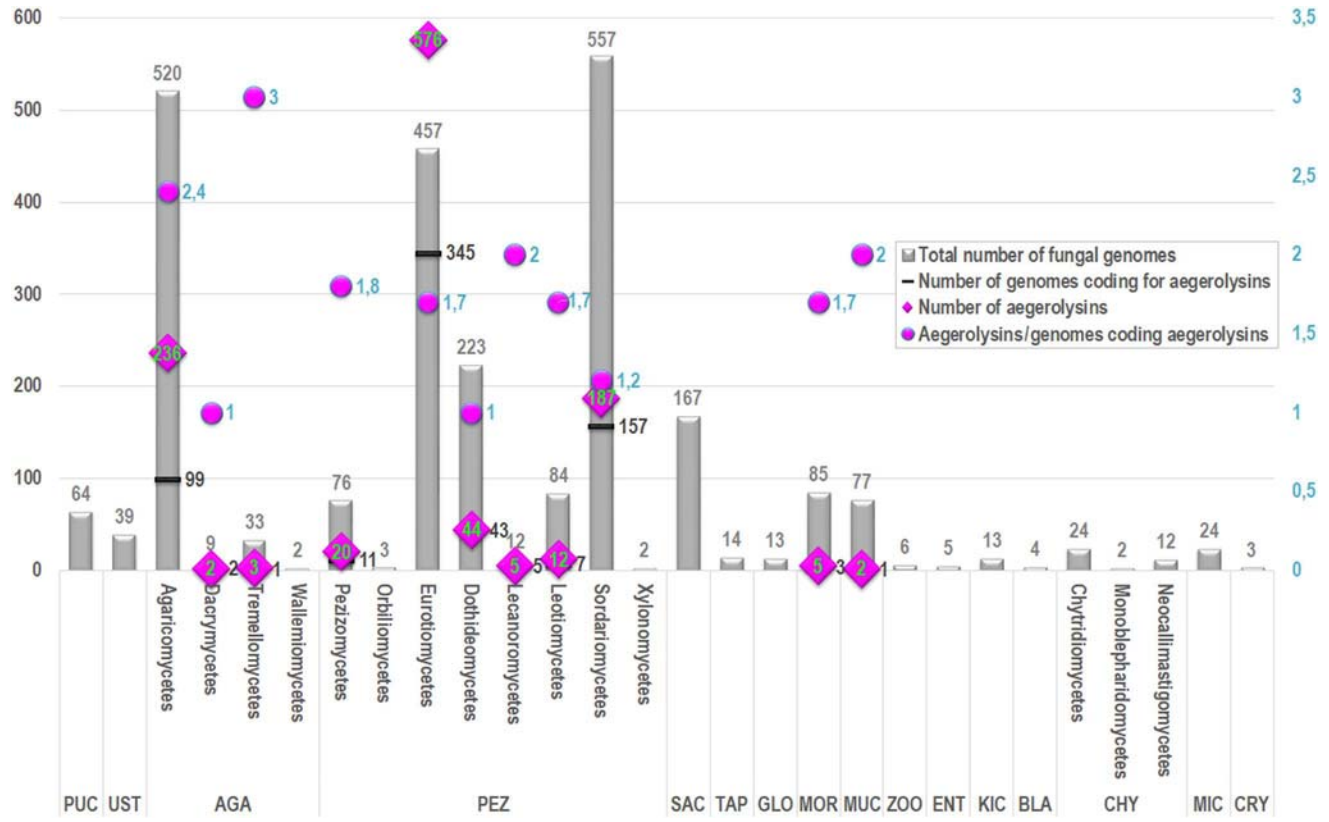

FIGURE 1 Number of aegerolysins in fungal genomes of different taxonomic categories. Basidiomycota: PUC, Pucciniomycotina; UST, Ustilaginomycotina; AGA, Agaricomycotina. Ascomycota: PEZ, Pezizomycotina; SAC, Saccharomycotina; TAP, Taphrinomycotina. Mucoromycota: GLO, Glomeromycotina; MOR, Mortierellomycotina; MUC, Mucoromycotina. Zoopagomycota: ZOO, Zoopagomycotina; ENT, Entomophthoromycotina; KIC, Kickxellomycotina. BLA, Blastocladiomycota. CHY, Chytridiomycota. MIC, Microsporidia. CRY, Cryptomycota. Data from the fungal genome database JGI MycoCosm.[22]

In the public fungal genome database MycoCosm (JGI), no genome was available for 40 of the identified extremotolerant and extremophilic species (37%). In total, there were 69 species with genomes (63%); of these species, about half were described as extremophilic (37 species, 54%) and the other half as extremotolerant (32 species, 46%) (Figure 3). A total of 127 genomes were identified, as several species have more sequenced strains (Table S1). For example, 17 genomes were available for *Fusarium oxysporum* strains, 11 for *Yarrowia lipolytica* strains and 6 for *A. niger* and *Saccharomyces cerevisiae* strains. For most species (52, 75%), only one genome was available. However, it is not necessary that any of the sequenced strains was isolated from the extreme environment.

3.4 | Taxonomic distribution of extremophilic and extremotolerant species in fungi

Most of extremophilic and extremotolerant species were found in the Sordariomycetes (30 species), followed by Eurotiomycetes (28 species), Dothideomycetes (15 species) and Saccharomycetes (12 species). Extremophilic and extremotolerant species were less abundant among Mucoromycetes (five species), Agaricomycetes, Leotiomycetes and Microbotryomycetes (four species), Wallemiomycetes (three species), Tremellomycetes (two species) and Cystobasidiomycetes and Schizosaccharomycetes (one species) (Figure 4) (Table S1). Compared to the top four taxonomic categories of fungi that have the highest number of species, in extremophilic and extremotolerant species, Saccharomycetes replaced Agaricomycetes (Eurotiomycetes, Sordariomycetes, Agaricomycetes and Dothideomycetes). However, the genomes of Saccharomycetes did not encode for aegerolysins and MACPF(i)s (with the exception of one species *Lipomyces tetrasporus* with four MACPF[i]s) (Figures 1 and 2). Also, the proportion of extremophilic and extremotolerant species encoding aegerolysins was lower in Eurotiomycetes (35% compared to 76%), Sordariomycetes (16% compared to 28%), Dothideomycetes (10% compared 19%) and Agaricomycetes (none in comparison to 19%) (Figures 1 and 4). The proportion of extremophilic and extremotolerant

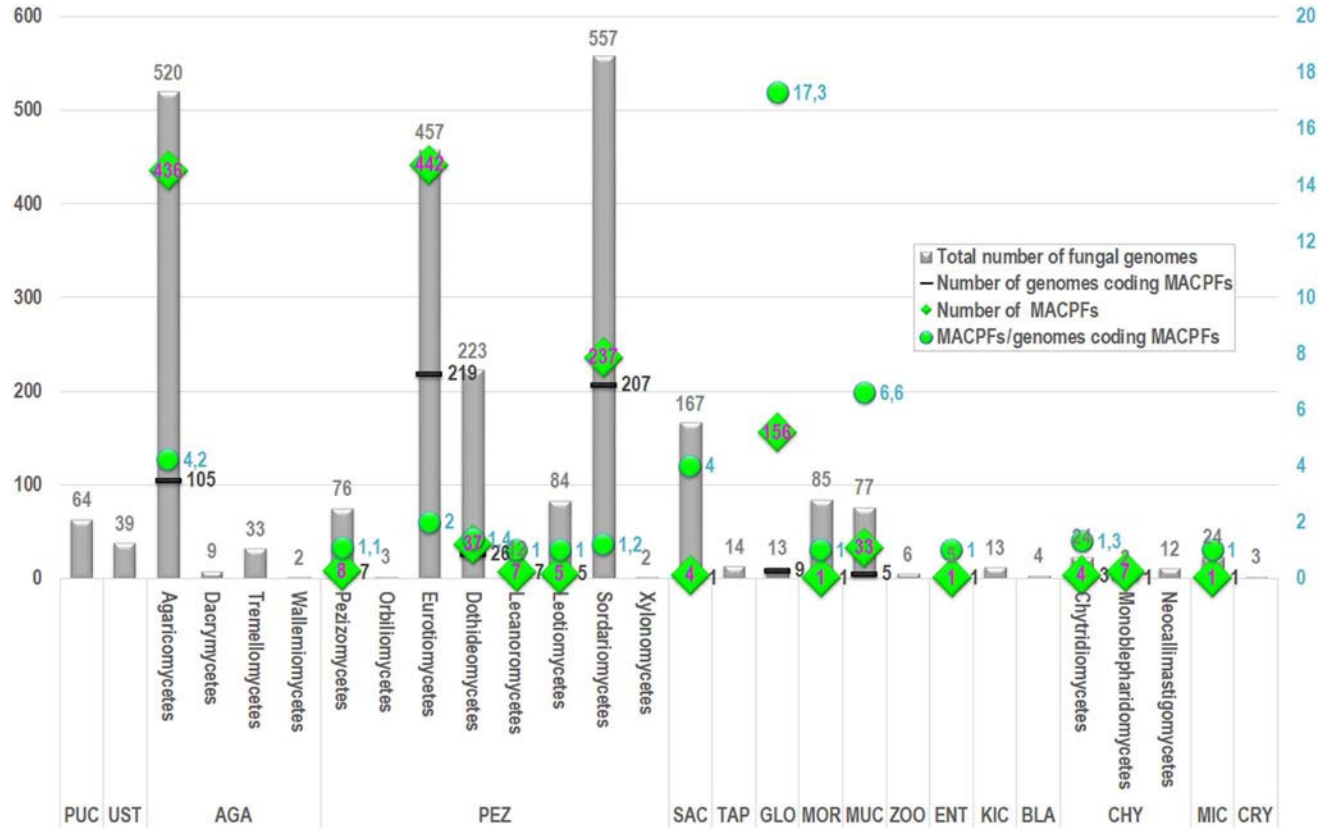

FIGURE 2 Number of membrane attack complex/perforins (MACPF)(i)s in fungal genomes of taxonomic categories. Basidiomycota: PUC, Pucciniomycotina; UST, Ustilaginomycotina; AGA, Agaricomycotina. Ascomycota: PEZ, Pezizomycotina; SAC, Saccharomycotina; TAP, Taphrinomycotina. Mucoromycota: GLO, Glomeromycotina; MOR, Mortierellomycotina; MUC, Mucoromycotina. Zoopagomycota: ZOO, Zoopagomycotina; ENT, Entomophthoromycotina; KIC, Kickxellomycotina. BLA, Blastocladiomycota. CHY, Chytridiomycota. MIC, Microsporidia. CRY, Cryptomycota. Data from the fungal genome database JGI MycoCosm.[22]

species encoding MACPFs was also lower in Eurotiomycetes (21% compared to 46%), Agaricomycetes (none compared to 20%), Dothideomycetes (none compared to 12%) and similar in Sordariomycetes (40% compared to 37%) compared to all fungal genomes (Figures 2 and 4).

3.5 | A minority of aegerolysins or MACPFs have been identified in extremophilic species

A total of 26 aegerolysins and 28 MACPF(i)s were encoded by 14 extremotolerant and extremophilic species of the Eurotiomycetes, Eurotiales: *Aspergillus glaucus*, *A. foetidus*, *A. niger*, *A. sydowii*, *Paecilomyces varioti*, *Thermomyces stellatus*, *A. thermomutatus*, *Exophiala viscosa* and *P. niveus*; of the Sordariomycetes, Hypocreales: *Fusarium oxysporum* and *F. solani*; of the Sordariales: *Thermocarpiscus australiensis* and *Thermothelomyces myriococcoides*; of the Dothideomycetes, Mycosphaerellales: *Acidomyces richmondensis*. Nine of these species were extremotolerant (13%): *T. stellatus* and *A. thermomutatus* were thermotolerant, *F. oxysporum* alkalitolerant, *A. foetidus*, *A. niger*, *A. sydowii* and *F. solani* metallotolerant and *E. viscosa* and *P. niveus* polyextremotolerant. Only five species were extremophilic (7%): *B. nivea*, *P. varioti*, *T. australiensis* and *T. myriococcoides* were thermophilic, *A. glaucus* halophilic and *A. richmondensis* acidophilic.

The majority of aegerolysins[21] and MACPF(i)s[26] belong to extremotolerant species. The minority, five aegerolysins (19%) and two MACPF(i)s (7%) belong to extremophilic species. Most of the aegerolysins and MACPF(i)s were encoded by 17 genomes of alkalitolerant, plant pathogenic, generalist *F. oxysporum* strains (4 aegerolysins and 21 MACPF[i]s) and 6 genomes of metallotolerant, saprotroph, opportunistic pathogen, generalist *A. niger* strains (7 aegerolysins and 1 MACPF[i]). As a metallotolerant strain, *A. niger* can grow in 2000 ppm of zinc, lead and mercury, 1200 and 1000 ppm arsenic (III) and (VI), 800 ppm of fluorine and cobalt and least in cadmium (400 ppm).[35] Although the genome of the biofilm-forming and chromium-resistant mangrove fungus strain *A. niger* BSC-1 was recently determined, it

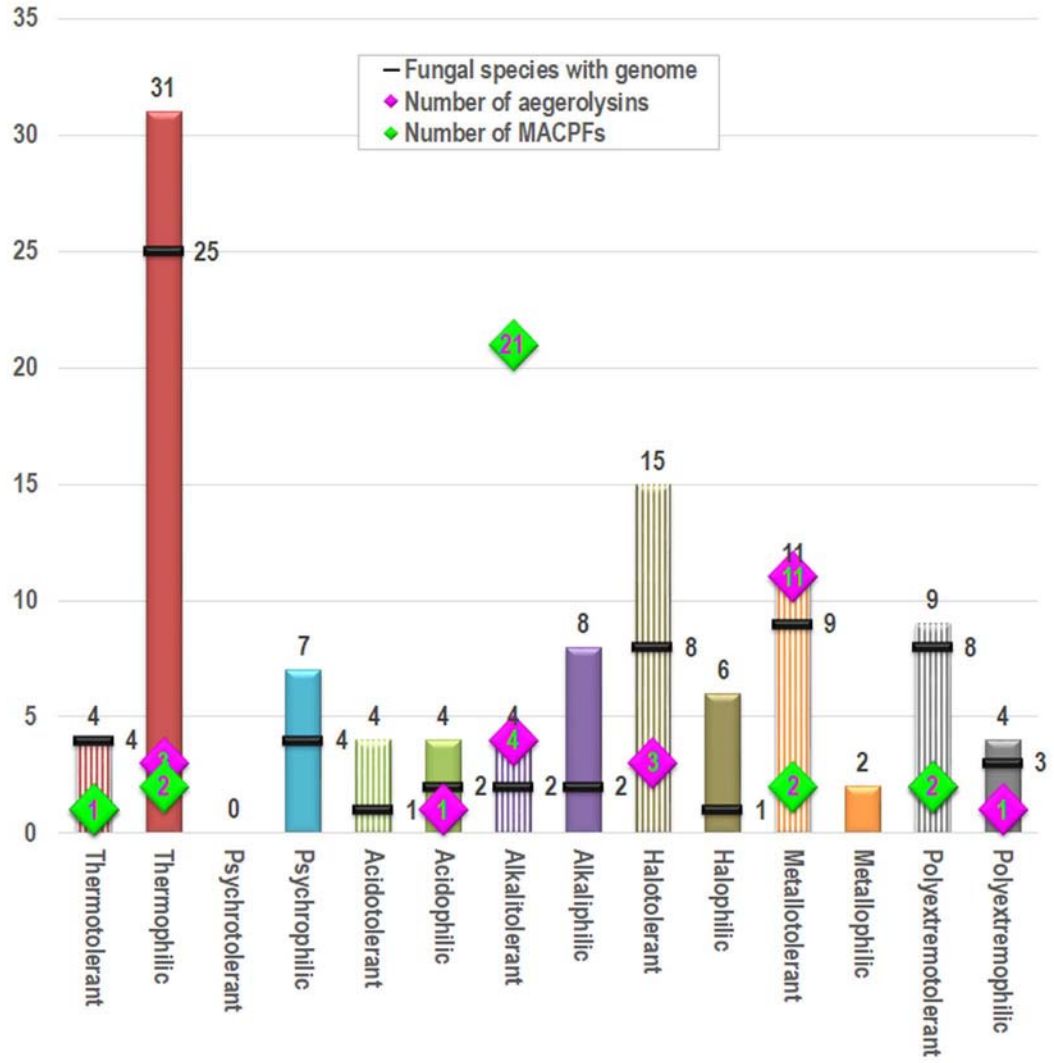

FIGURE 3 Fungal species according to extremotolerant and extremophilic properties. MACPF, membrane attack complex/perforin.

was not included in the public genome database MycoCosm.[36] PFAM terms search for specific protein domains showed that the genomes of the different *F. oxysporum* and *A. niger* strains encode different numbers of aegerolysins and MACPF(i)s.

Since MACPF(ii)s cannot be identified by the PFAM terms search algorithm, all 26 aegerolysin loci were manually analysed for MACPF(ii)s. A total of four possibilities were identified in these loci: aegerolysin only (A), aegerolysin and MACPF(i) on different contigs (B), aegerolysin and MACPF(ii) on the same contig (C) and MACPF(i) only (D) (Figure 5). Only aegerolysin was observed in three loci of halotolerant *A. sydowii*, two loci of thermotolerant *T. stellatus*, in the locus of acidophilic *A. richmondensis*, in halophilic *A. glaucus*, in thermophilic *T. australiensis* and in two loci of *T. myriococcoides* (Figure 5A). Only four species encode both, aegerolysin and MACPF(i), but none of them on the same scaffold: *A. niger*, *A. thermomutatus*, four strains of *F. oxysporum* and *F. solani* (Figure 5B). Aegerolysin and MACPF(ii)s were identified as bi-directional gene pairs with 5′–5′ orientation in two metallotolerant species: in three strains of *A. foetidus* and in six strains of *A. niger* (Figure 5C). Only MACPF(i)-encoded loci were present in the thermophilic *Byssochlamys nivea* (twice) and in the polyextremotolerant *E. viscosa* and *Paecilomyces niveus* (D).

3.6 | Inferred phylogenetic trees of aegerolysins or MACPFs partners from extremotolerant and extremophilic species

The aegerolysin phylogeny was inferred (Figure 6) and the presence of partner pairs was indicated by asterisks. Aegerolysin with MACPF(ii) partners appear to cluster also to the previously described NigA2, Asp-HS, L152

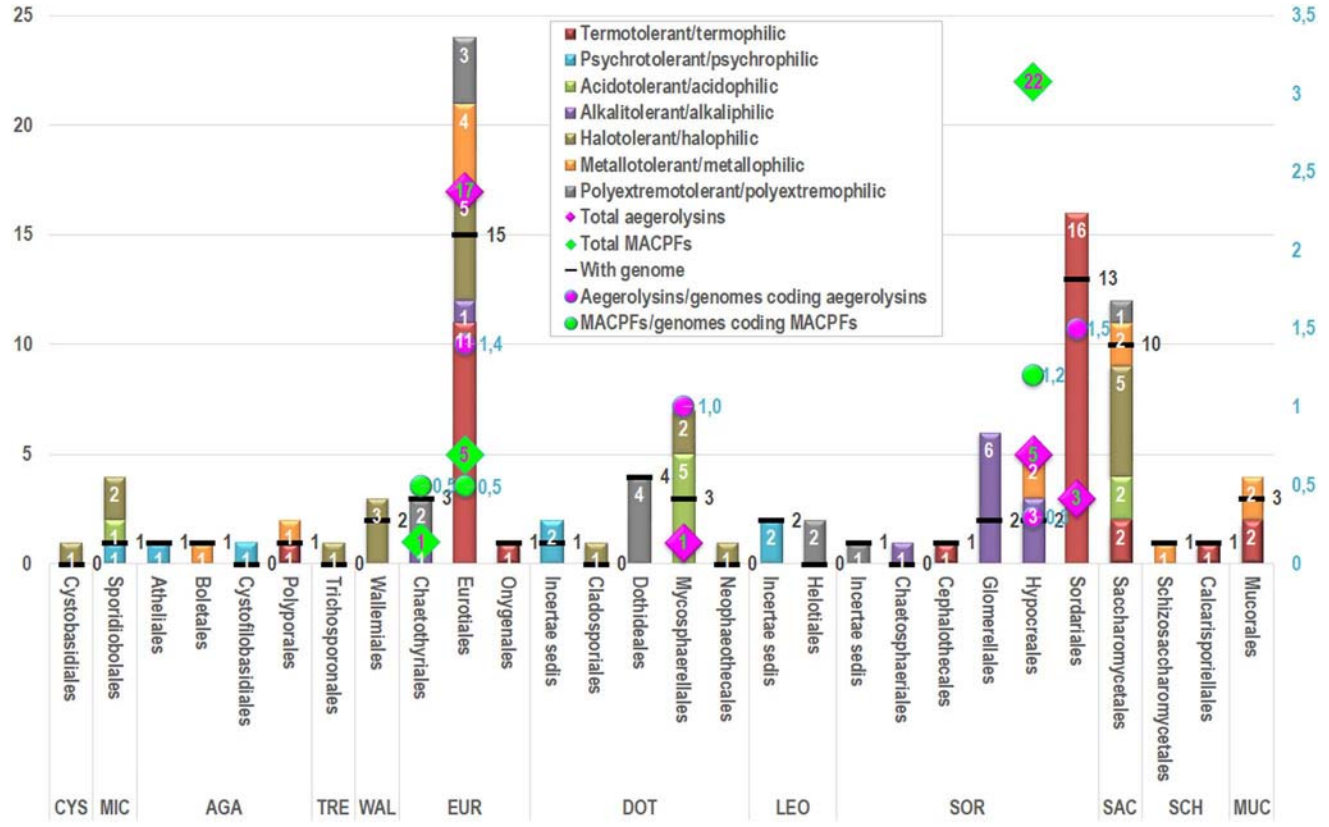

FIGURE 4 Different extremophilic and extremotolerant traits of fungi according to their taxa. Basidiomycota—Pucciniomycotina: CYS, Cystobasidiomycetes; MIC, Microbotryomycetes. Agaricomycotina: AGA, Agaricomycetes; TRE, Tremellomycetes; WAL, Wallemiomycetes. Ascomycota—Pezizomycotina: EUR, Eurotiomycetes; DOT, Dothideomycetes; LEO, Leotiomycetes; SOR, Sordariomycetes. Saccharomycotina: SAC, Saccharomycetes. Taphrinomycotina: SCH, Schizosaccharomycetes. Mucoromycota—Mucoromycotina: MUC, Mucoromycetes. MACPF, membrane attack complex/perforin. Data from the fungal genome database JGI MycoCosm.[22]

and BlyA, although they do not belong to the same taxonomic categories.[1] In fact, six aegerolysin sequences of *A. niger* strains were identical to each other and to NigA2.[24,25] When searching for PFAM terms (PF06355), a second aegerolysin sequence identical to NigA1 was found only in one of the *A. niger* strains (ATCC 13496), although the second aegerolysin sequences were identified also in some other *A. niger* strains (by Blast search).[25] Similar situation is observed for aegerolysins from *F. oxysporum* strains, three sequences from the three strains encoding for the aegerolysins were identical, a second one was found only in one of the *F. oxysporum* strains (MPI-CAGE-CH-0212). The sizes of the aegerolysins from extremotolerant and extremophilic species varied from 135 to 493 amino acids, averaging 143 amino acids, which was in line with expectations (if the two outliers with 165 amino acids and one with 493 amino acids were omitted); identity with the best studied fungal aegerolysin ostreolysin OlyA6 was low (12%–39%), as expected.

The phylogeny MACPFs partners was also inferred (Figure 7A), and the presence of aegerolysin partner pairs was indicated by asterisks, vice versa as in the previous figure. MACPF(ii)s with aegerolysin partners clustered together with NigB1, Asp-HSB, BlyB and L152B, again not from the same higher taxonomic categories.[1] Two groups of MACPF(i)s from *F. oxysporum* strains cover the majority, but not all, of the 21 MACPF(i)s identified in the genomes of these strains, other MACPF(i)s sequences were distributed across the phylogenetic tree.

MACPF domain protein sequences vary widely between species.[37] The sizes of MACPF(i)s from extremotolerant and extremophilic species appeared to divide in two groups, smaller ones from 469 to 793 amino acids and larger ones from 926 to 1180 amino acids. The sizes of MACPF(ii)s were the smallest, ranging from 463 to 599 amino acids. Identities with the best studied fungal MACPF, PlyB, were only 6%–17%, which was not unusual.

The only identified signature motif that is structurally conserved in MACPFs is Y/F-G-X_2-F/Y-X_6-G-G (X is any amino acid) and is assigned to one of the central β-strands. The significance of this motif is currently unclear; however, evolutionary conservation between

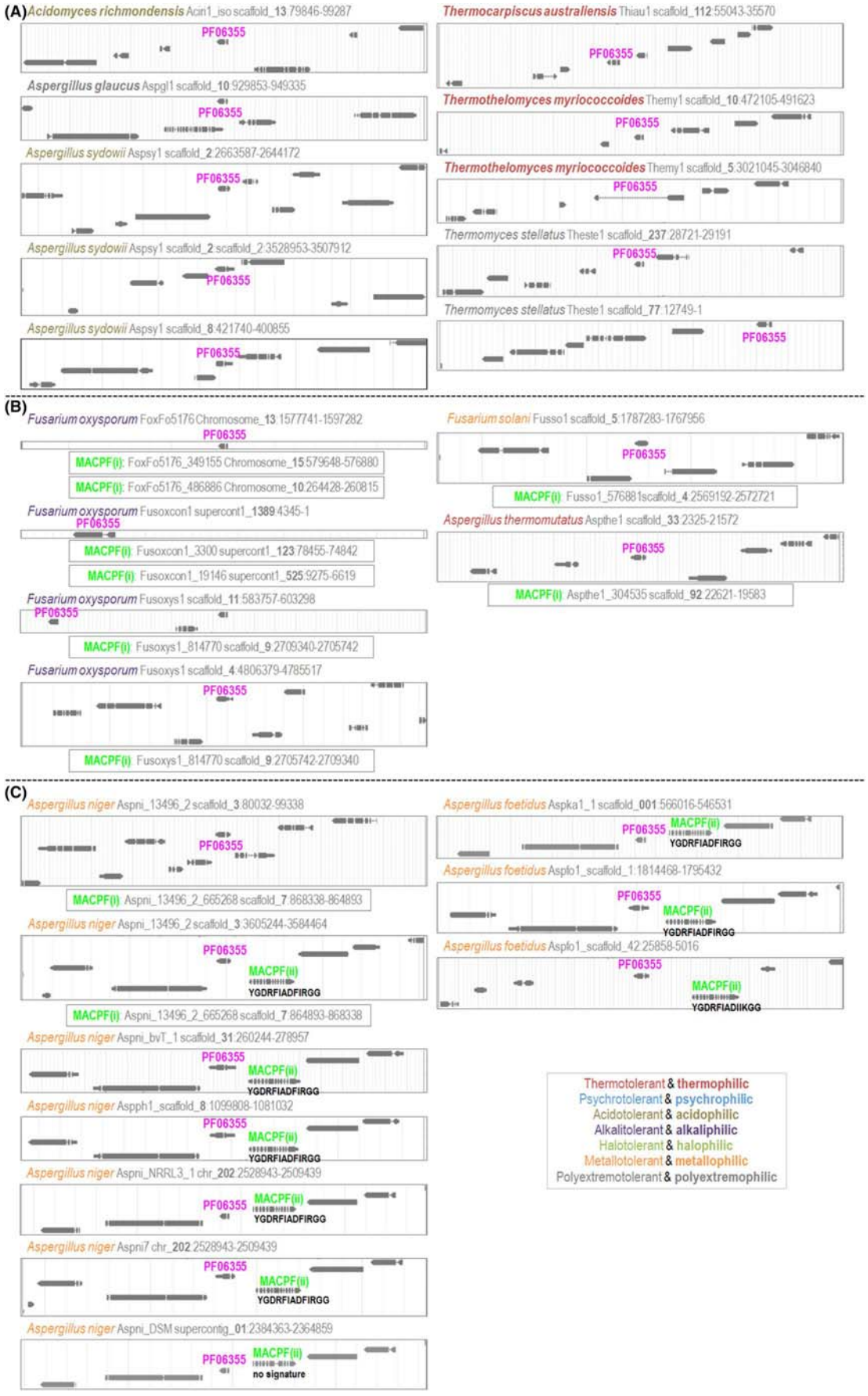

FIGURE 5 Legend on next page.

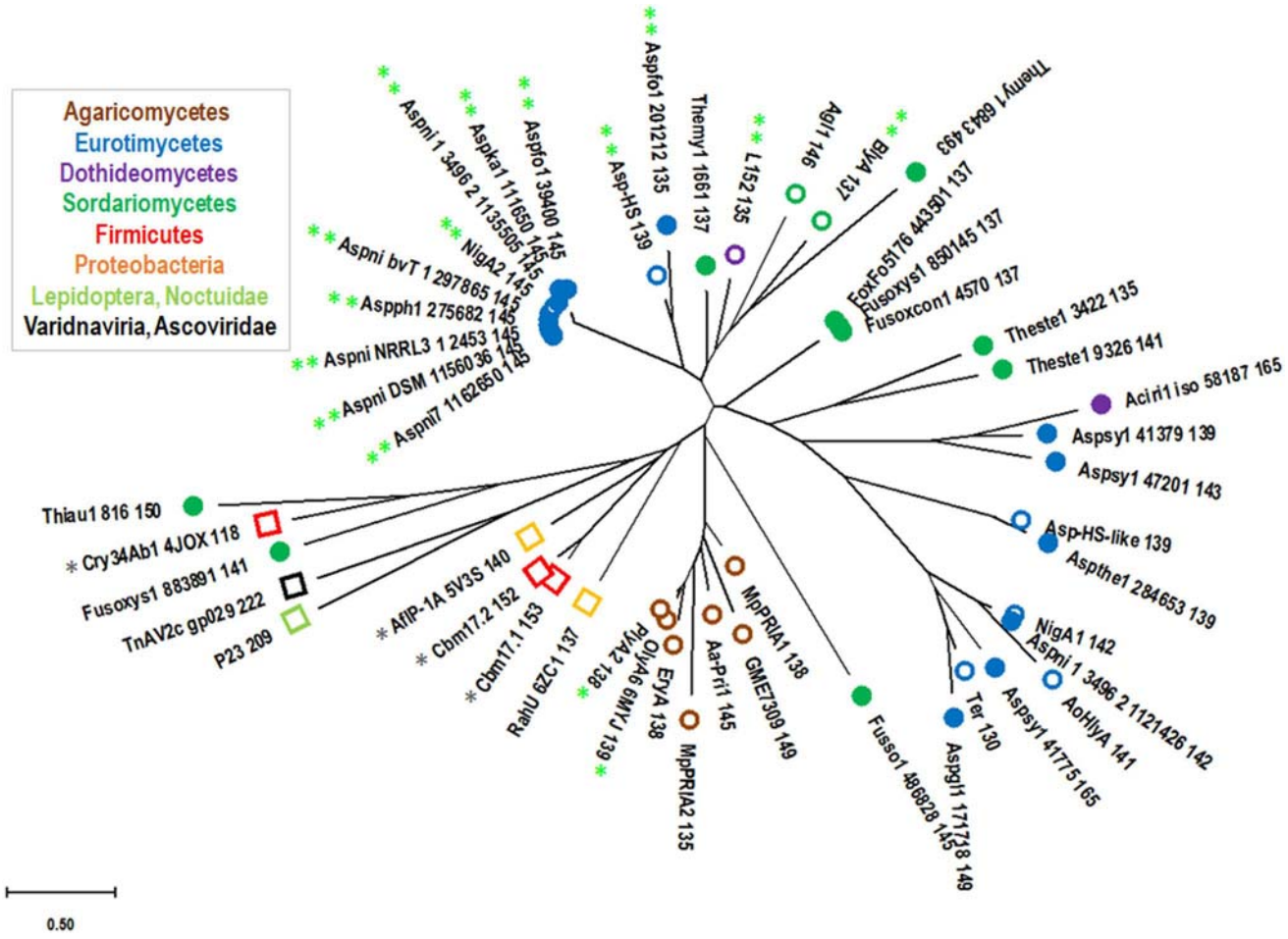

FIGURE 6 Aegerolysin phylogeny is not following taxonomical distribution. Open circles, already described fungal aegerolysins; full circles, aegerolysins from extremotolerant and extremophilic fungi; open squares, already described aegerolysins from other taxa (see colour legend). Aegerolysins: OlyA6/PlyA, ostreolysin A6/pleurotolysin A from *Pleurotus ostreatus* (PDB ID: 6MYJ); EryA, erylysin A, and PlyA2, pleurotolysin A2 from *P. eryngii*; Aa-Pri1, aegerolysin Aa-Pri1 from *Agrocybe aegerita*; MpPRIA1 and MpPRIA2, putative aegerolysin genes from *Moniliophthora perniciosa*; GME7309, *Lignosus rhinocerotis* aegerolysin-domain-containing protein; Asp-HS, Asp-hemolysin, and Asp-HS-like, Asp-hemolysin-like from *Aspergillus fumigatus*; Ter, terrelysin from *A. terreus*; AoHlyA, *A. oryzae* hemolysin; NigA1 and NigA2, nigerolysin A1 and A2 from *A. niger*; BlyA, beauveriolysin A from *Beauveria bassiana*; Agl1, *Trichoderma atroviride* aegerolysin; L152, *Alternaria geisen* aegerolysin; Cry34Ab1 (Gpp34Ab1) 13.6-kDa insecticidal crystal protein (PDB ID: 4JOX) from *Bacillus thuringiensis*; Cbm17.1 and Cbm17.2, hemolysin-like protein Cbm17.1 and Cbm17.2 from *Clostridium bifermentans*; AflP-1A, bicomponent insecticidal protein 16-kDa unit (PDB ID: 5V3S) from *Alcaligenes faecalis*; RahU, RahU protein (PDB ID: 6ZC1) from *Pseudomonas aeruginosa*; P23, protein 23 from *Pseudoplusia includes*, and TnAV6a1 gp029; ORF029 from *Trichoplusia ni ascovirus 2c*; aegerolysins from extremotolerant and extremophilic fungi (for identification see Table S1); number, size of the protein in amino acids; * in light green, membrane attack complex/perforin (MACPF)(i) bi-directional gene pair; ** in light green, MACPF(ii) bi-directional gene pair; * in grey, other gene pairs from other organisms. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model.[26] The tree with the highest log likelihood (−11,686.59) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 49 amino acid sequences. There were a total of 545 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.[27]

FIGURE 5 Aegerolysins and membrane attack complex/perforins (MACPFs) containing scaffolds. Only aegerolysins encoding scaffolds (A). Aegerolysins and MACPF(i) genes encoded on different scaffolds (B). Aegerolysin and MACPF(ii)s bi-directional pair of genes with 5′–5′ orientation (C). Only MACPF(i)-encoded loci polyextremotolerant *Exophiala viscosa* and *Paecilomyces niveus* and two from thermophilic *Byssochlamys nivea* are not presented.

humans and bacteria suggests a functional role. Conservation of several key glycine residues appears to be important for refolding and pore formation.[37–40] The generated logo produced by MACPFs partner proteins from extremotolerant and extremophilic species confirms the expected signature compared to other previously described MACPFs partner proteins from fungi (Figure 7B,C). The typical MACPF signature was present in most MACPF(i)s (except for EurotioJF033F_1_584745 and Fusoxvas1_11107 due to shorter sequences, and Fusoxlyc1_14938). In addition, typical MACPF signature was also not present in one of the sequences (Aspni_DSM_156035), which was 10 amino acids shorter and for this reason only 89% identical to other MACPF(ii) sequences from other *A. niger* strains.

3.7 | Predicted structural models of aegerolysins or MACPFs partners from extremotolerant and extremophilic species

Since three aegerolys in sequences from *F. oxysporum* strains (FoxFo5176_443501, Fusoxcon1_4570 and Fusoxys1_850145) were identical and six sequences from*A. niger* strains were identical (Aspni_1_3496_2_1135505, Aspni_bvT_1_297865, Aspph1_275682, Aspni_NRRL3_1_2453, Aspni7_1162650 and Aspni_DSM_1156036), a total of 19 different aegerolysins were identified in extremotolerant and extremophilic species. Superposition of six structural models of aegerolysins from metallotolerant and metallophilic species, four from thermotolerant and thermophilic species, three from halotolerant and halophilic species and from polyextremotolerant and polyextremophilic, two from alkalitolerant and alkaliphilic species and one from acidophilic species showed the typical β-sandwich fold compared to the best studied ostreolysin OlyA6 structure (Figure 8). One of the aegerolysins from the thermophilic *T. myriococcoides* showed a large α-helical extension at the N-terminus (Themy1_6843), another from the polyextremophilic *A. glaucus* a short α-helical extension at the C-terminus (Aspgl1_171718), one from the halotolerant *A. sydowii* two β-strand extensions at the C-terminus (Aspsy1_41775) and another from acidophilic the *A. richmondensis* an unstructured extension at the N-terminus (Aciri1_iso_58187).

Five MACPF(ii)s from *A. niger* strains had sequences that were 100% identical to previously described NigB1 (Aspni_13496_2_1135500, Aspni_bvT_1_372621, Aspph1_326308, Aspni_NRRL3_1_2452 and Aspni7_1182338), with the exception of Aspni_DSM_156035_463 (89% identical).[1] The structural models of three MACPF(ii)s from *A. foetidus* strains (Aspka1_1_11650, Aspfo1_39400 and Aspfo1_201212) showed good superposition with the previously described NigB1, Asp-HSB, BlyB and L152B (not shown).[1] However, all other 27 structural models showed many unfolded large loops, so that they did not allow reliable conclusions.

4 | DISCUSSION

Some organisms thrive in environments that were once considered completely inhospitable because they have extreme conditions such as heat, cold, acidity or pressure, that are lethal to most other life forms. Extreme conditions can be seen as those in which most species cannot grow or even survive. However, we must recognise that these organisms are only extreme from our anthropocentric point of view. These organisms can be categorised into two broad groups: extremophilic and extremotolerant organisms.[13] Extremophilic organisms require one or more extreme conditions to thrive, while extremotolerant organisms grow optimally under temperate conditions but can tolerate one or more extremes. Extremophilic specialists are rare in temperate environments, have a narrow range of ecological amplitudes and are often selective in their diet. Extremotolerant generalists are ubiquitous, commonly found in temperate environments, often have diverse diets and are usually able to tolerate a range of different extreme conditions, even if they are not particularly successful in any of them.[13,18] However, although extremotolerant species can grow in temperate conditions in the laboratory, some of them are never found in such conditions in nature. These species can be categorised as habitat specialists, along with true obligate extremophiles.[13] While generalists can compete with mesophilic species, specialists cannot.[13]

During this study, we identified several challenges that could affect these results. These challenges are the renaming of fungal species (i), the definition of extremotolerant/extremophilic traits (ii), the identification of extremotolerant/extremophilic traits (metadata) in the database (iii), the relationship between fungal isolates and fungal genomes (iv) and the automatic annotation of proteins (v).

The confusion in the nomenclature of extremophilic and extremotolerant fungal species has several reasons.[16] Some species have been repeatedly described under different names, as the early taxonomic literature was also written in different languages. Different names for the asexual (anamorphic) and sexual (teleomorphic) stages of the same fungus were used interchangeably. The 'one fungus one name' initiative has renamed many fungal species. Confusion arises from the merging (or splitting) of species that for many years were considered different (or same) species. Some extremophilic fungi are

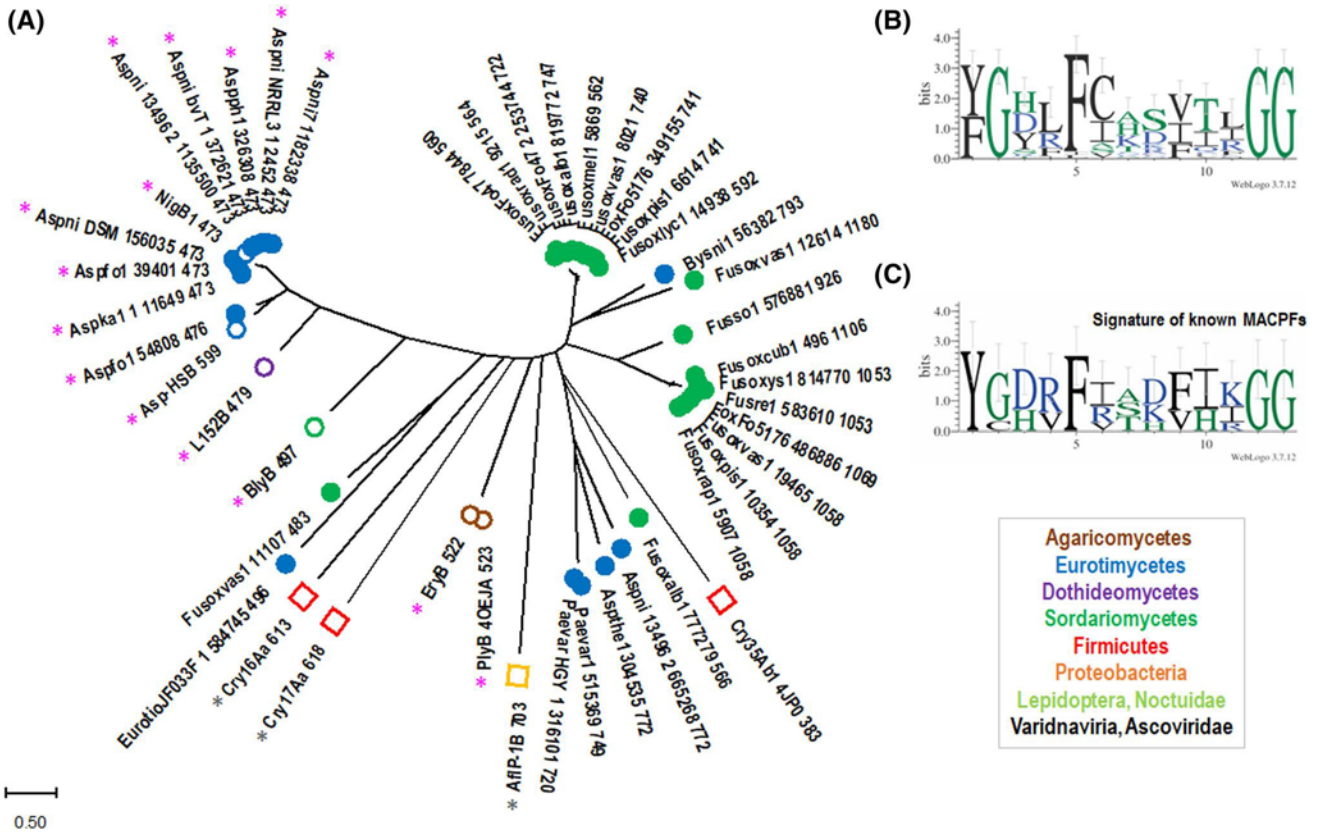

FIGURE 7 Membrane attack complex/perforin proteins (MACPFs) phylogeny is not following taxonomical distribution. Inferred phylogenetic tree (A). Signature of MACPFs from extremo-tolerant/-philic species (B). Signature of known MACPFs (C). Open circles, already described fungal MACPFs; full circles, those from extremotolerant and extremophilic fungi; open squares, already described partner proteins from other taxa (see colour legend). Partner proteins: PlyB, pleurotolysin B (PDB ID: 4OEJ) from *Pleurotus ostreatus*; EryB, erylysin B from *P. eryngii*; Asp-HSB, Asp-hemolysin partner protein from *Aspergillus fumigatus*; NigB1, nigerolysin B1 from *A. niger*; BlyB, beauveriolysin B from *Beauveria bassiana*; L152B, aegerolysin partner protein from *Alternaria geisen*; Cry35Ab1 (Tpp35Ab1), 43.8-kDa insecticidal crystal protein (PDB ID: 4JPO) from *Bacillus thuringiensis*; Cry16Aa and Cry17Aa, pesticidal crystal-like protein Cry16Aa and Cry17Aa from *Clostridium bifermentans*, and AfIP-1B from *Alcaligenes faecalis*, two-component insecticidal protein 77-kDa unit. MACPFs from extremotolerant and extremophilic fungi (for identification see Table S1); number, size of the protein in amino acids; * in magenta, aegerolysin bi-directional gene pair; * in grey, aegerolysin gene pairs from other organisms. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model.[26] The tree with the highest log likelihood (−38,856.34) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 45 amino acid sequences. There were a total of 1402 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.[27] MACPF signature (WebLogo).[28]

misidentified in the literature. In some cases, the specific epithet, such as *thermophilum* (or variants thereof), has been used without adhering to the proposed definition of extremophilic (thermophilic) fungi.

The threshold values that distinguish mesophilic from extremotolerant and extremophilic fungi are not always clearly defined. However, some of the commonly used values have been established but perhaps not sufficiently enforced.[13] Temperature: thermotolerant and thermophilic fungi grow above 45–50°C, but thermophilic fungi cannot grow below 20°C. Psychrotolerant (or psychrotrophic) and psychrophilic fungi can grow at or near 0°C, psychrophilic fungi grow optimally below 15°C but cannot grow above 20°C. pH tolerance: acidotolerant and acidophilic fungi grow under acidic conditions (sometimes below pH 4). Acidophilic fungi have a growth optimum below pH 3, they can even grow at pH 1, but no obligate acidophilic fungus has yet been described. Alkalitolerant and alkaliphilic fungi grow above pH 8, but alkaliphilic fungi have a growth optimum above pH 8 or 9. The extreme conditions may also depend on the concentration of dissolved substances, such as salt or heavy metals. Halotolerant and halophilic fungi grow above 17% NaCl (w/v), but halophilic fungi cannot grow in

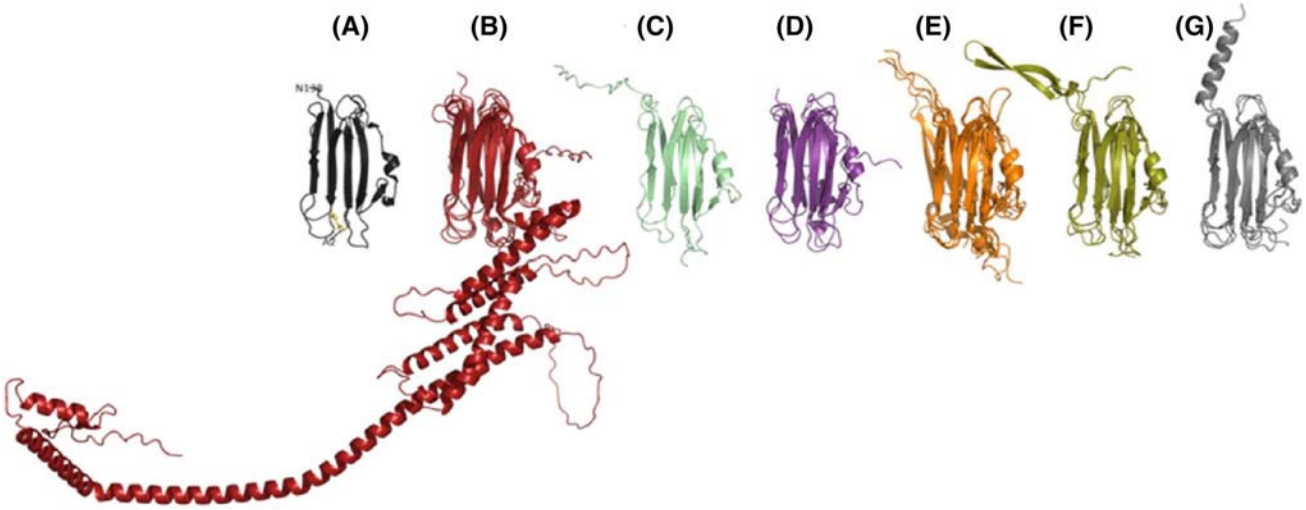

FIGURE 8 Predicted structural models of aegerolysins. OlyA6, ostreolysin A6 structure PDB ID: 6MYJ D from *Pleurotus ostreatus* (A). Superposition of four aegerolysins structural models from thermotolerant and thermophilic species (B), one aegerolysin from acidophilic species (C), two aegerolysins from alkalitolerant and alkaliphilic species (D), six aegerolysins from metallotolerant and metallophilic species (E), three aegerolysins from halotolerant and halophilic species (F) and three aegerolysins from polyextremotolerant and polyextremophilic species (G). Models of aegerolysins and protein partners calculated by AlphaFold2.[30] Cartoon presentation by PyMOL.[34]

normal mycological media. Metallotolerant and metallophilic fungi can tolerate high concentrations of dissolved (heavy) metals, such as arsenic, cadmium, cobalt, copper, fluorine, lead, mercury and zinc; definition limits must be set for each metal ion. Polyextremotolerant and polyextremophilic fungi can live under different forms of environmental stress.

However, there are some other extremes and organism types that have not been considered in this study, such as xerophilic based on water activity; osmophilic based on sugar concentration; sulphophilic based on sulphur concentration; radioresistant based on radiation; piezophilic (or barophilic) based on pressure; obligate aerobes, facultative anaerobes, aerotolerant anaerobes and obligate anaerobes based on oxygen concentration; capnophilic based on carbon dioxide concentration; cryptoendoliths, endoliths and hypoliths based on of growth area; oligotrophic based on nutrients, and hyperpiezophilic based on hydrostatic pressure.

There is no organised metadata to help identify extremotolerant or extremophilic species in databases, making it difficult to select and compare a large amount of genomic data. Some fungal species are curated in user groups, such as black yeasts, psychrophilic fungi, pyrophilous fungi and thermophilic fungi.[22]

It is possible that none, one or more genomes are available for the identified species. It is usually not clear whether the available fungal genome was isolated from a fungal strain growing under extreme conditions. This fact can be important as some aegerolysins appear to be encoded in the genome of one strain but not in others. For example, the genomes of different strains of *A. oryzae* encode different numbers of aegerolysins. One aegerolysin is encoded in *A. oryzae* strain 100-8 and is present in all four strains with only one amino acid variation. Another aegerolysin (AoHlyA) is encoded in another strain 3.042. A total of three aegerolysins (including AoHlyA) are encoded in the genome of strain RIB40. In strain BCC7051, there are three additional sequences, one shorter and two larger.[1]

The metallotolerant strain of *A. niger*, for example, was isolated from the polluted air of a petrol station.[35] Several genomes are available for the saprotrophic and human opportunistic pathogen *A. niger*. However, most of the genomes available in the public genome database MycoCosm originate from strains that have been used in the laboratory or in industry for a long time. Recently, the genome of the chromium-resistant *A. niger* BSC-1 strain was sequenced but it is not (yet) included in this database.[36]

There is no doubt that automatic annotation is irreplaceable. However, the absence of aegerolysins and MACPFs in some species or strains may also be an artefact in annotation and identification. There is a possibility that the number of aegerolysins and MACPFs was underestimated, but regardless of whether the species are extremotolerant or extremophilic or not.

Here, the uneven distribution of aegerolysins and MACPFs in the fungal kingdom was systematically demonstrated. Furthermore, it has also been systematically shown how the identified extremophilic and extremotolerant species are unevenly distributed among the

different taxa. However, there is no obvious overlap between species from extreme environments and the presence of aegerolysins or MACPFs. There were only a few aegerolysins and MACPFs encoded by genomes of extremotolerant or extremophilic fungi. However, only four extremotolerant species encode both, aegerolysin and MACPF(i), but none of them on the same scaffold: the metallotolerant *A. niger* and *F. solani*, the thermotolerant *A. thermomutatus* and the alkalitolerant *F. oxysporum*. Aegerolysin and MACPF(ii) were identified as bi-directional gene pairs with 5′–5′ orientation in only two metallotolerant species, beside *A. niger* also in *A. foetidus*. It would be interesting to see whether they are also expressed, and proteins are produced under extreme conditions. The function of other copies of aegerolysins that do not acquire MACPFs remains unclear; perhaps they serve as decoys.[1] The effects observed from MACPF proteins in general are primarily mediated by membrane interactions and the formation of transmembrane pores as important effectors in the immune system and in bacterial pathogenesis. However, their activity is not limited to membrane interactions, and the exact role and molecular mechanism of action of many representatives are also still unclear.[4]

As expected, the aegerolysins identified in the extremophilic and extremotolerant organisms also have typical β-sandwich fold, except for the four structural models, which have additional extensions: α-helical at the N- or C-terminus and β-stranded or unstructured at the C-terminus. It should be shown by expression studies that these extensions are not the result of incorrect annotation. The sequences of most of the identified MACPF domain-containing proteins were too different to create a realistic structural model. According to the PFAM terms searches for specific protein domain, the genomes of different strains may encode different numbers of aegerolysins and MACPFs, such as the genomes of *F. oxysporum* and *A. niger*.

5 | CONCLUSIONS

Aegerolysins and MACPFs do not contribute to the extreme survival ecology of extremophilic fungi and extremotolerant fungal specialists. The amount of these proteins is lower in extremotolerant and extremophilic fungi than in average fungal species, but both aegerolysins and their partners were encoded by only 7% of extremotolerant species. These results provide further evidence that aegerolysins and their partners belong to the specialised proteins with the presumed pore-forming role in the process of competitive exclusion, which allows further conclusions about their biological function.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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