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Duplications and losses of genes encoding known elements of the stress defence system of the Aspergilli contribute to the evolution of these filamentous fungi but do not directly influence their environmental stress tolerance

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Abstract: The contribution of stress protein duplication and deletion events to the evolution of the Aspergilli was studied. We performed a large-scale homology analysis of stress proteins and generated and analysed three stress defence system models based on *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Aspergillus nidulans*. Although both yeast-based and *A. nidulans*-based models were suitable to trace evolutionary changes, the *A. nidulans*-based model performed better in mapping stress protein radiations. The strong Mantel correlation found between the positions of species in the phylogenetic tree on the one hand and either in the *A. nidulans*-based or *S. cerevisiae*-based models on the other hand demonstrated that stress protein expansions and reductions contributed significantly to the evolution of the Aspergilli. Interestingly, stress tolerance attributes correlated well with the number of orthologs only for a few stress proteins. Notable examples are Ftr1 iron permease and Fet3 ferro-O_2-oxidoreductase, elements of the reductive iron assimilation pathway, in the *S. cerevisiae*-based model, as well as MpkC, a HogA-like mitogen activated protein kinase in the *A. nidulans*-based model. In the case of the iron assimilation proteins, the number of orthologs showed a positive correlation with H_2O_2-induced stress tolerance while the number of MpkC orthologs correlated positively with Congo Red induced cell wall stress, sorbitol induced osmotic stress and H_2O_2 induced oxidative stress tolerances. For most stress proteins, changes in the number of orthologs did not correlate well with any stress tolerance attributes. As a consequence, stress tolerance patterns of the studied Aspergilli did not correlate with either the sets of stress response proteins in general or with the phylogeny of the species studied. These observations suggest that stress protein duplication and deletion events significantly contributed to the evolution of stress tolerance attributes of Aspergilli. In contrast, there are other processes, which may counterbalance the effects of stress gene duplications or deletions including (i) alterations in the structures of stress proteins leading to changes in their biological activities, (ii) varying biosynthesis of stress proteins, (iii) rewiring stress response regulatory networks or even (iv) acquiring new stress response genes by horizontal gene transfer. All these multilevel changes are indispensable for the successful adaptation of filamentous fungi to altering environmental conditions, especially when these organisms are entering new ecological niches.

Key words: *Aspergillus* phylogeny, Environmental stress, Evolution of the Aspergilli, Fungal stress defence system, Gene deletion, Gene duplication, Stress protein radiation.

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INTRODUCTION

The Kingdom of Fungi is a large and diversified taxon with an estimated 2.2–3.8 million species (Lücking & Hawksworth 2018) occupying a breadth of ecological niches. Extensive fungal genome sequencing has led the construction of MycoCosm, a fungal genomics portal (https://genome.jgi.doe.gov/programs/fungi/index.jsf), which allows mycologists to gain a deeper and unique insight into the evolution of these organisms as new genome sequences continue to fill gaps in the Fungal Tree of Life (Grigoriev *et al.* 2014). These comparative genomics research projects are fuelled by the fact that the role of fungi in future bioeconomy including fermentation industry, biorefineries and agriculture cannot be overestimated (Baker *et al.* 2008, Grigoriev *et al.* 2011, Martin *et al.* 2011, Lange 2014, Meyer *et al.* 2016).

Among filamentous fungi, the ascomycetous genus *Aspergillus* includes several hundreds of cosmopolitan asexual species with world-wide distribution. Although these fungi seem to occupy various soil habitats with preference (*e.g.* the black Aspergilli *A. aculeatus*, *A. brasiliensis*, *A. niger*; Supplementary Table S1; Samson *et al.* 2007) some *Aspergillus* species are also well-known opportunistic colonisers of animals or even humans (*e.g. A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*; Sugui *et al.* 2014), and some others are indispensable production hosts for a wide spectrum of industrial fermentation and biotechnological processes (*e.g. A. niger*, *A. oryzae*, *A. terreus*; Park *et al.* 2017). Most Aspergilli have outstanding capabilities for biomass deconstruction with high efficiency due to their unique hydrolytic enzyme repertoire (*e.g. A. aculeatus*, *A. niger*, *A. oryzae*, *A. tubingensis*; Benoit *et al.* 2015, Park *et al.* 2017, Souza Guimarães & da Costa Souza 2017). Additionally, these fungi

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are also known to spoil corn, fruits as well as animal feed causing significant economic losses (*e.g. A. carbonarius*, *A. flavus*, *A. niger*; Perrone & Gallo 2016). The Aspergilli are ubiquitously present in indoor environments causing deterioration of artworks and also versatile health complications like asthma (*e.g. A. clavatus*, *A. fumigatus*, *A. niger*, *A. versicolor*; Egbuta *et al.* 2017, Mallo *et al.* 2017).

Not surprisingly, *Aspergillus* spp. have remarkable oxidative, osmotic, heavy metal and cell wall integrity stress tolerances, which help to explain the plethora of ecological niches these fungi occupy (de Vries *et al.* 2017, Orosz *et al.* 2018). As demonstrated by Orosz *et al.* (2018), some species grow remarkably fast at 37 °C (*A. fisheri*, *A. acidus* and *A. nidulans*) while others can tolerate osmolytes added at high concentrations or are even osmophilic in the presence of either non-ionic (sorbitol; *A. glaucus*, *A. wentii*, *A. versicolor*, *A. oryzae*) or ionic (NaCl; *A. glaucus*, *A. sydowii*, A. versicolor, *A. wentii*) osmolytes (Supplementary Table S2). Other species are surprisingly tolerant to other types of deleterious environmental stress like oxidative stress (*A. nidulans*, *A. niger*, *A. oryzae* to H_2O_2; *A. brasiliensis*, *A. aculeatus* to menadione sodium bisulfite), heavy metal stress (*A. sydowii*, *A. fumigatus*, *A. terreus*, *A. versicolor* and *A. wentii* to $CdCl_2$) and cell wall integrity stress (*A. niger* and *A. glaucus* to Congo Red) (de Vries *et al.* 2017, Orosz *et al.* 2018).

Previous work sheds light on the importance of both segmental and whole genome gene duplication events in the evolution of fungi (Wapinski *et al.* 2007). Gene duplications are important elements of evolutionary adaptation processes (Ames *et al.* 2010) and the duplicants produced by these events may undergo neofunctionalisation or subfunctionalisation processes (Levasseur & Pontarotti 2011) to avoid the disadvantageous consequences of increased and imbalanced gene dosages (Papp *et al.* 2003).

Gene duplication, diversification and differential gene loss processes also contributed significantly to the evolution of opportunistic human pathogenic fungi such as *A. fumigatus* (Fedorova *et al.* 2008). The rapid expansion and evolution of certain gene families functioning in the invasion of the host organisms by fungi typically takes place in genomic islands located at sub-telomeric regions, and which are also known as “gene factories” or “gene dumps” (Fedorova *et al.* 2008). Expansion of protein families, *e.g.* cell surface proteins and hydrolytic enzymes, was also reported in the near-obligate nematode endoparasitic fungus *Drechmeria coniospora* with the concomitant increase in the number of the orthologs of the *S. pombe* Mak1/2/3-type oxidative stress sensor kinases and also in that of the *A. nidulans* HogA-type mitogen activated protein kinases (MAPKs; Zhang *et al.* 2016). Importantly, the number of stress sensor proteins and stress response-related transcriptional regulators decreased, which indicated certain simplifications in the stress defence system of this endoparasite (Zhang *et al.* 2016). While core elements of stress signalling pathways seem to be evolutionarily well-conserved in fungi in general, upstream stress sensor proteins and down-stream transcriptional regulators evolve rapidly presumably as a way for these eukaryotes to tailor and fine-tune their stress defence systems for an ecological niche (Nikolaou *et al.* 2009).

Previously we collected and classified a large group of fungal stress response proteins with verified physiological functions, in order to generate the Fungal Stress Response Database version 2 (Karányi *et al.* 2013, Zhang *et al.* 2016, de Vries *et al.* 2017, http://internal.med.unideb.hu/fsrd2/default.aspx?p=consortium). Moreover, the Fungal Stress Database was also set up by us, and currently incorporates *Aspergillus* stress tolerance data recorded in a number of agar plate experiments performed under various types of stress conditions (oxidative stress, high-osmolarity stress, cell wall stress and heavy metal stress) as well as at different incubation temperatures (25 and 37 °C) (de Vries *et al.* 2017, Orosz *et al.* 2018; http://www.fung-stress.org/). Based on the plethora of fungal stress data accommodated mainly by these two databases, we set the following aims in this study: (i) To find any correlation between gene duplication, diversification and differential gene loss processes concerning stress response genes/proteins and the evolution of *Aspergillus* species. (ii) To assess whether evolutionary changes in the *Aspergillus* stress defence systems affect directly or indirectly the environmental stress tolerances of these important ascomycetes. (iii) To estimate the applicability of *Saccharomyces cerevisiae*-based, *Schizosaccharomyces pombe*-based and *Aspergillus nidulans*-based stress defence system models to describe the stress defence systems operating in the Aspergilli.

MATERIALS AND METHODS

Homology search and counting *Aspergillus* orthologs of fungal stress proteins

In stress protein homology search, our fungal stress protein (FSP) collection was utilised. Our FSP collection contains 2 150 proteins with known/verified physiological functions (Karányi *et al.* 2013; Zhang *et al.* 2016). The distribution of the proteins among fungal species was the following: *A. flavus*: 1; *A. fumigatus*: 83; *A. nidulans*: 145; *A. oryzae*: 13; *Candida glabrata*: 31; *C. neoformans*: 79; *F. graminearum*: 13; *F. oxysporum*: 14; *F. verticillioides*: 4; *N. crassa*: 78; *N. fischeri*: 2; *C. albicans*: 210; *S. cerevisiae*: 921; *S. pombe*: 534; *U. maydis*: 22 (see “Stress Database” in Supplementary Table S3). Within the scope of this study, the set of FSPs was manually curated, increasing the reliability of and making any background literature search easier in the “Stress Database”.

Homology searches were performed in the fully sequenced genomes of 25 *Aspergillus* and *Penicillium* strains representing 22 species (de Vries *et al.* 2017). The following species were included in the study: *A. aculeatus*, *A. brasiliensis*, *A. carbonarius*, *A. clavatus*, *A. fischeri*, *A. flavus*, *A. fumigatus*, *A. glaucus*, *A. kawachii*, *A. luchuensis*, *A. nidulans*, *A. niger* represented by three strains (CBS 113.46/ATCC 1015, CBS 513.88 and NRRL3), *A. oryzae*, *A. sydowii*, *A. terreus*, *A. tubingensis*, *A. versicolor*, *A. wentii*, *A. zonatus*, *Eurotium rubrum*, *P. chrysogenum*, *P. digitatum* and *P. rubens* (see “Stress Protein Orthologs” in Supplementary Table S3). After clicking on “Links to genome sequences” in Supplementary Table S3, the list of links to the appropriate genome sequence resources will appear.

In the identification and counting of stress homologs of FSPs, the protocol of Miskei *et al.* (2009) and Karányi *et al.* (2013) was used with modifications. Briefly, (i) the set of FSPs was blasted against the selected 25 *Aspergillus* and *Penicillium* species using Blastp (protein-protein BLAST), (ii) the set of potential homologs was reverse blasted *versus* the set of FSPs with Blastp, (iii) for each protein A from FSP, the list of b_0, b_1, ..., b_N was gained from each of the 25 *Aspergillus* and *Penicillium* species, ranked by e-