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The AFP from the fungi kingdom is under strong positive natural selection

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ABSTRACT

Fungal cells can colonize, virtually, in any surface of the planet and infect almost any multi cellular organism. Over the last few years, fungal infections in human patients have grown incredibly and the number of cases of deaths due to fungal infection in immunocompromised patients are surprisingly high. More than 300 species of fungus cause human diseases and a large number of commensal species are the cause of allergic reactions. Evolution has driven mechanisms associated with bio control agents produced either by fungus or other species. The antifungal protein precursor is a small biological entity with antimicrobial activity. Many species of fungus are able to produce this protein. has been pointed out as a possible antifungal drug because it destroy pathogenic fungi but has no effect on mammalian cells. In the present paper, we analyze the AFP nucleotide sequence of 61 fungal species in order to identify if natural selection is indeed acting upon AFP through dN/dS ration (number of non-synonymous and synonymous substitutions). We further discuss if selection is acting upon a specific site of the AFP nucleotide sequence. Our results show that selection is acting on the codon level of AFP, which means that there is a higher proportion of amino acid substitution in the population studied with dN/dS> 1. We hypothesize that the site 63 is an important region of variation of fungi AFP and could significantly contribute to the function of the protein and stabilization of PPIs and protein-membrane interaction. As a perspective for future projects, we are going to assess in silico mutation at site 63 and perform dynamical simulation in order to check if this specific site is essential for the conformational structure and function of AFP.

Keywords: *dN/dS; AFP; natural selection; Bioinformatics*

INTRODUCTION

Organisms from the fungi kingdom are able to grow, virtually, in any type of surface and colonize almost any multi cellular beings. Over the last few years fungal infections in human patients have grown incredibly (1) and the number of cases of deaths due to fungal infection in immune compromised patients are surprisingly high (2,3).There are over 300 species of fungus that can cause human infections (4) and a large number of harmless species can cause allergic reactions(5,6).Although the success of fungus in colonizing the most diverse environments, evolution has driven mechanisms associated with bio control agents produced either by fungus or other species (7–9).

The antifungal protein precursor (AFP) is a small protein with antimicrobial activity produced by a large variety of fungal species. AFP is a highly effective agent against pathogenic fungi(10,11). This protein has been pointed out as a possible antifungal drug because it destroy pathogenic fungi but has no effect on mammalian cells (12,13). The general structure of AFP consists of 50 amino acid residues with a conformational structure arranged into beta-sheets and stabilized by disulfide bonds (14). Moreover, AFP contains a γ-core motif, long known for its antifungal activity through membrane-protein interaction at the molecular level(15).

AFP has been identified in a large variety of fungal species (16–19). Antimicrobial proteins, such as AFP, has been positively selected during evolution as an advantage to organisms, which compete within similar nutritional and ecological niches (20). In addition, antifungal proteins produced by multi cellular organisms features an immunity mechanism in order to fight against possible fungal infections (21). An

important issue to address is the identification if natural selection is acting upon the gene sequence of AFP producing organisms. Understanding the evolutionary dynamics of such proteins should indicate how sequences change due to natural selection and drive the design of peptides and other synthetic compounds with more efficacies against fungal infection treatments.

In the present paper, we analyze the AFP nucleotide sequence of 61 fungal species in order to identify if natural selection is indeed acting upon AFP through dN/dS ration (number of non-synonymous and synonymous substitutions). We further discuss if selection is acting upon a specific site of the AFP nucleotide sequence. Our results show that selection is acting on the codon level of AFP, which means that there is a higher proportion of amino acid substitution in the population studied with dN/dS> 1.

MATERIALS AND METHODS

We retrieved the nucleotide sequence of 61 fungal species from The National Center for Biotechnology Information (NCBI). The inclusion criteria were based on sequences within at least 30% identity to the Aspergillus giganteus AFP amino acid sequence. Fungal species from the following genus: Aspergillus, Ophiocordyceps, Penicillium, Epichloe, Fusarium, Monascus, Colletotrichum, Isaria and Cordyceps.

The evolutionary history was inferred using the Neighbor-Joining method (22). The bootstrap consensus tree inferred from 500 replicates (23) is taken to represent the evolutionary history of the taxa analyzed(23). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method (24)and are in the units of the number of base substitutions per site. This analysis involved 61 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 390 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (25).

We performed a SLAC (Single Likelihood Ancestor Counting) using a maximum likelihoodancestral state reconstruction and minimum path substitution counting in order toestimate dS and dN at an amino acid residue level.We applied a binomial-basedtest to test to compare de number ofdSanddN. The statisticaestimates aggregate informationover all nucleotide sequence branches, thus the signal is from pervasivediversification or conservation characterizing the either positive of negative selection. The natural selection analysis and the dN/dS ratio test was performed by Data monkey(26).

RESULTS AND DISCUSSION

The phylogenetic classification of (putative) AFP in fungal species show 61AFPswith theγ-core motif. Branches with bootstrap values >50% is considered fairly reliable (Figure 1). To analyze the diversity of the phylogenetic clades of AFP, the well-documented structural features of AFP were checked for their presence in the sequence alignment. The nucleotide sequences of the 61protein sequenceshowed30–95% identity. The loops containing cysteine residues showed the most diversity (data not shown). The conservation of they-core motif across different clades indicates their critical roles in the structure and antifungal function of AFP.

Another approach is the natural selection analysis, which determines if the set of sequences under study is under positive selection, neutral or negative selection regarding the level of amino acid residues (26). Natural selection is intimately related to adaptive change that all beings undergo indisputably. This evolutionary process acts on the molecular level and understanding the evolutionary pressure nucleotide or proteins are submitted to, can shed some light on the way these macromolecules have evolved along the way. The so-called evolutionary pressure will either favor or oppose the preservation of genetic variation at a specific locus (27,28). Other evolutionary mechanisms, such as SNP (single nucleotide polymorphisms, homologous recombination, mutation, genetic drift and gene flow, contribute to favor or oppose variation within the genome of organisms. Certain, all of them are useful to tell the evolutionary history of a gene or a protein (29–31).

A useful way to determine the influence of natural selection at the molecular level is the dN/dS ratio (ω) test.The number of non-synonymous substitutions relative to the number of synonymous substitutions indicates if a certain group of genes, and consequently a protein, is undergoing positive, neutral or negative selection (32,33). Another important feature of such test is to pinpoint natural

selection acting at the level of specific aminoacids residues, such as SNPs that can increase disease susceptibility or hot spot residues that contribute to the overall free-energy of protein-protein interactions (PPIs) (27,34). To our knowledge there has not been a study conducted on the natural selection analysis for the AFP gene in fungi organisms.

We found strong negative values for the log likelihood scores ofNucleotide GTR and Global MG94xREV (Table 1), suggesting that both models had fitted the data properly and the analysis is rather reliable, with p value threshold of 0.1. Nucleotide GTR and Global MG94xREV are substitution models and the former is the most general neutral, independent, finite-sites, time-reversible model possible with parameters for nucleotides based on a frequency vector(35); the latter is based on a codon model allowing for a full GTR mutation rate matrix(26).

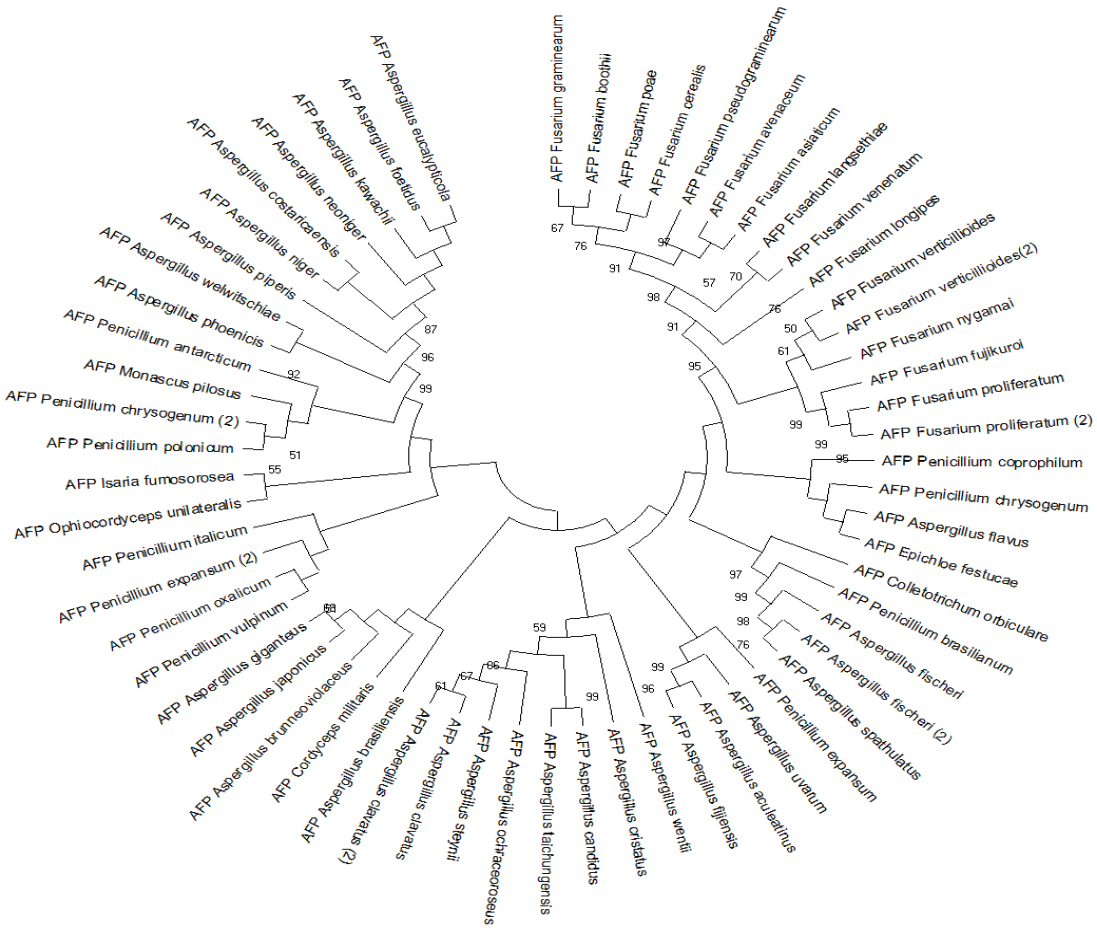

Figure1. *Evolutionary relationships of taxa - The evolutionary history was inferred based on the Neighbor-Joining method. The bootstrap consensus tree inferred from overall 500 replicates to represent the evolutionary history of the taxa. There were a total of 390 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. We included the following genus Aspergillus, Ophiocordyceps, Penicillium, Epichloe, Fusarium, Monascus, Colletotrichum, Isaria and Cordycepsto perform the analysis.*

Table1. *Mode fits for the dN/dS rate analysis.*

Model	AIC_C [a]	log L [b]	Parameters [c]	dN/dS	pvalue
Nucleotide GTR	25954.83	-12848.33	127	0.420	< 0.1
Global MG94xREV	25065.80	-12532.90	134		

a Sample information score

b Log likelihood of model fit

c Number of estimated paramateres

At this selected threshold, we found positive or diversifying selection at1site and negative or purifying selection at 29 sites. The strongest positive selection takes place at the amino acid

residue 63 (dN-dS = 0.308) (Table 2) and the strongest negative selection takes place at the amino acid residue 121 (dN-dS = -1.26) (Figure 2). We hypothesize that the site 63 is an important region of variation of fungi AFP and could significantly contribute to the function of the protein and stabilization of PPIs and protein-membrane interaction. As a perspective for future projects, we are going to assess in silico mutation at site 63 and perform dynamical simulation in order to check if this specific site is essential for the conformational structure and function of AFP.

Table2. *The five highest value of the difference between dN and dS.*

Site	dS	dN	dN-dS	P [dN/dS> 1]	P [dN/dS< 1]
63	9.54	20.3	0.308	0.0266	0.989
41	11.9	19.9	0.227	0.109	0.943
94	1.58	6.86	0.194	0.260	0.878
43	11.1	17.6	0.185	0.132	0.927
47	10.9	16.8	0.169	0.152	0.916

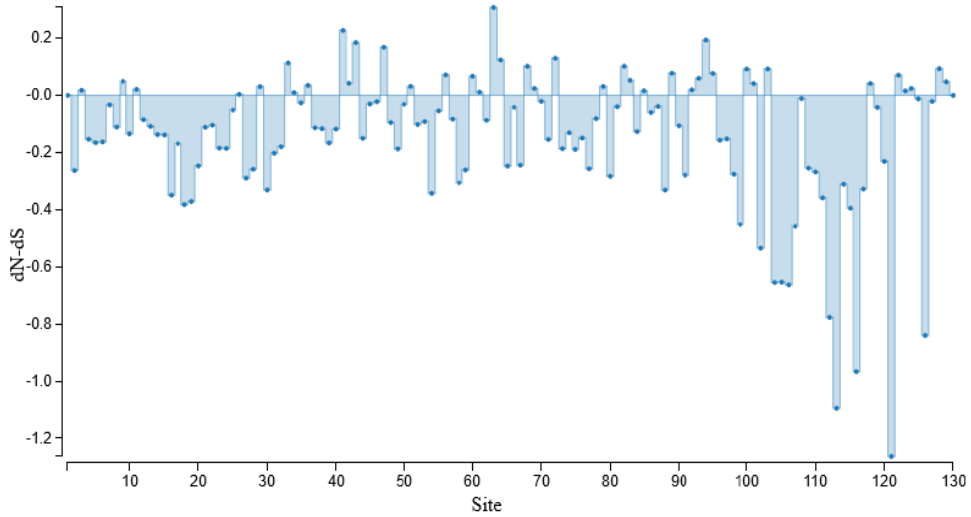

Figure2. *Difference between dN and dS for the amino acid residues–dN/dS ration for the 61 AFP nucleotide sequence. According to the analysis residue 63 is under strong positive selection and the residue 121 is under strong negative selection (dN number of non-synonymous and dS synonymous substitutions).*

Concluding Remarks

Currently, a dramatic increase in fungal resistance and infection in immune compromised patients have driven the sleek of antimicrobial drugs with novel mechanisms of action. Bioinformatics assessment has a raised as a promising approach to address this issue. Innumerous synthetic compounds and small molecules have been designed and tested against a large variety of fungal infections. A systems biology bottom-up approach was applied here to determine important regions of AFP from fungi organisms that could be modulated and used as a promissory antifungal agent.

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