Journal of Computational Intelligence in Bioinformatics
ISSN 0973-385X Volume 5, Number 1 (2012) pp. 1-11

http://www.ripublication.com/jcib.htm

Phylogenetic Analysis of Airborne Fungal Allergens using Bioinformatics Tools

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Abstract

A) Background: Fungi are ubiquitous in indoor and outdoor environment and nearly 10% of people worldwide have fungal allergy. Majority members of the Ascomycota and Basidiomycota induce Type I Allergy. An estimated 0.5-1% of proteins in a given fungal proteome may be allergens and appear to occur as functional groups. But still, little is understood about the structural basis for allergenicity.

B) Methods: Protein sequences of airborne fungal allergens were selected from the database created by us (http://www.mscwbif.org/afad/). BLAST was used to identify sequence identities. After aligning the sequences a phylogenetic tree was constructed using neighbour-joining method of MEGA 4.0.The percentage of bootstrap confidence levels for internal branches was calculated from 1000 random samplings.

C) Results: The 40 allergenic proteins selected for the study belonged to 13 fungal species from 8 orders representing 2 fungal phyla. Our analysis demonstrated that the fungal allergens have undergone a remarkable group-specific diversification. The combined tree showed two distinct groups. Group I had three clusters – A, B and C with cluster A having 5 clades and cluster C having 2 clades. Cluster B was branched out into 2 sub-branches. Group II had 2 branches each with sub-branches. Branch I had 6 clades while branch II had 3 clades. The Enolases of the fungi studied clustered together.

D) Conclusions: Data obtained during our analysis contributes towards better understanding of the diversity of the airborne fungal proteins and provide important clues about their possible cross-reactivity and ancestral lineage. Our results clearly support the notion that sequence identity is a useful predictor of cross-reactivity.

Introduction

The Phylogenetic analysis of amino acid sequences is used to address a number of biological questions. These methods enable the reconstruction of a cell and species evolution.

Fungi are common in indoor and outdoor environment, and nearly 10% of people worldwide have fungal allergy (1). Allergy is seen with increasing frequency in the developing world. (2, 3, 4, 5). Numerous studies have shown that exposure to fungi may be associated with acute toxic effects, allergies, and asthma (6, 1, 7, 8).

Out of over 100,000 fungal species reported, a few hundred occur as opportunists and about 100 are known to elicit mycoses in man and animals (9). More than 80 mold genera have been shown to induce type I allergies in susceptible persons, whereas allergenic proteins have been identified in only 23 fungal genera. The outdoor spore concentration ranges from 230 to 10^6 spores/m^3 (10, 11). Atmospheric fungal spore concentration exceeds mean pollen concentration by 100–1,000 times (12).

The most important allergy-causing fungal genera belonging to the ascomycota are *Alternaria* , *Aspergillus* , *Bipolaris* , *Candida* , *Cladosporium*, *Epicoccum* and *Phoma*, whereas *Calvatia* , *Coprinus*, *Ganoderma* , *Pleurotus* and *Psilocybe* are the most prominent genera of the basidiomycota

An estimated 0.5 – 1% of proteins in a given fungal proteome may be allergens. Despite intensive efforts to determine what distinguishes these proteins from the other non-allergens in the same proteome, little is understood about the structural basis for allergenicity. We also do not understand why some organisms are often associated with allergy and other closely related organisms are never or only rarely observed to cause allergy.

The known fungal allergens appear to occur as functional groups such as serine proteases, heat shock proteins or thioredoxins or orthologues of proteins such as Mn superoxide dismutase or enolase. Also most fungi may possess proteins that have potential to be allergens or to cross react with allergens. (13). Some of the fungal allergens show cross-reactivity within the phylum (14, 15, 16). Structure homology and phylogenic tree also throws light on the similarity in structures and thence on the cross-reactivity. This study was undertaken to study the structure homology and phylogenetic relationships between the airborne fungal allergens to add to the knowledge on cross-reactivity of fungal allergens and their ancestral lineage.

Methodology

Protein sequences of airborne fungal allergens were downloaded from the database

created by us: Airborne Fungal Allergen Database (AFAD), (http:// www.mscwbif.org/ index_db.html).BLAST (Basic Local Alignment Search Tool) server at our database was used to identify the sequence identity (17).

Protein sequences with more than 250 amino acids were selected. 40 sequences were considered for the study. The selected sequences were aligned using alignment explorer in MEGA 4.0 with default parameters. After aligning the selected sequences, the deduced amino acid sequences were further assembled into multiple sequence alignment. Phylogenetic tree on sequences was also constructed using Neighbor-Joining method of Molecular Evolutionary Genetic Analysis (MEGA) 4.0 (18). The percentage of bootstrap confidence levels for internal branches is defined by MEGA program and was calculated from 1000 random samplings.

Results

The combined tree (Fig 1) shows two distinct groups and they are as follows: Group I has three clusters A, B and C. Cluster A has 5 clades. Clade (a) has *P.chrysogenum* allergen Pen n 18 and *P. chrysogenum* vacuolar serine protease, which shows 100% bootstrap value. It also has a monoclade *P.oxalicum* vacuolar serine protease which is closer to clade (a) with bootstrap value of 99%. *Aspergillus fumigatus* serine proteinase precursor and *Aspergillus niger* serine protease formed clade (b) with well defined high bootstrap values (96%). The yeast *Rhodotorula mucilaginosa* vacuolar serine protease exists as monoclade. *Aspergillus fumigatus* alkaline protease and *Aspergillus oryzae* peptidase formed clade (c) and *P.chrysogenum* allergen Pen n 13 and *Trichophyton rubrum* allergen Tri r 2 formed clade (d). *Aspergillus niger* 3-phytase B composed a monoclade but was not supported by a high bootstrap value (26%). The aldehyde dehydrogenases of *Alternaria alternata* Alt a 10 and of *Davidiella jassiana* Cla h 10 formed a clade.

Cluster B has two branches with sub-branches. Branch I has a clade with *Malassezia furfur* Major allergen Malf1 and *Fusarium culmorum* helix-loop-helix protein. The branch II has a clade with the heat shock protein of *Penicillium citrinum* and the heat shock protein of *Davidiella jassiana*. The alcohol dehydrogenase Cand a 1 of *Candida albicans* forms a monoclade. Cluster C has two clades. Clade (a) has the aspergillo-pepsin I and F of *Aspergillus fumigatus*. Clade (b) has the major allergen Asp f 2 of *Aspergillus fumigatus*. There are three monoclades in this cluster. However the allergen Asp f 4 of *Aspergillus fumigatus* is closer to the clades of Calreticulin of *P. chrysogenum* and peptidyl peptidase 5. Tri r 4 of *T.rubrum* is the other monoclade in this cluster.

Group II has two branches, each with many sub-branches. Branch I has Cluster A which has a single clade and 5 monoclades. Branch II has Cluster B with three clades. The only clade in cluster A has the enolases of *A.fumigatus* with 100% identity. This cluster has the enolases of *P.citrinum, Alternaria alternata, Davidiella jassiana* and *Rhodotorula mucilaginosa.* Pen c 22, Alt a 11, Cla h 6 are closely related to the clade of *A.fumigatus* enolase Asp f 22 with bootstrap values of 98%, 63%, 99% and 100% respectively. Alt a 4, the isomerase of *A.alternata* is weakly supported (46%) in this cluster. Cluster B has two branches. Branch I has two clades, clade (a) has Asp f 23 of

A. fumigatus and Alt a 8 of *A.alternata*. The clade (b) has β-xylosidase of *A.niger* and catalase of *P.citrinum*. Branch II has 2 allergens of *A.fumigatus* Asp f 9 and *A.fumigatus* allergen in the lone clade. The extra cellular elastinolytic metalloproteinase of A.fumigatus is a monoclade with 91% bootstrap values. The heat shock protein Asp f 12 of *A.fumigatus* is also a monoclade though it is weakly supported.

The tree analysis shows that the group I and cluster A of group II have higher bootstrap values making evolutionary sense between the airborne fungal allergens. Similar allergens of different species clustered together. For instance, cluster A of group II comprises the enolase of *A.fumigatus. P.citrinum, A.alternata, Davidiella jassiana* and *Rhodotorula mucilaginosa*. Similarly the serine proteinase of *A.fumigatus* and *A.niger*; aldehyde dehydrogenase of *A.alternata* Alt a 10 and *Davidiella jassiana* Cla h 10; Heat shock proteins of *Davidiella jassiana* Cla h 4 and *P.citrinum* clustered to be in the same clades. But the vacuolar serine protease of *P.oxalicum* did not cluster in the clade (a) of Cluster A which comprised of serine protease and allergen Pen n 18 of *P.chrysogenum*. Likewise vacuolar serine protease of *Rhodotorula mucilaginosa* was placed as a sister clade to the cluster comprising the serine proteinase of *A.fumigatus* and *A.niger*.

Also the heat shock protein 90 Asp f 12 of *A.fumigatus* was a notable exception because it did not cluster with the heat chock protein of *Davidiella jassiana* and *P.citrinum*. Infact it belonged to cluster B of Group II while the heat shock proteins of *D.jassiana* and *P.citrinum* clustered in a clade in cluster B of Group I.

A close relationship between the enolase of *A.fumigatus* and the monoclade was supported by high bootstrap value of 98%, (*P. citrinum*) moderate bootstrap value (63%) *A.alternata*, high bootstrap value of 99% (*D.jassiana*), 100% of *Rhodotorula mucilaginosa*. A.niger 3-phytase B formed a polytony in group I.

Cluster B of Group II shows the relationship between the different allergens of *A.fumigatus, A.alternata, A.niger and P.citrinum*. The heat shock protein 90 Asp f 12 of *A.fumigatus* is an out-group while the elastolytic metalloproteinase of *A.fumigatus* is next most distantly related lineages. Asp f 9 and allergens of *A.fumigatus* are sister groups. Similarly Asp f 23 of *A.fumigatus* and Alt a 8 of *A.alternata* are sister groups just like β-xylosidase of *A.niger* and catalase of *P.citrinum*. They must have descended from same ancestral lineage.

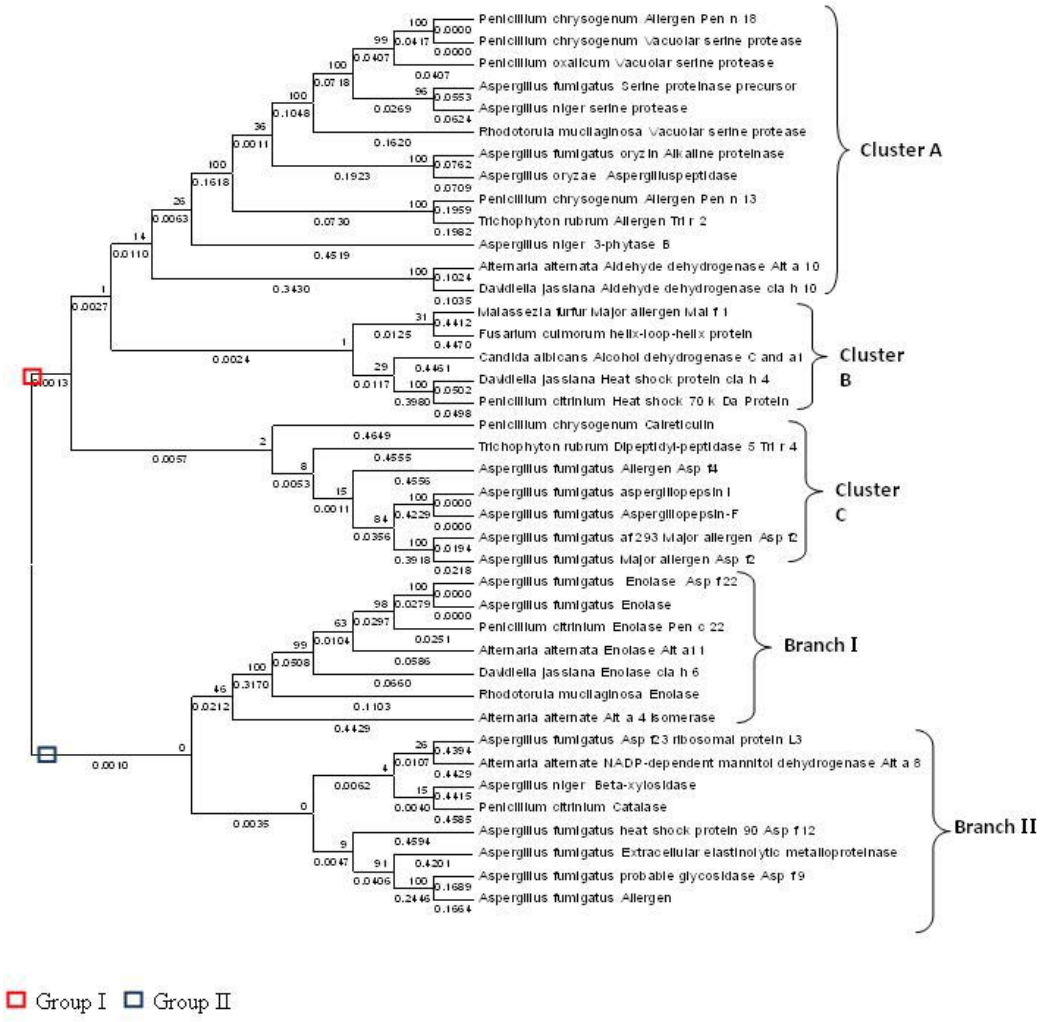

Figure 1: Phylogenetic tree showing the relationships among air-borne fungal allergens. The unrooted tree was constructed using Neighbor-Joining method of MEGA version 4. Numbers on the tree nodes indicate the levels of bootstrap support based on data for 1000 replicates. The scale bar indicates 0.1 amino acid substitution per site.

Discussion

Phylogenetics was used to compare allergens between allergenic fungal species and to study the presence of potential cross-reacting proteins or allergen homologues and orthologues. We found that many of the allergenic fungi possess a core set of allergen homologues and orthologues. Our results support the hypothesis that all fungi may contain allergen homologues and orthologues which could cross-react. Cross-reactivity occurs when homology levels between 40 and 70% is reported between fungal proteins. Even relatively small changes in protein sequence may affect cross

reaction. Allergens that show cross reactivity are closely related at the amino acid level which is evident in our phylogenetic tree.

Alternaria alternata is frequently encountered in outdoor environments. Thirteen allergens of *A.alternata* have been identified, most of which are intracellular housekeeping proteins. The allergens selected for this study: aldehyde dehydrogenase Alt a 10, Protein disulphide isomerase Alt a 4. (19). Enolase Alt a 11, NADP-dependent mannitol dehydrogenase Alt a 8 (20) belonged to different clusters and Groups. Most of the *A.alternata* allergens cloned and studied so far are major allergens except Alt a 1 which is although recognized by up to 98% of *A.alternata*-sensitized patients (21).

The *A.alternata* allergens are closely related to the mold *Cladosporium herbarum* (*Davidiella jassiana*) (19, 22, 20, 23). This was evident in our tree as the aldehyde dehydrogenase Alt a 10 and Cla h 10 of *A.alternata* and *C. herbarium* clustered to form sister allergens in the same clade and the enolases Alt a 11 and Cla h 6 clustered in the same cluster A of Group II.

So far, 14 allergens have been identified from *C.herbarum*. The allergens selected for our study Aldehyde dehydrogenase Cla h 10, enolase Cla h 6 (19) and the heat shock protein Cla h 4 are minor allergens. All the three allergens selected in this study belonged to different branches.

The saprophytic genus Aspergillus includes 132 different species. It is a dominant indoor pathogen. (24, 25). Aspergillus grows outdoors on decaying vegetation or indoors on the walls and has the ability to release large quantities of small conidiospores of 2-3 μm. *A.fumigatus* is implicated in about 80% of Aspergillus related functions (26). Fourteen allergens of *A.fumigatus* were selected for this study: Asp f 2 fibrinogen binding protein (27) Asp f 13 alkaline serine protease (28) Asp f 4 (29) Aspergillopepsin I, Aspergillopepsin F, Asp f 18 vacuolar serine protease (30) Asp f 22 enolase (31) Asp f 23 L3 ribosomal protein (32) Asp f 5 metalloprotease (33) Asp f 9 probable glycosidase (34). Among these Asp f 4, Aspergillopepsin I, Aspergillopepsin F, Asp f 2 were seen in the same cluster. Asp f 12, Asp f 5, Asp f 9 and allergen also clustered together indicating similar sequence homology. However, Asp f 23 was seen in different branch. On the contrary, alkaline serine protease Asp f 13 and Asp f 18 vacuolar serine protease though were in the same cluster A of Group I did not form sister allergens indicating no similarity. Among the allergens of *A.niger*, Asp n 14 β-xylosidases (35) Asp n 18 vacuolar serine protease (36) Asp n 25 3-phytase B phospatase (37) were selected in our study. As expected the Asp n 18 formed a clade with Asp f 18 and clustered with the serine protease of *Penicillium* species. Asp n 14 formed a clade with catalase of *P.citrinum* and Asp n 25 form an out-group. Asp O 13 alkaline serine protease of *A.oryzae* formed a clade with Asp f 13 alkaline serine protease of *A. fumigatus* which could be due to sequence similarity (38).

More than 150 Penicillium species exist, some of which have been described to be common indoor molds. Results from Shen *et. al.* (1999) showed that 80-93% of asthmatics displayed IgE reactivity to the 32-34 KD serine proteases from *P.citrinum, P.chrysogenum, P.oxalicum, P.brevicompactum, A.fumigatus, A.flavus, A.oryzae* and *A.niger*. In our phylogenetic analyses all the serine protease selected from

P.chrysogenum, P.oxalicum, A. fumigatus, A.niger and *Rhodotorula mucilaginosa* clustered together indicating sequence homology. Rho m 2, a vacuolar serine protease also cross-reacts with other fungal vacuolar serine proteases (16). Likewise Pen n 13, alkaline serine protease of *P.chrysogenum* formed a clade with Tri r 2, vacuolar serine protease of *Trichophyton rubrum*.

Enolases are another class of fungal pan-allergen with extensive cross reactivity and auto-reactivity (23). They are found in many fungal species e.g., *C.herbarum, A.alternata, C. albicans, S.cerevisiae, A.fumigatus, F.solani, C.lunata, R.mucilaginosa, Beauweria baniana* and *P.citrinum*.

Cross reactivity can be seen when IgE antibodies originally directed against a given allergen also bind to a structurally related allergen from another allergen source. Thus it is the result of shared B-cell epitopes among homologous proteins (39). A sequence identity of more than 50% between homologous allergens seems to be necessary in order to exhibit cross-reactivity. Cross-reactivity observed maybe ascribed to the close phylogenetic relationship of fungal species. This is very evident in this phylogenetic analysis. All the serine protease and enolases studied here clustered together. Aldehyde dehydrogenases of *A.alternata* and *Cladosporium herbarum* formed a clade; heat shock proteins of *C.herbarum* and *P.citrinum* formed a clade.

Lin *et al.* (2000) showed that vacuolar serine proteinase allergens from *Penicillium citrinum, P.notatum, P.oxalicum* and *Aspergillus fumigatus* cross reacted with the monoclonal antibodies. Chou *et al.* (2005) demonstrated cross-reactivity for the native and recombinant vacuolar serine proteases from *R.mucilaginosa* and *P.chrysogenum*.

The enolases of *C.herbarum, A.alternata, A.fumigatus* and *C.albicans* were shown to be cross-reactive by inhibition experiments (40). The enolases of our study Asp f 22, Pen c 22, Alt a 11, Cla h 6, Rho m 1 clustered together exhibiting sequence homology.

Clinically, allergic cross-reactivity is encountered as symptoms without prior exposure. It could also lead to occurrence of symptoms upon exposure to allergenic sources that are unlikely to sensitize. Cross-reactivity also describes the relation between two allergens. The closer the similarity between two allergens, the more likely it is to find a cross-reactive antibody. Cross-reactivity prediction algorithms should be developed, because our results support the notion that sequence identity is a useful predictor of cross-reactivity.

In a cross-reacting allergen couple it is difficult to decide which allergen is sensitizer. From our phylogenetic study, we can assume that symmetric cross-reactivity is a possibility when both allergens with sequence similarity can sensitize and both can largely (but not completely) inhibit the binding of IgE to the other allergen.

In the human body, polyclonal situation is encountered. Here a cross-reactive antigen will have a lower affinity than antigen the induced the antibody response. In clinical terms, this low affinity may translate into a high threshold for the cross-reactive allergen and/or milder symptoms. In this context, our results are noteworthy.

Our results also indicate that the presence of multiple close allergen homologues

and orthologues that are structurally indistinguishable may raise a considerable problem in diagnosis and classification of fungal allergy. Our results also help in preparing species specific allergens for effective skin prick testing or IgE binding assays.

Our results also revealed that several regions of the amino acid sequence of homologues are evolutionary highly conserved. Highly conserved primary sequences of allergenic homologues can be used to establish evolutionary relationships. This knowledge of sequence similarity of peptide allergens and phylogeny can be used to produce molecules with reduced allergenic activity and conserved antigenicity, which in turn can be used for vaccination for allergy treatment.

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