

IgE sensitization to fungi mirrors fungal phylogenetic systematics

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Background: Fungal allergy is an elusive disease, and little progress has been made in this field during recent years. Moreover, because of the complexity of the organisms, it is difficult to categorize fungi systematically on the basis of morphologic characterization. However, recent molecular phylogenetics studies have substantially improved fungal categorization. In parallel, new approaches to analyze large IgE antibody datasets enable identification and visualization of IgE sensitization patterns.

Objective: To study whether molecular phylogenetic relationships of fungal species, commonly used in allergy diagnosis, also are reflected in IgE sensitization profiles of individuals sensitized to fungi.

Methods: A dataset was compiled of recorded serum IgE antibody levels to 17 different fungal species from 668 individuals sensitized to at least 1 of the 17 species. By applying a clustering method to this dataset, the fungal species were grouped into a hierarchical organization. Finally, the resulting organization was compared with recently published fungal systematics.

Results: The hierarchical structure of fungi, based on the presence of IgE antibodies in sensitized individuals, very well reflected phylogenetic relationships. Examples include the distinct separation of basal fungi from the subkingdom Dikarya as well as individual cluster formations of fungi belonging to the subphylum Saccharomycotina and order Pleosporales.

Conclusion: To our knowledge, this is the first in-depth study that demonstrates a close relationship between molecular fungal systematics and IgE sensitization to fungal species. Because close evolutionary organisms typically have a higher degree of protein similarity, IgE cross-reactivity is likely the main reason for obtained organization. (*J Allergy Clin Immunol* 2010;■■■:■■■-■■■.)

Key words: Hierarchical clustering, fungal allergy, IgE sensitization, fungal systematics

Abbreviation used

CV: Coefficient of variance

Fungi are eukaryotic, nonchlorophyllous and heterotrophic organisms that commonly grow as saprophytes on nonliving organic material or as invasive pathogens in living tissue.¹ Because they have the capability to grow under highly variable conditions, they are ubiquitous throughout the environment.² Fungi are principally dispersed as sexual spores or asexual conidia and are common components in the atmosphere. In addition, unidentifiable fungal hyphae fragments may be aerosolized in large numbers, which further increases risk of human exposure through breathing. Counts of airborne fungi in indoor and outdoor environment fluctuate widely with genus as well as with environmental factors, and concentrations can range from 0 to 10⁶ colony-forming units per cubic meter of air.^{3,4}

Fungi can cause adverse health effects in human beings through harmful immune response, such as allergy and hypersensitivity pneumonitis, by toxic or irritant effects, or by direct infection. Thus, unlike many other airborne allergens, fungi are associated with a variety of illnesses besides IgE-mediated allergy. A common and undesirable feature of fungal allergies is the lack of clear evidence of disease, or a well defined pathology.⁵ There are several reasons for this diagnostic inadequacy, such as heterogeneous disease symptoms and differences in routes and amount of exposure, but an important contributor is the difficulties in characterization and identification of the allergenic species. Traditionally, species identification and evolutionary classification of fungal species are mainly founded on morphologic characterization. However, recently described revolutionary phylogenetic studies, based on sequence comparisons of PCR-amplified ribosomal RNA genes, have paved the way for more advanced characterization and classification of fungi.^{6,7} As a result of these achievements, a refined and improved view on fungal molecular systematics has been established and generally accepted within the scientific community.⁸

More than 80 fungal genera are currently recognized as being associated to allergy,^{2,9} and some of the most frequently occurring are *Cladosporium*, *Penicillium*, *Aspergillus*, *Alternaria*, and *Aureobasidium*.¹⁰⁻¹⁴ During the last decade, a variety of fungal allergens have been described in the literature, including those that are mainly genus-specific (such as the *Alternaria alternata* allergen Alt a 1 and its homologs in other species of genus *Alternaria*) as well as those present in various fungi.^{2,9} Examples of the latter include several protein families involved in IgE-cross-reactivity, such as enolases,¹⁵ nicotinamide adenine dinucleotide phosphate (NADP) mannitol dehydrogenase,¹⁶ and serin proteases.¹⁷

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In a recent report, we delineated and visualized the relationships among 89 allergens from sources such as foods, pollen, mites, epidermals, venoms and fungi with the aid of a large in-house database holding thousands of recorded IgE measurements.¹⁸ One among several findings in that study was that the cluster of allergenic fungi was well separated from other allergen groups. Moreover, although not studied in detail, the fungal cluster appeared to contain subclusters similar to the fungal systematics derived from the abovementioned phylogenetics studies. The currently proposed phylogeny of fungi is based on sequence similarities of ribosomal RNA genes being unrelated to allergens in human beings. However, it is possible that 2 phylogenetically close fungal species also have similar allergen components, thereby increasing the risk of IgE cross-reactivity. If this assumption is true, serologic studies of individuals sensitized to fungi should reveal a higher correlation of closely related molds compared with those that are phylogenetically distant. With the aid of a unique fungal sensitization dataset in combination with an advanced clustering algorithm, we have in this work investigated the association between fungal molecular systematics and IgE sensitization.

METHODS

Compilation of dataset holding specific IgE sensitization data to various fungi

A large repository of human blood serum, which currently holds more than 50,000 samples from nonidentifiable sensitized individuals, has been established over the last decades. The main purpose of this depot is to support research, development, and quality assessment of various ImmunoCAP laboratory systems (Phadia, Uppsala, Sweden). Over the last several years, a substantial amount of the collected sera has been screened for the presence of specific IgE responses to a wide range of allergen preparations. The results are stored in an internal database, which is unique in the sense that a vast number of serum samples have been tested for >100 various allergens, including many fungal species. For cluster analysis of fungal sensitization patterns, a subset of this database was compiled to include the maximal numbers of individual blood sera, which consistently had been tested across the largest panel of fungal allergen preparations. Moreover, only those individuals with at least 1 positive test (>0.35 kU_A/L [kilo units allergen-specific IgE per liter]) were considered. The compiled dataset included 668 unique samples (blood serum of human donors), each with recorded IgE antibody concentrations against 17 separate fungal species, but devoid of clinical history.

The assays for all of the analyzed 668 × 17 IgE measurements have been conducted as follows. Freeze-dried fungal source materials were provided by Allergon (Ångelholm, Sweden). Representative and well characterized strains were selected from Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands) or American Type Culture Collection (ATCC, Manassas, Va) and grown in surface cultures using a completely synthetic medium, thus containing both mycelia and spores. Before use of extracts, non-IgE-binding low-molecular-weight constituents were removed by using a Sephadex G-25 column (GE Healthcare, Uppsala, Sweden). For more information on the extraction protocol, see Karlsson-Borgå et al.¹⁹ To ensure consistency between extract batches over time, new batches of source materials were accepted only after comparison with a representative reference source material by using well defined positive and negative sera. Long-term trends are monitored internally and through external quality control programs. Measurements of specific IgE binding were performed by using the ImmunoCAP assay platform (Phadia, Uppsala, Sweden).^{20,21} Results are reported quantitatively using a kilounits per liter scale (kU/L). The calibrator is IgE bound to anti-IgE by using a 6-point quantitative curve. Calibration ranges from <0.35 kU/L to >100 kU/L. Because there have been changes in assignment of negative test results over the years (listed as <0.35, 0.0, or as the actual measured values between 0 and 0.35), all values below this

cut-off level were set to 0.35 kU_A/L. Experimental ImmunoCAP tests were prepared as described by the manufacturer.²²

Molecular taxonomic classification of fungi

The overall phylogenetic structure of the selected 17 fungal species, down to the level of order, was adapted from data based on sequence comparisons of PCR-amplified ribosomal RNA.⁸ The placing of the different species in their respective orders and families was adapted from 2 classification databases: National Center for Biotechnology Information (NCBI) and Index Fungorum (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=taxonomy> and <http://www.indexfungorum.org/Names/Names.asp>, respectively).

Cluster analysis of IgE sensitization data

An earlier study on clustering of allergen extracts concluded that best results were obtained by transforming IgE levels to their logarithm values and by measuring distances (or rather dissimilarities) in the clustering procedures based on the Pearson correlation coefficient.¹⁸ The use of logarithm IgE values reduces the impact of high extreme values on calculations of correlation coefficients. The principal analytical method used in this study is as a flexible multibranching divisive hierarchical clustering algorithm, which employs the classical k-means²³ for cluster formation and silhouette width²⁴ for selection of cluster numbers.²⁵ In contrast with standard binary agglomerative hierarchical clustering, which only merges clusters 2 at a time, this method is divisive, and the optimal number of subclusters is calculated at each division (branching).

Visualization of hierarchical structure

Overall and subordinate structures obtained by hierarchical clustering are typically visualized as dendrograms (family trees). At every hierarchical level of the dendrogram, samples are assigned into disjoint groups. Because standard dendrograms only allow binary splits at each hierarchical level, they are not applicable to the multibranching structure obtained here. Therefore, the hierarchical structure was visualized in a dendrogram featuring a flexible number of multiple branches.

Outline of hierarchical clustering procedure

The clustering procedure can be summarized as follows:

1. All IgE values in the 668 (individuals) × 17 (fungal allergens) were transformed by using the logarithm operator.
2. The dissimilarity measure between 2 objects x and y during the clustering was defined as

$$1 - r_{x,y},$$

where $r_{x,y}$ is the Pearson correlation coefficient. Thus, high correlation between 2 fungi returns a short distance (or rather dissimilarity). An example of this dissimilarity measure can be found in this article's Fig E1 in Online Repository at www.jacionline.org.

3. At the first hierarchical level, fungi are divided into 2 or more clusters (depending on which cluster number that maximizes the average silhouette criterion). Analogously, each resulting cluster is iteratively divided into subclusters until all fungal species represent a single cluster.
4. The resulting hierarchical structure is thereafter visualized as a dendrogram (family tree representation).

Analysis of cluster stability

If multiple recordings of the same serum sample are performed, each such measurement will vary slightly, and this variability is usually described with the coefficient of variance

TABLE I. Pairwise correlations of 17 allergenic fungal species*

Species names †	Species code names																
	f45	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10	m11	m12	m13	m14	m15	m16
<i>Saccharomyces cerevisiae</i> (337)	f45	0.73	0.71	0.76	0.63	0.80	0.52	0.82	0.74	0.78	0.65	0.67	0.85	0.73	0.67	0.68	0.71
<i>Penicillium chrysogenum</i> (310)	m1	1.00	0.72	0.85	0.60	0.63	0.57	0.84	0.76	0.77	0.73	0.62	0.82	0.71	0.73	0.76	0.74
<i>Cladosporium herbarum</i> (326)	m2		1.00	0.72	0.49	0.60	0.54	0.79	0.73	0.69	0.69	0.54	0.79	0.70	0.68	0.63	0.67
<i>Aspergillus fumigatus</i> (359)	m3			1.00	0.55	0.66	0.66	0.88	0.87	0.85	0.78	0.60	0.86	0.81	0.80	0.73	0.80
<i>Mucor racemosus</i> (319)	m4				1.00	0.50	0.34	0.60	0.53	0.56	0.48	0.91	0.62	0.53	0.52	0.60	0.58
<i>Candida albicans</i> (465)	m5					1.00	0.44	0.72	0.65	0.68	0.55	0.56	0.74	0.62	0.55	0.56	0.58
<i>Alternaria alternata</i> (384)	m6						1.00	0.70	0.78	0.70	0.87	0.39	0.68	0.78	0.78	0.59	0.74
<i>Botrytis cinerea</i> (341)	m7							1.00	0.91	0.86	0.84	0.66	0.94	0.87	0.83	0.75	0.84
<i>Setomelanomma rostrata</i> (352)	m8								1.00	0.87	0.90	0.59	0.89	0.93	0.88	0.71	0.87
<i>Fusarium proliferatum</i> (333)	m9									1.00	0.82	0.64	0.87	0.86	0.80	0.74	0.83
<i>Stemphylium herbarum</i> (367)	m10										1.00	0.54	0.82	0.88	0.90	0.71	0.86
<i>Rhizopus nigricans</i> (353)	m11											1.00	0.68	0.59	0.56	0.64	0.63
<i>Aureobasidium pullulans</i> (331)	m12												1.00	0.88	0.82	0.75	0.83
<i>Phoma betae</i> (342)	m13													1.00	0.90	0.74	0.90
<i>Epicoecum purpurascens</i> (315)	m14														1.00	0.74	0.90
<i>Trichoderma viride</i> (222)	m15															1.00	0.82
<i>Curvularia lunata</i> (325)	m16																1.00

*The color scheme for the estimated correlation coefficients *c* is as follow: $c < 0.70$ (yellow), $0.7 \leq c < 0.85$ (orange), and $0.85 \leq c < 1$ (red).

†The number within the parentheses indicates the number of individuals having a positive test (>0.35 kUA/L) to the specified fungus.

$$CV = 100 \times \frac{\sigma}{\bar{x}}$$

where σ is the SD and $\bar{x}$ is the average over several repeated measurements. Thus, because of variability of the test system, each IgE level recorded in the dataset contains some degree of measurement noise. The compiled dataset contains both IgE levels that are averages of several replicates as well as those that have been analyzed only once. Therefore, each recorded value was treated as an average value derived from a series of measurements. To study the stability of the hierarchical structure obtained, clustering was conducted of datasets with simulated noise added. Four different levels of noise were assessed, and for each such level, 20 noisy datasets were randomly generated as follows:

$$X_{noise} = X + R,$$

where each measurement x was added a noise variable r (drawn from a normal distribution with 0 mean and SD $x \cdot CV$). The 4 levels of noise were generated by varying the size of coefficient of variance (CV; 5%, 10%, 15%, or 20%). The highest simulated noise level corresponds to a CV level that exceeds those associated with variability of the ImmunoCAP laboratory systems, which for most allergens is less than 10% and rarely higher than 15%. Because all IgE analyses have been performed in 1 laboratory, the interlaboratory variability is assumed to be considerably lower. The noise variable should instead be regarded as simulated variability caused by temporal factors, such as differences in batches of fungal extracts or laboratory instruments over the years.

RESULTS

The number of allergen-specific IgE measurements above the cut-off (>0.35 kU_A/L) was fairly similar for all fungal species tested except for *Candida albicans* and *Trichoderma viride* (Table I). Among the 668 blood donors, almost 20% of the subjects were monosensitized (to the 17 fungi tested), whereas roughly 25% had IgE levels >0.35 kU_A/L against all 17 allergenic fungi (Fig 1). Among monosensitized individuals (in the context of the 17 allergens analyzed here), as many as 50% were sensitized to *C albicans* (data not shown). Inspection of *Candida*-sensitized individuals' records concluded that most of these tests were truly positive (ie, not a result of serum samples having extremely high total IgE levels or test values near the cut-off threshold [data not shown]). Although some individuals had only 1 positive test to the 17 fungal species studied in this work, it should be noted that many of them were multisensitized as regards allergens outside the kingdom Fungi (Fig 1). As also evident from Fig 1, the degree of general multisensitization is even higher in the group of people that had positive tests to all 17 fungal species.

As an introductory analysis of IgE sensitization relationships among the 17 fungal species, all pairwise correlation coefficients were calculated on the basis of log-transformed IgE values of 668 individuals (Table I). It is evident that *Mucor racemosus* and *Rhizopus nigricans* have a higher correlation coefficient than any of the other fungi, but such patterns are more difficult to identify for the other fungal species (Table I). Therefore, the organization of the fungal IgE sensitization dataset was delineated by means of flexible and multibranching hierarchical clustering. The dissimilarity measure used in clustering procedure was also based on correlation coefficients (see Methods). A detailed view of the resulting hierarchical tree is illustrated in Fig 2, whereas a schematic overview of the IgE-based relationships is depicted in Fig 3. Moreover, in Fig 3, the hierarchical pattern is compared with a phylogenetic structure of fungi that has been adopted from the literature, which in turn, is based on sequence comparisons of PCR-amplified ribosomal RNA genes of fungal genera. Both

the IgE-derived tree and that built on phylogeny have an identical split at the top hierarchical level, wherein the 17 fungal species are divided into 2 subclusters: 1 group holding 15 fungi of the subkingdom Dikarya, and the second cluster holding the 2 basal fungi species *M racemosus* and *R nigricans*. In the IgE-based tree, the Dikarya cluster is further partitioned into 3 subclusters: 1 holds the 2 allergenic yeasts of subphylum Saccharomycotina, whereas the 2 other subclusters hold 6 and 7 fungi, respectively, of subphylum Pezizomycotina. Whereas the latter Pezizomycotina group contains fungi of 4 mixed classes, the former exclusively holds fungal species belonging to the same class, subclass, and even order (Dothideomycetes, Pleosporomycetida, and Pleosporales, respectively).

To assess the stability of the hierarchical structure obtained for the 17 fungi, noise was randomly inserted to mimic the variability of the test system was performed. All noisy datasets were subsequently clustered, and the top 2 hierarchical levels were compared with those of the original dataset (Table II). Regardless of noise level, the first hierarchical branching (dividing Dikarya fungi from basal fungi) was consistently identified. The second level split of the Dikarya fungi into Saccharomycotina subphylum cluster, Pleosporales order cluster, and a Pezizomycotina cluster of mixed classes, respectively, was also largely robust to noise (Table II). Moreover, although the second level division into 3 subclasses was unidentified in 20% of the time at the highest noise level, the Saccharomycotina and Pleosporales subphylum clusters still mainly remained intact (data not shown).

DISCUSSION

Recently, we reported the organization of relationships among 89 allergens from a variety of sources, such as foods, pollen, and mites by using a large dataset covering IgE measurements from over 1000 multisensitized individuals.¹⁸ Besides establishing several known allergen relationships, the distinct cluster of fungal species seemed to contain potentially interesting substructures. Therefore, we have focused in the current study on delineating the hierarchical organization among allergy-related fungi with means of recorded IgE measurements to 17 fungal species. A number of recent studies have reported the use of molecular phylogenetics to delineate the higher level of the hierarchical structure within the kingdom Fungi.⁶⁻⁸ Extraction of this structure for the 17 fungi included in our analysis enabled comparison to our IgE-based hierarchical organization. Although founded on completely different data, there are large similarities between the hierarchical tree based on modern fungal phylogenetics and that based on fungal IgE sensitization patterns (Fig 3). Thus, the phylogenetic relationships of Fungi are also reflected by the immune system's response, as displayed in fungi-sensitized individuals. This is particularly interesting, considering that established phylogeny is founded on ribosomal RNA sequences, rather than genes coding for allergens recognized by IgE antibodies. Actually, a phylogenetic study of *Alternaria* species and a few other taxonomically close species was recently reported, wherein sequence similarities of genes coding for the Alt a 1 allergen (and its homologs) constituted the foundation for the identified hierarchy.²⁶ However, because *A alternata* is the only species that overlaps with the current study, it is difficult to compare our hierarchical structure with that reported by Hong et al.²⁵

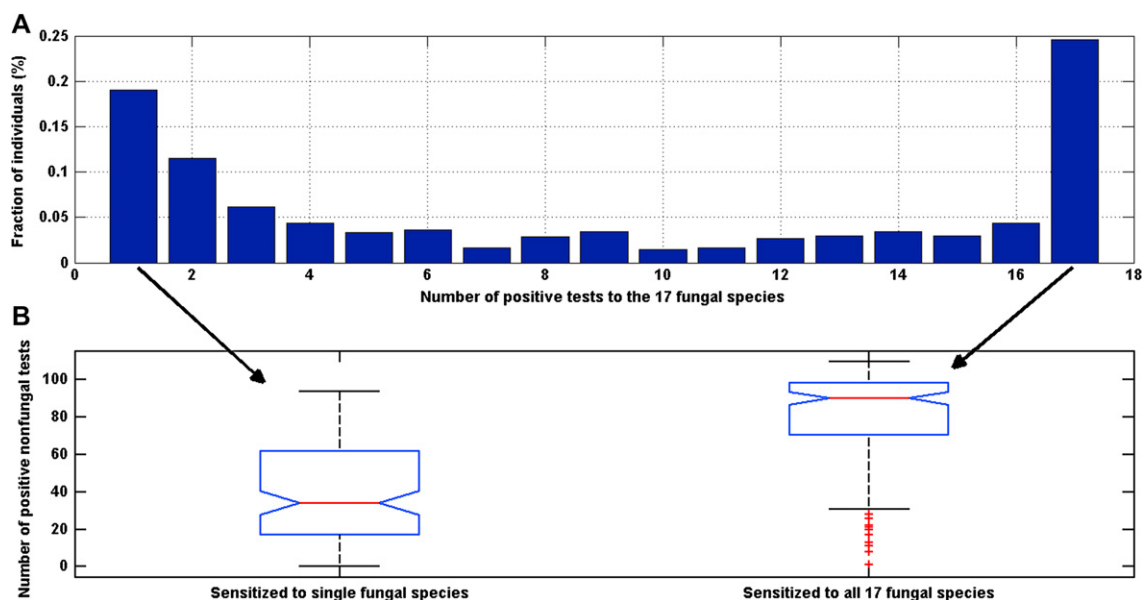


FIG 1. Degree of multisensitization within study subjects. The figure illustrates the degree of fungal multisensitivity of the 668 individuals as a histogram, ranging from individuals being positive to only 1 fungal allergen up to all 17 (A). Each bar represents the percentage of individuals who had detectable levels of specific IgE to a certain number of fungal species. The 2 groups (bars) being either monosensitized to only 1 fungus or having positive tests to all 17 fungal species are marked with arrows. Box plots of these 2 groups are depicted regarding multisensitivity to allergens in general (besides fungi; B). Each box represents the interquartile range (ie, the 25th to 75th percentile range). The horizontal bar in the box is the median, and the T-bars represent the main body of the data. Outliers are indicated by plus signs. The notches within the boxes display the variability of the median between samples. Because the notch widths of the 2 different groups do not overlap, those sensitized to all 17 fungi have more positive tests to nonfungal allergens than those sensitized to only 1 fungal species ($P < .05$ using Mann-Whitney test).

There are several plausible molecular interpretations of the observed statistical associations between fungi, as revealed by the IgE-based dendrogram (Figs 2 and 3). Theoretically, a strong association of 2 allergen extracts could be the result of a cosensitization event—joint exposure of the 2 allergen sources leading to mutually independent sensitization events. IgE cross-reactivity, which involves IgE antibody recognition of a protein with an epitope similar to that of the initially sensitizing allergen, is another plausible reason for serum associations,^{27,28} and several fungal protein families causing IgE-cross-reactivity have been described.⁹ Generally, IgE cross-reactivity typically involves allergenic proteins from phylogenetically related species, and commonly a relatively high degree of identity at the amino acid sequence level is typically seen between such proteins.²⁹ However, it should be stressed that homology to an allergenic protein does not automatically lead to cross-reactivity. A specific type of cross-reactivity arises from certain structures named cross-reacting carbohydrate determinants.³⁰ These reactions are a result of the high content of mannose glycan chains in Fungi, and their clinical relevance is disputed.⁹ Because IgE sensitization among the individuals studied here strikingly mirrors fungal phylogenetic relationships (Fig 3), we believe that observed associations are largely a result of cross-reactivity (including cross-reacting carbohydrate determinants). Examples include the cluster of Pleosporales order, as well as the cluster of Saccharomycotina subphylum. Thus, this work may pave the way for more specialized studies, wherein a subset of the 668 individual sera are further analyzed using a selection of recombinant or purified fungal allergens.

The IgE-based cluster analysis identifies *A alternata* as being the Pleosporales species with the least similarity to other members of the same order (Fig 2). *A alternata* expresses the clinically important allergen Alt a 1, whose homologs have been identified as allergens in primarily other *Alternaria* species,² although recent immunoblotting results indicate a certain degree of IgE-cross reactivity within the Pleosporales order.³¹ Thus, the lower similarity to other Pleosporales species could reflect that several of the individuals are sensitized to *A alternata* mainly through Alt a 1 and are thereby less likely to show positive IgE tests toward other fungal species. Furthermore, the correlation coefficient between *Candida* and *Saccharomyces* is not outstandingly high (compared with other values of Table I), and a plausible cause is that among the individuals with only 1 positive test, 50% are sensitized to *Candida* (data not shown). Nonetheless, the cluster analysis as-sorts these Saccharomycotina fungi into the same taxonomically intuitive cluster (Figs 3 and 4).

In line with our previous study on general allergen sensitization, this study is also characterized by a multivariate data analysis approach to large datasets of IgE antibody measurements. In the last 5 years, considerable analysis improvements have been made in component-resolved diagnostics,³²⁻³⁴ and platforms capable of simultaneously measuring hundreds of different allergen-specific IgE antibody levels from just 1 blood sample are now commercially available.³⁵⁻³⁷ With the aid of adequate multivariate data analysis, the interpretation of the resulting data is greatly simplified and can also be performed much faster. Therefore, the importance of development and application of methods

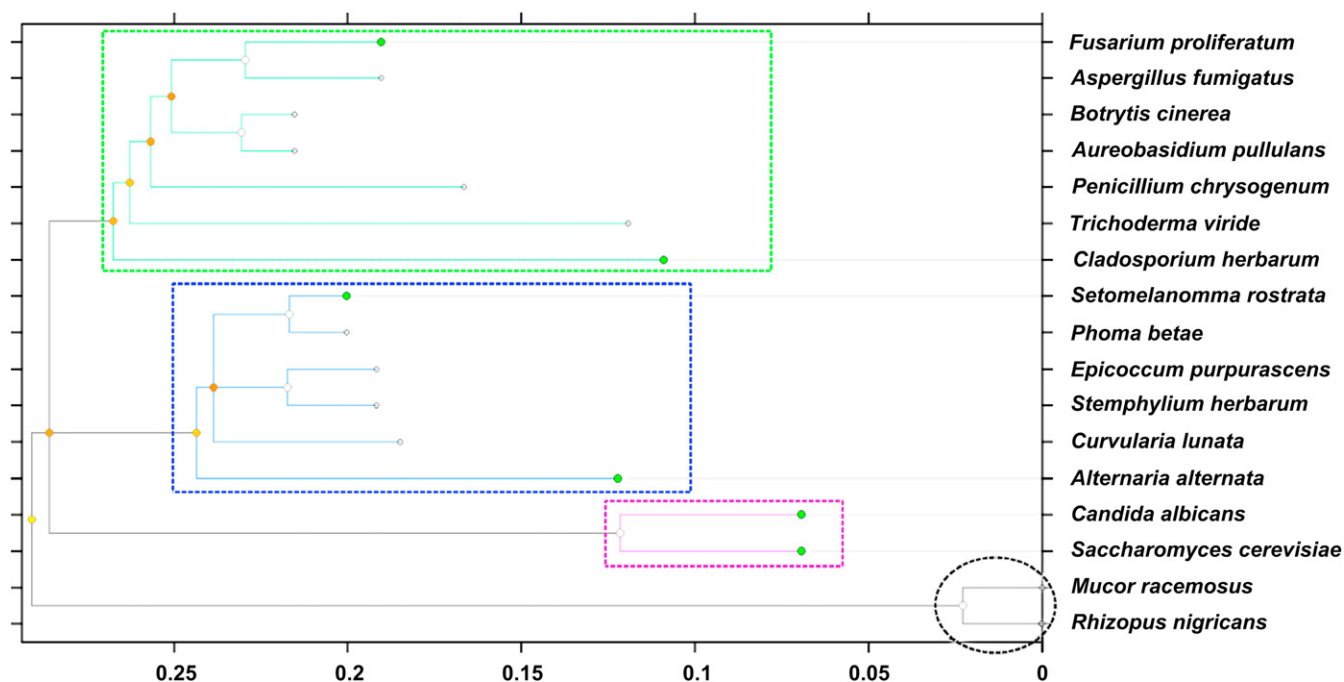


FIG 2. Hierarchical organization of the 17 fungi based on IgE sensitization data. A dendrogram (tree-like display) of molds, as obtained by the cluster analysis of IgE levels in sera of 668 individuals sensitized to fungi. Each horizontal line (branch) represents a cluster (or subcluster) at a given hierarchical level, whereas each branching level is represented by a small circle. The tree is read from left (where all species belong to 1 cluster, the stem) to right (where each species is a single cluster, leaves). At the first hierarchical level (far left), a first split can be seen in which *M racemosus* and *R nigricans* are divided from each other (black ellipse). The remaining fungal species are thereafter divided into 3 clusters (indicated by 3 squares in different colors). See also Fig 3 for a comparison with fungal systematics.

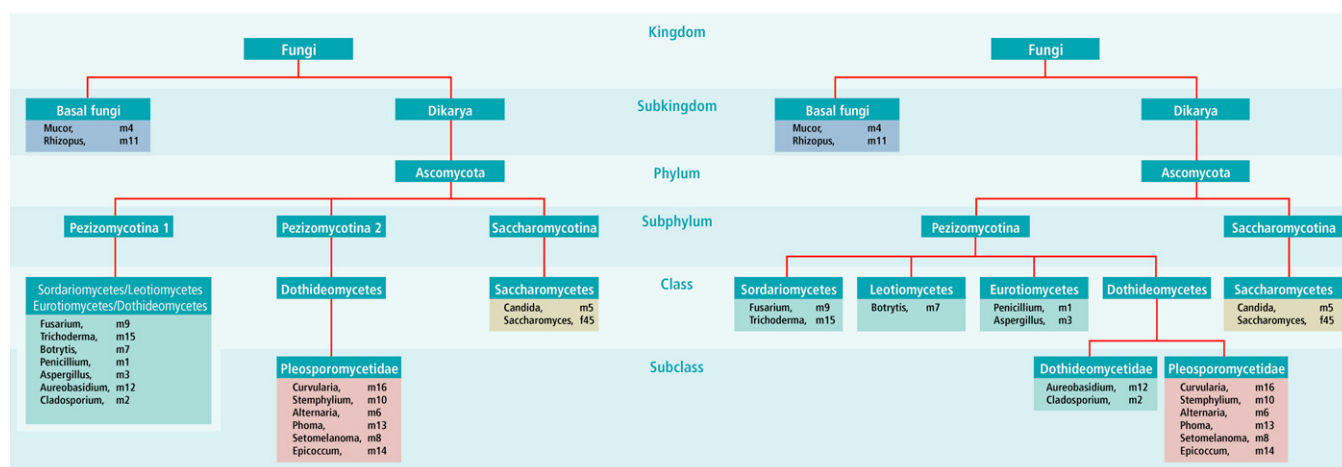


FIG 3. Schematic comparison of IgE sensitization hierarchical organization with that founded on phylogenetic data. The left tree is a schematic display of the IgE sensitization-based clustering, in which branch lengths are ignored, whereas the right tree represents phylogenetic structure of the 17 fungi adopted from the literature. The 2 separately created treelike representations are highly similar and almost identical at a higher-order level. Note that the IgE-based clustering divides the Ascomycota cluster into 2 separate Pezizomycotina clusters (1 and 2), where the second one contains those of subclass Pleosporomycetidae.

in bioinformatics and statistical learning, such as clustering, is quickly increasing within the allergology field.

Although not all allergy-related fungi are represented in the panel of 17 allergen extracts (eg, no species from phylum Basidiomycota, such as the allergy-related *Malassezia* species, are present), it is still likely to represent the most common and

relevant fungal IgE-mediated allergies. Moreover, the 17 allergen extracts spans over a broad taxonomical coverage, which enables comparisons with hierarchy obtained in phylogenetic studies. As mentioned, it is likely that cross-reacting carbohydrate determinants (with doubtful clinical relevance) to some extent contribute to the obtained hierarchical organization. Moreover, no confirming

TABLE II. Robustness tests to various amplitudes of simulated noise

Added noise level	Splitting events	
	First level All Fungi into 2 clusters	Second level Dikarya/Ascomycota into 3 clusters
CV = 5%	100%	100%
CV = 10%	100%	100%
CV = 15%	100%	85%*
CV = 20%	100%	80%*

symptom data on patients are available. Therefore, the relevance of the obtained clusters as regards clinical cross-reactivity needs to be further investigated. However, the functional relevance of the analysis is based on the fact that IgE sensitization is fundamental to IgE-mediated allergy (although IgE sensitization does not imply allergic disease). Moreover, the majority of sera have been collected from donors with a suspected allergy. Another problem of studying fungal allergy lies in the difficulties of preparing fungal extracts for allergy diagnosis, because fungi typically show a considerable variability as a result of interstrain genomic differences, varying culture conditions, and variable extraction procedures.^{38,39} However, as a result of a comprehensive internal quality program, the CV of IgE measurements rarely exceeds 15%. Moreover, the hierarchical structure was relatively robust to noise in simulated experiments wherein noise levels corresponding to CV had been added to the original dataset (Table I). To conclude, the robust and intuitive relationships among fungal preparations, in combination with the large size of the dataset (in total 11,356 IgE measurements), support the relevance of the obtained associations.

To our knowledge, this is the first study that clearly demonstrates a close relationship between fungal phylogenetics and fungal IgE sensitization. The 2 hierarchical structures presented are based on 2 entirely distinct types of input data: sequence comparisons of PCR-amplified ribosomal RNA genes and IgE sensitization profiles among 668 individuals sensitized to at least 1 fungal species. Since both hierarchical organizations demonstrate striking similarities, it is plausible that several subclusters are joined due to sharing similar allergens. Therefore, the hierarchical structure of fungi based on IgE sensitization constitutes a valuable foundation to reveal components involved in IgE cross-reactivity within obtained fungal subgroups. This may in turn lead to improved and simplified testing procedures for patients with suspected fungal allergy. Finally, the hierarchical organization presented in this study has been revealed with the aid of dedicated multivariate data analysis. We believe novel experimental profiling techniques, in combination with tailor-made multivariate methods, have potential to rapidly enhance our knowledge of IgE-mediated allergy.

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Clinical implications: The systematic organization of fungi based on IgE sensitization may be valuable for identifying proteins causing IgE cross-reactivity within the obtained fungal subgroups. This may in turn lead to improved and simplified testing procedures in allergy diagnostics.

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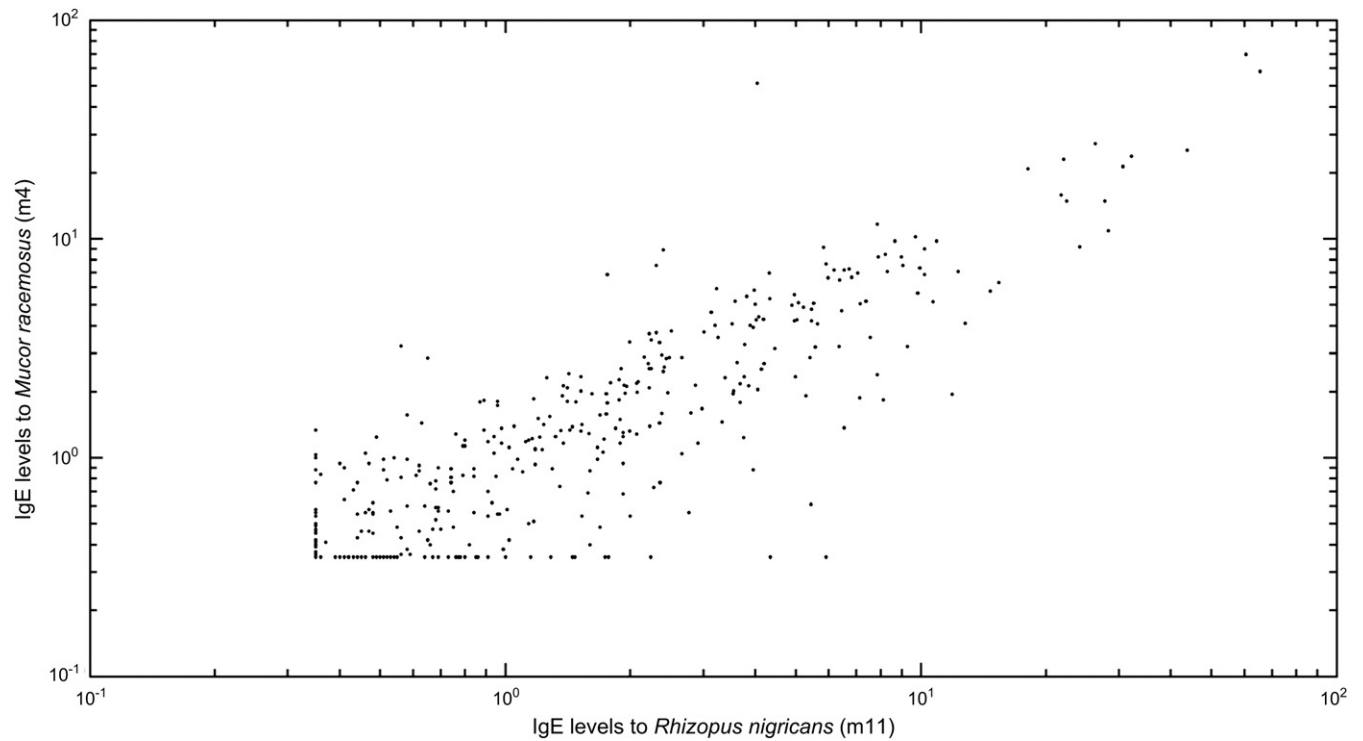


FIG E1. Estimation of correlation coefficients. A diagram over all recorded IgE measurements of the 668 individuals to *R nigricans* (abscissa) and *M racemosus* (ordinate). Note that logarithmic scales are used on both axes (log-log plot). The estimated correlation coefficient for these 2 fungi (based on logarithm values) is 0.91.