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Identification of *Ulocladium chartarum* as an important indoor allergen source

To the Editor,

The constant exposure to fungal spores, which constitute the largest proportion of aerobiological particles, can cause severe health problems, including allergic diseases (1-2). Appropriate management of fungal allergies is hampered by the fact that our knowledge about fungal allergy is still limited to a small number of thoroughly investigated fungi, whereas for the majority of species it is not yet known whether they play a role in allergic diseases (2). This lack of knowledge together with unreliable diagnostic results obtained with fungal allergen extracts of poor quality, contributes to a general underdiagnosis of fungal allergy (3-4). In the present study, we investigated the allergenic potential of *Ulocladium chartarum*, an opportunistic human pathogen (5) that can grow on various substrates (6-7). Exposure to *Ulocladium* species often occurs in the indoor environment as they are commonly detected inside damp buildings and are regarded as an indicator of water damages (6-9). The fact that *U. chartarum* belongs to the same family as the outdoor aeroallergen source *Alternaria alternata*, suggests that *U. chartarum* might also represent an allergen source (6-7).

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IgE immunoblots, performed with sera from patients sensitized to different mold species (Table S1), demonstrated the high allergenic potential of *U. chartarum*, since patients' IgE antibodies recognized several *U. chartarum* proteins (Figure 1A), whereas exposure of the blotted proteins to sera from non-allergic individuals or to immunodetection reagent did not lead to any unspecific binding (data not shown). Interestingly, despite their phylogenetic relationship, the IgE-binding profile of *A. alternata* (Figure S1) differed significantly from the one of *U. chartarum*. Mold-allergic patients not only recognized more proteins in *U. chartarum* than in *A. alternata* extract, but also showed an overall stronger reactivity to them.

Peptide mass fingerprinting allowed to identify a 15 kDa *U. chartarum* protein (A in Figure 1A) as an Alt a 1-homologous protein, a 41 kDa protein (B) as formate dehydrogenase, a protein not yet known as an allergen, and a 48 kDa protein (C) as an Alt a 6-homologous enolase. The proteins were designated Ulo c 1¹ (A), Ulo c 2² (B) and Ulo c 6³ (C). The IgE-reactive protein bands D, E and F could not yet be identified by mass spectrometry. cDNAs coding for the identified allergens were generated, cloned into a bacterial expression vector and recombinant proteins were produced in *Escherichia coli*. Circular dichroism spectroscopy revealed that the recombinant allergens contain considerable secondary structures. In case of rUlo c 1 mainly beta-sheet structures (minimum of far-UV spectrum at 213 nm) were found, whereas predominantly alpha-helical structures (minima at 207 nm and 220 nm) were detected for rUlo c 2 and rUlo c 6 (Figure S2).

ELISAs performed with sera from mold-allergic patients showed the IgE-binding capacity of the three recombinant allergens, with rUlo c 1 displaying the highest levels of IgE-reactivity (Figure 1B). Furthermore, IgE inhibition immunoblots, where nitrocellulose-blotted protein extracts from *U. chartarum* were exposed to serum pools that had been pre-incubated with the recombinant proteins, showed that the recombinant allergens were able to completely inhibit patients' IgE-binding to their natural counterparts (Figure 1C). This indicates that the recombinant allergens represent well-folded proteins which contain all the IgE-binding epitopes present in their natural counterparts and suggests

¹ NCBI Nucleotide Sequence Accession Number: MH716394

² NCBI Nucleotide Sequence Accession Number: MN953052

³ NCBI Nucleotide Sequence Accession Number: MH910061

that rUlo c 1, rUlo c 2 and rUlo c 6 could be used as tools for *in vitro* diagnosis of *U. chartarum* sensitization.

The prevalence of the molecules' IgE-reactivity was analyzed by ELISA using sera from 85 individuals sensitized to different mold species (Figure S3). rUlo c 1 was recognized by 58% of the patients, indicating that this molecule represents a major mold allergen, whereas rUlo c 2 was recognized by 43% and rUlo c 6 by 40% of the patients, suggesting minor mold allergens.

Sequence comparison of the three *U. chartarum* allergens with homologous *A. alternata* proteins revealed protein sequence identities of 89% between Ulo c 1 and Alt a 1, 99% between Ulo c 2 and its homologous *A. alternata* protein, and 100% between Ulo c 6 and Alt a 6. The high sequence homology between Ulo c 1 and Alt a 1 and the suggested presence of Alt a 1-homologous proteins in other species of the Pleosporaceae family (10), prompted us to investigate the potential cross-reactivity between rUlo c 1 and rAlt a 1 in IgE inhibition immunoblots. As depicted in Figure 2A, pre-incubation of patients' sera with one allergen always significantly reduced or even abolished IgE-binding to the other molecule, whereas IgE-binding was not reduced by pre-incubation of the sera with the irrelevant respiratory allergen rBet v 1. This reduction of IgE-binding obtained with rAlt a 1 and rUlo c 1 was comparable to the reduction obtained by self-inhibition and indicates that the two allergens share conserved IgE-epitopes.

Furthermore, both recombinant allergens, rUlo c 1 and rAlt a 1, induced a dose-dependent expression of CD63 in basophils from six of the eight analyzed mold sensitized individuals (Figure 2B), which provided evidence for the molecules' biological activity.

Analysis of the release of allergens from allergen sources can provide useful information about the potential exposure to these allergens. Interestingly, our investigations on the release kinetics of Ulo c 1 and Ulo c 6 from *U. chartarum* and Alt a 1 and Alt a 6 from *A. alternata* showed that the allergens were all immediately released from the spores (Figure S4). These findings suggest that rapid allergen elution might also occur when spores reach mucosal surfaces, where they can then elicit allergic reactions.

In conclusion, in this study we provide evidence that *Ulocladium chartarum* represents an important, so far underestimated, allergen source that shows at least partial cross-reactivity to the evolutionarily related species *A. alternata*. Owing to the fact that in contrast to the outdoor mold *A. alternata*, *U. chartarum* is an important component of the indoor environment, it is especially important to raise the awareness that *U. chartarum* represents a potential cause of respiratory allergic diseases and to include the species into routine allergy diagnosis. We identified the species' first allergens, Ulo c 1, Ulo c 2 and Ulo c 6, and produced them as IgE-reactive recombinant molecules. These recombinant allergens will expand the repertoire of fungal allergens available for fungal allergy diagnosis, thus improving diagnosis' specificity and sensitivity.

The description of the used methods can be found in the supporting information.

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CONFLICT OF INTEREST:

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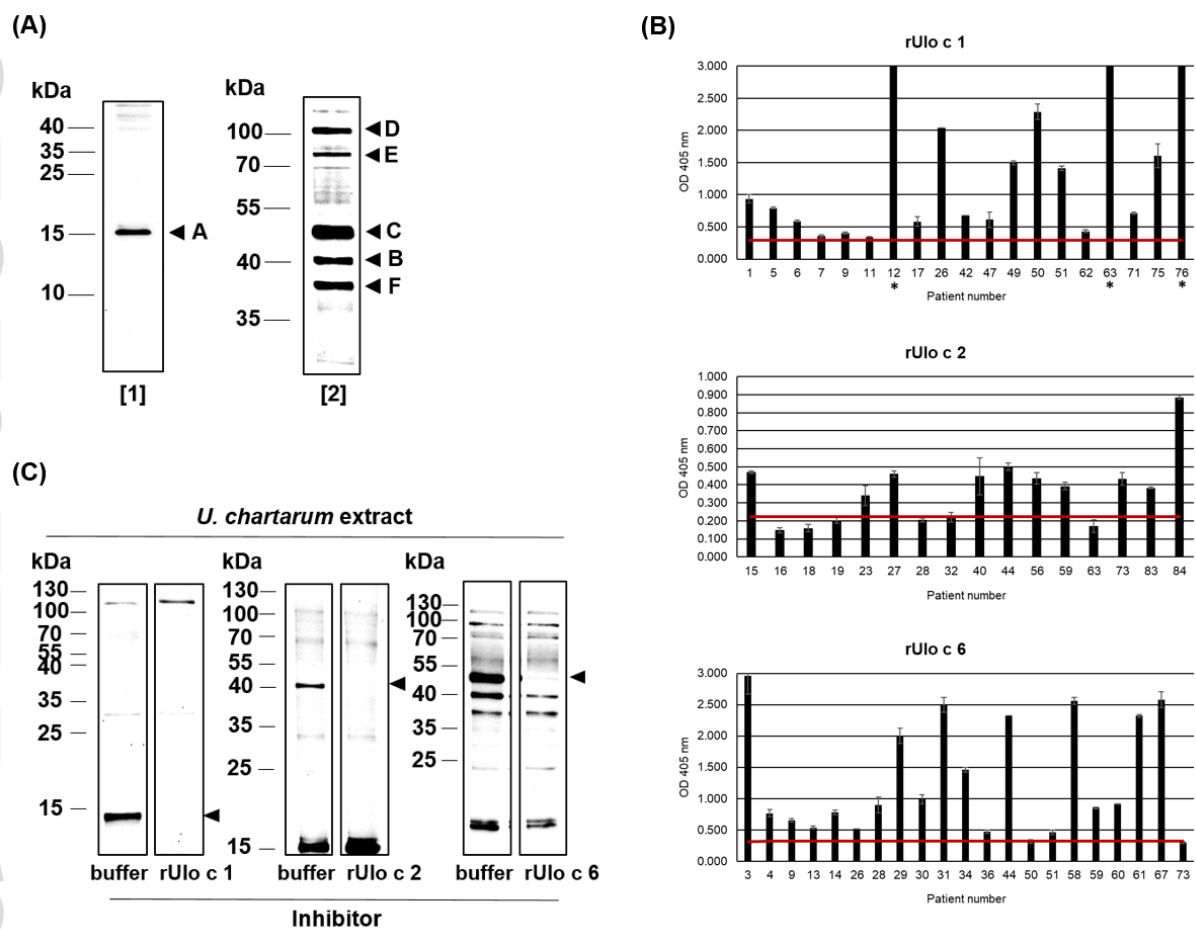
FIGURE LEGENDS:

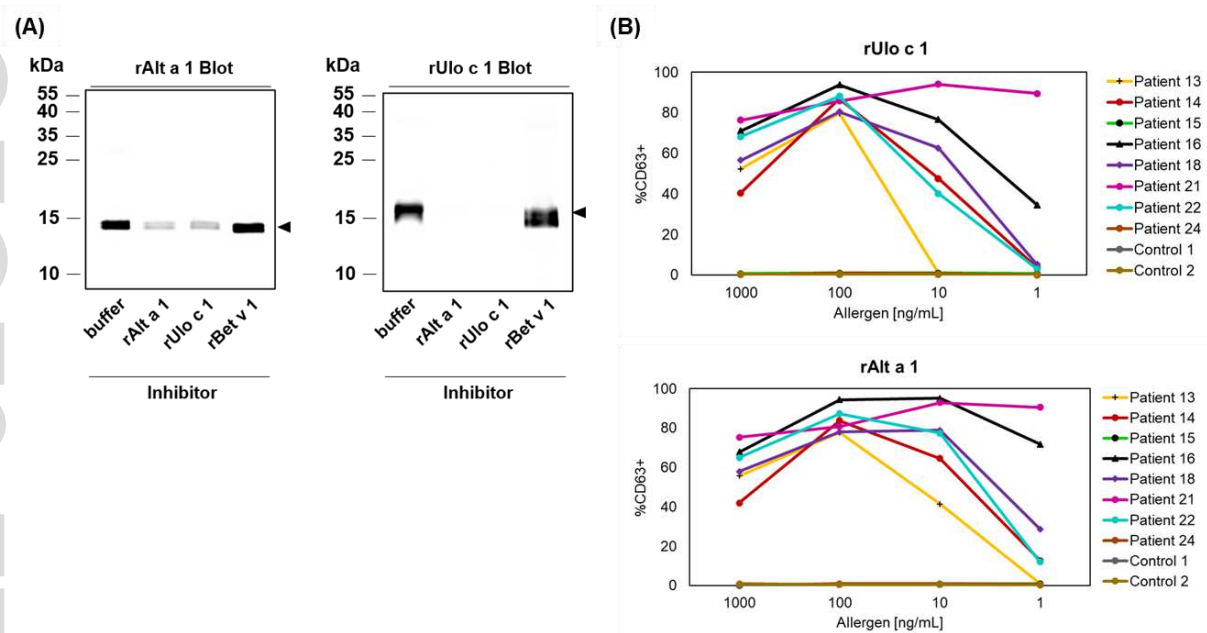
FIGURE 1. Identification and characterization of IgE-reactive proteins from

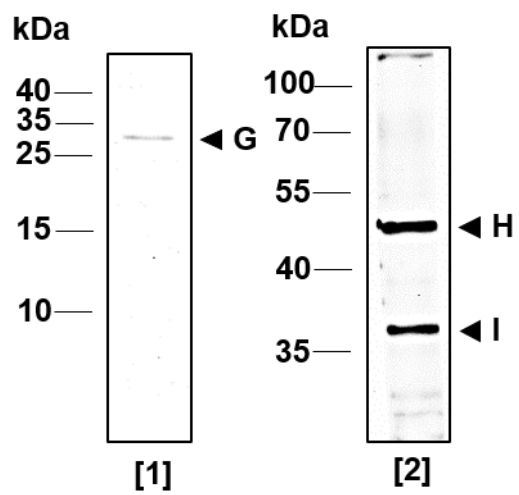
***Ulocladium chartarum*.** **A**, Nitrocellulose-blotted proteins from *U. chartarum* were exposed to two pools of sera [pool 1: sera 1, 2, 45-47 and 74; pool 2: sera 3-4, 13-14, 28-31, 34-36 and 58-61] from mold-allergic patients. IgE-reactive proteins identified by mass spectrometry are marked with A, B, C; strongly stained but unidentified bands are marked with D, E and F. Molecular weight markers are indicated in the left margins. **B**, Determination of the IgE-binding capacity of the recombinant allergens rUlo c 1, rUlo c 2 and rUlo c 6 by ELISA using sera from mold-allergic patients. Results are displayed as mean OD-values and standard deviations from two technical replicates are indicated as error bars in the bar charts. The red line represents the cut-off value, which was

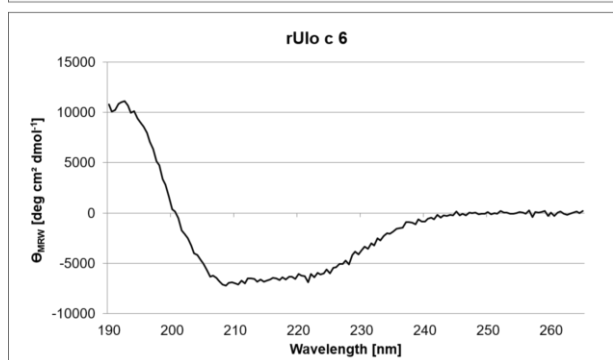
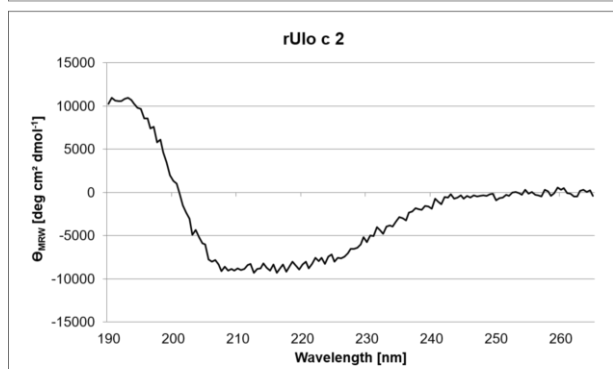
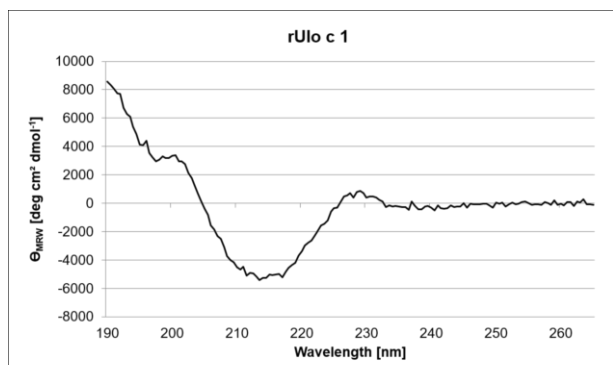
calculated from the mean OD-values plus two times the standard deviation of sera from three non-allergic patients. Sera that gave OD-values above 3.0 are marked with an asterisk (*). **C**, Inhibition immunoblot: blotted proteins of *U. chartarum* protein extracts were exposed to pools of sera (rUlo c 1: sera 25-27, 42, 78; rUlo c 2: sera 15, 28-32, 57; rUlo c 6: sera 3-4, 13-14, 28-31, 34-36, 58-61) that had been pre-incubated with rUlo c 1, rUlo c 2 or rUlo c 6 or, for control purposes, with buffer only. Molecular weight markers are indicated in the left margins. Arrows point to reduction of IgE-binding to natural Ulo c 1, Ulo c 2 and Ulo c 6 after pre-incubation with the recombinant proteins.

FIGURE 2. IgE cross-reactivity between the major fungal allergens rUlo c 1 and rAlt a 1. **A**, Two µg of the recombinant allergens were separated by SDS-PAGE, blotted onto nitrocellulose and exposed to a pool of sera (25-27, 42 and 78) from mold-allergic patients, which had been pre-incubated with rUlo c 1 or Alt a 1 or, for control purposes, with the non-relevant allergen rBet v 1 or with buffer only. Molecular weight markers are indicated in the left margins. **B**, Basophil activation of rUlo c 1 and rAlt a 1 was determined by measuring the expression of CD63 by flow cytometry after incubation of the whole blood from eight *Alternaria*-sensitized patients (patients 13-16, 18, 21, 22, 24) and from two non-atopic control individuals (control 1, 2) with increasing allergen concentrations. The percentage of CD63 positive basophils (y-axis), based on the total amount of basophils, is displayed against the concentration of the applied allergen (x-axis).



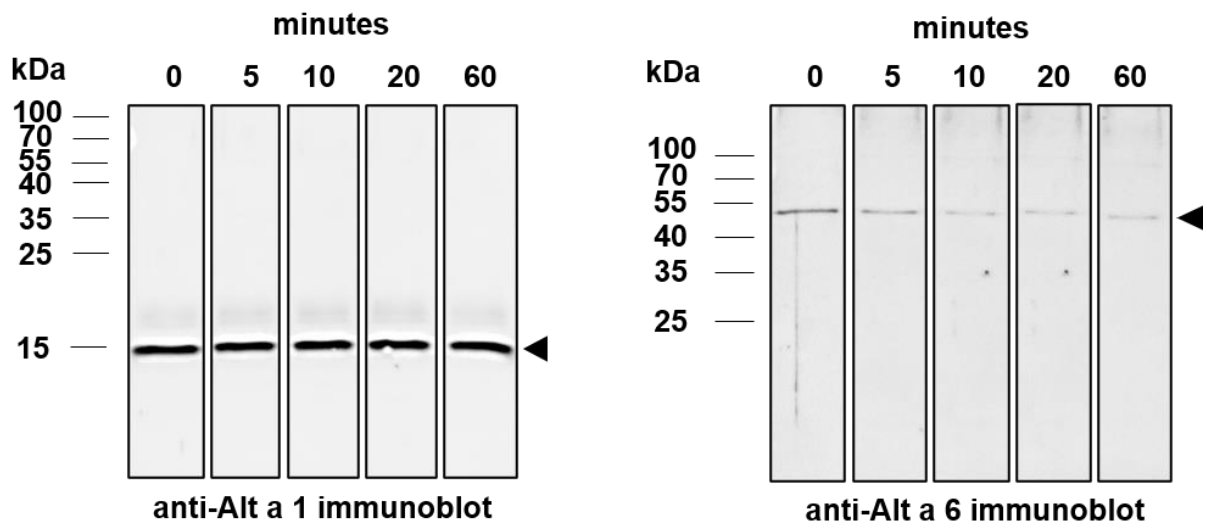






Patient number	rUloc1	rUloc2	rUloc6	Patient number	rUloc1	rUloc2	rUloc6
1				45			
2				47			
3				48			
4				49			
5				50			
6				51			
7				52			
8				53			
9				54			
10				55			
11				56			
12				57			
13				58			
14				59			
15				60			
16				61			
17				62			
18				63			
19				64			
20				65			
21				66			
22				67			
23				68			
24				69			
25				70			
26				71			
27				72			
28				73			
29				74			
30				75			
31				76			
32				77			
33				78			
34				79			
35				83			
36				84			
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44							

Ulocladium chartarum



Alternaria alternata

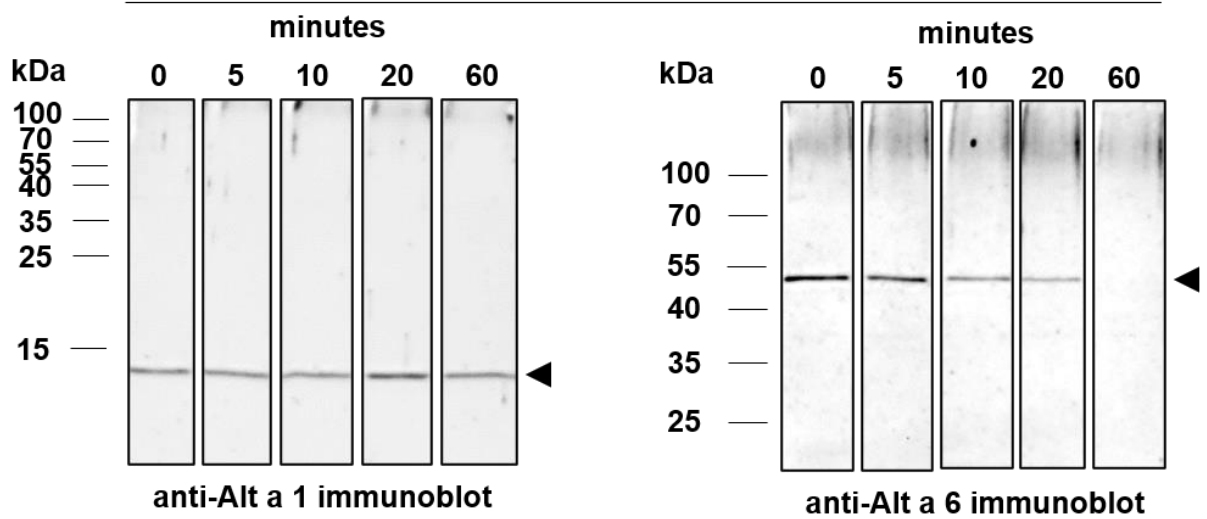


Table S1. Summary of demographic and serological data of mold-allergic patients grouped based on their IgE-reactivity profiles (n = 85). Total IgE (in kU/L) and specific IgE (sIgE; in kUA/L) against different mold species were determined by ImmunoCAP analysis.

Patient numbers	Allergen source	Age (yr)	Total IgE [kU/L]	sIgE mold [kUA/L]
1-27	<i>Alternaria</i>	4-83	25-646	1.54-66.60
28-38	<i>Alternaria</i> <i>Cladosporium</i>	7-36	108-2229	<i>Alternaria</i> : 3.50 - >100 <i>Cladosporium</i> : 0.89-80.10
39; 44	<i>Alternaria</i> <i>Aspergillus</i>	75; N/A	862; N/A	<i>Alternaria</i> : 1.92-3.62 <i>Aspergillus</i> : 0.01-3.99
40-41	<i>Alternaria</i> <i>Aspergillus</i> <i>Penicillium</i>	35-48	121-1679	<i>Alternaria</i> : 01.13-6.00 <i>Aspergillus</i> : 1.06-1.94 <i>Penicillium</i> : 0.49-0.81
42	<i>Alternaria</i> <i>Epicoccum</i>	N/A	N/A	<i>Alternaria</i> : 3.37 <i>Epicoccum</i> : 2.01
43	<i>Alternaria</i> <i>Aspergillus</i> <i>Cladosporium</i>	N/A	762	<i>Alternaria</i> : 23.3 <i>Aspergillus</i> : 22.5 <i>Cladosporium</i> : 5.73
45-56	<i>Aspergillus</i>	12-74	185-1874	2.11-19.60
57-70	<i>Cladosporium</i>	4-42	220-3625	3.61-23.60
71-72	<i>Penicillium</i>	4-13	66-291	4.04-6.30
73	<i>Fusarium</i>	N/A	N/A	4.10
74-77	<i>Aspergillus</i> <i>Cladosporium</i>	10-11	924-984	<i>Aspergillus</i> : 4.54-29.00 <i>Cladosporium</i> : 7.53-24.70
78-80	<i>Penicillium</i> <i>Cladosporium</i>	N/A	N/A	<i>Penicillium</i> : 1.28-9.10 <i>Cladosporium</i> : 1.42-8.10
81-82	<i>Penicillium</i> <i>Cladosporium</i> <i>Aspergillus</i>	N/A	N/A	<i>Penicillium</i> : 6.93-7.72 <i>Cladosporium</i> : 8.67-8.99 <i>Aspergillus</i> : 10.80-11.30
83-85			N/A	

Abbreviation: N/A, information not available

Methods

Patients' sera

Sera from mold-allergic patients were obtained from the Department of Allergy, La Paz, University Hospital, Madrid, Spain, from the Hospital Clinic de Barcelona, University of Barcelona, Spain and from the company AbBaltis (FDA-approved; Sittingbourne, United Kingdom). The demographic and serological characteristics of the patients are given in Table S1. Currently, *Ulocladium chartarum* is not included in the routine diagnosis of fungal allergy. Therefore, it was not possible to focus on sera of *U. chartarum* allergic individuals. Instead, patients with a sensitization to at least one of the fungal species used in routine diagnosis were included. All patients agreed to participate in this study and gave informed consent. This study was approved by the ethics committee of the Hospital Clínic de Barcelona (approval number 2011/6605) and of the La Paz University Hospital in Madrid (EK565/2007). We confirm that all methods were performed in accordance with the relevance guidelines and regulations.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblot and inhibition immunoblot experiments

Two µg of recombinant proteins or 5 µg of fungal protein extracts were separated on 10%, 12% or 15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGEs), as described by Laemmli (S1) and were transferred electrophoretically onto nitrocellulose membranes (0.2 µm; GE Healthcare, Chicago, IL) (S2). For IgE-immunoblots, membranes were blocked with PBS containing 0.5% Tween20 (PBS-T) and were then exposed to pools of sera from mold-allergic patients, or for control purposes, to a pool of sera from three non-allergic individuals or to PBS-T only.

These pools contained either 3, 5, 6, 9 or 16 sera (1:10 diluted in PBS-T). For IgE inhibition immunoblots, pools of sera were pre-incubated overnight with 20 µg of rUlo c 1 or rAlt a 1 or, for control purposes, with the unrelated respiratory allergen rBet v 1 (available in the laboratory) or with PBS-T and then proteins, blotted onto nitrocellulose membranes, were incubated with these pre-incubated sera. For the detection of protein-bound human IgEs, a mouse anti-human IgE antibody, conjugated to horseradish peroxidase (HRP), was used as detection antibody (final dilution 1:5,000; Southern Biotech, Birmingham, AL). The blots were developed using the SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions and imaged using the UVP ChemStudio device (Analytik Jena, Germany).

To detect Alt a 1, Alt a 6 and related proteins, nitrocellulose-blotted proteins were incubated with rabbit antisera either directed against a peptide of Alt a 1 (5'-KISEFYGRKPEGTYYNLSLG-3') or against the recombinant Alt a 6 (Charles River Laboratories, Écully, France). A goat anti-rabbit IgG HRP-labelled antibody (Vector Laboratories Inc, Burlingame, CA; diluted 1:10,000 in PBS-T) was used for detection and the blots were developed and imaged as described above for the IgE immunoblots.

cDNA cloning and recombinant expression of *U. chartarum* allergens

The full-length cDNAs coding for the *U. chartarum* proteins were generated from *U. chartarum* RNA by RT-PCR. For this, information on the full-length sequences of the Ulo c 1 and Ulo c 2 cDNAs first had to be obtained by 5'/3'-rapid amplification of cDNA ends (RACE) using the SMARTer RACE 5'/3' Kit (Takara Bio, Kyoto, Japan). Therefore, primers were designed based on the homologous *Alternaria alternata*

cDNA sequences of Alt a 1 (NCBI accession number: AY568627) and of the formate dehydrogenase (NCBI accession number: XM_018525579.1). To obtain the 5'- and 3'-end of the Ulo c 1 cDNA the following primers were used: 5'-AACGCATCCTGCCCTGTCACTACCGA-3' (for 3'-end amplification) and 5'-GCTGTTCTCGCCGCAAGAGTACCAC-3' (for 5'-end amplification). For the Ulo c 2 cDNA the following primers were used: 5'-GGAAAAGTTCTTCTCGTCCTCTACG-3' (for 3'-end amplification) and 5'-CTTGCGCTGACCGTACGCCTTGG-3' (for 5'-end amplification). Primers for full-length cDNA cloning of Ulo c 1 and Ulo c 2 by RT-PCR were then designed based on the sequences of the RACE-PCR-products.

Since enolases are very conserved proteins, the cDNA coding for Ulo c 6 could be obtained by RT-PCR using forward (5'-ACCATCACCAAGATCCACGCCCCGC-3') and reverse (5'-CAAGTTAACGGCAGTCCTGAA-3') primers designed based on the nucleotide sequence from the homologous *A. alternata* enolase Alt a 6 (NCBI accession number: U82437.2).

The obtained sequences were submitted to GenBank and received the following accession numbers: MH716394 (Ulo c 1), MN953052 (Ulo c 2) and MH910061 (Ulo c 6).

cDNAs coding for Ulo c 1, Ulo c 2 and Ulo c 6 were cloned with C-terminal hexa-Histidine-tags into the bacterial expression vector pET-17b. Following the successful expression of the allergens as soluble proteins in *E. coli*, the molecules were purified under native conditions by affinity chromatography. The protein concentrations were determined with the MicroBCA Protein Assay Kit (Thermo Fisher Scientific) using BSA as a standard.

For control purposes, rAlt a 1 from *Alternaria alternata* was also produced in *E. coli*. For this, the Alt a 1 cDNA (NCBI accession number: AY568627) was generated from

A. alternata RNA by RT-PCR and was cloned with a C-terminal hexa-Histidine-tag into the bacterial expression vector pET-17b. rAlt a 1 expression and purification were carried out as described for the *U. chartarum* allergens.

Secondary structure analysis by circular dichroism spectroscopy (CD-spectroscopy)

The secondary structure of rUlo c 1, rUlo c 2 and rUlo c 6 was evaluated by circular dichroism spectroscopy on a Chirascan Plus Spectrometer (Applied Photophysics, Leatherhead, United Kingdom) in 10 mM sodium phosphate buffer (PH 7.0) at 20°C and a protein concentration of 0.2 µg/µL using a quartz cuvette (Hellma Analytics, Müllheim, Germany) with a path length of 1 mm. CD-spectra were recorded from 190 to 280 nm with a resolution of 0.5 nm and results were the average of three scans. The final spectra were corrected by subtracting the buffer baseline spectrum, obtained under identical conditions, and normalized to the number of peptide bonds by using the extinction coefficient of the measured protein at 205 nm (ϵ_{205}) (S3). Results are expressed as mean residual ellipticity θ_{MRW} (deg cm² dmol⁻¹) at a given wavelength.

Enzyme-linked immunosorbent assay (ELISA) of recombinant fungal allergens

The IgE-reactivity of the recombinant *U. chartarum* allergens was analyzed in ELISAs performed with 85 sera from patients sensitized to different mold species. For this, 96-well nunc maxisorp ELISA plates (Thermo Fisher Scientific) were coated with 4 µg/mL of the recombinant proteins, diluted in bicarbonate buffer (pH = 9.6). Wells were blocked with phosphate buffered saline containing 0.5% Tween20 (PBS-T) and were then incubated with sera from mold-allergic patients (1:5 diluted in PBS-T) or, for control purposes, with sera from three non-allergic individuals (1:5 diluted in PBS-

T) or with PBS-T only. Bound IgE antibodies were detected with a HRP-labelled goat anti-human IgE antibody (SeraCare Life Sciences Inc., Milford, MA; diluted 1:2,500 in PBS-T) using 1.8 mM of 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich) in 60 mM citric acid, 77 mM $\text{Na}_2\text{HPO}_4(\text{H}_2\text{O})_2$ and 3 mM H_2O_2 as a substrate. After 60 minutes of incubation in substrate solution, the optical density of the samples was measured at a wavelength of 405 nm using the Multiskan FC Photometer (Thermo Fisher Scientific). Experiments were always carried out in duplicates and the results are expressed as mean OD-values. The cut-off, used to differentiate positive and negative IgE-reactivity, was calculated from the mean OD-values plus two times the standard deviation of sera from three non-allergic patients.

Basophil activation test (BAT)

To test the biological activity of the recombinant molecules, basophil activation tests were performed using the Flow Cast Kit (Bühlmann, Schönenbuch, Switzerland) according to the manufacturers' instructions. Whole blood from eight mold-sensitized patients and, for control purposes, from two patients with no fungal sensitization, was incubated with increasing concentrations (1 to 1000 ng/mL) of the recombinant allergens. Basophil activation was assessed by detecting the expression of CD63 by flow cytometry (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ). Activated basophils were gated as $\text{SSC}^{\text{low}}/\text{CCR3}^+/\text{CD63}^+$. At least 500 basophils were measured, and the percentage of CD63-expressing basophils, based on the total amount of basophils, was calculated.

Allergen Release Kinetics Experiments

Clones of *U. chartarum* and *A. alternata* were cultured on MEA-plates at 20-25°C for 7 days. Then, 10 mL of a buffer that mimics the natural nasal fluid (160 mM NaCl, 30 mM KCl, 5 mM $\text{MgCl}_2(\text{H}_2\text{O})_6$ and 2 mM $\text{CaCl}_2(\text{H}_2\text{O})_2$) (S4) was added to the plates and spores were detached using a Drigalski-spatula. The spore solutions were transferred into centrifugation tubes and were incubated under continuous shaking at 20-25°C. Aliquots of 1 mL each were taken after 0, 5, 10, 20 and 60 minutes and were immediately centrifuged to separate the supernatants, containing the released proteins, from the fungal spores. The supernatants were stored at -20°C and the protein concentrations were determined using the method of Bradford with BSA as a standard (S5). For the detection of released allergens, 0.1 µg of the supernatants were separated on 12% or 15% SDS-PAGEs, blotted onto nitrocellulose (as described before) and exposed to the antisera against Alt a 1 or rAlt a 6.

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FIGURE LEGENDS:

FIGURE S1. IgE-reactivity of *A. alternata* proteins. Nitrocellulose-blotted proteins from *A. alternata* were exposed to two pools of sera [pool 1: sera 1,2, 45-47 and 74; pool 2: sera 3-4, 13-14, 28-31, 34-36 and 58-61] from mold-allergic patients. IgE-reactive proteins are marked with G, H and I. Molecular weight markers are indicated in the left margins.

FIGURE S2. Structural analyses of recombinant *U. chartarum* allergens. Circular dichroism spectra were recorded for the recombinant allergens from 190 to 280 nm. Results shown are the average of three scans and are expressed as mean residual ellipticity θ_{MRW} (deg cm² dmol⁻¹) at a given wavelength.

FIGURE S3. IgE-reactivity profiles of mold-sensitized patients to different recombinant allergens. The binding of IgE antibodies from mold-sensitized patients to rUlo c 1, rUlo c 2 and rUlo c 6 was measured by ELISA and data are summarized in an IgE-reaction map. The optical density was measured in duplicates and the mean OD-value obtained with sera from three non-allergic individuals plus two times the standard deviation was set as a threshold for a positive reaction, indicated here in green. OD-values below the cut-off indicate negative results and are shown in red, whereas grey coloring indicates that the IgE-reactivity was not evaluated.

FIGURE S4. Ulo c 1, Alt a 1, Ulo c 6 and Alt a 6 are rapidly released from fungal spores. Proteins, released from *U. chartarum* and *A. alternata* spores after different times of hydration of the spores (0, 5, 10, 20, 60 min), were separated by SDS-PAGE and were blotted onto nitrocellulose membranes. Membranes were either exposed to

the anti-Alt a 1 or to the anti-rAlt a 6 antiserum. Molecular weight markers are indicated in the left margins.