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Identification of cross-reactive IgE-binding proteins from Philippine allergenic grass pollen extracts

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ABSTRACT

Background: Respiratory allergies are one of the most common allergic diseases that affect Filipinos. Grass pollen accounts for the majority of the outdoor allergens triggering these respiratory allergies. Cross-reactivity among the Philippine grass pollen grains has not been extensively studied.

Objective: This study aims to investigate the cross-reactivity of our local grasses and identify the cross-reactive allergens.

Methods: Grass pollen grains were collected and processed into crude allergenic extracts. The IgE-reactivity of these crude allergenic pollen extracts was studied using sera from patients who tested positive for the mentioned extracts. The proteins from the immunoblots of cross-reactive pollen allergen extracts were sequenced and identified.

Results. Allergenic pollen proteins were identified as cross-reactive among the grass pollen extracts. Four of these have not been listed yet as grass allergens in the World Health Organization/International Union of Immunological Societies allergen nomenclature database.

Conclusion: Local grass pollen allergens are cross-reactive with probable new allergens identified.

Keywords: Allergens; cross-reactivity; Poaceae; pollen

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Protocol development for the assessment of Philippine weed pollen extracts for the diagnosis of allergic diseases was presented in the poster session of the 2019 Education Conference of the International Union of Biochemistry and Molecular Biology held in Manila, Philippines last November 13-15, 2019. Pollen grain and pollen extract characterization of common allergenic plants in the Philippines, which was the first phase of the project, was presented in the poster session while the second phase on skin prick tests and enzyme-linked immunosorbent assays among allergic patients using allergenic local pollen extracts was presented in the oral presentation of the 34th Faculty Research Forum of the University of the Philippines College of Medicine last October 17-21, 2022.

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1. Introduction

Respiratory allergies are one of the most common allergic diseases that affect Filipinos. Approximately 20% of the Philippine adult population have allergic rhinitis (AR) [1]. Reported prevalence worldwide ranged from 1% to 63% because of the varying definitions and methods used in the diagnosis. Prevalence is also affected by geographic location. A review done in 2021 had the median AR prevalence at 29.4% (range 1.1%–63.3%), with a prevalence of 15% (1%–48%) in Asia [2]. Asthma, on the other hand, affects 8.7% of adult Filipinos [3]. Worldwide prevalence reported among adolescents, using the same definition as the Philippine data, is at 13.7% [4]. Both worldwide reviews reported increasing prevalence.

Pollen allergens were reported to be important sensitizing allergens in Australia, Middle Eastern countries, Southern Africa, and Turkey, while mite and cockroach allergens were more common sensitizing agents in the Asia-Pacific region [5]. A retrospective review of allergy skin tests done at the University of the Philippines–Philippine General Hospital (UP-PGH) between 2006 and 2011 showed that the most common sensitizing allergen is house dust mite (74%). Of the outdoor allergens, grasses accounted for 72% [6]. Grass pollen allergen extracts are used for allergy skin testing to confirm respiratory allergy diagnosis, such as AR and asthma, and for identifying the specific allergen triggers. These extracts are also used for allergen immunotherapy so that future encounters with the allergen will produce milder or no symptoms [7].

Grass allergens have been found to be highly cross-reactive. Cross-reactivity between allergens occurs when an antibody raised against 1 specific allergen has a competing high affinity

toward a different allergen; therefore, the antibody can recognize a protein that is different from the one it was raised against. Weber, in 2004, proposed 2 theories regarding pollen cross-reactivity. The first one considers phylogeny where plants come from a common progenitor and the other considers closely related plants as having more shared antigens than distantly related ones. It was also mentioned that an exception to these are the highly conserved proteins, such as profilins and pathogenesis-related proteins, that are shared by a wide range of plants [8]. The presence of significant cross-reactivity may result in adverse reactions because of the increased amount of the allergen present in the allergenic extracts that share the same allergen epitope. Knowledge of the cross-reactive allergens may help allergists decide which representative pollen extracts to use for skin testing and allergen immunotherapy to reduce the adverse reactions among patients (see Supplementary Figure, http://links.lww.com/PA9/A24 which shows the conceptual framework of the cross-reactivity study of the pollen extracts).

Most studies on grass pollen cross-reactivity involve temperate grasses from Western countries. This study aims to investigate the cross-reactivity of the Philippine local grasses and identify cross-reactive allergens.

2. Materials and methods

Ethical approval for the project entitled "Biochemical and Immunologic Characterization and Cross-reactivity Studies of Allergenic Local Pollen Extracts" (UPMREB 2016-528-01) was provided by the University of the Philippines Manila Research Ethics Board (Chair UPMREB Panel 1 Cecilia A. Jimeno, MD) on March 02, 2017. The project was conducted at the Allergy Clinic of the Outpatient Department (OPD) of the UP-PGH. The laboratory part was conducted at the Department of Biochemistry and Molecular Biology of the UP College of Medicine.

This article is the third phase of the project and will specifically deal with the cross-reactivity of the grass pollen extracts. The first phase of this project involved the pollen extraction and characterization of grass, weed, and tree pollen grains (KM Hate, MSc, unpublished data, 2023)[9]. The second phase of this project looked at the skin test and specific IgE reactivity of patients with respiratory allergies. This phase also investigated whether the pollen purity and protein concentration of the extracts will have a correlation with the skin test and sIgE reactivities [10]. Cross-reactivity of the pollen extracts from grasses such as *Imperata cylindrica* (cogon), *Oryza sativa* (rice), *Pennisetum polystachion* (foxtail), *Sorghum halepense* (Johnson grass), and *Saccharum spontaneum* (talahib) were investigated in this study.

2.1. Patient recruitment, allergy skin test, and sIgE through enzyme-linked immunosorbent assay

Patients, aged 19–50 years old, with AR and/or bronchial asthma, and whose symptoms are controlled, consulting at the Allergy Clinic of the OPD of UP-PGH were invited to participate in the study. Five healthy volunteers with no AR and bronchial asthma were recruited as negative control. Informed consent was obtained from each study participant and they were then screened for signs and symptoms of AR and/or asthma. Patients who did not give informed consent, had ongoing cough and colds, or intake antihistamines were excluded.

Skin prick tests (SPT) with crude pollen extracts, together with positive and negative controls, were performed on the volar surface of the forearm of the patients. The results of the SPT were read after 15 to 20 minutes. Reactions with a wheal size diameter of 3 mm more than that of the negative control are considered positive results.

Five mL of blood from 113 patients with positive skin test to one or more grass pollen extracts, and from 2 healthy volunteers with negative SPT to all pollen extracts, were collected for sIgE enzyme-linked immunosorbent assay (ELISA). Then another 50 mL of blood was collected from 37 participants with sera reacting positively to both SPT and sIgE ELISA.

Blood was collected into serum separator tubes, coded, and allowed to stand for 1 hour at room temperature to clot before centrifuging (3,000 rpm) at 4°C for 10 minutes. Sera were collected and coded. The sera were aliquoted afterward and stored in a –20°C freezer. Wastes were disinfected before disposal.

2.2. Glycoprotein detection

Protein components of the pollen extracts were separated on a 10% to 15% gradient sodium dodecyl sulfate (SDS)–polyacrylamide gel using a Mini vertical gel electrophoresis system (Major Science, United Kingdom) at constant 80 V. Proteins were visualized using Pierce 24612 silver stain kit (Thermo Fisher Scientific, Waltham, MA, USA). A tricolor broad-range prestained protein ladder (Vivantis PR064, Malaysia) was used to assess the molecular weight (MW) of the protein bands.

Periodic Acid-Schiff (PAS) staining was performed to identify glycoproteins among the resolved protein bands. The gel was fixed using a 3% acetic acid: 50% methanol solution for 1 hour at room temperature and washed with distilled water. The fixed gel was submerged in a 1% periodic acid: 3% acetic acid solution for 1 hour at room temperature with agitation in the dark, then washed with 3% acetic acid solution. The gel was stained with Schiff's reagent at room temperature, in the dark, for 30 minutes, submerged in 0.5% aqueous solution of sodium metabisulfite for 1 hour at room temperature, then washed with 3% acetic acid solution and rinsed with distilled water.

2.3. Determination of IgE binding components of pollen extract allergen through immunoblot

Proteins were separated in a 10% to 15% gradient SDS-polyacrylamide gel electrophoresis (PAGE) gel at a constant 100 V. The resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using the Mini PVDF Transblot kit (Bio-Rad, Hercules, CA, USA) and the Transblot-Turbo machine (Bio-Rad) at a constant 25 V for 10 minutes. The transfer was verified by reversible staining of the membrane with Ponceau S stain solution. The stain was removed using 1X Tris-buffered saline with Tween (TBST). The membrane was blocked with 1% nonfat dry milk in TBST for 2 hours at room temperature then incubated with a 1:1 ratio of pooled sera and 1X TBST for 18 hours at 4°C followed by 1:200 diluted horseradish peroxidase-conjugated goat anti-human IgE secondary antibody (Invitrogen A1870) for 3 hours at 4°C with shaking. The membrane was washed with 1X TBST after each step. The blot was developed with a 3,3',5,5'-tetramethylbenzidine (TMB) Western Blot Substrate solution (Promega, Madison, WI, USA). The reaction was stopped by washing with distilled water.

2.4. Two-dimensional SDS-PAGE

An 11 cm, nonlinear, pH 3-10 immobilized pH gradient (IPG) strip (Bio-Rad) was rehydrated with the pollen extracts in a rehydration solution for 18 hours at 20°C. Separation of proteins in the first dimension was carried out in the EttanIPGphor3 isoelectric focusing system (GE Healthcare, United Kingdom). The strips were focused at 500 V step until 0.5 kVh, 1000 V gradient until 0.8 kVh, 6000 V gradient until 9 kVh, 6000 V step until 3.7 kVh, and 6000 V step until 12 kVh. The IPG strips were stored at −80°C until use for the second dimension of electrophoresis.

Before performing the second dimension, the IPG strips were thawed, washed with ultrapure water, and equilibrated with urea equilibration solution with Dithiothreitol (2 mg/10 mL equilibration solution) for 10 minutes, followed by urea equilibration solution with iodoacetamide (2.5 mg/10 mL equilibration solution) for 10 minutes in a dark room. IPG strips were washed with ultrapure water and then placed on top of the discontinuous SDS-PAGE gel containing 4% stacking and 10% to 15% gradient resolving layers. Tricolor broad-range prestained protein ladder (Vivantis PR064, Malaysia) was placed beside the IPG strip. The strip and filter were sealed with 0.5% agarose gel solution containing 1% bromophenol blue. The second dimension was performed at a constant 125 V.

Resolved proteins were electro-transferred onto Midi PVDF membrane (Bio-Rad) using 1% nonfat dry milk as blocking solution, 2:1 pooled sera in 1X TBST as the primary antibody, 1:100 goat anti-human IgE (Invitrogen A1870) in TBST as secondary antibody, and TMB as the chromogenic substrate solution. The other set of the resolved proteins were stained with Pierce 24612 silver stain kit (Thermo Fisher Scientific), and the target protein spots were excised, placed in 1.5 mL microcentrifuge tubes, and stored in −80°C before shipment to the protein sequencing facility.

2.5. Allergenic protein identification

Target spots from western blot analysis were cut from silver-stained 2-dimensional electrophoresis gels and sent to 1st BASE proteomics services for protein identification by nano liquid chromatography-tandem mass spectrometry (LC-MS/MS). Samples were subjected to in-gel digestion and digested by trypsin.

The peptides were separated and analyzed using a Vanquish Neo ultra high performance liquid chromatography System coupled to an Orbitrap Exploris 480 (Thermo Fisher Scientific). Separation was performed on an EASY-Spray 75 µm × 15 cm column packed with PepMap Neo C18 2 µm, 100 Å (Thermo Fisher Scientific) using solvent A (0.1% formic acid) and solvent B (0.1% formic acid in 80% acetonitrile) at a flow rate of 300 nL/min with a 60-minute gradient. Peptides were then analyzed on an Orbitrap Exploris 480 apparatus with an EASY nanospray source (Thermo Fisher Scientific) at an electrospray potential of 2.0 kV. Raw data files were processed and searched using Proteome Discoverer 2.5 (Thermo Fisher Scientific). The Mascot algorithm was then used for data searching to identify proteins. Multiple Sequence Alignment (MSA) and percent identity matrix were done using UniProt (https://www.uniprot.org).

2.6. Data analysis

The results of the study were summarized using descriptive statistics. For categorical data, frequency and proportions were used and for quantitative data, mean with standard deviation or median and interquartile range were used. Minimum and maximum values were also reported.

3. Results

3.1. Physicochemical characterization of pollen extract proteins

At least 12 distinct protein bands were observed for the grass pollen extracts. *I. cylindrica*, *O. sativa*, *P. polystachion*, and *S. halepense* showed distinct bands at ~30 kDa and ~60 kDa. All 5 grasses showed a band at ~27 kDa (see Supplementary Figure, http://links.lww.com/PA9/A25, showing the total protein profile of pollen allergen extracts from silver-stained gradient SDS-PAGE gel). Grass pollen extracts showed the presence of glycoproteins (see Supplementary Figure, http://links.lww.com/PA9/A26, showing the glycoprotein profile of pollen allergen extracts from PAS-stained gradient SDS-PAGE gel).

3.2. Allergy skin testing and specific IgE through ELISA

Of the 165 patients who underwent allergy SPT to the grass pollen extracts, 108 (65.5%) had positive SPT results. Of the 108 patients, 25 had positive results for only 1 grass extract (monosensitized) while the remaining 83 were polysensitized (see Supplementary Table, http://links.lww.com/PA9/A27, which shows the number of patients with positive results for the grass pollen extracts by SPT).

The 108 patients who tested positive for the SPT had sIgE ELISA done. Among the 108 patients, only 40 (37.0%) had positive results for sIgE ELISA. Among the 40, 23 (57.5%) were monosensitized, and 17 (42.5%) were polysensitized. The healthy volunteers who served as controls did not have positive SPT results.

3.3. Determination of IgE-binding components of pollen extract allergen through immunoblot

Grass pollen extracts exhibited cross-reactive IgE-binding components situated within the 16 kDa–18 kDa, 25 kDa–32 kDa, 40 kDa, 46 kDa–47kDa, 50 kDa, 60 kDa, and 75 kDa. Unique IgE-binding components were found at approximately 65 kDa for *O. sativa* and at approximately 37 kDa for *S. halepense* grass pollen extracts (Fig. 1).

3.4. Two-dimensional SDS-PAGE

The 2-dimensional western blot of selected grass pollen extracts led to the identification of 22 protein spots recognized by serum IgE antibodies from allergic patients, which were in the range of 15 kDa–60 kDa molecular masses and isoelectric point (pI) range of 4.5–9.0 (Fig. 2).

Of the protein spots recognized, 5 were selected for identification by LC-MS based on the pI, reactivity to other allergen extracts (see Table 1), and not being published/listed yet to World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature database (http://allergen.org).

3.5. Allergenic protein identification

Raw data were processed and searched using Proteome Discoverer 2.5 and the SwissProt and Uniprot databases,

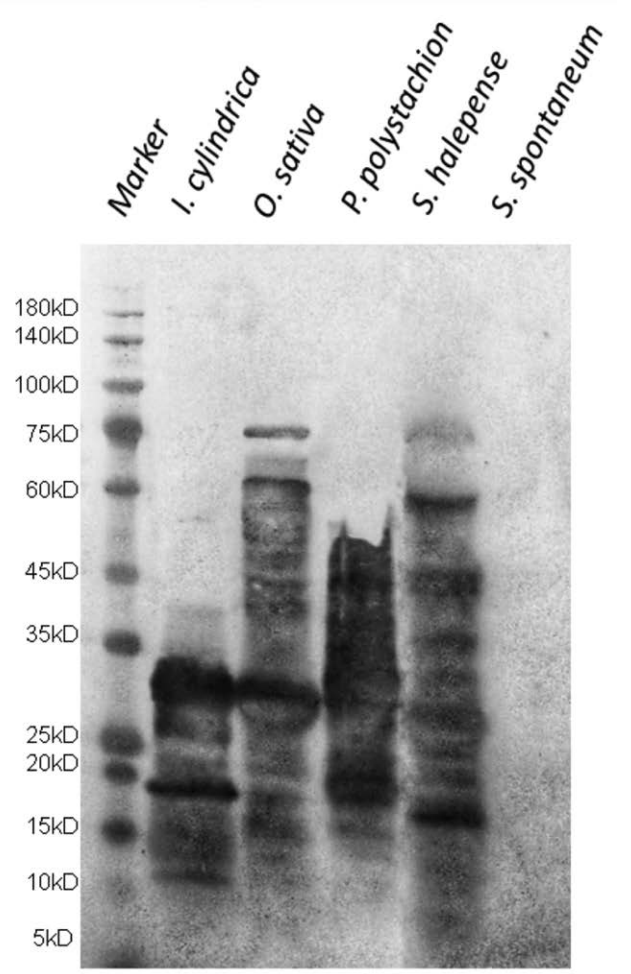

Figure 1. 1-D immunoblot of the grass pollen allergen extracts with the patients' pooled sera.

allowing the identification of proteins in the 5 immunodetected spots. Identified proteins are listed in Table 2. Their peptide sequences are shown in Figure 3.

4. Discussion

Grass pollen extracts are processed locally in the Philippines because the grass pollen sources are not the same as those in temperate countries. Knowledge of the cross-reactivity of these pollen allergens may help allergists decide which pollen extracts to include in their allergy skin test panel.

Among the 5 grass pollen allergen extracts initially collected, the first phase of the project showed that *S. spontaneum* had a low pollen purity and protein concentration (KM Hate, MSc, unpublished data, 2023) (see Supplementary Table, http://links.lww.com/PA9/A28, which shows the pollen characteristics, SPT and sIgE ELISA positivity rates of crude pollen extracts). This also had a smaller number of distinct protein bands on the immunoblot (see Fig. 1). A previous study also had similar results where *S. spontaneum* had the least number of bands despite pollen purity at >80% and high protein concentration [11]. This is also comparable to the electrophoretic results of the pollen extracts from the study of Cabauatan et al. [12] which showed degraded protein profiles. In their study, no protease inhibitor was utilized, and the purity of the pollen collections was not reported. It has been shown that pollen grains have protease activity. These include serine proteases, aspartic proteases, and cysteine proteases [13].

When the crude extracts were tested on patients with respiratory allergies through SPT and sIgE using ELISA, *S. spontaneum* also had the lowest positivity rate. The SPT results in this study showed that among the 108 patients who tested positive for the grass pollen extracts, 83 (76.9%) were polysensitized and 25 (23.8%) were monosensitized. Positive SPT means that these patients are sensitized or have IgE antibodies against the grass allergen. Sensitization can be to just one allergen (monosensitized) or to several allergens (polysensitized). A review by Migueres et al. [14] sought to identify definitions of terms related to polysensitization. In their review, polysensitization could either be due to cross-reactivity, where the same IgE binds to several different allergens with common structural features, or co-sensitization, which occurs when different IgEs will bind to allergens not necessarily having similar structures. To investigate whether there is cross-reactivity among the grass allergens, IgE-binding studies were done to see if the IgE-binding components of the different grass extracts have similar characteristics, in terms of MW, pI, and the presence of glycoproteins. *S. spontaneum* was excluded from the IgE-binding studies because of the previously mentioned findings.

The IgE-binding components of grass pollen are proteins that have been classified into several allergen groups according to their shared amino acid sequences. Major allergens belong to groups 1, 4, 11, and 13. Group 1 allergens are beta-expansins which are glycoproteins with MW of 27 kDa–35 kDa. They are considered ubiquitous grass pollen allergens. Group 4 allergens, 50 kDa–60 kDa, are also glycoproteins known as oxidoreductase. Group 11, 16 kDa–20 kDa, are glycoproteins known as Ole e 1-related proteins. Group 13 allergens, 45 kDa–60 kDa, are polygalacturonases.

Meanwhile, group 7 allergens, weighing 8 kDa–12 kDa, are polcalcins or Ca++-binding proteins. Group 12 allergens, with MW of 13 kDa–14 kDa, are profilins. Both polcalcins and profilins are pan-allergens. The functions of groups 2/3 (11 kDa–12 kDa), group 5 (27 kDa–35 kDa), and group 6 (12 kDa–13 kDa) allergens, are still unknown [15–17].

Most grass pollen allergens are glycoproteins in nature. All 5 grass pollen extracts in this study showed distinct magenta bands as an indication of the presence of glycoproteins through PAS staining. These glycoproteins have carbohydrate moieties known as cross-reactive carbohydrate determinants [18], but despite several studies investigating the significance of IgE-binding to these cross-reactive carbohydrate determinants, evidence is still lacking with regards to their role in clinical allergy [19].

The names of allergens or proteins that cause IgE-mediated reactions were standardized by the WHO/IUIS Allergen Nomenclature Subcommittee in 1986. In their 1994 revision, allergen names included the first 3 letters from the genus, 1 letter from the species, followed by an Arabic numeral. The same numeral across species are assigned to allergens that belong to the same biochemical protein family [20].

Among the grass pollen samples in this study, only *O. sativa* and *S. halepense* pollen have known allergens listed and verified in the WHO/IUIS Allergen Nomenclature database [21], the official database for allergen designations. No verified allergens were listed yet in the same database for the remaining 3 grass pollen extracts used in this study. For *O. sativa*, 2 allergens are listed: Ory s 1 (35 kDa), a beta-expansin; and Ory s 12 (14 kDa),

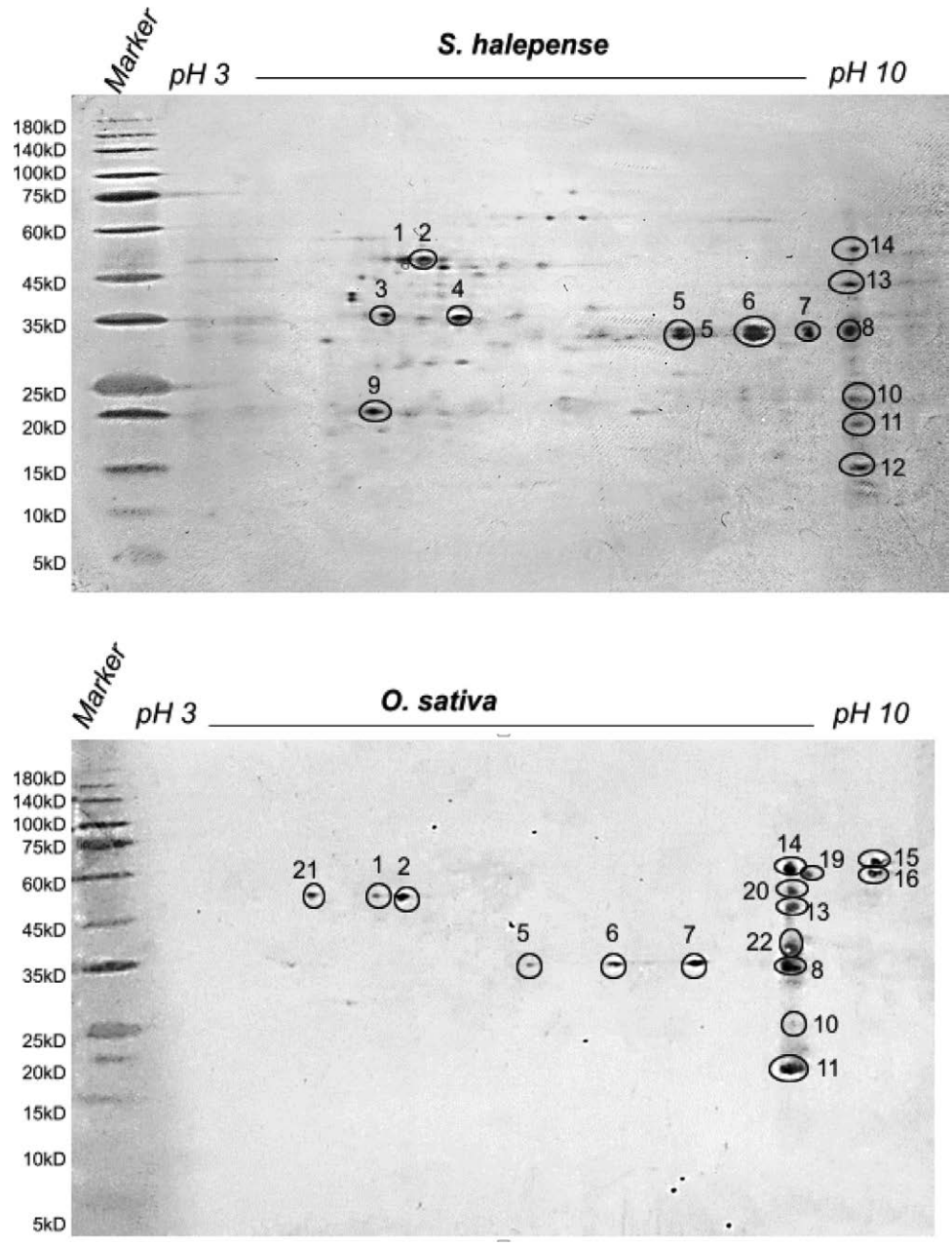

Figure 2. 2-D immunoblot blot of grass pollen allergen extracts against the patients' pooled sera.

a profilin. As for *S. halepense*, there are 3 allergens: Sor h 1 (30 kDa–35 kDa), an expansin-like protein; Sor h 2 (12 kDa), a grass pollen group 2 allergen; and Sor h 13 (54 kDa–55 kDa), an exopolygalacturonase (glycosyl hydrolase 28).

However, from the Allergome Database [22], *O. sativa* has 6 more allergens listed: Ory s 2; Ory s 3; Ory s 7, a polcalcin; Ory s 11, an Ole e 1-like trypsin inhibitor; Ory s 13, a polygalacturonase; and Ory s 23. *S. halepense* has 5 more allergens listed: Sor h CP, a 23 kDa cysteine protease [23]; Sor h 7, a polcalcin; Sor h 12, a profilin; Sor h 13 (54 kDa–55 kDa); and Sor h 23 (29 kDa) [24]. *I. cylindrica* has 4 allergens in their database: Imp c 4, a berberine bridge enzyme; Imp c 5, a ribonuclease; Imp c 7, a polcalcin; and Imp c VIIIe1, which has no name and function. There were 2 allergens for *S. spontaneum*: Sac sp 7, a polcalcin; and Sac sp 13, a polygalacturonase.

The pooled sera of the patients who tested positive for both the SPT and sIgE ELISA were utilized to identify the putative allergens through western blot. This study identified 22 protein spots among the 4 grass extracts. Five of these spots listed in Table 2 were selected for sequencing and protein identification based on these criteria: (1) must satisfy allergenic protein characteristics such as (a) acidic protein with pI range 4.0–6.0 or (b) glycoprotein (PAS staining-positive); (2) exhibit IgE-binding unique from a certain allergenic extract (western blot spot found in 1 allergenic extract); (3) exhibit IgE-binding found in at least 2 allergenic extracts (cross-reactive; western blot spot found in at least 2 allergenic extracts); and (4) not yet published in WHO/IUIS Allergen database. Isoelectric point was considered because a computer modeling study by Singh et al. [25] showed that aeroallergens were mostly acidic proteins with mean pI of 5.7 ± 0.15, while nonallergens have mean pI of 7.6 ± 0.16.

S. halepense, *O. sativa*, and *P. polystachion* have 2 cross-reactive IgE-binding proteins both with 33 kDa (both are glycoproteins; spot 5 has a pI of 5.80 while spot 7 has 6.50). These, most likely

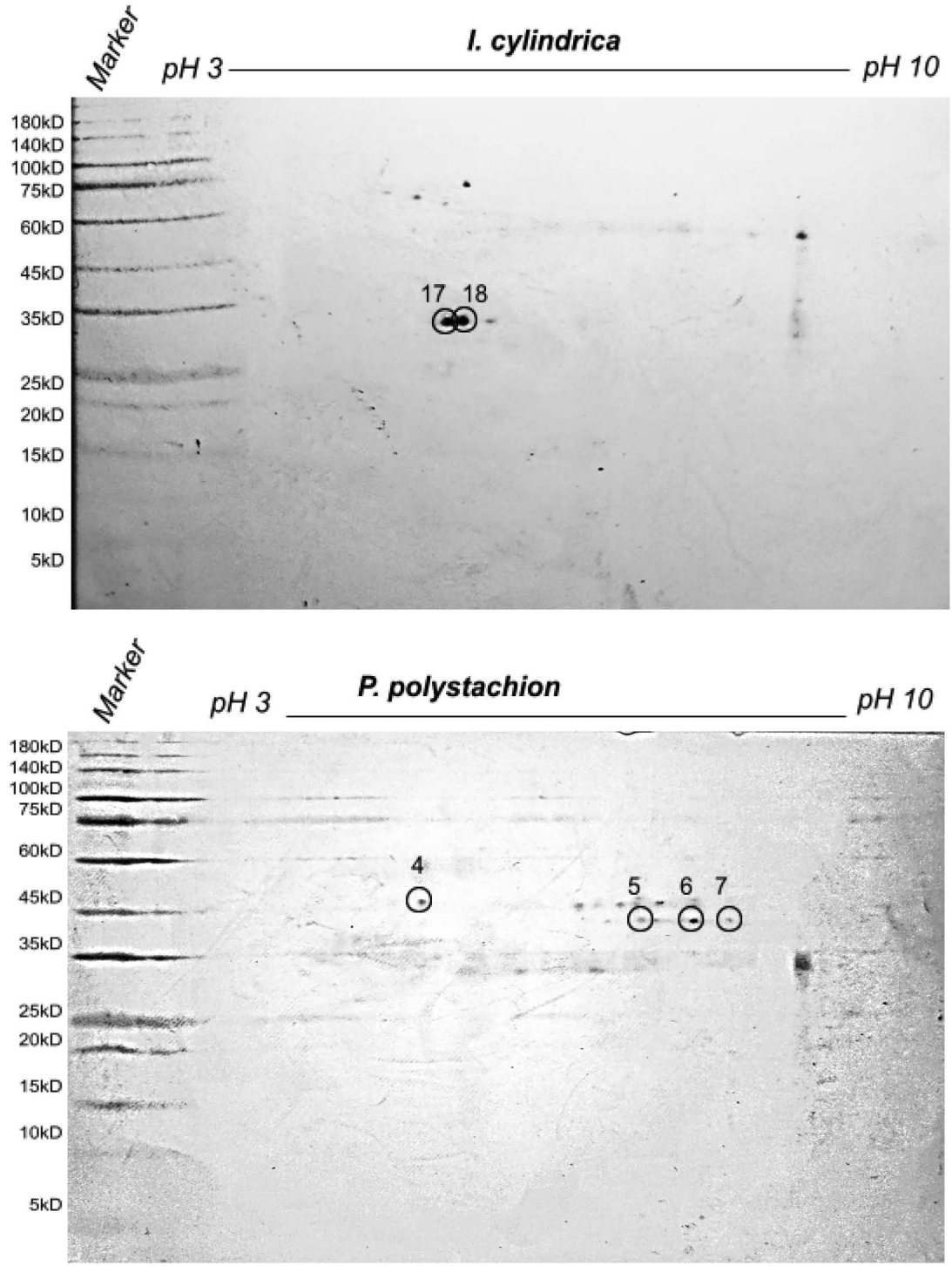

Figure 2. Continued

correspond to group 1 grass allergens which are beta-expansins. *S. halepense*, *O. sativa*, and *I. cylindrica* have 1 common IgE-binding protein at 33 kDa (a glycoprotein with pI of 7.00; spot 8). This may still be a group 1 allergen or beta-expansin, despite a basic pI. Although the *in-silico* study by Singh et al. [25] showed that a basic pI would seem rare in allergens [19], a study of 4 beta-expansins from corn pollen (Zea m 1 isoforms) showed all 4 to be basic glycoproteins with pI <9.0 [26]. Beta-expansins are a type of allergen predominantly found in grasses (WHO/IUIS; Allergome). They are small extracellular proteins that are responsible for plant cell wall loosening and expansion. Beta-expansin found in pollen differs from those vegetative beta-expansins in having an *N*-terminal motif extension in its 2nd domain. This motif is a posttranslational modification usually manifesting one or more hydroxyprolines and an asparagine-linked with xylose and fucose which serves as the epitope for IgE-binding and is responsible for IgE cross-reactivity [27].

S. halepense and *O. sativa* have several common IgE-binding proteins at 18 kDa (spot 11), 22 kDa (spot 10), 43 kDa (spot 13), 50 kDa (spots 1 and 2), and 65 kDa (spot 14) as shown in Figure 2. The 18 kDa probably corresponds to the group 11 grass allergens, also known as Ole e 1-related protein. Ole e 1-related

Table 1.

Molecular weight and isoelectric point of protein spots observed in 2-D immunoblot

Spot number	Source	Molecular weight (kDa)	Isoelectric point	With bands in PAS staining
1	*S. halepense*, *O. sativa*	50	5.10	✓
2	*S. halepense*, *O. sativa*	50	5.10	✓
3	*S. halepense*	36	5.00	
4	*S. halepense*, *P. polystachion*	35	5.25	
5	*S. halepense*, *O. sativa*, *P. polystachion*	33	5.80	✓
6	*S. halepense*, *O. sativa*	33	6.20	✓
7	*S. halepense*, *O. sativa*, *P. polystachion*	33	6.50	✓
8	*S. halepense*, *O. sativa*, *I. cylindrica*	33	7.00	✓
9	*S. halepense*	20	4.90	✓
10	*S. halepense*, *O. sativa*	22	7.00	
11	*S. halepense*, *O. sativa*	18	7.00	✓
12	*S. halepense*	15	7.00	
13	*S. halepense*, *O. sativa*	43	7.00	
14	*S. halepense*, *O. sativa*	65	7.00	✓
15	*O. sativa*	75	8.60	
16	*O. sativa*	60	8.60	✓
17	*I. cylindrica*	25	5.50	
18	*I. cylindrica*	25	5.50	
19	*O. sativa*	50	4.60	
20	*O. sativa*	64	7.10	
21	*O. sativa*	50	7.00	
22	*O. sativa*	40	7.00	

PAS, periodic acid-schiff.

Table 2.

Identified IgE-binding proteins from the selected sequenced protein spots

Spot	Protein	Accession number	MW/pI* (theoretical)	UP/PSM/SC†	Mascot Protein Score
4	Fructokinase-2	Q6XZ78	35.5/5.58	4/16/23	582
9	Triosephosphate isomerase, cytosolic	P12863	27/5.68	3/12/26	503
11	PMEI domain-containing protein	A0A0P0XZJ5	19.3/7.93	1/70/61	2598
14	FAD-binding PCMH-type domain-containing protein (glycoprotein)	A0A0E0HS70	58.3/9.2	16/31/34	1044
17‡	Haloacid dehalogenase-like hydrolase domain-containing protein	A0A453QD66	27.2/5.08	1/1/6	33

FAD, flavin adenine dinucleotide; PCMH, p-cresol methylhydroxylase; PMEI, pectin methylesterase inhibitor.

*Theoretical molecular mass (kDa)/isoelectric point.

†Number of unique peptides/number of identified peptide spectrum matches/ protein sequence coverage.

‡Protein putatively identified by one-scoring peptide.

protein is a major allergen similar to that of Ole e 1 in olive tree pollen with no known actual function yet. It has 2 main variants a nonglycosylated 18 kDa and a glycosylated 20 kDa component, with the latter being more prevalent. The glycosylated component of Ole e 1 is a single carbohydrate-containing peptide [28, 29]. Group 11 allergen was isolated and characterized as an 18-kDa IgE-binding glycoprotein from the pollen of *Lolium perenne* (perennial ryegrass) [30]. Meanwhile, the 50 kDa spot may correspond to group 4 (oxidoreductase) grass allergen or to group 13 (polygalacturonase) grass allergen. Oxidoreductase is a member of the berberine bridge enzyme family, and functions as a plant pathogen response system [17]. Group 4 grass allergens have been identified as IgE-binding glycoproteins in *Phleum pratense* (Timothy grass), *L. perenne* (perennial ryegrass), and *Dactylis glomerata* (orchard grass or cat grass) [30]. On the other hand, polygalacturonase (group 13) is one of the pectin-degrading allergens that are present in all pollen [31]. They are a relatively newly discovered group of grass pollen allergens [30].

S. halepense and *P. polystachion* have a common IgE-binding protein at 35 kDa. This was sent for protein sequencing (spot 4) as it fulfilled the criteria for selection (cross-reactive, IgE-binding spot observed in at least 2 allergen extracts and not published in the WHO/IUIS database). There were 38 (23.0%) patients who had positive SPT results for both allergen extracts. Spot 4 was identified as fructokinase-2. Fructokinases have been identified as allergenic in *Cocos nucifera* (coconut), *Carya illinoinensis* (pecan), and *Prosopis velutina* (velvet mesquite) pollen [32–34], where they may play a role in the regulation of pollen germination, probably by providing fructose-6-phosphate for glycolysis or through conversion to uridine diphosphate-glucose to assist the biosynthesis of cell wall material for pollen tube growth [35]. Fructokinase-2 from *S. halepense* and *P. polystachion* showed 90.75% sequence identity with the fructokinase-2 found in *O. sativa* (see Supplementary Figure, http://links.lww.com/PA9/A29, which demonstrates the MSA of Spot 4 and Fructokinase-2 found in *O. sativa*).

Spot 9 in *S. halepense* was identified as triosephosphate isomerase which is a homodimeric enzyme that catalyzes the interconversion of dihydroxyacetone phosphate into glyceraldehyde 3-phosphate [36]. This was chosen for sequencing since it has an acidic pI and is not yet published in the WHO/IUIS database. This protein has been described as an IgE-binding latex protein [37]. A similar protein in *P. pratense* (Timothy grass) pollen granules, described to be potentially allergenic [38], showed

Fructokinase-2	MAPLGDGGAA AAAASNNLVV SFGEMLIDFV PDVAGLSLAE SGGFVK**APGG APANVACAIA K**LGGSSAFVG K**FGDDEFGHM LVNILK**QNNV NAEGCLFDKH AR**TALAFVTL K**HDGEREFMF YRNPSADMLL TEAELDLGLV RRARVFHYGS ISLISEPCRS AHMAAMRAAK AAGVLCSYDP NVR**LPLWPSP DAAREGILSI WKEADFIK**VS DDEVAFLTRG DANDEKNVLS LWFDGLKLLV VTDGDKGCRY FTKDFKGSVP GFKVDTVDTT GAGDAFVGSL LVNVAKDDSI FHNEEKLREA LK**FSNACGAI CTTKK**GAIPA LPTVATAQDL IAKAN
Triosephosphate isomerase, cytosolic	MGRKFFVGGN WKCNGTTDQV EKIVKTLNEG QVPPSDVVEV VVSPPYVFLP VVKSQLRQEF HVAAQNCWVK KGGAFTGEVS AEMLVNLGVP WVILGHSERR **ALLGESNEFV GDKVAYALSQ GLKVIACVGE TLEQR**EAGST MDVVAAQTKA IAEKIKDWSN VVVAYEPVWA IGTGK**VATPA QAQEVHASLR DWLK**TNASPE VAESTR**IIYG** **GSVTAANCK**E LAAQPDVDGF LVGGASLKPE FIDIINAATV KSA
PMEI domain-containing protein	MAAVGLLVLL LVVGGWVAAQ EAGDAPASIV GPCSR**TGDKK ACVELLSGIP EARKATTVGP LAELYLRAIA** **NQTTEAKAMA TKLLATMK**GK GVPPVCLQQC TASVDTLSNA LAAFFSASAD VNKK**YRDLDG FLVGFLKQPP** **ICMSACPIRS CDMEEVTIAD KFHQAWKMLG VAHDLITQIL GTKS**
FAD-binding PCMH-type domain-containing protein (glycoprotein)	MATTSMTTTS RALALVLLSS CCLLVAVDAA YAKKPNLSKN DFLSCLAAGI PARQLYAK**GS PSYGSVLTST** **IR**NLRYLSSK TCNPLYIVTP TDVK**HIQVAV SCGR**RHNVRI RVRSGGHDYE GLSYR**SEIPE PFAIVDLVNM** **R**NVTVDGKAR **TAWVESGAQI GELYYGISK**A SPTLAFPAGV CPTIGVGGHF SGGGFGMLLR **KFGLASDNVL** **DVK**VVDANGK VQDRK**SMGED YLWAVR**GGGG SSFGIVVSWK LR**LLPVPATV TVIQMPKMVN EGAVDLLTKW** **QSLAPTFPED LMIR**VMAQAQ KAVFEGLYLG TCDALLPLVT SRFPELGVNR SHCNEMSWVQ SIAFIHLGKN ATVKDILNRT SSIR**AFGKYK SDYVTQPLSK** ATWDTIYKDW FSKPGSGIMI MDPYGATISK PGEADTPFPH RKGMLYNIQY ITFWFGEGAP AEAPIKWIRD FYAFMEPYVT KNPRQAYVNY RDLDLGVNAV EAGANVSCYQ VGKVWGEK**YF KGNFER**LART K**AKVDPTDFF RNEQSIPPLL A**
Haloacid dehalogenase-like hydrolase domain-containing protein	TSMAPTAGLL NPLAATVPVE AVLFDIDGTL CDSDPLHHIA FQELLLAIGY NNGVPIDDEF FINNIAGRSD AEAAQNLFPD WPLEKGLKFL EDKDVKYRSL AMEHLEPVKG LHKLVQWVKD HGYKRAAVTN APRINAELMI KLLGLSDFFQ AVIVGGECEK PKPAPFPYLK ALKELEVSAA HTFIFEDSAS GTR**AGVAAGM PVVAVSTR**NP EKSLQEAGAA LIISDYEDQK LWNALEEINR EEAKLKNGGA

Figure 3. Amino acid sequence of the allergenic proteins. The bold fonts are the peptide sequences identified in the LC-MS. LC-MS, liquid chromatography-tandem mass spectrometry.

88.14% sequence identity (see Supplementary Figure, http://links.lww.com/PA9/A30, which demonstrates the MSA of Spot 9 and P34947 [TPIS_HORVU]).

Pectin methylesterase inhibitor (PMEI)-domain-containing protein was from spot 11 found in *S. halepense* and *O. sativa*. It was chosen because of its cross-reactivity and has not been listed yet as an allergen. PMEI is implicated in the regulation of fruit development, carbohydrate metabolism, and cell wall extension [39]. Although PMEIs have been typically recognized as IgE-inducers of allergy symptoms [40], none has been reported to date of a PMEI domain-containing protein as an allergen in grasses.

Spot 14 is the second protein found in *S. halepense* and *O. sativa*. They were chosen for sequencing for the same reason as above. It is a glycosylated protein as shown by PAS staining and was identified as belonging to a family of flavin adenine dinucleotide (FAD) binding proteins that also included group 4 allergens. The MSA of spot 14 and other Group 4 FAD-binding p-cresol methylhydroxylase-type domain-containing pollen allergens such as Phl p 4 from *Phleum pratense* (Timothy grass) [41] and Sec c 4 from *Secale cereale* (Rye grass) showed 64.75% and 65.12% sequence identity, respectively (see Supplementary Figure, http://links.lww.com/PA9/A31, which demonstrates the MSA of Spot 14, Phl p 4, and Sec c 4).

Spot 17, found in *I. cylindrica*, was chosen because of the low pI and is not yet listed in the WHO/IUIS database. It was identified as belonging to haloacid dehalogenase-like hydrolases which represent one of the largest enzyme superfamilies found in all organisms [42]. A haloacid dehalogenase-like hydrolase domain-containing protein Sgpp from wheat showed 96.37% sequence identity with Spot 17 (see Supplementary Figure, http://links.lww.com/PA9/A32, which demonstrates the MSA of Spot 17 and A0A3B6I4I5). Even though spot 17 only had 1 identified peptide in the LC-MS, it cannot be disregarded in reports because the superiority of two identified peptides over one identified peptide has not been proven [43, 44].

This study was limited to 5 grasses due to the scarcity or seasonal availability of the other grass species. Furthermore, some grass species have relatively small pollen sizes that cannot be separated from impurities during sieving and thus have low quality. Of the 22 proteins that showed binding to the grass extracts, only 4 were selected based on the set criteria. The rest were not selected for protein sequencing because they were already listed in the database or were not cross-reactive (spots 3, 12, 15–22) and also due to nondistinct spots in the western blot.

The study showed that the Philippine grass pollen extracts were highly cross-reactive. Most of the patients with respiratory allergies were polysensitized, which was likely due to cross-reactivity. *S. halepense* and *O. sativa* pollen extracts were the most cross-reactive. In terms of the IgE-binding protein, spots 5, 7, and 8, which are putatively beta-expansins, were observed as the most predominant cross-reactive proteins, and are also present in both *S. halepense* and *O. sativa*. Some putative protein allergens, which are not yet listed in the allergen databases, were likewise identified. Recombinant protein studies using the protein sequence of the identified IgE-binding spots may be performed in future studies to evaluate their actual allergenic effect in vivo.

Based on the results of this study, the allergy SPT panel can contain *S. halepense* allergen extract only to represent both *S. halepense* and *O. sativa*, or vice versa. Patients who test positive for *S. halepense* may also be sensitized to *O. sativa,* hence, pollen avoidance measures for these two allergens should be advised. If allergen immunotherapy is prescribed to patients allergic to both of these allergens, using either one of these allergens only is recommended to avoid increased allergen dos,e which may cause adverse reactions.

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Conflicts of interest

The authors have no financial conflicts of interest.

Author contributions

Castor: provided the original idea, wrote the draft of the manuscript, performed significant revision of the draft, performed statistical analysis, substantial contributions to the conception or design of the work, the analysis, interpretation of data for the work, and contributed to discussion of the manuscript content. Cruz: performed significant revision of the draft, participated personally in data gathering, performed statistical analysis, substantial contributions to the conception or design of the work, the analysis, interpretation of data for the work, and contributed to discussion of the manuscript content. Balanag: participated personally in data gathering, performed statistical analysis, substantial contributions to the design of the work, the analysis, interpretation of data for the work, and contributed to discussion of the manuscript content. Hate: wrote the draft of the manuscript, performed significant revision of the draft, participated personally in data gathering, performed statistical analysis, substantial contributions to the design of the work, the analysis, interpretation of data for the work, and contributed to discussion of the manuscript content. Reyes: participated personally in data gathering, substantial contributions to the interpretation of data for the work and contributed to discussion of the manuscript content. De Jesus: contributed to the design of the work, the analysis, interpretation of data for the work and contributed to discussion of the manuscript content. Ocampo-Cervantes: contributed to the design of the work, the analysis, interpretation of data for the work and contributed to discussion of the manuscript content. Dalmacio: substantial contributions to the conception or design of the work, the analysis, interpretation of data for the work and contributed to discussion of the manuscript content, performed significant revision of the draft. All authors approved the final version for publication.

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