Relevance of allergenic sensitization to *Cynodon dactylon* and *Phragmites communis.* Cross-reactivity with the Pooideae grasses.

Running title: Sensitization to *Cynodon* and *Phragmites*

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Abstract

Background

Besides the Pooideae sub-family of grasses, there are other sub-families with allergenic members such as *Cynodon dactylon* (Chloridoideae) or *Phragmites communis* (Arundinoideae). Pooideae species belong to the same homologous group (sweet grasses). *C. dactylon* and *P. communis* are not included in this group because cross-reactivity with other grasses is not significant.

Objectives

The objectives were to investigate the sensitization profile to *C. dactylon* and *P. communis* in patients sensitized to grasses and to analyze the cross-reactivity between these two species and temperate grasses.

Methods

Patients were skin prick tested with a grass-mixture (GM). sIgE to GM, *C. dactylon*, *P. communis*, Cyn d 1 and Phl p 1 were measured by ImmunoCAP.

A pool of sera was used for immunoblot assays. Cross-reactivity was studied by ELISA and immunoblot inhibition.

Results

Thirty patients had sIgE to GM. Twenty-four (80%) were positives to *C. dactylon*, 27 (90%) to *P. communis*, 22 (73.3%) to nCyn d 1 and 92.9% were positive to rPhl p 1.

Bands were detected in the three extracts by immunoblot. No inhibition of GM was observed with the other two species by immunoblot inhibition or by ELISA inhibition. When *C. dactylon* or *P. communis* were used in the solid phase, GM produced an almost complete inhibition.

Conclusions

Eighty percent of the patients sensitized to grasses were also sensitized to *C. dactylon* and 90% to *P. communis*. Sensitization to these species seems to be induced by allergens different than to sweet grasses.

Keywords: grass allergy, cross-reactivity, *Cynodon dactylon*, *Phragmites communis*, Phl p 1, Cyn d 1

Resumen

Antecedentes

Desde un punto de vista taxonómico, el grupo homólogo de las Gramíneas pertenecen a la sub-familia Pooideae. Sin embargo, existen también otras especies de gramíneas alergénicas que pertenecen a sub-familias diferentes como son *Cynodon dactylon* (Chloridoideae) o *Phragmites communis* (Arundinoideae). *C. dactylon* y *P. communis* no están incluidas en este grupo homólogo debido a que la reactividad cruzada con otras gramíneas es limitada.

Objetivos

Los objetivos del estudio fueron investigar el perfil de sensibilización a *C. dactylon* y *P. communis* en pacientes sensibilizados a gramíneas y analizar la reactividad cruzada entre estas dos especies y las gramíneas más comunes.

Métodos

A los pacientes se les realizó una prueba cutánea con una mezcla de gramíneas (MG). Mediante ImmunoCAP se midió la IgE específica para MG, *C. dactylon*, *P. communis*, Cyn d 1 y Phl p 1.

Un pool de sueros se utilizó para ensayos de inmunoblot. La reactividad cruzada se estudió mediante ELISA e inmunoblot inhibición.

Resultados

Treinta pacientes tuvieron IgE específica para MG. Veinticuatro (80%) fueron positivos a *C. dactylon*, 27 (90%) a *P. communis*, 22 (73.3%) a nCyn d 1 y 92.9% fueron positivos a rPhl p 1.

Se detectaron bandas en los tres extractos mediante inmunoblot. No se observó inhibición de MG con las otras dos especies mediante inmunoblot o ELISA inhibición. Cuando *C. dactylon* o *P. communis* se usaron en fase sólida, MG produjo una inhibición casi completa.

Conclusiones

El 80% de los pacientes sensibilizados a gramíneas estaban también sensibilizados a *C. dactylon* y el 90% a *P. communis*. La sensibilización a estas especies parece estar inducida por diferentes alérgenos que en el caso de gramíneas.

Palabras clave: Alergia a gramíneas, reactividad cruzada, *Cynodon dactylon*, *Phragmites communis*, Phl p 1, Cyn d 1

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Introduction

Grasses are the major cause of pollen allergy worldwide, mainly the species of the Poaceae family. Although Poaceae family contains different sub-families, the most allergenic species belong to the temperate Pooideae sub-family. However, there are other sub-families with some members also identified according to their capacity to induce allergic symptoms such as *Cynodon dactylon* (Chloridoideae sub-family) or *Phragmites communis* (Arundinoideae sub-family). Both species have been identified in warm temperate and sub-tropical areas, including areas of Africa, Asia, Australia, America [1] and also in Europe to approximately 53°N latitude [2]. Although *C. dactylon* is now cosmopolitan, it is generally recognized that its present distribution has been achieved with the aid of man [3] as it is used as livestock herbage and turf. In recent years, due to climate change, their distribution and pollen release is being modified and different species are colonizing new areas, modifying the allergenic composition in the environment. In temperate climates such as Spain [4, 5] or Italy [6], Pooideae and sub-tropical grasses cohabit in the same areas. In Spain, *C. dactylon* is distributed in all areas of the country and *P. communis* is more associated with humid areas such as coastal regions or close to rivers [7].

Pollen from different grass families has similar morphology and it is very difficult to distinguish it based only on visual parameters. For that reason, deeper studies which include the flowering of the species may help in the identification of the pollination season of different species [8]. According to these studies, *C. dactylon* flowering occurs later than in temperate grasses [6], which could explain the onset of symptoms in grass sensitized patients in late June or July. For that reason, patterns of sensitization depend on the primary grass sensitization and differ depending on geographical areas [1].

Until now, species of the Pooideae family are grouped in the same homologous group (sweet grasses). Belonging to this group is based on the presence of three allergen families: group 1, 2 and 5 [9]. Moreover, cross-reactivity studies with different species showed that the majority of Pooideae grass species were highly cross-reactive [10, 11, 12]. *C. dactylon* is not included in this homologous group because cross-reactivity with other grasses is not substantial [9]. Recently, the inclusion of *C. dactylon* in this group has been proposed, but considering only Cyn d 1 [13]. Until now, seven allergens have been described in *C. dactylon*: Cyn d 1, Cyn d 7, Cyn d 12, Cyn d 15, Cyn d 22w, Cyn d 23 and Cyn d 24 (IUIS Allergen Nomenclature Subcommittee) [14]. There are 8 more described in Allergome.org [15]: Cyn d 2, Cyn d 3, Cyn d 5, Cyn d 6, Cyn d 11, Cyn d 13, Cyn d CP and Cyn d EXI.

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Several publications describe the lack of group 2 and 5 allergens in *C. dactylon*, though these are mentioned in Allergome [16]. Only Cyn d 1 and Cyn d 7 have some cross-reactivity with other grasses, but the results are not consistent [9]. Moreover, allergens of group 1 in *C. dactylon* have different epitopes compared to group 1 allergens of Pooideae grasses [17, 1]. These differences are probably responsible for the low cross-reactivity between *C. dactylon* and Pooideae grasses [18, 1].

Regarding *Phragmites*, five allergens have been described in *P. communis*: Phr a 1, Phr a 4, Phr a 5, Phr a 12 and Phr a 13, all of them in the allergome database [15]. Lack of group 2 and 6 allergens in *P. communis* has been described [19] and there is a low cross-reactivity with grasses from the Pooideae family [20]. Studies about *P. communis* were not enough to include this species in the homologous group of grasses.

According to these premises, the aims of the study were to investigate the sensitization profile to *C. dactylon* and *P. communis* in patients sensitized to grasses in the northwest of Spain (Catalonia Region), and to analyze the cross-reactivity between these two species and a mixture of temperate grasses.

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Materials and methods

Patient population

Patients were recruited in six hospitals from the northeast of Spain (Catalonia): Hospital Universitari Joan XXIII (Tarragona), Allergo Center (Barcelona), Hospital de Terrassa (Terrassa, Barcelona), Hospital Clinic (Barcelona), Hospital Vall d'Hebron (Barcelona) and Hospital Arnau de Vilanova (Lleida). The selection criteria were: respiratory symptoms (rhinitis and/or asthma) during grass pollen season and a positive skin prick test (diameter higher than 3 mm) with a standardized grass mixture (GM) that contains the equal amounts of *Dactylis glomerata, Festuca elatior, Lolium perenne, Phleum pratense* and *Poa pratensis* (Laboratorios LETI S.L., Tres Cantos, Spain). A serum sample was obtained after oral consent from each of the patients enrolled in the study.

All patients were also skin prick tested with a battery of biologically standardized aeroallergens including pollens such as *Olea europaea, Artemisia vulgaris, Parietaria judaica, Cupressus arizonica, Salsola kali, Platanus acerifolia* and *Plantago lanceolata,* as well as mites, molds and epithelia.

Extract manufacturing

Pollen extracts were prepared following internal manufacturing procedures (Laboratorios LETI). Briefly, grass pollen (*D. glomerata, F. elatior, L. perenne, P. pratense, P. pratensis, C. dactylon* and *P. communis*) were extracted consecutively for 4 and 8 hours in phosphate-buffered saline (PBS) 0.01M pH 7.4. After each extraction the sample was centrifuged and the supernatant recovered. Supernatants from both extractions were pooled, filtered and freeze dried. The protein content was measured using the Bradford method (Thermo Fisher Scientific, Rockford, IL, USA).

SDS-PAGE and 2-D electrophoresis

SDS-PAGE analysis was used to determine the protein profile of the grass extracts. Fifty micrograms of protein from every extract were loaded. Bands were analyzed via densitometry with the ImageQuant TL 8.1 software (GE Healthcare, Uppsala, Sweden).

For 2-dimensional electrophoresis, the extracts were purified and concentrated with a solution of ammonium sulfate in two different steps until reaching saturation percentage (40 and 80%) and then maintained at 4°C overnight. Thereafter, the samples were centrifuged, the pellets collected and reconstituted in ultrapure water. Concentrated extracts were washed using ReadyPrep 2-D Cleanup Kit (BioRad, Hercules, CA, USA), following the manufacturer's instructions. Proteins were separated according to their isoelectric point in ReadyStrip IPG strips (BioRad) in a pH range of 3–10, using Protean

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IEF Cell (BioRad). After the first dimension, the strips were equilibrated with the ReadyPrep 2-D Kit buffers (BioRad) and proteins were separated in the 2nd dimension according to their molecular weight (Mw). Gels were stained with Oriole fluorescent solution (BioRad) following the manufacturer's instructions.

Specific IgE

Specific IgE to GM, *C. dactylon, P. communis* and the allergens Phl p 1 and Cyn d 1 was determined for every serum by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) following the manufacturer's instructions. A sIgE >0.35 kUA/l was considered positive.

ELISA assays

Direct ELISA was performed with a pool of sera prepared by mixing equal quantities of those sera with sIgE to GM >1 kUA/l (27 sera). Briefly, microplate (Immulon 4HBX, Thermo Fisher Scientific) was coated with 20 μg of lyophilized extract/ml and the pool of sera (1:1 diluted in 0.01M PBS) and was incubated for two hours at room temperature. After 3 washes, peroxidase-conjugated monoclonal anti-human IgE (Ingenasa, Madrid, Spain) was added. After 2 hours the reaction was developed, stopped and read at 450 nm. Results were expressed in O.D.

ELISA inhibition was performed with the pool of sera as well as with individual sera. For the ELISA inhibition assay, sera were preincubated with the inhibitory extract for two hours before the addition to the microplate.

Immunoblot experiments

Fifty μg of protein of each extract were electrophoresed by SDS-PAGE and electrotransferred to a Trans Blot® Turbo™ Transfer Pack (BioRad). Membranes were incubated overnight with the pool of sera (dilution 1/5 in 0.01 M PBS Tween 0.1%). After washing, membranes were incubated with anti-human IgE-PO (Ingenasa), developed with luminol solutions (Immun-Star HRP Chemiluminescent Kit, BioRad) and detected by chemiluminiscence (ChemiDoc XRS, Bio-Rad).

The same procedure was used for the immunoblot inhibition experiments; in this case the pool of sera was incubated for 2 hours at room temperature with 500 μg of the inhibitory extract before its addition to the blot membrane.

Quantification of group 5 allergens

Group 5 allergens were quantified in the three extracts. Briefly, microplates (Maxisorp, Thermo Scientific, Roskilde, Denmark) were coated with MA-1D11 anti-Phl p 5 monoclonal antibody (Indoor Biotechnologies, VA, USA) at a dilution 1/1000. After

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blocking with 1% BSA, PBS-Tween 0.05%, the samples were added to the plate in serial dilutions from 1 μg/ml to 31.25 ng/ml. The European Pharmacopoeia Reference Standard (EDQM) was used as standard. After an hour of incubation, the biotinylated anti-Phl p 5 mAb Bo1 (Indoor Biotechnologies) was added and incubated for one hour. Finally, streptavidin-PO was added and the reaction developed and measured at 450 nm.

Statistical analysis

Descriptive statistical analyses were used for the calculation of variables and the Mann-Whitney Rank Sum Test for the comparison between sIgE values. Linear regression assay was performed to compare the relationship between sIgE values. SigmaStat 3.5 (Point Richmond, Calif., USA) software was used for the statistical analysis.

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Results

Patient population

A total of 31 patients were recruited (Table 1). All of them had rhinitis; 20 conjunctivitis (64.5%); 6 asthma (19.4%); and 6 cutaneous symptoms (19.4%). Only 3 individuals (9.7%) were monosensitized to grass pollen; 24 (77.4%) were sensitized to other pollen extracts, mainly olive tree pollen (19 patients, 61.3%); 16 to mites (51.6%); and 12 to animal dander (38.7%).

SDS-PAGE and 2-D electrophoresis

In SDS-PAGE, the three extracts (GM, *C. dactylon*, and *P. communis*) showed protein bands in a MW range between 10 and 100 kDa. Densitometry assay showed a very similar profile for the three extracts (Figure 1A). The most intense bands for the GM extract were those at 10 and 13 kDa. For the *C. dactylon* and *P. communis* extracts the most intense band was identified at 34 kDa (Figure 1). In 2-D electrophoresis, we observed that most proteins in the three extracts were located in the acidic region (left middle of the gel) and had different isoforms (Figure 1B). There were some spots with different intensity depending on the extracts, as two intense spots in *C. dactylon* and *P. communis* around 21 kDa that were unremarkable in the GM extract. Others as one of about 10 kDa found in the GM extracts had less intensity in *C. dactylon* and it was not present in the *P. communis* extract (in red in figure 1B). There were spots characteristic of some of the extracts, marked in green in the figure 1B. Other visible differences can be due to the specific method variations for the three gels.

Specific IgE

Positive specific IgE to GM was detected in 30 patients. One patient (number 24, see table 1) was negative to all the extracts and was not used in further assays. Twenty-four individuals were positive to *C. dactylon* (80%) and 27 to *P. communis* (90%). The highest IgE values were obtained with GM (23.8±33.4 kUA/l) and the lowest with *C. dactylon* (6.4±8.9 kUA/l). There was no statistical significant difference between the values obtained for the different extracts (Figure 2). For the group 1 allergens, 26 individuals (92.9%, 26/28) were positive to Phl p 1 (not performed in two patients due to lack of serum sample) and 22 (73.3%) to Cyn d 1 (Figure 2). In a regression analysis between sIgE values, we found the highest correlation between *P. communis* and Phl p 1 (R^2=0.8). For *C. dactylon*, the correlation with all the other extracts was low (0.2 to 0.4). Correlation among Cyn d 1 and Phl p 1 was 0.5 (Figure 3).

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ELISA and ELISA inhibition

The pool of sera recognized the three extracts by direct ELISA. Values obtained with GM in the solid phase were 2 to 4.4 times higher than those obtained with *C. dactylon* and 1.3 to 2.4 higher than with *P. communis* (Figure 4).

In ELISA inhibition, 0.05 ng of GM extract were necessary to obtain the 50% inhibition point; 5.7 and 4.9 μg were necessary to obtain a 50% inhibition with *C. dactylon* and *P. communis*, respectively. These assays were performed with GM in the solid phase.

Valid inhibition lines were obtained only with three individual serum samples (serum 3, 6 and 17). To obtain the 50% inhibition point with *C. dactylon*, we needed 650 times more quantity for serum 3, 353 for serum 6 and 337 for serum 17 than to obtain the 50% inhibition with GM. In the case of *P. communis* we needed 3360 times more quantity for serum 3, 436 for serum 6 and 300 for serum 17 to obtain the 50% inhibition than with GM. All the assays were performed with GM in the solid phase.

Immunoblot experiments

The pool of sera recognized two main bands in a molecular weight (MW) around 30 kDa in the GM extract, one band in *C. dactylon* extract and two in *P. communis* extract (Figure 5).

Inhibitions experiments

When GM extract was used in the solid phase, no inhibition was observed with the *C. dactylon* or *P. communis* extracts. Conversely, when *C. dactylon* or *P. communis* were used in the solid phase, GM inhibited the binding of the sera similar to when the inhibition was performed with the same extract. In both cases, the other extract produced an intermediate inhibition (Figure 6).

Quantification of group 5

GM extract contains 12.3 μg of group 5 allergens/mg of lyophilisate. However, allergens of group 5 were not detected in *C. dactylon* and in *P. communis* extracts.

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Discussion

In the present study we have assessed the cross-reactivity between a mixture of five grasses (GM) from the sweet grasses homologous group (*D. glomerata, F. elatior, L. perenne, P. pratense* and *P. pratensis*) and two species from different sub-families: *C. dactylon* (Chloridoideae sub-family) and *P. communis* (Arundinoideae sub-family), two sub-tropical grasses very abundant in the studied area (Catalonia, North-East of Spain). Patients included in the study had positive SPT to the GM extract. *C. dactylon* and *P. communis* extracts were unable to inhibit IgE binding to the GM extract; on the contrary, GM extract did inhibit IgE binding to the other two. These data are consistent with GM being the primary sensitizer in the population. The asymmetric cross-reactivity between temperate and sub-tropical grasses has been previously reported [21], and it is different according to the geographical origin of patients.

In areas where different Poaceae subfamilies share the same habitat and grow together, it is difficult to determine which species trigger symptoms in sensitized patients. All of them have similar pollen grain characteristics and are indistinguishable by their morphology. To overcome this problem, Frenguelli et al. [6] performed a phenology study in Italy and demonstrated that *C. dactylon* flowering was later than for other grasses. For a correct diagnosis it is important to know that patients with symptoms in summer (June-July) may be sensitized to *C. dactylon*. Comparisons of phenology data with symptoms and component resolved diagnosis (CRD) will aid in determining the individual sensitization profile and potentially aid in the selection of the most adequate specific immunotherapy.

Eighty percent of the individuals in this study had positive sIgE to *C. dactylon* and 90% to *P. communis*. For group 1 allergens, 92.9% were positive to Phl p 1 vs. 73.3% to Cyn d 1. There were individuals with high values to Phl p 1 that were negative to Cyn d 1 (number 12 and 14 in table 1). This data confirm previous studies that demonstrated immunologic differences between the two allergens [17], in the recognition of T-cell epitopes [21, 22] and in the amino acid sequence and 3D structure [13]. Also, the correlation coefficient (R^2) between sIgE to Phl p 1 and Cyn d 1 was 0.5, which corroborates the presence of different IgE binding epitopes. *P. communis* sIgE values showed the highest correlation with Phl p 1 sIgE (R^2=0.8); the correlation was also high with Cyn d 1 (R^2=0.7). Duffort el al. [23] developed a monoclonal antibody to quantify Cyn d 1 and it also recognized *P. communis*. Both results indicate that Phr a 1 would have epitopes in common with Cyn d 1 and with Phl p 1. Moreover, previous published data show that Phl p 1 has specific epitopes not present in the group 1 allergens of *C. dactylon* and *P. communis* [18]. Our findings

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confirm that IgE binding to Phl p 1 and to Cyn d 1 is different, probably due to differences in their epitopes. Although the Phr a 1 sequence is still unknown, our results suggest that it has more common epitopes with Phl p 1 than Cyn d 1. This hypothesis should be confirmed with purified allergens.

Group 5 allergen in the Poaceae subfamily is a major allergen [20, 24], and until now is the only allergen quantified in the immunotherapy extracts. However, no author has described group 5 allergens in *C. dactylon*. We confirmed that group 5 was undetectable in *C. dactylon* using the monoclonal antibody used for the quantification of group 5 in sweet grasses (EDQM standard). Although this standard consists on the Phl p 5a isoform, it can be used for the identification and quantification of other Pooideae members. With the same method, group 5 allergens were neither detectable in *P. communis*, which indicates that Phr a 5 has different antigenic determinants than group 5 allergen from sweet grasses. Therefore, patients sensitized to *C. dactylon* and/or *P. communis* do not recognize group 5 allergens. For that reason, it is important to know the primary sensitizer, since in patients sensitized to *C. dactylon* or *P. communis*, immunotherapy with an extract enriched in group 5 allergens (treatment with a mixture of sweet grasses) may not be effective; moreover it could induce new group 5 sensitizations [25, 26, 27].

One of the limitations of this study is the low number of patients included. It would be interesting to increase the number and to study a population selected by sensitization to *C. dactylon* and/or *P. communis*. The second limitation is the absence of clinical results which are the only way to confirm the immunological results obtained *in vitro*. It is important to know the primary sensitizer in a specific area to be able to apply the best immunotherapy treatment as proposed by Nony et al. for Australian patients [28]. In the studied population, immunotherapy with sweet grasses (Poaceae) would be adequate as all allergens recognized in *C. dactylon* and *P. communis* were inhibited by the GM extract. A population selected by sensitization to *C. dactylon* and/or *P. communis* as primary sensitizer could have different cross-reactivity behavior and would probably need a treatment with the species involved.

In summary, 80% of the patients sensitized to grasses were also sensitized to *C. dactylon* and 90% to *P. communis*, but with a lower sIgE. The grasses mixture and the *C. dactylon* and *P. communis* extracts have different IgE binding epitopes which rule out the possibility of including them in the sweet grasses homologous group. Sensitization to *C. dactylon* and *P. communis* seems to be induced by allergens different to those in sweet

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grasses. Further clinical studies should be considered to confirm the immunological results.

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Table 1: Characteristics of the population.

			IgE (kUA/l)						
Nº	**Age**	**Sex**	**GM**	**Phl p 1**	**Cyn**	**Cyn d 1**	**Phr**	**Symptoms**	**Other Sensitizations**
1	32	M	8.14	8.82	1.52	2.44	0.84	R, C	
2	44	F	3.53	0.94	N	0.51	0.4	R, C	
3	34	F	6.86	4.91	1.3	2.26	2.9	R, C, S	P, M, D
4	40	F	1.94	N	3.51	N	4.05	R, C, S	P, M, D
5	50	M	4.22	3.43	1.1	2.15	1.83	R, C, A	P
6	23	F	>100	-	21.4	65.8	>100	R, A	M, D
7	19	M	>100	-	6.82	33.4	55.4	R	P, M, D
8	25	M	24.9	N	N	N	2.53	R	P
9	52	M	21.8	2.3	5.43	2.51	6.34	R	P
10	9	M	>100	>100	6.77	62.2	48.7	R	M
11	37	M	29.6	10.3	1.11	0.4	3.32	R, C	P
12	29	M	1.55	14.2	N	N	0.58	R, C, S	P, M, F, D
13	20	F	84.5	92.9	28.3	17.6	32.1	R, C, S	P, M, D
14	39	F	14.1	17	1.46	N	1.77	R, C	M
15	36	M	1.23	2.04	0.45	N	0.61	R, C, A	P, M, F, D
16	42	F	3.16	4	1.14	0.93	1.14	R	P
17	8	M	27.7	42	15.8	11.2	13.9	R	P, D
18	62	F	2.82	0.99	0.62	1.66	1.56	R, C	P
19	27	M	0.82	0.79	N	N	N	R, C	M
20	60	F	0.59	1.36	N	N	N	R	
21	39	F	66.6	23.2	10.4	18.2	16.7	R, C	P, M, D
22	27	F	61.2	12.8	1.94	5.11	6.54	R, C	P, M, D
23	42	F	2.38	1.32	0.43	0.7	0.49	R, S	P, M, D
24	36	M	N	N	N	N	N	R, C	P
25	39	F	13.8	11.9	6.29	6.91	10.2	R	P, M
26	27	F	0.43	0.61	N	N	N	R, C	P, M
27	41	M	27.8	2,10	31.7	33.6	0.79	R, C, S	P, M, F, D
28	35	F	0.46	3.35	0.63	0.66	4.56	R, C, A	P
29	34	M	0.87	1.97	1.32	0.93	2.76	R	P
30	40	M	1.94	7.58	2.27	3.6	7.73	R, C, A	P
31	28	F	1.73	30.4	2.51	5.08	36.6	R, C, A	P

Sex: M, male, F, female; sIgE:-, not analyzed; N, negative (<0.35 kUA/L). GM, mixture of grasses, Cyn, *C. dactylon*, Phr, *P. communis*. Symptoms: R, rhinitis, C, conjunctivitis, A, asthma, S, skin symptoms. Sensitizations: P, other pollen, M, mites, F, fungi, D, animal dander.

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Figure Legends

Figure 1: Protein profile. A: Scanning densitometry for the comparison of the profile of the three extracts; Red-GM, Pink-*C. dactylon* and Purple-*P. communis*. B: 2-D electrophoresis for each extract of the study. SDS-PAGE of each extract was shown next to each 2-D gel. Spots with different intensity in the extracts are marked in red, spots that appears only in one extract are marked in green.

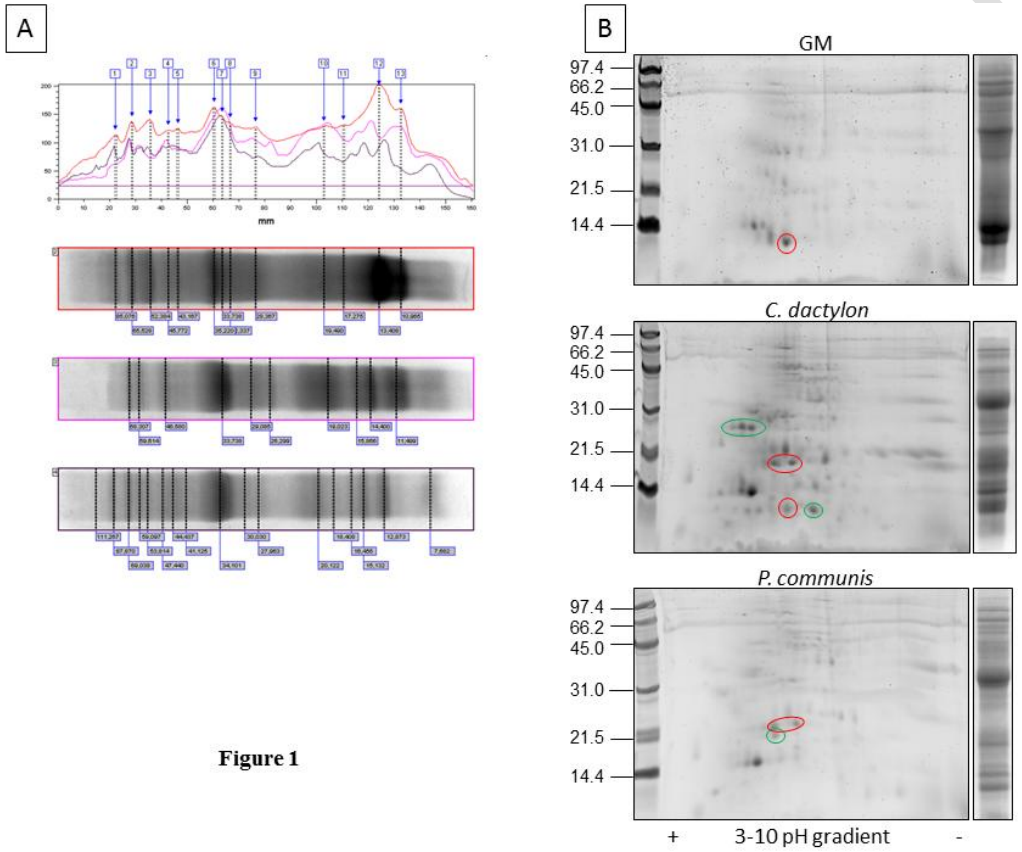
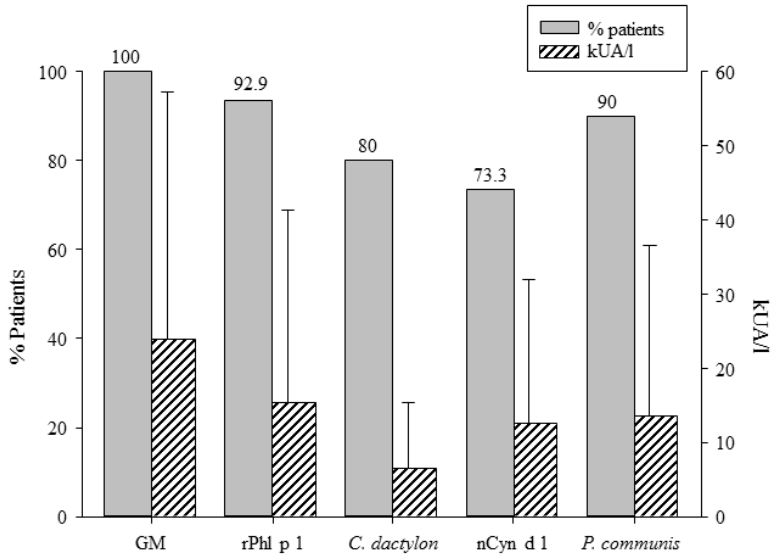


Figure 1

Figure 2: Percentage of patients sensitized to each extract and to the individual allergens and the mean value of sIgE (kUA/l). Error bars correspond to the standard deviation (SD). Mean values and standard deviations are shown in the table.



Mean	23.8	15.4	6.4	12.6	13.5
SD	33.4	25.9	8.9	19.3	23.0

Figure 2

Figure 3: Relation for every pair of sIgE data. The coefficient of determination (R2) for each pair of data is over the corresponding regression line.

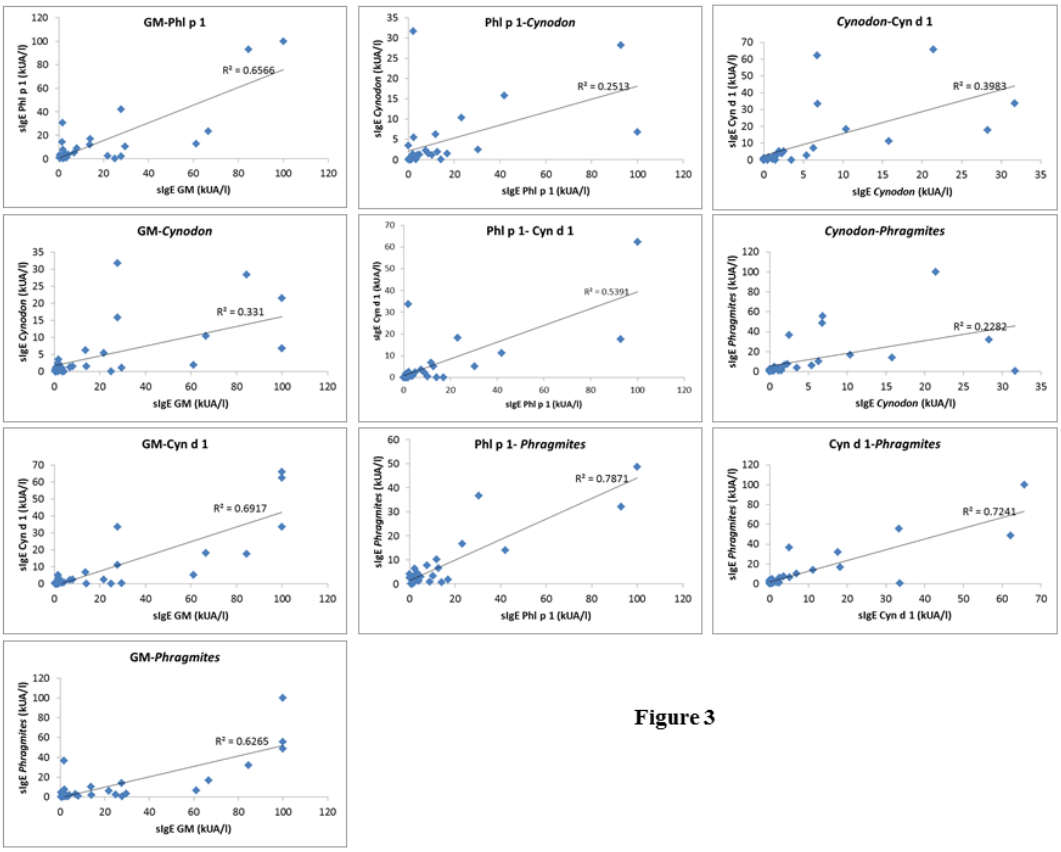
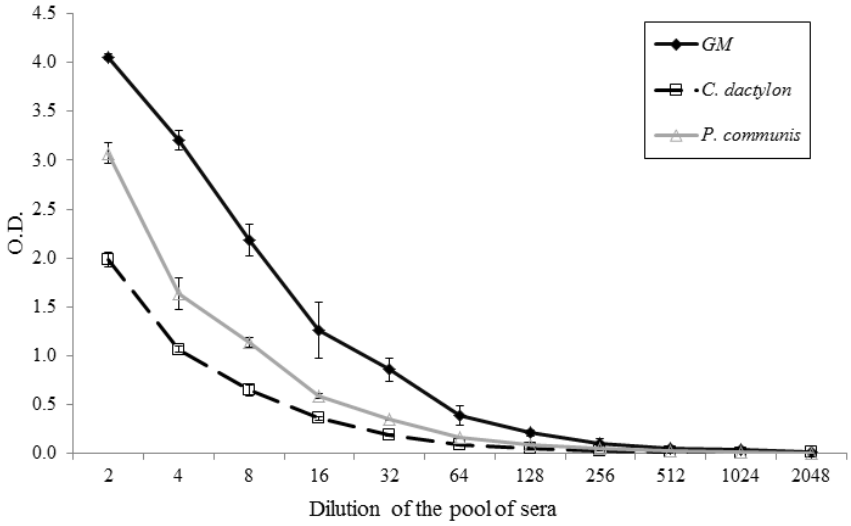


Figure 3

Figure 4: Direct ELISA with GM, *C. dactylon* or *P. communis* extracts in the solid phase and incubating with serial dilutions of the pool of sera. O.D. values are shown in the table.



GM	4.05	3.21	2.18	1.26	0.86	0.39	0.21	0.10	0.06	0.04	0.02
C. dactylon	1.98	1.07	0.64	0.36	0.19	0.09	0.05	0.02	0.01	0.01	0.01
P. communis	3.07	1.63	1.13	0.59	0.35	0.16	0.09	0.05	0.03	0.02	0.01

Figure 4

Figure 5: Immunoblot with the pool of sera (dilution 1/5). Fifty micrograms of each extract were run in the solid phase: 1-GM, 2-*C. dactylon* and 3-*P. communis* extracts.

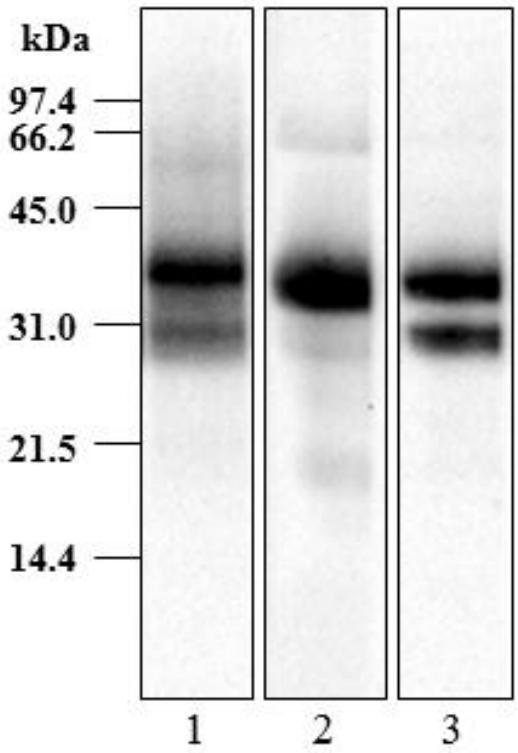


Figure 5

Figure 6: Immunoblot inhibition with the pool of sera (dilution 1/5). Fifty micrograms of each extract were run in the solid phase, the extract was indicated in the bottom part of the figure. Inhibitor extract was indicated in each line: GM-grass mixture, Cyn-*C. dactylon*, Phr-*P. communis* and W.I.- without inhibition.

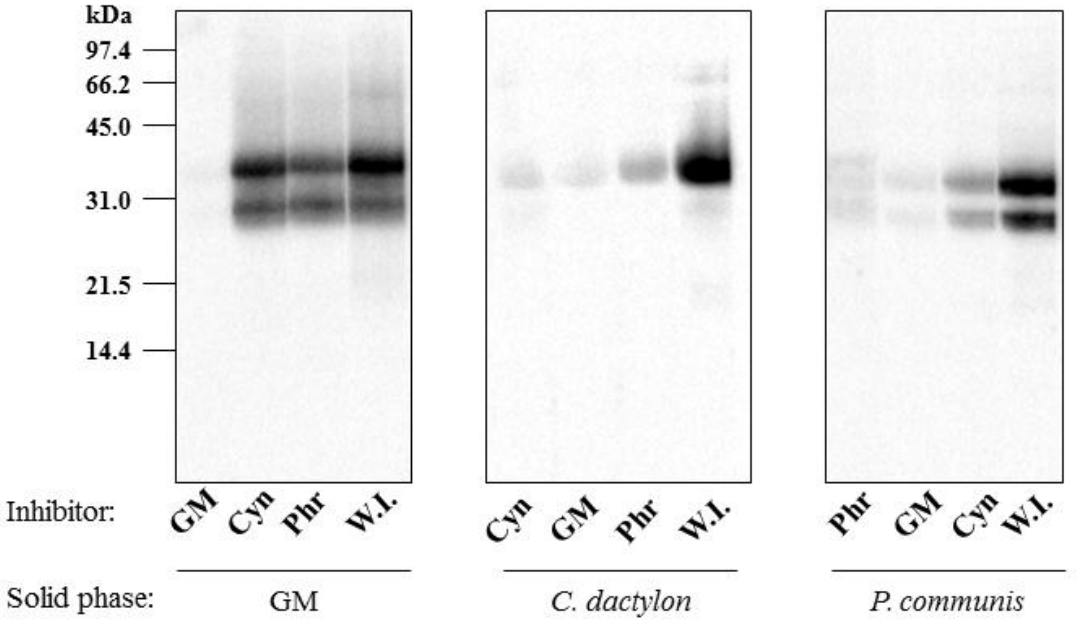


Figure 6