

Extensive IgE Cross-Reactivity towards the Pooideae Grasses Substantiated for a Large Number of Grass-Pollen-Sensitized Subjects

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Key Words

Allergen · Clades · Cross-reactivity · Grass pollen · IgE · Immunotherapy · *Phleum pratense* · Pooideae · Sequence homology · Taxonomy

Abstract

Background: Allergy to taxonomically related species is a common phenomenon caused by the same immunological receptor cross-reacting to homologous allergens from different species. Knowledge of patterns of cross-reactivity is crucial for the selection of optimal products for diagnosis and for specific immunotherapy. The objective of this study was to investigate patterns of serum IgE cross-reactivity towards pollens from various grass species. **Methods:** With grass group 1 allergens as the representative group, amino acid sequence alignment, structural modelling and comparison of 3D surface characteristics were performed to exemplify the molecular basis of IgE cross-reactivity. IgE binding to extracts from ten different grass species was determined (total number of data pairs >19,000), and IgE inhibition experiments using *Phleum pratense* were performed. **Results:** Analysis of surface topography for group 1 grass allergens demonstrated ample space for IgE binding epitopes in surface areas conserved among Pooideae grasses. Significant correlation was observed between the serum IgE response to *P. pratense* extract and extracts from the other Pooideae

grasses analyzed. *P. pratense* extract was demonstrated to inhibit the binding of IgE to the allergens in all of the extracts included in the investigation, indicating patient IgE to be primarily directed towards common epitopes. **Conclusion:** Extensive IgE cross-reactivity was observed towards the allergens of the Pooideae grasses, meaning that the immune system does not appear to distinguish based on the IgE level between the different species of this subfamily. The data suggest equal effect upon use of any of the Pooideae species for diagnostic as well as therapeutic purposes.

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Introduction

Allergy to taxonomically related species is a common phenomenon caused by the same immunological receptor, i.e. IgE antibody or T-cell receptor, cross-reacting with similar affinity to homologous allergens from different species [1–5].

Immunochemical cross-reactivity can arise from (i) homologous allergens from related species, (ii) pan-allergens such as profilin or lipid transfer proteins [5, 6] or (iii) cross-reacting carbohydrate determinants (CCDs) [7] which possess ubiquitous epitopes. The clinical relevance of pan-epitopes is still debated, and the influence of CCDs on different in vitro IgE determination assays is a

topic of ongoing discussion [7–11]. The present study concerns cross-reactivity towards grass pollen allergens.

Figure 1 shows a cladogram of the allergenic members of the grass family, depicting the taxons of subfamily, tribe and example species [2].

At the top of the cladogram in figure 1 is one of the larger groupings, called the PACC clade, including the subfamilies Chloridoideae, Arundinoideae and Panicoideae [5, 12]. This group probably originated in the Southern Hemisphere, with centers of distribution in Africa and Australia, which is supported by the observation that grasses of this group almost entirely utilize a type of photosynthesis metabolism (C4) that is advantageous in areas of high temperature and low soil moisture. In contrast, members of the Pooideae subfamily are distributed mostly in the temperate regions of the northern hemisphere and utilize C3 photosynthesis, which is more efficient in cooler and cold climates [12].

Based on present plant systematics, it would be expected that members of the PACC clade, such as *Cynodon*, *Sorghum* and *Phragmites*, are less similar to temperate pasture grasses (Pooideae), and by inference would show less cross-reactivity of allergenic proteins [5]. In contrast, the closer botanical and evolutionary relationship within the Pooideae subfamily is expected to give rise to a high degree of similarity between the allergens of the species listed in the bottom half of figure 1 [2–4, 6].

A number of allergens have been identified in pollen extracts of various grasses (www.allergen.org). The identification and classification into allergen groups was originally based on the detection of IgE-binding proteins with similar physiochemical properties in extracts of related grasses [13, 14]. Subsequently, the close relationship of the allergens has been confirmed by cloning and sequencing of the genes encoding the major allergens from several grass species [15, 16]. Each grass species contains a variety of isoallergens and variants of each individual allergen [17]. The sequence variation between isoallergens within the same species is in some cases similar in magnitude to sequence differences of homologous allergens between species. Thus allergens from the same group exhibit a high degree of sequence homology, particularly within subfamilies [17], and the classification of allergens into groups is also justified on the basis of the three-dimensional structure [3].

Thus, the distribution of grasses influences the sensitization and antibody cross-reactivity pattern of the individual patient, and for each allergen group the grass pollen-allergic population is presented with a large number of variants of what is essentially the same molecule.

The extent of IgE cross-reactivity towards allergens of botanically related grasses is the primary issue in the current report.

In this study, the differences and similarities between the allergens of the various grasses were illustrated by alignment of grass group 1 amino acid sequences available in the commonly accessible sequence databases. The results lead to a prediction of IgE cross-reactivity which has indeed been indicated by previous studies concerning the allergens of selected Pooideae grasses, conducted by van Ree et al. [18], with *Dactylis glomerata* and *Poa pratensis* as references. The present work seeks to investigate this cross-reactivity in depth by analyzing a very large set of data (>19,000 data pairs) for IgE cross-reactivity towards a large number of species. The grass species included in this study are mainly members of the Pooideae subfamily due to their widespread prevalence and importance as causes of allergy. *Phleum pratense* was chosen as the reference, since the clinical efficacy of allergen-specific immunotherapy with pollen extracts of this species is thoroughly documented [19–25]. Furthermore, elements of cross-reactivity of *P. pratense*-specific IgE towards a few species outside the Pooideae subfamily were also investigated. *Phragmites communis* was included as the Arundinoideae representative, and *Cynodon dactylon* was chosen as the Chloridoideae representative.

Methods

Study Populations and Samples

Determination of Grass Allergen-Specific IgE by the Magic Lite Method

Serum samples from an in-house serum bank were used (5,296 individual subjects from Northern Europe and North America). The subjects were all assessed as suffering from IgE-mediated allergy by a specialist or allergologist (positive skin prick test and a case history of allergic symptoms). For each of the species tested, results of 1,075–3,293 individual samples were obtained.

Determination of Grass Allergen-Specific IgE by the ADVIA Centaur Immunoassay System

The study included 29 serum samples from a randomized, double-blind, placebo-controlled, multicenter, phase I trial investigating the safety of GRAZAX® (*P. pratense* 75,000 SQ-T/2800 BAU) in children with grass pollen-induced rhinoconjunctivitis (with/without asthma) aged 5–12 years [26]. The samples were drawn prior to initiation of treatment. The trial was conducted in three centers in Germany in early 2006 outside the grass pollen season.

Relevant eligibility criteria are listed in the following:

- (1) boy or girl aged 5–12 years;

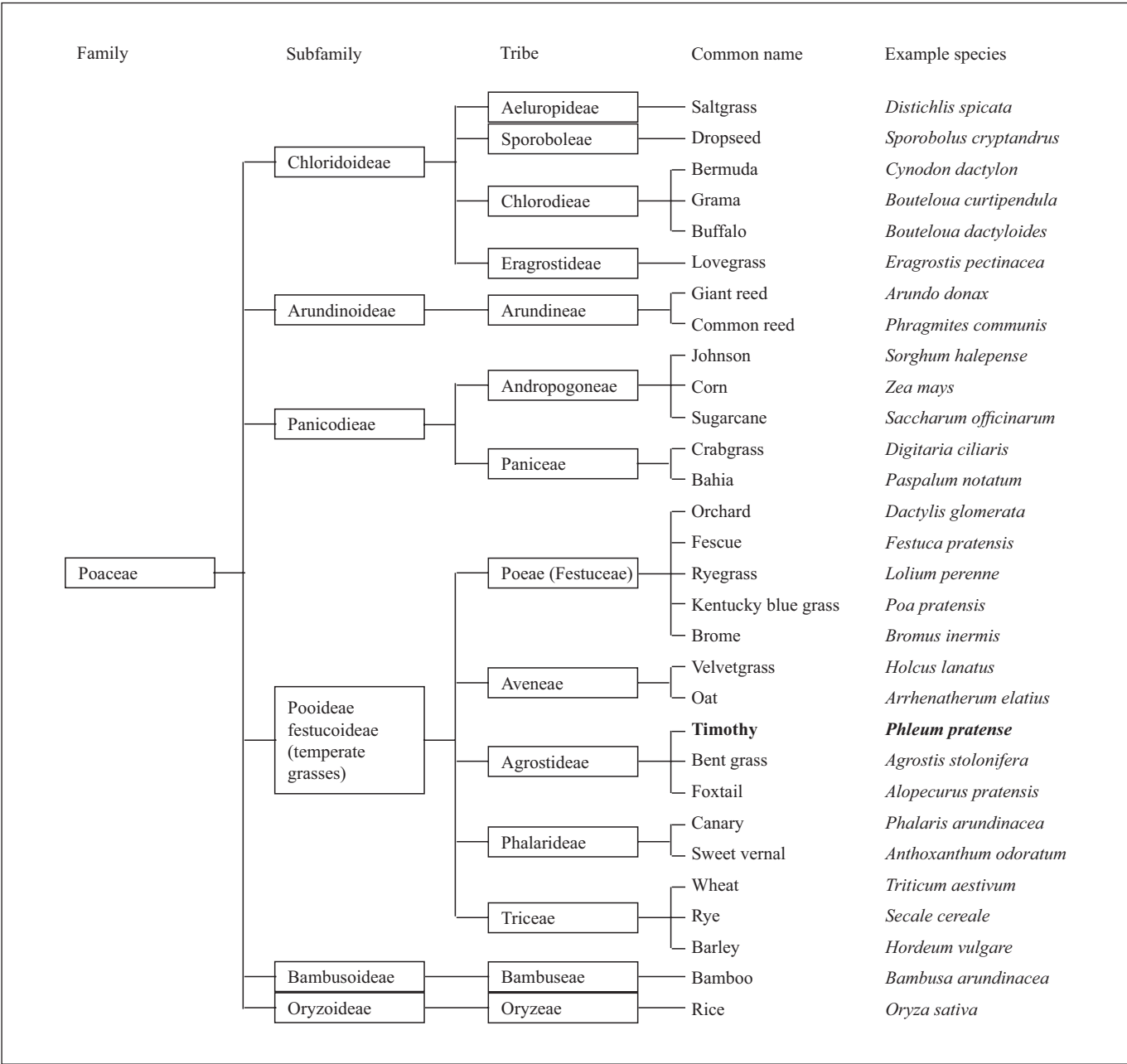


Fig. 1. Taxonomical classification scheme used in the present study.

- (2) written informed consent obtained from parents/guardians and from subjects if intellectual maturity is appropriate, at least when the subject is 12 years old, before entering the trial;
- (3) a clinical history of grass pollen-induced allergic rhinoconjunctivitis requiring treatment during at least one grass pollen season;
- (4) positive skin prick test response (wheal diameter ≥ 3 mm) to *P. pratense*;
- (5) positive specific IgE against *P. pratense* (≥ 0.7 kU/ml), and
- (6) no previous immunotherapy with grass pollen allergen or any other allergen within the previous 5 years.
- In addition, 20 serum samples were obtained from an in-house serum bank in Denmark, drawn prior to specific immunotherapy and all with specific IgE ≥ 3.5 kU/l against *P. pratense* (IgE class 3 or higher).
- These 49 serum samples were also used for the inhibition experiments.

Determination of Pan-Allergen- and Pan-Epitope-Specific IgE by the ADVIA Centaur Immunoassay System

Pan-allergen- and pan-epitope-specific IgE were analyzed as described elsewhere [27]. In brief, subjects were selected over a period of 3 months outside the pollen season, all with a clinical history of pollinosis, living in the same location in Southern Europe during the past 5 years or more, and without previous immunotherapy.

Statistics

IgE responses were analyzed by a Spearman rank correlation test. The Spearman rank correlation coefficients are presented with two-sided 95% confidence intervals. All stochastic probabilities <0.05 were considered significant.

Materials and Methods

Determination of Specific IgE Using the Magic Lite System

Specific IgE was determined with the Magic Lite method (ALK, Hørsholm, Denmark), as previously described by Kleine-Tebbe et al. [28].

The assay used a two-point adjustment based on an extended standard curve [29], was calibrated against a *P. pratense*-specific reference serum and measured specific IgE antibodies in concentrations from 1.43 to 800 standardized units per milliliter (SU/ml). For comparison with the results obtained by ADVIA Centaur, doses were converted after analysis to kilounits per liter, using the conversion 1 SU/ml = 4 kU/l.

Determination of Specific IgE Using the ADVIA Centaur Immunoassay System

Specific IgE was determined with the ADVIA Centaur immunoassay system (Siemens Medical Solutions, Diagnostics, Tarrytown, N.Y., USA), as previously described by Petersen et al. [30]. In the inhibition studies, specific IgE was determined before and after addition of non-labelled *P. pratense* extract (2 mg, 5,000-fold excess) to the biotinylated allergen extract reagent in question. Pollen extracts were obtained from ALK, manufactured as previously described [31]. Horseradish peroxidase (HRP) was obtained from Sigma-Aldrich (P8375). All reagents were used without further modification.

Bioinformatics

A BLAST search of the UniProtKB databases [32] was conducted for the amino acid sequences of Phl p 1 (P43213), Phl p 2 (P43214), Phl p 3 (Q69B42), Phl p 4 (Q2I6V7), Phl p 5 (Q40962), Phl p 7 (polcalcin O82040) and Phl p 12 (profilin P35079). Alignment was performed using the CLUSTAL-W program [33] provided by the ExPASy-CH server. The aligned sequences were used to calculate sequence identity/homology percentages and to identify differences in the sequences from those of *P. pratense*. The following amino acids were considered homologous: L = I = V = M, K = R = H, E = D, Q = E, F = Y = W and S = T = C. Structural comparison of the group 1 allergens was performed by mapping identical and homologous amino acids on the surface of the X-ray structure of Phl p 1 1n10. The three-dimensional structure of Phl p 1 was obtained from the PDB database (<http://www.rcsb.org/pdb/explore/explore.do?structureId=1n10>). The structure file contains a dimeric structure non-covalently linked by the first 14 N-terminal amino acids of the Phl p 1 sequence. When the monomer is isolated from the structure file this 14-membered stretch

protrudes from the surface justifying the omission of the segment for the investigation of the influence of amino acid substitution on the surface of the molecule.

Results

Sequence Homology and Structural Comparison

The change in amino acids as a function of isoform of Phl p 1 or in species is illustrated in figure 2, and the percentages of sequence identity and homology are listed in figure 3, which illustrates the change in surface-exposed amino acids. It is evident that a large portion of the Phl p 1 surface is relatively unaffected by the amino acid substitutions identified in both Phl p 1 isoforms and group 1 allergens from other species within the Pooideae subfamily, represented in figures 2 and 3 by Hol l 1, Poa p 1 and Lol p 1. The identical surface areas are large enough to harbor common, conserved IgE-binding epitopes within the group 1 allergens. The changes observed in the primary sequences of the Pooideae group 1 allergens seem to cluster within a discrete area of the surface representation of Phl p 1, revealing a confined region of diminished probability of shared epitopes.

In figures 2 and 3, the non-Pooideae species are represented by *C. dactylon*, and larger surface areas are markedly affected by the difference in amino acid sequence upon comparison of the Phl p 1 and Cyn d 1 sequences. The surface areas conserved between these species appear smaller, giving rise to a lower probability of cross-reacting IgE antibodies.

Sequence alignment of allergens of group 2–7 and 12 and of allergens from a larger selection of species reflecting those investigated in the present report is available as supplementary information. The results support the observations discussed above.

Grass Pollen Allergen-Specific IgE Binding – Magic Lite Data

Using the Magic Lite system, specific serum IgE detected by ten different grass pollen extracts (*P. pratense*, *P. pratensis*, *Festuca pratensis*, *D. glomerata*, *Lolium perenne*, *Holcus lanatus*, *Anthoxanthum odoratum*, *Secale cereale*, *C. dactylon* and *P. communis*) was quantified to investigate the correlation between IgE measurements performed with different related grasses. Responses for the nine latter species were correlated to the response for *P. pratense* (fig. 4).

For six of the species, the vast majority of the data points cluster around the line of identity, i.e. the IgE re-

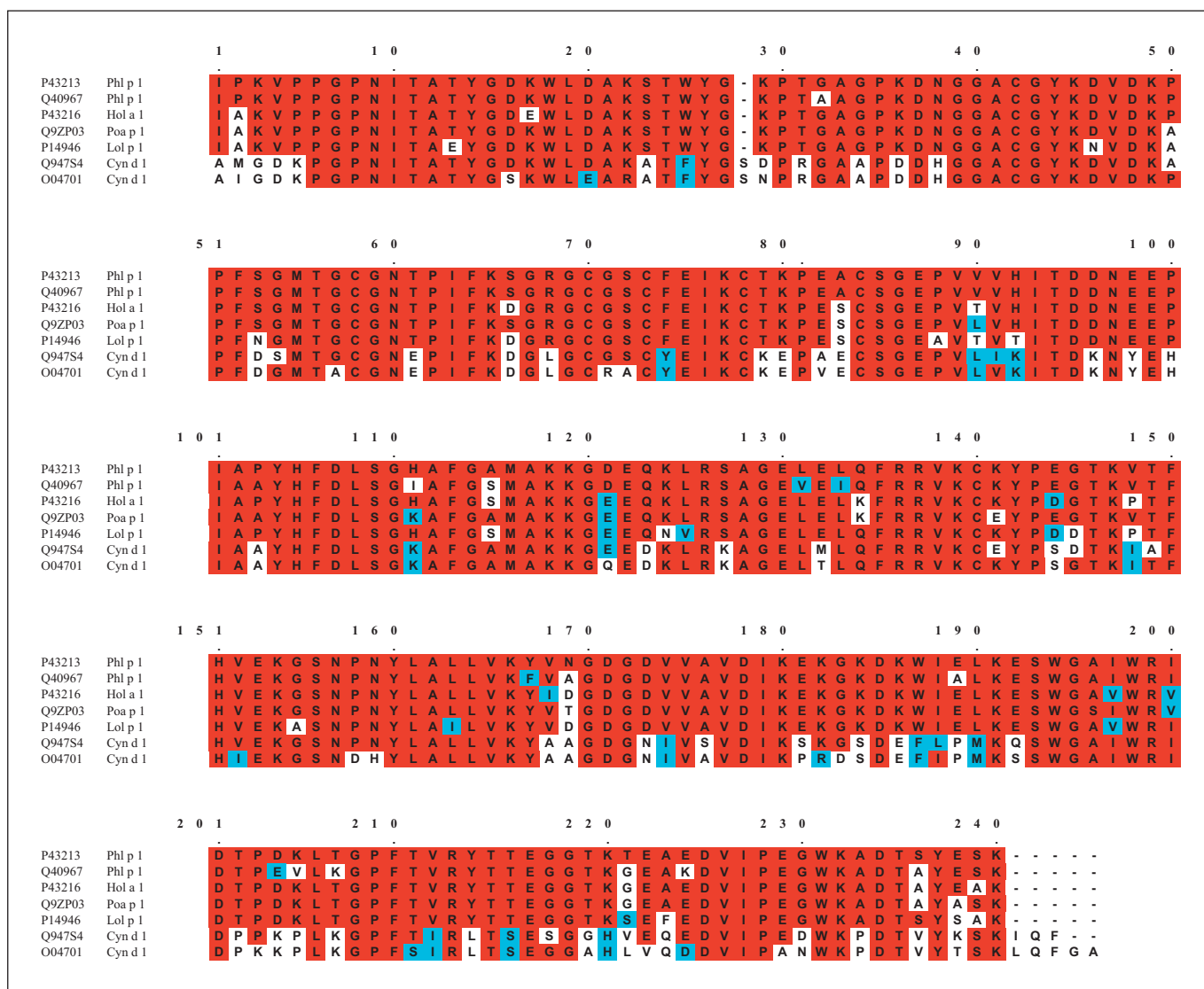


Fig. 2. Amino acid sequence alignment of selected grass group 1 allergens (Swiss-Prot/Tr-EMBL October 2007). Conserved amino acids are colored red (white in the printed version), whereas a change at a given position from the Phl p 1 sequence (P43213) is reflected as white (non-homologous substitution, light grey in print) or blue (homologous substitution, dark grey in print).

sponse to *P. pratense* pollen extract is numerically identical to the response to the extract in question. For *S. cereale*, correlation is also evident, but from visual inspection of the plot, the slope of the line of correlation seems to be slightly less than one. The Spearman rank correlation factors are listed in table 1.

These results strongly suggest a very high degree of cross-reactivity of the antibodies towards the allergens of six out of these seven Pooideae species and extensive cross-reactivity for *S. cereale* as well.

For the non-Pooideae species, *C. dactylon* and *P. communis*, a different pattern is observed, with a larger, biased dispersion of the data points and poorer correlation as indicated by the Spearman rank correlation factors (table 1).

Grass Pollen Allergen-Specific IgE Binding – ADVIA Centaur Data

Specific IgE binding was also investigated using the ADVIA Centaur immunoassay. Sera from 49 grass pol-

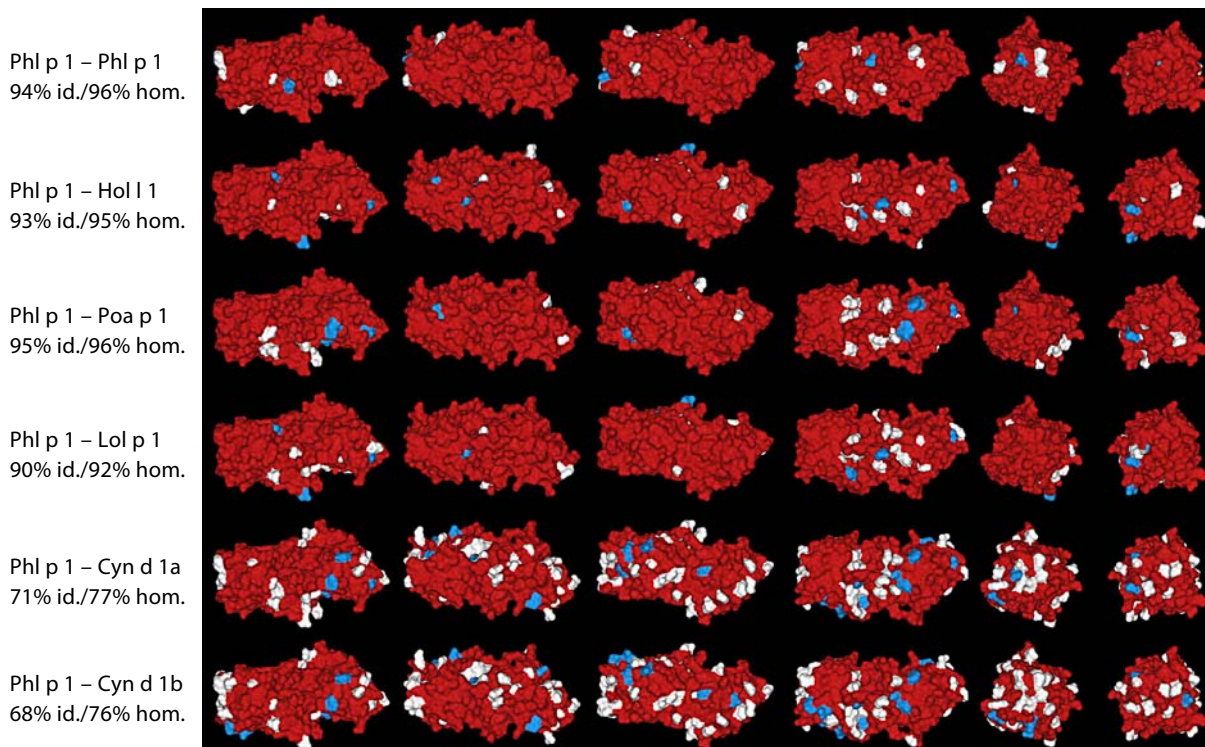


Fig. 3. 3D structures from different angles. Variation in surface-exposed amino acids gives rise to regional differences in surface characteristics. Conserved amino acids are colored red (white in print), whereas a change at a given position from the Phl p 1 sequence is reflected as white (non-homologous substitution, light grey in print) or blue (homologous substitution, dark grey in the printed version).

Table 1. Spearman rank correlation factors (95% confidence intervals) between the IgE response for the listed species and the IgE response to *P. pratense* pollen extract, along with the percentage of sera for which the IgE response was inhibited to a level below the limit of quantification by addition of *P. pratense* pollen extract

Species	Correlation factor		Inhibition, %
	Magic Lite	ADVIA Centaur	
<i>P. pratensis</i>	0.93 [0.92–0.94]	0.94 [0.90–0.97]	100
<i>F. pratensis</i>	0.97 [0.97–0.98]	0.97 [0.94–0.98]	100
<i>D. glomerata</i>	0.91 [0.90–0.91]	0.95 [0.91–0.98]	100
<i>A. elatius</i>		0.94 [0.89–0.97]	100
<i>L. perenne</i>	0.95 [0.95–0.96]	0.96 [0.93–0.98]	100
<i>H. lanatus</i>	0.95 [0.94–0.96]	0.93 [0.87–0.96]	98
<i>A. stolonifera</i>		0.94 [0.88–0.97]	100
<i>A. odoratum</i>	0.89 [0.88–0.89]	0.92 [0.85–0.96]	98
<i>S. cereale</i>	0.91 [0.90–0.92]	0.92 [0.86–0.96]	100
<i>C. dactylon</i>	0.43 [0.39–0.46]		94
<i>P. communis</i>	0.45 [0.42–0.48]		
<i>P. pratense</i>			100

Note that the number of sera tested was much higher for the Magic Lite experiments than for the ADVIA Centaur determinations.

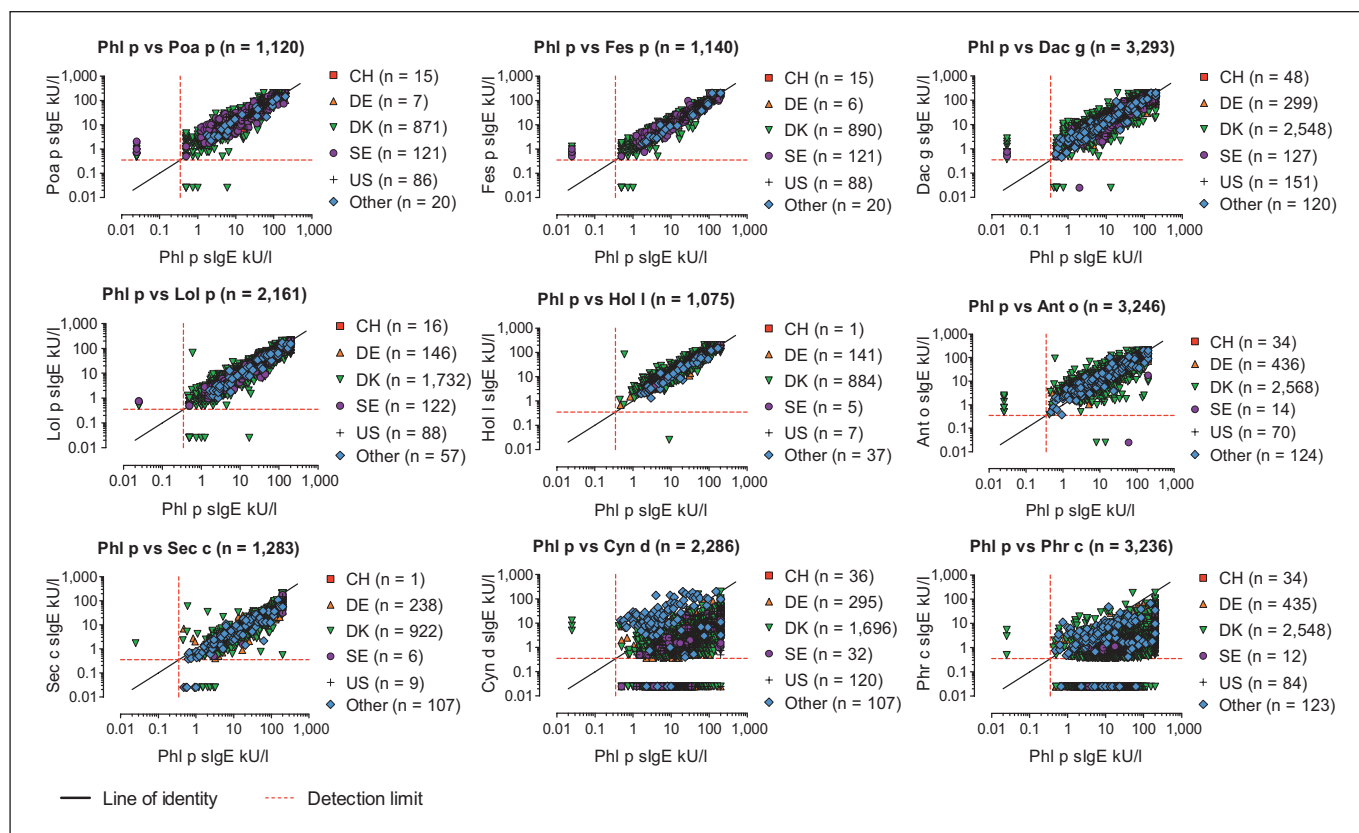


Fig. 4. Specific IgE in a large population, measured using the Magic Lite system. *P. pratense*-specific IgE plotted versus specific IgE detected by pollen extracts of nine selected species. The threshold for a positive result is 0.35 kU/l. A data point positioned on the line of identity suggests complete cross-reactivity of the *P.*

pratense-specific IgE antibodies towards the allergens of the tested extract. Different symbols are used for different countries: Switzerland (CH), Germany (DE), Denmark (DK), Sweden (SE) and USA (US).

len-sensitized subjects (*P. pratense* IgE class 3 or above) were analyzed for IgE specific towards the allergens in pollen extract of *P. pratense*, *P. pratensis*, *F. pratensis*, *D. glomerata*, *Arrhenatherum elatius*, *L. perenne*, *H. lanatus*, *Agrostis stolonifera*, *A. odoratum* and *S. cereale*. Responses for the nine latter were correlated to the response for *P. pratense* (fig. 5), yielding patterns in complete compliance with the results obtained using the Magic Lite system. Spearman rank correlation factors are listed in table 1.

IgE inhibition studies were then performed using the ADVIA Centaur immunoassay system. The results are seen in figure 6, and the percentage of sera for which the IgE response to the tested species was inhibited below the limit of quantification by addition of *P. pratense* extract is listed in table 1. From figure 6 and table 1, it is seen that addition of *P. pratense* pollen extract inhibits the IgE response in almost all sera for all of the species tested, in-

cluding *C. dactylon*, the only species tested in this assay which did not belong to the Pooideae subfamily.

Pan-Allergens – ADVIA Centaur Data

The influence of carbohydrate IgE-binding epitopes, CCDs, on the observed cross-reactivity was assessed by relating the number of sera displaying a response to HRP (a CCD representative [34]) to those displaying a positive IgE response to Phl p 1 and/or 5. Out of 651 serum samples from grass-pollen-allergic subjects, 31% displayed a positive IgE response towards HRP. This number is comparable to what has been described previously by Malandain [8]. An identical approach was taken to evaluate the role of profilin, represented by nPho d 2. Out of 136 tested serum samples from grass pollen-allergic subjects, 27% displayed reactivity towards profilin (nPho d 2).

Thus there is a possibility of the IgE response in figures 4 and 5 appearing quantitatively exaggerated with

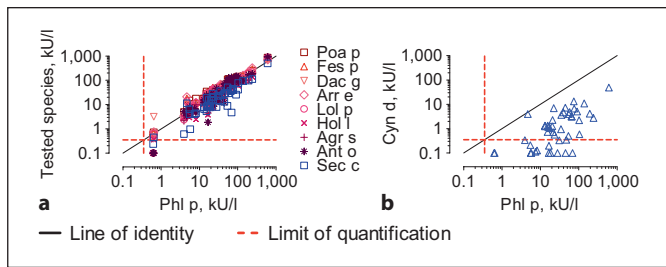


Fig. 5. Specific IgE (49 subjects) measured using the ADVIA Centaur assay. The threshold for a positive result is 0.35 kU/l. **a** Specific IgE detected by extracts of nine selected species plotted versus *P. pratense*-specific IgE. **b** Specific IgE detected by *C. dactylon* plotted versus *P. pratense*-specific IgE. A data point positioned on the line of identity suggests complete cross-reactivity of the *P. pratense*-specific IgE antibodies towards the allergens of the tested allergen extract.

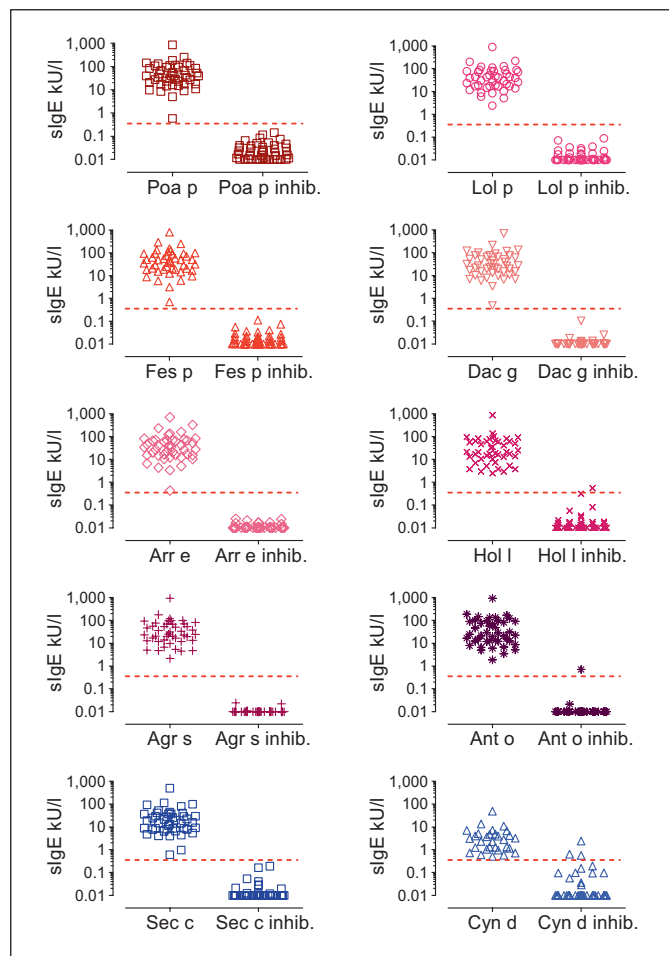


Fig. 6. Specific IgE measured before and after addition of non-labelled *P. pratense* pollen extract (ADVIA Centaur assay). The tests were performed only for sera displaying a positive IgE response to the species in question.

respect to clinical relevance [8]. However, this effect does not mask the possible presence of specific epitopes, as revealed by the data for *C. dactylon* and *P. communis*, which clearly show that *P. pratense* allergens possess specific epitopes not present in extracts of these two species.

Discussion

In accordance with botanical expectations, extensive cross-reactivity between the temperate grasses of the Pooideae subfamily has been indicated for some time [2, 4, 5, 17, 18] and is thoroughly substantiated in the present work based on the very large number of subjects analyzed (fig. 4).

The observed cross-reactivity was confirmed by IgE inhibition studies (fig. 6), with 98–100% of the samples with an IgE response to the Pooideae extract in question displaying complete inhibition of this response by addition of *P. pratense* extract in 5,000-fold excess. Human serum albumin, present in vast excess (10 mg/ml) in the washing buffer, displays no inhibitory capacity, ensuring the specificity of the assay.

The majority of the IgE determinations in the present study were performed using the Magic Lite assay. The results obtained with this assay were confirmed using a smaller number of sera in the ADVIA Centaur immunoassay system, and full correspondence between the results obtained with the two methods was observed.

The capability of the ADVIA Centaur assay of discriminating between monoclonal recombinant IgE antibodies of known allergen affinity has been shown previously [10, 35], and low-affinity IgE was only detected to a very small degree by this assay. Specific tests to detect pan-epitope- and CCD-specific IgE by the ADVIA Centaur immunoassay revealed that the presence of IgE directed towards such epitopes cannot account for the extensive correlation observed in the present study.

For both assays and all assessed species, the correlation matrices clustering around the line of identity display an ellipsoid distribution. In principle, the reaction pattern may be divided into three groups: (1) complete cross-reactivity (line of identity); (2) stronger IgE response towards *P. pratense*, and (3) stronger IgE response towards the second species. Since *P. pratense* completely inhibited the IgE binding to the second species the apparent differences between these three groups are likely to be explained by differences in the affinity of the IgE binding to the epitopes of allergens from different species.

True IgE cross-reactivity of IgE specific to allergens of the Pooideae grasses is thus established for a very large number of subjects in the present study. As seen in table 1 and figure 1, sequence homology is generally high within this subfamily, enhancing the probability of similar surface characteristics and common epitopes. In fact, identification of a single species as the sensitizing agent is probably not meaningful, and most likely cosensitization has occurred in most subjects due to the distribution of the various Pooideae grasses throughout the temperate areas. It is tempting to assume that the constant exposure to a variety of slightly differing allergens (isoallergens and variants from different species) promotes the formation of antibodies recognizing the epitopes *common* to all of these allergens. Based on the assumption that increased exposure leads to clonal selection of high-affinity antibodies dominating the response, such a funnel sensitization mechanism would lead to high levels of multi-cross-reacting antibodies [1].

The clinical implications are that patients experience symptoms in the presence of pollen from any of the Pooideae grasses during the grass pollen season. With regard to diagnosis, the observed cross-reactivity suggests that any one of these grasses may be used for skin prick testing or for the diagnosis by in vitro IgE determination of grass-pollen-allergic subjects living in the temperate regions of the world. Similarly, any one of these species may be used for specific immunotherapy. Studies of T-cell cross-reactivity based on standard T-cell proliferation assays and of the specificity of IgG₄ induced by specific immunotherapy with *P. pratense* pollen extract support this conclusion [36].

A slightly different pattern of reactivity was observed in the IgE binding studies (fig. 4) for *S. cereale* (cultivated rye). There is still clear correlation between the IgE response to *P. pratense* and *S. cereale* pollen extract; however, the slope of the correlation line appears to be less than unity. A lower IgE response to *S. cereale* has been observed previously by van Ree et al. [18] when compar-

ing more abundant species like *P. pratensis* and *D. glomerata*, even upon inclusion of sera from grass pollen-allergic subjects living in the vicinity of rye fields.

For *C. dactylon* (Bermuda grass) and *P. communis*, a biased distribution of the data points is observed (fig. 5), with a tendency for the population in the present study towards a much higher IgE response to *P. pratense* pollen extract. The bias may possibly be explained by the nature of the population, largely representing areas of the world where exposure to Chloridoideae and Arundinoideae grasses is limited. Similar results have been obtained in smaller populations of comparable geographical nature [17, 18], and the presence of specific allergens/epitopes in the Chloridoideae and Arundinoideae subfamilies have been demonstrated previously [4, 6]. The absence of group 5 allergen for the species belonging to these two subfamilies [1, 17] is clearly relevant in this context. However, correlation has been observed between the IgE response to *C. dactylon* and *P. pratensis* in sera from grass pollen-allergic subjects with high exposure to the former – or both species [18]. The latter observation is consistent with the theory of funnel sensitization. Part of the observed correlation may be explained by simple cosensitization but the existence of shared epitopes for allergens of the Pooideae and Chloridoideae subfamilies was confirmed by inhibition experiments. Comparison of cross-reactivity patterns between subjects specifically sensitized towards subtropical grasses, the present population and subjects living in areas with high abundance of both Pooideae and Chloridoideae/Arundinoideae grasses are called for.

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