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Component-resolved microarray analysis of IgE sensitization profiles to *Culicoides* recombinant allergens in horses with insect bite hypersensitivity

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Abstract

Background: Allergy to bites of blood-sucking insects, including biting midges, can affect both human and veterinary patients. Horses are often suffering from an IgE-mediated allergic dermatitis caused by bites of midges (*Culicoides spp*). With the aim to improve allergen immunotherapy (AIT), numerous *Culicoides* allergens have been produced as recombinant (r-) proteins. This study aimed to test a comprehensive panel of differently expressed *Culicoides* r-allergens on a cohort of IBH-affected and control horses using an allergen microarray.

Methods: IgE levels to 27 *Culicoides* r-allergens, including 8 previously unpublished allergens, of which 11 were expressed in more than one expression system, were determined in sera from 347 horses. ROC analyses were carried out, cut-offs selected using a specificity of 95% and seropositivity rates compared between horses affected with insect bite hypersensitivity (IBH) and control horses. The combination of r-allergens giving the best performing test was determined using logistic regression analysis.

Results: Seropositivity was significantly higher in IBH horses compared with controls for 25 r-allergens. Nine *Culicoides* r-allergens were major allergens for IBH with seven of them binding IgE in sera from > 70% of the IBH-affected horses. Combination of these top seven r-allergens could diagnose > 90% of IBH-affected horses with a specificity of > 95%. Correlation between differently expressed r-allergens was usually high (mean = 0.69, range: 0.28-0.91).

Conclusion: This microarray will be a powerful tool for the development of component-resolved, patient-tailored AIT for IBH and could be useful for the study of allergy to biting midges in humans and other species.

Abbreviations: IBH, equine insect bite hypersensitivity; r-allergen/protein, recombinant allergen/protein.

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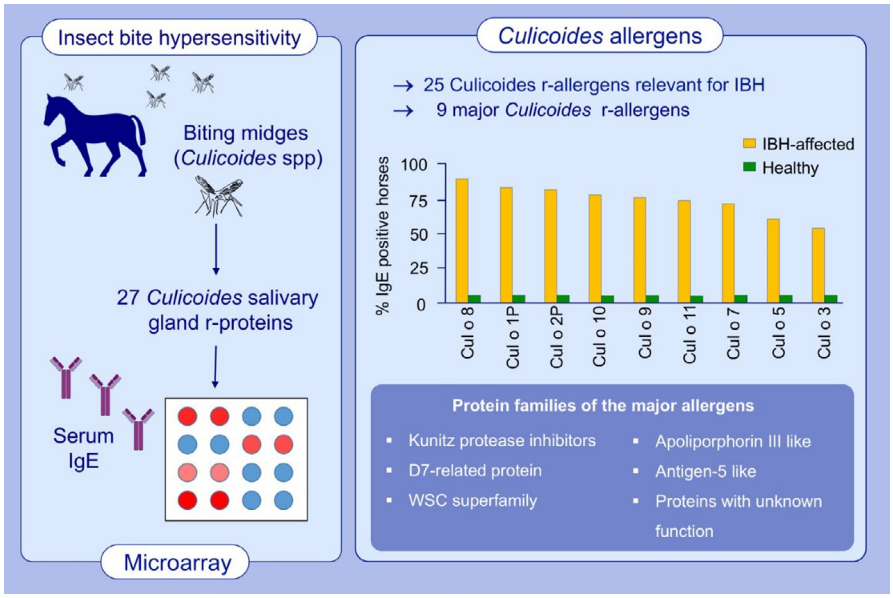
KEYWORDS

culicoides allergens, equine insect bite hypersensitivity, IgE, microarray



GRAPHICAL ABSTRACT

In this study, we identify and produce eight new *Culicoides* r-allergens, Cul o 8 to Cul o 15. Out of 27 tested *Culicoides* r-allergens, nine are major allergens for IBH and seven bind serum IgE in > 70% of the IBH-affected horses. Combination of these seven r-allergens could diagnose > 90% of IBH horses with a specificity > 95%.

1 | INTRODUCTION

Allergy to biting insects, including biting midges, is affecting both human and veterinary patients.[1] Insect bite hypersensitivity (IBH) is a seasonally recurrent, strongly pruritic allergic dermatitis[2-4] of horses caused by biting midges of the genus *Culicoides* and is thus also increasingly named Culicoides hypersensitivity (CH). IBH occurs nearly worldwide with a prevalence of 3% to 60%, depending on breed, family and environment.[5] IBH is not affecting horses in Iceland because of the absence of the causative *Culicoides* spp. However, horses imported as adults from Iceland to *Culicoides*-rich environments, for example, in mainland Europe, have a high prevalence of IBH (>50%), while the prevalence of IBH in horses of the Icelandic breed born in mainland Europe is only 8%-10% as reviewed in.[5] The *Culicoides* (C.) species causing IBH can vary depending on the geographical location: *C obsoletus* (Cul o) is the most common species found around horses in both North America and Europe.[6,7] *C nubeculosus* (Cul n) is found rather rarely in European countries[8] but can be bred under laboratory conditions.[9] IBH is characterized by type I, IgE-mediated reactions (reviewed in Ref. [5,10]) to salivary gland proteins from *Culicoides spp*.[11-13] IgE antibodies binding to the natural allergens[8,14,15] as well as to *Culicoides* recombinant (r-) allergens[16-18] have been demonstrated in sera of IBH-affected horses. Histamine and sulfidoleukotriene release assay with *Culicoides* allergens[19-21] further support type I reactions in IBH, usually followed by a late-phase reaction.[22-24]

Development of specific and sensitive diagnostic tests as well as of an efficacious allergen immunotherapy (AIT) for IBH has been hampered by the lack of pure *Culicoides* allergens. The use of *Culicoides* whole-body extracts (WBEs) usually leads to low specificity and sensitivity of serological tests[25-29] and to low efficacy of AIT.[30,31] While a recent study showed a better performance of IgE serology using a WBE from *Culicoides obsoletus* captured individually from horses in the field,[32] this collection is very laborious and does not allow for standardization of the extract.[33] *C obsoletus* cannot be bred under laboratory conditions.[9] IBH-affected horses also react to *Culicoides* species not present in the environment of the horses indicating the presence of cross-reactivity between allergens of *Culicoides* species.[34] Indeed, some allergens derived from different *Culicoides* species display high similarities (>70%) of the amino acid sequences,[18] while others do not. Consequently, various studies aiming at the characterization of *Culicoides* salivary proteins have been performed[8,35] and have resulted in the production of over 20 *Culicoides* r-allergens, derived from *C. nubeculosus*,[5,17] *C obsoletus*[16,18] and *C sonorensis*.[27] The majority of the *Culicoides* r-allergens have been produced in *E coli*, and few in insect

cells or barley.[18,27,36] A first study using a microarray including a limited panel of ten *C nubeculosus* and two *C obsoletus* r-allergens suggested a highly discriminatory potential for this technique. It showed that horses with IBH have a high probability of being classified separately from those affected with other allergic disease and from healthy control horses, suggesting that this approach is worth being refined for studying the sensitization pattern to *Culicoides* r-allergens in IBH.[37]

Using a large, comprehensive panel of *Culicoides r*-allergens printed on a microarray, the aim of the study was to determine which of these *Culicoides* r-allergens are most important for IBH and should be lead compounds for AIT and be included in diagnostic tests for IBH. A further aim was to compare IgE binding to r-allergens produced in different expression systems.

2 | MATERIALS AND METHODS

2.1 | Horses

Sera from 347 horses, 148 healthy controls and 199 IBH-affected horses were tested for allergen-specific IgE (Table 1). The serum samples came from five different countries and were collected during the IBH seasons in the years 2005 to 2017. All horses with IBH showed the typical, seasonally recurrent clinical signs,[5] while the healthy control horses (H), all living in the same environments as the IBH horses, had no clinical signs or history of skin diseases. Additionally, in the Swiss horses, clinical diagnosis of IBH had been confirmed in an in vitro sulfidoleukotriene release assay with *Culicoides* allergens.[20,38] The horses belonged to different breeds with a predominance of the Icelandic breed (Table 1). All IBH-affected Icelandic horses had been imported from Iceland as well as 89 of the 115 H Icelandic horses. The study was approved by the Animal Experimental Committee of the Canton of Berne, Switzerland (No. BE 121/05 and BE 2/17), and by an ethics committee and the State Office for Consumer Protection and Food Safety in accordance with the German Animal Welfare Law (LAVES—Reference Number: 33.14 42502-04-14/1718).

2.2 | New recombinant Culicoides allergens

Eight previously unpublished proteins from *C obsoletus* were included on the array. Their sequences were derived from cDNA synthesized using wild *C obsoletus* salivary glands prepared as described.[35] The cDNA was analysed by next-generation sequencing at the University of Bristol Genomics Facility, using ion proton sequencing. The data were assembled into full-length mRNA using the de novo assembly software "Trinity" and the University of Bristol Supercomputing Facility, BlueCrystal. They were named and numbered according to the allergen nomenclature (allergen.org), Cul o 8 to Cul o 15 (Table 2). The sequences were codon-optimized for expression in *E coli*, synthesized and cloned into pET100/D-TOPO (GeneArt® Gene Synthesis, Thermo Fisher). Proteins were expressed in *E coli* BL21(DE3) grown either in 100 mL of AIM (auto-induction medium super broth, www.formedium.com) for 18 hours,[39] or overnight in Cinnabar medium (www.teknova.com) with 100 μmol/L IPTG (isopropyl β-D-1-thiogalactopyranoside) added for a further 4 hours of culture before harvest. The culture was centrifuged at 8000 × *g* for 5 minutes at 4°C. To reduce LPS content, the pellet was washed with 100 mmol/L Tris (pH 8.0), followed by 100 mmol/L Tris (pH 8.0), 5 mmol/L $CaCl_2$ and then 100 mmol/L Tris (pH 8.0), and 10 mmol/L EDTA. The wash cycle was repeated with each buffer with two final washes in 100 mmol Tris to remove EDTA before freezing at −20°C.[40] After thawing, the pellet was resuspended in NZY lysis buffer with lysozyme, DNase (www.nzytech.com) and protease inhibitor cocktail (cOmplete Tablets, Roche) and incubated at RT for 1 hour before centrifugation at 16.000 × g for 10 minutes at 4°C. The pelleted inclusion bodies were washed with Tris buffer containing Triton X-114 for LPS removal according to Choi et al[40] and then resuspended in 50 mmol/L Tris (pH 8.0), 500 mmol/L NaCl, 20 mmol/L imidazole, 8 mol/L urea, 5 mmol/L TECP and rocket O/N at RT subsequently centrifuged at 16.000 × *g* and the supernatant harvested. The supernatant was applied to Ni-Sepharose™ 6 Fast Flow (GE Healthcare) for purification of His-tagged proteins according to manufacturer's procedures. After elution, the proteins were refolded in 400 mmol/L Arginine-HCL, 20 mmol/L Tris, pH 8, 500 mmol/L NaCl, 5 mmol/L b-cyclodextrin 100 mmol/L glycerol, 340 mmol/L imidazole, 0.2 mmol/L oxidized glutathione and 2 mmol/L reduced glutathione.

2.3 | Allergens used in the study

The allergens used included a total of 27 *Culicoides* r-allergen: eleven *C nubeculosus* (Cul n,[17,41] eight already published *C obsoletus* r-proteins (Cul o[16,18]) and the eight new *C obsoletus* r-allergens (Table 2). The allergens had been expressed not only in *E coli* as described[17,18,41] but also in barley, insect cells[36] and yeast (*Pichia*

TABLE 1 Origin of the horses affected with insect bite hypersensitivity (IBH) and of the healthy control (H) horses used for the determination of allergen-specific serum IgE on the microarray. A total of 347 horses were included, of which 235 horses belonged to the Icelandic breed. The other 112 horses belonged to various breeds

	Icelandic horses		Other breeds		All breeds		
Country	H	IBH	H	IBH	H	IBH	Total
Switzerland (CH)	67	59	22	25	89	84	173
Germany (DE)	11	5	—	5	11	10	21
Sweden (SE)[26]	37	56	—	—	37	56	93
Ireland (IRL)	—	—	11	21	11	21	32
United Kingdom (UK)	—	—	—	28	—	28	28
Total	115	120	33	79	148	199	347

TABLE 2 *Culicoides nubeculosus* (Cul n) and *Culicoides obsoletus* (Cul o) recombinant allergens produced in *E coli* (coli), baculovirus-infected insect cells (Baculo), yeast (Pichia) or barley. In a part of the analyses, only one expression system was chosen per allergen (in bold). Additionally, Cul n thorax extract (CN-TE), Cul o group whole-body extract (CO-WBE) and *Simulium vittatum* whole-body extract (SV-WBE) were used. *Alternaria alternate* 1 (alt a 1) and *Dermatophagoides farinae* (Der f) were used as control antigens, not relevant for IBH

Name	Expression system	Protein family	GenBank	Reference
Cul n 1	**Baculo**	PR1-like (antigen-5 like)	EU978899	Schaffartzik et al (2010)
Cul n 2	**Baculo**, Coli	Hyaluronidase	HM145950	Schaffartzik et al (2011)
Cul n 3	**Baculo**, Coli, Barley	DUF4803 superfamily[a]	HM145951.1	Schaffartzik et al (2011); Jonsdottir et al (2018)
Cul n 4	Baculo, Coli, **Barley**, Pichia	Unknown	HM145952	Schaffartzik et al (2011); Jonsdottir et al (2018)
Cul n 5	**Coli**	DUF4803 superfamily	HM145953	Schaffartzik et al (2011)
Cul n 6	**Coli**	Unknown	HM145954	Schaffartzik et al (2011)
Cul n 7	**Coli**	Unknown	HM145955	Schaffartzik et al (2011)
Cul n 8	**Baculo**, Coli, Pichia	Maltase (alpha amylase)	HM145956	Schaffartzik et al (2011)
Cul n 9	**Coli**	D7-related/OBP	HM145957	Schaffartzik et al (2011)
Cul n 10	**Coli**	DUF4803 superfamily	HM145958	Schaffartzik et al (2011)
Cul n 11	**Coli**	Serine protease/trypsin	HM145959	Schaffartzik et al (2011)
Cul o 1P	**Coli**	Kunitz protease inhibitor	JX512273	Peeters et al (2013)
Cul o 2	**Baculo**, Pichia	Hyaluronidase	KC339672	Van der Meide et al (2013)
Cul o 2P	Baculo, **Coli**	D7-related/OBP	JX512274	Peeters et al (2013)
Cul o 3	Baculo, **Coli**, Pichia	PR1-like (antigen-5 like)	KC339673	Van der Meide et al (2013)
Cul o 3P	**Coli**	D7-related/OBP	JX512275	Not published
Cul o 5	Baculo, **Coli**	Unknown	KC339675	Van der Meide et al (2013)
Cul o 6	Coli, **Pichia**	D7-related/ OBP	KC339676	Van der Meide et al (2013)
Cul o 7	**Baculo**, Coli, Pichia	Unknown	KC339677	Van der Meide et al (2013)
Cul o 8	**Coli**	Kunitz protease inhibitor	MN123710	New, clone CO145
Cul o 9	**Coli**	WSC superfamily, carbohydrate-binding domain	MN123712	New, clone CO120
Cul o 10	**Coli**	DUF4803 superfamily	MN123711	New, clone CO180
Cul o 11	**Coli**	Apolipophorin III-like	MN123713	New, clone CO167
Cul o 12	**Coli**	Leucine-rich repeat	MN123714	New, clone CO15
Cul o 13	**Coli**	D7-related/OBP	MN123715	New, clone CO13
Cul o 14	**Coli**	Serine protease/trypsin	MN123716	New, clone CO60
Cul o 15	**Coli**, Baculo	Apyrase	MN123717	New, clone CO147
CN-TE	**Extract**			Ziegler et al (2018)
SV-WBE	**Extract**			Torsteinsdottir et al (2018)
CO-WBE	**Extract**			
Alt a 1	**Coli**			Biomay
Der f	**Extract**			Stallergenes Greer

[a]Domain of unknown function.

pastoris, VALIDOGEN GMBH (formerly VTU Technology), Grambach, AT),[42] resulting in a total of 44 *Culicoides* r-proteins included on the array (Table 2 and Table S1).

Additionally, a Cul n thorax extract (CN-TE[43]) and a whole-body extract from Cul o group midges (CO-WBE)[16] as well as a black fly extract (*Simulium vittatum* [SV-WBE][44] were used. As negative control antigens, the recombinant mould allergen *Alternaria alternate* 1 (Alt a 1, Biomay, www.biomay.com) and a house dust mite extract (*Dermatophagoides farinae* [Der f], Stallergenes Greer, www.stallergenesgreer.com) were also assessed (Table 2).

2.4 | Serum IgE testing by protein microarray

IgE serum determination by protein microarray was performed as described.[45] All serum samples had been kept frozen until used and were tested in the same period, within few months.

Briefly, all proteins and extracts were normalized to 0.5 μg/mL protein before being printed onto Grace Bio-Labs Oncyte® Nova™ nitrocellulose film slides using a Marathon microarrayer (Arrayjet, Roslin, Scotland). The slides were blocked with 3% BSA in PBS for 3h with rotation at 37°C. Slides were subsequently washed in 0.05%

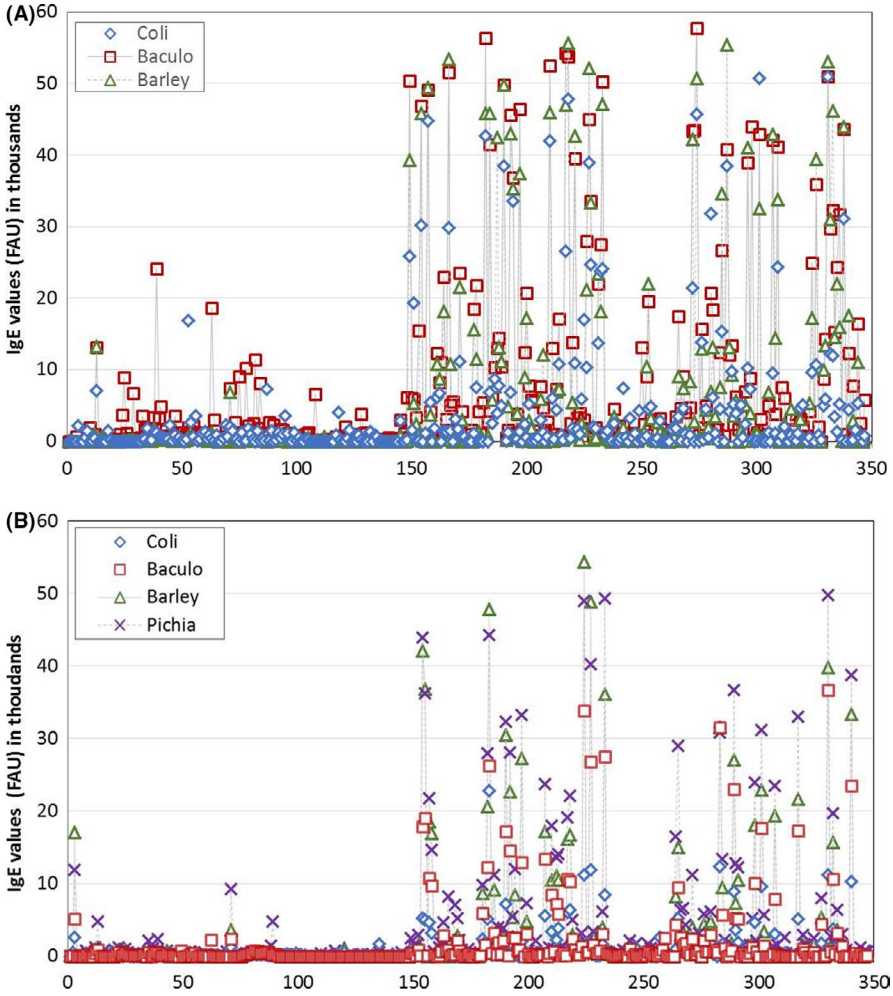


FIGURE 1 Comparison of IgE values in fluorescence arbitrary units (FAU) to recombinant allergens expressed in different expression systems, exemplified for Cul n 3 (A) and Cul n 4 (B). Every peak shows the IgE values of one horse on the allergen expressed in insect cells (Baculo; red square), in barley (green triangle), *E coli* (Coli; blue rhomboid) and *Pichia pastoris* (Pichia; violet cross; only Cul n 4). IgE values of the 148 healthy control horses are represented on the left side and of the 199 IBH-affected horses on the right side of the abscissa

PBS-T followed by washes with Milli-Q water (Millipore) before being dried via centrifugation at 300 × g for 10 minutes. Slides were fitted with ProPlate slide modules (Grace Bio-Labs), and the 16 nitrocellulose wells on the slide were washed three times with 150 μL/well 0.2% PBS-T. The sera diluted 1:2 in 4% BSA-0.4% PBS-T were applied to each well (100 μL/well), except for one used as blank (0.2% PBS-T). These slides were incubated O/N at 4°C at 13 oscillations/min (Stuart SSL4 See-saw Rocker). After washing with 0.05% PBS-T, anti-horse IgE mAb 3H10[46] diluted 1:400 in 0.2% PBS-T and 1% BSA was added (100 μL/well) and incubated at 37°C for 2 hours. After washing with 0.05% PBS-T, DyLight 649-conjugated anti-mouse IgG1 (Rockland, #610-443-040) was applied at dilution 1:400 in 0.2% PBS-T and 1% BSA. After final washes, the slides were dried by centrifugation. The pads were scanned in a GenePix4000B (Molecular Devices, Inc). The mean of the blanks with each allergen was subtracted from the values obtained with the serum samples before further analyses of the data. Data were presented as fluorescence arbitrary unit (FAU).

2.5 | Data analysis

For statistical analysis, NCSS software (NCSS 12 Statistical Software (2018), NCSS, LLC; ncss.com/software/ncss) was used. Since the data were not normally distributed, descriptive statistics using median and ranges and the Mann-Whitney U test to compare IgE values between IBH-affected and H control horses were performed.

In a second step, the capacity of the single allergens to discriminate IBH-affected from H horses was investigated by receiver operator characteristic (ROC) curves, with the accuracy of the test represented by the area under the curve (AUC).

Spearman rank correlation was used to compare IgE levels of r-allergens expressed in different expression systems.

When the same r-allergens were produced in different expression systems, one of them was chosen for further analyses: r-allergens with the best AUC were selected, and when the AUCs were similar, dot plot graphs helped to select the allergen giving the best separation between the IBH-affected and control group (not shown).

ROC curves were used to select the optimal cut-off values. As cut-off, values giving at least a specificity of 94% at the highest accuracy possible were selected. For each allergen, specific IgE values were then transformed in positive and negative (above and below cut-off level) results. The 2-sided Fisher's exact test was used to compare the proportion of IBH-affected and control horses with positive allergen-specific IgE results or to compare the proportion of positive results between Icelandic (ISL) and other breeds within IBH-affected or H horses.

TABLE 3 Spearman's correlations per allergen between the proteins expressed using different expression systems: *E coli* (coli), baculovirus-infected insect cells (Baculo), barley and yeast (Pichia). All correlations were significant ($P < .001$)

Allergen	Correlation Matrix	Baculo	Barley	Pichia
Cul n 2	Coli	0.50	n.a.[a]	n.a.
Cul n 3	Coli	0.63	0.73	n.a.
	Baculo	1.00	0.80	n.a.
Cul n 4	Coli	0.75	0.74	0.71
	Baculo	1.00	0.91	0.86
	Barley	0.91	1.00	0.89
Cul n 8	Coli	0.52	n.a	0.89
	Baculo	1.00	n.a.	0.48
Cul o 2	Baculo	1.00	n.a.	0.28
Cul o 2P	Coli	0.78	n.a.	n.a.
Cul o 3	Coli	0.70	n.a.	0.66
	Baculo	1.00	n.a.	0.76
Cul o 5	Coli	0.75	n.a.	n.a.
Cul o 6	Coli	n.a.	n.a.	0.38
Cul o 7	Coli	0.88	n.a.	0.76
	Baculo	1.00	n.a.	0.74
Cul o 15	Coli	0.32	n.a.	n.a.

[a]Not applicable.

To determine which combination of r-allergens would result in the best sensitivity and specificity, multivariable logistic regression analyses were performed with IBH status as the outcome and seropositivity to the different allergens as potential predictors. The model was built in a stepwise forward selection process, with the r-allergen with the best AUC entering the model first, followed by the allergen with the next best value. Inclusion of allergens was stopped after adding new allergens did not result in an improvement of sensitivity and specificity.

3 | RESULTS

3.1 | Comparison and selection of allergens produced in different expression systems

A total of 27 different *Culicoides* r-allergens, some of which were expressed in different expression systems, resulting in a total of 44 r-allergens, were analysed in this study. Median serum IgE values of IBH-affected and control horses and AUCs for each of these allergens are shown in Table S1. The horses usually displayed similar IgE reactivity patterns to the same r-allergen produced in the different expression systems (shown for Cul n 3 and Cul n 4 in Figure 1A,B), although insect cell–expressed Cul n 3 bound serum IgE from H horses more frequently than Cul n 3 expressed in the other systems. IgE levels to the same allergen expressed in various systems usually correlated rather well (R: 0.63-0.91, $P < .001$), with few exceptions, as shown in Table 3.

3.2 | Allergen-specific IgE levels in IBH-affected and control horses

Comparison of allergen-specific IgE levels between IBH-affected and H control horses was first performed country-wise by ROC curves and Mann-Whitney U test. The AUCs were statistically significant for all 27 recombinant *Culicoides* allergens in the horses from Switzerland (CH) and almost all r-allergens in the horses from Sweden (SE: 25/27 r-allergens), Ireland (IRL: 24/ 27 r-allergens) and Germany (DE: 26/ 27 r-allergens) (Table S2). Interestingly, the horses from Germany displayed higher AUC for most *Culicoides* r-allergens and in particular for the Cul n allergens compared with the horses from the other countries (Table S2).

The IgE-binding capacity of the allergens printed on the microarray varied greatly between allergens (Table 4), with allergens showing low values such as Cul n 2 or Cul n 5, while some other allergens gave a very strong signal with sera from IBH-affected horses, such as Cul o 1P and Cul o 8. For some of these allergens, IgE binding in the control sera was also higher. Irrespective of these differences between allergens, significant differences for median IgE levels between IBH-affected and control horses were found for all tested *Culicoides* allergens, except Cul n 7. Further analysis using ROC curves revealed that in our study population, some allergens had a very good performance to discriminate IBH-affected from H control horses with AUC > 0.90. This was the case for the six Cul o allergens, Cul o 1P, Cul o 2P, Cul o 8, Cul o 9, Cul o 11 and Cul o 10. AUC values between 0.80 and 0.90 were obtained for five *Culicoides* r-allergens (Cul o 3, Cul o 5, Cul o 7, Cul o 12, Cul o 13) as well as for the two *Culicoides* extracts.

3.3 | Analysis of the data after cut-off definition

For further analysis, the IgE results were grouped in positive or negative test results based on the cut-off value chosen at a specificity ≥ 94% (Table 4). Cut-offs selected based on Youden's index, that is, when the sum of the sensitivity and specificity is maximized, are shown in Table S3. For the three Cul o r-allergens, Cul o 1P, Cul o 8 and Cul o 10, the same cut-off values as when using a specificity > 94% were selected based on Youden's index. For the other r-allergens, the cut-off values were usually lower, resulting in an increased sensitivity with some loss of specificity. Comparison of the number of positive IgE reactions between IBH-affected and H control horses in Fisher's exact test revealed significant differences for all *Culicoides* r-allergens, except for Cul n 7 and Cul n 8. Figure 2 shows that the allergens Cul o 8, Cul o 1P and Cul o 2P bound serum IgE in > 80% of the IBH-affected horses followed by Cul o 10, Cul o 9, Cul o 11 and Cul o 7 with > 70% positive reactions. The allergens Cul o 5 and Cul o 3 bound IgE in > 50% of the allergic horse sera. The remaining r-allergens gave less frequently positive reactions in the

TABLE 4 Overview of allergen-specific IgE determined with a protein microarray in sera from 199 horses affected with insect bite hypersensitivity (IBH) and 148 healthy control (H) horses. Median serum IgE values (in fluorescence arbitrary unit) from H and IBH-affected horses and *P* values from the Mann-Whitney U test. Results from ROC analyses are as well-listed: pairwise accuracy (AUC) with lower and upper confidence limits, cut-off values selected to obtain a specificity ≥ 95% (CO-WBE 94%) and the resulting sensitivities (sens.)

	H	IBH	*P*		95% confidence limits			
Allergens	Median	Median	H vs IBH	AUC	Lower	Upper	Cut-off	Sens.
Cul n 1	44	859	<.001	0.74	0.69	0.79	2500	0.30
Cul n 2	0	106	<.001	0.71	0.66	0.76	230	0.37
Cul n 3	605	4038	<.001	0.79	0.74	0.84	7500	0.38
Cul n 4	0	588	<.001	0.79	0.74	0.83	820	0.42
Cul n 5	1	82	<.001	0.77	0.72	0.81	108	0.43
Cul n 6	0	280	<.001	0.70	0.64	0.74	800	0.28
Cul n 7	131	201	n.s.[a]	0.54	0.48	0.60	2200	0.07
Cul n 8	678	1089	<.001	0.62	0.56	0.68	3950	0.12
Cul n 9	393	1141	<.001	0.74	0.68	0.78	2400	0.31
Cul n 10	307	858	<.001	0.67	0.61	0.72	3500	0.20
Cul n 11	0	239	<.001	0.64	0.58	0.69	1500	0.26
Cul o 1P	**603**	**43 478**	**<.001**	**0.94**	**0.90**	**0.96**	**7300**	**0.83**
Cul o 2	7	77	<.001	0.73	0.67	0.77	150	0.34
Cul o 2P	**5**	**1549**	**<.001**	**0.94**	**0.91**	**0.96**	**300**	**0.82**
Cul o 3	193	1259	<.001	0.88	0.83	0.91	1070	0.54
Cul o 3P	0	522	<.001	0.75	0.70	0.80	870	0.37
Cul o 5	1249	7766	<.001	0.85	0.80	0.88	6000	0.60
Cul o 6	17	164	<.001	0.79	0.74	0.83	238	0.41
Cul o 7	**149**	**2854**	**<.001**	**0.88**	**0.84**	**0.91**	**900**	**0.71**
Cul o 8	**947**	**44 986**	**<.001**	**0.95**	**0.92**	**0.97**	**16 000**	**0.89**
Cul o 9	**37**	**19 070**	**<.001**	**0.92**	**0.89**	**0.95**	**1800**	**0.76**
Cul o 10	**0**	**6502**	**<.001**	**0.91**	**0.88**	**0.94**	**700**	**0.77**
Cul o 11	**3658**	**32 043**	**<.001**	**0.91**	**0.88**	**0.94**	**11 000**	**0.74**
Cul o 12	0	480	<.001	0.85	0.80	0.88	488	0.49
Cul o 13	804	3981	<.001	0.84	0.79	0.88	5600	0.42
Cul o 14	0	68	<.001	0.71	0.65	0.76	160	0.36
Cul o 15	53	171	<.001	0.81	0.77	0.85	1100	0.14
CN-TE	216	940	<.001	0.81	0.76	0.85	1150	0.42
CO-WBE	261	1503	<.001	0.84	0.79	0.87	1550	0.48
SV-WBE	254	598	<.001	0.71	0.65	0.76	1800	0.12
Alt a 1	20	18	n.s	0.50	0.44	0.55	300	0.07
Der f	518	519	n.s.	0.52	0.45	0.58	3700	0.09

Note: Bold indicates the seven top *Culicoides* r-alleysis.

[a]Not significant ($P > .05$).

IBH-affected group, with values ranging between 42% (Cul o 13) and 14% (Cul o 15). With the cut-offs selected to achieve a high specificity (>94%), the percentage of IBH-affected horses with positive IgE levels to the *Culicoides* extracts was below 50% (CO-WBE 48%, CN-TH 42%) and was negligible with SV-WBE (12%). In Figure S1, the data of IBH-affected horses are shown for each country. While there are differences in the percentages of positive IgE reactions to the different allergens between the countries, this figure also confirms that the seven top allergens from Figure 2 are major allergens for IBH in all countries (except for Cul o 7 in England and Ireland).

3.4 | IgE reactions to the r-Culicoides allergens in Icelandic horses compared with other breeds

Comparison of the IgE results in the sera from the IBH-affected horses belonging to the ISL breed to other breeds shows that ISL horses have significantly more often positive reactions than the horses from other breeds. This was performed for the horses living in Switzerland only, as there was often an overlap between breed and country (Table 1). The difference was significant for 14 r-allergens ($P \leq .05$). Nevertheless, the IgE results were very similar between ISL

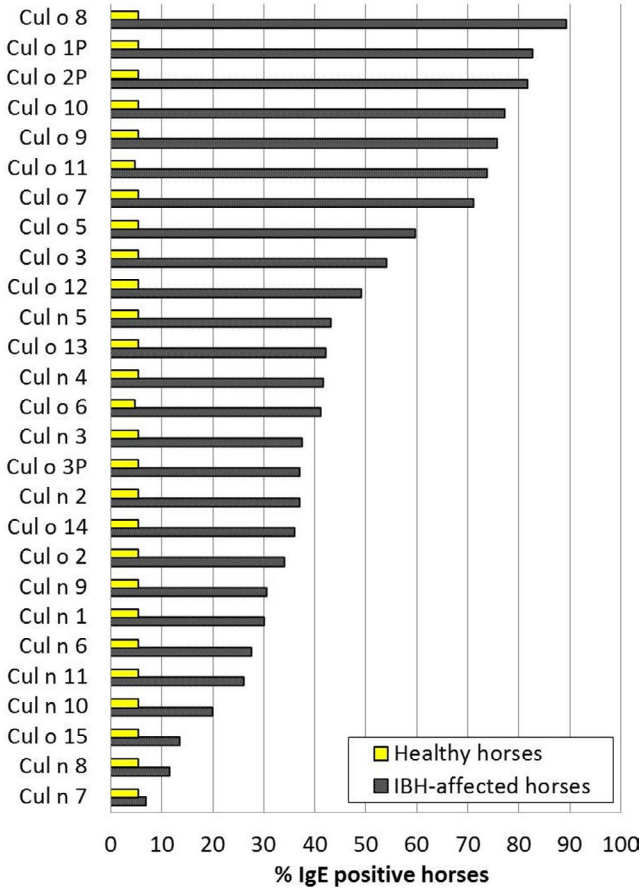


FIGURE 2 IgE levels to 27 recombinant *Culicoides* allergens. Percentage of horses with IgE levels above the cut-off values (as indicated in Table 4) in 199 IBH-affected and 148 healthy horses. The allergens are listed in decreasing order from those binding serum IgE in the highest number of IBH-affected horses. P values were calculated with Fisher's exact tests. The differences between healthy and IBH-affected horses were all significant ($P < .05$) except for Cul n 7 and Cul n 8

and other breeds for the top five allergens Cul o 8, Cul o 1P, Cul o 2P, Cul o 10 and Cul o 9 (Figure 3). Interestingly, IBH-affected ISL horses living in Switzerland or in Sweden, that is in different environments, showed very similar sensitization patterns (Figure S2). There were no significant differences between the breeds in the H control horses.

3.5 | Selection of the best combination of allergens for diagnosis of IBH

In the multivariate logistic regression model, combination of the three best performing r-allergens, Cul o 8, Cul o 1P and Cul o 2P, resulted in a sensitivity of 90% with a specificity of 94%. Addition of the next four top allergens Cul o 10, Cul o 9, Cul o 11 and Cul o 7 further increased the performance of the assay, resulting in a sensitivity of 90% and specificity of 96% (AUC = 0.957, 95% confidence interval: 0.899 to 0.982). Country-wise analysis was performed for CH, SE, IRL and DE and showed that using this combination of seven allergens also resulted in high sensitivities (92% to 100%) and specificities (97% to 100%) within these countries.

4 | DISCUSSION

Our aim was to test a most complete panel of all *Culicoides* r-allergens available using a newly developed microarray for IgE serology.[37,47] A total of 27 different Cul r-allergens of which eight were new allergens were thus analysed in this study. A first country-wise analysis of the results using AUCs indicated that nearly all tested r-allergens were relevant for IBH, independently of the country of origin of the horses, while AUCs for the control allergens were nonsignificant. Horses from Germany reacted more often with Cul n r-allergens than horses living in other countries. This might be due to a higher presence of *C nubeculosus* in this geographical area. However, as this group only consisted of 10 IBH-affected horses, larger numbers of horses will need to be tested in the future to define the sensitization pattern in horses from Germany. Ideally, *Culicoides* species should also be determined in the environments where the horses have been sampled. Horses from all countries were then pooled for an overall analysis. After ROC analysis and cut-off determination, seven r-allergens of *Culicoides obsoletus* group midges (Cul o 8, Cul o 1P, Cul o 2P, Cul o 10, Cul o 9, Cul o 11 and Cul o 7) were identified as the most relevant allergens for IBH, as between 74% and 89% of the IBH-affected horses had positive IgE values against these r-allergens. The specificity was high, as only 5% of the H horses had IgE above the cut-off values. Supporting earlier findings by van der Meide et al,[18] the best performing r-allergens were all derived from *Culicoides obsoletus* and not *Culicoides nubeculosus*, showing the importance to test horses against the native and most frequent *Culicoides* spp. from their environment. *Culicoides obsoletus* group midges are the most commonly found species in Switzerland,[48] Ireland,[49] England,[8] Germany[50] and Sweden.[51] Consideration of the results from each country separately shows that the *Culicoides* r-allergens Cul o 8, Cul o 1P, Cul o 2P, Cul o 10, Cul o 9 and Cul o 11 are major allergens for all tested horses independently of their origin or breed. Cul o 1P and Cul o 2P already showed good results for differentiation between IBH-affected and H horses in Belgian Warmblood horses,[16] but in that study no further r-Cul o allergens had been tested. Interestingly, the Irish group seems to be more often sensitized to Cul o3P, Cul o2P and Cul n 9 all belong to the D7 protein family, than the other horse groups. Comparison of the IgE results in IBH-affected horses from the ISL breed to those from other breeds shows that both groups are sensitized to a similar extend to the top r-allergens Cul o 8, Cul o 1P, Cul o 2P, Cul o 9 and Cul o 10. However, horses of the ISL breed have signficantly more often positive IgE results to other r-allergens than non-Icelandic horses. This suggests that imported Icelandic horses developing IBH in Europe are sensitized to more *Culicoides* r-allergens than horses born on the European continent. In particular, Cul o 7, Cul o 5 and Cul o 3 seem to be very important allergens in ISL horses. Although we tested a rather large number of horse sera, a limitation of our study is that breed and country are

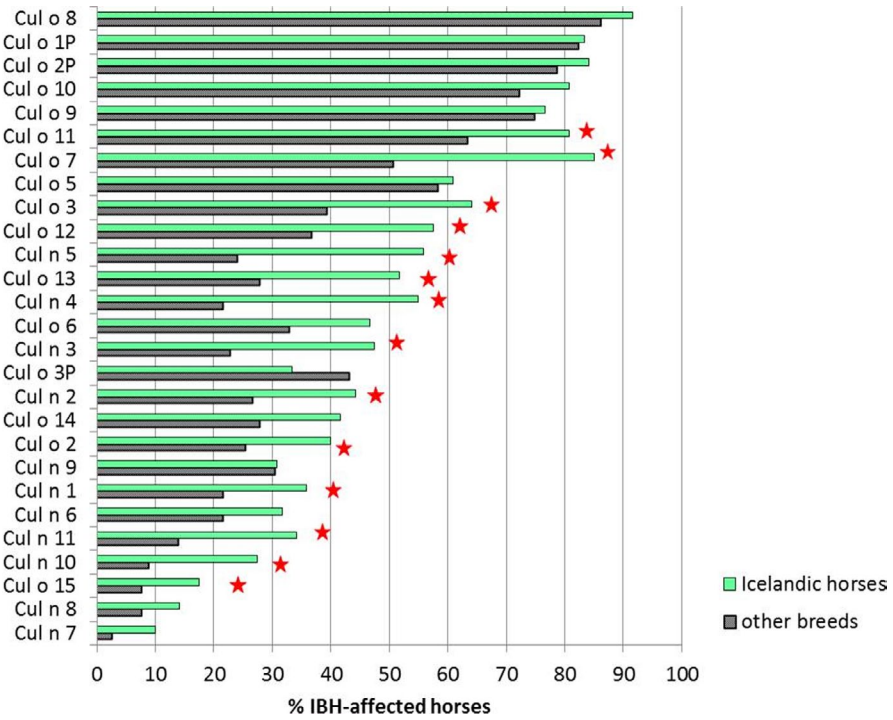


FIGURE 3 Comparison of the IgE levels (shown as % horses with IgE levels above cut off values) to the 27 recombinant *Culicoides* allergens between IBH-affected horses from the Icelandic breed (n = 120) versus horses belonging to other breeds (n = 79) within the horse group from Switzerland. Significant differences between groups in Fisher's exact test are shown with a star

often confounding factors, and the small groups from Ireland, United Kingdom and Germany may not be representative when analysed separately. Further studies with much larger numbers of samples are required to be able to investigate the effects of environment and breed on the sensitization pattern to *Culicoides* r-allergens.

Our study confirmed that a combination of r-allergens differentiates better between IBH-affected and nonaffected horses than the use of single r-allergens or WBE.[16,52,53] Only 45% and 42% of the IBH-affected horses were positive on CO-WBE and CN-TH, respectively, when a cut-off giving a high specificity was used. Combination of the top seven r-allergens in a logistic regression analysis could detect more than 90% of IBH-affected horses both overall and within each country, with a high specificity of > 95%. Combination of these 7 *Culicoides* r-allergens may thus be a powerful tool for diagnostic purposes and should replace the use of crude WBE. However, as a rather large part of the horses are sensitized to additional *Culicoides* salivary allergens, combination of these major allergens might not be sufficient for an effective AIT. This microarray will enable a component-resolved diagnosis as the basis for a patient-tailored AIT.

Most of our original panel of 44 r-allergens were expressed in *E coli*, followed by insect cells, yeast and barley. IgE binding to the same r-allergens expressed in different systems usually correlated well. Proteins can usually be expressed cheaply and in large quantities in *E coli*, but are often insoluble in inclusion bodies and have to be refolded, and lack critical posttranslational modification. Other systems such as yeast glycosylate or even overglycosylate proteins can also lead to false-positive results.[54] No clear trend towards one expression system could be seen when selecting the best performing r-allergen: From the 11 r-allergens that had been expressed in more than one expression system, five insect cell–expressed, four *E coli*–expressed and one each of yeast- and barley-expressed r-allergen were chosen. Van der Meide et al[18] compared the immunoreactivity between Cul o 1 expressed in *E coli* and insect cells and could not show an improved performance of the insect cell–expressed protein in IgE serology. Another study compared insect cell-, barley- and *E coli*–expressed allergens and showed that they performed quite similarly in serology, while *E coli* produced allergens were not suited for cellular assays.[36] However, as purified natural *Culicoides* allergens are not available, we cannot assess the IgE-binding capability of the r-*Culicoides* salivary proteins in comparison with the natural allergens. A limitation of our study is thus that some of the *Culicoides* r-proteins may be misfolded, resulting in a decrease in their IgE-binding capacity and thus to an under-estimation of the number of major *Culicoides* allergens for IBH. Methods to determine the biological activity of *Culicoides* salivary proteins need to be established in order to investigate whether and in which expression system correctly refolded *Culicoides* r-allergens can be produced.

In conclusion, this study shows that seven *Culicoides obsoletus* r-allergens, of which four are newly expressed allergens, are very important allergens for IBH as they bind serum IgE in > 70% of IBH-affected horses of various breeds and origins within central and northern Europe. Combination of these r-allergens results in a highly specific and sensitive serological test for in vitro diagnosis of IBH. In addition, this microarray with *Culicoides* r-allergens could be a powerful tool for the development of component-resolved, patient-tailored allergen-specific immunotherapy for IBH. Finally, this technique could also be useful to investigate sensitization profiles in human patients allergic to biting midges.

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CONFLICT OF INTEREST

All authors have nothing to disclose.

AUTHOR'S CONTRIBUTIONS

EM, MA and ST designed the study. SJ, SBS, ST, EM, DW, ET and CR produced the r-allergens. DR and HR provided the *Pichia*-expressed r-allergens, the serum samples and meta-data of the German horses. EM, RF and PT performed the clinical examinations of the horses and took the blood samples. EN, SW and MA performed the IgE determinations on the chip array. EN, EM and GSR carried out the statistical analyses. EN and EM wrote the manuscript. All authors have read, edited and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Table S1: IgE levels to *Culicoides nubeculosus* (Cul n) and *Culicoides obsoletus* (Cul o) recombinant allergens as well as to Cul n thorax extract (TE) Cul o whole body extract (WBE) and *Simulium vittatum* WBE. Two allergens irrelevant for IBH: Alt a1 and Der f extract were included as negative controls. Some of the allergens were expressed in various expression system, including *E.coli* (coli), insect cells (baculo), barley and yeast (pichia).Median, minimum and maximum allergen-specific IgE values in sera from healthy (n= 148) and insect bite hypersensitivity (IBH)-affected horses (n=199), with P-values from the Mann Whitney U test, and results of the ROC analyses, with area under the curve (AUC), standard error (SE) Z-value, P-value and 95% confidence limits. The allergens used in the final analyses (Table 4, Figure 2 & 3) are displayed in **bold**.

Allergen	Expression	U-test	Healthy horses			IBH-affected horses					Z-Value	2-Sided	95% Confidence Limits	
Name	System	H vs IBH	Median	Min	Max	Median	Min	Max	AUC	SE	AUC ≠ 0.5	P-Value	Lower	Upper
Cul n 1	**Baculo**	**<0.00001**	**44**	**0**	**17725**	**859**	**0**	**52049**	**0.742**	**0.026**	**9.393**	**<0.00001**	**0.687**	**0.788**
Cul n 2	**Baculo**	**<0.00001**	**0**	**0**	**589**	**106**	**0**	**4792**	**0.711**	**0.026**	**8.067**	**<0.00001**	**0.656**	**0.758**
Cul n 2	Coli	<0.00001	96	0	1740	228	0	5516	0.671	0.029	5.846	<0.00001	0.61	0.724
Cul n 3	**Baculo**	**<0.00001**	**605**	**0**	**24145**	**4038**	**0**	**57789**	**0.793**	**0.024**	**12.403**	**<0.00001**	**0.742**	**0.835**
Cul n 3	Barley	<0.00001	54	0	13263	1849	0	55649	0.812	0.023	13.838	<0.00001	0.763	0.852
Cul n 3	Coli	<0.00001	71	0	16926	1347	0	50963	0.726	0.026	8.614	<0.00001	0.67	0.773
Cul n 4	Baculo	<0.00001	0	0	5164	350	0	36738	0.771	0.024	11.267	<0.00001	0.72	0.814
Cul n 4	**Barley**	**<0.00001**	**0**	**0**	**17035**	**588**	**0**	**54464**	**0.787**	**0.024**	**12.154**	**<0.00001**	**0.736**	**0.829**
Cul n 4	Coli	<0.00001	35	0	2645	193	0	22808	0.682	0.028	6.6	<0.00001	0.624	0.732
Cul n 4	Pichia	<0.00001	140	0	11872	1174	0	49761	0.823	0.022	14.638	<0.00001	0.775	0.862
Cul n 5	**Coli**	**<0.00001**	**1**	**0**	**333**	**82**	**0**	**11620**	**0.769**	**0.024**	**11.05**	**<0.00001**	**0.717**	**0.813**
Cul n 6	**Coli**	**<0.00001**	**0**	**0**	**2174**	**280**	**0**	**45799**	**0.696**	**0.026**	**7.473**	**<0.00001**	**0.641**	**0.744**
Cul n 7	**Coli**	**ns**	**131**	**0**	**5888**	**201**	**0**	**23991**	**0.543**	**0.03**	**1.42**	**0.1555**	**0.481**	**0.6**
Cul n 8	**Baculo**	**< 0.001**	**678**	**0**	**8280**	**1089**	**0**	**24109**	**0.62**	**0.03**	**3.958**	**0.0001**	**0.557**	**0.676**
Cul n 8	Coli	< 0.001	1240	0	50011	1913	0	44443	0.626	0.03	4.204	<0.00001	0.564	0.681
Cul n 8	Pichia	< 0.001	698	0	48118	1312	0	39108	0.624	0.03	4.15	<0.00001	0.562	0.68
Cul n 9	**Coli**	**<0.00001**	**393**	**0**	**13932**	**1141**	**0**	**51165**	**0.735**	**0.027**	**8.844**	**<0.00001**	**0.678**	**0.783**
Cul n 10	**Coli**	**<0.00001**	**307**	**0**	**26208**	**858**	**0**	**51317**	**0.668**	**0.029**	**5.791**	**<0.00001**	**0.607**	**0.721**
Cul n 11	**Coli**	**< 0.0001**	**0**	**0**	**9229**	**239**	**0**	**45249**	**0.637**	**0.028**	**4.869**	**<0.00001**	**0.579**	**0.689**
Cul o 1P	**Coli**	**<0.00001**	**603**	**0**	**49359**	**43478**	**0**	**61226**	**0.935**	**0.014**	**32.163**	**<0.00001**	**0.903**	**0.957**
Cul o 2	**Baculo**	**<0.00001**	**7**	**0**	**458**	**77**	**0**	**13958**	**0.727**	**0.026**	**8.677**	**<0.00001**	**0.671**	**0.774**
Cul o 2	Pichia	< 0.001	668	0	5457	1060	0	32392	0.62	0.031	3.917	0.0001	0.556	0.677
Cul o 2P	Baculo	<0.00001	0	0	4033	519	0	61869	0.807	0.022	13.654	<0.00001	0.758	0.847

Cul o 2P	**Coli**	**<0.00001**	**5**	**0**	**2943**	**1549**	**0**	**53230**	**0.941**	**0.013**	**35.078**	**<0.00001**	**0.911**	**0.961**
Cul o 3	Baculo	<0.00001	556	0	26506	4817	18	55981	0.823	0.022	14.885	<0.00001	0.776	0.861
Cul o 3	**Coli**	**<0.00001**	**193**	**0**	**6718**	**1259**	**33**	**42304**	**0.875**	**0.018**	**20.457**	**<0.00001**	**0.834**	**0.907**
Cul o 3	Pichia	<0.00001	217	0	12797	1526	0	56530	0.812	0.022	13.92	<0.00001	0.763	0.852
Cul o 3P	**Coli**	**<0.00001**	**0**	**0**	**3523**	**522**	**0**	**51070**	**0.751**	**0.025**	**9.977**	**<0.00001**	**0.697**	**0.796**
Cul o 5	Baculo	<0.00001	729	0	21371	3269	0	47946	0.764	0.025	10.492	<0.00001	0.710	0.809
Cul o 5	**Coli**	**<0.00001**	**1249**	**0**	**22916**	**7766**	**0**	**57137**	**0.846**	**0.020**	**17.102**	**<0.00001**	**0.802**	**0.881**
Cul o 6	Coli	<0.00001	45	0	757	141	0	44984	0.711	0.027	7.738	<0.00001	0.653	0.760
Cul o 6	**Pichia**	**<0.00001**	**17**	**0**	**465**	**164**	**0**	**14442**	**0.789**	**0.024**	**12.112**	**<0.00001**	**0.737**	**0.831**
Cul o 7	**Baculo**	**<0.00001**	**149**	**0**	**3422**	**2854**	**0**	**60345**	**0.883**	**0.018**	**21.860**	**<0.00001**	**0.844**	**0.913**
Cul o 7	Coli	<0.00001	0	0	2592	2755	0	58921	0.898	0.017	23.866	<0.00001	0.860	0.927
Cul o 7	Pichia	<0.00001	34	0	2726	398	0	29689	0.814	0.023	13.819	<0.00001	0.765	0.854
Cul o 12	**Coli**	**<0.00001**	**0**	**0**	**3085**	**480**	**0**	**31436**	**0.846**	**0.021**	**16.835**	**<0.00001**	**0.801**	**0.882**
Cul o 9	**Coli**	**<0.00001**	**37**	**0**	**52460**	**19070**	**0**	**63028**	**0.921**	**0.015**	**28.569**	**<0.00001**	**0.887**	**0.946**
Cul o 8	**Coli**	**<0.00001**	**947**	**0**	**55489**	**44986**	**0**	**62609**	**0.949**	**0.013**	**33.463**	**<0.00001**	**0.915**	**0.969**
Cul o 10	**Coli**	**<0.00001**	**0**	**0**	**4056**	**6502**	**0**	**55173**	**0.91**	**0.015**	**26.787**	**<0.00001**	**0.875**	**0.936**
Cul o 15	**Coli**	**<0.00001**	**53**	**0**	**7776**	**171**	**0**	**20920**	**0.814**	**0.023**	**13.819**	**<0.00001**	**0.765**	**0.854**
Cul o 15	Pichia	< 0.001	734	0	45713	1196	0	50661	0.614	0.031	3.723	0.0002	0.550	0.670
Cul o 13	**Coli**	**<0.00001**	**804**	**0**	**22479**	**3981**	**31**	**53396**	**0.84**	**0.021**	**16.326**	**<0.00001**	**0.794**	**0.877**
Cul o 14	**Coli**	**<0.00001**	**0**	**0**	**1300**	**68**	**0**	**49812**	**0.709**	**0.026**	**8.051**	**<0.00001**	**0.654**	**0.756**
Cul o 11	**Coli**	**<0.00001**	**3658**	**0**	**34063**	**32043**	**113**	**63062**	**0.912**	**0.015**	**27.650**	**<0.00001**	**0.877**	**0.936**
CN-TE	**extract**	**<0.00001**	**216**	**0**	**5823**	**940**	**0**	**42325**	**0.808**	**0.023**	**13.262**	**<0.00001**	**0.757**	**0.849**
SV-WBE	**extract**	**<0.00001**	**254**	**0**	**7708**	**598**	**0**	**28460**	**0.707**	**0.029**	**7.233**	**<0.00001**	**0.646**	**0.758**
CO-WBE	**extract**	**<0.00001**	**261**	**0**	**8299**	**1503**	**0**	**36168**	**0.835**	**0.021**	**15.690**	**<0.00001**	**0.788**	**0.872**
Alt a 1	**Coli**	**ns**	**20**	**0**	**4353**	**18**	**0**	**4096**	**0.496**	**0.030**	**-0.118**	**0.9063**	**0.435**	**0.553**
Der f	**extract**	**ns**	**518**	**0**	**15170**	**519**	**0**	**13834**	**0.516**	**0.032**	**0.514**	**0.6072**	**0.451**	**0.576**

Table S2: Area under curve (pairwise accuracy; AUC) values for 27 recombinant *Culicoides nubeculosus* (Cul n) and *Culicoides obsoletus* (Cul o) allergens, as well as for Cul n thorax extract (CN-TE) and Cul o (CO-WBE) and *Simulium vittatum* (SV-WBE) whole body extracts in healthy (H) control horses and horses affected with insect bite hypersensitivity (IBH) from the different countries. Alt a 1 and Der f were used as control antigens, not relevant for IBH. Since the horses from UK were all IBH-affected, they cannot be included in this table. Values in bold show allergens with significant differences between IBH-affected and H horses within each country. *p<0.05; **p<0.01; ***p<0.001, CL= confidence limits

	Switzerland (n=173)			Sweden (=93)			Ireland (n=32)			Germany (n=21)		
Allergen	AUC	95% CL		AUC	95% CL		AUC	95% CL		AUC	95% CL	
		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper
Cul n 1	**0.70*** **	0.62	0.77	**0.76*** **	0.65	0.84	**0.76** **	0.54	0.88	**0.90*** **	0.66	0.97
Cul n 2	**0.69*** **	0.61	0.76	**0.77*** **	0.66	0.85	**0.76*** **	0.63	0.85	**0.95*** **	0.68	0.99
Cul n 3	**0.80*** **	0.72	0.86	**0.80*** **	0.70	0.88	**0.85*** **	0.61	0.95	**0.97*** **	0.81	1
Cul n 4	**0.79*** **	0.72	0.85	**0.89*** **	0.80	0.94	0.66	0.38	0.82	**0.85** **	0.55	0.95
Cul n 5	**0.80*** **	0.72	0.85	**0.80*** **	0.69	0.87	**0.72* **	0.48	0.86	**0.91*** **	0.63	0.98
Cul n 6	**0.67*** **	0.59	0.73	**0.75*** **	0.63	0.83	**0.68* **	0.46	0.83	**0.88*** **	0.62	0.97
Cul n 7	**0.59* **	0.5	0.67	0.51	0.38	0.62	0.49	0.28	0.66	0.69	0.38	0.86
Cul n 8	**0.65** **	0.57	0.73	0.54	0.41	0.65	**0.79** **	0.5	0.92	**0.89*** **	0.62	0.97
Cul n 9	**0.69*** **	0.6	0.76	**0.73*** **	0.61	0.82	**0.91*** **	0.65	0.98	**0.82** **	0.52	0.94
Cul n 10	**0.74*** **	0.66	0.81	**0.63* **	0.50	0.73	0.45	0.14	0.68	**0.91*** **	0.53	0.99
Cul n 11	**0.61** **	0.52	0.68	**0.66** **	0.54	0.76	**0.69* **	0.48	0.82	**0.89*** **	0.62	0.97
Cul o 1P	**0.94*** **	0.9	0.97	**0.97*** **	0.90	0.99	**0.92*** **	0.5	0.99	**0.96*** **	0.72	1.00
Cul o 2	**0.70*** **	0.62	0.77	**0.79*** **	0.69	0.87	**0.69* **	0.42	0.85	**0.86*** **	0.57	0.96
Cul o 2P	**0.93*** **	0.88	0.96	**0.98*** **	0.94	0.99	**0.92*** **	0.63	0.99	**0.99*** **	0.86	1.00
Cul o 3	**0.87*** **	0.81	0.91	**0.89*** **	0.81	0.94	**0.89*** **	0.7	0.96	**1.00*** **		
Cul o 3P	**0.69*** **	0.61	0.76	**0.76*** **	0.64	0.84	**0.88*** **	0.69	0.96	**0.90*** **	0.64	0.97
Cul o 5	**0.84*** **	0.77	0.89	**0.90*** **	0.81	0.94	**0.88*** **	0.69	0.96	**0.86*** **	0.58	0.96
Cul o 6	**0.77*** **	0.69	0.83	**0.80*** **	0.68	0.87	**0.90*** **	0.72	0.97	**0.94*** **	0.71	0.99
Cul o 7	**0.92*** **	0.87	0.95	**0.94*** **	0.87	0.97	**0.87*** **	0.64	0.95	**0.95*** **	0.67	0.99
Cul o 8	**0.97*** **	0.93	0.99	**0.98*** **	0.94	1.00	**0.94*** **	0.61	0.99	**1.00*** **		
Cul o 9	**0.93*** **	0.88	0.96	**0.96*** **	0.91	0.99	**0.92*** **	0.56	0.99	**0.94*** **	0.73	0.99
Cul o 10	**0.90*** **	0.84	0.94	**0.95*** **	0.87	0.98	**0.85*** **	0.58	0.95	**0.95*** **	0.65	0.99
Cul o 11	**0.88*** **	0.81	0.92	**0.96*** **	0.91	0.98	**0.98*** **	0.84	1.00	**0.97*** **	0.77	1.00
Cul o 12	**0.81*** **	0.74	0.87	**0.92*** **	0.84	0.96	**0.71* **	0.46	0.86	**0.94*** **	0.67	0.99
Cul o 13	**0.86*** **	0.79	0.9	**0.87*** **	0.78	0.93	**0.83** **	0.53	0.95	**0.94*** **	0.73	0.99
Cul o 14	**0.60** **	0.51	0.67	**0.80*** **	0.70	0.88	**0.87*** **	0.71	0.95	**0.85*** **	0.58	0.95
Cul o 15	**0.66** **	0.57	0.73	**0.74*** **	0.61	0.82	0.68	0.34	0.86	**0.92*** **	0.66	0.98
CN-TE	**0.78*** **	0.7	0.84	**0.84*** **	0.74	0.90	**0.94*** **	0.78	0.99	**0.94*** **	0.59	0.99
CO-WBE	**0.82*** **	0.74	0.87	**0.91*** **	0.83	0.95	**0.97*** **	0.84	0.99	**0.93*** **	0.61	0.99
SV-WBE	**0.69*** **	0.43	0.6	**0.63* **	0.25	0.48	**0.94*** **	0.45	0.91	**0.88*** **	0.21	0.74
Alt a 1	0.42	0.6	0.76	0.44	0.50	0.74	0.58	0.77	0.99	0.51	0.61	0.97
Der f	0.52	0.34	0.49	0.37	0.31	0.55	**0.76* **	0.44	0.69	0.52	0.23	0.71

Table S3: Cut-offs selected based on the highest Youden's index and not on a specificity >94%, as in table 4, with corresponding sensitivity, specificity and accuracy for the 10 major *Culicoides obsoletus* recombinant (r-)allergens and the four best performing *Culicoides nubeculosus* r-allergens. For comparison the cut-off used in the study, selected at a specificity >94%, as well as the corresponding sensitivities are given.

	Youden's	Cut-off selected based on Youden's Index				Cut-off at 95% specificity	
allergen	Index	cut off value	sensitivity	specificity	accuracy	cut off value	sensitivity
Cul o 8	0.847	16000	0.89	0.95	0.92	16000	0.89
Cul o 1P	0.782	7300	0.83	0.95	0.88	7300	0.83
Cul o 2P	0.790	211	0.86	0.93	0.89	300	0.82
Cul o 10	0.727	700	0.77	0.95	0.85	700	0.77
Cul o 9	0.753	732	0.83	0.92	0.87	1800	0.76
Cul o 11	0.701	9481	0.77	0.93	0.84	11000	0.74
Cul o 7	0.688	707	0.77	0.92	0.83	900	0.71
Cul o 5	0.566	5304	0.63	0.93	0.76	6000	0.60
Cul o 3	0.606	404	0.85	0.76	0.81	1070	0.54
Cul n 5	0.418	80	0.51	0.91	0.68	108	0.43
Cul n 4	0.470	162	0.71	0.76	0.73	820	0.42
Cul n 3	0.460	1530	0.76	0.73	0.70	7500	0.38
Cul n 2	0.362	78.5	0.58	0.78	0.67	230	0.37
Cul n 1	0.365	619	0.57	0.80	0.67	2500	0.30

Figure S1. Percentage of IBH-affected horses from Switzerland, Sweden, Ireland, Germany and the United Kingdom (England) with IgE values above cut-off. The seven top allergens (Cul o 8, Cul o 1P, Cul o 2P, Cul o 10, Cul o 9, Cul o and Cul o 7) are major allergen for IBH in all countries, (except Cul o 7 in England and Ireland) as >50% of the IBH-affected horses within each country had positive IgE values to these allergens.

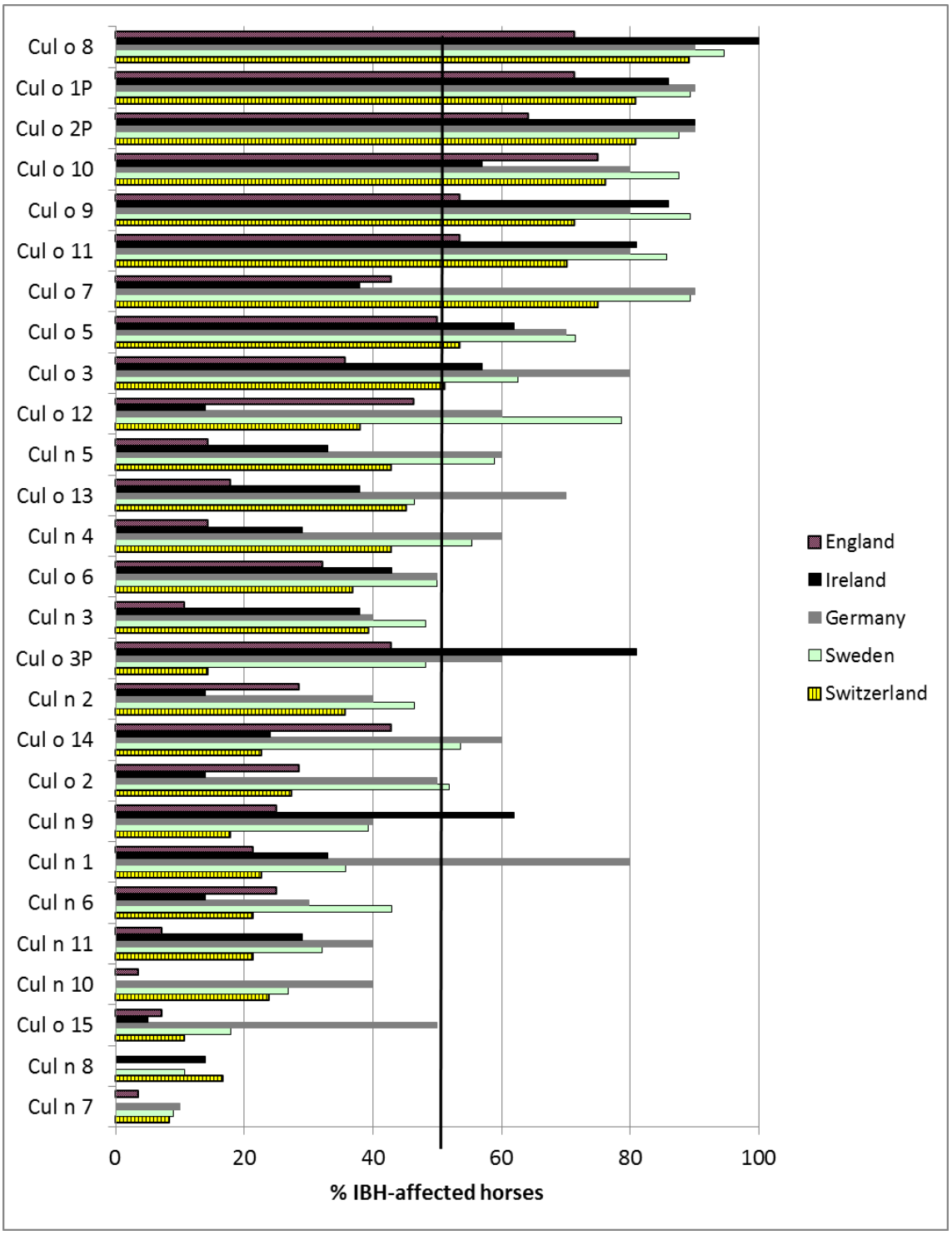
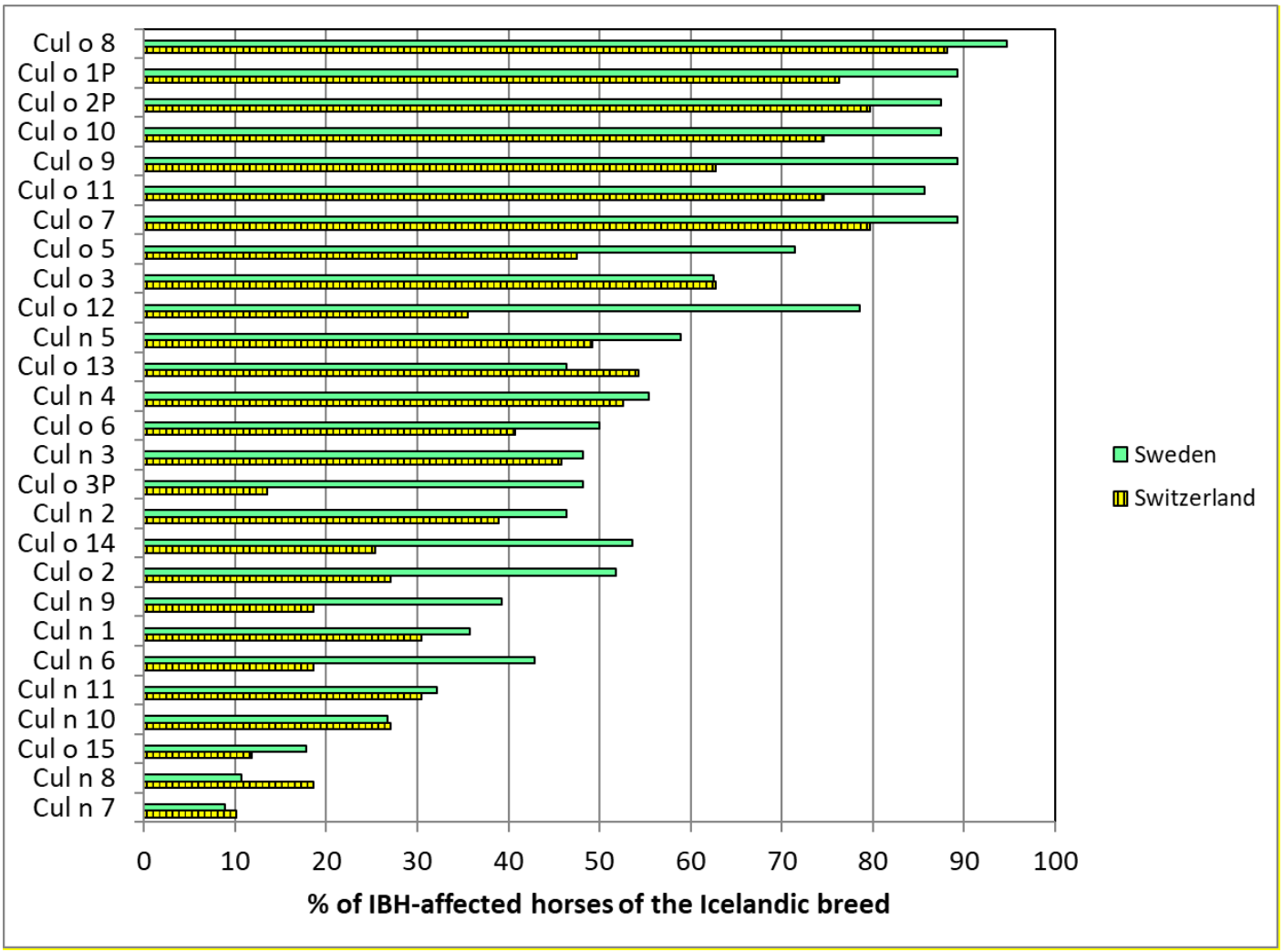
Figure S2. Percentages of positive IgE results in IBH-affected horses of the Icelandic breed from Switzerland (n=59) and Sweden (n=56). To investigate whether horses living in different countries, i.e. in different environments showed different IgE-binding patterns, horses from the same breed and origin (Iceland) but living in Switzerland or Sweden were compared. While the overall IgE-reactivity to the r-Cul allergens was weaker in the ISL IBH horses from Switzerland, the same seven top r-allergens as in Fig 1, Cul o 8, Cul o 1P, Cul o 2P, Cul o 10, Cul o 11, Cul o 7 and Cul o 9 were the best performing allergens in the ISL horses from both countries with ≥75% positive reactions, with the exception of Cul o 9 in ISL horses from Switzerland, with only 62% positive IgE reactions.