

Proteomic Analysis Reveals Allergen Variability among Breeds of the Dust Mite *Blomia tropicalis*

Eduardo Santos da Silva^{a-c} João Carlos Marques Ponte^{a, b}
Márcia Barbosa da Silva^{a, b} Carina Silva Pinheiro^a
Luis Gustavo Carvalho Pacheco^a Fatima Ferreira^b Peter Briza^b
Neuza Maria Alcantara-Neves^{a, c}

^aLaboratório de Alergia e Acarologia, Departamento de Ciências da Biointeração, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, Brazil; ^bDepartment of Biosciences, University of Salzburg, Salzburg, Austria; ^cPrograma de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia (RENORBIO), Natal, Brazil

Keywords

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Abstract

Background: The dawn of the “omics” technologies has changed allergy research, increasing the knowledge and identification of new allergens. However, these studies have been almost restricted to *Dermatophagoides* spp. Although *Blomia tropicalis* has long been established as a clinically important source of allergens, a thorough proteomic characterization is still lacking for this dust mite. **Objective:** To increase knowledge of *B. tropicalis* allergens through proteomic analysis. **Methods:** Eleven in-bred lineages of *B. tropicalis* were obtained from 11 unique different pregnant females. Their somatic extracts were analyzed and compared with a commercially available extract by liquid chromatography tandem mass spectrometry (LC-MS/MS). **Results:** Considerable differences in the protein expression profiles were found among the breeds, and most of them displayed higher expression levels of major allergens than the commercially available extract. Blo t 2 was the most

prominent allergenic protein in the analyzed extracts. Six identified allergens and 14 isoforms have not yet been recognized by IUIS. Conversely, 3 previously recognized *B. tropicalis* allergens were not found. **Conclusions:** The clear impact of inbreeding on allergen content shown by our study leads us to conclude that the quantification and/or identification of allergens from in-bred lines should be routinely considered for mite cultivation in order to select breeds with higher amounts of major allergens. In this sense, LC-MS/MS may be a useful method to achieve this quality control for research and commercial purposes.

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Introduction

House dust mites are important sources of allergens, representing a major cause of allergic respiratory diseases, such as perennial rhinitis and asthma. While *Dermatophagoides* spp. are widespread and predominate in tem-

E.S.S. and J.C.M.P. contributed equally to this study.

perate regions, *Blomia tropicalis*, initially described as a storage mite, is now widespread in households of tropical and sub-tropical regions where it is an important indoor allergen source that triggers allergic symptoms in humans [1–3]. Among *B. tropicalis* allergens, Blo t 5 and Blo t 21 are considered major allergens, with more than 90% IgE reactivity [4–6]. Despite their importance and the fact that Blo t 5 is the most well-studied allergen from *B. tropicalis*, the biological function of both allergens remains unknown. The WHO/IUIS Allergen Nomenclature Sub-Committee (www.allergen.org/) recognizes 14 allergenic proteins from *B. tropicalis*, most of which have been characterized at the molecular level, revealing diverse structures and biochemical functions [7, 8]. Besides Blo t 5 and Blo t 21, other allergens from *B. tropicalis* (namely Blo t 1, Blo t 2, Blo t 3, Blo t 4, Blo t 6, Blo t 8, Blo t 11, and Blo t 12) show moderate to high IgE reactivity (higher than 50%) in atopic individuals [8–11]. Low IgE reactivity (lower than 30%) was shown for Blo t 7, Blo t 10, Blo t 13, and Blo t 19 [7, 8, 12].

There is great variability in allergenic components among commercially available crude allergen extracts, which come from natural sources. High variation among 30 different batches of 15 skin prick tests and 15 sublingual immunotherapy products was observed [13]. Researchers have tested many different sources of in-house or commercially available allergens, and observed highly variable levels of allergen components, or absence of allergens such as dog dander, mold, and grass pollen [14–19]. This variability is also true for mites. For *D. pteronyssinus*, considerable differences in allergen levels were found between 10 different manufacturers: Der p 1 varied by 5- to 7-fold; Der p 2 varied by up to 26-fold, and Der p 7, Der p 10, and Der p 21 were absent in some extracts [20]. Our group has previously detected different concentrations of Blo t 5 by ELISA in *B. tropicalis* cultures isolated from dust of the same city (range 0–750 ng/mg of extract; unpubl. data). Therefore, highly variable results can be expected when using different allergen extracts for allergy diagnosis. Furthermore, use of variable allergen extracts for allergen-specific immunotherapy may increase the risk of side effects or new allergen sensitizations [13, 20–22]. In Europe, efforts have been made to standardize allergen extracts, but variability in total protein content and specific allergen levels in allergen extracts still seems to be an issue, since companies apply their own in-house methods for standardization and calculation, rather than following published recommendations [23]. The World Health Organization for instance recommends to manufacturers the quantification of ma-

ajor allergens in mass units for their allergen products, but not all extracts have ELISA systems based on monoclonal antibodies and these companies not always invest in mass spectrometry (MS) systems in their infrastructure [23, 24].

To overcome this issue, the use of purified natural allergens or their recombinant counterparts provides reproducible, safe, and patient-tailored treatments, and opens new avenues for the development of vaccines using hypoallergenic proteins. Nevertheless, limited information is available regarding different profiles of protein expression in different organisms of the same species. Therefore, allergy studies have recently used techniques, such as MS, to identify and characterize an entire set of proteins in a given organism [23, 24]. The advent of the “omics” era has accelerated progress in many scientific research areas. Acarology and allergology have embraced these approaches not only to answer scientific questions, but also to improve allergy diagnosis and immunotherapy. Although genome, transcriptome, and proteome analyses of various species of non-allergenic mites have been published [25–28], “omics” studies with allergenic mites have largely been restricted to certain species, particularly *Dermatophagoides* spp. [29–34].

Due to the importance of *B. tropicalis* in tropical and sub-tropical regions and the lack of proteomic studies [1, 2], and given our findings regarding the different concentration of Blo t 5 in our *B. tropicalis* cultures, in the present work we aimed at gaining insights into total allergenic content and intra-species expression variation of *B. tropicalis* breeds. We thus performed proteomic analyses for 11 *B. tropicalis* extracts – each one produced exclusively from a single adult female-derived breed – and a commercially-available *B. tropicalis* extract. This is the first descriptive study of *B. tropicalis* allergens at the proteomic level, unveiling a complete repertoire of allergens and the impact of inbreeding on allergenic protein expression in *B. tropicalis*.

Materials and Methods

Culture and Preparation of Mite Extracts

Mites were isolated from indoor dust samples collected in residences of the metropolitan area of Salvador, northeast Brazil (12°58'29-S 38°28'36-W). *B. tropicalis* cultures were maintained in BOD incubators at 25 °C and 75% relative humidity. The cultures were reared in cell culture containers and fed with a mixture containing biological yeast (Saf do Brasil Produtos Alimentícios Ltda, Rio de Janeiro, Brazil) and spirulina (Alcon® Pet, Camburiú, Brazil) at a ratio of 1:3. After the initial culture, 11 pregnant females

were picked up one by one and cultivated in 11 small containers, and each colony was considered to have an in-house breed. After expansion, and reaching the stationary phase, the mite breed cultures were purified from the culture medium by flotation in 5 M NaCl and desalting was carried out by filtration in sterile distilled water, reaching approximately >97.0% purity. Mites were resuspended in 0.15 mM of phosphate-buffered NaCl, pH 7.4 (PBS buffer) and ground for 3 min using a blender (Waring Commercial, Torrington, CT, USA) with subsequent centrifugation at 2,716 g for 15 min. Supernatants were then delipidified in the presence of diethyl ether (PA ACS; Hexis Científica, Jundá, Brazil) in equal volume in three cycles of incubation for 30 min and centrifugation at 11,000 g for 15 min, and stored at -20 °C. Commercially available *B. tropicalis* from Allergen® (Ängelholm, Sweden; Bt-Allergen®) was also subjected to this protein extraction procedure. The band profile of the extracts was evaluated by 12% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE), followed by Coomassie Brilliant Blue R-250 staining (Bio-Rad, Hercules, CA, USA).

In Solution Analysis of Mite Extracts

Mite extracts were digested in solution, using the ProteoExtract® trypsin digestion kit (EMD Millipore, Burlington, VT, USA) according to the manufacturer's instructions. After incubation, the samples were stored and peptides analyzed by MS coupled to liquid chromatography (LC-MS/MS). Peptides were loaded into the trap column (PepSwift Monolithic Trap Column, Dionex) and desalted with 0.1% (v/v) heptafluorobutyric acid at a flow rate of 10 µL/min. After 5 min, trap and separation columns (PepSwift Monolithic Nano Column, 100 µm × 25 cm, Dionex) were coupled with a switching valve and the peptides were eluted with an acetonitrile gradient (solvent A: 0.1% (v/v) FA/0.01% (v/v) TFA/5% (v/v) ACN; solvent B: 0.1% (v/v) FA/0.01% (v/v) TFA/90% (v/v) ACN; 5–45% B in 60 min) at flow rate of 1 µL/min at 55 °C. The HPLC was directly coupled via nano electrospray to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The capillary voltage was 2 kV. For peptide identification, a top 12 data-dependent analysis method was used. The instrument was tuned to maximum sensitivity. The normalized fragmentation energy was 27%.

Identification, Functional Classification, and Data Analyses of B. tropicalis Allergenic and Non-Allergenic Proteins

The generated MS/MS data were then submitted to the Proteome Discoverer version 1.4 (Thermo Fisher Scientific) with SequestHT as the search engine and PEAKS Studio 8 (Bioinformatics Solutions, Waterloo, ON, Canada). Searches were done with an in-house database with *B. tropicalis* sequences from UniProtKB (www.uniprot.org). For the search of total protein content (allergenic or non-allergenic) and analyses of biological process, molecular function, and cellular localization, only a representative in-house breed (No. 17) was evaluated. This breed was randomly selected since the total analyses in all 12 extracts would be time-consuming. Only peptides with high confidence scores (XCorr ≥ 2.3 for SequestHT, -10lgP ≥ 35 for PEAKS) were considered. Blast2GO (www.blast2go.de/b2ghome) was used for the functional classifications.

A second analysis was performed, in which all 11 breed extracts and the Bt-Allergen® extract were analyzed using both UniProtKB and the IUIS database (http://www.allergen.org). However, in this second analysis, the aim was identifying only allergenic proteins,

thus producing the allergome of the 12 different extracts. Non-allergenic proteins were not considered in this second search.

The percentage distribution of *B. tropicalis* allergens in each single extract was calculated using the parameter “Area” from the summarized proteomic search results. The percentage of a specific allergen in each single extract sample was calculated as (area of the specific allergen in the extract sample/sum of the area of all allergens in the extract sample) × 100. This percentage contribution of allergens in each single extract was then plotted in a heatmap.

In order to stratify the extracts as high or low for a specific allergen, a second heatmap analysis and a principal component analysis (PCA) were performed using ClustVis (https://biit.cs.ut.ee/clustvis/) [35]. In the case of the second heatmap, the best scaling was “unit variance scaling” and we could have classified some extracts. Since Blo t 2 isoform 1 was the most prominent allergen in the majority of extracts, we decided to perform the analysis using the following classification: extracts with a high (≥26.9%) or low (<26.9%) percentage contribution of Blo t 2. After that, the singular value decomposition (SVD) with imputation was the PCA method used to calculate principal components. PCA is a widely used method of reducing the dimensionality of high-dimensional data, such as ours [35]. Possible allergen correlations among all extracts were verified by Pearson correlation coefficient using GraphPad Prism (San Diego, CA, USA).

Results

SDS-PAGE Profiles of Crude Mite Extracts and Functional Annotation of the Identified Proteins

We first used SDS-PAGE analysis to compare the band patterns of 11 soluble extracts of *B. tropicalis* in-house breeds and a commercially available extract from Allergen (Bt-Allergen®). Figure 1 shows that the band profiles did not present any degradation pattern, and different band intensities were observed among the analyzed extracts. Breed 10 and 16, followed by Bt-Allergen® extracts, displayed weaker or absence of some bands when compared with the other extracts.

Using proteomic analysis, we found 60 proteins (non-allergenic and allergenic proteins) in a representative breed of our mite cultures (breed 17; online suppl. Excel spreadsheet; for all online suppl. material, see www.karger.com/doi/10.1159/000501964). We performed gene ontology term analysis to classify these breed 17 proteins according to their molecular function and their involvement in biological processes (Fig. 2). Nine protein categories were related to the molecular function (Fig. 2a), with 40.63% annotated as having catalytic activity; 15.63% with transporter activity, 25% without annotation available, and 18.78% with several activities (each of them with 3.3% participation). Furthermore, these proteins were classified into four biological processes (Fig. 2b): meta-

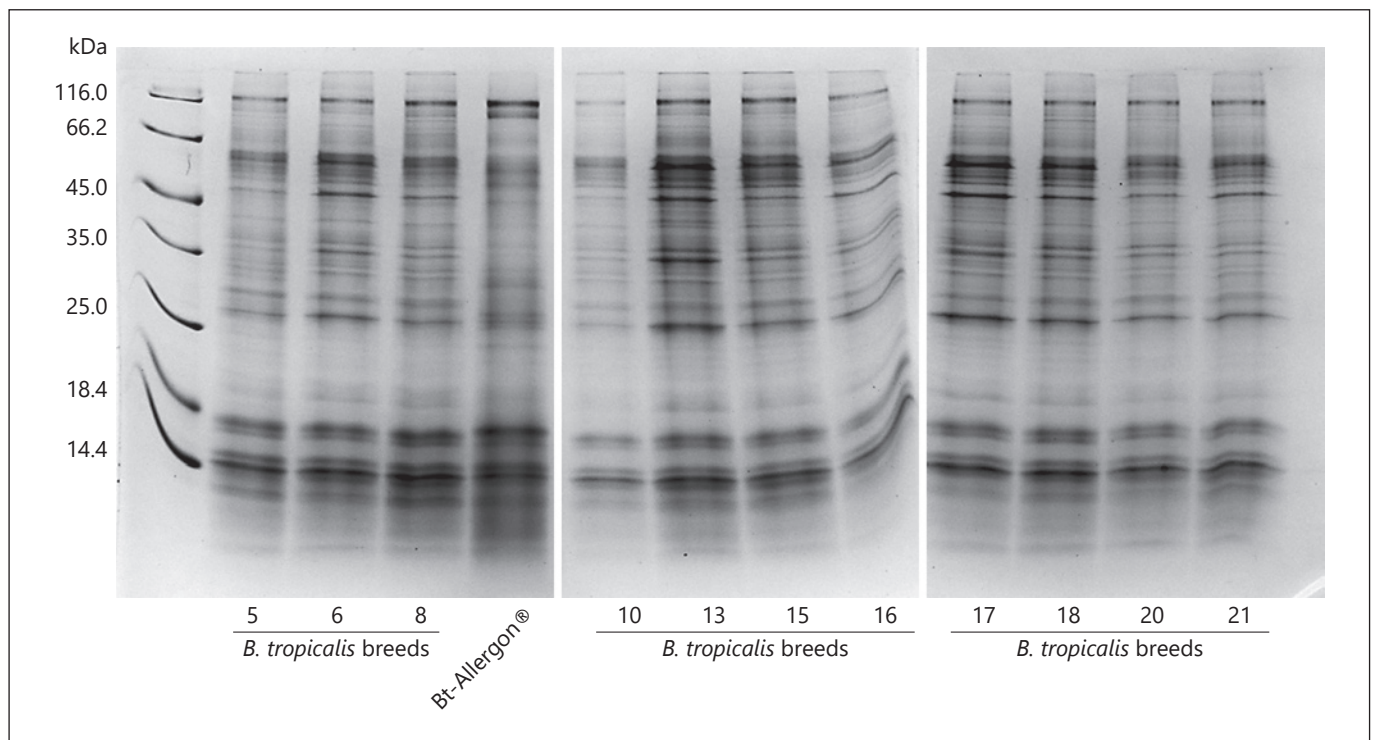


Fig. 1. Analysis of 11 *B. tropicalis* in-house breed crude extracts and Bt-Allergen® extract on a 12% SDS-PAGE gel; 10 µg of total protein extracts were loaded in each well. The protein molecular weight standard is shown to the left side of the image; 5, 6, 8, 10, 13, 15, 16, 17, 18, 20, and 21, arbitrarily assigned numbers of the crude extracts from *B. tropicalis* breeds, each one reared from a single pregnant female.

bolic (46.67%); no annotation available (26.66%); transport (16.67%); cell organization and biogenesis (6.67%), and regulation (3.3%). When cellular localization was considered (Fig. 2c), 58.82% of *B. tropicalis* proteins had no annotation available, 17.65% were present in cytoplasm, 11.76% were in membrane, 2.9% were in cytoskeleton, 2.9% were extracellular, 2.9% were in mitochondrion, and 2.9% were in ribosome.

Allergen Profiles in *B. tropicalis* Extracts

Table 1 shows the *B. tropicalis* allergenic proteins and their isoforms identified in the 11 in-house breeds and in the Bt-Allergen® extracts. A total of 23 allergenic proteins were identified; these allergens were present in at least one of the investigated extracts. Some of the allergens were found exclusively in the in-house breed extracts, such as Blo t 1.0101, Blo t 2 isoform 4, Blo t 3, Blo t Alt a 6 allergen, Blo t Mag29 allergen, and profilin. Of the Blo t 1, Blo t 2, Blo t 3, Blo t 4, Blo t 6, Blo t 10, Blo t 11, and Blo t 21 identified isoforms, 9 were already recognized by IUIS ([http://](http://www.allergen.org)

www.allergen.org). Meanwhile, no allergenic protein was found exclusively in Bt-Allergen® extract.

Blo t 2 isoform 1, Blo t 2 isoform 4, Blo t 3 A1KXI1, Blo t 5 A7IZF7, Blo t 8 A1KXG6, all 3 identified Blo t 13 isoforms, Blo t 14, Blo t 15, Blo t aldehyde dehydrogenase, Blo t Alt a 6 allergen, Blo t Mag29 allergen, and the *B. tropicalis* profilin have not yet been recognized by IUIS. Moreover, these latter 6 allergens have not yet been identified at the proteome level in extracts of this mite.

The allergen profiles of the analyzed *B. tropicalis* extracts are shown in Figure 3 and in online supplementary Figure S2–13. Figure 3 confirms that Blo t 2 isoform 1 was the most prominent allergen, with percentages ranging from 18.9 to 38.5% in the analyzed extracts. Even in breeds 5, 6, and 21, which had lower percentages (13.9, 14.0, and 11.0%, respectively), Blo t 2 isoform 1 was still one of the most abundant allergens. Blo t 2.0103 was found only in breeds 5, 6, and 18, as well as Bt-Allergen® extract, while Blo t 2 isoform 4 was found only in breeds 8, 10, and 17. The unique tropomyosin isoform identified by our study, Blo t 10.0101, had a medium representation

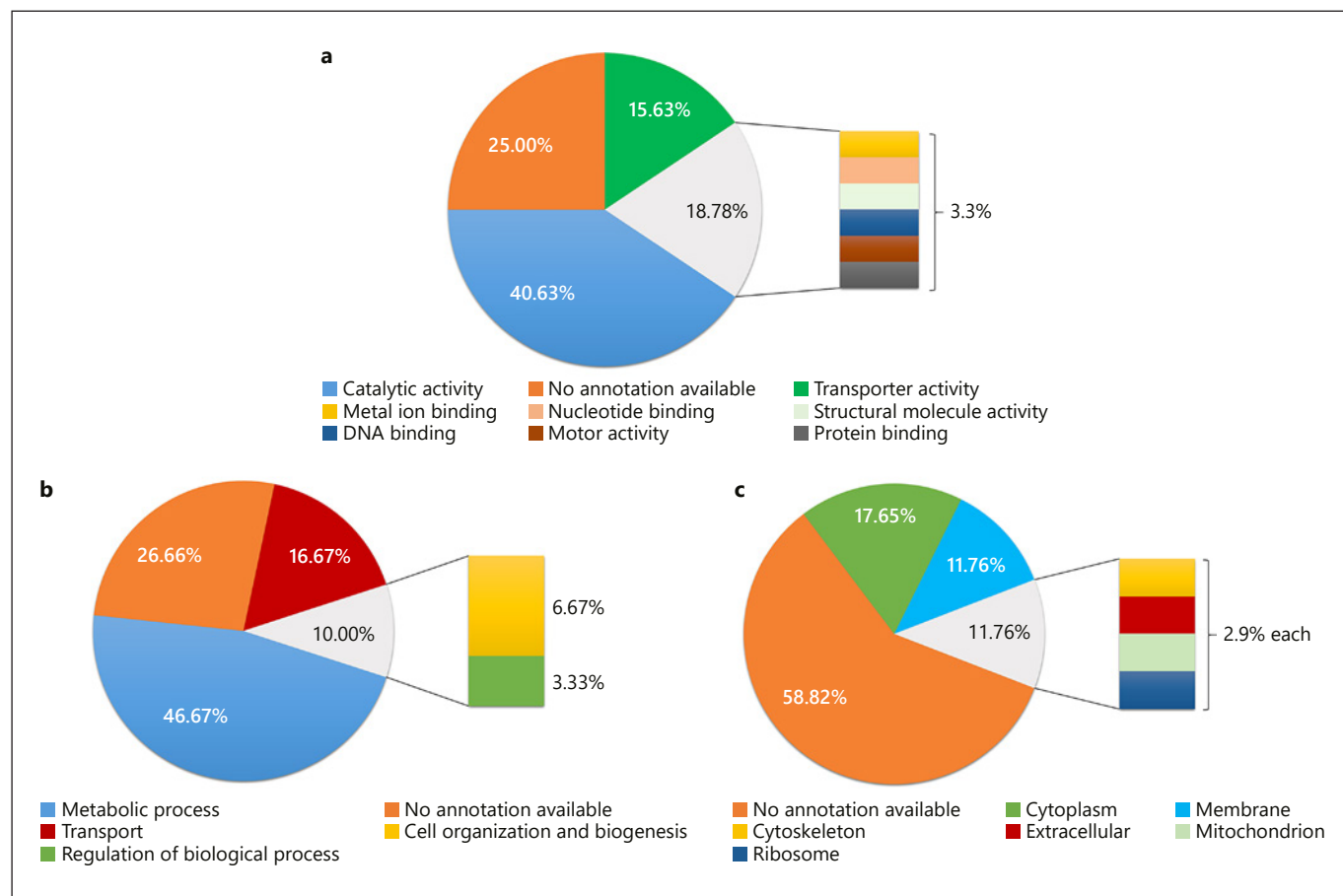


Fig. 2. Functional classification of the proteins identified by MS in a representative *B. tropicalis* extract in-house breed based on the gene ontology annotations of UniProt. **a** Classification according to molecular function. **b** Classification according to biological process. **c** Classification according to cellular localization.

percentage in almost all breeds, except for breeds 13 and 16, where its contribution was 20.4 and 24.6%, respectively. The major allergen Blo t 5 was modestly produced by most of the breeds (2.1–19.4%), although breeds 10, 17, and 20 showed low production, from 2.1 to 6.2%. While breed 6 produced high levels of Blo t 13 (A1KXG8; 19.5%), most of the breeds exhibited low to moderate representation percentages (3.8–15.5%). Blo t 21, another *B. tropicalis* major allergen, showed a variable pattern, in which some breeds (6, 8, 13, 15, 16, 20, and 21) showed a medium level of Blo t 21 production (5.8–18.2%) and others presented a low percentage (Bt-Allergen®, breeds 8, 10, 17, and 18). *B. tropicalis* aldehyde dehydrogenase percentage values were mostly low, but higher production was observed in breeds 8 and 15, both with 8.7%. All the other allergens displayed either low production, with values below 4%, or were not detected. However, Blo t 4

showed modest values of 7.9% and 7.5% in breeds 18 and 21, respectively.

The average abundance of the allergenic proteins in the all tested *B. tropicalis* extracts is shown as the mean plus standard deviation percentage in Figure 4. Blo t 2 isoform 1 represented the most abundant allergen in the tested extracts (23.4%). The additional isoforms of Blo t 2, Blo t 2.0103 and Blo t 2 isoform 4, both had a percentage of 5.9%. Therefore, Blo t 2 was the most abundant allergen in the *B. tropicalis* tested extracts with a total percentage of 35.2%. Blo t 10.0101 was the second most prominent allergen (14.7%), followed by Blo t 5 (10.0%), Blo t 13 (9.9%), and Blo t 21.0101 (8.7%). The aldehyde dehydrogenase from *B. tropicalis* represented 5.2% of the total allergens found in the extracts. The other allergens represented 16.2% of the identified proteins and included two more isoforms of Blo t 13, as well as other allergens

Table 1. MS analysis and database search summary of confirmed and putative allergenic proteins identified in *B. tropicalis* extracts obtained from different sources

Access No. UniProtKB	Allergen identity	Allergen presence in distinct tested samples ¹	Unique peptides	Score max.	Coverage max., %	MW, kDa	pI	WHO/IUIS isoform recognition	Protein existence (UniProt)	Gene ontology/ biological function ²
A1KXI0	Blo t 1.0201	both	5	16.49	22.22	38.1	5.96	yes	transcript level	cysteine protease
Q95PJ4	Blo t 1.0101	in-house	3	9.43	15.84	25.1	8.25		transcript level	
A6XEN8	Blo t 2 isoform 1	both	8	188.43	73.94	15.2	6.65	no	transcript level	NPC2 family
A6XEP1	Blo t 2 isoform 4	in-house	3	170.06	73.94	15.2	6.98	no	transcript level	
Q1M2P3	Blo t 2.0103	both	2	71.59	56.03	15.6	8.12	yes	transcript level	
A1KXI1	Blo t 3	in-house	3	4.92	13.16	27.5	7.93	no	transcript level	trypsin
Q8I9I6	Blo t 3.0101	both	4	16.75	20.68	27.5	8.15	yes	transcript level	
A1KXI2	Blo t 4.0101	both	11	60.15	30.83	57.4	6.93	yes	transcript level	alpha-amylase
A7IZF7	Blo t 5	both	4	47.33	55.22	14.8	5.81	no	transcript level	unknown
A1KXI3	Blo t 6.0101	both	2	2.13	12.73	29.9	6.67	yes	transcript level	chymotrypsin
A1KXG6	Blo t 8	both	7	20.08	27.54	27.6	5.14	no	transcript level	glutathione-S-transferase
A7XZI4	Blo t 10.0101	both	22	204.35	49.3	33	4.84	yes	transcript level	tropomyosin
Q8MUF6	Blo t 11.0101	both	17	63.55	24.8	102	5.86	yes	transcript level	paramyosin
A1KXG8	Blo t 13	both	10	122.26	68.46	14.8	5.76	no	transcript level	fatty acid-binding protein
A1KXG9		both	8	44.72	57.89	17.2	6.77		transcript level	
A1KXH5		both	6	28.87	62.6	14.9	5.63		transcript level	
A7XZI6	Blo t 14	both	11	22.55	17.88	86.5	8.97	no	transcript level	apolipoporphins
A1KXI8	Blo t 15/chitinase allergen	both	7	32.17	22.34	51.6	6.02	no	transcript level	chitinase
A7IZE9	Blo t 21.0101	both	5	31.4	50.39	14.8	5.81	yes	transcript level	unknown
A1KXI6	Blo t aldehyde dehydrogenase allergen	both	12	39.42	32.69	45.2	5.83	no	transcript level	oxidoreductase activity
A1KXI7	Blo t Alt a 6 allergen	in-house	4	11.85	28.07	11.7	4.68	no	transcript level	structural constituent of ribosome
A1KXJ1	Blo t Mag29 allergen	in-house	12	35.26	24.46	71.5	5.59	no	transcript level	heat shock protein
A1KXJ2	Profilin	in-house	3	5.12	27.69	14.1	6.51	no	transcript level	actin-binding protein

Extracts were prepared from 11 distinct *B. tropicalis* breeds reared and expanded from a single pregnant female, and Bt-Allergen® (*B. tropicalis* mites commercially available from Allergen®). Unique peptides, the number of peptide sequences unique to a protein group; score max., the protein score, which is the sum of the scores of the individual peptides; coverage max., calculated by dividing the number of amino acids in all found peptides by the total number of amino acids in the entire protein sequence; MW, molecular weight; pI, isoelectric point.

¹ Allergen identification in extracts. Both, allergens identified in the 11 in-bred mite extracts and in Bt-Allergen® extract; in-house, allergens identified exclusively in *B. tropicalis* breed extracts.

² Determined by IUIS (www.allergen.org/) or gene ontology annotations of UniProt (www.uniprot.org).

such as Blo t 4, Blo t chitinase allergen, Blo t 11.0101, Blo t Alt a 6 allergen, Blo t 8, Blo t Mag29 allergen, 2 isoforms each of Blo t 3 and Blo t 1, a *B. tropicalis* profilin, and Blo t 6.0101.

Figure 5 shows a comparison between Bt-Allergen® and the 11 in-house *B. tropicalis* breeds regarding the content of 2 major allergens (Blo t 5 and Blo t 21), as well as the 2 most abundant allergenic proteins (Blo t 2 isoform 1 and Blo t 10). Some of the in-house breeds had higher levels of Blo t 5, Blo t 10, and Blo t 21 than Bt-Allergen®. However, levels of Blo t 2 isoform 1 were lower in most of the in-house breeds than in Bt-Allergen®. Our second heatmap analysis (Fig. 6a) revealed that some extracts can be classified as having a high or low contribution of a specific allergen. In this sense, Bt-Allergen®, breeds 10, 13, 16, 17, and 20 were grouped as extracts with a high amount of Blo t 2 isoform 1. Interestingly, we have also noticed that this group displayed low levels of Blo t 5, Blo t 21, Blo t 11, Blo t 13 (A1KXG9), and Blo t aldehyde dehydrogenase allergen. It is also noteworthy that the group considered Blo t 2-low have produced high levels of Blo t 5, Blo t 21, Blo t 11, Blo t 13 (A1KXG9), and Blo t aldehyde dehydrogenase allergen. We also observed a clear distribution of 2 clusters in the PCA plot (Fig. 6b),

indicating that Blo t 2 isoform 1 had an influence in the percentage variability of total content of allergens. Remarkably, the production of Blo t 2 isoform 1 and Blo t 5 was the only significant negative correlation (Fig. 6c) when we tested correlations between the allergens (online suppl. Table S1). However, we observed some significantly positive correlations (online suppl. Table S1).

In addition, the percentage distributions of allergens – which were compared together in Figures 3 and 6 – are detailed for each breed extract and the Bt-Allergen® extract in online supplementary Figure S2–13. It is possible to notice in these Figures how some extracts showed a more homogenous presence of some allergens, whereas others displayed a more heterogeneous mixture. For instance, while online supplementary Figure S13 showed that Blo t 2 (2 isoforms) was 62.2% of Bt-Allergen® extract, breed 6 (online suppl. Fig. S3) was 26.4, 29.6, 19.1, and 24.9% of Blo t 2, the 2 *B. tropicalis* major allergens (Blo t 5 and Blo t 21), Blo t 13, and the other identified allergens, respectively.

The homogeneity or heterogeneity of allergens in the breeds was emphasized in our PCA plot (Fig. 6b). It was observed in the PCA plot that Bt-Allergen® extract was arranged distant not only from extracts of a different

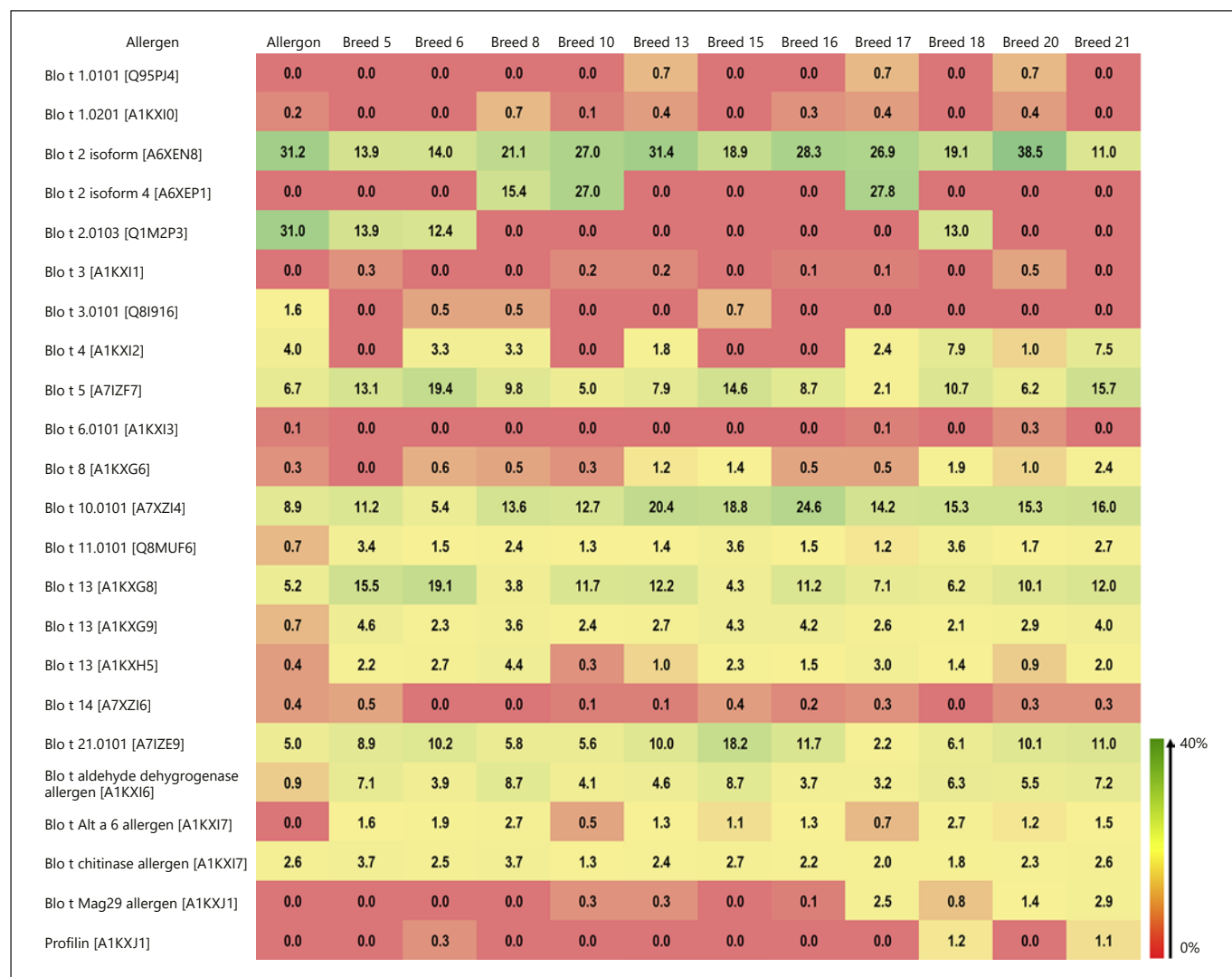


Fig. 3. Heatmap analysis of the percentage contribution of *B. tropicalis* allergens in extract analyzed by MS. The colored scale is shown in the lower right region of the frame. The percentage contribution of each allergen to the respective breed is shown in the center of each colored square.

group (Blo t 2-low), but also from extracts in its own group (Blo t 2-low).

Discussion

B. tropicalis studies have been restricted to transcriptomic analyses that focused on aquaporin identification and the use of sequence tagging (EST), a bioinformatic approach for the prediction of new allergens [36, 37]. However, no genome, transcriptome, or proteome studies on this acarid have been properly presented yet. There-

fore, we aimed in this work to expand the knowledge of *B. tropicalis* allergens through a proteomic analysis using 12 different extracts, for which 11 were in-house breed mites cultivated from 11 unique different pregnant females and 1 was from the Allergon company (Bt-Allergon®). Therefore, each breed of our study was a lineage from a unique female. We found a substantial difference in the protein content (presence of a protein or its isoforms, and amount) among the 12 extracts analyzed and some of the observed proteins or their isoforms have neither been recognized by IUIS or been reported.

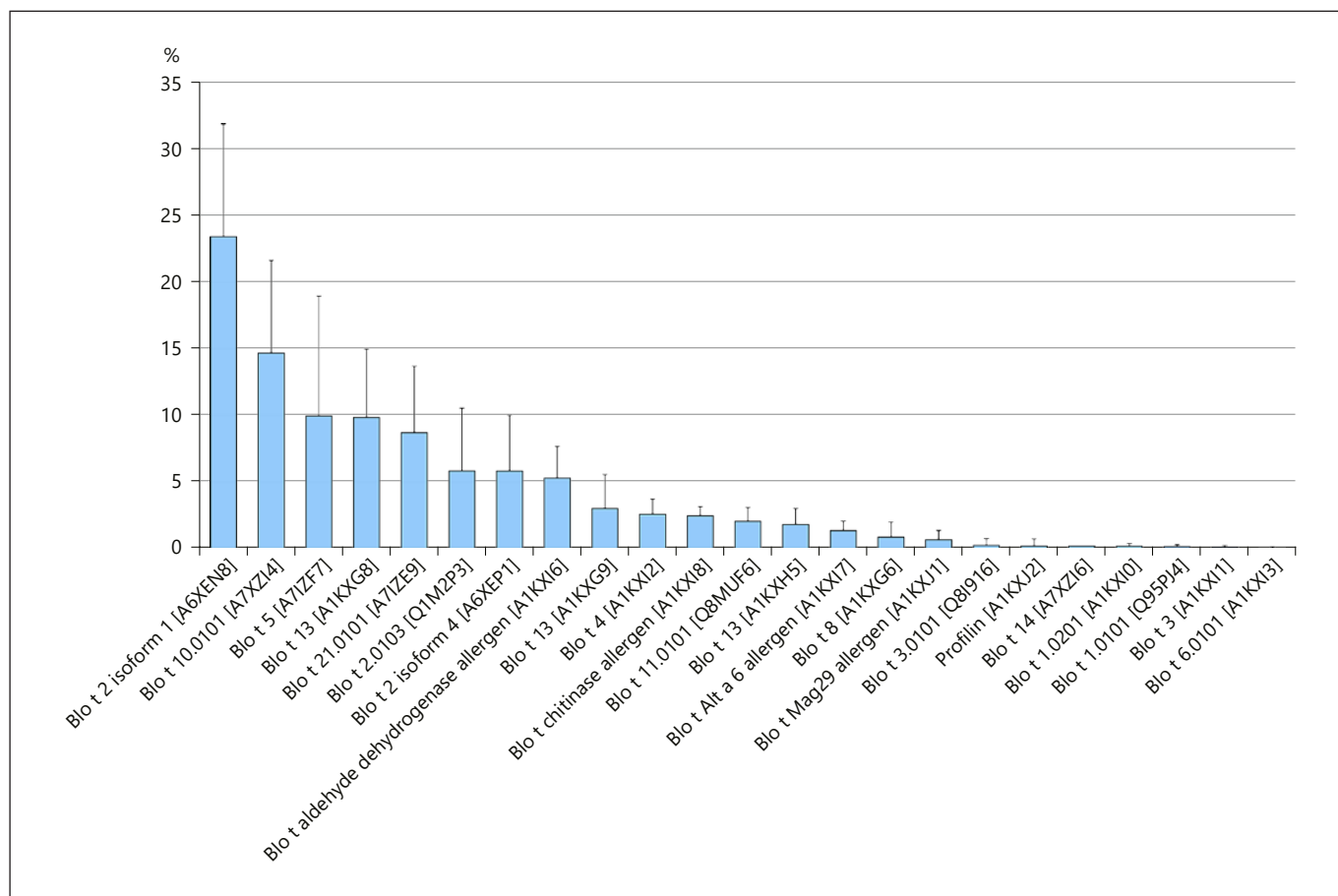
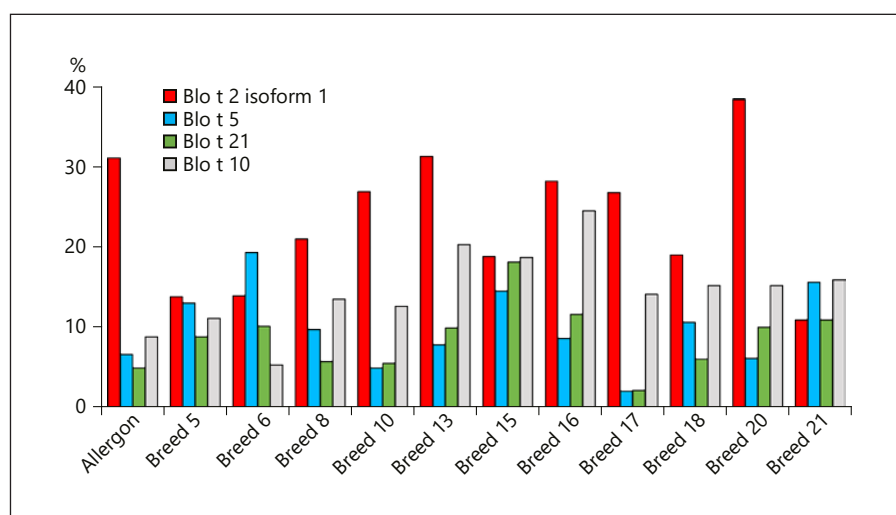


Fig. 4. Average percentage of *B. tropicalis* allergens and putative allergenic proteins present in the extracts analyzed by MS. The samples were prepared from 11 distinct *B. tropicalis* in-house breeds, each one reared and expanded from a single pregnant female, or from a commercially available extract (Bt-Allergen®).

Fig. 5. Comparison of the percentage of *B. tropicalis* major and most abundant allergens found in the MS analysis of the in-house breed extracts and Bt-Allergen® extract. The percentage of Blo t 2 isoform 1, Blo t 5, Blo t 21, and Blo t 10 are displayed in different colors, as indicated above the chart.



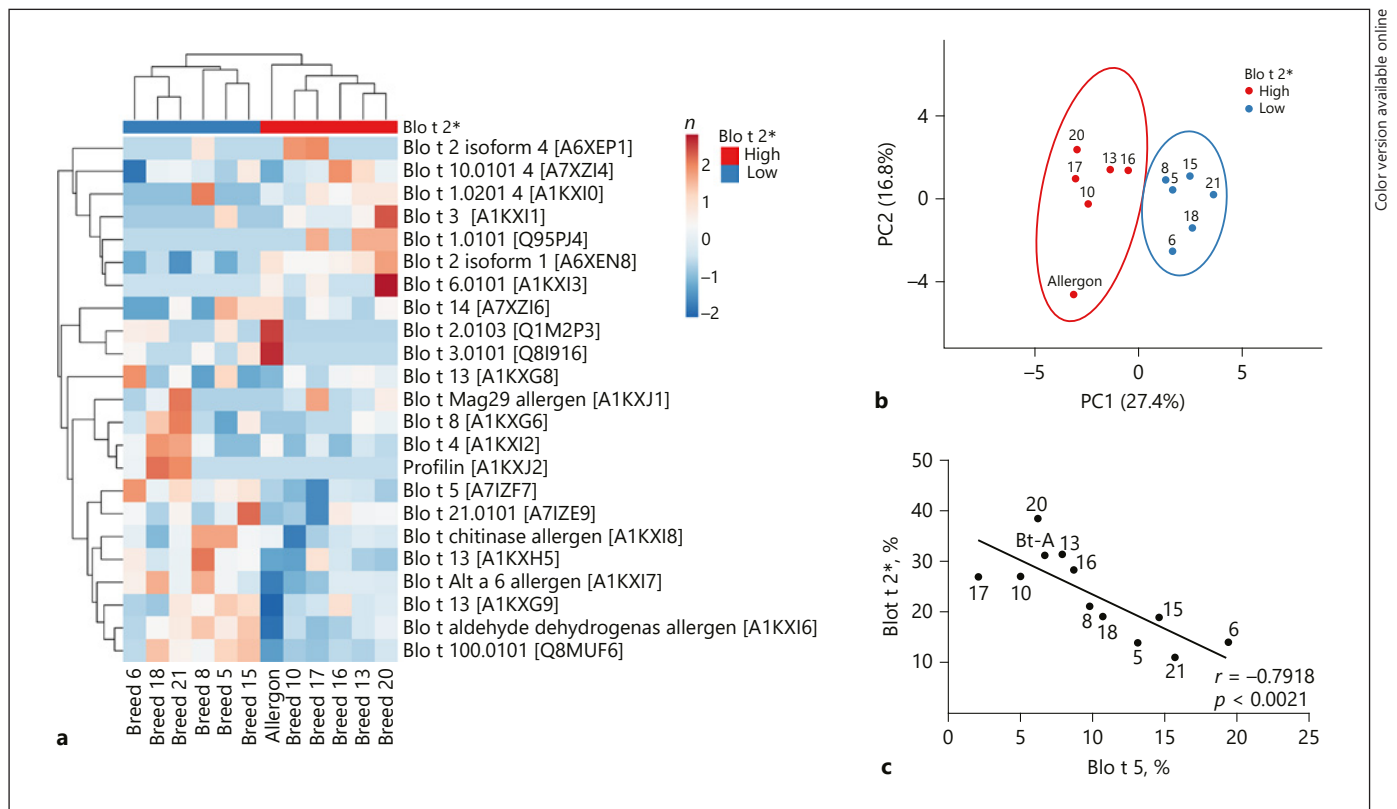


Fig. 6. Stratification of breeds by percentage of Blo t 2 in the in-house breed extracts and Bt-Allergon® extract. **a** Heatmap analysis using “unit variance scaling” of ClustVis (<https://biit.cs.ut.ee/clustvis/>). The percentage distribution data in Figure 4 were used as a basis to carry out the ClustVis unit variance scaling. Then, the extracts were grouped as having high amounts of Blo t 2 isoform 1 when its percentage contribution in the breed extract was $\geq 26.9\%$, and low when this value was $< 26.9\%$. **b** PCA plot of the analyzed

extracts using ClustVis. SVD with imputation was used for this analysis. Ellipses show clustering of the extracts, with their numbers or identification indicated. **c** Pearson correlation coefficient of Blo t 2 and Blo t 5. The comparison was performed using the percentage contribution for Blo t 2 and Blo t 5 in the extracts. Bt-A, Bt-Allergon® extract. * Only Blo t 2 isoform 1 was considered for these analyses.

Of 23 allergenic proteins identified in our study, 14 isoforms of 10 *B. tropicalis* allergens have not yet been recognized by IUIS. Since IUIS only recognizes the protein as an allergen if specific binding of IgE from patients is confirmed, in vitro and in vivo tests will be required to confirm the allergenicity of these 14 proteins [38].

Previous studies have highlighted the occurrence of isoforms of the most important mite allergens [32, 39]. Blo t 5 and Blo t 21 are considered major allergens of *B. tropicalis* [4–6, 10]; however, our study identified only a yet-to-be-acknowledged isoform of Blo t 5. The similar molecular weight but different pI (isoelectric point) of some of these isoforms may be due to variation in amino acid sequences or changes in glycosylation. Changes in amino acid sequences have been observed in major aller-

gens of *D. farinae* and *D. pteronyssinus* [32, 39–41]. Polymorphic sequences were also found in *B. tropicalis* allergens, such as Blo t 5 and Blo t 12, in some cases changing the IgE-binding capacity of those variants [42–44]. In addition, we have identified Blo t Alt a 6 allergen, which has homology to an allergenic protein described in non-mite species, particularly fungi [45, 46]. Since we did not perform 2D immunoblotting or Western blot assays, the allergenicity of this protein remains to be confirmed.

Among the 3 Blo t 2 isoforms found in our analysis, 2 are not documented by IUIS and isoform 1 was the most expressed protein in all breeds. Notably, Bt-Allergon® had the highest amount of this protein, compounding 62.2% of this mite extract. The importance of Blo t 2 for mite biology remains unknown, and further experimen-

tal works are required to unveil its role and to confirm if this protein belongs to the Niemann-Pick proteins of class C2 (NPC2) family, as it was shown in other allergenic mites [47]. NPC2 proteins are present in all vertebrates, acting as carriers of cholesterol and lipids [48]. In invertebrates, they are associated with both chemosensory activities and innate immune signaling pathways [49–52]. In fact, a study with variants of NPC2 showed that they bind LPS and activate the *Drosophila melanogaster* Toll pathway [50]. Additionally, during the determination of the tertiary structure of Der f 2, this protein was suggested to be related to the innate antibacterial defense system in mites [53]. Blo t 2 indeed binds to LPS [54], similar to what was previously predicted for Der p 2 [47]. We can infer that binding of hydrophobic molecules may be crucial for the physiological function of the group 2 mite proteins and this feature might be related to chemosensory activity as well as innate immune mechanisms in mites.

The second most abundant protein was the tropomyosin, Blo t 10; this protein family plays an important role in regulating the function of actin and muscle contraction, and their members are very conserved among invertebrates from diverse phyla. Anaphylactic reactions in patients due to IgE cross-reactivity of this protein with *Anisakis simplex* (a fish helminth that infects humans) and *Ascaris lumbricoides* tropomyosins have been extensively demonstrated [55–59]. Following this pan-allergen, Blo t 5 was the third most representative allergen in the proteome analysis. Blo t 5, together with Blo t 21 (fifth in position), are well established as clinically relevant *B. tropicalis* allergens [5, 6, 10, 12]. Additionally, Blo t 13, the fourth in position, is a fatty acid-binding protein, the function of which is one of the few experimentally confirmed in *B. tropicalis* reports. Other details about this allergen were given in a recent review from our group [8]. The allergenic enzymes, Blo t 1, Blo t 3, Blo t 4, Blo t 6, and Blo t 8, found in the present proteome are also described in this review. However, Blo t 15/chitinase allergen and Blo t aldehyde dehydrogenase allergen are other enzymes identified in the present work that have not yet been acknowledged by IUIS. Therefore, they are newly identified enzymes of *B. tropicalis*, and studies on their allergenicity shall be required. Kidon et al. [12] have produced Blo t 15 in its recombinant form and showed that 21% of the sera containing anti-BtE antibodies from 253 children reacted with this allergenic enzyme. In other mites, Der p 15 and Der f 15 were confirmed as major chitinase like-allergens for humans and dogs [60, 61]. Similarly, the aldehyde dehydrogenase mite allergens Der f 35

and Tyr p 35 displayed up to 80 and 70% IgE-binding frequencies, respectively [62, 63].

Interestingly, we noticed in our heatmap and PCA analyses that breeds with high levels of Blo t 2 isoform 1 showed low amounts of major allergens (Blo t 5 and Blo t 21) and vice versa. The function of these *B. tropicalis* allergens remains unknown, but inferences can be made using group mite allergen counterparts. Group 2 and 5 *Dermatophagoides* spp. allergen counterparts were immunolocalized in the gut and fecal pellets [64–67] and, coincidentally, these are the same sites for Blo t 5 and Blo t 21 [5], and probably Blo t 2 in *B. tropicalis*. Moreover, Der p 2 and Der p 5 were classified as lipid-binding proteins [47, 67]. Therefore, it is possible that Blo t 2, Blo t 5, and Blo t 21 may compete and end up nullifying each other's functions. However, genomic and transcriptomic studies of these breeds as well as immunoprobings techniques to localized Blo t 2 may help to shed light on the reasons for these inverse patterns of protein expression.

Other yet to be recognized *B. tropicalis* allergens identified in this work, Blo t 14, Blo t Mag29 allergen, and profilin, have been acknowledged in other mites, such as *D. farinae*, *D. pteronyssinus*, and *Tyrophagus putrescentiae*. Specifically, the apolipoproteins Der p 14 and Der f 14 showed low IgE-binding capacity in most studies, agreeing with a study that used Blo t 14 in an ELISA with a panel of *B. tropicalis* allergens [12, 68–70]. Amino acid sequence searches in the BLAST network using the access UniProtKB number of Blo t Mag29 allergen suggested that this allergen could be classified as a heat shock protein. So far, the other mite heat shock proteins acknowledged by IUIS, Der f 28 and Tyr p 28, have been shown to be major and intermediate allergens, respectively [32, 62]. Regarding the *B. tropicalis* profilin and for most other mite species, no data have been published yet, with the exception of Tyr p 36, which was considered a major allergen of *T. putrescentiae* [62]. However, in pollen, latex, and plant foods, profilins are considered pan-allergens [71]. Given that profilins usually show highly conserved structures, high levels of IgE cross-reactivity would also be expected among mite species. Future studies on IgE reactivity/cross-inhibition of these – newly identified – native and/or recombinant proteins will confirm whether the IgE responses display the same behavior as those in other mites.

Although many of the allergens uncovered by our analysis have been deposited in IUIS, we did not find the allergens Blo t 7, Blo t 12, or Blo t 19 in any of our in-house extracts or Bt-Allergen®. The latter has almost no information in the literature, as we previously noticed [8]. Blo t 7 was classified as an intermediate allergen, displaying

fatty acid binding and stimulating TLR2 signaling pathways in airway epithelial cells [7]. Patient reactivity against isoforms of Blo t 12 – a chitin-binding protein – varies depending on the geographical area [43]. The absence of these allergenic proteins in our extracts could be explained by the delipidation steps used in our protocol for obtaining the mite extracts. Possibly, in the case of Blo t 7, we lost the protein during centrifugation steps and removal of lipidic phase. Blo t 13 was identified, perhaps because the identified isoforms displayed more affinity to the soluble phase than to the lipidic phase. Blo t 12 and Blo t 19 may be extremely hydrophobic proteins and would not be part of the soluble phase of the mite extract. In fact, Blo t 12 isoforms have been shown to possess different affinities to chitin [72]. In this sense, if Blo t 12.0101 was part of the mite bodies, it would probably display more affinity to the debris rather than the soluble phase. Additionally, as many mite allergens are present in mite feces, it is possible that these proteins are more associated with such material than with the somatic extract [72–74].

Our proteomic data added new information about 6 *B. tropicalis* allergens unacknowledged in IUIS, which would increase the 14 deposited allergens to 20. There may be another 5 allergenic proteins of this mite, given that Caraballo et al. [75] identified 25 IgE-binding bands of *B. tropicalis* by immunoblotting. Moreover, the deposits for *Dermatophagoides* spp. in the IUIS database are more extensive than for *B. tropicalis*, implying that allergen counterparts remain to be discovered in this tropical mite. A combination of other “omics” approaches as well as molecular biology techniques may help to find all missing data on *B. tropicalis* allergens, as it did for another mites [26, 29–31, 34]. Interestingly, the major allergen of *D. pteronyssinus*, Der p 23, was discovered by immunoscreening of a cDNA library and classified as a peritrophin-like protein. The allergen was detected in high volumes on the peritrophic matrix of this mite’s fecal pellets [76]. *B. tropicalis* group 23 allergen has yet to be described and it is possible that this and other major mite allergen counterparts will only be acknowledged using such combined approaches, focusing also on the analysis of mite feces.

Another interesting evaluation of our study was the comparison of our in-house extracts with a commercially available product. Even though the key allergens of *B. tropicalis* were found in Bt-Allergen® extract, the percentage representation of major allergens, such as Blo t 5 and Blo t 21, were lower than in most of our in-house breeds. In fact, only 2 in-house breeds displayed lower levels of these 2 major allergens than the Bt-Allergen®

extract. Moreover, no allergen or isoform was unique to the Bt-Allergen® mites and we observed that our in-house breeds have mostly exhibited a more heterogeneous mixture of allergens than this commercially available extract. In fact, the Bt-Allergen® extract had more Blo t 2 than any breed extract, compounding 62.2% of the extract. However, this allergen IgE reactivity is not as high as Blo t 5 and Blo t 21 [6, 10, 54]. Pharmaceutical allergen preparations are variable in terms of the amount, presence, or complete absence of major allergens [20–22]. Since skin prick tests and immunotherapy for *B. tropicalis* as well as for other house dust mites have been typically based on natural allergen extracts [20–22], this discrepancy may have a direct influence in IgE reactivity of this Bt-Allergen® extract and raises important observations: (i) allergen standardization should not concentrate only on the safety aspect by determining the overall IgE-binding potency of the allergen extracts; (ii) tools that better quantify and identify allergens with clinical importance are needed – such tools include proteomics, ELISA kits with monoclonal antibodies against major allergens, or adoption of recombinant reference allergens; (iii) the allergen composition can indeed vary depending on culture conditions, extraction procedures, and storage conditions, since these procedures may change according to the company-specific production, and (iv) allergologists should keep in mind that genetic distances and polymorphisms among mites from different regions of the world may influence IgE responses [16, 20, 23, 24, 39, 40, 43, 77].

Our proteomics approach identified another possible mite culture issue, which should be considered for scientific and commercial purposes. Allergen content varied significantly from breed to breed in our in-house-generated lineages, although all breeds were cultivated in the same environmental conditions and the extracts were prepared from cultures in the stationary phase. Inbreeding can change gene expression to alleviate inbreeding depression, which is caused by an increase in the homozygosity of recessive deleterious alleles in the offspring, leading to a decline in survival, fecundity, growth, mating, and/or reproduction [78]. Although we did not implement a genetic model estimative of inbreeding depression, as previously carried out in genetic studies of in-bred acarids, the same studies addressed a relationship between gene regulation and inbreeding depression, showing up- and downregulation of certain proteins [78–80].

Two other genomic studies followed an approach similar to ours, using iso-female lines [25, 81]. In the case of the allergenic mite *D. pteronyssinus*, inbreeding led to the

production of new genetic variants of the three major allergens, Der p 1, Der p 2, and Der p 23, something that could change their IgE-binding behavior [81]. The group suggested, consequently, the possibility of using in-bred lines for industrial/pharmacological interests, focusing on the selection of in-bred lines according to a specific allergenic profile. Thus, inbreeding can produce: (i) reference in-bred lines for diagnosis purposes, (ii) reference breeds for immunotherapy, and (iii) reference stock colonies for general purpose research.

Despite our work focusing on allergen variation of iso-female lines, the culture medium can also influence allergen content [82], which represents a possible limitation of our study. However, in previous studies by our group and others, the content of Blo t 5 was usually high when this or similar culture medium was used [83–85].

In conclusion, the proteomic analysis undertaken in this work showed that Blo t 2 was the most prominent allergenic protein in many breeds and in Bt-Allergon[®], but the importance of this protein for mite biology and the reason for this high level of production will need further investigation. Since we have shown that inbreeding has had an evident impact on allergen content, the quantification and/or identification of allergens from in-bred lines should be routinely considered for establishing mite cultivation. To achieve this quality control of extracts, our results clearly strengthened the notion that MS can be a valuable tool to control the production of allergen extracts and, in combination with a mite inbreeding approach, breeds with a heterogeneous mixture of clinically relevant allergens could be used for commercial or research purposes, providing advances in diagnosis and immunotherapy of allergy caused by diverse mites, including *B. tropicalis*.

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Statement of Ethics

This research is not directly related to human or experimental animals; therefore, the authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicting interests to declare.

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Author Contributions

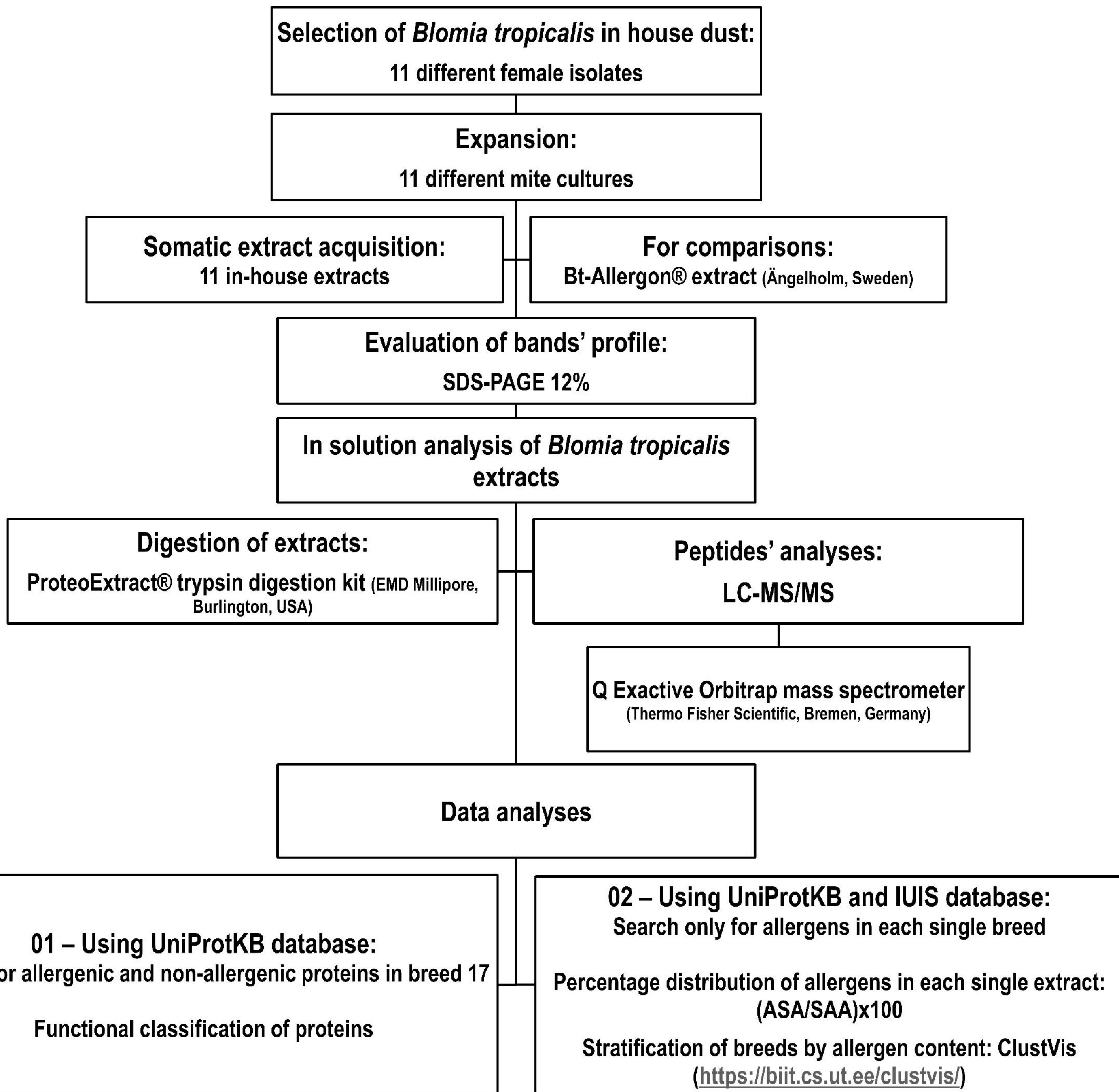
E.S.S. and J.C.M.P. carried out experiments and drafted the manuscript. M.B.S. participated in the analysis tool and helped to draft the manuscript. C.S.P. and L.G.C.P. participated in the design of the study. P.B. acquired and analyzed the MS data. N.M.A.-N., F.F., and P.B. conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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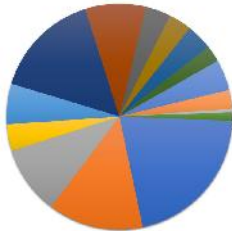


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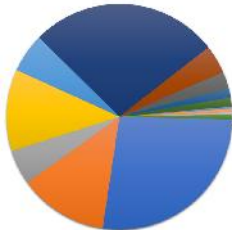
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Breed 10



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FULL-SCALE TESTS

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Abstract

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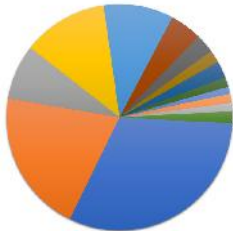
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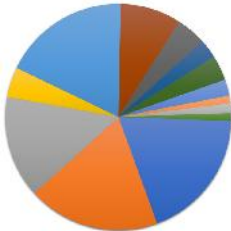
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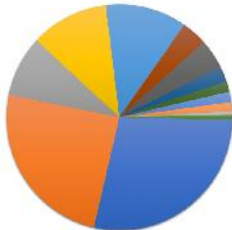
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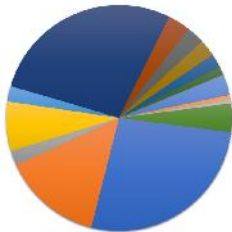
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- with 10.0% (100.0%) - 10.0%
- with 5.0% (100.0%) - 5.0%
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- with 20.0% (100.0%) - 20.0%

Breed 17



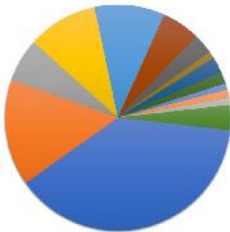
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Breed 18



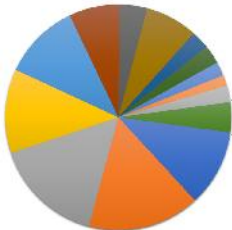
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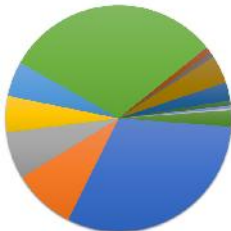
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Allergon



- Allergen 1: 35.2%
- Allergen 2: 28.7%
- Allergen 3: 15.1%
- Allergen 4: 12.3%
- Allergen 5: 3.8%
- Allergen 6: 2.5%
- Allergen 7: 1.9%
- Allergen 8: 1.2%
- Allergen 9: 0.8%
- Allergen 10: 0.5%
- Allergen 11: 0.3%
- Allergen 12: 0.2%
- Allergen 13: 0.1%
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- Allergen 17: 0.1%
- Allergen 18: 0.1%
- Allergen 19: 0.1%
- Allergen 20: 0.1%

Table S1 - Correlations of allergens expression in the in-house breed extracts and Bt-Allergen® extract

<i>Correlations (n=12)</i>	<i>Pearson r</i>	<i>p value</i>
Blo t 2* x Blo t 5	-0.7918	0.0021
Blo t 2* x Blo t 21	-0.2228	0.4863
Blo t 2* x Blo t 11	-0.0082	0.9799
Blo t 2* x Blot 13 [A1KXG9]	-0.5180	0.0845
Blo t 2* x Blo t aldehyde dehydrogenase allergen	-0.2051	0.5225
Blo t 5 x Blo t 21	0.6037	0.0377
Blo t 5 x Blo t 11	0.0751	0.8165
Blo t 5 x Blot 13 [A1KXG9]	0.2968	0.3488
Blo t 5 x Blo t aldehyde dehydrogenase allergen	0.0565	0.8616
Blo t 21 x Blo t 11	0.3460	0.2705
Blo t 21 x Blot 13 [A1KXG9]	0.2379	0.4565
Blo t 21 x Blo t aldehyde dehydrogenase allergen	0.1305	0.6860
Blo t 11 x Blot 13 [A1KXG9]	0.5862	0.0451
Blo t 11 x Blo t aldehyde dehydrogenase allergen	0.8444	0.0005
Blot 13 [A1KXG9] x Blo t aldehyde dehydrogenase allergen	0.7199	0.0083

All twelve extracts were taken into account for the analysis. Kolmogorov–Smirnov normality test was performed and, since the data was parametric, Pearson correlation coefficients were determined.

In bold significant correlations. $p < 0.05$ was considered significant.

* Only Blo t 2 isoform 1 was considered for the analysis