

Lep d 2 polymorphisms in wild and cultured *Lepidoglyphus destructor* mites

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We have previously cloned, expressed and characterized two variants of the major allergen Lep d 2 from cultured *Lepidoglyphus destructor* mites. These variants, Lep d 2.0101 and Lep d 2.0201, differ at 13 amino acid positions. In this study we investigated Lep d 2 sequence diversity between wild and cultured mites. PCR, Southern blot and DNA sequence analysis revealed the presence of two different Lep d 2 genes, one with and one without an intron. In addition, two new variants of Lep d 2, Lep d 2.0102 and Lep d 2.0202, were found at different frequencies in wild and cultured mites. When we expressed the Lep d 2 variants and compared their IgE binding properties by ELISA inhibition, we found that Lep d 2.0102 was a more potent inhibitor than Lep d 2.0101, and to a lesser extent Lep d 2.0202 was more potent than Lep d 2.0201. Long-

term cultures of peripheral blood mononuclear cells were used to assess the ability of the expressed Lep d 2 variants to induce cytokine release. Although cells from different individuals released different amounts of interferon- γ and interleukin-5, no consistent cytokine release pattern could be linked to any specific Lep d 2 variant. In conclusion, we show that both cultured and wild *Lepidoglyphus destructor* mites contain the same pattern of polymorphism. Furthermore, this Lep d 2 sequence diversity seems not to have any significant impact on the allergens IgE binding or its ability to induce T cell cytokine release.

Keywords: dust mite; *Lepidoglyphus destructor*; allergen; Lep d 2; polymorphism.

Traditionally, mites grown for years in culture have been the source of allergens for use in research, diagnostics and therapy. However, the actual source of mite sensitization is wild mites found in our environment. Therefore, it is important to study the possible differences between cultured and wild mites to assure adequate diagnosis and therapy. In this study we have investigated the occurrence of polymorphism in the dust mite *Lepidoglyphus destructor* (*L. destructor*) derived from different sources.

Polymorphisms in cultured and/or wild house dust mites *Dermatophagoides farinae* [1,2] and *Dermatophagoides pteronyssinus* [3,4] have been investigated previously. In these studies, genomic and cDNA sequences from group 2 allergens from cultured mites have shown the presence of missense mutations, resulting in three to five amino acid substitutions, and several silent mutations. Some of these mutations are also observed in mites taken straight from their natural environment. Compared to Der p 2, sequences

from Der p 1 clones show fewer base pair substitutions, but the base pair changes of Der p 1 more often lead to amino acid changes [4]. A recent study found no difference in the ability of four variants of Der p 2 to stimulate peripheral blood mononuclear cells (PBMC) in an *in vitro* proliferation assay [4]. On the other hand, peptides representing various Der p 1 T cell epitopes containing polymorphic residues differed in their ability to induce T cell proliferation.

L. destructor is a dust mite found both in rural [5–7] and urban [8] environments and has been shown to cause sensitization [9–14] and allergic disease [15–18]. Lep d 2, the major allergen of *L. destructor*, as well as other group 2 allergens from other dust mite species have been extensively investigated. Despite this, their function remains unknown. In a recent study of the crystal structure of the group two allergen Der p 2, a putative lipid binding cavity was found indicating that Der p 2 is a lipid binding protein [19]. We have previously cloned Lep d 2, the major allergen in *L. destructor*, using mites from a commercial source [20]. Lep d 2 was found as two isoforms, Lep d 2.01 and Lep d 2.02, differing in 13 amino acids and numerous nucleotides. Isoform Lep d 2.01 was found as two variants with identical amino acid sequence and differing only at the DNA level. In accordance with WHO/IUS allergen nomenclature [21], these were named Lep d 2.0101a accession no. X83876, formally Lep d 2.0101, and Lep d 2.0101b accession no. X89014, formally Lep d 2.0102. Lep d 2.02 was found only as one variant and named Lep d 2.0201 accession no. X83875. The two isoforms were later expressed as recombinant proteins, both in *Escherichia coli* and in a baculovirus expression system [22]. Subsequent studies showed that the IgE binding

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Abbreviations: IFN- γ , interferon- γ ; IL-5, interleukin 5; PBMC, peripheral blood mononuclear cells; RAST, radioallergosorbent test.

Note: The genomic sequences of Lep d 2.0102 and Lep d 2.0202 have been submitted to the EMBL nucleotide database and are available under the accession numbers AJ487972 and AJ487973, respectively.

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properties of the recombinant Lep d 2 isoforms from both expression systems were comparable to the native allergen as assessed by Western blot inhibition analysis [22]. In a histamine release assay the two expressed recombinant isoforms induced 70–84% of total histamine release compared to native Lep d 2, which induced close to 100% [22]. *In vivo* skin prick testing of 41 *L. destructor* sensitized individuals showed similar reactivity of Lep d 2.01 and Lep d 2.02 [23].

The objective of this study was to describe the genomic organization and polymorphisms of the major allergen Lep d 2 in wild and cultured *L. destructor* mites. In addition, the effects of polymorphisms on B and T cell epitopes were evaluated in inhibition ELISA and T cell cytokine release assays.

Materials and methods

Mites

Wild *L. destructor* mites were collected from a frozen hay dust sample from a farm on the Swedish island of Gotland. *L. destructor* mites were identified and isolated at the Swedish Museum of Natural History, Stockholm.

Cultured *L. destructor* mites were obtained from Allergon AB, Ängelholm, Sweden.

Human sera

Sera from six *L. destructor* allergic farmers from Gotland, who had previously participated in a study about respiratory diseases among farmers [16], were used in immunoblotting and ELISA inhibition experiments. The sera were analysed for specific IgE antibodies to *L. destructor* extract with the radioallergosorbent test® (RAST®) (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) (range: 5.0–94 PRU·mL⁻¹) and also to recombinant Lep d 2.0101 with Pharmacia CAP System™ Specific IgE FEIA (range: 6.1–88 kU·L⁻¹) [24]. One serum sample with a negative RAST® value to *L. destructor* was used as a negative control.

Peripheral blood mononuclear cells

PBMC were prepared from fresh whole blood drawn from six farmers from Gotland who had previously participated in an investigation regarding T cell responses to Lep d 2 [25]. All had positive skin prick test reactions to Lep d 2.0101, Lep d 2.0201 and to whole extract of

L. destructor [23]. PBMC were isolated by gradient centrifugation on Ficoll Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). The cells were kept at –140 °C until use.

Amplification, cloning and sequencing of genomic Lep d 2 DNA from wild and cultured mites

Genomic Lep d 2 DNA was amplified using PCR in a 50-μL reaction mixture containing one mite as DNA template, 20 mM Lep d 2 forward and Lep d 2 reverse primers (Table 1) (DNA Technologies A/S, Aarhus, Denmark), 0.4 mM dNTP (Amersham Pharmacia Biotech) and 5 μL 10 × *Pfu* PCR-buffer (Stratagene, La Jolla, CA, USA). After denaturation at 98 °C for 10 min, 2.5 U of *Pfu* polymerase (Stratagene) was added. Using a DNA Thermal Cycler 480 (Perkin Elmer, Foster City, CA, USA), 35 cycles of 94 °C 1 min, 50 °C 2 min and 72 °C 2 min 30 s were performed, the last cycle had its elongation step extended by 10 min. The PCR products were analysed by electrophoresis in 1.8% (w/v) agarose gels from which the amplified DNA was extracted by using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

The PCR products were cloned into pCR4-TOPO vector using TOPO TA Cloning Kit for Sequencing (Invitrogen, Groningen, the Netherlands), according to the manufacturer's protocol, and plasmids were transformed into *E. coli* TOP10 chemo-competent cells (Invitrogen). The recombinant clones were identified by restriction enzyme analysis of plasmid DNA isolated from the bacterial clones by using QIA spin Miniprep Kit (Qiagen). Sequencing of DNA was carried out on an ABI 377 Sequencer (Perkin Elmer) using an ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer), according to the manufacturer's instructions. M13 forward and M13 reverse primers (Table 1) (Invitrogen) were used to sequence both strands of the PCR products inserted into the pCR4-TOPO vector.

Genomic DNA extraction, Southern blot analysis and hybridization

DNA encoding Lep d 2 was amplified by PCR from one cultured mite as described above and used as a hybridization probe in Southern blot analysis. The PCR product was labelled with [³²P]dCTP using Ready To Go DNA Labelling Beads (-dCTP) (Amersham Pharmacia Biotech) and purified on ProbeQuant G-50 micro columns (Amersham

Table 1. Primers used for cloning, sequencing and mutagenesis.

Purpose	Primer	Nucleotide sequence in 5' to 3' orientation
PCR	Lep d 2 forward	ATGATGAAATTCATTGCTCT
	Lep d 2 reverse	TTCGACTTGTTTCGTGGA
Sequencing	M13 forward	GTAAAACGACGGCCAG
	M13 reverse	CAGGAAACAGCTATGAC
Site-directed mutagenesis	Lep d 2.01 forward A55T	CCATCAAGGTTTTGACCAAGGTTGCCGGTACC
	Lep d 2.01 reverse A55T	GGTACCGGCAACCTTTGGTCAAAACCTTGATGG
	Lep d 2.02 forward A102V	CCCCAAGATCAAGGTCGACGTCACCGCC
	Lep d 2.02 reverse A102V	GGCGGTGACGTCGACCTTGATCTTGGGG

Pharmacia Biotech) according to the manufacturer's protocols. High molecular mass genomic DNA was extracted from cultured *L. destructor* mites (Allergon AB) by using PUREGENE DNA isolation Kit (Gentra Systems, Minneapolis, MN, USA). Five micrograms of DNA were digested separately with the following restriction enzymes: *EcoRI*, *TseI*, *BsaI* and *BanI* (New England Biolab Inc., Beverly, MA, USA). The digestions were carried out overnight in 50 µL digestion mixtures at the temperature recommended by the manufacturer. DNA fragments were separated on a 0.8% (w/v) agarose gel containing ethidium bromide. Southern blot and hybridization were performed according to standard protocols [26] before autoradiography.

Site-directed mutagenesis

Site-directed mutagenesis was performed using primers (Table 1) designed to change one nucleotide in Lep d 2.0101 and Lep d 2.0201 resulting in codons found in Lep d 2.0102 and Lep d 2.0202, respectively. pET17b expression plasmids (Novagen, R&D Systems, Abingdon, UK) containing the full coding sequence of Lep d 2.0101 or Lep d 2.0201 cDNA, prepared earlier in our laboratory [22], were used as templates. Using QuickChange™ Site-Directed Mutagenesis Kit (Stratagene), a PCR amplification was performed according to the manufacturer's recommendations. The nucleotide exchange was confirmed by DNA sequencing, and plasmids containing the correct sequence were transformed into chemo-competent *E. coli* BL21(DE3) pLysS (Invitrogen) for expression.

Expression of recombinant proteins and immunoblotting

Recombinant proteins were expressed as C-terminal tagged hexahistidine fusion proteins using the pET-expression system and purified using metal chelate affinity chromatography as described previously [22], except that the proteins were eluted in 20 mM Tris/HCl pH 8.0, 100 mM NaCl containing 100 mM imidazole. The eluted recombinant proteins were dialysed against phosphate buffered saline (NaCl/P_i) and the concentrations were determined by total amino acid composition analysis. The analyses were carried out using a Biochrom 20 Plus ninhydrin-based analyser (Amersham Pharmacia Biotech) after hydrolysis at 110 °C for 24 h in evacuated tubes with 6.0 M HCl containing 0.5% (w/v) phenol. The recombinant proteins were subjected to SDS/PAGE and electroblotted onto a poly(vinylidene fluoride) membrane. Immunodetection with human sera was performed as described previously [27].

ELISA inhibition

IgE binding inhibition analysis was performed in 96-well ELISA plates. The wells were coated with Lep d 2.0101, Lep d 2.0102, Lep d 2.0201 or Lep d 2.0202, 10 µg·mL⁻¹ in carbonate buffer pH 9.6 overnight at 4 °C. All further incubations were performed at room temperature with washes between them with NaCl/P_i containing 0.05% (v/v) Tween 20. The wells were blocked for 2 h in 1.0% (w/v) BSA followed by a 2-h incubation with sera positive in

immuno-blotting to the Lep d 2 variants. The sera were pre-incubated for 2 h with 10-fold dilutions, 10–0.001 µg·mL⁻¹, of Lep d 2.0101, Lep d 2.0102, Lep d 2.0201, Lep d 2.0202 or with diluent only. The wells were then sequentially incubated with rabbit anti-human IgE (MIAB, Uppsala, Sweden) for 2 h and alkaline phosphatase conjugated goat anti-rabbit IgG (DAKO, Glostrup, Denmark) for 1 h. Finally, a 1.5-h incubation with substrate (*p*-nitrophenyl phosphate disodium) (Sigma Diagn., St Louis, MO, USA) was performed in the dark, where after the absorbance was measured at 405 nm. Inhibition values were calculated by using the following formula: % inhibition = 100–100(A/B), where A is the absorbance value obtained for a serum incubated with allergen and B is the value for the same serum incubated with diluent.

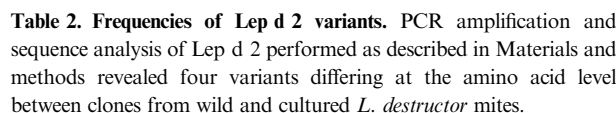
T cell responses

Cytokine release by PBMC in response to the four Lep d 2 variants Lep d 2.0101, Lep d 2.0102, Lep d 2.0201 and Lep d 2.0202, was assayed as described elsewhere [25]. Briefly, PBMC were cultured in Iscove's modified Dulbecco's medium supplemented with 5% pooled heat inactivated AB + serum, 25 µg·mL⁻¹ gentamicin, 2 mM L-glutamine, 100 IU·mL⁻¹ penicillin, 100 µg·mL⁻¹ streptomycin and 50 µM 2-mercaptoethanol. The allergens were purified from endotoxins using Affi-Prep Polymyxin Matrix (Bio-Rad, Richmond, CA, USA). PBMC cultures, 2.5 × 10⁶ cells per well in 2.5 mL, were set up in 12-well culture plates. The cells were stimulated with 5 µg·mL⁻¹ allergen at day zero and were further stimulated with interleukin 2 (20 U·mL⁻¹) at days 5 and 8 of culture. Supernatants were collected at day 11 and kept at –20 °C until assessment. Interferon γ (IFN-γ) and interleukin 5 (IL-5) were measured by ELISA (IFN-γ, MabTech AB, Stockholm, Sweden; IL-5, PharMingen Research Products, San Diego, CA, USA) as described in detail elsewhere [28] except that blocking was performed in NaCl/P_i containing 1.0% BSA. As standards IFN-γ and IL-5 from MabTech were used. The detection limit was for IFN-γ 0.03 pg·mL⁻¹ and for IL-5 0.06 pg·mL⁻¹.

Results

PCR amplification, cloning and sequencing of *L. destructor* genomic DNA encoding Lep d 2

Eight cultured mites from a commercial source and 10 wild mites from a hay dust sample were used as templates to amplify genomic Lep d 2 DNA. In each PCR reaction, a single mite was used as template to amplify the complete coding sequence of the Lep d 2 gene. Two PCR products, 400 and 480 bp in size, were obtained from each PCR reaction using both wild and cultured mites as template (data not shown). The 400 bp band corresponds well to the size of the known Lep d 2 cDNA [20]. Both PCR products were subsequently ligated into a cloning vector and sequenced. Sequence analysis revealed that the 400 bp PCR products were similar to the known Lep d 2 cDNA sequence [20]. Sequence analysis of clones containing the 480 bp PCR products revealed the presence of 76 and 75 nucleotides interrupting the Lep d 2.01 and Lep d 2.02



	Lep d 2.0101	Lep d 2.0102	Lep d 2.0201	Lep d 2.0202
Wild <i>L. destructor</i>	10	7	9	6
Cultured <i>L. destructor</i>	7	7	8	1

nomenclature rules [21]. Figure 2 shows the relationship between the different variants. More than one variant was often identified from a single mite. For the Lep d 2.01 isoform, variant Lep d 2.0101 was found in all the 10 mites while Lep d 2.0102 was found in seven out of 10 mites analysed. The distribution of isoform Lep d 2.02 variants showed that Lep d 2.0201 was present in nine out of the 10 mites and Lep d 2.0202 in six (Table 2). In addition to these variants with amino acid substitutions, several silent mutations were found in almost all sequences investigated.

DNA sequences of Lep d 2 in cultured mites

Sequences from six to eight clones originating from each of eight cultured mites from a commercial source resembled those from wild mites regarding the distribution of Lep d 2.0101- and Lep d 2.0201-like clones. The same patterns of base pair changes were seen in the cultured mites as in the wild mites. However, the frequencies of the amino acid substitutions were different. The variants Lep d 2.0101 and Lep d 2.0102 were both found in seven out of eight mites. Lep d 2.0201 was found in all eight mites and Lep d 2.0202 in one (Table 2).

Southern blot hybridization

Data from sequencing, showing the presence of Lep d 2 with and without an intron, indicated that multiple copies of the Lep d 2 gene are present in the *L. destructor* genome. To investigate this possibility, Southern blot analysis was performed on genomic *L. destructor* DNA. The DNA was digested with each of four enzymes: *EcoRI* recognizing no restriction site, *TseI* and *BsaI* recognizing single and *BanI* recognizing two restriction sites in the Lep d 2 complete DNA sequence. DNA encoding the full open reading frame of Lep d 2 was PCR amplified, [³²P]dCTP labelled and used as a probe. Hybridization of the probe with *EcoRI*-cleaved DNA gave rise to two bands (Fig. 3), and at least three bands were seen when the DNA was cleaved with *TseI*, *BsaI* or *BanI* (Fig. 3). These data support the idea that there is more than one copy of the Lep d 2 gene at different loci in the *L. destructor* genome.

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graph LR
    A[Lep d 2] --> B[Lep d 2.01]
    A --> C[Lep d 2.02]
    B --> D[Lep d 2.0101a/b]
    B --> E[Lep d 2.0102]
    C --> F[Lep d 2.0201]
    C --> G[Lep d 2.0202]
  
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The four main Lep d 2 variants, Lep d 2.0101, Lep d 2.0102, Lep d 2.0201 and Lep d 2.0202 were expressed as recombinant proteins and subjected to immunoblotting experiments in order to verify an IgE binding ability. All variants were recognized by six different sera

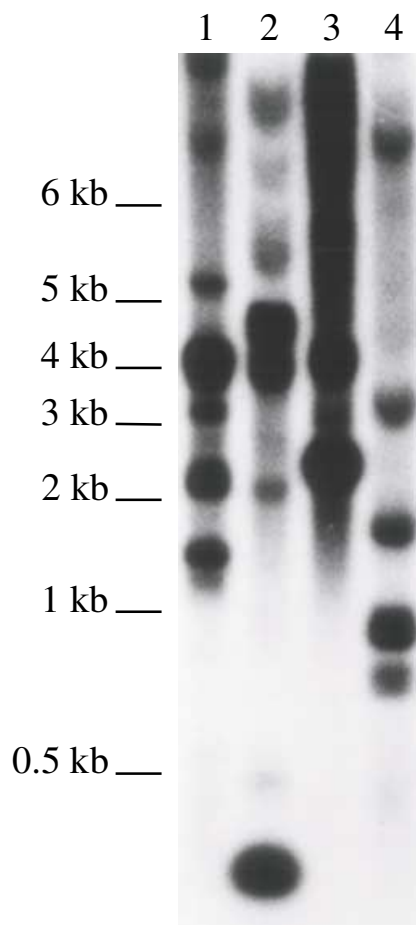


Fig. 3. Southern blot analysis of Lep d 2. Genomic *L. destructor* DNA digested with each of four enzymes: *EcoRI* recognizing no restriction site, *TseI* and *BsaI* recognizing single and *BamI* recognizing two restriction sites in the Lep d 2 complete DNA. After electrophoresis the DNA was transferred to a membrane and hybridized with [³²P]dCTP labelled, PCR amplified DNA encoding Lep d 2. Lane 1, *BsaI*; lane 2, *BamI*; lane 3, *EcoRI*; lane 4, *TseI*.

from subjects allergic to *L. destructor* (data not shown). An *L. destructor* negative control serum detected none of the variants.

ELISA inhibition

To investigate if the Lep d 2 variants found in cultured and wild mites have different IgE binding properties, we performed ELISA inhibition with the six sera positive to all variants in immunoblotting. A dose-dependent inhibition was observed with all variants in all six sera. The IgE binding capacity for the two isoforms was evaluated separately. In all experiments 100% inhibition was reached. Figure 4 shows the inhibition results obtained with two of the six sera using the two variants of the isoform Lep d 2.01. For both sera, a lower concentration of the variant Lep d 2.0102 was needed to reach 50% inhibition compared to the variant Lep d 2.0101 regardless of whether homologous or heterologous inhibition was performed. Similar results were obtained with the isoform

Lep d 2.02, where a lower concentration of the variant Lep d 2.0202 was needed to reach 50% inhibition compared to the Lep d 2.0201 variant, although the difference was less pronounced. To evaluate the overall inhibition results obtained with all six sera, we compared the concentrations of the different Lep d 2 variants needed for 50% IgE binding inhibition in each serum (Table 3). The same pattern of inhibition could be seen with a lower concentration needed of the variants Lep d 2.0102 and Lep d 2.0202 compared to the variants Lep d 2.0101 and Lep d 2.0201, respectively.

T cell responses

Interferon- γ and IL-5 were measured in culture supernatants from long-term PBMC cultures stimulated with Lep d 2.0101, Lep d 2.0102, Lep d 2.0201 or Lep d 2.0202. Although there were differences in the amounts of IFN- γ and IL-5 released by PBMC from individual patients, no consistent pattern of difference was found between the variants from either isoform, Lep d 2.0101 vs. Lep d 2.0102 or Lep d 2.0201 vs. Lep d 2.0202 in the six subjects investigated. However, the IFN- γ levels after stimulation with the variants from the Lep d 2.02 isoform (median 16.3 ng·mL⁻¹; range 7.57–105) were slightly higher compared to after stimulation with the Lep d 2.01 variants (median 14.1 ng·mL⁻¹; range 3.08–80.0). The levels for IL-5 in response to the Lep d 2.01 isoform (median 2.75 ng·mL⁻¹; range 0.42–6.05) was similar to Lep d 2.02 isoform (median 2.23 ng·mL⁻¹; range 0.53–3.93).

Discussion

It is well known that several dust mite allergens exist as different isoforms due to polymorphisms in the genes [30]. Differences between allergens from cultured and wild mites have also been reported [3,4]. In routine diagnostics, extracts from cultured *L. destructor* mites are used to detect sensitized individuals. However, it is not cultured mites that cause sensitization, but the wild mites found in our environment. Therefore, we decided to study polymorphism in the Lep d 2 gene of wild and cultured mites and what influence polymorphic residues might have on IgE binding and T cell responses.

In analogy with results obtained from sequence analyses of Der p 2 [4], sequencing of the Lep d 2 gene revealed several silent mutations but only a few mutations resulted in amino acid substitutions. Furthermore, all analysed clones were highly homologous to the previously published Lep d 2 cDNA sequences [20], either to Lep d 2.0101 or Lep d 2.0201. These results indicate the evolutionary divergence of two sequences corresponding to two main isoforms, a pattern similar to what has been found earlier for Der p 2 [4]. Our data show that the two most common substitutions of each isoform were found at positions 55 and 102 in clones otherwise identical to Lep d 2.0101 and Lep d 2.0201, respectively. Both substitutions were the result of single nucleotide exchanges. The possibility that these substitutions represent PCR artefacts is not likely, because they were found in several clones from different mites and different PCR amplifications. Moreover, we used *Pfu* polymerase that has a 3' to 5' exonuclease activity

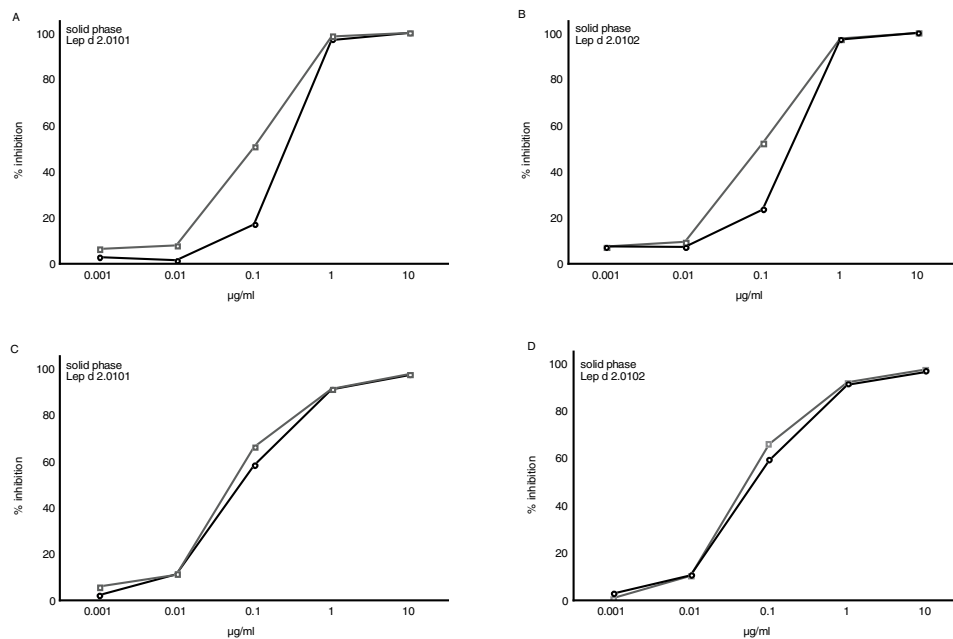


Fig. 4. ELISA inhibition. ELISA inhibition of IgE-binding to Lep d 2.0101 (A and C), and Lep d 2.0102 (B and D) on solid phase. Inhibition curves obtained with Lep d 2.0101 (○), Lep d 2.0102 (□) as indicated in the figure. Serum no. 6 was used in A and B and serum no. 4 in C and D.

Table 3. Concentrations ($\mu\text{g mL}^{-1}$) of the inhibiting allergen needed to reach 50% inhibition of IgE binding. ELISA inhibition was performed as described in Materials and methods. Concentrations of the inhibiting allergen needed to reach 50% inhibition of IgE binding to Lep d 2.0101 and Lep d 2.0102 and to Lep d 2.0201 and Lep d 2.0202 in *L. destructor* positive sera.

Serum no.	Lep d 2.0101 solid phase		Lep d 2.0102 solid phase		Lep d 2.0201 solid phase		Lep d 2.0202 solid phase	
	Lep d 2.0101	Lep d 2.0102	Lep d 2.0101	Lep d 2.0102	Lep d 2.0201	Lep d 2.0202	Lep d 2.0201	Lep d 2.0202
1	0.029	0.006	0.020	0.007	0.014	0.004	0.008	0.004
2	0.100	0.045	0.081	0.048	0.055	0.043	0.051	0.043
3	0.195	0.090	0.206	0.215	0.058	0.047	0.055	0.047
4	0.085	0.074	0.083	0.074	0.079	0.064	0.085	0.070
5	0.238	0.062	0.206	0.064	0.047	0.029	0.047	0.030
6	0.471	0.091	0.424	0.095	0.060	0.049	0.062	0.051

resulting in a low error rate. The source of the commercially available mites used in this study are cultured mites that have been grown isolated for years without introducing new mites from other sources (A. Anderson, Allergon AB, Ångelholm, Sweden, personal communication). This could explain the disparate frequency of the variants found in cultured and wild mites.

Two amplified Lep d 2 sequences were obtained in PCR amplifications with single mites as templates, and sequence analysis revealed the presence of a small intron in one of the amplified Lep d 2 PCR products. The size of the intron correlates well with introns previously reported in Der p 2 (80–83 bp) [3] and Der f 2 (87 bp) [2]. This finding indicates the presence of more than one copy of the Lep d 2 gene at different loci in the genome and was further supported by Southern blot hybridization experiments. In contrast to Der f 2, for which there is only a single gene in the genome [2], the substitutions found in the Lep d 2 gene are probably not only due to polymorphisms within the Lep d 2 gene, but also to multiple copies of the gene in individual mites.

The new Lep d 2 variants, Lep d 2.0102 and Lep d 2.0202 identified in the present study, were expressed as recombinant proteins and their IgE binding capacity evaluated by ELISA inhibition and compared to those of Lep d 2.0101 and Lep d 2.0201. The results did not reveal any major differences in IgE binding capacity between the variants. However, we found that Lep d 2.0102 inhibited the binding to Lep d 2.0101 to a somewhat higher degree than what was obtained with Lep d 2.0101. A possible explanation for this finding could be that IgE antibodies show higher avidity to Lep d 2.0102 than to Lep d 2.0101. Similar results were obtained with isoform Lep d 2.02 where Lep d 2.0202 was found to be slightly more effective as inhibitor than Lep d 2.0201. Studies of antibody epitopes in Der p 2, have shown that residues at position 55 and 102 are within the predicted epitopes and could be important in IgE binding [31]. The crystal structure of Der p 2 has been used in standard homology modelling to predict the secondary and tertiary structure of Lep d 2.0101 [32]. According to this model, amino acids

55 and 102 are buried inside the protein core of Lep d 2 and not exposed on the surface of the molecule (D. Benjamin, The Asthma and Allergic Disease Center, University of Virginia, VA, USA, personal communication). The difference in IgE binding can not be caused by direct antibody interaction of the side chains of the variable amino acid residues; rather it must be caused by more subtle changes in the tertiary structure.

The levels of two cytokines were measured in long-term PBMC cultures after stimulation with the four Lep d 2 variants. The cytokines measured were IFN- γ , a typical Th1 cytokine, and IL-5, a cytokine present in allergic inflammation. No consistent differences were found between the different variants. Using peptides that represent all parts of the mature Lep d 2, a previous study has shown that Lep d 2 contains two immuno-dominant regions spanning amino acids 11–25 and 61–75 [25]. The fact that we did not see any difference between the Lep d 2 variants differing at position 55 and 102 is therefore not surprising. However, individual differences between patients in cytokine responses could be detected.

In this study, we discovered new variants of Lep d 2 and found differences in the frequency of the variants between wild and cultured mites. In addition, our data suggest the presence of two genes, one with and one without an intron, encoding Lep d 2. After analysing the importance of these differences regarding IgE binding and T cell responses *in vitro*, we found that the differences between wild and cultured mites have no major impact on the allergenicity of Lep d 2. We can thus conclude that the commercially available cultured mites used in diagnostics and research today show the same general pattern of polymorphism in the Lep d 2 gene as the mites that are found in the environment and cause sensitization, at least in Sweden. Whether this holds true for other allergens in *L. destructor* and for mites from other geographical locations remains to be investigated.

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