# Setae from the pine processionary moth (*Thaumetopoea pityocampa*) contain several relevant allergens

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## Summary

**Background.** Pine processionary larvae produce urticating hairs (setae) that serve for protection against predators. Setae induce cutaneous reactions in animals and humans. The presence of toxic or allergic mechanisms is a matter of debate.

**Objectives.** To detect the presence of allergens in setae and to characterize them.

**Materials and methods.** Setae extracts were characterized by gel staining and immunoblot, with sera from patients with immediate reactions and positive prick test reactions, as well as a rabbit antiserum raised against setae. Setae proteins were fractionated by high-performance liquid chromatography. The most relevant allergen was analysed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS), and its sequence was deduced from an expressed sequence tag bank.

**Results.** Setae contained at least seven different allergens. The most intense detection corresponded to a protein of MW ~ 14 000 that was similar to thaumetopoein, a previously described protein with mast cell-degranulating properties. MALDI-MS-based *de novo* sequencing provided a partial amino acid sequence different from that of the previously described allergen Tha p 1, and it was named Tha p 2. This allergen was detected in 61% of patients, and it is therefore a new major caterpillar allergen.

**Conclusions.** Penetration of the setae from the pine processionary caterpillar delivers their allergenic content in addition to causing mechanical or toxic injury.

**Key words:** allergen; allergy; caterpillar; HPLC; immunoblot; MALDI-MS; setae; *Thaumetopoea pityocampa.*

Lepidoptera are among the most common insects, with ~175 000 described species worldwide (1). Accidental contact with the hairs/bristles of some larvae and adult moths induces symptoms that range from mild cutaneous lesions to severe systemic reactions, depending on the species involved, the severity of the contact, and the physical condition of the victim (2). Larvae of the genus *Thaumetopoea* (Lepidoptera: Nodontidae), commonly known as 'processionary moths', are found in Europe, and close relatives are also found in Australia, Asia, and Africa (1). *Thaumetopoea pityocampa* [pine processionary moth (PPM)] and *Thaumetopoea processionea* (oak processionary moth) seem to be most relevant sensitizers in Europe (3–9). To protect themselves against natural enemies, processionary larvae have evolved an urticating apparatus. The teguments of larvae at the fourth (L4) and fifth (L5) instar stages have different types of hair-like

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material: non-removable 'true' hairs, and removable setae disposed on cuticular plates (called mirrors) on the dorsal surfaces of the first eight abdominal segments (10). The urticating symptoms generally appear after contact with mature larvae, either alive or dead, and with airborne material produced by them (3, 10, 11). The usual reactions to processionary larvae appear on the areas contacted, but systemic manifestations, such as anaphylactic shock, have also been described (12–14); this suggests that an IgE-mediated mechanism can be operative, at least in some subjects. However, the presence of an immune or toxic mechanism is still a question of debate (15–21).

The sensitizing capacity of insects has been previously reported (22, 23). Allergic reactions after contact with biting or stinging insects are easy to understand, as the injection of a small amount of allergen can induce specific immune responses. However, the way in which processionary larvae can trigger immune responses remains unknown. Specific IgE in response to a crude extract of L5 larvae has been measured, and the agreement between symptoms and positive prick test reactions and/or *in vitro* determinations suggested that some of the allergens present in a crude larval extract are responsible for the allergic manifestations (15–21).

The aim of this work was to determine whether larval setae contain allergens and the different response patterns of the sera from subjects with clinical symptoms and positive prick test reactions. The present work shows that the setae of the pine processionary larva contain different allergens that can be released on contact with the larva.

## Patients, Materials and Methods

### Patients and prick tests

Eighteen patients from an area highly infested with processionary moths near Valladolid (Spain) showing immediate reactions to pine processionary larvae and positive prick test reactions were included. Prick tests were performed with a whole body extract and a setae extract, both from L5 larvae (Bial-Aristegui Laboratory, Bilbao, Spain). The extracts were tested on 10 non-exposed subjects (5 atopic and 5 non-atopic) as controls. Histamine dihydrochloride (10 mg/ml) and sterile 0.9% saline were used, respectively, as positive and negative controls. The response was considered to be positive if the mean wheal area was $>7$ mm$^2$ (wheal diameter $>3$ mm) higher than the negative control, measured 15 min after puncture. The prick response area was compared between larval and setae extracts for each patient, by use of a non-parametric Wilcoxon sign rank test. This study was approved by the Ethics Committee of the Hospital Universitario Río Hortega (Valladolid, Spain), and all patients included were asked to provide written informed consent.

### Setae collection

Colonies of *T. pityocampa* were collected in February 2009 from *Pinus nigra* trees in Tregnago (Verona, Italy), when all of the larvae were inside the nest, and immediately frozen at $-20^{\circ}$C. The nests were then opened in a hood, and the L5 larvae, still frozen, were transferred into vials in groups of 10. The vials were kept at $-20^{\circ}$C until being used for setae extraction. To prepare a sample of setae, a vial was taken inside a hood, and the setae of each larva were manually removed with forceps under a stereomicroscope with $\times$ 40 magnification. Care was taken to collect only the setae from the urticating apparatus, avoiding any contamination with true hairs and integument wounding. The best results in detaching the setae were obtained with partially thawed larvae.

### Protein extraction from the setae and crude larval extract preparation

The setae (0.6 g) were mixed with 0.6 ml of phosphate-buffered saline (PBS) and sonicated on ice for 10 seconds at 18 W. The resulting mixture was centrifuged for 10 min at 16 000 *g*. The supernatant was kept at $-20^{\circ}$C until being used. A crude larval extract was obtained as previously described (21).

### Antiserum production

An anti-setae antiserum was obtained by immunizing rabbits with a whole setae extract sent to Biomedal SL (Seville, Spain). This immunogen was obtained by washing 1.2 g of setae twice in PBS (2 ml) and sonicating the pellet with 2 ml of PBS. The slurry was allowed to settle for 5 min, for the removal of large debris. Finally, the supernatant obtained was aspirated with a 1-ml pipette with a standard tip, and the aspirated slurry was transferred to a new tube. Rabbits were immunized four times during a 2-month period.

### Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting

Electrophoresis was carried out on 16% Tris-tricine minigels, according to standard procedures. After electrophoresis, proteins were transferred to nitrocellulose membranes by overnight diffusion, and the membranes were incubated with the anti-setae rabbit antiserum or the sera from the 18 patients shown in Table 1 and the

**Table 1.** Clinical data of the patients

| Patient | Sex | Age (years) | Atopy status | Symptoms | Cutaneous lesion distribution | Crude larval prick area ($mm^2$) | Setae prick area ($mm^2$) |
|---|---|---|---|---|---|---|---|
| 1 | M | 27 | Atopic dermatitis | AU, CU, E | Neck, face, extremities | 50 | 28 |
| 2 | M | 43 | R, C, A (pollens, epithelia) | AU | Neck, face, extremities | 13 | 50 |
| 3 | M | 14 | — | AX (AU, CPD, E, D) | Neck, face, extremities | 20 | NT |
| 4 | F | 20 | R, C, A (pollens, epithelia), food allergy | AX (AU, E, C, R, D) | Neck, extremities, face, trunk | 20 | 28.3 |
| 5 | F | 32 | — | AU, E | Neck, extremities, face, trunk | 154 | 38.5 |
| 6 | M | 16 | — | AU, E | Neck, extremities, face, trunk | 113 | 133 |
| 7 | M | 20 | R, C (pollens, fungi, mites) | AU | Extremities | 13 | 28 |
| 8 | M | 38 | R, C (pollens, epithelia), food allergy | AU, APD, E | Neck, face, extremities | 13 | 13 |
| 9 | M | 27 | R, C, A (epithelia, pollens, mites), food allergy | AU | Extremities, trunk | 177 | 7 |
| 10 | F | 27 | — | AU, CU, E | Neck | 28 | 113 |
| 11 | F | 65 | R, C (pollens) | AU | Neck | 20 | 29 |
| 12 | F | 64 | — | AU, E | Neck, extremities, face, trunk | 20 | 50 |
| 13 | F | 21 | R, C (pollens, epithelia) | AU, E | Neck, face, extremities | 20 | 50 |
| 14 | M | 44 | R, C (pollens, mites) | AU, E | Neck, face, extremities | 13 | 20 |
| 15 | M | 34 | R, C (pollens) | AU | Neck, extremities | 28 | 64 |
| 16 | M | 37 | R, C (pollens, mites) | AU, C | Neck, trunk, extremities | 7 | 20 |
| 17 | M | 73 | R, C, A (pollens, mites), food allergy | AU, CU, E, C | Neck, face, extremities | 64 | 20 |
| 18 | M | 44 | — | AU, CU | Neck, trunk, extremities | 0 | 38.5 |

A, asthma; APD, airborne papular dermatitis; AU, airborne urticaria; AX, anaphylaxis; C, conjunctivitis; CPD, contact papular dermatitis; CU, contact urticaria; D, dyspnoea; E, oedema; NT, not tested; R, rhinitis.

corresponding antisera (anti-rabbit IgG, anti-human IgE, or anti-human IgG), according to previously published methods (24, 25). Sera from 2 non-atopic patients were used as negative controls.

### High-performance liquid chromatography (HPLC)

The setae extract (0.5 ml) was separated by reversed phase (RP)-HPLC on a Europa Protein 300 C4 column (20 × 0.46 cm; particle size 5 μm; Teknokroma, Barcelona, Spain). Sample injection and elution were performed with an Agilent 1200 Series liquid chromatograph (Agilent Technologies, Inc., CA, USA). Elution was carried out with a linear gradient of acetonitrile in 0.1% (vol/vol) trifluoroacetic acid (TFA) (0–5% for 10 min and 5% to 100% for 60 min, at a flow rate of 1 ml/min). Each fraction was tested for IgE binding by immunoblotting with a pool of positive sera.

### Protein digestion and mass spectrometry (MS)

Fractions 44, 45 and 48 from the RP-HPLC separation were digested with modified porcine trypsin (sequencing grade; Promega, Madison, WI, USA) at a final concentration of 300 ng/μl at 37°C for 4 h. The digestion solutions were mixed with an appropriate matrix solution, and the mixture was deposited onto a prestructured matrix-assisted laser desorption/ionization (MALDI) probe (Bruker Daltonik, Fremont, CA, USA) (26) and allowed to dry at room temperature. Samples were analysed in an Ultraflex MALDI time-of-flight (TOF)/TOF mass spectrometer (Bruker Daltonik) to obtain the corresponding MALDI-MS and MALDI-MS/MS spectra, as described previously (27).

### Database searching and *de novo* sequencing

MALDI-MS and MALDI-MS/MS data were combined by use of the BIOTOOLS 3.0 program (Bruker Daltonik) to search the National Center for Biotechnology Information (NCBI) non-redundant protein sequence database with MASCOT software (Matrix Science, London, UK) (28). Manual *de novo* sequencing was performed on the basis of MALDI-MS/MS spectra from selected peptides (29). The peptide sequences thus obtained were submitted to the BLAST search algorithm at the NCBI.

### Peptide N-terminal sulfonation and *de novo* sequencing

Derivatization with 4-sulfophenyl isothiocyanate (SPITC) (Sigma Chemical, St Louis, MO, USA) of peptides from the digestion solutions of fractions 44, 45 and 48 from

the RP-HPLC separation was performed according to the method described by Wang et al. (30). Briefly, the above-described digestion solutions were incubated with 10 mg/ml SPITC in 20 mM sodium bicarbonate (Sigma Chemical) for 1 hr at 55°C. The reaction was terminated by adding a small volume of 5% TFA. The resulting solution was vacuum-dried and resuspended in 20 µl of 0.1% TFA for clean-up with home-made reversed-phase columns (31) prior to MALDI analysis. Manual *de novo* sequencing was performed on the basis of MALDI-MS/MS spectra from selected derivatized peptides (29).

### Tha p 2 cloning

The peptide sequences obtained by MALDI-MS *de novo* sequencing were compared with the PPM expressed sequence tag (EST) library stored at Centre de Biologie et Gestion de Populations (INRA Montpellier, France).

Polymerase chain reaction (PCR) primers TP2 DF (5′-GTCCCGCAACTAAGTGAGAAAGC-3′) and TP2 DR (5′-TTACTAGGCCGAACAAGGACC-3′) were designed on the basis of the sequence of the mature protein (without the signal peptide) obtained from the translated EST sequence that matched the Tha p 2 peptides. PCR conditions were: 94°C for 1 min; 10 cycles of 94°C for 30 seconds, 70°C, decreasing by 1°C/cycle, for 30 seconds, and 72°C for 30 seconds; 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; and a final elongation step of 72°C for 5 min. PCR products were cloned in the vector pGEM T easy (Promega) and sequenced.

## Results

### Characterization of the patients' immune response

Table 1 shows the clinical data and Fig. 1 shows the IgE recognition patterns of 18 patients whose serum samples were used. There was no statistical difference in wheal size between the two extracts (Wilcoxon test; $p = 0.11$). No positive prick test reactions were found in the control group.

A high proportion of the positive prick test sera recognized allergens in the crude extract (Fig. 1). The most frequently detected allergen in the crude larval extract was a protein of MW 15 000 that could correspond to the major allergen Tha p 1 previously described by our group (21). Patients' sera also detected at least seven different allergens in the setae extract, with an allergen of MW ~14 000 being the most frequently recognized (11/18). The subjects who were positive for the crude larval extract were also positive for setae, with the exception of patient 9. This serum strongly detected several high

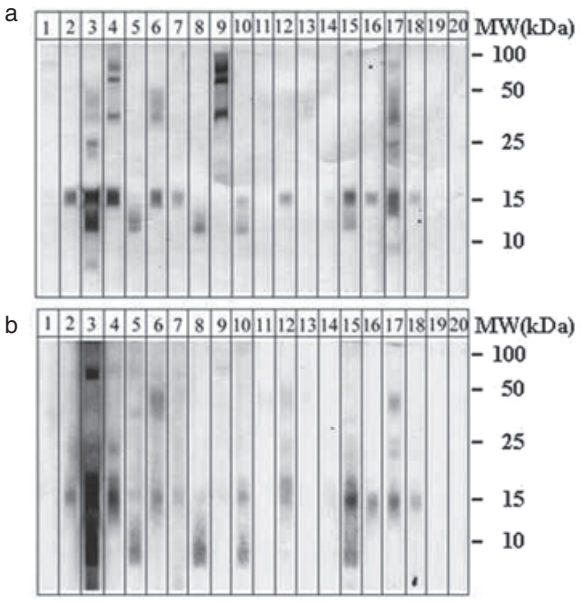


**Fig. 1**. IgE immunoblot of the 18 positive sera from the patients shown in Table 1 studied in the same order as presented in the table, with a crude larval extract (a) and the setae extract (b). Two unrelated human sera were used as controls (numbers 19 and 20).

molecular weight allergens in the crude extract, but none in the setae extract. This patient had a strong positive prick test reaction when the crude larval extract was tested, but gave negative results with the setae extract (Table 1).

Detection of specific IgG to proteins present in both the crude larvae and setae extracts was negative, with exception of serum 3, which was strongly positive to an antigen with a molecular weight similar to that of Tha p 1 (Fig. 2). This patient had a long history of severe reactions after exposure to larvae, some of them requiring treatment in emergency units.

### Fractionation by HPLC

RP-HPLC separated at least 70 fractions from the setae extract used. After lyophilization, all of the fractions were resuspended in 100 µl of PBS, and studied by immunoblotting with the rabbit antiserum and human sera as sources of IgE antibodies. Figure 3a shows the protein profile of the fractions with the highest $A_{280\ nm}$. The rabbit antiserum detected the presence of antigenic proteins mainly in three different peaks (fractions 44, 45, and 48) with the same molecular weight (Fig 3b). Two other proteins with slightly higher molecular weights were detected at lower intensity. Use of the sera from sensitized patients (Fig. 3c) showed that all of the proteins detected by the

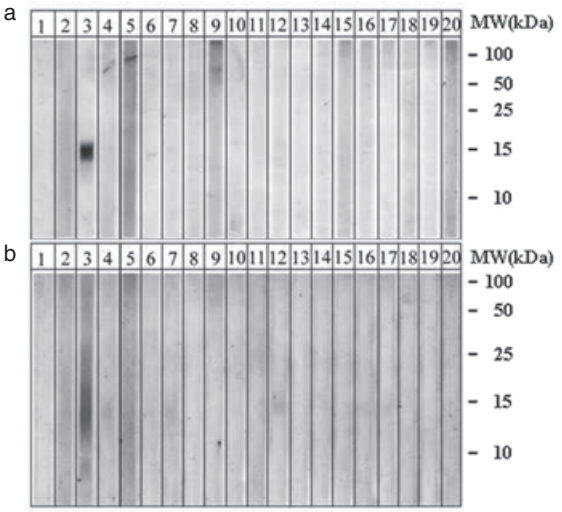


**Fig. 2**. IgG immunoblot of the 18 positive sera from the patients shown in Table 1 studied in the same order as presented in the table, with a crude larval extract (a) and the setae extract (b). Two unrelated human sera were used as controls (numbers 19 and 20).

rabbit anti-setae antiserum were, in fact, allergens. In addition, two other proteins of lower molecular weight bound specific IgE from the serum pool, and must be considered as allergens present in setae.

### MALDI-MS-based *de novo* sequencing

Given that the MASCOT searches performed with combined MALDI-MS and MALDI-MS/MS data obtained from the tryptic digests of fractions 44, 45 and 48 (Fig. 3) failed to assign any statistically significant peptide or protein matches, a manual *de novo* sequencing approach based on MALDI-MS/MS data was performed. This produced the putative sequences NNLFNLGSVAGDILSR at a mass-to-charge ratio ($m/z$) of 1689.892, KAEEAIDL TYQEK at $m/z = 1537.79$, SYSQSYSYVQCTQDSEC NGCWK at $m/z = 2737.08$, and DGCHVSFGCHK at $m/z = 1303.53$.

As these sequences showed no significant similarity to Tha p 1 or any other protein by BLAST, the digested fractions were derivatized with SPITC, which enabled confirmation of the $m/z$ 1689.892 sequence assignment. This suggested that the above sequences originated from a new caterpillar allergen, which was registered as Tha p 2 (UniProt Knowledgebase accession number: P86360).

### Tha p 2 cloning and expression

Peptide sequences obtained by proteomic approaches were compared with the PPM EST sequence library. They

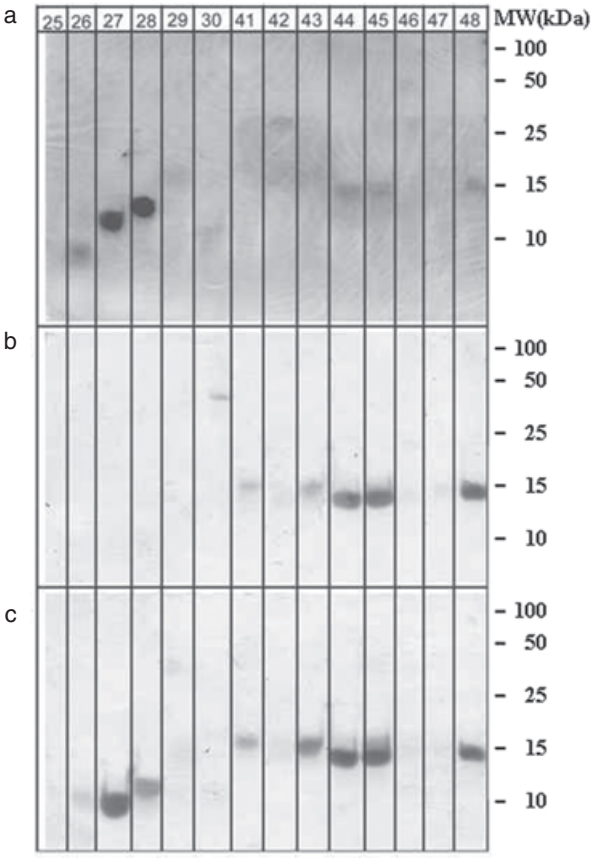


**Fig. 3**. High-performance liquid chromatography (HPLC) of the setae extract. Coomassie-stained sodium dodecylsulfate–polyacrylamide gel electrophoresis (a), and rabbit polyclonal (b) and human IgE (c) immunoblots, of selected fractions of the setae extract after reversed-phase HPLC separation.

matched to mRNAs coded by contigs 28088I454 and 28087I454. The translation of these contigs (Fig. 4a) resulted in a theoretical protein of 115 amino acids with a predicted signal peptide of 15 amino acids, deduced with SIGNALP 3.0 (www.cbs.dtu.dk/services/SignalP). The predicted mature Tha p 2 is a protein of MW 11 024, containing 100 amino acids. The alignment of the peptides sequenced and the predicted Tha p 2 is shown in Fig. 4b.

## Discussion

The nature of the urticating substances and their mechanism of action in the aetiology of the reactions to larval Lepidoptera have been a matter of controversy. Substances with enzymatic properties have been described (32). Pine and oak processionary larvae have an urticating apparatus known as the mirror, which may release up to 1 million setae to the air in order to


**Fig. 4**. (a) Sequences of contigs 28088I454 and 28087I454 and their translation to protein. (b) Alignment of the theoretical sequence of Tha p 2 and the peptides obtained by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) *de novo* sequencing of the allergen of MW 14 000 isolated from the setae extract by RP-HPLC. Tha p 2: sequence stored in the *Thaumetopoea pytiocampa* expressed sequence tag library. 1, 2, 3, and 4: sequences obtained by MALDI-MS *de novo* sequencing.

protect the larvae from their predators (33). The seta is very sharp in the distal part, and has backward spikes in the proximal part, in order to facilitate the penetration of the setae into the skin or mucosa of the attacking animal. Lamy et al. (34) extracted soluble proteins from setae, and isolated an urticating protein of MW 28 000, composed of two subunits of MW 13 000 and 15 000, known as thaumetopoein. This protein is present in large amounts in the glands that produce the setae (35, 36), it is recognized by a rabbit antiserum produced against setae (as in our case), and it has mast cell-degranulating properties suggesting a non-specific mechanism of inflammation (36). However, several years later, the same group reported that foresters heavily exposed to pine caterpillars can be sensitized to proteins extracted from setae, and that 3 of the 4 positive cases had specific IgE against thaumetopoein (37). They describe two allergens, one of them of MW 18 000, and the other migrating as a band of MW >45 000. According to our present data, thaumetopoein and our main setae allergen could correspond to the same protein, but unfortunately no information about the amino acid composition of thaumetopoein is available. In initial experiments performed by us, crude larval extracts did not activate basophils from non-sensitized controls (data not shown), a fact that argued against a non-specific mechanism as described for thaumetopoein, but the non-specific activation of thaumetopoein was tested on peritoneal mast cells instead of basophils (36).

The present study has shown that setae contain a complex mixture of at least 70 proteins, among them seven allergens that are delivered to humans by intradermal injection. Previous experimental work in rats has shown that intradermal administration of allergen causes the production of specific IgE after booster injections at a level 1000-fold lower than that obtained by the oral route (38). Larval allergens are delivered to the skin by penetration of the setae, which have minute amounts of protein contained in a chitinaceous structure, and this method of administration seems to be the second relevant fact that could polarize the immune system to a T helper 2 (Th2)

response. Chitin is a recognition element for tissue infiltration by innate cells implicated in allergic and helminth immunity (39). The role of chitin in allergic reactions after chitin exposure, owing to its ability to produce tissue infiltration with cells expressing interleukin (IL)-4 and IL-13, including Th2 cells, eosinophils, and basophils, has been recently described (39), and it has been suggested that exposure to chitin might be the primary external determinant in allergy development (40). In this regard, Fagrell et al. (33) recently reported that, following local application of setae of the northern PPM *Thaumetopoea pinivora*, setae penetrated the outer skin layer and remained in the skin for up to 3 weeks. During this time period, the setae could release allergens that could enhance an immune response in the affected individual. It is noteworthy that specific IgG was not found in the majority of our patients, suggesting that processionary caterpillars induce a predominantly IgE-mediated immune response in humans.

In conclusion, PPM setae have to be considered as a major source of allergens. As they can be delivered through various types of contact with the larvae, as well as being dispersed by wind over considerable distances (41), the risk for humans and animals is high in areas infested by these insects, such as forests, parks, and gardens. In infested rural areas of the Mediterranean region, up to 12% of people may show reactions to PPM (42). However, PPM may be of even more concern in the near future. Global warming is greatly favouring the survival of this winter-developing insect in more northerly areas, where it was unable to develop before. Thus, PPM is expanding towards urban, highly populated areas such as the Paris Basin, where more and more colonies are becoming established via natural dispersal as well as through accidental translocations by humans (43). Therefore, the allergen content of the setae constitutes an emerging serious health hazard.

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