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Original Article

Isolation and characterization of the major centipede allergen Sco m 5 from *Scolopendra subspinipes mutilans*Xin-Qiang Lan^{a, b, c, 1}, Feng Zhao^{a, b, 1}, Qi-Quan Wang^{a, c}, Jiang-Hua Li^e, Lin Zeng^a, Yun Zhang^{a, d, **}, Wen-Hui Lee^{a, *}^a Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences/Key Laboratory of Bio-active Peptides of Yunnan Province, Kunming Institute of Zoology, Kunming, Yunnan, China^b Key Laboratory of Ethnic Medical Resources Research and Southeast Asian International Cooperation of Yunnan Universities, Department of Biology and Chemistry, Puer University, Puer, Yunnan, China^c Kunming College of Life Science, University of Chinese Academy of Sciences, Kunming, Yunnan, China^d CAS Center for Excellence in Animal Evolution and Genetics, Kunming Institute of Zoology, The Chinese Academy of Sciences, Kunming, Yunnan, China^e Puer People's Hospital, Puer, Yunnan, China

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S. mutilans, *Scolopendra subspinipes mutilans*; *S. dehaani*, *Scolopendra subspinipes dehaani*; FPLC, fast protein liquid chromatography; SPT, skin prick test; ICTRP, International Clinical Trials Registry Platform; PVDF, polyvinylidene difluoride; MAT, Mast cell activation test

ABSTRACT

Background: Allergic reactions have been observed following both direct centipede bites and the clinical use of centipede-containing medicines, such as traditional Chinese medicines utilizing *Scolopendra subspinipes mutilans*; however, no natural centipede allergen has yet been characterized.

Methods: An allergen was purified from *S. s. mutilans* venom using Superdex 75 gel filtration and RESOURCE S ion chromatography, and its primary structure was determined via a combination of LC-MS-MS, MALDI-TOF/TOF and protein sequencing techniques. Its potential allergenicity was evaluated by immunoblotting, ELISAs, skin prick tests (SPTs) and mast cell activation assays.

Results: A novel allergen Sco m 5 (210 amino acids long) was successfully purified from crude *S. s. mutilans* venom. Sco m 5 could promote the degranulation of a human mast cell line, HMC-1. Among centipede-allergic patients, Sco m 5 showed an 83.3% IgE-binding frequency and a 66.7% positive reaction frequency, as detected by immunoblotting and SPTs, respectively. Sco m 5 IgE-binding frequencies of common Chinese population was found to be 9%–16%. Sera positive for Sco m 5 IgE-binding was cross-reactive against venom from the wasp *Vespa mandaeina*.

Conclusions: The present study isolated and characterized a novel allergen termed as Sco m 5 from the centipede *S. s. mutilans*. The use of Sco m 5 to identify centipede-allergic individuals could be important, given the high potential allergenicity of Sco m 5 among the general Chinese population, along with the likely possibility of cross-reactivity against wasp venom among centipede-allergic patients.

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Introduction

IgE-mediated allergy is a life-threatening condition affecting people in all countries.¹ Allergic diseases cause a heavy burden due to the direct or indirect therapy needed to treat the symptoms and the loss of work productivity.^{2,3} At present, numerous allergens are recognized, such as food allergens (e.g., milk, egg, peanut, fish, and

shellfish), aeroallergens (e.g., house dust mites, molds, pollens, and feathers), and other allergens (e.g., hornets, cats, dogs, rodents, and cattle).^{2,4–8} Various arthropods (e.g., honeybees, mosquitoes, houseflies, weevils, cockroaches, bugs, caterpillars, wasps, and ants) produce potent allergens that sensitize and induce IgE-mediated allergic reactions in humans, especially in response to bites or venom injection by these creatures.^{9–12}

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Centipedes, which are distributed worldwide and have a fossil record spanning 420 million years, can secrete venom from poisonous glands in their forceps, and venom plays an important role in their predation and defense.^{13,14} The most common reactions to centipede bites include redness, edema, severe itching, fever, chills, general rash, swelling, blistering, and eosinophilic cellulitis.^{15–18} Notably, many of the observed signs and symptoms following centipede envenomation, such as severe itching, fever, chills, general rash, and eosinophilic cellulitis, may be attributable to allergy-like complications.^{18,19} Envenomation symptoms can last several days and may lead to necrosis, hypersensitive reactions, shock, or even death if not treated properly.^{20–22} A type-I allergic reaction has been reported following envenomation by centipedes.²³ However, according to one case report, a centipede bite triggered a type-IV allergic reaction and caused the death of a 21-year-old female.¹⁷ Centipede venoms contain many active components and induce a variety of pharmacological and biomedical effects. Due to their unique characteristics, centipedes have been used to make traditional medicines in China and Korea for thousands of years.²⁴ These medicines have been reported to have therapeutic effects on convulsion, stroke, tetanus disease, rheumatism, hemorrhoids, scrofula, and snake bite.^{16,24,25} However, centipede-containing medicines sometimes cause adverse reactions, the most common of which are allergic reactions, represented by rashes, papules, redness, itching, swelling, nausea, dyspnea, and even anaphylactic shock.¹⁶

At present, nucleotide sequences of some suspected centipede allergens can be found in the NCBI database, but no natural centipede allergen has yet been purified and characterized. Our previous transcriptomic analysis identified five venom allergen-like proteins from the *Scolopendra subspinipes mutilans*, but only one of these allergen-like proteins could be identified based on a sequence similarity comparison of the *S. s. mutilans* proteomic data with known allergens from other arthropod species.²⁶ Moreover, venomic and transcriptomic analyses of *S. subspinipes dehaani* venom revealed that allergen-like proteins were also existed in the venom.²⁷ However, no natural allergens from *S. s. mutilans* or *S. s. dehaani* have been isolated and characterized. Thus, the novel allergen from *S. s. mutilans* reported in the present investigation represents the first natural centipede allergen to be characterized. Based on its similarity to allergen 5 from various wasp and bee venoms, the allergen was approved and registered as Sco m 5 by the WHO/I.U.I.S. Allergen Nomenclature Sub-committee.

Methods

Allergen purification and identification

Adult *S. s. mutilans* (both sexes, $n = 400$, length > 16 cm) were bought from Suizhou county, Hubei province, China. Venom collections were performed as previously described.²⁷ Lyophilized crude venom powder (500 mg) was dissolved with 2 ml of 20 mM Tris–HCl buffer; after centrifuging the mixture at 12,000 rpm for 10 min, the resulting supernatant was loaded onto a Superdex 75 column (2.6×100 cm, GE Healthcare, USA). The column was pre-equilibrated with the same buffer and eluted at a flow rate of 1.5 ml/min. Peak V from the gel filtration was collected and dialyzed against 20 mM phosphate buffer (NaH_2PO_4 , Na_2HPO_4 , pH 6.5), then further purified on a RESOURCE S column (1 ml, GE Healthcare). This column was pre-equilibrated with the same phosphate buffer and eluted with 20 mM phosphate buffer containing 1 M NaCl at a flow rate of 1 ml/min. Both the Superdex 75 and RESOURCE S columns were connected to a fast protein liquid chromatography (FPLC) system (ÄKTA Pure, GE Healthcare). Elution was monitored by the FPLC system at a wavelength of 280 nm. Each purification

step was monitored by an allergenicity assessment assay (immunoblotting), and the resulting purified allergens were stored at -20°C until use.

Mass spectra generation and N-terminal sequencing of the purified allergen were performed as previously described.²⁸ Internal peptide fragments were obtained by using MALDI-TOF/TOF (Autoflex speed TOF/TOF, Bruker, Germany) on a linear mode. The N-terminal sequence was determined by a protein sequencer (PPSQ-31A, Shimadzu, Japan), and the molecular weight was determined by an LC-MS-MS (Exactive Plus, ThermoFisher Scientific, USA).

Patients and ethics

All experiments were approved by the Ethics Committee of Puer People's Hospital (Authorization No. 2018pesyll-01). Informed consent was signed by each volunteer, and skin prick tests (SPTs) were performed on these subjects at Puer People's Hospital, Yunnan, China. The study conforms to the standards of the Declaration of Helsinki, and the related clinical trial was registered with the WHO International Clinical Trials Registry Platform (ICTRP) (name: identification and function of allergen from centipede, registration number: ChiCTR1900021604).

In this study, we recruited six centipede-allergic patients who were previously bitten by a centipede and displayed typical allergy symptoms, such as redness, itching, blistering, edema, or swelling (Supplementary Table 1). We also recruited 120 volunteers randomly selected from people undergoing a health examination (these individuals were not questioned or tested regarding centipede exposure or allergy) and 19 volunteers who self-reported that they had not ever been bitten by a centipede or used centipede-containing medicines (subjects answered this question prior to being selected).

Potential allergenicity assessment of Sco m 5

To evaluate the allergenicity of Sco m 5, SPTs,²⁹ immunoblotting,^{30,31} and ELISAs⁴ were performed on samples from the six centipede-allergic patients as previously described. Mast cell activation tests (MATs)¹ were performed using the HMC-1 cell line of human mast cells to validate the allergenicity of Sco m 5. The same ELISAs and immunoblotting assays were performed on samples from the 139 recruited volunteers to evaluate the potential allergenicity of Sco m 5 in the general Chinese population. For detailed information on these methods, see the Supplementary Methods.

Allergenicity of differently prepared *S. s. mutilans* samples

A variety of *S. s. mutilans* samples were prepared as follows: the head (1–2 segments containing the venom glands) and trunk (remaining segments of the body) of dried/live *S. s. mutilans* were collected and separately subjected to extraction using a juice extractor. The protein concentrations of these different samples were determined with a GENEQUANT 100 (Classic; GE Healthcare), using the assumption that an optical density at 280 nm (OD_{280}) of 1 is equal to 1 mg/ml. Thermally treated Sco m 5 samples were prepared by boiling at 100°C for 20 min. HMC1 cells were sensitized to serum IgE and stimulated with various processed *S. s. mutilans* samples. The β -hexosaminidase release was measured as previously described.^{1,32} The percentage of β -hexosaminidase release was calculated using the following formula: β -hexosaminidase release (%) = $(S-C)/(L-C) \times 100$, where C, S, and L represent the absorbance of cell-free medium, the supernatant from the lysate of treated HMC1 cells and the supernatant from the lysate of untreated control cells exposed to medium alone, respectively.

PCR assessment of the tissue distribution of allergens in *S. s. mutilans*

To clarify the tissue distributions of Sco m 5 and *S. s. mutilans* allergen-like proteins, PCR assays were performed as previously described.²⁶ Tissue samples were carefully dissected and pooled as follows: V (milked venom glands), H (head, 1–2 segments without venom glands), and T (trunk). RNAs from different tissues were extracted using the Eastep® Super Total RNA Extraction kit (Promega, USA) in accordance with the manufacturer's instructions. First-strand cDNA was synthesized using the PrimeScript™ RT reagent kit with gDNA Eraser (RR047A, TaKaRa Biotechnology Co., Ltd., China) following the manufacturer's protocol. The NCBI primer-blast tool was used to design primers and amplicon lengths varied from 100 to 300 bp (Supplementary Table 2).

Data analysis

All sequence alignments were performed using DNAMAN 8 software. All sequence data were deposited in the NCBI or UniProt

databases or the GenBank database under accession numbers MK440600, MK440601, MK440602, MK440603, MK440604, MK440606, MK440607, and MK440608. All data were analyzed in GraphPad Prism 5, and the criterion for statistical significance was defined as $p < 0.05$.

Results

Purification and identification of Sco m 5

To identify a natural allergen in *S. s. mutilans* venom, the allergenicity of each venom fraction was assessed via incubation with pooled serum from six centipede-allergic patients after each purification step. Superdex 75 gel filtration of crude *S. s. mutilans* venom resulted in the separation of six protein peaks (Fig. 1A, left), and the immunoblotting results for these peaks identified a positive reaction in peak V (Fig. 1A, right). The Superdex 75 gel filtration peak V was subsequently separated into five protein peaks by RESOURCE S cation exchange chromatography (Fig. 1B, left), and the immunoblotting results for these peaks identified a positive reaction in the peak V fraction (Fig. 1B, right). Sco m 5, with an apparent weight of

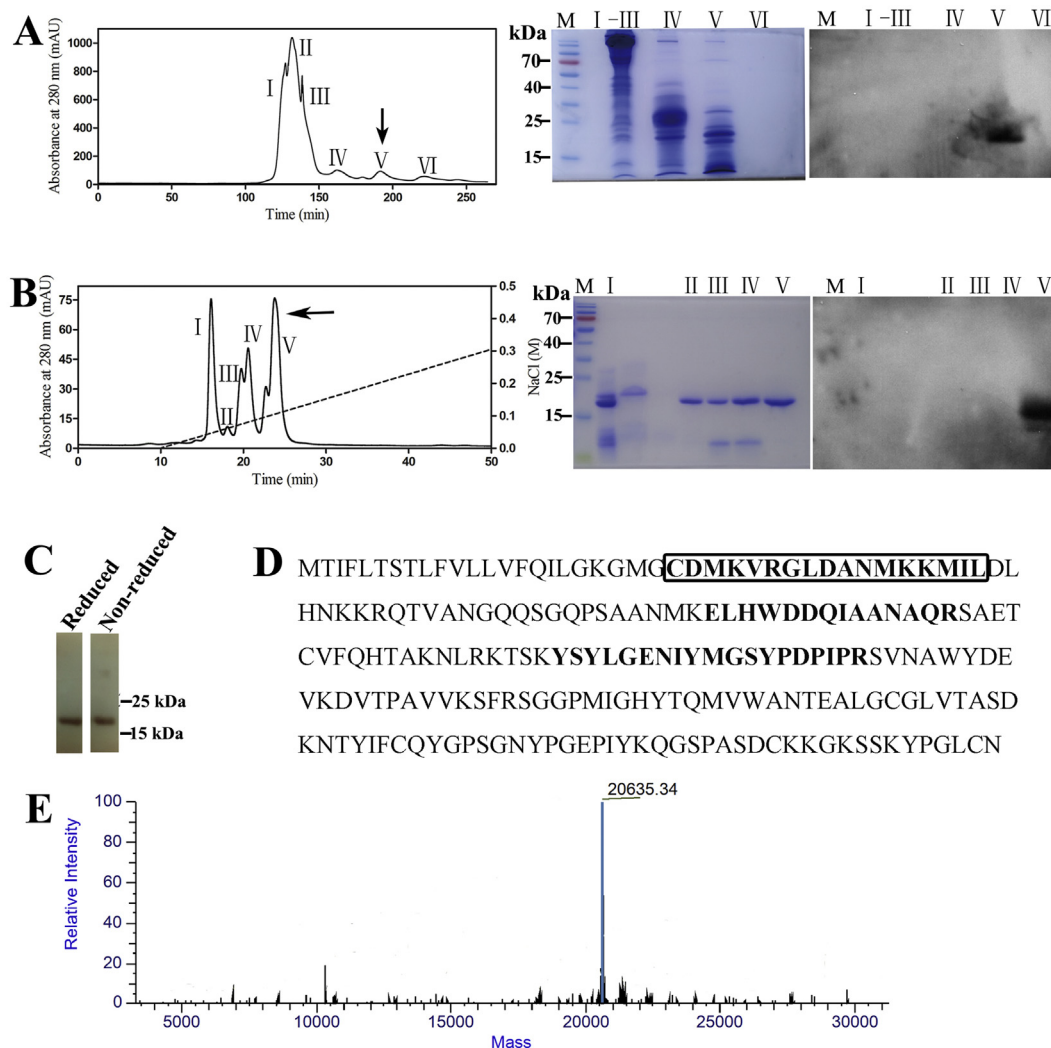


Fig. 1. Purification and identification of Sco m 5 from crude *S. s. mutilans* venom. **A.** Crude *S. s. mutilans* venom was divided into six fractions by a Superdex 75 gel filtration column (left). SDS-PAGE and immunoblotting for the six protein peaks (right). **B.** Peak V from (A) was divided into five fractions by RESOURCE S column (left). SDS-PAGE and immunoblotting for the five protein peaks (right). **C.** The purity and apparent molecular weight of Sco m 5 were determined by silver staining. **D.** The deduced amino acid sequence of Sco m 5. Boxed: N-terminal sequence, bold: sequences of two internal peptide fragments. **E.** The exact molecular weight of Sco m 5 was determined by LC-MS-MS.

20 kDa, was obtained from this fraction (Fig. 1C). The N-terminal sequence of Sco m 5 was determined to be CDMKVRGLDNAMK-MIL by N-terminal protein sequencing, and 2 amino acid sequences of ELHWDDQIAANAQR and YSYLGENIYMGSPDPIPR identified by MALDI-TOF mass spectrometry for interior peptides (Fig. 1D, Supplementary Fig. 1A and B). Transcriptomic and proteomic data analyses revealed that the deduced protein sequence of Sco m 5 is composed of 210 amino acid residues and has a 23-amino-acid signal peptide (Fig. 1D, accession number: MK300948.1). The exact molecular weight of Sco m 5 was determined by LC-MS-MS to be 20,635 Da (Fig. 1E). Overall, the identified N-terminal protein sequence, the determined amino acid residues of two fragments,

and the observed molecular weight (20,635 Da) matched well with the deduced protein sequence of Sco m 5 and its theoretical molecular weight (20,636 Da), unequivocally confirming the identities of Sco m 5 protein and its coding cDNA.

Interestingly, the primary structure of Sco m 5 shared 43%–49% sequence identity with other allergen-like proteins from the same species (Fig. 2A) and 33%–89% sequence identity with other centipede allergen-like proteins (Fig. 2B, Supplementary Fig. 2). The N-terminal sequence of allergen-like proteins from *S. s. dehaani* shared high sequence identity with the N-terminal sequence of Sco m 5, as did the N-terminal sequences of certain proteins from *Scolopendra viridicornis nigra* and *Scolopendra angulata*

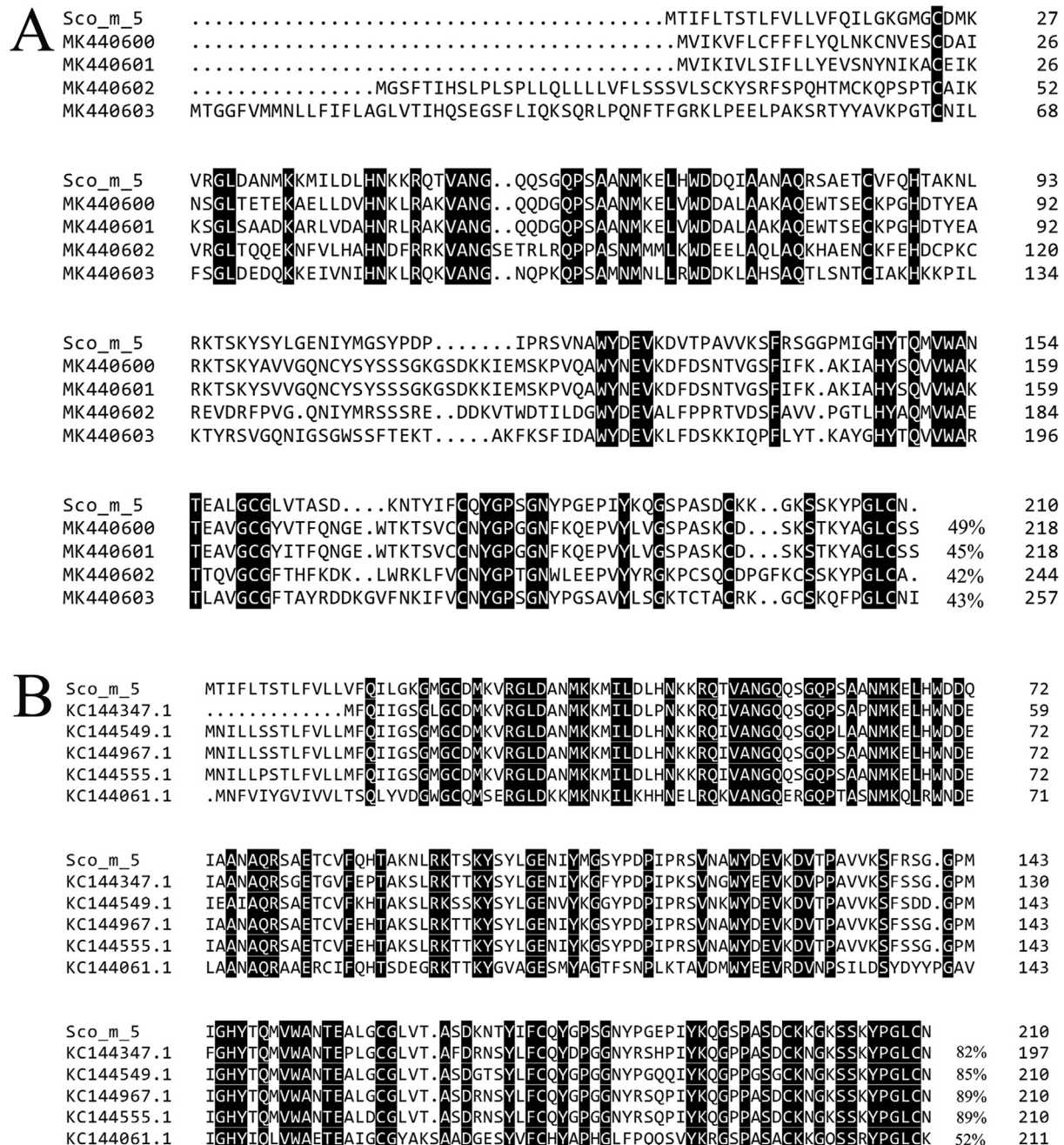


Fig. 2. Sequence alignment of Sco m 5 with allergen-like proteins from different centipede species. **A.** Comparison with four other venom allergen-like proteins from the same species: MK440600 (49%), MK440601 (45%), MK440602 (42%), and MK440603 (43%). **B.** Comparison with venom allergen-like proteins from *S. s. dehaani*: KC144347.1 (82%), KC144549.1 (85%), KC144967.1 (89%), KC144555.1 (89%), and KC144061.1 (52%). Identical amino acids are highlighted.

(Supplementary Fig. 3A). In addition, Sco m 5 shared 36%–41% sequence identity with allergens from non-centipede arthropods (Supplementary Fig. 3B).

Potential allergenicity frequency of Sco m 5

To assess the potential allergenicity of Sco m 5, we recruited six patients with known centipede allergies (Supplementary Table 1) and performed immunoblotting assays and SPTs. Sco m 5 had an IgE-binding frequency of 83.3% (5/6; Fig. 3A) and an SPT-positive reaction frequency of 66.7% was found among these patients (4/6; Fig. 3B). Competitive inhibition ELISAs, performed using sera from two of the centipede-allergic patients, revealed that the maximal inhibition of patient IgE binding to precoated crude *S. s. mutilans* venom was approximately 50% (Fig. 3C, D). In *in vitro* MAT tests, Sco m 5 induced approximately 2- and 2.5-fold increases in degranulation (as determined by CD63 and CD107a surface staining) following incubation

with centipede-allergic as opposed to nonallergic patient sera, respectively (Fig. 3E, F; Supplementary Fig. 4A and B).

Potential allergenicity of Sco m 5 among the general Chinese population

To assess the potential allergenicity of Sco m 5 in the general Chinese population, we first screened sera from 120 randomly selected individuals by performing immunoblotting assays, and Sco m 5 had a 9.2% (11/120) IgE-binding frequency in these subjects (Fig. 4A). Afterwards, we recruited 19 volunteers who self-reported that they had never been in contact with centipedes or taken centipede-containing medicines and repeated the same immunoblotting assays on their donated sera. Surprisingly, the IgE-binding frequency of Sco m 5 was 15.8% (3/19) in these volunteers (Fig. 4B). The immunoblotting results for both groups were supported by the results of ELISAs (Fig. 4C). In conclusion, our data indicate that the

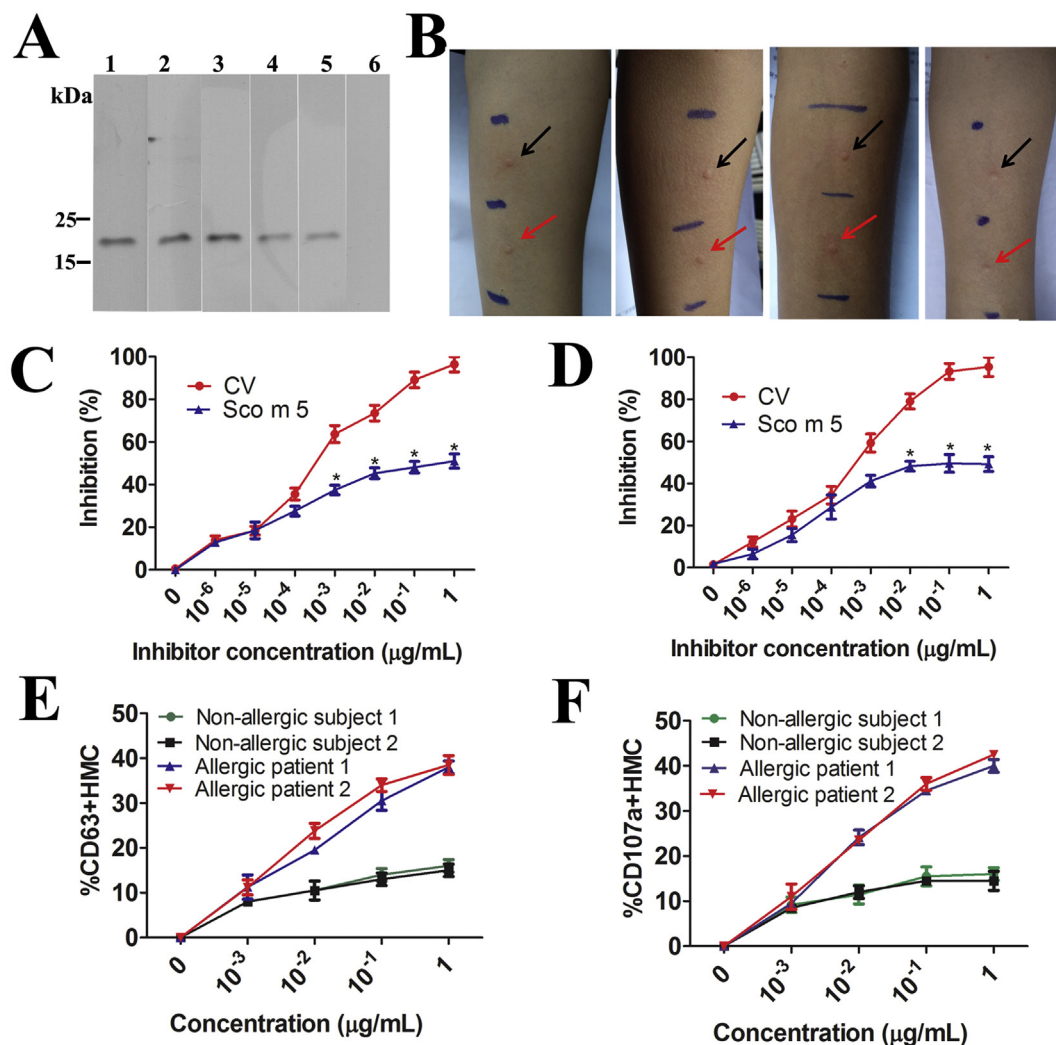


Fig. 3. Potential allergenicity assessment of Sco m 5. **A.** Immunoblotting of specific IgE binding to Sco m 5 using sera from six centipede-allergic patients. **B.** Skin prick test of Sco m 5 (The four patients are 1, 2, 3 and 5 in A; red arrows: Sco m 5; black arrows: histamine). **C, D.** The dose-dependent inhibition rate of Sco m 5 against the binding of IgE from allergic patient serum (from patients 2 [C] and 3 [D] in A) to precoated crude venom (CV). The data represent the mean $\pm$ SEM from three technical replicates, * $p < 0.05$ by unpaired *t*-test. **E, F.** Sco m 5 induced HMC-1 degranulation was measured based on CD63 (E) and CD107a (F) surface staining. HMC-1 sensitized with sera from centipede-allergic patients (patients 2, 3 in A) and non-allergic subjects (two randomly selected individuals among the 19 volunteers whose sera showed a negative IgE-binding reaction to Sco m 5). The data represent the mean $\pm$ SEM from three technical replicates.

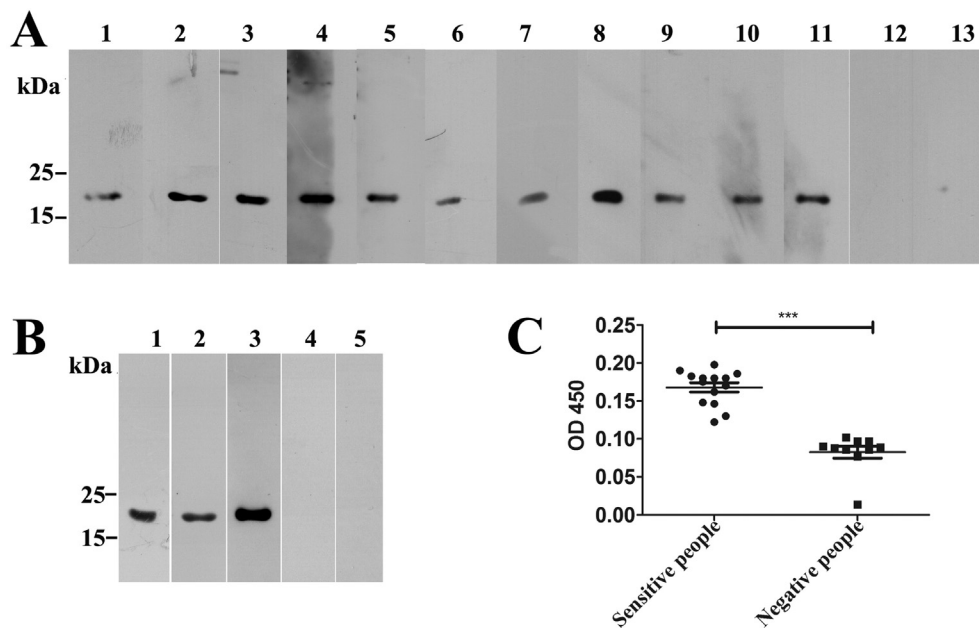


Fig. 4. Potential allergenicity of Sco m 5 in the general Chinese population. **A.** Immunoblotting for specific IgE binding to Sco m 5 in sera from 120 randomly selected volunteers; lanes 12 and 13 show representative negative reactions. **B.** Immunoblotting for specific IgE binding to Sco m 5 in sera from 19 volunteers who self-reportedly had never contacted a centipede or used centipede-containing medicines; lanes 4 and 5 show representative negative reactions. **C.** ELISA for specific IgE binding to Sco m 5. 14 Sco m 5-sensitive sera (lanes 1–11 of A and lanes 1–3 of B) and 10 specific IgE binding-negative sera samples were used. The data represent the mean $\pm$ SEM and were analyzed using a Mann–Whitney U test, *** p < 0.001.

IgE-binding frequency of Sco m 5 in the general Chinese population is approximately 9%–16%.

Allergenicity cross-reactivity between *S. s. mutilans* and *V. mandarinia*

To determine the allergenicity cross-reactivity between centipedes and Hymenoptera, we prepared the crude venom of *Vespa mandarinia*. Immunoblotting revealed that 14 of the 19 sera positive for allergy to Sco m 5 also showed a positive reaction to *V. mandarinia* venom (Fig. 5A). A competitive inhibition ELISA was conducted using sera from two of the centipede-allergic patients that had a positive immunoblotting reaction to *V. mandarinia* venom. The IgE reactivity to *V. mandarinia* venom was inhibited by up to 20% by Sco m 5 and was inhibited 40% by *S. s. mutilans* crude venom, respectively (Fig. 5B, C). These results indicate that many of the patients who are allergic to centipedes also displayed cross-reactivity against venom from the wasp *V. mandarinia*.

Allergenicity of various *S. s. mutilans* samples

The potential allergenicity of different *S. s. mutilans* samples were evaluated by the level of β -hexosaminidase release. The quantity of β -hexosaminidase released by HMC-1 cells in response to the positive (10 μ g/ml C48/80) and negative controls was approximately 40% and 3%, respectively (data not shown). However, different concentrations of variously prepared samples (head and trunk from live/dried centipedes, untreated/boiled Sco m 5) induced β -hexosaminidase release in a dose-dependent manner (Fig. 6A, B). Interestingly, there was no significant difference in the amount of β -hexosaminidase release triggered by equal doses of differently processed *S. s. mutilans* samples (i.e., using fresh, dried, or boiled preparations), which indicates that the various processing methods did not affect the allergenicity of *S. s. mutilans* allergens.

Tissue distribution of *S. s. mutilans* allergens

The tissue distribution of *S. s. mutilans* allergens was assessed via PCR (Fig. 6C), Sco m 5 and *S. s. mutilans* allergen-like proteins (MK440600, MK440601, MK440602, and MK440603) were widely distributed in different parts of the centipede body, but each allergen/allergen-like protein had a unique distribution. Sco m 5 was expressed mainly in the venom gland, with the second strongest signal coming from the rest of the head (which may have contained trace amounts of the venom gland). In contrast, MK440600, MK440602, and MK440601 were expressed mainly in the trunk and head but not in the venom gland, whereas MK440603 was equally expressed in the head, venom gland, and trunk.

Discussion

Allergic reactions are recognized as the most common adverse reaction to centipede bites or the clinical use of centipede-containing medicines.³³ The nucleotide sequences of suspected allergens from different centipede taxa are available from the NCBI database, based on their deduced sequences, these allergen-like proteins have molecular weights of approximately 20 kDa and belong to venom allergen families 3 and 5. Our lab previously demonstrated that as many as 21 allergen-like nucleotide sequences exist in the venom gland of *S. s. dehaani* and identified five allergen-like proteins via N-terminal protein sequencing of purified venom fractions.²⁷ Additionally, allergen-like proteins from *S. viridicornis nigra* have high sequence identity (40%–50%) with allergen 5 from wasp venom.¹⁸ Interestingly, Sco m 5 shares 43%–49% protein sequence identity with four other allergen-like proteins from the same species but showed the highest identities with four deduced proteins from *S. s. dehaani* (82%–89%) (Fig. 2) and one allergen-like protein from *S. subspinipes mojiangica* (87%) (Supplementary Fig. 2B). Thus, our results suggest that Sco m 5 and

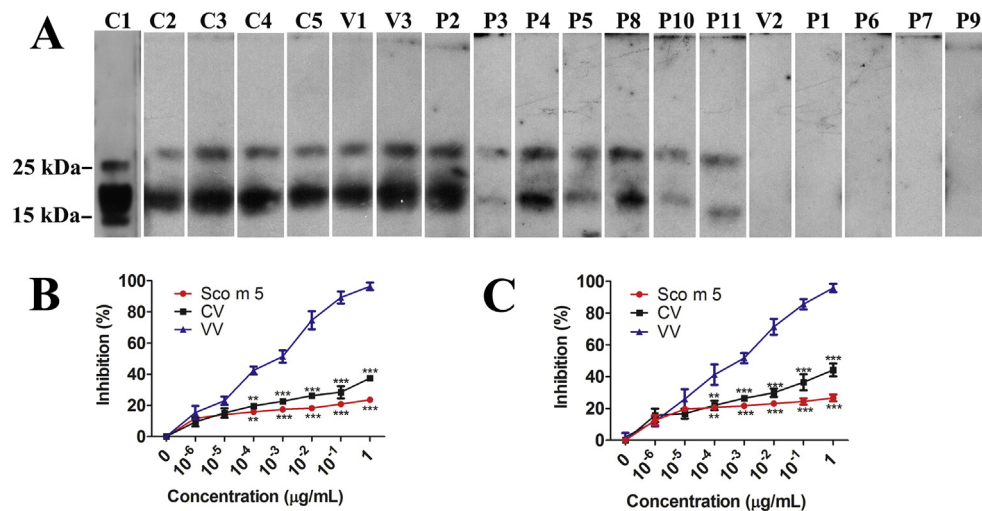


Fig. 5. Allergenicity cross-reactivity of Sco m 5 and *S. s. mutilans* crude venom against *Vespa mandarinia* venom. **A.** Immunoblotting of *V. mandarinia* venom after incubation with 19 sera previously identified as positive for specific IgE binding to Sco m 5. The right 5 lanes showed negative reactions. C: centipede-allergic patients, P: unscreened participants, V: self-reportedly non-centipede-exposed volunteers. The subject numbers match those shown in Fig. 3 and 4. **B, C.** The dose-dependent inhibition rate of Sco m 5 and *S. s. mutilans* crude venom (CV) against the binding of IgE from centipede-allergic patient serum (C2 [B] and C3 [C] in A) to pre-coated *V. mandarinia* venom (VV). The data represent the mean $\pm$ SEM from three technical replicates. ** $p < 0.01$, *** $p < 0.001$ by unpaired *t*-test.

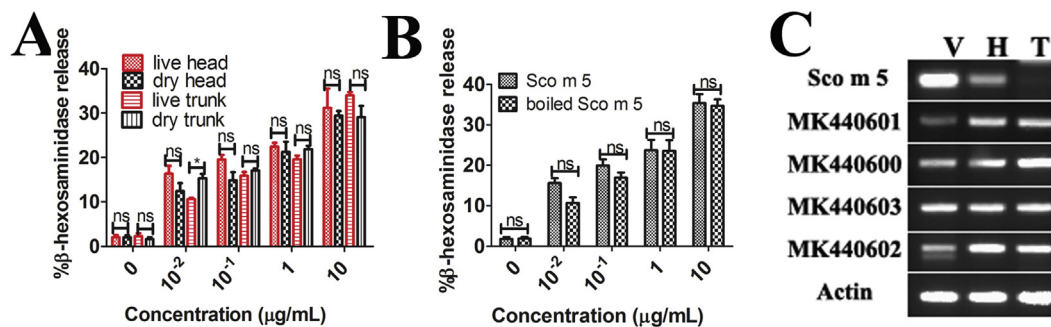


Fig. 6. Potential allergenicity assessment of *S. s. mutilans* samples processed in various manners and tissue distribution of *S. s. mutilans* Sco m 5/allergen-like proteins. **A, B.** β -hexosaminidase release in response to different *S. s. mutilans* samples or boiled Sco m 5. The data represent the mean $\pm$ SEM from three technical replicates. * $p < 0.05$ and ns = not significant by unpaired *t*-test. **C.** PCR of Sco m 5/allergen-like proteins from *S. s. mutilans* (V: venom gland, H: head, T: trunk).

its homologues are distributed across *S. s. mutilans*, *S. s. dehaani*, and *S. s. mojiangica*. Meanwhile, Sco m 5 also shared 36%–41% sequence identity with proteins from several non-centipede arthropod species (Supplementary Fig. 3B). The presence of highly similar allergen-like proteins across different centipede species suggests that certain allergens may be contained in a variety of centipede types and may play an important role in centipede allergenicity, at least for *S. s. mutilans*, *S. s. dehaani*, and *S. s. mojiangica*.

The difference in the measured IgE-binding frequency of Sco m 5 between an unscreened sampling of the general population (9.2%; 11/120) and a group of volunteers who had self-reportedly never been exposed to centipedes or centipede-containing medicines (15.8%; 3/19) might be caused by the dissimilarity in sample size (Fig. 4A, B). As such, the IgE-binding frequency of Sco m 5 in the general Chinese population is likely in the range of 9%–16%. For comparison, the frequencies of allergic reactions induced by flying Hymenoptera in general and by bee stings in particular were reported to be 2%–26% and 16%, respectively, in an unselected European population.^{34–36} Furthermore, proteins from *S. viridicornis nigra* have a strong similarity to allergen 5 from wasp venom, and patients with allergies to centipede venom have also shown allergic reactions to wasp, yellow-jacket, and honey bee venom.³⁷ The levels of sequence similarity are related to the cross-reactivity

levels of allergens.³⁸ Here, we used the sera that displayed a positive IgE-binding reaction to Sco m 5 to perform the same experiments against *V. mandarinia* venom, and results show that 14 of the 19 tested samples had positive reactions (Fig. 5). Allergic reactions to centipedes are rare, but allergies to wasps and bees are common. Taken together, a reasonable explanation for the high IgE-binding frequency of Sco m 5 among the Chinese population is a cross-reactivity between allergens from centipedes and those from various flying Hymenoptera.

Centipedes are an important animal source of traditional medicine, and a database search yielded 252 drugs derived from centipedes (<https://www.yaozh.com/>). Although there is still controversy over whether to keep or discard the heads of centipedes when they are used in clinical practice, all components of the preparation are required to be boiled before administration. Allergic reactions are reported to be the most common adverse reaction caused by centipede-containing medicines. Our present investigation demonstrates that different processing methods had no significant influence on centipede allergenicity (Fig. 6A, B). Sco m 5 and other allergen-like proteins were thermostable and were widely distributed throughout the bodies of *S. s. mutilans* (Fig. 6C). This thermostability may be the reason why centipede-containing medicines retain allergenicity after being boiled. Because 9%–16%

of the general Chinese population is susceptible to an allergic reaction against Sco m 5, the widespread clinical usage of centipede-containing medicines could contribute to a high frequency of allergic reactions against centipedes.

In conclusion, we purified and characterized a new allergen from centipede venom and evaluated its allergenicity. The discovery of Sco m 5 helps explain the occurrence of allergic reactions caused by centipede bites and the consumption of centipede-containing medicines. Allergic reactions caused by Sco m 5 should be noted as a possibility when a patient is bitten by a centipede or uses medicine that contains centipede parts. However, further research should be conducted to expand the known range of centipede allergens and to diagnose and treat allergic disease caused by centipede bites or centipede-containing medicines.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2020.06.003>.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

X-QL, W-HL and YZ conceived the study and wrote the paper. X-QL, Q-QW, FZ, J-HL, LZ performed the experiments. All authors read and approved the final version of the manuscript.

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Supplementary Materials

Isolation and characterization of the major centipede allergen Sco m 5 from *Scolopendra subspinipes mutilans*

Supplementary Methods

Allergen purification and identification

Venom collections were performed as previously described ¹. Briefly, electric stimulation-induced venom was washed with 20 mM Tris-HCl buffer (pH 7.8, containing 100 mM NaCl). The collected venom pools were centrifuged at 12,000 rpm for 10 min at 4 °C, and the resulting supernatants were vacuum freeze-dried and stored at –20 °C until use.

Potential allergenicity assessment of the Sco m 5

Skin prick test

SPTs were executed via the method of D'Avino ². Briefly, 100 µg/mL (final concentration) Sco m 5 dissolved in a 0.9% saline solution, 10 mg/ml histamine (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control, and a 0.9% saline solution was used as a negative control. All skin test liquids were freshly prepared by mixture with sterile glycerin in a ratio of 1:1 (v/v) and filter sterilized by being passed through 0.22 µm membrane filters (Millipore, Billerica, MA, USA). Testing was conducted at a dose of 50 µl/test point.

Immunoblotting

SDS-PAGE and immunoblotting were performed as previously described ^{3, 4}.

Briefly, 200 ng of purified Sco m 5 were subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). The membranes were blocked with 5% BSA (Sigma-Aldrich) and then further incubated with diluted (1:10, v/v) human sera and peroxidase-labeled anti-human IgE monoclonal antibody diluted 1:5000 (KPL, Inc., Gaithersburg, MD, USA). Finally, IgE-binding proteins were visualized with Super Signal chemiluminescence reagents (Thermo Fisher Scientific, USA). Each incubation step was followed by three 10-min washes with 0.1% TBST (100 mM Tris, 150 mM NaCl, pH 7.4, containing 0.1% tween 20).

ELISA

ELISAs were carried out as previously described with minor modifications ⁵. Briefly, 200 ng of purified Sco m 5 was pre-coated overnight onto a 96-well plate (Corning, USA) with 100 µl of coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) at 4 °C. The plate was then blocked with 5% BSA and incubated with 100 µl of diluted sera 1:10 (v/v) at 37 °C for 2 h. After IgE binding, the plate was incubated with 100 µl of the abovementioned peroxidase-labeled anti-human IgE monoclonal antibody at a dilution of 1:2000 for 1 h at 37 °C. Each incubation step was followed by three 1-min washes with 350 µl/well of 0.1% TBST. The plate was then incubated with 100 µl of colored substrate (Beyotime, China) at 37 °C for 15 min. An additional 50 µl of termination fluid (Beyotime) was added to each well to end the reaction. Finally, the plate was read at 450 nm with a microplate reader (TECAN, infinite M200 PRO NanoQuant).

Competitive inhibition ELISAs were performed in accordance with a previously

described method ⁵. Sera from two allergic patients were randomly chosen for use in the inhibition assay. After the sera were pre-incubated with different amounts of the purified proteins or crude venom (final concentration: 0–1 µg/ml) at 37 °C for 1 h, the mixtures were added to a 96-well plate previously coated with 2 µg of crude venom per well. The remaining steps of this assay were the same as those described in the ELISAs method section above.

Mast cell activation test

Human mast cells of the HMC-1 cell line were used to perform mast cell activation test (MAT), as described in a previous report ⁶. HMC-1 cells were purchased from Beijing Luyuan Bode Biotechnology Co. Ltd. The cells were cultured with supplementary medium [Iscove's modified Dulbecco's medium (Gibco, USA) + 10% fetal bovine serum (BI, Israel) + 0.5% penicillin-streptomycin solution] in a cell incubator (37 °C, 5% CO₂). The cells were sensitized overnight with diluted (1:10, v/v) sera from patients with centipede allergies, after which the cells were incubated with different concentrations of Sco m 5; goat anti-human IgE antibody (10 µg/ml) was used as a positive control. After 1 h of incubation, the cells were stained with CD107a-APC (clone H4A3, BD, PeproTech, USA) and CD63-FITC (clone H5C6, BD, PeproTech) antibodies and analyzed by flow cytometry (BD LSRFortessa™, BD Biosciences, USA) and FlowJo software (FlowJo 7.6-1).

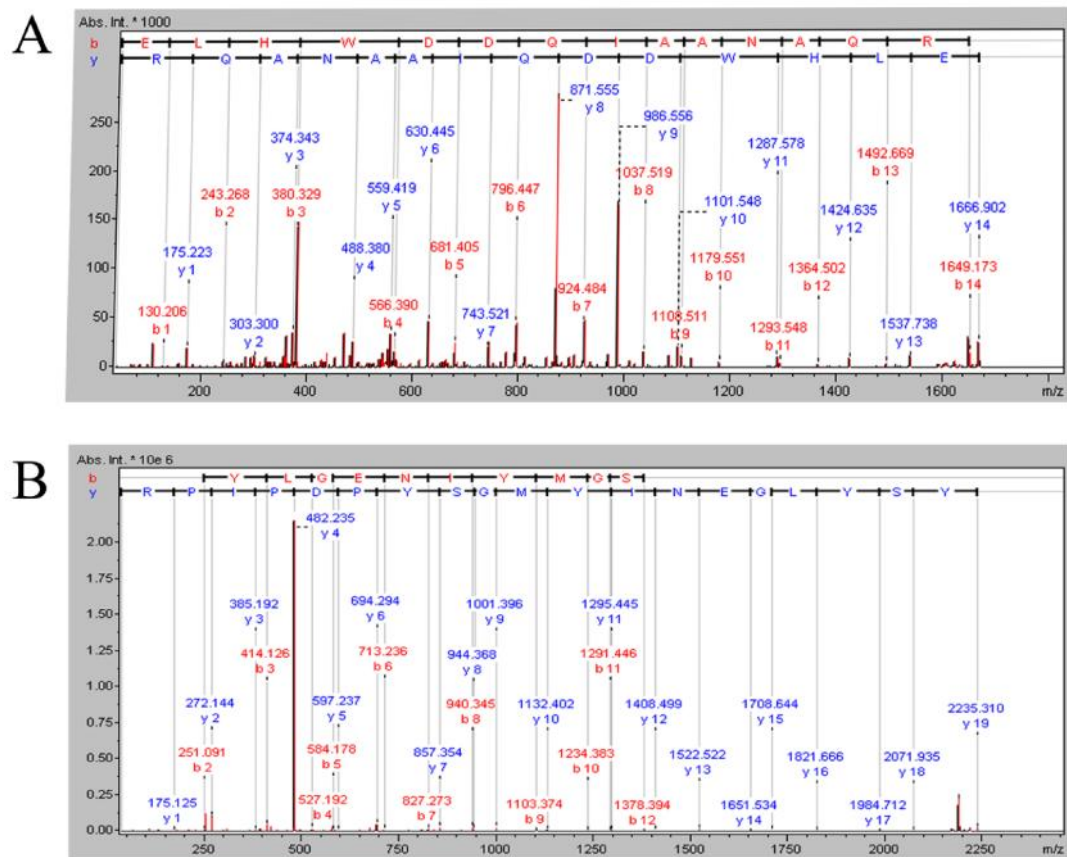
Allergenicity of variously prepared *S. mutilans* samples

The β-hexosaminidase release elicited by various *S. mutilans* samples was tested as previously described ^{6, 7}. Briefly, HMC-1 cells (1–5×10⁵ cells/well) were preincubated

with centipede-allergic patient sera overnight and incubated for 1 h with different *S. mutilans* samples. C48/80 (Sigma-Aldrich) at a final concentration of 10 µg/mL was used as a positive control. Afterward, the cells were centrifuged at 1,000 rpm for 5 min, and 100 µl of the resulting supernatant was taken and transferred to a new 48-well plate. An additional 100 µl of β-hexosaminidase substrate [1 mmol/L p-nitrophenyl N-acetyl-β-D-glucosamine (Sigma-Aldrich) in 0.05 mol/L citrate buffer (pH 4.5)] was added to each well and incubated for 2 h in a cell incubator (37 °C, 5% CO₂). Finally, the reaction was stopped by adding 300 µl of 0.05 mol/L sodium carbonate (pH 10), and the optical density at 450 nm was measured with a microplate reader.

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Supplementary Fig. 1. Identification of Sco m 5.

A, B. MALDI-TOF determination profiles of two internal peptide fragments.

A	Sco_m_5	MTIFLTSTLFVLLVFQILGKMGCDMKVRLDANMKKMI	LDLHNKKRQTVANGQQSGQPSAANMKELHWDQIAANAQRSA	81	
	tr T1JFC9	MRGGKTEEIHEIIRAHNKARQKVANGHQQAQPSASNMREVVSSELAIIAQRWA		55	
	tr T1IKB6	.MAKCDYEGFGKEHTMCKYTKHLDCVLASTSSTQKNLILRLHNEFRQSIANGSDPNFPAANMVEMAWDELEMIACKWT		80	
	Sco_m_5	ETGVFQHTAKNLRKTSKYSY.....LGENIYMGSYPDP	IP.....RSVNAMYDEVKDVTPAVVKSFRSGG.P	142	
	tr T1JFC9	EQIINAKDQNRRTSKLPYSLEKNGMPEKPKTFEHVGQNVFHSRSTNNPSADVTNVVEAMYNEVEKPGFNASINPFHNP		136	
	tr T1IKB6	DQCKQGPDAERRSYKYPISIG.....QNRFLDTEDDKVKKDVSGLAVRTMFSKKQGFSTNDVAEFKSSE..		145	
	Sco_m_5	MIGHYTMVWANTEALGCGLVTASD...KNTYIFCQYGPSGNYPGEPIYKQGS	PASDCKKKGSSKYPGLCN.....	210	
	tr T1JFC9	SVAHYTQLIWDIDLIGCGFVYFQTSDFTYHKHTICNYASGGNIKDGMMYKVG	PQCSNCPKGC	217	
	tr T1IKB6	KTNHFSQMIWATSESIGCGFTRYKEVF..FSNAITCNYGPAGNIPGNTAAGKEKEPN	GATRLLRNFMIYLCVPILFLIF	224	
	Sco_m_5			210	
	tr T1JFC9	ENYQLASSIGNPKTAVLQLDDEIISEKNESKVITEGGGLYGLLDNDEGTQDIKNSDPLHVST	TEKVLVDLVTETNK	36% 296	
	tr T1IKB6			33% 224	
B	Sco_m_5		MTIFLTSTLFVLLVFQILGKMGCDMK	27	
	MK440604		MVDYRLLEGGNPTSKMNIILLTFSLFVVFVHTLECGMGCDMK	42	
	MK440606	MLYTVPFVVFVFSQILQGMPQSTGAFIGRTSQQIPNNFTFGRKLPVELPAKTQ	PYSAMVKSETCNIL	68	
	MK440607		MMIRIALCFFFLNEVNRYNIEACELK	26	
	MK440608		MNFVIAVSALLTFQLYVDGWGCQMS	26	
	Sco_m_5	VRGLDANMKKMI	LDLHNKKRQTVANGQQSGQPSAANMKELHWDQIAANAQRSAETCVFQHTAKNLRK	95	
	MK440604	VRGLDANMKKMI	LDLHNKKRQTVANGQQSGQPSAANMKELHWDDEIASNAQRSAETCKFQHTAQSLRK	110	
	MK440606	FSGLNDQKKEIVNLHNKLRQKVASGNQPKOPSAINMNQLHWDNDLAHYAQILANRC	VAKHNSPTLKK	136	
	MK440607	NSGLTTSDKKAQLVDVHNR	LRAKVANGQQTGOPSAADMKQMKWDELA	AAQAEWANTCQGGHLDYDGRK	94
	MK440608	ERGLDKKMKNRIVKHNELRQKVAKQERGOPTASNMQLRNDELA	ANAQRAAERCVFQHSDEERT	94	
	Sco_m_5	TSKYSYLG	ENIYMGSYPDP.....IPRSVNAMYDEVKDVTPAVVKSFRSG.GPMIGHYTMVWANT	155	
	MK440604	SGKYSYLG	ENIYMGSYPDP.....IPRSVNAMYDEVKDVSPAVVKSFRSG.GPMIGHYTMVWANT	170	
	MK440606	YRSVGQ	NIAGWSSFSEKTP.....KFKSFDIGWYDEVKDFDSKKIQPFMYS..TVYGHYTMVWANT	197	
	MK440607	TSKYPVAGQNFYASYALGTGSANKINLYSPVQAWYGEVKDFDRNAVKS	SYFD..PKTGHYSQVWANT	160	
	MK440608	TTYKVGVTGESMYAGTFSNP.....LKTAVDRWYEEVRDVPNSIVDSYDYPGAVIGHYIQLVWAET		155	
	Sco_m_5	EALGCGLVT...ASDKNTYIFCQYGPSGNYPGEPIYKQGS	PASDCKKKGSSKYPGLCN.	210	
	MK440604	EALGCGLVT...ASDKNSYVF.....		87% 188	
	MK440606	QAVGCGFTAFRDDKGVFNKFVFCNYGPGGNYPGSSVYLSGKSCSNCKKGCSKQF	PSLCNV	42% 257	
	MK440607	EALGCGYVLFPR.GSWTEQHIICNYGPAGNFIGQPVYLEGTPASRCRS.KSTQYLG	LCSS	47% 218	
	MK440608	EALGCGYAKS...AADGQSYVFC	HYAPRGLYPQSSVYKRGSPASACKKGQSSRYPGLCK.	52% 211	

Supplementary Fig. 2. Sequence alignment of Sco m 5 with allergen-like proteins from centipede species.

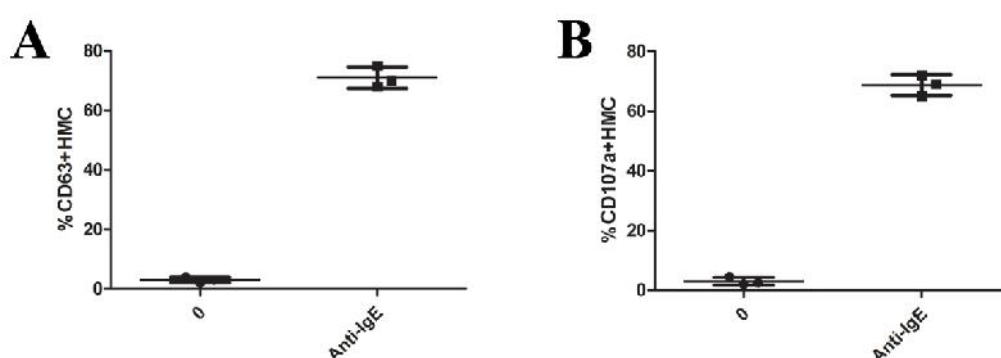
A. Comparison with tr|T1JFC9 (36%) and tr|T1IKB6 (33%) from *Strigamia maritima* (European centipede). **B.** Comparison with MK440604 (87%, incomplete), MK440606 (42%), MK440607 (47%), and MK440608 (52%) from *S. mojiangica*. Identical amino acids are highlighted.

A	Sco_m_5	..CDMKVRGLDANMKKMILDLHNKKRQTVANG.....	30
	KC144555.1	..CDMKVRGLDANMKKMILDLHNKKRQIVANG.....	97% 30
	KC144967.1	..CDMKVRGLDANMKKMILDLHNKKRQIV.....	96% 27
	KC144549.1	..CDMKVRGLDANMKKMILDLHNKKRQIVANG.....	97% 30
	KC144061.1	WGCQMSEERGLDKKMKNKILKHHNELR.....	57% 26
	KC144347.1	..CDMKVRGLDANMKKMILDL.....	100% 19
	sp P0C8D7	..XQMVERGLDAKAKAAMLD AHD XARQKVANGQEAGQPGAXNMKELH	57% 45
B	sp P0C8D4	..XQVVERGLDAKAKAAMLD AHNKARQKVANG.....	68% 30
	sp P0C8D5	..XKMSEQGLNAQMKQAQIVDLHNXARQGVANGQ.....	61% 31
	Sco_m_5	MTIFLTSTLFVLLVFQILGKG..MGCDMKVRGLDANMKKMILDLHNKKRQ	48
	XP_024085001.1	MSLFKMKVQLVSYLIVFSLMGLSCACSGAKLLKSGGLSCEEKRLIVDAHNRLRQ	54
	KFM60495.1	MVNHVIRIGLFFVLLGLSECAKCPYAKFSPDHSFCKDPNPKCTILERGLKSDDKQLRLDLHNMYRE	67
	XP_001845617.1	MEKQKGSPTLPATLPHIACNGLTTLATS..CGSGSQETLLDSSLQALILDAHNKLR	56
	XP_014222130.1	MTKSLIVCSLAVISLINFDAQNYCNLNKCGNSHTMCQYRSNNAGSKCGRVLSAG..LSENEKRTIVNVHNQLRQ	74
	tr A0A2P8YFD4	..MGNMELTSLAFLVVCVAVTVEANNVYCSLCKDHTLCKYPQGP..CNARKASGVDEASQKVIQVHNKLR	71
	tr A0A132AMK5	DLRGISKRDKLILDAHNYVRN	22
	sp P81657.1	NNYCKI..KCRSGIHTLCKFGISTKPCNGKVVVKASGLTKAEKLEILKQHNFRQ	53
	Sco_m_5	TVANG....QQSGQPSAANKKELHDDQIIPANQORSLETQVFQITAKNLKTSKYSYLGENIYMGSY.....	112
	XP_024085001.1	RVALLG....KVPQGSPAENMLEMSDDELARGAQKADQCIFQNSQNDRRVSRFLVG..QLAMTWSSPHPNLS	123
	KFM60495.1	KVASGKE..TQAGKLPATNMFEMADDELASTAQKMAEQIYDQDCDECRQVDRFDVG..QLYRAGSSD...K	135
	XP_001845617.1	KVASGQQTAAANALYPQAAARMATLQDNELATIAASMARRCVYGD..KCRNTVAFPYAGQNTAMFSYSQGT..F	127
	XP_014222130.1	KVANGREGRGLPGQPAAKNQNLQDNELATIAQRWANGCDFND..KCRNVERYSVG..QNIAYVGTG...D	142
	tr A0A2P8YFD4	VNTLKVESMVNNIYNEVKDYKNSVSKFTSLKGPNGKDVGYTQLVWAKTNRVGCG..AMR.....YNDGRFNK	209
	tr A0A132AMK5	NYSPEWENAINGIYNEVDKYKNQEVQS....YKFNSQTGHYTVVWADTSKVGCGYTAFR.....SANGGTVN	205
	sp P81657.1	SMKPQWKKIVKENYEEIEIAPPQVAVS....FQLFELNIGHFTQIAWATNRIIGCGFTSYQNDHSGNNAVRTV	161
	Sco_m_5	...DPIPRSVNAYDEVKDVTPAVVKSFRS....GGPMIGHYTMVWANTALGCG.....LVTASDKN	169
	XP_024085001.1	GDKPDFETQINNMFNEVRYRGGYSTQ.....VGHYTLQIWDITVLVGGG..YSY.....FLKGNQYT	179
	KFM60495.1	PTKHDWDRMVKKIYDEVSMFDKSYIKP.....FFSGVYNHFSQIVWATITWKLGGG..FVV.....YKEPEWYR	196
	XP_001845617.1	TVNELITGFVNDNFSEFKDANPNYVASYP...GYTGPDIHFQIIVSDRINRIGCS..LVK.....YDEGWTN	192
	XP_014222130.1	VNTLKVESMVNNIYNEVKDYKNSVSKFTSLKGPNGKDVGYTQLVWAKTNRVGCG..AMR.....YNDGRFNK	209
	tr A0A2P8YFD4	NYSPEWENAINGIYNEVDKYKNQEVQS....YKFNSQTGHYTVVWADTSKVGCGYTAFR.....SANGGTVN	205
	tr A0A132AMK5	QLYTQNYAPGGNPLGKMYEHGQ..AASQCPSRYRSRKRKSLCESVVENLGDISTNPSTVATQTQTSQAIPISST	235
	sp P81657.1	HYLVQNYGPAGNIGNEPIVERK.....	202
	Sco_m_5	TYIFQYQGPSGNYGPEPIYKQGS..PASDCKKGKSSKYPGLCN.....	210
	XP_024085001.1	KYYVQNYGPAGNVRGFPYKSGSPACAQHGTTPSRTYPGLCRNQKLSQPCSNFYYYYYY.....	239
	KFM60495.1	SYLVQNYGPTGNNDDEEEMVNVGR..TCSYCPTGSCCGADCTKKFKKTAKYPSLC.....	248
	XP_001845617.1	QYFVQNYALT..NIVGQPIYAGN..ACSKCTSGCNAAYPLCNANENVSPIP.....	241
	XP_014222130.1	FFLVQNYGPTGNYQGQPVYERR.....	231
	tr A0A2P8YFD4	KYYVQNYGKAGNIGETMYKKGK..ACSMCPNGTSCNAHGLCK.....	246
	tr A0A132AMK5	QLYTQNYAPGGNPLGKMYEHGQ..AASQCPSRYRSRKRKSLCESVVENLGDISTNPSTVATQTQTSQAIPISST	235
	sp P81657.1	HYLVQNYGPAGNIGNEPIVERK.....	202
	Sco_m_5		210
	XP_024085001.1		239
	KFM60495.1		248
	XP_001845617.1		241
	XP_014222130.1		231
	tr A0A2P8YFD4		246
	tr A0A132AMK5	VLIPSSPTTTIILNPENYNQSIGTDQITVTDNTNQSPASITNIIITTTIDNSDQKRTNYDQGGQITRTILRKKI	310
	sp P81657.1		202
	Sco_m_5		210
	XP_024085001.1		239
	KFM60495.1		248
	XP_001845617.1		241
	XP_014222130.1		231
	tr A0A2P8YFD4		246
	tr A0A132AMK5	ITHHNGQSESIDHLWQAKINNWKINIDSGNRQPTSRPKIARTDPPLSFSTWKKWRTSNYDWMKRWLTNYTQNLDT	385
	sp P81657.1		202
	Sco_m_5		36% 210
	XP_024085001.1		38% 239
	KFM60495.1		41% 248
	XP_001845617.1		41% 241
	XP_014222130.1		41% 231
	tr A0A2P8YFD4		37% 246
	tr A0A132AMK5	DLNRSWQKPLQSSVQNRQSTFDNNLDPSWHISNFSWYTNWTPKPNPKRLTTRIPTYTWALSV	36% 448
	sp P81657.1		36% 202

Supplementary Fig. 3. N-terminal sequence identities of Sco m 5 with other centipede allergen-like proteins and sequence alignment of Sco m 5 with allergens from non-centipede species.

A. N-terminal sequence identities of Sco m 5 with allergen-like proteins from *S. dehaani* (KC144347.1, KC144549.1, KC144967.1, KC144555.1, and KC144061.1), *S.*

viridicornis nigra (sp|P0C8D7), and *S. angulata* (sp|P0C8D4 and sp|P0C8D5). **B.** Comparison with other allergens from non-centipede species (sequences sourced from NCBI and UniProt database): venom allergen 5 isoform X3 from *Cimex lectularius* (36%, XP_024085001.1), venom allergen 5 partial from *Stegodyphus mimosarum* (38%, KFM60495.1), venom allergen 5 from *Culex quinquefasciatus* (41%, XP_001845617.1), venom allergen 3-like from *Trichogramma pretiosum* (41%, XP_014222130.1), venom allergen 3 from *Blattella germanica* (37%, tr|A0A2P8YFD4), Vespid v5 venom allergen-like protein (36%, tr|A0A132AMK5), and venom allergen 5 from *Vespa mandarinia* (36%, sp|P81657.1). Identical amino acids are highlighted.



Supplementary Fig. 4. HMC-1 activation by Sco m 5. Related to Fig. 3 E&F.

A, B. Sco m 5 induced HMC-1 degranulation was measured based on CD63 (A) and CD107a (B) surface staining. HMC-1 cells that had been sensitized with sera from centipede-allergic patients (2 and 3 in Fig. 3A) and non-allergic subjects (two randomly selected individuals among the 19 volunteers whose sera showed a negative IgE-binding reaction to Sco m 5). Either left untreated or exposed for 1 hour to anti-IgE (10 μ g/mL) as a positive control. The data represent the mean $\pm$ SEM from three technical replicates.

Supplementary Table 1. Information of the six centipede-allergic patients

No.	Age	Sex	Symptoms of allergy	Period from appearance to blood collection	Presence of toxic reaction	Experience of Hymenoptera sting
1	19	Male	Redness, itching, fever	1 week	Acute pain	Unknown
2	25	Female	Redness, itching, blistering	1 year	Pain	Unknown
3	38	Male	Redness, itching, edema	3 weeks	Acute pain	Unknown
4	42	Male	Redness, itching, blistering	2 months	Acute pain	Unknown
5	45	Female	Redness, itching, swelling	6 months	Acute pain	Unknown
6	48	Male	Redness, itching,	3 months	Acute pain	Unknown

Supplementary Table 2. Primers of Sco m 5 and 4 venom allergen-like proteins

Gene name	Forward primer Sequence (5'-3')	Reverse primer Sequence (5'-3')
Sco m 5	CGATCTGCTGAGACATGCGT	ACAGATCGCGGAATAGGGTC
MK440603	GCCAGGAACCTGCAACATTC	TAAACGACGACCACCCTGAG
MK440600	AAGCCCAAGAGTGGACGAGT	TGCCCAGACAACCTGTGAAT
MK440601	TGGACGAGTGAATGCAAGCC	TGCCCAGACAACCTGTGAAT
MK440602	AACCAGACTTCGACAACCCC	TCGGAGGAAAAAGAGCCACC
Actin	TCCCCGAGCTGTATTTCCCT	GGCCACTCGGAGTTCATTGT