



Shellfish Tropomyosin IgE Cross-reactivity Differs among Edible Insect Species

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Abbreviations

BSF	Black soldier fly
CRX	Cricket
Dro	<i>Drosophila Melanogaster</i>
GCR	German cockroach
GP	rabbit glycogen phosphorylase B
MCR	Madagascar hissing cockroach
HDM	House dust mite
MLW	Mealworm
pTM	partial Tropomyosin
SHR	Shrimp
SPW	Superworm
TM	Tropomyosin
WXW	Waxworm

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Abstract

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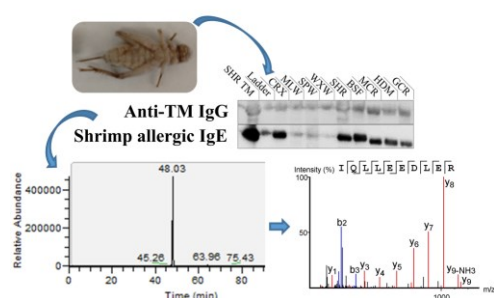
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Scope: Insects are a potentially environmentally friendly alternative dietary protein source to supplement mammalian and fish sources, but potential allergenic risks are a concern. Consumption of insects may result in anaphylaxis and has been implicated in cross-reactivity with shellfish. Many allergenic proteins may be involved in cross-reactivity including tropomyosin (TM). The uniformity of TM cross-reactivity among edible insects is unknown. We investigated candidate edible insects for variability in shellfish IgE cross-reactivity.

Methods and results: Selected insects and known related sources of allergens were extracted and probed by immunoblot with sera/plasma from patients sensitized to insects or shellfish.

Quantification of TM in these extracts was performed using MS/MS. A comparison of the quantity of TM and the IgE reactivity of TM from these insects was performed. Distinct patterns of IgE cross-reactivity were observed with three insect species showing diminished reactivity. This pattern was not consistent with the amount of TM present in these insects, or with overall sequence homology.

Conclusions: Insects display a diversity of TM-associated IgE reactivity. It is likely that minor sequence features and/or structural effects are primarily responsible. Additionally, we demonstrate that some insect species may present significantly less IgE cross-reactivity to shrimp than do others.



Insect and shellfish tropomyosins were investigated for their ability to bind shrimp allergic and sensitive IgE. Some insects were found be similarly reactive and others less reactive. Differences were not due to quantity of tropomyosins nor overall sequence identity. It is likely that small sequence and structural variations are responsible such as those observed among isoforms of tropomyosins.

1 Introduction

The growing human population, environmental concerns, and food inequality demand novel

solutions in food production. Insects can provide a protein-rich food source with low biomass, water, and energy inputs during production which offers sustainability advantages over traditional meat production.^[1] Edible insects are currently a dietary component for an estimated 2 billion consumers and although consumption in Europe and the Americas is low there have been initiatives to incorporate insects into Western diets.^[2] Shifting Western attitudes toward insects as a novel food will inevitably result in increases in exposure, necessitating the assessment of the risks posed, including food allergy.

In Asia, consumption of edible insects including silkworms as well as grasshoppers and locusts have resulted in reports of anaphylaxis.^[3] Mealworms (*Tenebrio molitor*; MLW) were recently identified as a source of both primary respiratory and food allergy in Northern Europe.^[4] Some individuals with shrimp (SHR) allergy may be at risk of allergic reactions to insects.^[5] Many insect-containing food products in the West contain language on their packaging indicating that shellfish allergic individuals should exercise caution upon consumption. Shellfish are major sources of food allergens with food challenge confirmed prevalence rates of up to 0.9%.^[6] Studies have demonstrated the potential for shellfish-allergic patients to be cross-reactive to insects and house dust mite via tropomyosin (TM), as well as other identified allergenic proteins such as arginine kinase.^[7]

Concern has focused on TM due to its resistance to denaturation and gastric digestion as well as abundance.^[8] TMs are known to form coiled-coil dimers that can associate to form polymers,^[9] which may theoretically result in IgE crosslinking through a single epitope repeated across a dimer or polymer. Current knowledge regarding insect TMs focuses on fruit fly (*Drosophila melanogaster*; Dro), which has two TM genes which together can generate several isoforms of TM through transcriptional alternative splicing.^[10] TMs are a family of closely related proteins, but minor sequence variation can lead to large differences in IgE binding and possibly allergic reactivity as seen in arthropods.^[11] Overall sequence identity may be informative but should be used cautiously. Jenkins *et al* identified 54-56% sequence identity shared among human and shrimp TMs, and found

that proteins with less than 62% identity were seldom allergenic.^[12] Aalberse noted that cross-reactivity is rare below 50% sequence identity and more common above 70%.^[13] Further information regarding allergic risks associated with consumption of edible insects is needed to adequately inform at-risk populations.

The study described herein addresses TM cross-reactivity at the IgE level across a range of edible and potentially edible insects. The quantity of TM present in each insect is pertinent to the potential risk of consumption and is addressed through IgG binding and through quantitative MS/MS. Partial cloning of TMs was performed to facilitate precise quantification by MS/MS. The aim was to better evaluate likely risks of TM cross-reactivity to shellfish allergic individuals.

2 Materials and methods

2.1 Samples

Adult crickets (*Acheta domesticus*; CRX), MLW larvae (*T. molitor*), superworm larvae (*Zophobas morio*; SPW), waxworm larvae (*Galleria mellonella*; WXW), and black soldier fly larvae (*Hermetia illucens*; BSF) were a gift from All Things Bugs™ (Midwest City, OK, USA). German cockroaches (*Blattella germanica*; GCR) and pork-medium-raised house dust mite (*Dermatophagoides pteronyssinus*; HDM) powder were obtained from Greer Laboratories™ (Lenoir, NC, USA). Adult Madagascar hissing cockroaches (*Gromphadorhina portentosa*; MCR) were a gift from the University of Nebraska-Lincoln entomology department (Lincoln, NE, USA). SHR were obtained from a local supermarket, sent to Applied Food Technologies (Alachua, FL, USA) for speciation, and identified as white shrimp (*Litopenaeus setiferus*). Samples were stored at -20 °C until use.

2.2 Protein Extraction

Samples were homogenized and defatted by mortar and pestle in the presence of acetone. Mixtures were brought to 5% (w/v) acetone, stirred (1 hour, 22 °C, 220 rpm), and dried. Dry powder was brought to 5% (w/v) hexane, stirred (2 hour, 22 °C, 220 rpm), and dried. The resultant defatted powder was further homogenized with a clean Braun™ KSM2 coffee grinder (Frankfurt, Germany)

and stored at -20 °C.

Samples were extracted as modified from Zhou *et al.*^[14] In brief, 50 mg of each defatted sample was extracted in 1 mL of 2D buffer (50 mM Tris-HCl, pH 8.8 containing 50 mM DTT, 5 M urea, 2 M thiourea, 10 mM EDTA, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 4% (w/v) PVP cross-linked). Suspensions were vortexed, incubated in a heated sonicating water bath (60 °C, 20 min), and the extracts were clarified by centrifugation (14,000 x g, 20 min). The supernatants (500 µL) were taken and an equal amount of extraction buffer was added to the pellet. Each 50 mg sample was sequentially extracted three times in order to maximize protein recovery, and the three supernatants of each pellet were pooled as a single replicate. Sequential extraction was performed in triplicate per species. Protein contents of extracts were determined using a 2D Quant-Kit™ per manufacturer's instructions (GE Healthcare Life Sciences, Chicago, IL, USA). Extracts were stored at -20 °C until further analysis.

2.3 Sera and Plasma

Sources of IgE used in this study were sera from five subjects who had positive oral challenge results for SHR, and plasma from nine subjects with self-reported adverse reactions to fish and/or shellfish and positive ImmunoCAP® values (>0.35 kU/L) to shellfish (crab F23, shrimp F24, lobster F80), dust mites (Der p D1, Der f D2), or German cockroach (I6). Two control subjects (one non-atopic and one atopic) without sensitivity to shrimp (Pen m 1, 2, 4), house dust mites (Der p 1, 2, 10; Der f 1, 2), or German cockroach (Bla g 1, 2, 5, 7) according to ImmunoCAP ISAC 112® (Table 1) were used. All subjects were adults and 93% were male. All oral challenge positive subjects were diagnosed after 18 years of age. Human sera were obtained and used as defined by University of Nebraska IRB Number: 200305289 FB Project ID: 6029 and as defined by clinical collaborators' ethics board guidelines for de-identified human serum samples and plasma obtained from a U.S. FDA licensed facility (PlasmaLab International, Everett, WA, USA) as defined by University of Nebraska IRB Number: 2009059345 EP. Written informed consent was obtained for all subjects whose sera

was used in this study, and plasma was from a facility that meets FDA regulations for collection of human plasma. Sera and plasma were frozen at -20 °C prior to use.

2.4 SDS-PAGE and Immunoblotting

SDS-PAGE was used to separate 4 µg of protein per sample using 4-12% NuPage Bis-Tris gels (Invitrogen, Carlsbad, Ca, USA). A protein ladder, Precision Plus Protein™ Dual Xtra standards (Biorad, Hercules, CA, USA), was used to estimate molecular weight. Immunoblotting was performed as described elsewhere using rabbit anti-shrimp TM IgG (Indoor Biotechnologies, Charlottesville, VA, USA),^[15] plasma, or sera each diluted in 2.5% non-fat dry milk in PBS with tween (0.05% Tween 20, 1x PBS, pH 7.4). Rabbit anti-shrimp TM IgG was diluted 1:4,000 followed by 1:4,000 goat anti-rabbit IgG peroxidase conjugated (Thermo Scientific, Waltham, WA, USA) and sera or plasma were diluted 1:10 followed by 1:1,000 mouse anti-human IgE peroxidase conjugated (Southern Biotech, Birmingham, AL, USA). Immunoblots were visualized by chemiluminescence using SuperSignal West Dura Extended Release Substrate (Thermo Scientific) detected using a Kodak Gel Logic 440 image station (Kodak, Rochester, NY) and SDS-PAGE was visualized with CBB R-250 (Biorad).

2.5 Cloning

Samples were frozen in liquid nitrogen and homogenized by mortar and pestle. RNA was isolated using TRIzol® (Life Technologies, Carlsbad, CA, USA) per manufacturer's instructions. Superscript® IV First-Strand Synthesis System (Invitrogen) was used to obtain cloned DNA. Primers were designed using NCBI tblastn queried using the *Litopenaeus vannamei* TM sequence (Lit v 1; NCBI, ACB38288.1) against each sample organism. Where DNA sequences were not present, searches were expanded up the taxonomic hierarchy until a match was found. Matches were used to generate primers targeting regions of high homology across hits (Supporting Information Table S1). PCR was performed using GoTaq DNA Polymerase (Promega, Madison, WI) according to manufacturer's instructions. PCR products were visualized with a 1% (w/v) agarose gel and the bands excised and extracted using a QIAquick gel extraction kit (Qiagen). DNA quality and identity were verified by NanoDrop 2000c (Thermo Scientific) and a second round of PCR.

A pGEM®-T Easy Vector System II (Promega) was used according to manufacturer's instructions to ligate amplified DNA into a pGEM vector, transform supplied JM109 *E. coli* cells, and screen cells for successful transformation. Transformed cells were incubated in sterile lysogeny broth with ampicillin (50 µg mL⁻¹) and plasmids purified using a Wizard® *plus* SV Minipreps DNA purification system (Promega) per manufacturer's instructions. Plasmids were sequenced by Genewiz (South Plainfield, NJ, USA) and sequences used to generate nested gene specific primers (Supporting Information Table S2) for use with a SMARTer® RACE 5'/3' Kit (Clontech, Mountain View, CA, USA) used per manufacturer's instructions. These sequence data have been submitted to GenBank under accession numbers MH999801-MH999809. Translated sequences were aligned using Clustal Omega (1.2.3) including known allergenic TMs from *L. vannamei*, *B. germanica* (Bla g 7; AAF72534.1), and *D. pteronyssinus* (Der p 10; ACI32128.1) as well as TMs from Dro (NP_732004.1, NP_732012.1).

2.6 Shrimp tropomyosin purification

Purification was conducted as modified from methods reported previously,^[16] with an additional gel filtration polishing step to improve protein purity. Briefly, SHR muscle was homogenized and used to produce an acetone muscle powder, subsequently extracted overnight, and the residual pellet reextracted for 2 hours. Supernatants were combined, underwent isoelectric precipitation at pH 4.6 and ammonium sulfate precipitation at 40% saturation. The pellet was resolubilized, boiled, dialyzed and passed through a 0.45 µm filter. Anion exchange was performed using a HiPrep Q Sepharose Fast Flow 16/10 column (GE Healthcare Life Sciences) and eluted at 5 mL min⁻¹ with linear NaCl gradients of 100 mL 0-300 mM 400 mL across 300-700 mM. Fractions enriched in a 37 kDa band, as determined by SDS-PAGE, were pooled, concentrated, and buffer-exchanged to 1 x PBS, pH 7.4 with an Amicon Ultra-15 10 kDa nominal molecular weight limit centrifugal filter (Merck Millipore, Billerica, MA, USA). The concentrate was loaded onto a pre-packed 90 mL Sephadex G-50 column (Sigma-Aldrich, St. Louis, MO, USA) equilibrated and eluted with 1 x PBS, pH

7.4 at a flow rate of 0.2 mL min^{-1} . Fractions containing TM were pooled and concentrated with an Amicon Ultra-15 filter.

2.7 Mass Spectrometry

Further details of the MS can be found in the supporting information. Briefly, extracts (prepared as above) were precipitated using acetone to remove salts and the pellet reduced, alkylated, and digested with trypsin for LC-MS/MS. Peptides were cleaned using Pierce C18 Spin columns (Thermo Scientific) according to manufacturer's instructions excluding final elution, which was performed using 50% ACN. Samples were vacuum evaporated and resolubilized to $1 \text{ ug } \mu\text{L}^{-1}$ in 0.1% formic acid with $50 \text{ fmol } \mu\text{L}^{-1}$ rabbit glycogen phosphorylase B (GP, P6635, Sigma-Aldrich; P00489.3) of which $2 \text{ } \mu\text{L}$ were injected into a Q Exactive PlusTM Hybrid Quadrupole-OrbitrapTM MS (Thermo Scientific).

2.8 Mass spectrometry data analysis

Data was analyzed using PEAKS 8.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) against an Arthropoda database (Uniprot, accessed 07/02/2017).^[17] PEAKS search parameters were 5 ppm mass error tolerance, 0.05 fragment mass error tolerance, fixed carbamidomethylation of cysteine, and variable oxidation of methionine. TM sequences identified were curated and added to a customized database along with predicted TM translation products cloned here and GP (33 sequences). Peptides selected for quantitation were selected for their occurrence in all insect species for which sequence data was available. For species for which sequence was not available, presence of the peptides in MS data was used as proof of presence. Common TM peptides were identified and their extracted ion chromatograms quantified using XcaliburTM 4.0 (Thermo Scientific) using a mass tolerance of 5 ppm and guided by PEAKS MS/MS identifications. For quantitation, data were normalized via the top three GP peptides using the label-free quant feature from PEAKS while excluding all GP peptides identified during the query against Arthropoda. Peptide values were analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test with significant p-values

below 0.05 using graphPad Prism version 4.03 for Windows (GraphPad Software, La Jolla California USA).

3 Results

3.1 IgE binding to tropomyosins

Binding of IgE from individual patient sera was evaluated by immunoblotting of proteins from various insect species. Coomassie staining of gels of extracted protein was also performed to evaluate protein extractions and presence of major insect proteins. Insect samples were prepared and extracted using a sequential extraction process to maximize protein extraction. Visualization of sample extracts via SDS-PAGE (Figure 1.A) demonstrate the diversity of extracted proteins across samples.

A representative immunoblot using sera from an allergic patient (A5) is provided (Figure 1.B). Complete immunoblots can be found in the supporting information (Supporting Information Figure S1-3). The principal pattern of IgE binding is between 32 and 37 kDa and sparse reactivity at 75 kDa for some samples. Rabbit anti-shrimp TM IgG was used to locate TM on blots (Figure 1.C). Two shrimp allergic subjects confirmed by oral food challenge (2 out of 5) and seven self-reported shellfish allergic subjects (7 out of 9) showed IgE binding to control SHR TM. Relatively lower IgE reactivity is present across MLW, SPW, and WXW among all patients, excluding A4. Patient A4, contrary to others, presents with consistent reactivity across all samples including MLW, WXW, and SPW. Negative control sera identified no IgE binding. Control SHR TM shows that, although principally reactive around 37 kDa, there is strong binding along a large range of molecular weights, which is mirrored to a lesser degree in SHR, BSF, and CRX.

3.2 Cloning and sequencing

Several cloned DNAs were cloned including 8 partial TM (pTM) clones from 5 species with two from CRX, MLW, and WXW and one from each of SHR and SPW as well as a full-length clone from MCR. Cloning of TMs from BSF was also attempted but unsuccessful. Predicted translation

products from cloned DNA sequences were aligned with known allergenic TMs and two Dro TMs representing each of the two Dro TM genes (Supporting Information Figure S4) and an identity matrix provided (Table 2). Inclusion of Dro TMs resulted in a segregation of the cloned and allergenic TMs where CRX pTM1, WXW pTM2, and Dro TM2 shared identities between 79-83% but shared identities of 58-70% beyond these three. Identities among isoforms within the same species followed the same pattern as Dro (61%) with CRX and WXW at 65% and 66% respectively. The isoforms of MLW were not similarly segregated due to peptides that were shared among the isoforms but dissimilar from the Dro TM2 cluster.

3.3 LC-MS/MS quantification of tropomyosin isoforms

Based on the separation of the TMs using the Dro TM gene structures, peptides were identified in conjunction with MS to appropriately quantify present TMs. Of the chosen peptides; [IQLLEEDLER]²⁺ targets an expressed exon shared by Dro TM1 sequences (see NCBI gene ID 41852, Genomic Sequence NT_033777.2), [MDQLTNQLK]²⁺ identifies Dro TM2 sequences (see NCBI gene ID 41853), and [SLEVSEEK]²⁺ was largely identified to be shared among both populations of TMs (Figure 2). Presence of each peptide used to quantify present TMs were not necessarily confirmed at the sequence level, but rather inferred according to their identification by MS. Leucine and isoleucine isomerism was observed in [IQLLEEDLER]²⁺ peptides (see Der p 10, Figure 2) but these peptides were treated equally due to isobaric masses of these residues resulting in identical m/z.

Label-free quantification of TMs indicated that on an equal protein basis SHR has the most [IQLLEEDLER]²⁺ followed by BSF and CRX (Figure 3). For [MDQLTNQLK]²⁺, BSF was relatively high but not significantly greater than MCR nor GCR; however, neither SHR nor HDM had detectable levels of [MDQLTNQLK]²⁺. Lastly for [SLEVSEEK]²⁺, both SHR and BSF had the highest concentrations, but not different from MCR nor GCR. Quantified levels of TM were not significantly different for any of the three peptides among the group of CRX, WXW, MLW, and SPW nor were they different between MCR and GCR.

4 Discussion

IgE reactivity is a function of both IgE binding and quantity of protein present. IgE binding to a protein is related to primary sequence, protein structure and possibly the presence of protein modifications. This study was primarily designed to examine differential IgE binding of food insect species, and to examine whether any such differences were due to quantity of TM present. Allergenicity of TMs does not decrease with processing and so use of raw samples here does not limit inference.^[18] There was clear diversity in the capacity of TM from the studied range of insect species to bind IgE from shrimp sensitized individuals. In particular, MLW, WXW and SPW show reduced binding compared to shrimp controls and other insect species. This effect was consistent for all of the sera used for which there was observed TM binding. Protein profiles observed in this study differed from other studies,^[5, 19] but these differences can most likely be explained by differences in extraction methodology in which insects were defatted and extracted under heated sonication,^[20] which can increase overall protein yield and potentially alter the representation of proteins present under equal protein loads. Despite this, the reactivity observed by Broekman *et al.* patients 2 and 9,^[5] primary SHR allergic subjects, were similar to those presented here. Further, insect TMs can be observed as polymers on reducing SDS-PAGE and thereby result in ancillary bands on an immunoblot.^[9]

We postulated that the most obvious cause of reduced IgE reactivity by the TM of MLW, WXW and SPW was lower TM abundance in these samples. Our polyclonal anti-TM IgG did not suggest significantly different levels of TM in MLW, WXW or SPW than in our other insect species. Additionally, relative quantitation by MS/MS revealed that quantity of TM cannot explain observed differences in IgE reactivity. This is consistent with TM being a major muscle protein and are likely to be highly conserved through evolution of insect species. This being the case, sequence differences may be the cause of observed differences in IgE reactivity. Ayuso *et al.* observed that single amino acid mutations to a synthesized IgE-binding peptide derived from Pen a 1 had the potential to mitigate that IgE-binding for some sera.^[11a] We performed sequence alignments of TM from insect

and shrimp species and determined that overall (sequence length) homology of proteins is not consistent with observed patterns of reactivity. We therefore now postulate that small, regionalized differences in protein sequence, most likely in IgE binding epitopes, are the most likely cause of differential IgE binding of insect TMs.

Isoforms of TM can result in such regionalized sequence differences. Isoform variation among Arthropodan TMs is well known,^[10, 21] but it is not well known how this affects allergic responses. The TM isoform represented by [IQLLEEDLER]²⁺ is the predominant isoform in SHR and presumably is the highly IgE reactive molecule for those with SHR allergies. This isoform is also present in insects. The other isoform represented by [MDQLTNQLK]²⁺ is absent from SHR and HDM but present in insects. The peptide [SLEVSEEK]²⁺ is then used as a proxy to represent both isoforms to indicate total TM. We speculate that higher amounts of [IQLLEEDLER]²⁺, and thus SHR-like TM, in BSF would indicate this insect food source would be the greatest risk to those with current SHR allergies. Likewise, high amounts of [SLEVSEEK]²⁺ in insects could potentially indicate that increasing consumption of insect food sources would lead to an allergic population arising to this source of TM in the future. The particular TM source of a subject's primary sensitization could potentially result in large differences in patterns of observed cross-reactivity. Insect TM isoforms may complicate assessing risk parity to shellfish as there may be a lack of complete sequence information for MS and/or unclear antibody reactivity for ELISA.

IgE binding by proteins is not of itself proof of the clinical allergenicity of a protein. However, the capacity to bind IgE is an essential component of the process of elicitation of an allergic reaction. The work presented here demonstrates that different insect species display different degrees of cross-reactivity when exposed to the IgE of shrimp TM sensitized individuals. It is therefore possible that consumption of some insect species (here MLW, WXW and SPW) present less risk to shrimp allergic individuals than do others. Further clinical investigation of this differential cross-reactivity is warranted, and in our opinion necessary, to more fully describe the risk that insect-derived foods pose to shellfish allergic individuals.

Acknowledgments

LP designed and performed immunoblotting experiments, analyzed proteomic data, and wrote the manuscript. JM designed and performed sequencing experiments and analyzed proteomic data. ML designed and performed purification experiments and screened plasma. RG provided plasma from self-reported allergic subjects. MZ conceived the study. PJ conceived the study and had primary responsibility for final content. All authors critically read, commented upon, and approved the final manuscript.

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Conflict of Interest

The authors report non-financial support from All Things Bugs™ and financial support from the Food Allergy Research & Resource Program during the conduct of the study. LP, JM, and PJ report a grant from the Defense Advanced Research Projects Agency and LP, PJ, and RG report a grant from the Aspire Food group outside of submitted work.

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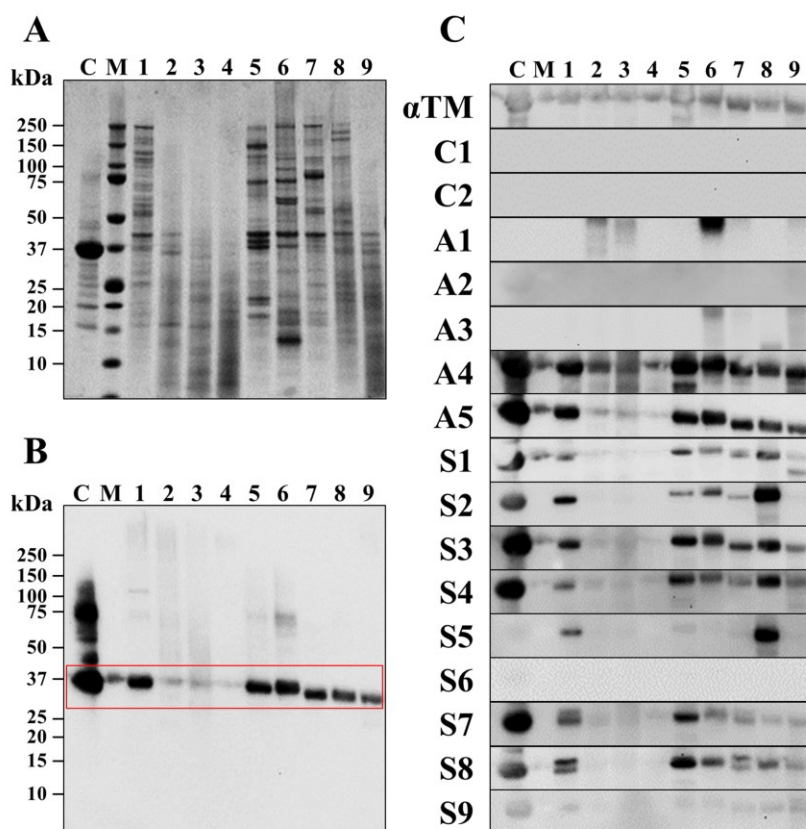


Figure 1. Gel and immunoblot analysis of pooled extracts

Pooled extracts visualized using SDS-PAGE (A) with lanes as follows: (C) Control purified shrimp tropomyosin, (M) molecular weight standards, (1) cricket, (2) mealworm, (3) superworm, (4) waxworm, (5) shrimp, (6) black soldier fly, (7) Madagascar hissing cockroach, (8) house dust mite, and (9) German cockroach. A representative immunoblot is presented (B) with the 37 kDa region of interest boxed, and a comparison of immunoblots focused around 37 kDa (C) with blots labeled as αTM, C1-2, A1-5, and S1-9 representing rabbit anti-tropomyosin IgG, control sera, shrimp oral challenge positive sera, and shrimp self-reported allergic sera respectively. Subjects with shrimp tropomyosin reactivity demonstrate a pattern of low reactivity to mealworm, superworm, and waxworm.

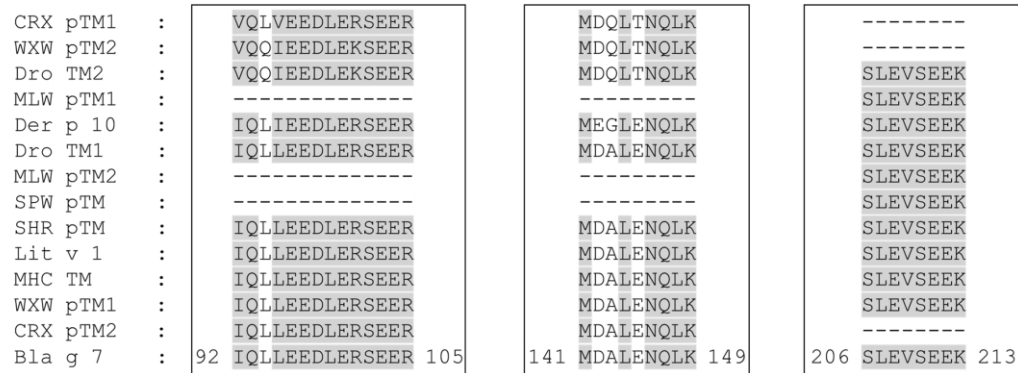


Figure 2. Partial protein sequence alignment of predicted cloned products, allergenic, and fly tropomyosins

Clustal omega alignment of cloned predicted translated tropomyosins, selected allergenic tropomyosins (Lit v 1, Bla g 7, and Der p 10), and *D. melanogaster* tropomyosins (1 and 2). Highly conserved residues are displayed in dark grey, less conserved residues are displayed in light grey, and missing residues are displayed with dashes. Alignment residue numbers are based on alignment to Bla g 7 and each alignment focuses on IQLLEEDLER, MDQLTNQLK, and SLEVSEEK respectively.

Alignment of cloned translationally predicted and representative tropomyosins demonstrates the presence of both *D. melanogaster* 1 and 2-type tropomyosins where cricket partial tropomyosin 1 and waxworm partial tropomyosin 2 are more like *D. melanogaster* tropomyosin 2 and the remainder are more similar to *D. melanogaster* tropomyosin 1.

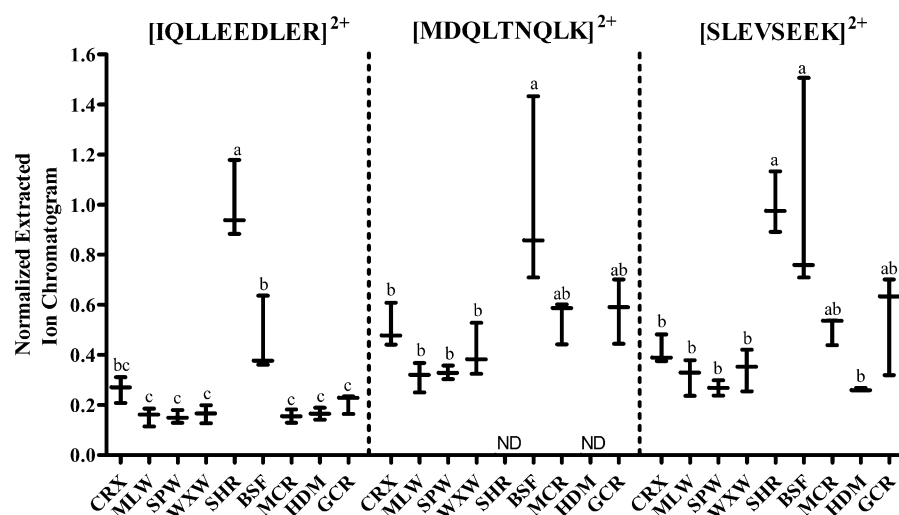


Figure 3. LC-MS/MS Label-free quantification of tropomyosin isoforms

Extracted ion chromatograms of tropomyosin specific peptides normalized first to internal rabbit glycogen phosphorylase B standard and second to the greatest species mean per peptide. Groups with different letters are significantly different. [MDQLTNQLK]²⁺ was not detected in neither shrimp or house dust mite. [IQLLEEDLER]²⁺ is tropomyosin 1 specific, [MDQLTNQLK]²⁺ is tropomyosin 2 specific, and [SLEVSEEK]²⁺ is general for either 1 or 2-type tropomyosins. Shrimp and black soldier fly had higher amounts of tropomyosins whereas mealworm, superworm, and waxworm had comparable tropomyosin levels to other insects.

Table 1. Serological data from clinically confirmed, self-reported, and control shrimp allergic or sensitive subjects

Seru	Sex	Specific IgE via ImmunoCAP® (kU/L)						Symptoms	Other Allergies
		Shrim F24	Cra F23	Lobste F80	G. I6	Der D1	Der D2		
Shrimp allergic subjects with positive oral challenge									
A1	M	25.3						P, U, N, AP, E	c
A2	M	2.6						AE, NC, L, DY, CD	ap, b, bn,
A3	M	3.0						AE, N, NC, L, SW	m, c
A4	M	81.1						P, AE, E, W	tn
A5	M	16.3						AE, LS, OAS	ap, p
Self-reported shellfish allergic and/or sensitized subjects									
S1	M		>10	>100				Itchy throat, rash	co, nuts,
S2	M				11.4	>10	>10	Respiratory/cutaneo	e, f,
S3	M	26.7			8.3	5.4	7.7	Itchy/swollen throat	c, f
S4	M	27.3	23.9					DY	c
S5	M	6.2			2.7	>10	>10	NI	c, f, nuts
S6	M		6.4	0.6	6.5	2.1		Nose and lung	c, f
S7	M	84.3	76.8	77.6	66.5			NI	c, f
S8	F	71.5	33.9			7.0		Oral itch	f
S9	M	21.9	13.0	9.3	9.8	6.6	5.9	Respiratory/cutaneo	c, f
Shellfish non-reactive control subjects									
		Specific IgE via ImmunoCAP ISAC 112® (ISU)							
C1	F	0 √			0 √	0 √	0 √	None	Mold,
C2	F	0 √			0 √	0 √	0 √	None	None

a) NI, no information; √, for all allergenic proteins per category;

b) AE, angioedema; AP, abdominal pain; CD, cardiovascular dysfunction; DY, dyspnea; E, emesis; L, laryngeal edema; LS, lip swelling; N, nausea; NC, nasal congestion; OAS, oral allergy symptoms; P, pruritus; SW, sweating; U, urticaria; W, wheezing;

c) ap, apple; b, banana; bn, bean; c, crustacean; ca, carrot; co, corn; f, fish; e, eggs; m, milk; p, peanut; pe, pea; st, strawberry; tn, tree nut

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Table 2. Percent identity matrix for predicted cloned products, allergenic, and fly TMs

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 CRX pTM1		80	79	60	63	62	69	70	68	68	66	69	67	66
2 WXW pTM2			83	58	64	62	67	68	65	65	64	66	65	64
3 Dro TM2				58	64	61	65	66	67	66	63	68	64	63
4 MLW pTM1					72	79	78	79	74	74	79	79	81	79
5 Der p 10						75	81	81	80	80	79	81	82	80
6 Dro TM1							93	95	80	78	85	91	88	86
7 MLW pTM2								99	91	85	89	98	100	88
8 SPW pTM									92	87	90	98	100	90
9 SHR pTM										100	83	85	83	84
10 Lit v 1											82	85	83	82
11 MCR TM												92	93	99
12 WXW pTM1													93	91
13 CRX pTM2														93
14 Bla g 7														

Received: 28/08/2019; Revised: 04/12/2019; Accepted: 24/01/2020

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Supporting Information

Materials and methods

Mass Spectrometry

For each replicate extract, 28 μg of protein were diluted to 1 $\mu\text{g } \mu\text{L}^{-1}$ with extraction buffer and precipitated with 4 volumes of ice-cold acetone at $-80\text{ }^{\circ}\text{C}$ for 2 hours. Samples were centrifuged (14,000 $\times g$, 20 min, $4\text{ }^{\circ}\text{C}$), the pellets washed with 80% (v/v) ice-cold acetone (10 min), re-centrifuged, and repeated twice. Pellets were dried for 1 hour.

Pellets were reduced by adding 45 μL of 50 mM ammonium bicarbonate, 4.5 μL of 100 mM DTT, 26.7 μL of water, and 4.8 μL of 100% ACN and incubating at $95\text{ }^{\circ}\text{C}$ for 5 minutes. The samples were alkylated by adding 9 μL of 100 mM of iodoacetamide and incubating at room temperature in the dark for 20 minutes. The samples were digested by adding 3 μL of 100 $\text{ng } \mu\text{L}^{-1}$ trypsin (Pierce, MS grade) and incubating at $37\text{ }^{\circ}\text{C}$ for 3 hours. A further 3 μL of 100 $\text{ng } \mu\text{L}^{-1}$ trypsin was added and incubated at $30\text{ }^{\circ}\text{C}$ for overnight. The reaction was quenched by freezing.

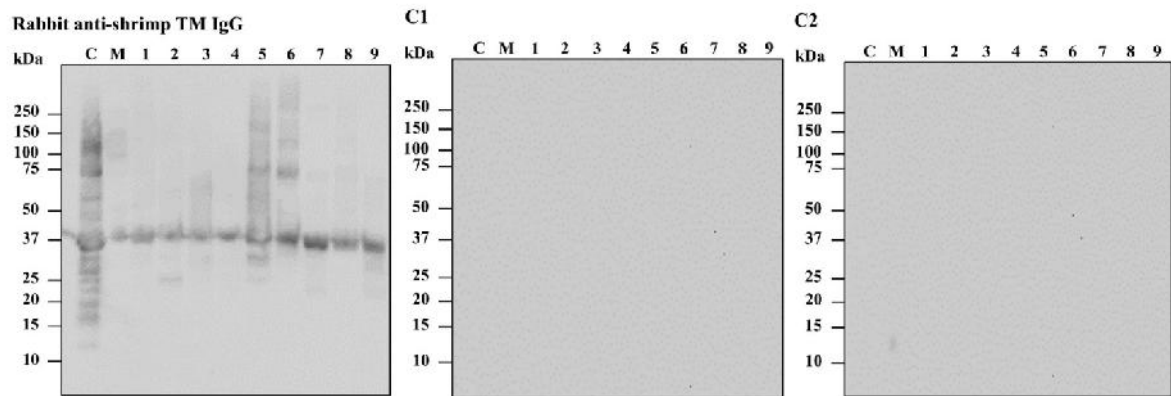
Pierce C-18 Spin columns (Thermo Scientific) were used according to manufacturer's instructions excluding the final elution, which was performed using 50% ACN. Eluted samples were dried by centrifugal vacuum evaporation (RC1010, Jouan, Saint-herblain, Pays de la Loire, France). Dried samples were resolubilized in 28 μL of 0.1% (v/v) formic acid containing 50 $\text{fmol } \mu\text{L}^{-1}$ of GP, where a 2 μL injection would be equivalent to 100 fmol of GP. Digests were aliquoted into vials and frozen until injection.

One-dimensional microscale liquid chromatography separation of tryptic peptides was performed with an UltiMate 3000RSL[®] liquid chromatography system (Thermo Scientific), equipped with a Hypersil Gold C18 1.9 μm , 100 $\times$ 1 mm analytical reversed phase column (Thermo Scientific). Mobile phase A consisted of 99.9% (v/v) water containing 0.1% (v/v) formic acid and mobile phase B was 99.9% (v/v) ACN containing 0.1% (v/v) formic acid. Two μL of the sample was injected on-column and peptides

25 were eluted from the analytical column and separated using a gradient of 2-40 % mobile phase B over
26 60 minutes at a flow rate of 60 μ L/min. The analytical column temperature was maintained at 35 °C.

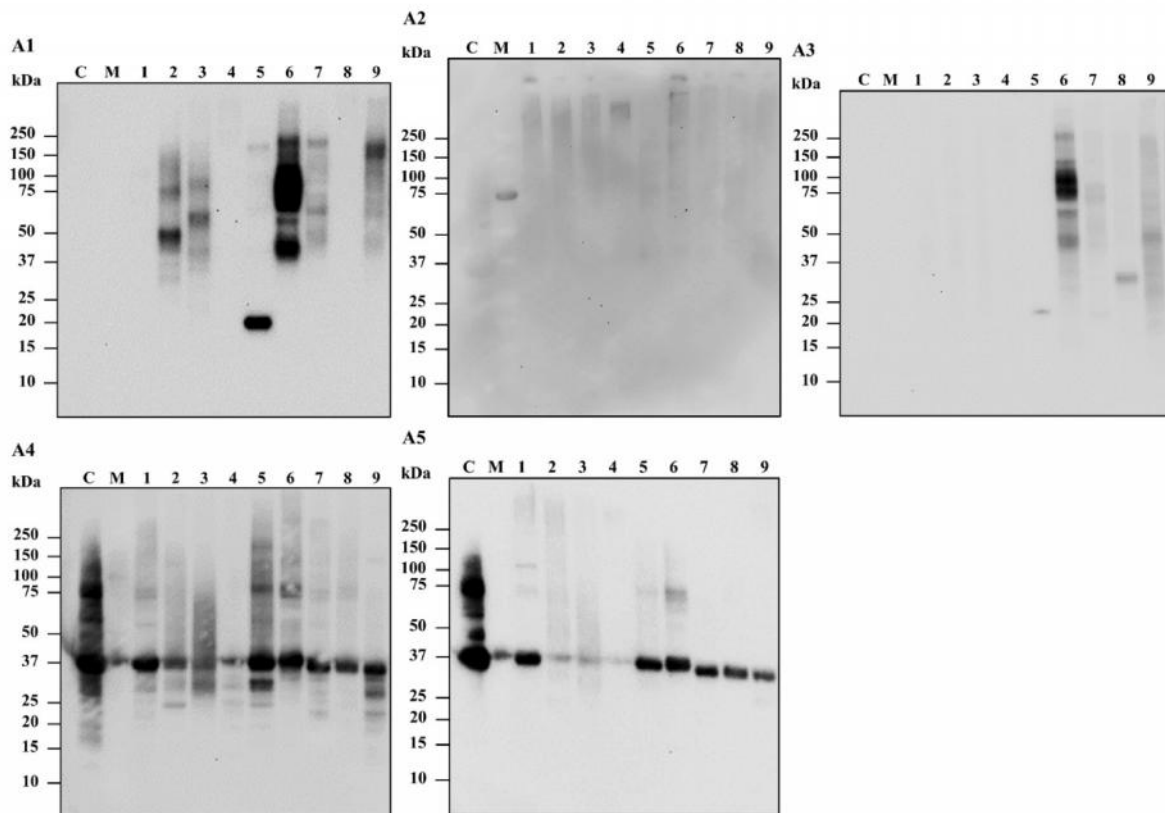
27 Mass spectrometric analysis utilized a Q Exactive PlusTM Hybrid Quadrupole-OrbitrapTM MS
28 (Thermo Scientific) in the data-independent mode with survey scans and fragment ion spectra acquired
29 at a resolution of 70,000 at m/z 400. Up to the top 20 most abundant isotope patterns with charge 2 to
30 4 from the survey scan were selected with an isolation window of 2 m/z with a window offset of -0.4
31 m/z and fragmented by higher energy collisional dissociation with normalized collision energies of 27.
32 The maximum ion injection times for the survey scan and the MS/MS scans were 100 and 240 ms,
33 respectively, and the ion target value for scan modes were set to 3E6 and 1E5, respectively. Repeat
34 sequencing of peptides was minimized by dynamic exclusion of the sequenced peptides for 20 s.

Supporting information Figure S1. Full immunoblots for positive and negative controls

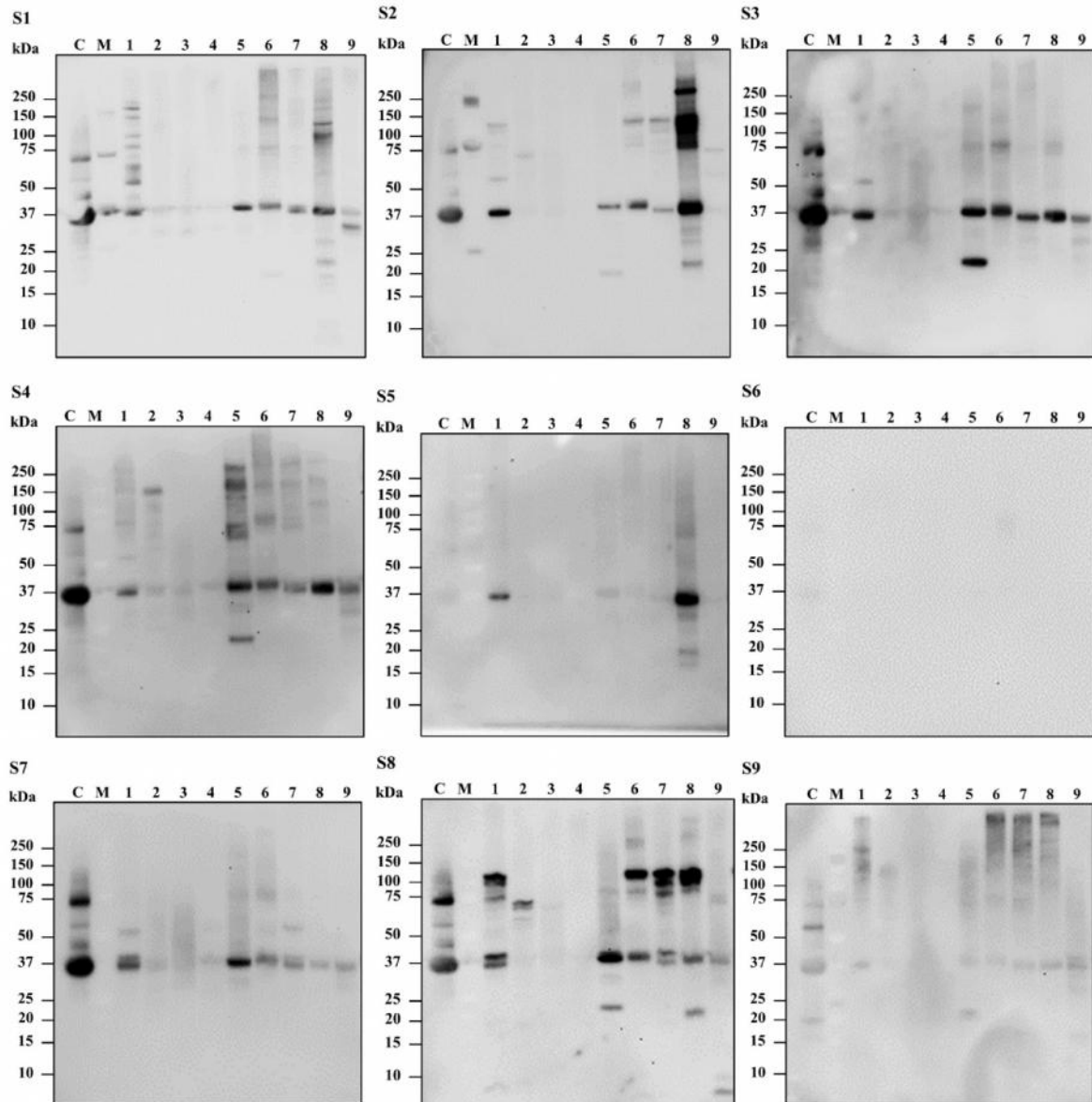


Lanes were as follows: (C) Control purified shrimp tropomyosin, (M) molecular weight standards, (1) cricket, (2) mealworm, (3) superworm, (4) waxworm, (5) shrimp, (6) black soldier fly, (7) Madagascar hissing cockroach, (8) house dust mite, and (9) German cockroach. Subjects with shrimp tropomyosin reactivity demonstrate a pattern of low reactivity to mealworm, superworm, and waxworm.

Supporting information Figure S2. Full immunoblots for oral challenge positive sera



Lanes were as follows: (C) Control purified shrimp tropomyosin, (M) molecular weight standards, (1) cricket, (2) mealworm, (3) superworm, (4) waxworm, (5) shrimp, (6) black soldier fly, (7) Madagascar hissing cockroach, (8) house dust mite, and (9) German cockroach. Subjects with shrimp tropomyosin reactivity demonstrate a pattern of low reactivity to mealworm, superworm, and waxworm.



50
 51 Lanes were as follows: (C) Control purified shrimp tropomyosin, (M) molecular weight standards, (1)
 52 cricket, (2) mealworm, (3) superworm, (4) waxworm, (5) shrimp, (6) black soldier fly, (7) Madagascar
 53 hissing cockroach, (8) house dust mite, and (9) German cockroach. Subjects with shrimp tropomyosin
 54 reactivity demonstrate a pattern of low reactivity to mealworm, superworm, and waxworm.

56 **Supporting information Figure S4.** Protein sequence alignment of predicted cloned products, allergenic,

57 and fly TMs

58	CRX pTM1	:	MDAIKKKMQAMKLEKDNAMDKADTCEGQAKDANNKADKINEDVQELTKKLAQVENDLITT	:	60
59	WXW pTM2	:	MDAIKKKMQAMKLEKDNAMDKADTCEQQARDANLRAEKVNEEVRELQKKLAQVEEDLTN	:	60
60	Dro TM2	:	MDAIKKKMQAMKLEKDNAIDKADTCENQAKDANSRADKLNNEEVRDLEKKFVQVEIDLVT	:	60
61	MLW pTM1	:	-----	:	-
62	Der p 10	:	MEAIKNKMQAMKLEKDNAIDRAEIAEQKARDANLRAEKSEEEVRLQKKIQQIENELDQV	:	60
63	Dro TM1	:	MDAIKKKMQAMKVDKDGALERALVCEQEARDANTRAEKAEEEARQLQKKIQTVENELDQT	:	60
64	MLW pTM2	:	-----	:	-
65	SPW pTM	:	-----	:	-
66	SHR pTM	:	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV	:	60
67	Lit v 1	:	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV	:	60
68	MCR TM	:	MDAIKKKMQAMKLEKDNAMDRALLCEQQARDANIRA EKAEEEARSLQKKIQQIENDLDQT	:	60
69	WXW pTM1	:	MDAIKKKMQAMKLEKDNALDRAAMCEQQAKDANLRAEKAEEEARQLQKKIQTIENTLDQT	:	60
70	CRX pTM2	:	MDAIKKKMQAMKLEKDNAMDRALLCEQQARDANLRAEKAEEEARGLQKKIQTIENTLDQT	:	60
71	Bla g 7	:	MDAIKKKMQAMKLEKDNAMDRALLCEQQARDANIRA EKAEEEARSLQKKIQQIENDLDQT	:	60
72					
73	CRX pTM1	:	KANLEQANKDLEDKEKALQAAESEMAALNRKVQLVEEDLERSEERAATAATKLQEASEAA	:	120
74	WXW pTM2	:	KNKLDQANKDLEEREKQLAATEAEVASLNRKVQIIEEDLEKSEERSGTAQQKLLAEASQA	:	120
75	Dro TM2	:	KEQLEKANTELEEKEKLLTATESEVATQNRKVQIIEEDLEKSEERSTTAQQKLLAEATQSA	:	120
76	MLW pTM1	:	-----	:	-
77	Der p 10	:	QEQLSAANTKLEEKEKALQTAEGDVAALNRRIQLIIEEDLERSEERLKIATAKLEASQSA	:	120
78	Dro TM1	:	QEALTLVTGKLEEKNKALQNAESEVAALNRRIQLLEEDLERSEERLGSATAKLSEASQA	:	120
79	MLW pTM2	:	-----	:	-
80	SPW pTM	:	-----	:	-
81	SHR pTM	:	QESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAEASQA	:	120
82	Lit v 1	:	QESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAEASQA	:	120
83	MCR TM	:	LEQLMQVNAKLDEKDKAFQNAESEVAALNRRIQLLEEDLERSEERLATATAKLAEASQA	:	120
84	WXW pTM1	:	QESLMQVNGKLEEKEKALQNAESEVAALNRRIQLLEEDLERSEERLATATAKLSEASQA	:	120
85	CRX pTM2	:	QEQLMQVNAKLEEKDKALQTAEGEIAALNRRIQLLEEDLERSEERLATATAKLAEASQA	:	120
86	Bla g 7	:	MEQLMQVNAKLDEKDKALQNAESEVAALNRRIQLLEEDLERSEERLATATAKLAEASQA	:	120
87					
88	CRX pTM1	:	DEAQRMCVKLENRSQQDEERMDQLTNQLKEARLLAEDADGKSDEVSRRKLAFVEDELEVAE	:	180
89	WXW pTM2	:	DENNRMCVKLENRAQQDEERMDQLTNQLKEARLLAEDADGKSDEVSRRKLAFVEDELEVAE	:	180
90	Dro TM2	:	DENNRMCVKLENRSQQDEERMDQLTNQLKEARMLAEDADTKSDEVSRRKLAFVEDELEVAE	:	180
91	MLW pTM1	:	-----AKLIAEEADKKYEEVARKLVLMEDLERAE	:	30
92	Der p 10	:	DESERMRKMLEHRSITDEERMELENQLKEARMMAEDADRKYDEVARKLAMVEADLERAE	:	180
93	Dro TM1	:	DESERARKILENRALADEERMDALENQLKEARFLAEEADKKYDEVARKLAMVEADLERAE	:	180
94	MLW pTM2	:	-----RKLAMVEADLERAE	:	14
95	SPW pTM	:	-----EARFLAEEADKKYDEVARKLAMVEADLERAE	:	31
96	SHR pTM	:	DESERMRKVLENRSLSDEERMDALENQLKEARFLAEEADRKYDEVARKLAMVEADLERAE	:	180
97	Lit v 1	:	DESERMRKVLENRSLSDEERMDALENQLKEARFLAEEADRKYDEVARKLAMVEADLERAE	:	180
98	MCR TM	:	DESERARKILESGLADEERMDALENQLKEARFMAEEADKKYDEVARKLAMVEADLERAE	:	180
99	WXW pTM1	:	DESERARKVLENRSLADEERMDALENQLKEARFLAEEADKKYDEVARKLAMVEADLERAE	:	180
100	CRX pTM2	:	DESERARKILENRSLADEERMDALENQLKEARFLAEEADKKYDEVARKLAMVEADLERAE	:	180
101	Bla g 7	:	DESERARKILESGLADEERMDALENQLKEARFMAEEADKKYDEVARKLAMVEADLERAE	:	180
102					
103	CRX pTM1	:	DRVKSGDSKIMELEEEELKVVGNSLK-----	:	205
104	WXW pTM2	:	DRVKSGDAKISELEEEELKVVGN-----	:	202
105	Dro TM2	:	DRVRSGESKIMELEEEELKVVGNSLKSLEVSEEKANQRVEEFKREMKTLISIKLKEAEQRAE	:	240
106	MLW pTM1	:	ERAEQSESKIVELEEEELRVVGNLKSLEVSEEKATLKKEEYSVTLKQVDQRLQEAERAE	:	90
107	Der p 10	:	ERAETGESKIVELEEEELRVVGNLKSLEVSEEKAQQREEAHEQQIRIMTTKLKEAEARAE	:	240
108	Dro TM1	:	ERAEQGENKIVELEEEELRVVGNLKSLEVSEEKANQREEEYKNQIKTLNTRLKEAEARAE	:	240
109	MLW pTM2	:	ERAEAGESKIVELEEEELRVVGNLKSLEVSEEKANQREEEYKNQIKNLTTTLKEAEARAE	:	74
110	SPW pTM	:	ERAEAGESKIVELEEEELRVVGNLKSLEVSEEKANQREEEYKNQIKNLTTTLKEAEARAE	:	91
111	SHR pTM	:	ERAETGESKIVELEEEELRVVGNLKSLEVSEEKANQREEAYKEQIKTLTNKLKAAEAEARAE	:	240
112	Lit v 1	:	ERAETGESKIVELEEEELRVVGNLKSLEVSEEKANQREEAYKEQIKTLTNKLKAAEAEARAE	:	240
113	MCR TM	:	ERAESGESKIVELEEEELRVVGNLKSLEVSEEKANLREEEYKQIKTLTTTLKEAEAEARAE	:	240
114	WXW pTM1	:	ERAESGESKIVELEEEELRVVGNLKSLEVSEEKANQREEEYKNQIKTLTTTLKEAEAEARAE	:	240
115	CRX pTM2	:	ERAEAGESKIVELEEEELRVVGN-----	:	205

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116 Bla g 7 : ERAETGESKIVELEEELRVVGNNLKSLEVSEEEKANLREEEYKQQIKTLNTRLKEAEARAE : 240
117
118
119 CRX pTM1 : ----- : -
120 WXW pTM2 : ----- : -
121 Dro TM2 : HAEKQVKRLQKEVDRLEDELGINKDRYKSLADEMDSTFAELAGY- : 284
122 MLW pTM1 : FAERSVQKLQKEVDRLEDDLLAE----- : 113
123 Der p 10 : FAERSVQKLQKEVGRLEDELVHEKEKYKSISDELDQTFaelTGY- : 284
124 Dro TM1 : FAERSVQKLQKEVDRLEDDLVEKERYKDIGDDLDTAFVELILKE : 285
125 MLW pTM2 : FAERSVQKLQKEVDRLEEEELVAEKERYKEIGDDLDTAFVELIL-- : 117
126 SPW pTM : FAERSVQKLQKEVDRLEDELVAEKERYKEIGDDLDTAFVELIL-- : 134
127 SHR pTM : FAERSVQKLQKEVDRLEDELVN----- : 262
128 Lit v 1 : FAERSVQKLQKEVDRLEDELVNEKEKYKSITDELDQTFSELSGY- : 284
129 MCR TM : FAERSVQKLQKEVDRLEDELVHEKEKYKYICDDLDMTFTELIGN- : 284
130 WXW pTM1 : FAERSVQKLQKEV----- : 253
131 CRX pTM2 : ----- : -
132 Bla g 7 : FAERSVQKLQKEVDRLEDELVHEKEKYKYICDDLDMTFTELIGN- : 284
133

```

134 Clustal omega alignment of cloned predicted translated tropomyosins, selected allergenic tropomyosins

135 (Lit v 1, Bla g 7, and Der p 10), and *D. melanogaster* tropomyosins (1 and 2). Highly conserved residues

136 are displayed in dark grey, less conserved residues are displayed in light grey, and missing residues are

137 displayed with dashes.

Target(s)	Sequence source	Primer Sequence (5'-3') <i>Translated target</i>	Direction (F/R)	T _m (°C)
CRX	AK284720 <i>Gryllus bimaculatus</i>	ATGGACGCGATCAAGAAGAAGATGCAG <i>MDAIKKKMQ</i>	F	60.7
CRX	AK284720 <i>Gryllus bimaculatus</i>	ATGGACCAGCTCACCAACCAACTGAAG <i>MDQL TNQLK</i>	F	62.5
CRX	AK281119 <i>Gryllus bimaculatus</i>	ATGGACGCTCTGGAGAACCAGCTCAAG <i>MDALENQLK</i>	F	63.7
MCR	AF106961 <i>Periplaneta americana</i>	ATGGACGCACTGGAGAACCAGCTGAAG <i>MDALENQLK</i>	F	64.1
MLW SPW	XM_008200702 <i>Tribolium castaneum</i>	ATGGACGCCCTCGAGAATCAGCTGAAG <i>MDALENQLK</i>	F	63.7
MLW SPW	XM_008200701 <i>Tribolium castaneum</i>	GTAGGCATCTTGGAAACCCAAGTGTCT <i>VGILETQLS</i>	F	60.5
MLW SPW WXW	XM_962035 <i>Tribolium castaneum</i>	ATGGACCAGCTCACCAACCAGCTGAAG <i>MDQL TNQLK</i>	F	64.2
SHR	AB270630 <i>Litopenaeus vannamei</i>	ATGGACGCCCTGGAGAACCAGCTCAAG <i>MDALENQLK</i>	F	65.7
BSF	XM_005181026.2 <i>Musca domestica</i>	ATGGACGCTCTTGAGAATCAATTGAAG <i>MDALENQLK</i>	F	57.3
BSF	XM_005181026.2 <i>Musca domestica</i>	GTAGCTGTTTTGGAAGCCCAATTAGCA <i>VAVLEAQLA</i>	F	59.6
WXW	XM_013327543.1 <i>Amyelois transitella</i>	GTGGCGATACTGGAGGCGCAACTAGTG <i>VAILEAQLV</i>	F	64.4
WXW	XM_013327529.1 <i>Amyelois transitella</i>	ATGGACGCCCTCGAGAACCAGCTGAAG <i>MDALENQLK</i>	F	65.5
CRX	AK281119.1 <i>Gryllus bimaculatus</i>	TCGCAGCTCTTCCTCAAGCTC <i>ELEEEELR</i>	R	59.3
CRX	AK284720.1 <i>Gryllus bimaculatus</i>	CTTCAACTCTTCCTCAAGCTC <i>ELEEEELK</i>	R	53
MCR	AF260897 <i>Blattella germanica</i>	ACGGAGCTCCTCCTCAAGCTC <i>ELEEEELR</i>	R	61
MLW SPW	XM_008200702.2 <i>Tribolium castaneum</i>	GCGAAGTTCCTCCTCAAGCTC <i>ELEEEELR</i>	R	57.9
BSF	XM_005181026.2 <i>Musca domestica</i>	GCGGAGTTCTTCTTCAAGTTC <i>ELEEEELR</i>	R	53.7
WXW	XM_013327567.1 <i>Amyelois transitella</i>	GCGCAGTTCTTCTTCAAGCTC <i>ELEEEELR</i>	R	56.6
CRX MCR SHR	AF106961.1 <i>Periplaneta americana</i> EU410072.1 <i>Litopenaeus vannamei</i>	CTTCTCCTCAGACACCTCAAGGG <i>LEVSEEK</i>	R	58.7

	AK281119.1 <i>Gryllus bimaculatus</i>			
CRX	AK284720.1 <i>Gryllus bimaculatus</i>	CTTCTCCTCGGACACTTCCAAGG LEVSEEK	R	59.1
MCR	AF260897.1 <i>Blattella germanica</i>	CTTCTCTTCAGACACCTCAAGGG LEVSEEK	R	56.9
MLW SPW	XM_008200702.2 <i>Tribolium castaneum</i>	CTTTTCTTCAGACACTTCAAGGG LEVSEEK	R	53.8
BSF	XM_005181026.2 <i>Musca domestica</i>	CTTTTCTTCTGAAACTTCAAGGG LEVSEEK	R	52.1
WXW	XM_013327567.1 <i>Amyelois transitella</i>	CTTCTCCTCAGAGACTTCCAGGG LEVSEEK	R	58.3

Primer	Target	Sequence (5'-3') <i>Translated target</i>	Direction (F/R)	Start(stop) position	T _m (°C)
MCR GSP1 3'	MCR	GCCAGGTTTCATGGCTGAAGAGGCA <i>ARFMAEEA</i>	F	4 (27)	68.5
MCR GSP2 3'		GCAGAAGAACGTGCCGAGAGTGGT <i>AEERAESG</i>	F	88 (111)	68.5
MCR GSP1 5'		GCCAACAACACGAAGTTCCTCCTCA <i>EEELRVVG</i>	R	156 (132)	67.4
MCR GSP2 5'		CGGCACGTTCTTCTGCTCTTTCCAA <i>LERAEERA</i>	R	103 (79)	67.4
CRX1 GSP1 3'	CRX	GAGGCGAGGCTGCTGGCTGAAGA <i>EARLLAE</i>	F	2 (23)	69.9
CRX1 GSP2 3'		CGCTGACGGCAAGTCCGACGAGG <i>ADGKSDE</i>	F	24 (46)	71.9
CRX1 GSP1 5'		AGCTGTTGCCGACGACCTTCAACT <i>LKVVGNS</i>	R	163 (140)	66.9
CRX1 GSP2 5'		CGATCCTCAGCCACTTCCAGCTCGT <i>ELEVAED</i>	R	98 (74)	70.7
CRX2 GSP1 3'	CRX	GGATCGCGCCCTTCTCTGCGAGC <i>DRALLCE</i>	F	30 (52)	71.9
CRX2 GSP2 3'		GCCCGCGATGCCAACTTGCGGGC <i>ARDANLR</i>	F	58 (78)	73.6
CRX2 GSP1 5'		CAGCAGCCTGGGAGGCTTCGGCCA <i>AEASQAA</i>	R	330 (311)	73.8
CRX2 GSP2 5'		TGGATGCGGCGGTTTCAGGGCAGC <i>AALNRRI</i>	R	248 (229)	71.9
SHR GSP1 3'	SHR	CGACGAGGTTGCCCGTAAGCTGGC <i>DEVARKL</i>	F	39 (62)	72.2
SHR GSP2 3'		CGTGCTGAGGAGCGTGCCGAGAC <i>RAEERAE</i>	F	85 (106)	71.9
SHR GSP1 5'		GCCAACGACACGCAGCTCTTCCT <i>EELRVVG</i>	R	156 (134)	68.3
SHR GSP2 5'		CTCGGCACGCTCCTCAGCACGCTC <i>ERAERAE</i>	R	103 (84)	73.8
WXW1 GSP1 3'	WXW	TCCTCGCTGAGGAAGCCGACAAGA <i>LAEEADK</i>	F	5 (28)	68.5
WXW1 GSP2 3'		CGATGAGGTGGCTCGTAAGCTGGC <i>DEVARKL</i>	F	33 (56)	70.6
WXW1 GSP1 5'		ACCAGATTCCGGCACGTTCTCCGC <i>AEERAESG</i>	R	105 (82)	70.6
WXW1 GSP2 5'		GTCAGCCTCGACCATGGCCAGCT <i>LAMVEAD</i>	R	71 (50)	69.9
WXW2 GSP1 3'	WXW	GAGGCCCGTCTCCTGGCTGAGGA <i>EARLLAE</i>	F	2 (21)	71.9
WXW2 GSP2 3'		CGCTGACGGCAAATCTGATGAGGT <i>ADGKSDE</i>	F	23 (47)	66.9
WXW2 GSP1 5'		CACGATCCTCAGCGACTTCGAGC	R	100 (78)	68.3

WXW2 GSP2 5'		LEVAEDR AACGAAGGCCAGCTTACGTGAAACC VSRKLAFV	R	69 (45)	67.4
SPW GSP1 3'	SPW	AGGCCCGCTTCTTGCTGAAGAAG ARFLAEE	F	2 (22)	68.5
SPW GSP2 3'		CTCGTAAATTGGCCATGGTGGAAGCC RKLAMVEA	F	48 (72)	69.5
SPW GSP1 5'		CCTGCCTCGGCGCGCTCTTCGGC AEERAEA	R	109 (90)	67.8
SPW GSP2 5'		GGCTTCCACCATGGCCAATTTACGA RKLAMVEA	R	75 (51)	67.4
MLW GSP1 3'	MLW	GCGAAACTAATAGCCGAAGAGGCCG AKLIAEEA	F	2 (26)	69.1
MLW GSP2 3'		CGAAGAGGTTGCCCGAAAATTGGTTC EEVARKLV	F	36 (60)	67.9
MLW GSP1 5'		GCTCTCGCTCTGTTCAGCGCGTTC ERAEQSES	R	115 (92)	70.6
MLW GSP2 5'		CCAATTTTCGGGCAACCTCTTCG EEVARKL	R	60 (36)	64.6