

# SHRIMP ALLERGENS, ENDOCRINE DISRUPTING CHEMICALS (EDCs) AND TRANSPOSABLE ELEMENT (TE) - LIKE REPEATS: A REVIEW

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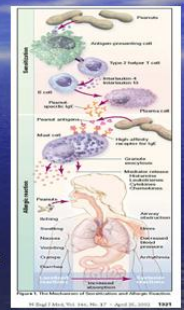


## Food Allergies

- Food allergies impact more than 11 million Americans each year
  - ~3 million are allergic to peanuts and/or shrimp
- The number of allergy-related incidences in the US doubled between 1997 and 2002.
  - Shrimp allergies in children are increasing
- Milk, egg, peanuts, soy, wheat, tree nuts, seafood (fish), shellfish (shrimp)

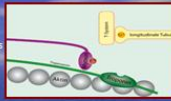
## Shellfish Allergy

- Shellfish allergy is an IgE-mediated, type I allergy
  - a long-lasting disorder, usually persisting throughout life
  - often associated with severe reactions, including life-threatening anaphylaxis
- To date, very little is known on how allergic reactions to seafood are initiated.
  - more research done with peanut allergy
- This shortage of information impedes development of new tests for adverse effects from new seafood allergens and the interpretation of data from toxicity and endocrine tests required by regulation.

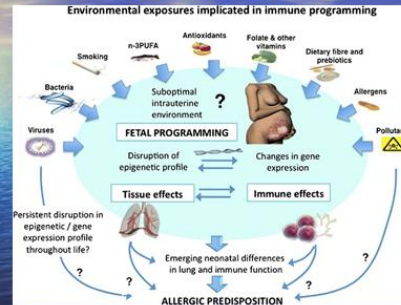


## Shrimp Allergens

- Tropomyosin (TM)**
  - is involved in muscle contraction.
  - along with **tropoinins**, it associates with the actin filaments and regulates actin mechanics
  - is an actin-binding protein
  - is regulated by the influx of calcium ions
- Arginine kinase (AK)**
  - is a member of the phosphagen kinase family that catalyzes the reversible transfer of a high-energy phosphate from the phosphagen arginine phosphate to ADP to form ATP.
- Myosin light chain (MLC)**
  - Smaller subunits of myosin that bind near the head groups of myosin heavy chains
  - Involved in muscle contraction
- Sarcoplasmic calcium-binding protein (SCP)**
  - is believed to function as the invertebrate equivalent of vertebrate parvalbumin, namely to "buffer" cytosolic Ca<sup>2+</sup>.



## Seafood consumption as a factor in allergic predisposition?



## Litopenaeus vannamei



- Litopenaeus vannamei* is an economically important commodity.
- L. vannamei* is the most widely cultured shrimp species in the Americas, now cultured globally (SE Asia).
- Most shrimp is imported, causing a yearly ~\$4.5 billion US seafood trade deficit.
- Specific pathogen-free (SPF) was developed by the US Marine Shrimp Farming Program (USMSFP) to develop shrimp aquaculture in the US – and abroad.

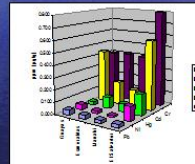
## Imported Shrimp Concerns

- Most of the shrimp consumed in the US is either farmed or wild caught in developing countries
  - often loaded with pathogens and persistent organic pollutants (POPs) such as endocrine-disrupting chemicals (EDCs): cadmium, PCBs, PAHs.
- Imported shrimp contain pollutants at low levels
  - viruses, heavy metals
  - PCBs, PAHs, antibiotics, etc.
- No compulsory commodity shrimp inspections
  - potential public health issues
  - risks to native crustacean populations
  - risks to human health



## Trace metals in *L. vannamei*

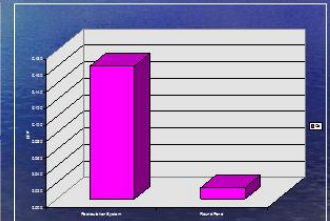
- 18 metals detected in shrimp from Latin America and SE Asia
- Cadmium (Cd) is the heavy metal of concern
- Cd was found in wild shrimp from 3 provinces of Ecuador: Guayas (now Santa Elena), Esmeraldas, Manabí
  - Highest Cd in Santa Elena shrimp
  - Santa Elena has most shrimp hatcheries
  - Santa Elena has an oil refinery
- Cd, which has been found present in crude oil, has negative health effects in most living organisms
- Cd accumulates in testes, prostate, renal epithelial cells and liver, and is linked to
  - kidney damage
  - prostate cancer
  - diabetes
  - may also affect immune and allergic responses



(Henry & Alcivar-Warren, 2004)

## SPF shrimp bioaccumulate cadmium

Cd levels increased 16-fold in Kona Line broodstock maintained in near zero-exchange recirculation system compared to a flow-through system.



(Alcivar-Warren & Meehan, 2004)

## Large number of Allergen genes isolated from TSV-challenged SPF *L. vannamei*



- No homology
- homology to ESTs of unknown function
- homology to hypothetical, unannotated, predicted or unknown proteins
- Ribosomal genes
- Chloroplast rRNAs
- homologies to viral immune function
- Dilatorion genes
- Transpositional regions

- Actin, myosin heavy chain, tropomyosin, tubulin, tropomyosin, AK, MLC, SCP, \*
- transcriptionally controlled tumor protein (TCTP, or fortin)
- Laminin receptor
- Chitinase, zinc proteinase
- heat shock protein 70
- antiviral protein hemocyanin,
- c-type lactins (PmAV & Kupffer cell receptor-like)
- 11.5 kDa antimicrobial peptide
- guanine nucleotide binding protein (rho pathway)
- nucleoside diphosphate kinase
- endoplasmic reticulum Ca<sup>2+</sup>+
- 16S rRNA, COI, COII
- 60S, 40S ribosomal proteins, 28S, 18S, translation elongation & initiation factors
- ATPase, preamylyase

\*known shrimp allergens

From Alcivar et al. 2011.

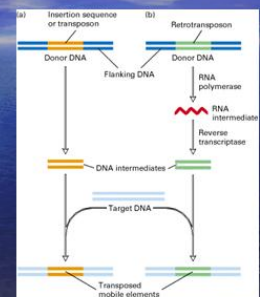


## Shrimp Allergens & Transposable Elements (TEs)

- Shrimp allergens contain repeats that resemble those of vertebrate TEs, including DNA transposons and retrotransposons (Alcivar-Warren et al. 2009, 2012; Das et al. 2009).
- TEs could play a role in the over expression of the shrimp allergens and in the body's recognition of foreign DNA.
- No biomarkers are available for shrimp allergies.
- We are searching for biomarkers associated with allergic reactions to shrimp consumption in order to develop a hypoallergenic shrimp.

## Classification of stress-sensitive TEs

- Transposon:** Transpose directly from DNA to DNA
- Retrotransposon:** Use reverse transcriptase to transpose by means of an RNA intermediate



## Genbank & Bioinformatics analyses

- Over 200 papers were accessed from Web of Science to write this review.
- The Unigene database was accessed at: <http://www.ncbi.nlm.nih.gov/unigene>
- A search for remnants of TE repeats in allergens was performed using CENSOR software: <http://www.girinst.org/>



## Allergy in Children

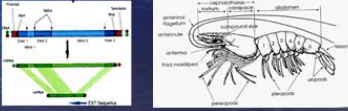
- Food allergy affects 6-8% of children in the United States (Kandyil et al. 2009).
- Onset of shellfish allergy occurs under the age of 18 years in 40% of people allergic to shellfish (Kandyil et al. 2009).
- Symptoms of seafood allergy may diminish with age (Belsler et al. 2001).
- Children with seafood allergy who have experienced allergy-related anaphylaxis have a significant amount of clinical cross-reactivity and higher rates of cross-sensitization both among fish and crustacean and between types of crustacean and fish (Turner et al. 2011).

## Allergy in Children, cont.

- Sensitization to shrimp in children is greater than sensitization to shrimp in adults (Ayuso et al. 2010).
  - The median IgE level in children was four-fold higher than in adults.
  - Children showed recognition to more shrimp proteins and individual peptides than adults.
  - Frequency of allergen recognition in children was higher than in adults
    - 81% for TM, 57% for MLC, 51% for AK, 45% for SCP.
  - TM may be associated in persistence of shrimp allergy into adulthood.

## Shrimp Allergens in UniGene

- There are ~20,000 EST sequences in the EST database of UniGene (Blastn est) of all penaeids
- There are only 7,738 gene clusters in UniGene (*L. vannamei* only)
  - 4 known allergen genes
    - ~4,000 ESTs of known allergen genes in the EST database
- Allergen-related ESTs were isolated from cDNA libraries of muscle, nerve cord, eyestalk, hemocyte, gills of SPF shrimp.



## 7,738 ESTs in the *L. vannamei* UniGene database

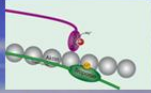
Arthropoda				
Arachnida				
<i>Ixodes scapularis</i> (black-legged tick)	19,405	Jul 30 2009	Jun 23 2010	
<i>Tetranychus urticae</i> (two-spotted spider mite)	7,177	Oct 27 2010	Jan 6 2011	
Branchiopoda				
<i>Daphnia pulex</i> (common water flea)	14,177	Jul 30 2009	May 26 2010	
Insecta				
<i>Acyrtosiphon plum</i> (pea aphid)	93,757	Nov 23 2010	Feb 17 2011	
<i>Aedes aegypti</i> (yellow fever mosquito)	16,580	Jun 2 2010	Aug 10 2011	
<i>Anopheles gambiae</i> (African malaria mosquito)	13,066	Oct 17 2010	Dec 21 2010	
<i>Aphis gossypii</i> (cotton aphid)	7,467	Oct 28 2010	Jan 26 2011	
<i>Apis mellifera</i> (honey bee)	24,292	Oct 21 2010	Nov 24 2010	
<i>Blyctus annectans</i> (squinting bush brown)	4,615	Nov 1 2010	Aug 15 2011	
<i>Bombus morio</i> (domestic silkworm)	12,467	Oct 24 2010	Dec 8 2010	
<i>Culex quinquefasciatus</i> (house mosquito)	5,021	Apr 20 2010	Sep 30 2010	
<i>Dendroctonus ponderosae</i> (mountain pine beetle)	6,783	May 24 2010	Mar 14 2011	
<i>Drosophila melanogaster</i> (fruit fly)	17,233	Jan 13 2011	Feb 26 2011	
<i>Drosophila simulans</i>	7,641	Dec 22 2009	Aug 3 2011	
<i>Glossina morsitans</i>	7,521	May 20 2010	Aug 3 2011	
<i>Nasonia vitripennis</i> (jewel wasp)	15,445	Jul 29 2010	Aug 16 2010	
<i>Tribolium castaneum</i> (red flour beetle)	6,855	Nov 2 2011	Dec 10 2011	
Mammalia				
<i>Litopenaeus vannamei</i> (Pacific white shrimp)	7,738	Jul 1 2011	Dec 27 2011	
Mollusca				
<i>Lentiginobolus subulnoides</i>	9,763	Nov 2 2010	Jan 6 2011	

## There are ~3,000 allergen-related ESTs in the UniGene database – most from SPF shrimp

Accession, TM	Arginine kinase, AK	Myosin Light Chain, MLC	Sarcoplasmic calcium-binding protein, SCP
Lit v 3.0101	Lit v 2	Lit v 3	Lit v 4
	Arginine kinase	Transcribed locus, moderately similar to XP_511049	Sarcoplasmic calcium-binding protein
Lit v 3.0101	Lit v 541; 994 seep	Lit v 296; 183 seep	Lit v 2176; 644 seep
		PREDICTED: similar to myosin regulatory light chain 2 [MRC2] (sea mussel)	
		Lit v 1302; 906	
		Transcribed locus, moderately similar to: XP_570203.1 similar to myosin I light chain isoform 2 [Thalassidroma]	
		Lit v 1350; 960	
		Lit v 9 allergen myosin light chain	
		Lit v 1216; 3 seep #12 are chimeric, with portions of both MLC 3 hypothetical protein	
		Transcribed locus, weakly similar to XP_524986.1	
		myosin light chain 2, isoform A [D. melanogaster]	
		Lit v 5509; 29	
		PREDICTED: locus, weakly similar to XP_393544.3	
		PREDICTED: myosin light chain alkali-like isoform 4 [Daph. mendicorum]	
		Lit v 2825; 3	
		Transcribed locus, weakly similar to XP_00194810.1	
		myosin light chain kinase [Culex quinquefasciatus]	
		Lit v 12264; 8	

## Tropomyosin (TM)

- The major shrimp allergen
  - More than 80% of shrimp-allergic subjects react (Lehrer et al., 2003).
  - First identified allergen in shrimp muscle
  - known as Pen a 1 (Daul et al., 1994.), Pen m 1 (Ayuso et al., 2002) and Lit v 1
- 10 TMs have been identified in crustacean shellfish
  - 6 have been identified in molluscan shellfish (Lopata et al., 2009)
- Exhibits cross-reactivity in many invertebrates including dustmites, cockroaches, and mollusks (Liu et al., 2007).
- Shrimp TM amino acid sequence shares nearly 60% sequence identity with TMs of vertebrates, molecules that are not allergenic (Lehrer et al., 2003).



## Myosin Light Chain (MLC)

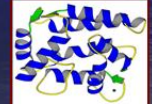
- MLC (Lit v 3.0101) was identified as a novel major shrimp allergen.
  - 177 amino acids
  - molecular weight of 20 kDa
- Five epitopes for IgE binding have been identified on MLC.



(Ayuso et al., 2010)

## Arginine Kinase (AK)

- AK Pen m 2 is similar to other food allergens.
  - plant profilins
  - iron transport protein allergen from egg white
  - animal serum albumins
  - fish parvalbumin allergen (Yu et al., 2003).
- L. vannamei* AK has a 96% identity to AK from *P. monodon*.
- A suggested pan allergen.



## Sarcoplasmic Calcium-binding Protein (SCP)

- Lit v 4.0101, has 194 amino acids, a molecular weight of 22 kDa, and a calculated isoelectric point of 4.7 (Ayuso et al., 2009).
- SCP contains an EF-hand-type Ca<sup>2+</sup> binding protein domain
  - like MLC and other allergens such as tree and grass pollen.
- Three IgE binding epitopes have been identified (Ayuso et al., 2010).
- Shows cross-reactivity among crustaceans and also with other arthropods (Ayuso et al., 2009).



## Tropomyosin and IgE binding

- Minor importance of linear epitopes for the IgE dependent allergen activity (Albrecht et al., 2009).
- The stability of a protein's 3-dimensional structure seems to play an important role in terms of allergenicity and immunogenicity (Albrecht et al., 2009).
- Amino acid substitutions in the epitope result in less IgE binding
  - Changes on the DNA level in the epitope coding region of the TM gene may impact allergenicity of the protein
- Importance of learning more about the polymorphisms of allergen genes, and the potential immunotherapy applications (Lehrer et al., 2003).

## Allergen Epitopes

- Allergenic determinants or epitopes represent the structures recognized by IgE.
  - An allergenic molecule can have linear epitopes making up a specific amino acid sequence along its primary structure, and conformational epitopes generated by the protein folding.
- Binding of IgE antibodies to specific regions of allergens is a prerequisite for triggering type I allergic reactions.

## TM Epitopes

	Pen a 1	Pen a 1	Pen i 1	Pen j 1
IgE-binding Region Identified	119-148, 153-179, 241-282	Regions 1-5: 143-57, 185-105, 1133-153, 1187-201, 1247-284	153-160, 50-56	Regions 3, 4, 5 (Ayuso et al 2002)
Note:	The center and the C terminus of the shrimp TM molecule contain most of the IgE-binding sites (Reese et al., 1999).	22 peptides were also identified as minor IgE-binding regions (Ayuso et al., 2002).	Corresponds to peptides LAEEADRYKDEVAR & MQQLENDLDQVQESLTKANLQLV EK (Shanti et al., 1993).	Region 5 a particularly important IgE binding epitope; 100% identity with Pen a 1 (Kunimoto et al., 2009).

## Allergens Cross Reactivity

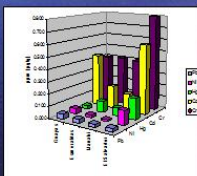
- IgE generated due to sensitization to an allergen can exhibit cross-reactivity to homologous proteins from different food sources.
- Cross-reactive TMs are present in crustaceans including shrimp, lobster, crab, and crawfish, in mollusks, in arachnids such as house dust mites, and in insects such as cockroaches (Besler et al., 2001).
- Though TM is present in vertebrates, IgE from allergic individuals generally do not bind to vertebrate TM (Besler et al., 2001).

## Cross Reactivity, cont.

- AK has also been suggested to be a pan-allergen that plays a role in cross-reactivity between shrimp (Pen m2) and mites (Der p 20) with 78% amino acid sequence homology.
- A new 20-kDa allergen in house dust mites and shrimp has been suggested to be responsible for cross-reactivity in only a subset of patients with crustacean allergy.
  - This protein has been speculated to be SCP (Villalta et al., 2010).

## Trace metals in *L. vannamei*

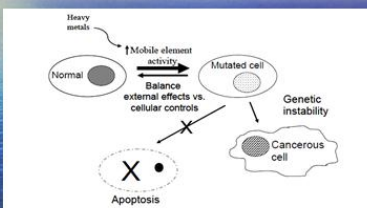
- 18 metals detected in shrimp from Latin America and SE Asia
- Cadmium (Cd) is the heavy metal of concern
- Cd was found in wild shrimp from 3 provinces of Ecuador: Guayas (now Santa Elena), Esmeraldas, Manabi
  - Highest Cd in Santa Elena shrimp
  - Santa Elena has most shrimp hatcheries
  - Santa Elena has an oil refinery
- Cd, which has been found present in crude oil, has negative health effects in most living organisms
- Cd accumulates in testes, prostate, renal epithelial cells and liver, and is linked to
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  - diabetes
  - may also affect immune and allergic responses



(Henry & Alcaivar-Warren, 2004)

## Why study Transposable Elements?

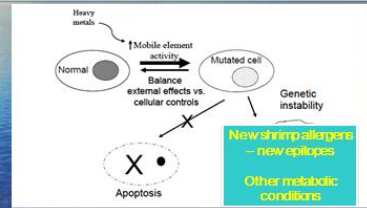
Because pollutants increase retrotransposon activity, which in turn affect fitness and disease susceptibility



LINE-1 non-LTR retrotransposon activity is induced by cadmium, mercury and nickel at very low levels, ppb (Kane et al., 2005)

## Hypothesis

Environmental stressors and viruses cause induction of transposable element activity, which cause host mutations



Is RTE-like, or LINE-1, or other non-LTR retrotransposons involved in virus infectivity, disease susceptibility?

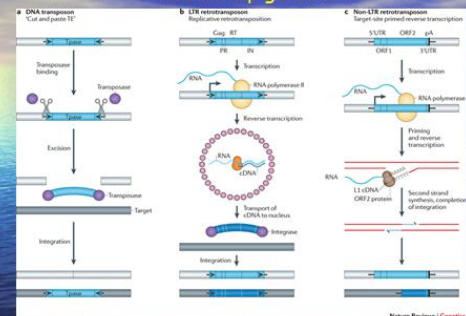
## Transposable Elements in shrimp

- Differential expression of TEs in response to viruses and other environmental factors has been reported in *L. vannamei*, *L. stylirostris* (Hizer, 2007) and *P. monodon* (de la Vega, 2006; Tang & Lightner, 2006).
- The reverse transcriptase-like (RT-like) non-LTR retrotransposon has been identified in *P. monodon* carrying non-infectious sequences of IHHNV inserted in the genome (Tang & Lightner, 2006), or IHHNV-infected *L. stylirostris* (Hizer, 2007).
- Remnants of TE repeats have been identified in SPF *L. vannamei* challenged with TSV and WWSV (Alcivar-Warren, 2009; Das, 2009) or exposed to Cd (Keating, 2007).
- It is possible that TEs increase the expression of allergens which are then perceived as "foreign antigens" by hypersensitive people, a hypothesis that merits testing

## TEs in Myosin Light Chain allergen-related ESTs - most from SPF shrimp

Myosin Light Chain, MLC	Classification statistics using Tefase software
Transposon locus, moderately similar to XP_311049: MLCcytoplasmic [D. melanogaster]	DNA transposons: 62
Transposon locus, moderately similar to XP_393171.2: PREDICTED: similar to Myosin regulatory light chain 2 (MLC-2) [ <i>Apis mellifera</i> ]	LTRs: 168
Transposon locus, moderately similar to XP_376209.1: similar to myosin 1 light chain isoform 2 (Tribolium castaneum) [Lva.12166: 960]	LINEs: 324
Transposon locus, moderately similar to XP_324586.1: myosin light chain 2, isoform A [D. melanogaster]	SINEs: 19
Transposon locus, moderately similar to XP_393544.3: PREDICTED: myosin light chain alkali-like isoform 4 [ <i>Apis mellifera</i> ]	Unclear: 387
Transposon locus, moderately similar to XP_00194910.1: myosin light chain kinase [ <i>Culex quinquefasciatus</i> ]	LINEs: 3
Lva.12166: 3 seqs	
Lva.6200: 20	
Lva.2292: 3	
Lva.12204: 8	

## Transposable element (TE)-like repeats are present in the shrimp genome



## TE-like repeats in Myosin 1 Light Chain, Lva.1350

most of the 960 ESTs originated from SPF shrimp

Repeat Class	Fragments	Length
<b>Transposable Element</b>	<b>71</b>	<b>5538</b>
DNA transposon	31	4900
EnSpM	5	284
Polinton	5	428
Sola	1	53
Transib	1	77
NAT	10	863
Endogenous Retrovirus	3	244
ERV1	2	159
ERV2	1	85
LTR Retrotransposon	32	3172
BEL	1	57
Copia	1	46
Gypsy	29	2988
Non-LTR Retrotransposon	5	362
CR1	2	148
Daphne	1	110
LINE 1	2	104
Interspersed Repeat	3	194

## TE-like repeats in Myosin 1 Light Chain Lva.12166 - 3 ESTs originated from SPF shrimp

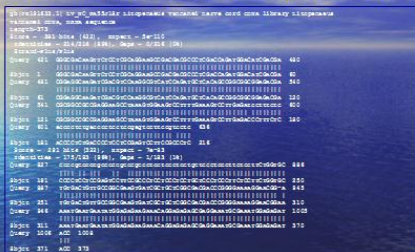
2 are chimeric, with portions of both MLC2 & hypothetical protein

Repeat Class	Fragments	Length
<b>Transposable Element</b>	<b>6</b>	<b>624</b>
DNA transposon	1	156
EnSpM	1	156
LTR Retrotransposon	5	468
Gypsy	5	468
Total	6	624

## TE-like repeats in Myosin 1 Light Chain Lva.12166 - 3 ESTs are the allergen Lit v3 unknown sample origin (supermarket), 2 are SPF shrimp [chimeric, with portions of MLC2 & hypothetical protein]

Name	From	To	Name	From	To	Class	Score
Lit v3 allergen EU449515	441	557	Gypsy19_PIT-1	2194	2289	LTR/Gypsy	219
	676	728	Gypsy118_LTR_DR	457	510	LTR/Gypsy	259
	933	1040	RETROSATS_LTR	1465	1566	LTR/Gypsy	317
nerve cord cDNA FE191634	5	77	RETROSATS_LTR	1465	1535	LTR/Gypsy	238
nerve cord cDNA FE191633	21	137	Gypsy19_PIT-1	2194	2289	LTR/Gypsy	218
	218	371	CACTA-O	750	908	DNA/EnSim	281

## Homology between allergen Lit v3 and chimeric clone FE191633 with portions of MLC2 & hypothetical protein



\*Results suggest that the allergen Lit v3 was caused by genome rearrangements -> new sequences perceived as foreign antigens

## Homology between nucleotides 191-373 of chimeric clone FE191633 with portions of polyprotein gene of Hepatitis C virus (tblastx)

```
AB429050.1 Hepatitis C virus gene for polyprotein, complete cds, isolate: AH1
Length=3474
Score = -1.0 bits (-1), Expect = 0.0
Identities = 14/33 (42%), Positives = 20/33 (61%),
Gaps = 0/33 (0%)
Frames = 42/142
Query 80 VTAARRRGGKRRKNDKINRDTGESEEMNGE 178
          VARRRGGKRRKNDKINRDTGESEEMNGE 178
Subject 746 VTAARRRGGKRRKNDKINRDTGESEEMNGE 844
```

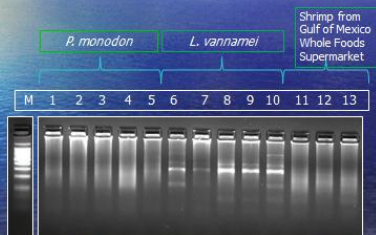
\*Results confirm gene rearrangements with an allergen - We need the complete shrimp genome sequence to figure out which transposable element is the polyprotein from - Gypsy?

## TE-like repeats in SCP allergen

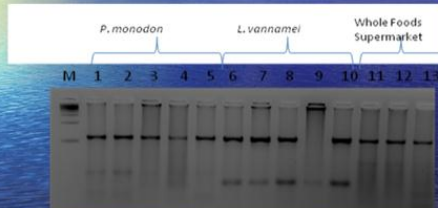
Repeat Class	Fragments	Length
<b>Transposable Element</b>	<b>6</b>	<b>624</b>
DNA transposon	1	156
EnSim	2	85
Member/ATC	1	48
IS611	1	333
IF	1	36
Polinton	1	91
Sola	1	787
Transib	4	210
NAT	10	1506
gypsy/RC	1	43
Endogenous Retrovirus	3	271
ERV1	1	106
ERV2	5	543
ERV3	1	54
LTR Retrotransposon	60	3368
IR1	2	127
Coop	33	709
Gypsy	60	2436
Non-LTR Retrotransposon	19	1139
CR1	8	311
Crack	1	74
LI	3	360
LC	4	270
IZ	1	78

## A hypoallergenic shrimp line?

PCR products amplified with primers for Tropomyosin



## PCR products amplified with primers for myosin light chain 2

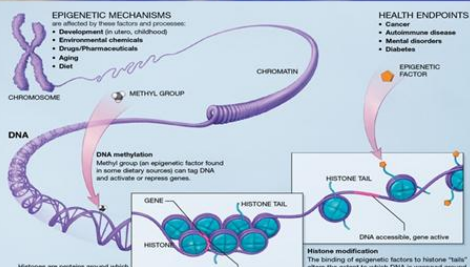


## EST-SSR markers could be useful for developing a hypoallergenic shrimp line, species identification, and traceability

- Polymorphic EST-SSRs identified in MLC, TM and AK among and within wild *P. monodon* of Thailand and wild and cultured *L. vannamei*.
  - single nucleotide polymorphisms (SNPs) in MLC were also identified among & within wild *P. monodon* of Thailand and wild & cultured *L. vannamei* of Ecuador and USMSFP, respectively.
- Allergen markers can identify penaeid species and will be useful for traceability.
- Allergen markers are being used to construct the second-generation *L. vannamei* (ShrimpMap2).
  - An EST-SSR for MLC is already mapped on to linkage group 7.

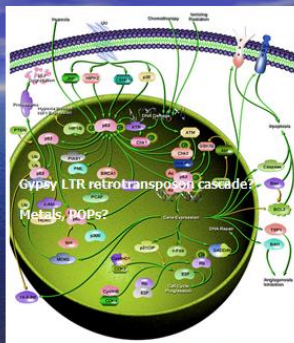
## Recommendations for Future Research

Test the hypothesis that Pollutant Load (viruses, heavy metals, POPs) affect the expression levels of allergens in shrimp muscle, and that changes in expression of allergen genes are caused by genome rearrangements caused by induction of stress-sensitive TEs.



## Future Research

- Investigate mechanisms of increased allergenicity from genetically improved, inbred, SPF shrimp.
- Test the hypothesis that TEs increase the expression of allergens, which are perceived as "foreign antigens" by hypersensitive people.



## Recommendations for Future research, cont.

- Clone cDNA and protein libraries to examine if there are differential allergic reactions to cultured SPF and wild *L. vannamei*.
- Continue research to develop a hypoallergenic shrimp.

