5-5-2015

Divergence of Vertebrate and Insect Specific Toxin Genes between Three Species of Widow Spider

Shannon G. Kahn
Georgia Southern University

Divergence of Vertebrate and Insect Specific Toxin Genes between Three Species of Widow Spider

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in Department of Biology.

By
Shannon G Kahn

Under the mentorship of Dr. J. Scott Harrison

ABSTRACT

The Brown widow spider, *Latrodectus geometricus*, is an introduced species to the southern United States (Brown 2008). The Brown widow is a member of the widow spider genus *Latrodectus* which includes the southern Black widow (*L. mactans*) and 29 other venomous species. All species of widow spiders produce venom which is used against both predator and prey. These venoms are composed of several different species-specific toxins, each encoded by a different gene (Graudins 2012). Previous research has shown that positive selection pressures affect the venom of snakes and snails, thus aiding in adaptive potential of the species (Gibbs 2008; Duda 1999). The study presented here was designed to sequence and characterize the α-latroinsectotoxin gene for the Brown and southern Black widow spiders. The sequence data was used to address two objectives: 1) quantify the nucleotide and amino acid divergence in α-latroinsectotoxin gene between the Brown widow, southern Black widow, and Mediterranean Black widow spiders; and 2) compare levels of divergence to that of α-latrotoxin and a non-toxin gene Cytochrome Oxidase I. Results showed that nucleotide difference lead to large amino acid differences in the toxin genes between species. Nucleotide divergence between species was similar at all three genes while amino acid divergence was 2.3 to 3.2 times higher in the toxin genes relative to the non-toxin gene (COI). High amino acid variation in the toxin genes between species suggests a structural basis exists for potential differences in functionality and toxicity.

Thesis Mentor:_____
Dr. J. Scott Harrison

Honors Director:_____
Dr. Steven Engel

April 2015
Department of Biology
University Honors Program
Georgia Southern University

Acknowledgments

This research was funded by the University Honors Program Undergraduate Research Fund and the Chandler Foundation Scholarship.

I would like to sincerely thank Dr. J. Scott Harrison for allowing me to work with him. You have been an incredible mentor with your patience and willingness to repeatedly show me and teach me both the most simple and sometimes complex techniques I have ever used in a laboratory. Thank you for allowing me to experience failure as well as success and working alongside me through all of it as I learned. You have taught me that starting back at the drawing board and dealing with the unexpected should be expected, and these are not things to be feared in the realm of research. I am grateful for the gamble you took on an undergrad whose passion and ideas were far bigger than her understanding of the work involved.

To my family: Thank you for being willing to listen to me ramble about spiders or complain about the “disasters” in lab, even if you had no idea what I was really talking about.

To Kayla Smith and Kyle Drew: Thank you for assisting me in catching new spiders, even if they frightened you as much as they frightened me.

To my roommates: Thank you for the support and encouragement during the times of research and writing. Thank you for your patience and your listening ear when I was overwhelmed and frustrated. Finally, thank you for always finding ways to make me laugh and reminding me that research is only a small part of understanding and enjoying the beautiful world around us.

Introduction

The field of ecology looks at the earth as one interconnected ecosystem, and the affects that individual organisms can have in relation to that ecosystem (Tripathi 2009). Advancements in technology have enabled ecologists to not only be broader in what they study, but also more in depth in specific areas (Tripathi 2009). One specific area of ecology is molecular ecology, which seeks to answer and understand ecological questions through the use of molecular and genetic approaches (Andrew *et al* 2013). The applications of molecular ecology include using molecular and genetic techniques to understand species interaction, the evolution of important ecological traits, relatedness of individuals, dispersal, behavior and movement of individuals, and the formation of new species (Andrew *et al* 2013).

In some cases, important traits can evolve at a genetic level due to outside factors in an organism's environment (Egg *et al* 2009). In addition to exogenous selective forces, intrinsic forces have been found to shape the evolution of genes that interact in function (DePristo *et al* 2005). When changes occur at the molecular level, even if these changes appear that they may negatively influence the organism, there can be compensatory molecular changes that work to increase the fitness of that organism (DePristo *et al* 2005). This has been proposed as a possible explanation for rapid adaptation of introduced and invasive species (Prentis *et al* 2008).

An introduced species is one that is found in a biogeographic region where it did not originate or evolve in, usually as a consequence of human activity (Prentis *et al* 2008). When a species is introduced, it may already possess the necessary traits to

survive in a particular area, or it may evolve and develop these necessary traits in response to selection pressures within the newly introduced region (Sargent & Lodge 2014) An example of an introduced species would be *Arabidopsis thaliana* (thale cress) which rapidly adapted to its environment due to environmental stress as a result of heritable genomic change (Prentis *et al* 2008). As the newly introduced species begins to establish itself within the new region, it can often cause dramatic changes in the ecosystem that lead to ecological and economic damage (Sargent & Lodge 2014). When this introduced species establishes thriving colonies that threaten ecosystems, habitats of native species or the native species themselves, the introduced species is labeled as invasive (Winfield *et al* 2011). Accounts of this have been documented in Scotland where several introduced species of fish have caused a decline in the native fish populations, and negatively impacted the migrating water birds (Winfield *et al* 2011). This study deals with *Latrodectus geometricus* (Brown widow spider), an introduced species in the United States (Brown *et al* 2008).

The spider genus *Latrodectus* (widow spiders) is comprised of thirty-one species (Garb & Hayashi 2013). *Latrodectus* species are found in areas inhabited by humans, and highly feared because of the neurotoxins released through their venomous bite (Garb & Hayashi 2013). The genus includes several species that have been commonly labeled as "Black widows" (Garb & Hayashi 2013). Black widows can be found on several continents (Ushkaryov *et al* 2004). Two of the Black widow species in this study (*L. mactans* and *L. tredecimguttatus*) are cosmopolitan species, with *L. mactans* being found in North America and *L. tredecimguttatus* having established populations in Asia and Europe (Ushkaryov *et al* 2004). These species tend to predominantly be found in

grass or dry stony places, where it is easy to capture their primary prey, insects (Vutchev 2001).

Latrodectus mactans (Southern Black widow) and *L. tredecimguttatus* (European Black widow) are part of the mactans (Black widow) clade within the genus *Latrodectus*, but are not closely related within the clade (Figure 1) (Garb & Hayashi 2013). The mactans clade includes approximately seventeen other species (Garb & Hayashi 2013). *Latrodectus tredecimguttatus* is the basal (oldest) species of the mactans clade, while *Latrodectus mactans* is grouped in a more derived clade with another *Latrodectus* species found in the Dominican Republic (Garb & Hayashi 2013). *Latrodectus geometricus* (Brown widow) is another widely distributed species of widow spider (Garb *et al.* 2004). This species is not part of the mactans clade and is part of the basal clade (oldest) to all species of *Latrodectus* (Figure 1) (Garb & Hayashi 2013).

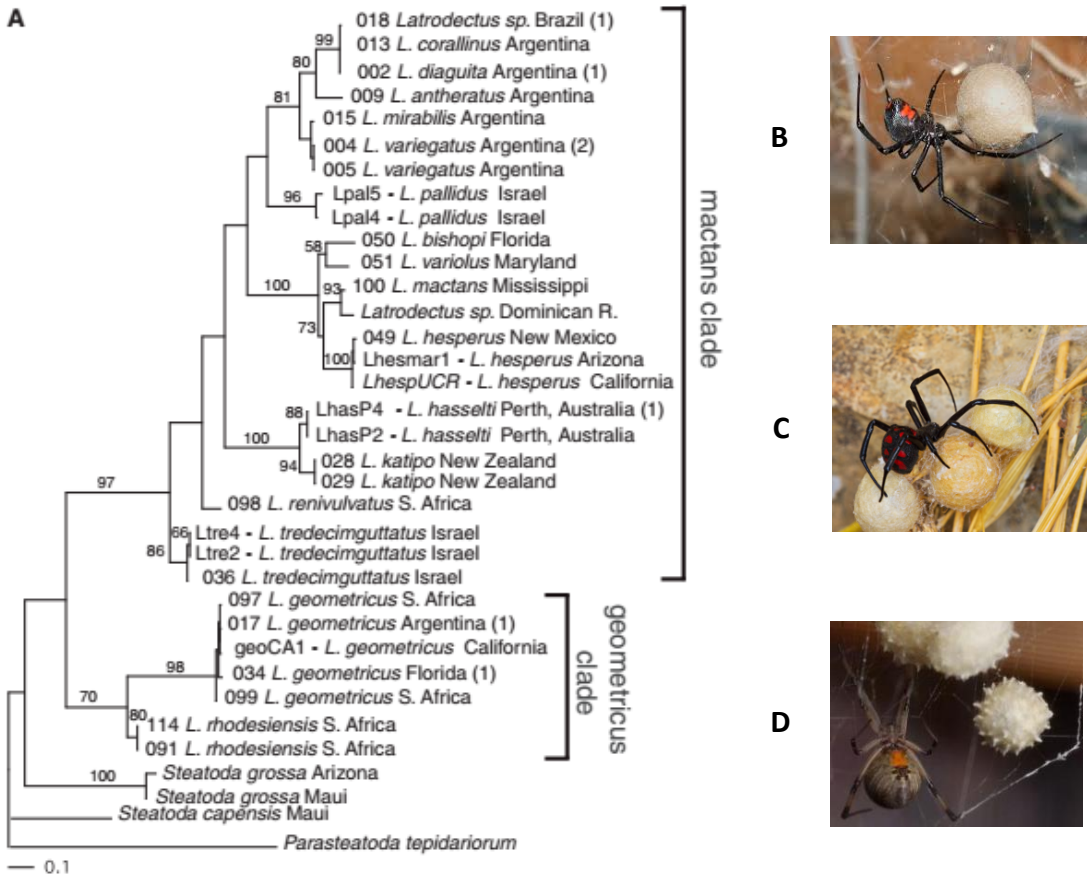

Figure 1. Phylogram based on ML analysis of 659 bp alignment of mt COI sequences (Garb & Hayashi 2013) with *L. mactans* (B), *L. tredecimguttatus* (C), and *L. geometricus* (D).

The Brown widow has been recognized as an introduced species to the United States, as the US is not its native region (Vetter *et al* 2012). Global commerce has often been the implicated means of the Brown widow's introduction into non-native region (Brown *et al* 2008). Australia, Papua New Guinea, Indonesia, and Japan are recent sites of human introductions of the Brown widow (Brown *et al* 2008).The suspected native range of the Brown widow is South Africa (Vetter *et al* 2012). The Brown widow was first documented in the United States in south Florida in 1935 (Vetter *et al* 2012). Starting in the 1990's this species started to expand its range throughout the southeast United States (Vetter *et al* 2012). A survey of the United States, conducted in 2008,

found established populations of the Brown widow had spread into Texas, Georgia, and multiple places in Louisiana and Mississippi (Figure 2) (Brown *et al* 2008).

Figure 2. Collection locations of *Latrodectus geometricus* in the southeastern United States. (Brown *et al* 2008).

Brown widows are considered one of the least dangerous widow spiders to humans (Brown *et al* 2008). The female Brown widow is non-aggressive and will often withdraw into her silken cone-shaped web, or ball up and drop from her web when she is disturbed (Brown *et al* 2008). In medically documented cases of a Brown widow spider bite, the cause was usually accidental trapping of the spider against bare skin (Brown *et al* 2008).

All species in the genus *Latrodectus* possesses venom that contains a group of protein toxins called latrotoxins (Guerrero *et al* 2010). Thus far, seven different latrotoxins have been identified in the venom of the Black widow *Latrodectus tredecimguttatus* (Ushkaryov *et al* 2004). Of these seven toxins, five have been identified

as specifically targeting insects (latroinsectotoxins), one has been identified as targeting to crustaceans (α-latrocrustotoxin), and one has been identified as specifically targeting vertebrates (α-latrotoxin) (Ushkaryov *et al* 2004).

Latrotoxins that are produced by widow spiders are protein neurotoxins (Vassilevski *et al* 2009). Of these protein neurotoxins, α-latrotoxin, α-latroinsectotoxin, and α-latrocrustotoxin consist of ~1100-1200 amino acids (Vassilevski *et al* 2009). The protein neurotoxin δ-latroinsectotoxin was found to be ~1000 amino acids in length (Vassilevski *et al* 2009). An N-terminal with two conservative hydrophobic regions (~30 amino acids in length) and a central region with ankyrin repeats are two distinguishing factors of all latrotoxin structure (Figure 3) (Vassilevski *et al* 2009). The spatial structure of α-latrotoxin is a symmetrical tetramer in the presence of either Ca^{2+} or Mg^{2+} ions, and a stable asymmetrical homodimer when no cations are present (Vassilevski *et al* 2009). This project specifically deals with α-latrotoxin and α-latroinsectotoxin.

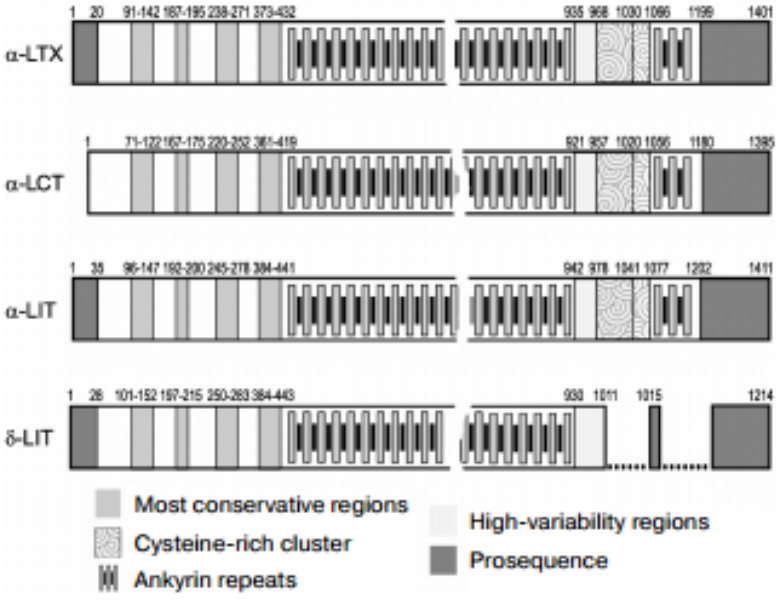

Figure 3. Amino acid sequences of different latrotoxins (Vassilevski *et al* 2009).

Latrotoxins are neurotoxins that induce the release of massive amounts of neurotransmitter at both the peripheral and central synapses after binding (Schiavo *et al* 2000). α-latrotoxin is selective in its action of triggering synaptic vesicle exocytosis on presynaptic nerve terminals (Südhof 2001). Specialized receptors on cell membranes determine the effect the latrotoxin will have on the cell (Vassilevski *et al* 2009). Mainly, α-latrotoxin will form an artificial membrane of pores that are permeable for cations (Vassilevski *et al* 2009). Looking at the toxin's structure, it is believed that in order to form the ion pore only the tetramer of the toxin is able to incorporate itself into the membrane and the amount of tetramer form of α-latrotoxin correlates with its ability to stimulate exocytosis of neuromediators (Vassilevski *et al* 2009). A membrane receptor is all that is necessary for α-latrotoxin to form a pore that leads to Ca^{2+} entry into the nerve terminals (Figure 4) (Vassilevski *et al* 2009). If α-latrotoxin binds with latrophilin or tyrosine phosphatase, it is then able to cause the release of Ca^{2+} ions from intracellular stores through the modulating of phospholipase C (Vassilevski *et al* 2009).

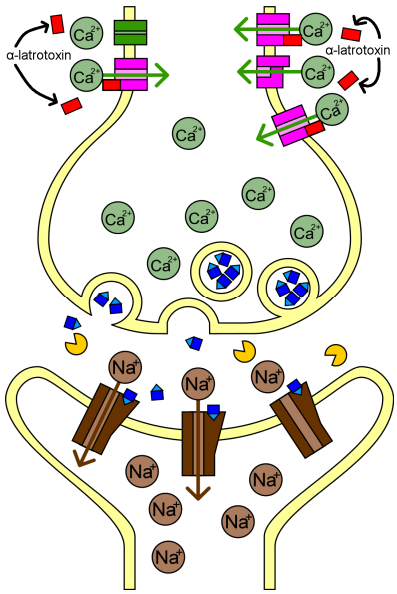

Figure 4. Binding of α-latrotoxin to presynaptic receptors.

Protein genes can be influenced by environment and are susceptible to molecular evolution as a way of adaptation (Garb & Hayashi 2013). Evolutionary changes can occur at the molecular level due to the influence of outside pressures like differing environmental conditions including differences in prey or predation. (DePristo *et al* 2005). The fitness of the organism can also be increased by additional compensatory molecular changes (DePristo *et al* 2005). Venom protein genes have been documented to be among the most rapidly evolving genes (Garb & Hayashi 2013). Several studies that have indicated selection playing a role in genetic variation in venom genes include snakes (Gibbs & Rossiter 2008), snails (Duda & Palumbi 1999), and parasitoid wasps (Werren *et al* 2010). It has been shown that the functional parts of venom genes in *Sistrurus* rattlesnakes can change due to positive selection (Gibbs & Rossiter 2008). Loci for conotoxins (neurotoxins in predatory snails of the genus *Conus*) have also shown signs of rapid adaptive evolution due to altered environmental factors, such as differing prey (Duda & Palumbi 1999). In parasitoid wasps, there are significant changes in the venom genes between three similar species when compared to the changes in non-venom genes (Werren *et al* 2010). The choice of host for these parasitoid wasps may be acting as a selective pressure that is causing these changes in the venom proteins (Werren *et al* 2010). It has also been suggested that there are important structural differences between the α-latrotoxins of different *Latrodectus* species (Graudins *et al* 2012).

Previous findings suggest that α-latrotoxin experienced an early and rapid evolution, followed by a purifying selection that increased its vertebrate toxicity (Garb & Hayashi 2013). This could be due to the fact that α-latrotoxin is utilized for predation of small vertebrates or defense from vertebrates (Garb & Hayashi 2013). If selective

pressures are driving the evolution of α-latrotoxin in this way, it is possible that these selection pressures could also be driving the evolution of α-latroinsectotoxin.

The objectives of this study are: 1) sequence the α-latroinsectotoxin gene for *Latrodectus geometricus* and *Latrodectus mactans*, as they have not been sequenced to date, and only *L. tredecimguttatus* has α-latroinsectotoxin data; 2) characterize the nucleotide and amino acid divergence of α-latroinsectotoxin between *L. geometricus*, *L. mactans, and L. tredecimguttatus*; and 3) compare levels of divergence in α-latroinsectotoxin to that of the vertebrate specific α-latrotoxin gene and a non-toxin gene, Cytochrome Oxidase I (COI). In completing these objectives I will address the following questions: 1) are there different levels of divergence in α-latroinsectotoxin and α-latrotoxin among the three species; and 2) do the toxin genes show higher levels of nucleotide and amino acid divergence than a non-toxin gene among the three species of *Latrodectus*.

Materials and Methods:

DNA was first extracted from *Latrodectus geometricus* and *Latrodectus mactans*. Two to three legs of each spider were dissected into pieces to allow for excess ethanol to be removed and to expose tissues within the exoskeleton to lysis buffers. DNA was extracted using the Qiagen DNeasy Tissue Kit following manufacture protocol (Qiagen Inc., Valencia, CA, U.S.A.).

Primers were designed using known α-latroinsectotoxin sequence from *Latrodectus tredecimguttatus* obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Primers were created by finding 18-24bp

segments of DNA with 50% AT and 50% GC content with a GC hook at the 3' end using the Primer3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). As sequence data was obtained for *L. geometricus* using the primers designed from *L. tredecimguttatus*, new primers were designed that were specific to *L. geometricus* to obtain sequences of additional gene segments.

Newly designed primers were tested using Polymerase Chain Reaction (PCR) with 6.0 μL diH_2O, 0.5 μM forward primer, 0.5 μM reverse primer, 1x concentration Apex Taq Master Mix [0.625 units of *Taq* DNA Polymerase, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ Genesee Scientific], and 2 μL DNA template in a final 20 μL volume. PCR amplifications were performed with the following protocol: One cycle of 5 minutes at 94.0°C; 35 cycles of 94.0°C for 30 seconds, primer specific annealing temperatures for 45 seconds, 72°C for 2.5 minutes; and one cycle at 72°C for 15 minutes for final elongation. Initially primers were tested at annealing temperatures ranging from 50.0°C to 62.0°C to determine the optimal annealing temperature. Optimal annealing temperatures are reported in Table 1. PCR products were confirmed using a 1.5% agarose gel electrophoresis in TAE (Tris-acetate-EDTA) buffer stained with GelRed. Electrophoresis gels were visualized with a UV light transilluminator. Primer pairs that produced single PCR products were used directly in sequencing reactions after purification (see below).

Table 1. Varying temperatures and primer combinations used in PCR for sequencing of α-latroinsectotoxin of *Latrodectus geometricus* and *Latrodectus mactans*

Latrodectus geometricus			
Combination	**Primers**	**Sequence**	**Temperatures**
A	Itox 193F	5'-TCCATTGAGGGAGCGAATCT	60.7°C
	Itox 750R	5'-GAACAGAGCGTAAAGAGCCTGA	
B	Ilat T1F	5'-AATTCCAAACAATGCTGCGG	59.8°C
	Itox 2330R	5'-CCCAAATGAAGGGCAGAAAA	
C	Ilat T2F	5'-GCAAGAACCAGCTCCTGGAA	57.2°C
	Itox 2330R	5'-CCCAAATGAAGGGCAGAAAA	
D	Ilat T4F	5'-TTATCGAACAAGAGTTGGCA	59.8°C
	Itox 2330R	5'-CCCAAATGAAGGGCAGAAAA	
E	Itox 3511F	5'-GCCGATGTCAATGCTAAAGG	58.6°C
	Itox 4048R	5'-CTGATATTACACTTCGTCTCCCACTC	
F	Itox 3542F	5'-TTAGGCCCATCGACATTGCT	61°C
	Itox 4048R	5'-CTGATATTACACTTCGTCTCCCACTC	
Latrodectus mactans			
Combination	**Primers**	**Sequence**	**Temperatures**
G	Itox 193F	5'-TCCATTGAGGGAGCGAATCT	56°C
	Itox 750R	5'-GAACAGAGCGTAAAGAGCCTGA	
H	Ilat T2F	5'-GCAAGAACCAGCTCCTGGAA	56°C
	Itox 2330R	5'-CCCAAATGAAGGGCAGAAAA	
I	Itox 1446F	5'-AAACGGTGCCAATGTGAGTG	60°C
	Itox 2330R	5'-CCCAAATGAAGGGCAGAAAA	
J	Itox 1446F	5'-AAACGGTGCCAATGTGAGTG	60°C
	Itox 2910R	5'-TTTTCCTCTGTGGGCTGCTT	
K	Itox 2271F	5'-AGCAAAGAATGCGGATGTGA	60°C
	Itox 2910R	5'-TTTTCCTCTGTGGGCTGCTT	
L	Itox 3511F	5'-GCCGATGTCAATGCTAAAGG	60°C
	Itox 4048R	5'-CTGATATTACACTTCGTCTCCCACTC	

Primer combination C (Table 1) produced more than one PCR product, but only one of the products was in the appropriate size range for the targeted product. In this case the PCR products were cloned before sequencing. The cloning reactions were made using combination C primers at 57.2 ℃ (Table 1). Volumes of 1, 2, 2.5, and 4μL of PCR product were used to clone products into the TOPO PCR.4 plasmid. The cloning reaction

followed the protocol according to TOPO TA Cloning Kit for Sequencing v.O (Invitrogen, Carlsbad, CA, U.S.A.). TOP10 competent cells were transformed and grown on standard Luria Agar plates supplemented with 100mg/ml Ampicillin. Bacterial colonies where then boiled in 50μL of diH_2O to extract plasmid DNA. Volumes of 0.5, 1, and 2μL of the supernatant of boiled cells were used as template DNA in PCR reactions as described above using combination C primers (Table 1).

PCR product was purified using 2.5 μL of Shrimp Alkaline Phosphotase (SAP) mixture (39.5 μL diH_2O, 5 units SAP, 1x SAP buffer, 0.2 units Exonuclease 1) in 15 μL of PCR product. Fragments were sequenced in both the forward and reverse direction using the primers used in PCR amplifications. Sequencing was conducted using Big Dye Terminator Kit, Version 3.1 (Applied Biosystems, Foster City, CA, U.S.A.) using 0.5 μM primer, .1 x sequencing buffer, 4 μL Big Dye, and 2.5μL of cleaned PCR product. Sequencing products were separated on an ABI 3500 Genetic Analyzer.

Sequences for the α-latrotoxin from all three species, as well as all sequences for *Latrodectus tredecimguttatus* were obtained from published data in GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). A 700bp portion of the mitochondrial COI gene was sequenced for all three species as a representative non-toxin gene.

All nucleotide and amino acid sequences were aligned using CLUSTALW in the SDSC Biology Workbench. The nucleotide and amino acid divergence were calculated using the Kimura models of evolution with DNADIST in the SDSC Biology Workbench. The amino acid divergence was calculated using the Kimura (unweighted) and PAM (weighted) models of evolution with PROTDIST in the SDSC Biology Workbench. The

Kimura evolution model calculates overall changes between the sequences. The PAM evolution model takes into account the types of changes, weighting changes between functional groups heavier than changes within functional groups, when calculating overall changes between the sequences.

Percent amino acid changes within and between groups were manually calculated. The changes were categorized based on the groups (Non-polar, polar, aromatic, positive, negative) (Nelson & Cox 2008) and whether the changes remained within the group or between groups. The percent of the classified changes was calculated with the overall number of changes as the base.

Results:

Comparison of *L. tredecimguttatus* and *L. mactans*, showed 16.41% nucleotide divergence in the COI gene, while α-latrotoxin and α-latroinsectotoxin showed 4.36% and 5.1% nucleotide variation respectively, making the nucleotide divergence for the non-toxin COI gene 12% higher than either of the toxin genes (Figure 5). The non-toxin COI gene showed 5.16% amino acid variation while α-latrotoxin showed 13.10% and α-latroinsectotoxin showed 11.62% amino acid variation (Figure 5). The amino acid divergence was 2.5 (α-latrotoxin) and 2.3 (α-latroinsectotoxin) times higher in the toxin genes than the COI genes (Figure 5). Additionally, the amino acid divergence was 1.2% higher in α-latrotoxin than in α-latroinsectotoxin (Figure 5).

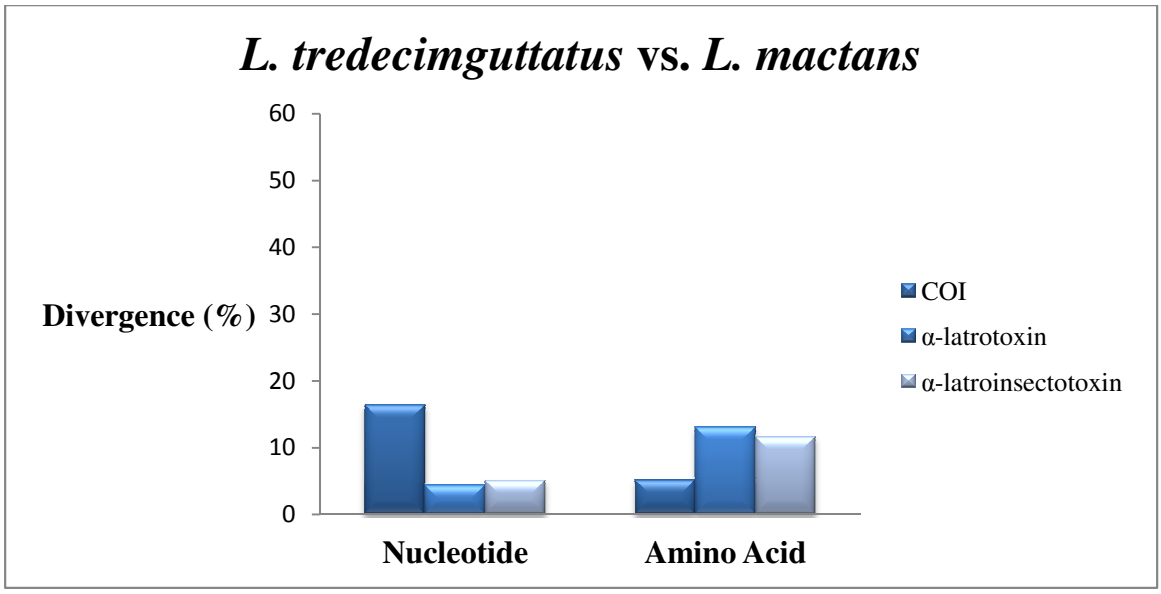

Figure 5. Divergence of toxin genes and COI gene for *L. tredecimguttatus* and *L. mactans*

For the comparisons between *L. tredecimguttatus* and *L. geometricus* the nucleotide divergence of COI (17.61%), α-latrotoxin (14.37%), and α-latroinsectotoxin (16.23%), are all within a close range of each other (Figure 6) with the non-toxin COI gene still being 3.1% higher than α-latrotoxin and 0.5% higher than α-latroinsectotoxin. The amino acid divergence is 12.60% for COI, while it is 39.68% and 44.82% for α-latrotoxin and α-latroinsectotoxin respectively (Figure 6). The amino acid divergence was 3.1 and 3.6 times higher in α-latrotoxin and α-latroinsectotoxin genes respectively, when compared to COI (Figure 6). When comparing the two toxin genes to each other, the amino acid divergence was 8.2% higher in α-latroinsectotoxin than α-latrotoxin.

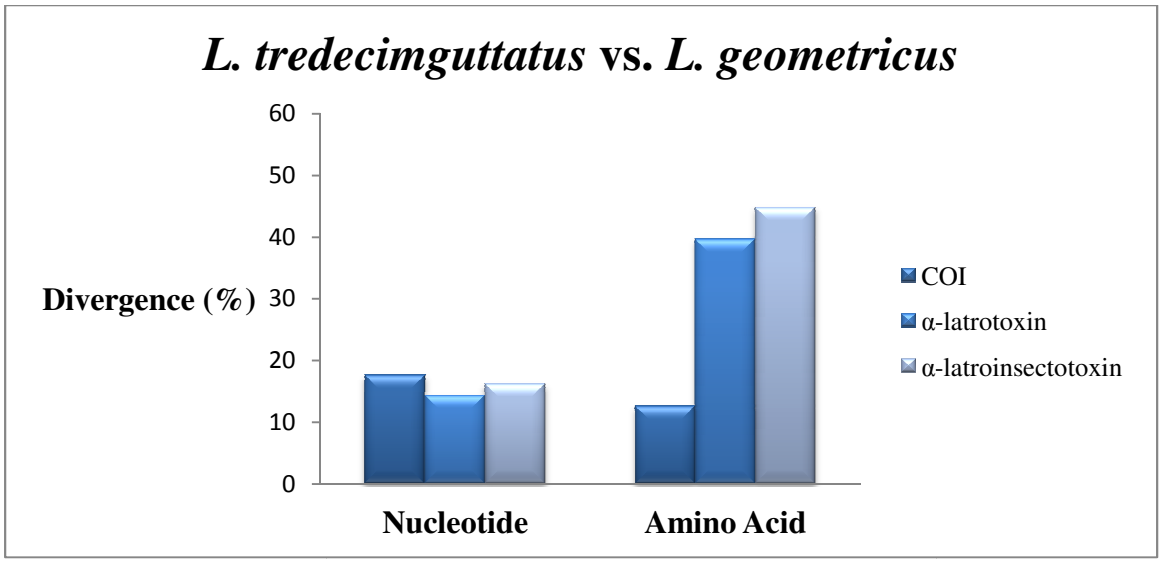

Figure 6. Divergence of toxin genes and COI gene for *L. tredecimguttatus* and *L. geometricus.*

The nucleotide divergence for the *L. geometricus* and *L. mactans* comparison was 18.53% for the COI nucleotide sequences, with α-latrotoxin varying 13.12% and α-latroinsectotoxin varying 16.78% (Figure 7) making COI 4.6% higher than α-latrotoxin and 2.1% higher than α-latroinsectotoxin in nucleotide divergence. The amino acid divergence for the COI genes was 12.65%. Toxin gene divergence was 36.13% for α-latrotoxin and 49.68% for α-latroinsectotoxin (Figure 7). The amino acid divergence was 2.9 (α-latrotoxin) and 3.9 (α-latroinsectotoxin) times higher than the divergence in the non-toxin COI gene. In comparing the two toxin genes, their amino acid divergence was 12.6% higher in α-latroinsectotoxin than α-latrotoxin.

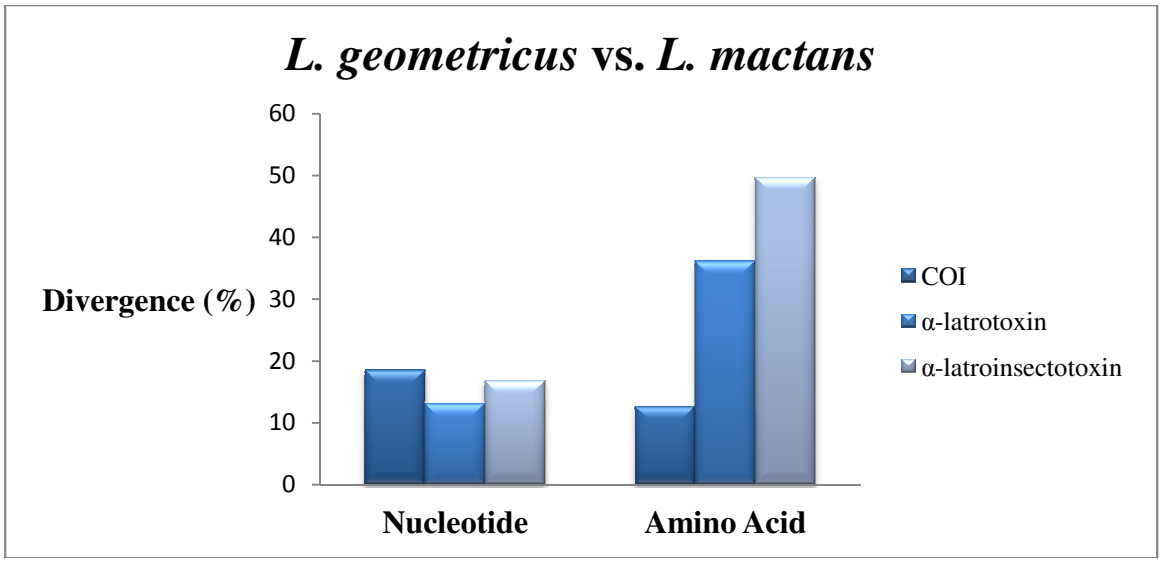

Figure 7. Divergence of toxin genes and COI gene for *L. geometricus* and *L. mactans.*

Upon examination of the amino acid distances in the amino acid changes between the two toxin genes using the PAM (weighted) and Kimura (unweighted) models, noticeable differences were detected. The Kimura results for *L. tredecimguttatus* and *L. mactans* comparisons found 6.31% and 5.69% divergence for α-latrotoxin and α-latroinsectotoxin , respectively (Figure 8). The PAM results showed 13.10% and 11.62% divergence for α-latrotoxin and α-latroinsectotoxin , respectively (Figure 8).

For the amino acid changes in *L. tredecimguttatus* and *L. geometricus*, Kimura models showed 16.70% divergence for α-latrotoxin and 18.01% divergence for α-latroinsectotoxin (Figure 8). The divergence for α-latrotoxin was 39.68% and 44.82% for α-latroinsectotoxin using the PAM model (Figure 8).

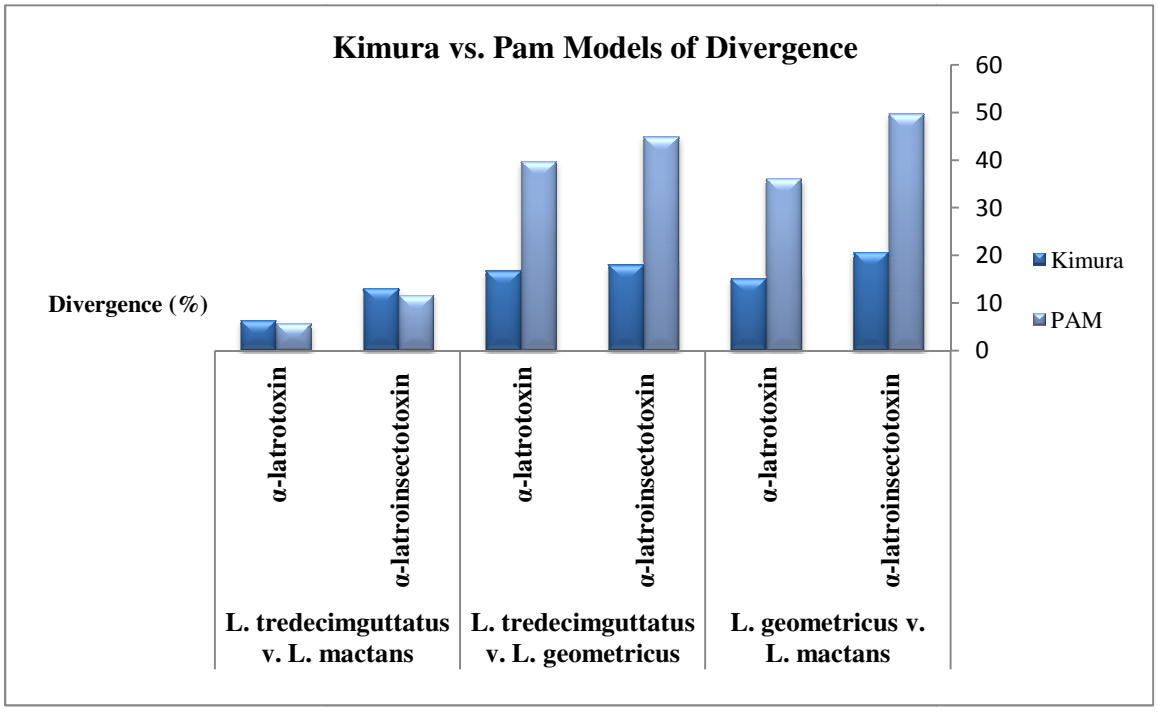

Figure 8. Analysis of amino acid divergence of the two toxin genes using Kimura and PAM models

Kimura models showed 15.06% amino acid divergence in α-latrotoxin and 20.67% divergence in α-latroinsectotoxin for *L. geometricus* and *L. mactans* comparisons (Figure 8). PAM models showed 36.13% divergence for α-latrotoxin and 49.68% divergence for α-latroinsectotoxin (Figure 8).

The amino acid changes in toxin genes and the non-toxin COI gene were broken down in terms of groups (Non-polar, aromatic, polar uncharged, positive, negative) and the percent of changes that occurred within the groups and between the groups were calculated. For *L. tredecimguttatus* and *L. mactans*, the non-toxin COI gene found 0% of the changes were between groups and all changes (100%) were within groups (Table 2). For the toxin genes, 63% of α-latrotoxin's changes were between groups, with the remaining 37% of changes occurring within groups (Table 2). α-latroinsectotoxin 's

changes occurred with 51% of the changes between groups and 49% of the changes within groups (Table 2).

Table 2. Percent amino acid differences between and within chemical categories

	Between Groups	**Within Groups**
L. tredecimguttatus v. L. mactans		
COI	0%	100%
α-latrotoxin	63%	37%
α-**latroinsectotoxin**	51%	49%
L. tredecimguttatus v. *L. geometricus*		
COI	36%	64%
α-latrotoxin	65%	35%
α-**latroinsectotoxin**	61%	39%
L. geometricus v. *L. mactans*		
COI	36%	64%
α-latrotoxin	61%	39%
α-**latroinsectotoxin**	68%	32%

For the non-toxin COI gene of the *L. tredecimguttatus* and *L. geometricus* comparisons, 36% of amino acid changes occurred between groups, with the remaining 64% of changes occurring within groups (Table 2). The changes between groups for α-latrotoxin was 65% and within groups 35% (Table 2). α-latroinsectotoxin 's amino acid changes were 61% between groups and 39% within groups (Table 2).

In the comparison of *L. geometricus* and *L. mactans*, the non-toxin COI gene's changes were 36% between groups and 64% within groups (Table 2). Changes between groups were 61% for α-latrotoxin and 39% within groups (Table 2). For α-latroinsectotoxin, changes between the groups occurred in 68% of the changes, and 32% of the changes were within groups (Table 2).

Discussion:

The objectives of this study were to sequence the α-latroinsectotoxin gene for *L. geometricus* and *L. mactans*, determine the nucleotide and amino acid divergence of α-latroinsectotoxin between our three studied species and compare the levels of divergence in α-latroinsectotoxin to the levels of divergence in α-latrotoxin and a non-toxin COI gene. I also address the questions of whether different levels of divergence in α-latroinsectotoxin and α-latrotoxin were present among the three species, and whether higher levels of nucleotide and amino acid divergence were found in the toxin genes or the non-toxin gene among the three species of *Latrodectus.*

In all cases of comparison, the toxin genes had lower levels of nucleotide divergence than the non-toxin gene (Figure 5-7). However, when that translated into amino acid sequence, the variation was much higher in the toxin genes (Figure 5-7). This suggests that most of the nucleotide changes in the COI gene were synonymous (neutral) changes and did not change the amino acid that the codon coded for. The low percentage of amino acid changes that were between groups (Table 2), and the lower percentages according to the Kimura and PAM models of analysis (Figure 8) suggest also that even though the amino acid sequences for the non-toxin COI gene are varied between the species, the general structure remains roughly the same. In the comparison between *L. tredecimguttatus* and *L. mactans*, which are both of the mactans clade, the amino acid sequence for the COI gene was low. This could be due to either low selection pressure, purifying selection, or their phylogenetic relationship.

Observations of the toxin genes show high levels of divergence in amino acid sequence as compared to the non-toxin COI gene, even if nucleotide sequence differences are lower in the toxin genes than that of the non-toxin COI gene (Figure 5-7). These findings are consistent with rapid positive selection observed in snakes, snails, and wasps (Gibbs & Rossiter 2008; Duda & Palumbi 1999; Werren *et al* 2010). In snails, the observed adaptive evolution in toxin genes was due to the necessity of the snails to produce a toxin that would effectively paralyze their prey as their prey began to adapt to the toxins (Duda & Palumbi 1999). In the same way, the amino acid divergence in toxin genes in snakes is due to positive selection affecting the function of the toxin on prey and whether the toxin functions in anticoagulant or hemolytic activity (Gibbs & Rossiter 2008). These same selection pressures in which predator and prey need to adapt in order to survive could be the driving mechanism behind the genetic divergence found in the spider toxin genes.

The amino acid changes observed in the toxin genes contained a high percentage between chemical groups relative to within groups. The amino acid sequence determines the structure of the protein, and structure is directly correlated with function. The differences observed in the amino acid sequence suggest a difference in functionality even though the mechanism of action is still the same (Graudins *et al* 2012). These differences in functionality could be the reason behind the differences in observed toxicity levels between the species (McCrone 1964). All comparisons with *L. geometricus* show higher levels of divergence in both toxin genes (Figure 5-8). *Latrodectus geometricus* has been reported be more toxic in volume to volume

comparisons of their toxins with other members of the *Latrodectus* species (McCrone 1964).

Of the toxin genes, α-latroinsectotoxin showed higher levels of amino acid divergence than α-latrotoxin when *L. geometricus* was in the comparison. One possible explanation for α-latroinsectotoxin showing higher levels of amino acid divergence than α-latrotoxin between *L. geometricus* and Black widow species is simply the phylogenetic relationships between the species. *Latrodectus geometricus* is in the geometricus clade and is basal to the other species of this study (Garb & Hayashi 2013). *Latrodectus mactans* and *L. tredecimguttatus* are both in the mactans clade and are more closely related to each other than either is to *L. geometricus* (Garb & Hayashi 2013). Nonetheless, the insect specific α-latroinsectotoxin appears to be accumulating differences at a higher rate than the vertebrate specific α-latrotoxin gene. A second possible explanation for this pattern is higher selective pressure acting on the α-latroinsectotoxin gene in *L. geometricus* than the α-latrotoxin gene. *Latrodectus geometricus* is an introduced species in the United States (Brown 2008) and may need a more effective toxin to capture a different kind of prey than is found in its native region. Comparisons of variation among worldwide introduced populations of this species might give additional insight into evolutionary mechanisms acting on this gene.

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