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α -Latrotoxin Genes are Highly Divergent Between Species of Widow Spiders (Genus *Latrodectus*)

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

By
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Under the mentorship of Professor J. Scott Harrison

Widow spiders (genus *Latrodectus*) possess neurotoxic venom that varies in potency among species. α -latrotoxin is the main protein in widow venom that affects vertebrates, including humans. The European black widow, *Latrodectus tredecimguttatus*, is currently the only species in this genus where the gene for α -latrotoxin has been characterized. The study presented here characterizes the genetic composition of α -latrotoxin from two additional species, the brown widow (*L. geometricus*) and the southern black widow (*L. mactans*). Genetic differences among the three species were quantified for α -latrotoxin. Between species genetic divergence in α -latrotoxin was also compared to that of a second gene, cytochrome oxidase I (COI), which is not associated with *Latrodectus* venom. Functional genetic differences among species were high with amino acid differences ranging from 14% - 58%. Amino acid divergence was approximately 3.7 times greater between species in α -latrotoxin than in COI.

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Introduction

Widow spiders (genus *Latrodectus*) have been documented as having a distribution that spans multiple continents and oceanic islands (Garb et al., 2004). The genus *Latrodectus* consists of approximately 30 species and includes species known as the black widow, the Australian red-back spider, and the cosmopolitan brown widow (Garb et al., 2004). Identification of different species of this genus has been historically challenging as scientists experienced difficulties in recognizing discrete morphological differences among species (Garb et al., 2004). In 1959, 22 species that were previously identified were consolidated into 6 because much of the observed variation in the different species was in fact continuous (Garb et al., 2004). Due to the complexity of studying the minimal morphological differences between species of the genus *Latrodectus*, scientists have recently begun to study the physiological and genetic differences of the species in the genus instead, including the study of their venom (Garb et al., 2004).

Spider venoms are known to be complex multicomponent mixtures of biologically active substances (Vassilevski et al., 2009). Venom composition is species-specific and is dependent on factors that include sex, nutrition, habitat condition, and climate such that the spider dispersing the venom carefully calculates an affective dosage based on components of its victim (Vassilevski et al., 2009). Venom is used in both protection and to acquire prey (Vassilevski et al., 2009). Spider venoms include various substances of different chemical nature (Vassilevski et al., 2009). These substances can be divided by molecular mass

into three different groups which are low molecular weight substances, small peptides, and high molecular weight substances including enzyme and neurotoxin proteins (Vassilevski et al., 2009). Latrotoxins are the functional high molecular weight neurotoxin proteins found in the venom of all widow spiders (Kiyatkin et al., 1990).

Spiders belonging to the genus *Latrodectus* are the most clinically significant group of spiders in the world due to the severe symptoms caused by their envenomation (Graudins et al., 2012). Due to the toxicity of their venom and their common occurrence in places where people frequent or live, members of this genus are some of the only spiders that cause medically significant bites (Garb et al, 2004). One species, *Latrodectus mactans*, more commonly known as the southern black widow, are identified by their red and black coloring and are native to North America (Upadhyay & Ahmad, 2011). They have historically been known as one of the most abundant toxin-bearing species of spiders in the US (Upadhyay & Ahmad, 2011). Widow spiders are known for the severe potency of the neurotoxic venom, which contains a cocktail of various forms of the protein latrotoxin including α -latrotoxin, α -latrocrustatoxin, and α -latroinsectotoxin (Garb et al., 2004).

Latrotoxin is responsible for symptoms of envenomation known as latrodectism (Graudins et al., 2012). Physiological affects to prey are caused latrotoxin selectively binding to presynaptic nerve endings and triggering an immense release of neurotransmitters (Upadhyay & Ahmad, 2011). The spiders of the genus *Latrodectus* possess multiple latrotoxin proteins that each target

specific taxonomic groups including vertebrates (α -latrotoxin), crustaceans (α -latrocrustatoxin), and insects (α -latroinsectotoxin) (Vassilevski et al., 2009). A comparison of the structure of the different latrotoxin proteins revealed an average of 30% identical amino acid residues. This high homology suggests these proteins likely evolved by gene duplication (Vassilevski et al., 2009).

All latrotoxins, α -latrotoxin included, trigger neurotransmitter release in organisms they are active in (Südhof, 2001). α -latrotoxin is a large ~130 kDa hydrophilic protein that targets neural and neuroendocrine nerve terminals to cause large amounts of spontaneous neurotransmitter release (Graudins et al., 2012). The structure of α -latrotoxin consists of 1100-1200 amino acids (Vassilevski et al., 2009). It's been proposed that α -latrotoxin is synthesized as a protein precursor in the venom gland, where it is cleaved by endoproteases to generate the mature toxin, which is composed of four different domains (Südhof, 2001). Domain I is a cleaved signal peptide. Domain II is a conserved N-terminal domain that is composed of 431 amino acid residues containing two hydrophobic sequences, each of 20-26 amino acids, and three invariant cysteine residues (Südhof, 2001). Domain III is a central domain composed of 22 imperfect ankyrin-like repeats. These repeats are characterized by 33-residue patterns that are made up of two alpha helices separated by loops covering 745 amino acids (Südhof, 2001). Domain IV is a C-terminal domain containing 206 amino acid residues. This domain is less conserved between latrotoxins and is most likely cleaved during the maturation of α -latrotoxin (Südhof, 2001).

The structure of α -latrotoxin has recently been studied extensively in *Latrodectus tredecimguttatus* to better understand its neurotoxic effect on vertebrates after the stimulation of neurotransmitter release (Ushkaryov et al., 2004). Two closely related species, the European black widow (*Latrodectus tredecimguttatus*) and the red-back spider (*Latrodectus hasseltii*), are the only two species that have been studied for genetic variation of α -latrotoxin (Graudins et al., 2012). An understanding of variation in α -latrotoxin structure among diverse species will be useful for fully understanding its function and differences in toxicity among species.

The purpose of this study is to characterize nucleotide and amino acid composition of the α -latrotoxin gene in *L. geometricus* and *L. mactans*. Gene sequences for these two species will also be compared to that of previously published data for *L. tredecimguttatus* (Kiyatkin et al., 1990). In doing so, this study will identify and quantify genetic differences that may underlie functional differences in the α -latrotoxin protein among species. Genetic differences among species in α -latrotoxin was also compared to that of a second gene, cytochrome oxidase I (COI), which is not associated with *Latrodectus* venom. The purpose of this comparison is to quantify differences in evolutionary rate between venom and non-venom related genes. Understanding the variation in structure of the α -latrotoxin gene is a first step in understanding differences in potency among species as well as in understanding the processes that drive the evolution of this gene.

Materials and Methods

DNA extraction

DNA was extracted from a single leg using the Qiagen DNeasy Tissue Kit following manufacture protocol (Qiagen Inc., Valencia, CA, U.S.A.).

Polymerase chain reaction

Polymerase chain reaction was used to amplify various segments of the *L. geometricus* and *L. mactans* α -latrotoxin gene. Primers were designed using the published sequence of α -latrotoxin from *L. tredecimguttatus* (Graudins et al., 2012). Samples for PCR were prepared by combining 6 μ l of DI water, 0.05 μ M of each primer, 10 Apex Taq Master Mix (Genesee Scientific), and 2 μ l of extracted DNA. PCR Amplification reactions were performed with the following cycling protocol: 94 °C for 30s, 55 °C for 60s, and 72 °C for 120s, and a final extension at 72 °C for 5min.

Following PCR, samples were run on a 1.5% agarose gel to confirm amplification.

DNA Sequencing

The PCR products were purified using 1 unit Shrimp Alkaline Phosphatase and 0.05 units Exonuclease 1 in 15 μ L of PCR product. Fragments were sequenced in both directions using the same primers used in PCR. Cycle sequencing was performed with the Big Dye Terminator Kit Version 3.1 (Applied

Biosystems, Foster City, CA, U.S.A.) and sequencing products were separated on an ABI 3500 Genetic Analyser at Georgia Southern University.

Genetic Analysis

Sequencher DNA sequence analysis software was used to align and edit DNA fragments from different primer set reactions for the same individual. The program was then used to analyze genetic sequences by performing a sequence alignment between *L. tredecimguttatus*, *L. geometricus*, and *L. mactans* α -latrotoxin sequences. Sequencher was also used to convert the DNA sequences into protein sequences for each of the three species.

SDSC Biology Workbench 3.2 (<http://workbench.sdsc.edu>) was used to align sequences and quantify variation in different DNA and protein strands between different species. After sequences were uploaded, the CLUSTALW-multiple sequence alignment tool was used within the protein tools option to align protein sequences. Alignments were used to quantify amino acid differences between the sequences being compared. Once differences were recorded, sequence alignments were imported and the 'PROTDIST' alignment tool was used to compute evolutionary distance between sequences using a categorization of amino acids on a chemical scale. This scale compares the chemical components of the amino acid differences between the two species being compared to calculate a protein divergence.

Nucleic tools in the SDSC Workbench were then used to compare nucleotide sequence differences between species. 'CLUSTALW- multiple

sequence alignment' tool was used to align the nucleotide sequences of the different species. Once differences were recorded, sequence alignments were imported and the 'DNADIST' tool was used to compute the evolutionary distance between sequences using the Kimura 2-Parameter model. DNA and protein sequences were compared between *L. tredecimguttatus*, *L. geometricus*, and *L. mactans* for both α -latrotoxin and COI.

Results

Amino acid changes were observed between α -latrotoxin and the COI mitochondrial gene protein sequences of *L. tredecimguttatus*, *L. mactans*, and *L. geometricus*. For α -latrotoxin, *L. tredecimguttatus* to *L. geometricus*, *L. tredecimguttatus* to *L. mactans*, and *L. mactans* to *L. geometricus* had 168, 24, and 51 total amino acid changes respectively. For COI, *L. tredecimguttatus* to *L. geometricus*, *L. tredecimguttatus* to *L. mactans*, and *L. mactans* to *L. geometricus*, had 7, 3, and 6 total amino acid changes respectively.

A total of 2,019 base pairs of the α -latrotoxin gene were compared between *L. tredecimguttatus* and *L. geometricus* (Figure 1). Of the 2,019 base pairs compared, 288 total nucleotide differences were observed with a calculated evolutionary distance of 0.152. A similar level of DNA sequence divergence was observed in the mitochondrial COI gene between *L. tredecimguttatus* and *L. geometricus* (Figure 1). There were 59 nucleotide differences observed and a calculated evolutionary distance of 0.160 between the two species.

The amino acid sequences of α -latrotoxin of *L. tredecimguttatus* and *L. geometricus* had a total of 161 amino acid differences out of 673 total amino acids. The calculated evolutionary distance was 0.479 (Figure 1). Amino acid differences between *L. tredecimguttatus* and *L. geometricus* for COI was much lower than that for α -latrotoxin. In the COI mitochondrial gene, 7 amino acid differences were observed and there was a calculated evolutionary distance of 0.128 (Figure 1).

A total of 982 base pairs of the α -latrotoxin gene were compared between *L. tredecimguttatus* and *L. mactans* (Figure 2). Of the 982 base pairs compared, 41 total nucleotide differences were observed with a calculated evolutionary distance of 0.049. A similar level of DNA sequence divergence was observed in the mitochondrial COI gene between *L. tredecimguttatus* and *L. mactans* (Figure 2). There were 57 nucleotide differences observed and a calculated evolutionary distance of 0.150 between the two species.

The amino acid sequences of α -latrotoxin of *L. tredecimguttatus* and *L. mactans* had a total of 24 amino acid differences out of 327 total amino acids. The calculated evolutionary distance was 0.150 (Figure 2). Amino acid differences between *L. tredecimguttatus* and *L. mactans* for COI was much lower than that for α -latrotoxin. In the COI mitochondrial gene, 3 amino acid differences were observed and there was a calculated evolutionary distance of 0.047 (Figure 2).

A total of 832 base pairs of the α -latrotoxin gene were compared between *L. mactans* and *L. geometricus* (Figure 3). Of the 832 base pairs compared, 111 total nucleotide differences were observed with a calculated evolutionary distance of 0.167. A similar level of DNA sequence divergence was observed in the mitochondrial COI gene between *L. mactans* and *L. geometricus* (Figure 3). There were 65 nucleotide differences observed and a calculated evolutionary distance of 0.183 between the two species.

The amino acid sequence of α -latrotoxin of *L. mactans* and *L. geometricus* had a total of 51 amino acid differences out of 277 total amino acids. The

calculated evolutionary distance was 0.601 (Figure 3). Amino acid differences between *L. mactans* and *L. geometricus* for COI was much lower than that for α -latrotoxin. In the COI mitochondrial gene, 6 amino acid differences were observed and there was a calculated evolutionary distance of 0.129 (Figure 3).

Of the 168 amino acid changes between α -latrotoxin *L. tredecimguttatus* and *L. geometricus*, 25.0% of amino acids changes were between non-polar and polar amino acids, 7.7% of changes were between non-polar and acidic amino acids, 8.3% of changes here between non-polar and basic amino acids, 10.1% of changes were between polar and acidic amino acids, 10.7% of changes were between polar and basic amino acids, 1.2% of changes were between acidic and basic amino acids, and 36.9% of amino acid changes remained in the same group (Table 1).

Of the 24 amino acid changes between α -latrotoxin *L. tredecimguttatus* and *L. mactans*, 16.7% of amino acids changes were between non-polar and polar amino acids, 8.3% of amino acid changes were between polar and acidic amino acids, 25.0% of amino acid changes were between polar and basic amino acids, 8.3% of amino acid changes were between acidic and basic amino acids, and 41.7% of amino acid changes remained in the same group (Table 1).

Of the 51 amino acid changes between α -latrotoxin *L. mactans* and *L. geometricus*, 23.5% of amino acids changes were between non-polar and polar amino acids, 2.0% of amino acid changes were between non-polar and acidic amino acids, 3.9% of amino acid changes here between non-polar and basic amino acids, 15.7% of amino acid changes were between polar and acidic amino

acids, 15.7% of amino acid changes were between polar and basic amino acids, 3.9% of amino acid changes were between acidic and basic amino acids, and 35.3% of amino acid changes remained in the same group (Table 1).

Of the 7 amino acid changes observed between COI mitochondrial gene of *L. tredecimguttatus* and *L. geometricus*, 14.3% of amino acid changes were between polar and non-polar amino acids and 85.7% of amino acid changes remained in the same group (Table 1).

Of the 3 amino acid changes observed between COI mitochondrial gene of *L. tredecimguttatus* and *L. mactans*, 33.3% of amino acid changes were between polar and non-polar amino acids and 66.7% of amino acid changes remained in the same group of amino acids (Table 1).

Of the 6 amino acid changes observed between COI mitochondrial gene of *L. mactans* and *L. geometricus*, 16.7% of amino acid changes were between polar and non-polar amino acids and 83.3% of amino acid changes remained in the same group (Table 1).

Discussion

This study characterized nucleotide and amino acid variation in α -latrotoxin among three species of widow spider. The lack of stop codons, similar nucleotide and protein sequence lengths, and fairly limited variability found between the gene sequences suggest that *L. geometricus* and *L. mactans* express a form of vertebrate-specific α -latrotoxin that is similar to that of the previously published sequence of *L. tredecimguttatus* (Kiyatkin et al., 1990).

The level of nucleotide and amino acid divergence in α -latrotoxin differed greatly from that of the mitochondrial gene cytochrome oxidase I (COI), a crucial subunit in the electron transport chain (Nelson & Cox, 2008). This suggests that very different evolutionary processes have shaped the structure of the two genes. The nucleotide divergence calculated between α -latrotoxin and mitochondrial COI were relatively similar. In comparison, α -latrotoxin had an amino acid divergence 3.7 times higher on average than that of the mitochondrial COI gene. If two genes are experiencing similar evolutionary or selective pressures, the length of time since divergence between species should be better reflected by a measure of amino acid differences than nucleotide differences (Zuckerandl & Pauling, 1965; Beebee & Rowe, 2008). The opposite patterns in nucleotide and amino divergence in COI and α -latrotoxin suggests different evolutionary processes have influenced the two genes. The evolution of α -latrotoxin has likely been driven by divergent selection. However, because of the importance of the function of the mitochondrial COI gene in overall metabolism, there is an incredibly low efficiency in evolutionary nucleotide substitution

changes. This results in less amino acid changes and less evolutionary divergence in COI than in α -latrotoxin (Gibson et al., 2010).

The protein divergence observed between species in the aligned sequences of α -latrotoxin and mitochondrial COI is greater in α -latrotoxin. Different patterns are also observed between the two genes in the types of amino acid changes that have occurred. Changes from one amino acid chemical group to another chemical group are observed more in α -latrotoxin between species than in COI. The protein changes that occur between hydrophobic and hydrophilic classes of amino acids are common which is consistent with that found by Garb et al., 2013. These changes between different physiological classes of amino acids suggest that these changes could result in functional differences, including different levels of toxicity in α -latrotoxin among species (Garb et al., 2013).

α -latrotoxin is the molecule responsible for the symptoms of widow spider envenomation and latrodectism in humans (Garb et al., 2013). Symptoms of latrodectism include generalized pain, nausea, abdominal pain, cramps, and tremors (Afshari et al., 2009). This occurs when the latrotoxin binds to neurons, which then signal massive neurotransmitter release (Garb et al., 2013). Different latrotoxins have specific and consistent shapes, which correspond to the species-specific neurons it binds to in different organisms (Garb et al., 2013). This explains the cocktail of various forms of the protein latrotoxin in venom, including α -latrotoxin, α -latrocrustatoxin, and α -latroinsectotoxin, each of which affects vertebrates, crustaceans, and insects respectively (Garb et al., 2004). Species-specific interactions between widow species and the different but

common species they envenomate could also explain the high rate of divergence in α -latrotoxin (Gibbs & Rossiter, 2008).

The high levels of divergence in spider venom genes shows similar patterns to that seen in snake venom genes. Because of the biomedical importance of the study of envenomation in snakes, there is a significant amount of knowledge on the structural composition and function of many of the proteins found in snake venom (Gibbs & Rossiter, 2008). The evolution of snake venom genes can be used as a comparison to gain a better understanding of the evolution of genes, like α -latrotoxin, found in spider venom. Snake venoms are considered one of the most high-level weapon delivery systems that represent a prime example of a predatory adaptation (Gibbs & Rossiter, 2008). The birth-and-death model is often used to describe and explain the evolution of genes that encode for venom of snakes and other animals. This model states that evolution occurs by repeated duplication events that are either maintained or deleted post mutation (Sunagar et al., 2013). This leads to a high diversity level in venom proteins of various species that often are due to prey-specific consequences (Gibbs & Rossiter, 2008).

The rapid evolution in snake venom suggests a strong selective pressure for it to evolve with its prey. Gibbs and Rossiter (2008) compared the variation of snake venom between four related species, each with different diet. They hypothesized that if the composition of venom of each species of snake was prey-specific and diet related, then high rates of variation of venom proteins would be observed between the related species. The study found that the various

genes are an essential evolutionary determinant in the adaptive evolution of these various related species (Gibbs & Rossiter, 2008). This is consistent with the birth-and-death model in that the divergence between the venom genes of these species allows venom snakes the ability to adapt to their species-specific prey (Jiang et al., 2011). These findings are similar to the high levels of amino acid divergence between the various species in genus *Latrodectus*. Each of these species evolved in different geographic areas and their venom would have evolved to work efficiently with the different diets or enemies they are exposed to.

Structural protein genes are strongly affected by the process of natural selection in order to maintain their function (Beebee & Rowe, 2008). Because of this, the species-specific shape of the binding latrotoxin could drive the higher rate of evolution in the α -latrotoxin observed between species. As the prey of the various species of *Latrodectus* evolve, like the prey of snakes, this would force the latrotoxin to evolve along with it or it will no longer have the ability to bind to the neurons.

Unlike α -latrotoxin which seems to have evolved under the influence of divergent selection, mitochondrial COI differences are consistent with negative or stabilizing selection (Nelson & Cox, 2008). This is due to the role it plays in the critical function of the electron transport chain (Nelson & Cox, 2008). Mutations and evolutionary changes in the COI gene sequence would likely result in negative or even fatal results (Nelson & Cox, 2008). Thus, these changes are unlikely to remain in a population.

The first goal of this project was to characterize nucleotide and amino acid variation in the α -latrotoxin gene in *L. geometricus*, *L. mactans*, and *L. tredecimguttatus*. The second goal was to compare patterns of divergence in α -latrotoxin with that of a non-venom gene; the mitochondrial COI gene. The two genes show very different patterns of divergence, suggesting different evolutionary processes have influenced their structure. When comparing the nucleotide and amino acid divergence of α -latrotoxin with that of the mitochondrial COI gene, there was a relatively similar divergence in the nucleotide sequences, but a higher divergence in the protein sequences. This suggests that α -latrotoxin has a faster evolutionary rate. Many of the amino acid differences observed among species would likely result in functional differences. Further characterization of these differences could shed light on the molecular mechanisms by which this neurotoxin functions as well as the mechanisms behind different levels of toxicity among widow species

L. tredecimguttatus* vs *L. geometricus

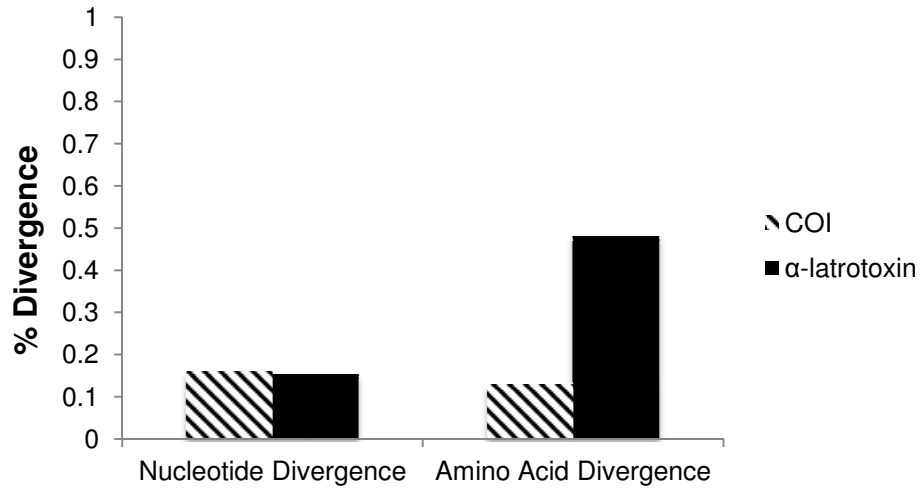


Figure 1: Nucleotide and amino acid divergence of the α -latrotoxin gene and COI mitochondrial gene between *L. tredecimguttatus* and *L. geometricus*.

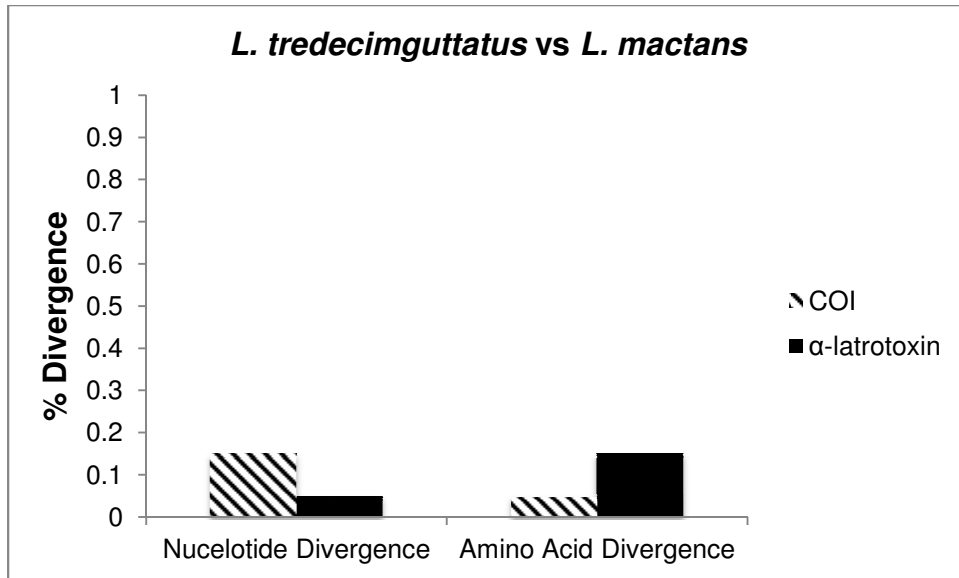


Figure 2: Nucleotide and amino acid divergence of the α -latrotoxin gene and COI mitochondrial gene between *L. tredecimguttatus* and *L. mactans*.

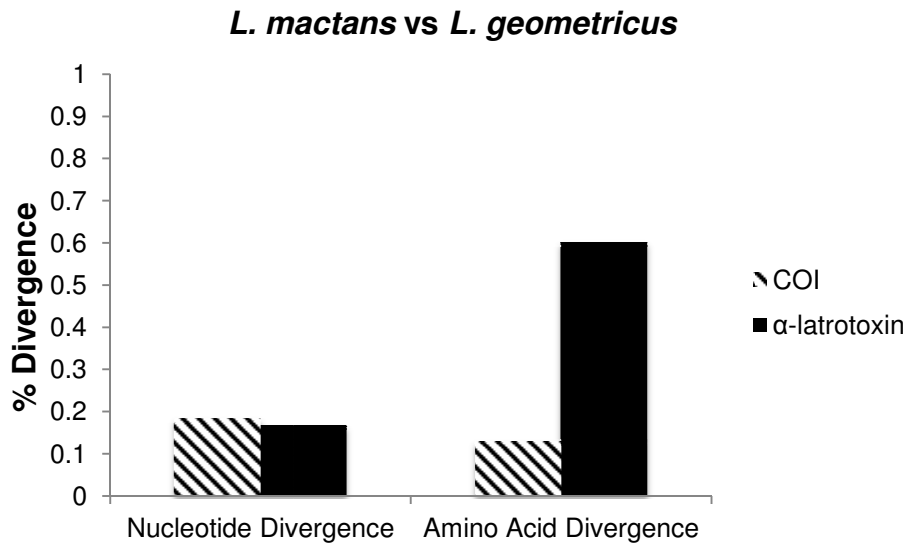


Figure 3: Nucleotide and amino acid divergence of the α -latrotoxin gene and COI mitochondrial gene between *L. mactans* and *L. geometricus*.

	NP \leftrightarrow P	NP \leftrightarrow A	NP \leftrightarrow B	P \leftrightarrow A	P \leftrightarrow B	A \leftrightarrow B	SG
α -LTX							
LT \rightarrow LG	25.0%	7.7%	8.3%	10.1%	10.7%	1.2%	37.0%
α -LTX							
LT \rightarrow LM	16.7%	0.0%	0.0%	8.3%	25.0%	8.3%	41.7%
α -LTX							
LM \rightarrow LG	23.5%	2.0%	3.92%	15.7%	15.7%	3.9%	35.3%
COI							
LT \rightarrow LG	14.3%	0.0%	0.0%	0.0%	0.0%	0.0%	85.7%
COI							
LT \rightarrow LM	33.3%	0.0%	0.0%	0.0%	0.0%	0.0%	66.7%
COI							
LM \rightarrow LG	16.7%	0.0%	0.0%	0.0%	0.0%	0.0%	83.3%

Table 1: Changes between physiological classes of amino acids in α -latrotoxin (α -LTX) and COI mitochondrial gene. Amino acid changes are from *L. tredecimguttatus* (LT) to *L. geometricus* (LG), *L. tredecimguttatus* to *L. mactans* (LM), and *L. mactans* to *L. geometricus*. Amino acid changes include changes to the following groups: non-polar (NP), polar (P), acidic (A), basic (B), and same group (SG).

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