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Development of Microsatellite Markers for Loxosceles devia Gertsch & Mulaik (Araneae: Sicariidae) using Next Generation Sequencing

Tanya Y. Garza

DEVELOPMENT OF MICROSATELLITE MARKERS FOR *LOXOSCELES DEVIA* GERTSCH & MULAIK (ARANEAE: SICARIIDAE) USING NEXT GENERATION SEQUENCING

A Thesis

by

TANYA YVETTE GARZA

Submitted to Texas A&M International University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCES

May 2016

Major Subject: Biology

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Approved as to style and content by:

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ABSTRACT

Development of Microsatellite Markers for *Loxosceles devia* Gertsch & Mulaik (Araneae: Sicariidae) using Next Generation Sequencing (May 2016)

Tanya Yvette Garza, B.S., Texas A&M International University;

Chair of Committee: Dr. Dan Mott

Brown recluse spiders in the genus *Loxosceles* are known for causing severe necrotic skin lesions. Despite their impact on human health, very little is known concerning the genetic diversity or population structure of this genus. The development of a suite of molecular markers (microsatellites) would allow us to obtain genetic data and expand the resources available to study *Loxosceles devia*, the species found throughout south Texas. Microsatellites are long tandem repeats about 1-6 nucleotides long of DNA, also known as simple sequence repeats. Their co-dominant method of inheritance, wide spread distribution across the nuclear genome, ease of scoring, and high mutation rates make microsatellites an ideal molecular marker. Unfortunately, the cost of developing microsatellites has limited their availability until now. The Ion Torrent Personal Genome Machine® was used to shotgun sequence libraries of genomic DNA derived from *L. devia*. The program MSATCOMMANDER™ was used to identify and design primers for di-, tri-, and tetra- microsatellite repeats from these shotgun sequences. Seventeen microsatellite markers were designed and polymerase chain reaction conditions were optimized for each primer to develop a suite of novel molecular markers for this species.

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INTRODUCTION

Arachnids.—The arthropods are a diverse group of taxa that includes crustaceans, insects, and arachnids. Within the arachnids, the spiders (Araneae) makeup over 45,700 species worldwide (WSC 2015). The family Sicariidae consists of two genera, *Loxosceles* and *Sicarius*, which are widely distributed. The two genera originated from a common sicariid ancestor and spread throughout Western Gondwana, before Africa and South America split (Gremski et al. 2014; Chaim et al. 2011; Binford et al. 2008). The genus *Loxosceles* is distributed worldwide in warm temperate areas, and they prefer to live in dry, dark, and sheltered areas. They tend to seek shelter in abandoned buildings and areas such as basements and under stairways. The genus *Loxosceles* is known to be inactive, non-aggressive, and nocturnal (Chaim et al. 2011; Andrade et al. 1999). The name *Loxosceles* comes from the Greek meaning "slanted legs" due to the way the spider holds its legs at rest (Vetter 2015).

Loxosceles rufescens Dufour 1820, the Mediterranean recluse, is the type species for the entire genus since it was the first *Loxosceles* spider to be described in 1820 (Vetter 2015). There are over 120 identified *Loxosceles* species distributed worldwide, however, there are only 6 species found in North America (Fig. 1) (Platnick 2013). *Loxosceles reclusa* Gertsch & Mulaik 1940 has the widest distribution in North America extending through southwestern Nebraska to southern Iowa, Illinois, and Indiana to Ohio. They extend south to eastern Texas and through west Georgia and Florida. The species with the second widest distribution is *L. deserta* Gertsch 1973 that extends from central Arizona into California, and into southern Nevada. In addition, two species *L. laeta* Nicolet 1849 and *L. rufescens*, are "cosmopolitan" since they are widely distributed and have been transported through trade vehicles (Gertsch & Ennik 1983). The Texas

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This thesis follows the style of Journal of Arachnology.

recluse, *L. devia* Gertsch & Mulaik 1940, is found only in south Texas.

Brown recluse spiders are commonly found during the summer, between May and August, and tend to disappear in the winter. However, heat can be fatal which results in them retreating far underground utilizing existing burrows, allowing them to only be active at night (Vetter 2015). In areas where they do occur, brown recluse spiders can be found in large numbers.

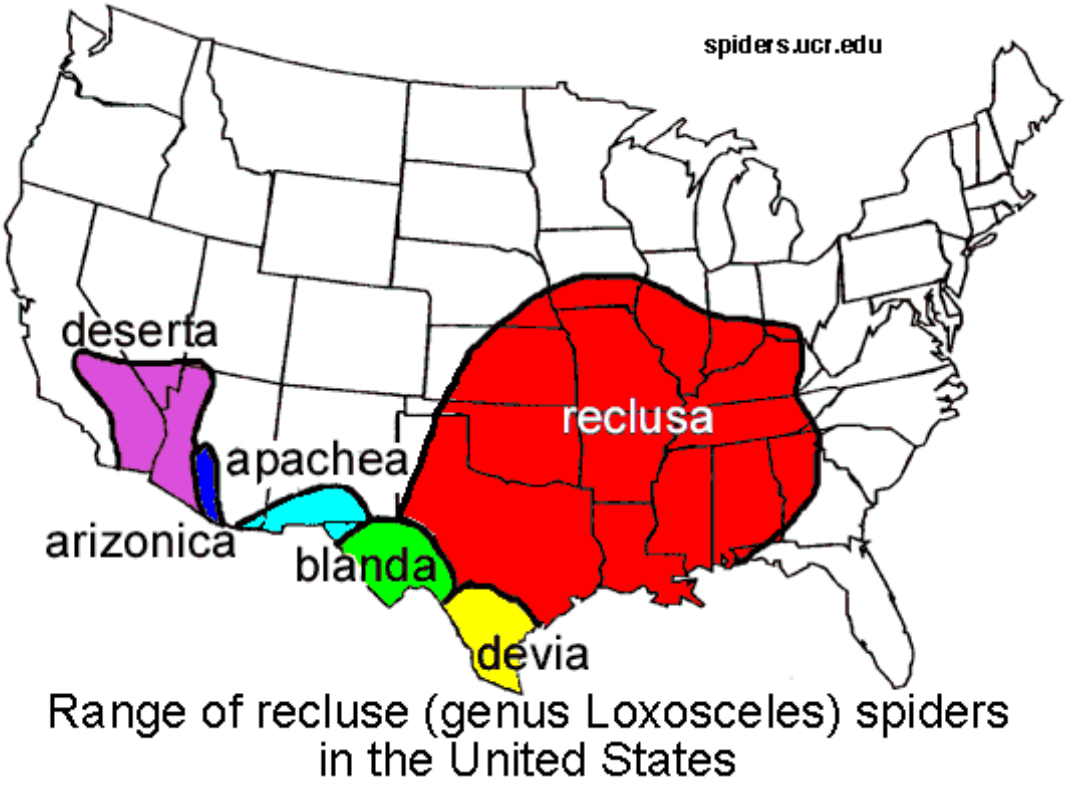

Figure 1. Map of the distribution of the most common *Loxosceles* species found in North America. As seen in yellow, *L. devia* is found only in south Texas (spiders.ucr.edu 2009).

The genus *Loxosceles* has been transferred among three families over the past 74 years. They were originally placed in the family Sicariidae (Simon 1893). They were then placed in their own family, Loxoscelidae (Gertsch 1949) and subsequently transferred to Scytodidae

(Gertsch 1967). However, based on synapomorphic spinneret morphology, they are currently placed back in the Sicariidae (Platnick et al. 1991). The genus is known by common names such as brown, fiddle-back, violin, and recluse spiders. There are two definite ways to identify a recluse spider; one being their dark pigmented violin pattern on their carapace (Vetter 2008a). The carapace is pale yellow to reddish brown, with a dusky dark patch in front of the median groove. The patch is connected to the front of the carapace by brown stripes, resulting in a violin shape (Fig. 2). At times the pattern isn't distinct on young recluses since their pigments haven't fully developed (Edwards 2003). *Loxosceles* also have six eyes arranged in three dyads, forming a U-shape (Fig. 3) (Chaim et al. 2011). This distinctive eye arrangement is used to help identify recluse spiders.

Figure 2. Spider of the genus, *Loxosceles* (Edwards 2015).

Loxosceles range from 7-12mm in body length with females (mean 9mm) slightly larger than the males (mean 8mm) (Forks 2000). All *Loxosceles* have thin, uniformly colored legs with

fine hairs covering them. They have two-segmented chelicerae that have two short fangs attached to stout basal segments capable of limited lateral motion (Gertsch 1967). They have a particular fang orientation known as “uncate,” in which the fang is short and a small spike projects off the chelicera to form a pincer-like arrangement (Fig. 3) (Vetter 2015). Their abdomen is oval shaped, and varies in color from light cream to dark brown depending upon their food intake. Light cream abdomens come from feeding on crickets or termites; dark abdomens come from feeding on houseflies (Vetter 2015). It has been reported that *Loxosceles* spiders prefer to scavenge on dead prey rather than consuming live prey (Sandidge 2003). Feeding on dead prey that was killed with an insecticide does not have any effect on the consumer (Vetter 2011). Brown recluses don’t build webs to capture prey, but they use silk to build a retreat in which they hide in during the day (Vetter 2008b).

Figure 3. Close-up of the genus *Loxosceles* eye pattern and fang orientation. They have six eyes arranged in three dyads and an “uncate” fang orientation (Walker 2011).

Loxosceles have relatively long life spans (1.5-2 years) for small spiders. They have the ability to slow their heart rate, which allows them to live longer (Vetter 2015). They can also go

long periods of time without food. Recluse spiders are haplogynes (simple male and female genitalia) and have simple reproductive structures (Platnick et al. 1991). Females lack hardened genitalia and both genders have simple reproductive structures that are similar to a lock and key mechanism, where the male mating organ only fits the female reproductive openings of the same species (Vetter 2015).

Male recluses have their reproductive structures on the terminal end of their pedipalps. The males have a pointed embolus that is inserted into the females reproductive system. *Loxosceles reclusa, L. deserta, L. rufescens,* and *L. devia* have slightly different reproductive structures (Fig. 4) (Vetter 2015). The emboli in males vary in size, length, and curvature based on the species (Gertsch 1967). In *L. reclusa,* the tip of the embolus looks like the beveled point of a hypodermic needle with its end cut off. *Loxoscles deserta* has a thicker bottom portion of the embolus with a curved tip. In *L. rufescens*, the embolus is shorter and more curved with a small bulb on top. Lastly, in *L. devia*, the embolus is curved and longer than the bulb (Gertsch & Ennik 1983).

The female reproductive organs are difficult to examine since viewing them is a delicate operation. The reproductive organs need to be dissected from the abdomen and fat tissues must be cleaned before viewing under a microscope since they're haplogynes. The reproductive organs of the female *L. reclusa* consist of a thin, hardened ridge with many bumps (Fig. 5). *Loxosceles deserta* females contain only one central bump, while the *L. rufescens* has a hardened ridge on the top parts of its organs (Vetter 2015). *Loxosceles devia* has a genital groove that is surmounted by a finger-like lobe directed toward the midline with additional small folds (Gertsch 1983). All species have a slightly different reproductive structure.

Brown recluse spiders are most active from May-October in cooler climates and tend to mate and reproduce during June and July (Cramer 2015; Edwards 2003). The female recluse uses her spinnerets to lay down a fine white silk to make an egg sac. She positions herself on top of the silk and the eggs are dropped as a moist, gooey mass from the reproductive opening. After she has completed laying her eggs, a similar white layer of silk is produced to enclose the eggs in the sac. A looser silk is then produced to cover the outer surface of the egg sac. The female recluse protects the egg sac from any predators until the eggs dry out inside the sac and become free from each other. Female fecundity for *Loxosceles* ranges from 30-50 eggs per sac (Fischer & Neto 2005). Females have frequently been found with more than one egg sac. The young spiderlings molt at least 8 times before reaching adulthood and becoming a mature adult. They undergo their first true molt inside the egg sac. Since spiders have a rigid exoskeleton, there are limits to how large an individual can grow before it has reached the maximum that an exoskeleton of a particular size will allow. A few days before molting, the legs darken as new setae can be seen under the old cuticle (Vetter & Rust 2010). As the brown recluses prepare to molt, they stretch their legs apart evenly known as splay-leg molting position, and use their claws to grab onto the silk to help pull themselves out of their old exoskeleton (Vetter & Rust 2010; Vetter 2015). The spider gets in molting position two hours before the active molting process and remains motionless. The just-molted recluse spider has pale legs and cephalothorax, and the typical violin pattern lacks pigments. During the fourth molt, the genital openings are still closed making male and female undistinguishable. By the fifth and sixth molt, male pedipalps are distinguishable (Margraf et al. 2011). Molting typically occurs in the spider's retreat since it's vulnerable to predators.

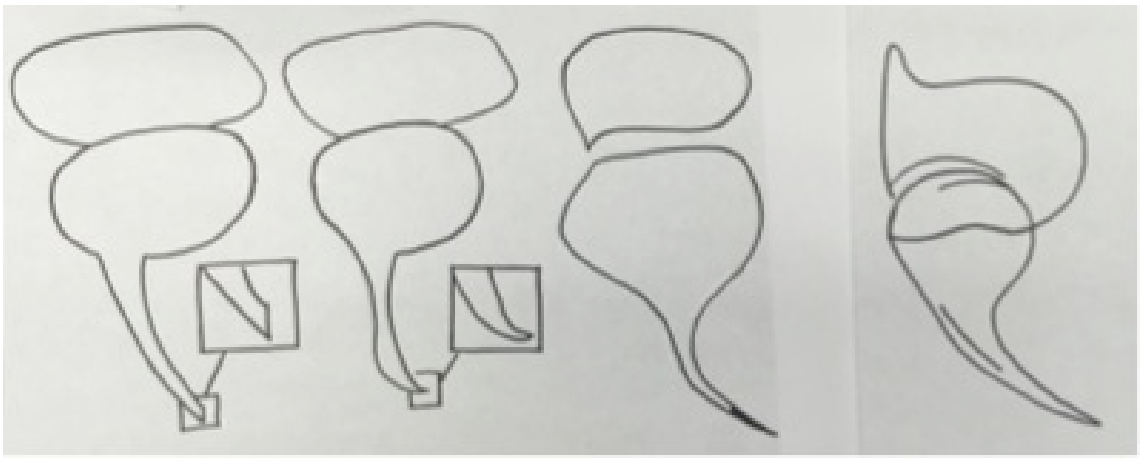

Figure 4. Male reproductive organs of (from left to right*) L. reclusa, L. deserta, L. rufescens,* and *L. devia* (Vetter 2015).

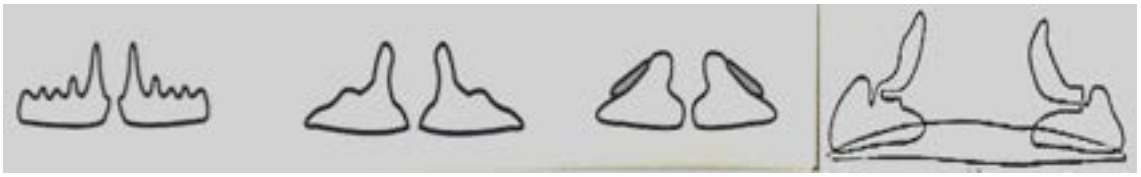

Figure 5. Female reproductive organs of (from left to right) *L. reclusa, L. deserta, L. rufescens,* and *L. devia.* As shown, reproductive structures slightly differ in both males (Fig. 3) and females (bottom) (Vetter 2015).

Loxosceles spiders are known for their tissue destroying venom that contains sphingomyelinase D, an enzyme that catalyzes hydrolysis to yield ceramide-1-phosphate, C1P (Rivera et al. 2015). Ceramide-1-phosphate promotes cell growth and stimulates cell proliferation. The enzyme destroys cell membranes and causes necrosis of the tissue. *Loxosceles* venom contains SMase D isoforms that makeup a family of homologs with over 100 redundant sequences (Dias-Lopez et al. 2014). Although purified brown recluse venom contains at least 8 different enzymes, SMase D is considered the active component of spider venom in the sicariids (Rivera et al. 2015). The compound begins to develop after the third molt and increases until the spider is mature (Vetter 2015).

Loxosceles spider bites are referred to as loxoscelism, which is the cause of dermonecrosis (Swanson & Vetter 2006). The spiders are known for leaving a classic bulls-eye lesion. A red blister forms in the center, surrounded by a bluish area, with a white separation between the red and the blue colors. The bites are usually painless so people are unaware when they are bitten. Bites occur most frequently on the legs and on the arms (Vetter 2015). They usually occur when spiders feel trapped or threatened. Since females are a bit larger than males, they produce a larger amount of venom, which results in a more serious bite (Forks 2000). However, misdiagnosis is highly common with *Loxosceles*, along with misidentification. There are many possible conditions that have been or could be misdiagnosed as a brown recluse spider bite such as infections (bacterial, fungal, and viral), topical agents (burns and plant toxins), and diabetic ulcers.

Microsatellite markers.—Microsatellites are di-, tri-, or tetra nucleotide tandem repeats of DNA sequences ranging from 2-6 base pairs (bp). They are also known as simple sequence repeats, SSRs. Every repeat is vastly over-represented in the genome, however, they are considered to be unique DNA (Ellegren 2004). Microsatellites are distributed throughout the genome on all chromosomes and regions, including introns and non-gene sequences (Liu 2011). At each locus, individuals possess two alleles of varying lengths each inherited from a parent. Dinucleotide repeats are the most common repeats found in DNA, while repeats greater than tetranucleotides are less likely to be found (Liu 2011). The variation of repeats results from slippage of base-pairing DNA strands during replication and misalignment (Chen 2012). Evidence shows that microsatellites are nonrandom due to the effects on chromatin organization, regulation of gene activity and DNA replication (Li et al. 2004). Microsatellite markers can be used for many types of studies such as family linkages, population genetics, genome mapping,

and molecular ecology (Planas et al. 2015). Microsatellites have a high mutation rate, which aid in understanding how much environmental factors have affected genomic mutation rates (Ellegren 2004). High polymorphism is a major feature that makes microsatellites of interest for genetic studies. The co-dominant method of inheritance, wide spread distribution across the nuclear genome, ease of scoring and high mutation rates make microsatellites an ideal molecular marker. Unfortunately, in the past the development of microsatellite markers was limited due to the cost. Next generation sequencing technology has made sequencing easy and cheap. It has become an efficient and cost-effective procedure for a quick development of microsatellite markers on non-model organisms. It's an easy approach to explore selected genomes. Next generation sequencing aids in obtaining SSRs and describing the step-by-step process from DNA extraction to characterization of selected markers (Planas et al. 2014).

In arthropods, AC is the most frequently found dinucleotide repeat and AAT is the most abundant trinucleotide repeat (Toth et al. 2000). *Loxosceles rufescens* is the only *Loxosceles* species in which microsatellite markers have been developed (Planas et al. 2014) who supports that AAT is the most abundant repeat as *L. rufescens* have it on 6 different loci. DNA surrounding a microsatellite locus is called the flanking region. Flanking regions are usually conserved across individuals of the same species. Therefore, a microsatellite locus can often be identified by its flanking sequence. The region containing the microsatellite is amplified using primers that flank the microsatellite. The development of a suite of molecular markers (microsatellites) would allow us to obtain genetic data and expand the resources available to study this venomous species.

Purpose. —In this study, microsatellite markers were developed for *L. devia* using next generation sequencing. The project aim was to identify repetitive DNA elements within the

genome of *L. devia* (di-, tri-, and tetranucleotide repeats) that can be used as molecular markers. Specific primers were designed and optimized to isolate and amplify certain regions. The purpose of this was to understand the genetics and genome of this species by testing the microsatellite markers for variability, heterozygosity, and Hardy-Weinberg Equilibrium.

METHODS

Collecting spiders.—In order to conduct this study, a collection of 35 *L. devia* were used for DNA extraction. The spiders that were used had been collected throughout the past 5 years from Laredo, TX and Zapata, TX. They were collected in pit-fall traps using propylene gylcol, along with hand collection methods. They were preserved in 70% ethyl alcohol in individual vials.

DNA extraction and quantification.—Once the specimens were obtained, genomic DNA was extracted using the protocol from the NucleoSpin™ Tissue XS Kit. DNA samples were homogenized in Buffer B5 and extracted using silica-membrane technology. DNA was quantified using a Thermo Scientific NanoDrop™ 2000 Spectrophotometer. One µL of nuclease-free water was pipetted onto the measurement surface and used as the "Blank." Nucleic acid concentration was measured for each individual by testing 1µL of sample. After measurement, each sample was wiped off the measurement surface with a Kimwipe™. Libraries were created using Ion Xpress™ Plus gDNA & DNA Library Preparation (Revision 3 Jan 2012) following the manufacturer's protocol. Genomic DNA was fragmented with Ion Shear™ Plus reagents following the 1µg protocol. Continuing by ligating adaptors, using nick-repair, and purifying the ligated DNA by still following the 1µg protocol. Then, the unamplified library was size-selected using E-Gel® SizeSelect™ Agarose Gel. The target size was between 200-250 bp. The library was amplified and purified before dilution factors were calculated. Dilutions were calculated using the Ion OneTouch Library Dilution Calculator (Table 1). The dilutions were made using the amplified library and Low TE, storing in a twist top reaction tube in the freezer (-20˚C).

Table 1. Dilution factors for *Loxosceles* species calculated by Ion OneTouch Library Dilution Calculator.

LD-98	Dilution 1	Dilution 2	Dilution 3
Library	2µL	5µL	5µL
Low TE	4µL	45µL	500µL

Template preparation.—The Ion PGM™ Template OT2 200 Kit protocol was used to prepare templates for 200 base-read libraries to be used on Ion OneTouch™ 2 System (Elmusion PCR). The diluted library consisted of 5µL of Dilution 1 and 20µL of water for amplification. A quality control check using the Qubit® 2.0 Fluorometer was performed after the amplification to determine the percent template. The Ion Sphere™ Quality Control assay on the Qubit® 2.0 Fluorometer labels the Ion Sphere™ Particles (ISPs) 200 with two different fluorophores: Alexa Fluor® 488 (AF488) and Alexa Fluor® 647 (AF647). The ratio of Alexa Fluor® 647 fluorescence (template ISPs) to the Alexa Fluor® 488 fluorescence (all ISPs present) yields the % template. Once the template reached 10 percent or above, an enrichment was performed using Ion OneTouch™ ES.

Sequencing DNA.—The Ion Torrent Personal Genome Machine® (PGM™) was used to sequence libraries of genomic DNA derived from *L. devia.* The Ion Torrent PGM™ Sequencing 200 Kit v2 was used along with the Ion 316™ Chip following the manufacturers protocol. The Ion Torrent PGM™ protocol formats the sequence in FASTQ format, which is a text-based format storing both a nucleotide sequence and its quality scores. The FASTQ format was converted to FASTA format using an online open-source program called Galaxy®. Galaxy® groomed the FASTQ sequence, filtered FASTQ reads by quality score and length, groomed FASTQ again, and finally converted FASTQ to FASTA. The quality scores and information

were not required for future use. Then, the DNA sequence in FASTA format was input into MSATCOMMANDER®. The program MSATCOMMANDER® was used to identify and design primers for di-, tri-, and tetra- microsatellite repeats from these shotgun sequences.

Primers and primer design.—MSATCOMMANDER® identified each primer and its motifs. The nucleotide fragments generated by these primers were between 100-200 base pairs. Primers were selected and ordered from Integrated DNA Technologies®. Once primers were received, they were resuspended by adding the desired amount of .1 TE Buffer for 100µM. Each primer required a different amount of .1 TE Buffer. Primers were vortexed and refrigerated for 24 hours. The following day, they were stored in the freezer (-20˚C) and aliquots for each primer were made. Aliquots were made up of 5µg of each primer (forward and reverse) being diluted in 95µg of .1 TE Buffer. With 17 primers, a total of 34 aliquots were made.

PCR optimization.—Microsatellite loci were isolated and scored using a polymerase chain reaction (PCR); conditions were optimized for each primer to amplify specific sequences and develop a suite of novel molecular markers for *L. devia* species. PrimeSTAR® GXL DNA Polymerase (Takara) was used for amplification. The optimization of primers varied by adjusting annealing time, annealing temperature, primer concentration and number of cycles. A master mix was created for all primers that consisted of 135.5µL of nuclease-free water, 45µL of 5xPS GXL Buffer, 20µL of dNTPs, 5-6µL each of forward and reverse primer, and 4.5µL of PrimeSTAR® GXL Taq polymerase (Takara) yielding about 217µL. The master mix was then divided into eight different reactions; 24µL into each reaction tube with 1.0µL of an individuals DNA added into each. The extra 24µL was placed into a reaction tube with 1µL of nuclease-free water to act as a negative control. Once vortexed and spun down, the PCR reaction was ready to be placed

into the Bio-Rad® Thermal Cycler. The optimal temperatures and times for each primer varied in accordance to their melting temperatures.

Gel electrophoresis.—For gel electrophoresis, 1% agarose was coated with ethidium bromide to detect the presence of amplified DNA using a Bio-Rad® GelDoc transilluminator. 0.5g of agarose and 50mL of 1x TAE buffer were combined in a flask and heated for 1.5 minutes to ensure the agarose dissolved completely and uniformly for the DNA to pass through. The flask was left to cool for about 5 minutes and 2µL of ethidium bromide was added. It was then poured into a gel tray and a comb was inserted to form the wells. The gel was left to harden for about 15 minutes. Samples were then prepped to load into the gel wells. 6µL of each PCR product was then mixed with 3µL of loading dye. Loading dye was made up of 5mL of 50% glycerol, 2.9mL of nuclease-free water, 1mL of 0.5M EDTA, 1mL of 2% orange G, and 0.1mL of 10% SDS. Orange G loading dye facilitated the viewing of the PCR products during electrophoresis. The ladder was made up of 280µL of nuclease-free water, 80µL of loading dye, and 40µL of size standard 1 kb ladder. The ladder was used as a comparison to determine the size and quantity of the tested DNA. After the gel hardened, it was placed into the gel box filled with 1 x TAE buffer. 4µL of ladder was placed into the first well. The PCR product with the loading dye (totaling 9µL) was pipetted into each gel well (wells 2-10), then left to run for 30 minutes at 100 volts and 500mA. The gel was visualized and analyzed under the Bio-Rad® GelDoc transillumilator, and images were saved on the computer.

Microcapillary electrophoresis.—The Experion™ Automated Electrophoresis was used for DNA separation and analysis. A green microfluidic chip was used following the DNA 1K analysis kit protocol. A gel stain was created and used to prime the chips. Chips were loaded with loading buffer, Experion™ ladder, and PCR samples. One chip analyzed up to 11 individual

samples. Chips were used to further quantify DNA and identify alleles for each primer that displayed a clean amplification. The software subtracted background noise, identified and integrated peaks, and assigned base pair sizes and concentration.

RESULTS

Quality control assay.—The Qubit® 2.0 fluorometer measured all ISPs present with the AF488 assay and measured the template ISPs with the AF647 assay (Table 2). It took 5 trials of amplification using the emulsion PCR to generate a percent template over 10, which is recommended for sequencing.

Table 2. Quality control check for *Loxosceles devia.*

	AF488	AF647	% Template
Blank	31.9	8.0	-
LD-98-E	536.6	186.4	12.82

Ion Torrent Personal Genome Machine® sequencing run.—The Ion Torrent PGM™ generated 134.8 million base pairs of *de novo* genomic sequences for a *Loxosceles* species with 1 sequencing run. It filtered polyclonal, primer dimers, and low quality to give a final library read. Of those 134.8 Mbp generated, 92.81 million base pairs were of high quality (Q20). There was a total of 1,512,703 reads and the longest read was 297 base pairs with a mean length of 89 base pairs. There was a 47% ISP loading density (Fig. 6), 98% enrichment, 65% clonal ISPs with 35% polyclonal ISPs, and 80% final library read. They key signal which is the percentage of LiveISPs with a key signal identical to the library key signal was 60. The red-orange colors in Fig. 6 display the ISPs loaded into the wells portraying a high loading density as the blue represents poor loading density. Only one ISP can fit into each well, however some wells remain empty resulting to poor loading density.

Primers.—The MSATCOMMANDER® program looked over the 1.5 million sequence reads generated from the Ion Torrent PGM™ and determined how many had some form of microsatellite region. Ion Torrent PGM™ identified a total of 665 microsatellite regions for *L. devia*. Of the 665 microsatellite regions identified, 578 were dinucleotide, 84 were trinucleotide, and 64 were tetranucleotide (Fig. 7). There were no penta-, or hexanucleotide repeats present for *L. devia*. The most common dinucleotide motifs were AC and AG, which are highly common in most species. ATC was the most common trinucleotide having 77 recurrences (Fig. 8). AAT was the second most common trinucleotide motif for this species.

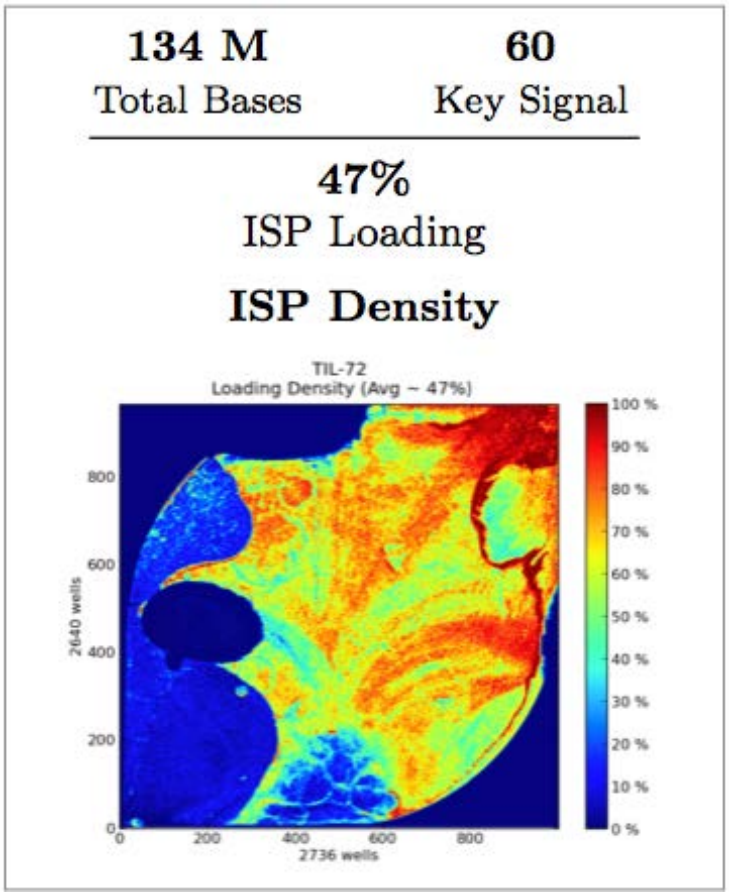

Figure 6. Summary of sequencing run for *L. devia*. The red-orange color shows a higher percent loading density.

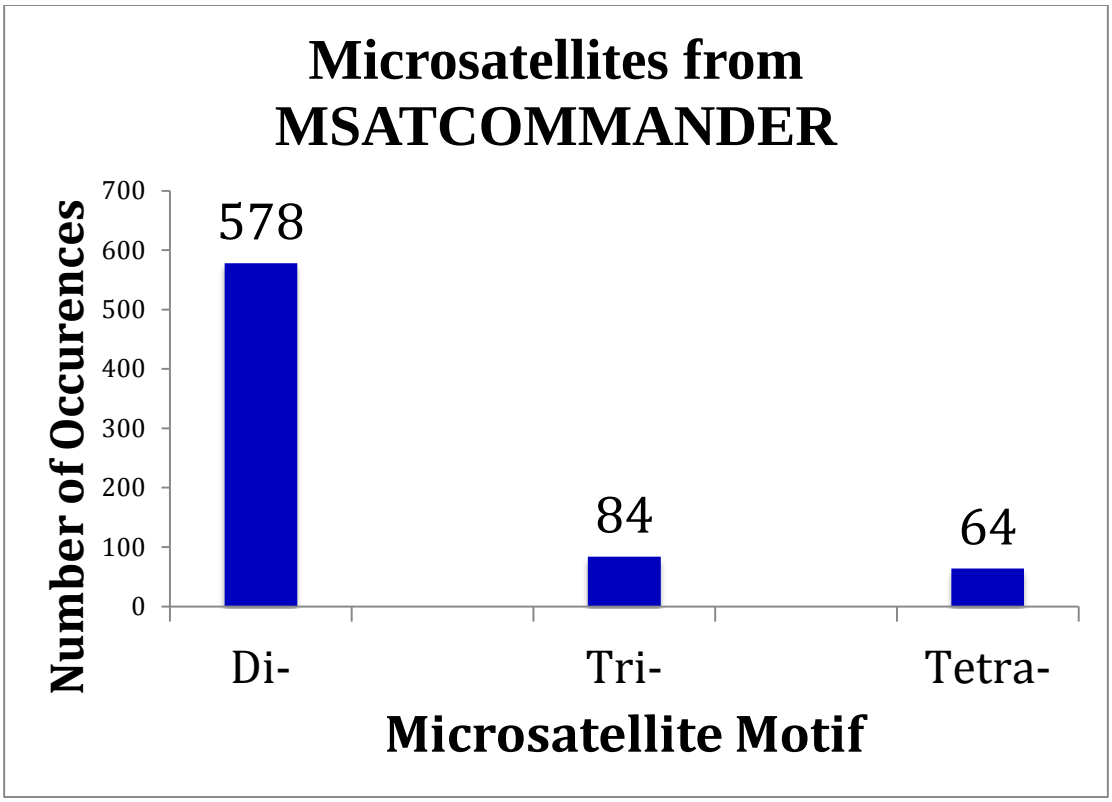

Figure 7. Occurrence of repeats. Dinucleotide motifs were most common.

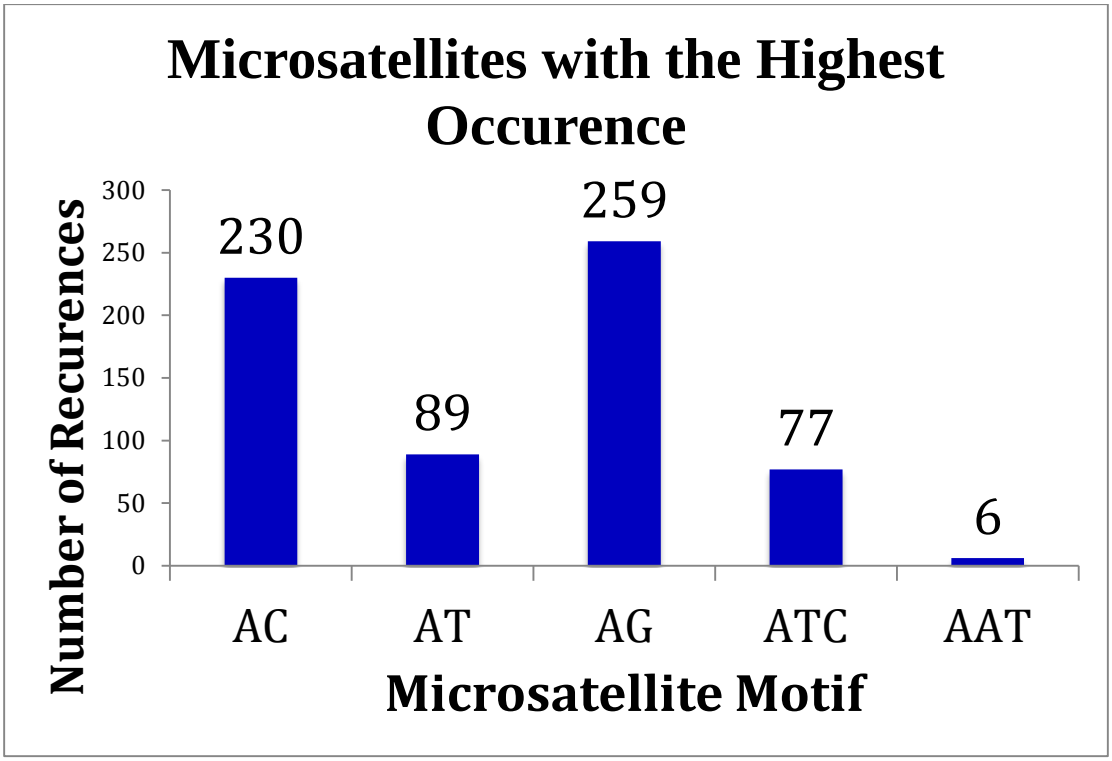

Figure 8. Motifs with the highest occurrence. AG and AC were the most common dinucleotide motifs. ATC was the most common trinucleotide.

MSATCOMMANDER® generated primers (upstream and downstream) of each repeat (Table 3). The program generated broad melting temperatures for each primer to aid in PCR optimization. It isolated the motifs for each primer, along with the pair product size. Of the 665 microsatellite regions identified, primers for 17 novel molecular markers were designed and ordered from Integrated DNA Technologies® (IDT™). IDT™ also generated melting temperatures for the primers, which were more specific. The IDT™ melting temperature was used to set up PCR's.

Table 3. Microsatellite primers designed by MSATCOMMANDER™.

Primer Name	Motif	Forward Sequence	FTp (˚C)	Reverse Sequence	RTp (˚C)
Lox001	AC	CCACCTTTATACCGGCGAAG	55.6	TTCATGCGAAACAACCTGCC	56.5
Lox002	AC	TGAGCCCAGGTTAGCAAGAC	57.1	GCTAATTGGCGCTTCTCTGG	56.4
Lox003	AC	ACTGTTAAGATCATGCTGTGCC	55.4	ACTGTGTCGAGGAGTCCAAG	56.4
Lox004	AC	TCTATTTCTTTCGTTGGTGCCG	55.5	GTCAACGCGTCTACTGTGTG	56.0
Lox005	AC	TCCTCACTCTGGTTCTTCGC	56.7	TCCTTTGCTTCCCTTTATCACG	55.1
Lox006	AC	ACACAACACTCGGAATCTTCG	55.1	CAAGTGCTCAAAGTGGCGAC	56.9
Lox007	AC	AAGAGTTGCCGAATTCCTGG	55.3	GTCGTTTGATGCTCGGTCTG	56.1
Lox008	AC	TTTCCAGCAGTACACCGCC	57.9	AGTGATACTGGAGACCGCAC	56.5
Lox009	AC	GTATACCAACCCTGCTCTCG	56.0	CAACTGTGATGCTTCCTTCATG	54.2
Lox010	AC	AATTGAAACCGAAGTCATAGCG	53.5	TTGGCATTTGTCAGTACGGC	56.0
Lox011	AC	GTCCGTGGTGTTTGATCTC	56.5	GACCCGGTCGTTTAAACAGG	55.9
Lox012	AC	TAAACCAGCCCAGAAGACCG	57.1	TGGCTGTCTGGTGATCGTG	57.3
Lox013	AC	CACCCATTACATCAGTAGGAGC	55.0	GCTCTCGTCTCGTGTGAAATG	55.8
Lox014	ATC	CGACCGAGTACATTGCAGG	55.7	CCTGCAGTGGATTGATAAAGGC	56.2
Lox015	ATC	ATTTCTCTTCACCTTGGTTTGC	53.8	CTGCAGTGGATTGATAGGGC	55.7
Lox016	ATC	GGCCTACATCCTGCAGTGG	58.2	AGATGTGGATTTGACAACGGC	55.8
Lox017	ATC	GGCCTACATCCTGCAGTGG	58.2	AACCAGTTCCACGTCCCAG	57.4

PCR optimization and gel electrophoresis.—Optimization for each primer varied in accordance to its annealing temperature. Adjusting annealing temperature, elongation time, and/ or primer concentration optimized PCR conditions for each primer. The elongation time

fluctuated between 0:30-0:55 seconds (Table 4). The annealing temperatures for the PCR cycles were 0.5-0.8˚C lower than the primers lowest melting temperature. Cycles varied between 35 and 37, as reactions with 35 cycles resulted in fragments that were more defined.

Table 4. Optimization protocol for PCR thermal cycler.

Primer	Denaturation temp. (°C)	Denaturation time	Annealing temp. (°C)	Annealing time	Elongation temp. (°C)	Elongation time	Amplification cycles
Lox001	95.0	0:45	55.0	0:45	72.0	0:55	35
Lox002	95.0	0:45	55.6	0.45	72.0	0:55	35
Lox003	95.0	0:45	54.9	0:45	72.0	0:55	35
Lox004	95.0	0:45	55.0	0:45	72.0	0:55	35
Lox005	95.0	0:45	54.5	0:45	72.0	0:55	35
Lox006	95.0	0:45	54.3	0:45	72.0	0:30	35
Lox007	95.0	0:45	54.7	0:45	72.0	0:55	35
Lox008	95.0	0:45	56.1	0:45	72.0	0:30	36
Lox009	95.0	0:45	53.7	0:45	72.0	0:30	35
Lox010	95.0	0:45	53.0	0:45	72.0	0:55	35
Lox011	95.0	0:45	55.4	0:45	72.0	0:55	35
Lox012	95.0	0:45	56.6	0:45	72.0	0:30	35
Lox013	95.0	0:45	54.5	0:45	72.0	0:55	35
Lox014	95.0	0:45	55.2	0:45	72.0	0:55	35
Lox015	95.0	0:45	53.3	0:45	72.0	0:30	37
Lox016	95.0	0:45	55.3	0:45	72.0	0:55	35
Lox017	95.0	0:45	56.9	0:45	72.0	0:55	35

Gel electrophoresis was used to separate DNA according to molecular size. An electric current was applied across the gel resulting with a positive charge on one end and a negative charge at the other. The current allows molecules to migrate across the gel. DNA is negatively charged, which means it will be pulled toward the positively charged end of the gel. Smaller fragments migrate through the gel more quickly and travel further than larger fragments. The ethidium bromide enables the DNA on the gel to be seen under the transilluminator. In the images obtained of the agarose gels by the Bio-Rad® GelDoc transilluminator, the amplified PCR product for each primer measured approximately 100-200 base pairs (Figs. 9-12). The ladder (on the far right) served as a guide to estimate and determine the size of the unknown PCR fragments being tested.

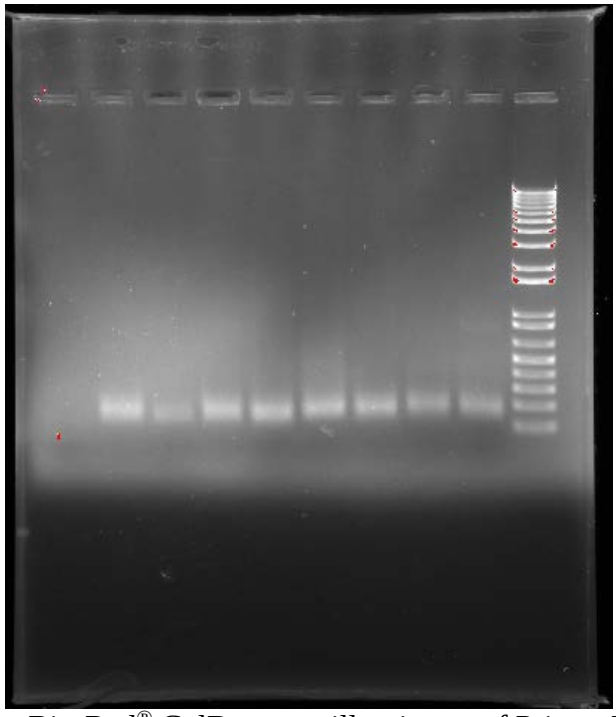

Figure 9. Gel image from Bio-Rad® GelDoc transilluminator of Primer Lox001. Different individual samples in lanes 2-9 and DNA ladder in lane 10. All individuals show a positive and clean look.

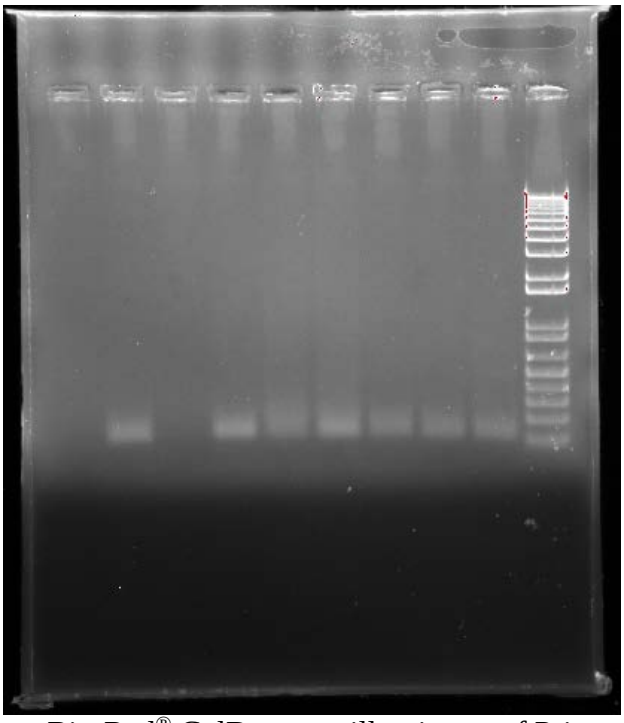

Figure 10. Gel image from Bio-Rad® GelDoc transilluminator of Primer Lox003. Different individual samples in lanes 2-9 and DNA ladder in lane 10. Bands shown are clean and amplified. Lane 3 shows no amplification for that individual.

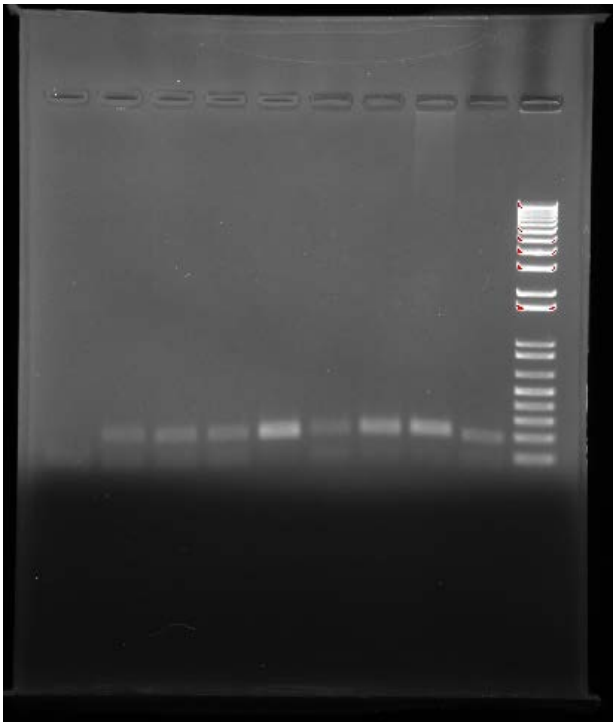

Figure 11. Gel image from Bio-Rad® GelDoc transilluminator of Primer Lox0013. A negative control in lane 1, different individual samples in lanes 2-9, and DNA ladder in lane 10. Primer dimers appear to be present at the top of each amplified band.

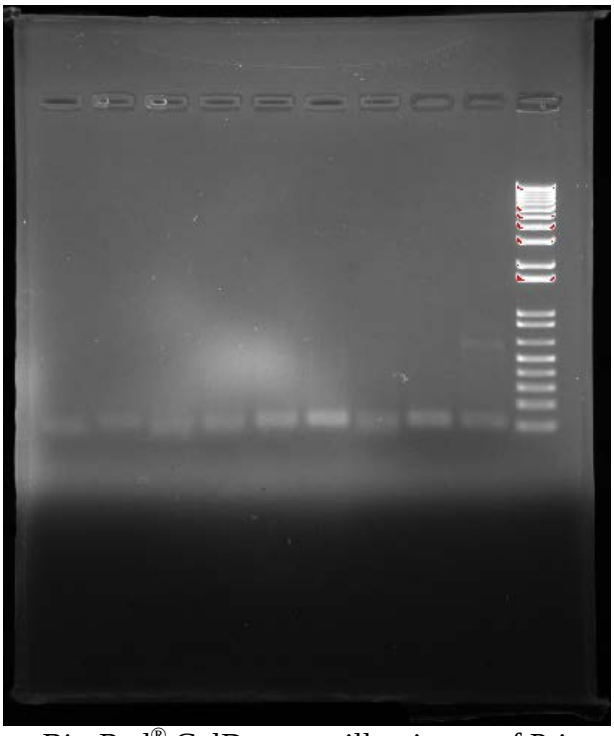

Figure 12. Gel image from Bio-Rad® GelDoc transilluminator of Primer Lox0014. A negative control in lane 1, different individual samples in lanes 2-9, and DNA ladder in lane 10.

Experion™ automated electrophoresis.—Microcapillary electrophoresis was performed on all primers that showed positive and clean bands during gel electrophoresis. It identified several alleles for each tested primer pair, suggesting healthy allelic diversity for local populations of *L. devia.* The Experion™ software displays data analysis in three different forms, an electropherogram, a virtual gel, and a results table. The software integrates peaks and assigns their sizes and concentrations. The microcapillary machine generated a virtual gel report with an electropherogram for each well that was loaded with sample (Figs. 13 & 14). Each lane of the virtual gel corresponds to a different sample, and all samples from a chip are shown in a single gel view. The Experion™ software converted electropherogram data into bands, which appear in the virtual gel.

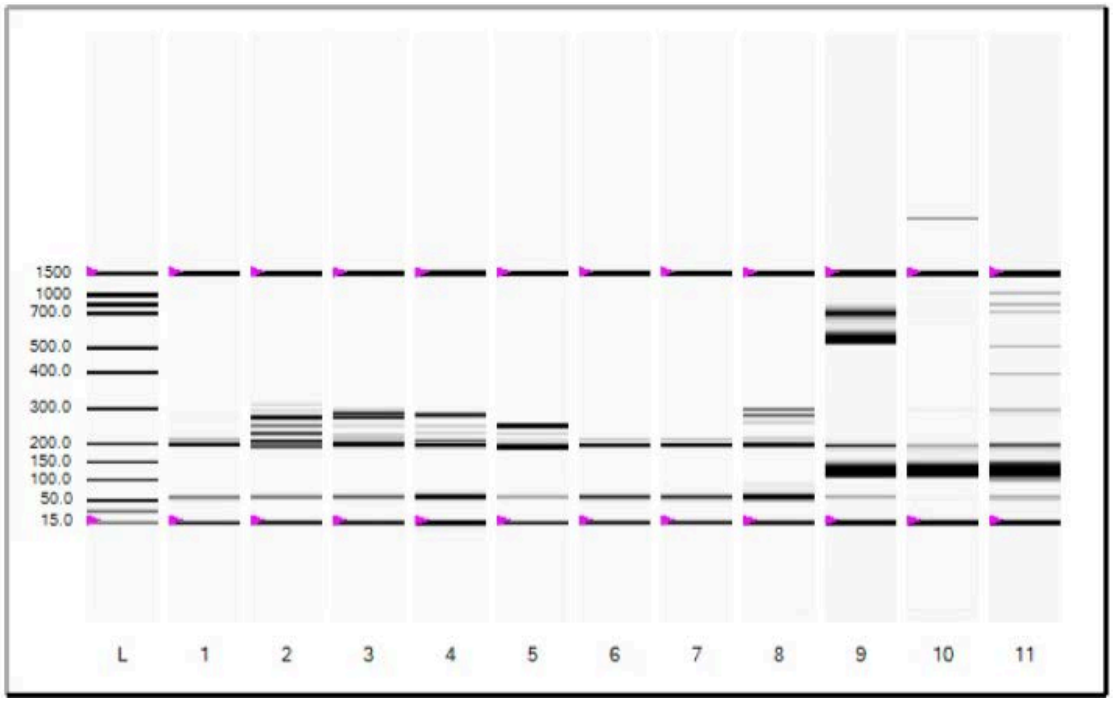

Figure 13. Virtual Gel Report of Lox013. Ladder is in L lane, individual PCR samples of Lox013 were loaded into lanes 1-11. Product size is between 100-200bp.

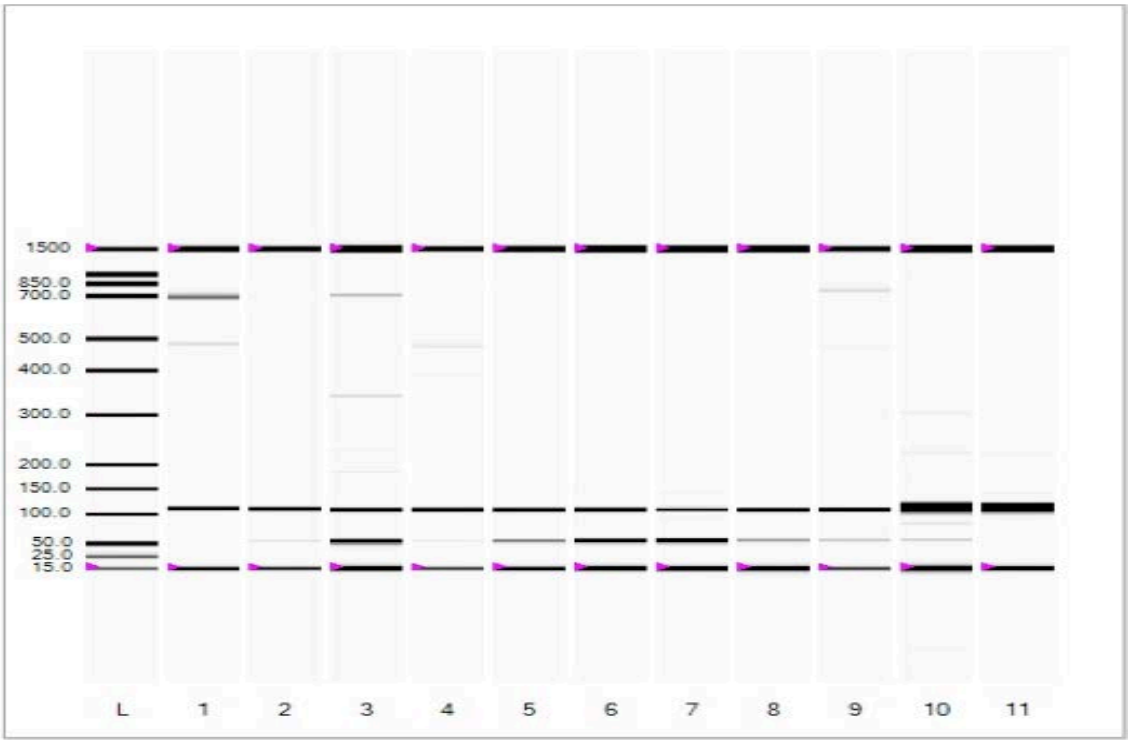

Figure 14. Virtual Gel Report of Lox014 and Lox003. Ladder is in L lane, Lox014 samples are in lanes 1-8 and Lox003 samples are in lanes 9-11. Product size is between 100-150bp.

DNA fragments could be separated, sized, quantified, and analyzed using the Experion™ system. The electropherogram (Egram) aids in distinguishing alleles and determining if they are homozygote or heterozygote for that gene. In population genetics, homozygotes have two identical alleles (one from the mother and one from the father) present at the same locus. Heterozygotes have two different alleles (one from each parent) at a locus. Homozygotes show only one peak on the Egram; while heterozygotes have two peaks (Figs. 15 & 16). Alleles ranged from 103-253 base pairs for each individual. The Experion™ software detected and labeled peaks with an upper and lower marker used for normalization. All peaks identified by Experion™ software are numbered, and the numbers appear under the peaks. The peak number, calculated size, concentration, and percentage of total DNA are identified with the software program.

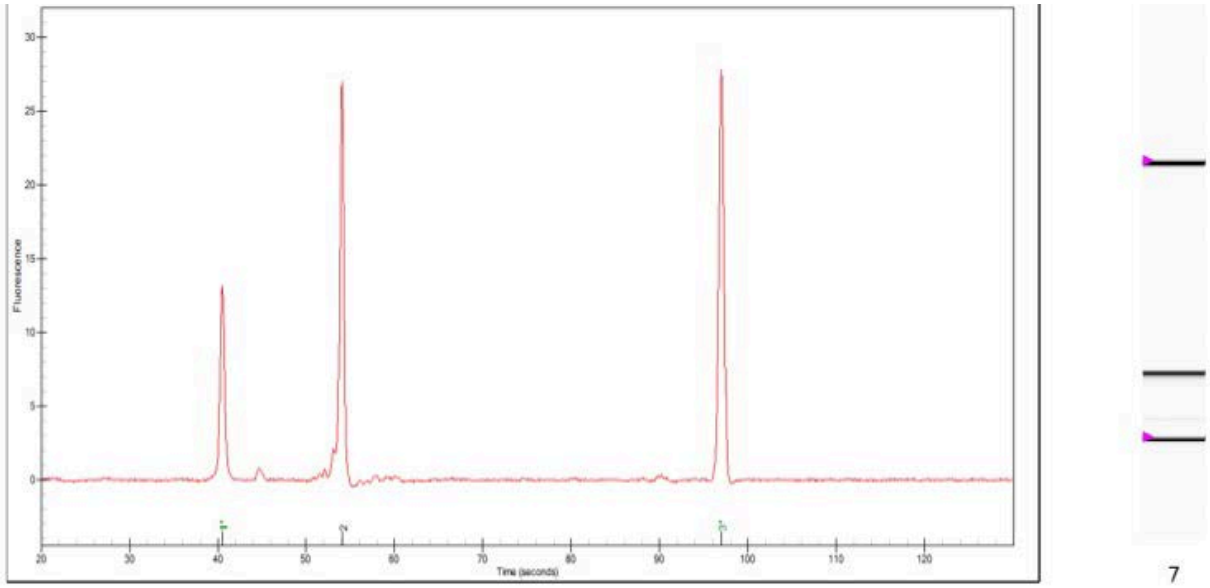

Figure 15. Electropherogram of the total fluorescence displayed by an individual using primer Lox001 displaying homozygosity. The single middle peak represents that the individual is homozygous. The first and third peaks are standard markers from the ladder.

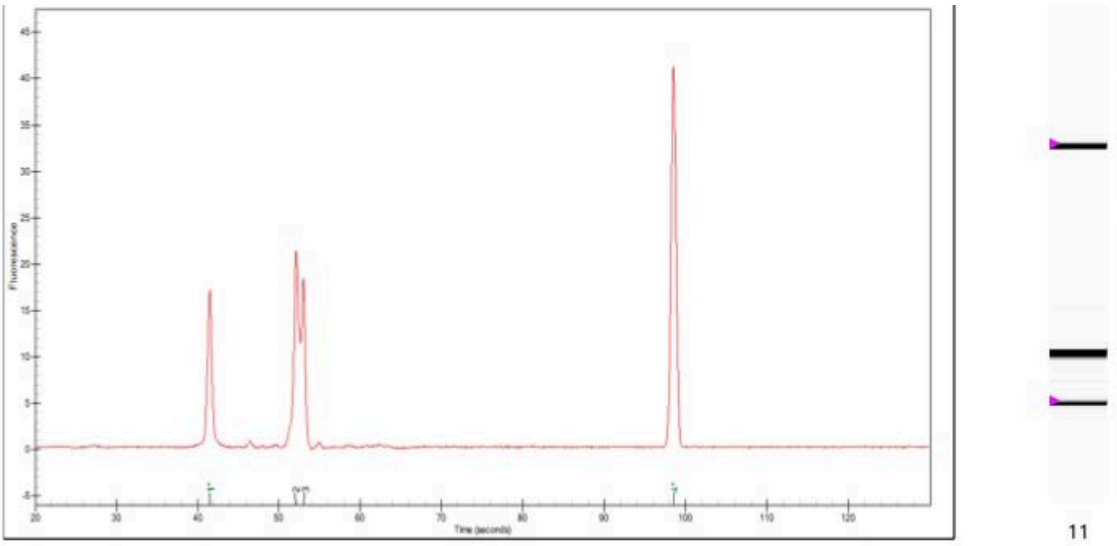

Figure 16. Electropherogram of the total fluorescence displayed by an individual using primer Lox003 displaying heterozygosity. The middle peak has two peaks representing that the individual is heterozygous. The lowest and highest peaks are standard markers from the ladder.

Each microsatellite marker tested resulted in 2-5 alleles being found. Alleles ranged from 103- 253 base pairs. There were 3-11 individuals tested for each primer pair. Heterozygote individuals were divided by homozygotes for each marker to determine observed heterozygotes, H_O (Table 5). Two markers Lox005 and Lox014 showed only homozygotes, thus far 2 alleles were found from testing only 9 individuals. There were no heterozygotes amplified during microcapillary electrophoresis for those 2 markers. The most alleles found were with marker Lox013, with a total of 5 alleles. Obtaining a larger sample size would alter the observed heterozygotes percentage for these markers.

Lox001 had a total of 3 alleles ranging from 118-152; their allelic frequencies were $p_{(147\text{-}149)}$ = .85, $q_{(152)}$ =.1, and $r_{(118)}$ =.05. For Lox002 allelic frequencies were $p_{(109\text{-}111)}$ = .45, $q_{(103\text{-}105)}$ =.27, $r_{(122\text{-}123)}$ =.18, and $s_{(106\text{-}108)}$ = .09. Lox003 had a total of 2 alleles ranging from 111-120; allele frequencies were $p_{(111)}$ = .5 and $q_{(119\text{-}120)}$ =.5. For Lox004 allelic frequencies were $p_{(112\text{-}114)}$ = .6, $q_{(106)}$ =.2, $r_{(109)}$ =.1, and $s_{(115)}$ = .1. Lox005 had a total of 2 alleles ranging from 114-117;

allelic frequencies were $p_{(114\text{-}116)}$ = .67 and $q_{(117)}$ =.33. For Lox006 allelic frequencies were $p_{(122\text{-}123)}$ = .83 and $q_{(132)}$ =.17. Lox007 had frequencies of $p_{(114\text{-}115)}$ = .5, $q_{(119)}$ =.33, and $r_{(131)}$ =.17. For Lox009 allelic frequencies were $p_{(133\text{-}135)}$ = .67, $q_{(136\text{-}138)}$ =.25, and $r_{(144)}$ =.08. Lox011 had a total of 2 alleles ranging from 165-175; allelic frequencies were $p_{(165\text{-}167)}$ = .93 and $q_{(175)}$ =.07. For Lox013 allelic frequencies were $p_{(196\text{-}198)}$ = .5, $q_{(199)}$ =.25, $r_{(209)}$ =.125, $s_{(253)}$ = .0625, and $t_{(189)}$ = .0625. Lastly, Lox014 had allelic frequencies of $p_{(109\text{-}110)}$ = .78 and $q_{(111\text{-}112)}$ =.22. Allele frequencies were used to determine expected heterozygotes, H_E for each marker.

Table 5. Characteristics of markers that were tested using Experion™ electrophoresis for *L. devia*. Markers, total # of alleles, allele range (bp), observed heterozygotes (H_O), expected heterozygotes (H_E), and the total # of individuals tested were reported.

Markers	Total # of Alleles	Allele Range (bp)	H_O	H_E	Total # of individuals tested
Lox001	3	118-152	.10	.335	10
Lox002	4	103-123	.18	.6642	11
Lox003	2	111-120	.33	.5	6
Lox004	4	106-115	.20	.58	4
Lox005	2	114-117	0	.4422	6
Lox006	2	122-132	.33	.2822	3
Lox007	3	114-131	.33	.6122	3
Lox009	3	133-144	.17	.4822	6
Lox011	2	165-175	.14	.1302	7
Lox013	5	189-253	.11	.6641	9
Lox014	2	109-112	0	.3432	9

DISCUSSION

This is the first successful attempt to develop and test microsatellite markers for *L. devia*. Currently, there are no sequences for this venomous species in NCBI, suggesting that this may be the first study to examine the DNA of *L. devia*. The development of these markers will, for the first time enable us to examine diversity and understand the level of genetic diversity for every population of this species. We can look at gene flow and how these spiders disperse from location to location. We will be able to determine if they are sharing genes or if they're isolated subpopulations each of which would then potentially be protected or treated differently. We have developed a resource that anyone can now use to answer genetic and population level questions. This work provides a methodology that any researcher can use for designing microsatellites.

Whereas microsatellites used to be in the domain of big model organisms only, these markers can now be used by the spider biologist to answer evolutionary, population structure, and biogeography questions. These molecular markers will expand our resources available to understand this genus. Microsatellite markers reveal differences between genotypes by the presence of alleles at target loci. Since microsatellites can be found throughout the genome including noncoding regions, selection does not affect them and they can still evolve. Microsatellites have an advantage over other types of markers because they can detect heterozygosity and they have a high number of alleles per locus making polymorphism identifiable. Next generation sequencing technology has made it easy to identify microsatellites and fully optimize them.

The Ion Torrent PGM™ generated nearly 135 million base pairs of *de novo* genomic sequence for *Loxosceles devia*. Toth (2000) states AC is the most frequently found dinucleotide repeat and AAT is the most abundant trinucleotide repeat in arthropods. However, we found that

AG is the most frequently found dinucleotide along with AC and ATC was the most common trinucleotide found in *L. devia*. The second most common trinucleotide was AAT. This was interesting as the most common di- and tri- nucleotides of arthropods were the second most common for *L. devia.* Of the 665 microsatellite regions identified, primers were designed for 17 novel molecular markers. Primers were selected based on which markers produced product at different sizes. Of those 17 primers chosen, 13 of them amplified an AC motif region. The reason for choosing AC was because they evolve at a fast rate. When studying population level differences of the species, fast evolving motifs is what creates polymorphism and new alleles. Trinucleotide repeats evolve at a slower rate, which is why only 4 trinucleotide repeats were selected to be amplified. Thirteen primers have been amplified to their respective region, and seven have exhibited heterozygosity. The marker Lox010 showed no amplification and was disregarded. Multiple adjustments such as decreasing primer concentration and increasing annealing temperature were made and a band was never present. Microcapillary electrophoresis has been able to identify several alleles for each tested primer. However, after multiple runs on the machine, it shows that it's not consistent to a single base pair or even two base pairs. Alleles were not identified for markers Lox012, Lox016, or Lox017 since they did not show clean amplification during gel electrophoresis. Markers Lox008 and Lox015 have been amplified and are pending optimization by the microcapillary electrophoresis. The individuals tested were of the same species, but not from one local population, which may have affected our observed heterozygosity. The number of individuals tested (sample size) was not consistent and could possibly have had an affect on heterozygosity and the number of alleles found. A few possible reasons for not observing heterozygotes in Markers Lox05 and Lox014 include not having a large enough sample size, presence of over-dominant selection, or presence of null alleles

leading to false observation (Csencsics et al. 2010). The sample size for those 2 markers ranged from 3-9 individuals tested and 2 alleles were found for each. With a larger sample size, more alleles could be observed along with heterozygotes.

Planas et al. (2014) reported *L. rufescens* as the only *Loxosceles* species in which microsatellite markers have been developed. Both species used next generation technology; Ion Torrent Personal Genome Machine® was used for this study of *L. devia* and Roche 454 Sequencing™ was used for *L. rufescens.* Only 1 specimen of *L. devia* was used for sequencing and a total of 1,512,703 reads were generated. A total of 384,166 reads were generated using 3 *Loxosceles* specimens for the *L. rufescens* sequencing run. Allele base pair sizes varied for *L. devia* from 100-255. For *L. rufescens,* alleles varied from 89-279 base pair size. The microsatellite markers developed for *L. devia* ranged within the same allele base pair sizes as the markers developed for *L. rufescens.* Seventeen microsatellite markers were tested for *L. devia* while fifty-eight were tested for *L. rufescens*. Of those 17 primers, 13 primers have been successful for *L. devia,* while 18 primers were successful for *L. rufescens* from the 58. However, mainly dinucleotide motifs and a few trinucleotides were amplified for *L. devia*. *Loxosceles rufescens* amplified tri- and tetranucleotide motifs; there were only three dinucleotide motifs that were amplified. The reason the *L. rufescens* markers were larger than *L. devia* was because Roche 454 Sequencer™ obtains larger fragment sizes. Dinucleotides were frequently found within the *L. rufescens* loci, but most of them did not have a successful PCR amplification and were disregarded. Of the 17 primers used for PCR amplification for *L. devia,* 13 of them were successfully amplified as they resulted in clean and clear bands. PCR conditions were altered several times to optimize primers. Only 1 primer (Lox010) was disregarded after several failed PCR runs. Planas et al. (2014) used a different approach as they tested a broad amount of primers

and only had a small success rate for *L. rufescens.* The success rate of *L. devia* over *L. rufescens* points to improved quality of the Ion Torrent PGM™ over the Roche 454 Sequencer™. If this trend continues for Ion Torrent PGM™ sequencing it may suggest that this platform may be better for developing microsatellite markers for other non-model species.

Microsatellites were also developed for an endangered plant, *Typha minima* using next generation sequencing technology (Csencsics 2010). The use of next generation sequencing technology has become a useful tool for population genetics in non-model species. Next generation sequencing is a fast and cost-effective method for developing microsatellite markers. The Roche 454 Sequencer™ was also used for this study as trinucleotide repeats were the most common repeats found. Thirty potential markers were tested and 17 microsatellite markers were successfully developed for *T. minima,* testing 20 individuals for each marker. Of the 17 markers successfully developed, 14 of the markers optimized were trinucleotide motifs. It was observed that a few markers deviated from Hardy-Weinberg Equilibrium possibly because samples were pooled from different regions for analysis.

For future research, using fluorescently labeled primers to score allele sizes would help in allele identification. Also, having a larger sample size would allow us to verify variability and heterozygosity for each primer. More comprehensive analyses at the population level are needed to provide reliable estimates of marker viability. Lastly, testing these 17 microsatellite markers with a different *Loxosceles* species to determine if they're utility for this species. By using shotgun sequencing of a genomic sequence, robust microsatellite markers can be identified and assess population genetics for this species for the first time.

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