

Amplification and sequencing of transcript encoding the shaker homologue in *Ixodes Scapularis*

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Abstract

In light of the efforts to sequence the tick genome, here is presented the results of an effort to contribute to this project by determining partial sequence for the tick (*Ixodes scapularis*) shaker-like homologue mRNA transcript. The shaker gene, as discovered in *Drosophila melanogaster* is conserved throughout the plant and animal kingdoms. *Ixodes scapularis* is closely related to *Drosophila melanogaster*, *Apis mellifera*, and *Anopheles gambiae* and primers to the *Ixodes scapularis* were designed based on the most conserved regions in the mRNA transcripts of the three aforementioned species. These primers were used to amplify these regions from tick and *Drosophila* DNA and these PCR products were sequenced.

Introduction

The shaker gene, or voltage gated potassium channel gene in mammals, represents a highly conserved ancient protein family discovered in most organisms and predicted to be present in almost every organism (1). The diversity of these channels is so expansive in humans, *C. elegans*, and *Drosophila* that each organism contains between 30-100 potassium channels (2). The potassium channels are divided into two subsets: voltage gated and resting channels. Voltage gated potassium channels are six pass

transmembrane proteins with remarkable specificity for a single ion that is only activated upon membrane potential fluctuations (3).

The definitive feature of the voltage gated potassium channels is the S4 segment which regulates the electrical signaling capabilities. This membrane residing S4 segment is categorized by a series of basic amino acids at every third position (4,5). With an influx of positive ions into the cell, the positively charged S4 segment shifts from the previously negative interior of the cell to the newly negative exterior forcing the channel to open its selectivity filter to potassium channel entrance(6).

The channels specificity for K^+ ions is determined by a selectivity filter composed of a highly conserved amino acid sequence Gly-Tyr-Gly which guides the dehydrated K^+ ion through the pore along the amino acids' carbonyl backbones (7). The Gly-Tyr-Gly motif preferentially allows for passage of a larger dehydrated K^+ ion over a smaller dehydrated Na^+ ion (6,8).

The prevalence of the voltage gated potassium channels in all organisms, and their high homology allows for reliable detection of homologues from various organisms whose genes have not been sequenced. Here is provided the experimental approach to amplification and sequencing of partial transcripts of *Ixodes scapularis* shaker like homologue. The mRNA sequences for shaker from the three related arthropods *Drosophila melanogaster* (cg12348-RB), *Anopheles gambiae* (ensangg 00000006066), and *Apis mellifera* (ensapmg 00000003378) were aligned and primers were designed to the most highly conserved regions, but wholly specific to *Drosophila melanogaster*. Primers were designed around the S5 segment, specifically to exon 10, and also in regions of exons 3 and 4. Sequence homology was then studied for each region.

Materials and Methods

Ixodes and *Drosophila* collection and storage

Adult ticks were collected and individually stored at -80°C until total genomic DNA purification. *Drosophila* adults were grown from larvae and then stored at -4°C until total genomic DNA purification.

Genomic DNA extraction

Ten adult ticks and 25 mg of *Drosophila melanogaster* adults were first frozen with liquid nitrogen and crushed until the insects were noticeably homogenized and subsequently lysed for 16 hours in the presence of proteinase K and lysis buffer provided by Qiagen. The total genomic DNA was extracted from the fruit fly and tick homogenates using the DNeasy purification kit (Qiagen) according to protocol, except that the DNA was eluted once in 70 µl of room temperature dH₂O. The DNA was quantified by spectrophotometry.

Primers and PCR

The sequence of shaker and shaker homologue mRNA transcripts of *Anopheles gambiae*, *Apis mellifera*, and *Drosophila melanogaster* were aligned via clustal w (1.81) multiple sequence alignment (MacVector). Primers were designed to amplify between 100-150bps. Three pairs of primers were designed to amplify regions of exons 3, 4 and 10 which have the highest homology among the three organisms. Primers were designed to recognize *Drosophila melanogaster* DNA completely because *Drosophila* was used as a positive control for PCR. Characterization of exon 10 was chosen because it contains the site of the S5 segment. Exons 3 and 4 were studied because they are present in every

isoform discovered in the three organisms. The primers that generated products from exon 3, 4 and 10 are presented in table 1.

Table 1.

Primer Sites	Forward primer	Reverse primer
Exon 3	TTGAGCAGTCAAGACGAAGA-31A	TCGCAGAAATCATGATCGTG-32B
Exon 4	AGCGGATTAAGGTTTGAGACA-42F	ATCGCATCGAAGCTCGGT-41R
Exon 10	GTACTCTTCTCATCGGCGGT-101A	CAACGGTGGTCATGGTAAC-102B

PCR was performed with the one step RT-PCR kit (Qiagen), making note to skip the reverse transcription step, and the elongase kit (Invitrogen). For the one step RT-PCR kit, reaction volumes were 15 µl containing 5x buffer, 10 nM dNTP mix, 10 pmol each primer, RT enzyme mix, 4 µl DNA, and dH₂O. Reaction conditions were as follows: 95°C for 15 minutes for 1 cycle, 95°C for 15 seconds, 56°C for 30 seconds, 72°C for 30 seconds for 50 cycles and 72°C for 5 minutes. 6 µl of each reaction was analyzed on a 1.5% agarose gel.

For the elongase kit, reaction volume was 50 µl containing 10 mM dNTP, 10 pmol each primer, 5 µl DNA, dH₂O, 5x buffer A & 5x buffer B (Mg²⁺ concentration ratio 1.7), and elongase enzyme mix. Reaction conditions were as follows: 94°C-30 seconds for 1 cycle, 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 30 seconds for 50 cycles, and finally 68°C for 1 minute. 10 µl of each reaction was analyzed on a 1.5% agarose gel.

PCR product purification

PCR products were purified and eluted in 25 μ l of 70°C dH₂O using the PCR purification kit (Marligen) and the concentrations of DNA were determined via spectrophotometry.

The *Ixodes scapularis* PCR products from exon 4 were gel purified and eluted in 25 μ l of 70°C dH₂O using the rapid gel extraction kit (Marligen).

Sequencing

The PCR purified products were sequenced by the Sanger dideoxy method using an amplicycle kit (Perkin Elmer). The reaction volume for each reaction was 8 μ l total and contained 5 ng of DNA, 10x cycling mix, primer, dH₂O, and 30 μ Ci α ³³P dATP. The reaction conditions were as follows: 94°C for 2 minutes for 1 cycle, 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute for 35 cycles. At the end of the reaction 4 μ l of stop solution was added to each sample, they and were denatured at 94°C for 3 minutes, loaded on a denaturing polyacrylamide gel and run for 1.5 hrs at 85 watts. The gel was dried onto filter paper and exposed to film for 18 hours. The sequences read were aligned using MacVetor (Accelrys) and compared to known sequences using the NCBI blast database.

Results

The primers designed to amplify a region from exon 4 generated the expected 150 bp products from tick and *Drosophila* as well as a 100 bp fragment from the tick using a one step RT-PCR kit (Figure 1).

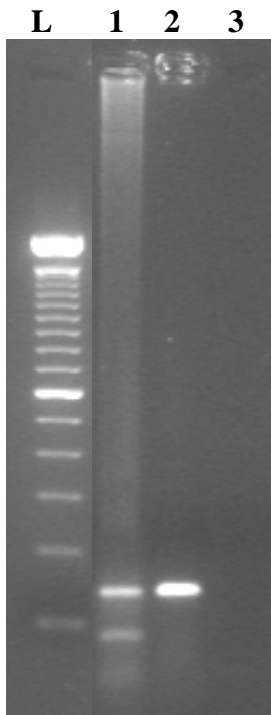


Figure 1. PCR products using primers 42F and 41R. Sizes of PCR products are determined by comparing the size with that of the 100 bp ladder. Lane 1 presents the PCR products generated from the purified Tick genomic DNA. The product at 150 bp is the expected size while the 100 bp products was determined to be nonspecific. Lane 2 presents the product generated from the purified fruit fly genomic DNA, which is of the expected size. Lane 3 represents the non-template control.

The products in figure 1 were purified and sequenced as described in the Materials and Methods. The sequence similarities between the *Drosophila shaker* cDNA and the *Ixodes* transcript were very close with few differences. This is not unexpected as the primers were designed around regions that are highly conserved. A clustal W alignment presented in figure 2 reveals 97% identity in the sequence. The 100 bp PCR product generated from the tick genomic DNA was sequenced and found to have no significant similarities with any known sequences.

Formatted Alignments

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                                10                20                30
IS exon 4  G A G A C A C A A C T A C G T A C G T T A A A T C A A T T C
DM exon 4  G A G A C A C A A C T A C G T A C G T T A A A T C A A T T C
          G A G A C A C A A C T A C G T A C G T T A A A T C A A T T C

                                40                50                60
IS exon 4  C C G G A C A C G C T G C T T G G G G A T C C A G C T C G G
DM exon 4  C C G G A C A C G C T G C T T G G G G A T C C A G C T C G G
          C C G G A C A C G C T G C T T G G G G A T C C A G C T C G G

                                70                80                90
IS exon 4  A G A T T A C G G T A C T T T G A C C C G C T T A G A A A T
DM exon 4  A G A T T A C G G T A C T T T G A C C C G C T T A G A A A T
          A G A T T A C G G T A C T T T G A C C C G C T T A G A A A T

                                100               110               120
IS exon 4  G A A T A T T T T T T T G A C C G T A G T C G A C C G A G C
DM exon 4  G A A T A T T T T T T T G A C C G T A G T C G A C C G A G C
          G A A T A T T T T T T T G A C C G T A G T C G A C C G A G C

                                130               140               150
IS exon 4  T T C G A T G C C G A
DM exon 4  T T C G A T G C
          T T C G A T G C C G A
  
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Figure 2. Clustal w alignment of *Ixodes* and *Drosophila* sequences.

The PCR products generated from regions of exons 3 and 10 are presented in figure 3. Panel A of figure 3 presents amplicons of exon 3 which were generated using the one step RT-PCR kit and panel B presents amplicons from exon 10 generated using the elongase kit.

PANEL A **PANEL B**
IS DM NTC IS DM NTC L

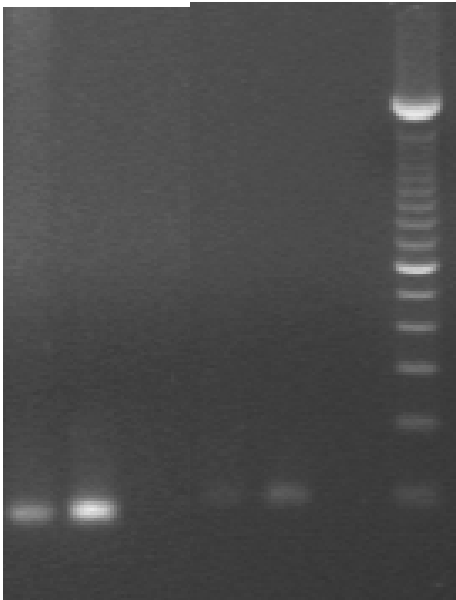


Figure 3. PCR products using primers 101A & 102B and 31A & 32B. Panel A: Exon 3 amplicons for the tick (IS) and *Drosophila* (DM) with a non-template control (NTC) Panel B: exon 10 amplicons from tick (IS) and *Drosophila* (DM) with a non-template control (NTC). The size of both sets of amplicons is ~100 bp. Size comparison was done using a 100 bp ladder (L).

The fragments were purified and sequenced as described in Materials and Methods and sequence comparisons between the tick and the *Drosophila* showed high homology. The alignments presented in figure 4 reveal 100% sequence homology for exon and a 98% sequence homology for exon 10.

Formatted Alignments

	10	20	30
<i>IS exon 3</i>	T T G A G C A G T C A A G A C G A A G A A G G G G G G G C T		
<i>DM exon 3</i>	T T G A G C A G T C A A G A C G A A G A A G G G G G G G C T		
	T T G A G C A G T C A A G A C G A A G A A G G G G G G G C T		
	40	50	60
<i>IS exon 3</i>	G G T C A T G G C T T T G G T G G C G G A C C G C A A C A C		
<i>DM exon 3</i>	G G T C A T G G C T T T G G T G G C G G A C C G C A A C A C		
	G G T C A T G G C T T T G G T G G C G G A C C G C A A C A C		
	70	80	90
<i>IS exon 3</i>	T T T G A A C C C A T T C C T C A C G A T C A T G A T T T C		
<i>DM exon 3</i>	T T T G A A C C C A T T C C T C A C G A T C A T G A T T T C		
	T T T G A A C C C A T T C C T C A C G A T C A T G A T T T C		
	100	110	120
<i>IS exon 3</i>	T G C G A		
<i>DM exon 3</i>	T G C G A		
	T G C G A		

Formatted Alignments

	10	20	30
<i>IS exon 10</i>	C T C T T C T C A T C G G C G G T T T A T T T T G C G G A A		
<i>DM exon 10</i>	C T C T T C T C A T C G G C G G T T T A T T T T G C G G A A		
	C T C T T C T C A T C G G C G G T T T A T T T T G C G G A A		
	40	50	60
<i>IS exon 10</i>	G C T G G A A G C G A A A A T T C C T T C T T C A A G T C C		
<i>DM exon 10</i>	G C T G G A A G C G A A A A T T C C T T C T T C A A G T C C		
	G C T G G A A G C G A A A A T T C C T T C T T C A A G T C C		
	70	80	90
<i>IS exon 10</i>	A T A C C C G A T G C A T T T T G G T G G G C		A G T C G T T
<i>DM exon 10</i>	A T A C C C G A T G C A T T T T G G T G G G C		G G T C G T T
	A T A C C C G A T G C A T T T T G G T G G G C R G T C G T T		
	100	110	120
<i>IS exon 10</i>	A C C T A G A C C A C C		
<i>DM exon 10</i>	A C C A T G A C C A C C		
	A C C W W G A C C A C C		

Figure 4. Clustal W alignments for *Ixodes* and *Drosophila* exonic sequences.

Discussion

The National Institute of Allergy and Infectious Diseases has begun funding the DNA sequencing of the deer tick genome in hopes of understanding the role ticks play in passing pathogens to humans that cause Lyme disease, Rocky Mountain spotted fever and tularemia (9). The research project undertaken provides an easy and efficient means to begin the tick DNA sequencing on a small scale, which can be done in almost any molecular biology laboratory. The sequence homology between organisms for the voltage-gated potassium channels provided an easy passage for primer design. Since the gene was undoubtedly expected to be present in the tick, the project easily provided a partial sequence of the voltage-gated potassium channel.

Although the elongase kit had been used, it had only been used twice within the entire experiment so the conditions had not been optimized for amplifying the tick and fruit fly DNA. The elongase kit had been designed to amplify templates up to 12 kb, so with the correct conditions, large fragments of the shaker homologue as well as other genes could be amplified in the future.

The genomic DNA from the tick and the fruit fly had been amplified using a one-step RT-PCR kit which may appear to be wasteful, however the amplification had been attempted using a PCR kit (Qiagen) but to no avail. Speculation on this matter had led me to believe that the buffer provided in the RT-PCR kit is more suitable to carrying out PCR in the presence of substances that inhibit the reactions in the standard buffers. Future research efforts are likely to lead to more improved methodologies for tick DNA purification and PCR amplification.

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