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Bioinformatic analyses of male and female *Amblyomma americanum* tick expressed serine protease inhibitors (serpins)

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Abstract

Serine protease inhibitors (serpins) are a diverse family of proteins that is conserved across taxa. The diversity of *Amblyomma americanum* serpins (AAS) is far more complex than previously thought as revealed by discovery of 57 and 33 AAS transcripts that are respectively expressed in male and female *A. americanum* ticks, with 30 found in both. While distinct reproductively, both male and female metastriate ticks, such as *A. americanum*, require a blood meal. Thus, 30 AAS sequences found in both male and female ticks could play important role(s) in regulating tick feeding and thus represent attractive candidates for anti-tick vaccine development. Of significant interest, 19 AAS sequences expressed in male and female ticks are also part of the 48 AAS sequences expressed in fed female tick salivary glands or midguts; two organs through which the tick interacts with host blood and immune response factors. Considered the most important domain for serpin function, the reactive center loop (RCL) is further characterized by a single 'P1' site amino acid residue, which is central to determining the protease regulated by the serpin. In this study, a diversity of 17 different P1 site amino acid residues were predicted, suggesting that *A. americanum* serpins potentially regulate a large number of proteolytic pathways. Our data also indicate that some serpins in this study could regulate target protease common to all tick species, in that more than 40% of AAS show 58–97% inter-species amino acid conservation. Of significance, 24% of AAS showed 62–100% inter-species conservation within the functional RCL domain, with 10 RCLs showing ≥90–100% conservation. In vertebrates, serpins with basic residues at the P1 site regulate key host defense pathways, which the tick must evade to feed successfully. Interestingly, we found that AAS sequences with basic or polar uncharged residues at the putative P1 site are more likely to be conserved across tick species. Another notable observation from our data is that AAS sequences found only in female ticks and those found in both males and females, but not those found only in male ticks, were highly conserved in other tick species. While descriptive, this study provides the basis for more in-depth studies exploring the roles of serpins in tick feeding physiology.

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Keywords

Amblyomma americanum; serine protease inhibitors (serpins); tick feeding physiology; orthologous serpins

INTRODUCTION

Ticks are among the most successful ectoparasites of humans and animals. In livestock production, an estimated 80% of the world's cattle are affected by tick-borne diseases (TBD), of which the most important include theileriosis, babesiosis, anaplasmosis, and heartwater (Marcelino et al., 2012). In addition to pathogen transmission, tick feeding has been documented to cause severe direct effects including paralysis, exsanguination, reduced livestock productivity, and damage to skin impacting the economic value of hides (Klompen et al., 1996; Jongejan and Uilenberg, 2004). In public health, ticks have been implicated in the transmission of 17 diseases affecting humans, and the list continues to grow (Day, 2011; Dantas-Torres et al., 2012; Savage et al., 2013).

Amblyomma americanum has emerged among the most important tick species in public health in the United States. *A. americanum* transmits multiple TBD agents, including *Ehrlichia chaffeensis, E. ewingii, Rickettsia amblyommii, Francisella tularensis*, the as yet undescribed causative agent of southern tick associated rash illness (STARI), *Cytauxzoon felis, Theileria cervi*, and the emerging human Heartland virus (Waldrup et al., 1992; James et al., 2001; Childs and Paddock, 2003; Telford and Goethert, 2004; Telford III et al., 2008; de la Fuente et al., 2008; Goddard, 2009; Schulze et al., 2011; Savage et al., 2013). For many years, this tick was mainly distributed in the southeastern United States, but has now been reported as established in 32 states throughout the Southeast, South Central, and Midwest regions as well as along the eastern seaboard as far north as Maine (Springer et al., 2014). Its emerging geographic expansion and role as a vector of many important human disease agents makes *A. americanum* an important consideration in strategies to improve public health.

With a lack of effective vaccines against TBD agents, the prevention of these infections in humans and animals depends on the control of ticks, which is currently acaricide based. However, acaricide use comes with many disadvantages, including the threat of food and environmental contamination and resistance development to these chemicals by ticks. A promising alternative strategy to the chemical control of ticks is to vaccinate hosts against the ticks themselves. The prerequisite to the development of an effective vaccine however, is the identification of effective molecular targets against tick feeding success and/or pathogen transmission. Among the emerging candidates for vaccine target antigens are members of the serine protease inhibitor (serpin) family. In animals, pathways critical to life, such as blood coagulation, complement activation, and inflammation are tightly regulated by serpins (Moore et al., 1993; Gettins, 2002; Tekin et al., 2005; Huntington, 2006; Huntington, 2011; Gatto et al., 2013). Furthermore, dysfunctional serpin activity in humans has been cited to cause numerous diseases including cirrhosis, emphysema, blood coagulation disorders, and dementia (Stein and Carrell, 1995; Davis et al., 1999; Gooptu and Lomas, 2009;

Mocchegiani et al., 2011; Benson and Wilkes, 2012; Bosche et al., 2012; Gatto et al., 2013). The tick feeding style of lacerating host tissue and imbibing host blood which bleeds into the feeding site, is expected to provoke tissue repair and immune response mechanisms such as platelet aggregation, inflammation, blood clotting, and complement pathways (Ribeiro, 1989; Wikel et al., 1994), all of which are serpin-regulated. Thus, to complete feeding, ticks have to overcome serpin-regulated host defense pathways. From this perspective, it is conceivable that ticks may utilize serpins to block these host defenses against tick feeding (Muleng et al., 2001; Mulenga et al., 2002).

Serpin-encoding cDNAs have now been cloned from several tick species (Nene et al., 2002; Sugino et al., 2003; Mulenga et al., 2003a; Mulenga et al., 2003b; Imamura et al., 2005; Ribeiro et al., 2006; Imamura et al., 2006; Prevot et al., 2007; Chalaire et al., 2011; Yu et al., 2013). On the basis of unique putative functional domain reactive center loops (RCLs), at least 45 serpins are expressed in *I. scapularis* (Mulenga et al., 2009). Similarly, Mulenga et al., (Mulenga et al., 2007) and Karim et al., (Karim et al., 2011) have reported at least 17 and 32 different serpin transcripts expressed in *A. americanum* and *A. maculatum*, respectively. Data are now emerging which support the idea that some tick-encoded serpins are functional inhibitors associated with counter defense against anti-tick responses in the host, such as inflammation, complement activation, platelet aggregation, and blood clotting (Imamura et al., 2005; Prevot et al., 2009; Chmelar et al., 2011; Chmelar et al., 2012; Mulenga et al., 2013). In other studies, a significant reduction in feeding efficiency has been observed in ticks which fed on animals immunized with recombinant tick serpins (Imamura et al., 2005; Imamura et al., 2006; Prevot et al., 2007; Imamura et al., 2008; Kaewhom et al., 2009; Jittapalapong et al., 2010) suggesting a prime importance of serpins in tick feeding physiology.

An observation in our lab is that while overall amino acid conservation levels for serpins are around 35–45%, there are some serpins which show much higher conservation across all tick species investigated. We believe that highly conserved serpins could play crucial role(s) in tick physiology. The goal of this study was two-fold: first, to identify serpin transcripts expressed in unfed and fed *A. americanum* ticks, and second, to conduct a global intra- and inter-tick species bioinformatic analysis of *A. americanum* serpins (AAS) and other tick serpins. This study has described 57 and 33 AAS sequences that were respectively found only in male and female ticks, and a further 30 that were found in both. Nearly half of the serpin sequences expressed in *A. americanum* are predicted to regulate pathways important to all tick species, as they show 58–97% amino acid conservation in both metastriate and prostriate ticks. Although this study is descriptive, data presented here provide a foundation for further in depth studies on the roles of serpins in tick physiology.

MATERIALS AND METHODS

Identification and sequence analysis of A. americanum serpin (AAS) transcripts

AAS sequences used in this study were obtained by data mining of *de novo* assembled *A. americanum* transcriptomes (*unpublished data*), serendipitously while attempting to clone other targets, and from GenBank (Mulenga et al., 2007). *A. americanum* transcriptomes were assembled from Illumina sequence reads (BioProject accession number

PRJNA226980) of 24 and 96h fed female phage display cDNA expression libraries, unfed and fed male and unfed and 24h fed female whole ticks, as well as 48, 96 and 120h fed tick dissected salivary gland (SG) and midgut (MG) tissues, using two approaches. In the first approach libraries were individually assembled with source library information for each contig retained, and in the second approach reads from all sources were combined and assembled (unpublished).

Mining and identification of putative AAS sequences was accomplished in two steps. In the first step, assembled contigs were subjected to batch blastx screening against tick sequences in GenBank. In the second step, contig sequences with matches to serpin sequences were manually inspected to confirm the presence of two consensus amino acid motifs: the reactive center loop (RCL) “"p17 [E]-p16 [E/K/R]-p15 [G]-p14 [T/S]-p13 [X]-p12-9 [AGS]-p8-1 [X]-p1' -4'” in the C-terminus, and the ‘NAVYFKG’ motif in the N-terminus (Carrell et al., 1987; Miura et al., 1995; Gettins, 2002). Sequences with a unique RCL sequence were identified as new and assigned an AAS number. Sequences without an RCL region were declared partial in the C-terminus region. For these sequences, two comparisons were made using Bl2seq-blastp (NCBI). First, these sequences were compared to each other to cluster contigs representing the same serpin. Next, we compared these clusters to AAS containing an RCL to eliminate redundancy between these groups. In addition to consensus amino acid motifs and secondary structure, a typical serpin ranges from 350–450 amino acids long (Gettins, 2002). Thus, sequences that had a starting methionine and were at least 350 amino acid residues long were considered putatively full-length. Full-length sequences were subjected to SignalP Version 4 web server to detect signal peptides (Petersen et al., 2011).

Relative AAS transcript abundance

To get insight into relative abundance, Illumina reads were mapped back to assembled contigs using the map reads to reference option in CLC genomics workbench vers. 6.4.2. Relative abundance values were adjusted to account for contig size and total library reads using the following equation: $e_y = (n_y N_x L_{1x}/n_x N_y L_y) \times e_x$, where e_x and e_y represent normalized relative abundance levels of AAS transcript in libraries X and Y, N_x and N_y represent the total number of reads in libraries X and Y, n_x and n_y represent the number of reads related to the specific AAS transcript in libraries X and Y, and L_x and L_y represent the length of the contig related to the specific AAS transcript in libraries X and Y.

Phylogeny and comparative sequence analysis among AAS sequences

To determine relationships between AAS sequences, a guide phylogeny tree was constructed using the neighbor-joining method in MacVector vers 12 DNA analysis software (MacVector Inc., Cary, North Carolina). Sequences were first aligned using T-coffee, then a phylogeny tree out-rooted from human antithrombin (CAA48690), was constructed using the neighbor-joining method set to the default bootstrap setting of 1000 replications and differences adjusted using the absolute # differences setting. Subsequently, AAS sequences that clustered together on the phylogeny tree were subjected to pairwise sequence alignment analyses using MacVector. For partial sequences, amino acid identity levels were determined based on available sequences.

Comparative analyses of AAS to other tick serpin sequences

To investigate relationships among all available tick serpins, AAS and other tick serpins from publically available databases were subjected to phylogeny analysis and multiple sequence alignment analyses using alignment tools at NCBI and MacVector vers 12. This analysis was done at the whole amino acid sequence and RCL levels. At the whole amino acid sequence level, amino acid sequences were subjected to batch pairwise comparisons using Bl2seq-blastp (NCBI) to identify AAS orthologs in other tick species. The serpin RCL is an important functional domain, which determines what protease is regulated by a candidate serpin. Thus, to investigate the relationship of AAS sequences with other tick serpins at the functional level, the neighbor-joining method in MacVector (MacVector Inc.), was used to construct guide phylogeny trees using putative RCLs. To manage the huge dataset, RCLs were first divided into four groups based on charge and polarity characteristics of the amino acid residue at the putative P1 site: polar basic, polar acidic, polar uncharged, and hydrophobic. A separate tree was constructed for each group. Next intra-clade pairwise alignments of RCLs were performed using MacVector to determine identity levels.

RESULTS

Amblyomma americanum male and female ticks express large numbers of serpin transcripts

Data mining of transcriptomes from fed and unfed male and female whole ticks, as well as dissected 48, 96 and 120h female SG and MG transcriptomes, identified 28 and 57 AAS sequences respectively found only in females and males, respectively, and an additional 30 found in both (Table 1, Supplemental Tables 1 and 2). Mulenga et al., (2007) described 17 AAS (here after identified as AAS1–17) sequences that were expressed in 120h fed ticks. Of these 17 AAS sequences, this study found 7 sequences in both males and females, and five AAS sequences in females, while the remaining five were not found at all. Taken together, these studies show the total number of AAS sequences found only in female ticks to be 33 (Table 1, Supplemental Table 1). Please note that AAS 77 and 78 were found only in the combined *A. americanum* transcriptome were source library information for assembled contigs was not retained, and thus source information is unknown (Supplemental Table 1). Overall, 87 and 63 AAS sequences were found in male and female ticks, respectively (Supplemental Table 1 and 2).

Assignment of AAS identification numbers was done arbitrarily in three steps. In the first step, assembled *A. americanum* transcriptomes were subjected to batch blastx scanning against tick serpin sequence entries at NCBI. This analysis identified 388 contigs that encoded putative serpins. A typical serpin is characterized by a unique RCL region (Gettins, 2002). Thus in the second step, we conducted a manual inspection of all 388 contigs and found 61 previously unknown AAS RCL sequences that did not show identity to previously described AAS1–17 (Mulenga et al., 2007). AAS sequences encoding the 61 new RCLs were assigned the identifications of AAS18–78, according to the order in which they were discovered. 233 of the 388 contigs did not have RCL regions. These sequences were subjected to intra-contig comparisons using the bl2seq-blastn function at NCBI. This

analysis identified 44 contig sequences that encoded previously unreported AAS sequences and were designated as AAS79–122 (Table 1). This brought the total number of unique transcripts identified in this study to 105. A typical serpin molecule is 350–450 amino acids long (Gettins, 2002). On this basis, 40 of the 122 AAS sequences were determined to have complete open reading frames (ORF), as well as the consensus serpin amino-terminus motif (NAVYFKG), and the start methionine. Of the 40 AAS ORFs, 23 are predicted to have signal peptides (Table 1).

Majority of female AAS transcripts expressed in fed salivary glands and midgut tissues

Figures 1A–C summarizes the different AAS sequences found in MG and SG of 48, 96 and 120h fed female ticks. Data previously reported for AAS1–17 are indicated with an asterisk in Figure1A, and data for MG and SG at 120h is taken from Mulenga et. al., (2007). Of the 63 AAS sequences found in female tick transcriptomes to date, 48 have been found in 48, 96, and 120h MG and/or SG. Of these 48, 10 and 12 AAS sequences were respectively found only in MG or in SG, while the remaining 26 were found in both (Figure 1A). This translates to a total of 36 and 38 different AAS sequences found in MG and in SG, respectively. Of the 36 AAS found in MG, seven (AAS1, 4, 7, 8, 21, 31, and 109) were found at all-time points, four (AAS27, 28, 72, and 110) were found at both 48 and 96h time points, and four (AAS19, 20, 22 and 43), one (AAS108), and 16 (AAS9–18, 23, 29, 32, 36, 45, and 115) were found at the 48, 96, or 120h time points, respectively (Figure 1B). Likewise, of the 38 AAS found in SG (Figure 1C), five (AAS7, 19, 21, 23, and 110) were found at all-time points, seven (AAS3, 9, 25, 27, 28, 31, and 39) were found at the 96 and 120h time points, and five (AAS20, 36, 116, 117, and 118), five (AAS24, 26, 37, 38 and 40), and 12 (AAS1, 2, 5, 8, 10–17, 41, and 42) were found in 48, 96, or 120h SG, respectively.

Relative AAS transcript abundance

We successfully mapped reads back to *de novo* assembled AAS contigs. This analysis identified 20 of 122 AAS transcripts with variable sequence reads in different libraries (Figure 2A and 2B, Supplemental Table3). For 8 (AAS25, 28, 39, 51, 66, 108, 110, and 119) of the 20 AAS sequences, differences between sequence reads were minimal (Supplemental Table 3) and we concluded that these apparently occurred in equivalent abundance in all libraries. Figure 2A summarizes differential abundance for ten (AAS19, 21, 23, 27, 29, 30, 31, 50, 54, 67, 72, 121) in unfed and fed, male and female whole tick libraries. Of ten AAS detected at unfed and fed time points, five are more abundant in unfed ticks (AAS19, 30, 31, 54, and 67), four are more abundant in fed ticks (AAS21, 29, 50, and 121), and one (AAS72) showed a mixed pattern with abundance in unfed females, but not in unfed males, and a transcript increase in fed male ticks (Figure 2A). It is also notable that AAS19, 21, 29, and 30 are abundant in female ticks, while AAS50, 67, and 121 are predominant in male ticks. Of the 31 AAS sequences summarized in Figure 1, we determined differential abundance in SG and MG for five transcripts, AAS19, 21, 23, 27, and 31 (Figure 2B), with differences for the remaining sequences being minimal to negligible (Supplemental Table 3). While AAS23 transcript abundance increases with feeding in both SG and MG, the remaining show an apparent dichotomous pattern. Transcripts for AAS19, 21, 27, and 31 are highest at the 96h time point, and lower at the 120h time point in SG. In MG, AAS19 and 21

are abundant at 48h, and absent or low at 96 and 120h time points, while AAS27 and 31 increase with feeding (Figure 2B).

A diversity of seventeen amino acid residues is predicted at P1 sites of AAS putative RCLs

Table 2 lists 78 predicted reactive center loops (RCLs) from 27 (Table 2A) and 22 (Table 2B) AAS sequences found in female and male ticks, the 27 found in both (Table 2C), and 2 of an undetermined source (Table 2D). Please note that Table 2A includes previously characterized AAS1–17 (Mulenga et al., 2007). We would like to note that the RCL regions for AAS68 and 71 (Table 2B), and AAS77 (Table 2D) are partial (marked with an asterisk in Table 2), however based on the available sequence we were still able to conclude these RCLs to be unique. Additionally, while the predicted RCL for AAS8 and 9, 4 and 12, and 13 and 15 are identical, these sequences differ in the N-terminus region by 13–25 amino acids (Mulenga et al., 2007). Numbering of amino acid residues in the RCL is based on the standard nomenclature developed by Schechter and Berger (Schechter and Berger, 1967), in which amino acid residues at the N-terminal end of the scissile bond (P1-P1) are not primed and those on the C-terminal end are primed: “"p17 [E]-p16 [E/K/R]-p15 [G]-p14 [T/S]-p13 [X]-p12-9 [AGS]-p8-1 [X]-p1' -4'” (Miura et al., 1995; Gettins, 2002). Molecular analysis predictions of the P1 site assume that there are 17 amino acid residues between the beginning of the RCL hinge region (P17), and the scissile bond (P1-P1’), (Hopkins and Stone, 1995). Based on these conventions, a diversity of 17 different amino acid residues is predicted at the P1 sites of AAS1–78 (except for AAS71 which has a partial RCL excluding the P1 site, Table 2B). The P1 residues for AAS1–78 have the following charge and polarity properties: 27 (~ 35%) are polar uncharged [S (10/26), C (5/26), T (6/26), Q (4/26), Y (2/26)], 25 (~32%) are hydrophobic [L (8/25), I (4/25), P (5/25), G (3/25), M (1/25), A (3/25), V (1/25)], 21 (27%) are polar basic [R (11/21), K (9/21), H (1/21)], and four residues (~ 5%) are polar acidic [D (3/4), E (1/4)] (Figure 3). It is noteworthy that 11 of the 22 AAS sequences found in MG and SG have basic residues at the predicted P1 site.

Some AAS sequences are highly identical

To gauge the relationships between AAS sequences, amino acid sequences were subjected to phylogeny analysis, except for AAS87 and 109, which were too short for an informative alignment (Figure 4). We would like to note that due to the large number of sequences, we split the tree into two parts, Figures 4P1 and 4P2. As shown in Figure 4, 68 of the 122 AAS sequences segregated into 19 clusters labeled A-S, and the remaining sequences did not cluster. Of the 19 clusters, eight have more than two sequences: E (AAS8–10, 18, 22, 41, 46, and 67), F (AAS4–6, 11–17, 24, 30, and 112), G (AAS1–3, 23), H (AAS75, 87, and 95), M (AAS32, 43, and 62), O (AAS33, 35, 52 and 79), P (AAS28, 36, 47, and 108), and R (AAS7, 25, 26, 37, 45, and 78), and the remaining 11 clusters have single pairs. When subjected to pairwise sequence alignment analysis, sequences in clusters A, B, D, E, F, G, K, M, O, P, R, and S showed variable amino acid identity levels of 15, 96, 93, 66–98, 61–98, 40–93, 98, 84 (excluding AAS62), 66–98, 56–78, 61–98, and 96%, respectively (Figure 4P1 and 4P2). In the remaining clusters (marked with asterisks), amino acid identity levels were below 15%. We would like to caution here that some sequences being compared are partial, and therefore the picture of these relationships might be incomplete. From pairwise alignment analyses, two general patterns emerged. In the first pattern, differences between

two sequences were scattered throughout the alignment (not shown). In the second pattern, found between AAS25 and AAS45, and AAS33 and AAS52, differences were restricted to the C-terminus region within the RCL (not shown).

Close to half of AAS sequences have orthologs in other tick species

To determine if any AAS sequences in this study had orthologs in other tick species, inter-species comparisons were performed. A search for other tick serpin sequences in publically available databases retrieved 165 serpin sequences across nine tick species (Table 3). Pairwise comparisons of these serpins to AAS1–122 identified 50 AAS sequences that were conserved in other tick species. To manage the high number of sequences, the data are presented in separate tables: *A. americanum* versus *A. maculatum* (Karim et al., 2011) and *A. variegatum* (Ribeiro et al., 2011) (Table 4A), and versus *R. pulchellus* (direct submission), *R. appendiculatus* (Mulenga et al. 2003b), *R. microplus* (Tirloni et al., 2014), The Gene Index Project, http://compbio.dfci.harvard.edu/tgi/], *R. haemaphysaloides* (direct submission), *H. longicornis* (Imamura et al., 2005), *I. scapularis* (GenBank direct submissions, The Gene Index Project), and *I. ricinus* (Leboulle et al., 2002b) (Table 4B). As shown in Table 4A, AAS4–6, 8–19, 22–27, 29, 30, 41, 46, and 47 show ≥75–96% amino acid identity to *A. maculatum* serpin sequences AEO35533, AEO35520, AEO34312, AEO34447, AEO34313, AEO34314, AEO32541, AEO34349, AEO34279, AEO34218, AEO34217, AEO33019, and AEO32217, while the remaining sequences showed <75% amino acid conservation. Likewise, in Table 4B, AAS19–22, 41, 42, and 65 show amino acid identities of ≥75–91% to 7 *R. pulchellus* serpins: JAA54307, JAA54309, JAA543410, JAA54167, JAA54314, JAA54313, respectively, while the rest showed amino acid identities of <75%. Additionally, AAS7 shows 75 and 77% identity to *R. microplus* EST89704 and *R. pulchellus* JAA54312, and AAS19 and 21 show 82–96% identity to *R. microplus* TC17409, TC22658, and EST767976, while AAS41, 42, and 54 show 65, 79 and 90% identity to partially characterized *R. appendiculatus* AAK61378, and AAK61376, and *R. microplus* TC16456, respectively. Four AAS (24, 38, 69, and 70) showed 70% or greater identity to the same partially characterized *R. appendiculatus* AAK61377. Of the 50 AAS sequences conserved in other tick species, only 11 sequences (AAS19–21, 25, 37, 42, 44, 45, 54, 66 and 78) appear to be conserved in prostriate ticks (Table 4B). Except for AAS19, which showed 81 and 82% amino acid identity to *I. ricinus* ABI94058 and *I. scapularis* XP_00245308, respectively, all other AAS sequences showed 58–70% conservation with prostriate tick sequences (Table 4B).

Table 5 summarizes the 29 AAS RCL sequences which show at least 62% sequence conservation in other tick species. Preliminary manual inspection of RCLs showed high identities between sequences where the predicted P1 site is of the same charge and polarity, therefore all 212 tick serpin sequences for which an RCL could be determined were first divided into one of four groups: polar uncharged, polar basic, polar acidic, and hydrophobic. RCL sequences (Figure 5A–D) were then subjected to phylogeny analysis (not shown). Lastly, RCLs from all tick species were subjected to pairwise sequence alignments. Identity levels for the 29 highly conserved AAS RCLs, (AAS4, 7, 12, 14, 18–23, 25–29, 31, 37, 38, 42, 44, 45, 47, 50, 52, 54, 58, 65, 69, and 70) ranged from approximately 62–100% (Table 5). Of these 29 RCLs, seven (AAS7, 19–21, 23, 27, and 42) are conserved in both

metastriate and prostriate ticks, with the remaining being conserved only in metastriate ticks. Seven AAS RCLs (AAS18, 20–22, 27, 42, and 50) showed 95–100% conservation with at least one RCL from another species. For AAS25, RCL identity to its partially characterized ortholog in *R. appendiculatus* (AAK61375), was higher, at 81% identity, than for whole sequence identity, at 62%. Three AAS RCLs (AAS7, 19, and 20) showed >80% identity to at least one RCL from a prostriate species. The identities between remaining RCL sequences ranged between ~62–76% (Table 5). It is notable that the RCL for AAS19 is 100% identical to serpin RCLs across several tick species in multiple genera, and including both metastriate and prostriate ticks. Of the 29 conserved AAS RCLs, 38% (11/29) (AAS18–20, 22, 23, 27, 28, 31, 37, 50, 65) have basic amino acid residues, 35% (10/29) (AAS21, 25, 42, 44, 47, 52, 54, 58, 69, and 70) have polar uncharged residues, and 24% (7/29) (AAS4, 7, 12, 14, 26, 29, 38, and 42) have hydrophobic residues, while only one sequence, AAS45, has a polar acidic residue at the putative P1 site. It is interesting to note that for the 10 most highly inter-species conserved RCL sequences (those which are ≥90%), the majority (7/10) have basic P1 residues, while the remaining three have polar uncharged P1 residues.

DISCUSSION

This study provides an update of unique serpin-coding sequences expressed in unfed and fed *A. americanum* male and female ticks. While this is the first report of a very large number of serpin transcripts from male ticks, data presented here are not unusual. High numbers of serpin sequences were reported in ticks *I. scapularis* (Mulenga et al., 2009), *R. pulchellus* (direct submission), and *A. maculatum* (Karim et al., 2011), in mosquitoes *Anopheles gambiae*, *Culex quinquefasciatus*, and *Aedes aegypti* (Rawlings et al., 2012), in *Bombyx mori* (Zou et al., 2009), *Tribolium* (Zou et al., 2007), *Drosophila* (Reichhart, 2005), in mouse and human genomes (Puente and López-Otín, 2004; Gatto et al., 2013; Heit et al., 2013), and in *Arabidopsis* and *Oryza sativa* (Fluhr et al., 2012). Such large serpin counts across a great diversity of taxa indicate the importance of this protein family in regulating homeostasis in most branches of life. While serpin counts in other tick species do not approach the number for *A. americanum* as reported here, the discrepancy is likely due to the fact that this is the first analysis of tick transcriptomes of its scope with both male and female tissues at various time points having been explored.

Although based on the design of this study we are unable to conclude expression patterns, the observation of a greater diversity of AAS transcripts in male than female ticks is interesting. We speculate that the high diversity of AAS transcripts found in male ticks could be explained by the timing of reproductive physiological changes. Male metastriate tick species such as *A. americanum* feed for only a short time prior to completion of spermatogenesis and mating (Kiszewski et al., 2001). On the other hand, many female reproductive activities including vitellogenin synthesis and deposition into oocytes, ovulation, fertilization, and oviposition do not occur until after several days of feeding (Kiszewski et al., 2001; Sonenshine and Roe, 2013). The majority of AAS which were found exclusively in males in this study, were from male ticks fed for at least three days and were potentially ready to mate, while female ticks in this study were fed up to 120h with reproductive activities just beginning. As a result, it is possible that there are additional serpins important for female reproductive physiology, that are expressed at later feeding

time points than those identified in this study. Although empirical data will be needed, it's interesting to note that AAS50 and 121 both found in male ticks were abundant after feeding when the male tick has entered its physiologically reproductive state. It will be interesting to investigate expression of serpins in the immature stages, where there is a lack of sexual dimorphism. This will inform on which serpins are indeed involved in sex-specific physiology, and which are involved in other feeding-related physiology such as host-defense modulation. Evidence in insects, *Drosophila* and *Aedes aegypti*, indicated that serpins are among the male reproductive gland proteins transferred to females during mating (Coleman et al., 1995; Sirot et al., 2008). Although there are distinct differences between biology of ticks and insects, it is plausible that some male *A. americanum* serpins found in this study could serve as reproductive proteins.

The SG and MG represent two major organs through which the tick interacts with its host and with pathogens. Thus, the identification of 31 previously unknown AAS transcripts in SG and MG is interesting. Although relative expression analysis done in this study is limited, the expression patterns of AAS21 and AAS27 are notable. Based on relative abundance determined in this study AAS21 abundance is 1,000-fold higher at 48h MG compared to SG, while AAS27 increases 500 fold at 120h in MG more than SG. It will be interesting to investigate role(s) of AAS21 and AAS27 in tick feeding. Another important goal of this study was to identify *A. americanum* tick serpins that are expressed in both male and female metastriata ticks. We believe that these could represent those that are important to tick feeding regulation. Despite obvious differences in their biology, both male and female ticks must interact with host defense mechanisms before mating. Indeed, there is evidence that like females, male ticks express anti-inflammatory molecules such as histamine-binding proteins (Paesen et al., 1999; Bior et al., 2002), which are potentially involved in facilitating male tick feeding. Thus, the 16 AAS transcripts found in both male and female ticks, and in 48–120h SG and MG could represent those that are important to tick feeding success.

The high number of AAS sequences in this study could be explained by gene duplication and exon shuffling in the RCL region as suggested by high amino acid identity among some AAS sequences that clustered together on the phylogeny tree and those that showed differences restricted to the RCL region. Serpin diversity by gene duplication and subsequent divergence has been reported in a number of organisms including humans (Heit et al., 2013), mice (Borriello and Krauter, 1990; Hancock, 2005) and *B. mori* (Zou et al., 2009). In *An. gambiae* serpin genes clustering phylogenetically were found in clusters on the same chromosome, indicating that they could be duplicated genes (Suwanchaichinda and Kanost, 2009). Similarly, in *I. scapularis* 11 highly identical serpins were found on the same supercontig (Mulenga et al, 2009). The observation of differences restricted to the RCL has been observed in transcripts that are products of alternatively spliced exons. This phenomenon was reported in *Manduca sexta* (Jiang and Kanost, 1997), in *B. mori* (Zou et al., 2009), and in *Ctenocephalides felis* (Brandt et al., 2004). We also observed a very curious pattern between AAS4 and 12, and AAS13 and 15, in which RCLs were identical with differences restricted to outside of the RCL (Mulenga et al., 2007). Whether or not these observations are consistent with events *in vivo* requires further investigation. However,

it is interesting to note that in this study we observed a similar pattern between serpin sequences in other tick species: *A. maculatum* AEO34217 and AEO34218, and *R. pulchellus* JAA62387 and JAA63611, where the RCL is the same and differences in sequences are outside of the RCL region. We speculate that if consistent with events *in vivo*, AAS transcripts that share the same RCL sequence could function as redundant proteins, or could regulate the same protease under different spatio-temporal conditions.

Within the RCL region, the amino acid at the P1 site is considered most important in determining the target protease(s) that is/are regulated by a candidate serpin (Gettins, 2002; Huntington, 2006; Huntington, 2011). Accordingly, the observation that 17 different amino acid residues were predicted at P1 sites of putative RCLs identified in this study indicates the potential diversity of proteases that may be regulated by these serpins. Our analysis of AAS RCL P1 sites showed a near-even distribution of residues across charge/polarity types with the exception of polar acidic similar to *M. sexta*, *B. mori* (Zou et al., 2009), plants (Roberts et al., 2004; Roberts and Hejgaard, 2008), and humans (Cassar and Hunter, 2013) where polar acidic P1 residues in serpins appear to be rare. Prediction of the P1 site amino acid residue is not in itself sufficient to determine the protease that may be regulated by a candidate serpin; empirical evidence is required. However, there is ample evidence that serpins with basic residues at the P1 site regulate trypsin and tryspin-like proteases (Gettins, 2002, Li et al., 1999, Leboulle et al., 2002a; Leboulle et al., 2002b; Prevot et al., 2006). Thus it is interesting that the most highly conserved AAS RCLs in this study have basic P1 residues. From the perspective of tick feeding biology, serpins with basic P1 residues could represent those that ticks use to evade trypsin-like protease-mediated host defense pathways such as blood clotting. We would like to caution the reader here that in both plants and in non-hematophagous organisms such as *Drosophila*, the majority of serpins have basic P1 amino acid residues (Reichhart, 2005; Fluhr et al., 2012). It is also interesting to note that two tick anti-coagulant serpins, HLS2 (orthologous to AAS12), in *H. longicornis* (BAD11156) (Imamura et. a., 2005), and Iris in *I. ricinus* (AJ269658) (Prevot et. al., 2006), contain hydrophobic residues at their P1 sites. Further experiments are therefore required in order to determine proteases/pathways that may be regulated by the serpins described in this study.

In nature, different tick species may infest the same animal host and implicitly, these different tick species will face the same host defense mechanisms. It is conceivable that different tick species could utilize conserved proteins to interact with the same host, such as the 50 cross-tick species conserved AAS sequences identified in this study. It is notable that only ~22% (11/50) were conserved in both metastriate and prostriate tick species. These could be particularly interesting candidates for further functional studies given that in general, amino acid identities between metastriate and prostriate tick proteins sequences are low. In particular, AAS25, which was detected in fed males and females, and in SG, is orthologous to Iris, found in SG and saliva of *I. ricinus*. Iris was demonstrated to inhibit lymphocyte proliferation, as well as the immune system cytokines IFN-γ and IL-6 (Leboulle et. al., 2002a; Leboulle et. al., 2002b). Similarly, Highly conserved AAS proteins represent very interesting candidates for development of universal anti-tick vaccines as advocated (Maritz-Olivier et al., 2007).

The RCL is important to serpin function (Gettins, 2002), and thus it is interesting to note that some AAS RCLs are 90–100% conserved in other tick species. These serpins could be involved in regulating target proteases that are important to all ticks. Based on our inter-species comparative sequence analysis, it is interesting to note that AAS sequences with polar basic P1 sites, followed by polar uncharged P1 sites, were likely to be conserved in other tick species. It is noteworthy that hydrophobic P1 residues, though prevalent, tended to show little conservation in other tick species. This suggests that proteases that can interact with hydrophobic Pl residues may be involved in species-specific physiology, and might be diverging at a fast rate. Although the present study is descriptive, it contributes significantly to the picture of serpin transcript diversity expressed by male and female *A. americanum* ticks. Additionally, based on the data in this analysis, the picture of serpin transcript diversity in other tick species is likely to be far from complete.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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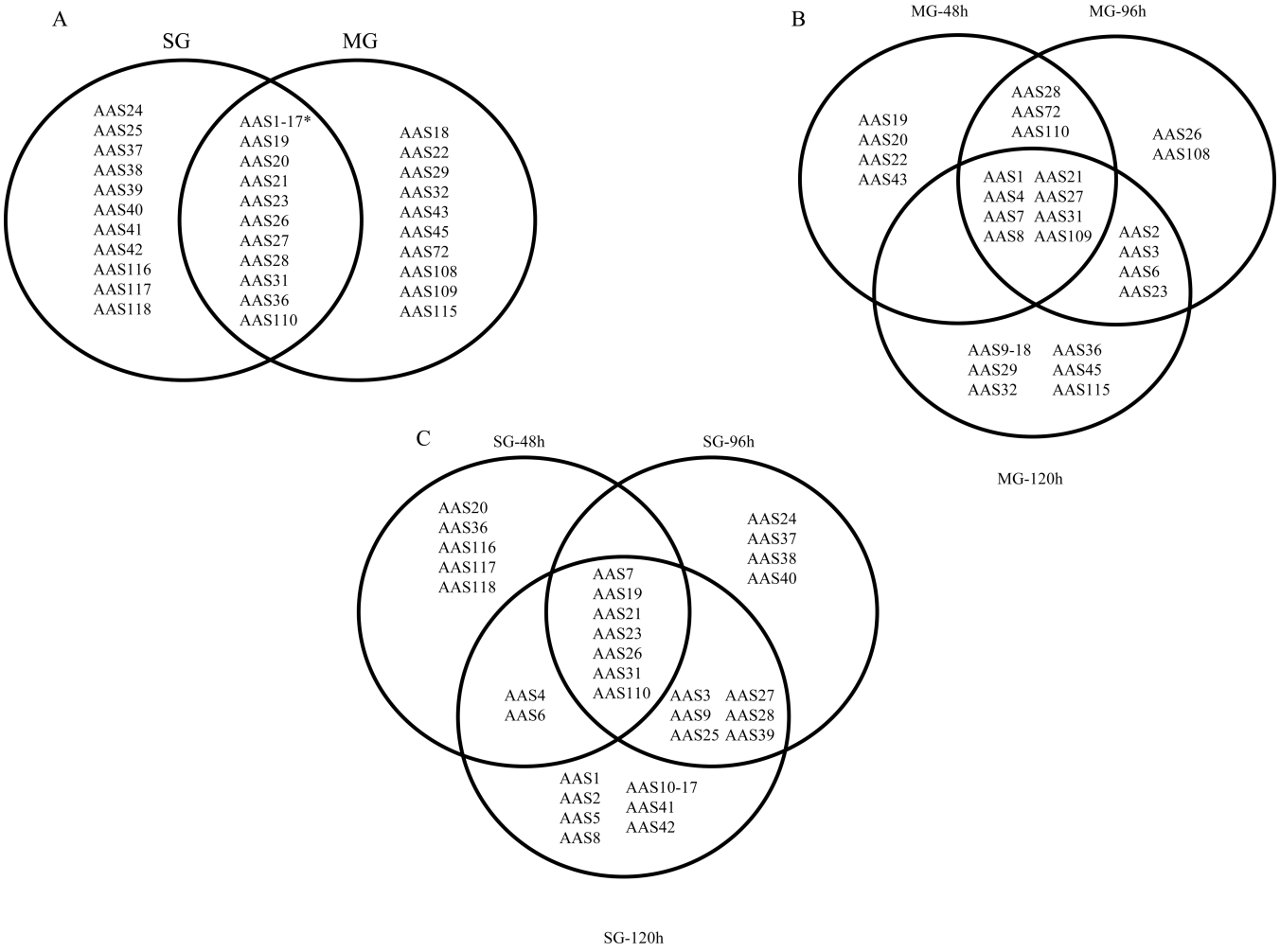
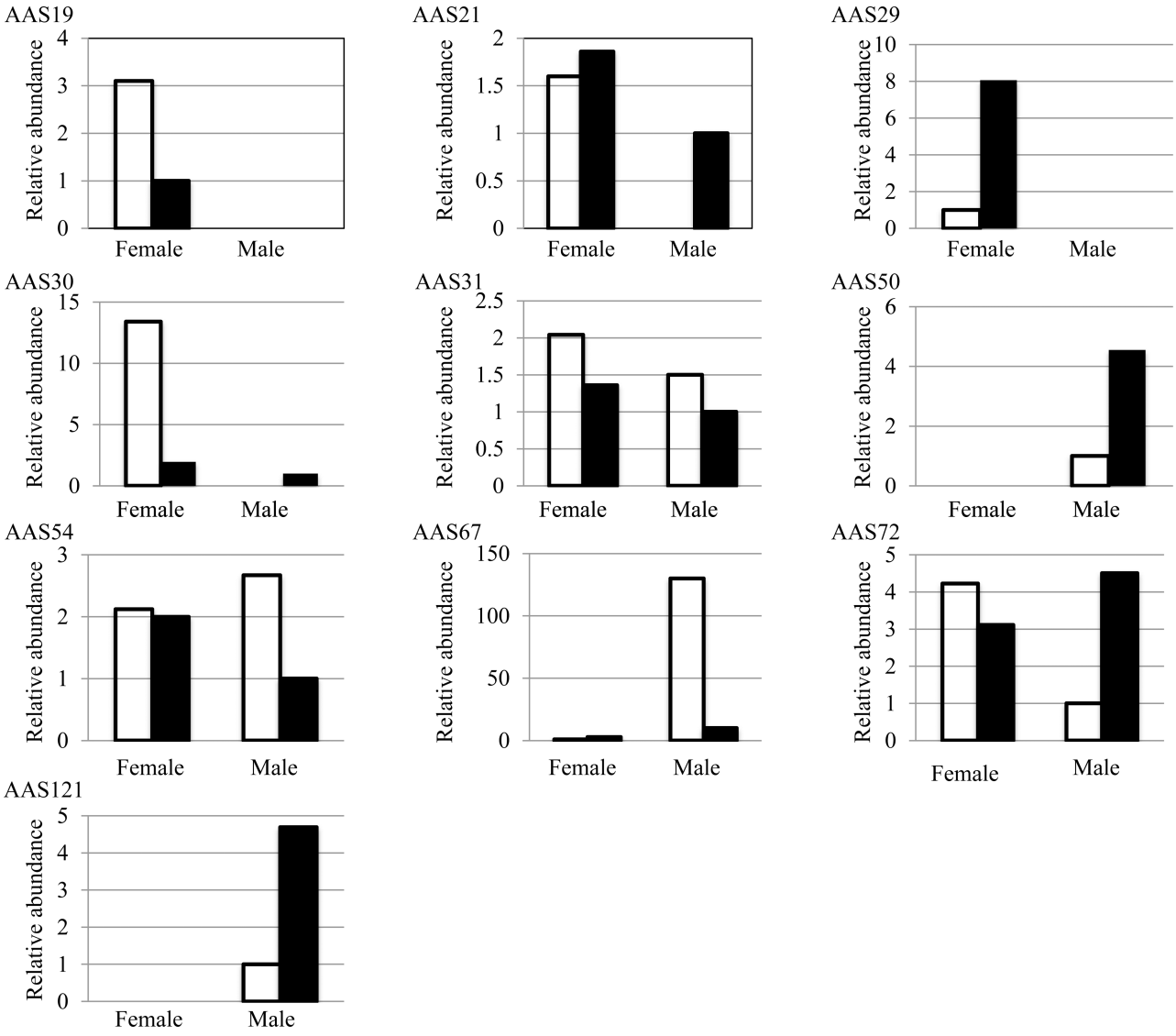
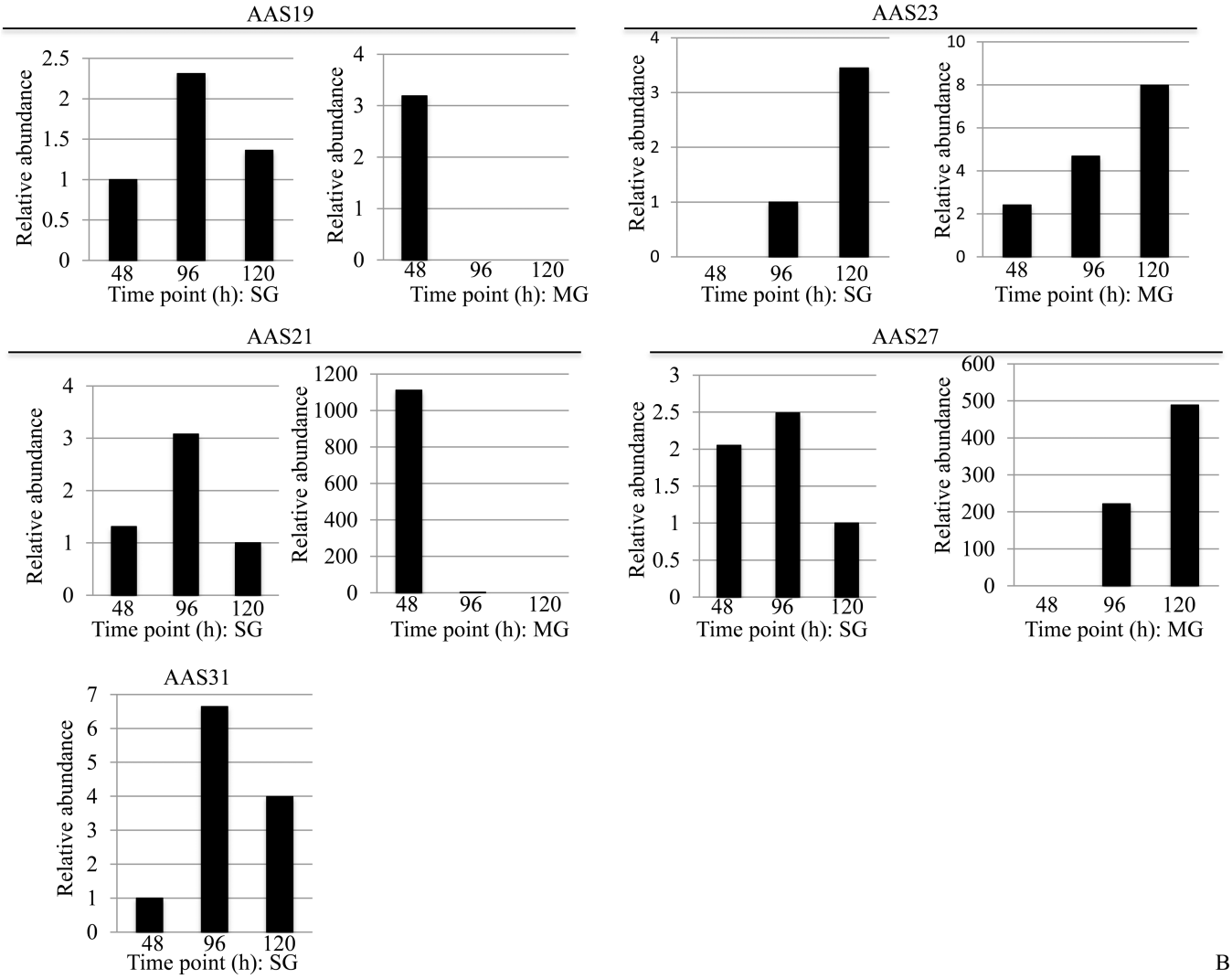


Figure 1. Adult female *A. americanum* salivary gland (SG) and midgut (MG) expressed serpin (AAS) transcripts

(A) Total AAS sequences found in MG and SG and both at all tested time points, (B) Apparent temporal and spatial distribution of AAS transcripts found in MG (B), and SG (C). Please note that 17 previously characterized SG and MG expressed AAS transcripts [42] are not included here.



A



B

Figure 2. Relative abundance of *A. americanum* serpin (AAS) transcripts: Sequence reads were mapped back to *de novo* assembled contigs in different libraries

Relative abundance values in: (2A) unfed (empty bars) and fed (black filled bars) male and female ticks, and (2B) dissected 48–120h salivary glands (SG) and midguts (MG) were calculated using the formula described in materials and methods..

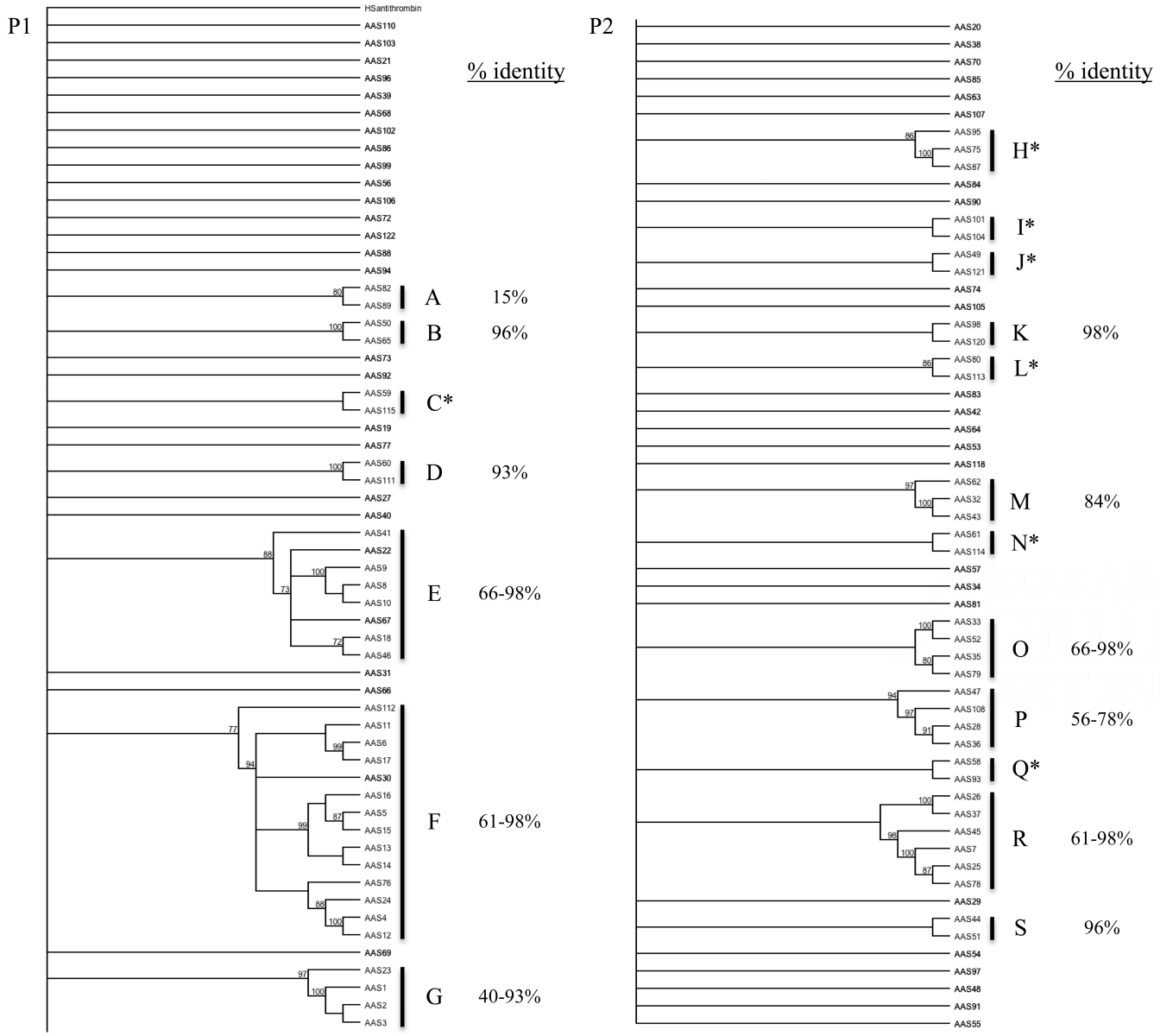
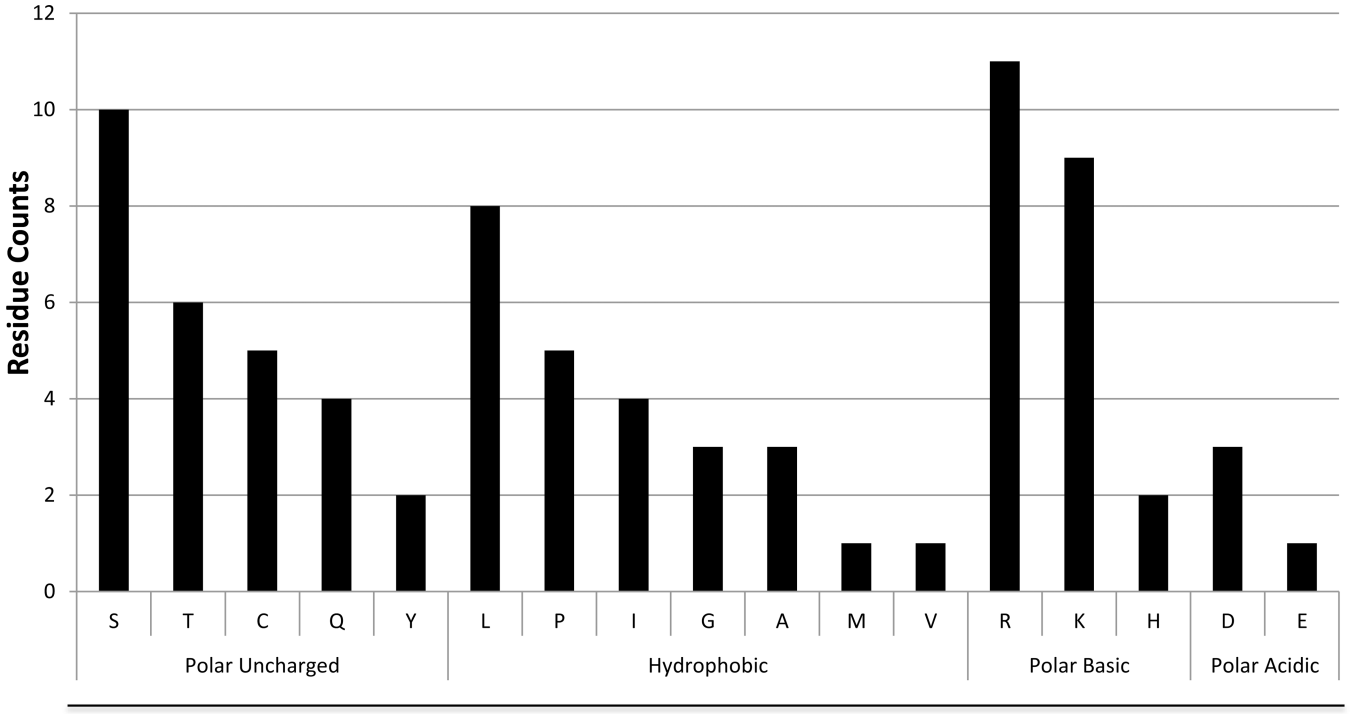


Figure 3. Diversity and counts for amino acid residues at the putative P1 position in reactive center loops of the first 78 *A. americanum* serpin sequences

Amino acid residues at putative P1 sites were determined based on molecular analysis predictions of the P1 site that assume that there are 17 amino acid residues between the beginning of the RCL hinge region (P17), and the scissile bond (P1-P1') [54].



Predicted P1 sites in putative *A. americanum* serpin reactive center loops

Figure 4. Phylogeny relationship of *A. americanum* serpin (AAS) sequences

Translated AAS amino acid sequences and human antithrombin were aligned using T-coffee in in MacVector version 12. A bootstrap supported phylogeny tree was then constructed with human antithrombin (CAA48690) as the out-group using neighbor-joining method. Clades containing more than 2 AAS sequences are labeled “A” to “T”. Amino acid identity levels within each cluster are indicated. Clusters where amino acid identities were below 15% are marked with an asterisk (*) sign.

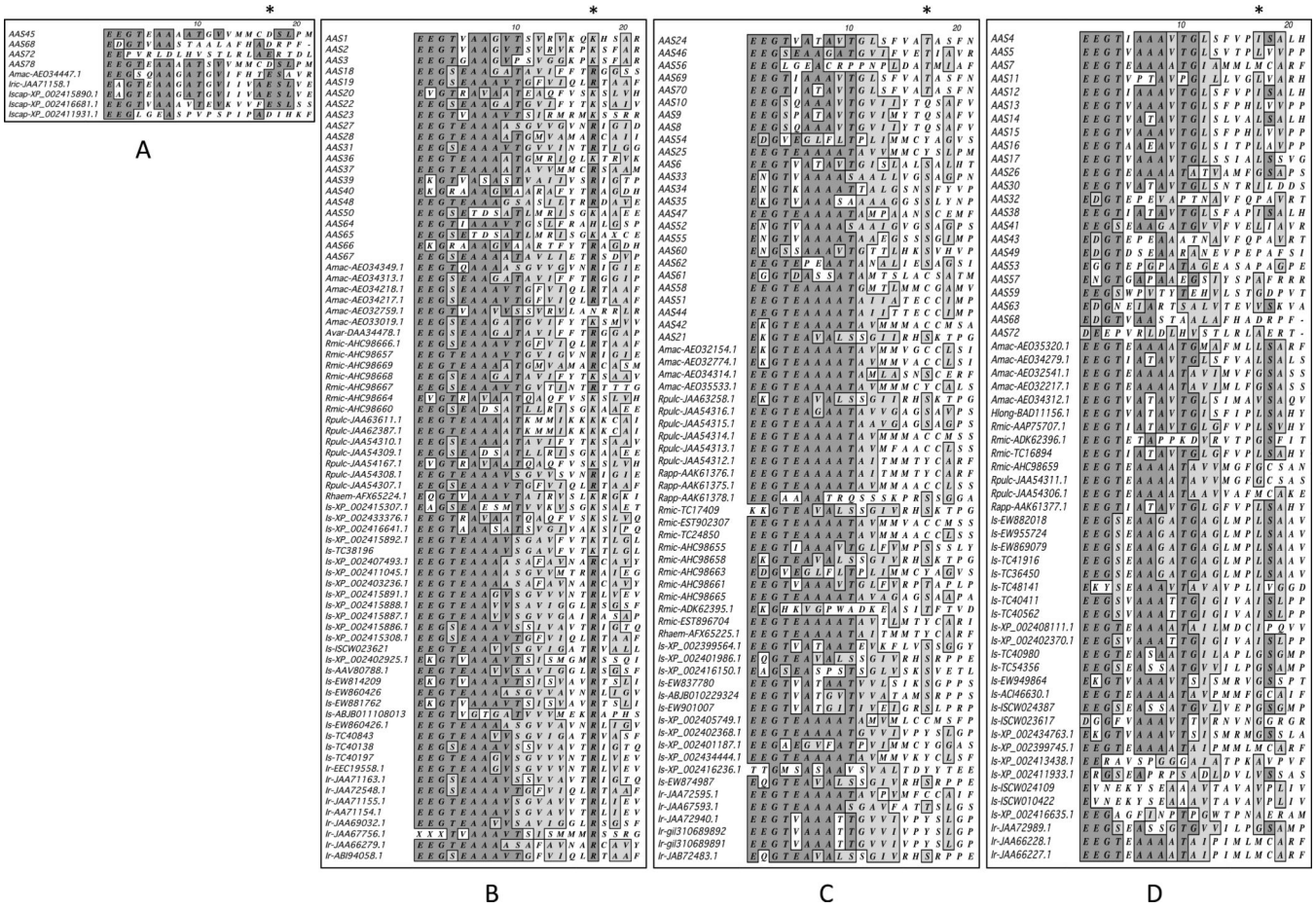


Figure 5. Multiple sequence alignment of tick serpin reactive center loops (RCL)

Predicted RCLs in serpins found in this study and other tick serpins downloaded from GenBank were subjected to multiple sequence alignment using T-coffee in MacVector vrsion 12. Asterisk (*) sign denotes predicted amino acid residues at P1 sites: (A) polar acidic, (B) polar basic, (C) hydrophobic, and (D) polar uncharged. Identical amino acid residues are shaded gray. AAS = *Amblyomma americanum* serpins, Amac = *A. maculatum*, Avar = *A. variegatum*, Rmic = *Rhipicephalus microplus*, Rpulc = *R. pulchellus*, Rhaem= *R. haemaphysalis*, Rapp = *R. appendiculatus*, Hlong = *Haemaphysalis longicornis*, Is = *Ixodes scapularis*, Ir = *I. ricinus*.

Table 1

Updated list of *Amblyomma americanum*serpin (AAS) transcripts

Table 1A: AAS sequences found in male ticks

AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment
44	GAYW01000312	Partial	73	GAYW01000241	Partial	95	GAYW01000268	Partial
46	GAYW01000194°	Partial	74	GAYW01000254	Partial	96	GAYW01000269	Partial
48	GAYW01000205°	Full, NSP	75	GAYW01000255	Partial	97	GAYW01000270	Partial
49	GAYW01000207	Partial	79	GAYW01000231	Partial	98	GAYW01000271	Partial
50	GAYW01000208°	Partial	80	GAYW01000243	Partial	99	GAYW01000272	Partial
52	GAYW01000285	Partial	81	GAYW01000232	Partial	100	GAYW01000273	Partial
53	GAYW01000287	Full, NSP	82	GAYW01000247	Partial	101	GAYW01000275	Partial
55	GAYW01000292°	Partial	83	GAYW01000249	Partial	102	GAYW01000277	Partial
56	GAYW01000294	Full, NSP	84	GAYW01000251	Partial	103	GAYW01000279	Partial
57	GAYW01000295	Full, NSP	85	GAYW01000235	Partial	104	GAYW01000280	Partial
58	GAYW01000298	Full, NSP	86	GAYW01000237	Partial	105	GAYW01000281	Partial
59	GAYW01000302	Full, NSP	87	GAYW01000256	Partial	106	GAYW01000282	Partial
60	GAYW01000304	Full, NSP	88	GAYW01000258	Partial	107	GAYW01000283	Partial
61	GAYW01000310	Partial	89	GAYW01000259	Partial	111	GAYW01000225	Partial
62	GAYW01000311	Partial	90	GAYW01000262	Partial	113	GAYW01000240	Partial
64	GAYW01000246	Partial	91	GAYW01000264	Partial	114	GAYW01000230	Partial
68	GAYW01000308°	Partial	92	GAYW01000265	Partial	120	GAYW01000253°	Partial
70	GAYW01000197	Partial	93	GAYW01000266	Partial	121	GAYW01000257°	Partial
71	GAYW01000313	Partial	94	GAYW01000267	Partial	122	GAYW01000239	Partial

Table 1B: AAS sequences found in female ticks

AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment
2	ABS87354	Full, SP	20	GAYW01000324°	Full, SP	43	GAYW01000156	Partial
5	ABS87357	Full, SP	22	GAYW01000149°	Partial	45	GAYW01000131	Partial
9	ABS87361	Full, SP	26	GAYW01000039°	Partial	65	GAYW01000368	Partial
11	ABS87363	Full, NSP	29	GAYW01000365°	Partial	69	GAYW01000330	Partial

Table 1B: AAS sequences found in female ticks

AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment
12	ABS87364	Full, SP	32	GAYW01000130	Partial	76	^	Full, NSP
13	ABS87365	Full, SP	36	GAYW01000364°	Full, NSP	109	GAYW01000172°	Partial
14	ABS87366	Full, SP	37	GAYW01000322°	Partial	112	GAYW01000397	Partial
15	ABS87367	Full, SP	38	GAYW01000044	Partial	115	GAYW01000125	Partial
16	ABS87368	Full, SP	40	GAYW01000047	Partial	116	GAYW01000070	Partial
17	ABS87369	Full, SP	41	GAYW01000021	Partial	117	GAYW01000067	Partial
19	GAYW01000076°	Full, SP	42	GAYW01000022	Partial	118	GAYW01000073	Partial

Table 1C: AAS sequences found in both male and female ticks

AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment
1	ABS87353	Full, SP	24	GAYW01000037°	Partial	47	GAYW01000316°	Full, NSP
3	ABS87355	Full, SP	25	GAYW01000015°	Full, NSP	51	GAYW01000367°	Partial
4	ABS87356	Full, SP	27	GAYW01000017°	Full, SP	54	GAYW01000363°	Partial
6	ABS87358	Full, SP	28	GAYW01000019°	Full, NSP	63	GAYW01000286°	Partial
7	ABS87359	Full, NSP	30	GAYW01000321°	Full, SP	66	GAYW01000309°	Partial
8	ABS87360	Full, SP	31	GAYW01000315°	Full, SP	67	GAYW01000317°	Full, NSP
10	ABS87362	Full, SP	33	GAYW01000184	Partial	72	GAYW01000344°	Partial
18	GAYW01000325°	Full, NSP	34	GAYW01000293°	Full, NSP	108	GAYW01000260°	Partial
21	GAYW01000077°	Full, SP	35	GAYW01000288°	Partial	110	GAYW01000276°	Partial
23	GAYW01000078°	Full, SP	39	GAYW01000018°	Full, SP	119	GAYW01000221°	Partial

Table 1D: AAS sequences with no source information

AAS# ID	Accession#	Comment	AAS# ID	Accession#	Comment
77	^	Partial	78	Clust1-1-153	Partial

Full = full length open reading frame, SP = signal peptide is present, NSP = no signal peptide;

° indicates more than one accession number associated with AAS (see Supplemental Table 1);

^ contig sequence was too short for GenBank submission (<200bp), hyperlinked in Supplemental Table 1.

Table 2

Reactive Center Loops (RCLs) of *A. americanum*serpins

Table 2A: Predicted RCLs found in female ticks

AAS ID	RCL amino acid sequence	AAS ID	RCL amino acid sequence
2	EEGTVAAGVTSVRVKP**K**SFAR	29	EEGTEAAAATAVTVVD**G**CMPR
5	EEGTVAAAVTGLSVTP**L**VVPP	32	EDGTEPEVAPTNAVFQ**P**AVRT
9	EEGSPATAVTGVIMYT**Q**SAFV	36	EEGTEAAAATGMRIQL**K**TRVK
11	EEGTVPTAVPGILLVG**L**VARH	37	EEGTEAAAATAVVMMC**R**SAAM
12	EEGTIAAAVTGLSFVP**I**SALH	38	EEGTIATAVTGLSFAP**I**SALH
13	EEGTVAAAVTGLSFPH**L**VVPP	40	EKGRAAAGVAARAFYT**R**AGDH
14	EEGTVATAVTGISLVA**L**SALH	41	EEGSEAAGATGVVFVE**L**IAVR
15	EEGTVAAAVTGLSFPH**L**VVPP	42	EKGTEAAAATAVMMMA**C**CMSA
16	EEGTAAEAVTGLSITP**L**AVPP	43	EDGTEPEAAATNAVFQ**P**AVRT
17	EEGTVAAAVTGLSSIA**L**SSVG	45	EEGTEAAAATGVVMMC**D**SLPM
19	EEGSEAAAVTGFVIQL**R**TAAF	65	EEGSETDSATLMRISG**K**AXCE
20	EVGTRAVAATEAQFVS**K**SLVH	69	EEGTIAAAVTGLSFVA**T**ASFN
22	EEGSEAAGATGVIFYT**K**SAIV	76	EEGTIAAAVTGSLFRA**H**LGSP
26	EEGTEAAAATATVAMF**G**SAPS		

Table 2B: Predicted RCLs Found in male ticks

AAS ID	RCL amino acid sequence	AAS ID	RCL amino acid sequence
44	EEGTEAAAATAIITTE**C**CIMP	59	EEGSWPVTYTEHVLST**G**DPVT
46	EEGSEAAGATGVIFVE**T**IAVR	60	ENGSSAAAVTGTTLHK**S**VHVP
48	EEGTEAAAGSASILTR**R**DAVE	61	EGGTDASSATAMTSLA**C**SATM
49	EDGTDSEAARANEVPE**P**AFSI	62	EEGTEPEAATANALIE**S**AGSI
50	EEGSETDSATLMRISG**K**AAEE	64	ARGGRAVSNEVQSTTS**A**TTAA
52	ENGTVAAAASAAIGVG**S**AGPS	68	EDGTVAASTAALAFHA**D**RPF*
53	EGGTEPGPATAGEASA**P**AGPE	70	EEGTIATAVTGLSFVA**T**ASFN
55	ENGTVAAAATAAEGGS**S**SGIM	71	EVGTKA*

Table 2B: Predicted RCLs Found in male ticks

AAS ID	RCL amino acid sequence	AAS ID	RCL amino acid sequence
56	EEGLGEACRPPNPLPA**T**MIAF	73	GAGGRPPSSNDSREAG**T**SPAK
57	ENGTGAPAAEGSIYSP**A**FRRR	74	ERSTSRMPKYTGAQGA**P**GTSS
58	EEGTEAAAATGMTLMM**C**GAMV	75	RRGPKTVAAQAVAKEA**A**RTAK

Table 2C: Predicted RCLs found in both male and female ticks

AAS ID	RCL amino acid sequence	AAS ID	RCL amino acid sequence
1	EEGTVAAGVTSVRVKQ**K**HSAR	30	EEGTVATAVTGLSNTR**I**LDDS
3	EEGTGAAGVPSVGGKP**K**SFAR	31	EEGSEAAAVTGVVINT**R**TIGG
4	EEGTIAAAVTGLSFVP**I**SALH	33	ENGTVAAAASAALLVG**S**AGPN
6	EEGTVATAVTGISLAL**S**ALHT	34	ENGTKAAAATTALGSN**S**FYVP
7	EEGTEAAAATGIAMML**M**CARF	35	EKGTVAAASAAAAGGS**S**LYNP
8	EEGSQAAAVTGVIIYT**Q**SAFV	39	EKGTVASASTVAIIVS**R**IGTP
10	EEGSQAAAVTGVIIYT**Q**SAFV	47	EEGTEAAAATAMPAAN**S**CEMF
18	EEGSEAAGATAVIFFT**R**GGSS	51	EEGTEAAAATAIIATE**C**CIMP
21	EKGTEAVALSSGIIRH**S**KTPG	54	EDGVEGLFLTPLIMMC**Y**AGVS
23	EEGTVAAAVTSIRMRM**K**SSRR	63	EDGNEIARTSALVTEV**V**SKVA
24	EEGTVATAVTGLSFVA**T**ASFN	66	EKGRAAAGVAARTFYT**R**AGDH
25	EEGTEAAAATAVVMMC**Y**SLPM	67	EEGSEAAAATAVLIET**R**SDVP
27	EEGTEAAAASGVVGVN**R**IGID	72	EEPVRLDLHVSTLRLA**E**RTDL
28	EEGTEAAAATGMVAMA**R**CAII		

Table 2D: Predicted RCLs, source undetermined

AAS ID	RCL amino acid sequence	AAS ID	RCL amino acid sequence
77	EEGSQATAVTGVIIYT**Q**S*	78	EEGTEAAAATSVVMMC**D**SLPM

Amino acid residues at putative P1 site are bolded;

* indicates a partial RCL sequence.

Table 3

Other tick serpins downloaded from publically available databases

Source tick species	Accession#	Source tick species	Accession#	Source tick species	Accession#
	AEO35533		AHC98664	*I. scapularis*	TC36982
	AEO35320		AHC98665		TC38196
	AEO34447		AHC98666		TC40138
	AEO34349		AHC98667		TC40197
	AE034314		AHC98668		TC40226
	AE034313	*R. microplus*	AHC98669		TC40411
	AE034312		AAP75707		TC40562
	AE034279		TC16456_2		TC40843
Amblyomma maculatum	AE034218		EST767976_2		TC40980
	AE032154		EST896705_3		TC41916
	AE032160		ADK62395		TC48141
	AE032217		ADK62396		TC50198
	AE032541	*Ixodesricinus*	ABI94058		XP_002402925
	AE032759		JAA66227		EW874987
	AE032774		JAA66279		DN974443
	AE033019		JAA66964		EW860426
	AE034217		JAA67593		XP_002416236
	DAA34267		JAA67756		XP_002415307
A. variegatum	DAA34257		JAA69032		XP_002407493
	DAA34183		JAA71154		XP_002399564
	DAA34478		JAA71155		XP_002435393
Haemaphysalislongicornis	BAD11156		JAA71156 JAA72548		XP_002434763 EW869079
Rhipicephalushaemaphysaloides	AFX65224		gi|310689892		XP_002434444
	AFX65225		gi|310689891		XP_002433376
R. appendiculatus	AAK61377		JAA71163		XP_002415208

Source tick species	Accession#	Source tick species	Accession#	Source tick species	Accession#
	AAK61378		JAA66228		XP_002413438
	AAK61376		JAA73759		XP_002416263
	AAK61375		JAA72989		XP_002411933
	JAA63611		JAA72940		XP_002411931
	JAA63258		JAA72595		XP_002402370
	JAA54387		EW949864		XP_002411045
R. pulchellus	JAA54315	*I. scapularis*	EW837780		XP_002401187
	JAA54314		EW881762		XP_002400954
	JAA54313		EW901007		XP_002403236
	JAA54312		EW814209		XP_002401986
	JAA54311		EW860426		XP_002415891
	JAA54310		EW882018		XP_002415890
	JAA54309		ISCW017295		XP_002415888
	JAA54308		ISCW011017		XP_002415887
	JAA54307		ISCW024435		XP_002415886
	JAA54306		ISCW006062		XP_002415308
	JAA54167		ISCW023208		XP_002416681
	JAA53966		ISCW024387		XP_002416635
R. microplus	AHC98652		ISCW023617		XP_002408111
	AHC98653		ISCW024109		XP_002416150
	AHC98654		ISCW023621		XP_002415892
	AHC98655		ISCW010422		XP_002411932
	AHC98656		EW851734		XP_002402368
	AHC98657		EW959899		XP_002399745
	AHC98658		EW955724		XP_002405749
	AHC98659		AAM93649		ABJB011030283
	AHC98660		AAV80788		ABJB010229324
	AHC98661		ACI446630		ABJB011108013
	AHC98662		TC36450		

Source tick species	Accession#	Source tick species	Accession#	Source tick species	Accession#
	AHC98663		TC54356		

Table 4

***A. americanum* serpins (AAS) that have orthologs in other *Amblyomma* spp ticks**

AAS ID	Other tick best match	% ID
1	Amac-AEO32759	**71**
2	Amac-AEO32759	**71**
3	Amac-AEO32759	**70**
4	Amac-AEO34312	**79**
	Amac-AEO34279	**89**
5	Amac-AEO34312	**75**
	Amac-AEO34279	77
6	Amac-AEO34312	**75**
	Amac-AEO34279	**82**
7	Amac-AEO35533	**87**
	Amac-AEO35520	**86**
	Amac-AEO32774	**72**
	Amac-AEO32541	**65**
	Amac-AEO32217	**66**
	Amac-AEO32160	**70**
	Amac-AEO32154	**71**
8	Amac-AEO34447	**82**
	Amac-AEO34313	**64**
	Amac-AEO33019	**71**
9	Amac-AEO34447	**79**
	Amac-AEO34313	**62**
	Amac-AEO33019	**69**
25	Amac-AEO35533	**81**
	Amac-AEO35520	**76**
	Amac-AEO32774	**70**
	Amac-AEO32541	**63**
	Amac-AEO32217	**64**
	Amac-AEO32160	**67**
	Amac-AEO32154	**70**
26	Amac-AEO35533	**64**
	Amac-AEO35520	**64**
	Amac-AEO32774	**62**
	Amac-AEO32541	**77**
	Amac-AEO32217	**79**
	Amac-AEO32160	**62**
	Amac-AEO32154	**61**
27	Amac-AEO34349	**81**
28	Amac-AEO34314	**62**
29	Amac-AEO35533	**62**
	Amac-AEO35520	**59**
	Amac-AEO32774	**67**
	Amac-AEO32160	**61**
	Amac-AEO32154	**65**
30	Amac-AEO34312	**76**

A. americanum serpins (AAS) that have orthologs in other _Amblyomma_ spp ticks

AAS ID	Other tick best match	% ID
10	Amac-AEO34447	**82**
	Amac-AEO34313	**64**
	Amac-AEO33019	**72**
11	Amac-AEO34447	**63**
	Amac-AEO34313	**69**
12	Amac-AEO34447	**78**
	Amac-AEO34313	**89**
13	Amac-AEO34447	**74**
	Amac-AEO34313	**78**
14	Amac-AEO34447	**74**
	Amac-AEO34313	**80**
15	Amac-AEO34447	**73**
	Amac-AEO34313	**77**
16	Amac-AEO33019	**75**
	Amac-AEO34447	**72**
	Amac-AEO34313	**75**
17	Amac-AEO34447	**75**
	Amac-AEO34313	**79**
18	Amac-AEO34447	**68**
	Amac-AEO34313	**76**
19	Amac-AEO32217	**91**
	Amac-AEO34218	**91**
22	Avar-DAA34478	**71**
	Amac-AEO34279	**76**
36	Amac-AEO34314	**59**
37	Amac-AEO35533	**67**
	Amac-AEO35520	**65**
	Amac-AEO32774	**71**
	Amac-AEO32541	**70**
41	Amac-AEO32217	**73**
	Amac-AEO32160	**68**
	Amac-AEO32154	**71**
46	Avar-DAA34478	**73**
	Amac-AEO34447	**69**
	Amac-AEO34313	**76**
	Amac-AEO33019	**75**
47	Amac-AEO34314	**79**
67	Amac-AEO34313	**70**
	Amac-AEO33019	**74**

A. *americanum* serpins (AAS) that have orthologs in other *Amblyomma* spp ticks

AAS ID	Other tick best match	% ID	AAS ID	Other tick best match	% ID
	Amac-AEO34447	**69**			
	Amac-AEO34313	**74**			
	Amac-AEO33019	**86**			
23	Amac-AEO32759	**66**			
24	Amac-AEO34312	**77**			
	Amac-AEO34279	**86**			

B: AAS amino acid identity to serpins in metastriata and prostriata ticks

AAS ID	Other tick best match	% ID	AAS ID	Other tick best match	% ID	AAS ID	Other tick best match	%ID
	Rhaem-AFX65224	**61**					Rpulc-JAA54306	**66**
1	Rapp-AAK61377	**69**		Rapp-AAK61377	**66**		Rpulc-JAA54314	**71**
	Rmic-AAP75707	**68**		Rapp-AAK61375	**62**	45	Rpulc-JAA54313	**71**
	Rmic-TC16466	**73**		Rmic-TC24850	**66**		Rpulc-JAA54312	**68**
				Rmic-EST89704	**69**		Ir-XP_02399745	**61**
	Rhaem-AFX65224	**59**		Rpulc-JAA54306	**70**		Ir-XP_002408111	**60**
	Rapp-AAK61377	**66**		Rpulc-JAA54314	**63**			
4	Rmic-AAP75707	**65**	25	Rpulc-JAA54313	**66**	46	Rapp-AAK61378	**64**
	Rmic-TC16466	**67**		Rpulc-JAA54312	**71**		Rpulc-JAA54312	**73**
	Hlong-BAD11156	**73**		Rpulc-JAA54311	**64**			
				Ir-CAB55818.2	**61**	47	Rpulc-JAA63611	**59**
	Rapp-AAK61377	**68**		Is-XP_002434444	**59**			
	Rmic-AAP75707	**66**		Ir-00245308	**70**	50	Rpulc-JAA54309	**76**
6	Rmic-TC16466	**69**		Ir-EW874987	**70**			
	Hlong-BAD11156	**69**				54	Rmic-TC16466 Is-XP_002401187	**90** **58**
						65	Rpulc-JAA54309	**80**
	Rhaem-AFX65224	**74**		Rmic-TC24850	**61**			
	Rmic-TC24850	**69**		Rmic-EST89704	**61**	66	Rpulc-JAA54310 Is-XP_002415891	**70** **59**

B: AAS amino acid identity to serpins in metastriata and prostriata ticks

AAS ID	Other tick best match	% ID	AAS ID	Other tick best match	% ID	AAS ID	Other tick best match	%ID
	Rmic-EST89704	**75**		Rpulc-JAA54306	**61**			
7	Rpulc-JAA54306	**72**	26	Rpulc-JAA54314	**66**	67	Rpulc-JAA54310	**68**
	Rpulc-JAA54314	**69**		Rpulc-JAA54313	**63**			
	Rpulc-JAA54313	**69**		Rpulc-JAA54312	**62**		Rapp-AAK61377	**70**
	Rpulc-JAA54312	**77**		Rpulc-JAA54311	**66**	69	Rmic-AAP75707	**70**
	Hlong-BAD11156	**74**					Hlong-BAD11156	**68**
				Rmic-TC24850	**60**		Rapp-AAK61377	**71**
8	Rpulc-JAA54310	**68**	29	Rmic-EST89704	**62**	70	Rmic-AAP75707	**71**
9	Rpulc-JAA54310	**66**		Rpulc-JAA54310	**74**		Hlong-BAD11156	**73**
10	Rpulc-JAA54310	**66**	30	Rapp-AAK61377	**66**	78	Rpulc-JAA54306	**65**
11	Rmic-TC16466 Hlong-BAD11156	**62** **62**		Rmic-AAP75707 Rmic-TC16466	**66** **68**		Rpulc-JAA54313 Rpulc-JAA54312	**61** **70**
				Hlong-BAD11156	**70**		Rpulc-JAA54314	**63**
12	Rmic-TC16466 Hlong-BAD11156	**72** **74**					Rmic-AHC98652	**62**
			36	Rpulc-JAA63611	**69**		Rmic-AHC98653	**68**
13	Rmic-TC16466	**67**		Rpuc-JAA62387	**68**		Rmic-AHC98662	**61**
14	Rmic-TC16466	**70**					Rapp-AAK61376	**61**
15	Rmic-TC16466	**66**		Rmic-EST89704	**65**		Rhaem-AFX65225	**68**
				Rpulc-JAA54306	**63**		Is-XP_00240811	**61**
16	Rmic-TC16466	**66**						
				Rpulc-JAA54306	**60**		Is-XP_002399745	**61**
17	Rmic-TC16466	**69**		Rpulc-JAA54314	**67**		Ir-JAA66228	**61**
18	Rpulc-JAA54310	**72**	37	Rpulc-JAA54313	**64**		Ir-JAA72595	**61**
				Rpulc-JAA54312	**63**		Ir-JAA66227	**61**
	Rmic-TC22658	**95**		Rpulc-JAA54311	**67**			
	Rmic-EST67697	**82**		Ir-CAB55818.2	**60**			
19	Rpulc-JAA54307	**87**		Is-ACI46630	**61**			

B: AAS amino acid identity to serpins in metastriata and prostriata ticks

AAS ID	Other tick best match	% ID	AAS ID	Other tick best match	% ID	AAS ID	Other tick best match	%ID
	Ir-ABI94058	**81**				114	Rpulc-JAA54310	**75**
	Is-XP_00245308	**82**						
				Rapp-AAK61377	**71**		Rpulc-JAA63611	**67**
			38	Rmic-AAP75707	**70**	121	Rpulc-JAA62387	**67**
	Rpulc-JAA54310	**79**						
20	Is-XP_002401986	**58**	39	Ir-XP_002401986	**68**			
	Rmic-TC17409	**96**	40	Rpulc-JAA54310	**67**			
	Rpulc-JAA63258	**91**		Rapp-AAK61378	**65**			
21	Ir-JAB72483	**67**	41	Rpulc-JAA54310	**76**			
	Is-XP_002401986	**70**						
				Rapp-AAK61376	**79**			
22	Rapp-AAK61378	**61**		Rpulc-JAA54314	**79**			
23	Rhaem-AFX65224	**71**	42	Rpulc-JAA54313	**77**			
	Rpulc-JAA54310	**75**		Is-XP_002403236	**61**			
				Is-XP_002407493	**61**			
	Rapp-AAK61377	**71**						
24	Rmic-AAP75707	**71**	44	Rpulc-JAA54314	**59**			
	Rmic-TC16466	**73**		Ir-XP_02399745	**58**			

Amac = *A. maculatum*, Avar = *A. variegatum*, % amino acid identities are bed

Rapp = *R. appendiculatus*, Rmic = *R. microplus*, Rhaem = *R. haemaphysaloides*, Rpulc = *R. pulchellus*, Ir = *I. ricinus*, Is = *I. scapularis*, and Hlong = *H. longicornis.* % amino acid identities are bolded

Ticks Tick Borne Dis. Author manuscript; available in PMC 2016 February 01.

Table 5

Cross-tick species conserved *A. americanum* serpin reactive center loops

Serpin ID	Conserved RCL amino acid sequence	*% ID	Serpin ID	Conserved RCL amino acid sequence	*% ID
AAS4*	EEGTIAAAVTGLSFVP**I**SALH	-	AAS26	EEGTEAAAATATVAMF**G**SAPS	-
Amac-AE034279	EEGTIATAVTGLSFVA**L**SALS	81	Amac-AE032541	EEGTEAAAATAVIMVF**G**SASS	76
Rapp-AAK61377	EEGTIATAVTGLGFVP**L**SAHY	76	Amac-AE032217	EEGTEAAAATAVIMLF**G**SASS	76
Rmic-AAP75707	EEGTIATAVTGLGFVP**L**SVHY	71	AAS27	EEGTEAAAASGVVGVN**R**IGID	-
Rmic-AHC98654	EEGTIATAVTGLGFVP**L**SAHY	71	Is-EW860426	EEGTEAAAASGVVAVN**R**LIGV	66
Rmic-AHC98661	EEGTVAAAVTGLFVRP**T**APLP	71	Is-XP_002411045	EEGTEAAAASGVVMTR**R**AIEG	75
Rmic-AHC98655	EEGTIAAAVTGLFVMP**S**SSLY	81	Amac-AE033019	EEGTQAAAASGVVGVN**R**IGIE	90
Hlong-BAD11156	EEGTVATAVTGISFIP**L**SAHY	67	Rpulc-JAA54308	EEGTEAAAVSGVVSVN**R**IGIE	86
AAS7	EEGTEAAAATGIAMML**M**CARF	-	Rmic-AHC98657	EEGTEAAAVTGVIGVN**R**IGIE	81
Amac-AE035320	EEGTEAAAATGMAFML**L**SARF	81	AAS28	EEGTEAAAATGMVAMA**R**CAII	-
Ir-JAA66227	EEGTEAAAATAIPIML**M**CARF	86	Rmic-AHC98669	EEGTEAAAATGMVAMA**R**CASM	90
Ir-JAA72595	EEGTEAAAATAVPVMF**C**CAIF	66	AAS29	EEGTEAAAATAVTVVD**G**CMPR	-
Rhaem-AFX65225	EEGTEAAAATAITMMT**Y**CARF	81	Rmic-AHC98662	EEGTEAAAATAVMMVA**C**CMSS	71
Rapp-AAK61376	EEGTEAAAATAITMMT**Y**CARF	81	AAS31	EEGSEAAAVTGVVINT**R**TIGG	-
Rpulc-JAA54312	EEGTEAAAATAITMMT**Y**CARF	81	Rmic-AHC98667	EEGSEAAAVTGVTINT**R**TTTG	81
AAS12	EEGTIAAAVTGLSFVP**I**SALH	-	Rmic-AHC98657	EEGTEAAAVTGVIGVN**R**IGIE	71
Amac-AE034279	EEGTIATAVTGLSFVA**L**SALS	81	AAS37	EEGTEAAAATAVVMMC**R**SAAM	-
Rapp-AAK61377	EEGTIATAVTGLGFVP**L**SAHY	71	Rpul-JAA54314	EEGTEAAAATAVMMMA**C**CMSS	66
Rmic-AAP75707	EEGTIATAVTGLGFVP**L**SVHY	71	Rmic-AHC98662	EEGTEAAAATAVMMVA**C**CMSS	66
Rmic-AHC98654	EEGTIATAVTGLGFVP**L**SAHY	71	Rmic-AHC98669	EEGTEAAAATGMVAMA**R**CASM	71
Rmic-AHC98661	EEGTVAAAVTGLFVRP**T**APLP	71	AAS38	EEGTIATAVTGLSFAP**I**SALH	-
Rmic-AHC98655	EEGTIAAAVTGLFVMP**S**SSLY	81	Amac-AE034279	EEGTIATAVTGLSFVA**L**SALS	86

Serpin ID	Conserved RCL amino acid sequence	*% ID	Serpin ID	Conserved RCL amino acid sequence	*% ID
Hlong-BAD11156	EEGTVATAVTGISFIP**L**SAHY	67	Rapp-AAK61377	EEGTIATAVTGLGFVP**L**SAHY	81
AAS14	EEGTVATAVTGISLVA**L**SALH	-	Rmic-AHC98654	EEGTIATAVTGLGFVP**L**SAHY	71
Amac-AE034279	EEGTIATAVTGLSFVA**L**SALS	81	AAS42	EKGTEAAAATAVMMMA**C**CMSA	-
Rapp-AAK61377	EEGTIATAVTGLGFVP**L**SAHY	67	Rpulc-JAA54314	EEGTEAAAATAVMMMA**C**CMSS	90
Rmic-AAP75707	EEGTIATAVTGLGFVP**L**SVHY	62	Amac-AE032774.1	EKGTEAAAATAVMMVA**C**CLSI	86
Hlong-BAD11156	EEGTVATAVTGISFIP**L**SAHY	Amac-AE032154.1	EKGTEAAAATAVMMVG**C**CLSI	81	
AAS18	EEGSEAAGATAVIFFT**R**GGSS	-	Rmic-AHC98662	EEGTEAAAATAVMMVA**C**CMSS	86
Amac-AE034313	EEGSEAAGATAVIFFT**R**GGIP	90	Rmic-AHC98652	EEGTEAAAATAVMMAA**C**CLSS	81
Avar-DAA34478	EEGSEAAGATAVIFFT**R**GGAP	90	Rapp-AAK61375	EEGTEAAAATAVMMAA**C**CLSS	81
Rmic-AHC98668	EEGTIAAAVTGLFVMP**S**SSLY	71	Is-XP_002405749	EEGTEAAAATAMVMLC**C**MSFP	76
AAS19	EEGSEAAAVTGFVIQL**R**TAAF	-	AAS44	EEGTEAAAATAIITTE**C**CIMP	-
Rpulc-JAA54307	EEGSEAAAVTGFVIQL**R**TAAF	100	Rmic-AHC98662	EEGTEAAAATAVMMVA**C**CMSS	66
Amac-AE034218	EEGSEAAAVTGFVIQL**R**TAAF	100	AAS45	EEGTEAAAATGVVMMC**D**SLPM	-
Amac-AE034217	EEGSEAAAVTGFVIQL**R**TAAF	100	Rmic-AHC98669	EEGTEAAAATGMVAMA**R**CASM	71
Ir-JAA72548	EEGSEAAAVTGFVIQL**R**TAAF	100	AAS47	EEGTEAAAATAMPAAN**S**CEMF	-
Ir-ABI94058	EEGSEAAAVTGFVIQL**R**TAAF	100	Amac-AE034314	EEGTEAAAATAMLASN**S**CERF	86
Is-XP_002415308	EEGSEAAAVTGFVIQL**R**TAAF	100	AAS50	EEGSETDSATLMRISG**K**AAEE	-
Rmic-TC22658	EEGSEAAAVTGFVIQL**R**TAAF	100	Rpulc-JAA54309	EEGSEADSATLLRISG**K**AAEE	90
AAS20	EVGTRAVAATEAQFVS**K**SLVH	-	Rmic-AHC98660	EEGSEADSATLLRISG**K**AAEE	90
Rpulc-JAA54167	EVGTRAVAATQAQFVS**K**SLVH	95	AAS52	ENGTVAAAASAAIGVG**S**AGPS	-
Is-ISCW016489	EEGTRAVAATQAQFVS**K**SLVQ	90	Rpulc-JAA54315	EEGTEAAAATAAVGAG**S**AGPS	76
Rmic-AHC98664	EVGTRAVAATQAQFVS**K**SLVH	95	AAS54	EDGVEGLFLTPLIMMC**Y**AGVS	-
AAS21	EKGTEAVALSSGIIRH**S**KTPG	-	Rmic-AHC98663	EDGVEGLFLTPLIMMC**Y**AGVS	100

Serpin ID	Conserved RCL amino acid sequence	*% ID	Serpin ID	Conserved RCL amino acid sequence	*% ID
Rpulc-JAA63258	EKGTEAVALSSGIIRH**S**KTPG	100	AAS58	EEGTEAAAATGMTLMM**C**GAMV	-
Rmic-AHC98658	EKGTEAVALSSGIVRH**S**KTPG	95	Rmic-AHC98669	EEGTEAAAATGMVAMA**R**CASM	71
Is-XP_002401986	EQGTEAVALSSGIVRH**S**RPPE	95	AAS65	EEGSETDSATLMRISG**K**A-CE	-
Ir-JAB72483	EQGTEAVALSSGIVRH**S**RPPE	90	Rpulc-JAA54309	EEGSEADSATLLRISG**K**AAEE	81
Rmic-AHC98658	EKGTEAVALSSGIVRH**S**KTPG	95	Rmic-AHC98660	EEGSEADSATLLRISG**K**AAEE	88
AAS22	EEGSEAAGATGVIFYT**K**SAIV	-	AAS69	EEGTIAAAVTGLSFVA**T**ASFN	-
Amac-AE034349	EEGSEAAGATGVIFYT**K**SMVV	90	Rmic-AHC98661	EEGTVAAAVTGLFVRP**T**APLP	71
Rpulc-JAA54315	EEGSEAAAATAVIFYT**K**SAAV	86	AAS70	EEGTIATAVTGLSFVA**T**ASFN	-
Rmic-AHC98668	EEGTIAAAVTGLFVMP**S**SSLY	90	Rmic-AHC98654	EEGTIATAVTGLGFVP**L**SAHY	66
AAS23	EEGTVAAAVTSIRMRM**K**SSRR	-			
Is-XP_002434763	EKGTVAAAVTSISMRM**G**SSLA	76			
AAS25	EEGTEAAAATAVVMMC**Y**SLPM	-			
Rmic-AHC98653	EEGTEAAAATAVTLMT**Y**CARI	66			
Rmic-AHC98662	EEGTEAAAATAVMMVA**C**CMSS	66			

Amac = *A. maculatum*, Is = *I. scapularis*, Ir = *I. ricinus*, Rapp= *R. appendiculatus*, Rmic = *R. microplus*, Rpulc = *R. pulchellus*, Rhaem = *R. haemaphyssaloides*, Avar = *A. variegatum*, Hlong= *H. longicornis*. Amino acid residues at P1 sites are bed.