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Using comparative genomics to decode the genetics of acaricide resistance

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1. ABSTRACT

The availability of genome assemblies and other genomic resources is facilitating investigations of complex genetic traits for several species of ticks. Understanding the genetics of acaricide resistance is a priority for tick and tick-borne disease control. The synaptic enzyme acetylcholinesterase (ACE) is recognized as the target of organophosphates (OPs) and carbamates, and mutations in ACE have been tied to resistance. Multiple studies support three ACE (*ace*) loci in *R. microplus* but the molecular basis of OP-resistance in this tick remains elusive. Here, we exploited the genome assembly of the black-legged tick *Ixodes scapularis* and comparative genomic analyses to explore the complement of tick ACEs and their potential roles in OP resistance. We identified eight putative *ace* loci (*IscaACE1a, 1b, 2a-c, 3a-c*) in *I. scapularis*. Molecular analyses and homology modeling suggest ACE activity for IscaACE1a. Our analyses reveal the molecular complexity of the *I. scapularis ace* gene family, highlight the need for functional studies of ACEs in species of the Ixodidae, and reveal potential challenges to management of OP resistance in ticks.

2. INTRODUCTION

2.1. The changing landscape of tick genomics: decoding the genetics of complex traits in the Ixodidae

An improved understanding of complex genetic traits is expected to drive the development of novel tick and tick-borne disease control strategies and is the primary justification for investment in genome research. Traits considered priorities for forward tick genetics research include vector competence and capacity, host preference, and acaricide resistance. The goal to enable tick genomics, first established in the early 2000's (1–4) has become reality. Notable developments include the publication of genome assemblies for the black-legged tick *Ixodes scapularis* (5), the castor bean tick *Ixodes ricinus* (6) and the cattle tick *Rhipicephalus microplus* (2) (Table 1). These resources, together with transcriptome and proteome datasets (6–9), have enabled the first comparative genomic and functional analyses between species of pro- and metastriate ticks. Genome assemblies are also available for several species of mites (10, 11), thus expanding capabilities for genome research to major lineages of the subclass Acari (ticks and mites). Next generation sequencing (NGS) technologies have provided large collections of modern genetic markers for elucidating the molecular basis of phenotypes (5), and deep sequencing is facilitating a greater understanding of the complexity of the tick microbiome (12–14) and its role in determining infectious disease outcomes in the vertebrate host.

2.2. The genetics of acaricide resistance in the Ixodidae

Pesticide resistance is a serious threat to continued tick control. There is a pressing need to better understand the complex molecular mechanisms that underpin resistance in ticks that impact both human and animal health. Resistance studies have focused on the southern cattle tick *Rhipicephalus microplus,* a vector of several bovine pathogens and a major pest of cattle worldwide. Populations of *R. microplus* have been reported with resistance to organophosphates (OPs), synthetic pyrethroids (SPs) and formamidines (15). One population of *R. microplus* from Mexico exhibited resistance to SPs, OPs, the formamidine, amitraz and the macrocyclic lactone, ivermectin (16). This situation highlights the need to decode the molecular systems associated with *R. microplus* resistance to the major acaricide classes and better understand the potential for resistance development in other tick species. Such information will guide development of molecular diagnostics and resistance management strategies. It will also have utility for the discovery of new classes of acaricidal chemistries that operate at non-conserved orthosteric or allosteric sites on existing targets or via novel molecular targets. In short, a "systems biology" approach is required to understand the complement of gene products that produce resistance phenotypes in ticks, and genomics resources provide much-needed tools to achieve this goal.

Insecticide resistance is a complex response involving target-site insensitivity, metabolic detoxification and drug efflux, reduced insecticide penetration as a result of cuticular synthesis, and behavioral avoidance by the arthropod. Multiple adaptive steps have been observed in different arthropods and include point mutations in the acetylcholinesterase (*ace*) and voltage-gated sodium channel (*Kdr*) genes, and amplification and up-regulation of detoxification enzymes, transporters and channels (17–21) The picture that is emerging from studies in mosquitoes suggests a complex interplay between the above mechanisms, and involving multiple genes and alleles (21). While comprehensive, genome-wide studies are lacking in ticks, it is assumed that the "molecular culprits" will include point mutations in the *ace* and *Kdr* loci, and gene amplification and overexpression of members of the major detoxifying (e.g., carboxyl esterases, UDP glucornyltransferases, glutathione S transferases, and cytochrome P450s) and non-detoxifying (e.g., ABC transporters, aquaporins and cuticular synthesis enzymes) proteins implicated in pesticide resistance (22, 23).

Studies of the *I. scapularis* genome revealed an expansion of the genes involved in detoxification processes, with 75 gene models for carboxylesterase (COE)-like proteins and more than 200 cytochrome P450s (CYP450s) (5), suggesting an expanded arsenal of molecular weapons to metabolize pesticides and the potential for rapid resistance development in this tick. Presumably, these and other families of detoxifying genes will be similarly expanded in species of ixodid ticks. The current challenge for the field of tick research is to define the repertoire of detoxifying and non-detoxifying gene products that act in concert to produce resistance phenotypes in key pest species. The availability of genomic resources will enable much needed "systems biology" research and progress towards this goal.

2.3. Acetylcholinesterase loci in the Ixodidae

The OP coumaphos is the backbone of the U.S. Cattle Fever Tick Eradication Program (CFTEP) (15) and thus, an understanding of OP-resistance is a high priority for tick research. The synaptic enzyme acetylcholinesterase (ACE) (E.C. 3.1.1.7.) is an essential enzyme in the transmission of nerve impulses in arthropods, vertebrates and nematodes (24) and the main target of OPs and carbamates (25, 26). ACE catalyzes the hydrolysis of the neurotransmitter acetylcholine, producing acetate and choline, and thus

Table 1. Summary of genome sequencing projects for species comprising the subclass Acari

Species	Common name	Sequencing Technology; Fold-Coverage	GenBank Assembly Accession	Total No. Scaffolds/ Contigs; N_{50} (bp)
Superorder Parasitiformes; Order Ixodida (Ticks)				
Ixodes scapularis	Black legged tick	Sanger WGS; 3.8 X	ABJB010000000	369,495; 51,000bp
Ixodes ricinus	Castor bean tick	PacBio; 6 X	GCA_000973045.2.	204,516; 3,065bp
Rhipcephalus microplus	Southern cattle tick	454; 3.4 X	GCA_000181235.2.	175,211[1]; 827bp
Superorders Acariformes and Parasitiformes (Mites)				
Varroa destructor	Varroa mite	454; 5 X	GCA_000181155.1.	184,190[1]; 2,262bp
Sarcoptes scabiei ovis	Scabies mite	Illumina; 174 X	GCA_000828355.1.	19,811[1]; 11,197bp
Metasielius occidentalis	Predatory mite	NA; in progress	NA; in progress	NA; in progress
Tetranychus urticae	Two spotted spider mite	WGS; 8 X	GCA_000239435.1.	640; 2,993,488bp
Dermatophagoides farinae	House dust mite	Illumina HiSeq; 436 X	GCA_000767015.1.	515; 186,342bp
Achipteria coleoptrata	-	Illumina HiSeq; 90 X	GCA_000988765.1.	72,776[1]; 3,360
Hypochthonius rufulus	-	Illumina HiSeq; 70 X	GCA_000988845.1.	153,462[1]; 3,126bp
Platynothrus peltifer	-	Illumina GAIIx; 19 X	GCA_000988905.1.	126,756[1]; 1,236
Steganacarus magnus	-	Illumina GAIIx; 17 X	GCA_000988885.1.	126,765[1]; 1,617bp

[1]denotes contigs; NA, not available; WGS, whole genome shotgun sequencing

terminating the transmission of the nerve impulse (27). Multiple efforts have investigated mechanisms of OP resistance in *R. microplus* (28–33). Point mutations in *ace* genes have been implicated in OP resistance in several species of insects and ticks (34, 35). Alternative resistance mechanisms involving alternative *ace* loci and/or post-translational modifications have also been proposed (31).

The *ace* genes are members of the esterase (EST) gene family that includes COE and butyrylcholinesterase (BCE) genes (36). Many COEs are recognized for their roles in metabolic resistance (19). Variable copy numbers of *ace* genes have been identified among eukaryotes. Vertebrates typically have one copy of *ace*, while most invertebrates have two (37). Three putative *ace* loci have been identified in *R. microplus* and are referred to as *AChE1* (31), *AChE2* (24), and *AChE3* (29). Baculovirus expression was used to produce recombinant proteins of the three *R. microplus* ACEs and allowed the biochemical confirmation of enzyme function. Mutations in the *ace* sequences produced OP-insensitive enzymes, providing further evidence for the involvement of these ACEs in resistance (34). Studies of putative *ace*s identified from the *I. scapularis* and *R. microplus* genomes have reinforced the hypothesis of gene duplication and multiple alleles, which could reduce fitness costs associated with OP-resistance (38). The expression of ACEs in neural and non-neural tissues also suggests separation of structure and function (34). However, there is ambiguity around the contribution of specific *ace* loci to OP resistance in *R. microplus* and the role of ACEs in other tick species (34, 35, 38, 39).

The *I. scapularis* genome assembly IscaW1.1 (5) offers an opportunity to investigate the repertoire of *ace* loci and the suite of molecular mechanisms that could operate in OP resistant ticks. In this "genome to gene" study we present a comprehensive analysis of the *I. scapularis ace*s in an effort to better define the tick cholinergic system and identify candidate OP resistance loci in this tick. Our analyses revealed eight, possibly more, *ace* loci in *I. scapularis*. The ACE family comprised diverse sequences with several members predicted to bind and catalyze acetylcholine and one gene (*IscapACE1a*) that may function as the major *ace* locus. These findings suggest a gene family prone to duplication and with potential for functional redundancy. Our analyses highlight the need for complementary investigations in other species of ixodid ticks and potential challenges to implicating resistance loci and managing acaricide resistance.

3. MATERIALS AND METHODS

3.1. Identification of putative *ace* genes in *I. scapularis*

Protein sequences for the three *R. microplus ace* genes (AChE1, AChE2 and AChE3; accession numbers CAA11702, CAB93511 and AAP92139) were downloaded from the NCBI database and used in tBLASTn searches against the 20,486 *I. scapularis* gene models (IscaW1.1) predicted by automated annotation of the genome and available at VectorBase (www.vectorbase.org). Supercontigs (DS611849-DS981340) from the *Ixodes scapularis* assembly (ABJB010000001-ABJB011141594) were downloaded from VectorBase and candidate *I. scapularis ace*s

were manually annotated using Artemis software V8 (40) (www.sanger.ac.uk/Software/Artemis/v8/). The nucleotide and amino acid sequences were aligned using MultAlign and ClustalW software with default settings (41, 42). ClustalW2 was used to generate alignment files for the putative ACEs, and the COE sequences were used as out-group for the phylogenetic analyses. *Ixodes scapularis ace* genes were named based on similarity to orthologous *R. microplus ace*s. The *I. scapularis ace* gene models were used in iterative tBLASTn searches of the NCBI non-redundant database to identify additional candidate *ace* genes in *I. scapularis* that were subsequently manually annotated and included in phylogenetic analyses.

3.2. Vertebrate and invertebrate *ace* genes

ACE sequences from vertebrates and invertebrates were downloaded from NCBI and used in phylogenetic analyses. Preference was given to sequences predicted from species with genome assemblies or with evidence of ACE biochemical function. In addition, EST-encoding (*est*) genes identified in a study of gene duplication (5, 43, 44) were also included in the phylogenetic analysis.

3.3. Phylogenetic analysis of *ace* genes in the Ixodidae

Two phylogenetic trees were constructed (Figures 5 and 6), one focusing on the EST superfamily and a second on the ACE subfamily. The ACE phylogenetic tree contained 45 ACEs from vertebrates, invertebrates and nematodes. COEs from *C. elegans* (CeleCAR) and *R. microplus* (RmicCzEST9) were used as an out-group. The tree was constructed using the FigTree program (http://tree.bio.ed.ac.uk/software/figtree/). Bootstrap values shown in nodes were based on 100 repetitions. Branch colors and small brackets represent species-specific lineages or lineages within the same clade. The bar shown at the base of the Figures represents the amino acid distance between the sequences. For the EST phylogenetic tree, sequences were first aligned using MUSCLE (45) and the output alignment file was subsequently used to construct a phylogenetic tree using the neighbor-joining method with ProtTest (46). A bootstrap of 1000 replications was used to estimate confidence levels for the tree topology. Trees were visualized using FigTree v1.2.2. (http://tree.bio.ed.ac.uk/software/figtree/).

3.4. Analysis of key amino acid residues associated with *Ixodes scapularis* ACE activity

The putative *I. scapularis* ACEs were aligned to compare amino acid features of functional ACEs and COEs from other species. Site-specific mutagenesis of the *ace* gene of *Torpedo californica* (Pacific eel ray) implicated the following amino acid residues and motifs in catalytic activity and substrate specificity: (i) the choline binding site, a tryptophan residue located at position 84 (W84); (ii) the three residues of the catalytic triad, namely serine, glutamic acid and histidine (S200, E327 and H440) which are located at the bottom of a narrow, deep gorge lined by 14 aromatic residues; (iii) the six cysteines residues (C67, C94, C254, C265, C402 and C521) involved in the three disulphide bonds; (iv) the canonical "FGESAG' motif (position S200), characteristic of the active site of cholinesterases; (v) the phenylalanine residues (F288 and F290) which are conserved in all invertebrate ACE sequences, and (vi) the oxyanion hole composed of two glycines and one alanine residue (G118, G119 and A201). These amino acid residues and motifs were investigated in the *I. scapularis* and *R. microplus* ACEs. Sequences were aligned using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and key residues were identified by comparison to *T. californica*. Amino acid numbering was based on the conventional classification employed for the *T. californica* ACE sequence.

3.5. Three dimensional modeling of *Ixodes scapularis* and *Rhipicephalus microplus* ACEs and hypothetical acetylcholine docking

3.5.1. Modeling of *Ixodes scapularis* and *R. microplus* ACEs

The macromolecular structure of the *T. californica* ACE was obtained from the Protein Data Bank (PDB database) (http://www.rcsb.org/pdb/home/home.do) and used to predict the 3D structure of *I. scapularis* and *R. microplus* ACEs by homology modeling. Hypothetical 3D protein structures were generated using the Python script Modeller 9v5 (http://salilab.org/modeller/release.html). The model quality was estimated using https://prosa.services.came.sbg.ac.at/prosa.php, which compares properties of the model such as surface charge (expressed as the z-score) with known protein homologs. The database used for comparison of models employed crystal structures and NMR data. In the majority of cases, all three models matched in their z-scores. The best z-score was selected for substrate docking. Three dimensional structure modeling and substrate docking experiments were not performed with incomplete sequences for IscaACE2b, 2c, 3b and 3c.

3.5.2. Substrate docking

Substrate docking was performed using the MEDOCK program (http://medock.csbb.ntu.edu.tw). PDB files of all proteins were translated into .pdbq files using the PDB2PQR server (http://pdb2pqr.sourceforge.net). PDB files do not consider charges of amino acid residues and molecule docking was based both on spatial fit as well as charge. Therefore, the PDB file was

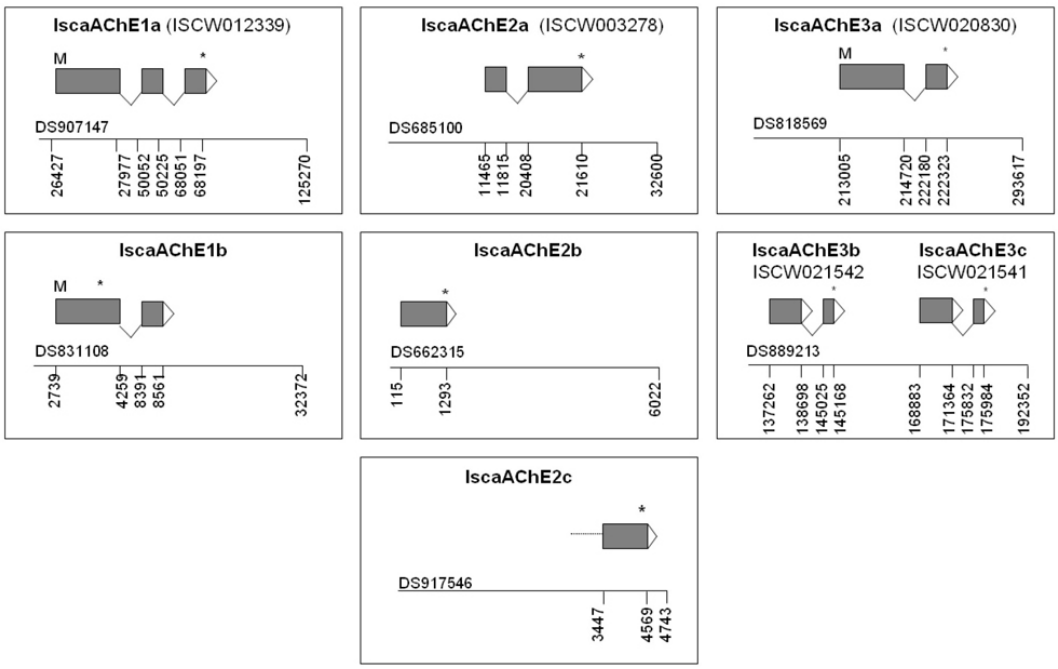

Figure 1. Schematic representation of eight putative ace genes identified in the Ixodes scapularis genome. The gene structure and position of each gene model within the supercontig is shown. The start methionine (M) and stop codon (*), and corresponding VectorBase gene model is shown, where possible. Supercontig and gene model identifiers start with "DS" and "ISCW" prefixes respectively.

transformed into PDBQ (Q designates charge). pdbq files of putative substrates were produced with the Dundee PRODRG2 Server (http://davapc1.bioch.dundee.ac.uk/prodrg) (47). Docking was replicated five times for each of four runs, resulting in a total of 20 molecule-docking predictions for each protein structure under investigation. For each run, the start position of the substrate was selected randomly and the program searched for a global minimum (consideration of space as well as charge) and the position with the lowest energy necessary to retain the docked substrate was selected. Molecules were considered of good fit when docking occurred multiple times at the same position, independent of start site. Protein and docking models were displayed using PyMol (http://pymol.sourceforge.net).

4. RESULTS

4.1. Identification of putative *ace* genes in *I. scapularis*

Eight putative *ace* genes (*IscaACE1a, 1b, 2a-c*, and *3a-c*) were identified in the *I. scapularis* genome by tBLASTn using the *R. microplus* AChE1–3 genes as query (Figure 1; Table 2). The *I. scapularis aces* share less than 70% similarity at the nucleotide level, suggesting that they are not alleles. The amino acid similarity between the *I. scapularis* ACEs identified in this study and the homologous *R. microplus* ACEs, is shown in Table 3. The schematic representation of the *I. scapularis ace* genes and their nucleotide position within supercontigs is shown in Figure 1. Two *I. scapularis* genes (*IscaACE1a* and *IscaACE1b*) were orthologous the *R. microplus* AChE1 gene. The *IscaACE1a* sequence appears to be full-length; the conceptual protein is 623 amino acids in length, has three exons, and contains a start methionine and stop codon. IscaACE1b is 678 amino acids in length, comprises two exons and a start methionine but is missing the stop codon. The first exon of this gene contains a stop codon at amino acid position 404. *IscaACE2a-c* are possible orthologs of the *R. microplus AChE2.* It was not possible to identify the putative start methionine of these genes due to the quality of the *I. scapularis* draft genome. The conceptual proteins are 517, 392 and 374 amino acids in length, respectively. *IscaACE3a-c* are putative orthologs of the *R. microplus AChE3.* IscaACE3a is 620 amino acids in length, possesses both a start methionine and stop codon, and comprises two exons. IscaACE3b and IscaACE3c are 544 and 525 amino acids in length, respectively; both lack a putative start methionine but possess a stop codon. Alignment of the *I. scapularis* and *R. microplus* ACEs with the *T. californica* ACE revealed limited amino acid similarity between sequences (Figures 2–4, 7; Table 4).

4.2. Molecular and phylogenetic analyses

4.2.1. Phylogenetic analysis of *est* loci in the Ixodidae

A phylogenetic analysis of the EST superfamily was constructed using a total of 148 sequences, including ACEs, BCEs, COEs and ESTs,

Table 2. Putative *ace* genes identified in the *Ixodes scapularis* IscaW1.1 assembly

I. scapularis Gene	VectorBase Gene Model	Supercontig	Base Pair Start-End Position	Amino acid length
IscaACE1a	ISCW012339	DS907147	26427- 68197	623
IscaACE1b	NA	DS831108	2739–8561	562
IscaACE2a	ISCW003278	DS685100	11465–21614	517
IscaACE2b	NA	DS662315	115–1293	390
IscaACE2c	NA	DS917546	3447–4569	374
IscaACE3a	ISCW020830	DS818569	213005–222323	620
IscaACE3b	ISCW021542	DS889213	137262–145168	544
IscaACE3c	ISCW021541	DS889213	168883–175984	525

NA, not available

Table 3. Amino acid similarity of *Ixodes scapularis* ACEs to homologs identified in *Rhipicephalus microplus*

I. scapularis ACE	*R. microplus* Homolog (top tBLASTn hit)	GenBank Accession Number	E-value[1]	Percent Amino Acid Identity[2]
IscaACE1a	AChE1 (31)	AJ 223965	0.0.	54%
IscaACE1b	AChE1 (31)	CAA11702.1	$1e^{-139}$	49%
IscaACE2a	AChE2 (24)	AJ278345	$1e^{-145}$	47%
IscaACE2b	AChE2 (24)	CAB93511.1	$3e^{-108}$	47%
IscaACE2c	AChE2 (24)	CAB93511.1	$1e^{-96}$	46%
IscaACE3a	AChE3 (29)	AAP92139	0.0	80%
IscaACE3b	AChE3 (50)	TK215336.1	$4e^{-101}$	40%
IscaACE3c	AChE3 (50)	TK215341.1	$4e^{-176}$	45%

[1]E-value (expected value); [2]Percent amino acid identity = number of residues identical at corresponding positions/number of aligned positions

and the putative *I.* scapularis ACEs identified in this study. A comparative study was performed using ACE sequences from 21 insect species, representing eight different insect orders, as well as seven sequences from the class Arachnida (spiders, scorpions, mites and ticks). Sequences for BCEs, COEs and ESTs were used as out-groups (Figure 5; Table 5).

A comprehensive search of the Iscaw1.1 scaffolds identified multiple candidate *est* genes in addition to the *IscaACE1–3* genes. We identified and selected 17 full-length genes predicted to encode for products with amino acid similarity to ESTs from vertebrates and invertebrates, and employed these in a comprehensive phylogenetic analysis (Figure 5) that identified nine major clades. The ACE1 "locus 1" included three clades comprising tick ACEs and invertebrate and vertebrate ACE1s. The ACE1 "locus 2" clade comprised only invertebrate gene products. Nematode ACE2, 3 and 4 formed a separate clade. All but one (ISCW013301) of the 17 full-length *I. scapularis* gene products formed clades within the putative ACE2 and ACE3 tick clades. ISCW013301 clustered with sequences from the hemichordate, *Saccoglossus kowalevskii* (acorn worm) at the base of the ACE1 and ACE2 clade. Additionally, we identified a basal clade composed of ESTs and a clade comprising BCEs.

The tick sequences formed a separate clade within the ACE1 "locus 1" clade. IscaACE1a clustered with insect ACE "locus 1" sequences, whereas IscaACE1b clustered in a tick specific clade within the ACE1 "locus 1" clade. The *C. elegans* ACE1 (CeleACE1) was placed at the base of the arthropod clade for ACE1, while the CeleACE2, CeleACE3 and CeleACE4 sequences clustered separately at a more basal position of the clade that includes ACE1 "locus 1" sequences from arthropods, mammals and BCE sequences.

The *I. scapularis* and *R. microplus* ACE2 and ACE3 clades, as well as the 17 putative *I. scapularis* ESTs employed in this study, clustered in separate clades that were basal to the invertebrate ACE "locus 1" and "locus 2" clades. The EST sequences were the most basal sequences in the phylogenetic analysis.

4.2.2. Phylogenetic analysis of *ace* loci in the Ixodidae

Invertebrate ACEs formed distinct clades corresponding to the first and second *ace* loci (ACE1 locus 1 and 2 clades) (Figure 6). IscaACE1a clustered with insect sequences in the ACE1 clade. ACE1 sequences from other tick species formed a separate

Figure 2. Amino acid alignment of the ACE1-like acetylcholinesterases from *Rhipicephalus microplus* (RmicAChE1), *Ixodes scapularis* (IscaACE1a and IscaACE1b) and *Torpedo californica* (TcalACE) genes. * amino acid conserved among all sequences; : conserved amino acid substitution; . semi-conserved amino acid substitution.

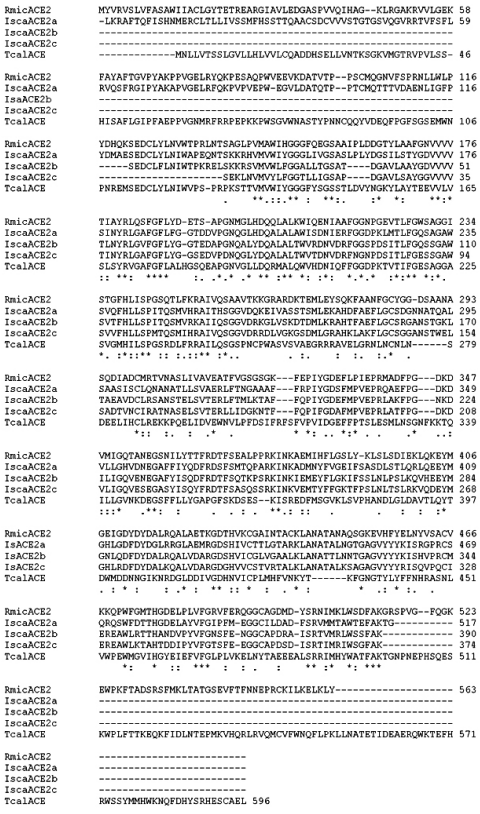

Figure 3. Amino acid alignment of the ACE2-like acetylcholinesterases from *Rhipicephalus microplus* (RmicAChE2), *Ixodes scapularis* (IscaACE2a, IscaACE2b and IscaACE2c) and *Torpedo californica* (TcalACE) genes. * amino acid conserved among all sequences; : conserved amino acid substitution;. semi-conserved amino acid substitution.

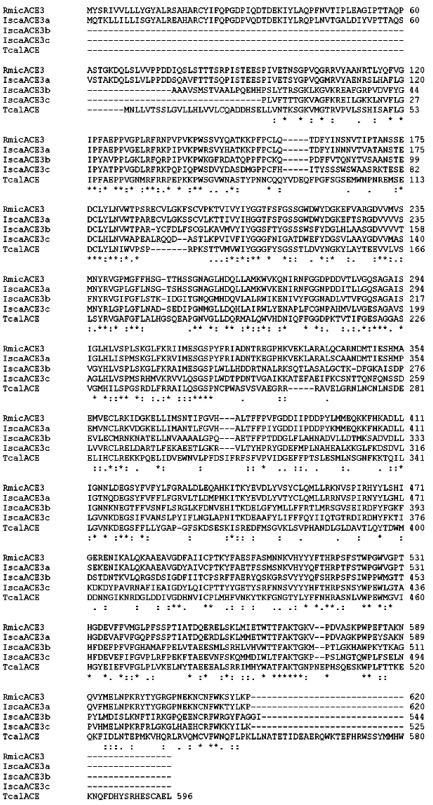

Figure 4. Amino acid alignment of the ACE3-like acetylcholinesterases from *Rhipicephalus microplus* (RmicAChE3), *Ixodes scapularis* (IscaACE3a, IscaACE3b and IscaACE3c) and *Torpedo californica* (TcalACE) genes. * amino acid conserved among all sequences; : conserved amino acid substitution;. semi-conserved amino acid substitution.

Table 4. Key amino acids associated with acetylcholinesterase catalytic activity in the ACEs of *Ixodes scapularis* and *Rhipicephalus microplus*

Species	***T. californica***	***R. microplus***			***I. scapularis***							
Protein	ACE	AChE1	AChE2	AChE3	ACE1a	ACE1b	ACE2a	ACE2b	ACE2c	ACE3a	ACE3b	ACE3c
No. amino acids	586	595	563	620	623	562	517	390	374	620	544	525
Catalytic triad [1]	S200	S222	S230	S269	S237	S233	S231	S106	S90	S289	S212	S194
	E327	E347	E356	E398	E363	E358	E358	E23	E217	E418	E340	E323
	H440	H460	H476	H512	H477	H464	H479	H354	H338	H532	H454	H437
Disulfide bond [2]	C67	C86	C101	C135	C105	C98	C101	-	-	C156	C79	C62
	C94	C113	C125	C157	C132	C125	C125	C4	-	C177	C101	C84
	C254	-	C284	C322	C291	C284	C28	C160	C144	C342	C265	C247
	C265	-	C300	C339	C304	C298	C302	C177	C161	C359	C281	C264
	C402	C422	C432	C474	C439	C426	C435	C310	C294	C494	C416	C399
	C521	C544	C554	C591	C561	C550	-	-	-	C611	C533	C516
Choline binding site[3]	W84	W103	W114	-	W122	W115	-	-	-	-	-	-
Oxyanion hole [4]	G118	G149	G149	G187	G155	G148	G149	G28	G12	G207	G130	G112
	G119	G150	G150	G188	G156	G149	G150	G29	G13	G208	G131	G113
	A201	A223	A231	A270	A238	A231	A232	A107	A91	A290	A213	A195
Acyl pocket [5]	F288	F309	-	-	F326	F321	-	-	-	-	-	-
	F290	F311	F324	F364	F328	F323	F326	F201	F185	F383	F305	-

The tick ACEs are shown in comparison to the *Torpedo californica* ACE. Dashes (-) represent positions in the amino acid sequence that lack the expected amino acid. [1]Catalytic triad (51); [2] Disulfide bonds (52); [3] Choline binding site (53); [4] Oxyanion hole (54); [5]Acyl pocket (37). Single letter amino acid code: A: Alanine; C: Cysteine; E: Glutamic acid; F: Phenylalanine; G: Glycine; H: Histidine; S: Serine; W: Tryptophan

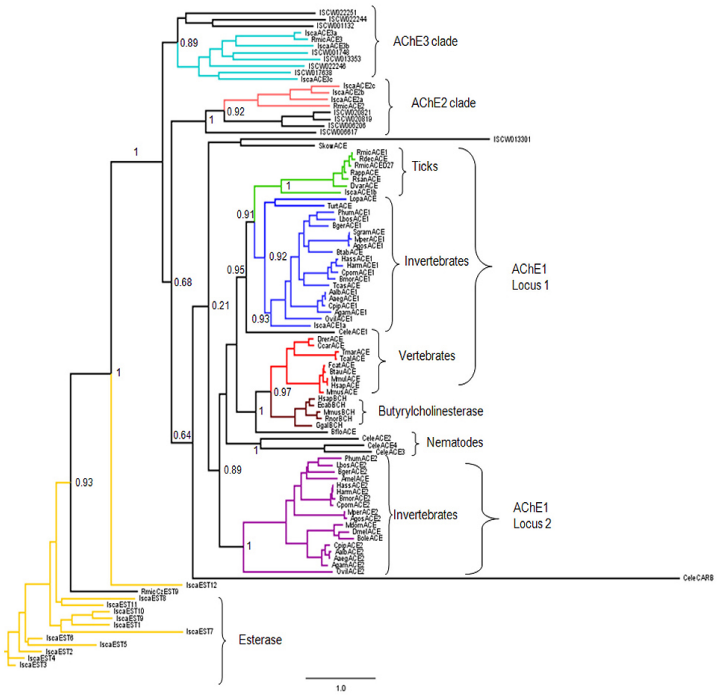

Figure 5. Unrooted maximum likelihood tree showing the phylogenetic relationships between the putative ACE proteins of *Ixodes scapularis* and *Rhipicephalus microplus* and ESTs from vertebrates and invertebrates. The invertebrate ACE1 clade is divided into locus 1 and locus 2 based on catalytic activity of proteins.

tick-specific clade within this clade. The *C. elegans* ACE 1 (CeleACE1) was positioned at the base of this clade. Vertebrate ACEs clustered together, as did the remaining three ACEs from *C. elegans* (CeleACE2, CeleACE3 and CeleACE4). Invertebrate sequences corresponding to the second *ace* locus (ACE2 clade) clustered together, forming a clade basal to the ACE1 clade. Two COEs used as an out-group (CeleCARB and RmicCzEST9) were positioned at the base of the ACE1 and ACE2 clades. ACE2 and ACE3 orthologs from *I. scapularis* and *R. microplus* formed two separate clades, basal to ACE1 sequences. ACE3 orthologs were the most basal among all ACE sequences analyzed in this tree.

4.3. Analysis of key amino acids residues associated with *I. scpaularis* ACE activity

Site-specific mutagenesis studies of the *ace* gene of *T. californica* implicated the multiple amino acid residues and six motifs in catalytic activity and substrate specificity. Each of the *I. scapularis* and *R. microplus* gene models were reviewed for the presence of conserved residues associated with the six ACE motifs identified in *T. californica*. The *I. scapularis* ACE sequences ranged from 500–600 amino acids in length. The amino acids comprising the catalytic triad in *T. californica* (S200, E327 and H440) were conserved in *I. scapularis* and *R. microplus* ACEs (Figure 7). Amino acids associated with the formation of the three disulfide bonds (C67, C94, C254, C265, C402 and C521) and the residues forming the oxyanion hole (G18, G119 and A201) were partially conserved in *I. scapularis* ACEs. The anionic choline binding site (W84) and the acyl pocket formed by two phenylalanine residues (F288 and F290) implicated in acetylcholine substrate specificity and inhibition, were present only in the ACE1 copies of both ticks. The canonical motif "FGESAG", identified in *T. californica* and conserved in invertebrate ACEs, was also conserved in the *I. scapularis* and *R. microplus* ACE1 sequences. However, an E to Q substitution was observed in this motif in IscaACE2a, IscaACE2b, IscaACE3a and IscaACE3b and the *R. microplus* ACE3. In *T. californica,* this particular substitution is believed to confer loss of substrate inhibition (27).

4.4. Three dimensional modeling of *I. scapularis* and *R. microplus* ACEs and hypothetical acetylcholine substrate docking

Three dimensional structure modeling and substrate docking experiments predicted docking of acetylcholine at the main catalytic site (S200 of the catalytic triad) in IscaACE1a and b (Figure 8) and an alternative entry site to the catalytic center. This characteristic is observed in the *T. californica* ACE and is believed to increase the enzyme turnover rate and substrate specificity (27). Modeling of the *R. microplus* ACE1 did not produce a high quality three-dimensional structure suitable for docking experiments. Three dimensional structure modeling and substrate docking

Table 5. Vertebrate and invertebrate EST and ACE sequences used in phylogenetic analyses

Phylogenetic Classification	Species	GenBank Accession Number	Abbreviation Shown in Tree
Acetylcholinesterase Proteins			
Vertebrata	*Homo sapiens*	AAA68151.1	HsapACE
	Bos taurus	P23795.2	BtauACE
	Felis catus	062763.1	FcatACE
	Mus musculus	P21836.1	MmusACE
	Macaca mulatta	NP_001121560.1	MmulACE
	Cyprinus carpio	BAH11081.1	CcarACE
	Danio rerio	Q9DDE3.1	DrenACE
	Torpedo californica	P04058.2	TcalACE
	Torpedo marmorata	P07692.2	TmarACE
Nematoda	*Caenorhabditis elegans*	P38433	CeleACE1
	"	O61371	CeleACE2
	"	O61459	CeleACE3
	"	O61372	CeleACE4
Cephalochordata	*Branchiostoma floridae*	XP_002208203	BfloACE
Hemichordata	*Saccoglossus kowalevskii*	ACH73233	SkowACE
Mollusca	*Loligo opalescens*	AAD15886	LopaACE
Insecta, Diptera	*Aedes aegypti*	ABN09911.1	AaegACE1
	"	S66236	AaegACE2
	Aedes albopictus	BAE71346.1	AalbACE1
	"	BAE71347.1	AalbACE2
	Anopheles gambiae	CAD29865.2	AgamACE1
	"	AGAP000466	AgamACE2
	Culex pipiens	CAD56155.1	CpipACE1
	"	CAJ43752.1	CpipACE2
	Drosophila melanogaster	P07140.1	DmelACE
	Musca domestica	CAC39209.1	MdomACE
	Bactrocera oleae	AAM69920	BoleACE
Hemiptera	*Aphis gossipii*	AAM94376	AgosACE1
	"	AAM94375	AgosACE2
	Bemisia tabaci	ABV45412	BtabACE
	Myzus persicae	AAN71600	MperACE1
	"	JC7990	MperACE2
	Schizaphis graminum	AAK09373	SgraACE
Hymenoptera	*Apis mellifera*	AAG43568	AmelACE
Blatteria	*Blattella germanica*	ABV45412	BgerACE1
	"	ABB89946	BgerACE2
Lepidoptera	*Bombyx mori*	BAF33337	BmorACE1
	"	BAF33338	BmorACE2
	Cydia pomonella	ABB76666	CpomACE1
	"	ABB76665	CpomACE2
	Helicoperva armigera	AAY59530	HarmACE1
	"	AAN37403	harmACE2
	Helicoperva assulta	AAY42136	HassACE1
	"	AAV65638	HassACE2
Psocoptera	*Liposcelis bostryvhophila*	ACN78619	LbosACE1
	"	ABO31937	LbosACE2

Collembola	*Orchesella villosa*	ACL27226	OvilACE1
	"	ACL27227	OvilACE2
Phtiraptera	*Pediculus humanus*	BAF46107	PhumACE1
	"	BAF46106	PhumACE2
Coleoptera	*Tribolium castaneum*	XP_973462.2	TcasACE
Acari	*Tetranychus urticae*	AAO73450	TurtACE
	Dermacentor variabilis	AAP49303	DvarACE
	Rhipicephalus appendiculatus	O62563	RappACE
	Rhipicephalus decoloratus	O61987	RdecACE
	Rhipicephalus sanguineus	AAP49301	RsanACE
	Rhipicephalus (B.) microplus	CAA11702	RmicACE1
	"	CAB93511	RmicACE2
	"	AAP92139	RmicACE3
	Ixodes scapularis	215507026	IscaACE1a
	"	NA	IscaACE1b
	"	215494737	IscaACE2a
	"	NA	IscaACE2b
	"	NA	IscaACE2c
	"	215502075	IscaACE3a
	"	215506018	IscaACE3b
	"	215506017	IscaACE3c
Butyrylcholinesterase Proteins			
Vertebrata	*Equus caballus*	NP_001075319.1	EcabBCH
	Gallus gallus	Q90ZK8	GgalBCH
	Homo sapiens	NP_000046.1	HsapBCH
	Mus musculus	AAH99977.1	MmusBCH
	Rattus morvegicus	NP_075231.1	RmorBCH
Carboxylesterase Proteins			
Acari, Ixodidae	*R. microplus* CzEST9	AAF00497	RmicCzEST9
	Rmic1D27	NA	RmicACED27
Esterase Proteins			
Acari, Ixodidae	*Ixodes scapularis*	215506248	ISCW013301[1]
		215497786	ISCW006617[1]
		215503327	ISCW020819[1]
		215503328	ISCW020821[1]
		215499589	ISCW006206[1]
		215496648	ISCW017638[1]
		215507869	ISCW022246[1]
		215491672	ISCW001748[1]
		215506923	ISCW013353[1]
		215507873	ISCW022251[1]
		215492097	ISCW001132[1]
		215507867	ISCW022244[1]
		215507872	ISCW022250
		215508751	ISCW014233
		215509148	ISCW014784
		215500523	ISCW007945
		215500524	ISCW007946
		215503326	ISCW020818

		215504955	ISCW024669
		215497505	ISCW003776
		215499588	ISCW006205
		215492043	ISCW001079
		215510603	ISCW022937
		215499033	ISCW005431
		215501420	ISCW007846
		215501422	ISCW007848
		215501423	ISCW007849
		215501730	ISCW020835
		215502070	ISCW020825
		215502071	ISCW020826
		215502072	ISCW020827
		215502074	ISCW020829
		215502076	ISCW020831
		215502077	ISCW020832
		215502078	ISCW020833
		215502079	ISCW020834
		215506719	ISCW012483
		215506804	ISCW022870
		215507870	ISCW022248
		215507874	ISCW022252
		215493581	ISCW002384
		215493670	ISCW024191
		215495281	ISCW004315
		215496944	ISCW004387
		215497297	ISCW003637
		215497539	ISCW024384
		215499744	ISCW024484
		215501022	ISCW019926
		215501511	ISCW019823
		215502150	ISCW010310
		215502666	ISCW009205
		215502964	ISCW010323
		215506019	ISCW021543
		215507580	ISCW022051
		215508210	ISCW022036
		215509271	ISCW014999
		215510830	ISCW015340
		215510995	ISCW023963
		215511097	ISCW015477
		215490879	ISCW015800
		215493951	ISCW001875

ACE: acetylcholinesterase; NA, not available. 1Sequences used to construct the esterase/acetylcholinesterase phylogenetic trees shown in Figures 5 and 6

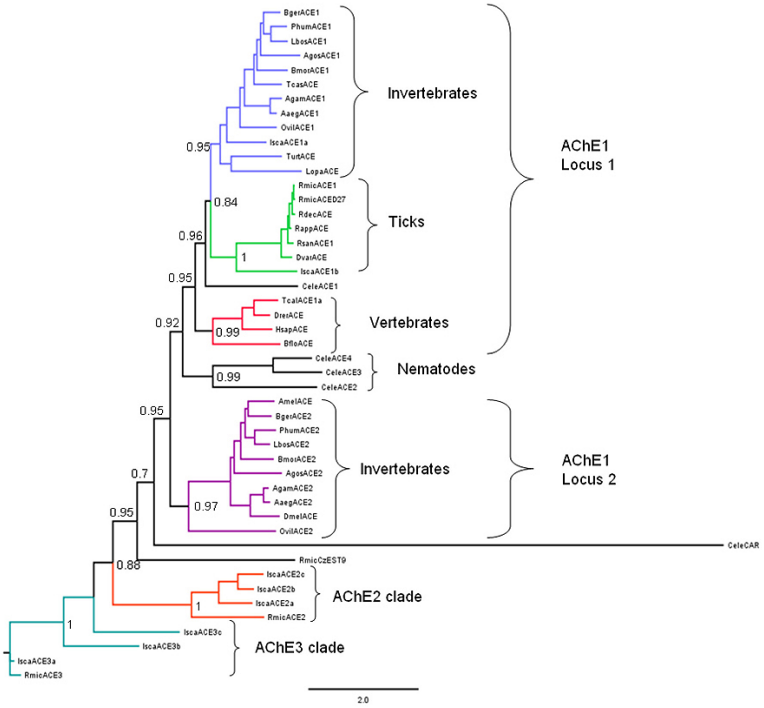

Figure 6. Unrooted maximum likelihood tree showing the phylogenetic relationships between the putative ACE proteins of *Ixodes scapularis* and *Rhipicephalus microplus* and ACEs from invertebrates and vertebrates. The ACE1 clade is divided into locus 1 and locus 2 based on the catalytic activity of ACEs.

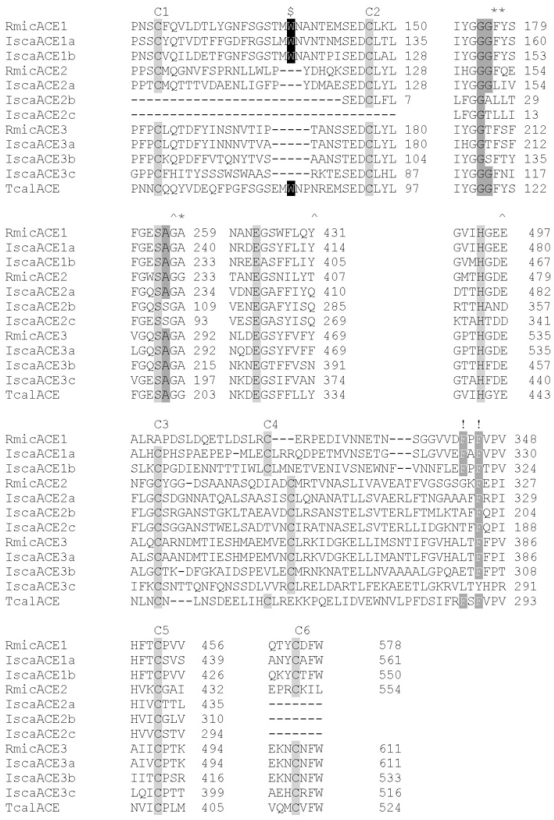

Figure 7. Partial sequence alignment of *Ixodes scapularis, Rhipicephalus microplus* and *Torpedo californica* ACE proteins, highlighting key amino acids involved in functional enzymes.

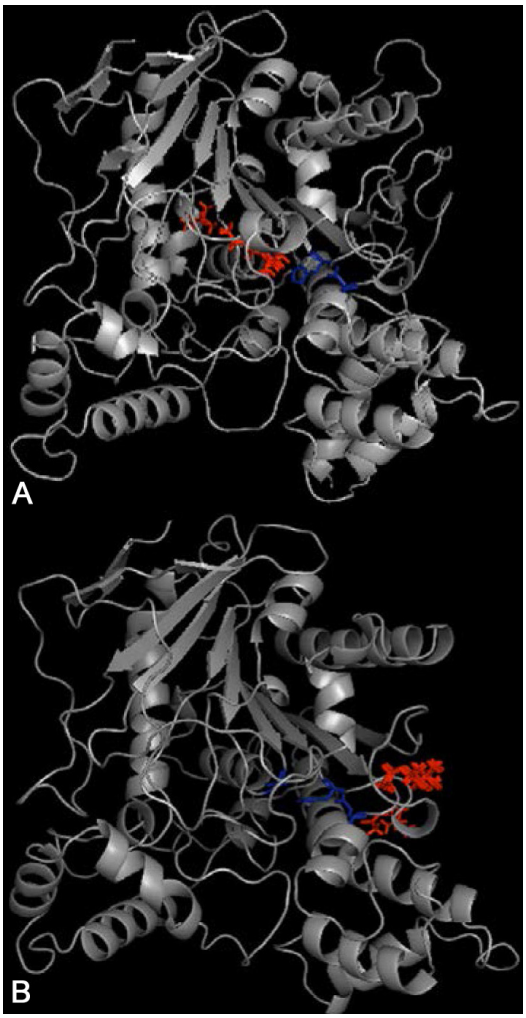

Figure 8. Predicted three-dimensional structure of the *Ixodes scapularis* ACEs. (A) Docking of the acetylcholine (red) molecule at the catalytic ACE catalytic site (blue) in IscaACE1a; (B) Improper docking of the acetylcholine (red) molecule at the catalytic ACE catalytic site (blue) of IscaACE1b. ACE 3-D structure in ticks was predicted by homology modeling using the *Torpedo californica* ACE.

experiments were not performed with the incomplete IscaACE2b, 2c, 3b and 3c. Modeling did not support the binding of acetylcholine to IscaACE2a or the *R. microplus* AChE2. Modeling predicted binding of acetylcholine and choline in the main active binding site of IscaACE3a but did not support the docking of acetylcholine or choline at the catalytic activity site of the *R. microplus* AChE3.

5. DISCUSSION

Point mutations in insect *ace*s confer target-site insensitivity to OPs. The ACE1 locus, one of two *ace* genes in insects, has been associated with the majority (~95%) of catalytic activity in many species (37). We identified eight putative *ace* genes in the *I. scapularis* genome (*IscaACE1a, b, 2a-c*, *3a-c*; Figure 1). Two putative orthologs of the *R. microplus AChE1* were identified in the *I. scapularis* genome *(IscaACE1a and 1b)*. IscaACE1a had 52% amino acid sequence identity to the *R. microplus* AChE1 and clustered with invertebrate ACEs in the ACE 1 “locus 1” clade (Figures 2, 5–6). IscaACE1b had 49% amino acid identity to the *R. microplus* AChE1 and clustered with tick ACEs in a separate clade within the invertebrate ACE 1 “locus 1” clade. The presence of an in-frame stop codon suggests that *IscaACE1b* is a pseudogene, although molecular studies are required to investigate this possibility, especially in field populations of *I. scapularis*. *IscaACE2a-c* are putative orthologs of the *R. microplus AChE2*. IscaACE2a had 49% amino acid identity to the *R. microplus* AChE2 (Figure 3). *IscaACE2b* and *IscaACE2c* are incomplete gene models; the identification of the complete CDS for these genes was complicated by their location in short scaffolds and the presence of scaffolds in gaps. *IscaACE3a-c* are putative orthologs of the *R. microplus AChE3*. IscaACE3a had 82% amino acid identity to the *R. microplus* AChE3 (Figure 4). The *I. scapularis* and *R. microplus* AChE2 and 3 sequences clustered

in separate clades at the base of the ACE tree (ACE2 and ACE3 clades) and shared greatest similarity to invertebrate sequences in the ACE 1 "locus 2" clade (Figures 5–6).

Phylogenetic analysis was used to investigate the *I.* scapularis ACEs and their relationship to members of the EST superfamily, which comprises ACEs, COEs and BCEs (Figure 5). The *I. scapularis* ACEs cluster in three clades (ACE locus 1; ACE2 and ACE3) with invertebrate and vertebrate ACEs and BCEs, and separate from tick putative EST sequences, including the *R. microplus* czEST9 implicated in metabolic detoxification of SPs (30, 39). Baxter and Barker reported that *R. microplus AChE1* gene products had amino acid similarity to vertebrate ACEs (31). This result is supported by our analyses which revealed that the *R. microplus* AChE1 clustered with other tick ACE1 sequences in an invertebrate clade comprising members that share similarity to the vertebrate ACE 1 "locus 1" and BCEs (Figure 6). However, the identification of additional vertebrate and invertebrate *ace*s could change the topology of the phylogenetic trees. Only IscaACE1a and 1b clustered with functional vertebrate and invertebrate ACEs (ACE locus 1 clade) and no tick sequences clustered with sequences forming the invertebrate ACE1 "locus 2" clade, suggesting that this lineage evolved separately in some invertebrates. The location of the tick ACE2 and ACE3 sequences at the base of the ACE lineage suggests ancestral genes and gene products that may be associated with EST functions, although refinements to tick *ace* gene models could change phylogenetic assignment. Seven and 10 full length *I. scapularis* putative *ace*-like genes were identified and clustered in these clades (Figure 5), respectively, suggesting subfamilies prone to gene duplication and increasing the number of candidate loci for functional evaluation.

The *I. scapularis* ACEs possessed key amino acids associated with functional ACEs and ESTs including the catalytic triad, aromatic residues and glycosylation sites (Figure 7). The choline-binding site is essential for the catalytic activity and substrate specificity of ACE. Residues associated with the choline-binding site and acyl pocket were identified only in Isca1a and 1b and the *R. microplus* AChE1, implicating these proteins as functional ACEs. Further support for the above hypothesis is provided by homology modeling of tick ACEs (Figure 8). Despite the substantial divergence among ACEs and esterases at the nucleotide and amino acid level, tertiary structures are conserved among these enzymes. Three-dimensional structure analyses of tick ACEs suggests that the IscaACE1 and 1b and the *R. microplus* ACE1 are capable of binding acetylcholine at the catalytic activity center. Moreover, the alternative entry site identified in IscaACE1a and IscaACE1b was observed in *T. californica* and is believed to increase both enzyme turnover rate and substrate specificity (27). These findings raise questions regarding the enzyme kinetics of tick ACEs. Modeling did not support docking of acetylcholine with IscaACE2a and *R. microplus* AChE2, despite biochemical evidence to the contrary for the latter (34). Acetylcholine must enter through and bind resides at the bottom of the catalytic gorge. The narrow, deep gorge and outer binding site identified in proteins of the ACE2 clade are expected to prevent substrate entry and docking, consistent with EST but not ACE function. Interestingly, modeling revealed docking of both acetylcholine and choline at the main catalytic activity center of IscaACE3a, suggesting an expanded functional role for this protein, and possibly other ACE3 isoforms. Further molecular characterization of the *IscaACE2a* and *IscaACE3a* gene models, together with an increased repertoire of crystallized invertebrate ACEs, could improve the predictive power of the homology-modeling approach for *I. scapularis* and other tick ACEs.

Our analyses of the *I. scapularis ace* loci reinforce the notion of a gene family prone to duplication and functional diversification and raise questions regarding the involvement of tick ACEs in the hydrolysis of acetylcholine and detoxification of xenobiotic compounds. The ACE1 locus 1 is responsible for the majority of catalytic activity in arthropods (37). Phylogenetic and amino acid analyses and 3D structure modeling suggest that *I. scapularis* may possess at least one functional ACE (IscaACE1a). This protein clusters with invertebrate ACE1 proteins in the locus 1 clade that are associated with target-site insensitivity to OPs, making this protein a candidate for functional studies. One ACE1 (*AChE1*) gene has been identified in *R. microplus* to date and further genome sequencing is needed to explore the possibility of additional *ace1* loci in this tick. Biochemical studies and mutational analyses suggest ACE activity of *R. microplus* AChE1, 2 and 3 (34). Interestingly, in phylogenetic analyses, the *R. microplus* AChE1 and ACEs from other tick species clustered separately from functional invertebrate ACE "locus 1" sequences in a basal, tick-specific clade indicative of potential functional diversification in these proteins. Taken together, phylogenetic and modeling data support EST function for tick ACE2s and ACE3s, but the issue of ACE activity in orthologous gene products of species across the phylum remains unresolved, except in the case of *R. microplus*. Future genetic and biochemical studies must clarify the functional roles of tick ACE1–3 proteins and identify the major *ace* locus. This information will be essential to diagnose and track mutations associated with OP resistance in the field.

The *ace* genes identified in this study appear to be the result of gene duplication events in *I.*

scapularis. Our finding that *IscaACE3b* and *IscaACE3c* are tandemly arrayed on the DS889213 supercontig supports this hypothesis and demonstrates that these copies are not haplotypes. Duplicated sequences offer potential for differentiation of physiological functions or compensatory gene effects (19, 48). Evidence suggests that gene duplication is a common phenomenon in *I. scapularis* (43) and population genetic studies support the idea of neo-functionalization as one outcome for genes experiencing positive selection in this species (49). Multiple *ace* loci could generate enzymes capable of functional substitution in ticks. Based on our finding of eight potential *ace* loci in *I. scapularis*, we speculate that *R. microplus* may possess additional *ace*s that could contribute to OP-resistance. The *ace* expansion observed in *I. scapularis* (eight genes) and *R. microplus* (three genes) relative to other invertebrates (typically two genes) challenges notions regarding the *ace* complement in invertebrates. Studies to investigate this phenomenon must be expanded to other species of ixodid ticks and will be facilitated by the generation of genomic resources.

The contribution of detoxifying and non-detoxifying proteins to acaricide resistance also deserves consideration in the case of *R. microplus* and other ixodid ticks. ACE is a member of the EST superfamily, which includes multiple COE species associated with pesticide metabolism in insects. Tick ACEs could confer resistance by overproduction of EST activity and pesticide inactivation, consequently sparing the neural synaptic ACE (29). The COE gene family is significantly expanded in many organisms, often with more than 200 copies per genome (36). Genome analyses identified at least 75 gene models for COE-like gene products in *I. scapularis* (5). Genome-wide studies, coupled with biochemical analyses, are needed to resolve the contribution of ACEs, COEs and other detoxifying enzymes to metabolic resistance in *I. scapularis* and other tick pests.

Our analyses support studies of *ace* genes in *R. microplus* (34). The identification of multiple candidate *ace*s in *I. scapularis* provides an important framework for systematic evaluation of this gene family in multiple ticks of medical and veterinary importance, and for understanding the OP resistance mechanisms. Questions remain regarding the role of the *ace* family in ticks. Future genetic, genomic and functional studies to determine the mechanisms of tick OP resistance must consider a “multi-genic” response and the interplay between insensitivity at ACE targets and metabolic detoxification. This information will enable the development of molecular diagnostic probes to monitor the prevalence of resistance genes within a tick population, and support chemical and genetic management strategies that could reverse the metabolism-based resistant condition and further control the spread of resistance.

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