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A Comparative Analysis of Serpin Genes and Recombinant Proteins from the Salivary Glands of Thai Cattle Ticks, *Rhipicephalus microplus*

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Abstract

The cattle tick, *Rhipicephalus microplus*, is the main ectoparasite of livestock in tropical countries, in Thailand particularly. This tick causes a range of problems, including anemia, widespread production losses, as well as lethal tick-borne diseases in animals. Control of tick infestations is based on the use of chemical acaricides, which have numerous undesirable side-effects, for example, environmental pollution and contamination of the food animals eat. This has led to the development of alternative, environmentally friendly methods of tick control, such as anti-tick vaccines. Tick salivary gland (TSG) proteins are one potential source of vaccine candidates. Molecules secreted from the TSG modulate the vertebrate host immune response, and are thus potential targets for novel tick-control measures. TSG serine protease inhibitor (serpin) is one such molecule, which may facilitate tick feeding, blood meal digestion, and pathogen transmission. In this study, we cloned serpin cDNA from the TSG of the cattle tick (*R. microplus*) by reverse transcriptase-PCR, and analyzed their nucleotides and deduced amino-acid sequences. The results demonstrated that 10 serpin cDNA 1,200 bp in length encoded a serpin protein with 399 amino acid residues, which were 96-98% identical to each other. Based on this result, recombinant serpin protein might be used as an antigen in anti-tick vaccines against *R. microplus* in numerous regions. TSG serpins of Thai *R. microplus* were clustered into groups of serpins belonging to each tick species. Phylogenic analysis of other serpins in the GenBank database indicated that Thai serpin sequences contained minor variations in their amino-acid residues, compared with other tick serpins. Greater numbers of variations have been shown for other arthropods.

Keywords: serpin gene, *Rhipicephalus microplus*, phylogenetic analysis, salivary gland, Thailand

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Introduction

Ticks have a variety of direct effects on cattle in tropical areas, and serve as vectors for etiologic agents of tick-borne diseases, like anaplasmosis,

babesiosis and theileriosis. *Rhipicephalus (Boophilus) microplus*, the 'cattle tick', regularly infests cattle all over the world, and results in significant economic losses. Acaricide treatment is a common means of controlling these parasites, but such chemicals have several disadvantages, including their expense, environmental contamination, chemical residues in animal products, and the development of tick resistance. However, advances in molecular cloning and expression of eukaryotic transcripts over recent decades has made it feasible to consider defined anti-tick vaccines which do not suffer the same disadvantages as chemical acaricides, thus offering promising alternatives for tick control.

One major consequence of the vaccines in current use is a continual decline in tick numbers due to a reduction in tick fecundity. Two categories of candidate in relation to vaccine antigens are, first, 'exposed' antigens, which enter the host during the course of normal tick feeding, and secondly, 'concealed' or 'novel' antigens, which are not normally subjected to the adaptive host immune response [1,2]. Tick saliva contains pharmacologically active molecules, some of which modulate the host immune response. Immuno-modulation at the attachment site facilitates tick feeding, and is likely to enhance pathogen transmission between ticks and vertebrate hosts. However, hosts immunized with tick salivarian antigens can induce anti-tick resistance [3].

Serine protease inhibitors (serpins) are a component of tick saliva, and could be important serine protease regulators with a role to play in inflammation, blood coagulation, and fibrinolysis, and could complement activation in the vertebrate host [4]. Serpins are thought to play an important role in the arthropod immune system; their presence may block the proliferation of pathogens which use proteinase for the invasion of host tissues, for the acquisition of nutrients, and/or for evasion of the arthropod immune system [5]. Serpins are also recognized as a promising vaccine candidate antigen, due to evidence of delayed coagulation time and the inhibition of the thrombin activity associated with significant decreases in tick numbers and egg-mass weights [6-8]. To confirm variations in the efficacy of prospective vaccines, this study cloned and compared variations of serpin genes from the salivary glands of cattle ticks from different parts of Thailand.

Materials and methods

1. Ticks strains

Ticks (*R. microplus*) were collected from cattle in Buriram, Chiang Rai, Chaiyaphum, Khon Kaen, Lampang, Nakhon Phanom, Phayao, Roi Et, Sakhon Nakhon, and Udon Thani provinces, in Thailand.

2. Salivary gland dissection

Ticks were dissected as described by Jittapalapong *et al* [9]. Briefly, under a dissection light microscope, partially-fed ticks were submerged in phosphate buffered saline (PBS; pH 7.4), and held down with a pair of soft tissue forceps. The dorsal cuticle was excised, and salivary glands separated from the other organs by an 18-guage needle. Following dissection, tissues were transferred into RNA stabilizer reagents and kept frozen at -80 °C until use.

3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from adult female *R. microplus'* salivary glands by the acid phenol-chloroform method [10]. RT-PCR was performed using two-step RT-PCR kits (Invitrogen®). Briefly, first strand cDNAs were obtained by reverse transcription using 50 ng of total RNA from TSG, 13 µl of distilled water, 10 mM dNTPs, 2.5 µM Oligo-dT primers, 4 µl of reverse transcriptase buffer, 0.1 M DTT, 1 U Superscript III reverse transcriptase, and 1 U RNase inhibitor (Finnzymes®) at 50 °C for 50 min. The resulting cDNA was amplified by polymerase chain reaction using the specific forward primer 5'-ATGCTCGCCAAATTTCTCTTTCTCG-3' and the specific reverse primer 5'-TAGTGTGTTAACCTCTCCGATGAAA-3'.

Polymerase chain reaction was performed for 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; a final extension was performed at 72 °C for 7 min in a solution 100 µl of 10 µl cDNA templates, 10 µl buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.02 mM dNTPs, 0.10 mM $MgCl_2$, 0.6 pgmol of sense and anti-sense primer, and 5 U DNA polymerase (Invitrogen®) in a Primus 96 plus thermocycler.

4. Construct to cloning vector

The amplified serpin gene was purified using a QIAquick Gel Extraction Kit (QIAGEN®), and ligated to a pGEM-T easy cloning vector. This vector contains the ampicillin resistance gene for positive selection in *E. coli* (Invitrogen®). The ligated plasmids were used to transform *E. coli* strain DH5∝ competent cells. Positive clones were selected using colony screening in LB agar plates containing ampicillin (100 mg/ml) and confirmed using PCR assay.

5. DNA sequencing and computer-assisted sequence analysis

A single colony of *E. coli* positive clones was selected and subcultured in LB media. After overnight growth, plasmid DNA was purified from bacteria culture using QIAprep Spin Miniprep Kit (QIAGEN®) and confirmed by PCR technique. Nucleotide sequencing was performed by the BioService Unit (BSU), National Science and Technology Development Agency (NSTDA), Thailand. The diversity of serpin genes of TSG from different locations was analyzed by MEGA version 3.0 using the neighbor-joining method. Different serpin genes of cattle ticks were compared using the ClustalW program, version 1.83.

Results

Total RNAs were extracted from adult female *R. microplus*' salivary glands and amplified by RT-PCR. RT-PCR products were used in a PCR reaction with serpin gene-specific primers (Fig 1).

Ten serpin genes were cloned in pGEM-T easy vector and transformed to *E. coli* strain DH5∝. Positive clones were confirmed using PCR and corrected for sequencing assay. A combination of 3' and 5' prime T7 and SP6 was used to amplify full-length cDNA encoding serpins. All serpin cDNA were 1,200 bp in length, encoding a serpin protein of 399 amino acid residues (Fig 2).

Comparing the deduced amino-acid sequence with the 10 serpins from each province, the

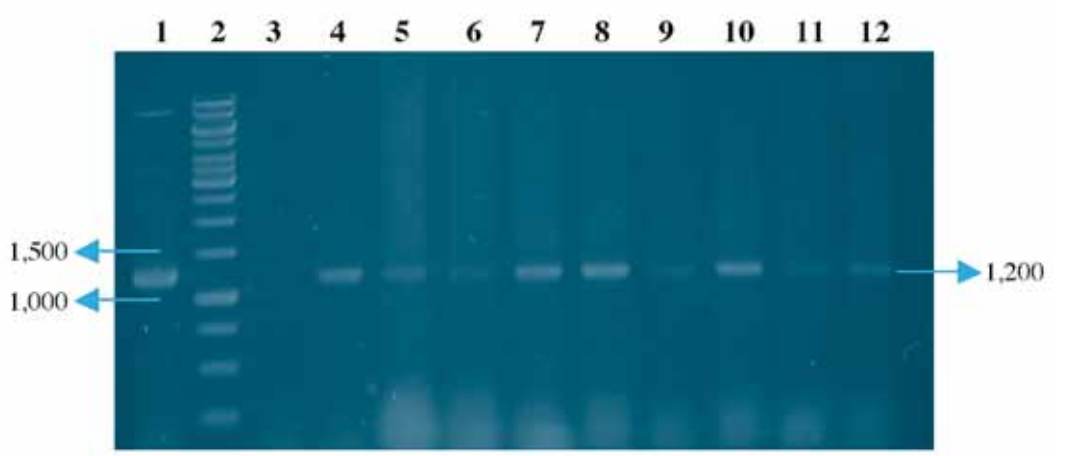

Fig 1 Analysis of PCR products of the serpin gene. Lane 1, 4-12 = PCR products of the serpin gene from Buriram, Chaiyaphum, Chiang Rai, Khon Kaen, Lampang, Nakhon Phanom, Phayao, Roi Et, Sakhon Nakhon and Udon Thani, respectively. Lane 2 = DNA marker and lane 3 = negative control.

nucleotides and amino-acid sequences of Khon Kaen were 98% identical to those from Buriram, Chaiyaphum, Lampang, and 97% identical to those from Chiang Rai, Nakhon Phanom, Phayao, Roi Et, Sakhon Nakhon, and Udon Thani.

For the 10 Thai serpins, their nucleotides and amino acid sequences were 95% identical to other *R. microplus* coming from RNA extracted from the whole tick (accession number AY312432); were 70% identical to *Haemaphysalis longicornis*' rHLS-2 (accession number AB162827); were 92-93% identical to *Rhiphicephalus appendiculatus*' serpin-3 (accession number AAK61377); were 30-31% identical to *R. appendiculatus'* serpin-2 (accession number AAK61376); were 31-32% identical to *R. appendiculatus*' serpin-1 (accession number AAK61375); were 32-33% identical to *Ixodes ricinus*' serpin (accession number CAB55818); were 68-69% identical to *Amblyomma americanum* (accession number EU072742); and were 45-46% identical to *I. scapularis* (accession number XM_002416596).

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KKserpin        MLAKFLFLASALAVAHCETDDSTLLARAHNQFAVNLLKQLATENPSSNVFFSPTSIAAAF  60
CYserpin        MLAKFLFLASALAVAHCETDDSTLLARAHNQFAINLLKELATENPSSNVFFSPTSIAAAF  60
CRserpin        MLAKFLFLASAIAVAQCETDDSTLLARAHNQFAVNLLKELATENPSPNVFFSPTSIAAAF  60
NPserpin        MLAKFLFLASAIAVAHCETDDSTLLARAHNQFAVNLLKELATENPSSNVFFSPTSIAAAF  60
UDserpin        MLAKFLFLASAIAVAHCETDDSTLLARAHNQFAVNLLKELATENPSSNVFFSPTSIAAAF  60
BRserpin        MLAKFLFLASAIAVAHCETDDSTLLARAHNQFAVNLLKELATENPSSNVFFSPTSIAAAF  60
REserpin        MLAKFLFLASAIAVAHCETDDSTLLARAHNQFAVNLLKELATENPSSNVFFSPTSIAAAF  60
PYserpin        MLAKFLFLASALAVAHCETDDSTLLARAHNQFAVNMLKELATENPSSNVFFSPTSIAAAF  60
SKserpin        MLAKFLFLASALAVAHCETDDSTLLARAHNQFAVNLLKELATENPSSNVFFSPTSIAAAF  60
LPserpin        MLAKFLFLASALAVAHCETDDSTLLARAHNQFAVNLLKELATENPSSNVFFSPTSIAAAF  60
Rmi_AY312432    MLAKFLFLASALAVAHCDTDDSTLLARAHNQFAVNLLKQLATENPSSNVFFSPTSIAAAF  60

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CYserpin        GMAYVGARGGSESELNSVFGHTDVGLTDQSRLLTAYKNLLELSASPNVTLDVANMVLAQD  120
CRserpin        GMAYVGARGGSESELNSVFGHADVGLTDRSRLLTAYKNLLELSASPNVTLDVANMVLAQD  120
NPserpin        GMAYVGARGGSESELNSVFGHTDVGLTDRSRLLTAYKNLLELSASPNVTLDVANMVLAQD  120
UDserpin        GMAYVGARGGSESELNSVFGHTDVGLTDRSRLLTAYKNLLELSASPNVTLDVANIVLAQD  120
BRserpin        GMAYVGARGGSESELNSVFGHTDVGLTDRSRLLTAYKNLLELSASPNVTLDVANIVLAQD  120
REserpin        GMAYVGARGGSESELNSVFGHTDVGLTDRSRLLTAYKNLLELSASPNVTLDVANMVLAQD  120
PYserpin        GMAYLGARGGSESELNSVFGHTDVGLTDRSRLLTAYKNLLELSASPNVTLDVANMVLAQD  120
SKserpin        GMAYLGARGGSESELNSVFGHADVGLTDRSRLLTAYKNLLELSASPNVTLDVANMVLAQD  120
LPserpin        GMAYLGARGGSESELNSVFGHTDVGLTDRNRLLTAYKNLLELSASPNVTLDVANMVLAQD  120
Rmi_AY312432    GMAYLGARGGSESELNSVFGHADVGLTDRSRLLTAYKNLLELSASPNVTLDVANMVLAQD  120

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CYserpin        RFPISDSYKQQLREIFDADLRSANFVEDGPRVAAEVNAWVREKTRGKISGILPEGQPLDI  180
CRserpin        RFPISDSYKQQLREIFDADLRSANFVEDGPRVAAEVNAWVREKTRGKISGILPEGQPLDI  180
NPserpin        RFPISDSYKQQLREIFDADLRSANFVEDGPRVAAEVNAWVREKTRGKISGILPEGQPLDI  180
UDserpin        RFPISDSYKQQLREIFDADLRSANFAEDGPRVAAEVNAWVREKTRGKISGILPEGQPLDI  180
BRserpin        RFPISDSYKQQLREIFDADLRSANFAEDGPRVAAEVNAWVREKTRGKISGILPEGQPLDI  180
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SKserpin        RFPISDSYKQQLREIFDADVRSTNFAEDGPRVAAEVNAWVREKTRGKISGILPEGQPLDI  180
LPserpin        RFPISDSYKQQLREIFDADLRSANFVEDGPRVAAEVNAWVREKTRGKISGILPEGQPLDI  180
Rmi_AY312432    RFPISDSYKQQLREIFDADLRSANFVEDGPRVAAEVNAWVRER-QGARSRYPPEGQPLDI  179
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KKserpin        VLFILNAVYFKGTWVTKFDAHRTINKPFLNLGTTEVSKPAMHLKARFPYARVEPLHASAL 240
CYserpin        VLFILNAVYFKGTWVTKFDTHRTINKPFLNLGTTEVSKPAMHLRARFPYARVEPLHASAL 240
CRserpin        VLFILNAVYFKGTWVTKFDAHRTINKPFLNLGTTEVSKPAMHLKARFPYARVEPLHASAL 240
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UDserpin        VLFILNAVYFKGTWVTKFDAHRTINKPFLNLGTTEVSKPAMHLRARFPYARVEPLHASAL 240
BRserpin        VLFILNAVYFKGTWVTKFDAHRTINKPFLNLGTTEVSKPAMHLRARFPYARVEPLHASAL 240
REserpin        VLFILNAVYFKGTWVTKFDAHRTINKPFLNLGTTEVSKPAMHLRARFPYARVEPLHASAL 240
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LPserpin        VLFILNAVYFKGTWVTKFDAHRTINKPFLNLGTTEVSKPAMHLKARFPYARVEPLHASAL 240
Rmi_AY312432    VLFILNAVYFKGTWVT-FDAHRTINKPSS-PGTTEVSKPAMHLKARFPYARVEPLHASAL 237

KKserpin        EIPYEGDRFTMVVLLPDNATGLAAVRNGLSLAALEDVGSRLSFRDVILQLPKFDMSLSYG 300
CYserpin        EIPYEGDRFTMVVLLPDNATGLAAVRNGLSLAALEDVGSRLSFRDVILQLPKFDMSLSYG 300
CRserpin        EIPYEGDRFTMVVLLPDNATGLAAVRNGLSLATLEDVGSRLSFREVILQLPKFDMSLSYG 300
NPserpin        EIPYEGDRFTMVVLLPDNATGLAAVRNGLSLATLEDVGSSLSFREVILQLPKFDMSLSYI 300
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REserpin        EIPYEGDRFTMVVLLPDNATGLAAVRNGLSLAALEDVGSRLSFRDVILQLPKFDMSLSYG 300
PYserpin        EIPYEGDRFTMVVLLPDNATGLAVVRNGLSLAALEDVGSRLSFRDVILQLPKFDMSLSYG 300
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Rmi_AY312432    EIPYEGDRFTMVVLLPDNATGLAAVRNGLSLAALEDVGSRLSFRDVILQLPKFDMSLSYG 297

KKserpin        LVPAMKAIGLNSVFGGSADFSGISEAVPLVISDVLHKAAVEVNEEGTIATAVTGLGFVPL 360
CYserpin        LVPAMKAIGLNSVFGGSADFSGISEAVPLVISDVLHKAAVEVNEEGTIATAVTGLGFVPL 360
CRserpin        LVPAMKAIGLNSVFGGSADFSGISEAVPLVISDVLHKAAVEVNEEGTIATAVTGLGFVPL 360
NPserpin        LVPAMKAIGLNSVFGGSADFSGISEAVPLVISDVLHKAAVEVNEEGTIATAVTGLGFVPL 360
UDserpin        LVPAMKAIGLNSVFGGSADFSGISEAVPLVISDVLHKAAVEVNEEGTIATAVTDLGFVPL 360
BRserpin        LVPAMKAIGLNSVFGGSADFSGISEAVPLVISDVLHKAAVEVNEEGTIATAVTGLGFVPL 360
REserpin        LVPAMKAIGLNSVFGGSADFSGISEAVPLVISDVLHKAAVQVNEEGTIATAVTGLGFVPL 360
PYserpin        LVPAMKAIGLNSVFGGSADFSGISEAVPLVVSDVLHKAAVEVNEEGTIATAVTGLGFVPL 360
SKserpin        LVPAMKAIGLNSVFGGSADFSGISEAVPLVISDVLHKAAVEVNEEGTIATAVTGLGFVPL 360
LPserpin        LVPAMKAIGLNSVFGGSADFSGISEAVPLVISDVLHKAAVEVNEEGTIATAVTGLGFVPL 360
Rmi_AY312432_   LVPAMKAIGLNSVFGGSADFSGISEAVPLVISDVLHKAAVEVNEEGTIATAVTGLGFVPL 357

KKserpin        SAHYNPPPPIEFTVDHPFIFYIRDRSTNRVLFIGEVNTL 399
CYserpin        SAHYNPPPPIEFTVDHPFIFYIRDRSTNRVLFIGEVNTL 399
CRserpin        SAHYNPPPPIEFTVDHPFIFYIRDRSTNRVLFIGEVNTL 399
NPserpin        SAHYNPPPPIEFTVDHPFIFYIRDRSTNRVLFIGEVNTL 399
UDserpin        SAHYNPPPPIELTVDHPFIFYIRDRSTNRVLFIGEVNTL 399
BRserpin        SAHYNPPPPIEFTVDHPFIFYIRDRSTNRVLFIGEVNTL 399
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LPserpin        SAHYNPPPPIEFTVDHPFIFYIRDRSTNRVLFIGEVNTL 399
Rmi_AY312432    SVHYNPPPPIEFTVDHPFIFYIRDRSTNRVLFIGEVNTL 396
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Fig 2 Multiple alignments of the deduced amino acid sequences of Thai *R. microplus* serpin from Buriram, BR; Chaiyaphum, CY; Chiang Rai, CR; Khon Kaen, KK; Lampang, LP; Nakhon Phanom, NP; Phayao, PY; Roi Et, RE; Sakhon Nakhon, SK; and Udon Thani, UD. RNA extracted from tick salivary glands and known serpin sequences from *R. microplus*; RNA extracted from whole tick (accession numbers AY312432) using ClustalW. Reactive center loop (RCL) indicated as double-underlined; consensus regions of serpins in boxes; and predicted possible cleavable signal peptide (amino acid 1-17) indicated as a dot-line.

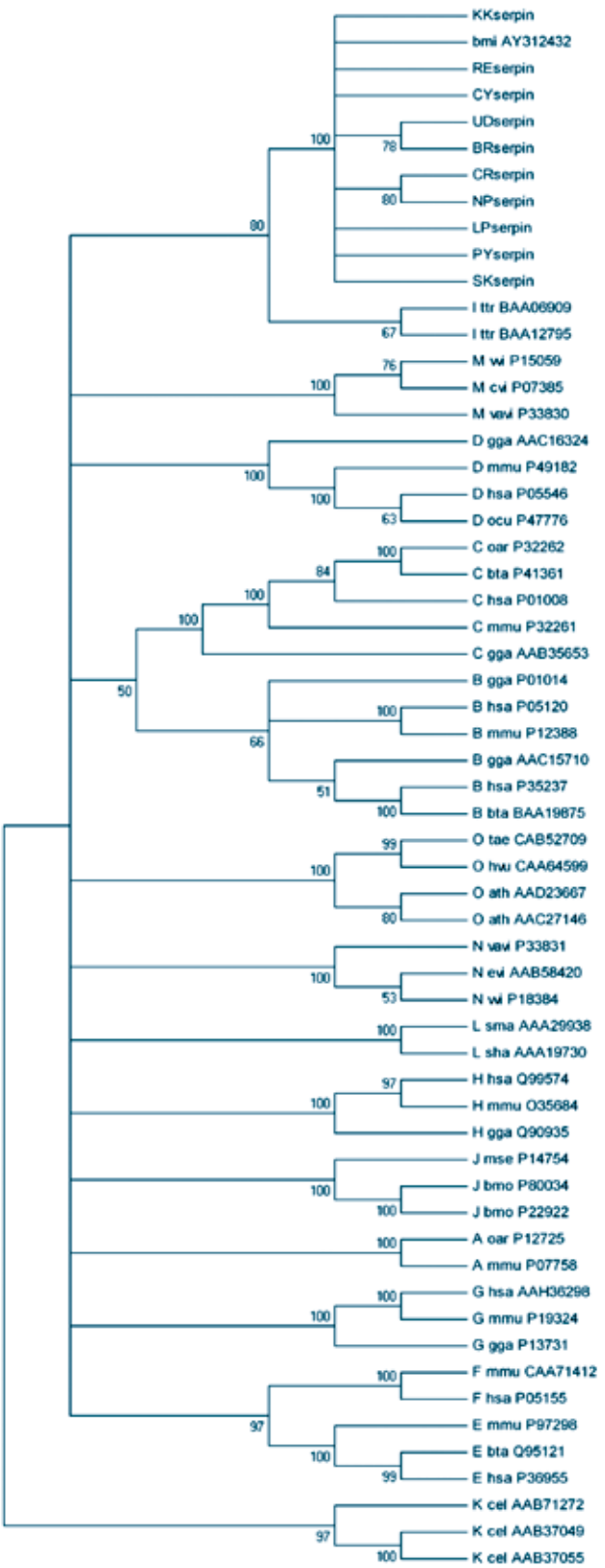
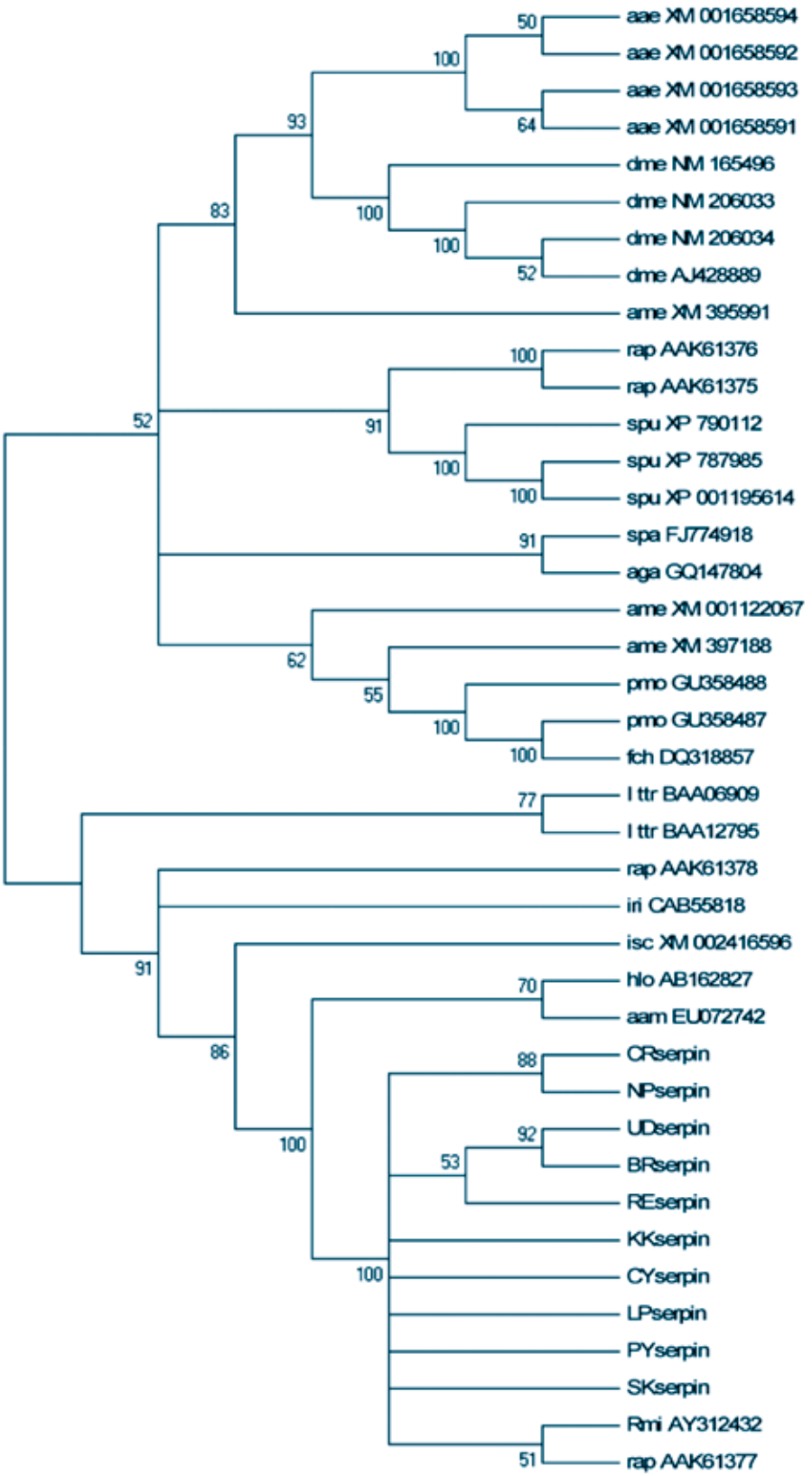


Fig 3 **Phylogeny of *R. microplus* serpin (Buriram, BR; Chaiyaphum, CY; Chiang Rai, CR; Khon Kaen, KK; Lampang, LP; Nakhon Phanom, NP; Phayao, PY; Roi Et, RE; Sakhon Nakhon, SK; and Udon Thani, UD), and of a known serpin superfamily from the GenBank database, using the neighbor-joining method (NJ-JTT model of amino acid substitution). Bootstrap support values > 50% are shown at the nodes (n = 1,000) and drawn using MEGA version. Serpin clades are indicated as capital letters. Sequences are identified by scientific name abbreviations, followed by the GenBank accession number. Species abbreviations: (ath) *Arabidopsis thaliana*; (bmo) *Bombyx mori*; (bta) *Bos taurus*; (cel) *Caenorhabditis elegana*; (cvi) *Cowpox virus*; (evi) *Ectromelia virus*; (gga) *Gallus gallus*; (hsa) *Homo sapiens*; (hvu) *Hordeum vulgare*; (mmu) *Mus musculus*; (mse) *Manduca sexta*; (ocu) *Oryctolagus cuniculus*; (oar) *Ovis aries*; (sha) *Schistosoma haematobium*; (sma) *Schistosoma mansoni*; (ttr) *Tachypleus tridentatus*; (tae) *Triticum aestivum*; (vvi) *Vaccinia virus*; and (vavi) *Variola virus*.**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NPserpin
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BRserpin
REserpin
KKserpin
CYserpin
LPserpin
PYserpin
SKserpin
Rmi AY312432
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83
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62
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70
86
88
100
92
53
100
51

Fig 4 Phylogeny of *R. microplus* serpin (Buri Ram, BR; Chaiyaphum, CY; Chiang Rai, CR; Khon Kaen, KK; Lampang, LP; Nakhon Phanom, NP; Phayao, PY; Roi Et, RE; Sakhon Nakhon, SK; and Udon Thani, UD) and known arthropod serpin sequences from the GenBank database using the neighbor-joining method (NJ-PAM model of amino acid substitution). Bootstrap support values higher than 50% are shown at the nodes (n = 1,000) and drawn using MEGA version. Sequences are identified by scientific name abbreviations, followed by the GenBank accession number. Species abbreviations: (aae) *Aedes aegypti*; (aam) *Amblyomma americanum*; (aga) *Anopheles gambiae*; (ame) *Apis mellifera*; (dme) *Drosophila melanogaster*; (fch) *Fenneropenaeus chinensis*; (hlo) *Haemaphysalis longicornis*; (isc) *Ixodes scapularis*; (oca) *Opisthacanthus cayaporum*; (rap) *Rhipicephalus appendiculatus*; (rmi) *Rhipicephalus microplus* (whole tick); (pmo) *penaeus monodon*; (spa) *Scylla paramamosain*; (spu) *Strongylocentrotus purpuratus*; and (ttr) *Tachypleus tridentatus*.

According to phylogenetic analysis of the serpin superfamily, Thai *R. microplus* serpins – from Buriram, BR; Chaiyaphum, CY; Chiang Rai, CR; Khon Kaen, KK; Phayao, PY; Lampang, LP; Nakhon Phanom, NP; Roi Et, RE; Sakhon Nakhon, SK; and Udon Thani, UD – were a closely related and separate cluster from serpin clades (α1-proteinase inhibitor, A; Intracellular, ov-serpin, B; Antithrombin, C; Heparin cofactor II, D; α2-antiplasmin, PEDF (pigment epithelium derived factor), E; C1 inhibitor, F; Heat shock protein 47, G; Neuroserpin, H; Horseshoe crab, I; Insect, J; Nematode, K; Blood fluke, L; Viral serpin 1 and 2, M; Viral serpin 3, N; Plant, O; and Unclassified (orphans), P; [11] and closely related to the Horseshoe crab, I; serpin clades. The UD serpin was more closely related to the BR serpin, and the CR serpin was more closely related to the NP serpin (Fig 3).

The phylogeny of *R. microplus*' serpin (RNA extracted from salivary glands) and known arthropod serpin sequences from the GenBank database are shown in Fig 4. The results indicate there are two groups of serpin, tick serpins and other arthropod serpins (crab, shrimp, horseshoe crab, spider, mosquito, and fly) (Fig 4).

Discussion

Serpins have been genetically cloned from several tick species, including *R. appendiculatus* [12], *H. longicornis* [13], *B. microplus* [14] and *I. scapularis* [15]. Results have revealed there is potential for using recombinant serpin proteins as anti-tick vaccine antigens for controlling tick infestations in cattle [7,13,16]. Recently, Kaewhom *et al* [17] reported the complete sequence of serpin from *R. microplus* collected from cattle in Thailand. Serpin is one of the potential candidate proteins for an anti-tick vaccine; numerous trials have demonstrated their outstanding efficacy [5,7,13]. Therefore, the biodiversity of serpin genes in different tick locations is required for analysis, since these variations may correlate with efficacy properties.

A possible signal peptide of 10 serpin proteins was present at N-terminal (amino acid position 1-17). The 9 sequences also contained two serpin consensus motifs (NAVYFKG and EVNEEG), which exist in arthropod and mammalian serpin consensus motifs [18,19]. The first E amino acid position of EVNEEG consensus motifs of serpin from Roi Et was substituted by Q amino acid. A comparison of the reactive center loop (RCL) among the 10 serpins showed both nucleotides and amino-acid sequences from the different provinces were 96-98% and 100% identical, respectively. The RCL of serpins plays an essential role in the inhibitor mechanism, by acting as a substrate for their target proteases [20]. Based on a consensus of amino-acid residues in the RCL, Thai serpins are putatively inhibitory since they are predicted at the PI and PI′ positions [16]. This result confirms the potential of using serpin protein due to their having fewer variations as a

candidate antigen for anti-tick vaccine against *R. microplus* in Thailand.

In a phylogenic analysis of the serpin Superfamily, serpins from Thai *R. microplus* revealed a close relationship. Thai salivarian serpins from *R. microplus* were a clearly separated cluster among the serpin clades. Strong statistical support for grouping serpins was divided into 16 clades (A-P) [21]. However, the remaining serpins are orphans, but these might be in additional clades, as more serpin sequences are identified [21]. Although ticks belong to the same phylum (Arthropoda) as insects [22], tick serpin is not classified as part of the same cluster of insect clades (J serpin clade in Fig 3). It remains, however, closely related to the horseshoe crab (I serpin clades) [21]. Ticks and horseshoe crabs are members of the same phylum, Arthropoda, but they are in a different class–Arachnida and Merostomata, respectively [22]. Ticks and other arthropods are classified into different classes within the phylum Arthropoda, and tick serpins are a clearly separated cluster from other arthropod serpins as well (*eg* crab, shrimp, horseshoe crab, spider, mosquito and fly). This suggests, therefore, that tick serpins are a clearly separated cluster from serpin clades and other serpin arthropods.

In summary, 10 cDNA clones of serpins from TSG were collected from cattle in Thailand and isolated by RT-PCR. Ten serpin cDNA, 1,200 bp in length, encoded a serpin protein with 399 amino-acid residues; the deduced amino acids were 96-98% identical to each other. A sequence analysis of the serpins demonstrated Thai serpin sequences have fewer amino-acid variations in most amino-acid residue positions, but more variation than other arthropods. Salivarian serpins from Thai *R. microplus* are clustered into a group of serpins from each of the tick species. This strongly supports the use of a locally made vaccine antigen against Thai cattle ticks, which may also be developed as a vaccine antigen for other ticks.

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