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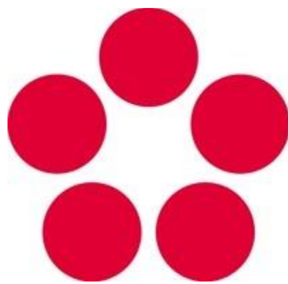

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Characterisation and functional analysis of defensins from the ticks *Ixodes ricinus* and *Ixodes scapularis*

Ph.D. Thesis

MVDr. Miray Tonk, MSc.

Supervisor: Prof. RNDr. Libor Grubhoffer, CSc.

Institute of Parasitology
Biology Centre, Academy of Sciences of the Czech Republic
České Budějovice, 2014

I dedicate this thesis to my family

Herşeyimi borçlu olduğum Aileme ithafen...

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Annotation

The hard-bodied tick *Ixodes ricinus* (castor bean tick) is the most common tick species in Europe and *Ixodes scapularis* (deer tick) is the most common tick species in North America. They are vectors of the causative agents of diseases that affect humans and animals including tick-borne encephalitis, borreliosis, tick-borne fever, anaplasmosis and babesiosis. Ticks are bloodsucking arthropods that have an innate immune defence system that provides them with an efficient defence against pathogenic microorganisms in the event of their penetration into the tick body. Antimicrobial peptides (AMPs) constitute an important feature of the tick immune system. Defensins are a well-known class of AMPs. They exhibit antimicrobial activity against Gram-positive (Gram+) and Gram-negative (Gram-) bacteria, viruses, fungi, yeasts and protozoan parasites. In this study, the identification, functional characterisation of six novel defensins from *I. ricinus* (DefMT2, DefMT3, DefMT4, DefMT5, DefMT6, DefMT7) and functional characterisation of two defensins from *I. scapularis* (Scapularisin-3 and Scapularisin-6) are reported.

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Miray Tonk

Contents

Annotation i

Acknowledgements ii

Contents iv

List of Figures vi

List of Tables vii

Abbreviations viii

Chapter 1. Introduction 1

1.1 *Ixodes ricinus* and *Ixodes scapularis* 1

1.2 Importance of ticks 4

1.3 Tick innate immune system 5

1.3.1 Antimicrobial peptides 6

1.3.2 Defensins 7

1.3.2.1 Characterisation of defensins 7

1.3.2.2 Biosynthesis, gene structure and expression 9

1.3.2.3 Biological activities of defensins 10

1.3.2.4 Defensins against tick-borne diseases 11

1.3.3 Tick defensins 12

1.3.3.1 Hard tick defensins 12

1.3.3.2 Soft tick defensins 17

1.3.4 Application of defensins 19

1.3.4.1 Defensins as novel drugs 19

1.3.4.2 Outlook: defensins 19

Chapter 2. Project Objectives 21

Chapter 3. Materials and Methods 22

3.1 Sample collection 24

3.1.1 Ticks 24

3.1.2 Organ collection 24

3.1.3 Haemolymph collection 25

3.1.4 Cell culture 25
3.2 Nucleic acid analysis 26
3.2.1 RNA extraction 26
3.2.2 Complementary DNA (cDNA) synthesis 27
3.2.3 Isolation of genomic DNA extraction 27
3.2.4 Polymerase chain reaction 28
3.2.5 Agarose gel electrophoresis 30
3.2.6 Cloning and bacteria transformation 31
3.2.7 Miniprep 32
3.2.8 Restriction digest analysis 32
3.2.9 Sequencing 33
3.2.10 Sequence analysis 33
3.2.10.1 Phylogenetic analysis 33
3.2.10.2 Tertiary structure analysis 34
3.2.11 Biochemical property prediction of new defensins 34
3.2.12 Antibacterial assays 34
3.2.13 Antifungal assays 36
Chapter 4. Results **37**
4.1 Characterisation of *I. ricinus* and *I. scapularis* defensins 37
4.2 Phylogenetic and tertiary structure analysis 45
4.3 Expression analysis 50
4.4 Functional analysis 51
Chapter 5. Discussion and Conclusions **59**
5.1 Discussion 59
5.2 Conclusions 67
Chapter 6. Final Remarks **68**
Chapter 7. Published and Submitted Manuscripts **69**

Appendix I A
References I

List of Figures

Figure 1	Distribution of *Ixodes ricinus*	2
Figure 2	Distribution of *Ixodes scapularis*	3
Figure 3	γ-core motif of selected *I. ricinus* defensins	22
Figure 4	Illustration of DefMT4 genomic structure	37
Figure 5	Alignment of nucleotides sequences of eight *I. ricinus* defensins	39
Figure 6A	Amino acid sequences of novel *I. ricinus* defensins	42
Figure 6B	Amino acid alignment of hard and soft tick defensins with *I. ricinus* defensins	43
Figure 7	Comparison of *I. ricinus* isoforms Def1, Def2, DefMT3 and DefMT4 amino acid sequences	44
Figure 8	The primary structures of Scapularisin-3 and Scapularisin-6	44
Figure 9	Phylogenetic tree and predicted 3D structure of representative defensins	45
Figure 10	3D structure of *I. ricinus* defensins	47
Figure 11	Tertiary structure of Scapularisin-3 and Scapularisin-6	49
Figure 12	Expression profile of novel *I. ricinus* defensins	50
Figure 13	Expression profile of Scapularisin-3 and Scapularisin-6	51
Figure 14	Antifungal activity of DefMT3 and DefMT6 against *Fusarium spp.*	53
Figure 15	Antimicrobial activity of Scapularisin-6 against *Listeria grayi*	55
Figure 16	Antifungal activity of Scapularisin-3 and Scapularisin-6 against *Fusarium spp.*	58

List of Tables

Table 1	Molecular mass of γ-core motif of selected *I. ricinus* defensins	23
Table 2A	Primers for *I. ricinus* defensins	29
Table 2B	Primers for *I. scapularis* defensins	30
Table 3	Predicted structure of disulphide bond between cysteine residues	38
Table 4	Amino acid and nucleotide similarities between *I. ricinus* defensins	40
Table 5	Half maximal inhibitory concentration of synthetic DefMT3 and DefMT6	54
Table 6	Minimum inhibitory concentration of synthetic DefMT3 and DefMT5	54
Table 7	Minimum inhibitory concentration of synthetic *I. scapularis* defensins	56
Table 8	Half maximal inhibitory concentration of synthetic *I. scapularis* defensins	57

Abbreviations

aa	Amino acid(s)
AGE	Agarose gel electrophoresis
AMP	Antimicrobial peptide
bp	Base pair(s)
^{o}C	Degrees Celsius
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
gDNA	Genomic DNA
kDa	Kilodalton(s)
M	Molar
mM	Milli molar
ml	Millilitre
µl	Microliter
mRNA	Messenger ribonucleic acid
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
TAE	Tris-acetate-EDTA

Chapter 1: Introduction

1.1 *Ixodes ricinus* and *Ixodes scapularis*

Kingdom: Animal
Phylum: Arthropoda
Class: Arachnida
Subclass: Acari
Superorder: Parasitiformes
Order: Ixodida
Superfamily: Ixodoidea
Families: Argasidae, **Ixodidae**

Ticks are arthropods (phylum Arthropoda) of great medical and veterinary importance (Bell-Sakyi et al., 2007). *Ixodes ricinus* and *Ixodes scapularis* ticks are vectors of a variety of pathogens including bacteria (*Borrelia burgdorferi, Anaplasma phagocytophilum*), viruses (tick-borne encephalitis virus (TBE), louping ill virus), protozoa (*Babesia* spp.) and other organisms pathogenic to both animals and humans with variable degrees of pathogenicity (Dumler et al., 2001; Gray, 1991; Randolph and Storey, 1999; Saito et al., 2009; Schicht et. al., 2012; Schwan and Piesman, 2002; Stanek, 2009; Stiller et al., 2002; Zeidner et al., 2009; Zheng et al., 2012). Additionally, ticks harbour a number of bacterial species including of the genera *Francisella* (Scoles, 2004; Zheng et al., 2012), *Ehrlichia* (Johnson et al., 1998; Murphy et al., 1998; Zheng et al., 2012) and *Rickettsia* (Azad and Beard, 1998; Zheng et al., 2012).

Figure 1. Distribution of *I. ricinus.*

I. ricinus, the castor bean tick, is a three-host tick and can feed on humans and animals. *I. ricinus* is found in Europe, the Northern part of Africa, through Russia and the Middle East **(Figure 1)**. *I. ricinus* undergoes four developmental stages: egg, larva, nymph, and adult requiring three blood meals to complete its development, once each as a larva, nymph and adult (Sonenshine, 1991). It has a three-host life cycle. After hatching from eggs in leaf litter, larvae spread into the vegetation and parasitise the first host. Once engorged, they drop off the host and moult to nymphs. Nymphs seek a second host, feed and moult again. Adults search for a third host, mate and females feed (males do not need to feed as their gametogenesis begins with the moulting, not after feeding as in other genera); after detachment females find an optimal environment, lay eggs and die (Sonenshine, 1991). The life cycle lasts under 1 year favourable conditions but in Central Europe it usually takes 3 years because of a winter diapause in each developmental stage. Over 300 animal species can be parasitised by *I. ricinus.* Typical hosts are small mammals, birds and lizards for larvae and nymphs; bigger animals like deer, sheep, cattle and other ruminants are typical hosts for adult ticks. Humans are rather occasional hosts. *I. ricinus* is the primary vector of significant microorganisms and able to transmit a great variety of important pathogens and cause diseases including Lyme disease and tick-borne

encephalitis (Chrudimská et al., 2011; Rudenko et al., 2007; Schicht et al., 2012). *I. ricinus* is an important vector of some animal pathogens; for example sheep are endangered by a *Flavivirus* of the TBE complex causing louping ill, dogs by canine ehrlichiosis caused by *E. canis* and ruminants by tick-borne fever after infection by A. *phagocytophilum*. Different borrelioses can be a big problem for multiple animal species: dogs, cats, cattle and horses. Tularemia is a typical disease of hares, rabbits and rodents (Hillyard 1996; Sonenshine et al., 2002).

Figure 2. Distribution of *I. scapularis.*

I. scapularis is commonly known as the deer tick or blacklegged tick and is found in North America (Drummond, 2004) **(Figure 2)**. It is a medically important tick species and the vector of several diseases of animals, including humans (Lyme disease, babesiosis, anaplasmosis and Powassan encephalitis) and is known as the deer tick owing to its habit of parasitising the white-tailed deer. The distribution of *I. scapularis* is linked to the distribution and abundance of its primary reproductive host, white-tailed deer (Wilson et al., 1988). It is also known to parasitise lizards, mice and migratory birds especially while the tick is in the larva or nymph stage (Aliota et al., 2014; Levine et al., 1997; Manneli et al., 1994; Ogden et al., 2008).

I. scapularis is a three-host tick; each developmental stage feeds upon a different host animal. In June and July, eggs deposited earlier in the spring hatch into tiny six-legged larvae. Peak larval activity occurs in August, when larvae attach and feed on a wide variety of mammals and birds, primarily on mice (Anderson and Magnarelli, 1980). After feeding for three to five days, engorged larvae drop from the host to the ground where they overwinter. In May, larvae moult into nymphs, which feed on a variety of hosts for three to four days. In a similar manner, engorged nymphs detach and drop to the forest ground where they moult into the adult stage, which becomes active in October. Adult ticks remain active throughout the winter on days when the ground and ambient temperatures are above freezing. Adult female ticks feed for five to seven days while the male tick feeds only sparingly, if at all. Adult ticks feed on large mammals, primarily upon white-tailed deer (Carey et al., 1980; Piesman et al., 1979; Troughton and Levin, 2007; Wilson et al., 1990). Beginning in May, engorged adult females typically lay between 1000 and 3000 eggs on the forest floor at the site where they detached from their hosts. Due to their low probability of finding a host, starvation would be a major cause of mortality of ticks. Host immunity and grooming activity may also affect mortality (Brown, 1988; Randolph, 1979). Other effects on lifespan are density-independent such as temperature and humidity changes. (Kiszewski and Spielman, 2002).

1.2 Importance of ticks

The medical and economic importance of ticks has long been recognised due to their ability to transmit diseases to humans and animals and the threat they pose to their hosts' health worldwide (de la Fuente et al., 2008; Furman and Loomis, 1984; Jongejan and Uilenberg, 2004). Ticks cause great economic losses to livestock globally and have deleterious effects on their livestock hosts in several ways (Oliver, 1989; Snelson, 1975). The major losses caused by ticks are due to their ability to transmit protozoan, rickettsial and viral diseases of livestock, which are of great economic importance world-wide; for example protozoan diseases (theileriosis and

babesiosis) and rickettsial diseases (e.g. anaplasmosis and heartwater) are major health and management problems of livestock in many developing countries. Although the global economic importance of ticks is expecially high for livestock, there is also a great impact on public health, primarily due to Lyme borreliosis but also other zoonotic tick-borne illnesses, with those of viral origin, characterised by encephalitis and haemorrhagic fevers, causing the highest morbidity and mortality in humans (Jongejan and Uilenberg, 2004).

1.3 Tick innate immune system

Despite the importance of tick-borne diseases worldwide, little is known about the tick immune system (Yu et al., 2006). However the tick immune system has been receiving great interest in the last few years as a source of molecules with potential antimicrobial activity (Kopaček et al., 2012). Reports suggest that ticks have the ability to control infections when challenged with various bacteria (Rego et al., 2006; Yu et al., 2006). From these results it could be inferred that an efficient defence mechanism is required for ticks to eliminate microbial infections, or to mitigate infections to tolerable levels. Therefore, the ticks must defend themselves from infectious pathogens without antibodies, thymus or lymphocytes which are part of the adaptive immune response of vertebrates (Zasloff, 1992). Ticks have been shown to possess defence mechanisms directed against invading microorganisms with an innate immune response which consists of effective cellular and humoral responses (Nakajima et al., 2002a, 2002b; Saito et al., 2009; Taylor, 2006; Yu et al., 2006).

Cellular responses represented by haemocytes include encapsulation, nodulation and phagocytosis, and humoral responses to invading pathogens consist of three main types: humoral encapsulation, haemagglutination and synthesis of antimicrobial peptides (AMPs) (Taylor, 2006). One of the major features of innate immunity in ticks is a rapid and transient synthesis of AMPs (Grubhoffer et al., 2013).

1.3.1 Antimicrobial peptides

AMPs are well-conserved evolutionarily ancient tools and pervasive contributors of the innate immune response that is conserved in many organisms such as mammals, birds, amphibians, insects, scorpions, molluscs, plants and ticks (Bulet et al., 1999; Fogaça et al., 2004; Lai et al., 2004; Taylor 2006; Tsuji, et al., 2007; Yu et al., 2006; Zheng et al., 2012). Recently, different types of AMPs with different functions involved in the tick innate immune response have been described. Ticks have developed various antimicrobial factors because they encounter a variety of pathogenic organisms during their life cycle. It is important for ticks to produce AMPs to eliminate or modulate detrimental pathogens to which they are exposed (Fogaça et al., 2004; Lai et al., 2004; Taylor, 2006; Yu et al., 2006; Zheng et al., 2012).

AMPs typically present a low molecular weight, heat stable (below 5 kDa) positively-charged short peptide structure (12 to 100 aa) with an amphiphilic nature (Wang and Lai, 2010). They have been also termed "natural antibiotics" because they are active against a large spectrum of microorganisms, including bacteria (including strains that are resistant to conventional antibiotics), viruses, filamentous fungi - in addition to protozoan and metazoan parasites and even transformed or cancerous cells (Chrudimská et al., 2011; Isogai et al., 2009; Izadpanah and Gallo, 2005). AMPs are excellent candidates for developing novel therapeutic agents complementary to conventional antibiotic therapy (Chrudimská et al., 2011; Yu et al., 2006). Currently the amount of bacterial resistence to conventional antibiotics is rapidly growing and represents a serious health problem wordwide. The generation of native peptides derived from invertebrates, namely ticks, with novel mechanisms of action against a broad spectrum of microrganisms will present a potential and effective source of pharmaceutical substances as an alternative for treatment of infections (Yu et al., 2006). Although the development of pathogen resistance might also constitute a threat to bactericidal effect of AMPs in the future, a complete resistance has not yet emerged.

Understanding the mechanism of action of AMPs is also paramount to answering the question of how pathogens survive in ticks (Chrudimská et al., 2010; Lowenberger et al., 1995; Nakajima et al., 2002a; Pichu et al., 2009; Rudenko et al., 2007).

Known tick antimicrobial peptides possess a wide variety of structural motifs and can be divided into several groups that include defensins, cystatins, haemoglobin fragments, lectins, serine proteinase, lysozymes, cecropins, attacins, proline-rich peptides, glycine-rich peptides and tick-specific antimicrobial compounds (Ceraul et al., 2003; Grunclova et al., 2003; Hajdušek et al., 2013; Kotsyfakis et al., 2006; Nakajima et al., 2001; Rudenko et al., 2007; Simser et al., 2004; Taylor, 2006; Yu et al., 2006; Zheng et al., 2012). The research on defensins conducted to date in ticks is briefly outlined below.

1.3.2 Defensins

1.3.2.1 Characterisation of defensins

Defensins are naturally-occurring AMPs and represent the most widely characterised class of antimicrobial molecules occurring in arthropods (Grubhoffer et al., 2013; Nakajima et al., 2002a; Sonenshine and Hynes, 2008; Taylor, 2006). Defensins are synthesised as prepropeptides and characterised by the conserved cysteine residues in the mature peptide that form disulphide bridges (Fogaça et al., 2004; Hynes et al., 2005). Defensins are small peptides of approximately 4 kDa in size. They present a furin motif (RVRR) conserved between the pro-peptide and mature peptide (Hynes et al., 2005). The function of the pro-peptide is speculative; however, it is suggested that it may help during the biogenesis of the mature peptide. It was observed that defensins of plesiomorphic groups of arthropods, including scorpions and ticks, have N-terminal segments shorter than defensins of more apomorphic groups, such as the superior orders of insects (Fogaça et al., 2004). Mature defensins are cyclic peptides possessing a pattern of six paired cysteine residues in their primary structure with

three or four disulphide bridges, forming conserved cysteine-stabilised α-helix and β-sheet (CSαβ) structural motifs that are crucial for the antimicrobial activity (Galay et al., 2012; Grubhoffer et al., 2013; Hajdušek et al., 2013; Hazlett and Wu, 2011; Isogai et al., 2009; Pichu et al., 2009; Taylor, 2006; Viljakainen and Pamilo, 2005; Wang and Zhu, 2011). Previous studies have shown that the functional region of defensins is primarily located in the C-terminal β-sheet domain, called the 'γ -core motif' (Wang and Zhu, 2011). Therefore, the variable γ-core domain of defensins contains the antimicrobial activities which have been found in *I. scapularis* (Wang and Zhu, 2011). The γ-core domain represents the antimicrobially active peptide that varies considerably between individual molecules in sequence, length (roughly 14 residues), and function (Wang and Zhu, 2011). Several structural features have been identified as relevant for the microbicidal function of antimicrobial peptides: size, sequence, charge, degree of structuring (helicity), overall hydrophobicity, amphipathicity and the angles subtended by hydrophobic and hydrophilic surfaces of the helical molecule (Giangaspero et al., 2001).

Most defensins are cationic however some anionic peptides have been reported (Ganz and Lehrer, 1994; Lai et al., 2004, Tonk et al., 2014). When the net charge becomes more positive, binding to negatively-charged surfaces of microorganisms is increased; however, the formation of trans-membrane pores is inhibited (Dathe et al., 1997; Dathe and Wieprecht, 1999).

In ticks, the first record of defensins resulted from the cloning and sequencing of two isoforms from *Ornithodoros moubata* haemolymph (Nakajima et al., 2001). Subsequently, many other defensins have been reported in ticks (Fogaça et al., 2004; Hynes et al., 2005; Johns et al., 2001; Lai et al., 2004; Nakajima et al., 2001; Saito et al., 2009; Todd et al., 2007; Tonk et al., 2014; Tsuji et al., 2007; Rudenko et al., 2005, 2007; Wang and Zhu, 2011).

Defensins have been identified in several species of ticks such as *I. ricinus* (Rudenko et al., 2005, 2007; Tonk et al., 2014), *I. scapularis* (Hynes et al., 2005), *Amblyomma americanum* (Todd et al., 2007), *Amblyomma hebraeum* (Lai et al., 2004), *Amblyomma cajennense* (Batista et al., 2008), *Rhipicephalus* (*Boophilus*) *microplus* (Esteves et al., 2008; Fogaça et al., 2004), *Rhipicephalus bursa* (Arolas et al., 2005), *Dermacentor andersoni* (Mattila et al., 2007), *Dermacentor variabilis* (Ceraul et al., 2003, 2007; Johns et al., 2001; Sonenshine et al., 2002), *Haemaphysalis longicornis* (Saito et al., 2009; Tsuji et al., 2007; Zhou et al., 2007) and many others. More than one defensin isoform has been detected in several tick species, such as *O. moubata*, *I. ricinus*, *A. hebraeum* and *H. longicornis* ticks (Chrudimská et al., 2010; Lai et al., 2004; Nakajima et al., 2002a; Rudenko et al., 2005, 2007; Tonk et al., 2014; Zhou et al., 2007).

Tick defensins have been reported to be active against Gram+ and Gram- bacteria, as well as against intracellular rickettsiae and protozoa (Kocan et al., 2008). Although their mode of action has not yet been unequivocally demonstrated, it was suggested that their mode of action involves electrostatic interaction with the bacterial membrane by introducing voltage-dependent channels into bacterial walls, leading to permeability changes or pore formation (Hajdušek et al., 2013; Sonehshine and Hynes, 2008; Todd et al., 2007; Tsuji et al., 2007). Therefore, the detailed knowledge of tick AMPs especially defensins is crucial to understanding the mechanism of the innate immune system of ticks.

1.3.2.2 Biosynthesis, gene structure and expression

Nakajima and colleagues found for the first time an intron/exon structure in the soft tick *O. moubata* defensin gene (Nakajima et al., 2002a). After that, other introns were identified in the hard tick *I. ricinus* (Rudenko et al., 2007; Tonk et al., 2014). The intron/exon genomic organisation of the gene is similar to the organisation in *O. moubata*, but not to that of the intronless defensins of *D. variabilis* and *I. scapularis*

(Hynes et al., 2005; Rudenko et al., 2007). In addition to the diversity of structure and mode of action, the site and the regulation of the synthesis of defensins also differs in tick species.

In insects, defensins are generally induced by septic injury and blood feeding, then released into the haemolymph (Nakajima et al., 2002b; Taylor, 2006). On the other hand, recent studies have revealed that some haematophagous insect defensins are involved in midgut immune mechanisms (Nakajima et al., 2002b). Defensin secretion into the gut lumen has been demonstrated in *Stomoxys calcitrans* (Lehane et al., 1997) and *Anopheles gambiae* (Nakajima et al., 2002b; Vizioli and Salzet, 2002). Gene expression of defensins in the midgut was shown in *Aedes aegypti* (Lowenberger et al., 1999; Nakajima et al., 2002b). Initially, defensins were isolated from the haemolymph of various tick species. In recent years, several defensins have been identified in the midgut, salivary glands, ovaries and Malpighian tubules and even in the male accessory glands of *H. longicornis*, indicating that these molecules have a role in host defence (Yu et al., 2006; Zheng et al., 2012). The expression patterns of specific defensins are described below.

1.3.2.3 Biological activities of defensins

Antimicrobial activity of defensins occurs through several mechanisms. Interactions between the peptide and surface membranes of the target organisms play an important role. The initial binding is thought to depend on electrostatic interactions between the positively-charged peptides and the negatively-charged molecules at the surface of the target. The biophysical features of the membrane are destroyed by direct interactions with the peptide (Ladokhin et al., 1997; Rozek et al., 2000). Another explanation for the mechanism of killing bacteria by tick defensins could be through depolarisation of the cytoplasmic membrane (Chrudimská et al., 2010; Nakajima et al., 2002b).

Defensins mainly kill Gram+ bacteria with a high affinity for the major phospholipids of bacteria (Gillespie et al., 1997; Taylor, 2006). The antibacterial activity of defensins is generally ascribed to their effects on microbial membranes, although the actual antibacterial mechanism is not clear (Lai et al., 2004). Cationic defensins interact with negatively-charged components of microbial membranes that include lipopolysaccharide in Gram- bacteria, polysaccharides in Gram+ bacteria and phospholipids (Lai et al., 2004).

Generally, tick defensins showed antibacterial activity against many Gram+ bacteria (e.g., *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus cereus*, *Corynebacterium renale*, *Enterococcus faecalis*, *Listeria grayi*, *Micrococcus luteus*, *Staphyloccocus xylosus* and multi-drug resistant strain of *Staphyloccocus aureus*); some defensins have also shown activity against Gram- bacteria (*Cedecea lapagei*, *Escherichia coli*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Serratia rubidaea*), yeasts (*Pichia pastoris*), protozoa (*Babesia equi, Babesia microti*) and fungi (*Candina albicans*) (Bulet et al., 2004; Chrudimská et al., 2010, 2011; Fogaça et al., 2004; Isogai et al., 2009; Lu et al., 2010; Nakajima et al., 2002a; Saito et al., 2009; Taylor, 2006; Tonk et al., submitted manuscript; Tsuji et al., 2007; Yu et al., 2006; Zheng et al. 2012).

1.3.2.4 Defensins against tick-borne diseases

In humoral defence, borreliacidal activity has been demonstrated with the plasma portion of haemolymph from *D. variabilis* (Johns et al., 2001). In *D. variabilis*, a synergistic effect between defensin and lysozyme against Lyme disease spirochaetes was shown (Johns et al., 2001). A cationic defensin (varisin) identified from haemocytes of the hard tick, *D. variabilis* showed antimicrobial activity against Gram+ bacteria; varisin is also active against *Borrelia burgdorferi* (Ceraul et al., 2003; Hynes et al., 2005; Johns et al., 2001). Longicin was found to exert a remarkable ability to inhibit the proliferation of merozoites, an erythrocyte blood stage of equine *Babesia equi*, by killing the parasites (Tsuji et al., 2007).

I. scapularis transmits the agent of human granulocytic anaplasmosis, *A. phagocytophilum*, among other pathogens. The mechanisms used by the tick to control *A. phagocytophilum* are not known. A salivary gland gene family encoding 5.3 kDa antimicrobial peptides (to which *I. ricinus* 5.3 kDa IRAMP revealed significant identity) is highly induced upon *A. phagocytophilum* infection of tick salivary glands. Silencing of these genes increased *A. phagocytophilum* infection of tick salivary glands and transmission to the mammalian host (Liu et al., 2012).

1.3.3 Tick defensins

1.3.3.1 Hard tick defensins

Ixodes ricinus

So far, two defensins have been identified from *I. ricinus*. Rudenko and colleagues (2005) found two new isoforms, Def1 and Def2. Def1 and Def2 only differ by one amino acid substitution in the defensin molecule and this has impact on antimicrobial activity (Chrudimská et al., 2011). This substitution results in slightly different antimicrobial potential of Def1 and Def2. The Def2 isoform is more effective in the inhibition of bacterial cell growth and killing of bacteria than the Def1 peptide (Chrudimská et al., 2011). However, no significant difference was noticed in the degree of erythrocyte haemolysis (Chrudimská et al., 2011). Expression of both Def1 and Def2 was induced in different tick organs by blood feeding or pathogen injection (Chrudimská et al., 2011; Rudenko et al., 2005). Recently, six novel *I. ricinus* defensins have been identified (Tonk et al., 2014).

Ixodes scapularis

Scapularisin

Hynes and colleagues (2005) found twenty-five defensins with significant sequence similarity to Scapularisin, named Scapularisins -1 to -25, following the name of the first defensin discovered in *I. scapularis.* Most of the *I. scapularis* defensins contain a complete γ-core of 11 or 14 amino acids with a GXC motif. All peptides have six conserved cysteine residues. A fragment corresponding to the γ-core of Scapularisin-20 showed slightly more effect on Gram+ bacteria than on Gram- bacteria (Wang and Zu, 2011).

Ixodes perculcatus

Persulcatusin

A cDNA library was constructed from the whole bodies of fed nymphs of *I. persulcatus*. From this library one defensin (Persulcatusin) was identified. The amino acid sequence showed high similarity to other tick and arthropod defensins. *I. persulcatus* defensin mRNA transcripts were detected in all life cycle stages of fed ticks and found to be predominantly expressed in the midgut of adult female ticks, but not in the salivary glands, a finding verified by Western blotting analysis. Synthetic Persulcatusin showed activity against Gram+ bacteria including *S. aureus*, *B. subtilis* and *C. renale*, but not Gram- bacteria except *E. coli* O157 (Saito et al., 2009).

Ixodes sinensis

Ixosin

Ixosin was isolated from the salivary glands of *I. sinensis* by ion exchange chromatography, gel filtration and reverse-phase high-performance liquid chromatography (RP-HPLC) (Yu et al., 2006). The molecular weight of Ixosin was 2.9 kDa analysed by fast atom bombardment (FAB) mass spectrometry (Yu et al., 2006). Ixosin is the first known antimicrobial peptide with less than six cysteines in its primary structure. Ixosin was active against *S. aureus*, *E. coli* and *C. albicans* (Yu et al., 2006).

Rhipicephalus* (*Boophilus*) *microplus

Microplusin

Microplusin has a molecular mass of 10.2 kDa and contains six cysteine residues involved in three internal disulphide bridges. Microplusin was obtained from cell-free haemolymph (Fogaça et al., 2004). The complete cDNA sequence showed that microplusin is synthesised as a precursor molecule with a signal peptide of 20 residues (Fogaça et al., 2004). Gene expression of microplusin was observed in the ovaries, fat body and haemocytes (Fogaça et al., 2004). Virtually, no expression of microplusin was observed in the midgut (Fogaça et al., 2004). Despite the fact that microplusin gene expression has been observed in haemocytes, the presence of the mature polypeptide was not detected in haemocyte acidic extract, as demonstrated by RP-HPLC. This result suggests that the mature peptide may be released to haemolymph, and for this reason its concentration inside the cell is below the detectable concentration of the inhibitory growth assay (Fogaça et al., 2004).

Haemaphysalis longicornis

HlMS-defensin

As a first time from the tick genital system, a novel gene was identified and characterised from a cDNA library from the male accessory glands (MAG) and named HlMS-defensin. The full-length cDNA of the HlMS was 349 bp, encoding 79 amino acids (48 amino acids mature portion) (Zheng et al., 2012). The putative mature peptide had a predicted molecular weight of 5.5 kDa and an isoelectric point (pI) value of 9.21 (Zheng et al., 2012). The gene expression was determined only in the MAG, and its mRNA level was up-regulated with increasing feeding duration (Zheng et al., 2012). Real-time PCR (RT-PCR) was performed to determine where, in midgut, salivary glands, testis/vas deferens, accessory glands and the remaining carcass of the engorged male, the HlMS-defensin gene was expressed at the highest level (Zheng et al., 2012). In the fed male tick tissues, HlMS-defensin was predominantly expressed in the accessory glands, suggesting that the peptide plays an important role in protection of the male reproductive tract (Zheng et al., 2012). The gene was also expressed at low levels in the midgut, salivary glands and testis/vas deferens. During the entire feeding period, HlMS-defensin mRNA levels increased gradually and reached a distinct peak in fed males, indicating that the gene expression was up-regulated during the course of mating preparation, and reached the highest level in mating-ready males (Zheng et al., 2012).

The HlMS-defensin gene was expressed exclusively in pre-feeding males, suggesting that it has the potential to offer protection against microorganisms for male ticks (Zheng et al., 2012). Excellent fertility of ticks is tightly linked with their genital system; it is therefore important for them to maintain a hostile environment for infecting microbes (Zheng et al., 2012). Therefore Zheng and colleagues have presented a new aspect of the tick defence system from the genital system (Zheng et al., 2012).

Longicornsin

A novel AMP was isolated from the salivary glands of the hard tick, *H. longicornis* and named as longicornsin (Lu et al., 2010). Its molecular weight was 5.4 kDa (Lu et al., 2010). There were six half-cysteines in its sequence as in other defensin-like peptides (Lu et al., 2010). The protein predicted from the cDNA sequence was composed of 78 amino acids including a mature longicornsin (Lu et al., 2010).

Purified longicornsin showed potent antimicrobial activity against *E. coli* and *C. albicans*; longicornsin also had strong antimicrobial ability against drug-resistant strains of *S. aureus* and *P. aeruginosa*. Longicornsin was also active against *H. pylori* (Lu et al., 2010).

Amblyomma americanum

Amercin

The full-length transcript (556 bp) sequence from *A. americanum* haemocytes was determined, named as amercin and the 219 bp coding region was identified. The gene encodes a 72 amino acids prepropeptide with a putative 37 amino acids mature peptide. Amercin transcripts were found in the midgut, fat body and salivary gland tissues, as well as in the haemocytes. Transcripts of this defensin were also present in early-stage eggs, late-stage eggs, larvae and nymphs of *A. americanum* (Todd et al., 2007).

Dermacentor variabilis

Varisin

Varisin is 624 bp long with a 225 bp region that translates to a 74 amino acids preprodefensin. It was isolated from partially-fed *D. variabilis* females and was collected following *B. burgdorferi* challenge and assayed for antimicrobial activity against *B. subtilis* and *B. burgdorferi* (Johns et al., 2001). Injection of *B. burgdorferi* results in the secretion of defensin into the haemolymph of *D. variabilis*. The presence of the peptide is observed as early as 15 min post-challenge and remains present for 18 hours post-challenge. However, Varisin was not effective against *B. burgdorferi* unless combined with lysozyme (Taylor, 2006). Ceraul and colleagues (2003) reported the identification of a defensin expressed in the haemocytes of *D. variabilis* and suggested that defensin is stored in the haemocytes and released into the haemolymph when stimulated by bacterial challenge (Taylor, 2006).

Interestingly, silencing by RNA interference of Varisin resulted in a significant reduction in *Anaplasma marginale* infection, indicating the impact of defence mechanisms on certain pathogens (Kocan et al., 2008).

1.3.3.2 Soft tick defensins

Ornithodoros moubata

A total of four defensins have been identified in *O. moubata* (isoforms: A, B, C, and D). All four *Ornithodoros* defensins are coded as preprodefensins. *Ornithodoros* defensin genes consist of four exons and three introns (Nakajima et al., 2001, 2002b). *Ornithodoros* defensin C and D genes are predominantly expressed in the midgut and up-regulated in response to blood feeding (Nakajima et al., 2002b). The mature peptide of the previously cloned *Ornithodoros* defensin A was purified from the midgut lumen. This is the first demonstration of defensin secretion into the midgut

lumen of ticks (Nakajima et al., 2002b). These findings confirm the involvement of *Ornithodoros* defensin in midgut immunity (Nakajima et al., 2002b). An upregulation of the isoforms A and B was also observed in the midgut after an experimental bacterial infection. RT-PCR analyses of these defensin genes revealed they are expressed in all stages from eggs to adults and appear to be constitutively expressed (Nakajima et al., 2001).

Other soft tick defensins

Three isoforms (A, B, and D) of a defensin gene were identified in engorged females of *Ornithodoros papillipes.* Isoforms A and B were isolated from fully engorged females of *Ornithodoros tartakovskyi* and *Ornithodoros puertoricensis*. Only one isoform of a defensin gene was found in *Ornithodoros rostratus*. All sequenced cDNAs encoding new *Ornithodoros* preprodefensins are 222 bp long. The deduced protein sequences are 73 amino acids long (Chrudimská et al., 2010). Isoform defA from *O. tartakovskyi* was identical to *O. moubata* defA. Isoform B (defB) of defensin genes from *O. papillipes*, *O. puertoricensis* and *O. tartakovskyi* was also identical to *O. moubata* defB (Nakajima et al., 2001). The *O. papillipes* defD deduced amino acid sequence was also identical to *O. moubata* defD (Nakajima et al., 2002a). Six conserved cysteine residues that form the three disulphide bridges in mature defensin molecules were also present (Chrudimská et al., 2010). Recently Prinsloo and colleagues have identified two defensin isoforms (OsDef1 and OsDef2) from *Ornithodoros savignyi.* OsDef2, was found to be active against *B. subtilis* and *M. luteus*, but activity could not determined for *E. coli* and *P. aeruginosa* (Prinsloo et al., 2013).

1.3.4 Applications of defensins

1.3.4.1 Defensins as novel drugs

Development of new drugs most of the time brings unavoidable emergence of bacterial resistance to antibiotics following widespread clinical usage. The pharmaceutical industry has continuously produced drugs by modifying existing antibiotics and developing newer antibiotics. However, the rapid increase of resistance is a greater problem for life-threatening infections. The effective solution to this problem could be introducing novel defensins.

Defensins appear to be effector substances of the innate immune system. Therefore identification, functional analysis and evaluation of the functions of novel defensins in diseases might provide information about the corresponding pathogenesis, to develop novel drugs to overcome drug resistance problems worldwide (Zanetti et al., 2002).

1.3.4.2 Outlook: defensins

Although the actual antimicrobial mechanism remains still unclear, as a novel class of antibiotics, AMPs have attracted considerable attention, especially for the treatment of antibiotic-resistant pathogens (Lu et al., 2010). More diversity of antimicrobial peptides provides more opportunities for designing novel and effective antimicrobial agents (Lu et al., 2010).

The success of pathogen transmission by ticks is due to the long period of co-evolution of pathogen and tick and to specific aspects of tick biology (Zheng et al., 2012). As with other arthropods, ticks need the capacity to respond to microorganisms invading their tissues with a variety of innate immunity molecules (Zheng et al., 2012).

The above-mentioned studies clearly show the importance of defensins in the immune responses of both soft and hard ticks (Taylor, 2006). They also show that there are some differences in the responses between tick species and these differences may result in the transmission of disease by some species and not others. Although we have a good start for understanding the mechanisms by which defensins function, much remains to be clarified (Taylor, 2006). Further studies at the molecular level, protein level and organismal level are needed to understand these mechanisms (Taylor, 2006).

Despite their importance as vectors of disease the immune mechanisms of ticks have only recently attracted the attention of researchers so there is much less known about the innate immunity of ticks (Taylor, 2006).

Advantages of defensins: (i) they rapidly kill target microorganisms, (ii) they have broad activity spectra, (iii) they are ineffective against mammalian cells, (iv) they have a mode of action against resistant strains (Bulet et al., 1999).

Chapter 2: Project Objectives

This PhD thesis was designed to analyse six novel defensin genes that were named as DefMT2, DefMT3, DefMT4, DefMT5, DefMT6 and DefMT7. They were identified *in silico* from the recently published salivary gland shotgun assembly transcriptome (GenBank Bioproject PRJNA177622) of *I. ricinus* adult females and nymphs, and two defensins from *I. scapularis* (Scapularisin-3 and Scapularisin-6).

The main goals of the study were to identify and characterise these defensins, then commercially synthesise only the γ-core of selected defensins from *I. ricinus* (DefMT3, DefMT6 and DefMT7) and the whole mature peptides of *I. ricinus* defensins and *I. scapularis* defensins to analyse the presence of antimicrobial and antifungal activities. An essential part of this study is devoted to analysis of their antimicrobial potential and to analysis of the protein expression pattern in different tick organs.

The main goals are briefly summarised below:

- ❖ To identify defensin gene sequences encoding antimicrobial proteins (defensins), characterise and compare new *I. ricinus* defensins with other known tick defensins at the structural level. Characterisation of two *I. scapularis* defensins.
- ❖ To obtain commercially synthesised peptides from *I.* ricinus; γ-core of DefMT3, DefMT6 and DefMT7 and mature peptides of DefMT2, DefMT3, DefMT5, DefMT6, DefMT7 and mature peptides of *I. scapularis* defensins; Scapularisin-3 and Scapularisin-6.
- ❖ To test for the presence of antimicrobial and antifungal activities of the synthetic peptides.
- ❖ To determine the tissue expression patterns of *I. ricinus* and *I. scapularis* defensins.

Chapter 3: Materials and Methods

Sequences of *I. ricinus* defensins: An attempt was made to identify new members of the *I. ricinus* defensin family from the recently published shotgun assembly transcriptome of the salivary glands of *I. ricinus* adult females and nymphs within GenBank (NCBI — National Center for Biotechnology Information—Bethesda, MD, USA) (Schwarz et al., 2013). For sequence identification the strategy reported by Wang and Zhu (2011) that they used for identification of defensins in *I. scapularis* ticks was basically followed. The only modification to the strategy of Wang and Zhu (2011) was that the search was done by using BLASTP in the Database "Transcriptome Shotgun assembly proteins" from NCBI and not TBLASTN and genomic database as in Wang and Zhu (2011).

Preparation of synthetic γ-core of DefMT3, DefMT6 and DefMT7: DefMT3, DefMT6 and DefMT7 sequences were aligned with the γ-core motif of Scapularisin-20 (Wang and Zhu, 2011) and the resultant γ-core motifs of DefMT3, DefMT6 and DefMT7 were synthesised as below for functional analysis (**Figure 3**).

```
core*   CGGFLKKTCICVMK 14
DefMT3  CGNFLKRTCICVKK 14
DefMT6  CSGIIKQTCTCYRK 14
DefMT7  CNGPFNIVCSCY-- 12
```

Figure 3. γ-core motif of selected novel *I. ricinus* defensins. Synthesised γ-core motif of selected novel *I. ricinus* defensins aligned with Scapularisin-20 γ-core motif**.** (* Scapularisin-20 γ-core motif)

The peptides of the above sequences with reduced cysteine residues were provided on the basis of custom synthesis in the service department of the Institute of Organic Chemistry and Biochemistry of the Academy of Sciences of the Czech Republic. Peptides were prepared in amounts of 5 - 10 mg using a protocol of Fmoc solid phase

peptide synthesis. Their purities were checked by RP-HPLC analysis to be more than 95% pure and identities were verified by mass spectrometry **(Table 1)**.

Table 1. Molecular mass of γ-core motif of selected novel *I. ricinus* defensins**.**

	Calculated molecular mass *	**Determined molecular mass**
DefMT3	1611.84	1611.6
DefMT6	1602.77	1603.0
DefMT7	1318.51	1318.4

* monoisotopic

Preparation of synthetic DefMT2, DefMT3, DefMT5 DefMT6 and DefMT7: Mature peptides were chemically synthesised with more than 95% purity (Pepmic, China). Lyophilised peptides were stored at -20°C until use.

Defensin sequences and preparation of synthetic Scapularisins: Using publicly available sequence data (Wang and Zhu, 2011), 38 amino acids of the mature peptides of each of Scapularisin-3 (Genbank accession number: EEC13914) and Scapularisin-6 (Genbank accession number: EEC08935) were chemically synthesised with ~95% purity (Peptide 2.0, USA). Lyophilised peptides were stored at -20°C until use.

3.1 Sample Collection

3.1.1 Ticks

I. ricinus and *I. scapularis* ticks were provided by the Institute of Parasitology, Academy of Sciences of the Czech Republic. Uninfected ticks were reared for several generations in the animal facilities of the Institute and fed on adult guinea pigs that were raised to be free of infection under the strict hygiene regulations of the Central Commission for the Protection of Animals (§21, section 3e, the Animal Protection Law of the Czech Republic No. 246/1992 sb., ethics approval number 137/2008). Blood-fed ticks were obtained by attaching a container with ticks to a shaved area on the back of an adult guinea pig. Ten partially engorged (6 days post-attachment) female *I. ricinus* and *I. scapularis* ticks were collected from the guinea pig for further studies.

3.1.2 Organ collection

Salivary glands, midguts, ovaries and Malpighian tubules were collected from *I. ricinus* and only salivary glands and midguts were collected from *I. scapularis*. The ticks were affixed to the bottom of a petri dish with wax, submerged in PBS and incised along the dorsal-lateral margin and the dorsal integument was removed under a binocular microscope. The organs were excised, washed in PBS to remove excess blood, transferred into RNAlater® solution (Ambion, USA) and kept at -80°C for later use (modified from Fogaça et al., 2004).

3.1.3 Haemolymph collection

The forelegs of *I. ricinus* ticks were cut the coxal-trochanteral joint and gentle pressure applied on the tick's body, the clear haemolymph was drawn into a plastic micropipette, transferred into RA1 buffer (NucleoSpin® RNA II, Macherey Nagel, Germany) and stored at -80°C for later use.

3.1.4 Cell culture

The *I. ricinus* embryo-derived cell line IRE/CTVM19 (Bell-Sakyi et al., 2007) was used in this study. Briefly, IRE/CTVM19 cells were maintained at 28°C in L-15 (Leibovitz) medium supplemented with 20% heat-inactivated foetal bovine serum, 10% tryptose phosphate broth, 2mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (Bell-Sakyi, 2004). Medium was changed weekly by removal and replacement of ¾ of the medium volume.

The *I. scapularis* embryo-derived cell lines ISE6 and ISE18 (Kurtti et al., 1996; Munderloh et al., 1994) were maintained at 28 °C in L-15 (Leibovitz) medium supplemented with 20% heat-inactivated foetal bovine serum, 10% tryptose phosphate broth, 2mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. Medium was changed weekly by removal and replacement of ¾ of the medium volume and subcultures were carried out when required (Bell-Sakyi et al., 2007).

3.2 Nucleic acid analysis

3.2.1 RNA extraction

Total RNA was extracted using the RNeasy® Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, tick organs and harvested tick cells were centrifuged at 300 x g for 5 min. To lyse the cells, the pellet was resuspended in 350-600 µl of Buffer RLT containing dithiothreitol (DTT) and vortexed. An equal volume of 70% ethanol was added to the lysate and mixed. Up to 700 µl of the sample were transferred to an RNeasy Mini spin column placed in a 2 ml collection tube, centrifuged at ≥8000 x g for 15 sec and the flowthrough discarded. An extra step was carried out to remove contaminating DNA using the RNase-Free DNase Set (Qiagen) as follows: 350 µl of Buffer RW1 was added to the column, centrifuged for 15 sec at ≥8000 x g and the flowthrough discarded. Ten µl of DNase I stock solution was added to 70 µl of Buffer RDD and gently mixed. Eighty µl of this mix was added directly to the column membrane and incubated at room temperature for 15 min. Three hundred µl of Buffer RW1 was added to the column and centrifuged at ≥8000 x g for 15 sec, discarding the flowthrough afterwards. To wash the membrane, 500 µl of Buffer RPE was added, centrifuged at ≥8000 x g for 15 sec and the flow-through discarded. An additional 500 µl of Buffer RPE was added, centrifuged at ≥8000 g for 2 min and the flow-through discarded. To dry the membrane, the column was centrifuged at 13300 x g for 1 min. RNA was eluted into a 1.5 ml collection tube by adding 50 µl of RNase-free water and centrifuging at ≥8000 x g for 1 min. Following the RNA extraction, quantity and quality were assessed using a nanodrop spectrophotometer (NanoDrop® ND-1000, Peqlab, Germany). The RNA was stored at -80°C for further use.

3.2.2 Complementary DNA (cDNA) synthesis

cDNA was synthesised from the extracted RNA as follows. Briefly, a 13 µl first reaction mix was prepared with 1 µl of 50 µM Oligo(dT)$_{15}$ (Promega), 1 µl of 10 mM dNTP Mix (Promega), 0.5-2.0 µg of RNA and RNase-free water up to the final volume. The mixture was heated at 65°C for 5 min and cooled down to 4°C for 1 min. A second reaction mix was prepared with 4 µl of 5x First-Strand buffer (Invitrogen), 1 µl of 0.1 M DTT (Invitrogen) and 1 µl of RNaseIN^{TM} recombinant ribonuclease inhibitor (Invitrogen). The mixture was heated at 50°C for 2 min and 1 µl of SuperScript^{TM} III reverse transcriptase (Invitrogen) was added. The mixture was heated at 50°C for 58 min, with a final incubation of 15 min at 70°C. cDNA was stored at -20°C for further use.

3.2.3 Isolation of genomic DNA extraction from *I. ricinus*

DNA extraction was carried out using the DNeasy® Blood and Tissue Kit (Qiagen) according to the protocol for purification of total DNA from two unfed adult female *I. ricinus* ticks (Spin-Column protocol). Briefly, the whole tick bodies were cut into pieces using a sterile scalpel blade and homogenised by passing through a 0.9 mm needle (20 gauge) fitted to a syringe, and DNA was extracted from the homogenate according to the manufacturer's recommendations. Twenty µl of proteinase K (Qiagen) and 200 µl of Buffer AL were added and the suspension mixed thoroughly by vortexing. Two hundred µl of 100% ethanol (Sigma) was added, the suspension mixed by vortexing and transferred to a DNeasy Mini Spin column. After centrifugation at room temperature for 1 min at 6000 x g, 500 µl of Buffer AW1 was added and the column centrifuged using the same conditions. Five hundred µl of Buffer AW2 was added and the column centrifuged at room temperature for 3 min at 13300 x g. DNA was eluted by adding 50 µl of Buffer AE to the centre of the spin column membrane, followed by incubation for 1 min at RT and then centrifugation at RT for 1 min at 6000 x g. This last step was repeated with the eluate to increase the DNA yield. DNA concentration and purity were determined by measuring the optical

density at both 260 and 280 nm with a DNA-RNA calculator (NanoDrop® ND-1000, Peqlab, Germany). Genomic DNA was dispensed in aliquots of 10 µl each and kept frozen until use in a PCR reaction.

3.2.4 Polymerase chain reaction

The oligonucleotide primers used for the amplification of defensin genes are shown in **Table 2 (A and B)**. Primers were designed using software (PrimerSelect; DNAStar, USA). Beta-actin (β-actin) was used as a control in the PCR. Two independent PCR reactions were performed for each gene. For each PCR amplification, 1 µl of cDNA or gDNA was used as the template in a 20 µl reaction mixture containing 20 pmol of each primer and 2x PCR Master Mix (Promega, USA) (shown below). The reactions were conducted in an Eppendorf Mastercycler Personal (Eppendorf, Germany) with the following parameters: 5 min at 96°C, followed by 35 cycles at 96°C for 30 s, 55°C for 30 s and 72°C for 1 min. The final extension step was at 72°C for 10 min. The PCR products were visualised by agarose gel electrophoresis (AGE) as described below (Section 3.2.5).

PCR reaction:

2x GoTaq Colorless Master Mix (Promega, USA) 10µl

Forward primer ♦ (10 µM) 1µl

Reverse primer ♦ (10 µM) 1µl

DNA template...0.5-1µl

dH_2O .... up to 20 µl

♦ Table 2 (A and B)

Table 2A. Primers for *I. ricinus* defensins.

Primers used in this study for the amplification of the DefMT2, DefMT3, DefMT4, DefMT5, DefMT6 and DefMT7 genes from *I. ricinus.*

Target	Primers	Sequence	Size (bp)
DefMT2	DefMT2-5-F DefMT2-R	5'- ATGAAGGTTTGGCTGGTTG -3’ 5'- TTAGCCCCGAACGCAGAT-3’	222
DefMT3	DefMT3-F DefMT3-4-R	5’ ATGAAGGTCCTTGCTGTCTC- 3’ 5’- CTATTTCTTGACACAGATGCAGG-3’	231
DefMT4	DefMT4-F DefMT3-4-R	5’- ATGAAGGTCCTTGCCGTCT-3’ 5’- CTATTTCTTGACACAGATGCAGG-3’	231
DefMT5	DefMT2-5-F DefMT5-R	5’- ATGAAGGTTTGGCTGGTTG - 3’ 5’- TTAGTTCATAATGCAGATGCACG- 3’	222
DefMT6	DefMT6-F DefMT6-R	5’- ATGAAGGCCGTTGCTATC-3’ 5’- TTAAATTGCCAACCAATTCAG-3’	339
DefMT7	DefMT7-F DefMT7-R	5’-TACGTCTTCATTATTCACGGTG-3’ 5’-CAGTAGCAGCTACAGACAATATTG-3’	210
β-Actin	Actin-F Actin-R	5’- ATGTGTGACGACGAGGTTGCCGC-3’ 5’- GTACAGCGACAGCACGGCCTGG -3’	530

Table 2B. Primers for *I. scapularis* defensins.

Primers used in this study for the amplification of the Scapularisin-3 and Scapularisin-6 genes from *I. scapularis*.

Target	Primer	Sequence
Scapularisin-3	Sca3-F	5'-ATGAAGGTCGTTGGAATTGCTCTT-3’
	Sca3-R	5'-TTATTTCTGGTAACAGGTGCAAGTTC-3’
Scapularisin-6	Sca6-F	5'-ATGAGGGTCATTGCTGTTACCTTGA-3’
	Sca6-R	5'-TTAGTTGTGGTAGCATGTGCACGTC-3’
β-Actin	Actin-F	5’- ATGTGTGACGACGAGGTTGCCGC-3’
	Actin-R	5’- GTACAGCGACAGCACGGCCTGG -3’

<u>Chemicals used for PCR</u>:

Buffer	**Composition**
Agarose	1.5 % agarose dissolved in 1x TAE buffer
50x TAE buffer	2M Tris-acetate; 50mM EDTA
6x loading dye	20% Ficoll; 10mM Tris-HCl (pH 8.0); 1mg/ml ‘Orange G’
SYBR Green	500x ‘SYBR Green’(Sigma) in 5x loading buffer

3.2.5 Agarose gel electrophoresis (AGE)

PCR and restriction digest analysis products were visualised by AGE as follows. To prepare 1% agarose gel as required, 1 g of agarose (Serva) was added to 100 ml of Tris-acetate-EDTA (TAE) buffer and heated in a microwave oven for 2.5 min to dissolve the agarose powder. PCR and restriction digest analysis products were visualised using 1% and 0.7% AGE, respectively. Between 10 and 20 µl of each sample mixed with 3.35 µl of 6x loading dye (Promega) was loaded directly into the wells. A 100 bp ladder (ThermoScientific) was loaded depending on the expected

fragment size. Both the ladder and loading dye contain SYBR® GreenER™ (Invitrogen, USA) allowing the visualization on the agarose gel. Gels were run at 100 V for 60 min. The products were visualised using a UV transilluminator.

3.2.6 Cloning and bacteria transformation

The amplified and purified PCR products were cloned into the pCR™4-TOPO® vector using the TOPO® TA Cloning® Kit for Sequencing (Invitrogen, USA), according to the manufacturer's instructions. To perform the TOPO® Cloning reaction, 4 µl of the purified DNA was added to 1 µl of salt solution and 1 µl of pCR™4-TOPO® vector, according to the kit used. The reaction was mixed gently and incubated at room temperature for 30 min and thereafter placed on ice.

Transformation was carried out using One Shot® TOP10 Competent Cells (Invitrogen, USA), as follows. One Shot® TOP10 competent cells were thawed on ice. Two µl of the TOPO® Cloning reaction was added 50 µl of One Shot® TOP10 competent cells. Cells were gently mixed and incubated on ice for 30 min. Cells were heat-shocked for 30 sec at 42°C and immediately transferred to ice. Two hundred and fifty µl of Super Optimal broth with catabolite repression medium was added and cells incubated at 37°C for 1 hour in a shaking incubator. Cultures were centrifuged at 13300 x g for 1 min and most of the supernatant was removed leaving 50 µl. Fifty µl of L-broth was added and the pellet resuspended. Transformations were plated onto LB plates containing 50 µg/ml ampicillin and incubated overnight at 37°C. Single colonies from each plate were chosen and placed in 4 ml of L-broth containing 50 µg/mL of ampicillin. Cultures were incubated overnight at 37°C in a shaking incubator.

3.2.7 Miniprep

Plasmid purification was carried out using the QIAprep Spin Miniprep Kit (Qiagen), according to the manufacturer's instructions. In brief, bacteria grown overnight were centrifuged at 11000 x g for 30 sec. Supernatant was discarded, the pellet resuspended in 250 µl of Resuspension Buffer P1 and cells lysed by adding 250 µl of Lysis Buffer P2 and gently mixed by inverting each microcentrifuge tube. 350 µl of Neutralisation Buffer N3 was added and the solution mixed by inverting each microcentrifuge tube. Lysate was clarified by centrifuging at 11000 x g for 10 min. To bind the DNA, 750 µl of each sample was transferred to an QIAprep Spin Column, placed in a 2 ml Collection Tube, and centrifuged at 11000 x g for 1 min. Residual sample was added to the respective column and the previous step repeated. To wash the silica membrane, 750 µl of Wash Buffer PE was added to the column, centrifuged at 11000 x g for 1 min and the flowthrough discarded and the centrifugation step was repeated. To remove residual ethanol, the column was centrifuged at 11000 x g for 2 min. To elute the DNA, the column was placed in a new 1.5 ml microcentrifuge tube, 50 µl of Elution Buffer P was added directly to the centre of the silica membrane and incubated at room temperature for 1 min. A last centrifugation was performed at 11000 x g for 1 min. DNA concentration were determined by measuring the optical density at both 260 and 280 nm with a DNA-RNA calculator (NanoDrop® ND-1000, Peqlab, Germany).

3.2.8 Restriction digest analysis

Restriction digest analyses were carried out using the endonucleases *EcoR* I (New England Biolabs) and product sizes analysed according to the vector maps. For *EcoR* I digests were carried out in a 10 µl reaction and prepared as follows: 7 µl of plasmid DNA was added to 1 µl of *EcoR* I, 1 µl of buffer 3 and 1 µl of 10x acetylated BSA (BSA, New England Biolabs). Reactions were incubated at 37°C in a heat-block for 1 h (*EcoR* I). Products were analysed by AGE (Section 3.2.5) using a 100 bp DNA ladder (ThermoScientific).

3.2.9 Sequencing

The sequencing was done at the "in-house" sequence facility of the Faculty of Science, University of South Bohemia and Biology Centre of the Academy of Sciences of the Czech Republic in České Budějovice.

3.2.10 Sequence analysis

The nucleotide sequences were aligned using BLAST (Zhang et al., 2000). Deduced protein sequences (using ExPASy: http://expasy.hcuge.ch/tools/dna.html) were aligned using CLUSTALW (Thompson et al., 1994). The homology between sequences was analysed using MegAlign, (DNASTAR, USA). The signal peptide cleavage sites of the deduced amino acid sequences of the six defensins were predicted with SignalP 4.0 (Petersen et al., 2011). For comparison between species, *I. ricinus* defensins were aligned with the defensins from other hard tick species using the programme ClustalX (Thompson et al., 1994).

3.2.10.1 Phylogenetic analysis

The phylogenetic analysis was performed as follows: sequences were aligned with MUSCLE (v3.7) configured for highest accuracy (Edgar, 2004). After alignment, ambiguous regions were removed with Gblocks (v 0.91b) (Castresana, 2000). The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML programme (v3.0 aLRT) (Anisimova and Gascuel, 2006; Guindon and Gascuel, 2003). Reliability for internal branch was assessed using the bootstrapping method (1000 bootstrap replicates). Graphical representation and editing of the phylogenetic tree were performed with TreeDyn (v 198.3) (Chevenet et al., 2006).

3.2.10.2 Tertiary structure analysis

Tertiary protein modeling for *I. ricinus* defensins

3-D structures of *I. ricinus* defensins were predicted using the Swiss-Model Protein Modeling Server (Arnold et al., 2006; Guex and Peitsch, 1997; Schwede et al., 2003).

Tertiary protein modeling for *I. scapularis* defensins

Predicted tertiary models of the mature peptides were generated using the Phyre2 server (Kelley and Sternberg, 2009). The predicted models were then refined via minimisation and the hydrogen-bond network optimised using the Schrodinger's Maestro Protein Preparation Wizard (Li et al., 2007). The electrostatic potentials for each structure were calculated using the implemented Poisson-Boltzmann equation in the Maestro software.

3.2.11 Biochemical property prediction of new defensins

The molecular weights, isoelectric points and disulphide bridges between cysteine residues of each putative mature peptide were predicted using the Sequence Manipulation Suite (Stothard, 2000), ExPASy tools server (Gasteiger et al., 2005) and DiANNA server (Ferré and Clote, 2005) respectively.

3.2.12 Antibacterial assays

Antibacterial assays for γ-cores of DefMT3, DefMT6 and DefMT7

A quick, qualitative estimate of antimicrobial properties was made using the drop-diffusion test on Petri dishes for Gram+ bacteria *M. luteus*, *B. subtilis*, *S. aureus*, and

Gram- bacteria *E. coli* and *P. aeruginosa*. Quantitative minimum inhibitory concentrations (MICs) were established by observing bacterial growth in multiwell plates. Mid-exponential phase bacteria were added to individual wells containing solutions of the peptides at different concentrations in Luria-Bertani (LB) broth (final volume 0.2 ml, final peptide concentration in the range of 0.5–100 μM). The plates were incubated at 37 °C for 20 h while being continuously shaken in a Bioscreen C instrument (Oy Growth Curves AB Ltd., Finland). The absorbance was measured at 540 nm every 15 min and each peptide was tested at least three times in duplicate. Routinely, 1×10^4 to 5×10^4 CFU of bacteria per well were used for activity determination.

Antibacterial assays for γ-cores of DefMT3, DefMT6, DefMT7, Scapularisin-3 and Scapularisin-6

Concentrations of the peptides ranging from 0.03 to 250 μM were used to test antibacterial activity and MIC (minimum inhibitory concentration) of the peptides against Gram+ bacterial species including *L. fleishmannii*, *L. grayi*, *L. marthii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. rocourtiae*, *S. aureus* and *S. epidermidis* as well as the Gram- bacteria *E. coli* and *P. aeruginosa*. The assays were performed in either Brain Heart Infusion Broth (BHIB) medium in case of *Listeria spp*. or Tryptic Soy Broth (TSB) (Roth, Germany) for others, in 384-well plates (Griener Bio One, Germany). Bacteria species, in the mid-logarithmic phase, were used for all growth inhibition assays. The initial optical density (OD), at 600 nm, for *Listeria* spp. assays was set to 0.01 and for the rest of the bacteria to 0.001 to ensure full contact of each bacterial cell with defensins added to the suspensions. Changes in OD were monitored at 20 min intervals over a 24 h period using an Eon™ Microplate Spectrophotometer (BioTel Instruments, USA). For each assay, a medium-only control culture was included. Antimicrobial activity of the peptides was tested against all bacteria in dublicate.

Antibacterial assays for DefMT2, DefMT3, DefMT5 DefMT6 and DefMT7

Concentrations of the peptides ranging from 0.03 to 250 µM were used to test antibacterial activity and MIC of the peptides against Gram- bacteria *E. coli* and *P. aeruginosa*. The assays were performed in Tryptic Soy Broth (TSB) (Roth, Germany) in 384-well plates (Griener Bio One, Germany). Bacteria species, in the mid-logarithmic phase, were used for all growth inhibition assays. The initial optical density (OD), at 600 nm, for antimicrobial assays was set to 0.001 to ensure full contact of each bacterial cell with defensins added to the suspensions. Changes in OD were monitored at 20 min intervals over a 24 h period using an Eon™ Microplate Spectrophotometer (BioTel Instruments, USA). For each assay, a medium-only control culture was included. Antimicrobial activity of the peptides was tested against all bacteria in duplicate. (All microorganism strains were available at the Fraunhofer Institute for Molecular Biology and Applied Ecology, Department of Bioresources.)

3.2.13 Antifungal assays

Fungicidal activities of synthetic peptides were determined using an inhibition assay reported previously (Rahnamaeian et al., 2009). *Fusarium culmorum* and *Fusarium graminearum* strain 8/1 (Miedaner et al., 2000) were cultured in the dark on Nirenberg Synthetic Nutrient Agar (SNA) medium at 18 °C for 1–2 weeks (Rahnamaeian and Vilcinskas, 2012). Briefly, *F. culmorum* and *F. graminearum* were incubated with different concentrations of peptides from 0.1 µM to 20 µM at room temperature for 24 h under humid conditions. Spore germination and growth were monitored on an inverted microscope Motic AE21 (Motic, China). The IC50 values were recorded when only 50% of the spores were germinated in the solution.

Chapter 4: Results

4.1 Characterisation of *I. ricinus* and *I. scapularis* defensins

Characterisation of novel genes encoding *I. ricinus* defensins

Six novel defensin genes, DefMT2, DefMT3, DefMT4, DefMT5, DefMT6 and DefMT7, were identified *in silico* from the recently published salivary gland shotgun assembly transcriptome (GenBank Bioproject PRJNA177622) of *I. ricinus* adult females and nymphs. GenBank accession numbers for identified novel genes are presented in (Appendix I). To analyse the genomic organisation of novel defensin genes, genomic DNA from two adult female ticks was used as template in PCR. Out of six identified defensin fragments, only DefMT3, DefMT4 and DefMT7 were successfully amplified. The sequences of the PCR products of both DefMT3 and DefMT4 obtained from genomic DNAwere 841 bp long. Sequence analysis showed that DefMT3 and DefMT4 genes include three exons of 45, 62 and 124 bp **(Figure 4)**. Nevertheless, even though both genes contain two introns, these differ in size being 119 and 491 bp for DefMT3 and 110 and 500 bp for DefMT4. The genomic amplified PCR product for DefMT7 was 216 bp. No introns were identified within the sequence of DefMT7.

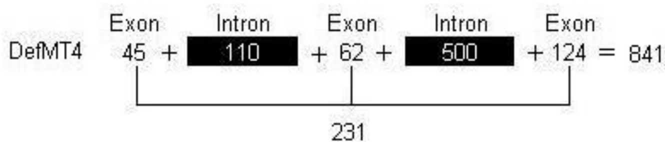

Figure 4. Illustration of DefMT4 genomic structure (Exon: 42 bp + 62 bp + 124 bp, Intron: 110 bp + 500 bp).

The same primers **(Table 2A and 2B)** were used for PCR reactions with cDNA as templates and all the putative defensins were amplified and sequenced. The molecular sizes of the cDNAs ranged from 216 bp to 231 bp **(Figure 5)**.

Five different patterns of disulphide bridges between cysteine residues were found in the novel *I. ricinus* defensins **(Table 3)**. Def1, DefMT3, DefMT4 and DefMT5 had the same disulphide bridge structure and the rest of the defensins had different disulphide bridge structures; DefMT6 contained "insect-type" disulphide bridges.

Table 3. Predicted structure of disulphide bonds between cysteine residues.

***Ixodes ricinus* defensins**	**Disulphide bonds**		
Def2	C1-C3	C2-C6	C4-C5
DefMT2	C1-C6	C2-C4	C3-C5
DefMT6*	C1-C4	C2-C5	C3-C6
DefMT7	C1-C5	C2-C3	C4-C6
Def1, DefMT3, DefMT4, DefMT5	C1-C4	C2-C6	C3-C5

*Same disulphide bond structure as in insect defensins (Taylor, 2006)

The predicted isoelectric points ranged from 9.54 to 10.01 in mature defensins DefMT2 - DefMT6 while the predicted isoelectric point of DefMT7 was 6.48. Thus, five of the novel *I. ricinus* defensins were found to be cationic, while DefMT7 represents an anionic member of the defensin family.

```
Def1   ATGAAG--GTCCTTGCCG-TCTCGCTTGCCTTTTTGCTGATCGCTGGTCTGATCAGCACATCGCTGGCCCAAAATGAGGAAGGAGGAGAAAAAGAGCTTGTTCGAGTTCGTCGCGGTGGT
Def2   ATGAAG--GTCCTTGCCG-TCTCGCTTGCCTTTTTGCTGATCGCTGGTCTGATCAGTACATCGTTGGCTGAAAATGACGAGGGAGGAGAAAAAGAGCTCGTCCGAGTTCGCCGCGGTGGT
DefMT2 ATGAAG--GTT-TGGCTGGTTGCAGCACTCATAATATCAGCTTTAGGATGCCTTGGTGCGTTTCCGGCA------GAAGGGAACAACGAACTTGTACATCACAGAGTCCGTCGAGG---A
DefMT3 ATGAAG--GTCCTTGCTG-TCTCGCTTGCCTTTTTGCTGATCGCTGGTCTGATCACTTCATCGCTGGCTGAAAATGACGAGGGAGGAGAAAAAGAGCTCGTTCGAGTTCGCCGCGGTGGT
DefMT4 ATGAAG--GTCCTTGCCG-TCTCGCTTGCCTTTTTGCTGATCGCTGGTCTGATCACTACATCGCTGGCTGAAAATGACGAGGGAGGAGAAAAAGAGCTCGTTCGAGTTCGCCGCGGTGGT
DefMT5 ATGAAG--GTT-TGGCTGGTTGCAGCGCTCATAATATCAGCTTTAGGATGCCTTGCTGCGTTTCCGGCT------GAAGGCAACAGCCAACTTGCACATCACAGAGTCCGCCGAGG---G
DefMT6 ATGAAG--GCCGTTGCTA-TCGCTCTCGTCGTCGTGATGATTGCGGTCTTATCAGCACTTC-CTG-CTCACAA-GAAGACGATAGCCAGGTGGCTCATGTCAGAGTTCGTCGTGG--GT
DefMT7 ATGAATACGTCTTCATTATTCACGGTGGCGCTGATTGTCTTTGCTGGGTTCATGACGATTCAGCTTGTTTCC-------CCGTA-CGTGATTCAACCTTTC----TTCGACATAG--GT
       *****   *   *      *  *        *  *          **     *                                           *         * ** *   *

Def1   TACTACTGCCC--ATTTTTTCAAGATAA-ATG-CCACCGTCACTGCAGG---AGCTTCGGGCGAAAAGCAGGCTACTGC--GGCGGTTTTCTCAAAAAGACCTGCATCTGCGTCATGAAA--- 228
Def2   TACTACTGCCC--ATTCCGGCAGGATAA-ATG-CCACCGTCACTGCAGG---AGCTTCGGGCGAAAAGCAGGCTACTGC--GGCGGTTTTCTCAAAAAGACCTGCATCTGCGTCTG------- 224
DefMT2 TACTTTTGCCC--ATACAAT--GGCTACTGTGATCACCACTGTCGCAAG---AAACTCCGTTGGCGTGGAGGTTACTGC--GGAGGACGTTGGAAGCTGACGTGCATCTGCGTTCGGGGCTAA 222
DefMT3 TATTACTGCCC--ATTCCGGCAAGATAA-ATG-CCACCGTCACTGCAGG---AGCTTCGGGAGAAAAGCAGGCTACTGC--GGCAATTTTCTGAAAAGGACCTGCATCTGTGTCAAGAAATAG 231
DefMT4 TACTACTGCCC--ATTCCGGCAAGATAA-ATG-CCACCGTCACTGCAGG---AGCTTCGGGCGAAAAGCAGGCTACTGC--GGCAATTTTCTGAAAAGGACCTGCATCTGTGTCAAGAAATAG 231
DefMT5 TTCTTTTGCCC--ATACAAT--GGATATTGTGATCGCCACTGCCGTAAA---AAACTCCGTCGCCGTGGAGGTTACTGT--GGAGGACGCTGGAAGTTGACGTGCATCTGCATTATGAACTAA 222
DefMT6 T-CGGCTGTCC--ACTCAACCAGGGGGC-GTG-TCACAACCACTGCAGA---AGCATCAAGCGCCGGGGTGGTTACTGCTCGGGAATCATC--AAGCAAACCTGCACCTGCTACCGGAAGTGA 225
DefMT7 TTCGGTTGTCCGAAGTCTGCC---TTGTCCTGCTC-CCAGCAATGTAGAGAAAACAACACACACAGTGGAGGATACTGC--AACGGACCCTTCAATATTGTCTGTAGCTGCTACTGA------ 216
       *     ** **  *                ** * *       * *     *    *         *  ** *****              **       ** * ***
```

Figure 5. Alignment of nucleotide sequences of the eight *I. ricinus* defensins. Asterisks indicate the conserved nucleotide residues.

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The nucleotide sequences of the six novel defensins were aligned with two previously reported defensins from *I. ricinus* (Def1 and Def2) (Rudenko et al., 2007; Chrudimská et al., 2011). At the amino acid and nucleotide levels the most dissimilar defensin was DefMT7 and the closest to each other were DefMT3 and DefMT4 as they are 98% similar at the nucleotide and amino acid levels. The recently described Def1 and Def2 are 95% similar at the amino acid level. In addition, DefMT2 and DefMT5 are 88% similar at the nucleotide level and 86% similar at the amino acid level. Overall the percentages of nucleotide and amino acid similarity were above 45% and 15%, respectively **(Table 4)**.

Table 4. Amino acid and nucleotide similarities between *I. ricinus* defensins.

	DefMT2	DefMT3	DefMT4	DefMT5	DefMT6	DefMT7	Def1	Def2
DefMT2	***	54.0 nt	56.0 nt	88.0 nt	54.0 nt	45.0 nt	60.0 nt	58.0 nt
DefMT3	31.0 aa	***	98.0 nt	88.0 nt	63.0 nt	48.0 nt	90.0 nt	93.0 nt
DefMT4	31.0 aa	98.0 aa	***	58.0 nt	63.0 nt	47.0 nt	92.0 nt	95.0 nt
DefMT5	86.0 aa	28.0 aa	28.0 aa	***	59.0 nt	56.0 nt	57.0 nt	56.0 nt
DefMT6	30.0 aa	41.0 aa	43.0 aa	34.0 aa	***	54.0 nt	63.0 nt	60.0 nt
DefMT7	18.0 aa	15.0 aa	15.0 aa	19.0 aa	22.0 aa	***	54.0 nt	50.0 nt
Def1	32.0 aa	89.0 aa	90.0 aa	38.0 aa	45.0 aa	16.0 aa	***	92.0 nt
Def2	32.0 aa	94.0 aa	95.0 aa	30.0 aa	44.0 aa	16.0 aa	95.0 aa	***

- The values are % of nucleotide and amino acid sequence similarity

- nt nucleotide sequence similarities

- aa amino acid sequence similarities

Deduced amino acid sequences of preprodefensins ranged from 71 to 74 amino acids **(Figure 6A and 6B)**, while mature proteins, after signal peptide cleavage, contained 54 amino acid residues for DefMT2, DefMT3, DefMT4, DefMT5, DefMT6 and 56 amino acids for DefMT7 with the predicted molecular weights being 6.43, 6.36, 6.36, 6.39, 5.9 and 5.06 kDa respectively. The predicted signal peptides are shown in **Appendix I**.

The ClustalW alignment shows conserved positions in *I. ricinus* defensins, compared with the previously reported *I. ricinus* defensins (Def1 and Def2), and six conserved cysteine residues that form intramolecular disulphide bridges, essential for tick defensin function (Zheng et al., 2012). Amino acid similarity among *I. ricinus* defensins ranged from 15% to 95% (**Table 4**).

Similarity between DefMT3 and DefMT4 was found to be 98% at the amino acid and nucleotide levels **(Table 4- Figure 7)** with only one amino acid difference at position 19 of the signal peptide. Serine substitution was found in DefMT3 and threonine in DefMT4. In addition, DefMT3 and DefMT4 were similar to Def1 and Def2 at both the amino acid and nucleotide levels **(Figure 7)**, therefore they were considered to be isoforms. **Table 4** shows that DefMT2 and DefMT5 have high similarity at both amino acid (86%) and nucleotide (88%) levels. When compared with defensins from other hard ticks, *I. ricinus* defensins showed conserved amino acid positions, particularly in cysteine residues **(Figure 6A and 6B)**.

```
DefMT-2  -MKVWLVAALIISALGCLGAFPAEGNNE--LVHHRVRRG-YFCPYN-GYCDHHCRKKLRWRGGYCGGRWKLTCICVRG 73
DefMT-5  -MKVWLVAALIISALGCLAAFPAEGNSQ--LAHHRVRRG-FFCPYN-GYCDRHCRKKLRRRGGYCGGRWKLTCICIMN 73
DefMT-3  -MKVLAVSLAFLLIAGLITSSLAENDEGGEKELVRVRRGGYYCPFRQDKCHRHCR-SFGRKAGYCGNFLKRTCICVKK 76
DefMT-4  -MKVLAVSLAFLLIAGLITTSLAENDEGGEKELVRVRRGGYYCPFRQDKCHRHCR-SFGRKAGYCGNFLKRTCICVKK 76
DefMT-6  -MKAVAIALVVVMIAGLISTSCSQEDDS-QVAHVRVRRG-FGCPLNQGACHNHCR-SIKRRGGYCSGIIKQTCTCYRK 74
DefMT-7  MNTSSLFTVALIVFAGFMTIQLVSP----YVIQPFFDIG-FGCPKSALSCSQQCRENNTHSGGYCNGPFNIVCSCY-- 71
```

Figure 6A. Amino acid sequences of novel *I. ricinus* defensins. DefMT3, DefMT4, DefMT5, DefMT6 and DefMT7.

```
DefMT-2.JAA65352             --------MKVWLVA-ALIISALGCLGAFPA-EGN-NELVHHRVRR-GYFCPYN--GYCDHHCRKKLRWRGGYCGGRWKLTCICVRG 73
DefMT-3.JAA71488             --------MKVLAVS-LAFLLIAGLITSSLAENDEGGEKELVRVRRGGYYCPFRQ-DKCHRHCRSFG-RKAGYCGNFLKRTCICVKK 76
DefMT-4.JAA7177              --------MKVLAVS-LAFLLIAGLITTSLAENDEGGEKELVRVRRGGYYCPFRQ-DKCHRHCRSFG-RKAGYCGNFLKRTCICVKK 76
DefMT-5.JAA66832             --------MKVWLVA-ALIISALGCLAAFPA-EGN-SQLAHHRVRR-GFFCPYN--GYCDRHCRKKLRRRGGYCGGRWKLTCICIMN 73
DefMT-6.JAA71516             --------MKAVAIA-LVVVMIAGLISTSCS-QEDDSQVAHVRVRR-GFGCPLNQ-GACHNHCRSIK-RRGGYCSGIIKQTCTCYRK 74
DefMT-7.JAA69779             --------MNTSSLFTVALIVFAGFMTIQLV-----SPYVIQPFFDIGFGCPKSA-LSCSQQCRENNTHSGGYCNGPFNIVCSCY-- 71
I.ricinus.AY335442           --------MKVLAVS-LAFLLIAGLISTSLAQNEEGGEKELVRVRRGGYYCPFFQ-DKCHRHCRSFG-RKAGYCGGFLKKTCICVMK 76
I.ricinus.EF067917           --------MKVLAVS-LAFLLIAGLISTSLAQNEEGGEKELVRVRRGGYYCPFFQ-DKCHRHCRSFG-RKAGYCGGFLKKTCICV-- 74
I.ricinus.DQ361064           --------MKVLAVS-LAFLLIAGLISTSLAENDEGGEKELVRVRRGGYYCPFRQ-DKCHRHCRSFG-RKAGYCGGFLKKTCICV-- 74
I.scapularis.AY660970        --------MRVIAVT-LIALLVAGAFMTSSA-QEEENQVAHVRVRR-GFGCPFDQ-GACHRHCQSIG-RRGGYCAGFIKQTCTCYHN 74
I.scapularis.EEC17916        --------MNTSSLFTVALIAFAGFMTIQLV-----SPYVIQPFFDIGFGCPKSA-LSCSQQCRENNTHSGGYCNGPFNIVCSCY-- 71
I.scapularis.EEC01374        --------MKVLAVS-LAFLLITGLISTSLAENDEGGEKELVRVRRTSYNCPFQK-HKCHRHCKSIG-HIAGYCGGFRNRTCICVKK 76
I.persulcatus.AB469201       --------MRVVAVA-LIALLVAGAFMTSSA-QEEENQVAHVRVRR-GFGCPFNQ-GACHRHCRSIG-RRGGYCAGLFKQTCTCYSR 74
D.andersoni.EF060192         --------MRGLCIC-LVFLLVCGLVSATAA-APAESEVAHLRVRR-GFGCPLNQ-GACHNHCRSIR-RRGGYCSGIIKQTCTCYRN 74
D.variabilis.AY181027        --------MRGLCIC-LVFLLVCGLVSATAA-APAESEVAHLRVRR-GFGCPLNQ-GACHNHCRSIR-RRGGYCSGIIKQTCTCYRN 74
R.microplus.AY233213         --------MRGIYIC-LXFVLXCGLVSGLAD-VPAESEMAHLRVRR-GFGCPFNQ-GACHRHCRSIR-RRGGYCAGLIKQTCTCYRN 74
R.microplus.Q86LE4           --------MRGIYIC-LVFVLVCGLVSGLAD-VPAESEMAHLRVRR-GFGCPFNQ-GACHRHCRSIR-RRGGYCAGLIKQTCTCYRN 74
H.longicornis.EU627689       --------MAESTTT-CFLLLVTGYVTAVMS------EEAHLRSRR-DFGCGQGMIFMCQRRCMRLYPGSTGFCRG---FRCMCDTH 68
H.longicornis.HQ908088       --------MARSNLL-LLLLLAAVTIVAVAE------EAAHHRARR-DFGCARGMIFVCMRRCARMYPGSTGYCQG---FRCMCDTH 68
H.longicornis.AB105544       --------MKVLAVA-LIFVLVAGLFCTAAA-QDDESDVPHVRVRR-GFGCPLNQ-GACHNHCRSIG-RRGGYCAGIIKQTCTCYRK 74
H.longicornis.EF432731       --------MKVFSAL-FLVGLLLAFLAFAAG-DEEDSSKPLVRVRR-GFGCPFDE-RACHAHCQSVG-RRGGYC-GNFRMTCYCYKN 73
H.longicornis.EU035972       --------MKVFSAL-FLVGLLLAFLAFAAG-VKEDSSKPLVRVRR-GFGCPFDE-RACHAHCQSVG-RRGGYC-GNFRMTCYCYKN 73
H.longicornis.EU035973       --------MKVLAVA-LIFVLVAGLFCTSAA-QDDESDVPHVRVRR-GFGCPLNQ-GACHNHCRSIG-RRGGYCAGIIKQTCTCYRK 74
H.longicornis.AB028926       --------MKLLVVC-AALTVLGGLLQGATCDDSDHG----FRTAHVDLVCPDNP-DNCIQQCVSKG-AQGGYCT---NEKCTCYEK 69
A.hebraeum.AY437137          MATVRNSRPEAAGEP-SGVSSTEGDWRHIEKRDVSYQGEGNTRRFDNPFGCPADE-GKCFDHCNNKA-YDIGYCGGSYRATCVCYRK 84
A.hebraeum.AY437138          MATQRREISWTFGPL-YTWRTTKGYGTTLETTNATSTSSKPSRRYENPYGCPTDE-GKCFDRCNDSE-FEGGYCGGSYRATCVCYRT 84
A.americanum.DQ864986        --------MKVLAVA-FIFVLVAGLVST-AD-EEDKSQVPLVRVRR-GFGCPFNQ-YQCHSHCLSIG-RRGGYCGGSFKTTCTCYN- 72
O.moubata.Q9BLJ3             --------MNKLFIV-ALVVALAVATMAQEV-HNDVEEQSVPRVRR-GYGCPFNQ-YQCHSHCSGIRGYKGGYCKGTFKQTCKCY-- 73
O.moubata.Q9BLJ4             --------MNKLFIV-ALVVALAVATMAQEV-HDDVEEQSVPRVRR-GYGCPFNQ-YQCHSHCRGIRGYKGGYCTGRFKQTCKCY-- 73
O.moubata.Q8MY08             --------MNKLFIV-ALVLALAVATMAHEV-YDDVEEPSVPRVRR-GYGCPFNQ-YQCHSHCSGIRGYKGGYCKGLFKQTCNCY-- 73
O.moubata.Q8MY07             --------MNKLFIV-ALVLALAVATMAHEV-HDDIEEPSVPRVRR-GFGCPFNQ-YECHAHCSGVPGYKGGYCKGLFKQTCNCY-- 73
O.tartakovskyi.ACJ04431      --------MNKLFIV-ALVVALAVATMAQEV-HNDVEEQSVPRVRR-GYGCPFNQ-YQCHSHCSGIRGYKGGYCKGTFKQTCKCY-- 73
O.tartakovskyi.ACJ04432      --------MNKLFIV-ALVVALAVATMAQEV-HDDVEEQSVPRVRR-GYGCPFNQ-YQCHSHCRGIRGYKGGYCTGRFKQTCKCY-- 73
O.papillipes.ACJ04425        --------MNKLFIV-ALVVALAVATMAQEV-HDDVEEQSVPRVRR-GYGCPFNQ-YQCHSHCSGIRGYKGGYCKGTFKQTCKCY-- 73
O.papillipes.ACJ04426        --------MNKLFIV-ALVVALAVATMAQEV-HDDVEEQSVPRVRR-GYGCPFNQ-YQCHSHCRGIRGYKGGYCTGRFKQTCKCY-- 73
O.papillipes.ACJ04427        --------MNKLFIV-ALVLALAVATMAHEV-HDDIEEPSVPRVRR-GFGCPFNQ-YECHAHCSGVPGYKGGYCKGLFKQTCNCY-- 73
O.puertoricensis.ACJ04429    --------MNKLFIV-ALVAALAVATMAQEV-HNDVEEQSVPRVRR-GYGCPFNQ-YQCHSHCSGIRGYKGGYCKGTFKQTCKCY-- 73
O.puertoricensis.ACJ04430    --------MNKLFIV-ALVVALAVATMAQEV-HDDVEEQSVPRVRR-GYGCPFNQ-YQCHSHCRGIRGYKGGYCTGRFKQTCKCY-- 73
O.rostratus.ACJ04428         --------MNKLFIV-ALVVAPAVATMAQEV-HNDVEEQSVPRVRR-GYGCPFNQ-YQCHSHCSGIRGYKGGYCKGTFKQTCKCY-- 73
A.monolakensis.Q09JJ7        --------MKYLAVL-VFLLISSTVQVSAQD-DDGDDDAALTRVRR-GFGCPFNQ-GACHRHCQSIG-RKGGYCSGLFKQTCTCYRH 74
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Figure 6B. Amino acid alignment of hard and soft tick defensins with *I. ricinus* defensins.
Six conserved cysteine residues are indicated by rectanges.

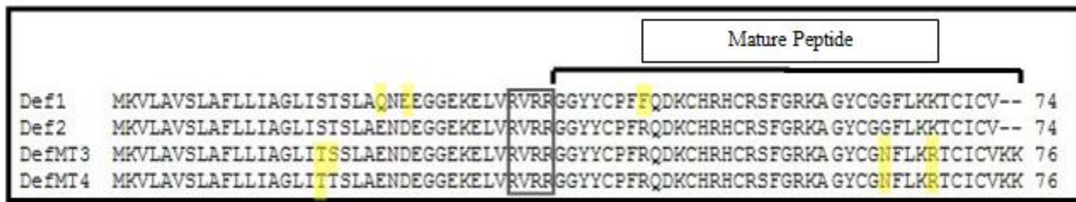

Figure 7: Comparison of amino acid sequences of *I. ricinus* isoforms Def1, Def2, DefMT3 and DefMT4. Mature peptides of the isoform are indicated after the furin motif (RVRR-rectangled), yellow highlights indicates amino acid changes between the isoforms.

Characterisation of Scapularisin-3 and Scapularisin-6

The primary structures of Scapularisin-3 and Scapularisin-6 are shown in **Figure 8**. The deduced amino acid sequences of the prodefensins are 70 amino acids for Scapularisin-3 and 74 amino acids for Scapularisin-6. As indicated in **Figure 8**, the two prodefensins contain a furin motif, indicating putative enzymatic cleavage *in vivo* by the enzyme Furin. After cleavage, both mature proteins contain 38 amino acid residues. The mature peptides of Scapularisin-3 and Scapularisin-6 have predicted molecular weights of 4.3 kDa and 4.1 kDa, respectively.

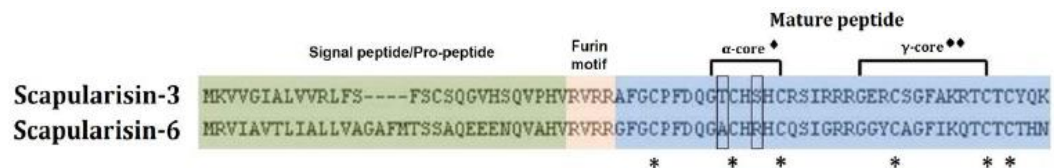

Figure 8. The primary structures of Scapularisin-3 and Scapularisin-6. Alignment of the primary structure of the defensins Scapularisin-3 and Scapularisin-6. Deduced amino acid sequences of prodefensins are 70 and 74 amino acids for Scapularisin-3 and Scapularisin-6, respectively. After cleavage, both mature proteins contain 38 amino acid residues (mature peptides). Within the α-core motif residues Thr10 and Ser13 in Scapularisin-3 are substituted with Ala10 and Arg13 in Scapularisin-6, enclosed in rectangles, and the six conserved cysteine residues are indicated by stars (♦ (Sagaram et al., 2011), ♦♦ (Wang and Zhu, 2011)).

4.2 Phylogenetic and tertiary structure analysis

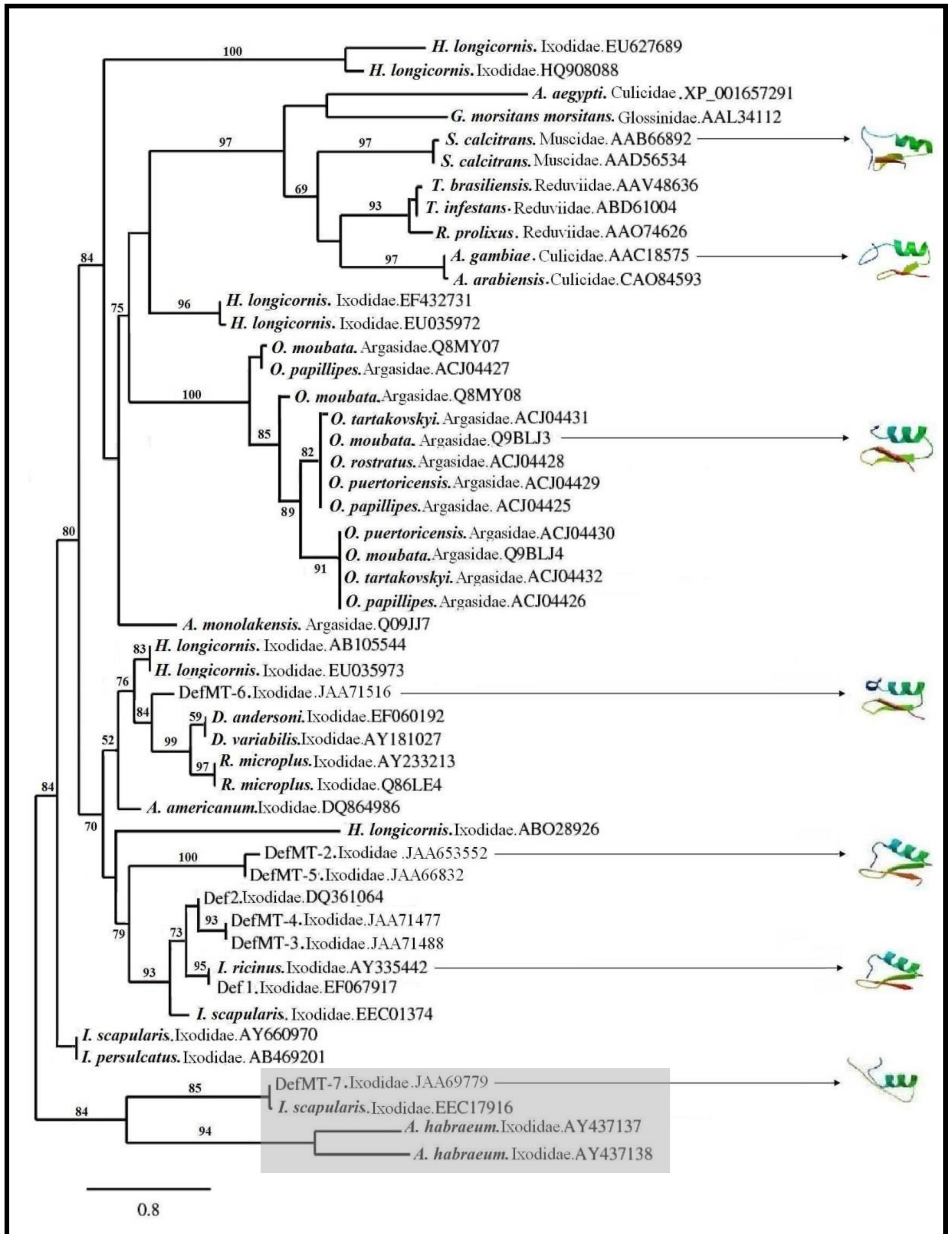

Figure 9. Phylogenetic tree and predicted 3D structure of representative defensins. Maximum likelihood phylogenetic tree. Name of the organism, genus/family and accession numbers are presented for each sequence used. Defensins from soft (genera: *Ornithodoros* and *Argas*) and hard (genera: *Haemaphysalis*, *Dermacentor*, *Ixodes*, *Amblyomma* and *Rhipicephalus*) ticks and members of different blood-feeding families of arthropod vectors (*Reduviidae*, *Culicidae*, *Muscidae* and *Glossinidae*) are shown. Bootstrap valuesmore than 50% are indicated. Blue and red areas indicate the N-terminus and C-terminus of the putative 3D structures of representative defensins of themain phylogenetic clusters. α-Helix is to the N-terminus and antiparallel β strands to the C-terminus of the protein 3D structures. All the structures are similar except for the structure of DefMT7 which lacks the antiparallel β strands to the C-terminus of the protein.

Figure 9 shows the phylogenetic tree generated using maximum likelihood and the 3D structures of representative defensins from different phylogenetic clades. Our phylogenetic analysis shows evolutionary diversity among defensins which did not form a unique clade. DefMT6 clustered together with Dermacentor spp. and R. microplus defensins while DefMT2, DefMT3, DefMT4 and DefMT5 clustered together with other defensins from I. ricinus that were previously characterised (Chrudimská et al., 2011; Rudenko et al., 2007). DefMT7 is a noncationic molecule (pI 6.48) while the rest are cationic (pI ranging from 9.54 to 10.01). In the tree, it is shown that DefMT7 clusters together with other non-cationic defensins included in the phylogenetic analysis (**Figure 9**, grey box).

Predicted 3D structures of defensins belonging to different phylogenetic clusters are also shown in **Figure 9**. The 3D structure of the defensins among the presented phylogenetic clusters barely changes. All contain an α-helix at the N-terminus while an antiparallel β strand is observed at the C-terminus. One exception to this rule is DefMT7 which does not have the characteristic β strand at the C-terminus.

In general, hard tick defensins have a mixed α-helix and β-sheet structure (data not shown). Predicted 3D structures of *I. ricinus* defensins DefMT3, DefMT4, Def1 and Def2 showed structural similarities. DefMT7 does not have the characteristic β strand at the C terminus.

The other *I. ricinus* defensins contain anti parallel β strands **(Figure 10)** at the end of the molecule**.** The position of anionic DefMT7 in the tree supports its low primary, secondary and tertiary structural similarities with other cationic members of *I. ricinus* defensin family.

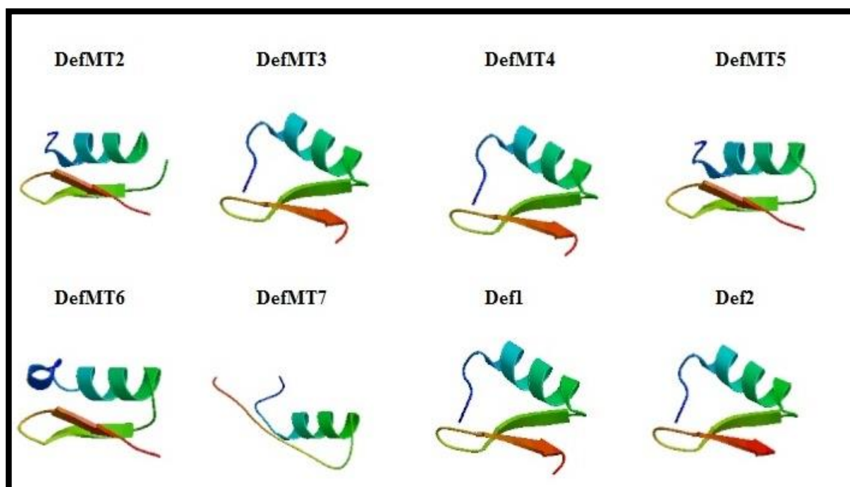

Figure 10. 3D structure of *I. ricinus* defensins. New defensins DefMT2, DefMT3, DefMT4, DefMT5, DefMT6, DefMT7 and previously reported Def1, Def2. DefMT3, DefMT4, Def1 and Def2 show structural similarities as shown in their primary structures. In addition DefMT7 shows a different 3D structure from the other *I. ricinus* defensins. 3D structure was predicted using the SWISS-MODEL programme.

Tertiary structure of Scapularisin-3 and Scapularisin-6

The tertiary structures of the *I. scapularis* defensins are similar and are conserved when compared to the NMR structure of the antibacterial defensin DEF-AAA from *A. gambiae* (Landon et al., 2008; **Figure 11A**). The defensins depicted in Figure 11, however, are missing the archetypal disulphide bond that links both termini, and

deletions in the α-core motif and insertions in the γ-core motifs are shorter. The overall backbone deviation does not exceed 3.2Å and the deviation between the two *I. scapularis* defensins is <1Å **(Figure 11B)**. Figure 11A also shows that the cationic electrostatic surface of Scapularisin-3 (pI 9.4) and Scapularisin-6 (pI 8.7) is mainly basic (blue); however, Scapularisin-6 possesses slightly more acidic residues (red). These acidic surfaces are within the α-core motif with the residues Thr10 and Ser13 in Scapularisin-3 substituted by Ala10 and Arg13 in Scapularisin-6 (GxCHxHC), indicated by the yellow arrowhead in **Figure 11A**.

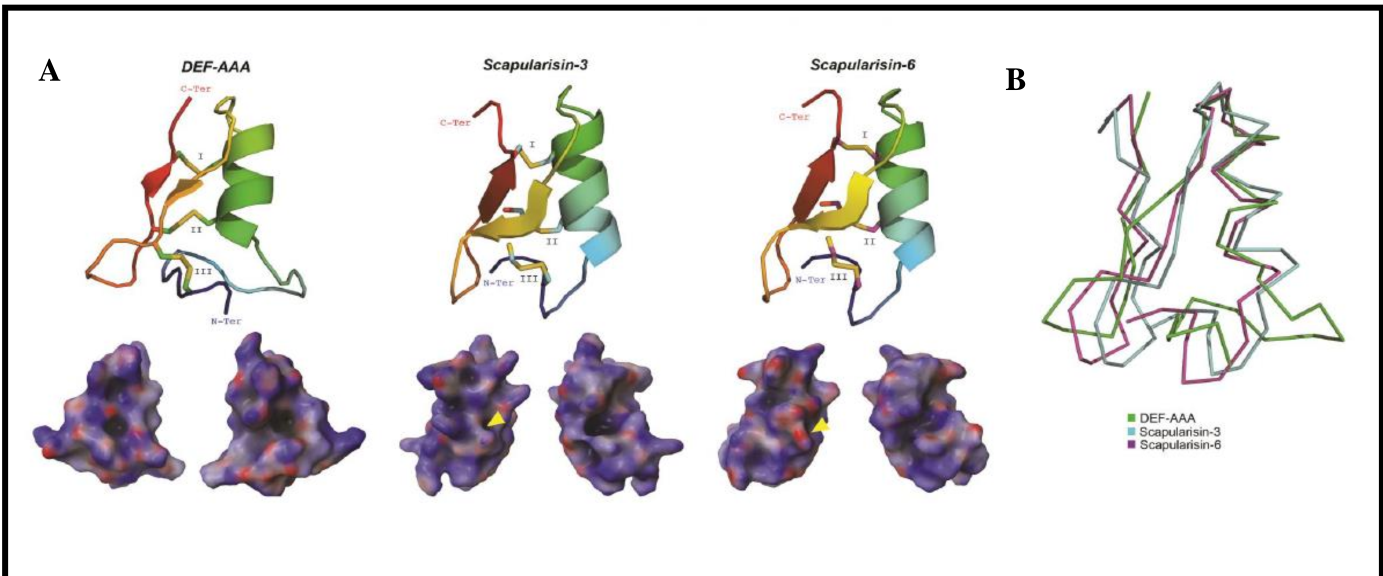

Figure 11. Tertiary structure of Scapularisin-3 and Scapularisin-6. Tertiary structure of defensin peptides. Panel A displays the NMR defensin structure from *A. gambiae* (DEF-AAA; PDB: 2NY8) and the two predicted tertiary structures of Scapularisin-3 (GenBank: EEC13914) and Scapularisin-6 (GenBank: EEC08935). The tertiary structures depict the conserved disulphide bridges (Roman numerals), loops, β-sheets, and the α-helix. All structures are coloured from the N-terminus (blue) to the C-terminus (red). Below are the respective electrostatic potentials for each structure in 180˚ turns (blue = positive; red = negative; white = neutral). (B) The protein backbone alignment in Panel B depicts each structure colour-coded as indicated.

4.3 Expression analysis

DefMT3, DefMT4, DefMT5, DefMT6 and DefMT7 expression patterns

To determine the expression profiles of the defensins in different *I. ricinus* tissues, PCR was performed. DefMT3, DefMT4, DefMT7 gene expression was observed in all organs tested (salivary glands, ovaries, Malpighian tubules, midgut and haemolymph). DefMT6 gene expression was observed in the salivary glands, ovaries and Malpighian tubules. No DefMT6 transcripts were detected in the midgut and haemolymph. Expression of DefMT2 and DefMT5 was only observed in the salivary glands **(Figure 12)**. Transcripts from all defensins reported in this study were found in IRE/CTVM19 cells, except for DefMT5 **(Figure 12)**.

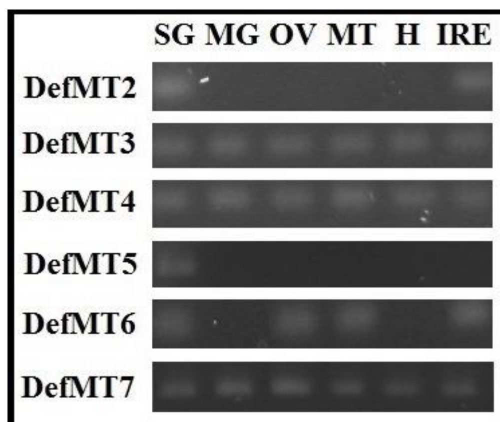

Figure 12. Expression profile of novel *I. ricinus* defensins. The expression pattern of the different defensins isolated in this study is shown. *I. ricinus* SG: salivary gland, MG: midgut, OV: ovary, MT: Malpighian tubule, H: haemolymph and IRE: embryo-derived tick cell line IRE/CTVM19 were used in order to test the presence of transcripts of the defensins. DefMT2, 5 and 6 were found to be expressed in a tissue specific manner while DefMT3, 4 and 7 were found to be ubiquitous.

Scapularisin-3 and Scapularisin-6 expression patterns

In order to determine the expression profiles of Scapularisin-3 and Scapularisin-6 in *I. scapularis* salivary gland, midgut and embryo-derived cell lines, RNA was extracted from these tissues and PCR amplification of transcripts of the Scapularisin-3, Scapularisin-6 and β-actin genes was performed. Scapularisin-6 gene expression

was observed in both salivary glands and midgut (**Figure 13**). However, Scapularisin-3 gene expression was only observed in the salivary glands. No transcripts from either defensin were amplified from the ISE6 or ISE18 tick cell lines (**Figure 13**).

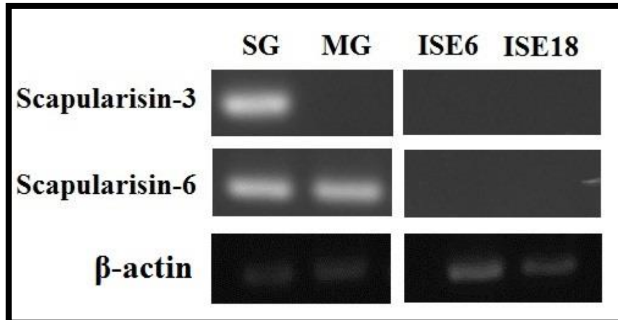

Figure 13. Expression profile of Scapularisin-3 and Scapularisin-6. The expression pattern determined by PCR and visualised by agarose gel electrophoresis of Scapularisin-3 and Scapularisin-6 in *I. scapularis* salivary glands (SG), midgut (MG) and embryo-derived tick cell lines ISE6 and ISE18 is shown. Both defensins were expressed in SG. Scapularisin-6 was expressed in MG and neither of the two defensins was expressed by embryo-derived tick cells (ISE6 and ISE18) *in vitro*. (β-actin used as a positive control)

4.4 Functional analysis

DefMT3 possesses inhibitory activity against *Multicoccus luteus*

The antimicrobial activities of the γ-cores of DefMT3, DefMT6 and DefMT7 were evaluated against Gram+ bacteria *M. luteus*, *B. subtilis*, *S. aureus*, *S. epidermidis* and seven *Listeria* spp. and Gram- bacteria *E. coli* and *P. aeruginosa*. Only *M. luteus* emerged as susceptible to increasing concentrations of DefMT3, being totally inhibited in the presence of 15 µM DefMT3. However, no activity against any of the bacteria species used in the antimicrobial assays was detected for DefMT6 and DefMT7, tested in a range of concentrations up to 250 µM.

DefMT3 and DefMT6 are potent Antifungal agents

The antifungal activity of the γ-cores of DefMT3, DefMT6 and DefMT7 was assessed using two phytopathogenic fungi, *F. culmorum and F. graminearum*. While DefMT7 was inactive, the other two defensins show high antifungal activity. DefMT3 totally inhibited the spore germination of *F. culmorum* and *F. graminearum* at, respectively 1 and 2 μM concentrations (**Table 5- Figure 14**). In the case of DefMT6, maximum activity was recorded at 2 μM and 5 μM against *F. culmorum* and *F. graminearum*, respectively (**Table 5- Figure 14**).

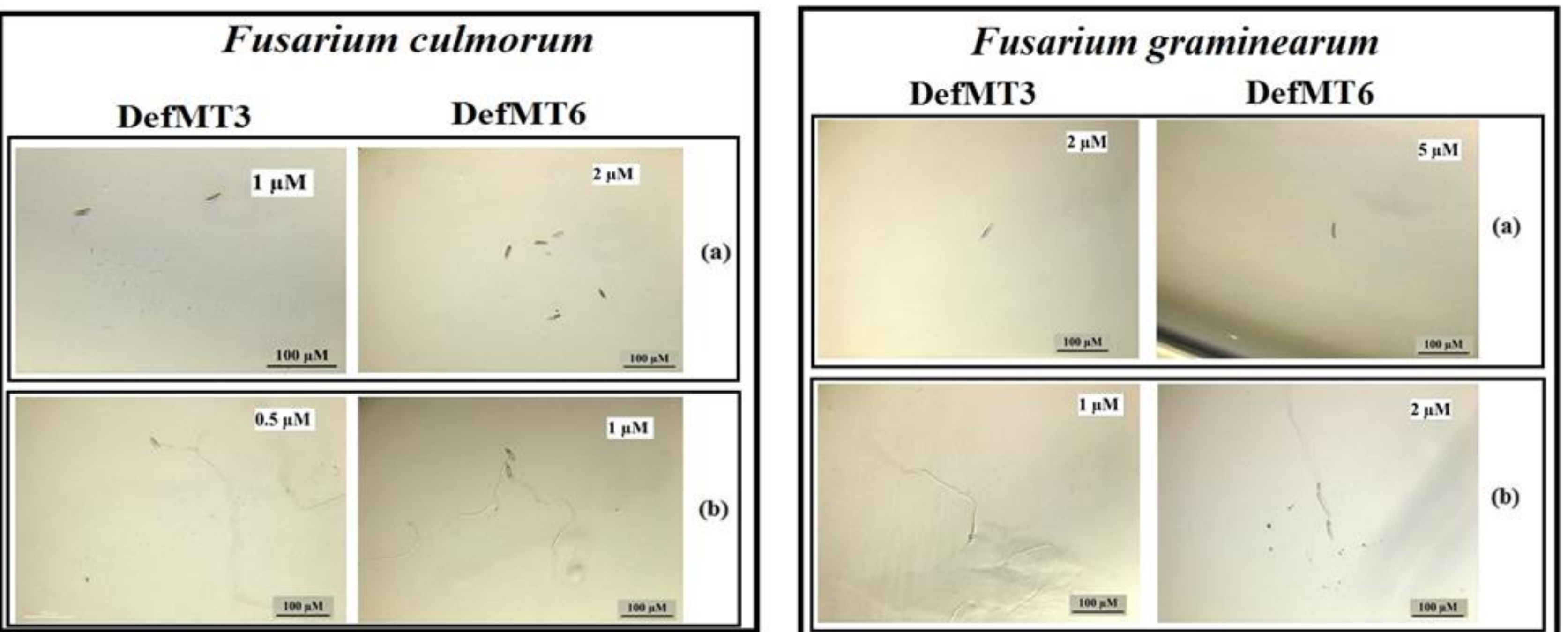

Figure 14. Antifungal activity of DefMT3 and DefMT6 against *Fusarium* spp. Images show (a) inhibition of spore germination and (b) spore germination (presence of hyphae) at different concentrations of DefMT3 and DefMT6. Pictures of the respective fungal cultures were taken after 24 hours of incubation of *F. culmorum* and *F. germinearum* with the peptides.

Table 5. Half maximal inhibitory concentration of synthetic DefMT3and DefMT6.

Peptide	IC_{50} value (µM)	
	F. culmorum	*F. graminearum*
DefMT3	1	2
DefMT6	2	5

IC_{50}: Half maximal inhibitory concentration

Preliminary results of antimicrobial activity of whole peptides of DefMT2, DefMT3, DefMT5, DefMT6 and DefMT7

The antimicrobial activities of all synthetic defensins were evaluated against the Gram- bacteria *E. coli* and *P. aeruginosa*. Both *E. coli* and *P. aeruginosa* were susceptible to increasing concentrations of DefMT3 and DefMT5 (**Table 6**). No activity against *E. coli* and *P. aeruginosa* used in the antimicrobial assays was detected for DefMT2, DefMT6 and DefMT7, tested at a range of concentrations up to 250 µM.

Table 6. Minimum inhibitory concentration of synthetic DefMT3 and DefMT5.

Peptide	MIC (µM)	
	E. coli	*P.aeruginosa*
DefMT3	120	60
DefMT5	120	6

MIC: minimum inhibitory concentration

Scapularisin-6 possesses inhibitory activity against *L. grayi*

The antimicrobial activities of Scapularisin-3 and Scapularisin-6 were evaluated against the Gram+ bacteria *S. aureus*, *S. epidermidis* and seven *Listeria* spp., and the Gram- bacteria *E. coli* and *P. aeruginosa*. Of these, only *L. grayi* was susceptible to increasing concentrations of Scapularisin-6, being totally inhibited in the presence of 120 μM Scapularisin-6 **(Figure 15- Table 6)**. No activity against any of the bacteria species used in the antimicrobial assays was detected for Scapularisin-3, tested at a range of concentrations up to 250 μM.

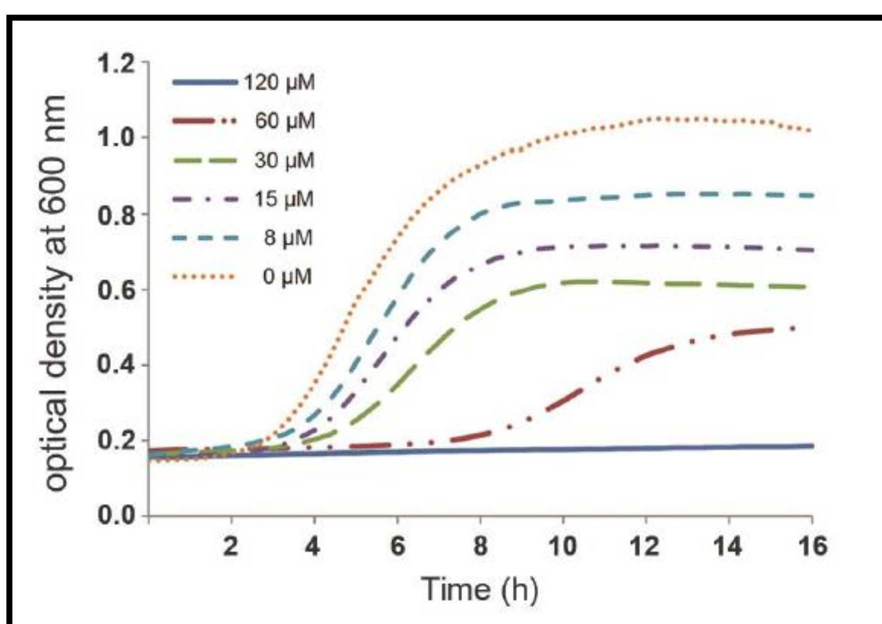

Figure 15. Antimicrobial activity of Scapularisin-6 against *L. grayi*. The figure represents bacterial growth curves at different concentrations of Scapularisin-6 peptide. Maximal growth inhibition was observed at 120 μM of peptide concentration (blue line).

Table 7. Minimum inhibitory concentration of synthetic *I. scapularis* defensins

Peptide	MIC (μM)						
	L. fleishmannii	*L. grayi*	*L. marthii*	*L. innocua*	*L. welshimeri*	*L. seeligeri*	*L. rocourtiae*
Scapularisin-3	> 250	> 250	> 250	> 250	> 250	> 250	> 250
Scapularisin-6	> 250	**120**	> 250	> 250	> 250	> 250	> 250

MIC: minimum inhibitory concentration

Scapularisin-3 and Scapularisin-6 are potent antifungal agents

Additionally, the antifungal activity of Scapularisin-3 and Scapularisin-6 was assessed using two major phytopathogenic fungi, *F. culmorum and F. graminearum*. Both Scapularisin-3 and Scapularisin-6 showed high levels of antifungal activity. Scapularisin-3 totally inhibited germination of *F. culmorum* and *F. graminearum* spores at concentrations of, respectively, 0.5 and 1 µM (**Table 8- Figure 16**). In case of Scapularisin-6, maximum activity was recorded at 1 µM and 2 µM against *F. culmorum* and *F. graminearum*, respectively (**Table 8- Figure 16**).

Table 8. Half maximal inhibitory concentration of synthetic *I. scapularis* defensins.

	IC_{50} value (µM)	
Peptide	*F. culmorum*	*F. graminearum*
Scapularisin-3	0.5	1
Scapularisin-6	1	2

IC_{50}: Half maximal inhibitory concentration

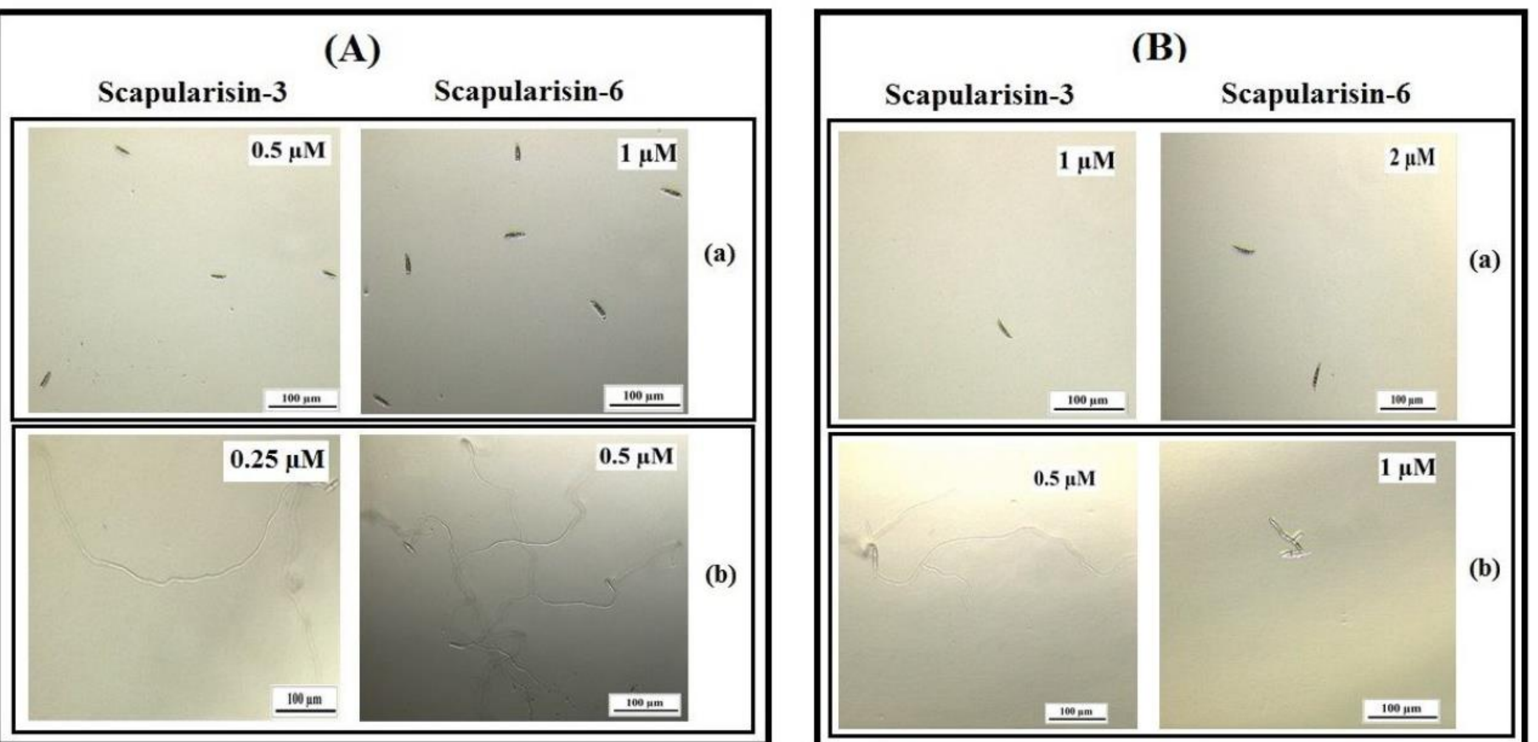

Figure 16. Antifungal activity of Scapularisin-3 and Scapularisin-6 against *Fusarium* spp.. Images show (a) inhibition of spore germination and (b) spore germination (presence of hyphae) at different concentrations of Scapularisin-3 and Scapularisin-6. Pictures of the respective fungal cultures were taken after 24 hours of incubation of *F. culmorum* (A) and *F. germinearum* (B) with the peptides

Chapter 5. Discussion and Conclusions

5.1 Discussion

Discussion of *I. ricinus* defensins

Defensin families with multiple members have been reported in hard and soft ticks (Johns et al., 2001; Nakajima et al., 2001; Saito et al., 2009; Wang and Zhu, 2011). Earlier reports described two defensins from *I. ricinus* (Rudenko et al., 2005, 2007). Six genes encoding novel members of the highly diverse defensin family from *I. ricinus* were successfully identified and partially characterised in the present study: DefMT2, DefMT3, DefMT4, DefMT5, DefMT6 and DefMT7. It is interesting to note that possible defensin isoforms were detected among the family; DefMT3 and DefMT4 shared 98% of similarity at the nucleotide and protein level. Previously identified *I. ricinus* defensins Def1 and Def2 were reported as isoforms (Chrudimská et al., 2011; Rudenko et al., 2007). The similarities among DefMT3, DefMT4, Def1 and Def2, suggest that they are isoforms. Defensin isoforms have been identified in *O. moubata*, *I. ricinus*, *I. scapularis*, *D. variabilis, A. hebraeum* and *H. longicornis* ticks (Ceraul et al., 2007; Hynes et al., 2005; Lai et al., 2004; Nakajima et al., 2002a; Rudenko et al., 2005, 2007; Zhou et al., 2007).

Exon-intron genomic structure has been previously described for vector arthropods including in *I. ricinus* defensins (Rudenko et al., 2007), defensins from the soft tick *O. moubata* (Nakajima et al., 2002a), and defensins from the insects *Aedes aegypti*, the vector of yellow fever virus (Lowenberger et al., 1999) and *A. gambiae*, a vector of malaria (Eggleston et al., 2000). DefMT4 contains two introns, but DefMT7 was not found to contain introns. Intronless defensins in blood feeding arthropods have been previously reported in *I. scapularis* and *D. variabilis* (Hynes et al., 2005) and in *Stomoxys calcitrans* (Munks et al., 2001); it is possible that introns were lost during evolution to decrease the genome size (Froy and Gurevitz, 2003). Introns are found most prominently in genes that are highly expressed in a wide range of tissues (Parra

et al., 2011). The question of whether the presence of introns has an impact on the biological significance of tick defensins remains unanswered.

Defensins primarily attack Gram+ bacteria, leading to cell lysis by the formation of membrane-penetrating channels (Gillespie et al., 1997) or intramolecular bridges (Beerntsen et al., 2000). Bactericidal potential against several pathogens was observed in defensins from *A. hebraeum* (Lai et al., 2004), *I. persulcatus* (Isogai et al., 2010), *D. variabilis* (Johns et al., 2001) and *O. moubata* (Nakajima et al., 2002a). In addition, several AMPs have been shown to display antiparasitic activity against malaria, leishmaniasis (Vizioli and Salzet, 2002), African sleeping sickness (Pascholati et al., 2009) and *Toxoplasma gondii* (Tanaka et al., 2010). Anti-*Plasmodium* activity has been demonstrated by insect defensins (Shahabuddin et al., 1998).

DefMT2 and DefMT6 were also predicted to be active against Gram- bacteria, fungi and parasites; these two defensins share similar tertiary structure that might contribute to their functional activity against protozoans, while the secondary structure of DefMT6 is different from other *I. ricinus* defensins which might also influence its microbial activity.

Based on their similar structure, DefMT3 and DefMT4 may be functionally similar to Def1 and Def2. Def1 and Def2 were shown to possess effective antimicrobial activity against the Gram+ bacteria *S. xylosus, M. luteus, B. subtilis* and a clinical isolate of multi-drug resistant strain *S. aureus* (Chrudimská et al., 2011). One amino acid substitution in mature Def1 and Def2 resulted in slightly different antimicrobial potential of the two peptides (Rudenko et al., 2007). The Def2 isoform, detected in every tissue of the tick body examined, was more effective in the inhibition of bacterial cell growth and killing of bacteria than the Def1 peptide expressed predominantly in the midgut (Chrudimská et al., 2011). Presumably, DefMT3 and DefMT4 might show a similar effect on Gram+ bacteria. Six cysteine residues, well-

conserved among insect and other tick defensins (Zheng et al., 2012), were also present in the mature peptides of *I. ricinus* defensins. Using the DiANNA server, 5 different predicted forms of disulphide bridges between the cysteine residues were found. Disulphide bonds play an important role in the folding and stability of proteins (Sevier and Kaiser, 2002). The importance of disulphide bond-formation in the function of defensins has also been reported (Ganz and Lehrer, 1994; Isogai et al., 2010; Wanniarachchi et al., 2011). The pattern of disulphide bonds was shown to be variable in hard and soft tick lipocalin, where only two disulphide bonds out of five were conserved (Cheng et al., 2010). In insect defensins examined so far, the same cysteine pairing (Cys1-Cys4, Cys2-Cys5, Cys3-Cys6) has been shown (Taylor, 2006). Different models of disulphide bridges were predicted in *I. ricinus* defensins that might affect the functional activities of these defensins. Prediction of isoelectric points of the defensins showed that, except for anionic DefMT7, all the novel defensins are cationic. The basic isoelectric point in defensins is an important factor for function (Sagaram et al., 2011). In addition, Gram- bacteria are more resistant to treatment with cationic antimicrobial peptides. Their cell wall, mainly the outer membrane, is known to be an effective permeability barrier (Isogai et al., 2009; Nikaido, 2001); thus DefMT7 might have antimicrobial activity against pathogens different from the other defensins.

We found high phylogenetic variability among members of *I. ricinus* defensins. This is in agreement with the results previously reported for the defensin family of *I. scapularis* (Wang and Zhu, 2011). This phylogenetic variability may be caused by the various types of microbes and environments that different tick species encountered during their evolutionary development and geographical distribution (Hynes et al., 2005; Zheng et al., 2012). Sequences from soft and hard ticks (genera: *Ornithodoros*, *Argas*, *Haemaphysalis*, *Dermacentor*, *Ixodes*, *Amblyomma and Rhipicephalus*) and also different groups of blood feeding arthropods (*Triatominae*, *Rhodnius*, *Stomoxys*, *Glossina*, *Anopheles*, *Aedes*) were introduced in the phylogenetic analysis. The inclusion of sequences from several taxa (phylogenetic information) rendered a reliable tree, with most of the bootstrap values for internal

branches above 50%. Our phylogenetic tree is consistent with previous reports where Ornithodoros genus defensins fall apart from Ixodidae having *Argas monolakensis* as basal group (Chrudimská et al., 2010). The tree shows that DefMT6 and DefMT7 fall also apart from *I. ricinus* defensins but still they cluster with defensins from other Ixodidae ticks. This suggests that they emerged before the divergence of different tick species in Ixodidae and that they may be ancestral compared with the other defensins from *I. ricinus*. DefMT3 and DefMT4 cluster togetherwith previously characterised defensins from *I. ricinus* (Rudenko et al., 2005., 2007), forming a monophyletic clade, suggesting that these defensins are more diverged members of the *I. ricinus* defensin family and may represent a recent expansion of this group of proteins in *I. ricinus*. Expansion of the defensin family of proteins was previously reported for *I. scapularis* (Wang and Zhu, 2011). The position of DefMT3 and DefMT4 in the tree is consistent with our conclusion that DefMT3 and DefMT4 are isoforms of the previously characterised defensins from *I. ricinus* Def 1 and Def 2 (Rudenko et al., 2005, 2007). The phylogenetic positions of the different *I. ricinus* defensins show phylogenetic diversity in defensins fromthis tick species as it was previously reported for defensins from *I. scapularis* (Wang and Zhu, 2011). It is worthy to mention that DefMT7 which we found to be a non-cationic defensin, cluster together with other non-cationic defensins from Ixodidae ticks which is consistent with previous findings (Chrudimská et al., 2010).

Most AMPs display an amphipathic structure (α-helix, β-hairpin-like β-sheet, β-sheet, or α-helix/β-sheet mixed structures) that is believed to be essential for their antimicrobial action (Bulet et al., 2004). The α-helix and β-sheet regions are considered to be the active sites of defensins (Tsuji et al., 2007). It was shown, for example, that the well-developed β-sheet of longicin from *H. longicornis* is responsible for its antimicrobial activity (Rahman et al., 2010). Analysis of DefMT2-DefMT7 from *I. ricinus* reveals their high variability in secondary structure. Diverse rearrangements of α-helix and β-sheet regions in protein sequences of novel defensins most probably will affect their antimicrobial potential.

The expression of tick defensins seems to be tissue-specific and ubiquitous for others (Baumann et al., 2010). *D. variabilis* defensins had distinct tissue distribution patterns. Defensin-1 was expressed exclusively in the haemocytes, whereas defensin-2 was expressed mostly in the ovary, midgut, and fat body. *I. persulcatus* defensin was predominantly expressed in midguts of adult female ticks, but not in salivary glands and tick carcasses (Ceraul et al., 2007; Saito et al., 2009). AMPs found in tick haemocytes, midgut and fat body were suggested to offer protection against microorganisms (Fogaça et al., 2004; Lu et al., 2010; Zhou et al., 2007). Studies have shown that some insect defensins are involved in the midgut immune response (Nakajima et al., 2002b). Differential expression was confirmed in our study for novel defensin genes from *I. ricinus*.

Differential tissue expression patterns for defensins have been previously reported (Baumann et al., 2010). *A. americanum* defensin, Amercin was expressed in haemocytes, midgut, fat body and salivary gland tissues (Todd et al., 2007). *Ornithodoros* defensins A, B, C and D were expressed in the fat body and midgut. Defensins A, B and C displayed higher levels of expression in the midgut, while defensin D displayed a higher level of expression in the fat body only (Nakajima et al., 2002a). Def1 was predominantly expressed in the midgut, whereas Def2 was expressed in salivary gland, midgut and ovary of *I. ricinus* ticks (Chrudimská et al., 2011). In the present study expression of DefMT2 and DefMT5 was only detected in salivary glands. DefMT6 was expressed in salivary glands, ovaries and Malpighian tubules. In contrast to the above-mentioned defensins, DefMT3, DefMT4 and DefMT7 were expressed in every tick tissue analysed in our study. Such differences in site of tissue expression reflect, most probably, the antibacterial abilities of each protein. The question "Does the site of expression determine the function of tick defensin (s)" needs to be answered.

All the novel defensins were constitutively expressed in the *I. ricinus* embryo-derived cell line IRE/CTVM19 except for DefMT5. Development of *in vitro*

IRE/CTVM19 assays will give us an invaluable tool for study of gene regulation and functional potential of the *I.ricinus* defensins identified.

Discussion of *I. scapularis* defensins

Defensins have been identified in both soft and hard ticks (Chrudimská et al., 2010; Wang and Zhu, 2011). Defensins from several members of the genus *Ixodes* have been previously characterised, including Persulcatusin from *I. persulcatus* (Saito et al, 2009), and defensins recently identified from *I. ricinus* (Tonk et al., 2014). However, one of the largest families of tick defensins were found in *I. scapularis*. This tick species presents two phylogenetically divergent groups of defensins named Scapularisin and Scasin, with 25 and 21 members respectively. Of the 25 Scapularisins, only Scapularisin-20 has been functionally characterised so far, and it shows antimicrobial activity against Gram− and Gram+ bacteria (Wang and Zhu, 2011). Thus a gap exists regarding the spectrum of biological functions of *I. scapularis* defensins. The results of the present study expand the knowledge on the antimicrobial spectrum of *I. scapularis* defensins since they showed that scapularisins are effective not only against bacteria, but also against fungi. Antifungal activity against *Candida albicans* has been previously reported in tick defensins from *H. longicornis* (Lu et al., 2010) and *I. sinensis* (Yu et al., 2006). However, this is the first study showing that tick defensins are effective against the bacterium *L. grayi* and two fungi of the genus *Fusarium*, *F. culmorum* and *F. graminearum.*

The antibacterial activity of tick defensins has been reported to be mainly directed against Gram+ bacteria, but some isoforms were reported to also have activity against Gram- bacteria (Nakajima et al., 2001; Tsuji et al., 2007). The γ-core of Scapularisin-20 from *I. scapularis* showed slightly more effect on Gram+ bacteria than on Gram- bacteria (Wang and Zhu, 2011). In agreement with those reports, Scapularisin-3 and Scapularisin-6 were not effective against the Gram- bacteria tested in the present study. However, they were also not effective against most of the Gram+ bacteria tested in this study. Interestingly, Scapularisin-6 showed

antibacterial activity against *L. grayi*. A growing number of *Listeria* strains resistant to antimicrobial compounds and particularly to antibiotics have been reported (Salimnia et al., 2010; Schwaiger et al., 2009). Thus, the identification of anti-listerial agents has become extremely important (Mukherjee et al., 2011).

The ascomycete *F. graminearum* has major economic impact in the agriculture industry (Nielsen et al., 2014). Production losses worldwide have been estimated to be as much as 50%. Furthermore, *F. graminearum* was fourth in a list of the top 10 fungal pathogens in molecular plant pathology (Dean et al., 2013). In addition to the direct effect of this fungus on grains, it produces mycotoxins that make the grains unsafe for human consumption, animal feed or malting purposes (Dean et al., 2013). In general, the control of *Fusarium spp* has become challenging; for example azole, one of the available fungicides, is only moderately effective against *F. graminearum* (Dean et al., 2012). Thus, new control methods are needed. Some results suggest that defensins in general are effective against *Fusarium spp*. The expression of radish defensin in transgenic wheat confers resistance to *F. graminearum* (Li et al., 2011). In addition, defensins isolated from the venom of the snake *Bothrops jararaca* (Gomes et al., 2005), from the plant *Nicotiana alata* (Dracatos et al., 2014) and from mice (Kolar et al., 2013) have been shown to be effective against some species of *Fusarium*. In the present study, the IC_{50} against *F. culmorum* and *F. graminearum* of both Scapularisin-3 and Scapularisin-6 was low, suggesting that they are potent antifungal agents. When compared to Scapularisin-6, Scapularisin-3 caused growth inhibition of fungi at a lower concentration, suggesting that Scapularisin-3 is more potent that Scapularisin-6, at least under the conditions of the assay used. Interestingly, Scapularisin-3 has many substitutions to Arg when compared to Scapularisin-6. A recent study found that cationic residues (such as Arg) within the α-core and γ-core motifs found in plant defensins are important in antifungal activity against *F. graminearum* (Sagaram et al., 2011). Further studies should be carried out in order to clarify the mechanistic actions of *I. scapularis* defensins against fungi of the genus *Fusarium*.

In addition to examining the antimicrobial activity of Scapularisin-3 and Scapularisin-6, the expression pattern of these proteins in tick tissues was determined. Scapularisin-3 and Scapularisin-6 were shown to be transcribed differentially in *I. scapularis* salivary glands and midgut, with the former defensin being transcribed in salivary glands and the latter in both tissues. This is in agreement with the expression patterns of defensins identified recently in *I. ricinus*, some of which some seem to be tissue-specific and others ubiquitous (Tonk et al., 2014). In contrast to the novel defensins from *I. ricinus* that were all constitutively expressed in the *I. ricinus* embryo-derived cell line IRE/CTVM19 except for DefMT5 (Tonk et al., 2014), expression of the two *I. scapularis* Scapularisin-3 and Scapularisin-6 was not detected in either of the embryo-derived tick cell lines ISE6 and ISE18. Although tick cell lines are generally quite heterogeneous, having been derived from multiple tissue types (Bell-Sakyi et al., 2007; Munderloh et al., 1994), they do not necessarily represent cells from the full complement of tick organs present in the starting material. ISE6 and ISE18 are relatively homogeneous lines compared to the *I. ricinus* line IRE/CTVM19 (Bell-Sakyi, personal communication) and it may be that the cell types expressing Scapularisin-3 and Scapularisin-6 in whole tick salivary glands and midgut are not present in the *I. scapularis* cell lines, while some of the cell types expressing the *I. ricinus* defensins are present in the more heterogeneous cell line derived from the latter species. Moreover, the pattern of defensin expression may differ between cells of tick embryos that have never experienced bacterial challenge or even a bloodmeal, and partially-fed adult female ticks removed from a vertebrate host. Further studies involving *in vitro* microbial challenge of these and additional *I. scapularis* and *I. ricinus* cell lines (Bell-Sakyi et al., 2007) are needed to fully elucidate the expression patterns of defensins in embryo-derived cells.

In summary, tick defensins play important roles in the innate immune response of ticks to pathogen invasion, recently reviewed by Hajdušek et al. (2013). Further *in vivo* studies are required to clarify the tissue specificity and physiological roles of Scapularisin-3 and Scapularisin-6 in response to pathogens in *I. scapularis*.

5.2 Conclusions

Conclusions for *I. ricinus* defensins

Six novel defensin genes from *I. ricinus* were structurally and phylogenetically characterised. They show a high degree of evolutionary diversity. The transcriptional expression pattern of these defensins in salivary glands, midguts, ovaries, haemolymph and Malpighian tubules is diverse as some were found to have tissue-specific expression while others were ubiquitously expressed. Except for DefMT5, all the new putative defensins from *I. ricinus* were also expressed in the embryo-derived *I. ricinus* cell line (IRE/CTVM19). Three of the novel defensins were characterised at the genomic level; two contain introns and one is an intronless gene. Finally, from the molecular and structural analysis and antimicrobial assays it was concluded that they are antimicrobial peptides belonging to the family of defensins.

Conclusions for *I. scapularis* defensins

Two defensins from *I. scapularis* were functionally characterised and they showed activity against fungi and Gram+ bacteria. Both Scapularisin-3 and Scapularisin-6 were highly effective against *F. graminearum* and *F. culmorum* suggesting their promise for plant protection purposes. In addition, Scapularisin-6 has activity against the bacterium *L. grayi*. Our results confirm the wide antimicrobial spectrum of tick defensins and the potential of these defensins to be used as drugs against important agricultural and medical pathogens.

Chapter 6. Final Remarks

By identifying and performing the characterisation of six novel defensin genes, present within the *I. ricinus* genome, and functional characterisation of both *I. ricunus* and *I. scapularis* defensins this study broadens the scope of research on antimicrobial peptides in arthropod vectors as well as on the arthropod innate immune system.

Further studies, including RNA interference *in vitro* and *in vivo*, are needed to identify the response of these defensins to pathogen infection in *I. ricinus* and *I. scapularis*. The study highlights the great structural diversity of novel AMPs that might reflect their different functional capability.

Defensins have emerged as effector substances of the innate immune system involving not only activities as endogenous antibiotics but also as mediators of inflammation, wound healing and tissue repair. Several important topics will have to be addressed in the future: (i) identification of defensins; (ii) analysis of the biologically relevant functions of defensins. Besides experiments that give the first insight into the function of peptide antibiotics, a broader approach involving animal models is necessary to interpret *in vitro* data in the context of a whole organism; (iii) evaluation of the function of defensins in diseases might provide information about the corresponding pathogenesis; (iv) development of defensins as drugs. Studying the biology of antimicrobial peptides may allow the development of novel therapeutics including anti-infectious, anti-inflammatory or anti-fungal drugs.

Therefore, they can be considered as an alternative therapeutic agent to overcome drug-resistance and new emerging pathogens.

Chapter 7. Published and Submitted Manuscripts

Tonk, M., Cabezas-Cruz, A., Valdés, J.J., Rego, R.O.M, Rudenko, N., Golovchenko, M., Bell-Sakyi, L., de la Fuente, J., Grubhoffer, L. (2014). Identification and partial characterisation of new members of the *Ixodes ricinus* defensin family. *Gene,* 540: 146-152. http://dx.doi.org/10.1016/j.gene.2014.03.002

Tonk, M., Cabezas-Cruz, A., Valdés, J.J, Rego, R.O.M, Chrudimská, T., Strnad, M., Šíma, R., Bell-Sakyi, L., Franta, Z., Vilcinskas, A., Grubhoffer, L., Rahnamaeian, M. Defensins from the tick *Ixodes scapularis* are effective against phytopathogenic fungi and the human bacterial pathogen *Listeria grayi* (Submitted to *Parasites and Vectors*).

Appendix I – *I. ricinus* defensins used in this study

			aa	aa	bp	
Species	**Name**	**Accession Number**	**Signal Peptide**	**Mature Peptide**	**Full Length**	**References**
I. ricinus	DefMT2	JAA65352	19	54	222	Tonk et al., 2014
I. ricinus	DefMT3	JAA71488	22	54	231	Tonk et al., 2014
I. ricinus	DefMT4	JAA71477	22	54	231	Tonk et al., 2014
I. ricinus	DefMT5	JAA66832	19	54	222	Tonk et al., 2014
I. ricinus	DefMT6	JAA71516	22	52	225	Tonk et al., 2014
I. ricinus	DefMT7	JAA69779	25	46	216	Tonk et al., 2014
I. ricinus	Def1	EF067917	22	54	228	Rudenko et al., 2007
I. ricinus	Def2	DQ361064	22	52	224	Rudenko et al., 2007

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miraytonk@gmail.com

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University of South Bohemia in České Budějovice
Faculty of Science
Branišovská 31
CZ- 37005, České Budějovice, Czech Republic

Phone: +420 387 772 244
www.prf.jcu.cz, e-mail: sekret@prf.jcu.cz