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Evolution of the tissue factor pathway inhibitor-like Kunitz domain-containing protein family in *Rhipicephalus microplus*

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ABSTRACT

One of the principle mechanisms utilised by ticks to obtain a blood meal is the subversion of the host's haemostatic response. This is achieved through the secretion of saliva containing anti-haemostatic proteins into the feeding lesion. Lineage-specific expansion of predicted secretory protein families have been observed in all previously studied ticks and occurred in response to adaptation to a blood-feeding environment. Of these, the predominant families are common between both hard and soft ticks. One of these families, namely the Kunitz domain-containing protein family, includes proven tissue factor pathway inhibitor-like (TFPI-like) anti-haemostatics such as ixolaris and penthalaris that play a crucial role during tick feeding. Although Kunitz-type proteins have been found in *Rhipicephalus microplus*, the TFPI-like Kunitz protein family has not yet been studied. We report a comprehensive search for TFPI-like Kunitz domain-containing proteins in *R. microplus* expressed sequence tag libraries, resulting in the identification of 42 homologues. The homologues were bioinformatically and phylogenetically studied, including the application of an intensive Bayesian Markov Chain Monte Carlo (MCMC) analysis of the individual Kunitz domain nucleotide sequences. We show that the *R. microplus* TFPI-like Kunitz protein family groups into two main clades that presumably underwent ancient duplication, which indicates that a whole genome duplication event occurred at least 150 million years ago. Evidence for recent and ancient gene and domain duplication events was also found. Furthermore, the divergence times of the various tick lineages estimated in this paper correspond with those presented in previous studies. The elucidation of this large protein family's evolution within *R. microplus* adds to current knowledge of this economically important tick.

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1. Introduction

Rhipicephalus microplus is considered to be the most important tick parasite of cattle worldwide, resulting in profit losses and financial expenditures due to both direct and indirect damage to the animals. Infestation by these ticks causes reductions in weight gain and milk production. This tick species is also responsible for indirect damage to cattle due to its vectoring capability for anaplasmosis and babesiosis pathogens. Losses due to *R. microplus* infestations have been estimated at US $7 billion annually (Peconick et al., 2008) and these losses are expected to rise due to expansion of the tick's habitable range into non-endemic areas (White et al., 2003; Estrada-Peña et al., 2005; Lynen et al., 2008). Currently, only one tick vaccine has been developed and is marketed as Gavac in Central and South America, and as TickGARD in Australia (de la Fuente et al., 2007a). It is a recombinant vaccine based on a *R. microplus* gut antigen (Bm86) and while it has been successfully used in conjunction with other acaricides it exhibits limited and varying efficacy against different tick species and strains (reviewed by Willadsen, 2004). Therefore, the identification of novel tick vaccine candidates is of great importance to the global cattle industry.

Recently, tick research has benefitted greatly from genetic technologies such as expressed sequence tag (EST) studies and whole-genome sequencing. Before the advent of such technologies, gene discovery had been achieved through time-consuming and laborious experiments, often resulting in the identification of only a single gene. The recent release of first-draft genome sequencing data for *R. microplus* (Bellgard et al., 2012), together with genome data already available for *Ixodes scapularis* (Hill and Wikel, 2005; van Zee et al., 2007), has stimulated gene discovery in these and other ticks. However, these sequencing data sets need to undergo additional analyses before useful information may be extracted. Additionally, bioinformatic analyses and results should be confirmed by biological evidence such as protein characterisation and functional studies.

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Previous EST studies showed lineage-specific expansion of several predicted secretory protein families for both hard and soft ticks (Mans et al., 2002b, 2008a; Mans and Neitz, 2004). These studies pointed to a series of gene duplication events after the divergence of the Ixodidae and Argasidae families. Such duplications provide the genetic variance that enables phenotypic hypervariability and may result in many paralogues with slightly altered specificities that can target a range of host and native proteins (Ohta, 1994, 2000; Christeller, 2005). The predominant predicted secretory protein families, i.e. lipocalins, basic tail secretory protein, Kunitz domain-containing peptides and metalloproteases are common between hard and soft ticks. Of these, the Kunitz domain-containing protein family alone accounts for between 5% and 20% of all predicted secretory proteins (Ribeiro et al., 2006; Chmelař et al., 2008; Francischetti et al., 2008a,b; Mans et al., 2008a). In *I. scapularis*, arguably the most intensively studied tick in North America due to its vector capacity for Lyme disease, the Kunitz domain-containing protein family comprises 10% of all predicted secretory proteins (Ribeiro et al., 2006). Even though several Kunitz proteins have been identified and/or characterised in *R. microplus* (Tanaka et al., 1999; de Miranda Santos et al., 2004; Sasaki et al., 2004; Rachinsky et al., 2007; Macedo-Ribeiro et al., 2008), the larger Kunitz domain-containing protein family has not been specifically studied within this tick species.

Kunitz-type proteins also have significant roles within other parasites. In the black fly, *Simulium vittatum*, they have been shown to inhibit coagulation and have a possible role in modulating inflammatory responses (Tsujimoto et al., 2012). In the flatworm, *Echinococcus granulosus*, a family of Kunitz domain-containing proteins have been implicated in the initial stages of infection (González et al., 2009), while many other parasites have recruited the Kunitz-scaffold into their venom proteins (reviewed by Fry et al. (2009)). In hookworms such as *Ancylostoma ceylanicum* it has been shown that Kunitz-type inhibitors protect the parasite against digestive serine proteases present in the host's small intestine (Costa et al., 2009; Tsujimoto et al., 2012). Immunisation against the *A. ceylanicum* Kunitz-type inhibitors confers partial protection against hookworm-associated growth delay, lending support for their possible use as parasite vaccine candidates (Chu et al., 2004).

The Kunitz protein family members, of which bovine pancreatic trypsin inhibitor (BPTI) is the type member (Burck et al., 1967; Cerwinsky et al., 1967), are typically serine protease inhibitors. One of their crucial functions in vertebrates is the control of haemostasis. Human tissue factor pathway inhibitor (TFPI) is a potent anticoagulant that achieves inhibition of essential serine proteases of the blood coagulation cascade, such as factor Xa (fXa) and factor VIIa (fVIIa), through its three Kunitz domains (Lwaleed and Bass, 2006). As blood coagulation is one of the primary and most robust defences of vertebrates against blood-feeding parasites, it is unsurprising that all ticks secrete an assortment of anti-haemostatic proteins (reviewed by Maritz-Olivier et al. (2007) and Corral-Rodríguez et al. (2009)). These anticoagulants serve to keep blood in its fluid form in both the feeding lesion and the tick's midgut, enabling the parasite to both obtain and digest its blood meal.

Various tick-derived Kunitz proteins that inhibit the serine proteases of the blood coagulation cascade, as well as soft tick-derived platelet aggregation inhibitors, have been identified. Ornithodorin and savignin are both bi-Kunitz thrombin inhibitors isolated from the soft ticks, *Ornithodoros moubata* (van de Locht et al., 1996) and *Ornithodoros savignyi* (Mans et al., 2002a). Despite having similarity to BPTI, they do not bind to thrombin via the Kunitz substrate-binding loop, but instead insert their N- and C-terminal residues into thrombin's active site cleft and basic fibrinogen recognition exosite, respectively. Similar binding modes have been inferred for the hard tick-derived thrombin inhibitors, boophilin from *R. microplus* (Macedo-Ribeiro et al., 2008) and hemalin from *Haemaphysalis longicornis* (Liao et al., 2009), as well as for the soft tick-derived fX(a) inhibitors, tick anticoagulant protein from *O. moubata* (Wei et al., 1998) and fXa inhibitor (fXaI) from *O. savignyi* (Joubert et al., 1998). Multi-Kunitz inhibitors of fX(a) have also been isolated from the hard tick *I. scapularis* (Francischetti et al., 2002, 2004). Platelet aggregation inhibitors from soft ticks that use the Kunitz domain substrate-binding loop to present an RGD integrin recognition motif have also been characterised. Savignygrin isolated from *O. savignyi* is the most well-known of these (Mans et al., 2002c), but several savignygrin homologues have been identified in other soft ticks, including monogrin in *Argas monolakensis* (Mans et al., 2008b), disagregin in *O. moubata* (even though it presents RED instead of RGD (Karczewski et al., 1994; Karczewski and Connolly, 1997)) and RGD-presenting Kunitz proteins have also been found in *Ornithodoros parkeri* (Francischetti et al., 2008a).

Two TFPI homologues, ixolaris and penthalaris, have been identified and characterised in *I. scapularis* and have been shown to be potent anticoagulants (Francischetti et al., 2002, 2004; Monteiro et al., 2005, 2008; Nazareth et al., 2006). They share sequence homology with TFPI and have anticoagulant capabilities within the nanomolar range. In the case of ixolaris, this is achieved through inhibition of the tissue factor/fVIIa complex by using fX(a) as a scaffold (Francischetti et al., 2002). Penthalaris and ixolaris also form part of the larger Kunitz domain-containing protein family in *I. scapularis* (Ribeiro et al., 2006) which has developed through lineage-specific expansion.

Lineage-specific expansion of the Kunitz protein family has been previously observed in all studied ticks (Valenzuela et al., 2002; Francischetti et al., 2005, 2008a,b; Ribeiro et al., 2006; Alarcon-Chaidez et al., 2007; Batista et al., 2008; Chmelař et al., 2008; Mans et al., 2008a; Dai et al., 2012). Previous studies have shown that this protein family has evolved through gene duplication within the main tick families but after the divergence of hard and soft ticks (Mans et al., 2002b, 2008a,b; Mans and Neitz, 2004). The Kunitz protein family has therefore expanded in response to the blood-feeding environment that the Ixodidae and Argasidae encountered individually. Recently, Dai et al. (2012) showed that a greater expansion of the Kunitz-type proteins within hard ticks, specifically *I. scapularis*, likely facilitates the long-term blood-feeding behaviour of these ticks. It is likely that a similar, expanded Kunitz domain-containing protein family is present in *R. microplus*, with the members showing homology to the TFPI-like proteins, ixolaris and penthalaris.

Due to a lack of knowledge regarding a TFPI-like Kunitz protein family in the economically important *R. microplus*, we undertook to identify TFPI-like Kunitz domain-containing proteins in the cattle tick. Our preliminary identification of this protein family, using available *R. microplus* sequence databases, revealed a family of 42 homologues. Phylogenetic analysis uncovered both ancient and recent gene- and domain duplications, supporting previous observations that lineage-specific expansions of the Kunitz protein family occurred in all studied ticks. More exhaustive studies on this family of proteins will be of great significance once the complete genome of the cattle tick becomes available. This will allow intensive cross-genera analyses, facilitating a deeper insight into the evolution of ticks and their independent adaptation to a blood-feeding lifestyle.

2. Materials and methods

2.1. Identification of TFPI-like Kunitz domain-containing proteins in R. microplus

In order to assemble a data set of TFPI-like Kunitz domain-containing homologues, the Basic Local Alignment Search Tool

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