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Comparative genomic study of arachnid immune systems indicates loss of β GRPs and the IMD pathway

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Abstract

Analyses of arthropod genomes have shown that the genes in the different innate humoral immune responses are conserved. These genes encode proteins that are involved in immune signalling pathways that recognize pathogens and activate immune responses. These immune responses include phagocytosis, encapsulation of the pathogen, and production of effector molecules for pathogen elimination. So far, most studies have focused on insects leaving other major arthropod groups largely unexplored. Here we annotate the immune related genes of six arachnid genomes and present evidence for a conserved pattern of some immune genes, but also evolutionary changes in the arachnid immune system. Specifically, our results suggest that the family of recognition molecules of Beta-1,3-glucanase-related proteins (β GRPs) and the genes from the immune deficiency (IMD) signalling pathway have been lost in a common ancestor of arachnids. These findings are consistent with previous work suggesting that the humoral immune effector proteins are constitutively produced in arachnids in contrast to insects, where these have to be induced. Further functional studies are needed to verify this. We further show that the full hemolymph clotting cascade found in the horseshoe crab is retrieved in most arachnid genomes. *Tetranychus* lacks at least one major component, although it is possible that this cascade could still function through recruitment of a different protein. The gel-forming protein in horseshoe crabs, coagulogen, was not recovered in any of the arachnid genomes, however, it is possible that the arachnid clot consists of a related protein, spätzle, that is present in all of the genomes.

Keywords

Arachnida; immunity; signalling pathway; genomics; coagulation

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Introduction

The innate immune system consists of a range of cellular and humoral defences against parasites and pathogens (Rowley & Powell, 2007). Defence strategies are based on the recognition of infectious agents by a variety of pattern recognition molecules that are either present in a soluble form in plasma/hemolymph or associated with various cell types. Comparative studies of insects and vertebrates suggest that pathogen recognition proteins of the innate immune system are conserved (Dziarski, 2004, Kim et al., 2000). These proteins typically recognize surface components of intruding agents and subsequently activate immune responses such as phagocytosis and encapsulation, or the elimination of pathogens by antimicrobial peptides or other effector molecules like lysozymes (Janeway & Medzhitov, 2002). Our knowledge of the arthropod immune system is predominantly based on insights from *Drosophila* and a few other insect or crustacean model systems (Lemaitre & Hoffmann, 2007, Imler, 2014), leaving other major arthropod taxa such as arachnids largely unexplored. Recent sequencing of whole arachnid genomes now makes it possible to explore immune system evolution in arachnids. The aim of this study was to analyse patterns of arachnid immune system diversity and evolution through comparative analysis of arachnid and insect genomes. In particular, we focused on three types of humoral responses: coagulation, melanisation, and release of antimicrobial peptides (AMPs) via signalling pathways.

Coagulation

Hemolymph coagulation properties of the tri-spine horseshoe crab (*Tachypleus tridentatus*) has attracted scientific attention for more than 50 years due to its extreme sensitivity to the presence of bacterial endotoxins (Levin & Bang, 1964a, Levin & Bang, 1964b). Since *T. tridentatus* belongs to the Xiphosura, which constitutes a sister group to the arachnids, it can be used for an outgroup comparison. Clotting cascade proteins of *T. tridentatus* include the serine proteases factor B, factor C, factor G and proclotting enzyme (Iwanaga et al., 1998). See Figure 1 for an overview of the clotting pathway. Factor C is activated in the presence of lipopolysaccharide, the main component of the outer membrane of Gram-negative bacteria. The active factor C will activate factor B, which in turn converts the proclotting enzyme to clotting enzyme. Factor G is activated by the presence of beta-1,3-glucan, a component of the cell wall of fungi, which also converts proclotting enzyme to clotting enzyme. The clotting enzyme, activated either by factor B or factor G, will convert coagulogen to coagulin (Iwanaga et al., 1998). Coagulin is the main component of the clot in horseshoe crabs, and formation of the clot stops hemolymph loss and traps pathogens to prevent them to proliferate further into the hemocoel (Theopold *et al.*, 2004). The clot is stabilized as coagulin homopolymers are cross-linked with proline-rich proteins 'proxins', which are antigens on the surface of hemocytes (Osaki & Kawabata, 2004, Osaki et al., 2002). This cross-linkage is mediated by transglutaminase activity. The horseshoe crab coagulation cascade is linked to prophenoloxidase activation, since specific factors from the coagulation cascade leads to conversion of hemocyanin to phenoloxidas (see below) (Nagai & Kawabata, 2000).

Less is known about the proteins involved in hemolymph coagulation in crustaceans and insects. In the crayfish, *Pacifastacus leniusculus*, hemolymph clotting is mediated by transglutaminase activity which cross-links a plasma protein (Hall et al., 1995, Kopacek et al., 1993). This plasma protein is homologous to a protein found in the spiny lobster, *Panulirus interruptus*, called lobster fibrinogen (Hall et al., 1995, Kopacek et al., 1993, Fuller & Doolittle, 1971). As opposed to horseshoe crab clotting, the cross-links occur intermolecularly between the plasma clotting proteins, and not between plasma clotting proteins and hemocyte surface antigens. The plasma proteins in the crayfish and the spiny lobster are also found to be homologous to vitellogenins, a major component of the insect clot (Hall et al., 1995), however, even though they are functionally equivalent to coagulogen in the tri-spine horseshoe crab *T. tridentatus*, vitellogenins and coagulogen are not homologous (Iwanaga & Lee, 2005).

In *Drosophila* there is evidence that the cascade involved in formation of dorso-ventral polarity in the embryo shows several similarities with the hemolymph coagulation cascade in *T. tridentatus* (Krem & Di Cera, 2002), and homology between the factor B and proclotting enzyme in *T. tridentatus* and *Drosophila* easter and spätzle (also functional in the Toll pathway, see below) was suggested (Krem & Di Cera, 2002, Smith & Delotto, 1992). There is evidence that lipophorin proteins and hexamerin and its receptor protein Fondue are involved in clot formation in some insects (Loof et al., 2011, Theopold et al., 2014, Theopold et al., 2004). However, analyses of the clot in *Drosophila* showed that the most abundant protein is hemolectin (Goto et al., 2003, Kotani et al., 1995, Scherfer et al., 2004), and the importance of this protein is substantiated by hemolectin-knock down experiments causing bleeding defects (Scherfer et al., 2004). In *Drosophila* the clot is stabilized by cross-linking caused by transglutaminase activity, but there is also a discussion of whether or not phenoloxidase plays a role in clot stabilization in insects (Eleftherianos & Revenis, 2011, Wang et al., 2010).

The mammalian coagulation cascade has two pathways: the intrinsic and the extrinsic. The extrinsic pathway is activated by trauma, which leads to the activation of the serine protease thrombin. Thrombin cleaves fibrinogen to fibrin, which is the main component of the clot in mammals (Doolittle et al., 2009). Fibrinogen is functionally equivalent to coagulogen in *T. tridentatus* but not homologous (Theopold et al., 2004). The thrombin that has arisen from the extrinsic pathway will activate several different serine proteases in the intrinsic pathway, which again will lead to cleavage of prothrombin to thrombin and the production of fibrin. Fibrin is stabilized by transglutaminase activity, which creates intermolecular cross-links as in the crayfish. In summary, this section has demonstrated that transglutaminases show a remarkably conserved pattern across species, and that these protein-protein cross-linking enzymes have been identified in all hitherto characterized clotting mechanisms (Theopold et al., 2014).

Melanisation

Melanisation, the encapsulation of pathogens with melanin, and killing of pathogens through toxic quinone precursors, is an important part of the innate immune system in arthropods (Cerenius & Söderhäll, 2004). Crucial for the melanisation pathway is the enzyme

prophenoloxidase (proPO), which in the activated form converts ortho-diphenols such as dopamine into quinones that serve as a precursor for melanin that encapsulates the pathogen. In a common ancestor of arthropods the oxygen carrying molecule hemocyanin likely evolved from a PO-like protein (Burmester, 2002). PO has been found in insects and crustaceans, but specific POs have not been found in the investigated chelicerates, as e.g. in the horseshoe crab *T. tridentatus*, where the oxygen bearing protein hemocyanin demonstrates PO activity after activation (Nagai et al., 2001). Similarly, hemocyanin with PO activity has been identified in one tarantula species *Eurypelma californicum* (Decker & Rimke, 1998). These observations indicate that arachnids may utilize hemocyanin in the melanisation process.

Recognition molecules, signalling pathways and antimicrobial peptides (AMPs)

Pathogens are also targeted by antimicrobial peptides (AMPs), which act by increasing membrane permeability of the pathogen leading to cell lysis (Bala & Kumar, 2014). The proteins responsible for recognition of intruding agents consist of two groups of receptors, the beta-1,3-glucanase-related proteins (β GRPs) and the peptidoglycan recognition proteins (PGRPs) (Steiner, 2004, Gobert et al., 2003). Expression and release of AMPs is induced upon recognition of intruding agents and activation of one or more signalling pathways: the Toll, immune deficiency (IMD), JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) and JNK (c-Jun N-terminal kinase) (Myllymaki et al., 2014, Tanji et al., 2007, Schmidtberg et al., 2013), see Figure 1 for an overview of the signalling pathways. However, in some species (the horseshoe crab *T. tridentatus*, the spider *Acanthoscurria gomesiana*, the penaeid shrimps, and the bivalve *Crassostrea gigas*) a number of AMPs are constitutively expressed, stored in granulocytes in the hemolymph, and then released when pathogens are recognized (Iwanaga & Lee, 2005, Iwanaga et al., 1998, Bachere et al., 2004, Kuhn-Nentwig & Nentwig, 2013, Lorenzini et al., 2003). Whether constitutive expression of AMPs is the general pattern in arachnids remains to be investigated. The proteins and pathways involved in immune system responses are remarkably conserved, and homologies among arthropods, vertebrates and even plants immune responses exist. Comparative analyses suggest an ancient origin that predates arthropods at least for some of the immune pathways (Gerardo et al., 2010, McTaggart et al., 2009, Zou et al., 2007, Christophides et al., 2004). For example, the AMPs of the defensin family found in plants, animals and fungi, were proposed to originate from a bacteria-like ancestor (Silva et al., 2014). Nevertheless, different species show striking differences in gene diversity and the number of gene copies with the pea aphid having lost the entire IMD pathway as an extreme example (Gerardo et al., 2010). Current knowledge suggests that immune response genes are conserved in the pancrustacean arthropods (crustaceans + hexapods). In the present study, we annotated the genes underlying the innate immune responses and determined whether immune response genes are also conserved in arachnids. Among the arthropods, the *Drosophila melanogaster* genome has the most complete annotation of genes involved in the immune response. Therefore, we used a set of *D. melanogaster* genes to computationally annotate genes involved in the immune response in a set of arachnid genomes including the scorpion *Mesobuthus martensii*, the spider mite *Tetranychus urticae*, the deer tick *Ixodes scapularis*, and the three spiders *Stegodyphus mimosarum*, *Acanthoscurria geniculata* (based on transcriptome (Sanggaard et al., 2014)) and *Parasteatoda tepidariorum*. Similarly, we used

the genes involved in hemolymph coagulation in the tri-spine horseshoe crab *T. tridentatus* to annotate the genes encoding the clotting factors in the same set of arachnid genomes. The genomes of three insects, *D. melanogaster*, *Tribolium castaneum* and *Aedes aegypti* were included as outgroups. The phylogenetic relationships of the major groups of arthropod discussed in this paper is shown in Figure 2 including detailed phylogenetic relationships of the relevant arachnids. Additionally, we performed an inhibition assay using the horseshoe crab *Limulus polyphemus*, the scorpion *Babycurus jacksoni* and two spiders, *Stegodyphus africanus* and *Tegenaria domestica*, that were unchallenged by bacteria to investigate whether their hemolymph could inhibit bacterial growth, which would indicate a constitutive AMP expression.

Methods

Data sets

Genes encoding proteins involved in different humoral immune responses in *D. melanogaster* were downloaded from www.flybase.com (October 2014). The set of immune related proteins included in our analyses is not exhaustive for all *D. melanogaster* immune responses, but were selected to represent a set of key genes (Table 1) from each pathway (see for example Bier & Guichard (2012)). The set of genes encoding effector molecules were chosen to represent all classes; AMPs (defensins, glycine-rich peptides) induced by Toll, IMD and JNK in *D. melanogaster*, lysozymes induced by Toll in *D. melanogaster*, and TEPs induced by JAK/STAT in *D. melanogaster*.

We included the genes encoding clotting factors from the pathway of the tri-spine horseshoe crab *T. tridentatus* described in Iwanaga & Lee (2005). In addition, key molecules (transglutaminase, hemolysin and Fibrinogen) from insect clotting were included (Lindgren et al., 2008, Goto et al., 2003).

Gene lists based on annotation of the individual genome sequences by the authors conducting each sequencing project respectively were downloaded for each species (see Supplementary Information for web pages), and used for blastp analyses (see below). Besides *D. melanogaster*, these included two insects (the mosquito *Aedes aegypti* (Nene et al., 2007), the beetle *Tribolium castaneum* (Richards et al., 2008)) and six arachnids (the spider mite *Tetranychus urticae* (Grbic et al., 2011), the deer tick *Ixodes scapularis* (Vectorbase, IscaW1.4), the scorpion *Mesobuthus martensii* (Cao et al., 2013), the mygalomorph spider *Acanthoscurria geniculata* (Sanggaard et al., 2014), the two araneomorph spiders *Parasteatoda tepidariorum* (Augustus 3, SpiderWeb, Clarke et al, *in prep*) and *Stegodyphus mimosarum* (Sanggaard et al., 2014)). Defensins from *S. mimosarum* were found by tblastx to the genome sequence (see below), since no defensins were found in the gene list.

Sequence comparison analyses

Blastp analyses were performed using Bioedit 7.0.8.0 (Hall, 1999) by first creating local databases for each of the above mentioned gene lists, and subsequently blasting the *D. melanogaster* or *T. tridentatus* genes of interest to each of the local databases. For each blast

run all genes with an E-value lower than e^{-5} were blasted back to the *D. melanogaster* gene list at NCBI, and genes that did not hit the *D. melanogaster* genes initially used to search the arachnid, *Aedes aegypti* and *Tribolium castaneum* gene lists were discarded. This was done to avoid mischaracterizing loci that have closer evolutionary relationships, and therefore high similarities, to *D. melanogaster* genes that are not the actual gene or class of genes searched for. This approach has the limitation that genes that are not identified cannot be unequivocally identified as missing. For example, the gene lists may be incomplete, and certain genes may be little conserved or fast evolving making them difficult to detect using a similarity search.

The above mentioned approach did not yield any genes with similarity to defensins for *S. mimosarum* and we did not find β GRPs in any of the arachnid genomes. We therefore searched for defensins in The Antimicrobial Peptide Database (<http://aps.unmc.edu/AP/main.php>) using the search categories “arthropod”, “insect”, “arachnid”, “spider”, “scorpion” and “tick” and retained unique sequences. These were blasted to the genome sequences of *S. mimosarum* using the tblastx tool in CLC Genomics Workbench v. 7.0. To verify the absence of β GRPs in the arachnids we performed a more extensive blastp search using representative sequences spanning all subfamilies of the β GRPs, GNBP and beta-1,3-glucanase family. The sequences were recovered from Hughes (2012), and accession numbers can be found in Supplementary Table 1. This search was limited to the arachnid genomes.

Domain structure analysis

The proteins involved in the clotting cascade in the tri-spine horseshoe crab *T. tridentatus* (factor B, C, G and proclotting enzyme) are all serine proteases. Serine proteases constitute a large family, and we identified a large number of them in our genomes searches. However, based on BLAST searches, orthology to the clotting cascade proteins of *T. tridentatus* was difficult to determine. Domain structure analyses of the three best blast hits for each protein from each species were therefore conducted using the program SMART (Normal mode) to identify protein features specific for serine proteases of the clotting cascade (Normal mode) (Schultz et al., 1998). Using a similar approach to the proteins of the clotting cascade, we performed a domain analysis of the PGRPs to investigate the presence of a transmembrane domain, signal peptide and zinc binding residues, as these are crucial for correct identification.

Phylogenetics

Phylogenetic trees were constructed for all groups of genes/gene classes involved in the signalling pathways of the humoral immune responses from all nine species investigated. A phylogenetic tree was not constructed when genes were absent in the arachnids. All sequences interpreted to belong to a certain gene class from blast analyses were included. No outgroup sequences were used. Sequences were aligned using the ClustalOmega algorithm (Sievers et al., 2011). All alignments were manually inspected. Phylogenies were constructed using the Maximum Likelihood approach implemented in Mega6 (Tamura et al., 2013). We used Jones-Taylor-Thornton (JTT) substitution model, partial deletions and modelled rate variation with a gamma distribution (5 discrete categories). Tree inference

method was Nearest-Neighbor-Interchange (NNI) with 1000 bootstrap replications to obtain node support values.

Inhibition assay

Hemolymph samples were collected from horseshoe crab (*Limulus polyphemus*), scorpion (*Babycurus jacksoni*) and two spiders (*Stegodyphus africanus* and *Tegenaria domestica*) by piercing the book lungs with a sterile minuten needle. Prior to hemolymph sampling the animals were anaesthetized with CO₂ and fixed under a mesh lying on their dorsal side. The book lungs were disinfected in 70 % ethanol, which was allowed to evaporate before piercing. The hemolymph was collected by a 20 µl Drummond micro capillary and allocated on an agar plate. For *Limulus polyphemus* a 1 ml syringe, 25 G needle was used to pierce the soft integument of the dorsal hinge between prosoma and opisthosoma, withdrawing a hemolymph sample from the dorsal heart. The hinge was disinfected with 70 % ethanol before sampling. An average of 1-5 µl of hemolymph was collected from each animal and applied to the agar plates.

Nutrient broth (NB) plates (0.5 % bacteriological agar) were used, each plate containing a hemolymph sample from each of the experimental animals and a droplet of Ringer solution for control. Each plate was covered with a lawn of bacterial solution (100 µl per plate), after the hemolymph was absorbed into the agar. Two Gram-negative bacteria, *Agrobacterium* sp. and *E. coli* were used. The bacteria were all reared in 10 ml liquid NB media while standing in an incubation shaker (Innova 4000) at 30 °C and 120 rpm. The plates were incubated for 20 h at room temperature. After incubation it was observed whether or not the hemolymph had inhibited the bacterial growth on the agar plates, and photos of the extending of the inhibition zones were obtained with a Nikon DS-Fi1 camera mounted on a Leica stereo microscope.

Results and discussion

Coagulation

Our analyses show that all arachnid genomes contain genes with high similarity to the clotting factors of the tri-spine horseshoe crab *T. tridentatus* (Figure 3). Clotting in *T. tridentatus* is initiated by recognition of lipopolysaccharides or beta-1,3-glucans, molecules from bacterial and fungal cell walls. These molecules bind to factor C and factor G, respectively, which transforms them into activated forms, and factor C subsequently activates factor B, which like factor G, activates the proclotting enzyme. Finally, this protease cleaves soluble coagulogen resulting in the formation of the clot-forming coagulin (Iwanaga, 2002). Factor B, G and proclotting enzyme of the *T. tridentatus* all have a domain structure consisting of a signal peptide, a CLIP domain and a trypsin-like serine protease domain (see Figure 4B). This domain structure is common among serine proteases, and we found blast hits showing the domain structure for all proteins in all species searched. Factor C from *T. tridentatus*, on the other hand, has a more complex domain structure consisting of 6 different predicted domains, including the LCCL domain characteristic of *T. tridentatus* factor C (named after Factor C from the American horseshoe crab *Limulus polyphemus*), and 10 predicted domains in total (see Figure 4A). Blast hits with this specific domain structure

were identified in the deer tick *Ixodes scapularis* and the three spider species. A sequence with high similarity to *T. tridentatus* factor C (e-147) was found in the scorpion *Mesobuthus martensii*, but this sequence is incomplete by lacking the 5' end. Alignment of this sequence and the complete *T. tridentatus* factor C sequence reveals that the LCCL domain in *T. tridentatus*, is located in the 5' part of the sequence and that the *M. martensii* sequence is lacking. We therefore hypothesize that factor C is present in *M. martensii*. These findings suggest that all arachnids, but the spider mite *Tetranychus urticae*, use the same complete clotting cascade as the horseshoe crab *T. tridentatus*.

The clottable protein coagulogen was absent in all arachnid genomes included in this study, but the structure of coagulogen shows similarities to spätzle in e.g. the fruit fly *D. melanogaster* (Bergner et al., 1997, Bergner et al., 1996). Since both proteins are activated by potential homologous serine proteases, it has been suggested that one protease cascade evolved into two cascades with very different functions, and that horseshoe crabs have co-opted a spätzle-like protein for blood coagulation (Krem & Di Cera, 2002). As we find spätzle-like genes (see below), coagulation in arachnids should be explored using a proteomics approach to decisively demonstrate the role of spätzle in arachnid clot composition.

We identified hemolectin encoding genes in all arachnid genomes, apart from *T. urticae*. This suggests that these proteins may be involved in clot-formation in arachnids, similar to their function in some insects and crustaceans. We find transglutaminase encoding genes in all investigated genomes indicating that these enzymes, in the arachnids, stabilize the clot. Our findings are consistent with previous findings of no coagulation activity in a species of the *Tetranychus* genus, *T. neocaledonicus* (Rockett & Woodring, 1972).

Melanisation

No proPO-encoding genes were identified in any of the arachnid genomes, in contrast to the insect genomes where multiple copies are found, for example three and four copies in *D. melanogaster* and the beetle *T. castaneum* respectively, and as many as 16 in the mosquito *A. aegypti* (Figure 3). Also the centipede *Strigamia maritima* has a gene encoding proPO (Chipman et al., 2014). The lack of proPO-encoding genes in arachnid genomes is corroborated by a separate study of the immune-related genes in the deer tick *Ixodes scapularis* (Smith & Pal, 2014). The role of melanisation in immune system functioning (and not just colouration and/or sclerotization) in spiders was supported when black particles were observed in the hemolymph after bacterial introduction in *Stegodyphus dumicola* (pers. obs. Reut Berger-Tal). Also, the insertion of a thread in the abdomen of a male wolf spider lead to melanisation of the intruding thread (Ahtiainen et al., 2006). The absence of PO-encoding genes and the observation of melanisation indicate that arachnid hemocyanin, a member of the same gene family as proPO (Terwilliger & Ryan, 2006), displays PO-activity, as observed in the tri-spine horseshoe crab *T.* and the spider *Eurypelma californicum* (Decker & Rimke, 1998, Nagai et al., 2001). In *T. tridentatus*, the AMP tachyplesin convert hemocyanin to have prophenoloxidase activity after binding to pathogen chitin (Nagai et al., 2001). In *E. californicum*, hemocyanin obtained prophenoloxidase activity after treatment with trypsin and chymotrypsin (Decker & Rimke, 1998). See

Supplementary Figure 2T for a phylogenetic tree of arachnid hemocyanins, some insect hemocyanins and insect proPOs, arachnid hemocyanins form a monophyletic group with high support as do the insect proPOs.

Recognition molecules, signalling pathways and antimicrobial peptides (AMPs)

Peptidoglycan Recognition Proteins (PGRPs)—We identified genes encoding PGRPs in all arachnid genomes investigated except for the scorpion *Mesobuthus martensii*. The absence of PGRPs was also reported for the crustacean *Daphnia pulex* (McTaggart et al., 2009) and the pea aphid *Acyrtosiphon pisum* (Gerardo et al., 2010). The copy numbers of PGRP genes (both incomplete and complete) in spiders were 8, 12, and 7 in *Acanthoscurria geniculata*, *Parasteatoda tepidariorum* and *Stegodyphus mimosarum*, respectively, which is comparable to insects, but appears higher than those of other arachnids (Figure 3). No transmembrane domains were predicted based on motif search (Reiser et al., 2004) in any of the complete PGRP sequences identified in the arachnids, except for a single *S. mimosarum* PGRP sequence (Table 2). No transmembrane domains were predicted in the incomplete PGRPs of arachnids either. In the insects, on the other hand, about half of the PGRPs have transmembrane domains. The transmembrane PGRPs are involved in the activation of the IMD pathway through pathogen recognition (see below) suggesting that this pathway is important for the insects, but not for the arachnids. Another group of PGRPs are the ones that are catalytic, with amidase activity that break down peptidoglycans and thereby kill bacteria (Dziarski, 2004), and are characterized by having three conserved residues in insects (Reiser et al., 2004). These residues bind zinc that is required for amidase activity (Mellroth et al., 2003, Reiser et al., 2004). We find that most of the insect PGRPs that have predicted zinc binding residues also have a predicted signal peptide, suggesting an extracellular localization. In the arachnids, the picture is different with many PGRPs that do not contain conserved zinc-binding residues and signal peptides, or have just one or the other (Table 2) (Dostert et al., 2005, Souza-Neto et al., 2009, Myllymaki & Ramet, 2014). The arachnid PGRPs form a monophyletic group, however, only with low support (Supplementary Figure 2A). This tentatively suggests that the variation observed in arachnids arose after the origin of this group.

Beta-1,3-glucanase related proteins (βGRPs)—In the fruit fly *D. melanogaster* (where the term Gram Negative Binding Proteins (GNBP) is used) and other arthropods, βGRPs function by binding the polysaccharide beta-1,3 glucans from the cell wall of fungi and lipopolysaccharides from the cell wall of Gram-positive bacteria (Kim et al., 2000). This leads to activation of the Toll cascade. Our analyses show that *D. melanogaster* and the beetle *T. castaneum* have three copies of βGRPs, and the mosquito *A. aegypti* has seven copies. While βGRPs are identified in all the insect species investigated, in the centipede *Strigamia maritima* (Chipman et al., 2014) and in the crustacean *Daphnia pulex* (McTaggart et al., 2009), we did not identify βGRPs in any of the arachnids (Figure 3), also not after including a more extensive search for genes similar to members representing the entire Beta-1,3 glucanase family. This suggests that βGRPs have been lost in the common ancestor of chelicerates, since βGRPs are also not found in two species of horseshoe crabs (*Limulus polyphemus* and *Tachypleus tridentatus*, results not shown), but have been found in

Mollusca (Zhang et al., 2007), and therefore that their function was lost, or taken over by different proteins in chelicerates.

Toll pathway—The Toll pathway has functions in both development and immunity. The immune function of the Toll pathway is initiated by both fungi, Gram-negative and Gram-positive bacteria in the fruit fly *D. melanogaster* (Valanne et al., 2011) and Gram-negative and -positive bacteria in the Coleoptera and Lepidoptera (Yu et al., 2010, Tanaka et al., 2008). Fungal and bacterial cell wall molecules are recognized by PGRPs and/or β GRPs that cause cleavage of the extracellular protein, spätzle, which subsequently activates the transmembrane Toll receptor. An active Toll receptor initiates an intracellular cascade that results in transcription of antimicrobial peptides and proPO, through the transcription factor dorsal. The proteins from the Toll signalling cascade are conserved among insects and other arthropods (Chipman et al., 2014, Gerardo et al., 2010, McTaggart et al., 2009, Valanne et al., 2011). We recovered all the genes encoding the proteins of the Toll cascade searched for in the three insect species. In the arachnids, the Toll cascade seems to be conserved as well. One of the genes searched for, tube, appears to be missing in all of the arachnid species, which is in accordance with the absence of tube in the centipede *Strigamia maritima* (Chipman et al., 2014) and in the crustacean *Daphnia pulex* (McTaggart et al., 2009). This is however based on the search with *D. melanogaster* tube, which has lost its kinase domain. If the tube sequence from the mosquito *A. aegypti* is used, blast hits are obtained of proteins that have retained the kinase domain in the arachnids. However, tube and pelle are members of the same gene family both having a death domain and a kinase domain, and orthology is difficult to infer.

Multiple gene copies encoding Toll receptors and spätzle are found in both insects and arachnids. The variation in both genes seems to be older than the split between mandibulata and chelicerates, and have likely originated in a common ancestor (Supplementary Figure 2K-L). The scorpion *M. martensii* and the spiders have high numbers of Toll receptors with *P. tepidariorum* and *S. mimosarum* exhibiting the highest number with 16 and 17 copies, respectively. It appears that there is some variation in copy number across taxa as *A. aegypti* has 14, which are higher than both *D. melanogaster* and the beetle *T. castaneum*. The highest known diversity is found in the centipede *S. maritima* which has 36 copies (Chipman et al., 2014). The functions of the different copies of Toll receptors and spätzle are not all well characterized. However, besides the dual function in development and immunity, it is possible that several variants facilitate the recognition of a broader range of pathogens. Phylogenetic analyses of the copies of Toll receptors and spätzle show that variants are not unique to either species or insects/arachnids, but must have originated before the common ancestor of arthropods (Supplementary Figure 2 K-L). The transcription factor *dorsal* that is activated by the Toll pathway is mostly found in a single copy in the arachnids. In the araneomorph spiders, *P. tepidariorum* and *S. mimosarum*, however, we find three and two copies, respectively. As mentioned above, we do not find β GRPs in the arachnids. β GRPs are used by insects to initiate the Toll pathway suggesting that this pathway may either not be activated in the arachnid immune response, or that PGRPs or other recognition molecules can initiate Toll based immune responses.

IMD pathway—The IMD signalling pathway is active against Gram-negative and Gram-positive bacteria in insects, (Myllymaki et al., 2014, Buchon et al., 2014, Yu et al., 2010). In the fruit fly *D. melanogaster*, transmembrane PGRPs are activated by bacterial peptidoglycans, which cause the PGRP to interact with the IMD protein. This initiates the IMD signalling pathway resulting in the activation of the transcription factor relish, which induces the production of several antimicrobial peptides. While the IMD pathway seems to be conserved among insects, several of the components of this pathway were not identified in the arachnids. Especially the upstream components (IMD, dFADD and Dredd) are missing, while several downstream components like IRD5, TAK and relish are found. This finding suggests that relish is still functional as transcription factor of certain AMPs, but is activated differently in arachnids. As mentioned above, the transmembrane PGRPs are lost in arachnids, which is congruent with the absence of most upstream components of the IMD pathway in arachnids, suggesting that IMD induced immunity may not occur in arachnids. However, this needs further verification as it is unknown whether insect and arachnid IMD pathways share a similar upstream component, and thus could be activated differently. IMD pathway genes also differ in amino acid substitution rate which challenges the identification of orthologous genes, especially for fast evolving genes (Obbard et al., 2009).

It has been reported that the IMD pathway has been lost in the pea aphid, *Acyrtosiphon pisum* (Gerardo et al., 2010), and the authors of that study speculated that the close symbiosis with especially Gram-negative bacteria has driven this development. *A. pisum* has also lost relish, which suggests that the antimicrobial peptides that were ancestrally expressed by the activation of the IMD pathway are no longer expressed.

JNK pathway—Our understanding of the role of the JNK pathway in arthropods is currently limited, but in the fruit fly *D. melanogaster* it is involved in various processes including development, apoptosis and immunity (Kockel et al., 2001). Several genes can initiate the JNK pathway, such as TAK that is also part of the IMD pathway (Park et al., 2004). In *D. melanogaster*, JNK genes are up-regulated by the infection of bacteria (Boutros et al., 2002, Chen et al., 2002), and overexpression leads to increased survival when challenged with mainly Gram-negative bacteria (Libert et al., 2008). The part of the JNK pathway investigated here seems to be conserved among the arachnids. Since TAK is found in all arachnids investigated, it suggests that the JNK pathway can be functional in arachnids. Insects appear to have only one copy of each of the genes involved in the JNK pathway, whereas two copies of some of the genes are found in the arachnids (Figure 3). An exception is that the mosquito *Aedes aegypti* seems to have two copies of Basket/JNK, but one variant (AAEL008622) seems to be highly divergent, and may not serve the function of Basket/JNK (Supplementary Figure 2B). Genes with two copies in arachnids originated after the split between mandibulates and chelicerates, but predate the split between spiders and scorpions (Supplementary Figure 2B-E). The spider *A. geniculata* deviate by apparently lacking a copy of hep, a protein found in the JNK pathway. However, we note that this could be due to the fact that the gene list of *A. geniculata* is based on transcriptome sequencing, and therefore is less complete compared to the other gene lists.

JAK/STAT pathway—Similar to the JNK pathway, the JAK/STAT pathway has been shown to be involved in several biological processes, especially in development and immunity (Lebedeva et al., 2013). In insects, the JAK/STAT pathway is a signalling cascade mainly triggered by the recognition of viruses, but it is also involved in the immune response against bacteria in both insects, e.g. the fruit fly *D. melanogaster* (Boutros et al., 2002) and arachnids, e.g. the deer tick *Ixodes scapularis* (Liu et al., 2012). The part of the JAK/STAT pathway investigated here is conserved with all insects showing a single copy of each of the involved genes (Figure 3). Arachnids such as the scorpion *M. martensii* and the two spiders *A. geniculata* and *S. mimosarum* are all missing one or more genes involved in the insect JAK/STAT cascade.

Antimicrobial peptides (AMPs) and other effectors—In insects, effector molecules, or pathogen killing molecules, are expressed and released from fat bodies (Bulet & Stocklin, 2005) following infection by the activation of one or more of the signalling pathways. Activation of the IMD pathway results in the expression of antimicrobial peptides like defensins, and the Toll pathway in the expression of other antimicrobial peptides and lysozymes (Gerardo et al., 2010). The JAK/STAT pathway causes the expression of thiolester containing proteins (TEPs) (Lagueux et al., 2000). However, there are exceptions, for example in mosquitoes, where TEP1 is released into the hemolymph upon infection of *Plasmodium* and causes lysis of the bacteria via the activation of the IMD or Toll pathway (Blandin et al., 2004).

Defensins are AMPs that are used to fight pathogens across all eukaryotes (Silva et al., 2014). They are relatively small peptides containing six to eight conserved cysteines required for appropriate folding of the protein. In invertebrates five types can be distinguished (Tassanakajon et al., 2015), arthropod and mollusc 6-cysteine defensins (Froy & Gurevitz, 2003), mollusc 8-cysteine (Yang et al., 2000), nematode 8-cysteine (Froy, 2005), invertebrate big defensins (horseshoe crab, Saito et al., 1995, amphioxus, Teng et al., 2012, molluscs, Gerdol et al., 2012) and beta-defensin-like peptides (crustaceans, Tassanakajon et al., 2015). Visual inspection of the alignment of the defensins identified in the species studied in this paper reveals that they all belong to the 6-cysteine type (see Figure 5).

We did not find evidence for the presence of defensins in the genomes of the spider mite *T. urticae* and the spider *A. geniculata*. The gene list of *A. geniculata* originates from transcriptome sequencing (Sanggaard et al., 2014), and since defensins are small they may have been missed or discarded in the assembly process. Similarly, the spider *S. mimosarum* gene list did not contain any defensins, but six defensins were found by searching the genome sequence. This suggests that defensins may easily be missed in the genome annotation process, and that the numbers of defensins reported here probably are underestimated. Even though defensins are much older than the origin of arthropods (Silva et al., 2014), they seem to be monophyletic for both insects and arachnids (Supplementary Figure 2S).

Glycine-rich peptides are another group of AMPs. They have previously been found in spiders, where they are constitutively expressed (Fukuzawa et al., 2008, Lorenzini et al.,

2003, Baumann et al., 2010). Their function has not been investigated in great detail, but the results indicate that they are active against bacteria and fungi (Baumann et al., 2010, Fukuzawa et al., 2008, Lorenzini et al., 2003). In the two araneomorph spiders, *P. tepidarium* and *S. mimosarum*, we found four and six copies of putative glycine-rich peptides respectively (Figure 3). These are characterized by 20-40 N-terminal non-glycine-rich residues and 100-800 highly glycine-rich residues. This pattern is similar to both the mygalomorph acanthoscurrins and the araneomorph ctenidins (Kuhn-Nentwig & Nentwig, 2013). The four sequences found in *P. tepidarium* have easily identifiable homologs to four of the *S. mimosarum* sequences based on highly similar N-terminal in all four pairs and almost identical central sequences of ~15 residues interrupting the glycine-rich regions of the proteins in three of the four pairs. A central sequence of 10 residues interrupting the glycine-rich region is also present in ctenidins (Kuhn-Nentwig & Nentwig, 2013, Baumann et al., 2010). The repetitive nature of glycine-rich peptides makes them difficult to assemble from short read sequencing, and the numbers presented here are likely underestimates. Future functional studies are needed to verify if the putative glycine-rich peptides identified in the araneomorph spiders have an antimicrobial function.

Lysozymes function by hydrolysis of peptidoglycans, which causes killing of bacteria by degradation of the bacterial cell wall. All genomes investigated have several genes encoding lysozymes except the mygalomorph spider *Acanthoscurria geniculata*, where only one was found.

TEPs are thought to be transcribed by activation of the JAK/STAT pathway in insects (Gerardo et al., 2010). They function indirectly by marking pathogens for phagocytosis (Lagueux et al., 2000). All genomes investigated had one or two genes encoding TEPs except the fruit fly *D. melanogaster* that has three copies (Figure 3). Two other types of antimicrobial peptides have previously been identified in spiders, small open-end cyclic peptide (gomesin) (Silva et al., 2000) and hemocyanin fragment (rondonin) (Riciluca et al., 2012). We did not identify any of those peptides in the species investigated here. The variation of lysozymes seems very old and predates the split between mandibulates and chelicerates (Supplementary Figure 2R).

Inhibition assay—We analysed whether the hemolymph from a scorpion (*Babycurus jacksoni*), a horseshoe crab (*Limulus polyphemus*) and two spiders (*Stegodyphus africanus* and *Tegenaria domestica*) can inhibit growth of the Gram-negative bacteria *Agrobacterium* sp. and *Escherichia coli*. Using a standardised zone of inhibition assay, all four species showed ability to inhibit the growth of *Agrobacterium* sp., while only *B. jacksoni* and *L. polyphemus* inhibited *Escherichia coli* (Supplementary Figure 1). As these individuals were not previously challenged by us with bacteria, these results are consistent with a constitutive expression of antimicrobial effector molecules. However, the identity of these has to be further verified. In a mygalomorph spider, an antimicrobial peptide, acanthoscurrin, has been isolated from hemocytes and shown to be active against *E. coli* (Fukuzawa et al., 2008, Lorenzini et al., 2003).

Conclusion

Until now, our knowledge on the origin of the arthropod innate immune system suggests that several aspects predate the most recent common ancestor of arthropods, and parts of the immune system is to a large extent conserved among insects, crustaceans and myriapods. We present data indicating fundamental differences in how the humoral innate immune responses functions in arachnids compared with previously investigated arthropods. First, our analysis suggests that the coagulation cascade in arachnids, except for the spider mite *T. urticae* that seems to lack key genes (factor C and hemolactins), is similar to the well-described mechanism in the tri-spine horseshoe crab *T. tridentatus*, however the clot forming protein is not retrieved, and arachnid clot proteomes remain to be investigated. Second, all arachnids lacked the genes encoding proPO that catalyse the formation of melanin, and hemocyanin is likely to contain PO activity. This is in contrast to mandibulates (insects, crustaceans and myriapods), where at least one copy was found in all species. Third, the arachnids differ from the other arthropods by missing pathogen recognition molecules from the β GRP family, and they appear to have lost transmembrane PGRPs. Fourth, we show the absence in arachnids of the genes encoding the upstream part of the IMD pathway, suggesting that IMD signalling has been lost in an ancestor to arachnids. Taken together, these different lines of evidence demonstrate that the arachnid humoral innate immune response differ from that of other arthropods.

The AMPs previously investigated in arachnids were shown to be constitutively expressed (Kuhn-Nentwig & Nentwig, 2013, Lorenzini et al., 2003). Several results presented in this study are consistent with a constitutive expression of AMPs. Transmembrane PGRPs, the IMD gene and several other genes from the IMD signalling pathway are missing in the arachnids, and this pathway is involved in a bacteria-mediated response inducing expression and secretion of AMPs in other arthropods. Also, no β GRPs were identified in arachnids. These molecules function to initiate the signalling pathway in insects, especially the Toll pathway. Moreover, a functional assay shows that hemolymph originating from unchallenged arachnids inhibits the growth of two Gram-negative bacteria, further supporting constitutive expression of AMPs. Future work should include functional studies to reveal if AMPs are generally constitutively expressed, or if the signalling pathways function to also induce their expression.

Using this multifaceted approach, we obtained orthologous immune system genes from six arachnid genomes. However, we cannot exclude that for certain genes we lack the power for accurate detection. This is especially the case for fast evolving genes where similarity may be low. Therefore, we are cautious in the interpretation of searches where genes are not identified. The qualities of assemblies and gene annotations may differ among species, which may also complicate detection of certain genes. However, the highly similar results for the insects and arachnids respectively provide confidence in our conclusions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Key components searched for in five investigated immune related pathways. The tri-spine horseshoe crab *Tachypleus tridentatus* model of clotting is redrawn from (Iwanaga & Lee, 2005), and the fruit fly *Drosophila melanogaster* models of signalling pathways are redrawn from (Bier & Guichard, 2012). Clotting: dark blue represents pathogen surface molecules that initiate the clotting pathway, brown represents cascade molecules, and grey represents end product. Toll: dark blue represents pathogen surface molecules that initiate the Toll pathway, dark green represents recognition molecules, blue represents signalling molecules, and red represents transcription factor. IMD: dark blue represents pathogen surface molecules that initiate the Toll pathway, green represents recognition molecules, orange represents signalling molecules, and purple represents transcription factor. JNK: light green represents signalling molecules, and light brown represents transcription factors. JAK/STAT: yellow represents ligand of domeless released upon infection (not searched for), pink represents signalling molecules, and turquoise represents transcription factor.

Figure 2. Phylogenetic relationships of the major arthropod groups including the six arachnids of this study. The relationships among the arachnids are based on (Sanggaard et al., 2014).

Figure 3.

Copy numbers identified in six arachnid and three insect genomes (in *Drosophila melanogaster* numbers originate from www.flybase.org) of the genes involved in different humoral innate immune responses. On top is a representation of the phylogeny of the focal species. The relationships among arachnids are based on (Sanggaard et al., 2014). The identification of tube is difficult (see text), and it is uncertain whether we identified a tube homolog in the arachnids, indicated by 0/1 in this figure. The colour coding is the same as in Figure 1, where a detailed description can be found.

Figure 4.

Domain structure of the clotting cascade factors of the tri-spine horseshoe crab *Tachypleus tridentatus*. A) shows the domain structure of factor C, and B) shows the domain structure of factor B, factor G and proclotting enzyme. Signal: signal peptide, EGF: epidermal growth factor-like domain, CCP: Domain abundant in complement control proteins; SUSHI repeats; short complement-like repeats (SCR), LCCL: named after Limulus Factor C, CLECT: C-type lectin (CTL) or carbohydrate-recognition domain, Tryp_SPc: trypsin-like serine protease, CLIP: clip or disulphide knot domain.

Drosophila melanogaster Def-PA (FBgn0010385) -----MK---FFVLVAIAFALLACVACQPVSDVDPIPEDH-----VLVHEIAHQEVLQHSRQKRAICDLLSKWN-----WNHTAQAGHCIAKG--FKGYCND--KAVCVCRN
 Aedes aegyti AAEL003857 -----MQP---LTVICFIALCTGAI--TSAYPQEPVIADARPFANSLEDELPEETVCAAVENFRLKRAICDLLSGFG-----VGDSACAAHCIARG--NRGGYCNS--KKVCVCRN
 Aedes aegyti AAEL003849 -----MKSITTLCLAVVCFIALLSVGAAPQESVSIQAEHFAEFDTIQVQHDCAEQIPVEFQRRKRIICDLLSGLG-----WGHISIAAGHCIAISWRYRGGYCND--QGVCVCRT
 Aedes aegyti AAEL003841 -----MKS---ITVICFIALCTVAI--TSAYPQEPVIADARPFANSLEDELPEETVCAAVENFRLKRAICDLLSGFG-----VGDSACAAHCIARG--NRGGYCNS--KKVCVCRN
 Aedes aegyti AAEL003832 -----MRT---LTVVCFVALCLSAITFTGNALPEELADVRSYANSLEDELPEESVCAAVENFRLKRAICDLLSGFG-----VGDSACAAHCIARR--NRGGYCNA--KKVCVCRN
 Tribolium castaneum gi|270014296| -----MK---FIVFVVISL-CVFAITAYP-----LDQV-----EEQDEHQVAHVRVVRVTCDLLSAEA--KGV--KVNHAACAAHCLLKR--KRGGYCNA--RRICVCRN
 Tribolium castaneum gi|270012772| -----MIKPDNALKMK-----LIILIALFCVFEITAF-----ADG-----EHVRVKRRTCDVLSAEGGFRGVSIKLNHAAACAAHCLYLK--KRGGYCND--KAVCVCRK
 Tribolium castaneum gi|270004928| -----MK-----LLIIVALVALFCIFETIAFP-----TDG-----EHIRVKRRTCDVLSAEGGFRGVSVKLNHSAACAAHCLFLK--KRGGYCNA--KAVCVCRN
 Mesobuthus martensii Mma36626 -----MKVIALFFELFAFIF-----CTLEVAIVEAGFGCPFN-----QAGCHRHCLIR--RRGGYCSGFFKQCTCTYR
 Mesobuthus martensii Mma39355 -----RIANADPKIMKTIVILFVLALVF-----CTLEMCMVEAGFGCPFN-----QAGCHRHCLIR--RRGGYCSGFLKQRCVCR
 Mesobuthus martensii Mma39356 -----MKTIVILFVLALVF-----CTLEMCMVEAGFGCPFN-----QAGCHRHCLIR--RRGGYCSGFLKQRCVCR
 Ixodes scapularis ISC005926 -----EHRITINLEIMKVIAVALIALLVAGAFMTSS-----AQEEDQVAHVRVVRVRRFGCPFD-----QAGCHRHCLIR--RRGGYCSGFLKQRCVCR
 Ixodes scapularis ISC005927 -----MKAVIALVMMIAGLISTSC-----SQEEDQVAHVRVVRVRRFGCPFN-----QAGCHRHCLIR--RRGGYCSGFLKQRCVCR
 Ixodes scapularis ISC005928 -----MKVIAVTLLIALLVAGAFMTSS-----AQEEDQVAHVRVVRVRRFGCPFD-----QAGCHRHCLIR--RRGGYCSGFLKQRCVCR
 Ixodes scapularis ISC011162 -----MKVVGIALVVRFLFS-----FSC-----SQGVHSCVPHVRVVRVRRFGCPFD-----QAGCHRHCLIR--RRGGYCSGFLKQRCVCR
 Parasteatoda tepidariorum aug3.g3278.t1 -----MIAKYIVLLVAVLL-----SFAFAVAICADHGGPHD-----EPSCHSYCRNG--FAFGWGGFLNFCNCHE
 Parasteatoda tepidariorum aug3.g12738.t1 -----MLPKILIIIVCLT-----IALTTTKAARNLCPTN-----WNECHELCRQG--RRGGYCSGFLKQRCVCR
 Stegodyphus mimosarum scaffold2575 -----EAGFGCPFD-----QAGCHRHCLIR--RRGGYCSGFLKQRCVCR
 Stegodyphus mimosarum scaffold2373 -----EAGFGCPGN-----QAGCHRHCLIR--RRGGYCSGFLKQRCVCR
 Stegodyphus mimosarum scaffold2035 -----SGNMCPSN-----QAGCHRHCLIR--RRGGYCSGFLKQRCVCR
 Stegodyphus mimosarum scaffold1762 -----YGCPLL-----QAGCHRHCLIR--RRGGYCSGFLKQRCVCR
 Stegodyphus mimosarum scaffold2089 -----CPYD-----QAGCHRHCLIR--RRGGYCSGFLKQRCVCR
 Stegodyphus mimosarum scaffold2175 -----CRED-----QAGCHRHCLIR--RRGGYCSGFLKQRCVCR

Figure 5.

Alignment of identified defensins. Six cysteines (marked in red) are conserved among all loci. The species names are followed by identifiers from the respective gene lists, except for *Stegodyphus mimosarum*, where the genome sequence scaffold carrying the defensin loci is given.

Table 1

Overview of the set of key immune related genes analysed.

Recognition	Toll	IMD	JNK	JAK/STAT	Effectors	Clotting	Melanisation
PGRPs	Toll	IMD	Hep	Domeless	TEPs	Factor C ^b	Prophenol oxidase
βGRPs	Spätzle	dFADD	Basket/JNK	Hop/JAK	Lysozyme	Factor B ^b	
	MyD88	Dredd	JRA	STAT	Defensins	Proclotting enzyme ^b	
	Pelle	TAK	Kayak		Glycine-rich peptide ^a	Factor G ^b	
	Cactus	Kenny				Coagulogen ^b	
	Tube	IRD5				Transglutaminase	
	Dorsal	Relish				Fondue	
						Hemolectin	

^a only searched for in the araneomorph spiders.

^b from the tri-spine horseshoe crab *Tachypleus tridentatus*.

Table 2

Numbers of PGRPs with predicted transmembrane domain (T), signal peptide (S) and zinc binding residues (Z) of the complete PGRPs sequences identified.

	Aedes	Tribolium	Tetranychus	Ixodes	Parasteatoda	Stegodyphus
-SZ	3	2	0	1	2	3
T--	5	3	0	0	0	1
-S-	0	1	1	0	5	2
--Z	0	0	0	0	2	1
---	1	1	0	0	2	0