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Review paper

Evolutionary genetics of insect innate immunity

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Abstract

Patterns of evolution in immune defense genes help to understand the evolutionary dynamics between hosts and pathogens. Multiple insect genomes have been sequenced, with many of them having annotated immune genes, which paves the way for a comparative genomic analysis of insect immunity. In this review, I summarize the current state of comparative and evolutionary genomics of insect innate immune defense. The focus is on the conserved and divergent components of immunity with an emphasis on gene family evolution and evolution at the sequence level; both population genetics and molecular evolution frameworks are considered.

Key words: Toll; Imd; AMP; gene family evolution; positive selection

Introduction

In defense against pathogens, insects rely mainly on their innate immune system. In addition, many insects are better protected against a specific pathogen on recurrent encounter, even though insects do not have adaptive immunity such as the somatic recombination of antibody encoding genes used by vertebrates [e.g. 1, 2]. Functional studies in *Drosophila* have elucidated the details of insect immunity. The core of the inducible immune response builds on two signaling pathways, Toll and Imd [3]. The Toll pathway has a dual function, being central in developmental processes, whereas Imd functions exclusively in immunity. Toll and Imd pathways are interconnected and together work synergistically [4]. Both pathways form part of the humoral immune response that is triggered by the recognition of microbes and results, via multiphase signal transduction, in the secretion of antimicrobial peptides, lysozymes and other microbe-targeting substances. Cellular response is another component of insect immunity. Hemocytes participating in phagocytosis or encapsulation of foreign particles and activation of phenoloxidase cascade leading to melanization of macroparasites are the hallmarks of cellular response [5]. Multipurpose pathways JNK and JAK/STAT also contribute to immunity, and RNA interference is essential to viral defense [5].

A multitude of insect genomes have been sequenced and most of these genomes have annotated immune genes. The annotation has been facilitated by the development of databases such as ImmunoDB [6] and the Insect Innate Immunity Database [7]. These databases are excellent starting points for characterizing immunity genes in non-model species, but it should be noted that both databases rely on information derived from dipterans and, therefore, are to some extent restricted for immune gene identification. Studies of comparative genomics of insect immunity are rapidly accumulating. This review summarizes the current state of evolutionary genetics of insect immunity focusing on genetic components conserved across insect taxa and components that have diverged in terms of gene presence/absence, gene family evolution by copy-number changes and variation in nucleotide sequences.

The conserved pathways

Evolutionarily conserved components involved in a biological process typically are recognized by the presence of orthologous

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Table 1. Glossary of molecular evolution

Term	Definition
Homologous genes	Genes found in different species that have shared ancestry, includes both orthologous and paralogous genes.
Orthologous genes	Homologous genes in different species as a result of speciation.
Paralogous genes	Homologous genes that have duplicated within a genome.
Substitution	Mutation that has become fixed in a population, i.e. replaced all other nucleotide variants at the same position.
Nonsynonymous substitution	Fixed mutation in protein-coding sequence that results in amino acid change.
Synonymous substitution	Fixed mutation in protein-coding sequence that does not alter the amino acid.

Box 1. Types of selection

Inference of natural selection is based on the neutral theory of molecular evolution [27, 28], whereby most nucleotide substitutions are assumed to be selectively neutral and random genetic drift is the major evolutionary force driving mutations to extinction or fixation. The neutral theory provides the null hypothesis against which alternative hypotheses of positive or purifying selection can be tested. Positive selection is inferred when more amino acid changes have taken place than would be expected for neutral sites; conversely, fewer changes than expected are likely the result of purifying selection. Nucleotide substitutions that lead to deleterious amino acid changes typically are pruned rapidly and thus do not contribute to polymorphism within species or divergence between species.

genes across wide taxonomical distribution (Tables 1 and 2). Single-copy orthologous genes have likely retained the same function even over long evolutionary timescales [6]. Newly sequenced insect genomes frequently have their immune genes annotated based on sequence homology to known genes from other species (Table 1). The fruit fly *Drosophila melanogaster* has long been the primary model species of insect immunity, and its immune genes have served as the principal catalog to search for homologs in other species. The first genome-level comparison of immune genes in *D. melanogaster* and the mosquito *Anopheles gambiae* indicated that both species have single-copy orthologous signal transduction genes characteristic to the central immune pathways Toll and Imd [8]. Since then, almost every insect with genome sequence accompanied by immune gene annotations has reported the presence of these two signaling pathways, and the complementary pathways JAK/STAT and JNK (Table 2). Exceptions are the pea aphid *Acyrthosiphon pisum* and the body louse *Pediculus humanus*, which both lack most or some key genes of the Imd pathway, respectively [9, 10] (Table 2). It is noteworthy, that the homology-based approach is biased toward species that have been targets of elaborate functional studies, namely, *Drosophila* and *Anopheles*, which are both from the order Diptera. While the homology-based approach is valuable in detecting the strictly conserved components of immune response, it inevitably misses any new immune genes or pathways that a certain species might have [11]. It is also good to keep in mind that genes present in these model organisms may not exist in other species or may not share the same function.

The divergent components

The raw materials of evolution are mutations, such as gene gain, gene loss and single nucleotide changes that alter the protein-coding sequence. In the long term, only mutations that are fixed in a species are relevant. A fixed mutation, or in other words substitution, may occur by chance (neutral evolution) or be driven by selection (Table 1). Positive selection is the type of selection leading to fixation of advantageous mutations (directional selection), maintained polymorphism (balancing selection) or the persistence of a recently duplicated gene, whereas deleterious mutations and harmful gene duplicates are removed by purifying selection (Box 1). Evolution of immune genes is dominated by expansions and contractions of gene families and the host–pathogen arms race is driven by positive selection, including both directional and balancing selection.

Gene family evolution

The prominent mode of evolution in immune gene families is birth-and-death evolution [29]. New genes are born by gene duplication, and some gene family members are lost by the accumulation of detrimental mutations. Christophides *et al.* (2002) put forth a hypothesis according to which immune genes adapt, are lost, or duplicate and then diversify to meet new ecological and physiological requirements. A detailed comparative study of immune genes in *D. melanogaster* and the mosquitoes *Aedes aegypti* and *A. gambiae* showed that gene families vary in the degree of diversification measured as the proportion of single-copy orthologs and species- or lineage-specific paralogs [6, 30] (Table 1). Signaling genes are mostly present as single-copy orthologs and evolve in concert, i.e. show similar levels of divergence, most likely to maintain functionality of the pathways [6]. Gene family expansions are extensive in the categories of recognition and modulator proteins, and the expansions may be species- or lineage-specific [6]. The observation of large gene families and family size variation due to birth–death dynamics within the recognition and modulator gene categories applies also to a wider taxonomical sample (Table 2).

Antimicrobial peptides (AMPs) are short cationic molecules that can be classified into families on the basis of their protein structure and/or amino acid composition [31]. AMPs have undergone extensive gene duplication and loss as well as exon duplication and exon shuffling [11, 17, 32, 33]. As a result, AMPs in insects show lineage specificity both in copy numbers within a gene family and the presence/absence of an entire gene family [6, 13]. For example, the coleoptericin family of AMPs is present only in the order Coleoptera [34] and Drosomycin family in certain *Drosophila* [13]; the gene family comprising defensins is the only one present in all insect orders studied thus far (Table 2). Because of lineage specificity and the potential of sequence divergence to rapidly erode signals of homology, particularly within a short sequence, the identification of novel AMPs in a newly sequenced genome is challenging. Tian *et al.* (2010) used an integrated computational approach to uncover 44 AMPs in the *Nasonia vitripennis* genome, which is the largest repertoire of AMPs reported thus far in an insect. The AMP content of *N. vitripennis* might be even larger, as Sackton *et al.* (2013) found 14 additional proteins that have the characteristics of AMPs. Moreover, the transcriptome of infected *Drosophila virilis* yielded five putative

Table 2. Summary of immune genes across selected insect orders. Information on immune gene contents were obtained from [6, 8–26]

			Coleoptera		Diptera		Hemiptera		Hymenoptera			Lepidoptera		Odonata	Phthiraptera
Gene function	Pathway	Gene	T. mol	T. cas	A. gam	D. mel	A. pis	N. lug	A. mel	N. vit	L. hum	B. mor	M. sex	C. pue	P. hum
Recognition		*C-type lectin*	*12*[a]	16	25	34	5	9	10	31	12	21	4	*?*	9
		Dscam	*?*	1	1	1	1	9	1	*?*	1	1	1	1	1
		Eater	*?*	*?*	1	1	0	1	0	1	0	0	*?*	*?*	*?*
		GNBPs[b]	3	3	7	6	2	7	2	3	4	4	4	2	0
		PGRPs	6	7	11	13	0	2	4	12	6	12	10	4	1
		TEPs	3	4	13	10	2	*?*	4	3	3	3	2	1	3
Signaling	Toll	*Spätzle*	7	7	6	6	6	8	2	9	5	3	1	1	3
		Toll	2	9	10	9	6	6	5	9	6	14	1	3	6
		MyD88	1	1	1	1	1	2	1	1	1	1	1	1	1
		tube	1	1	1	1	1	1	1	1	1	1	1	*?*	1
		pelle	1	1	1	1	1	1	1	1	1	1	1	1	1
		cactus	1	1	1	1	1	1	3	1	2	1	1	1	1
		dorsal	1	1	1	2	2	1	2	1	1	1	1	1	1
Signaling	Imd	*imd*	1	1	1	1	0	1	1	1	1	1	1	1	0
		FADD	1	1	1	1	0	*?*	1	1	1	1	1	*?*	0
		Dredd	1	1	1	1	0	1	1	1	1	1	1	1	1
		IAP2	1	1	1	1	1	1	1	1	1	1	1	1	1
		TAK1	1	1	1	1	1	1	1	1	1	1	1	1	1
		Tab2	1	1	1	1	1	1	1	1	1	1	1	1	1
		IKKβ/ird5	2	1	1	1	1	1	1	1	1	1	1	1	1
		IKKγ/key	1	1	1	1	0	1	1	*?*	1	1	1	1	*?*
		Relish	2	1	1	1	0	1	2	6	1	1	2	1	1
Signaling	JAK/STAT	*domeless*	1	1	1	1	4	1	1	1	1	1	1	1	1
		hopscotch (JAK)	*?*	1	1	1	1	1	1	1	1	1	1	1	1
		Stat92E	1	1	2	1	2	1	1	1	1	1	1	1	1
Signaling	JNK	*JNK/basket*	3	1	1	1	1	*?*	1	1	1	1	1	1	1
		hemipterous	1	1	1	1	1	*?*	1	1	1	1	1	1	1
		Jra/Jun	*?*	1	1	1	1	*?*	1	1	1	1	1	1	1
Effector		*defensin*	1	4	4	1	0	2	2	5	1	1	*?*	*?*	2
		other AMPs	11	8	5	19	6	0	4	39	5	21	19	*?*	0
		lysozyme	4	4	8	17	3	8	3	2	2	4	2	*?*	
		NOS	*?*	1	1	1	1	1	1	1	1	2	1	*?*	
Modulator		*cSP*	19	48	59	47	6	12	18	13	8	15	6	*?*	6
		serpin	6	31	21	30	14	9	7	12	7	26	12	*?*	16
Melanization		*PPO*	2	3	9	3	2	*?*	1	9	1	2	2	*?*	1
RNA interference		*Ago-2*	*?*	1	1	1	*?*	*?*	1	1	1	1	1	1	*?*
		Dcr-2	*?*	1	1	1	*?*	*?*	1	1	1	1	1	1	*?*

[a]Numbers in italics indicate they are derived from transcriptome data because genome sequence for that species is not published.
[b]This category involves also genes encoding β-glucan binding proteins (βGBPs).
Question marks indicate that this gene/gene family has not been reported in the insect's genome but does not necessarily imply its absence in the genome.

novel AMPs [35]. In conclusion, homology-based identification is not expected to be a fruitful method to characterize AMPs across many insect taxa but thorough computational and functional approaches are required.

Immune function has been well characterized for AMPs and pattern recognition proteins in several insect species. For other immune gene families, the comparison of gene numbers only between different insect species and drawing conclusions based on that is problematic for three reasons. First, all of the members of a gene family may not have been identified [36]. Second, it is not always known if all the gene family members have an immune function, as sequence similarity *per se* is not sufficient to infer conserved function [37], and there is evidence that the function of apparently homologous genes (based on their sequence) may vary between closely related species [38]. Third, similar copy numbers do not necessarily imply that there is comparable immunocompetence, as the nucleotide sequence divergence among different family members may result in different functional properties of the encoded proteins [30].

Evolution at the sequence level

Immune genes are a classic example of genes in which positive selection is expected to occur [39–41]. Microbes are capable of rapid evolution to evade the host immune system, creating a selection pressure on the host to evolve counteradaptations. Typically, some inference about the level of historic positive selection uses the ratio of nonsynonymous to synonymous substitution rates (d_N/d_S, Table 1). The most commonly used methods rely on codon substitution models that allow variation in the intensity of selection among codon sites within a gene [42, 43]. Nucleotide sequence comparisons are valid only between relatively closely related species because of saturation, the accumulation of multiple mutations in a nucleotide site that obscures true level of evolutionary divergence. This places limits on the taxonomical breadth of species that can be sampled for comparative genomic studies. Of course, caution should be exercised also when applying d_N/d_S as a measure of selection to closely related species, as shared and lineage-specific polymorphisms may bias the estimate [44]. Recent and/or

ongoing selection regimes, as well as balancing selection, can be tested using population genetic approaches based on allele frequencies [45].

The first comprehensive genome-level molecular evolutionary study of insect immunity used genome data of six *D. melanogaster* group species and discovered that immune genes evolve at a faster rate (measured as the d_N/d_S ratio) than nonimmune genes [13]. While a faster rate of evolution does not necessarily imply positive selection, this was a likely explanation in *D. melanogaster*, as Sackton *et al*. (2007) also observed a significantly higher proportion of positively selected genes in the immunity category compared with all other nonimmunity protein-coding genes. There has been an indication of rapid evolution in a small subset of immune genes in ants and bees, but evidence for positive selection only in one ant gene [46]. Such rapid evolution could be attributed to either positive selection or relaxed purifying selection. The latter scenario was supported in a population genetic study of Toll pathway genes in honeybees [47], whereas the former scenario was supported in a recent genome-wide scan for positive selection in ants and bees [48]. The study by Roux *et al*. (2014) used gene ontology annotation, and several categories with immunity-related functions were enriched for positively selected genes.

Based on the *Drosophila* study, microbial recognition genes have been affected strongly by positive selection [13]. In all, 9 of the 10 recognition genes affected by selection participate in phagocytosis, with *TepI* found to be positively selected in several population genetic studies of closely related *Drosophila* species and in *A. gambiae* [49–52]. Peptidoglycan recognition proteins (PGRPs) and gram-negative bacteria-binding proteins (GNBPs) recognize microbial surface structures and some of them act upstream of the Toll and Imd signaling pathways. Positive selection on different PGRPs has been reported in ants (PGRP-S) and *Drosophila* (PGRP-LC and PGRP-LD), whereas selection on GNBPs has been found only in termites [13, 46, 51, 53]. It is notable that several short PGRPs (PGRP-S) have experienced purifying selection in *Drosophila* [54]. The inconsistent results regarding selection on PGRPs and GNBPs suggest that these groups of genes are not common targets of host–pathogen coevolution across insects, but may rather be involved in bouts of lineage-specific arms races. Moreover, the differences between the studies may result from the application of different statistical tests based on either (i) models of molecular evolution that operate on long timescale or (ii) population genetic models that consider shorter timescale.

The overall pattern of evolution in signal transduction genes does not show signs of positive selection in *Drosophila* [13]. Supporting this, a network analysis of immune signaling pathways in *Drosophila* found purifying selection as the predominant mode of evolution, and that this effect was greatest on the downstream components [55]. However, lineage-specific positive selection has been detected in several genes along the Imd pathway in certain *Drosophila* species [13, 51, 56]. Of these, the transcription factor Relish has been identified as under strong positive selection in termites also [53]. In both *Drosophila* and termites, the selected sites were located in the caspase cleavage site that is required to activate Relish [13, 53]. This is an interesting example of pathogen-driven positive selection on a protein with no direct interaction with microbes.

In Diptera, there is little evidence of positive selection in AMPs [13, 41, 51, 57, 58], with the only documented case being in *gambicin* of *A. gambiae* [59]. In other insects, positive selection has been found in ant and termite defensins [60–62]. However, in another study, three AMPs, abaecin and defensin in ants and hymenoptaecin and defensin in honeybees, did not show signs of positive selection [46]. The widespread lack of positive selection in a class of genes where it intuitively should be detected has been explained by the difficulty of microbes to evolve resistance to an arsenal of AMPs with distinct activities expressed in a single insect host [63]. Instead, selection may operate at functional level, for example, in boosting the expression of AMPs [13].

In insects, the evolution of genes involved in viral defense has been thus far studied only in *Drosophila* and *A. aegypti*. Several genes of the RNA interference pathway show strong positive selection as a signature of host–pathogen arms races [51, 64–67]. Similar to studies of viral defense genes, evolutionary studies of cellular defense genes are still scarce. Nonetheless, one interesting example in *Drosophila* is the rapid and adaptive evolution of a gene that is induced on parasitic wasp attack [68].

In summary, molecular evolution and population genetics of insect immunity in non-model insects remain an understudied research field. More studies are needed to better understand the evolutionary dynamics between hosts and pathogens. For example, is it a general phenomenon that microbes exert the strongest selective pressure on recognition components of the immune system, as we see in *Drosophila*? Moreover, it is of primary importance to validate the immune function of candidate genes and characterize new immune components in non-model organisms. RNA-seq has already been successfully used for this purpose in several insects [10, 19–22, 24, 25].

Key points

- Toll and Imd signaling pathways are well conserved across taxonomically wide distribution of insects.
- AMPs are the most labile component of insect immunity showing rapid gene birth–death dynamics and lineage-specific gene families.
- Immune genes and especially recognition genes are frequently targets of positive selection driven by host–pathogen arms races.
- Homology-based annotation is useful but to some extent restricted approach to find immune-related genes in a newly sequenced genome.
- Novel immune genes have been found in many insects and should be looked for in future research.

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