How Did Arthropod Sesquiterpenoids and Ecdysteroids Arise? Comparison of Hormonal Pathway Genes in Noninsect Arthropod Genomes

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Abstract

The phylum Arthropoda contains the largest number of described living animal species, with insects and crustaceans dominating the terrestrial and aquatic environments, respectively. Their successful radiations have long been linked to their rigid exoskeleton in conjunction with their specialized endocrine systems. In order to understand how hormones can contribute to the evolution of these animals, here, we have categorized the sesquiterpenoid and ecdysteroid pathway genes in the noninsect arthropod genomes, which are known to play important roles in the regulation of molting and metamorphosis in insects. In our analyses, the majority of gene homologs involved in the biosynthetic, degradative, and signaling pathways of sesquiterpenoids and ecdysteroids can be identified, implying these two hormonal systems were present in the last common ancestor of arthropods. Moreover, we found that the “Broad-Complex” was specifically gained in the Pancrustacea, and the innovation of juvenile hormone (JH) in the insect lineage correlates with the gain of the JH epoxidase (CYP15A1/C1) and the key residue changes in the binding domain of JH receptor (“Methoprene-tolerant”). Furthermore, the gain of “Phantom” differentiates chelicerates from the other arthropods in using ponasterone A rather than 20-hydroxyecdysone as molting hormone. This study establishes a comprehensive framework for interpreting the evolution of these vital hormonal pathways in these most successful animals, the arthropods, for the first time.

Key words: juvenile hormone, methyl farnesoate, ecdysteroid, Myriapoda, Chelicerata, Arthropoda.

Introduction

The arthropods or joint-legged animals, in terms of described living and fossil species numbers, are certainly the most successful animals on the earth. They exhibit a diverse range of body plans and can be found in the widest possible range of habitats worldwide. One of the reasons linked to their success is their hardened chitinous exoskeleton, which provides them with the protection from their often harsh environment and ecosystems (Minelli et al. 2013). In order to grow and increase in body size, arthropods have evolved a complex endocrine cassette. These hormones include sesquiterpenoids (such as methyl farnesoate [MF] and juvenile hormone [JH]) and the ecdysteroids (such as 20-hydroxyecdysone [20E]), which coordinate play vital roles in regulating the maturation, growth, and development of arthropods. Their actions and metabolism have been particularly well-investigated in the Insecta, in which high titer of JH generally restrains insects from undergoing metamorphosis, and high titer of 20E triggers molting/ecdysis (Jindra et al. 2013). Nevertheless, their functions and regulation of production and degradation in noninsect arthropods are poorly understood (Hui et al. 2013; Kenny et al. 2013). With the advent of affordable next-generation sequencing, genomes of noninsect arthropods are now available, including the crustaceans (water flea and shrimp) (Colbourne et al. 2011; Kenny et al. 2014), myriapods (centipede and millipede) (Chipman et al. 2014; Kenny, Shen, et al. 2015), and chelicerates (scorpion, horseshoe crab, mite, and...
Materials and Methods

Identification of Sesquiterpenoid and Ecdysteroid Pathway Genes

Amino acid sequences of known components in sesquiterpenoid and ecdysteroid systems from insect species including Drosophila melanogaster, Bombyx mori, and Tribolium castaneum were retrieved from FlyBase (http://flybase.org/, last accessed July 4, 2015), SilkDB database (http://silkworm.genomics.org.cn/, last accessed July 4, 2015), and BeetleBase (http://beetlebase.org/, last accessed July 4, 2015) and used as queries to identify putative gene homologs in noninsect arthropod genomes via TBLASTN search (Altschul et al. 1990) with the cutoff E-value of 1 × 10^-6. The noninsect arthropod genomes included those of the water flea (Daphnia pulex; http://www.jgi.doe.gov/, last accessed July 4, 2015) (Kenny, Shen, et al. 2015); tick (Strigamia maritima; http://www.vectorbase.org/, last accessed July 4, 2015) (Kenny et al. 2014); millipede (Trigoniulus corallinus; http://www.tetranychusurticae.org, last accessed July 4, 2015) (Kenny, Shen, et al. 2015); tick (Ixodes scapularis; https://www.vectorbase.org, last accessed July 4, 2015); shrimp (Neocaridina denticulata; http://www.hgsc.bcm.tmc.edu/, last accessed July 4, 2015) (Colbourne et al. 2011); shrimp (Penaeus japonicus; http://bioinformatics.psb.ugent.be/genomes/, last accessed July 4, 2015) (Kenny et al. 2014); centipede (Scolopendra subspinipes; http://beetlebase.org/, last accessed July 4, 2015); and mite (Tetranychus urticae; http://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed July 4, 2015) for initial confirmation of identity.

Phylogenetic Confirmation of Gene Identity

To confirm the orthology of retrieved gene sequences, phylogenetic analyses using both maximum-likelihood and Bayesian inference methods were carried out. Gene sequences were first translated into amino acid sequences and aligned to sequences with known identity using MAFFT (Katoh and Standley 2013) under appropriate models as noted in figure legends. Maximum-likelihood phylogeny was carried out by MEGA 6 (Tamura et al. 2013) using the LG + 4G + I model and 1,000 bootstrap replicates. Bayesian inference was performed on alignments using MrBayes (Ronquist and Huelsenbeck 2003). The model jumping command in MrBayes was implemented, which selected substitution models in proportion to their posterior probability. A Markov chain Monte Carlo search was initiated with random trees and run for 1,000,000 generations with sampling every 100 generations (as noted when generation number differed). Convergence was indicated when the average standard deviation of split frequencies was less than 0.01 (Ronquist and Huelsenbeck 2003). Convergence was checked by plotting the likelihood scores against generations and the first 25% of the generations were discarded as burn-in. Trees were visualized in FigTree for annotation and display.

Results and Discussion

Sesquiterpenoid Biosynthetic and Degradative Pathway Genes in Noninsect Arthropods

Sesquiterpenoids are a class of hydrocarbon/terpenes found in arthropods and plants; in arthropods, they are derived from the conversion of acetate via the mevalonate pathway (Tobe 1999; Belles et al. 2005; Hui et al. 2010, 2013). In insects, there are two alternative pathways for the biosynthesis of JH: Either through the conversion of farnesoid acid (FA) to JH-III acid (JHA) by an epoxidase (CYP15C1) followed by methylation by JH acid methyltransferase (JHAMT) in the lepidopterans; or methylation of FA to MF by JHAMT followed by oxidation by another epoxidase (e.g., CYP15A1) as found in cockroaches and locust (Helvig et al. 2004; Belles et al. 2005; Marchal et al. 2011; Daimon et al. 2012; fig. 14). In crustaceans, MF is synthesized through methylation of FA (Tobe et al. 1989). In addition to playing defensive roles in some social insects such as ants and bees, sesquiterpenoids including MF and JH are endogenously produced as master controllers of molting and sexual maturity in crustaceans and insects, respectively (Nagaraju 2007; Riddiford 2008). Although JH has never been identified in noninsect arthropods, JH mimics have been observed to play a role in controlling sex development and predator response in D. pulex (Olmedast and Leblanc 2002; Tatarazako et al. 2003; Miyakawa, Gotoh, et al. 2013). Recently, it has also been noted that the genes involved in JH production and degradation pathways, previously thought to be insect specific, are identified in water flea, shrimp, and centipede (Chipman et al. 2014; Sin et al. 2014). Here, we have identified JHAMT orthologs in the genomes of all investigated arthropods (fig. 1C and supplementary figs. S1 and S20, Supplemental Material online), suggesting the existence of a sesquiterpenoid biosynthetic system in the last common ancestor of all arthropods. However, no homologs of insect JH epoxidase (CYP15A1 or C1) could be identified in any of these noninsect arthropod genomes (fig. 1C). On the basis of this finding, we conclude, for the first time, that JH epoxidase was specifically gained in the insect lineage.

In addition to the biosynthetic pathway genes for insect JH, we could also identify genes encoding enzymes (JH esterase
A schematic diagram showing (A) biosynthetic and (B) degradative pathways of sesquiterpenoid hormones in arthropods (Hui et al. 2010, 2013; Sin et al. 2014; for details, refer to the main text); (C) summary of the presence of the sesquiterpenoid biosynthetic, degradative, and signaling pathway genes in the investigated arthropod genomes. “+” denotes presence and “−” represents the absence of supporting evidence.
[JHE] and JH epoxide hydrolase [JHEH]) and associated protein (JH esterase binding protein [JHEBP]) involved in insect JH degradative pathway in these investigated arthropod genomes (fig. 1C and supplementary figs. S2–S4, Supplementary Material online). In insects, JHE and JHEH contribute to the reduction of JH titer and induce metamorphosis (fig. 1B) (Share and Roe 1988; Anand et al. 2008), and JHEBP functions in JH transportation and degradation that can indirectly modulate JH titer (Hao et al. 2013).

The esterases are a diverse and rapidly evolving class of genes in arthropods, and their roles in regulation of hormonal action can be vastly different between species. In insects, dipterans and hymenopterans possess “Dipteran-type” JHE genes, whereas lepidopterans use another type of JHE (Mackert et al. 2008). In our sequence and phylogenetic analyses, Dipteran-type JHEs are found in crustaceans including N. denticulata (Sin et al. 2014) and Pandalopsis japonica (Lee et al. 2011), and myriapods such as the centipede S. maritima (Chipman et al. 2014) and the millipede T. corallinus (supplementary fig. S21, Supplementary Material online). Although bootstrap support is weak at the base of the Dipteran-type JHE node in this tree, these genes share the diagnostic residues that suggest JHE identity (Mackert et al. 2008) and a clear lack of affinity to any other esterase gene present in the Arthropoda (represented in supplementary fig. S21, Supplementary Material online, by Dr. melanogaster sequences). Although there is a tremendous diversity in esterase gene number and sequence in all sequenced chelicerate genomes to date, there are no genes which share strong phylogenetic signal with the JHE genes of the Dipteran-like clade, suggesting that chelicerates may use another type of esterase.

JHEBP was first described in Manduca sexta (Shanmugavelu et al. 2000) and has since been found in a number of insects, crustaceans, and a centipede (Chipman et al. 2014; Sin et al. 2014). Of the genes examined here, only Mesobuthus martensii lacks a clear JHEBP homolog, although sequence MMa33864 may represent a highly divergent member of this clade. This sequence contains the Timm-44-like domain at the C terminus (supplementary fig. S4, Supplementary Material online; Liu et al. 2007; Hao et al. 2013).

JHE plays an important role in regulating insect JH titer by conversion of JH into JH diol or JHA into JHA diol (Mackert et al. 2010). It was first cloned from M. sexta, and belongs to the microsomal epoxide hydrolase (EC 3.3.2.9) family (Wojtasek and Prestwich 1996; Zhou et al. 2014). Here, we identified JHE orthologs in both myriapods and chelicerates (fig. 1C and supplementary fig. S3, Supplementary Material online), suggesting that JHE may have evolved in the last common ancestor of arthropods.

Sesquiterpenoid Signaling Genes
A range of gene homologs involved in the JH signaling pathway can be identified in the investigated arthropod genomes. These include “Methoprene-tolerant” (Met), “cytosolic juvenile hormone binding protein” (cytosolic JHBP, FKBP39, and Chd64 (fig. 1C and supplementary figs. S5–S8, Supplementary Material online).

Met is a member of the basic Helix-Loop-Helix Per-Arm-Sim family that is assumed to function as a JH intracellular receptor, binding with JH via its C-terminal Per-Arm-Sim (PAS) domain; this activates downstream gene transcription in insects (Konopova and Jindra 2007; Charles et al. 2011; Li et al. 2011). Met orthologs have been previously identified in crustaceans such as the water flea (LeBlanc et al. 2013; Miyakawa, Toyota, et al. 2013) and the shrimp (Sin et al. 2014). A single amino acid mutation in the PAS domain (change from T to V) of daphnid Met significantly changed the JH responsiveness, and the authors suggested that this mutation contributed to the evolution of the insect JH receptor system (Miyakawa, Toyota et al. 2013). In our sequence analysis, we found that a Met ortholog is also present in myriapods and chelicerates, and the conservation of the valine position could only be detected in the insect lineage, consistent with the previous suggestion that the valine substitution in the insect ancestor coevolved with JH production (fig. 1C and supplementary figs. S6 and S23, Supplementary Material online).

Cytosolic JHBP genes have been described in the shrimp N. denticulata (Sin et al. 2014), centipede S. maritima (Chipman et al. 2014), and in a number of other arthropod species, particularly in the Lepidoptera (Touhara and Prestwich 1993; Touhara et al. 1994; Carter et al. 2013). However, to date little has been done to describe the evolutionary origin of these genes. In our analyses, we have identified cytosolic JHBP in a range of noninsect arthropods which do not produce JH (fig. 1C; Sin et al. 2014). Initial examination of its domain structure and sequences suggests strong affinity with the “glyoxalase domain containing 4” (GLOD4) genes which are known to bind to a range of ligands (supplementary fig. S5, Supplementary Material online). Because protein with binding affinity toward MF has been identified in the hemolymph of crustaceans (Li and Borst 1991; King et al. 1995; Tamone et al. 1997), we suggest that these cytosolic JHBP orthologs in the noninsect arthropods could potentially function as FA or MF binding proteins.

FKBP39 is a member of the FK506 binding protein family that serves as protein folding chaperones in insects; this protein binds to juvenile hormone response elements (JHRE) that regulate the cross-talk between ecdysteroids and JH (Li et al. 2007). We have also identified FKBP39 orthologs in almost all of the noninsect arthropods investigated here, which share the two characteristic residue signatures including a 3-nucleotide indel and a six residue “GMKVGG” domain (supplementary fig. S7, Supplementary Material online). Chd64 is another protein previously known to bind to the JHRE and modulates the responses to JH exposure (Li et al. 2007). Although Chd64 possesses a cleavage calponin homology (CH) domain (pfam id: pfam00307), its relationship to the wider family of other CH-
containing genes is largely uncataloged (Kozłowska et al. 2014). In our analysis, we have also identified putative Chd64 orthologs in the noninsect arthropods (supplementary fig. S24, Supplementary Material online). Our identification of JHRE-related genes in noninsect arthropods provides new potential targets for dissecting the regulation of sesquiterpenoid action.

The Ecdysteroid Pathway Genes of Noninsect Arthropods

Ecdysteroids play a variety of roles in regulating the growth (in particular molting) and sexual maturation of insects (Parthasarathy et al. 2010; Tennesen and Thummel 2011) and crustaceans (Chávez et al. 2000; Warren et al. 2002, 2004; Petryk et al. 2003; Niwa et al. 2004; Mykles 2011). Other than in insects, their biosynthetic pathway has only been described in two crustaceans (Rewitz and Gilbert 2008; Sin et al. 2014) and a mite (Cabrera et al. 2014). In both insects and crustaceans, the ecdysteroid 20E is synthesized from dietary cholesterol via cytochrome P450 enzymes encoded by the Halloween genes (Rewitz et al. 2007; Rewitz and Gilbert 2008; Sin et al. 2014; fig. 2A). Initially, the conserved Rieske-like oxygenase neverland converts cholesterol into 7-dehydrocholesterol which is then processed into bioactive 20E through a series of reactions catalyzed by Spook/Spookier (CYP307A1/2), Phantom (CYP306A1), Disembodied (CYP302A1), Shadow (CYP315A1), and Shade (CYP314A1), respectively (fig. 2A). Here, we have identified the majority of these gene homologs in all investigated arthropod genomes (fig. 2B and supplementary figs. S9–S16, 25, and 26, Supplementary Material online), suggesting that myriapods and chelicerates could also synthesize ecdysteroids. Nevertheless, we could not identify any CYP306A1 ortholog in any chelicerate genomes. Although it has been previously suggested that the ortholog of CYP306A1 is present in the scorpion (Cao et al. 2013), our phylogenetic analysis strongly supports the view that the gene sequence belongs to the CYP18A1 family instead (supplementary figs. S16 and S26, Supplementary Material online). We therefore suggest that the broad gene ortholog in the noninsect/crustacean arthropod genomes examined here (supplementary fig. S28, Supplementary Material online). We therefore suggest that the broad gene is a pancrustacean innovation.

Concluding Remarks: The Panarthropod Sesquiterpenoid and Ecdysteroid Pathways

In this study, we utilized newly available noninsect arthropod genomes to gain the first global picture of the componentry of the sesquiterpenoid and ecdysteroid pathways in arthropods. Major gene orthologs involved in the biosynthetic, degradative, and signaling pathways of sesquiterpenoids and ecdysteroids can be found in crustaceans (shrimp and water flea), myriapods (centipede and millipede), and chelicerates (mite, tick, scorpion, and horseshoe crab) revealing the existence of these hormonal systems in the common ancestor of arthropods (fig. 3). Lineage-specific gene gains that probably contributed to their successful evolution have also been identified, including Br-C in the Pancrustacea, JH epoxidease (CYP15A1/C1), and key residue changes in the binding domain of the JH receptor (Met) in the insect common ancestor, and the appearance of “Phantom” in the mandibulate lineage after the
**FIG. 2.**—(A) Schematic diagram showing the biosynthetic and degradative pathways of ecdysteroids in arthropods (Sin et al. 2014; for details, refer to the main text); (B) summary of the presence of ecdysteroid biosynthetic, inactivation, and signaling pathway genes in the investigated arthropods. “+” denotes presence and “-/0” represents the absence of supporting evidence. Nvd: neverland.

| Ecdysteroid related pathway genes | Insects | Water Flea | Shrimp | Millipede | Spider Mite | Tick | Dust Mite | Scorpion | Horseshoe Crabs |
|----------------------------------|---------|------------|--------|-----------|-------------|------|-----------|-----------|----------------|
|                                  | Hexapoda| Crustacea  | Myriapoda| Chelicerata|             |      |           |           |                |
| **Nvd**                          | +       | +          | +       | +          | +           | +    | +         | +         | +              |
| **Spook (CYP307A1)**             | +       | +          | +       | +          | +           | +    | +         | +         | +              |
| **Phantom (CYP306A1)**           | +       | +          | +       | +          | -           | -    | -         | -         | -              |
| **Disembodied (CYP302A1)**       | +       | +          | +       | +          | -           | +    | +         | +         | +              |
| **Shadow (CYP315A1)**            | +       | +          | +       | +          | -           | +    | -         | +         | +              |
| **Shade (CYP314A1)**             | +       | +          | -       | +          | +           | +    | +         | +         | +              |
| **CYP18A1**                      | +       | +          | -       | +          | +           | +    | +         | +         | +              |
| **Ecr**                          | +       | +          | +       | +          | +           | +    | +         | +         | +              |
| **RXR/USP**                      | +       | +          | +       | +          | +           | +    | +         | +         | +              |
| **Br-C**                         | +       | +          | +       | -          | -           | -    | -         | -         | -              |
split of chelicerates from other arthropods, resulting in the appearance of different molting hormones. Considering that previous studies showed the existence of functional ecdysteroid responsive systems in nematodes (Parihar et al. 2010; Liu et al. 2012), it is likely that the ecdysteroid system coevolved with the emergence of molting in the Ecdysozoa (fig. 3). The comprehensive framework established in this study will allow further dissection of the functional roles played by these crucial pathways in vivo in noninsect arthropods.

Supplementary Material
Supplementary figures S1–S28 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

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