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CYP450s analysis across spiny lobster metamorphosis identifies a long sought missing link in crustacean development

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ABSTRACT

Cytochrome P450s (CYP450s) are a rapidly evolving family of enzymes, making it difficult to identify *bona fide* orthologs with notable lineage-specific exceptions. In ecdysozoans, a small number of the most conserved orthologs include enzymes which metabolize ecdysteroids. Ecdysone pathway components were recently shown in a decapod crustacean but with a notable absence of shade, which is important for converting ecdysone to its active form, 20-hydroxyecdysone (20HE), suggesting that another CYP450 performs a similar function in crustaceans. A CYPome temporal expression analysis throughout metamorphosis performed in this research highlights several un-annotated CYP450s displaying differential expression and provides information into expression patterns of annotated CYP450s. Using the expression patterns in the Eastern spiny lobster *Sagmariasus verreauxi*, followed by 3D modelling and finally activity assays *in vitro*, we were able to conclude that a group of CYP450s, conserved across decapod crustaceans, function as the insect shade. To emphasize the fact that these genes share the function with shade but are phylogenetically distinct, we name this enzyme system Shed.

1. Introduction

1.1. Cytochrome P450 versatility

Cytochrome P450s (CYP450s) form an ancient family of enzymes with versatile roles [1]. In ecdysozoa, CYP450s are known to be involved in the metabolism of key compounds that regulate development, growth and reproduction [2–4]. CYP450s are also involved in detoxification; this may range from promiscuous enzymes, which can metabolize multiple substrates [5], to those specialized in metabolizing one or a few substrates [6,7]. It is perhaps for these reasons that CYP450s form one of the most versatile enzyme families, where their rapid evolution enables the organism to cope with a changing environment on the one hand, yet on the other hand, it makes it hard to clearly define orthologs. An example for the high evolutionary rate of this family is evident from the observation that insect CYP450s alone range in number from 36 to 180 [8].

1.2. Conserved arthropod CYP450 orthologs: the Halloween genes

A small number of CYP450s are known to participate in the biosynthesis of the active form of the molt hormone, 20-hydroxyecdysone (20HE; (2β,3β,5β,22R)-2,3,14,20,22,25-hexahydroxycholest-7-en-6-one), one key factor which generates the active juvenile hormone (JH). In addition, several other CYP450s have been partially annotated based on phylogeny and their role deduced in one or more species [8]. In a sense, the CYP450 complement (also referred to as CYPome) of a species is a unique signature defined by its interaction with the environment and the mechanism by which it regulates development and reproduction.

A high evolutionary change rate might, in part, be associated with the need to cope with various toxins in the changing environment but, from a mechanistic point of view, it is also associated with the fact that the CYP450s tend to form clusters in the genome. Some duplicated gene clusters can be maintained over very long timescales. The head to tail pair of the close paralogs CYP306A1 and CYP18A1 is conserved as a cluster in all insects studied so far (except in *Anopheles gambiae* that has lost the CYP18A1 gene), and is even found in the crustacean *Daphnia*

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pulex, thus dating this cluster to well over 500 MY [8]. Stable duplication events like this increase the evolutionary change rate, since chromosome rearrangement events might lead to exponential duplications [8]. While versatile and rapidly changing, all the protostome CYP genes identified to date can be assigned to one of four clans: CYP2, CYP3, CYP4 and the mitochondrial CYP clan [9]. While clan nomenclature is inferred by phylogeny [10], members of each clan can have various roles. The CYP4 clan in insects for instance, includes members associated with pheromone synthesis and breakdown [11], as well as cuticle hardening [12]. From a substrate perspective, several stages in the synthesis of a bio-active compound can involve CYP450s from different clans; such is the case of the CYP450s which synthesize 20HE.

The conserved arthropod CYP450 orthologs are those involved in 20HE biosynthesis and degradation as well as juvenile hormone biosynthesis. The primary source of ecdysteroid biosynthesis is the Y-organ in crustaceans, which is analogous to the insect prothoracic gland [13]. The synthesis of 20HE is negatively regulated in crustaceans by the molt inhibiting hormone (MIH), which acts through an as yet unidentified receptor on the YO membrane to block its function. MIH is produced predominantly in the X-organ, then transported to the sinus gland where it is stored until secretion [14]. This neuroendocrine complex, known as the X-organ-sinus gland complex (XO-SG), resides in the crustacean eyestalk. While in crustaceans 20HE is negatively regulated by MIH derived from the XO-SG, in insects 20HE synthesis is positively regulated by neurosecretory cells in the brain which produce the prothoracicotropic hormone (PTTH) [15]. In the biosynthesis pathway of 20HE, five CYP450s were discovered to be conserved in insects. They were named the Halloween genes due to the embryonic lethal effect of null mutations, resulting in disfigured flies, probably due to low titer of ecdysteroids and inability to properly form a cuticle. Spook (CYP307A1) (and the diptera lineage specific paralogs spookier (CYP307A2) [16] and spookiest (CYP307B1) [17]) are expressed in the insect prothoracic gland in a stage-specific manner, regulating the first steps in 20HE synthesis [18,19]. The *phantom* gene (CYP306A1) is also expressed predominantly in the insect prothoracic gland and the enzyme follows spook in the biosynthesis of 20HE [20]. These stages are followed by enzymatic reactions catalyzed by disembodied (CYP302A1), shadow (CYP315A1) [21] and shade (CYP314A1); the latter enzyme catalyzes the final step in the 20HE biosynthetic pathway in the target cells [6]. Degradation of 20HE is facilitated by CYP18A1 [22], which clusters with its paralog CYP306A1 in most insects studied as well as in *D. pulex* [23]. While spook, phantom and CYP18A1 are part of clan 2, the other three enzymes are part of the mitochondrial clan. A recent study has also identified orthologs of five out of the six genes (with the notable exception of shade) in the decapod cherry shrimp *Neocaridina denticulata* [24].

1.3. Metamorphosis in spiny lobsters

Metamorphosis in spiny lobsters is a dual phase process where an oceanic transparent, alien-like larva (phyllosoma) metamorphose into a nektonic miniature transparent version of the lobster (puerulus), manifesting massive restructuring of anatomy and physiology in a single step [35]. The puerulus swims towards the shore where it completes metamorphosis into the benthic juvenile form [38]. Our research to date shows that the phyllosoma-puerulus metamorphic transition in the Eastern spiny lobster *S. verreauxi* is accompanied by vast transcriptomic changes exceeding 25% of the transcriptome [35]. The lengthy transition, the large-sized larvae and their transparency enable clear molt staging by gut retraction. The availability of transcriptomic data for both the larval metamorphic transition at high resolution [35], alongside tissues of juveniles and adults from this species [44–49] enables thorough examination of expression patterns and correlation with spatial-temporal expression.

In this research we characterized the differential expression of CYP450s across the phyllosoma-puerulus transition in the Eastern spiny lobster *S. verreauxi*. We identified 43 putative CYP450s with clear phylogenetic annotation for eight of them. Expression throughout metamorphosis varied significantly for 11 out of the 43 CYP450s, with four predominant expression patterns. Inferred from expression pattern, 3D modelling, *in vitro* assay and cross-species analysis, we predict that a clan 4 CYP450, conserved in crustaceans, is the putative ecdysone to 20HE hydroxylase. By using ultra-high pressure liquid chromatography-quadrupole time of flight-mass spectrometry (UHPLC-QToF-MS), one enzyme from this group was shown to produce hydroxyecdysone (HE) *in vitro*. We thus conclude that this is the Shade ortholog in crustaceans and thus named it 'Shed'.

2. Materials and methods

2.1. Bioinformatics analysis

The transcriptome of whole individuals sampled from five developmental stages throughout metamorphosis of *S. verreauxi* (in duplicates, including six phyllosoma and 4 puerulus) [35], was converted to amino acids (aa) of the most probable open reading frame (ORF) using OrfPredictor (proteomics.ysu.edu/tools/OrfPredictor.html). Where ORF was predicted to be partial, iterative tBLASTn searches using CLC Genomics Workbench (Qiagen, version 8.0.3), against the transcriptome of both developmental stages [35] and juvenile and mature tissues [44,48], were performed in order to identify flanking regions. The predicted ORFs were searched for CYP450 domains using PFAM database in CLC Genomics Workbench. Mapping and quantification were previously performed using CLC Genomics Workbench and Partek Genomics Suit [35], and a fold-change ≥ 2 between the five sub-stages with P value ≤ 0.05 [with FDR; as previously calculated [35]] was considered as differential expression.

Multiple sequence alignments followed by phylogenetic trees constructed using Neighbor Joining (NJ) method (bootstrap = 1000) were performed using the CLC Genomics Workbench. The aligned sequences were tested using Maximum Likelihood and Maximum Parsimony (100 bootstraps for each) in MEGA 6.0 (Supplementary file S2).

Three dimensional modelling was performed using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER) followed by rendering in CLC Genomics Workbench. Only models with C-score > 0.75 were considered.

2.2. Cell culture and transient transfection of cells

Transient transfection and cell culture protocols were undertaken according to Aizen et al. [46]. Briefly, COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 100 U/mL nystatin (Life Technologies). Cells were grown at 37 °C, with 5% CO_2 until 80% confluent, followed by transfection with either an empty pCDNA3.1+ vector (Promega), or a pCDNA3.1+ vector expressing Sv-Unigene1882 (Genscript), using TransIT®-LT1 Transfection Reagent (Mirus), according to the manufacturer's instructions. The cells were cultured for 8 h, then split into various groups in triplicates with or without the addition of 20 μg/mL ecdysone (Sigma).

2.3. Collection and isolation of samples for UHPLC-QtoF-MS analysis

Following 2 h of incubation with or without 20 μg/mL ecdysone, COS-7 cells and culture medium were administered with an equivalent volume of methanol, vortexed thoroughly and then centrifuged at 16,000 × *g* for 10 min at 4 °C. The supernatant was collected (cells removed) and subjected to freeze-drying, and the lyophilized samples stored at −80 °C until subsequent analysis. Three biological replicates from two sample groups were used for LC–MS analysis.