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Authors for correspondence:
Jin-Hua Xiao
e-mail: xiaojh@ioz.ac.cn
Da-Wei Huang
e-mail: huangdw@ioz.ac.cn

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Molecular evolution

Adaptive evolution of vertebrate-type cryptochrome in the ancestors of Hymenoptera

Bo Wang[1,2], Jin-Hua Xiao[1], Sheng-Nan Bian[3], Hai-Feng Gu[1,2] and Da-Wei Huang[1,3]

[1]Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China
[2]Graduate School of the Chinese Academy of Sciences, Beijing 100039, People's Republic of China
[3]Plant Protection College, Shandong Agricultural University, Tai'an 271018, Republic of China

One of the most mysterious aspects of insect clock mechanisms is that some insects, including Hymenoptera and *Tribolium*, only express a vertebrate-type cryptochrome (*cry2*). It is unknown whether or not *cry2* underwent adaptive evolution in these insects. In the present study, we cloned and sequenced the full-length *cry2* from a fig pollinator species, *Ceratosolen solmsi* (Hymenoptera: Chalcidoidea: Agaonidae), and examined the molecular evolution and daily expression of this gene. Our results suggest that *cry2* underwent positive selection in the branch leading to hymenopteran insects. The function of CRY2 might have been fixed since undergoing natural selection in the ancestor of Hymenoptera. Male pollinators showed stronger rhythmicity in the host figs, which reflect an adaptation to their life cycles.

1. Introduction

Cryptochrome (CRY) is a photolyase-like flavoprotein that shows no DNA-repair activity [1]. Animal CRY proteins are phylogenetically divided into two clusters: one contains *Drosophila*-type CRY (CRY1) and the other includes all the vertebrate CRY (CRY2) [2,3]. *Drosophila* species only possess *cry1*, mosquitoes and butterflies have both genes, and hymenopteran insects and *Tribolium* have lost *cry1* [2–4]. CRY1 is a blue-light sensor that plays a major role in photic entrainment in *Drosophila* [5–7]. In contrast, insect CRY2 is an important transcriptional repressor in the circadian clock, which shows no light sensitivity in culture [3,8]. Recently, both CRYs were reported to have light-dependent magnetosensitivity [9–11]. However, we have no knowledge of whether or not the CRYs have different evolutionary patterns in the diverse insect taxa. As the protein encoded by *cry1* serves as an important light sensor in the circadian systems of insects, we hypothesize that natural selection might have driven the evolution of *cry2* to compensate for loss of *cry1* in hymenopteran insects. To test this hypothesis, we amplified and sequenced the full-length *cry2* of a fig pollinator species, *Ceratosolen solmsi*, whose life cycle is strictly synchronized to its host fig tree *Ficus hispida*, and characterized the daily expression of this gene.

2. Material and methods

Fig fruits of *F. hispida*, were sampled from Danzhou (19°30′29′ N, 109°29′6′ E), Hainan province, China in October 2011. Both female and male pollinators were collected.

Full-length *cry2* of *C. solmsi* was amplified by RT-PCR and RACE PCR from cDNA samples and sequenced. Additional insect *cry2* gene sequences were acquired from NCBI (www. ncbi.nlm.nih.gov), as listed in electronic supplementary material, table S1.

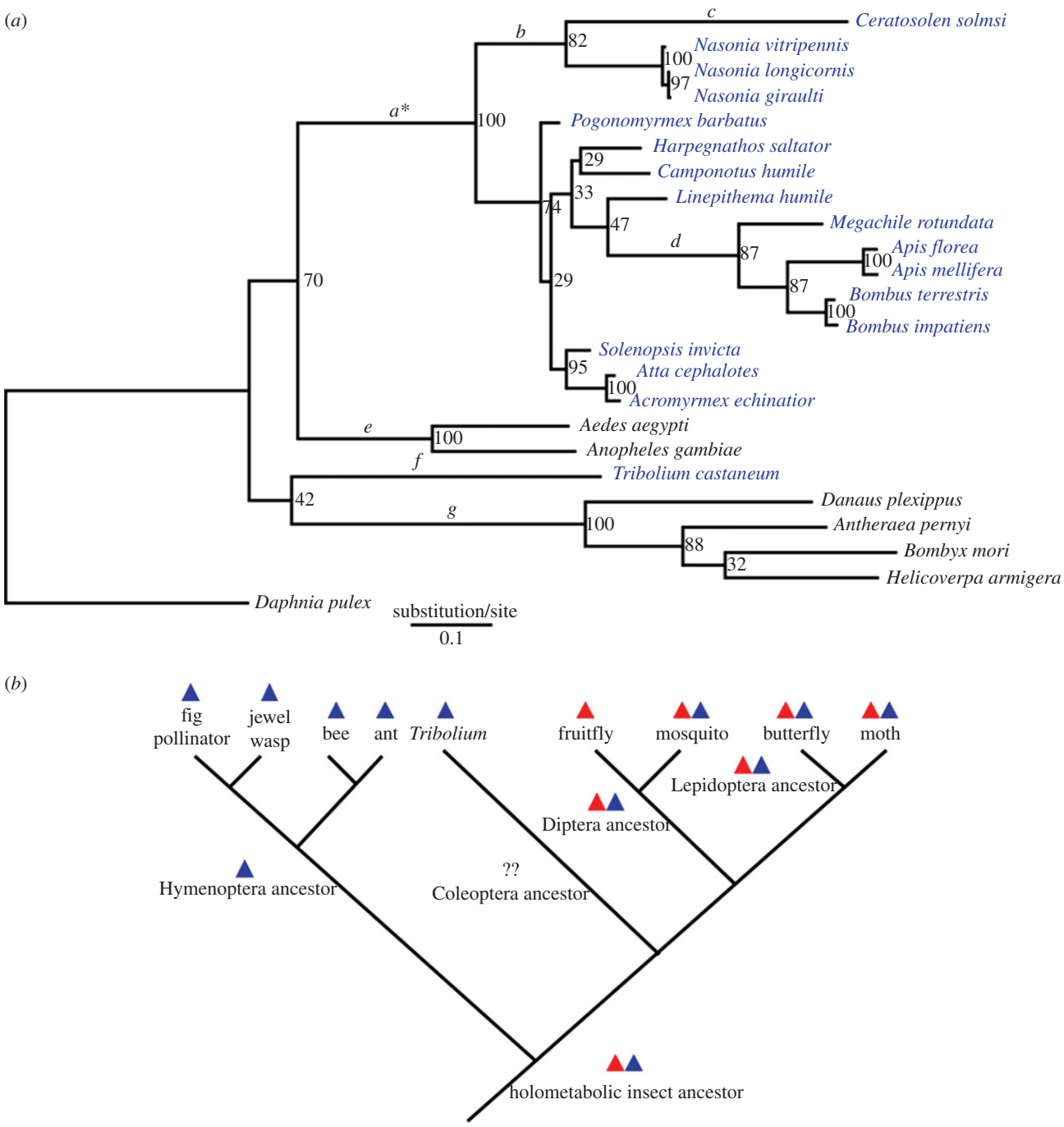

Figure 1. (*a*) Phylogenetic reconstruction of *cry2* in holometabolic insects. The ML tree is constructed from full-length coding sequences of *cry2*. Bootstrap values are shown at the nodes. The branches tested for positive selection are labelled as *a*–*g*. Asterisk (*) indicates that positively selected sites are detected. The species that lack *cry1* are highlighted in blue. (*b*) Phylogeny of the holometabolic insects, character mapping of possession of cryptochrome genes and proposed ancestral state. Triangles indicate *cry1* (red) and *cry2* (blue).

To study sequence evolution of *cry2* in holometabolic insects, we reconstructed a gene tree for *cry2*, and based on which we performed tests for positive selection using maximum-likelihood estimation by CODEML in PAML v. 4.5 [12]. Two types of selection models, Site models and Branch-site models [13–16] were used to test for episodic evolution of *cry2* along branches *a*–*g* (figure 1*a*). Since seven separate likelihood ratio tests (LRTs) were performed in this analysis, Bonferroni adjustment was used for multiple testing correction. The results are shown in electronic supplementary material, tables S2 and S3. Detailed methods were given in the electronic supplementary material.

RT-qPCR was used to analyse daily transcript levels of *cry2* in female and male wasps under different light conditions (females in natural light-treated figs, females in dark-treated figs, females exposed to natural light, males in natural light-treated figs, and males in dark-treated figs).

3. Results

(a) Episodic evolution of *cry2*

We obtained the 1704 bp full-length *cry2* sequence (Genbank accession no. JX409893) from *C. solmsi*. Combined with published data from other holometabolic insect species, we reconstructed the gene tree of *cry2* (figure 1*a*). The sequences of cryptochrome genes have been mapped onto the species phylogeny of holometabolic insects based on previous

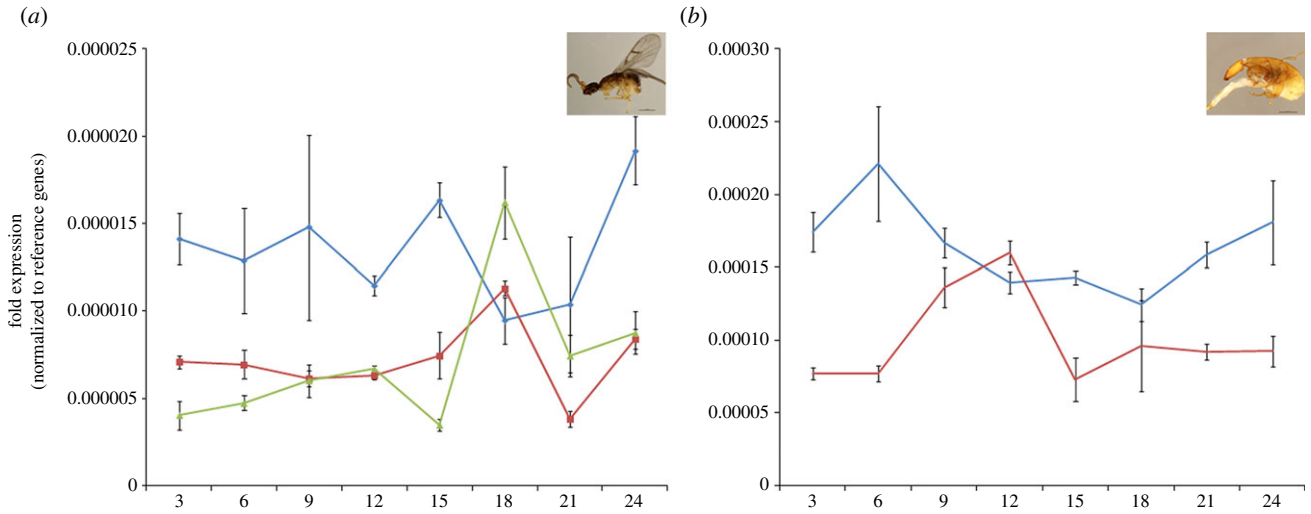

Figure 2. (*a*) Daily expression of *cry2* in female and (*b*) male *C. solmsi*. Fold expression of *cry2* (mRNA abundance of opsin genes relative to reference genes) at each time point are represented by bars. LF (blue), fig-female light; DF (red), fig-female dark; F (green), emerged females light; LM (blue), fig-male light; DM (red), fig-male dark. Asterisk (*) indicates significant rhythmic expression. Morphological dimorphism between female and male of *C. solmsi* is present.

Table 1. Statistical values of *cry2* expression in *C. solmsi*. d.f., degree of freedom; *F*, value of *F*-test; *p*-value, probability.

source	d.f.	*F*	*p*-value	% of total variation
female:				
light	2	190.9	<0.0001	44.73
time	7	9.772	<0.0001	15.31
light × time	14	9.524	<0.0001	29.85
male:				
light	1	412.4	<0.0001	49.58
time	7	5.837	$=0.0003$	13.30
light × time	7	12.08	<0.0001	27.52

studies [17]. We also inferred the distribution of both genes in the ancestor of the four main holometabolic insect orders: Hymenoptera, Coleoptera, Lepidoptera and Diptera (figure 1*b*). Results of the evolutionary analysis of site models indicated that both *cry1* and *cry2* experienced strong purifying selection (*cry2*: $\omega = 0.01536$ for all taxa; *cry1*: $\omega = 0.02130$ for mosquitoes and Lepidoptera), although there is evidence that *cry2* (*cry2*: $\omega = 0.01624$ for mosquitoes and Lepidoptera) is even more constrained than *cry1* ($p = 0.015$; electronic supplementary material, table S2). Branch-site models identified a group of amino acid sites that underwent positive selection along the branches leading to Hymenoptera (17 sites for branch a) (see the electronic supplementary material, table S2, S3). However, we found no evidence of positive selection along other branches we tested.

(b) Daily expression of *cry2* in *Ceratosolen solmsi*

Daily levels of *cry2* mRNA in both female and male *C. solmsi* varied as a function of the time × light interaction (table 1). Cosinor analyses [18] indicated that the *cry2* was rhythmically expressed in emerged females exposed to natural light (emerged female light; one way ANOVA: $p < 0.001$; Cosinor: $p = 0.016$) and males in both natural light-treated (fig-male light; one way ANOVA: $p = 0.001$; Cosinor: $p < 0.001$) and dark-treated (fig-male dark; one way ANOVA: $p < 0.001$; Cosinor: $p = 0.004$) figs (figure 2).

4. Discussion

The crustacean species *Daphnia pulex* has both *cry1* and *cry2*, suggesting that the gene duplication of cryptochrome occurred before the emergence of insects. Therefore, possession of only one cryptochrome in some holometabolic insects should be attributed to lineage-specific gene loss events. Since all the hymenopteran species (ants, bees and wasps) that we sampled only possess *cry2*, it is likely that loss of *cry1* occurred in the ancestor of Hymenoptera. *Tribolium castaneum* also lacked *cry1*, but we could not determine when the gene loss event occurred in the coleopteran lineage. Based on our selective pressure test of cryptochrome in holometabolic insects, *cry2* seemed to have undergone stronger purifying selection than *cry1* in the same set of species (mosquitos and lepidopteran insects). This was predictable because the function of CRY2 as a transcriptional repressive component was relatively conservative. However, branch-site tests suggested that during the evolutionary history of holometabolic insects, at least some residues of CRY2 experienced positive selection in the ancestor of hymenopteran species, after gene loss of *cry1*. This evolutionary pattern supports our hypothesis that *cry2* was subjected to more positive selection in the absence of the function conferred by CRY1. We did not find evidence of positive selection for branches leading to extant hymenopteran species such as bees, wasps and the fig pollinator, *C. Solmsi*. It seems that the

function of CRY2 has been conserved since undergoing natural selection in the ancestor of Hymenoptera. Rhythmic expression of *cry2* has been recorded in several holometabolic species [2,19,20]. In some cases, expression of this gene showed stronger plasticity than *cry1* not only across species [19] but also in different tissues within the same organism [20]. This suggests that the expression pattern of *cry2* has the potential to reveal species-specific adaptation to the light environment or lifestyle, especially in the insects that lack photosensitive CRY1.

Figs (*Ficus*: Moraceae) and their pollinators (Family Agaonidae) form one of the best-known examples of obligate mutualism [21]. The wasps develop in the galls formed within the syconia of figs. Upon maturation, males emerge from the galls before females, and they chew holes in the galls containing females so that the latter can disperse to pollinate in other receptive fig fruits [22]. Apparently, males initiate the life cycles in the fig pollinators. Because males seldom leave the fig fruits and the females are responsible for colonizing new hosts [22,23], it is reasonable to anticipate that males will show stronger rhythmicity within the fig fruits than females. Although it remains unclear how CRY2 functions, hymenopteran's *cry2* mRNA levels should reflect some rhythmicity as a core clock-element [2,3]. This prediction allows for testing our hypothesis on the adaptation of rhythms of fig pollinators to their host. Consistent with our hypothesis, the results from RT-qPCR suggested that males maintain rhythmic expression of their *cry2*, yet females maintain the rhythmicity only when they are outside the fig fruits. Such sex-specific expression patterns of *cry2* could be the result from the adaptation of pollinators to their host figs during their long-term coevolution [24,25].

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