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

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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, Centosolen 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, Centosolen solmsi, whose life cycle is strictly synchronized to its host fig tree Ficus hispida, and characterized the daily expression of this gene.

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 1a). 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 1a). The sequences of cryptochrome genes have been mapped onto the species phylogeny of holometabolic insects based on previous
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 1b). 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 (mosquitoes 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

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

<table>
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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.
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 rhythmicity within the fig 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 by fig species (Agaonidae) 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 RNA 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 rhythm 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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References