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ORIGINAL ARTICLE

The clock gene *cryptochrome* of *Bactrocera cucurbitae* (Diptera: Tephritidae) in strains with different mating times

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Differences in mating time between populations can give rise to premating reproductive isolation. Tephritid fruit flies exhibit large variation in mating time among intra- or inter-specific populations. We previously cloned the clock gene *period* from two strains of melon fly, *Bactrocera cucurbitae*; in one the individuals mate early during the day, whereas in the other the individuals mate later. These strains were originally established by divergent artificial selection for developmental time, 'short' and 'long', with early and late mating times, respectively. The deduced amino acid sequences of PERIOD proteins for these two strains were reported to be identical. Here we cloned another clock gene *cryptochrome* (*cry*) from the two strains, and found two stable amino acid substitutions in the strains. In addition, the allele frequency at the two polymorphic sites of *cry* gene correlated with the circadian locomotor period (τ) across strains, whereas the expression pattern of *cry* mRNA in the heads of flies taken from the short strain significantly differed from that from the long strain. These findings suggest that variation in the *cry* gene is related to differences in the circadian behaviour in the two strains, thus implying that the *cry* gene may have an important role in reproductive isolation.
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Introduction

Reproductive isolation among sympatric populations can be maintained by breeding at different time schedules (Miyatake, 2002b; Coyne and Orr, 2004). In the wild, temporal reproductive isolation results from differences in mating season in birds (Friesen *et al*., 2007), mating period in insects (Bush, 1969; Hendry *et al*., 2000), timing of gamete release in corals (Levitan *et al*., 2004), timing of migration and breeding in fishes (Quinn *et al*., 2000), and time of mating in flies (An *et al*., 2004).

Variation in the time of day at which mating occurs, has been widely observed in tephritid species, between both inter- and intra-species (see Miyatake, 1997a; Matsumoto *et al*., 2008). For example, there is a specific time of mating in two sibling species of Australian native tephritid fruit flies, *Bactrocera tryoni* and *Bactrocera neohumeralis* (Lewontin and Birch, 1966; An *et al*., 2002, 2004). Mating time may also be a reproductive barrier among three sympatric *Anastrepha* tephritid fly species in South America (Malavasi *et al*., 1983; Henning and Matioli, 2006).

For the melon fly *Bactrocera cucurbitae* (Coquillett), previous reports showed that there is a lack of synchrony in the timing of male courtship and copulation across natural populations (Suzuki and Koyama, 1980; Matsuyama and Kuba, 2009). Employing two-way artificial selection for developmental period in the flies, Miyatake (1995) established two strains of *B. cucurbitae* with short (S strain) and long (L strain) development times and found that the time of mating differed between the two strains; the S flies always mated at an earlier time than the L flies (Miyatake, 1997a). In addition, large variation in the free-running period of the circadian locomotor rhythm of the adult flies was observed between the two strains (Shimizu *et al*., 1997), thus suggesting that variation in the free-running period might control the difference in time of mating (Miyatake, 2002b). The results of crossing experiments between S and L flies suggest the presence of a major gene that controls the time of mating (Miyatake, 1997a).

In *Drosophila*, null mutants of the clock gene *period* (*per*) (*per*^{01}) lost the circadian rhythm in mating activity (Sakai and Ishida, 2001). In addition, a different study, which used transformants of *per* derived from *Drosophila melanogaster* or *Drosophila pseudoobscura*, clarified that *per* transgenes carry species-specific information concerning

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the phases of both circadian locomotor and mating activity (Tauber *et al*., 2003). Thus, *per* can potentially lead to temporal mating isolation via changes in mating rhythms.

Our previous study of *per* in *B. cucurbitae* revealed that the two (S and L) strains differ in circadian periods of *per* mRNA cycling under constant dark (DD) conditions (S: 22 h, L: 30 h) (Miyatake *et al*., 2002). The full-length *per* gene of the two strains in *B. cucurbitae* were subsequently cloned and sequenced, however, the putative protein sequences were identical (Matsumoto *et al*., 2008, EMBL/GenBank/DDBJ accession no. AB517621 and AB517622, S and L strains, respectively). An *et al*. (2002) investigated the *per* gene in *B. tryoni* and *B. neohumeralis*, which also exhibit inter-specific differences in mating time, but similarly, no difference was found in the *per* gene sequence. Therefore, we observed differences in mating times in tephritid flies, which may be under the control of other clock genes (An *et al*., 2002, 2004; Matsumoto *et al*., 2008).

In insects, the circadian clock is described as a negative feedback loop involving rhythmic *per* and other clock genes transcription (Giebultowicz, 1999). *Cryptochrome* (*cry*) is a key circadian clock gene in *Drosophila*, encoding a photoreceptor (Stanewsky *et al*., 1998). The *cry* gene is expressed in the *Drosophila* adult brain, and is thought to function as a circadian pacemaker for behavioural rhythms (Emery *et al*., 2000). *Cry^b* mutant flies show normal behavioural rhythms but exhibit poor synchronization to light–dark cycles (Stanewsky *et al*., 1998). These facts suggest that the *cry* gene has an important role in the entrainment of *Drosophila's* clock to light–dark cycles. In *B. tryoni* and *B. neohumeralis,* it has been suggested that the *cry* gene is related to mating isolation, as the expression patterns of *cry* transcripts in the brain and antennae differ in the two sibling species (An *et al*., 2004).

In this study, we aim to clarify the differences in characteristics of the *cry* gene between the two strains of *B. cucurbitae*. We compare the *cry* sequence in the two strains and analyse the correlation of the allele-type of the gene with circadian behaviour. In addition, we compare the *cry* mRNA expression pattern between the strains.

Materials and methods

Cloning and sequencing

We separated 15 individuals from each strain during the photophase and purified the total RNA from their heads using a Fast RNA kit (Q-BIO gene, Carlsbad, CA, USA) according to the manufacturer's instructions. After DNase treatment, 5 μg of total RNA was used for reverse transcription to synthesize cDNA with Superscript II (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) was carried out to amplify the *cry* gene, using cDNA as a template and a degenerate primer set based on the *cry* gene sequence of *B. tryoni*. The amplified fragments were cloned into a *pCR*II plasmid (Invitrogen) and sequenced using a BigDye terminator v3.1 Cycle sequencing kit (Applied Biosystems, Tokyo, Japan) with an ABI PRISM 3100 sequencer (Applied Biosystems). Untranslated regions were determined by the 5′ and 3′ RACE using a FirstChoice RLM-RACE kit (Ambion, Tokyo, Japan). Sequence analyses were carried out with at least three clones for each PCR fragment.

Allele characterization at sites 1212 and 1865 of the *cry* gene by PCR

To examine whether the two common amino acid polymorphisms found in the S and L strains relate to overt behavioural rhythmicity, we investigated individual genotypes at the two polymorphic sites using PCR and their free-running period (τ) in locomotor activity across strains. *B. cucurbitae* strains were established by selecting for S and L development time from a mass-reared stock of *B. cucurbitae* (Mass) maintained at the Okinawa Prefectural Plant Protection Center, Okinawa, Japan (for a detailed description of the protocol see Miyatake, 1995; Miyatake and Shimizu, 1999). Two replicate 'young' and 'old' strains (Y1, Y2, O1 and O2) were also established from the base population by selecting for young and old age at reproduction (for a detailed description of the protocol see Miyatake, 1997b, 2002a).

Two single-nucleotide polymorphisms at sites 1212 (cry1212) and 1865 (cry1865) of the *cry* gene (EMBL/GenBank/DDBJ accession no. AB517607 and AB517608, respectively), were characterized from a single fly by employing the PCR-amplification refractory mutations system method. In the first polymorphic site (cry1212), the following allele specific forward primers were used: *l-cry* primer 5′-GCTTGAAGAGATCATGTAC**ACT**-3′ or *s-cry* primer 5′-GCTTGAAGAGATCATGTAC**ACG**-3′ (positions 1233–1212, boldface represents 3′ tails allowing the selective amplification of *l-cry* or *s-cry* alleles, respectively). The common reverse primer was 5′-GT GCGTTCTCTATGTAACGACTT-3′ (positions 726–748). In the second polymorphic site (cry1865), the following allele-specific forward primers were used: *l-cry* primer 5′-GCGTGTACATTACGTTTTGATAC**A**-3′ or *s-cry* primer 5′-GCGTGTACATTACGTTTTGATAC**T**-3′ (positions 1887–1865, boldface represents 3′ tails allowing the selective amplification of *l-cry* or *s-cry* alleles respectively). The common reverse primer was 5′-GCGTGAGTACTTT TATACAATGT-3′ (positions 1307–1329). The PCR reaction was carried out for 28 cycles with 94 °C for 30 s, 61 °C for 30 s and 72 °C for 1 min using Taq DNA polymerase (Takara, Japan). To examine the correlation between the allele frequency and circadian locomotor period across strains, Spearman's rank correlation was carried out (STATVIEW 5.0).

Monitoring of circadian locomotor activity

Locomotor (walking) activity of individual flies was recorded as described previously (Shimizu *et al*., 1997). In brief, adults were kept singly in containers and provided with water and sugar at 25 ± 2 °C. Their locomotor activities were monitored via the interruptions of an infrared beam and a photoelectric switch (OMRON, Tokyo, Japan). The free-running period was computed from the least-square spectrum of free-running data that was collected for 2 weeks.

Whole head measurements of *cry* mRNA abundance

Before the analysis of the *cry* mRNA expression pattern, S and L flies were kept for at least 1 week in an environmental chamber, maintained at 25 °C, 60–80% RH

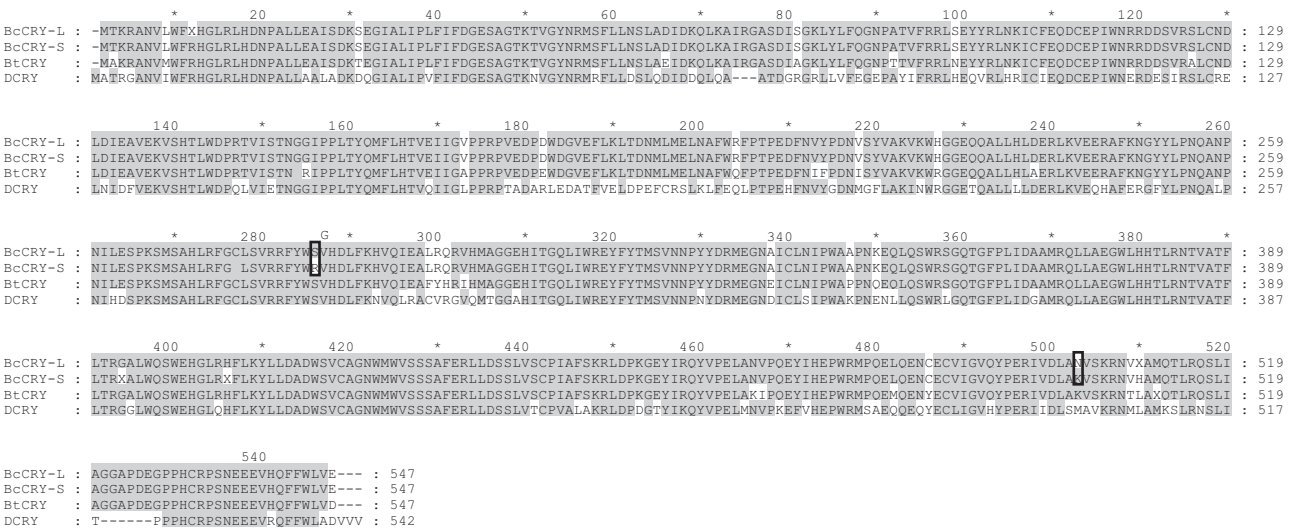

Figure 1 Alignment of the putative *Bactrocera cucurbitae* cryptochrome (CRY) amino acid sequences with CRY from other species. BcCRY-L, *B. cucurbitae* long (L)-strain CRY; BcCRY-S, *B. cucurbitae* short (S)-strain CRY; BtCRY, *B. tryoni* CRY; DCRY, *Drosophila melanogaster* CRY. The highlighted amino acids are conserved between *B. cucurbitae* and other species. Polymorphic sites are indicated in bold. The polymorphic amino acids of the S/L strains are indicated by bold squares.

and light–dark cycle 12:12 h conditions. Subsequently, over a period of 24 h five to twelve individuals were removed every 4 h. After the flies had been captured, they were immediately transferred into a freezer and stored at −80 °C until required. Two series of total RNA were extracted from each fly head using a Sepasol RNA I Super (Nacalai tesque, Kyoto, Japan). After extracting the RNA mixture, we carried out DNase treatment. The abundance of *cry* mRNA was measured by quantitative real-time PCR with a MyiQ (Bio-Rad, Tokyo, Japan). We used the primer set for the *cry* gene of *B. cucurbitae*, 5′-AAACGAGCGAACGTCTTGTG-3′ and 5′-ATATCAC TCGCTCCGCGTATGG-3′. As a control, we also used the primer set for the *Gpdh* gene, 5′-GTCGGGACATTAAGT ATGCCAAAA-3′ and 5′-CCTAGACCATCGACGAAAC CAG-3′. *Gpdh* is a general housekeeping gene of *B. cucurbitae*, expression of which should not change through time. The relative abundance of *cry* mRNA to *Gpdh* mRNA in each sample was thus obtained. The difference in mean *cry* mRNA expression across the S and L strains was tested using a *t*-test at each sampling point.

Results

Differences in the CRY sequence in S and L strains

To examine differences in the CRY sequence between the S and L strains, we first amplified and cloned a partial fragment of the *cry* gene in *Bactrocera cucurbitae* using RT-PCR. We used a degenerate primer set based on the *cry* sequence of *B. tryoni*. Two cDNA sequences of 2190 bp (S fly) and 2154 bp (L fly), respectively, were obtained (EMBL/GenBank/DDBJ accession no. AB517607 and AB517608, respectively). The coding region spanned nucleotide positions 360–2000 (S and L flies), which encodes 547 amino acids. CRY belongs to the Cryptochrome/Photolyase protein family, so we compared the putative amino acid sequence of *Bactrocera* CRY with CRY from other fly species (*B. tryoni* and *D. melanogaster*). The amino acid sequences showed high similarity to the *cry* genes in *B. tryoni* (94% identity) and *D. melanogaster* (73% identity). However, four stable nucleotide substitutions were detected between the S and L strains of *Bactrocera*'s *cry* gene, two of them non-synonymous single-nucleotide polymorphisms. The former base-pair substitution (C1212A) changes an arginine (285) to a serine, the latter (A1865T) changes a lysine (502) to an asparagine in the S and L strains, respectively. The other two nucleotide substitutions were positioned within the 3′ untranslated region (Figure 1).

The frequency of the 1212 and 1865 base substitutions in the *cry* gene correlates with the period of behavioural rhythm

To examine whether the two common amino acid polymorphisms between the S and L strains relate to overt behavioural rhythmicity, we investigated individual genotypes at the two polymorphic sites by PCR and their free-running period (τ) in locomotor activity. The results showed that, in S and L strains, all individuals (100%) were S-type homozygous for both cry1212 and cry1865, whereas in the L strain, all individuals (100%) were L-type homozygous for both sites (Table 1). In the other strains, some individuals (20–45%) were S- and L-type heterozygous for each site, whereas others were S- or L-type homozygous for each site (Figure 2, Table 1). At both cry1212 and cry1865 sites, the S-type allele frequency in each strain showed a significant negative correlation with the mean free-running period of circadian locomotor rhythms (Spearman's rank test, cry1212: $Rs = -0.857$, $P < 0.05$; cry1865: $Rs = -0.857$, $P < 0.05$; Figure 3).

The expression pattern of *cry* mRNA differs in the two strains

To determine whether the expression patterns of *cry* mRNA are different between the two strains, we measured mRNA expression cycles in S- and L-type

Table 1 Allele frequency of *cryptochrome* (*cry*) 1212 and *cry*1865 and circadian locomotor period in several strains (Y1, Y2, O1, O2, Mass, short (S) and long (L)) of *Bactrocera cucurbitae*

Selected strains	*Genotype (No. (%)) at cry1212*			*Genotype (No. (%)) at cry1865*			*Free-running period (h)*	*Total* (n)
	SS	*SL*	*LL*	*SS*	*SL*	*LL*		
Y1	50 (100)	0 (0)	0 (0)	50 (100)	0 (0)	0 (0)	22.1 ± 0.6	50
Y2	50 (100)	0 (0)	0 (0)	50 (100)	0 (0)	0 (0)	23.9 ± 2.1	50
O1	22 (44.0)	20 (40.0)	8 (16.0)	22 (44.0)	19 (38.0)	9 (18.0)	26.4 ± 3.0	50
O2	39 (78.0)	11 (22.0)	0 (0)	39 (78.0)	11 (22.0)	0 (0)	26.0 ± 1.9	50
Mass	52 (52.0)	42 (42.0)	6 (6.0)	60 (60.0)	34 (34.0)	6 (6.0)	24.7 ± 2.7	100
S	50 (100)	0 (0)	0 (0)	50 (100)	0 (0)	0 (0)	22.6 ± 0.6	50
L	0 (0)	0 (0)	50 (100)	0 (0)	0 (0)	50 (100)	30.9 ± 0.9	50

SS or LL means the number of homozygous of S- or L-type allele examined, respectively, at the site. SL means the heterozygous of S- or L-type allele at the site.

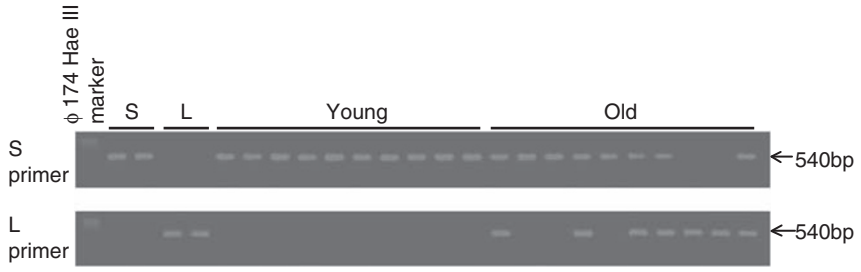

Figure 2 The short (S)- and long (L)-type allele at 1865th position of *cry* gene is detectable by S- and L-type specific primers, respectively. The expected PCR products are 540 bp (S type) in the upper picture and 540 bp (L type) in the lower picture.

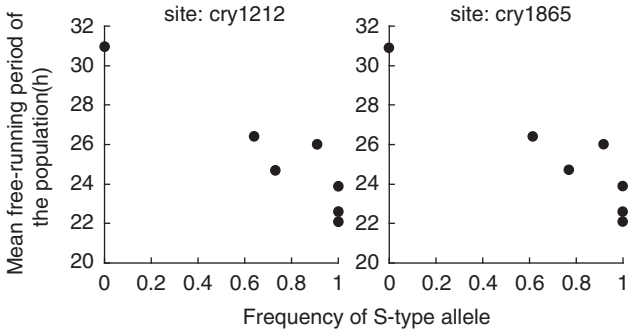

Figure 3 The relationship between circadian locomotor period and allele frequency of cry1212 (left) and cry1865 (right) in several strains of *Bactrocera cucurbitae*. The horizontal axis indicates the short (S)-type allele frequency of each strain. The vertical axis indicates the mean free-running period of each strain.

B. cucurbitae. The *cry* mRNA of S flies oscillated with approximately twofold amplitude between peak and through (analysis of variance $P<0.01$). In contrast, that of the L flies varied with little amplitude, and this was not statistically significant (analysis of variance $P=0.16$, Figure 4). In the S flies, the peak occurred at the end of the photophase (Bonferroni test, $P<0.01$). In the L flies, there was no peak at the end of the photophase, but a weak local peak occurred at the beginning of the scotophase. The amount of *cry* mRNA expression in the L flies was significantly higher than in the S flies at ZT2, 6 and 10, but not at ZT14, 20 and 22 (*t*-test, $P<0.05$).

Discussion

We identified two common amino acid substitutions in the clock gene *cryptochrome* in the tephritid fly, *B. cucurbitae*, in flies from the S strain, which mate early during the day, and flies from the L strain, which mate later (Miyatake *et al*., 2002). Both substitutions, at nucleotide positions 1212 and 1865, resulted in the replacement of amino acids: arginine with serine and lysine with asparagine at amino acid positions 285 and 502 in the S and L strains, respectively.

One of the two amino acid substitutions, is known to be located within the carboxy-terminal side of the DM1 region (Hemsley *et al*., 2007). Neither of the two amino acid substitutions were at cofactor binding sites, 5, 10-methenyltetra-hydrofolate, FAD and cyclobutane pyrimidine dimer (Figure 1). They were also different from the mutation site reported for the cry^b mutant (Stanewsky *et al*., 1998).

We measured the two allele frequencies at sites 1212 and 1865 of the *cry* gene in the seven different strains of *B. cucurbitae*. All flies of the S and L strains were S-type homozygous for cry1212 and cry1865, whereas those from the L strains were L-type homozygous for cry1212 and cry1865. Flies from the remaining four strains have intermediate free-running periods between those of S and L strains (Shimizu *et al*., 1997; Miyatake, 2002a); these were heterozygous for cry1212 and cry1865, and homozygous for cry1212 and cry1865. In an inter-strain comparison, the mean circadian free-running periods across strains was negatively correlated with the S-type allele frequency (Figure 3). This suggests that these substitutions may alter the circadian free-running period in *B. cucurbitae*.

The insect CRY can be generally classified into two groups: one group (CRY1) functions primarily as a blue-light photoreceptor in clock cells (Emery *et al*., 2000), whereas the other (CRY2) constitutes potent transcriptional repressors, which could act within the clockwork

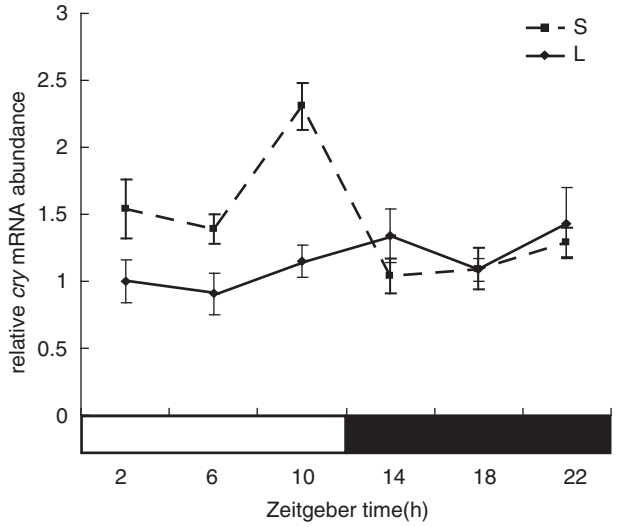

Figure 4 Difference in *cry* mRNA expression pattern in the whole head of *Bactrocera cucurbitae* in long (L) and short (S) strains. Vertical bars represent the s.e.m. ($n = 5$–12). The horizontal bar at the bottom of the graph represents the illumination regime (white bar, photophase; black bar, scotophase).

itself (Zhu *et al*., 2005; Yuan *et al*., 2007). *Drosophila*'s CRY (CRY1) is also reported to function as a transcriptional repressor for the clockwork itself (Ivanchenko *et al*., 2001; Collins *et al*., 2006). The *B. cucurbitae* CRY shows greater similarity to *Drosophila* CRY1 (73% identity) than *Tribolium* CRY2 (40% identity) and *Apis* CRY2 (39% identity). The CRY of *Bactrocera* appears to be a member of the *Drosophila* CRY group.

Transformed *Drosophila* flies, in which the CRY (CRY1) function was genetically manipulated by overexpression of the carboxy terminus-removed CRY, showed a longer free-running period of locomotor activity than wild-type flies (Dissel *et al*., 2004). This carboxy terminus region consists of two parts: one is involved in direct interactions with other molecules, the other represses these interactions in a light-dependent manner (Hemsley *et al*., 2007). One of the two amino acid substitutions (CRY502) in *B. cucurbitae*, was located in the carboxy-terminal side, positioned within the former part of the carboxy terminus region. This amino acid substitution (CRY502) might affect interactions with other molecules. Further, the change such interactions with other molecules could alter the circadian period between S- and L-strain.

We demonstrated a daily rhythm of *cry* mRNA expression in the adult head of S flies in *B. cucurbitae*: *cry* mRNA level was downregulated during scotophase and upregulated at the end of photophase. However, the mRNA level in flies from the L strain was less than in the S strain (Figure 4). In two closely related tephritid species, *B. neohumeralis* that mates at midday and *B. tryoni* that mates at dusk, expression levels of the *cry* gene are higher in the former species than in the latter, in both the brain and the antennae (An *et al*., 2004). As in *B. cucurbitae* S strain flies mate earlier than L strain flies (Miyatake *et al*., 2002), our results are consistent with the relationship between *B. neohumeralis* and *B. tryoni*.

The lower levels of *cry* mRNA in the L strain may be because the light–dark cycle of 24 h does not permit the natural long *cry* cycle to take place. It has previously been reported that L flies mate after the onset of the dark phase of the light–dark cycle (Miyatake, 1997a). One possible explanation for this is that a reduction in photosensitivity resulting from low levels of *cry* mRNA L flies may cause insensitivity to the light–dark transition.

It is possible that the *cry* gene might have an important role in altering the time of mating by lengthening of the circadian period and reduction of light–dark transition sensitivity in the L strain of *B. cucurbitae*. The *Cry* gene, as well as the clock gene *period* (Tauber *et al*., 2003), could thus be an indispensable gene for maintaining reproductive isolation.

Challenges for the future include determining whether the two amino acid substitutions in *B. cucurbitae*'s CRY are the principal cause of variation in circadian locomotor period and mating time, by employing a reverse genetic approach similar to that previously utilized in *Drosophila*. We think that the two substitutions of the *B. cucurbitae*'s *cry* gene reported here, could be responsible for the observed differences in circadian locomotor period and mating time in the two strains. However it is possible that other minor genes affect them (see Miyatake, 1997a). Further analysis involving other genes will further clarify what is potentially a novel mechanism of reproductive isolation.

Here we have demonstrated variation in mRNA sequence and expression for the circadian photoreceptor gene CRY in two strains of a Tephritid fly. Our findings imply that CRY might have an important role in maintaining allochronic reproductive isolation through shifting the time of mating.

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