The clock gene *period* in the medfly *Ceratitis capitata*

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Summary

We have isolated the clock gene *period* (*per*) from the medfly *Ceratitis capitata*, one of the most economically important insect pest species. The overall pattern of conserved, non-conserved and functional domains that are observed within dipteran and lepidopteran *per* orthologues is preserved within the coding sequence. Expression analysis from fly heads revealed a daily oscillation in *per* mRNA in both light : dark cycles and in constant darkness. However PER protein levels from head extracts did not show any significant evidence for cycling in either of these two conditions. When the *Ceratitis per* transgene under the control of the *Drosophila per* promoter and 3′UTR was introduced into *Drosophila per*-null mutant hosts, the transformants revealed a low level of rescue of behavioural rhythmicity. Nevertheless, the behaviour of the rhythmic transformants showed some similarities to that of *Ceratitis*, suggesting that *Ceratitis per* carries species-specific information that can evidently affect the *Drosophila* host’s downstream rhythmic behaviour.

1. Introduction

In *Drosophila*, the clock gene *period* (*per*) plays a key role in the generation and maintenance of a number of biological rhythms, including the circadian periodicity of locomotor activity and adult emergence from the pupal case (Hall, 2003). *Drosophila per* is expressed rhythmically at both mRNA and protein levels (Siwicki et al., 1988; Hardin et al., 1990; Edery et al., 1994) and the protein also shows a temporal regulation in its subcellular localization (Shafer et al., 2002). PER is phosphorylated by DOUBLETIME (DBT), the *Drosophila* homologue of human Casein Kinase 1ε (Kloss et al., 1998; Price et al., 1998), and this phosphorylation targets the protein for degradation. PER degradation is prevented by the formation of a complex with TIMELESS (TIM), another key component of the *Drosophila* circadian clock (Hall, 2003). The DBT/TIM complex enters the nucleus, where it represses the positive regulation of the dCLK/CYC dimer on *per* and *tim* transcription (Hall, 2003). Recent findings have demonstrated that PER monomers alone can efficiently inhibit dCLK/CYC transcriptional activation (Rothenfluh et al., 2000) and PER mediated inhibition of dCLK/CYC is enhanced but not dependent on TIM (Weber & Kay, 2003).

The comparison of *per* coding regions from *D. melanogaster*, *D. pseudoobscura* and *D. virilis* reveals that the translation products consist of interspersed blocks of six conserved (c1–c6) and five non-conserved (n1–n5) regions (Colot et al., 1988). The longest conserved region (c2) includes those sites to which *perL*, *perS* and *per01* mutations have been mapped (Baylies et al., 1987; Yu et al., 1987). In addition it encompasses the PAS domain, which is protein–protein dimerization domains (Crews et al., 1988) and is particularly prevalent in proteins that act as environmental sensors via light, oxygen...
and redox potential (Ponting & Aravind, 1997; Gu et al., 2000). The conserved region 2 (c2) also includes the cytoplasmic localization domain (CLD) which is C-terminal to PAS, and is involved in the retention of PER in the cytoplasm (Saez & Young, 1996). The non-conserved region n2 includes, in D. melanogaster, the Thr-Gly repeat, which shows dramatic sequence and length variation among dipterans and is known to be under selection (Costa & Kyriacou, 1998).

Orthologues of Drosophila per have been cloned in several dipterans, including the housefly Musca domestica (Piccin et al., 2000), the sheep blow fly Lucilia cuprina (Warman et al., 2000), the melon fly Bactrocera cucurbitae (Miyatake et al., 2002) and two related species, the Queensland fruit fly B. tryoni and its sibling B. neohumeralis (An et al., 2002). In addition, the giant silkmoth Antheraea pernyi has been the focus of several studies within Lepidoptera (Reppert et al., 1994; Chang et al., 2003). Here we describe the isolation and characterization of the clock gene period from the medfly Ceratitis capitata. The Mediterranean fruit fly, Ceratitis capitata (Wiedemann) is one of the world’s most destructive fruit pests. The species originated in the Mediterranean region of Europe and North Africa and is now known to be established in Florida, California, Argentina, Bermuda, Brazil, Costa Rica, Hawaii, Uruguay, Western Australia, many countries in Europe and Africa, and in Middle Eastern countries in the Mediterranean area. Because of its worldwide distribution, its ability to tolerate cooler climates and its wide range of hosts, it is considered the most economically important fruit fly species.

2. Methods

(i) Fly strains

All Drosophila melanogaster strains were reared in 12 h:12 h light:dark cycles (12:12 LD, where Zeitgeber time (ZT)0 corresponds to lights-on and ZT12 to lights-off) at 23 °C. Drosophila and Ceratitis were fed on standard sugar medium. C. capitata (ISPSRA strain) were provided at the pupal stage by Prof. G. Gasperi (Department of Animal Biology, University of Pavia, Italy).

(ii) Identifying Ceratitis per

Genomic DNA was extracted from adult Ceratitis as in Straus (1994), partially digested with Sau3A1 and cloned in Lambda EMBL3/BamHI, using the Gigapack III Gold Cloning Kit (Stratagene) according to the manufacturer’s instructions. A Thr-Gly encoding fragment from Ceratitis (amino acids 552 to 657) was used to screen 1·3 × 10^9 pfu of the EMBL3 Ceratitis library and the largest clone identified, approximately 20 kb, was subcloned into pBluescript and partially sequenced. A 14 kb region corresponding to C. capitata period was identified, but lacked the 5’ end. By chromosome walking, an approximately 15 kb positive clone was isolated, subcloned and partially sequenced.

(iii) Sequence comparison and phylogenetic analysis

Multiple sequence alignment was performed with the ClustalW software suite (Thompson et al., 1994). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar et al., 2001), the ‘Number of Differences’ model to calculate distance and the UPGMA phylogeny inference method.

(iv) RNA isolation and Ceratitis per cDNA isolation

Fifteen adult heads were collected at ZT12, ZT14 and ZT16 and mixed in a single sample. Total RNA was then prepared using the TRIZOL reagent (Life Technologies) according to the manufacturer’s instructions. Subsequently, CDNA was prepared with SUPERSCRIPT II RNase H− Reverse Transcriptase (GIBCO BRL) according to the manufacturer’s instructions. Ceratitis per coding sequence was obtained as two amplified fragments: (i) a 1·8 kb 5’ fragment, using the 5’ primer 5’-CAGCATATATTTCCATTG-3’ (positions 306–324) and the 3’ primer 5’-TTCTTCA-TTGGGTCCAGCC-3’ (positions 2095–2119; boldface represents the unique internal SalI restriction site); (ii) a 1·45 kb 3’ fragment, with the 5’ primer 5’-GGTGACCAATGAAGAA-3’ (positions 2095–2119; boldface represents the unique internal SalI restriction site), and the 3’ primer 5’-CATGTA-TGTATGACATATATGTATG-3’ (positions 3470–3498). The primer positions refer to the C. capitata per sequence reported in Fig. 1. All the PCR reactions were performed with a high-fidelity DNA polymerase (Expand High Fidelity PCR System; Roche) in order to minimize PCR-induced errors. The two fragments were cloned into the pCRII-TOPO vector (Invitrogen) and sequenced in order to ensure the absence of PCR mutations. Subsequently, in a three-way ligation reaction they were joined at the SalI site in the pBluescript vector, in order to produce an approximately 3·1 kb fragment containing the whole C. capitata period CDNA.

(v) Construction of the C. capitata and D. melanogaster transgene MC1

The C. capitata per construct pMC1 for P-element transformation was prepared by joining the 7·3 kb
D. melanogaster per 5' regulatory region and the 3 kb 3'UTR to the coding sequence of the Ceratitis gene, using a series of cloning steps.

First step. The approximately 7 kb D. melanogaster per BamHI–XbaI fragment, which contains the 5' flanking DNA, the first, untranslated exon and the first large intron, was extracted from the 13.2 genomic clone (Petersen et al., 1988; Peixoto et al., 1998) and subcloned in pBluescriptKS. The immediately downstream 330 bp XbaI–SpeI fragment, was mutagenized via PCR, by using the 5' primer 5'-GAATTG-AGTGAATCG-3' (positions 49436–49457 in the GenBank AE003425 sequence; the SpeI site is in boldface) and the 3' primer 5'-CAGTTGG-TTG TCTAGA GCGAG-3' (54335–54356 in the GenBank AE003425; the XbaI site is in boldface), in order

| Start | End |
|-------|-----|
| TTAAGCAGTGTAAACAGCGAGATGCAGCGGAGAGGTCTTTTAGAACTCTACGATGCTAAGAGAAGAAAGATTTACTAATAATT | 54 |
| AGTGGTGGCGTGTGGTTTGAAGACCAAAGCTAAGACATGCTATGACATGATCCTTCTACAGCTCAATTGGA | 61 |
| ATATATTATATAATGAAAGAATGACTTGGAAATCTTATACATATATAACTATTTGTGAA | 121 |
| CGGTATTTCTAACTAAAGTGTAAACACACAAAAAAAAGTGTAGCAGCTTTTCTAA | 181 |
| AAGACATTTTTTATACAAAAATGAAAGAAAAAAATATTATATACAAAGATTTAAAAA | 241 |
| GTATAACGCTATATTTCCATTTAGAAAAAATACGCCAAGGAAAAGTGGG | 301 |

Fig. 1. (Cont.)
to convert it to a 330 bp Spel–XbaI fragment. This fragment was subcloned in the pCRII-TOPO vector (Invitrogen) and automatically sequenced to check for errors. Subsequently, it was extracted and fused to the approximately 7 kb upstream D. melanogaster per region in pBluescriptKS, via the compatible XbaI/SpeI junction, obtaining a 7.3 kb D. melanogaster per fragment.

Second step. The 3.1 kb C. capitata per cDNA was then extracted as a SpeI–XbaI fragment and subcloned downstream to the 7.3 kb D. melanogaster 5' per region in the pBluescript vector using the compatible SpeI/XbaI junction, giving rise to a 10.4 kb chimeric fragment.

Third step. The approximately 3 kb D. melanogaster 3' UTR per region was amplified using the 13.2 kb genomic clone as template and introducing the NotI and SacI restriction sites at the 5' and 3' termini respectively, with the following primers:

5' k primer:

1381 GACGGGAATCATTTCGCACGTGGACAGCGCTGCGGTATCAACATTGGGTTATTTACCACA
1441 GACATTTATTGGACGTTCAATATTTGACTTTTATCATCCGGAAGATCTTATGGTATTGAA
1501 GGAATAAATCACAGAATCAGTTGAAAGTGAAGTTTCTCGATACGACCTGCTATACATACATAC
1561 ACCGTACGTCCTCTAACAATAGGCTACATATATTGTATGGTACACACGACATTTGTTATCA
1621 TTTTCGATACGGCCTGCTATGAGGTACGTAGTCGAAATATACGCATTAAAGACGAAACTCCAT
1681 GGTCCCAAAACATTATTAAAAATGATTITCGATACGACCGCAGGAAACAAACAAACAAACTCTG
1741 TGAGGTAACGTCGTAAGGATACGATAGCTACACACAGAAAGACAAGGAGATCAGGCTTGTAAGCTG
1801 CATATCAGGTCCTCGAGACACCTGTAAGAAGAAGGAGATCAGGCTAGGTTGAGCTAAGCTG
1861 TCATCTGGTGAAACTCTCATGAGGAAGATCTTATGGTATTGAA
1921 GGAAATTTACGAAACCGTCATGAAGAAGGGACAAACAGCGGGCGCTTCTTTTTGCAGCAA
1981 CATATCGCGGCCTTCGGACACGGTAAAGCAAGAGGTGTCACGCCGTTGTCAAGCGTTAGC
2041 GACCTTATGGAATCATTTCGCACGTGGACAGCGCTGCGGTATCAACATTGGGTTATTTACCACA
2101 GACATTTATTGGACGTTCAATATTTGACTTTTATCATCCGGAAGATCTTATGGTATTGAA
2161 GGAATAAATCACAGAATCAGTTGAAAGTGAAGTTTCTCGATACGACCTGCTATACATACATAC
2221 TTTTCGATACGGCCTGCTATGAGGTACGTAGTCGAAATATACGCATTAAAGACGAAACTCCAT
2281 GGTCCCAAAACATTATTAAAAATGATTITCGATACGACCGCAGGAAACAAACAAACAAACTCTG
2341 TGAGGTAACGTCGTAAGGATACGATAGCTACACACAGAAAGACAAGGAGATCAGGCTTGTAAGCTG
2401 CATATCAGGTCCTCGAGACACCTGTAAGAAGAAGGAGATCAGGCTAGGTTGAGCTAAGCTG
2461 TGCCGCCATCAACCATACCATGAACTAGTATATTACGAAACACAGTAACCGCGACGCGACGCTT
2521 GGTCCCAAAACATTATTAAAAATGATTITCGATACGACCGCAGGAAACAAACAAACAAACTCTG

Fig. 1. (Cont.)
**Fig. 1.** Coding and deduced amino acid sequence of *C. capitata* period gene. Nucleotides are numbered on the left and amino acids on the right. Introns positions are indicated by filled triangles and numbered above. The end of translation is marked by an asterisk (*). A second stop codon, in frame with the first, is shown in boldface type and underlined. Two overlapping polyadenylation signals are double underlined. Regions conserved in PER proteins of other insect species, C1 through C6, as defined by Colot and colleagues (1988), are indicated on the top of the coding sequence. The PAS (PAS A and PAS B), CLD, Thr-Gly (TG), CCID and NLS domains are indicated (according to Huang et al., 1993; Vosshall et al., 1994; Saez & Young, 1996; Chang & Reppert, 2003).
pCRII-TOPO vector (Invitrogen) and automatically sequenced to check for errors. Subsequently, it was extracted and fused downstream to the approximately 10.4 kb chimeric fragment in the pBluescript vector.

Fourth step. The resulting approximately 13.4 kb fragment, containing the 7.3 kb 5′ D. melanogaster per region, the 3.1 kb C. capitata per cDNA and the 3 kb D. melanogaster 3′UTR, was finally subcloned as Kpn I–SacII segment in the pW8 vector, obtaining the pMCI construct for P-element transformation.

Transformation of Drosophila was carried out according to Spradling (1986). Six independent transgenic lines were obtained (21, 34, 119, 192 and 197), chromosomally mapped, balanced and then made homozygous. In situ hybridization on third instar salivary gland chromosomes was used to map the position and the number of inserts in each line using a 2 kb fragment of white cDNA as a probe and the DIG-DNA Labelling and Detection Kit (Roche).

(vi) Northern analysis
Adult Ceratitis flies 3–4 days old were rearred for at least 3 days in 12 : 12 LD at 23 °C and heads were collected by freezing at 3 h intervals on day 4. Adult flies reared in 12 : 12 LD for 3 days were also placed in constant darkness (DD), and harvested over the first and the second day of DD. Approximately 30 μg of total RNA extracted from 15 heads per time point were subjected to electrophoresis through a 1% agarose-formaldehyde gel, blotted onto nylon membrane and hybridized with DNA probes labelled with [α-32P]dCTP by random priming (New England Biolabs). The probes used were the same 1.8kb [a-32P]dCTP by random priming (New England Biolabs). The probes used were the same 1.8 kb fragment of C. capitata period gene described in Section (iv), and an approximately 0.9 kb fragment corresponding to the 5′end of C. capitata actin cDNA (act; positions from 626 to 1555 according to GenBank M76614). For quantification of the signals, each film was analysed with Quantity One 4.2.0 Software (Bio-Rad). Relative abundance of per mRNA was defined as a per/act ratio and normalized to the highest time point value for each experiment.

(vii) Western blotting
Samples of 15 fly heads were taken at 3 h intervals in 12 : 12 LD at 23 °C for at least 3 days and collected on day 4. Adult flies reared in 12 : 12 LD for 3 days were also placed in DD, and heads harvested at 3 h intervals over two circadian cycles in the first and second day of DD. For each sample, approximately 100 μg of total proteins was extracted and subjected to western blotting as in Edery et al. (1994). The anti-PER primary antibody used was an affinity-purified rabbit polyclonal antibody against a synthetic 17 amino acid peptide KKHRESRGRTGK corresponding to residues 808–823 of the D. melanogaster PER protein (364 anti-DmPER, 1 : 50; Neosystem, France). This region is highly conserved in C. capitata PER (16 of 17 amino acids), and corresponds to residues 670–685 (Fig. 1). A mouse anti-HSP70 (1 : 10000, Sigma) was employed to immunodetect HPS70, used as a loading control. An anti-rabbit IgG-HRP (1 : 3000; Bio-Rad) and an anti-mouse IgG-HRP (1 : 5000; Sigma) were used as secondary antibodies. Positive immunoreactivity was visualized using a chemiluminescence system. For quantization of the immunodetected signals, each film was analysed with the Quantity One 4.2.0 Software (Bio-Rad). Relative abundance of PER was defined as a ratio with HSP70 (PER/HSP) and normalized to the highest time point value for each experiment.

(viii) Behavioural analysis
Adult male flies 4–5 days old of wild-type (Oregon-R), perΔ and pMCI Drosophila transgenic lines in perΔ and perΔ backgrounds, were analysed with respect to their circadian locomotor activity as described previously (Piccin et al., 2000). Locomotor activity of Ceratitis capitata adult flies was studied by using the TriKinetics activity monitoring system (Waltham, MA). Flies were entrained in a 12 : 12 LD cycle for 3 days and subsequently free-run in DD for 7 days at 18, 23 and 28 °C. Periodicity was calculated by spectral analysis performed with the CLEAN algorithm of Roberts et al. (1987), enhanced by a Monte Carlo simulation to generate 95% and 99% confidence limits for the estimation of the significance of the peaks, as described in Zordan et al. (2005). In addition, all the behavioural data were analysed by autocorrelation, and only flies that showed significant periods with both statistical analyses were judged as ‘rhythmic’, as described in Peixoto et al. (1998). Mean phase and onset values were calculated for morning and evening activity peaks on the third day of 12 : 12 LD, and given in hours from the last lights-on and lights-off transition (values are negative when occurring before the transition), respectively. Onset was defined as the mean time between the beginning of locomotor activity and the maximum of the peak as previously described in Majercak et al. (2004). In order to compare profiles between individuals and between genotypes, the data for locomotor activity in DD for each fly were standardized according to the procedure of Tauber et al. (2003).

(ix) Statistical analyses
Analysis of variance was performed using a one-way ANOVA for northern and western blot data and a two-way ANOVA for behavioural activity data, using the STATISTICA 5.0 package (Statsoft).
3. Results

(i) Cloning of the C. capitata per homologue

C. capitata per (Ceper) spans approximately 15 kb from the starting codon to the putative polyadenylation signal. This dramatic increase in size compared with its Drosophila orthologues (Citri et al., 1987; Colot et al., 1988; Thackeray & Kyriacou, 1990) has also been documented in B. neohumeralis, B. tryoni and Musca domestica per (Piccin et al., 2000; An et al., 2002), and is due to changes in intron–exon structure. RT-PCR and 5'k-RACE performed on head RNA extracts provided a coding sequence of approximately 3.1 kb which includes 352 bp of 5’ untranslated sequence (Fig. 1). A second stop codon, in frame with the first, has been identified from nucleotide 3578. Two overlapping polyadenylation signals are predicted from nucleotides 3753 to 3763 (Fig. 1). The Ceratitis per transcript contains an open reading frame (ORF) encoding an inferred protein of 1035 residues with a putative molecular weight of 116 kDa (Fig. 1).

(a) Intron–exon structure

C. capitata per has 12 introns, the first localized in the 5’UTR and the second revealing the presence of a 68 bp sequence that shares 82% identity with the mariner transposase pseudogene from Rivellia quadri-fasciata (Accession number: U91383; Robertson et al., 1998). These data support the ability of the mariner transposon family to undergo horizontal transfer even between taxonomically rather distant organisms (Lampe et al., 2003). A comparison of intron number and size between the relevant species is illustrated in Fig. 2 and Table 1. It shows five additional introns in C. capitata compared with Drosophila, and one additional intron compared with Musca (intron 5), as well as a general increase of intron size with respect to both Drosophila and Musca (Table 1). Except for intron 5, all other introns within the coding region are located in well-conserved intron/exon boundaries in C. capitata and M. domestica.

(b) Coding sequence

The overall pattern of conserved (c) and non conserved (nc) regions, introduced by Colot et al. (1988), is preserved in Ceratitis (Fig. 1). The nuclear localization signal (NLS; Vosshall et al., 1994; Saez & Young, 1996), amino acids (aa) 72 to 76; the two PAS regions (PAS A aa 194 to 243 and PAS B aa 344 to 396; Huang et al., 1993); the cytoplasmic localization domain (CLD) that spans aa 399 to 458; the CKL:CYC inhibition domain (CCID) with the

![Diagram of intron/exon comparison](https://example.com/diagram.png)
Table 1. Size (bp) of period introns in C. capitata, B. neohumeralis/B. tryoni, M. domestica and D. melanogaster

| Intron | C. capitata | B. neohumeralis/B. tryoni | M. domestica | D. melanogaster |
|--------|-------------|---------------------------|--------------|----------------|
| 1      | Not sequenced | Not sequenced             | Not sequenced | 2000           |
| 2      | >7000        | Not sequenced             | 5000         | 61             |
| 3      | 184          | 155                       | 72           | Absent         |
| 4      | 111          | 107                       | 64           | Absent         |
| 5      | 620          | 1100                      | Absent       | Absent         |
| 6      | 641          | 1400                      | 63           | Absent         |
| 7      | 74           | 86                        | 56           | 64             |
| 8      | 73           | 56                        | 63           | 62             |
| 9      | 92           | 66                        | 68           | 70             |
| 10     | 71           | 800                       | 71           | Absent         |
| 11     | 2498         | 1100                      | 61           | 64             |
| 12     | 199          | 166                       | 60           | 58             |

We were interested in the evolution of the conserved regions (Colot et al., 1988), and therefore compared the PAS domain, implicated in protein dimerization, with the evolution of non-PAS sequences.

Fig. 3A and B represents phylogenetic trees obtained with the amino acids from the PAS region (which also includes the Ceratitis PER CLD domain: aa 184 to 442; Fig. 1), and the Ceratitis PER conserved non-PAS sequence (aa 1–78, 179–183, 443–637, 649–721, 762–932, 947–1035; Fig. 1). The PAS tree places D. melanogaster and D. yakuba closer to Ceratitis and Bactrocera, and farther from the other Drosophila species. Although the bootstrap value associated with the ancestral node common to these species is low, a similar result was obtained by Piccin et al. (2000) with the Musca sequence, whose unorthodox position relative to D. melanogaster/yakuba and D. pseudoobscura/D. viridis is maintained in this phylogeny. On the other hand the non-PAS tree follows the species tree, as also shown in Piccin et al. (2000).

(iii) C. capitata per mRNA levels oscillate both in LD and in DD

The temporal expression of Ccper mRNA was determined by northern blot in 12:12 LD at 23 °C. In these conditions, no alternatively spliced forms were revealed in any of the sampled ZTs. The approximately 6 kb unique per mRNA form showed a significant daily oscillation in abundance, with a peak at ZT9 and a mean fivefold amplitude (∆F_{8,18}=7.29, P<0.0003; Fig. 4A, B). A significant daily rhythm in per mRNA expression was maintained also in DD for the first 48 h after switching from 12:12 LD, but with maximum levels at CT15 and CT39, revealing a 6 h phase difference after shifting to DD (∆F_{16,4}=4.08, P<0.0003; Fig. 4C, D). However, an increase in the baseline values leads to a dampening in the amplitude of the oscillation during the second day of DD,

internal NLS that span aa 647 to 858 and aa 692 to 719 respectively (Chang & Reppert, 2003), are all conserved in C. capitata. The sites to which the Drosophila per, per and per mutations have been mapped (Baylies et al., 1987; Yu et al., 1987) are perfectly conserved in CcPER. The Cc PER threonine–glycine (TG) repeat (aa 640 to 646), as in Bactrocera and Musca, has not undergone the dramatic expansion in size observed in the Drosophila genus (Costa et al., 1991; Peixoto et al., 1992, 1993). In general, levels of identity of CcPER compared with Drosophila PER were very high (>70%) for each domain that we examined, and dropped only for the CCID (48%) and DBT interacting domain (aa 1 to 311; 62%).

We also calculated the Relative Synonymous Codon Usage (RSCU) index for C. capitata period coding sequence (GCUA, v1.0; McInerney, 1998). This analysis was performed for the full-length cDNA, for the PAS, the non-PAS-conserved and the non-conserved coding sequences separately. A general low codon bias was observed for the full-length cDNA, except for CGU (Arg) codon, which showed a RSCU index of 2.36 (out of 6 codons coding for Arg) in the full-length cDNA analysis, and reached 4.24 in the PAS coding sequence when analysed separately. These numbers appear to be consistent with the fact that per is not a highly expressed gene (Grantham et al., 1981).

(ii) Molecular phylogeny of the PER proteins

Sequences from C. capitata, four species of Drosophila (D. melanogaster, D. viridis, D. yakuba and D. pseudoobscura), two species of Bactrocera (B. tryoni and B. neohumeralis), Lucilia cuprina, Musca domestica, Chymomiza costata and the lepidopteran Antheraea pernyi were aligned using Clustal W (Thompson et al., 1994), in order to reveal any significant differences between the species tree and the PER protein tree.

The temporal expression of Ccper mRNA was determined by northern blot in 12:12 LD at 23 °C. In these conditions, no alternatively spliced forms were revealed in any of the sampled ZTs. The approximately 6 kb unique per mRNA form showed a significant daily oscillation in abundance, with a peak at ZT9 and a mean fivefold amplitude (∆F_{8,18}=7.29, P<0.0003; Fig. 4A, B). A significant daily rhythm in per mRNA expression was maintained also in DD for the first 48 h after switching from 12:12 LD, but with maximum levels at CT15 and CT39, revealing a 6 h phase difference after shifting to DD (∆F_{16,4}=4.08, P<0.0003; Fig. 4C, D). However, an increase in the baseline values leads to a dampening in the amplitude of the oscillation during the second day of DD,
probably reflecting a gradual loss of synchrony between individual heads.

(iv) C. capitata PER protein does not oscillate in LD or DD

Western blot of Ceratitis head extracts using anti-DmPER antibody 364 identifies an approximately 120 kDa band that can be ascribed to Ceratitis PER (Fig. 5A, lane 1). This band is in fact observed in D. melanogaster flies transgenic for C. capitata PER (lane 2), but is absent in both D. melanogaster arrhythmic mutant per$^{01}$ (lane 3) and Oregon-R (lanes 4 and 5). Analysis of C. capitata PER protein levels was performed in 12:12 LD as well as during 2 days of DD at 23 °C. No evidence for rhythmic oscillation of protein levels was observed, either in LD ($F_{7,16}=1.74; P=0.17$; Fig. 5B, C) or in DD ($F_{16,33}=0.99; P=0.48$; Fig. 5D, E). In all cases data were taken from three replicate experiments.

(v) Behavioural rhythms in C. capitata and transgenic D. melanogaster

The locomotor activity of males from C. capitata ISPRA strain and six D. melanogaster transgenic lines bearing the C. capitata per gene was monitored at 18, 23 and 28 °C for 3 days in 12:12 LD followed by 7 days in DD. The histograms in 12:12 LD for C. capitata were compared with those for D. melanogaster Oregon-R, which shows similar activity patterns to per$^{+}$ transformants (Sawyer et al., 1997). Unlike Oregon-R, which shows a bimodal pattern at the higher temperatures, no bimodality of locomotor activity was found in C. capitata, but a single broad peak during the daytime was present at every temperature (Fig. 6A, B). Mean locomotor phase and onset values were recorded on the third day of 12:12 LD at 18, 23 and 28 °C. No significant differences in the phase values were observed ($F_{2,60}=0.70; P=0.50$) but a significant change in onset was observed, which at 18 °C fell 2 h after lights-on, and at 29 °C occurred...
1.5 h before lights-on \( (F_{2,69} = 26.90, \ P < 0.0001; \) Table 2).

All the transgenic lines that were behaviourally tested carried one autosomal copy of the \( pMC1 \) construct in a \( per^{04} \) background. The locomotor activity patterns in 12:12 LD were analysed for those transgenic \( pMC1 \) flies that showed a circadian activity in the subsequent free-running condition. In many cases (40–80\% of the rhythmic \( pMC1 \) transgenics, depending on the line tested) the flies showed a bimodal locomotor activity profile, very similar to \( D. \ melano- \) gaster control flies, with anticipatory activity corresponding to the lights-on and -off transitions (morning and evening peaks) at every temperature.
Fig. 5. PER protein in *Ceratitis capitata*. (A) Western blot (with purified 364 anti-DmPER antibody) on adult head extracts from *C. capitata* collected at ZT21 (lane 1), 192MC1 *D. melanogaster* transgenic line in per<sup>+</sup> background, collected at ZT18 (lane 2), *D. melanogaster* arrhythmic mutant per<sup>01</sup> (lane 3) and *D. melanogaster* Oregon-R flies collected at ZT18 (lane 4; expected peak of expression) and ZT9 (lane 5; expected trough of expression). Empty arrow indicates the approximately 180 kDa signal corresponding to *Drosophila* PER and the filled arrow shows a band of estimated 120 kDa, detected solely in *Ceratitis* and in the *D. m.* 192MC1 transgenic line. (B) Representative western blot of *Ceratitis* adult head extracts probed with 364 anti-DmPER antibody (top) and an anti-HSP70 (bottom). Flies' heads were collected every 3 h over the first and second day of DD, at 23 °C. Circadian Time refers to subjective time under constant conditions; grey bars indicate the subjective day and black bars the subjective night. (C) Quantification of PER levels. Relative abundance of PER was defined as a ratio with HSP70 (PER/HSP), normalized to the highest value and mean levels ± standard deviation of three independent experiments are shown. No significant PER cycling was observed ($F_{7,16} = 1.74$, $P = 0.17$). (D) Representative western blot of *Ceratitis* adult head extracts probed with 364 anti-DmPER antibody (top) and an anti-HSP70 (bottom). Flies' heads were collected every 3 h or over the first and second day of DD, at 23 °C. Circadian Time refers to subjective time under constant conditions; grey bars indicate the subjective day and black bars the subjective night. (E) Quantification of PER levels. Relative abundance of PER was defined as a ratio with HSP70 (PER/HSP), normalized to the highest value. Mean levels ± standard deviation of three independent experiments are shown. No significant PER cycling was observed ($F_{16,33} = 0.99$, $P = 0.48$).
tested (Fig. 6C). On the other hand, in the remaining cases it was not possible to determine the morning and/or evening peak, because any possible underlying rhythmicity appeared to be masked by light-stimulated locomotor activity. Mean phase and onset for morning and evening peaks at 18, 23 and 28 °C were calculated on the third day of 12 : 12 LD for pMC1 transgenic lines and compared with those of D. melanogaster Oregon-R control flies (Table 2).

Focusing attention on 156 MC1 transgenic flies, which showed bimodal activity at every temperature tested, significant temperature-dependent variations were observed only for the evening activity peak (Evening Onset: $F_{2,46}$ = 15.58; $P < 0.0001$; Evening Phase: $F_{2,47}$ = 16.53; $P < 0.0001$), not for morning activity (Morning Onset: $F_{2,46}$ = 0.50; $P = 0.61$; Morning Phase: $F_{2,46}$ = 0.63, $P = 0.54$).

C. capitata adults were also monitored in DD. The percentage of rhythmic flies in DD varied with temperature, with lower values observed in the coldest condition (Table 3). Among the rhythmic flies, the mean period values were around 22–23 h, with no significant differences between temperatures ($F_{2,46}$ = 0.62, $P = 0.54$). Fig. 7 shows an analysis of locomotor activity in DD, in which each individual fly’s period is normalized to a 24 h period. This analysis confirms the unimodal nature of Ceratitis locomotor activity (Fig. 7A) that was also observed in LD. Transgenic lines were also analysed for their ability to rescue circadian locomotor activity rhythms in DD at different temperatures (Table 3). In the per$^{at}$ background, the Ccper transgene showed low rescue, from 0 to 68%, depending on the line and the temperature considered. Periods were highly variable between 18 and 34 h, although the means were within the circadian range and no significant differences in period were observed between the three temperatures for any of the lines. Fig. 7C shows histograms in DD for the transgenic line 156MC1, which gave the best rescue. Only significantly rhythmic animals are included in the analysis. At 18 °C we observed a subjective morning peak, as in C. capitata (but not Oregon-R; Fig. 7A–C), as well as an early evening peak, as in Oregon-R (but not Ceratitis).
Table 2. Morning and evening peaks (onset and phase values) of homozygous pMC1 transgenic lines, D. melanogaster Oregon-R control flies and C. capitata on the third day of 12:12 LD at 18, 23 and 28 °C

| Line          | Morning peak                      | Evening peak                     |
|---------------|-----------------------------------|----------------------------------|
|               | Onset ± SEM Phase ± SEM (N)       | Onset ± SEM Phase ± SEM (N)      |
| w. per<sup>AB</sup>; 21MC1 | 18 °C 23 °C 28 °C | 18 °C 23 °C 28 °C |
| w. per<sup>AB</sup>; 34MC1 | 18 °C 23 °C 28 °C | 18 °C 23 °C 28 °C |
| w. per<sup>AB</sup>; 119MC1 | 18 °C 23 °C 28 °C | 18 °C 23 °C 28 °C |
| w. per<sup>AB</sup>; 156MC1 | 18 °C 23 °C 28 °C | 18 °C 23 °C 28 °C |
| w. per<sup>AB</sup>; 192MC1 | 18 °C 23 °C 28 °C | 18 °C 23 °C 28 °C |
| w. per<sup>AB</sup>; 197MC1 | 18 °C 23 °C 28 °C | 18 °C 23 °C 28 °C |
| OR-R          | 18 °C 23 °C 28 °C | 18 °C 23 °C 28 °C |
| C. capitata   | 18 °C 23 °C 28 °C | 18 °C 23 °C 28 °C |

Values are given in hours from the last lights-on (morning peak) and lights-off (evening peak) transition, with negative values indicating that they occur before the transition. (N) indicates the number of flies tested.

- Significant delay of the evening peak between 18 °C and 28 °C in 156MC1 transgenic line (Evening Onset: F<sub>x</sub> = 15.58, P < 0.0001; Evening Phase: F<sub>x</sub> = 16.53, P < 0.0001).
- Significant variations of the morning and evening peaks between 18 °C and 28 °C in D. melanogaster Oregon-R control flies (Morning Onset: F<sub>x</sub> = 10.77, P < 0.0001; Morning Phase: F<sub>x</sub> = 21.80, P < 0.0001; Evening Onset: F<sub>x</sub> = 39.10, P < 0.0001; Evening Peak: F<sub>x</sub> = 78.82, P < 0.0001).
- Significant difference in the onset values between 18 °C and 28 °C in C. capitata (F<sub>x</sub> = 26.90, P < 0.0001).

At 23 and 28 °C, the profiles for the transformants seemed to be intermediate between the two parental species, giving rather ‘flatter’ (average) profiles.

4. Discussion

The cloning of the C. capitata period orthologue and the comparative analysis with D. melanogaster, M. domestica, B. tryoni and B. neohumeralis shows that the Ceratitis gene is very closely related to Bactrocera per, with regard to number, position and length of introns. Such a tight correlation between the two species has already been observed in the organization of the Adhl and white loci (Gomulski et al., 1997, 2001). All these genes share a dramatic increase in size, which is due to an increase in both number and size of introns. Several studies have indicated that there may be a positive correlation between intron length and genome size, which is stronger when homologous genes are considered (Moriyama et al., 1998; Deutsch & Long, 1999). C. capitata and B. tryoni have similarly sized genomes of approximately 5 × 10<sup>8</sup> bp, and therefore longer introns would be expected in these species compared with D. melanogaster, whose genome size is 1.7 × 10<sup>8</sup> bp (Adams et al., 2000). Moreover, such long introns tend to accumulate insertions, such as repetitive sequences and transposable elements (Stephan et al., 2001).
Table 3. Free-running periods of homozygous pMC1 transgenic lines, in per\textsuperscript{01} and per + background, and C. capitata males in DD at different temperatures

| Line                      | % Rescue (N) | Period (in hours) ± SEM |
|---------------------------|--------------|------------------------|
|                           | 18 °C | 23 °C | 28 °C | 18 °C | 23 °C | 28 °C |
| w, per\textsuperscript{01}; 21MC1 | 12 (25) | 28 (39) | 16 (32) | 25.57 ± 0.69 | 22.88 ± 1.13 | 21.99 ± 0.97 |
| w, per\textsuperscript{01}; 34MC1 | 28 (40) | 47 (74) | 9 (44) | 24.81 ± 0.79 | 25.17 ± 0.69 | 22.81 ± 0.83 |
| w, per\textsuperscript{01}; 119MC1 | 20 (20) | 27 (48) | 0 (11) | 26.81 ± 0.83 | 24.21 ± 1.22 | – |
| w, per\textsuperscript{01}; 156MC1 | 68 (22) | 24 (29) | 38 (20) | 24.03 ± 0.37 | 24.55 ± 1.13 | 22.97 ± 0.68 |
| w, per\textsuperscript{01}; 192MC1 | 31 (16) | 9 (11) | 10 (10) | 22.12 ± 0.9 | 22.34 | 23 |
| w, per\textsuperscript{01}; 197MC1 | 21 (47) | 13 (30) | 0 (10) | 25.71 ± 1.28 | 22.86 ± 1.16 | – |
| w, per\textsuperscript{01}; 3 | 3 (31) | 3 (29) | 0 (27) | 18:01 | 24:01 | – |
| w, per\textsuperscript{+}; 21MC1 | – | 90 (20) | – | – | 24.41 ± 0.19 | – |
| w, per\textsuperscript{+}; 34MC1 | – | 97 (33) | – | – | 24.41 ± 0.14 | – |
| w, per\textsuperscript{+}; 119MC1 | – | 94 (29) | – | – | 24.33 ± 0.14 | – |
| w, per\textsuperscript{+}; 156MC1 | – | 95 (19) | – | – | 23.87 ± 0.1 | – |
| w, per\textsuperscript{+}; 192MC1 | – | 100 (15) | – | – | 24.02 ± 0.1 | – |
| w, per\textsuperscript{+}; 197MC1 | – | 95 (20) | – | – | 24.32 ± 0.14 | – |
| OR-R                     | 100 (24) | 100 (21) | 100 (30) | 23.73 ± 0.01 | 23.71 ± 0.02 | 24.84 ± 0.04 |
| C. capitata              | 41 (29) | 50 (10) | 86 (28) | 22.66 ± 0.18 | 23.23 ± 0.67 | 22.27 ± 0.36 |

OR-R and w; per\textsuperscript{01} represent the D. melanogaster Oregon-R control flies and the arrhythmic mutant per\textsuperscript{01} strain respectively. No significant differences in the period length between 18 and 28 °C have been found (ANOVA test w, per\textsuperscript{01}; 21MC1 F\textsubscript{2, 41} = 1.32, P = 0.29; w, per\textsuperscript{01}; 34MC1 F\textsubscript{2, 41} = 0.71, P = 0.49; w, per\textsuperscript{01}; 156MC1 F\textsubscript{2, 41} = 1.04, P = 0.36; C. capitata F\textsubscript{2, 41} = 0.26, P = 0.76).

(N) indicates the number of flies tested.

Fig. 7. Free-running locomotor activity profiles of C. capitata, D. melanogaster Oregon-R control flies and homozygous 156MC1 transgenic line at 18, 23 and 28 °C. Activity events were collected in 30 min bins. Black and grey bars correspond to the activity during subjective night and subjective day respectively. (A) Mean activity histograms for C. capitata in DD at 18 °C (29 flies), 23 °C (5 flies) and 28 °C (28 flies). (B) Mean activity histograms for D. melanogaster Oregon-R control flies in DD at 18 °C (27 flies), 23 °C (23 flies) and 28 °C (30 flies). (C) Mean activity histograms of transgenic line 156MC1 at 18 °C (15 flies), 23 °C (9 flies) and 28 °C (10 flies).

et al., 1994). It is noteworthy that degenerate forms of mariner transposable elements have been observed in long introns of both white and period genes, both in Ceratitis (Gomulski et al., 2001; this study) and in Bactrocera (Bennett & Frommer, 1997; An et al., 2002).
Sequence comparison of PER proteins reveals high levels of conservation, mostly in the N-terminal region, indicating that important PER functions are performed by this part of the protein. This region contains the PAS domain, a protein–protein interaction module which is also known to have important sensory and signalling functions (Gu et al., 2000). In the Drosophila clock the PAS domain promotes the dimerization of PER with its molecular partners TIM, CLOCK and BMAL. The phylogeny of the PAS regions shows that Ceratitis PAS clusters more closely to D. melanogaster and D. yakuba PAS regions than suggested by the phylogenetic distance of Ceratitis from these Drosophila species, as already observed with the Musca sequence (Piccin et al., 2000). This is probably due to the fact that most of the amino acids in the PAS domain are under selective constraint and that this region of the PER protein cannot evolve independently, but instead is constrained to coevolve in concert with the dimerization domains of its conspecific molecular partners.

At the transcriptional level, we observed a clear daily rhythm for C. capitata per mRNA, both in LD and in DD. However, in LD the mRNA peak occurs during the day, resembling those of mammalian Per1 mRNA (ZT7; Hastings et al., 1999) and the marine snail Bulla gouldiana (ZT5; Constance et al., 2002), rather than those of other insects, which peak at the light–dark transition or early in the night phase (e.g. Drosophila (ZT13; Hardin et al., 1990), L. cuprina (ZT12; Warman et al., 2000) or Chyomyzyna costata (ZT15; Kostal & Shimada, 2001)). The heterogeneity between organisms in the per mRNA peak in LD supports the hypothesis of species-specific regulatory mechanisms in clock gene expression (Constance et al., 2002). Furthermore, the unexpected 6 h shift of the CcPer mRNA peak observed immediately after transfer from LD to DD conditions suggests the possibility of the presence in C. capitata of two different regulatory mechanisms in modulating per expression, which would act alternatively when a strong Zeitgeber such as light is present or in constant darkness. Nonetheless, as the environmental conditions change from LD to DD, rather intriguingly, the 6 h shift in the mRNA peak does not seem to have direct effects on the behavioural circadian output (i.e. the locomotor activity). One needs to bear in mind here that mRNA profiles from the head are averaging the dynamics of the CcPer transcript over several tissue types, and we expect that the tissue that contributes most is probably the eyes. The neuronal populations that express CcPer and generate the circadian behaviour (see Grima et al., 2004; Stoleru et al., 2004) may well show rather different mRNA profiles (e.g. Peng et al., 2003).

On the other hand, CePER protein is maintained at constant levels both in LD and in DD. This is in contrast to the data from other insect species such as Drosophila (Hall, 2003), L. cuprina (Warman et al., 2000) or Thermobia domestica (Zavodska et al., 2003), where studies carried on whole head extracts revealed a circadian oscillation in PER levels, with a peak during the dark period. However, western blots of head extracts inevitably include the eyes, which, at least in Drosophila, carry the major portion of PER antigenicity. Thus, as explained in the case of mRNA analysis, it could be that in the central pacemaker cells of Ceratitis, PER protein cryptically cycles in the canonical way expected for the Diptera. Immunocytochemical analysis with appropriate reagents would therefore represent the next step in this comparative analysis.

To our knowledge, this is the first study on the circadian locomotor activity of Ceratitis capitata. Our results show a diurnal activity with a unimodal profile in LD cycles (Drosophila, under the same conditions, shows a bimodal profile of activity) and with little evidence for the modulation of behaviour by temperature (Majercak et al., 1999, 2004; Collins et al., 2004). This might suggest that the temperature-sensitive 3' splicing that occurs in D. melanogaster and which moves evening locomotor activity earlier into the day phase under colder conditions is not present in Ceratitis (Majercak et al., 1999, 2004; Collins et al., 2004). Other Drosophila species such as D. pseudoobscura and D. virilis do not have the same 3' thermosensitive per splicing mechanism (B. Collins & C. P. Kyriacou, unpublished observations), so as Ceratitis generally inhabits warmer regions than the cosmopolitan D. melanogaster, it is perhaps not altogether surprising that Ceratitis does not have such a mechanism. This hypothesis seems to be sustained also by the ability of the chimeric period transgene to drive the temperature-dependent modulations of the evening component of LD activity. As the chimeric transgene carries the D. melanogaster thermally regulated 3' intron (Majercak et al., 1999, 2004; Collins et al., 2004), we might imagine that in transformant flies the splicing of this intron is controlled efficiently at level of the dorsal lateral neurons (LN\(_{\text{DH}}\)), which drive the evening peak in 12:12 LD conditions (Grima et al., 2004; Stoleru et al., 2004). Moreover, we also observed a temperature-sensitive modulation of morning activity in wild-type flies. While this phenomenon has not been commented on previously, we have detected in the data of both Majercak et al., 1999 (see their figure 1A) and Collins et al. 2004 (see their figure 4) similar thermally regulated morning behaviour in D. melanogaster. However, this is not carried over into the pMC1 transformants. It would therefore be of considerable interest to examine whether the 3' per splicing is relevant to this morning peak modulation in D. melanogaster.
The transformant lines carrying CePER, like those carrying A. pernyi and D. pseudoobscura per (Petersen et al., 1988; Levine et al., 1995; Peixoto et al., 1998), did not give robust rescue of rhythms in arrhythmic mutant pernull in DD. This could be due to the fact that the Ceratitis per cDNA was used, so regulatory regions that might be present in intronic sequences were lacking. Alternatively, the poor level of rescue might imply an alteration of the interactions of CePER with the Drosophila nuclear translocation and/or phosphorylation machinery(ies). Nevertheless, in the transgenic line in which rhythmicity was re-established, a bimodal locomotor activity was observed at 18 °C, with a clear subjective morning peak which was reminiscent of Ceratitis diurnal activity, but not D. melanogaster behaviour. While this result taken in isolation might be considered as not very significant or relevant, the fact that D. pseudoobscura per transfers the D. pseudoobscura species-specific behavioural profile to D. melanogaster hosts (Petersen et al., 1988; Tauber et al., 2003) suggests that a similar phenomenon is occurring with Ceratitis per. This effect is not seen as dramatically at higher temperatures, where the transformants appear to show a generally intermediate phenotype between those of the parental species. This suggests that either the transgene conveys basic rhythmicity to the host which is not strong enough to provide a fully wild-type behavioural profile, or that the transgene conveys species-specific aspects of locomotor patterns but is not robust enough to fully ‘convert’ the host to a Ceratitis pattern.

The study of such transgenes in the heterospecific host offers opportunities to examine the evolution of gene regulation, because unlike de novo mutants, which are usually identified and isolated because of their dramatic effects on the phenotype, their phenotypes are usually much more subtle. This is because the gene or protein under study has coevolved with its partner molecules under natural selection, and changed its interactions with these partners along the different lineages. Thus, unlike an amorph, which renders its normal molecular interactions null and void, an interspecific transgene would probably maintain many of these interactions but at different levels, thereby providing a paradigm by which to test the evolution of these interactions.

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