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Genomic identification of a putative circadian system in the cladoceran crustacean *Daphnia pulex*

Andrea R. Tilden[1,*], **Matthew D. McCoole**[2], **Sarah M. Harmon**[3], **Kevin N. Baer**[2], and **Andrew E. Christie**[3,*]

[1]Department of Biology, Colby College, 5720 Mayflower Hill, Waterville, Maine 04901, USA

[2]College of Pharmacy, Department of Toxicology, University of Louisiana at Monroe, 700 University Avenue, Monroe, Louisiana 71209 USA

[3]Neuroscience Program, John W. and Jean C. Boylan Center for Cellular and Molecular Physiology, Mount Desert Island Biological Laboratory, P.O. Box 35, Old Bar Harbor Road, Salisbury Cove, Maine 04672 USA

Abstract

Essentially nothing is known about the molecular underpinnings of crustacean circadian clocks. The genome of *Daphnia pulex*, the only crustacean genome available for public use, provides a unique resource for identifying putative circadian proteins in this species. Here, the *Daphnia* genome was mined for putative circadian protein genes using *Drosophila melanogaster* queries. The sequences of core clock (*e.g.* CLOCK, CYCLE, PERIOD, TIMELESS and CRYPTOCHROME 2), clock input (CRYPTOCHROME 1) and clock output (PIGMENT DISPERSING HORMONE RECEPTOR) proteins were deduced. Structural analyses and alignment of the *Daphnia* proteins with their *Drosophila* counterparts revealed extensive sequence conservation, particularly in functional domains. Comparisons of the *Daphnia* proteins with other sequences showed that they are, in most cases, more similar to homologs from other species, including vertebrates, than they are to those of *Drosophila*. The presence of both CRYPTOCHROME 1 and 2 in *Daphnia* suggests the organization of its clock may be more similar to that of the butterfly *Danaus plexippus* than to that of *Drosophila* (which possesses CRYPTOCHROME 1 but not CRYPTOCHROME 2). These data represent the first description of a putative circadian system from any crustacean, and provide a foundation for future molecular, anatomical and physiological investigations of circadian signaling in *Daphnia*.

[*]Correspondence to either: Dr. Andrea R. Tilden, Department of Biology, Colby College, 5720 Mayflower Hill, Waterville, ME 04901 USA. Phone: 207-859-5743; FAX: 207-859-5705, atilden@colby.edu. Dr. Andrew E. Christie, John W. and Jean C. Boylan Center for Cellular and Molecular Physiology, Mount Desert Island Biological Laboratory, P.O. Box 35, Old Bar Harbor Road, Salisbury Cove, ME 04672 USA. Phone: 207-288-9880 ext. 284; FAX: 207-288-2130; achristi@mdibl.org.

Keywords

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1. Introduction

Virtually all organisms exhibit physiological and behavioral rhythms oscillating with a period of approximately 24-h. Regardless of species, these physiological/behavioral patterns, commonly referred to as circadian rhythms, are characterized by four basic properties (Allada and Chung, 2010): I. they persist under constant conditions (indicating the presence of a self-sustaining clock), II. the clock-driven activity reoccurs approximately every 24-h, III. the activity pattern is entrained by the solar day, and IV. the period of the activity, while sensitive to changes in environmental conditions, is stable over a wide range of temperatures. In addition, all circadian systems have three functional components (Allada and Chung, 2010): I. a core clock, which is responsible for time keeping, II. input pathways that act to synchronize the clock to the environment, and III. output pathways that transmit the timing information for the control of physiology and behavior (see Table 1 for the proteins in each category). Depending on the system in question, the cellular location of these components may be distinct or contained within a common locus.

While a number of species have been the subjects of investigations into the molecular mechanisms underlying the core circadian clock, perhaps the best studied are insects, and in particular, the fruit fly *Drosophila melanogaster* (Allada and Chung, 2010; Tomioka and Matsumoto, 2010). In *Drosophila*, work from many laboratories has elucidated several interacting molecular feedback loops, which form the core of a molecular clock. As recently reviewed by Allada and Chung (2010), a heterodimer formed by the CLOCK (CLK) and CYCLE (CYC) proteins binds to E-box elements in the promoter regions of the *period* (*per*) and *timeless* (*tim*) genes, activating their transcription (typically peaking late in the day). Due to this activation, PERIOD (PER) and TIMELESS (TIM) proteins are produced, accumulate and dimerize in the cytoplasm during the early evening hours, are translocated to the nucleus at approximately midnight, ultimately binding to the CLK/CYC heterodimer. The binding of the PER/TIM heterodimer to CLK/CYC inhibits this complex's DNA binding to, and hence activation of, the *per* and *tim* genes during the late night.

In addition to the core clock proteins CLK, CYC, PER and TIM, a number of others are also involved in the control of the core clock feedback loop of *Drosophila* (Allada and Chung, 2010). Specifically, PER, TIM and CLK each exhibit rhythmic phosphorylation, with the peak in this state occurring in the late night or early day; PER is phosphorylated by casein kinase Iε (DOUBLETIME [DBT]) and CASEIN KINASE II (CKII), while TIM is phosphorylated by GLYCOGEN SYNTHASE KINASE 3B (SHAGGY [SGG]) and CKII. CLK is phosphorylated by a nuclear complex of PER and DBT. The phosphorylation of PER is known to enhance its repressor activity. In addition, phosphorylated PER and TIM are targets of the phosphatases PROTEIN PHOSPHATASE 2A (PP2A) and PROTEIN PHOSPHATASE 1 (PP1), the former of which is believed to be involved in generation of PER's phosphorylation rhythm. The peak in phosphorylation of these proteins is known to

precede their disappearance, which at least partially involves the ubiquitination of DBT-phosphorylated PER (and its resulting degradation via the ubiquitin-proteasome pathway) by the E3 ubiquitin ligase SUPERNUMERARY LIMBS (SLIMB); the proteolysis of PER removes repression of the CLK/CYC complex allowing for a new cycle of *per* and *tim* transcription. TIM is the target for CYPTOCHROME (CRY), a cell autonomous blue-light photoreceptor protein, which triggers its degradation (in the remainder of this paper *Drosophila*-type CRY is referred to as CRY1 to distinguish it from vertebrate-type CRY or CRY2, which is present both with and without CRY1 in non-drosophalid insects; *e.g.* Yuan et al., 2007).

In addition to their roles in regulating the PER-TIM feedback loop, the CLK/CYC heterodimer also activates several other interdependent feedback loops that are hypothesized to play roles in setting the phase and amplitude of the *Drosophila* core clock, as well as its rhythmic output (Allada and Chung, 2010). Specifically, CLK/CYC bind to E-box elements in the promoters of the *par domain protein 1* (*pdp1*) and *vrille* (vri) genes, activating their transcription. In turn, the PAR DOMAIN PROTEIN 1 (PDP1) and VRILLE (VRI) proteins activate and repress, respectively, the transcription of the *clock* (*clk*) and *cryptochrome* (*cry*) genes. Because the accumulation of PDP1 is delayed relative to that of VRI, the rhythms of *clk* and *cry* activation are antiphase (peaking in early day) to those of *per* and *tim*. In addition the CLK/CYC heterodimer also activates transcription of the *clockwork orange* (*cwo*) gene. The CLOCKWORK ORANGE (CWO) protein, a basic helix-loop-helix (HLH) repressor, in turn targets the E-box elements of CLK/CYC target genes, repressing their activation.

Interestingly, while the *Drosophila* circadian system is arguably the best understood in the animal kingdom, it may not be stereotypical, even within insects (*e.g.* Zhu et al., 2005; Yuan et al., 2007; Zhu et al., 2008). Based on the complement of CRYs present, several models have been proposed for clock systems in insects (Yuan et al., 2007). Specifically, whereas *D. melanogaster* possesses a single CRY, in many insects, two CRYs have been identified, one similar to that of *Drosophila*, commonly referred to as dCRY or CRY1, and the other similar to that present in vertebrates, commonly referred to as CRY2; in several insects, only CRY2 has been found. In essentially all systems where it is present, CRY1 is proposed as a photosensitive input to the clock, providing a mechanism for entraining the clock to the solar day (Yuan et al., 2007). In contrast, CRY2 does not appear to play a role in photic entrainment, but rather appears to be a core clock protein, functioning as a repressor of CLK/CYC-mediated transcription (*e.g.* Zhu et al., 2005; Yuan et al., 2007; Zhu et al., 2008). Thus, in *Drosophila*, CRY likely functions solely as an input to the clock system, whereas in other insects members of the CRY family appear to serve both as inputs to the clock (CRY1) and as members of the core clock ensemble itself (CRY2); in insects with only CRY2, novel photic entrainment pathways are hypothesized, with CRY2 proposed to function primarily, perhaps solely, as a transcriptional repressor (*e.g.* Zhu et al., 2005; Yuan et al., 2007; Zhu et al., 2008). Evolutionary studies of CRY gene duplication and loss suggest that the clock system possessing both CRY1 and CRY2 is the most ancestral organization (Yuan et al., 2007).

As in other organisms, many crustaceans are known to display circadian patterns in physiology and behavior. As recently reviewed by Strauss and Dircksen (2010), known/postulated crustacean circadian behaviors include, but are not limited to, locomotion, feeding, moulting, reproduction, hatching/larval release, color change, and diel vertical migration. Interestingly, and despite the rich repertoire of circadian rhythms exhibited by crustaceans, essentially nothing is known about the molecular underpinnings of circadian clocks in these animals. While many laboratories have attempted to molecularly clone crustacean circadian proteins via reverse transcription polymerase chain reaction using degenerate primers, only two putative circadian proteins have thus far been identified and characterized from crustaceans, *i.e.* a putative homolog CLK from the freshwater prawn *Macrobrachium rosenbergii* (Yang et al., 2006) and a CRY homolog from the Antarctic krill *Euphausia superba* (Mazzotta et al., 2010).

The recent sequencing of the genome of the cladoceran crustacean *Daphnia pulex* provides an alternative avenue for identifying putative crustacean homologs of known insect circadian proteins, namely identification via genome mining; members of the genus *Daphnia*, like many other planktonic crustaceans, are known to exhibit pronounced diel migratory behaviors (*e.g.* Lampert, 1989; Loose, 1993; Loose and Dawidowicz, 1994). In the study presented here, we have used such a strategy to predict a large suite of *D. pulex* proteins that show significant homology to those that form the molecular underpinnings of the *D. melanogaster* circadian clock. Structural analysis of the identified proteins, which include, among others, putative homologs of PER, TIM, CLK and CYC, revealed that essentially all contain the domains known to be required for function in the fruit fly. Moreover, putative homologs of both CRY1 and CRY2 were identified, suggesting that the clock system of *Daphnia* is organized more like that proposed for lepidopterans and mosquitoes, than it is to the *Drosophila* system (Yuan et al., 2007), *i.e.* CRY1 acting as a circadian photoreceptor to the clock and CRY2 participating in the establishment of the core clock itself. In addition, a protein likely involved in mediating the output signaling of the clock, *i.e.* a receptor for pigment dispersing hormone, was identified and characterized. Taken collectively, the data presented here represent the first description of a putative circadian system from any crustacean, and provide a foundation for future molecular, anatomical and physiological investigations of circadian signaling in *D. pulex* and other crustacean species.

2. Materials and methods

2.1. Genome sequencing and gene modeling

For current descriptions of the preparation, sequencing and modeling of the *D. pulex* genome, readers are referred to http://wfleabase.org/ (Colbourne et al., 2005; 2011), which is maintained by the Indiana University Genome Informatics Laboratory (Indiana University, Bloomington, IN, USA).

2.2. Genome mining

Genome mining was accomplished using BLAST+ 2.2.23 software (downloadable from the National Center for Biotechnology Information, Bethesda, MD, USA; ftp://

ftp.ncbi.nlm.nih.gov/blast/executables/blast+/) and the beta-release of the *D. pulex* Genes 2010 frozen genome assembly (Indiana University Genome Informatics Laboratory, and Center for Genomics and Bioinformatics at Indiana University, Bloomington, IN, USA; http://wfleabase.org/) as described in several earlier publications (Christie et al., 2011; McCoole et al., 2011); *D. melanogaster* proteins were used to query the genome. For all searches, the BLAST score and BLAST-generated E-value for significant alignment are provided in Table 1. To strengthen our gene identifications, the sequence of the *Daphnia* protein deduced from each gene was reciprocally blasted against the *Drosophila* proteins curated in FlyBase (Tweedie et al., 2009); the results of these analyses are shown in Table 2. In addition, each protein was blasted against all non-redundant protein sequences curated at NCBI (excluding *Daphnia* proteins, obvious partial proteins, synthetic constructs and provisional protein sequences) using the online program protein blast (blastp algorithm used; http://blast.ncbi.nlm.nih.gov/Blast.cgi); the top five hits for each protein are shown in Table 3.

2.3. Analyses of protein structure

Analyses of protein structural motifs were accomplished using the online program SMART (http://smart.embl-heidelberg.de/ [Schultz et al., 1998; Letunic et al., 2009]) and homology to the structural motifs of previously described insect circadian proteins, predominantly ones from *D. melanogaster*. Alignment of all proteins shown in our figures was done using the online program MAFFT version 6 (http://align.bmr.kyushu-u.ak.jp/mafft/online/server/; [Katoh and Toh, 2008]). Amino acid identity was calculated as number of identical amino acids (denoted by [*]) divided by the total number of amino acids in the longest sequence, while amino acid similarity was calculated as number of identical and similar amino acids (the latter denoted by the [:] and [.] symbols in the protein alignments) divided by the total number of amino acids in longest sequence.

2.4. Figure production

Alignments generated in MAFFT were copied and pasted into Microsoft Word, and the structural domains identified by SMART analyses, colored using this program. For all figures, a common coloring scheme was used to highlight each structural domain: serine/threonine kinase catalytic, red; HLH, green; PAS, light blue; PAC, blue; coiled-coil, pink; orange, yellow; basic region leucine zipper, dark blue; protein phosphatase 2A, dark green; WD40, dark red; FBOX, dark gray; transmembrane, light gray; hormone receptor, black.

3. Results

As stated in Section 1, all known circadian systems are composed of three functional components: a core clock, input pathways that act to synchronize the clock to the environment, and output pathways that transmit timing information. With one exception, the results of genome searches and protein analyses are grouped according their putative role within the theoretical *Daphnia* circadian system, *i.e.* core clock proteins, input pathway proteins, or output pathway proteins; the results for CRY are presented under “input pathway proteins”, though, in some species, family members also serve as key components of the core clock as well. Within each of these grouping, data are presented in alphabetical

order based on the *Drosophila* protein name. It should be noted that the *Daphnia* protein sequences reported in this study are based on the Genes 2010 gene model algorithm, which, in a previous study (Christie et al., 2011), was found to typically have the best fit with the extant *D. pulex* transcriptome data, at least for peptide precursor protein genes. This said, other gene model algorithms (*i.e.* JGI, Gnomon, PASA and SNAP) did in some cases predict slightly different protein sequences from those shown here, and readers should take heed of this and treat the sequences presented as theoretical rather than biochemically-confirmed.

3.1. Core clock proteins

3.1.1. CASEIN KINASE II (CKII)

3.1.1.1. CKII α-subunit: A single *D. pulex* gene (*dappu-ckII α*) was identified as encoding a putative CKII α-subunit protein via a query using a *D. melanogaster* CKII α (Accession no. **AAN11415**; Adams et al., 2000). The Genes 2010 gene model shows *dappu-ckII α* to be located on Scaffold 17 of the genome, with a predicted length of 3693 nucleotides (Table 1).

Figure 1A shows the alignment of the protein deduced from *dappu-ckII α* (Dappu-CKII α; 365 amino acids in overall length) with that of the *Drosophila* query (336 amino acids long).

Comparison of the sequence of Dappu-CKII α with that of Drome-CKII α revealed 81.1% amino acid identity/89.6 % amino acid similarity between the two proteins. SMART analyses of Dappu-CKII α and Drome-CKII α identified a single, highly conserved (93.4% identical/97.9% similar) serine/threonine kinase catalytic domain within each protein (Fig. 1A).

Reciprocal blasting of Dappu-CKII α against all proteins curated in FlyBase revealed CKII α (Flybase no. **FBpp0070043**) as the *D. melanogaster* protein most similar to the *Daphnia* query (Table 2). Interestingly, blastp comparison of Dappu-CKII α with all non-redundant protein sequences curated by NCBI revealed the catalytic subunit of human CK II (**1NA7_A**) to be the most similar protein match (Table 3); the remaining top five blastp hits are all insect CKII α proteins, though *Drosophila* CKII α is not among them (Table 3).

3.1.1.2. CKII β-subunit: A single *D. pulex* gene (*dappu-ckII β*) was identified as encoding a putative CKII β-subunit protein via a query using a *D. melanogaster* CKII β (**AAF48093**; Adams et al., 2000). The Genes 2010 gene model shows *dappu-ckII β* to be located on Scaffold 41 of the genome, with a predicted length of 2093 nucleotides (Table 1).

Figure 1B shows the alignment of the protein deduced from *dappu-ckII β* (Dappu-CKII β; 221 amino acids in overall length) with that of the *Drosophila* query (215 amino acids long). Comparison of the sequence of Dappu-CKII β with that of Drome-CKII β revealed 84.2% amino acid identity/94.6 % amino acid similarity between the two proteins. No functional domains were identified in either Dappu-CKII β or Drome-CKII β via SMART analysis.

Reciprocal blasting of Dappu-CKII β against all proteins curated in FlyBase identified CKII β (Flybase No. **FBpp0089135**) as the *D. melanogaster* protein most similar to the *Daphnia* query (Table 2). Interestingly, and similar to the result found for Dappu-CKII α, a human CKII β protein (**CAI18393**) was found to have the highest homology score when Dappu-

CKII β was blasted against all non-redundant protein sequences curated by NCBI (Table 3). Perhaps even more surprising was the finding that none of the remaining top five blastp hits from the NCBI database were from insects. In fact, only one invertebrate protein, a CKII β from the bivalve mollusc *Mytilus galloprovincialis* (**CBK3891**), was among these hits (Table 3).

3.1.2. CLOCK (CLK)—A single *D. pulex* gene (*dappu-clk*) was identified as encoding a putative CLK protein via a query using a *D. melanogaster* CLK (**AAC62234**; Bae et al., 1998). The Genes 2010 gene model shows *dappu-clk* to be located on Scaffold 27 of the genome, with a predicted length of 5939 nucleotides (Table 1).

Figure 2A shows the alignment of the protein deduced from *dappu-clk* (Dappu-CLK; 890 amino acids in overall length) with that of the *Drosophila* query (1027 amino acids long). Comparison of the sequence of Dappu-CLK with that of Drome-CLK revealed 30.8% amino acid identity/57.4% amino acid similarity between the two proteins. SMART analyses of Dappu-CLK and Drome-CLK identified similar, though not identical, sets of structural domains within each protein. Specifically, both Dappu-CLK and Drome-CLK are predicted to contain a single HLH domain, two PAS domains, and a single PAC domain (Fig. 2A). In addition, Drome-CLK is predicted to contain three coiled-coil regions; this motif is absent in Dappu-CLK (Fig. 2A). For those domains that are shared between Dappu-CLK and Drome-CLK, extensive amino acid conservation is evident: HLH, 60.8% identity/96.1 % similarity; PAS1, 43.3 % identity/80.6 % similarity; PAS2, 73.1 % identity/92.5 % similarity; PAC, 70.5 % identity/93.2 % similarity. Essentially no conservation of sequence is seen between the two proteins in any of the coiled-coil regions (Fig. 2A).

As stated in Section 1, CLK is one of the few circadian proteins for which a putative crustacean family member has been identified, *i.e. M. rosenbergii* CLK (Yang et al., 2006). Comparison of the sequences of Dappu-CLK and Macro-CLK (**AAX44045**; Yang et al., 2006), revealed a level of amino acid identity/similarity similar to that seen for Dappu-CLK and Drome-CLK (*i.e.* 30.4 % identity/56.0 % similarity; Fig. 2B). As for Dappu-CLK and Drome-CLK, SMART analysis of Macro-CLK identified single HLH domain, two PAS domains, and a single PAC domain within this protein (Fig. 2B). In addition, this analysis identified a single coiled-coil region in Macro-CLK (Fig. 2B). Comparison of the HLH, PAS and PAC domains of Macro-CLK and Dappu-CLK revealed slightly higher levels of conservation to those reported above for Drome-CLK and Dappu-CLK (HLH, 62.7% identity/100 % similarity; PAS1, 60.0% identity/85.0% similarity; PAS2, 80.0% identity/ 95.0% similarity; PAC, 81.8% identity/93.2% similarity). Little sequence conservation is seen between the *Macrobrachium* and *Daphnia* CLKs in the coiled-coil region of the former protein.

Reciprocal blasting of Dappu-CLK against the proteins curated in FlyBase identified CLK (Flybase no. **FBpp0076500**) as the *D. melanogaster* protein most similar to the *Daphnia* query (Table 2). Comparison of Dappu-CLK with all non-redundant protein sequences curated by NCBI revealed the top five blastp hits to be insect CLK sequences, with a CLK from the firebrat *Thermobia domestica* (**BAJ16353**) showing the highest similarity (Table 3); *Drosophila* CLK was not among these proteins (Table 3).

3.1.3. CLOCKWORK ORANGE (CWO)—A single *D. pulex* gene (*dappu-cwo*) was identified as encoding a putative CWO protein via a query using a *D. melanogaster* CWO (**AAF54527**; Adams et al., 2000). The Genes 2010 gene model shows *dappu-cwo* to be located on Scaffold 19 of the genome, with a predicted length of 3773 nucleotides (Table 1).

Figure 3 shows the alignment of the protein deduced from *dappu-cwo* (Dappu-CWO; 809 amino acids in overall length) with that of the *Drosophila* query (698 amino acids long). Comparison of the sequence of Dappu-CWO with that of Drome-CWO revealed 23.9% amino acid identity/51.4% amino acid similarity between the two proteins. SMART analyses of Dappu-CWO and Drome-CWO identified a single HLH and a single orange domain within each protein (Fig. 3); high levels of amino acid conservation were evident when the *Daphnia* domains were compared to their *Drosophila* counter parts (HLH, 78.2% identity/ 92.7% similarity; ORANGE, 37.5% identity/85% similarity).

Reciprocal blasting of Dappu-CWO against all proteins curated in FlyBase identified CWO (Flybase No. **FBpp0081723**) as the *D. melanogaster* protein most similar to the *Daphnia* query. Moreover, blastp comparison of Dappu-CWO with all non-redundant protein sequences curated by NCBI revealed *D. melanogaster* CWO (**AAF54527**) as most similar to this protein as well (Table 3); three of the top five blastp hits are *D. melanogaster* sequences, the remaining two are also insect proteins (Table 3).

3.1.4. CYCLE (CYC)—A single *D. pulex* gene (*dappu-cyc*) was identified as encoding a putative CYC protein via a query using a *D. melanogaster* CYC (**AAF49107**; Adams et al., 2000). The Genes 2010 gene model shows *dappu-cyc* to be located on Scaffold 1 of the genome, with a predicted length of 6473 nucleotides (Table 1).

Figure 4 shows the alignment of the protein deduced from *dappu-cyc* (Dappu-CYC; 654 amino acids in overall length) with that of the *Drosophila* query (413 amino acids long). Comparison of the sequence of Dappu-CYC with that of Drome-CYC revealed 33.9% amino acid identity/49.7% amino acid similarity between the two proteins. SMART analyses of Dappu- and Drome-CYC identified a single HLH domain, two PAS domains and a single PAC domain in each protein (Fig. 4). Comparisons of the *D. pulex* domains with those of *D. melanogaster* show high levels of amino acid conservation in these portions of the proteins: HLH, 71.4 % identity/95.2 % similarity; PAS1, 75.0 % identity/94.1 % similarity; PAS2, 59.7 % identity/91.9 % similarity; PAC, 53.8% identity/94.9 % similarity.

Reciprocal blasting of Dappu-CYC against all proteins curated in FlyBase identified CYC (Flybase no. **FBpp0074693**) as the *D. melanogaster* protein most similar to the *Daphnia* query (Table 2). Comparison of Dappu-CYC with all non-redundant protein sequences curated by NCBI identified a CYC from the firebrat *T. domestica* (**BAJ16354**) to be the most similar protein match (Table 3); no *Drosophila* proteins were among the top five blastp hits (Table 3).

3.1.5. DOUBLETIME (DBT)—A single *D. pulex* gene (*dappu-dbt*) was identified as encoding a putative DBT protein via a query using a *D. melanogaster* DBT (**AAF57110**;

Adams et al., 2000). The Genes 2010 gene model shows *dappu-dbt* to be located on Scaffold 1 of the genome, with a predicted length of 2124 nucleotides (Table 1).

Figure 5 shows the alignment of the protein deduced from *dappu-dbt* (Dappu-DBT; 409 amino acids in overall length) with that of the *Drosophila* query (440 amino acids long). Comparisons of the sequence of Dappu-DBT with Drome-DBT revealed 61.4% amino acid identity/78.4% amino acid similarity between the two proteins. SMART analyses of Dappu-DBT and Drome-DBT identified a serine/threonine kinase domain in each protein, the sequences of which were nearly identical, *i.e.* 82.6% amino acid identity/96.2% amino acid similarity (Fig. 5).

Reciprocal blasting of Dappu-DBT against all proteins curated in FlyBase identified DBT (Flybase no. **FBpp0085106**) as the most similar *D. melanogaster* protein to the *Daphnia* query (Table 2). Comparison of Dappu-DBT with all non-redundant protein sequences curated by NCBI revealed a casein kinase Iε (an alternative name for DBT) from the ant *Camponotus floridanus* (**EFN64010**) to be most similar to *Daphnia* DBT (Table 3); no *Drosophila* proteins were among the top five blastp hits (Table 3).

3.1.6. PAR DOMAINE PROTEIN 1ε (PDP1ε)—A single *D. pulex* gene (*dappu-pdp1ε*) was identified as encoding a putative PDP1ε protein via a query using a *D. melanogaster* PDP1ε (**AAF04509**; Lin et al., 1997). The Genes 2010 gene model shows *dappu-pdp1ε* to be located on Scaffold 21 of the genome, with a predicted length of 4794 nucleotides (Table 1).

Figure 6 shows the alignment of the protein deduced from *dappu-pdp1ε* (Dappu-PDP1ε; 350 amino acids in overall length) with that of the *Drosophila* query (351 amino acids long). Comparisons of the sequence of Dappu-PDP1ε with that of Drome-PDP1ε revealed 39.6% amino acid identity/63.5% amino acid similarity between the two proteins. SMART analyses of Dappu-PDP1ε and Drome-PDP1ε identified a single basic region leucine zipper domain in each protein, which were 67.7% identical/81.5% similar in amino acid composition (Fig. 6).

Reciprocal blasting of Dappu-PDP1ε against all proteins curated in FlyBase identified PDP1 (Flybase No. **FBpp0076495**) as the *D. melanogaster* protein most similar to the *Daphnia* query (Table 2). Interestingly, comparison of Dappu-PDP1ε with all non-redundant protein sequences curated by NCBI identified a rat protein (**EDM05669**), likely a PDP1, to be most similar to Dappu-PDP1ε (Table 3), though the next three of the top five blastp hits were *D. melanogaster* PDP1 isoforms (Table 3).

3.1.7. PERIOD (PER)—A single *D. pulex* gene (*dappu-per*) was identified as encoding a putative PER protein via a query using a *D. melanogaster* PER (**AAF45804**; Adams et al., 2000). The Genes 2010 gene model shows *dappu-per* to be located on Scaffold 47 of the genome, with a predicted length of 5860 nucleotides (Table 1).

Figure 7 shows the alignment of the protein deduced from *dappu-per* (Dappu-PER; 1286 amino acids in overall length) with that of the *Drosophila* query (1218 amino acids long).

Comparisons of the sequence of Dappu-PER with Drome-PER revealed 27.4% amino acid identity/59.6% amino acid similarity between the two proteins. SMART analyses of Dappu-PER and Drome-PER identified two PAS domains in each protein (Fig. 7), both of which showed considerable amino acid conservation between the two proteins: PAS-1, 50.0% amino acid identity/82.3% amino acid similarity; PAS-2, 47.3% amino acid identity/84.2% amino acid similarity. In addition, SMART analysis identified a PAC domain within the Drome-PER (Fig. 7) but not in Dappu-PER. Interestingly, the portion of Dappu-PER that overlaps with the *Drosophila* PAC domain is 72.7% identical/93.2% similar in amino acid composition to that of the *Drosophila* protein (Fig. 7).

Reciprocal blasting of Dappu-PER against the proteins curated in FlyBase identified PER (Flybase No. **FBpp0070455**) as the *D. melanogaster* protein most similar to the *Daphnia* query (Table 2). Moreover, blastp comparison of Dappu-PER with all non-redundant protein sequences curated by NCBI showed the top five hits to be insect PER proteins (Table 3), with an isoform from the cockroach *Blattella germanica* (**AAN02439**; Table 3) exhibiting the highest similarity.

3.1.8. PROTEIN PHOSPHATASE 1 (PP1)—Two *D. pulex* genes (*dappu-pp1 a* and *dappu-pp1 b*) were identified as encoding putative PP1 proteins via a query using a *D. melanogaster* PP1 (**CAA39820**; Dombradi et al., 1990). The Genes 2010 gene model shows *dappu-pp1 a* and *dappu-pp1 b* to be located on Scaffolds 145 and 12 of the genome, respectively, with lengths of 2591 and 1972 nucleotides (Table 1).

Figure 8A shows the alignment of the protein deduced from *dappu-pp1 a* (Dappu-PP1 A; 332 amino acids in overall length) with that of the *Drosophila* query (327 amino acids long). Comparison of the sequences of Dappu-PP1 A with Drome-PP1 revealed 84.0% amino acid identity/90.7% amino acid similarity between the two proteins. Comparison of the sequence of Dappu-PP1 B (325 amino acids in overall length) with that of the *Drosophila* query with that of Drome-PP1 revealed a similar level of amino acid conservation, *i.e.* 81.3% identity/89.6% similarity (alignment not shown). Figure 8B shows the alignment of the two *Daphnia* PP1s with one another. As can be seen from this panel, the two proteins are nearly identical in amino acid sequences (84.3% identity/96.9% similarity in amino acid composition). SMART analyses of Dappu-PP1 A, Dappu-PP1 B, and Drome-PP1 identified a single serine/threonine protein kinase domain in each protein (Fig. 8A–B). The serine/threonine protein kinase domain in each of the *Daphnia* proteins is nearly identical to that present in their *Drosophila* counterpart: Dappu-PP1 A vs. Drome-PP1, 95.9% amino acid identity/100% amino acid similarity; Dappu-PP1 B vs. Drome-PP1, 89.6% amino acid identity/99.3% amino acid similarity. Similarly, this domain is highly conserved between the two *Daphnia* proteins (91.5% identity/98.5% similarity).

Reciprocal blasting of Dappu-PP1 A and B against all proteins curated in FlyBase identified isoforms of PP1 (Flybase nos. **FBpp0084026** and **FBpp0071382**, respectively; Table 2) as the most similar *D. melanogaster* proteins to the *Daphnia* queries. Comparison of the Dappu-PP1s with all non-redundant protein sequences curated by NCBI identified a serine/threonine-protein phosphatase alpha-1 isoform (of which PP1 is a family member) from the ant *C. floridanus* (**EFN69572**) to possess the highest similarity score to Dappu-PP1 A

(Table 3), with a PP1 from the zebra fish *Danio rerio* (**CAD61270**) being most similar to Dappu-PP1 B (Table 3); no *Drosophila* proteins were among the top five blastp hits for either of the Dappu-PP1s (Table 3).

3.1.9. PROTEIN PHOSPHATASE 2A (PP2A)

3.1.9.1. PP2A catalytic subunit – MICROTUBULE STAR (MTS): A single *D. pulex* gene (*dappu-mtr*) was identified as encoding a putative PP2A catalytic subunit protein (MTS) via a query using a *D. melanogaster* MTS sequence (**AAF52567;** Adams et al., 2000). The Genes 2010 gene model shows *dappu-mts* to be located on Scaffold 13 of the genome, with a predicted length of 2099 nucleotides (Table 1).

Figure 9A shows the alignment of the protein deduced from *dappu-mts* (Dappu-MTS; 308 amino acids in overall length) with that of the *Drosophila* query (309 amino acids long). Comparisons of the sequence of Dappu-MTS with Drome-MTS revealed 64.4% amino acid identity/90.3% amino acid similarity between the two proteins. SMART analyses of Dappu-MTS and Drome-MTS identified a single protein phosphatase 2A catalytic domain in each protein (Fig. 9A). Comparison of the sequences of these domains revealed 66.9% amino acid identity/92.6% amino acid similarity between these two regions of the proteins (Fig. 9A).

Reciprocal blasting of Dappu-MTS against all proteins curated in FlyBase identified MTS (Flybase no. **FBpp0077017**) as the *D. melanogaster* protein most similar to the *Daphnia* query (Table 2). Comparison of Dappu-MTS with all non-redundant protein sequences curated by NCBI revealed a serine/threonine-protein phosphatase 4 catalytic subunit from the ant *Harpegnathos saltator* (**EFN85419**) to be most similar to *Daphnia* MTS (Table 3); no *Drosophila* proteins were among the top five blastp hits (Table 3).

3.1.9.2. PP2A regulatory subunit

3.1.9.2.1. WIDERBORST (WBT): Two *D. pulex* genes (*dappu-wbt a* and *dappu-wbt b*) were identified as encoding putative PP2A regulatory subunit proteins (WBTs) via a query using a *D. melanogaster* WBT sequence (**AAF56720;** Adams et al., 2000). The Genes 2010 gene model shows *dappu-wbt a* and *b* to be located on Scaffolds 8 and 2 of the genome, respectively, with a predicted lengths of 6199 and 4882 nucleotides (Table 1).

Figure 9B1 shows the alignment of the protein deduced from *dappu-wbt a* (Dappu-WBT A; 481 amino acids in overall length) with that of the *Drosophila* query (524 amino acids long). Comparisons of the sequence of Dappu-WBT with Drome-WBT revealed 73.7% amino acid identity/85.5% amino acid similarity between the two proteins. Alignment of Dappu-WBT B with Drome-WBT revealed a lower level of amino acid conservation between these two proteins, 52.6% identity/74.1% similarity (alignment not shown). Figure 9B2 shows the alignment of the two *Daphnia* WBTs; these proteins are 50.5% identical/69.7% similar in amino acid sequence. SMART analyses of Dappu-WBT A identified a single coiled-coil domain; this domain was not predicted by SMART analyses in either Dappu-WBT B or Drome-WBT, though in the former protein this region is 45.5% identical/69.7% similar in

amino acid composition to that of Dappu-WBT A and in the latter protein 72.7%identical/ 97.0% similar to that of Dappu-WBT A (Fig. 9).

Reciprocal blasting of Dappu-WBT A and B against all proteins curated in FlyBase identified PP2A regulatory subunit isoforms (Flybase nos. **FBpp0084579** and **FBpp0288759**, respectively; Table 2) as the most similar *D. melanogaster* proteins to the Daphnia queries. Comparison of the Dappu-WBTs with all non-redundant protein sequences curated by NCBI identified serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit isoforms from the ant *C. floridanus* to show the highest similarity scores to each of Dappu-WBTs (Accession nos. **EFN66909** and **EFN69797**, respectively; Table 3); while a *Drosophila* isoform of WBT was among the top five blastp hits for Dappu-WBT A, none were among the top hits for Dappu-WBT B (Table 3).

3.1.9.2.2. TWINS (TWS): A single *D. pulex* gene (*dappu-tws*) was identified as encoding a putative PP2A regulatory subunit protein (TWS) via a query using a *D. melanogaster* TWS sequence (**AAF54498;** Adams et al., 2000). The Genes 2010 gene model shows *dappu-tws* to be located on Scaffold 43 of the genome, with a predicted length of 4157 nucleotides (Table 1).

Figure 9C shows the alignment of the protein deduced from *dappu-tws* (Dappu-TWS; 443 amino acids in overall length) with that of the *Drosophila* query (499 amino acids long). Comparisons of the sequence of Dappu-TWS with Drome-TWS revealed 71.5% amino acid identity/83.6% amino acid similarity between the two proteins. SMART analyses of Dappu-TWS and Drome-TWS identified six and seven WD40 domains in these proteins, respectively (Fig. 9C). Comparison of the sequences of the shared WD40 domains, as well as the region of the *Daphnia* protein corresponding to the sixth of the seven *Drosophila* domains, revealed high degrees of amino acid conservation in these regions of the two proteins: WD40 1, 87.2% identity/100% similarity; WD40 2, 90.2% identity/100% similarity; WD40 3, 90.0% identity/95.0% similarity; WD40 4, 89.5% identity/94.7% similarity; WD40 5, 87.2% identity/100 similarity; WD40 6 (no domain formally identified by SMART in *Daphnia*), 75% identity/100% similarity; WD40 7, 94.7% identity/100% similarity (Fig. 9C).

Reciprocal blasting of Dappu-TWS against all proteins curated in FlyBase identified TWS (Flybase No. **FBpp0081671**) as the *D. melanogaster* protein most similar to the *Daphnia* query (Table 2). Comparison of Dappu-TWS to all non-redundant protein sequences curated by NCBI identified a beetle *Tribolium castaneum* protein (**EFA10095**) as showing the highest similarity to the *Daphnia* query (Table 3); three Drosophila TWS isoforms were among the top five blastp hits (Table 3).

3.1.10. SHAGGY (SGG)—A single *D. pulex* gene (*dappu-sgg*) was identified as encoding a putative SGG protein via a query using a *D. melanogaster* SGG (**AAN09084**; Adams et al., 2000). The Genes 2010 gene model shows *dappu-sgg* to be located on Scaffold 76 of the genome, with a predicted length of 5712 nucleotides (Table 1).

Figure 10 shows the alignment of the protein deduced from *dappu-sgg* (Dappu-SGG; 439 amino acids in overall length) with that of the *Drosophila* query (514 amino acids long). Comparison of the sequence of Dappu-SGG with Drome-SGG revealed 64.8% amino acid identity/77.6% amino acid similarity between the two proteins. SMART analyses of Dappu-SGG and Drome-SGG identified a single serine/threonine kinase domain in each protein (Fig. 10); the two serine/threonine kinase domains are nearly identical in amino acid sequence, *i.e.* 89.5% identity/98.2% similarity. Reciprocal blasting of Dappu-SGG against all proteins curated in FlyBase identified SGG (Flybase no. **FBpp0070450**) as *D. melanogaster* protein most similar to the *Daphnia* query (Table 2). Comparison of Dappu-SGG with all non-redundant protein sequences curated by NCBI revealed a glycogen synthase kinase (of which SGG is family member) from the tick *Rhipicephalus microplus* (**ABO61882**) to be the most similar protein match (Table 3); no *Drosophila* proteins were among the top five blastp hits (Table 3).

3.1.11. SUPERNUMERARY LIMBS (SLIMB)—A single *D. pulex* gene (*dappu-slimb*) was identified as encoding a putative SLIMB protein via a query using a *D. melanogaster* SLIMB (**AAF55853**; Adams et al., 2000). The Genes 2010 gene model shows *dappu-slimb* to be located on Scaffold 169 of the genome, with a predicted length of 2880 nucleotides (Table 1).

Figure 11 shows the alignment of the protein deduced from *dappu-slimb* (Dappu-SLIMB; 510 amino acids in overall length) with that of the *Drosophila* query (510 amino acids long). Comparison of the sequence of Dappu-SLIMB with Drome-SLIMB revealed 76.3% amino acid identity/91.8% amino acid similarity between the two proteins. SMART analyses of Dappu-SLIMB and Drome-SLIMB identified an FBOX domain and seven WD40 domains in each protein (Fig. 11); the amino acid sequences of each of these domains is highly conserved between the two species: FBOX domain, 80.0% amino acid identity/95.0% similarity; WD40-1, 89.5% amino acid identity/94.7% amino acid similarity; WD40-2, 84.2% amino acid identity/94.7% amino acid similarity; WD40-3, 89.5% amino acid identity/97.4% amino acid similarity; WD40-4, 94.7% amino acid identity/100% amino acid similarity; WD40-5, 97.4% amino acid identity/100% amino acid similarity; WD40-6, 94.7% amino acid identity/100% amino acid similarity; WD40-7, 94.7% amino acid identity/100% amino acid similarity.

Reciprocal blasting of Dappu-SLIMB against all proteins curated in FlyBase identified SLIMB (Flybase No. **FBpp0083434**) as the *D. melanogaster* protein most similar to the *Daphnia* query (Table 2). Interestingly, comparison of Dappu-SLIMB to all non-redundant protein sequences curated by NCBI identified vertebrate proteins, all apparent members of the E3 ubiquitin ligase family, as the top five blastp hits, with a mouse protein (**BAE26547**) being the best match with the *Daphnia* sequence (Table 3).

3.1.12. TIMELESS—Eight *D. pulex* genes (*dappu-tim a, dappu-tim b, dappu-tim c, dappu-tim d, dappu-tim e, dappu-tim f, dappu-tim g,* and *dappu-tim h*) were identified as encoding putative TIM proteins via a query using a *D. melanogaster* TIM (**AAN10371;** Adams et al., 2000). The Genes 2010 gene model shows *dappu-tim a, dappu-tim b, dappu-tim c, dappu-tim d, dappu-tim e, dappu-tim f, dappu-tim g,* and *dappu-tim h* to be located on Scaffolds 24,

6, 10, 6, 6, 24, 75, and 91 of the genome, respectively, with lengths of 14166, 5052, 3650, 5438, 5542, 11674, 2798, and 4537 nucleotides (Table 1).

Figure 12 shows the alignments of the protein deduced from *dappu-tim a* (Dappu-TIM A; 1197 amino acids in overall length) with that of the *Drosophila* query (1421 amino acids in length). Comparison of the amino acid sequences of these two proteins revealed 29.8% identity/60.1% similarity between the two TIMs. Comparisons of the other *Daphnia* TIMs with Drome-TIM showed varying levels of amino acid conservation, with some nearly as high as that seen between Dappu-TIM A and Drome-TIM, and others considerably lower: Dappu-TIM B, 23.6% amino acid identity/52.0% amino acid similarity; Dappu-TIM C, 23.7% amino acid identity/47.8% amino acid similarity; Dappu-TIM D, 26.4% amino acid identity/55.9% amino acid similarity; Dappu-TIM E, 23.4% amino acid identity/47.9% amino acid similarity; Dappu-TIM F, 22.3% amino acid identity/51.1% amino acid similarity; Dappu-TIM G, 14.2% amino acid identity/30.8% amino acid similarity; and Dappu-TIM H, 21.3% amino acid identity/45.6% amino acid similarity (alignments not shown). Alignment of the eight *Daphnia* TIM proteins to one another shows considerable variation in sequence composition between the proteins (in the interest of space, this alignment is provided only as an online supplemental figure [Supplemental Figure 1]). Table 3 provides pairwise comparisons of the amino acid identity/similarity of Dappu-TIM A-H. No functional domains were identified by SMART analyses in Drome-TIM or any of the Dappu-TIMs. Reciprocal blasting of eight Dappu-TIMs against all proteins curated in FlyBase identified an isoform of TIM as the *D. melanogaster* protein most similar to each *Daphnia* query (Flybase nos. **FBpp0291971**, **FBpp0291971**, **FBpp0291971**, **FBpp0077254**, **FBpp0291970**, **FBpp0077254**, **FBpp0291970** and **FBpp0291970**, respectively; Table 2). Comparison of the Dappu-TIMs with all non-redundant protein sequences curated by NCBI revealed each to be most similar to an insect TIM isoform (*i.e.* the butterfly *Danaus plexippus* [**AAR15505**], the moth *Antheraea pernyi* [**AAF66996**], the beetle *T. castaneum* [**EFA04644**], *T. castaneum* [**EFA04644**], *D. melanogaster* [**ADV36936**], *D. melanogaster* [**P49021**], the fruit fly *Drosophila virilis* [**O17482**], and the cricket *Gryllus bimaculatus* [**BAJ16356**] for Dappu-TIM A-H, respectively; Table 3). In fact, the top five hits for each of the *Daphnia* proteins were insect isoforms of TIM (Table 3).

3.1.13. VRILLE (VRI)—A single *D. pulex* gene (*dappu-vri*) was identified as encoding a putative VRI protein via a query using a *D. melanogaster* VRI (**AAF52237**; Adams et al., 2000). The Genes 2010 gene model shows *dappu-vri* to be located on Scaffold 92 of the genome, with a predicted length of 2225 nucleotides (Table 1).

Figure 13 shows the alignment of the protein deduced from *dappu-vri* (Dappu-VRI; 676 amino acids in overall length) with that of the *Drosophila* query (729 amino acids long). Comparison of the sequences of Dappu-VRI and Drome-VRI revealed 27.7% amino acid identity/49.5% amino acid similarity between the two proteins. SMART analyses of Dappu-VRI and Drome-VRI identified a single basic region leucine zipper domain in each protein (Fig. 13); the amino acid sequence of this domain is highly conserved between the two VRIs, *i.e.* 76.9% amino acid identity and 96.9% amino acid similarity.

Reciprocal blasting of Dappu-VRI against all proteins curated in FlyBase identified VRI (Flybase no. **FBpp0289297**) as the most similar *D. melanogaster* protein to the *Daphnia* query (Table 2). Comparison of Dappu-VRI with all non-redundant protein sequences curated by NCBI revealed a VRI from the butterfly *D. plexippus* (**ATT86041**) to be most similar to *Daphnia* VRI (Table 3); two *Drosophila* VRIs are among the top five blastp hits for this query (Table 3).

3.2. Input pathway proteins

3.2.1. CRYPTOCHROME (CRY)—Four *D. pulex* genes (*dappu-cry a*, *dappu-cry b*, *dappu-cry c*, and *dappu-cry d*) were identified as encoding putative CRY proteins via a query using a *D. melanogaster* CRY (**AAC83828;** Emery et al., 1998). The Genes 2010 gene model shows that these genes are located on Scaffolds 40, 18, 10, and 7 of the genome, respectively, with lengths of 2706, 4661, 3072 and 2052 nucleotides (Table 1).

Figure 14A shows the alignment of the protein deduced from *dappu-cry a* (Dappu-CRY A; 525 amino acids in overall length) with that of the *Drosophila* query (542 amino acids long). Comparison of the sequence of Dappu-CRY A with Drome-CRY revealed 44.8% amino acid identity/76.4% amino acid similarity between the two proteins (Figure 14A). Alignments of Dappu-CRY B, Dappu-CRY C and Dappu-CRY D with Drome-CRY also revealed high levels of structural homology between the proteins: Dappu-CRY B vs. Drome-CRY, 37.6% amino acid identity/69.1%; Dappu-CRY C vs. Drome-CRY, 38.2% amino acid identity/69.9% amino acid similarity; Dappu-CRY D vs. Drome-CRY, 24.4% amino acid identity/59.6% amino acid similarity (alignments not shown). Figure 14B shows the alignment of the four *Daphnia* CRYs with one another. As this panel shows, the four proteins show considerable variation in amino acid composition. Table 4 provides pairwise comparisons of the amino acid identity/similarity of Dappu-CRY A-D. No functional domains were identified by SMART analyses in Drome-CRY or any of the Dappu-CRYs.

As discussed in Section 1, along with CLK, CRY is the only other circadian protein for which a crustacean family member is known, *i.e.* an isoform from the Antarctic krill *E. superba* (Mazzotta et al., 2010). Alignments of the *Daphnia* CRYs with Eupsu-CRY, show similar levels of amino acid conservation to that seen for alignments with the *Drosophila* protein: Dappu-CRY A vs. Eupsu-CRY, 36.0% identity/69.5% similarity; Dappu-CRY B vs. Eupsu-CRY, 67.1% identity/88.6% similarity; Dappu-CRY C vs. Eupsu-CRY, 47.2% identity/76.5% similarity; Dappu-CRY D vs. Eupsu-CRY, 26.8% identity/58.9% similarity (alignments not shown).

Reciprocal blasting of four Dappu-CRYs against all proteins curated in FlyBase identified members of the CRY/6-4 photolyase family as the most similar *D. melanogaster* proteins to the *Daphnia* queries. For Dappu-CRY A, CRY (Flybase no. **FBpp0083150**) was found to be the most similar *Drosophila* protein to the query sequence, while the remaining three sequences were found to be most similar to 6-4 photolyase (Flybase no. **FBpp0080935**). Comparison of the Dappu-CRYs with all non-redundant protein sequences curated by NCBI revealed each to be most similar to a CRY protein, though all are more similar to isoforms from other species than they are to *Drosophila* proteins (*i.e.* the cricket *Dianemobius*

nigrofasciatus [**BAF45421**], the mosquito *Anopheles darlingi* [**EFR20390**], the clawed frog *Xenopus tropicalis* [**AAI66277**], and the European seabass *Dicentrarchus labrax* [**CBN81995**]) for Dappu-CRY A-D, respectively). Based on the results of our blastp analyses (Table 3), it would appear that Dappu-CRY A is a homolog of the *Drosophila*-type or CRY1 subfamily, with Dappu-CRY B being a homolog of the vertebrate-type or CRY2 subfamily; alignments of Dappu-CRY A and B with CRY1 and CRY2 of the butterfly *D. plexippus*, respectively, are shown in Figure 15. Dappu-CRY D appears most similar to members of the CRY DASH subfamily (Table 3). It is unclear as to which subfamily of the CRY/6-4 photolyase superfamily Dappu-CRY C is a member (Table 3).

3.3. Output pathway proteins

3.3.1. PIGMENT DISPERSING HORMONE RECEPTOR (PDHR)—A single *D. pulex* gene (*dappu-pdhr*) was identified as encoding a putative PDHR via a query using a *D. melanogaster* pigment dispersing factor receptor (PDFR; **AAF45788**; Adams et al., 2000). The Genes 2010 gene model shows *dappu-pdhr* to be located on Scaffold 1 of the genome, with a predicted length of 4621 nucleotides (Table 1).

Figure 16 shows the alignment of the protein deduced from *dappu-pdhr* (Dappu-PDHR; 516 amino acids in overall length) with that of the *Drosophila* query (669 amino acids long). Comparison of the sequence of Dappu-PDHR with that of Drome-PDFR revealed 32.7% amino acid identity/55.0% amino acid similarity between the two proteins. SMART analyses of Dappu-PDHR and Drome-PDFR identified seven transmembrane domains (TMDs) in each protein (Fig. 16). The amino acid sequences of these TMDs are highly conserved between the two proteins: TMD1, 52.2% amino acid identity and 87.0% amino acid similarity; TMD2, 72.2% amino acid identity and 94.4% amino acid similarity; TMD3, 72.7% amino acid identity and 95.5% amino acid similarity; TMD4, 47.4% amino acid identity and 78.9% amino acid similarity; TMD5, 55.5% amino acid identity and 95.0% amino acid similarity; TMD6, 72.2% amino acid identity and 83.3% amino acid similarity; TMD7, 81.8% amino acid identity and 100% amino acid similarity. In addition, a single hormone receptor domain was identified in Dappu-PDHR; this domain is absent in Drome-PDFR, though the corresponding region of this protein is 35.8% identical/58.9% similar to its *Daphnia* counterpart (Fig. 16).

Reciprocal blasting of Dappu-PDHR against all proteins curated in FlyBase identified PDFR (Flybase No. **FBpp0099841**) as the *D. melanogaster* protein most similar to the *Daphnia* query (Table 2). Comparison of Dappu-PDHR to all non-redundant protein sequences curated by NCBI identified a PDHR from the penaeid shrimp *Marsupenaeus japonicus* (BAH85843) as the top protein match for the query; three *Drosophila* proteins were also among the top five blastp hits identified via the *Daphnia* protein (Table 3).

4. Discussion

4.1. Genome mining identifies a putative set of putative circadian proteins in Daphnia pulex

Over the last decade, genome mining has become a major method for protein discovery in both vertebrates and invertebrates. At present, the only crustacean genome that has been fully sequenced and is available for public use is that of the cladoceran *D. pulex* (Colbourne et al., 2005; Bauer, 2007; Stollewerk, 2010; Colbourne et al., 2011; Tautz, 2011). Recently, this resource has been used for protein discovery in this species, providing detailed information on the structures of molecules involved in many physiological/behavioral processes, for example, steroid biosynthesis and innate immunity (Rewitz and Gilbert, 2008; McTaggart et al., 2009).

In the study presented here, we have used the *D. pulex* genome to mine for proteins that may be involved in the control of circadian rhythmicity in this species. Specifically, we used the sequences of known *Drosophila* circadian proteins to query the *Daphnia* genome for putative ortholog genes and their encoded proteins. Using this strategy, a number of putative *D. pulex* circadian genes and their proteins were identified and characterized, including those likely involved in the establishment of the core clock, *i.e.* PER, TIM, CLK, CYC and CRY2, as well as proteins in their post-translational modifications and degradation, *i.e.* DBT, CK2, SGG, PP2A, PP1 and SLIMB. Moreover, genes and proteins putatively involved in setting the phase and amplitude of the core clock were identified, *i.e.* PDP1, VRI and CWO, as were orthologs of the blue-light receptor protein CRY1, which likely function as input pathways to the core clock, synchronizing it to the solar day, and PDHR, which may serve to transduce one of the clock's output signals. Taken collectively, this collection of genes/proteins represents the first putative set of circadian proteins thus far described from any crustacean.

4.2. Several Daphnia circadian genes appear to exhibit extensive gene duplications

Overall, *Daphnia* have an unusually high level of gene duplication in comparison with the other arthropods for which genomic databases exist (Colbourne et al., 2011). In fact, only in aphids has a similar level of gene duplication been noted (Huerta-Cepas et al., 2010; Ollivier et al., 2010; Shigenobu et al., 2010); both *Daphnia* and aphids are cyclical parthenogens (Cortés et al., 2008). The purpose of gene redundancy in these and other species is generally not well understood. For some genes, the encoded protein isoforms may be nonfunctional, may have different kinetic properties for the same substrate, or may have novel functions (Force et al., 1999). It is also possible that the protein isoforms may be expressed in a life stage-specific manner (development, diapause, reproduction), or that their expression is tissue-specific. Multiple genes, and hence protein isoforms, may help an organism adjust to shifting environmental conditions, *e.g.* changes in salinity, oxygen levels, or temperature.

As discussed in Section 1, a defining parameter of circadian rhythms is temperature compensation, the molecular basis of which is not well understood (Salomé and McClung, 2005; Salomé et al, 2010). Tomaiuolo et al. (2008) constructed a mathematical model based on two splice variant isoforms of the β-subunit of *Drosophila* CKII that differ in kinetic

rates of PER phosphorylation. This model suggests that through dynamic regulation of the proportions of the two β-subunit isoforms expressed, an increase in robustness of the circadian clock can be predicted. Two *per* alleles exist in wild populations of *D. melanogaster*, with population differences in frequency that vary by latitude (Sawyer et al., 1997). The PER proteins have different thermokinetic properties that may be involved in the circadian clock temperature compensation. Likewise, in a northern-latitude *Drosophila* population, the *tim* mutation, *ls-tim*, has been shown to adjust photoresponsiveness in this more seasonally-variable environment (Sandrelli et al., 2007). The multiple *Daphnia* PP1, PP2A-WBT and TIM variants predicted here may likewise possess different kinetic parameters to offset temperature, salinity, oxygen, and/or other environmental variables.

4.3. Conservation of structural domains suggests Daphnia possesses an insect-like molecular clock, but organized more like that of butterflies and mosquitoes than of Drosophila

In our study, putative homologs to most of the known *Drosophila* circadian proteins were identified in *D. pulex*. Structural domains in both the *Daphnia* and *Drosophila* proteins were analyzed via the online program SMART (Schultz et al., 1998; Letunic et al., 2009). While we realize that not all of the domains/functional regions that have been reported for the *Drosophila* circadian proteins are detected via this program (*e.g.* Saez and Young, 1996; Ousley et al., 1998; Chang and Reppert, 2003; Lin and Todo, 2005), those that were, for the most part, appear to be highly conserved between the two species' putative homologs; significant amino acid variation was noted outside of functional regions for several proteins. Even where discrepancies were noted, *e.g.* a PAC domain identified in Drome-PER but not in Dappu-PER, the corresponding regions of the two proteins were often very similar in amino acid composition (in the case of the PER PAC domain 72.7% identical/93.2% similar). Interestingly, blast analyses of the *Daphnia* sequences show them to be, for the most part, more similar to proteins identified from other species than they are to *Drosophila*. Moreover, the presence of both CRY1 and CRY2 in *Daphnia* suggests that its molecular clock is likely organized more similar to that recently described for butterfly and mosquito (*e.g.* Zhu et al., 2005; Yuan et al., 2007; Zhu et al., 2008) where CRY1 is proposed as a photosenstive input to the clock and CRY2 is core clock protein (functioning to repress of CLK/CYC-mediated transcription), than it is to *Drosophila* (which possesses only CRY1).

4.4. Potential cellular locus and output signals of a Daphnia neuronal clock

All circadian systems have three functional components: a core clock, which is responsible for time keeping, input pathways that act to synchronize the clock to the environment, and output pathways that transmit the timing information from the clock for the control of physiology and behavior. Here we have identified a protein that may function as the input to a *Daphnia* clock, *i.e.* an isoform of CRY1, as well as proteins that may act to establish the core molecular clock itself, *i.e.* PER, TIM, CLK, CYC, CRY2, DBT, SGG, VRI, etc. To be determined, however, are possible output pathways from the *Daphnia* clock that would signal the timing information necessary to establish circadian rhythms in physiology and behavior in this species.

As the cellular location of the core circadian clock (or clocks) in *D. pulex* is unknown, it is difficult to postulate how output signals would be generated in, and transmitted from, this timekeeper. This said, work conducted on other species (both invertebrate and vertebrate) would suggest that circulating hormones are an important part of the *Daphnia* clock's output pathway, mediating the expression of the overt circadian rhythms present in this species. One possibility is that hormones are released directly from the clock cells themselves; alternatively, the clock cells may project to and innervate relay sites, likely endocrine organs, which are the sources of the hormonal signals. Regardless of locus, in insects, several peptide hormones have been shown to be key components of circadian signaling systems, particularly pigment dispersing factor (PDF), a member of the pigment dispersing hormone (PDH) family; PDF is present in a number of known circadian clock neurons in *Drosophila* (for review see: Allada and Chung, 2010; Tomioka and Matsumoto, 2010).

Recent transcriptome and genome mining in *D. pulex* has identified a homolog of PDF/PDH in this species, NSELINSLLGLPRFMKVVamide (Gard et al., 2009; Christie et al., 2011). Moreover, immunohistochemistry using an antibody generated against β-PDH (NSELINSILGLPKVMNDAamide) labels a small set of neurons (~8 somata) that are distributed throughout the brain/optic ganglia of *D. pulex* (Gard et al., 2009). While currently speculation, the role of PDF as a signaling agent, and hence marker, for some clock cells in the brain of *Drosophila* suggests that PDH-immunopositive neurons in the brain of *D. pulex* may represent at least a subset of the cellular loci for a circadian neuronal pacemaker in this species, a hypothesis recently strengthened by the finding of circadian patterns of activity in at least some of these cells (Strauβ et al., 2011).

In addition to PDF, a number of other hormones, primarily peptides, have been implicated in circadian signaling in insects. For example, corazonin, crustacean cardioactive peptide (CCAP) and diapause hormone have all been suggested as possible output signals from insect clock systems (*e.g.* Sehadová et al., 2007); isoforms of both corazonin and CCAP have been predicted from the *D. pulex* transcriptome and/or genome (Gard et al., 2009; Christie et al., 2011). Likewise, several peptide hormones have been shown to, or are postulated to show, circadian rhythms in their cycling in decapod crustaceans (Strauss and Dircksen, 2010), *i.e.* red pigment concentrating hormone and crustacean hyperglycemic hormone. Transcriptome and genome mining in *D. pulex* suggests that these hormonal systems too are present in this species (Gard et al., 2009; Christie et al., 2011). In fact, via transcriptome and genome mining over 100 peptide hormones have recently been identified in *D. pulex* (Gard et al., 2009; Christie et al., 2011). Here we have identified a putative PDH receptor protein, which, if we are correct in the peptide being a circadian signal in *Daphnia*, may function to transduce at least one of the core clocks output signals for the control of physiology and behavior in this species.

5. Conclusions and future directions

Circadian rhythms in physiology and behavior have been documented in numerous crustacean species; however, little is known about the molecular and/or cellular machinery underlying them in any member of this arthropod subphylum (Strauss and Dircksen, 2010). This said, their well-mapped nervous systems and amenability to in-depth

electrophysiological and molecular investigations make them an optimal group of animals for studying circadian biology. Moreover, the fact that many intertidal crustaceans exhibit both circadian and circatidal rhythms make these animals an ideal model to explore possible interactions between circadian and circatidal signaling systems, including whether these two timekeeping systems use common or distinct molecular and/or cellular components.

Clearly the first step toward understanding circadian signaling in any species is obtaining knowledge of the molecules required for the establishment of the core clock. Here, we have achieved this milepost for the cladoceran crustacean *D. pulex* using a strategy combining genome mining and phylogenetic comparisons to known previously identified circadian proteins. *D. pulex* is now the only crustacean for which a putative set of circadian genes and proteins are known. With these data, we are now positioned to begin functional studies directed at determining if the mRNAs of the identified genes cycle in a circadian fashion and, if so, whether these rhythms are similar to those seen in insects. Similarly, the proteins deduced from these identified genes now allows for the generation of *Daphnia*-specific antibodies to these molecules, which will be useful both for mapping the distribution of these proteins and for determining if they cycle in manners similar to their insect counterparts. Finally, the identification of the *D. pulex* circadian genes described here now provide targets for knockdown experiments designed to elucidate the functional roles their encoded proteins play in the establishment of circadian signaling in this and other crustacean species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. *Drosophila* vs. *Daphnia* CASEIN KINASE II (CKII) α-subunit

```
Drome-CKII α     MTLPSAARVYTDVNAHKPDEYWDYENYVVDWGNQDDYQLVRKLGRGKYSEVFEAINITTT
Dappu-CKII α     MPLASQARVYADVNLHRAREYWDYESHVIEWGEQDDYQLVRKLGRGKYSEVFEAINVVTN
                 *.*.* ****:*** *:. ******.:*::**:************************:.*.

Drome-CKII α     EKCVVKILKPVKKKKIKREIKILENLRGGTNIITLLAVVKDPVSRTPALIFEHVNNTDFK
Dappu-CKII α     EKCVVKILKPVKKKKIKREIKILENLRGGTNIITLQAVVKDPVSRTPALIFEHVNNTDFK
                 ********************************** ************************

Drome-CKII α     QLYQTLTDYEIRYYLFELLKALDYCHSMGIMHRDVKPHNVMIDHENRKLRLIDWGLAEFY
Dappu-CKII α     LLYQTLTDYDIRFYLYELLKALDYCHSMGIMHRDVKPHNVMIDHENRKLRLIDWGLAEFY
                  ********:**:**:*********************************************

Drome-CKII α     HPGQEYNVRVASRYFKGPELLVDYQMYDYSLDMWSLGCMLASMIFRKEPFFHGHDNYDQL
Dappu-CKII α     HPGQEYNVRVASRYFKGPELLVDYQMYDYSLDMWSLGCMLASMIFRKEPFFHGHDNYDQL
                 ************************************************************

Drome-CKII α     VRIAKVLGTEELYAYLDKYNIDLDPRFHDILQRHSRKRWERFVHSDNQHLVSPEALDFLD
Dappu-CKII α     VRIAKVLGTEELFEYLDKYQIELDPRFNDILGRHSRKRWERFVHSENQHLISPEALDFLD
                 ************: *****:*:*****:*** *************:****:*********

Drome-CKII α     KLLRYDHVDRLTAREAMAHPYFLPIVNGQ-----------MNPNN--------------
Dappu-CKII α     KLLRYDHQERLTAREAMDHPYFYPIVKEQNRLASLSTSPTLLPPGTSVGGGTSGGAGPSG
                 ******* :******** **** ***: *            : *..

Drome-CKII α     ---QQ
Dappu-CKII α     GGPSQ
                    .*
```

B. *Drosophila* vs. *Daphnia* CKII β-subunit

```
Drome-CKII β     MSSSEEVSWVTWFCGLRGNEFFCEVDEDYIQDKFNLTGLNEQVPNYRQALDMILDLEPED
Dappu-CKII β     MSSSEEVSWISWFCGLRGNEFFCEVDEDYIQDKFNLTGLNEQVPHYRQALDMILDLEPDD
                 *********::*********************************:**************:*

Drome-CKII β     ELEDNPLQSDMTEQAAEMLYGLIHARYILTNRGIAQMIEKYQTGDFGHCPRVYCESQPML
Dappu-CKII β     DVEDNPNQSDLIEQAAEMLYGLIHARYILTNRGILQMLEKYHAGDFGHCPRVYCENQLML
                 ::**** ***: ********************** **:***::************.* **

Drome-CKII β     PLGLSDIPGEAMVKTYCPKCIDVYTPKSSRHHHTDGAYFGTGFPHMLFMVHPEYRPKRPT
Dappu-CKII β     PIGLSDVPGEAMVKLYCPKCMDVYTPKSSRHHHTDGAYFGTGFPHMLFMVHPEYRPKKPT
                 *:****:******* ******:**************************************:**

Drome-CKII β     NQFVPRLYGFKIHSLAYQIQLQAAANFKMPLRA------KN
Dappu-CKII β     NQFVPRLYGFKIHPLAYQIQQQGASNFKVPMRTVSYNNGRK
                 *************.****** *.*:***:*:*:      ::
```

Figure 1.

Putative *Daphnia pulex* CASEIN KINASE II (CKII) α- and β-subunit proteins. (**A**) Alignment of *Drosophila melanogaster* CKII α-subunit (Drome-CKII α) with *D. pulex* CKII α-subunit (Dappu-CKII α). (**B**). Alignment of *D. melanogaster* CKII β-subunit (Drome-CKII β) with *D. pulex* CKII β-subunit (Dappu-CKII β). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure. In this figure, serine/threonine kinase catalytic domains predicted by SMART analyses are highlighted in red.

A. ***Drosophila*** **vs.** ***Daphnia*** **CLOCK (CLK)**

```
Drome-CLK       M-----------------------DDESDDKDDTKSFLCRKSRNLSEKKRRDQFNSLVDD
Dappu-CLK       MSVKSQPCGLTSSKLKKATASKISDDGLEDEVDEKGVIKRKSRNLSEKKRRDQFNILINE
                *                        **  :*: * *..: ***************** *:::

Drome-CLK       LSALISTSSRKMDKSTVLKSTIAFLKNHNEATDRSKVFE-IQQDWKPAFLSNDEYTHLML
Dappu-CLK       LCSMVCTGKRKMDKSTILKSAISFIRNHNQVTMQSHCQESVQEDWKPSFLSNEEFTHLML
                *.::::*..*******:***:*:*::***:.* :*:  * :*:****:****:*:*****

Drome-CLK       ESLDGFMMVFSSMGSIFYASESITSQLGYLPQDLYNMTIYDLAYEMDHEALLNIFMNPTP
Dappu-CLK       EALDEFIIVFSSTGKILYVSENITCLLGHTPSDLIGSSLSDLVWEEERIVVESLLGSWGA
                *:** *::**** *.*:*.**.**. **: *.** . :: **.:* :: .: .:: .  .

Drome-CLK       VIEPRQTDISSSNQITFYTHLRRGGME--KVDANAYELVKFVGYFRNDTNTSTGSSSEVS
Dappu-CLK       DHESSQVTGNKENHISLSCHLRRGNLSDANFESSNYELVFFSGYYRVQGNPDISSVSRVS
                  *. *.  ...*:*::  *****.:.  :.::. **** * **:* : *.. .* *.**

Drome-CLK       NG----SNGQPAVLPRIFQQNPNAEVDKKLVFVGTGRVQNPQLIREMSIIDPTSNEFTSK
Dappu-CLK       SSWGDDSKESTNFGDALSQYN-------GLVFVASARLQTPQLSVEMSIVDVSKSEFTSR
                ..    *: .. .   : * *         ****.:.*:*.***  ****:* :..****:

Drome-CLK       HSMEWKFLFLDHRAPPIIGYMPFEVLGTSGYDYYHFDDLDSIVACHEELRQTGEGKSCYY
Dappu-CLK       HSLEWKFLFLDHRGPPIIGYLPFEVLGTSGYDYYHVDDLEKVSTCHEALMQKGEVTSCCY
                **:**********.******:**************.***:.: :*** * *.** .** *

Drome-CLK       RFLTKGQQWIWLQTDYYVSYHQFNSKPDYVVCTHKVVSYAEVLKDSRK-EGQKSGNSNSI
Dappu-CLK       RFLTKGQQWIWLQTKYYITYHQWYSKPEFIVCSHRVISYNEVTGHPLKIESEESCDQVPG
                ***************.**::***: ***:::**:*:*:** **  .. * *.::* :. .

Drome-CLK       T-NNGSSKVIASTGTSSKSASATTTLRDFEL----SSQNLDSTLLGNSL-----------
Dappu-CLK       TPNTPTSKQLKSEYKSGLSHGKNAKTDDRNVLQDDSNRNQKRNMITNQSNRDRMKNNKQN
                * *. :** : *  .*. * . .:.  * ::    *.:* . .:: *.

Drome-CLK       -----------ASLGTETAATSPAVDS-----SPMWSASAV-----QPSGSCQINPLKTS
Dappu-CLK       YHHNVRQHQPNSSMSDAQPPESPSGESVMLRPPPLPTTESMPQHSRHPSGSSRV-----G
                           :*:.   .. **: :*     .*: ::.::     :****.::     .

Drome-CLK       RPASSYGNISSTGISPKAKRKCYFYNNRGNDSDSTSMSTDSVTSRQSMMTHVSSQSQRQR
Dappu-CLK       TALSDTGSISSSG-----------------SFQSAASMQSDQSMHSIHSHNSMQSVHGQ
                 . *. *.***:*                 * .:: * :*  * :*: :* * ** : :

Drome-CLK       SHHREHHRENHHNQS---HHHMQQQQQHQNQQQQHQ----------QHQQLQQ--QLQHT
Dappu-CLK       QTQQATPTFRSSNQSCSVHSCVNTNHQHQNKQPQPQFVNQNSFTSANNMQMRNTGQLVHT
                . ::     .  ***   *  :: ::****:* * *          :: *:::  ** **

Drome-CLK       VGTPKMV----PLLPIASTQIMAGNACQFPQP-----------------AYPLASPQLVA
Dappu-CLK       QSRGGEVHSNLSLSTPVSTSSISNTGHRFLQPRVNSGSSVRRTFLSGTSPTPVPTSNSAS
                 .    *    .* . .**. ::... :* **                 . *:.:.: .:

Drome-CLK       PTFLEPPQYLTAIPMQPVIAPFPVAPVLSPLPVQSQTDMLP-DTVVMTPTQSQLQDQLQR
Dappu-CLK       SSGASFTYQSVSIGMEGSGVALPVQRIIEGLPVVTLPGIVAHEPIIMTAGQREFHERLRI
                .:  . .   .:* *:   ..:**  ::. *** : ..::. :.::**. * :::::*:

Drome-CLK       KHDELQKLILQQQNELRIVSEQLLLSRYTYLQPM---MSMGFAPGNMTAAAV-GNLGASG
Dappu-CLK       KHLEIQKSILAQQEELRRVETELLLAQYGAWGPTVLKMTVPYAETDGTASATQPTVLSTG
                ** *:** ** **:*** *. :***::*    *    *:: :*  : **:*.  .: ::*

Drome-CLK       QRGLNFTGSNAV---QPQFNQYGFALNSEQMLNQQDQQMMMQQQQNLHTQHQHNLQQQHQ
Dappu-CLK       GLVTCLTTENSISLGQPSSSQVGNS-SAGSMTFSPGTPILVQSPPELHTRSPERDFLSHE
                     :* .*::   **. .* * : .: .*  . .  :::*.  :***:  ..   .*:

Drome-CLK       SHSQLQQHTQQQHQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQLQLQQQNDILLRED
Dappu-CLK       IQILLAQSLLQDN-------------------------------------------------
                 :  * *   *::

Drome-CLK       IDDIDAFLNLSPLHSLGSQSTINPFNSSSNNNNQSYNGGSNLNNGNQNNNNRSSNPPQNN
Dappu-CLK       ------------------------------------------------------------

Drome-CLK       NEDSLLSCMQMATESSPSINFHMGISDDGSETQSEDNKMMHTSGSNLVQQQQQQQQQQQI
Dappu-CLK       ------------------------------------------------------------

Drome-CLK       LQQHQQQSNSFFSSNPFLNSQNQNQNQLPNDLEILPYQMSQEQSQNLFNSPHTAPGSSQ
Dappu-CLK       ---------------------------PTSIEYLP-----------------------
                                          *..:* **
```

B. ***Macrobrachium*** **vs.** ***Daphnia*** **CLK**

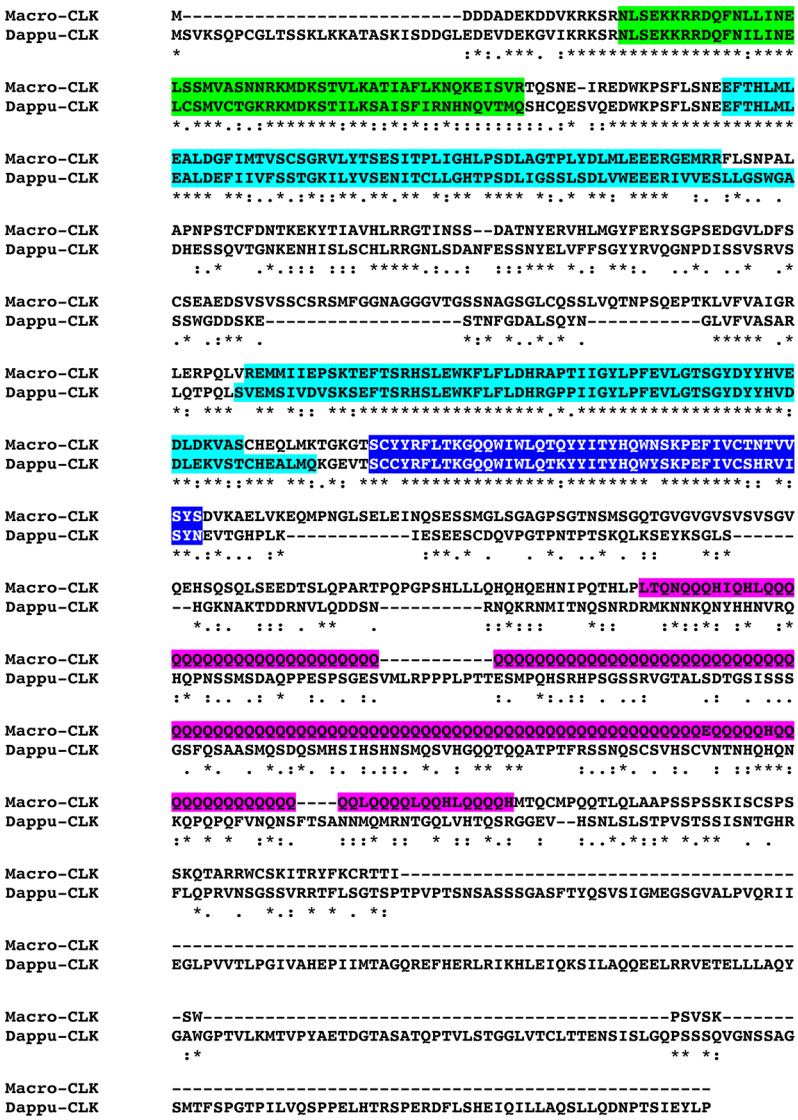

Figure 2.

Putative *Daphnia pulex* CLOCK (CLK) protein. (**A**) Alignment of *Drosophila melanogaster* CLK (Drome-CLK) with *D. pulex* CLK (Dappu-CLK). (**B**). Alignment of *Macrobrachium rosenbergii* CLK (Macro-CLK) with Dappu-CLK. In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure. In this figure, helix-loop-helix, PAS, PAC and coiled-coil domains identified by SMART analyses are highlighted in green, light blue, blue, and pink, respectively.

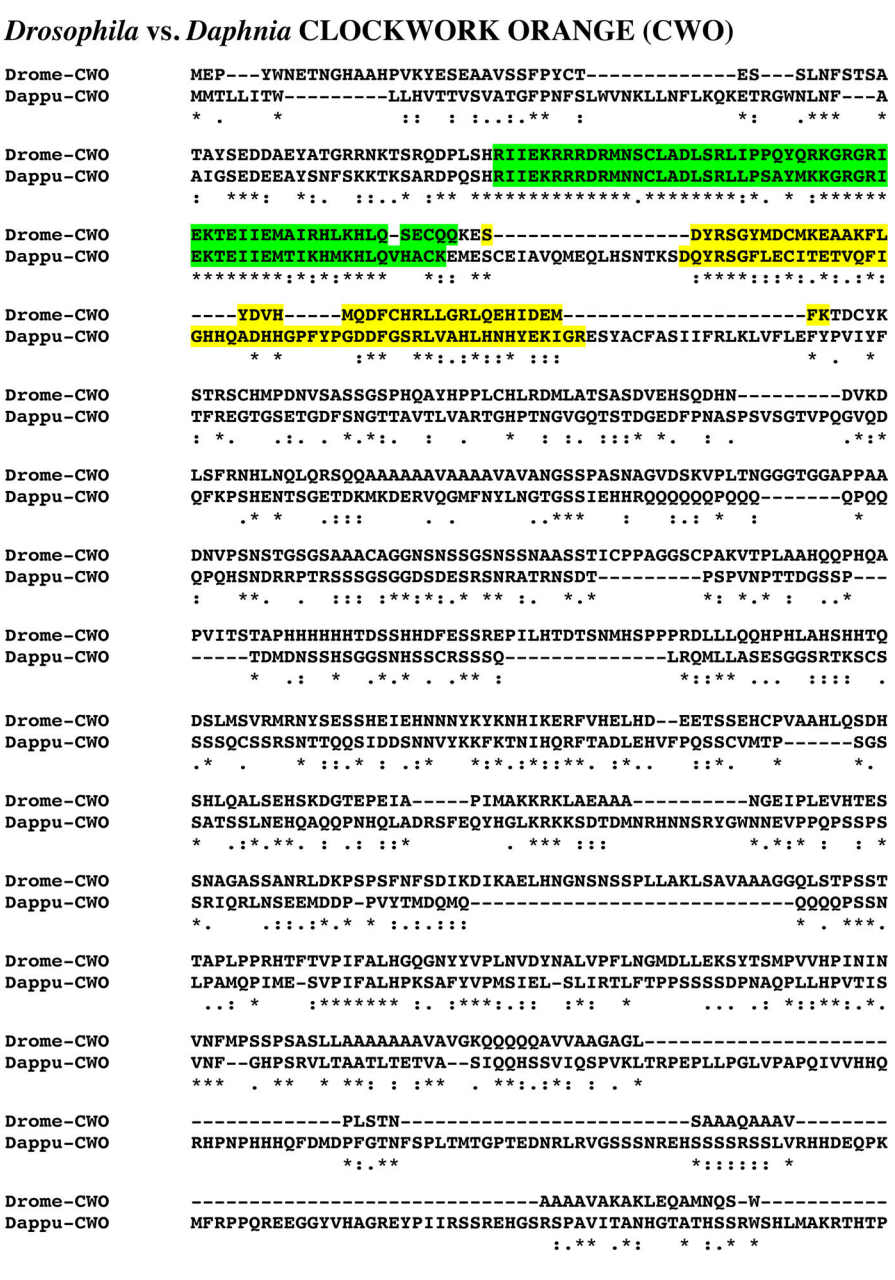

Figure 3.

Putative *Daphnia pulex* CLOCKWORK ORANGE (CWO) protein. Alignment of *Drosophila melanogaster* CWO (Drome-CWO) with *D. pulex* CWO (Dappu-CWO). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure. In this figure, helix-loop-helix and orange domains identified by SMART analyses are highlighted in green and yellow, respectively.

Drosophila vs. *Daphnia* CYCLE (CYC)

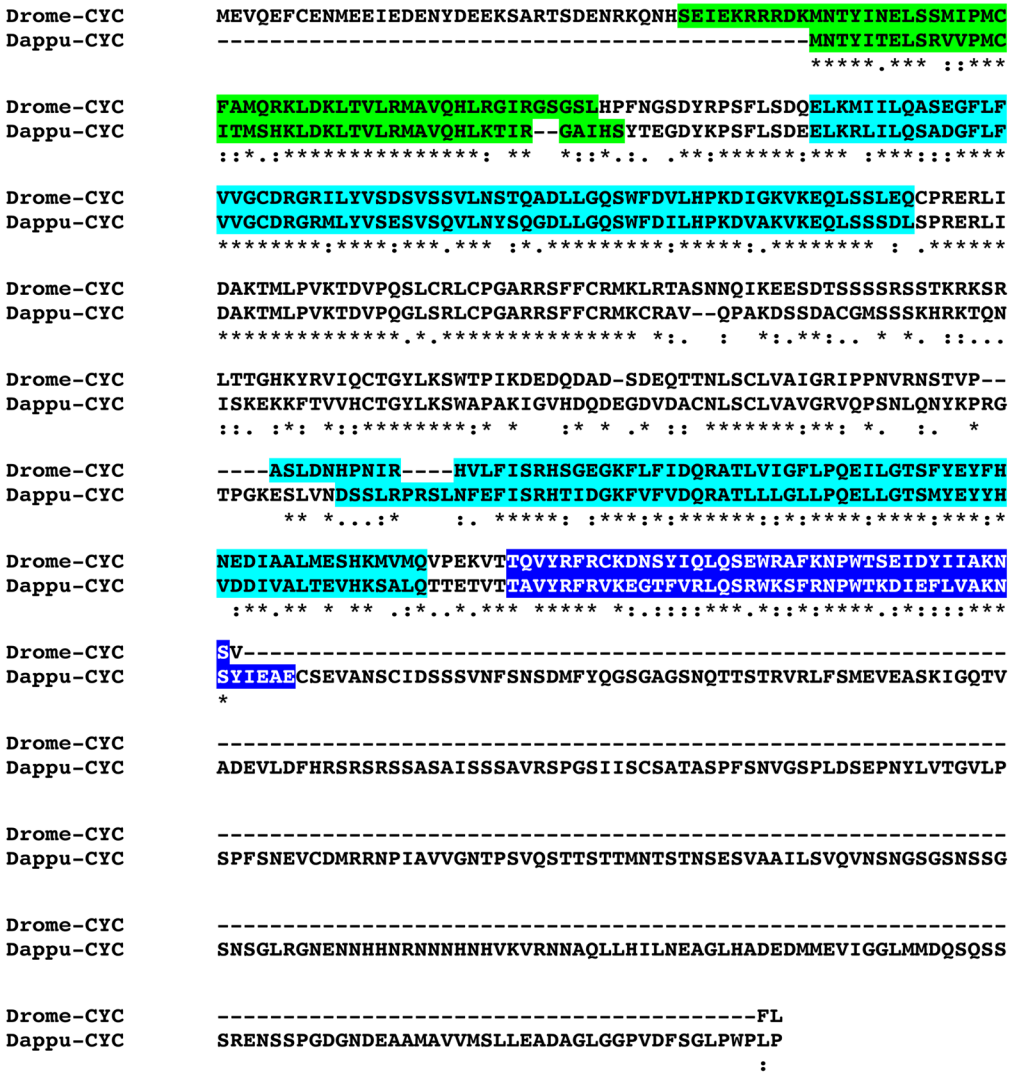

Figure 4.

Putative *Daphnia pulex* CYCLE (CYC) protein. Alignment of *Drosophila melanogaster* CYC (Drome-CYC) with *D. pulex* CYC (Dappu-CYC). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure. In this figure, helix-loop-helix, PAS and PAC domains identified by SMART analyses are highlighted in green, light blue and blue respectively.

Drosophila vs. *Daphnia* DOUBLETIME (DBT)

```
Drome-DBT       --MELRVGNKYRLGRKIGSGSFGDIYLGTTINTGEEVAIKLECIRTKHPQLHIESKFYKT
Dappu-DBT       MTMELRVGNKYRLGRKIGSGSFGDIYLGTNIATGEEVAIKLECIKTKHPQLHIESRFYKM
                  *************************.* ************:**********:***

Drome-DBT       MQGGIGIPRIIWCGSEGDYNVMVMELLGPSLEDLFNFCSRRFSLKTVLLLADQMISRIDY
Dappu-DBT       LQGGVGIPAVKWCGSEGDYNVMVMELLGPSLEDLFNFCSRRFSLKTVLLLADQLVCRIEY
                :***:*** : ****************************************::.**:*

Drome-DBT       IHSRDFIHRDIKPDNFLMGLGKKGNLVYIIDFGLAKKFRDARSLKHIPYRENKNLTGTAR
Dappu-DBT       IHSKNFIHRDIKPDNFLMGLGKRGNLVYIIDFGLAKKYRDARTNAHIPYRENKNLTGTAR
                ***::*****************:**************:****:  ***************

Drome-DBT       YASINTHLGIEQSRRDDLESLGYVLMYFNLGALPWQGLKAANKRQKYERISEKKLSTSIV
Dappu-DBT       YASIHTHVGIEQSRRDDLESLGYVLMYFNRGSLPWQGLRAATKRQKYEIISEKKMSTSVE
                ****:**:********************** *:******:**.****** *****:***:

Drome-DBT       VLCKGFPSEFVNYLNFCRQMHFDQRPDYCHLRKLFRNLFHRLGFTYDYVFDWNLLKFGGP
Dappu-DBT       ELCRGYPAEFATYLNYCRSLRFEEKPDYPYLRHLFRNLFHRQRFTYDYVFDWNMLKFGGA
                 **:*:*:**..***:**.:::*:::*** :**:*********  **********:*****.

Drome-DBT       RNPQAIQQAQDGADGQAGHDAVAAAAAVAAA---------AAASSHQQQQHK-------V
Dappu-DBT       QKPAS------GANGSSSNGLATANSALPVAPNPVSMPVDVAASSEDKEQRARAYRNTPI
                ::* :      **:*.:.:. .:* :*:..*          .****.:::*:        :

Drome-DBT       NAALGGGGGSAAQQQLQGGQTLAMLGGNGGGNGSQLIGGNGLNMDDSMAATN---SSRPP
Dappu-DBT       AQWVGQTATLPPERPVQ-------------------------------PATNPPAPTRPS
                   :*  .  ..:: :*                                 .***   .:**.

Drome-DBT       YDTP------------ERRPSIRMRQGGGGGGGGVGVGGMPSGGGGGGVGNAK
Dappu-DBT       FSHPTNPLPATSASWAARSRAARTQQ--------------------------
                :. *             *  : * :*
```

Figure 5.

Putative *Daphnia pulex* DOUBLETIME (DBT) protein. Alignment of *Drosophila melanogaster* DBT (Drome-DBT) with *D. pulex* DBT (Dappu-DBT). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure. In this figure, serine/threonine kinase domains identified by SMART analyses are highlighted in red.

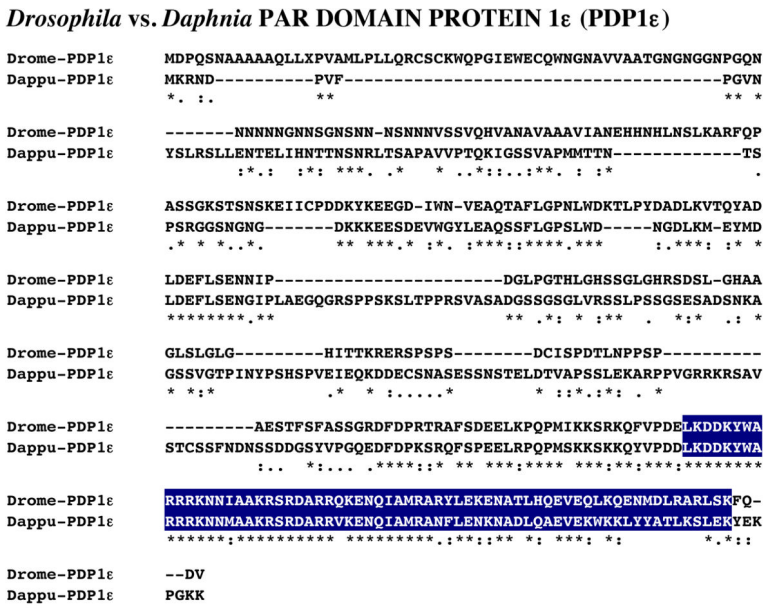

Figure 6.

Putative *Daphnia pulex* PAR DOMAIN PROTEIN 1ε (PDP1ε) protein. Alignment of *Drosophila melanogaster* PDP1ε (Drome-PDP1ε) with *D. pulex* PDP1ε (Dappu-PDP1ε). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure. In this figure, basic region leucine zipper domains identified by SMART analyses are highlighted in dark blue.

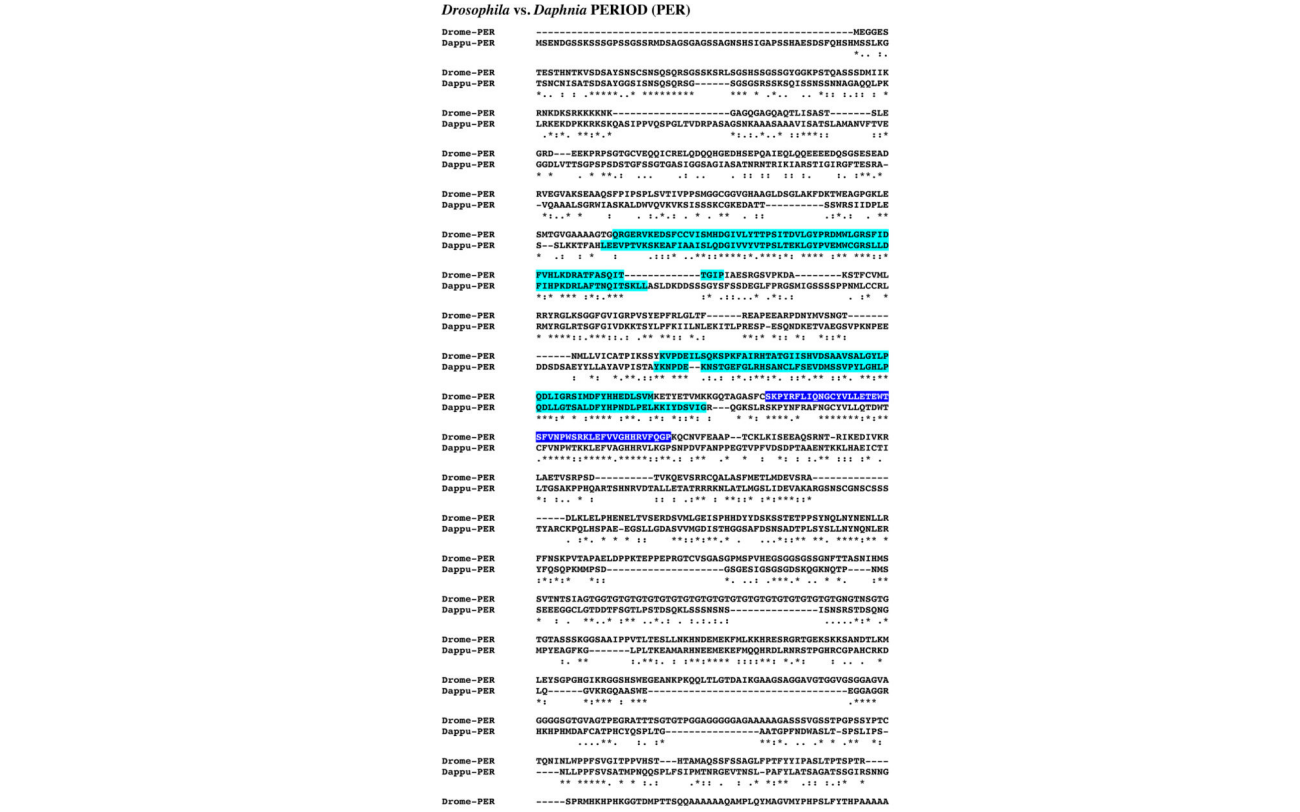

Figure 7.

Putative *Daphnia pulex* PERIOD (PER) protein. Alignment of *Drosophila melanogaster* PER (Drome-PER) with *D. pulex* PER (Dappu-PER). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved,

while single and double dots denote amino acids that are similar in structure. In this figure, PAS and PAC domains identified by SMART analyses are highlighted in light blue and blue, respectively.

A. *Drosophila* vs. *Daphnia* PROTEIN PHOSPHATASE 1 (PP1)

```
Drome-PP1        M--SDIMNIDSIISRLLEVRGARPGKNVQLSESEIRSLCLKSREIFLSQPILLELEAPLK
Dappu-PP1 A      MAETDKLNIDSIIARLLEVRGSRPGKNVQLTENEIRGLCLKSREIFLSQPILLELEAPLK
                 *  :* :******:*******:********:*.***.**********************

Drome-PP1        ICGDIHGQYYDLLRLFEYGGFPPESNYLFLGDYVDRGKQSLETICLLLAYKIKYAENFFL
Dappu-PP1 A      ICGDIHGQYYDLLRLFEYGGFPPESNYLFLGDYVDRGKQSLETICLLLAYKIKYPENFFL
                 ************************************************.*****

Drome-PP1        LRGNHECASINRIYGFYDECKRRYTIKLWKTFTDCFNCLPVAAIVDEKIFCCHGGLSPDL
Dappu-PP1 A      LRGNHECASINRIYGFYDECKRRYNIKLWKTFTDCFNCLPVAAIVDEKIFCCHGGLSPDL
                 ************************.**********************************

Drome-PP1        SSMEQIRRIMRPTDVPDQGLLCDLLWSDPDKDTMGWGENDRGVSFTFGAEVVGKFLQKHE
Dappu-PP1 A      QSMEQIRRIMRPTDVPDQGLLCDLLWSDPDKDTMGWGENDRGVSFTFGAEVVAKFLHKHD
                 .************************************************.***:**:

Drome-PP1        FDLICRAHQVVEDGYEFFAKRQLVTLFSAPNYCGEFDNAGAMMSVDDTLMCSFQILKPAD
Dappu-PP1 A      MDLICRAHQVVEDGYEFFAKRQLVTLFSAPNYCGEFDNAGAMMSVDETLMCSFQILKPAD
                 :*********************************************:*************

Drome-PP1        KRRFVYPNFGSSGRPLTPPRGA----NNKNKKK
Dappu-PP1 A      KKKFPYGGL-NTGRPMTPPRGGPQAKQNKGKNK
                 *::* * .: .:***:*****.    :**.*:*
```

B. *Daphnia* PP1 A vs. PP1 B

```
Dappu-PP1 A      MAETDKLNIDSIIARLLEVRGSRPGKNVQLTENEIRGLCLKSREIFLSQPILLELEAPLK
Dappu-PP1 B      M--ADDLNVDSIISRLLEVRGCRPGKSVQMTEAEVRGLCLKSREIFLQQPILLELEAPLK
                 *  :*.**:****:*******.****.**:** *:************.************

Dappu-PP1 A      ICGDIHGQYYDLLRLFEYGGFPPESNYLFLGDYVDRGKQSLETICLLLAYKIKYPENFFL
Dappu-PP1 B      ICGDIHGQYTDLLRLFEYGGFPPEANYLFLGDYVDRGKQSLETICLLLAYKIKYPENFFL
                 ********* *************:**********************************

Dappu-PP1 A      LRGNHECASINRIYGFYDECKRRYNIKLWKTFTDCFNCLPVAAIVDEKIFCCHGGLSPDL
Dappu-PP1 B      LRGNHECASINRIYGFYDECKRRYNIKLWKTFTDCFNCLPIAAIIDEKIFCCHGGLSPDL
                 ****************************************:***:****************

Dappu-PP1 A      QSMEQIRRIMRPTDVPDQGLLCDLLWSDPDKDTMGWGENDRGVSFTFGAEVVAKFLHKHD
Dappu-PP1 B      QNMDQIKRIMRPTDVPDTGLLCDLLWSDPDKDVQGWSENDRGVSFTFGADVVSKFLNRHD
                 *.*:**:********** ***************.  **.*************:**:***::**

Dappu-PP1 A      MDLICRAHQVVEDGYEFFAKRQLVTLFSAPNYCGEFDNAGAMMSVDETLMCSFQILKPAD
Dappu-PP1 B      LDLICRAHQVVEDGYEFFAKRQLVTLFSAPNYCGEFDNAGGMMSVDETLMCSFQILKPSE
                 :*************************************.*****************::

Dappu-PP1 A      KK-KFPYGGLNTGRPMTPPRGGPQAKQNK--GKNK
Dappu-PP1 B      KKAKYQYSGINATKP--------NAAANKPVPKKK
                 ** *: *.*:*: :*        :*  **   *:*
```

Figure 8.

Putative *Daphnia pulex* PROTEIN PHOSPHATASE 1 (PP1) proteins. (**A**) Alignment of *Drosophila melanogaster* PP1 (Drome-PP1) with *D. pulex* PP1 A (Dappu-PP1 A). (**B**). Alignment of Dappu-PP1 A and PP1 B. In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure. In this figure, serine/threonine kinase domains identified by SMART analyses are highlighted in red.

PROTEIN PHOSPHATASE 2A (PP2A)

A. *Drosophila* vs. *Daphnia* PP2A catalytic subunit – MICROTUBULE STAR (MTS)

```
Drome-MTS       MEDKATTKDLDQWIEQLNECNQLTETQVRTLCDKAKEILSKESNVQEVKCPVTVCGDVHG
Dappu-MTS       MSD---FSDLDRQIEQLRRCDIIKESEVKALCAKAREILVEESNVQRVDSPVTVCGDIHG
                *.*    .***: ****..*: :.*::*::** **:** :*****.*..********:**

Drome-MTS       QFHDLMELFRIGGKSPDTNYLFMGDYVDRGYYSVETVTLLVALKVRYRERITILRGNHES
Dappu-MTS       QFYDLKELFKVGGDVPDTNYLFMGDFVDRGFYSVETFLLLLALKVRYPDRITLIRGNHES
                **:** ***::**. **********:****:*****. **:****** :***::******

Drome-MTS       RQITQVYGFYDECLRKYGNANVWKYFTDLFDYLPLTALVDGQIFCLHGGLSPSIDSLDHI
Dappu-MTS       RQITQVYGFYDECFRKYGSVTVWRYCTEIFDYLSLSAIIDGKIFCVHGGLSPSIQTLDQI
                ************:****...***:* *::****.*:*::**:***:********::***:*

Drome-MTS       RALDRLQEVPHEGPMCDLLWSDPDDRGGWGISPRGAGYTFGQDISETFNNTNGLTLVSR
Dappu-MTS       RIIDRKQEVPHDGPMCDLLWSDPEDTQQGWGVSPRGAGYLFGSDVVQHFNTSNDIEMICR
                * :** *****:**********:* : ***:******* **.*: : **.:*.: ::.*

Drome-MTS       AHQLVMEGYNWCHDRNVVTIFSAPNYCYRCGNQAALMELDDSLKFSFLQFDPAPR--RGE
Dappu-MTS       AHQLVMEGYKWHFNETVLTVWSAPNYCYRCGNVAAILELDENLHREFTIFEAAPQETRGI
                *********:* .:..*:*::********** **::***:.*: .*  *:.**:  **

Drome-MTS       PHVTRRTPDYFL
Dappu-MTS       PS-KKPQADYFL
                *  .:  .****
```

B1. *Drosophila* vs. *Daphnia* PP2A regulatory subunit – WIDERBORST (WBT)

```
Drome-WBT       MSSGTFVDRIDPFAKRSLKKKGKKSQGSSRYRNSQDVELQQLPPLKADCSSLEQEELFIR
Dappu-WBT A     MSAGPLVDKIDPF-KRSPKKKPKKSQGSSRYHSGSEADLQPLPLLK-DVPAGEQEELFIR
                **:*.:**:**** *** *** *********:...:.:** ** ** * .: ********

Drome-WBT       KLRQCCVSFDPMDPVTDLKGKEIKRAALNDLSTYITHGRGVLTEPVYPEIIRMISCNLFR
Dappu-WBT A     KLRQCCLGFDPLDPVADLKGKEIKRASLTELVDYITAGRGVLTEPVYPEIIRTITSNLFR
                ******:.***:***:*********:*.:*  *** *************** *:.****

Drome-WBT       TLPPSENPDFDPEEDDPTLEASWPHLQLVYEVFLRFL-----ESQDFQATIGKRVIDQKF
Dappu-WBT A     TLPPSDNPDFDPEEDDPTLEASWPHLQVIYPVDFYLVCLFCRESPDFQPGIGKKVIDQKF
                *****:********************::* * : ::     ** ***. ***:******

Drome-WBT       VLQLLELFDSEDPRERDFLKTVLHRIYGKFLGLRAFIRKQINNIFLRFIYETEHFNGVGE
Dappu-WBT A     VLQLLELFDSEDPRERDFLKTVLHRIYGKFLGLRAFIRKQINNIFLRFIYETEHFNGVSE
                ************************************************************.*

Drome-WBT       LLEILGSIINGFALPLKAEHKQFLVKVLLPLHKVKCLSLYHAQLAYCIVQFLEKDPFLTE
Dappu-WBT A     LLEILGSIINGFALPLKVEHKQFLVKVLLPLHKVKCLSLYHAQLAYCVVQFLEKDPSLTE
                ***************.*************************:******** ***

Drome-WBT       PVVRGLLKFWPKTCSQKEVMFLGEIEEILDVIDPFQFVKIQEPLFRQIAKCVSSPHFQVA
Dappu-WBT A     EVIRGLLKYWPKTCSQKEVMFLGEIEEILDVTEPAQFIKIQEPLFKQISRCVSSPHFQVA
                 *:*****:********************** :*.**:*******:**::**********

Drome-WBT       ERALYLWNNEYAMSLIEENNAVIMPIMFPALYRISKEHWNQTIVALVYNVLKTFMEMNSK
Dappu-WBT A     ERALYLWNNETVMSLIEENSAAIMPIMFPALYRISKEHWNQTIVALVYNVLKTFMEMNSK
                **********.*******.*.**************************************

Drome-WBT       LFDELTATYKTERQKEKKKEKERDELWKKLAELE---ISHRRREPSAKT-NAAGGVALAK
Dappu-WBT A     LFDELTATYKTERQKEKKKEKERDELWKKLAELE---ISHRRREPSAKT-NAAGGVALAK
                ******::**:*******:*::*:***:** ***    * *    **.* *:*...* :.

Drome-WBT       LQPPSSAGLNSHQQQSNSGSSGSLSSGGAGGDNNPATTNAKIKQDKADN
Dappu-WBT A     QSPPTL-------------------------------------------K
                 .**:                                            :
```

B2. *Daphnia* WBT A vs. WBT B

```
Dappu-WBT A     M----------SAGPLVDKIDPFKR-----------------SPKKKP----------
Dappu-WBT B     MPNKKKEKGSEDTGKPKVGNGKSSKEGSSTENGEESGRTSPGSSPSSGPKLATGPPPPAQ
                *           :. * *.: .. *.                **.. *

Dappu-WBT A     -------------KKSQGSSRYHSGSEADLQPLPLLKDVPAGEQEELFIRKLRQCCLGFD
Dappu-WBT B     INKIKFTGTTVVKNRRQSSSRFNITKNREIQKLPLLKDAAPAREELFVQKIQQCCVLFD
                             :: *.***::  .: ::* ******....*:****::*::***: **
```

Figure 9.

Putative *Daphnia pulex* PROTEIN PHOSPHATASE 2A (PP2A) proteins. (**A**) Alignment of *Drosophila melanogaster* PP2A catalytic subunit MICROTUBULE STAR (MTS) protein (Drome-MTS) with *D. pulex* MTS (Dappu-MTS). (**B1**). Alignment of *D. melanogaster* PP2A regulatory subunit WIDERBORST (WBT) protein (Drome-WBT) with *D. pulex* WBT A (Dappu-WBT A). (**B2**). Alignment of Dappu-WBT A and Dappu-WBT B. (**C**). Alignment of *D. melanogaster* PP2A regulatory subunit TWINS (TWS) protein (Drome-WBT) with *D. pulex* TWS (Dappu-TWS). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure. In this figure, protein phosphatase 2A catalytic, coiled-coil and WD40 domains identified by SMART analyses are highlighted in dark green, pink and dark red, respectively.

Drosophila vs. *Daphnia* SHAGGY (SGG)

```
Drome-SGG       MSGRPRTSSFAEGNKQSPSLVLGGVKTCSRDGSKITTVVATPGQGTDRVQEVSYTDTKVI
Dappu-SGG       MSGRPRTTSFADKTSLNPSF--------GKDGSKVTTVVATPGQGPDRAQEVAYTDAKVI
                *******:***: .. .**:         .:*****:**********.**.***:***:***

Drome-SGG       GNGSFGVVFQAKLCDTGELVAIKKVLQDRRFKNRELQIMRKLEHCNIVKLLYFFYSSGEK
Dappu-SGG       GNGSFGVVYQAKLCETGELVAIKKVLQDKRFKNRELQIMRRLEHCNIVKLKYFFYSSGEK
                ********:*****:************:**********:********* *********

Drome-SGG       RDEVFLNLVLEYIPETVYKVARQYAKTKQTIPINFIRLYMYQLFRSLAYIHSLGICHRDI
Dappu-SGG       KDEVFLNLVLEFIPETVYKVARHYSKSKQTIPISFIKLYMYQLFRSLAYIHSLGICHRDI
                :**********:**********:*:*:******.**:************************

Drome-SGG       KPQNLLLDPETAVLKLCDFGSAKQLLHGEPNVSYICSRYYRAPELIFGAINYTTKIDVWS
Dappu-SGG       KPQNLLLDPESGVLKLCDFGSAKHLVQGEPNVSYICSRYYRAPELIFGATDYTTNIDVWS
                **********:.***********:*::********************** :***:*****

Drome-SGG       AGCVLAELLLGQPIFPGDSGVDQLVEVIKVLGTPTREQIREMNPNYTEFKFPQIKSHPWQ
Dappu-SGG       AGCVLAELLLGQPIFPGDSGVDQLVEIIKVLGTPTREQIREMNPNYTEFKFPQIKAHPWQ
                **************************:****************************:****

Drome-SGG       KVFRIRTPTEAINLVSLLLEYTPSARITPLKACAHPFFDELRMEGNHTLPNGRDMPPLFN
Dappu-SGG       KVFRARTPLEAIDLVSRLLEYTPSARISPLEACAHTFFEELR-DPHTRLPNGRELPVLFN
                **** *** ***:*** **********:**:*****.**:*** : :  ******:* ***

Drome-SGG       FTEHELSIQPSLVPQLLPKHLQNASGPGGNRPSAGGAASIAASGSTSVSSTGSGASVEGS
Dappu-SGG       FTEHELKIQPALNAVLIPPHMRGASGLMG--------VMNATSPSQDSSATGSPNSAGGA
                ******.***:* . *:* *::.***  *         .  *:* * . *:***  *. *:

Drome-SGG       AQPQSQGTAAAAGS---------GSGGATAGTGGASAGGPGSGNNSSSGGASGAPSAVAA
Dappu-SGG       EGSSAAGASGAAGGHIHSPPDVCSSTGETLGAVGTA-----------------------
                  ..: *::.***.          .* * * *: *::

Drome-SGG       GGANAAVAGGAGGGGGAGAATAAATATGAIGATNAGGANVTDS
Dappu-SGG       -------------------------------------------
```

Figure 10.

Putative *Daphnia pulex* SHAGGY (SGG) protein. Alignment of *Drosophila melanogaster* SGG (Drome-SGG) with *D. pulex* SGG (Dappu-SGG). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure. In this figure, serine/threonine kinase domains identified by SMART analyses are highlighted in red.

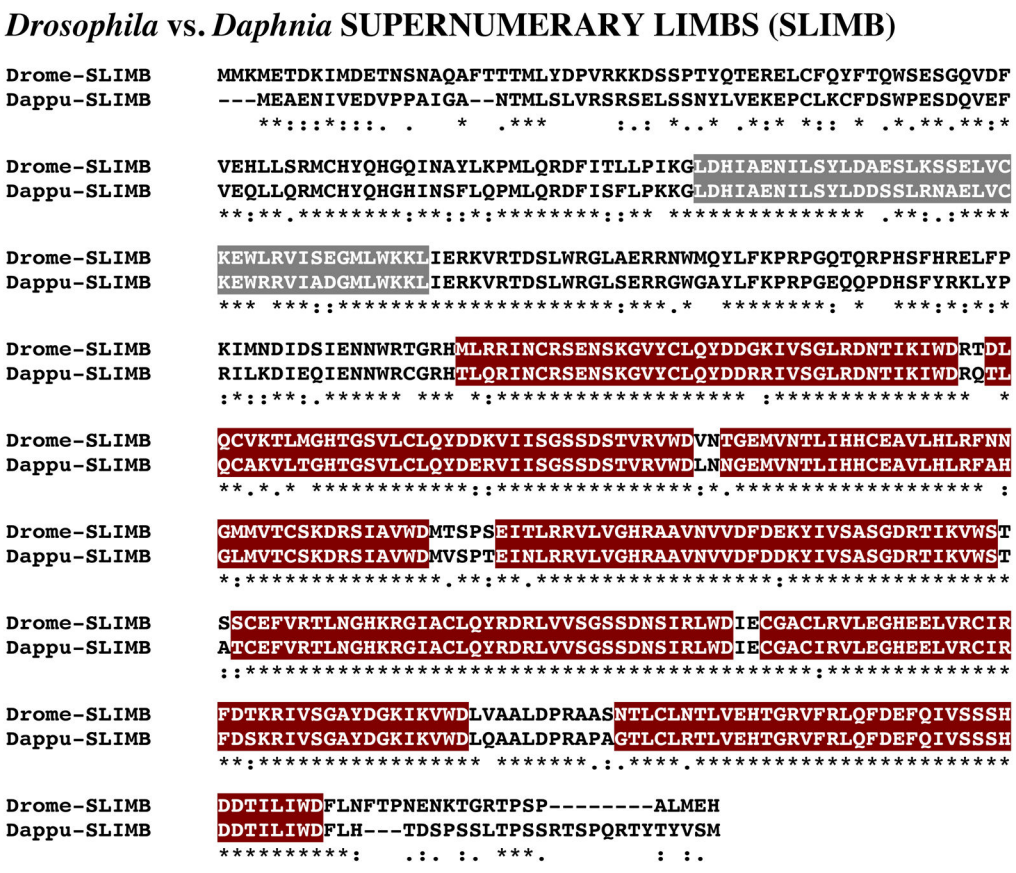

Figure 11.

Putative *Daphnia pulex* SUPERNUMERARY LIMBS (SLIMB) protein. Alignment of *Drosophila melanogaster* SLIMB (Drome-SLIMB) with *D. pulex* SLIMB (Dappu-SLIMB). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure. In this figure, FBOX and WD40 domains identified by SMART analyses are highlighted in dark gray and dark red, respectively.

***Drosophila* vs. *Daphnia* TIMELESS (TIM)**

```
Drome-TIM       MSRVRQLHNHIWNNQNFDKVKSVMDWLLATPQLYS-AFSSLGCLEGDTYVVNPNALAILE
Dappu-TIM A     ----------------------MDWLTLSGGVPSLSLIPLGILHNNKYYISDDCLFHLE
                                      ****  :  : * :: .** *..:.* :. :.*  **

Drome-TIM       EINYKLTYEDQTLRTFRRAIGFGQNVRSDLIPLLENAKDD-AVLESVIRILVNLTVPVEC
Dappu-TIM A     EIVVKLNCEDQTTRPLRCALGFMDILNKDLIPILINSKDQPSVFCSTIKLLVGLTVPVEC
                **  **. **** *.:* *:** : :..****:* *:**: :*: *.*::**.*******

Drome-TIM       LFSVDVMYRTDVGRHTIFELNKLLYTSKEAFTEARSTKSVVEYMKHILESDPKLSPHKCD
Dappu-TIM A     LVCVDTSNQSPTSQHIVHELTQLLYNAKEAFLDPRATRAVIDHLHRLLEKQP-LSSDDSE
                *..**.  :: ..:* :.**.:***.:**** :.*:*::*:::::::::**.:* **....:

Drome-TIM       QINNCLLLLRNILHIPETHAHCV--------------------MPMM--QSMPHGI----
Dappu-TIM A     SVNHSLLLIRNILHAPERPPNMTEVGDNEAATASAQPGSNNSQTPYLGPHSHSHGFTADC
                .:*:.***:***** **  .: .                      * :  :* .**:

Drome-TIM       SMQNTILWNLFIQSIDKLLLYLMTCPQRAFWGVTMVQLIALIYKDQHVSTLQKLLSLWFE
Dappu-TIM A     SQQNRLLWNLFSQGLDRLLINLLASSQKNEWVVTIVQLIALFYKDQHVENMQKLLGTWIN
                * ** :***** *.:*:**: *::..*:  * **:******:******..:****. *::

Drome-TIM       ASLSESSE-DNESNTSPPKQGSGDSSPMLT--SDPTSDSSDNGSNGRGMGGGMREGTAAT
Dappu-TIM A     PTTSESSEDDDESNTSPP-IGPCNSPQILTSSSDPTSDSSDS------------------
                .: ***** *:******* *. :*. :**  *********.

Drome-TIM       LQEVSRKGQEYQNAMARVPADKPDGSEEASDMTGNDSEQPGSPEQSQPAGESMDDGDYED
Dappu-TIM A     ------------------------AQRKASALAPQDENSEANPNESQ-------------
                                        ...:** :: :*.:. ..*::**

Drome-TIM       QRHRQLNEHGEEDEDEDEVEEEEYL-QLGPASEPLNLTQQPADKVNNTTNPTSSAPQGCL
Dappu-TIM A     ------------TETMTTIEKKAVVTNAASTTSPGATTQVATGKSSRSKGHKFSTVSGAP
                             *    :*::  : : ..::.*   ** .:.* ..:.. . *: .*.

Drome-TIM       GNEPFKPPPPLPVRASTSAHAQMQKFNESSYASHVSAVKLGQKSPHAGQLQLTKGKCCPQ
Dappu-TIM A     GGD--------TASTSSTAPSQYSSQSDKSRRSSQHSVSSGFHSENSGNQHPSHHAKDSD
                *.:         .. :*::* :* .. .:.*  *   :*. * :* ::*: : ::    .:

Drome-TIM       KRECPSSQSELSDCGYGTQVENQESISTS------------SNDDDGPQGKPQHQKPPCN
Dappu-TIM A     DHKEKSSKRRCHGNSQNSERRNETVIPTTIQTPVIIKEEILSTSFSGTPTVTLQQTEDTN
                .::  **: .  . . .:: .*:  *.*:             *.. .*.   . :*.   *

Drome-TIM       TKPRNK--PRTIMSPMDKKELRRKKLVKRSKS-SLINMKGLVQHTPTDDDISNLLKEFTV
Dappu-TIM A     NKRSNKVTHKTGKVQPPEKEQRRKKLSKRSRTMGHIKMKA-VQYTPTEEDISQLLKEFTV
                .*  **   :*      :** ***** ***:: . *:**. **:***::***:*******

Drome-TIM       DFLLKGYSYLVEELHMQLLSNAKVPIDTSHFFWLVTYFLKFAAQLELDMEHIDTILTYDV
Dappu-TIM A     DFLLNGYNALVGELHQQLLQQDDLPLDKSHFLWLITYFLRFASQLELDMEHFKDVFTIDL
                ****:**. ** *** ***.: .:*:*.***:**:****:**:********:. ::* *:

Drome-TIM       LSYLTYEGVSLCEQLELNARQEGSDLKPYLRRMHLVVTAIREFLQAIDTYNKV-------
Dappu-TIM A     LCYLTWEAVRETEEFEMNSLRPSIDLKPCLRRLHLGVTAIREYLQALETYSRLGSSQNAA
                *.***:*.*   *::*:*: : . **** ***:** ******:***::**.::

Drome-TIM       -THLNEDDKAHLRQLQLQISEMSDLRCLFVLLLRRFNPSIHSKQYLQDLVVTNHILLLIL
Dappu-TIM A     GNGSSQGYEERICQLRGYLPAIRDLRQLFLLQLRHFNPIIQSRRYLRDVITANHVLLLTL
                 .  .:. : :: **:  :. : *** **:* **:*** *:*::**:*::.:**:*** *

Drome-TIM       DSSAKLGG-CQTIRLSEHITQFATLEVMHYYGILLEDFNNNGEFVNDCIFTMMHHIGGDL
Dappu-TIM A     ERAAQQSTYGPSFDLRDHLHQFCSKTILTRYGTALEDFKTNGPFVNDCILTVLHHIGADL
                : :*: .    :: * :*: **.:  ::  **  ****:.** ******:*::*****.**

Drome-TIM       GQIGVLFQPIILKTYSRIWEADYELCDDWSDLIEYVIHKFM---NTPPKSPLTIPTTSLT
Dappu-TIM A     GRADLLCEPVILRSFSKIWEEEFNMCDDWDDLIEYVVQKFLRNFQTGGCYDVGSPQRSVS
                *: .:* :*:**:::*:*** ::::*****.******::**:   :*     :  *  *::

Drome-TIM       EMTKEHNQEHTVCSWSQEEMDTLYWYYVQSKKNNDIVGKIVKLFSNNGNKLKTRISIIQQ
Dappu-TIM A     GSPDQEGGSPATDTEAQQQFD------AQASPAGD------------------------
                  ..:.. . :. : :*:::*      .*:.  .*

Drome-TIM       LLQQDIITLLEYDDLMKFEDAEYQRTLLTTPTSATTESGIEIKECAYGKPSDDVQILLDL
Dappu-TIM A     ------------DDPF-----------------------------FDTPRTDIESLRGQ
                           ** :                              :..*  *:: * .

Drome-TIM       IIKENKAQHLLWLQRILIECCFVKLTLRSGLKVPEGDHIMEPVAYHCICKQKSIPVVQWN
Dappu-TIM A     LMDSGFQKQLDWIQSSLLVSCSAR------LGTYNGQEFRNPIASLSRKMKVSCPIIPWT
                ::...  ::* *:*  *: .* .:      * . :*:.: :*:*  .   : * *:: *.

Drome-TIM       NEQSTTMLYQPFVLLLHKLGIQLPADAGSIFARIPDYWTPETMYGLAKKLGPLDKLNLKF
```

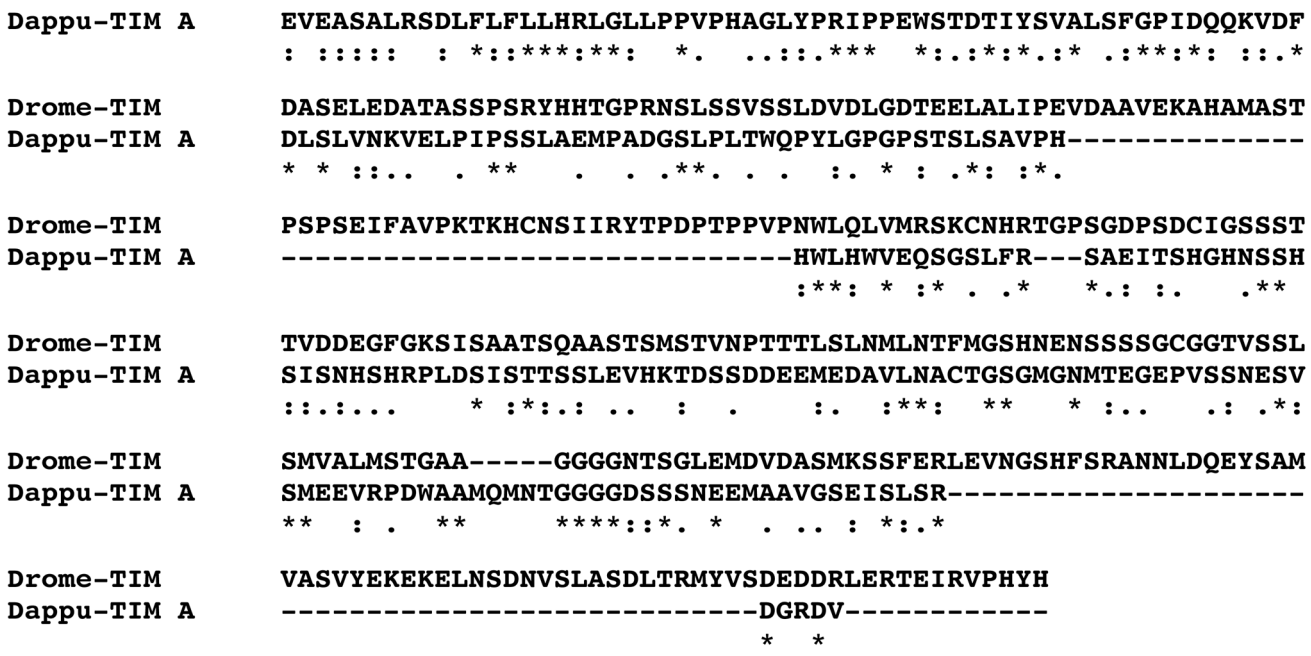

Figure 12.

Putative *Daphnia pulex* TIMELESS (TIM) protein. Alignment of *Drosophila melanogaster* TIM (Drome-TIM) with *D. pulex* TIM A (Dappu-TIM A). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure.

***Drosophila* vs. *Daphnia* VRILLE (VRI)**

```
Drome-VRI       MSIVCTLEQKVNFFKAAATTKNLLILKNNTDTNYINNYKQDNPSNNKFPRIQAQSNNSHL
Dappu-VRI       M------------------------------------------------------------
                *

Drome-VRI       QHQQQLQQKLAQLHHYSQQKLSGSDFPYGPRPPTGGKEEKLLLLAPPGKLYPEASVSTAM
Dappu-VRI       ------------------------------------------------------------


Drome-VRI       PEVLSGTPTNSHNKANIAMMNNVRLSNISPTLSMNGSSNEASNLHPLSMYGGSISPQSND
Dappu-VRI       ----SGEPQRT--------------------------------------------SPSGNS
                    ** * .:                                            **..*.

Drome-VRI       SGMSDSLGKYVPGSGYGDGMM--AQSPSQGGNGPQSALTAAQKELFSQRKQREFTPDNKK
Dappu-VRI       QQHVNSLSHHNAAMEERAAALQAAAAVAAAVSGFSSANLSVGMDLSGLRKQREFIPDNKK
                .   :**.:: ..     . :  * : : . .* .**  :.  :* . ****** *****

Drome-VRI       DESYWDRRRRNNEAAKRSREKRRYNDMVLEQRVIELTKENHVLKAQLDAIRDKFNISGEN
Dappu-VRI       DDSYWDRRRRNNEAAKRSREKRRLNDMVLESRVLELTKENHILRAQLSAVREKYGINPDG
                *:********************* ******.**:*******:*:***.*:*:*:.*. :.

Drome-VRI       LVSVEKILASLPTSEQVLSNTK-RAKMSGSGGSSSGSSPS-GSGSGEGSPQGGHNGYPVG
Dappu-VRI       LVSIDQVLATLPSPDQVLSLPRPRSRL------LSGLSPSMGSGRGGGSVSPAPSNRSLS
                ***::::**:**:.:**** .: *:::       ** *** *** * ** . . .. .:.

Drome-VRI       PPLSPLIYGPN-GNARPEATVKSVHHIHHAGVAPPPTHLQQLVVPQSQTQHLYQPQP---
Dappu-VRI       PPISP--SPPNVGSGQGSGSLSMQQQQQHAYASNGHHHQQQ----QQQHQHGYRYQSVGE
                **:**    ** *..: ..::.  :: :** .:    * **    *.* ** *: *.

Drome-VRI       ---------QQHQPHQQQQISQPPQQQQQQQEPSPSAGSSSPVISDPHNRPPSTTIANLQ
Dappu-VRI       SAAAGANVFQHHQHHQHHQHAQPMKAQQRVMERS--------------------------
                         *:** **::* :** : **:  * *

Drome-VRI       VQLQQALNRNVRPEDLDSLRKVVAAGALYNAAAVVGAPPPPPSAGLYVPAPSAYKDHLEA
Dappu-VRI       -------------------------------------LPPLPALTPVPGISP-NHHLEA
                                                    ** .*   **. *. :.****

Drome-VRI       AAAWSHNVEAAVSSSAVDAVSSSSVSGSAASVLNLSRRACSPSYEHMLSSTTSSTLSSAS
Dappu-VRI       GASPAEGAHPQHGLAVVDRTGSSGNVGPVAAFFDL----CS-------SSSSSSSSSSS
                .*: :.....  . :.** ..**.  *..*:.::*    **       **::**: **:*

Drome-VRI       SSGAVSGDDEQEHEPAHMAPLQLQRSSPQQGSDANNCLPLKLRHKSHLGDKD------AA
Dappu-VRI       HPGSSSGED-GSHSPIDPA------------VAAGRLLPLKLRHKTHLGDRDVTAATASA
                 .*: **:*  .*.* . *              *.. ********:****:*      :*

Drome-VRI       ATALLSLQHIKQEPNCSRASPPAWNDGGDN-----------------------------
Dappu-VRI       AAVLLTLNEIKHEPEGIDDSPSAVESTVENVTSEMNNNVCDHHSQVHHHGLHHHPEHHHH
                *:.**:*:.**:**:    **.* :.  :*

Drome-VRI       -------------------------------SSDERDSGISIASAEWTAQLQRKLLAPKE
Dappu-VRI       HHHPQPHLQSHPNPHPHQQLHQHQSGRRSTESSDDRDSGIS-SGGDWSLPRSSSRFSSSS
                                             ***:****** :..:*:   . . ::...

Drome-VRI       ANVVTS---------AERDQMLKSQLERLESEVASIKMILAE------------------
Dappu-VRI       SGSTASVTGNHGNNPAHSYQPLQQQQQQQQQQPASKRMRMSSNSPPIQHHAQQHQQAVQR
                :. .:*         *.  * *:.* :: :.: ** :* ::.

Drome-VRI       ------------------------------------------------------------
Dappu-VRI       RFVGSRVNQQQQQQQQQQLMGNQETEKEENEAASGINHERLVIVGGNESSDENNDELRSH


Drome-VRI       -------------------------------------------
Dappu-VRI       IARLASELESLKTMMLGTGSASTMSSSSAMRTNKTNSTSNFRLN
```

Figure 13.

Putative *Daphnia pulex* VRILLE (VRI) protein. Alignment of *Drosophila melanogaster* VRI (Drome-VRI) with *D. pulex* VRI (Dappu-VRI). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure. In this figure, basic region leucine zipper domains identified by SMART analyses are highlighted in dark blue.

Figure 14.

Putative *Daphnia pulex* CRYPTOCHROME (CRY) proteins. (**A**) Alignment of *Drosophila melanogaster* CRY (Drome-CRY) with *D. pulex* CRY A (Dappu-CRY A). (**B**). Alignment of Dappu-CRY A–D. In the line immediately below each sequence

grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure.

A. ***Danaus*** **CRYPTOCHROME (CRY) 1 vs.** ***Daphnia*** **CRY A**

```
Danpl-CRY1      MLGGNVIWFRHGLRLHDNPSLHSALEDASSPFFPIFIFDGETAGTKMVGYNRMRYLLEAL
Dappu-CRY A     M---NVLWFRRGLRIHDNPALLSALEN-SKDFIALFVFDTTFQDPGYKPYHMNGFLLECL
                *   **:****:***:****:* ****: *. *:.:*:**    ..    *:   :***.*

Danpl-CRY1      NDLDQQFRKYGGKLLMIKGRPDLIFRRLWEEFGIRTLCFEQDCEPIWRPRDASVRALCRD
Dappu-CRY A     HDLNESLESVGTKLHVFQGCPLEVFRHLHNIKPINKLCFIQDCEPIFHERDIAAKNLCSE
                :**::.:.. * ** :::* *  :**:* :   *..*** ******:: ** :.: ** :

Danpl-CRY1      IGVSCREHVAHTLWNPDTVIKANGGIPPLTYQMFLHTVEIIGNPPRPVDDVDLNGVNFGS
Dappu-CRY A     LDIEVYEHVAHTLWDPMDIIASNGGTPPLTYEMFVHVAMSVGDPPKPVADPEWKGVKFLT
                :.:.  ********:*  :* :*** *****:**:*..  :*:**:** * : :**:* :

Danpl-CRY1      LPESFYREFVVFDKAPKPEDLGVFLENEDIRMIRWVGGETAALKQMQERLAVEYETFCRG
Dappu-CRY A     LEPFESNRFTLFGGVPTLSQIGVPENPVGCRGRRIFGGETNALKHFAIRLQAEETAFRSG
                *     ..*.:*. .*. .::**  :  . *  * .**** ***::  ** .*  :*  *

Danpl-CRY1      SYLPTHGNPDLLGPPISLSPALRFGCLSVRRFYWSLQDLFQQVHQGRLASTQFITGQLIW
Dappu-CRY A     FYQPNQARPDLLGPPLSLSAAISVGAISVRLFYWRIHEIFDKVNRGNPPAWLGITGQIIW
                 * *.:..*******:***.*: .*.:*** *** ::::*:::*::*. .:   ****:**

Danpl-CRY1      REYFYTMSVNNPNYAQMSGNPICLDIPWKEPENDELQRWKEGRTGFPFVDAAMRQLRTEG
Dappu-CRY A     RDYFYAMSRMNPKFDKEVDNPICLQIPWVENE-EFFEKWKNGQTGYPFIDAGMRQLNQEG
                *:***:**  **:: :  .*****:*** * * : :::**:*:**:**:**.****. **

Danpl-CRY1      WLHHVVRNTVASFLTRGTLWLSWEHGLQHFLKYLLDADWSVCAGNWMWVSSSAFEALLDS
Dappu-CRY A     WMHHSVRNAVAMFLTRGDLWLNWDIGAEYMANQLVDSDWSVNSGNWMWVSSSAFERLLDC
                *:** ***:** ***** ***.*: * ::: : *:*:**** :************ ***.

Danpl-CRY1      GECACPVRLGRRLEPTGHYVRRYVPELARMPGEYIYEPWRAPLEVQEAAGCVIGRDYPAP
Dappu-CRY A     SVCINSVLYGKRLEPSGDYIRRYVPELANFEFEYIHEPWKAPIDIQRTANCIIGQDYPAQ
                . *  .*  *:****:*.*:********.:  ***:***:**:::*.:*.*:**:****

Danpl-CRY1      VVDHTAAAARNRANMQELRRLLEKAPPHCCPSSEDEVRQFMWLGDDSQPELTTT
Dappu-CRY A     MVVHEEVLPRNLEWMKEFRQKFKETPAHCQPSSNSEVYKFFCLPDDSLP----F
                :* *  . .**   *:*:*: ::::*.** ***:.** :*: * *** *
```

B. ***Danaus*** **CRY2 vs.** ***Daphnia*** **CRY B**

```
Danpl-CRY2      MSVAETLPLRARSPTAQKSSQPAGVPKEKHTVHWFRKGLRLHDNPALREGLVDATTFRCV
Dappu-CRY B     MSGYDS------------------EPREKQVVHWFRKGLRLHDNPSLKDGLKGCSTYRCI
                **  ::                   *:**:.**************:*::** ..:*:**:

Danpl-CRY2      FIIDPWFASSSNVGINKWRFLLQCLEDLDKNLRKLNSRLFVVRGQPADALPKLFREWGTT
Dappu-CRY B     FILDPWFAGSSNVDINKWRFLLESLEDLDQNLRKLNSRLFVIRGQPAGVLPKLFKEWETT
                **:*****.****.********:.*****:**********:******..*****:** **

Danpl-CRY2      ALTFEEDPEPYGRVRDHNIMTKCREVGIQVTSRVSHTLYKLDDIIEKNGGKAPLTYHQFQ
Dappu-CRY B     CLTFEEDPEPFGRVRDQNIITMCKDFNIEVITRASHTLYHPQKIIEKNGGKAPLTYRQFQ
                .*********:*****:**:* *:::..*:* :*.*****: :.*************:***

Danpl-CRY2      ALIASMPPPPSAEPTISLETLNRAVTPISDNHDERFGVPTLEELGFDTEGLKPPIWIGGE
Dappu-CRY B     NIIASVDAPPPPESDITFESIGRGYTPMDESMDDRFSVPTLEELGFDTDGLMPAVWHGGE
                 :***: .**..*. *:::*::.*. **:.:. *:**.***********:** *.:* ***

Danpl-CRY2      NEALLRLERHLERKAWVASFGRPKMTPESLLSSQTGLSPYLRFGCLSTRLFYYQLSELYK
Dappu-CRY B     TEALTRLERHLERKAWVASFGRPKMTPQSLLASQTGLSPYLRFGCLSVRLFHQQLTNLYK
                .*** **********************:***:***************.***: **::***

Danpl-CRY2      RIKQERPPLSLHGQILWREFFYCAATRNPNFDRMEGNPICVQIPWEKNQEALKKWANGQT
Dappu-CRY B     KIKKAQPPLSLHGQVLWREFFYCAATNNPNFDKMIGNPICVQIPWDSNAEALAKWANGQT
                :**: :********:***********.*****:* **********:.* *** *******

Danpl-CRY2      GFPWIDAIMIQLRNDGWIHHLARHAVACFLTRGDLWISWEEGMKVFDELLLDADWSVNAG
Dappu-CRY B     GFPWIDAIMTQLREEGWIHHLARHAVACFLTRGDLWISWEEGMKVFEELLLDADWSVNAG
                ********* ***::**********************************:************

Danpl-CRY2      MWMWLSCSSFFQQFFHCYCPVRFGRKTDPNGDFIRKYIPVLKNMPTRYIHEPWVCPEEIQ
Dappu-CRY B     TWMWLSCSSFFHQFFHCYCPVRFGRKVDPNGDFIKKYQPVLKNFPLQYIHEPWNAPESVQ
                 **********:***************.*******:** *****:* :****** .**.:*

Danpl-CRY2      KSIRCIIGKDYPMPIVDHTKASEINLERIKQVYAQLAKFKPQGALIPQMLQRPNVLQSSP
Dappu-CRY B     RAAKCVIGKDYPLPMVNHLEVSQLNIERMKQVYQRLTQYRGTGLM----------SHSPQ
                :: :*:*******:*:*:* :.*::*:**:**** :*::::  * :           :*.

Danpl-CRY2      SPTSIIANINQSNYLCSQSSDVPTPTNQTTNQFKEDAVFLKPTVNNIKSNVDKQQQFKQV
Dappu-CRY B     SDHGIIINVGNKN---------KNENSHAKQFRTDE-LRQNAVQRNQSNLN--------
                *  .** *:.:.*          .  *. ::**: *  : : :*:. :**::
```

```
Danpl-CRY2      VIVQEDKHSENQRHSVGNKYIVNEINKNINDIPVKQNNYDFKALTLNLNKFSNEPLTFLN
Dappu-CRY B     ------------------------------------------------------------

Danpl-CRY2      QTPNKNESFGQDVNNVIDVYSTSKPKFYFTDNGVITHNENAQTFKRDSYSDNYNKESTGS
Dappu-CRY B     ------------------------------------------------------------

Danpl-CRY2      NRVGEVHSNNPQTDKISSEKKN
Dappu-CRY B     ----------------------
```

Figure 15.

Alignment of *Daphnia pulex* CRYPTOCHROME (CRY) A and B with their *Danaus plexippus* homologs. (**A**) Alignment of *D. plexipus* CRY1 (Danpl-CRY1) with *D. pulex* CRY A (Dappu-CRY A). (**B**). Alignment of *D. plexipus* CRY2 (Danpl-CRY2) with *D. pulex* CRY B (Dappu-CRY B). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure.

Drosophila vs. *Daphnia* PIGMENT DISPERSING FACTOR/ HORMONE RECEPTOR (PDF/HR)

```
Drome-PDFR      MTLLSNILDCGGCISAQRFTRLLRQSGSSGPSPSAPTAGTFESKSMLEPTSSHSLATGRV
Dappu-PDHR      M------------------------------------------------------------
                *

Drome-PDFR      PLLHDFDASTTESPGTYVLDGVARVAQLALEPTVMDALPDSDTEQVLGNLNSSAPWNLTL
Dappu-PDHR      ------------------------------------------------------------


Drome-PDFR      ASAAATNFENCSALFVN-----YTLPQTGLYCNWTWDTLLCWPPTPAGVLARMNCPGGFH
Dappu-PDHR      --------EQCRQLYQEAQRDLEETSQFQVFCRVTWDTLLCWPPTRPGETVHLPCPPR-Q
                        *:*  *: :         .*  ::*. ********** .*  .:: **   :

Drome-PDFR      GVDTRKFAIRKCELDGRWGSRP----------------NATEVNPPGWTDYGPCYKPEII
Dappu-PDHR      GIDPTQWAERRCLDSGIWEGPPPSDASDAAAAAVALQNDADDMQQQGWTNYSQCFLPEIR
                *:*. ::* *:*  .* * . *                 :* :::  ***:*. *: ***

Drome-PDFR      RLMQQMGS---KDFDAYIDIARRTRTLEIVGLCLSLFALIVSLLIFCTFRSLRNNRTKIH
Dappu-PDHR      DLMKRLDSGSGQDAENKLIVAQATRVLELTGLTVSLISLMISLFIFTYFRSLQNHRTRIH
                 **::::.*   :* :  : :*: **.**:.** :**::*::**:**  ****:*:**:**

Drome-PDFR      KNLFVAMVLQVIIRLTLYLDQF-----------RRGNKEAATNTSLSVIENTPYLCEASY
Dappu-PDHR      KNLFAAIGIQVIIRMTLYLDQAVFVSEMVGGGSHQTLIDSSTVAAARGIHETPILCEWFY
                ****.*: :*****:******             ::   :::* ::   *.:** ***  *

Drome-PDFR      VLLEYARTAMFMWMFIEGLYLHNMVTVAVFQGSFPLKFFSRLGWCVPILMTTVWARCTVM
Dappu-PDHR      IVLEYARTTMFVWMFIEGLFLHNLITVMVFRPDTYHKLYLALGWGIPIILTAIWAAFTAT
                ::******:**:*******:***::** **: .   *::  *** :**::*::**  *.

Drome-PDFR      YMDTSLGECLWNYNLTPYYWILEGPRLAVILLNFCFLVNIIRVLVMKLRQSQASDIEQTR
Dappu-PDHR      HQTTS--ACWLGYNLTPSYWILEGPRLTIIFINLLYLLNILRVLVTKLRNSQCSEAEQLR
                :  **   *  .***** *********::*::*: :*:**:**** ***:**.*: ** *

Drome-PDFR      KAVRAAIVLLPLLGITNLLHQL-APL-KTATNFAVWSYGTHFLTSFQGFFIALIYCFLNG
Dappu-PDHR      KSVKAAMVLLPLLGITNALVMIKAPLDRSVVEFALWSYSSHFLTSFQGFFVALLYCFLNG
                *:*:**:********** *  : *** ::..:**:***.:**********:**:******

Drome-PDFR      EVRAVLLKSLATQLSVR----GHPEWAPKRASMYSGAYNTAPDTDAVQPAGDPSATGKRI
Dappu-PDHR      EVRSTLAKKMRNYLTDRQLGTSFLGFGPGSTRLMSQFANPSTQVETEREA--------RT
                ***:.* *.: . *: *    ..  :.*  : : *   *.:.:.:: : *        *

Drome-PDFR      SPPNKRLNGRKPSSASIVMIHEPQQRQRLMPRLQNKAREKGKDRVEKTDAEAEPDPTISH
Dappu-PDHR      VLDDQQLQQLQPGSTA-----EKHPRHLLRASFSLSAAAQ-----QHQDTSLEP------
                   :::*:  :*.*::     * : *: * . :. .*  :     :: *:. **

Drome-PDFR      IHSKEAGSARSRTRGSKWIMGICFRGQKVLRVPSASSVPPESVVFELSEQ
Dappu-PDHR      ----------------------------INKMMTTTLV------------
                                          : :: ::: *
```

Figure 16.

Putative *Daphnia pulex* PIGMENT DISPERSING HORMONE RECEPTOR (PDHR) protein. Alignment of *Drosophila melanogaster* pigment dispersing factor receptor (Drome-PDFR) with *D. pulex* PDHR (Dappu-PDHR). In the line immediately below each sequence grouping, stars indicated amino acids that are identically conserved, while single and double dots denote amino acids that are similar in structure. In this figure, hormone receptor and transmembrane domains identified by SMART analyses are highlighted in black and light gray, respectively.

Table 1

Putative *Daphnia pulex* genes identified via genome mining

Protein	Gene name*	Gene location/length		Homology to query	
		Scaffold	Length (nucleotides)	Blast score	E-value
CORE CLOCK PROTEINS[1]					
CASEIN KINASE II α-subunit (CKII α)	*dappu-ckII α*	17	3693	576	1e-164
CASEIN KINASE II β-subunit (CK II β)	*dappu-ckII β*	41	2093	410	5e-115
CLOCK (CLK)	*dappu-clk*	27	5939	407	2e-113
CLOCKWORK ORANGE (CWO)	*dappu-cwo*†	19	3773	130	3e-30
CYCLE (CYC)	*dappu-cyc*	1	6473	437	1e-122
DOUBLETIME (DBT)	*dappu-dbt*	1	2124	550	1e-156
PAR DOMAIN PROTEIN 1e (PDP1e)	*dappu-pdp1ε*	21	4794	139	3e-33
PERIOD (PER)	*dappu-per*	47	5860	289	1e-77
PROTEIN PHOSPHATASE 1 (PP1) A	*dappu-pp1 a*	145	2591	584	3e-167
PROTEIN PHOSPHATASE 1 (PP1) B	*dappu-pp1 b*	12	1972	566	1e-161
PROTEIN PHOSPHATASE 2A (PP2A) catalytic subunit – MICROTUBULE STAR (MTS)	*dappu-mts*	13	2099	427	5e-120
PROTEIN PHOSPHATASE 2A (PP2A) regulatory subunit – WIDERBORST (WBT) A	*dappu-wbt a*	8	6199	746	0.0
PROTEIN PHOSPHATASE 2A (PP2A) regulatory subunit – WIDERBORST (WBT) B	*dappu-wbt b*	2	4882	614	5e-176
PROTEIN PHOSPHATASE 2A (PP2A) regulatory subunit – TWINS (TWS)	*dappu-tws*	43	4157	777	0.0
SHAGGY (SGG)	*dappu-sgg*	76	5712	665	0.0
SUPERNUMERARY LIMBS (SLIMB)	*dappu-slimb*	169	2880	807	0.0
TIMELESS 1 (TIM1) A	*dappu-tim1 a*	24	14166	326	8e-89
TIMELESS 1 (TIM1) B	*dappu-tim1 b*	6	5052	282	2e-75
TIMELESS 1 (TIM1) C	*dappu-tim1 c*	10	3650	276	1e-73
TIMELESS 1 (TIM1) D	*dappu-tim1 d*	6	5438	270	5e-72

Protein	Gene name*	Gene location/length		Homology to query	
		Scaffold	Length (nucleotides)	Blast score	E-value
TIMELESS 1 (TIM1) E	*dappu-tim1 e*	6	5542	258	3e-68
TIMELESS 1 (TIM1) F	*dappu-tim1 f*	24	11674	235	2e-61
TIMELESS 1 (TIM1) G	*dappu-tim1 g*	75	2798	218	3e-56
TIMELESS 1 (TIM1) H	*dappu-tim1 h*	91	4537	181	5e-45
VRILLE (VRI)	*dappu-vri*	92	2225	153	5e-37
INPUT PATHWAY PROTEINS[1]					
CRYPTOCHROME (CRY) A – (CRY1)	*dappu-cry a*≠	40	2706	474	6e-134
CRYPTOCHROME (CRY) B – (CRY2)	*dappu-cry b*Δ	18	4661	358	7e-99
CRYPTOCHROME (CRY) C	*dappu-cry c*‡	10	3072	355	5e-98
CRYPTOCHROME (CRY) D – (CRY DASH)	*dappu-cry d*+	7	2052	129	5e-30
OUTPUT PATHWAY PROTEINS					
PIGMENT DISPERSING HORMONE RECEPTOR (PDHR)	*dappu-pdhr*£	1	4621	362	5e-100

[1] It should be noted that the group designations are based on the organization of the *Drosophila melanogaster* clock, whose proteins were used for querying the *Daphnia* genome. Unlike *Drosophila*, which possess only CRY1 (an input pathway protein), *D. pulex* appears to contain multiple CRY isoforms, including both CRY1 and CRY2 (a core clock protein), though in this table, all are presented under the "INPUT PATHWAY PROTEIN" heading.

* All genes appear correctly annotated (Genes2010 Annotation) in wFleaBase (http://wfleabase.org/) unless otherwise noted.

† Gene annotated as "conserved protein" in wFleaBase.

≠ Gene annotated as "dna photolyase" in wFleaBase.

Δ Gene annotated as "phr6-4, cryptochrome-1" in wFleaBase.

‡ Gene annotated as "phr6-4, cryptochrome-1" in wFleaBase.

+ Gene annotated as "Cryptochrome-1" in wFleaBase.

£ Gene annotated as "class b secretin-like g-protein coupled receptor gprcal2" in wFleaBase.

Table 2

Reciprocal blasting of deduced *Daphnia pulex* proteins versus known *Drosophila melanogaster* protein sequences in FlyBase

	Top named protein hit in FlyBase			
			Homology to query	
***Daphnia* protein**	**Protein name**	**FlyBase ID**	**Blast score**	**E-value**
CORE CLOCK PROTEINS[1]				
Dappu-CKII α	CKIIalpha-PC	FBpp0070043	566.2	1.06e-161
Dappu-CKII β	CKIIbeta-PE	FBpp0089135	409.1	9.91e-115
Dappu-CLK	CLK-PA	FBpp0076500	397.9	1.64e-110
Dappu-CWO	CWO-PA	FBpp0081723	106.3	7.84e-23
Dappu-CYC	CYC-PA	FBpp0074693	432.6	3.92e-73
Dappu-DBT	DCO-PC (DBT)	FBpp0085106	540.8	5.61e-154
Dappu-PDP1e	PDP1-PD	FBpp0076495	109.8	2.72e-24
Dappu-PER	PER-PA	FBpp0070455	264.6	3.30e-70
Dappu-PP1 A	PP1alpha-96A-PA	FBpp0084026	598.2	2.36e-171
Dappu-PP1 B	FLW-PB (PP1)	FBpp0071382	599.7	8.31e-172
Dappu-PP2A MTS	PP4-19C-PE (MTS)	FBpp0077017	592.8	1.02e-169
Dappu-PP2A WBT A	WDB-PE	FBpp0084579	625.9	1.67e-179
Dappu-PP2A WBT B	PP2A-B′-PJ	FBpp0288759	744.2	0
Dappu-PP2A TWS	TWS-PH	FBpp0081671	755.0	0
Dappu-SGG	SGG-PA	FBpp0070450	658.7	0
Dappu-SLIMB	SLMB-PA (SLIMB)	FBpp0083434	803.1	0
Dappu-TIM1 A	TIM-PJ	FBpp0291971	308.1	2.47e-83
Dappu-TIM1 B	TIM-PJ	FBpp0291971	261.9	1.69e-69
Dappu-TIM1 C	TIM-PJ	FBpp0291971	275.8	9.85e-74

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Daphnia protein	Top named protein hit in FlyBase			
			Homology to query	
	Protein name	FlyBase ID	Blast score	E-value
Dappu-TIM1 D	TIM-PC	FBpp0077254	251.1	3.03e-66
Dappu-TIM1 E	TIM-PI	FBpp0291970	255.8	1.10e-67
Dappu-TIM1 F	TIM-PC	FBpp0077254	212.2	2.43e-54
Dappu-TIM1 G	TIM-PI	FBpp0291970	219.2	7.13e-57
Dappu-TIM1 H	TIM-PI	FBpp0291970	193.7	5.21e-49
Dappu-VRI	VRI-PD	FBpp0289297	149.1	8.29e-36
INPUT PATHWAY PROTEINS[1]				
Dappu-CRY A	CRY-PA	FBpp0083150	476.1	5.56e-140
Dappu-CRY B	PHR6-4-PB	FBpp0080935	495.0	2.59e-134
Dappu-CRY C	PHR6-4-PB	FBpp0080935	594.7	5.15e-170
Dappu-CRY D	PHR6-4-PB	FBpp0080935	167.5	2.12e-41
OUTPUT PATHWAY PROTEINS				
Dappu-PDHR	PDFR-PA	FBpp0099841	345.5	5.62e-95

Abbreviations: CKII, casein kinase II; CLK, CLOCK; CRY, CRYPTOCHROME; CWO, CLOCKWORK ORANGE; CYC, CYCLE; DBT, DOUBLETIME; MTS, MICROTUBULE STAR; PDFR, PIGMENT DISPERSING FACTOR RECEPTOR; PDHR, PIGMENT DISPERSING HOROMONE RECEPTOR; PDP1ε; PAR DOMAIN PROTEIN 1ε; PER, PERIOD; PHR, PHOTOLYASE; PP1, PROTEIN PHOSPHATASE 1; PP2A, PROTEIN PHOSPHATASE 2A; SGG, SHAGGY; SLIMB, SUPERNUMERARY LIMBS; TIM, TIMELESS; TWS, TWINS; VRI, VRILLE; WBT, WIDERBORST.

[1]It should be noted that the group designations are based on the organization of the *Drosophila melanogaster* clock, whose proteins were used for querying the *Daphnia* genome. Unlike *Drosophila*, which possesses only CRY1 (an input pathway protein), *D. pulex* appears to contain multiple CRY isoforms, including both CRY1 and CRY2 (a core clock protein), though in this table, all are presented under the "INPUT PATHWAY PROTEIN" heading.

Table 3

blastp analyses of putative *Daphnia pulex* circadian system proteins vs. all NCBI curated non-redundant protein sequences*

Daphnia protein	Top five blastp protein hits				
	Accession no.	**Species**	**Protein description**	**Blast score**	**E-value**
CASEIN KINASE II (CKII) – α subunit					
Dappu-CKII α	1NA7_A	*Homo sapiens*	Chain A, Catalytic Subunit of Protein Kinase Ck2	624	9e-177
	EFN84867	*Harpegnathos saltator*	Casein kinase II subunit alpha	621	6e-176
	EFN62439	*Camponotus floridanus*	Casein kinase II subunit alpha	620	6e-176
	EGI62250	*Acromyrmex echinatior*	Casein kinase II subunit alpha	619	2e-175
	O76484	*Spodoptera frugiperda*	Casein kinase II subunit alpha	605	3e-171
CASEIN KINASE II (CKII) – β subunit					
Dappu-CKII β	CAI18393	*Homo sapiens*	casein kinase 2, beta polypeptide	425	2e-117
	EDL26660	*Mus musculus*	casein kinase II, beta subunit, isoform CRA_c	424	3e-117
	EDL83523	*Rattus norvegicus*	casein kinase 2, beta subunit, isoform CRA_b	424	4e-117
	CBK38916	*Mytilus galloprovincialis*	protein kinase CK2beta regulatory subunit	422	2e-116
	CAJ83806	*Xenopus tropicalis*	casein kinase 2, beta subunit	421	4e-116
CLOCK (CLK)					
Dappu-CLK	BAJ16353	*Thermobia domestica*	CLOCK	467	5e-129
	AAR14936	*Antheraea pernyi*	CLOCK	461	2e-127
	EGI62057	*Acromyrmex echinatior*	Circadian locomoter output cycles protein kaput	444	2e-122
	EFN76178	*Hapregnathos saltator*	Circadian locomoter output cycles protein kaput	442	1e-121
	EFN61630	*Camponotus floridanus*	Circadian locomoter output cycles protein kaput	441	3e-121
CLOCKWORK ORANGE (CWO)					
Dappu-CWO	AAF54527	*Drosophila melanogaster*	Clockwork orange	132	3e-28
	AAF24476	*Drosophila melanogaster*	Sticky ch1	132	3e-28
	EFN67685	*Camponotus floridanus*	Hairy/enhancer-of-split related with YRPW motif protein 2	131	4e-28
	ACK77653	*Drosophila melanogaster*	RE11081p	131	5e-28
	EGI62806	*Acromyrmex echinatior*	Hairy/enhancer-of-split related with YRPW motif protein 1	130	9e-28
CYCLE (CYC)					

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Daphnia protein	Top five blastp protein hits				
	Accession no.	**Species**	**Protein description**	**Blast score**	**E-value**
Dappu-CYC	BAJ16354	*Thermobia domestica*	CYCLE	612	8e-173
	ABI21880	*Lutzomyia longipalpis*	Cycle	592	5e-167
	EFN72014	*Camponotus floridanus*	Aryl hydrocarbon receptor nuclear translocator-like protein 1	567	2e-159
	AAW80970	*Xenopus laevis*	BMAL1	483	5e-134
	Q6YGZ5	*Tyto alba*	Aryl hydrocarbon receptor nuclear translocator-like protein 1	474	3e-131
DOUBLETIME (DBT)/CASEIN KINASE Iε					
Dappu-DBT	EFN64010	*Camponotus floridanus*	Casein kinase I isoform epsilon	590	9e-167
	EGI65788	*Acromyrmex echinatior*	Casein kinase I isoform epsilon	584	6e-165
	Q5ZLL1	*Gallus gallus*	Casein kinase I isoform epsilon	582	4e-164
	AAF65549	*Mesocricetus auratus*	casein kinase I epsilon	582	4e-164
	EFB26732	*Ailuropoda melanoleuca*	PANDA_002512	582	5e-164
PAR DOMAIN PROTEIN 1ε (PDP 1ε)					
Dappu-PDP 1ε	EDM05669	*Rattus norvegicus*	rCG33934	176	5e-42
	AAN12026	*Drosophila melanogaster*	PAR-domain protein 1, isoform J	175	7e-42
	AAN12025	*Drosophila melanogaster*	PAR-domain protein 1, isoform D	175	8e-42
	AAN12027	*Drosophila melanogaster*	PAR-domain protein 1, isoform C	174	2e-41
	EFB19547	*Ailuropoda melanoleuca*	PANDA_015107	174	2e-41
PERIOD (PER)					
Dappu-PER	AAN02439	*Blattella germanica*	circadian clock protein PERIOD	397	4e-108
	BAG48878	*Gryllus bimaculatus*	period	370	6e-100
	BAI47546	*Modicogryllus siamensis*	period clock protein	367	6e-99
	Q25637	*Periplaneta americana*	Period circadian protein	366	1e-98
	ADO24377	*Laupala paranigra*	period	353	7e-95
PROTEIN PHOSPHATASE 1 (PP1)					
Dappu-PP1 A	EFN69572	*Camponotus floridanus*	Serine/threonine-protein phosphatase alpha-1 isoform	654	0.0
	ADY40510	*Ascaris suum*	Serine/threonine-protein phosphatase PP1-beta	643	0.0
	EFA05189	*Tribolium castaneum*	TcasGA2_TC015321	642	0.0
	ACO15366	*Caligus clemensi*	Serine/threonine-protein phosphatase PP1-beta	641	0.0

Daphnia protein	Top five blastp protein hits				
	Accession no.	**Species**	**Protein description**	**Blast score**	**E-value**
	BAJ85104	*Hordeum vulgare*	predicted protein	640	0.0
Dappu-PP1 B	CAD61270	*Danio rerio*	similar to human protein phosphatase 1, catalytic subunit, beta isoform	624	4e-177
	AAM88380	*Canis lupus familiaris*	protein phosphatase type 1 catalytic subunit delta isoform	622	2e-176
	ABQ18261	*Carassius auratus*	protein serine/threonine phosphatase-1 catalytic subunit beta isoform	622	2e-176
	EFN76901	*Harpegnathos saltator*	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	622	2e-176
	BAE38902	*Mus musculus*	unnamed protein product	622	3e-176
PROTEIN PHOSPHATASE 2A – catalytic subunit MICROTUBULE STAR (MTS)					
Dappu-MTS	EFN85419	*Harpegnathos saltator*	Serine/threonine-protein phosphatase 4 catalytic subunit	605	3e-171
	AAD01262	*Takifugu rubripes*	serine/threonine phosphatase	601	4e-170
	AAV38551	*Homo sapiens*	protein phosphatase 4 (formerly X), catalytic subunit	598	2e-169
	ACO10717	*Caligus rogercresseyi*	Serine/threonine-protein phosphatase 4 catalytic subunit	595	3e-168
	ACO10485	*Caligus rogercresseyi*	Serine/threonine-protein phosphatase 4 catalytic subunit	595	4e-168
PROTEIN PHOSPHATASE 2A – regulatory subunit WIDERBORST (WBT)					
Dappu-WBT A	EFN66909	*Camponotus floridanus*	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit epsilon isoform	769	0.0
	EGI66726	*Acromyrmex echinatior*	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit alpha isoform	761	0.0
	EFA06323	*Tribolium castaneum*	TcasGA2_TC009194	747	0.0
	AAN14114	*Drosophila melanogaster*	widerborst, isoform C	742	0.0
	AAH64358	*Homo sapiens*	PPP2R5E protein	678	0.0
Dappu-WBT B	EFN69797	*Camponotus floridanus*	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform	855	0.0
	EFN83893	*Harpegnathos saltator*	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform	848	0.0
	BAG61599	*Homo sapiens*	unnamed protein product	836	0.0
	AAQ01559	*Mus musculus*	protein phosphatase 2A B56 delta subunit	830	0.0
	BAF84486	*Homo sapiens*	unnamed protein product	828	0.0
PROTEIN PHOSPHATASE 2A – regulatory subunit TWINS (TWS)					
Dappu-TWS	EFA10095	*Tribolium castaneum*	TcasGA2_TC012273	823	0.0
	AAN13458	*Drosophila melanogaster*	twins, isoform E	777	0.0
	AAF54498	*Drosophila melanogaster*	twins, isoform A	776	0.0
	AAA99871	*Drosophila melanogaster*	phosphoprotein phosphatase 2A 55 lDa regulatory subunit	775	0.0

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Daphnia protein	Top five blastp protein hits				
	Accession no.	**Species**	**Protein description**	**Blast score**	**E-value**
	P56932	*Rattus norvegicus*	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B delta isoform	761	0.0
SHAGGY (SGG)/GLYCOGEN SYNTHASE KINASE 3B					
Dappu-SGG	ABO61882	*Rhipicephalus microplus*	glycogen synthase kinase	676	0.0
	EFN78376	*Harpegnathos saltator*	Protein kinase shaggy	676	0.0
	EGI58906	*Acromyrmex echinatior*	Protein kinase shaggy	673	0.0
	Q5YJC2	*Spermophilus citellus*	Glycogen synthase kinase-3 beta	670	0.0
	P18266	*Rattus norvegicus*	Glycogen synthase kinase-3 beta	666	0.0
SUPERNUMERARY LIMBS (SLIMB)/E3 UBIQUITIN LIGASE					
Dappu-SLIMB	BAE26547	*Mus musculus*	unnamed protein product	840	0.0
	EAW49771	*Homo sapiens*	beta-transducin repeat containing, isoform CRA_b	839	0.0
	EDL94307	*Rattus norvegicus*	beta-transducin repeat containing	839	0.0
	DAA14816	*Bos taurus*	beta-tranducin repeat containing protein	838	0.0
	BAG36210	*Homo sapiens*	unnamed protein product	838	0.0
TIMELESS (TIM)					
Dappu-TIM A	AAR15505	*Danaus plexippus*	timeless	468	3e-129
	BAB85487	*Sarcophaga bullata*	timeless	344	4e-92
	AAB94890	*Drosophila melanogaster*	circadian clock protein	344	6e-92
	AAF51098	*Drosophila melanogaster*	timeless, isoform B	343	7e-92
	AAN10371	*Drosophila melanogaster*	timeless, isoform D	343	7e-92
Dappu-TIM B	AAF66996	*Antheraea pernyi*	timeless	394	4e-107
	AAR15505	*Danaus plexippus*	timeless	383	7e-104
	AAB94930	*Drosophila virilis*	circadian clock protein	313	1e-82
	ABW71828	*Chymomyza costata*	timeless	303	1e-79
	EFA04644	*Tribolium castaneum*	timeless	302	2e-79
Dappu-TIM C	EFA04644	*Tribolium castaneum*	timeless	397	4e-108
	AAR15505	*Danaus plexippus*	timeless	371	3e-100
	AAN10371	*Drosophila melanogaster*	timeless, isoform D	302	1e-79

Daphnia protein	Top five blastp protein hits				
	Accession no.	**Species**	**Protein description**	**Blast score**	**E-value**
	P49021	*Drosophila melanogaster*	Protein timeless	302	1e-79
	AAB94890	*Drosophila melanogaster*	circadian clock protein	302	2e-79
Dappu-TIM D	EFA04644	*Tribolium castaneum*	timeless	387	4e-105
	AAF66996	*Antheraea pernyi*	timeless	386	1e-104
	AAR15505	*Danaus plexippus*	timeless	382	2e-103
	AAB94890	*Drosophila melanogaster*	circadian clock protein	293	8e-77
	AAF51098	*Drosophila melanogaster*	timeless, isoform B	293	8e-77
Dappu-TIM E	ADV36936	*Drosophila melanogaster*	timeless, isoform I	282	2e-73
	AAB94890	*Drosophila melanogaster*	circadian clock protein	282	2e-73
	AAF51098	*Drosophila melanogaster*	timeless, isoform B	282	2e-73
	AAN10372	*Drosophila melanogaster*	timeless, isoform C	281	3e-73
	AAL13507	*Drosophila melanogaster*	GH03106p	281	3e-73
Dappu-TIM F	P49021	*Drosophila melanogaster*	Protein timeless	236	2e-59
	AAN10371	*Drosophila melanogaster*	timeless, isoform D	236	2e-59
	AAF51098	*Drosophila melanogaster*	timeless, isoform B	236	2e-59
	AAB94890	*Drosophila melanogaster*	circadian clock protein	236	2e-59
	AAF51097	*Drosophila melanogaster*	timeless, isoform K	236	3e-59
Dappu-TIM G	O17482	*Drosophila virilis*	Protein timeless	236	6e-60
	AAB94930	*Drosophila virilis*	tim	236	9e-60
	BAB85487	*Sarcophaga bullata*	timeless	232	2e-58
	AAY40757	*Aedes aegypti*	TIMELESS	231	3e-58
	AAF51098	*Drosophila melanogaster*	timeless, isoform B	231	4e-58
Dappu-TIM H	BAJ16356	*Gryllus bimaculatus*	TIMELESS	216	1e-53
	O17482	*Drosophila virilis*	Protein timeless	214	6e-53
	AAB94930	*Drosophila virilis*	tim	213	9e-53
	BAB85471	*Sarcophaga crassipalpis*	TIMELESS	212	3e-52
	BAB85487	*Sarcophaga bullata*	timeless	211	7e-52

Daphnia protein	Top five blastp protein hits				
	Accession no.	**Species**	**Protein description**	**Blast score**	**E-value**
			VRILLE (VRI)		
Dappu-VRI	AAT86041	*Danaus plexippus*	vrille	169	2e-39
	AAS92609	*Antheraea pernyi*	vrille	166	1e-38
	CAX37108	*Acyrthosiphon pisum*	vrille	159	1e-36
	CAA72535	*Drosophila melanogaster*	bZIP transcription factor	155	3e-35
	AAF52237	*Drosophila melanogaster*	vrille, isoform A	155	3e-35
			CRYPTOCHROME (CRY)		
Dappu-CRY A	BAF45421	*Dianemobius nigrofasciatus*	cryptochrome precursor	523	2e-146
	AAK11644	*Antheraea pernyi*	cryptochrome	516	2e-144
	AAX58599	*Danaus plexippus*	cryptochrome	511	1e-142
	BAI67362	*Bactrocera cucurbitae*	cryptochrome	506	5e-141
	BAI67363	*Bactrocera cucurbitae*	cryptochrome	504	9e-141
Dappu-CRY B	EFR20390	*Anopheles darlingi*	AND_20159	882	0.0
	ABB29887	*Anopheles gambiae*	cryptochrome 2	877	0.0
	ABO31112	*Bombus impatiens*	cryptochrome 2 protein	851	0.0
	EFN85258	*Harpegnathos saltator*	Cryptochrome-1	850	0.0
	BAG07408	*Riptotus pedestris*	cryptochrome-m	844	0.0
Dappu-CRY C	AAI66277	*Xenopus tropicalis*	LOC100144974 protein	669	0.0
	EFR25332	*Anopheles darlingi*	AND_09443	620	1e-175
	ACN10565	*Salmo salar*	Cryptochrome-1	612	5e-173
	CAG02357	*Tetraodon nigroviridis*	unnamed protein product	612	6e-173
	AAL90322	*Drosophila melanogaster*	RE11660p	595	8e-168
Dappu-CRY D	CBN81995	*Dicentrarchus labrax*	Cryptochrome DASH	634	1e-179
	Q4KML2	*Danio rerio*	Cryptochrome DASH	623	2e-176
	AAH98514	*Danio rerio*	Cryptochrome DASH	619	3e-175
	CAO90052	*Microcystis aeruginosa*	unnamed protein product	475	8e-132
	P77967	*Synechocystis sp.*	Cryptochrome DASH	452	5e-125

Daphnia protein	Top five blastp protein hits				
	Accession no.	**Species**	**Protein description**	**Blast score**	**E-value**
PIGMENT DISPERSING HORMONE RECEPTOR (PDHR)					
Dappu-PDHR	BAH85843	*Marsupenaeus japonicus*	pigment dispersing hormone receptor	385	8e-105
	ADG46049	*Drosophila melanogaster*	MIP16931p	380	3e-103
	AAF45788	*Drosophila melanogaster*	PDF receptor	380	3e-103
	CAB72288	*Drosophila melanogaster*	EG:BACR25B3.3	347	2e-93
	CAP34509	*Caenorhabditis briggsae*	CBG_16586	227	7e-71

*excluding *Daphnia* proteins, partial proteins, synthetic constructs and provisional protein sequences.

Table 4

Matrix of percent amino acid identity/similarity between putative *Daphnia pulex* TIMELESS (TIM) proteins

	TIM-A	TIM-B	TIM-C	TIM-D	TIM-E	TIM-F	TIM-G	TIM-H
TIM-A								
TIM-B	47.1/70.9							
TIM-C	47.7/68.3	44.1/71.2						
TIM-D	48.6/73.9	46.0/70.3	41.7/67.8					
TIM-E	45.0/65.8	45.0/68.8	44.0/72.4	58.7/75.8				
TIM-F	31.5/55.2	26.5/49.3	25.8/44.6	27.0/50.3	22.8/42.4			
TIM-G	28.8/45.9	31.9/53.1	33.9/54.2	26.6/46.4	28.3/50.5	16.7/29.9		
TIM-H	37.8/62.2	36.9/63.4	37.8/68.5	36.5/64.2	38.1/68.3	19.8/40.1	26.8/48.9	

Values shown are percent amino acid identity/similarity.

Percent identity = number of amino acids identically conserved between the two proteins divided by the total number of amino acids in the longer protein.

Percent similarity = number of identical and similar amino acids in the two proteins divided by the total number of amino acids in the longer protein

Table 5

Matrix of percent amino acid identity/similarity between putative *Daphnia pulex* CRYPTOCHROME (CRY) proteins

	CRY-A	CRY-B	CRY-C	CRY-D
CRY-A				
CRY-B	37.4/70.7			
CRY-C	38.3/71.2	48.1/78.7		
CRY-D	26.3/61.0	26.4/58.8	27.7/64.3	

Values shown are percent amino acid identity/similarity.

Percent identity = number of amino acids identically conserved between the two proteins divided by the total number of amino acids in the longer protein.

Percent similarity = number of identical and similar amino acids in the two proteins divided by the total number of amino acids in the longer protein

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