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# Time Enough At Last: Identification and Analysis of Core-Clock Proteins and the Evolution of ARNT and PERIOD in the Lower Bilateria

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April 3, 2015

To the Dean of the Graduate School:

We are submitting a thesis written by Daniel Stanton entitled Time Enough At Last: Identification and Analysis of Core-Clock Proteins and the Evolution of ARNT and PERIOD in the Lower Bilateria. We recommend acceptance in partial fulfillment of the requirements for the degree of Master of Science in Biology.

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**TIME ENOUGH AT LAST: IDENTIFICATION AND ANALYSIS OF CORE-  
CLOCK PROTEINS AND THE EVOLUTION OF ARNT AND PERIOD IN THE  
LOWER BILATERIA**

A Thesis  
Presented to the Faculty  
Of the  
College of Arts and Sciences  
In Partial Fulfillment  
Of the  
Requirements for the Degree  
Of  
Master of Science  
In Biology  
Winthrop University

April 3, 2015

By

Daniel Stanton

## ABSTRACT

The circadian rhythm is important to all organisms and plays a key role in physiology. Although much is known about the genetic regulation of the circadian clock in *Drosophila melanogaster* and in *Mus musculus*, less is known about the circadian clock in cnidarians, let alone in the lower Bilateria. This study attempts to fill the information gap between Cnidaria and higher metazoans by characterizing the core circadian clock of *Isodiametra pulchra* (Acoelomorpha). These animals live in between sand grains above the sulfide zone on low wave-action beaches, exposing them to a wide array of daily environmental changes. *I. pulchra* circadian core-clock proteins were identified by comparing known *Drosophila* and *Mus* orthologues. Final orthology assignments were chosen based on protein domain characteristics, sequence similarities, and phylogenetic analyses. By comparing known orthologues of clock proteins found in insects and vertebrates, three full sequences and two incomplete sequences were identified. I examined the mRNA expression of two of these proteins (IPUL1\_3616 and IPUL1\_2961) using a semi-quantitative RT\_PCR approach. I found that IPUL1\_3616 varied in a circadian fashion where expression was highest one hour before lights on, but IPUL1\_2961 did not vary significantly in expression. This study is the first study of the circadian clock in a basal bilaterian.

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## 1. INTRODUCTION

### 1.1 The Circadian Rhythm

The circadian rhythm is important for physiological and psychological function (Pittendrigh and Minis 1964; Kinmonth-Schultz *et al.* 2013), which includes: metabolism (Asher and Schibler 2011; Froy 2011a; Rey and Reddy 2013), sleep regulation (Franken and Dijk 2009), memory (Eckel-Mahan and Storm 2009), aging (Froy 2011b), reproduction and development (Brooks and Canal 2013; Boden *et al.* 2013), and immune regulation (Scheiremann *et al.* 2013). An organism's circadian rhythm is regulated by multiple mechanisms including light, chemical signaling, and gene regulation (Dugay and Cermakian 2009; Tessmar-Raible *et al.* 2011). Disruption of this rhythm can have wide effects across tissue systems including disruption of tissue homeostasis, premature aging, chronic inflammation, acceleration of tumor progression, and even an increased predisposition for cancer (Canaple *et al.* 2003; Gauger and Sancar 2005; Hunt and Sassone-Corsi 2007; Froy 2011b). In fact, cancer cells have a circadian rhythm that is out of phase with normal cells (Canaple *et al.* 2003).

Mechanisms of the circadian clocks may have evolved to protect an organism from photo-oxidative damage by free radicals and/or hyperoxic/hypoxic conditions, both of which influence the regulation of cell cycle events (Canaple *et al.* 2003; Gauger and Sancar 2005; Hunt and Sassone-Corsi 2007; Khapre *et al.* 2010; Masri *et al.* 2013). In fact early studies in *Spirogyra* cell division suggested



that cells divide mostly at night (Campbell 1890). This effect has been observed in the lower bilaterians. For instance, Bowie and others (2012) from the Smith lab demonstrated that the number of mitoses was significantly higher during the scotophase and lower during the photophase in *Aeolosoma headleyi* (Annelida).

Because the circadian rhythm has wide effects across various tissues, it is important to understand the underlying genetic regulation involved in these systems. The genetic regulatory mechanism is well described in *Drosophila melanogaster* and *Mus musculus* (Hardin and Panda 2013). Scientists have also begun to describe circadian regulation mechanisms in the cnidarian *Nematostella vectensis* (Reitzel *et al.* 2010; Reitzel *et al.* 2013), in the marine annelid *Platynereis dumerilii* (Zantke *et al.* 2013), and in the marine crustacean *Eurydice pulchra* (Zhang *et al.* 2013). Although the circadian effects on melatonin production, asexual reproduction, and cell division are apparent in lower Bilaterians (Morita and Best 1993; Stanton and Smith 2009; Bowie *et al.* 2012), nothing is known about the genetic regulation of the circadian clock in organisms between cnidarians and arthropods (Reitzel *et al.* 2010; Reitzel *et al.* 2013), with the exception of a recent paper describing the circadian clock in *P. dumerilii* and how circalunar changes can influence the circadian clock (Zantke *et al.* 2013).

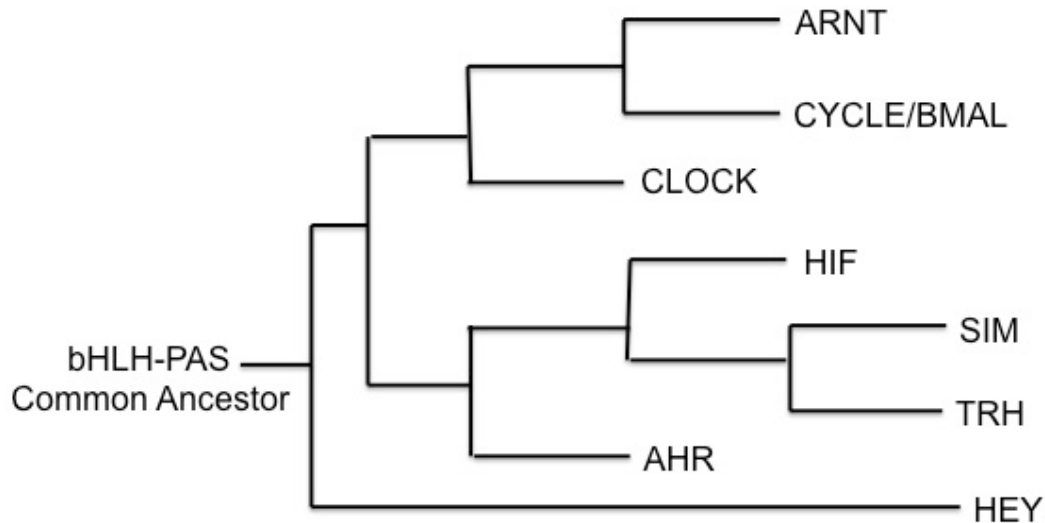
By comparing the amino acid sequences of circadian proteins, one can begin to search for orthologous proteins in lower Bilateria. Although domain conservation can be observed between insect and vertebrate circadian orthologues, little is known about how the circadian clock evolved in the lower Metazoa, let alone in basal bilaterians.

## 1.2 Domain Structures of Circadian Core-Clock Proteins

Protein binding domains allow for protein-protein interactions or protein-DNA interactions. The identification of these domains can lead to the identification of the correct protein family, which can ultimately lead to a predicted orthology assignment. Many proteins have a similar domain structure, so it is difficult to predict protein orthologues beyond protein family generalizations. In order to assign a final orthology, one must use a combination of bioinformatics, protein structure comparisons, phylogenetic comparisons, and mechanistic analyses. Each of the circadian core-clock proteins (described below) has at least one or more of the following protein domains in its structure: a basic Helix-Loop-Helix (bHLH) domain, a PER-ARNT-SIM (PAS) domain; or a Timeless domain (Panda *et al.* 2002; Yu *et al.* 2006; Yu *et al.* 2009; Peschel *et al.* 2009; Reitzel *et al.* 2010). These domains are important in understanding interaction of circadian proteins in the formation of dimers and trimers.

Proteins containing bHLH domains are found in a variety of organisms (Massarri and Murre 2000). These domains allow for protein-protein interactions as well as protein-DNA interactions (Jones 2004). A large array of bHLH domain-containing protein makes up the bHLH superfamily. The bHLH-PAS protein subfamily (Figure 1) was not identified until the bHLH superfamily was classified by similarities in protein sequence (Simionato *et al.* 2007), and later strengthened by phylogenetic analysis (Skinner *et al.* 2010). Because these proteins are not tissue-specific, they were not classified under the original bHLH

family classification system based on tissue location (Massari and Murre 2000; Jones 2004).



**Figure 1. The bHLH-PAS clade from the larger bHLH superfamily tree contains two of the proteins (CLOCK and CYCLE/BMAL) that are included in the circadian core-clock. (Adapted from Simionato *et al.* 2007).**

The number of bHLH-PAS proteins has expanded from one protein to seven proteins over the course of the evolution of Animalia (Simionato *et al.* 2007). The demosponge *Amphimedon queenslandica* has three bHLH-PAS proteins (ARNT, CLOCK, and AHR). Cnidarians have five bHLH-PAS proteins that include CLOCK, CYCLE, AHR, ARNT, and HIF proteins (Reitzel *et al.* 2010). Deuterostomes, on the other hand, have seven bHLH-PAS proteins and include CLOCK, BMAL (CYCLE), ARNT, AHR, HIF, THR, and SIM.

The second domain commonly found in circadian core-clock proteins is the PAS domain. Proteins that contain this domain are found in organisms ranging from bacteria to mammals (McIntosh *et al.* 2010). The PAS domain was named after a region of high similarity found in the amino acid sequences of PER (period circadian protein), ARNT (aryl hydrocarbon receptor nuclear translocator protein), and SIM (single-minded protein) (McIntosh *et al.* 2010). The PAS domain facilitates interactions to allow for the binding of multiple proteins in one complex (Partch and Gardner 2010). Although the definition of a PAS domain has varied in the literature over time, I adopt McIntosh and colleagues' definition where a PAS domain is defined as two PAS repeats (PAS A and PAS B) that are about 70 amino acids long each (McIntosh *et al.* 2010).

The TIM superfamily is the third domain found in the circadian clock proteins. The group is subdivided based on domain structure into TIMELESS (TIM1) and TIMEOUT (TIM2) (Rubin *et al.* 2006). TIM1 has a TIM domain and is important in forming a heterodimer with PER to increase the stability of PER for nuclear import. TIM2 has a TIM domain and a Timeless-C domain and is involved in cascade responses to DNA damage (Hunter-Ensor *et al.* 1996; Panda *et al.* 2002; Yu *et al.* 2006; Yu *et al.* 2009; Engelen *et al.* 2013). From the literature is clear that TIM2 is the primitive member of the TIM protein family. The TIM1 is a paralogue that likely arose from an escaped TIM domain of a primitive TIM2 orthologue (Benna *et al.* 2000; Rubin *et al.* 2006; McFarlane *et al.* 2010). TIM 1 is predominately found in higher protostomes with a few exceptions -- *Apis mellifera* (Insecta) lacks the TIM1 (Rubin *et al.* 2006). Deuterostomes only have a

TIM2 orthologue, however *Strongylocentrotus purpuratus* (Echinodermata) is an exception because it has both TIM1 and TIM2 orthologues (Benna *et al.* 2000; Rubin *et al.* 2006; McFarlane *et al.* 2010; Engelen *et al.* 2013). The TIM1 paralogue evolved to have a specific role in the circadian clock (McFarlane *et al.* 2010). Engelen and others (2013) recently proposed that the core-clock mechanism differs from being light-driven (direct TIM1 involvement) in insects to protein-driven (indirect TIM2 involvement via post-transcriptional modification) in mammals, where proteins adapted to this change (Engelen *et al.* 2013).

To date, TIM2 orthologues have been found in every animal species in which they have been studied (Benna *et al.* 2000; Rubin *et al.* 2006; Engelen *et al.* 2013). *Mus* has a TIM2 orthologue that varies in expression (highest during late photophase and lowest during late scotophase), but it is not directly involved in the regulation of the circadian core-clock (Gillette and Tyan 2009). Protostome and deuterostome TIM2 are both involved in the cell cycle and DNA damage pathways (Ünsal-Kacmaz *et al.* 2005; McFarlane *et al.* 2010; Engelen *et al.* 2013). The role of TIM family members in the phosphorylation of S-phase checkpoint kinases is highly conserved among the Metazoa (McFarlane *et al.* 2010). A *Saccharomyces cerevisiae* TIMELESS family member was the first TIM family protein discovered to have a role in cell cycle regulation through direct interactions with cell cycle checkpoint kinases (Foss 2001; McFarlane *et al.* 2010). Additionally, TIM2 is also required for CRY1 and CHECKPOINT KINASE 1 (CHK1) association and dimerization in a signal transduction cascade induced by DNA damage (Engelen *et al.* 2013). There is also evidence to support the

hypothesis that TIM2 is involved in DNA repair by bridging helicases and polymerases (McFarlane *et al.* 2010).

### 1.3 Mechanisms of the Circadian Clock

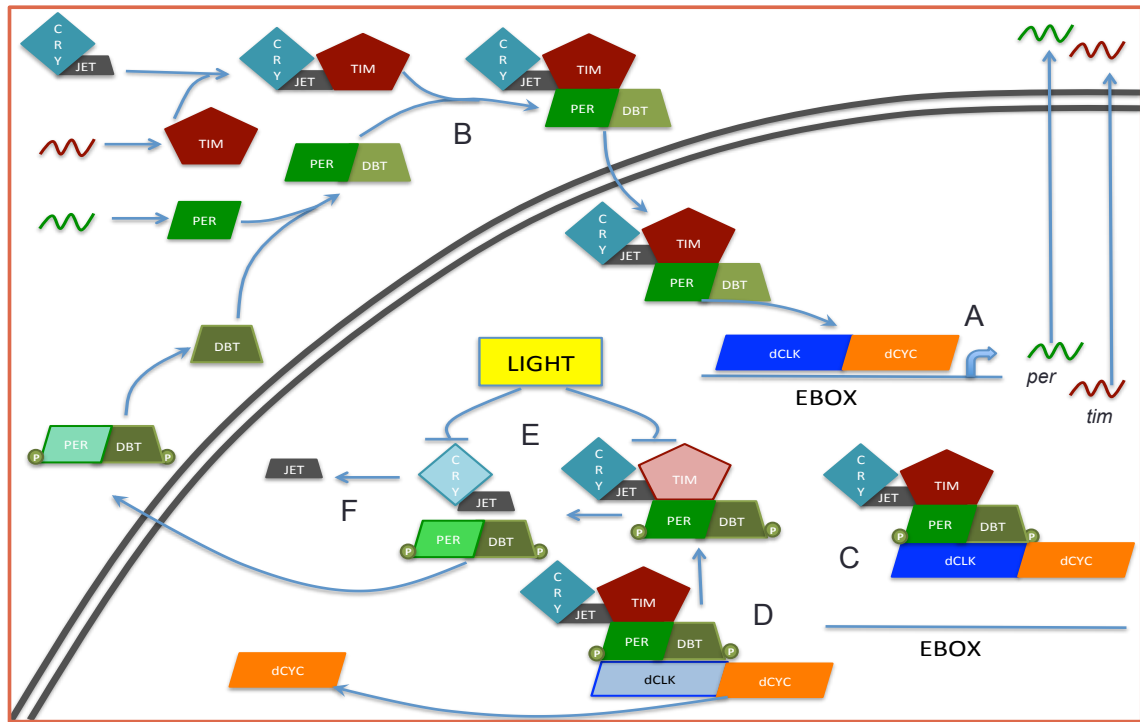
The circadian core-clock proteins conserved between higher insects and vertebrates are CLOCK (CLK), CYCLE (CYC) or Brain-and-Muscle-ARNT-like (BMAL), and PERIOD (PER) (Brown *et al.* 2012; Hardin and Panda 2013). There are other proteins also involved in the regulatory mechanism of the circadian clock such as JETLAG (JET), DOUBLE-TIME (DBT), and CRYPTOCHROME (CRY) in *Drosophila* and casein kinase 1 $_{\xi}$  (CK1 $_{\xi}$ , orthologous to DBT) and CRY proteins in vertebrates (Yu *et al.* 2006; Peschel *et al.* 2009; Brown *et al.* 2012; Hardin and Panda 2013). Additionally some of the clocks have subsidiary loops; however, the focal point for this study is the comparison of core-clocks across phyla to understand how the circadian clock evolved (Brown *et al.* 2012; Hardin and Panda 2013). Here, I concentrate on the four core-clock proteins (CLK, CYC/BMAL, PER, and TIM1 or CRY).

One of the most well-studied model organisms for genetic regulation of circadian rhythms is *Drosophila melanogaster*, which has two circadian core-clock proteins (CLK and CYC) that promote transcription of the *per* and *tim* genes and two core-clock proteins (PER and TIM1) that repress transcription of the *per* and *tim* genes (Figure 2). CYC mRNA is constitutively expressed, although CLK, PER, and TIM1 mRNA cycle because translated proteins suppress transcription as they form dimers and trimers (as described below). As

each protein is degraded, transcription resumes. CLK mRNA expression is highest during early photophase and lowest during early scotophase, whereas PER and TIM1 mRNA expression are highest during the early to mid-scotophase and lowest during mid-photophase (Hardin *et al.* 1990; Price *et al.* 1995; Lee *et al.* 1999; McDonald and Rosbash 2001; Panda *et al.* 2002; Yu *et al.* 2006; Brown *et al.* 2012; Hardin and Panda 2013).

CLK and CYC form a heterodimer and bind to the E-box in the regulatory region of the *per* and *tim1* genes (Figure 2A) to start transcription (Lee *et al.* 1999; Yu *et al.* 2006; Taylor and Hardin 2008; Brown *et al.* 2012). PER dimerizes with DBT in the cytoplasm. PER also binds to TIM1 forming a stable heterotrimer (Yu *et al.* 2006). This allows for the stabilization of PER by TIM1 for translocation into the nucleus (Figure 2B) where it hyperphosphorylates CLK (Vosshall *et al.* 1994; Price *et al.* 1995; Lee *et al.* 1999; Hardin and Panda 2013). The hyperphosphorylation of CLK by PER represses transcription (Figure 2C) by removing the CLK : CYC heterodimer from the E-box (Lee *et al.* 1999; Yu *et al.* 2006; Hardin and Panda 2013). CLK is destabilized by DBT (Figure 2D) and degrades after being hyperphosphorylated (Yu *et al.* 2006). JETLAG (JET) binds to CRY and forms a heterotrimer with TIM1 (Figure 2E). When induced by light, JET undergoes a conformational change and has a higher affinity to bind to TIM1. TIM1 becomes phosphorylated and is degraded in the nucleus (Peschel *et al.* 2009). PER requires TIM1 for nuclear transport (Vosshall *et al.* 1994) Without TIM1, PER becomes unstable (Figure 2F) and is transported out of the nucleus (Vosshall *et al.* 1994; Yu *et al.* 2006; Peschel *et al.* 2009). CLK and CYC form a

heterodimer in the cytoplasm (Lee *et al.* 1999; Yu *et al.* 2006; Taylor and Hardin 2008; Brown *et al.* 2012). The CLK : CYC heterodimer bind to the E-box, which starts transcription of PER and TIM1 mRNA again (Panda *et al.* 2002; Peschel *et al.* 2009).



**Figure 2.** The Figure shows the basic mechanism of the circadian clock in *D. melanogaster*.

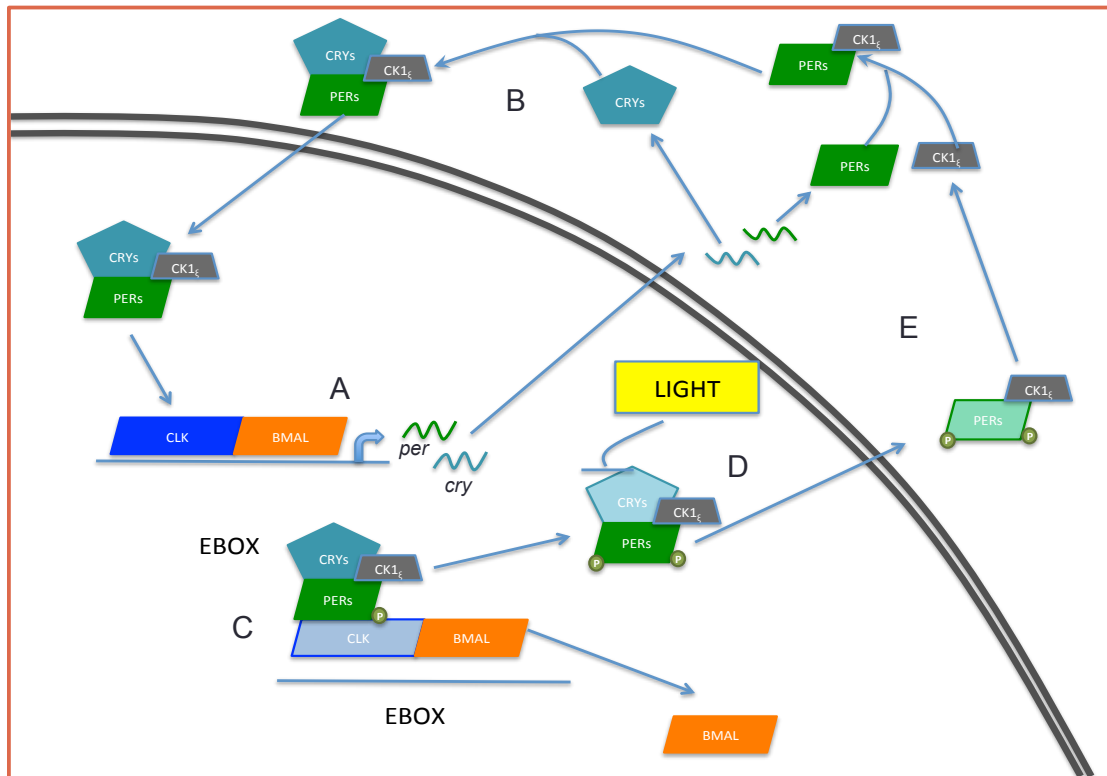
In mammals, the suprachiasmatic nucleus (SCN) found within the hypothalamus is the master regulator of mammalian circadian rhythms. The master regulator resets all primary and secondary circadian clocks (Gillette and Tyan 2009). The mammalian clock also has subsidiary loops; however, the comparison of the circadian core-clocks is the focal point for this study (Doherty



and Kay 2010; Brown *et al.* 2012; Hardin and Panda 2013). Most of the circadian core-clock proteins in *Mus* have temporal variations in expression. BMAL mRNA expression is highest during mid-scotophase and lowest during early photophase (Shearman *et al.* 2000; Akhtar *et al.* 2002; Karolczak *et al.* 2004; Gillette and Tyan 2009). *Mus* has two PER paralogs that participate in the SCN core-clock and a third paralog that participates in tissue specific peripheral clocks. All mRNA expression of PER paralogs is highest during the mid-photophase and lowest during early scotophase. CRY1 expression is highest during the late photophase and lowest during mid scotophase, whereas CRY 2 expression is highest during the early scotophase and lowest during early photophase (Shearman *et al.* 2000; Akhtar *et al.* 2002; Gillette and Tyan 2009).

The mechanism for *Mus musculus* is similar to the mechanism for *Drosophila* (Yu *et al.* 2006; Gillette and Tyan 2009; Yu *et al.* 2009; Hardin and Panda 2013). *Mus* has a CLK and a BMAL ortholog that form a heterodimer and bind to the E-Box (Figure 3A) (Doherty and Kay.2010; Brown *et al.* 2012; Hardin and Panda 2013). This up-regulates transcription of two PERIOD paralogs, and a third paralog in peripheral clocks. It also up-regulates transcription of two CRY paralogs (Gillette and Tyan 2009; Hardin and Panda 2013). PER becomes stable for nuclear transport by forming a heterotrimer with CK1 $_{\xi}$  and CRY in the cytoplasm (Figure 3B). The heterotrimer is translocated into the nucleus where it hyperphosphorylates CLK (Figure 3C) and functions as described in the *Drosophila* mechanism (Yu *et al.* 2006, Gillette and Tyan 2009; Yu *et al.* 2009; Doherty and Kay 2010; Hardin and Panda 2013). Light degrades

CRY (Figure 3D), reducing the stability of PER. This causes the dephosphorylation and subsequent degradation of CLK and moves PER back to the cytoplasm (Figure 3E), where it is degraded by CK1 $_{\xi}$  (Panda *et al.* 2002, Yu *et al.* 2009; Gillette and Tyan 2009; Hardin and Panda 2013).



**Figure 3. This Figure shows the basic mechanism of the circadian clock in *M. musculus*.**

Biologists have begun to understand the circadian core-clock proteins and variations in their expression in cnidarians. *Nematostella vectensis* has a CLK orthologue, which varies in expression in a circadian fashion that is highest during late photophase and lowest during late scotophase. *Nematostella* also has

a CYC/BMAL orthologue that does not significantly vary in expression (Reitzel *et al.* 2010; Reitzel *et al.* 2013). Additionally, *Nematostella* has two CRY proteins, indicating the ability to respond to light cues at the molecular level (Reitzel *et al.* 2013). However, *N. vectensis* lacks PER and TIM1, but it does have TIM2. Because *Drosophila* has a PER orthologue but PER is absent in *Nematostella*, Reitzel and others (2010) have suggested that PER may have evolved in the lower bilaterians.

#### 1.4 Evolution of the Circadian Clock

As previously discussed, we have well-developed models for *Drosophila* and *Mus*, and have partially discovered the core-clock mechanism for *Nematostella*. Recently, the core circadian clock of *Eurydice pulchra* (Crustacea) and *Platynereis dumerilii* (Annelida) has been described to have a CLOCK, BMAL, PER, and TIM1 orthologue (Zantke *et al.* 2013; Zhang *et al.* 2013). This leaves a large taxonomic gap between Cnidaria and the higher Lophotrochozoa. This gap includes all of the Turbellaria and the Acoelomorpha (Egger *et al.* 2009). It is clear that the number of proteins involved in the regulation of the circadian clock has increased over the course of evolution (Simionato *et al.* 2007; Brown *et al.* 2012).

Three hypotheses exist to explain the evolution of the circadian clock (Reitzel *et al.* 2013), two of which are centered on the role of light. The first hypothesis states that clocks arose to minimize damage to DNA caused by UV light by timing DNA replication with the scotophase (Reitzel *et al.* 2013). CRYs

are found in a variety of organisms and are thought to originate from photolyases, which repair DNA through the use of blue light. (Tóth *et al.* 2001; Gehring and Rosbosh 2003; Losi and Gärtner 2012). The evidence that CRY degradation is light-dependent and that CRY proteins relay environmental timing cues to entrain the circadian clock suggests that this hypothesis is correct. (Reitzel *et al.* 2010; Reitzel *et al.* 2013). CRY likely acts a negative regulator in *Nematostella*, as it does in *Mus*. This indicates that CRY's action as a negative regulator may be the primitive condition retained by vertebrates, whereas TIM1 is a derived characteristic and functions as a negative regulator only in higher protostomes (Reitzel *et al.* 2010).

The second hypothesis states that the circadian clock arose as a homeostatic mechanism responsive to photo-oxidative damage caused by the production of free radicals (Edgar *et al.* 2012; Reitzel *et al.* 3013). The environment may shape the clock mechanism differently in aquatic animals than in terrestrial organisms. For example, the mechanism may be regulated by oxygen availability or by aryl hydrocarbon concentrations more so than light. *Nematostella*, *Drosophila*, and *Mus* all have other bHLH-PAS proteins such as ARNT, HIF and AHR that respond to environmental changes in light, lunar cycles and aryl hydrocarbon concentrations (Dugay and Cermakian 2009; Tessmar-Raible *et al.* 2011; Kronfeld-Schor *et al.* 2013; Reitzel *et al.* 3013; Zantke *et al.* 2013; Reitzel *et al.* 2014), These proteins may act as a redundancy system, as circadian regulation is essential for an organism's survival.

The third hypothesis states that circadian clocks may have arisen as a result of symbiotic relationships. Evolution of a centralized circadian pacemaker may have been driven by symbiosis between a prokaryote and an Archaea species when these two cells fused to form the first eukaryote. The onset of an internal pacemaker allowed for the coordination of timing of metabolic processes and cell cycles of each organism (Reitzel *et al.* 2013).

## 2. Rationale

The mechanism by which the circadian clock operates in *Drosophila* and *Mus* has been well established. Additionally work has been done to elucidate a circadian mechanism in cnidarians. This leaves a huge taxonomic gap (which contains the acoelomorphans and the turbellarians) between cnidarians and higher lophotrochozoans.

Daily rhythms have been observed in lophotrochozoans. For example, fission (asexual reproduction) occurs at night in triclad and catenulid flatworms (Morita and Best 1993; Stanton and Smith 2009). Additionally in the annelid *Aeolosoma headleyi*, the cell cycle progresses in a circadian fashion (Bowie *et al.* 2012). The cell cycle becomes altered under the influence of melatonin (a light-dependent hormone produced in a circadian rhythm) in triclad flatworms and in the catenulid *Stenostomum virginianum* (Morita and Best 1993; Stanton and Smith 2009). Although a daily rhythm may be apparent in basal Bilaterians (Morita and Best 1993), the genetic mechanism that regulates this rhythm in acoelomorphans and turbellarians is unknown (Reitzel *et al.* 2010).

*Isodiametra pulchra* – SMITH AND BUSH is an Acoelomorph worm that lives between sand grains approximately one centimeter above the sulfide zone on low wave-action beaches. It feeds on diatoms, which are abundant in the sand (Smith and Bush 1991). Given its changing habitat, *I. pulchra* may be exposed to a wide array of daily environmental changes including: changes in aryl hydrocarbons and oxygen concentrations, tidal levels, lunar phases, and

diurnal solar exposure. Additionally, preliminary data suggest that *I. pulchra* lay their eggs at night, indicating that they have a daily rhythm. When cultured in constant light environment, egg production stops (Ladurner and Smith unpubl.). Known circadian core-clock proteins can be used to identify orthologues in unpublished transcriptomes for *Isodiametra pulchra*, *Macrostomum lignano*, and *Stenostomum virginianum*. Additionally there are unpublished genomes available for *M. lignano* and *I. pulchra* however the genome for *I. pulchra* is unfinished.

In addition, recent publications argue between the position of Acoelomorpha as the base of the Bilateria (Egger *et al.* 2009) and Acoelomorpha as the base of the deuterostomes (Rundell and Leander 2010; Philippe *et al.* 2011). Identification of circadian clock proteins in *I. pulchra* could help to define the position of Acoelomorpha. Accordingly, I sought to elucidate the evolution of the circadian core-clock in the lower Bilateria, specifically in *I. pulchra*, by identifying potential core-clock protein orthologues, by identifying similarities and differences in protein structure and phylogeny, and by analyzing variations in mRNA expression. This is the first study of the circadian clock in a basal bilaterian.

### 3. Hypotheses

I predict that *I. pulchra* will have four core-clock-proteins including the following orthologues and their subsequent conserved protein domains: CLOCK (bHLH-PAS-PAS), CYCLE/BMAL (bHLH-PAS-PAS), TIMEOUT (TIM-TIM-C), and PERIOD (PAS-PAS). I expect this because the primitive cnidarian outgroup has three of the above-mentioned core-clock proteins (Reitzel *et al.* 2010) whereas the more advanced Arthropoda has all four core-clock proteins. Reitzel and others (2010) found that *Nematostella* lacked both PERIOD and TIM1. They were also the first to suggest that the PERIOD may have evolved within the Bilateria. I predict that they are correct and that *I. pulchra* has a PERIOD orthologue, indicating that PERIOD first appeared at the base of the Bilateria. I also predict that *I. pulchra* will have a TIM2 orthologue and that this is the primitive condition seen across the animal kingdom.

Because the analyses of the transcriptome, protein structure, and phylogenetic trees are not enough to correctly assign orthology, mRNA expression studies are needed to understand the behavior of the circadian core-clock-proteins. Expression studies of mRNA or protein temporal variation are often measured over one or two complete light/dark cycles. Zeitgeber time (ZT) is the twenty-four hour period in which one light/dark cycle is completed where at ZT0 the circadian rhythm is synchronized by lights on. The mRNA expression patterns are well understood in both *Drosophila* and *Mus*, and partially understood in *Nematostella*. These models can be used to compare known



patterns of mRNA expression to the patterns of PER mRNA expression in *I. pulchra*. It is difficult to predict when mRNA expression will be highest because *Nematostella* does not have a PER orthologue. Because the split between deuterostomes and protostomes occurred later in evolution, the mRNAs corresponding to circadian clock genes expressed in *I. pulchra* could resemble either the protostomes or the deuterostomes. Based on the temporal variation in mRNA expression in *Drosophila* and *Mus* discussed above, I predict that the circadian expression of PER in *I. pulchra* will be lowest during late photophase (ZT11) and highest during the late Scotophase (ZT23).

## 4. METHODS

### 4.1 Bioinformatics and Primer Design

*Drosophila melanogaster* core-clock-proteins were used to identify orthologous sequences in the *Isodiametra pulchra* transcriptome (Ladurner and Agata, unpubl. data). Amino acid sequences were obtained from the National Center For Biotechnology Information (NCBI) protein database (<http://www.ncbi.nlm.nih.gov>) for CLOCK, CYCLE, PERIOD, TIM1 and TIM2 proteins. Using the basic local alignment search tool or BLAST (Altschul *et al.* 1990), each *D. melanogaster* circadian clock protein sequence was blasted against the *I. pulchra* transcriptome using tblastn (protein sequence to mRNA sequence blast query) to identify likely *I. pulchra* orthologues. The blast results were ranked according to similarity by e-values. An e-value is the number of different alignments equal to or greater than a particular score that would be expected to occur randomly in a database search by chance (Altschul *et al.* 1990; Pevsner 2003). The *I. pulchra* mRNA transcript with the top e-value was recorded as the most likely orthologue for each of the *D. melanogaster* circadian clock proteins. If there were two or more top *I. pulchra* transcriptomic hits, then the top hits were aligned using Clustal W (Thompson *et al.* 1994) in MacVector 12.7.5 (MacVector Inc. 2013) to check for splice variants or duplicated sequences. When two or more sequences were identical, the most complete sequence was used for all further bioinformatics analyses and primer design.

The top sequence results from the transcriptome were compared against protein sequences in GenBank (Bilofsky and Burks 1988), using blastx to identify the reading frames. If a shift in reading frame occurred, all blastx hits in GenBank had a segment of the sequence in one reading frame (ex. +1) and the other portions of the sequence in a different reading frame (ex. +2). Any shift in the reading frame was a result of insertion or deletion errors in the *I. pulchra* transcriptomic assembly. If a shift in the reading frame occurred, it was corrected by deleting a base or by inserting a base (the added base was determined by a pattern/series of the same bases or, if there was no pattern, an adenine was inserted). The mRNA sequence was translated into a peptide sequence in the reading frame identified by the blastx results. The protein was complete if the first M after a stop codon in the sequence and the stop codon at the end of the sequence could be identified. The five prime and three prime untranslated regions (UTRs) were trimmed away so that the complete protein sequence could be used for further analysis. If the protein sequence was incomplete, then the amino acid sequence was trimmed either before the first M or after the stop. The coding region of the protein was blasted against other protein sequences in GenBank using BLASTP to verify orthology. All proteins were evaluated for domain structure using Expasy Prosite (Gasteiger *et al.* 2003).

Primers were designed for each potential *I. pulchra* orthologue using Primer3 software (Rozen and Skaletsky 2000) within MacVector. A single pair of primers was used to recover an amplicon of 2500 bases or fewer. If the sequence was larger than 2500 bases, then multiple overlapping primer sets

were designed (Table 1). Additional overlapping internal PCR products were designed for direct sequencing (Appendix 9.4) because the upper limit for direct sequencing was 900-1,000 base reads. Additionally, primers were designed for semi-quantitative Reverse-Transcriptase PCR (sqRT-PCR) to recover *I. pulchra* sequences of 330-360 base pairs for ARNT, PER, and EF-1 $\alpha$  orthologues (Table 2). All primers were synthesized by IDT (Integrated DNA Technologies) and diluted to 100mM stock solutions. Working stocks were further diluted to 20mM for PCR.

Sequence	Forward Primer	Reverse Primer	Product Size
IPUL1_3616	TCCTCCACACAGTTAATGGTGG	GCAGTAGAGGTAGAACTCTGGC	2226
IPUL1_2961	TGTCCGTACAGACATCAATTGC	TTTTTGTCCGGATTGTTGCTGC	1836
IPUL1_6832A	AGGCATGGATCACTTAGCAGAG	CGCAGGATAAGAGAATAAGCGC	2260
IPUL1_6832B	GATTTCCGGCAGTTTATGGAGC	ATTGCACGAATTCAGCACACTC	2390
IPUL1_26016	CTGGATGCAGAGAACTGAAGC	CTGGATGCAGAGAACTGAAGC	367
IPUL1_17690	AGAATGCTCTACGCCGAGACAG	GTACAGGAGTTTTGCATTGCTG	539
IPUL1_923	GTCAGTATTGTCGTCATTGGCC	CCAGCGAGAAGACTCAATTATG	1386

**Table 1. These primer sets above were used to obtain full sequence products. Full sequence products were analyzed on a gel for size verification.**

Sequence	Forward Primer	Reverse Primer	Product Size
sqlPUL1_3616	GTGTGTTTCGATTTCTGTGCTCC	GAAGTGAAGATATGCCGATCGC	335
sqlPUL1_2961	TTCCCTCAACTTGGATCTCGTG	GAAGTGAAGATATGCCGATCGC	358
sqlPUL1_923	ATCTCATCTACAAGTGCGGTGG	AGGGCATGCTCTCTAGTTTGTC	337

**Table 2. These primer sets were designed for sqRT-PCR expression experiments.**

## 4.2 Alignments and Phylogenetic Tree Construction

Using known *Drosophila* and *Mus* orthologues, NCBI blastp queries were conducted to identify orthologues in representative metazoans (see results). Amino acid sequences for each protein family (bHLH-PAS family protein sequences, PERIOD family protein sequences, and TIM family protein sequences) were aligned using MUSCLE (Edgar 2004) with default settings. Neighbor-joining trees were initially constructed in MacVector 12.7.5 and final Maximum likelihood trees were constructed in MEGA 5.1 (Tamura *et al.* 2011). The bHLH-PAS representatives were aligned with the *Amphimedon* sequences identified in Simionato (2007) and the *Nematostella* sequences identified in Reitzel (2010), whereas the PERIOD family and TIM family representatives were aligned with the *Nematostella* sequences identified in Reitzel (2010) and *Amphimedon* sequences identified by NCBI BLASTP queries. The *I. pulchra* orthologues listed above as well as the top blast hits for each circadian clock orthologue from the *Macrostomum lignano* transcriptome (Berezikov *et al.* unpubl. data) obtained by Stanton and Smith, and those from the *Stenostomum virginianum* transcriptome (Reddien *et al.* unpubl. data) obtained by Smith were also added to the alignments (Stanton and Smith 2013). These sequences were derived using the same method described above. Additional orthologues were included in the bHLH-PAS alignments and tree construction. These include ARYL HYDROCARBON RECEPTOR (AHR), SINGLE-MINDED proteins (SIMs), HYPOXIA-INDUCIBLE FACTOR proteins (HIFs), and THYROTROPIN-RELEASING HORMONE (TRH).

Neighbor-Joining phylogenetic trees were constructed in MacVector for initial topology and evaluated at 10,000 bootstraps for bHLH-PAS proteins rooted in *Amphimedon* HEY (Hairy/Enhancer-of-split related with YRPW protein--XP\_003387453), PERIOD proteins rooted in *Amphimedon* HEY (XP\_003387453), and a TIM tree rooted in *Arabidopsis* Timeless (AED96277). These trees allowed for the identification of duplicate sequences and for some editing of divergent sequences. Bootstrap values were calculated by the tree construction software and are an estimate of the probability that a node will appear over a given number of iterations (Felsenstein 1985). Tree construction was repeated using a Jones-Taylor-Thornton+G modeled Maximum Likelihood tree. This was the recommended best-fit model determined by MEGA 5.1. The Maximum Likelihood tree was constructed with 75% partial deletion (75% site-coverage cut-off where if there are more than 25% gaps at a particular position, then the position is considered uninformative and ignored when constructing a tree). The trees were evaluated at 1,000 bootstraps using MEGA 5.1.

#### 4.3 Culture of *Isodiametra pulchra*

*Isodiametra pulchra* SMITH and BUSH 1991 were obtained from the Institute of Zoology (University of Innsbruck, Austria) and cultured in F/2 media that contained artificial nutrient-enriched seawater (Vizoso 2010 -- adapted from Andersen *et al.* 2005). The master cultures were kept in petri dishes under fluorescent lights on a 14:10 light : dark) cycle at room temperature and fed on the diatom *Nitzschia curvilineata* (obtained from the Institute of Zoology,



University of Innsbruck) once a week. The cultures were undisturbed except for a media change with diatom replenishment after two weeks. RNA was extracted as described below for sequence verification from animals that were in culture for nine months. The cultures died a few months after the extraction requiring the collection of more animals as described below.

#### 4.4 Collection and Culture for sqRT-PCR

Wild-type (WT) *I. pulchra* were collected at four points along the kayak path behind the North Carolina Aquarium at Pine Knoll Shores from a saltmarsh on the outskirts of the Intracoastal Waterway in Atlantic Beach, NC (Appendix 1). This site was chosen because it was a north-facing beach with low wave action. To minimize sample contamination by the sulfide zone (indicated by the presence of black sand), only the upper layer of sand was collected (approximately one centimeter deep). Worms were extracted using a magnesium chloride anesthesia/decantation technique (Hulings and Gray 1971). The dishes were undisturbed for six to twelve hours to allow meiofauna to crawl through the extraction screen. Animals were selected based on morphological characteristics unique to *I. pulchra* and placed in fresh artificial saltwater in a petri dish. Animals were allowed to acclimate for twelve hours before being placed in a petri dish with F/2 culture medium and transported in 50ml screw-cap conical centrifuge tubes until returning to Winthrop University where master cultures were prepared. Cultures were prepared as described above. The cultures were kept in an incubator set at 20°C with a 12:12 cycle. Animals remained undisturbed except

for approximate worm counts under a dissecting microscope every week until there were enough animals for an RNA extraction at each time point. Animals were in culture for approximately one month before animals were collected for an RNA extraction described below. Remaining animals were cultured for an additional month before a second RNA extraction was performed.

#### 4.5 Extraction, Purification, and Quantification of RNA

Total RNA was extracted for Reverse Transcriptase PCR (RT-PCR) afternoon isolation around 2pm (ZT7) and for Semi-quantitative Reverse Transcriptase PCR (sqRT-PCR) at each isolation at 6am (ZT23) and 6pm (ZT11) as described below. Animals were moved to a petri dish with fresh F/2 media and starved for 36 hours to prevent RNA contamination from food. Total RNA was extracted by placing two hundred and fifty animals in a 1.5mL microcentrifuge tube. The animals were digested with Trizol (Sigma-Aldrich), and the total RNA was precipitated with chloroform using the protocol adapted by Smith from De Mulder (2009). A 1 $\mu$ l aliquot of the extracted total RNA was analyzed for concentration and purity using a Nanodrop spectrophotometer and the total RNA was stored at -80°C (Appendix 2A). The total RNA yield for the 2PM sample was 317ng. The total RNA yield for the 6AM (ZT23) sample was 367.3ng and the total RNA yield for the 6PM (ZT11) sample was 50% less (170.1ng).

#### 4.6 cDNA Synthesis

A Superscript III First strand cDNA synthesis kit (Invitrogen) was used to make cDNA from the isolated RNA according to the Superscript III kit instructions. An aliquot of total RNA was diluted 1:5 so that approximately 63ng of the 2pm (ZT7) sample RNA was used in the cDNA synthesis reactions. Both oligo (dt)<sub>20</sub> and random hexamer primers were used to synthesize cDNA for subsequent recovery of amplicons by PCR for sequencing. cDNA synthesis reactions for semi-quantitative PCR used an aliquot of the 6am (ZT23) sample and the 6pm (ZT11) sample of total RNA was also diluted 1:5 so that approximately 73ng of 6am (ZT23) RNA sample and approximately 34ng of 6pm (ZT11) RNA sample was used in cDNA syntheses. Only random hexamer primers were used to synthesize cDNA for semi-quantitative PCR. The cDNA products were stored at -20°C until used for RT-PCR or sqRT-PCR as described below.

#### 4.7 Phusion PCR for sequencing and Gel Electrophoresis

PCR amplicons for each orthologue were synthesized from cDNA using primers (designed as described above) and a Phusion High-Fidelity PCR kit (New England Biolabs). Each PCR reaction contained 1µl of cDNA (diluted if the concentration was greater than 10ng/µl), Phusion master mix, 1µl each of the 20mM forward and reverse primers (see above for primer design), and molecular grade water (Appendix 2B). Both 25µl and 50µl reactions were used depending on the purification preparation as described below. In the case of 50µl reactions,

all aliquots of reagents, cDNA, and primers were doubled. This protocol is further outlined in De Mulder's dissertation (2009). The tubes were loaded into a programmed (Appendix 2B) dual-block C1000 Touch thermocycler (Bio-Rad Laboratories Inc.). A 1% agarose gel was made using 0.5x TBE and stained with a 2 $\mu$ l aliquot (small 40ml gel) or a 5 $\mu$ l aliquot (large 90ml gel) of GelRed (Biotium) and a sample of each PCR product for each orthologue was loaded onto a gel (Appendix 2D). The gel was run at 45mA (150V). The gel was visualized using a Gel Doc <sup>TM</sup> XR+ gel imager (Bio-Rad Laboratories Inc.) and observed for predicted band size by comparing the results to a 100bp DNA ladder (Life Technologies). The first gel was used to establish that the primers worked; this was determined by checking the observed bands were the expected size for each PCR product. If the bands matched the expected size and there was a single band, then a second gel was prepared by loading the PCR products in every other lane to allow for band isolation as described below. If one clear band was present, then a QIAquick PCR spin-column purification kit (Qiagen) was used to purify the PCR product. If there was more than one band present in a lane, the band at the expected product size was cut out of the gel using a sterile razor blade and placed in a pre-weighed sterile microcentrifuge tube. The gel slices were purified using a Wizard SV Gel and PCR Clean-Up System (Promega). Purified PCR products were quantified using a Nanodrop spectrophotometer, and prepared for sequencing (see below). In the event that the concentration of PCR product was too low for sequencing, another PCR

reaction was run using a 50 $\mu$ l reaction. The same purification procedures were used for 50 $\mu$ l PCR reactions as were used for the 25 $\mu$ l PCR reactions.

#### 4.8 Direct Sequencing

After the PCR products were purified they were diluted, if needed, in molecular grade water and prepared according to the ENGECORE sequencing guidelines. The PCR products along with specific primers were sent to the University of South Carolina's EnGecore facility in Columbia, SC for sequencing. The chromatograms were visualized in FinchTV (Geospiza Inc.) and the nucleotide sequences were aligned and compared with the transcript from the transcriptome to identify any sequence differences. Purified PCR products using sqRT-PCR primers were also prepared and shipped to Eurofins (Eurofins MWG Operon) in preloaded sample tubes according to pre-loading instructions provided by the sequencing service or prepared as directed and sent to the University of Florida Interdisciplinary Center for Biotechnology Research (UF ICBR) sequencing facility.

#### 4.9 Semi-quantitative Reverse Transcriptase PCR

An amplification curve was conducted by running PCR reactions for three genes (two experimental and one housekeeping) at different cycle numbers to identify the point at which the PCR reaction plateaus. The inflection point was determined by the results as described below. A master mix was made using

standard Taq Polymerase (New England Biolabs), 10x reaction buffer (New England Biolabs), 10mM dNTPs mix (New England Biolabs), and molecular grade RNase-free water (Appendix 2C). An eight-tube PCR tube strip was cut in half so that there were four tubes per strip. The reaction mix was aliquoted into the four tubes. Each 25 $\mu$ l reaction contained 22 $\mu$ l of master mix, 1 $\mu$ l of 6AM cDNA, and 1 $\mu$ l each of sequence-specific primers for each orthologue (Ipul1\_3616- ARNT and Ipul1\_2961 - PER) and a housekeeping gene (IPUL1\_923 - EF-1 $\alpha$ ). The order of the tubes was as follows: tubes one through three contained primers for the genes (ARNT primers in tube one, PER primers in tube two, and EF-1 $\alpha$  primers in tube three), although tube four did not contain any primers in order to serve as a control. The four-tube PCR strip was prepared for each cycle measured (15, 18, 21, 24, 27, 30, 33 and 36). Each strip was taken at the completion of its designated cycle and placed on ice. The gel was prepared and stained with GelRed as described above (Appendix 2C). A 2 $\mu$ l aliquot of product from each PCR reaction of a given gene was loaded on a gel for electrophoresis for each cycle listed above so that each gel only contained the products of one gene.

The gels were imaged as described above. The intensity of the each band was analyzed by the amount of fluorescence emission in Image Lab 5.0 (Bio-Rad Laboratories 2013). EF-1 $\alpha$  (IPUL1\_923) was selected as the housekeeping gene because it is constitutively expressed in all cell types. Because it is endogenous, the housekeeping gene controlled for all variables such as pre-mortem factors (such as disease, genetic responses to environmental changes, and culture

contamination), as well as post-mortem factors (such as sample contamination, error in RNA extraction and cDNA synthesis, and genetic variations between worms). Because the housekeeping gene is constitutively expressed and controls for various external factors, it can be used to compare an experimental gene derived from the same extraction and cDNA synthesis to look at variations in the relative expression of the experimental gene. EF-1 $\alpha$  (IPUL1\_923) was analyzed in tandem with experimental genes. The amplification results were graphed by plotting band intensity against the cycle number and fit to a sigmoid curve. PCR amplification is exponential until the shape of the curve changes at the infection point. The curve then continues until a plateau is reached when the limiting reagent has been used up (Marone *et al.* 2001).

Due to the limited number of animals in culture, there were not enough worms to yield enough RNA for use in a quantitative Real-Time PCR method. This study meets the criteria for choosing a semi-quantitative RT-PCR method (Marone and others 2001), because a quantitative Real-Time PCR method would require multiple RNA extractions. Accordingly, a semi-replication method was used to look for variation in mRNA expression of ARNT and PER *I. pulchra* orthologues. six 6 AM (ZT23) and six 6 PM (ZT 11) cDNA syntheses carried out from a single AM or PM RNA extraction (described above in section 4.6). Three sqRT-PCR reactions were performed from each of the six 6am and six 6pm cDNA syntheses for a total of 36 PCR reactions. Three gels of each PCR reaction were analyzed in adjacent lanes that included a lane for the ladder, a lane for each 6am orthologue (ARNT, PER, and EF-1 $\alpha$ ), and a lane for each 6pm

orthologue for a total of 108 lanes. The PCR products were analyzed as described below to determine variations in mRNA expression between the two time points.

All sq-RT PCR reactions were run prepared as described above using similar cycle conditions (Appendix 2D) and stopped after 30 cycles (see results below). The three amplicons from the 6AM and 6pm extraction were analyzed in parallel for each PCR reaction and for each gel. Each strip contained six tubes of reaction mix, three with 6AM (ZT23), three with 6PM (ZT11) cDNA, and one of three respective primer pairs (IPUL1\_3616--ARNT, IPUL1\_2961--PER, or IPUL1\_923--EF-1 $\alpha$ ) for each cDNA time point. The order of the tubes were as follows: tubes one through three contained 6AM cDNA, ARNT primers in tube one, PER primers in tube two, and EF-1 $\alpha$  primers in tube three. Tubes four and five were skipped. Tubes six through eight contained 6PM cDNA, ARNT primers in tube six, PER primers in tube seven, and EF-1 $\alpha$  primers in tube eight. A no-primer negative control was used in tube 4 of the first strip for every run. There were a total of 18 strips. A 1% agarose gel was prepared as described above. The gels were visualized using either the Gel Doc <sup>TM</sup> XR+ (Bio-Rad Laboratories Inc.) or an Alphascreen EP (Alpha Innotech). Each gel image was exported for analysis as a tiff file and analyzed using FIJI (Schindelin *et al.* 2012). The file was opened in FIJI and the LUT was inverted so that the bands were black. The bands of interest were isolated using the rectangle tool. The band intensity peaks were plotted and the peaks were separated using the line tool. The area of under each peak was measured. The size of the peak was indicated as a percent of the



total size of all of the highlighted peaks. (Ferreira and Rasband 2012). The peak percentage of each experimental gene was divided by the peak percentage of the housekeeping gene to give the relative percent of expression for each experimental gene at each time point. The relative expression was used in statistical analyses as described below.

#### 4.10 Experimental Analyses

The sequences with the best e-value during BLAST searches were chosen for MUSCLE alignments (Edgar 2004) with MacVector and with MEGA 5 (Tamura *et al.* 2011). Orthologues were selected for the alignments by selecting the top orthologue that had an e-value smaller than an e-value of 0.001. The strength of each node on the Neighbor Joining and Maximum Likelihood trees were evaluated by the bootstrap value of that node (Reitzel *et al.* 2010; Tamura *et al.* 2011). Any bootstrap value of 50% or above was considered a weak relationship. If the bootstrap value was above 70%, then it was considered a good relationship (Hillis and Bull 1993).

To generate sigmoid curves, Ipul1\_3616 - ARNT (AM RNA extraction), Ipul1\_2961 (AM RNA extraction), and Ipul1\_923 – EF-1 $\alpha$  (PM RNA extraction) band intensities were plotted and graphed against cycle number from amplification data collected at cycles 15, 18, 21, 24, 27, 30, 33 and 36 using PRISM 6 (GraphPad Software Inc.) To identify the inflection point for the sqRT-PCR amplification curve for each orthologue, the standard curve was fitted to a sigmoid function by selecting analyze and interpolating the standard curve with

the Sigmoidal, 4PL X is log model using default settings. The default settings included the calculation of a 95% confidence interval (C.I.) and reported the C.I. in the graph. Because the band intensity on a sqRT-PCR gel and sqRT-PCR reactions are not independent of the cDNA syntheses, a nested ANOVA was analyzed in JMP (Statistical Discovery™ from SAS; SAS Institute Inc.). The effects tested in the ANOVA were as follows: time-point, time-point nested within cDNA, and time-point nested within cDNA nested within PCR.

## 5. Results

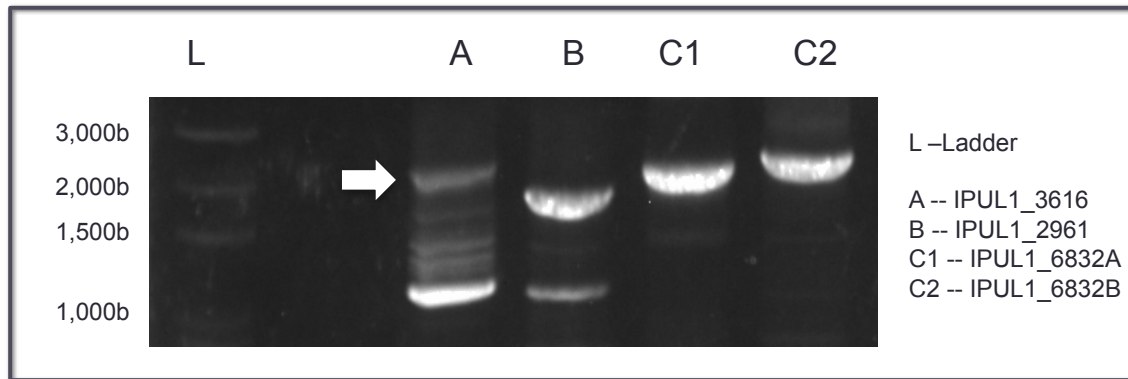
### 5.1 Orthology and Sequence Analysis

The preliminary orthologies that were assigned to *Isodiametra pulchra* from exhaustive blast searching and Expasy domain analysis are listed below (Table 3). Orthology was also preliminarily assigned to *Stenostomum virginianum* circadian clock sequences (Table 3), as established by Smith, and *Macrostomum lignano* circadian clock sequences (Table 3), as established by Stanton and Smith (Stanton and Smith 2013). The orthologies were later verified by domain analyses and phylogenetic analyses -- see results below. Each transcriptome contained incomplete sequences. For these sequences, orthology can be predicted based on the domains present in the fragment, but not with high accuracy. There was a shift in the reading frame in the IPUL1\_3616 and IPUL1\_6832 sequences due to a transcriptomic sequencing error. Neither *I. pulchra* nor *S. virginianum* had a CLOCK orthologue or a CYCLE/BMAL orthologue in their transcriptome.

	Sequence	AA Size	Complete/ Structure	blastp Hits	Orthology
<i>Isodiametra pulchra</i>	IPUL1_13616	689	YES / BHLH PASA PASB	ARNT e-99	ARNT
	IPUL1_2961	656	YES / PASB	MET e-09	MET
	IPUL1_26016	126	NO 5' /...PASB	HIF e-04	HIF
	IPUL1_6832	1558	YES / TIM TIM-C	TIMELESS e-64	TIMEOUT
	IPUL1_17690	148	NO 3' / bHLH...	SIM e-18	SIM
<i>Macrostomum lignano</i>	RNA815-29296	222	NO 3' / bHLH...	CLOCK e-25	CLOCK
	RNA815-29465	287	NO 3' / bHLH...	BMAL e-14	BMAL
	RNA815-12606	417	NO 3' / bHLH PASA...	ARNT e-128	ARNT
<i>Stenostomum virginianum</i>	67021992	577	YES/ bHLH PASA PASB	ARNT e-119	ARNT
	67019705	601	YES/ bHLH PASA PASB	SIM e-120	SIM
	6708625	201	NO 3' / BHLH PASA...	SIM/AHR e-42	AHR
	6702987	1553	YES / TIM TIM-C	TIMEOUT e-60	TIMEOUT

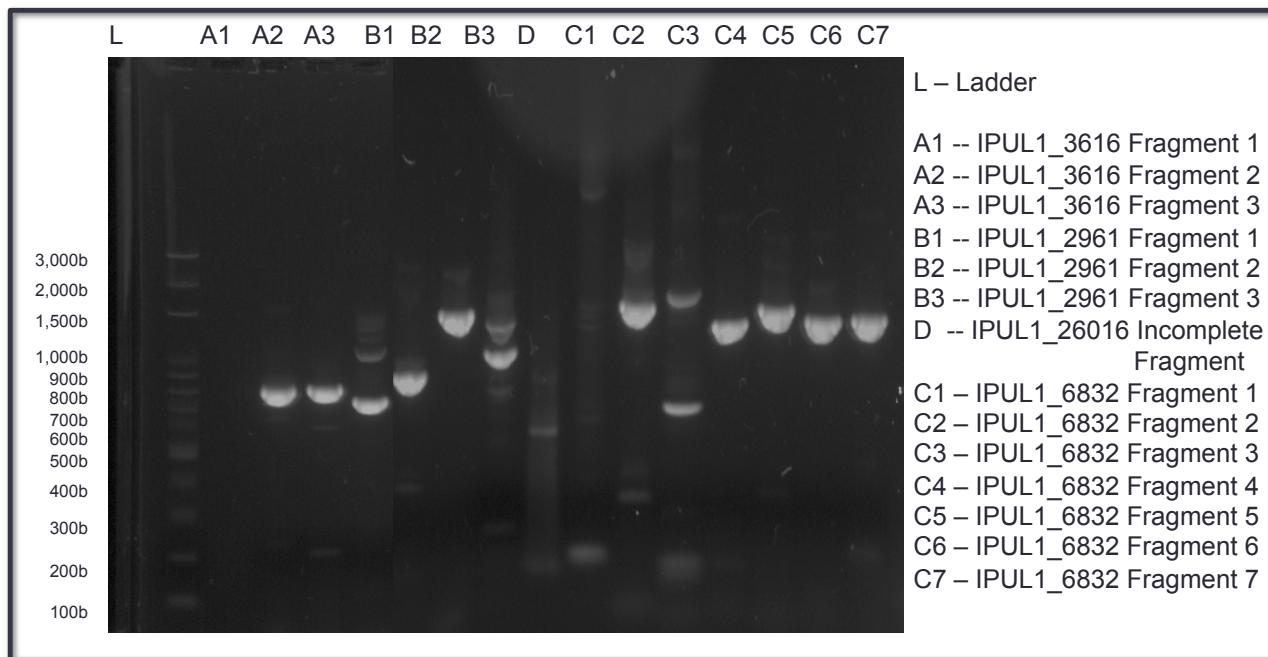
**Table 3. The table shows the preliminary orthologues assigned to *Isodiametra pulchra*, *Macrostomum lignano*, and *Stenostomum virginianum* based on BLAST searches and protein structure analyses (Stanton and Smith 2013).**

The *I. pulchra* transcriptomic sequences (IPUL1\_3616, IPUL1\_2961, IPUL1\_6832 and IPUL1\_26016) were first verified by comparing the actual band size of the full sequence (Figure 4) to the predicted band size of the PCR product. The actual band sizes and the predicted band sizes of IPUL1\_3616, IPUL1\_2961, IPUL1\_6832 and IPUL1\_26016 were approximately the same (Figure 4 - see also figure 6). The annealing temperature for IPUL1\_3616 was approximately five degrees higher than the annealing temperature for the rest of the sequences. Because of this there is not a clear band for the gel in figure 4 (lane A). The upper band (see arrow in figure 4) became the dominant band as the annealing temperature was raised. Additionally in Lane A, there was a second weaker band present in the same position as the bright band of figure 4. These bands were excised, purified, and sequenced.



**Figure 4. PCR products were generated to verify the amplification of the predicted sized bands. The bands above were recovered from full sequence PCR. Because IPUL1\_6832 was large, it was divided in half with overlapping primer sets. The arrow in lane A indicates the correct band for Ipu11\_3616.**

Internal primer sets were needed to break up the larger sequences into overlapping fragments for sequencing. The actual band sizes of the internal fragments (Appendix 4) were approximately the same size as their respective predicted band sizes (Figure 5). All three fragments of IPUL1\_3616 and IPUL1\_2961 resulted in dominant bright bands as well as the bands for IPUL1\_6832 fragment 2 and fragments 4-7. A weak band was noted for IPUL1\_26016 and there were two bands for IPUL1\_6832 fragment C3. A band was not present for the first internal primer set of IPUL1\_6832 (TIM2), Because the forward primer was different than the forward primer set of the first full sequence segment, a band was not present for the first internal primer set of IPUL1\_6832 (TIM2),



**Figure 5. The gel shows that PCR products obtained were comparable to their respective predicted band size.**



Although bands were present for each fragment of IPUL1\_3616, none of the internal fragments was sequenced successfully. However, the semi-quantitative PCR products were recovered successfully and used in the analyses of mRNA expression (see section 5.5). Sequencing failures likely occurred because of contamination or because the concentration of amplicon was below concentration limits for sequencing.

The sequencing results were compared to the *I. pulchra* transcriptome by aligning the successful forward and reverse reads for each portion of the sequence (Figure 6 – see also Appendix 9.5). Approximately 60% of the sequence was recovered for *I. pulchra* PER (IPUL1\_2961) and TIM2 (IPUL1\_6832). Although a band of the appropriate size was obtained for *I. pulchra* ARNT, but only about 15% of the sequence was recovered through direct sequencing and aligned with the transcriptome (Figure 6). The shifts in reading frame likely resulted from an error in the transcriptome where a base was mistakenly inserted or deleted. Unfortunately, there was not a successful read for either sequence in the region of the frame shift to determine if it was corrected in the right position.

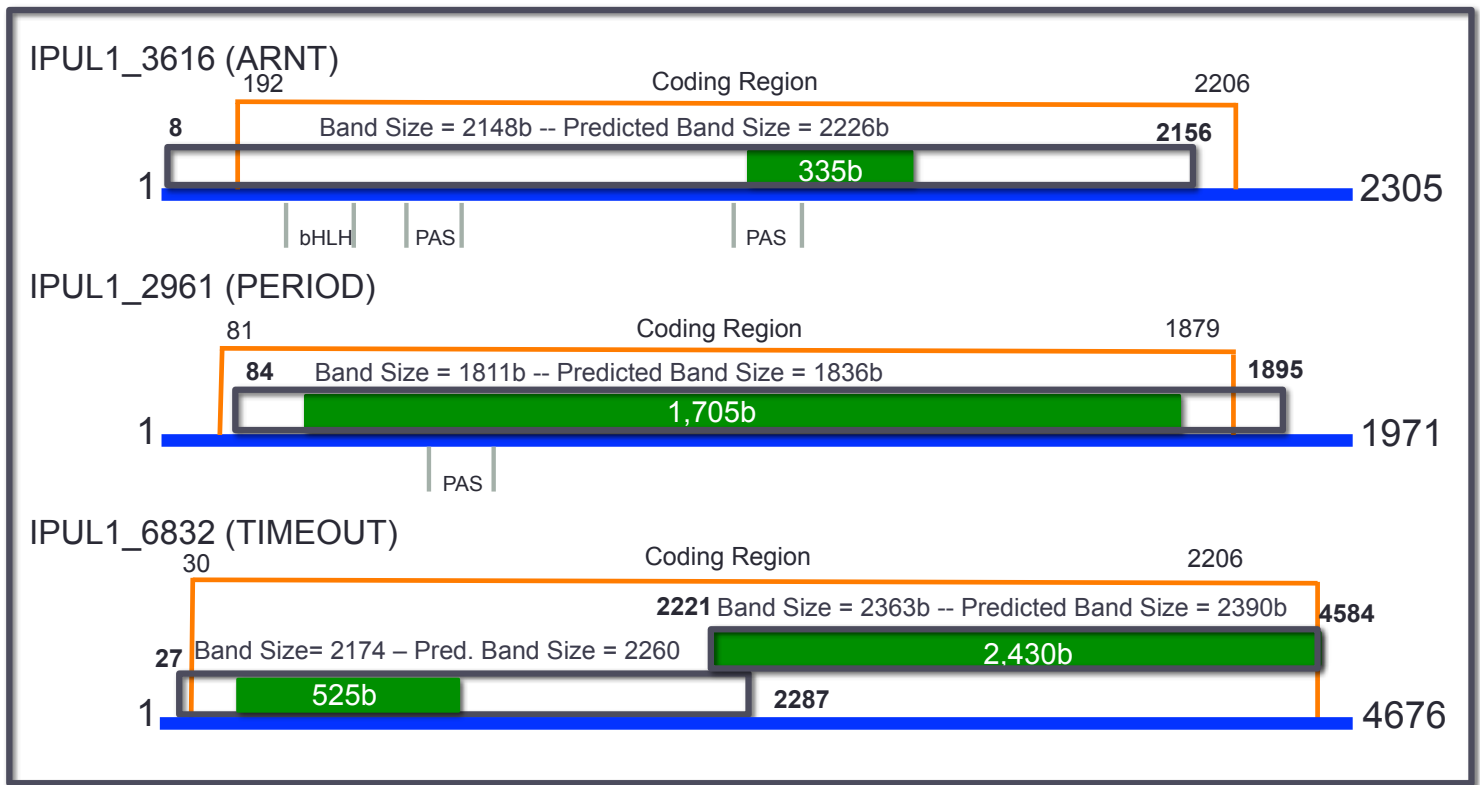


Figure 6. The sequencing map above shows that a portion of each of the three transcriptomic sequences was verified by band size (dark outlined box) and by direct sequencing (green)

## 5.2 Evolution of bHLH-PAS Orthologues

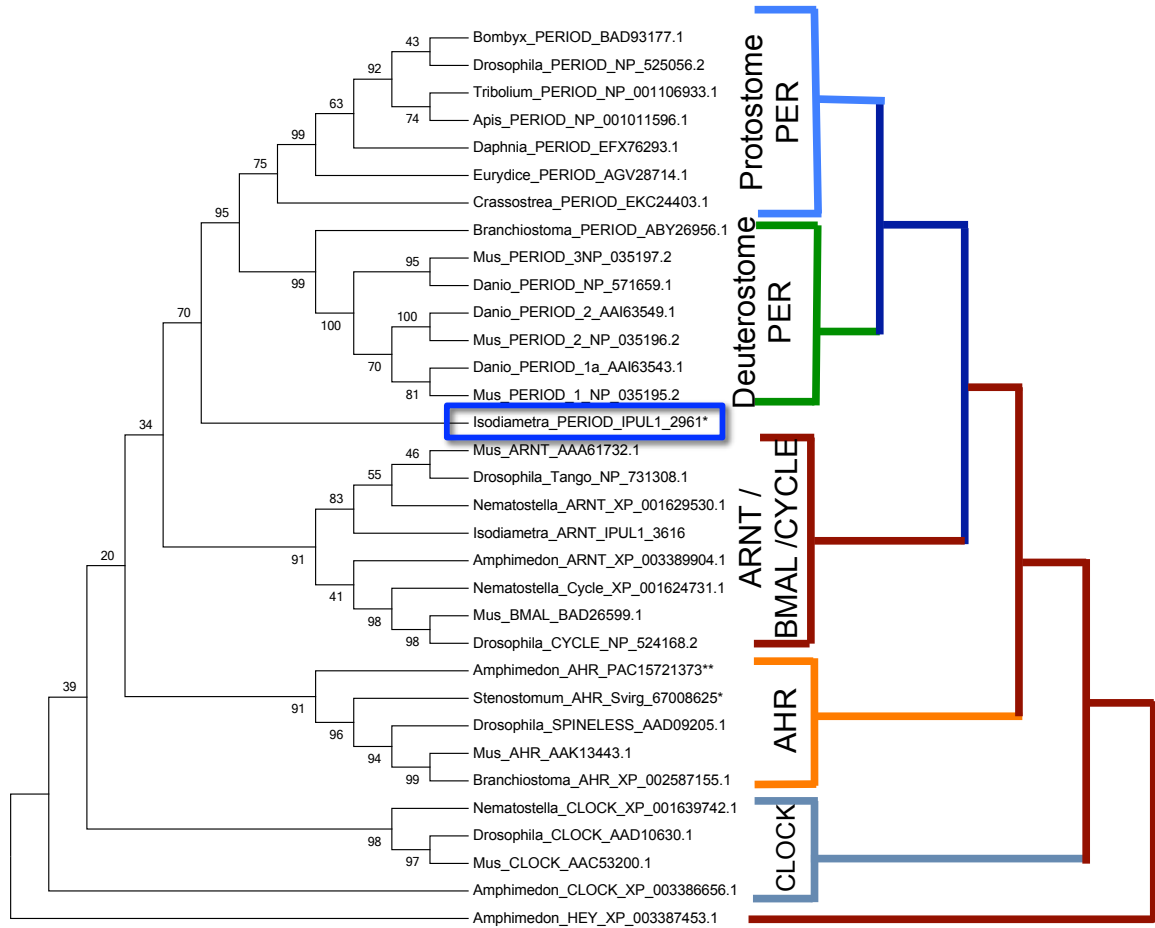
The tree results showed that IPUL1\_3616 is an ARNT orthologue and grouped with *M. lignano* (Mlig\_RNA815\_12606) and *S. virginianum* (Svirg\_67021992) ARNT (Figures 7 and 8). Additionally the results confirmed that Smith correctly identified Svirg\_67019705 as AHR (Stanton and Smith 2013). Not all of the identified orthologues in Table 3 could be verified by means of tree construction because some of orthologue sequences were either incomplete or did not group with a particular protein family. These sequences were removed from the tree. Although the *M. lignano* transcriptome appeared to contain orthologues for CLK and CYC/BMAL (Table 3), the orthologues did not group in their respective clades indicating that the sequences were divergent. Because of this, *M. lignano* CLK and CYC/BMAL were not included in the alignment or the tree below. The topology of the bHLH-PAS tree was similar to the tree topology that Simionato and others (2007) found by aligning just the bHLH domains (Figure 1).

The full sequence bHLH-PAS tree solidified the position of the *Amphimedon* ARNT and CLK sequences. The results clearly show that the *Amphimedon* ARNT sequence is the root of the ARNT clade with a bootstrap value of 73. The results also show that *Amphimedon* CLK also forms the root of the CLK clade with a bootstrap value of 75. The bHLH-PAS tree also indicates that CYC first evolved in *Nematostella* (supported by a bootstrap value of 91), as *Amphimedon* does not have a CYC orthologue. Additionally the bHLH-PAS tree



### 5.3 Evolution of PERIOD Orthologues

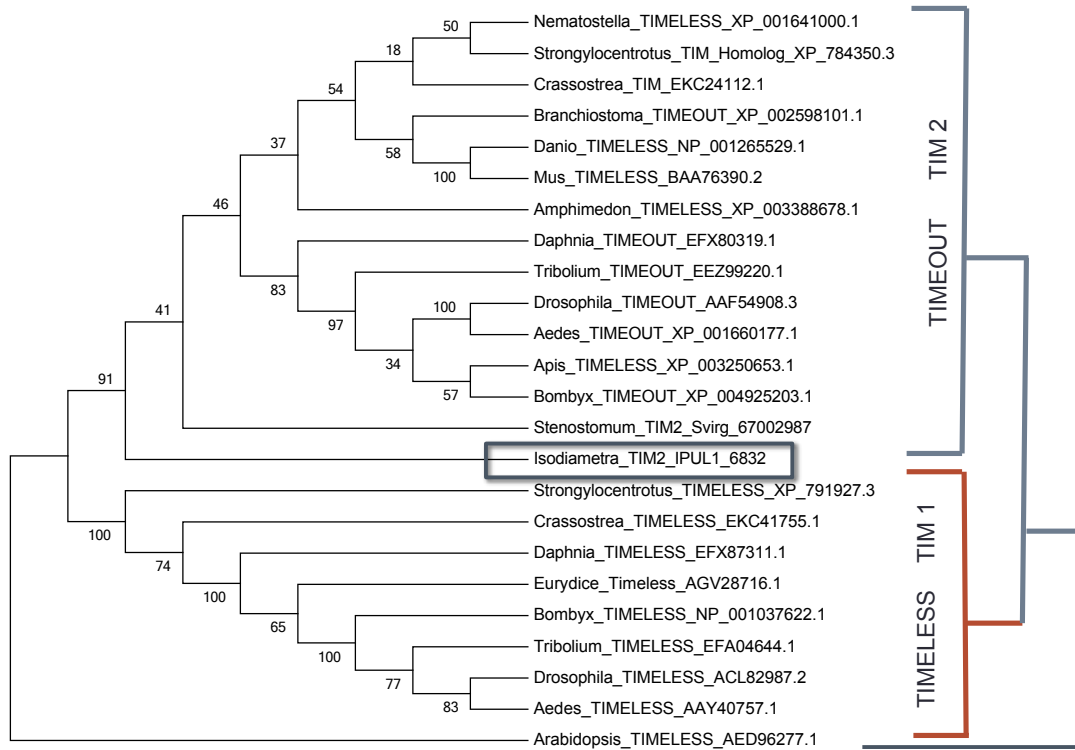
From exhaustive BLAST searching, it was found that cnidarians and sponges did not have a PER orthologue; however *I. pulchra* had a primitive PER orthologue (IPUL1\_2961). Additionally, the blast searches revealed that that hemichordates, echinoderms, some mollusks (specifically cephalopods) and platyhelminths appear to have lost PER. The phylogenetic results show that PER evolved at the base of the Bilateria. *I. pulchra* is the primitive sister group to the PER clades (Figure 9). The results show bootstrap support for a protostome clade (73) and a deuterostome clade (99), which indicates that they diverged along different lines.



**Figure 8. The maximum likelihood tree (1,000 bootstraps) shows that IPUL1\_2961 is the primitive PERIOD of the Bilateria.**

#### 5.4 Evolution of TIMELESS Orthologues

A TIM ortholog (IPUL1\_6832) in the *I. pulchra* transcriptome was identified using BLAST. The domain structure of IPUL1\_6832 was indicative of TIM2 because it has both a TIM domain and a TIM-C domain. After constructing a phylogenetic tree (Figure 9), it is clear that IPUL1\_6832 is a member of the TIM2 family. Additionally, deuterostome TIMELESS orthologues, as well as *Apis* TIMELESS and *Nematostella* TIMELESS orthologues grouped within the TIM2 clade. Blast searches also revealed that deuterostomes (specifically *Danio rerio*, *Xenopus laevis*, and *Mus musculus*) and platyhelminths (specifically *Macrostomum lignano*, *Stenostomum virginianum*, *Schistosoma mansoni*, and *Hymenolepis microstoma*) lost TIM1.

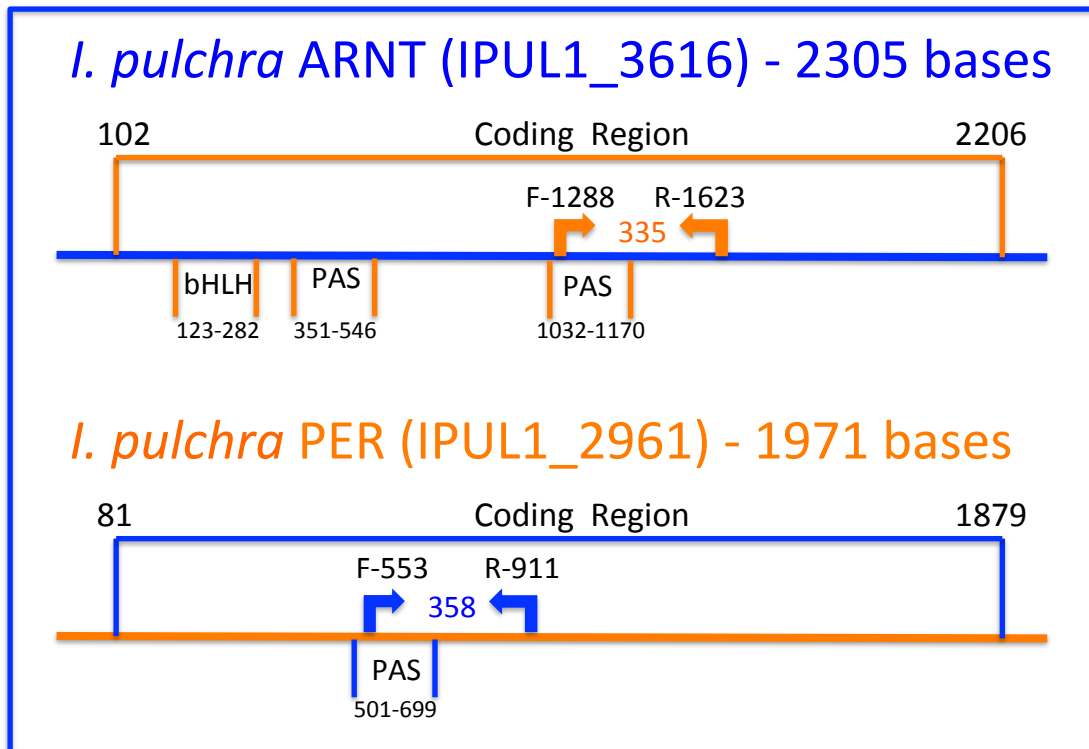


**Figure 9. The maximum likelihood tree (1,000 bootstraps) shows that IPUL1\_6832 groups with the TIM2 protein family.**



## 5.5 Standard Amplification Curves for sqRT-PCR

Each sqRT-PCR primer set flanked a region of sequence that encoded for a portion of the non-conserved region and a portion of a conserved PAS domain (Figure 10). The forward and reverse sequencing reads were aligned with the transcriptome and primers to verify that the sqRT-PCR products for ARNT (using the primer set IPARNTB) and for PER (using the IPPERA primer set) matched their respective transcripts identified in the transcriptome (Figures 11 and 12). Sequences were verified against the transcriptome using two reads (a forward sequencing read and a reverse sequencing read) at every position. Direct sequencing results showed that the *I. pulchra* ARNT sqRT-PCR amplicon (IPARNTB) was an identical match (Figure 11) to the primer-flanked region of the transcriptomic sequence. Likewise the *I. pulchra* PERIOD sqRT-PCR amplicon (IPPERA) was an exact match to the primer-flanked region of the transcriptomic sequence (Figure 12).



**Figure 10.** The primer blueprints show where the sqRT-PCR primer sets for *I. pulchra* ARNT (IPARNTB) and PER (IPPERA) bind to the cDNA transcript in a region that included a portion of a conserved PAS domain.

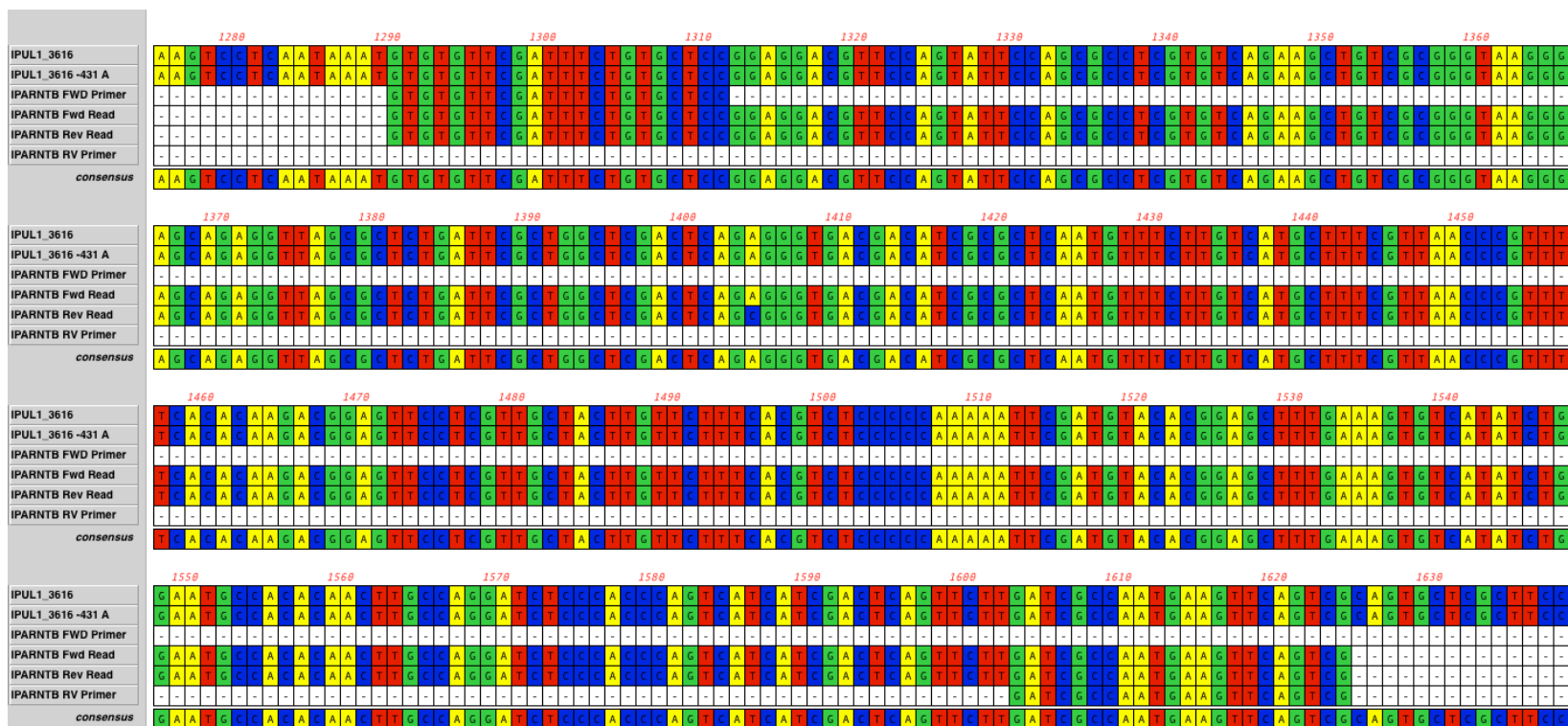


Figure 11. The alignment of *I. pulchra* ARNT (IPUL1\_3616) and the IPARNTB sqRT-PCR fragment shows that the primers were successful in generating a product, and that the recovered sequence for IPARNTB aligned perfectly with the transcriptome.

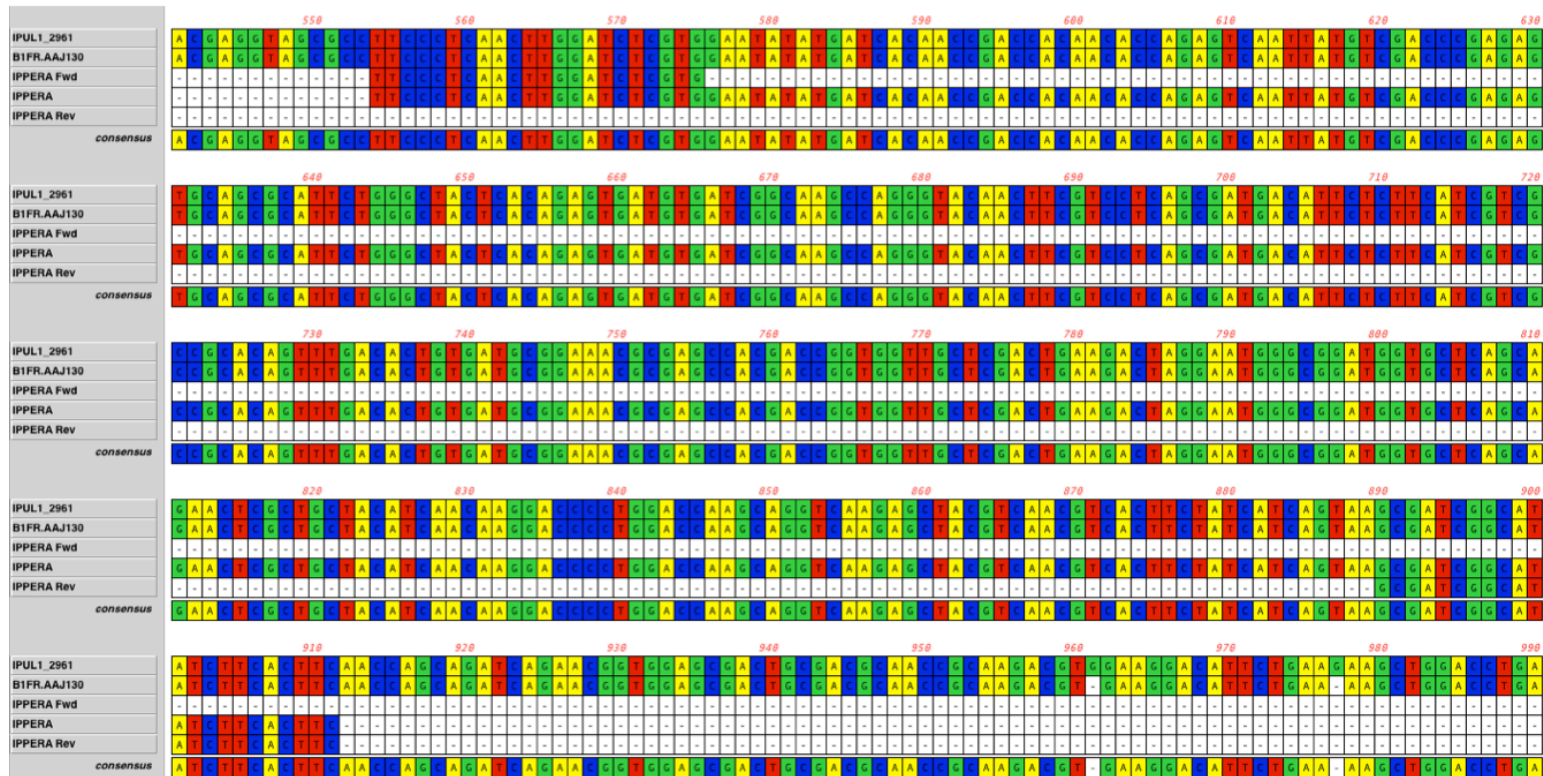
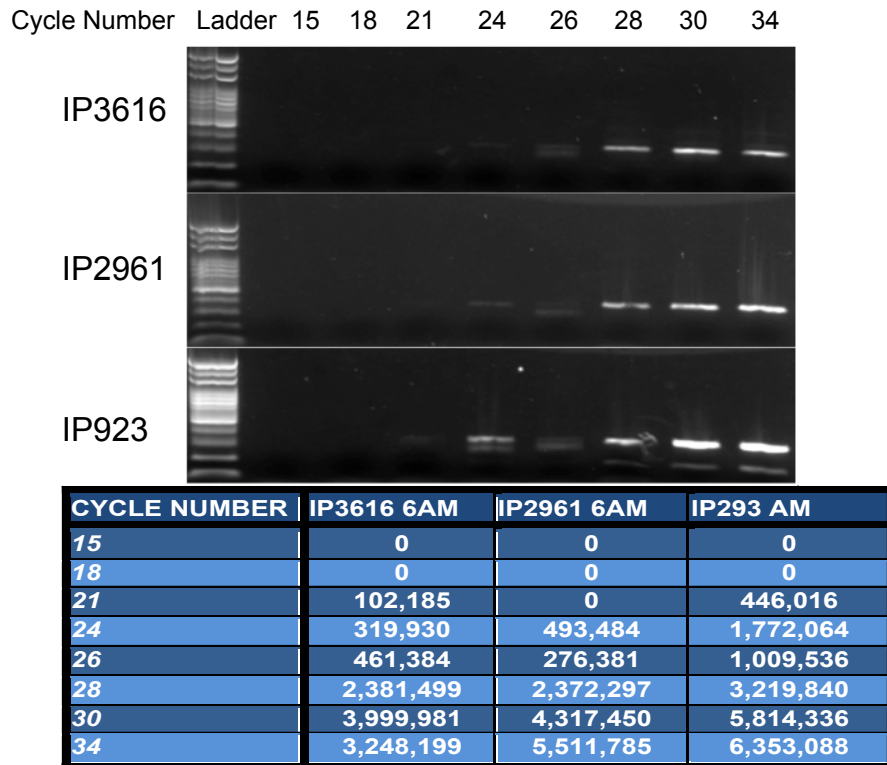


Figure 12. Likewise, the alignment of *I. pulchra* PER (IPUL1\_2961) also shows that the primers were successful in producing a PCR product and that the recovered sequence for *I. pulchra* PER aligned perfectly with the transcriptome.

Amplification curves for the visualization of amplicon concentration changes were generated from gel data (Figure 13). Amplification gel data revealed that all three sequences (*I. pulchra* ARNT, PER, and EF-1 $\alpha$ ) and their respective primer pairs resulted in bands. Additionally, Figure 14 shows that amplification was exponential as expected, and that the PCR reaction under the specific cycle conditions plateaued between 25 and 35 cycles. The amplification curves validated the use of 30 cycles for sqRT-PCR experiments based on the inflection point (the point at which the shape of the curve changed) in all sigmoid amplification curves (Figure 14).



**Figure 13. Amplification curves for the visualization of amplicon concentrations. Each of the bands on the gels above were quantified to construct an amplification curve for *I. pulchra* ARNT (IP3616), PER (IP2961) and EF-1 $\alpha$  (IP923). The table shows the amount of product (represented in arbitrary units based on the intensity of a single band) that formed after every three cycles.**

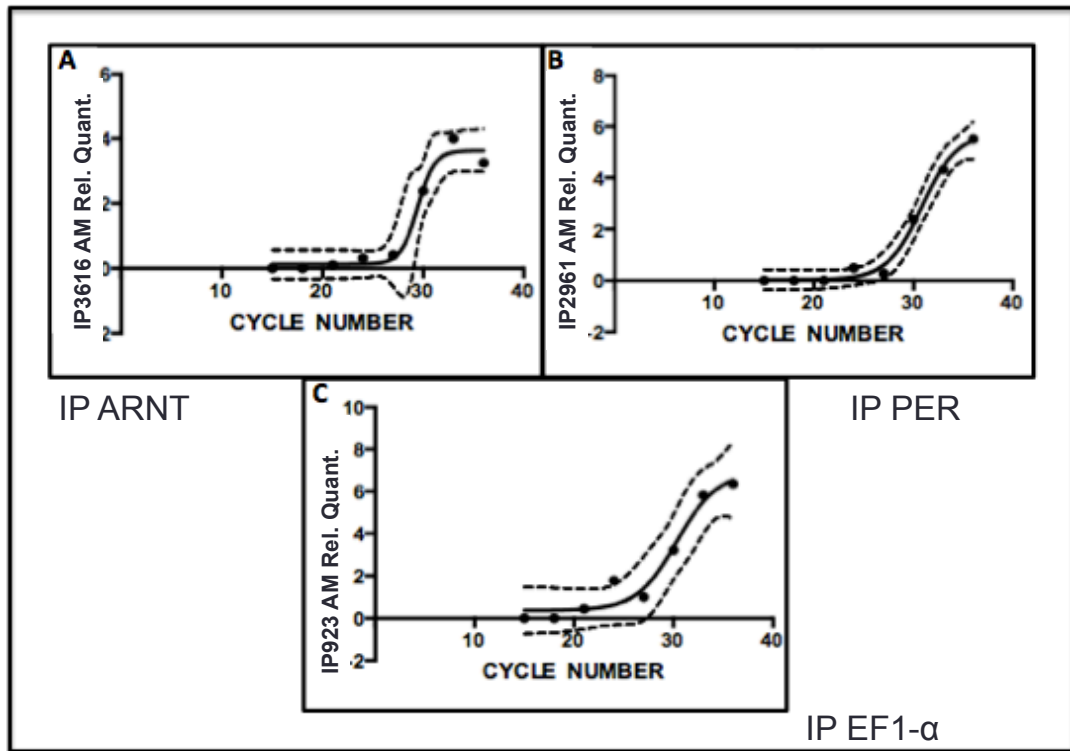
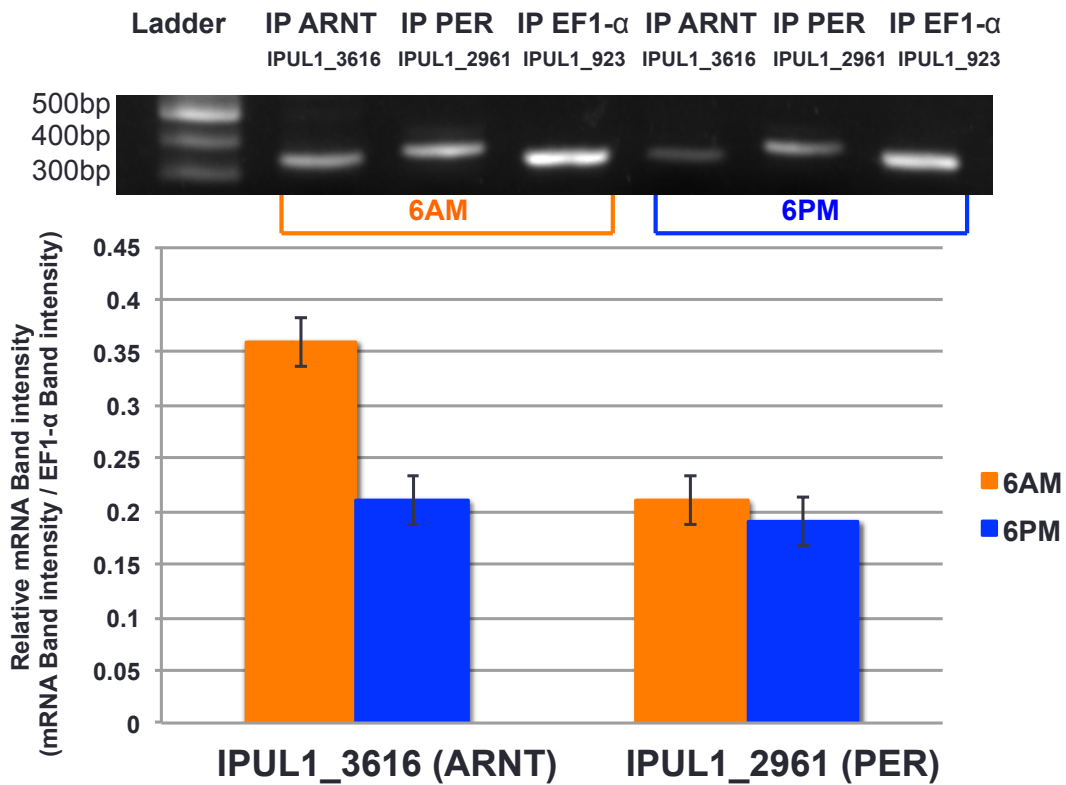


Figure 14. Each graph of each orthologue shows that the PCR amplification curve changes shape at 30 cycles. Because of this finding, sqRT-PCR runs were completed at 30 cycles during mRNA expression analysis experiments.

## 5.6 Analysis of mRNA Expression

Analyses of mRNA expression levels were carried out by comparing *I. pulchra* ARNT (IPUL13616) and *I. pulchra* PERIOD (IPUL1\_2961) to the constitutively expressed housekeeping gene *I. pulchra* EF-1 $\alpha$  (IPUL1\_923) at two time points (12 hours apart). The results of the nested ANOVA (Figure 15) revealed that ARNT (IPUL1\_3616) mRNA expression varied between 6am (ZT23) when expression was high and 6pm (ZT11) when expression was low ( $F=3.8$ ,  $MSE=0.11$ ,  $p=0.0001$ ). All effects were significant; time-point ( $F=23.84$ ,  $p=0.0001$ ), cDNA [time-point] ( $F=3.41$ ,  $p=0.0019$ ), and PCR [time-point, cDNA] ( $F=2.57$ ,  $p=0.0029$ ). The nested ANOVA revealed that PERIOD (IPUL\_2961) expression did not vary, as the effect of time-point was not significant ( $F=1.58$ ,  $p=0.2148$ ). The ANOVA appeared to be significant ( $F=4.24$ ,  $MSE=0.10$ ,  $p<0.0001$ ), however, the variability in cDNA synthesis and PCR runs accounted for the statistical significance of the overall effect (Figure 15), as the effects of cDNA [time-point] ( $F=5.28$ ,  $p<0.0001$ ) and PCR [Time-point, cDNA] ( $F=3.82$ ,  $p<0.0001$ ) were significant.





**Figure 15. Analyses of mRNA expression levels by cDNA synthesis for ARNT and PER. The graph shows that the average *I. pulchra* ARNT mRNA expression decreased during the day, but the average *I. pulchra* PER mRNA expression only slightly decreased between morning and night.**

## 6. Discussion

### 6.1 Circadian Clock Orthologues in *Isodiametra pulchra*

It is clear that the addition of *I. pulchra* orthologues helps to elucidate the evolution of the circadian clock between the cnidarians and arthropods. BLAST searching and analyses of protein domains using ExPASy Prosite were valuable bioinformatics tools in obtaining the correct preliminary orthology for three core-clock sequences (*I. pulchra* ARNT, PER, and TIM2) and led to the identification of additional partial sequences in the *I. pulchra* transcriptome. The results of the phylogenetic tree confirmed that the correct orthology was assigned to the three complete transcriptomic sequences.

It is possible that CLK and/or CYC/BMAL orthologues were not identified because the RNA extracted for the transcriptomic assembly was isolated when the CLK and CYC/BMAL transcripts were either partially degraded or the RNA for the transcriptome assembly was isolated at a time in which the transcription of these orthologues was inhibited by their negative regulators. In this case, it is possible that the failure to identify a core-clock orthologue in the transcriptome does not mean that an orthologue is not present in this animal. A future investigation using at least two transcriptomes (one assembled from RNA extracted at lights-on and the other assembled from RNA extracted at lights off) is warranted to confirm that *I. pulchra* lacks both CLK and CYC.

In addition to bioinformatics and phylogenetic analyses, expression

studies help to further validate correct orthology assignment based on the pattern of expression of particular gene, as some of these genes vary in expression across the circadian cycle. In order to assess variation in expression using sqRT-PCR, it was essential to establish a standard curve to identify the cycle number at which the expression of each sample was measured. The standard curve established a baseline for amplification by relating the amplicon concentration to the cycle number. Amplification plateaued as a result of either the loss of Taq polymerase activity or the limiting reagent (likely the dNTP or primers) was used up. It is also possible that exogenous double-stranded DNA and the endogenous amplicon inhibited formation of additional PCR product, and caused the reaction to plateau (Kainz 2000). The inflection point of the curve occurred at 30 cycles and was consistent across the three amplification curves, one for each orthologue (*I. pulchra* ARNT, PER, and EF-1 $\alpha$ ).

The results of the mRNA expression study show that *I. pulchra* ARNT may vary in a circadian fashion where expression is highest one hour before lights on (ZT23) and lowest one hour before lights off (ZT11). *I. pulchra* PER did not vary in expression, however temporal variation cannot be ruled out with just two time points. PER expression in *Drosophila* is highest during the early to mid-scotophase and lowest during mid-photophase (Hardin *et al.* 1990; Price *et al.* 1995; Yu *et al.* 2006; Hardin and Panda 2013). The mRNA expression of two *Mus* PER paralogues in the suprachiasmatic nucleus are highest during the mid-photophase and lowest during early scotophase (Gillette and Tyan 2009). It is possible that *I. pulchra* PER mRNA varies in a pattern more similar to *Mus* than

*Drosophila*. If this is true, then it would be expected that *I. pulchra* PER peaks during the mid-photophase. In this case, PER would not be at its peak of expression at the times that the RNA was isolated for this study. It is possible that PER may vary; unfortunately there were not enough animals to acquire data for the additional mid-scotophase and mid-photophase time points required to investigate this hypothesis. A second mRNA variation study is needed to test the hypothesis that PER expression is highest during mid-photophase.

## 6.2 Evolution of bHLH-PAS Proteins

The bHLH-PAS tree results expand upon the bHLH tree results of Simionato and others (2007). By including the full sequence in the alignments and not just the bHLH domain, the bootstrap support was strengthened for the position of *Amphimedon* CLK and ARNT within their respective clades. *Amphimedon* ARNT grouped outside of the ARNT- CYC clade with a bootstrap support of 60 in Simionato and others' (2007) bHLH tree; while the results of bHLH-PAS tree indicates that *Amphimedon* ARNT is the primitive member of the ARNT clade with a bootstrap support of 73 (Figure 7). The bootstrap value of Simionato and other's (2007) tree did not support the position of *Amphimedon* CLK within the CLK clade (Simionato *et al.* 2007), however the bHLH-PAS tree shows that *Amphimedon* CLK is the most primitive orthologue of the CLK clade (Figure 7). Given the results of the bHLH-PAS tree, it is not surprising that the bHLH-PAS components of the animal circadian clock likely evolved from three

primitive *Amphimedon* orthologues identified in Simionato and others' (2007) bHLH tree - ARNT, CLK, and AHR.

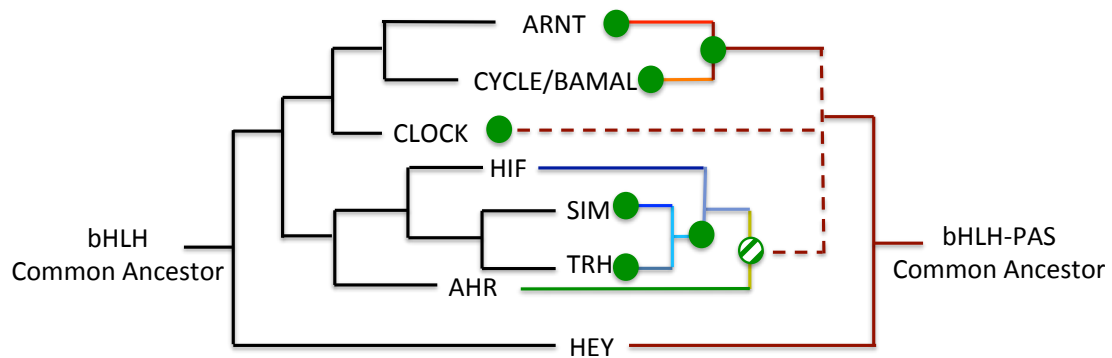
The *I. pulchra* orthologues and the results of the bHLH-PAS tree added critical data needed to elucidate the evolution of ARNT and CYC. It is clear that *Amphimedon* has the most primitive ARNT orthologue in the animal kingdom. The data also supports the hypothesis that the *Amphimedon* ARNT is the common ancestor of the ARNT and CYC/BMAL clades because *Amphimedon* lacks a CYC orthologue. This supports the hypothesis that CYC/BMAL evolved from *arnt* gene duplication (Simionato *et al.* 2007). As expected, the results of the bHLH-PAS tree confirmed that *Nematostella* ARNT grouped within the ARNT clade (Reitzel *et al.* 2010). *I. pulchra* and two other basal bilaterians (*Stenostomum virginianum* and *Macrostomum lignano*) also have ARNT orthologues that grouped within the ARNT clade. The results suggest that ARNT diverged along different lines multiple times, indicated by a supported flatworm clade, a supported arthropod clade, and a supported vertebrate clade (Figure 7). However, the bootstrap support is weak for lower protostome, higher protostome, and deuterostome clades, resulting in an ARNT polytomy.

In addition, these results of the bHLH-PAS tree expand upon the work of Reitzel and others (2010) to include a larger group of metazoans. This strengthened the relationship between *Nematostella* CYC and the rest of the CYC clade from a bootstrap support of 55 in Reitzel and others' tree (2010) to a bootstrap support of 91 in the bHLH-PAS tree (Figure 7). The data suggests that the duplication event that gave rise to CYC occurred in the cnidarians, as

*Nematostella* CYC is the most primitive orthologue in the CYC/BMAL clade. Additionally, *Platynereis* has a CYC orthologue. Given that CYC is present in both cnidarians and annelids, it would be expected that *I. pulchra* would also have a CYC orthologue. However, it appears that *I. pulchra* and *S. virginianum* either lost CYC/BMAL or the orthologue was not successfully identified in the transcriptome as described above. Although there was a CYC orthologue present in the *M. lignano* transcriptome, it did not group within the CYC/BMAL clade and was removed from the tree. It is clear from the results of the bHLH-PAS tree that CYC diverged along different lines in higher arthropods and vertebrates. This also likely occurred in parallel with changes in circadian clock function as directed by the change in the negative regulator as described below.

Due to very weak bootstrap support, the bHLH-PAS tree resulted in a polytomy among the ARNT-CYC/BMAL, CLK, and HIF-SIM-TRH-AHR clades (Figure 16). The bootstrap support for the internal nodes of the bHLH-PAS tree could not be compared to Simionato's and others' bHLH tree because they did not report those values in their paper and the original data were lost post-publication (Dr. Michael Vervoort, Paris Diderot University, Per. Com. 2014). The topology of CLK in the bHLH-PAS tree was different than the topology of Simionato and others' (2007) bHLH tree. Due to a lack of bootstrap support at the root of the CLK clade, the bHLH-PAS tree resulted in a polytomy. The weak bootstrap support for the CLK clade in the bHLH-PAS tree may have been a result of a disproportionate number of higher metazoan orthologues included in the tree compared to the limited number of lower metazoan orthologues available

for inclusion in the bHLH-PAS tree. *M. lignano* was one of the only basal bilaterians to have a CLK orthologue, but it was removed from the tree because it did not group within the CLOCK clade indicating that it was divergent or was possibly poorly assembled.



**Figure 16. The bHLH-PAS tree included full sequences, which gave rise to a different tree topology than the bHLH tree (adapted from Simionato *et al.* 2007). The bHLH-PAS tree resulted in a polytomy (dotted line). Green circles indicate the supported nodes of the bHLH-PAS tree and striped circles indicate nodes that are weakly supported.**

The bootstrap support of the CLK clade may be strengthened with the inclusion of a larger number of lower metazoan orthologues. A future study is warranted to re-examine this topology and should include a PAS-only tree like that of McIntosh and others (2010) to examine the lineages of the circadian clock PAS domains. The lineage of the PAS domains will further elucidate the origins

of each bHLH-PAS protein and may have implications in circadian clock evolution, specifically the change in binding affinities for the different negative regulators

### 6.3 Evolution of PER

The results show that *I. pulchra* PER is the most primitive PER discovered to date. The PER phylogenetic tree supports the hypothesis that PER first appeared at the base of the Bilateria in the Acoelomorpha (Figure 8), as *Amphimedon* and *Nematostella* do not have a PER orthologue (Reitzel *et al.* 2010). *I. pulchra* PER did not group with any other PER orthologues, which likely indicates that acoelomorphan PER is the sister group to the rest of the PER clade (Figure 8). Additionally *I. pulchra* (representing the Acoelomorpha) is the root of a bifurcation between the protostome PER clade and the deuterostome PER clade, indicating that the protostome and deuterostome PERs are divergent. The differences between the higher protostome and deuterostome PER clades could be explained by the difference in affinity to bind to different negative co-regulators. The negative co-regulator for higher protostomes is TIM1 (which forms a heterodimer with PER), but the negative co-regulator in higher deuterostomes is CRY (which also forms a heterodimer with PER).

Like ARNT and CYC/BMAL, PER likely evolved as a result of duplication events. The origin of PER is still elusive, although it is possible that PER evolved at the base of the Bilateria from an escaped PAS domain. Because the bootstrap support at the node shared by ARNT and PER is low (Figure 8), the tree does



not support the hypothesis that PER likely originated as an escaped ARNT PAS domain. Further analysis is needed to support the hypothesis that the variable regions of the sequence contribute to the low bootstrap value. Using a concatenated alignment that links the conserved domains and removes the variable regions, the sequence variation would be reduced and likely improve the bootstrap support for weak or unsupported nodes.

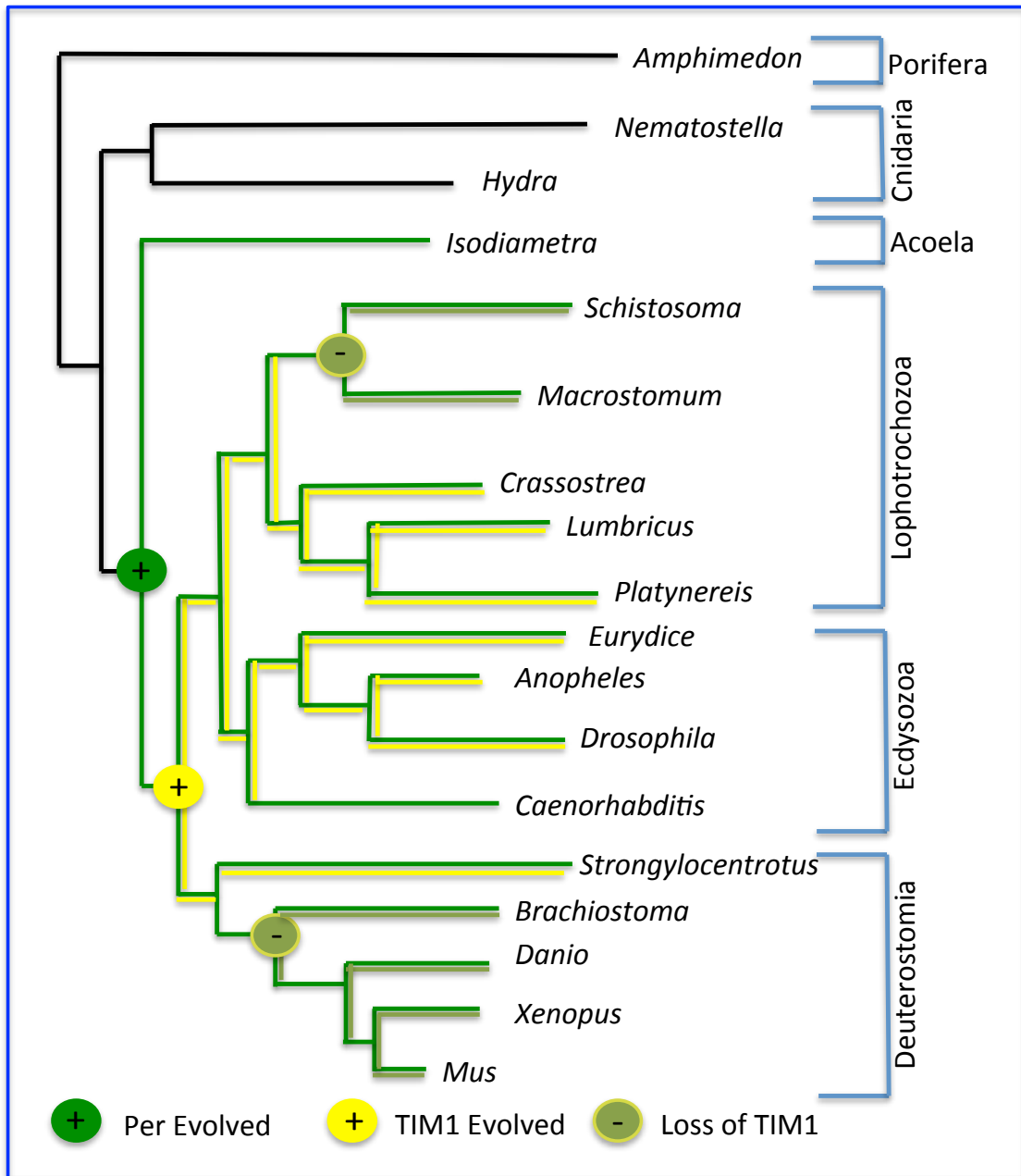
Because *I. pulchra* PER is the most primitive PER identified to date, it would be beneficial to include it in a PAS alignment to determine the origin of PER. A PAS-only tree, as suggested in section 6.2, would also help to determine if the PAS domain in *I. pulchra* PER escaped from an *I. pulchra* ARNT PAS domain or if it evolved from a different orthologue completely.

#### 6.4 Evolution of TIM Proteins

BLAST searches revealed that *I. pulchra* only has one TIM orthologue, TIMEOUT (TIM2). The orthology was first assigned based on domain structure alone because IPUL1\_6832 has both a TIM domain and a TIM-C domain, which is indicative of TIM2 because TIMELESS (TIM1) only has a TIM domain. This orthology assignment was confirmed by the TIM phylogenetic tree, which revealed that *I. pulchra* TIM2 grouped within the TIM2 clade (Figure 9). The results confirmed that TIM2 is the most primitive TIM orthologue found in the animal kingdom. Additionally, it appears that higher protostome TIM2 orthologues are divergent because they form their own clade, but share a common ancestor

with the other TIM2 clade that contains deuterostome and lower metazoan TIM2 orthologues (Figure 9). The TIM phylogenetic tree confirmed that TIM1 evolved at the bifurcation of the protostomes and the deuterostomes (Figure 9), indicating that TIM1 is likely a synapomorphy of the protostomes and deuterostomes (Figure 17) that was lost in the Platyhelminthes and lost in the higher deuterostomes (Rubin *et al.* 2006; McFarlane *et al.* 2010; Engelen *et al.* 2013). This provides additional support to the conclusions of Rubin and others (2006) by confirming that higher arthropods and echinoderms have a TIM1 orthologue and that TIM1 and TIM2 are paralogous. Additionally the results improve upon previous work (Rubin *et al.* 2006) by identifying the point at which TIM1 evolved (Figure 17).

The evolution of TIM proteins likely parallels changes in the molecular regulation of the circadian clock (Engelen *et al.* 2013). PER does not interact directly with TIM2 in deuterostomes, as it does with TIM1 in higher protostomes (Gillette and Tyan 2009). In mammals, PER can negatively regulate TIM2 indirectly through affinity competition of CRYPTOCHROME (CRY) binding because the formation of a PER:CRY heterodimer is favored (Engelen *et al.* 2013). Likewise, the different roles of each TIM paralogue may have played a role in shaping the circadian core-clock mechanism, specifically the evolution of PER as discussed below.



**Figure 17.** The tree above indicates where PER and TIM1 evolved, and where TIM1 was lost. The tree and the representative nodes were modified after Egger and others (2009) to include data from this study.

## 6.5 Evolution of the Core-Circadian Clock

The results of this study provide additional information to elucidate how the molecular clock evolved in the Metazoa. The results corroborate previous data demonstrating that the number of core-clock proteins increased (Table 4) as the circadian clock evolved (Simionato *et al.* 2007; Brown *et al.* 2012; Hardin and Panda 2013). The increase in number of circadian proteins likely arose from duplication in order to provide a more precise response to environmental signals.

The circadian clock also likely evolved from a single feedback loop to secondary loops and peripheral clocks as the need to make fine-tuned adjustments to maintain homeostasis in a changing environment. These environmental changes include changes in cycles (such as lunar cycles, tidal cycles), changes in temperature, changes in oxygen availability, or changes in the timing of physiological activities (such as food consumption) according to various stimuli (Bell-Pederson *et al.* 2005; Peschel *et al.* 2009; Tessmar-Raible *et al.* 2011; Sujino *et al.* 2012; Hardin and Panda 2013; Kronfeld-Schor *et al.* 2013; Leuck *et al.* 2013; Zantke *et al.* 2013). The evolutionary shift from a centralized clock to a clock with secondary feedback loops could have been selected for based on the need to adjust to other signals in order to maintain homeostasis. It is possible that these environmental variables may override the need for photoperiodic timing ultimately leading to the loss of the classical core clock (Tessmar-Raible *et al.* 2011; Zantke *et al.* 2013). It is also possible that secondary loops evolved independently and converged with the core clock so that the secondary loops could be re-set by a diurnal master regulator (Bell-

Pederson *et al.* 2005; Tessmar-Raible *et al.* 2011; Zantke *et al.* 2013).

Additionally a shift from secondary loops to peripheral clocks also allowed for tissue-specific responses that gave rise to a change in the entrainment and negative regulation of the circadian clock (Bell-Pederson *et al.* 2005; Engelen *et al.* 2013; Masri *et al.* 2013). For example, peripheral clocks in mammals are often entrained by the core clock, but have tissue-specific effects (such as metabolic effects) as well as system-wide effects (such as adjustment of cell cycle events around photoperiod) (Bell-Pederson *et al.* 2005; Masri *et al.* 2013).

ORGANISM	ORTHOLOGUES						
	CLOCK	CYCLE/BMAL	PER	TIM1	TIM2	CRY's	ARNT
<i>Amphimedon</i>	✓	N <sup>P</sup>	N <sup>P</sup>	N <sup>G</sup>	✓	✓	✓
<i>Nematostella</i>	✓	✓	N <sup>GP</sup>	N <sup>GP</sup>	✓	✓	✓
<i>Isodiametra</i>	? <sup>T</sup>	? <sup>T</sup>	✓	N <sup>T</sup>	✓	P	✓
<i>Stenostomum</i>	? <sup>T</sup>	? <sup>T</sup>	? <sup>T</sup>	N <sup>T</sup>	✓	P	✓
<i>Macrostomum</i>	P	P	? <sup>T</sup>	N <sup>T</sup>	P	P	✓
<i>Crassostrea</i>	✓	? <sup>P</sup>	✓	✓	✓	✓	✓
<i>Platynereis</i>	✓	✓	✓	✓	✓	✓	✓
<i>Eurydice</i>	✓	✓	✓	✓	N <sup>GP</sup>	✓	✓
<i>Apis</i>	✓	✓	✓	N <sup>GP</sup>	✓	✓	✓
<i>Drosophila</i>	✓	✓	✓	✓	✓	✓	✓
<i>Strongylocentrotus</i>	✓	✓	✓	✓	✓	✓	✓
<i>Branchiostoma</i>	✓	✓	✓	N <sup>G</sup>	✓	✓	✓
<i>Danio</i>	✓	✓	✓	N <sup>G</sup>	✓	✓	✓
<i>Mus</i>	✓	✓	✓	N <sup>GP</sup>	✓	✓	✓
<b>Key</b>	✓ = Orthologue Present		P = Preliminary Data		? = Inconclusive Data		N = Negative

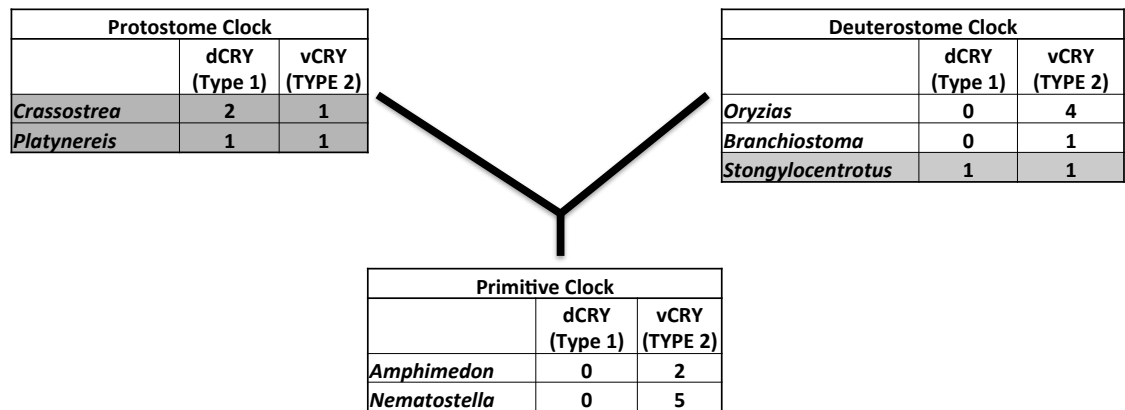
Table 4. The comparison table of circadian clock proteins shows how the clock changed over the course of evolution. A superscript designator (G – GENBANK, T- Transcriptome, and P – Publication) was given to an orthologue that was negative or inconclusive to describe the validation of the data.

### *The Primitive Clock*

*Amphimedon* has three bHLH-PAS proteins (ARNT, CLK, and AHR), it has a TIM2 orthologue, and it has a CRY orthologue (Simionato *et al.* 2007; Müller *et al.* 2013). Given that this is the basal condition of the circadian core-clock proteins of the Metazoa, all metazoan core-clock orthologues likely originated from one of these five proteins by gene duplication. It is likely that ARNT and CLK form a heterodimer that acts as the positive element in the *Amphimedon* clock mechanism.

Additionally, sponges have multiple CRY proteins that function as photoreceptors (Müller *et al.* 2013; Oliveri *et al.* 2014). The blue-light activation of CRY proteins and their structural organization as a photoreceptor appears to be conserved, as CRY proteins act as blue-light photoreceptors in plants and fungi (Chaves *et al.* 2011; Heintzen 2012; Oliveri *et al.* 2014). Specifically, *Amphimedon* has two paralogous Type 2 CRY (CRY2) proteins that it shares with animals and three photolyases that it share in common with algae and plants (Oliveri *et al.* 2014). In fact, CRY2 proteins found in Demospongiae are primitive to the split between Type 1 CRY (CRY1) and CRY2 proteins (Figure 18) that are involved in the protostome and deuterostome circadian clocks (Müller *et al.* 2010 Oliveri *et al.* 2014). The cnidarian CRY2 proteins are primitive to the rest of the CRY2 lineages in the Bilateria (Hoadley *et al.* 2011). Furthermore, cnidarians have a CRY2 orthologue derived from 6-4 photolyase that likely acts as a negative regulator in the cnidarian clock because CRY2 proteins are repressors in the mammalian circadian clock as suggested by Reitzel and others (2010;

2013). This supports the hypothesis that CRY2 is the primitive negative regulator in the circadian clock in animals and that the animal negative feedback loop originated at the base of the Metazoa in *Amphimedon*.



**Figure 18. CRY proteins follow a similar pattern of evolution where CRY1s are only found in organisms that have a TIM1 orthologue (Gray boxes). This figure was modified from Oliveri and others (2014) and arranged accordingly to show the parallel evolution of CRY1 and TIM1. This further supports the hypothesis that the change in negative regulator shaped the evolution of the core-clock.**

Although the circadian mechanism for *Amphimedon* has not been described, the circadian mechanism for *Nematostella* has been partially described. Cnidarians have more bHLH-PAS proteins than sponges (Simionato *et al.* 2007; Reitzel *et al.* 2010). *Nematostella* has a primitive CYC orthologue that likely resulted from ARNT duplication (Simionato *et al.* 2007; Reitzel *et al.*



2010). CLK and CYC form a heterodimer and act as positive regulators (Reitzel *et al.* 2010). It is clear that *Nematostella* does not have a PER orthologue, but it does have a CRY 2 (Figure 18). The regulation of CRY2 may be different in cnidarians than in sponges. For instance, CRY2s show cycling in corals, but not in *Nematostella* indicating that the CRY2 may not act as a photoreceptor (Hoadley *et al.* 2011; Reitzel *et al.* 2013). It is possible that CRY2 evolved from a primitive CRY protein with a photoreceptor function so that it could interact directly with the negative regulation of the core-clock. CRY2 likely acts as a negative regulator in the cnidarian clock, as described above (Reitzel *et al.* 2010). However the heterodimer of the negative regulatory loop, if any is unknown. It is possible that CRY2 may regulate the circadian clock alone or form a dimer with another CRY protein. In this case it is possible that type 2 would form a dimer with another CRY family member that acts as a photoreceptor. It is equally possible that there are other negative regulatory mechanisms at work such as negative regulation by other proteins.

Unlike *Nematostella*, *I. pulchra* has a primitive PER orthologue. This is one of the biggest differences between the cnidarian circadian clock and the circadian clock at the base of the Bilateria. It is also clear that *I. pulchra* has a TIM2 orthologue, but lacks a TIM1 orthologue, indicating that TIM1 evolved at the base of the protostome/deuterostome split (Figure 17). The hypothesis that CRY likely acts as the negative regulator in *I. pulchra* is supported by the lack of a TIM1 in *I. pulchra* and the evidence described above indicating that the negative regulator in both *Amphimedon* and *Nematostella* is likely CRY driven.

Preliminary BLAST results indicate that the *I. pulchra* transcriptome has at least one CRY protein (Unpubl. data). It is possible that PER and CRY form a heterodimer and that the dimer acts as the negative regulator of the *I. pulchra* circadian clock. The positive regulator is more elusive given the fact that an ARNT orthologue was identified, but a CLK or CYC orthologue could not be identified. Before any further work can be done to elucidate the positive regulator loop in *I. pulchra*, work needs to be done to confirm that these orthologues are truly missing and that they are not missing because of error as described in section 6.1.

### *The Protostome Clock*

The number of bHLH-PAS proteins increased between the Urmetazoa and Ureumetazoa split and between the Ureumetazoa and the Urbilateria split (Simionato *et al.* 2007). This is important because the urbilaterian ancestor to the protostome-deuterostome split appears to be the Acoelomorpha (Egger *et al.* 2009). The data confirm that PER first appeared at the base of the Bilateria in Acoelomorpha (represented by *I. pulchra*). Additionally, acoelomorphan PER is the sister group to protostome PER, as PER divergent in the higher protostomes as described in the 'Evolution of PER' section above. The circadian clock of *Platynereis dumerilii* is the most primitive protostome clock model described to date that contains a TIM1 orthologue (Zantke *et al.* 2013). However, the results of the TIM tree confirm that mollusks are the first group to have a TIM1 orthologue in the protostomes, as *Crassostrea* likely has a PER and a TIM1

orthologue (Zhang *et al.* 2012; Shoguchi *et al.* 2013). Furthermore, the PER and TIM tree data confirm the assignment of *Crassostrea* orthologues, which could possibly form a heterodimer that acts as the negative regulator of the mollusk core-clock. This improves upon the hypothesis that TIM duplication occurred after the cnidarian-bilaterian split (Reitzel *et al.* 2010), as the results indicate that TIM1 evolved at the base of the protostome-deuterostome split.

Furthermore the base of the protostome-deuterostome split may be the point in evolution where the negative regulator changed from a CRY:PER heterodimer to a TIM1:PER heterodimer (Figure 17). The data confirmed that the Platyhelminthes lost TIM1. Because *Crassostrea* and *Platynereis* both have a PER and TIM1 orthologue, it is likely that TIM1 first functioned as a negative regulator in the mollusks. As discussed above, the primitive negative regulator likely involved CRY2. While the higher deuterostomes retained the primitive negative regulator (PER:CRY2), the change to TIM1:PER negative elements first appeared in the protostomes in the annelids, as *Platynereis dumerilii* has a TIM1 orthologue (Zantke *et al.* 2013). The change was solidified in the Crustacea and evidence supports that *Eurydice pulchra* only has a TIM1 orthologue and lacks a TIM2 orthologue, but TIM1 functions as a negative regulator similarly to TIM1 in *Drosophila* (Zhang *et al.* 2013) TIM1 is functionally present in the the Crustacea, as) *Eurydice pulchra* Has a TIM1 orthologue, but lacks a TIM2 orthologue. Additionally, *E. pulchra* TIM1 and PER repress CLK and BMAL transcription (Zhang *et al.* 2013). The data indicates that the negative regulator in *Eurydice* functions differently than the negative regulator in the *Drosophila* clock. The

TIM1:PER negative regulatory mechanism is well known in *Drosophila* (Peschel *et al.* 2009; Brown *et al.* 2012; Hardin and Panda 2013). Furthermore, the mRNA and protein expression patterns in higher ecdysozoans likely changed as the negative regulator shifted to a PER:TIM1 heterodimer so that PER and TIM1 mRNA expression became relatively parallel (Brown *et al.* 2012; Hardin and Panda 2013).

CRY1 is also involved in the negative regulation of the higher protostome clock. TIM1 is degraded by CRY1 through an intermediate protein (JET), where CRY1 first degrades JET and then degrades TIM1 (Peschel *et al.* 2009). CRY1 likely arose from CRY2 duplication. It appears that CRY1 co-evolved with TIM1 at the base of the protostome-deuterostome split because organisms that have a TIM1 orthologue have a CRY1 orthologue. Organisms that do not have a TIM1 orthologue only have CRY2 orthologues (Oliveri *et al.* 2014). It is important to note that the *Platynereis* clock functions with a CRY1 orthologue (Zantke *et al.* 2013). However, *Eurydice* clock functions with a CRY2 and not CRY1, as compared to the *Drosophila* clock (Zhang *et al.* 2013). This further indicates that CRY1 evolved first in the mollusks (Oliveri *et al.* 2014). CRY1 has been shown to be functional in annelids ((Zhang *et al.* 2013), and that *Eurydice* has lost CRY1 (Zantke *et al.* 2013). It is likely that the common ancestor to the CRY1 and dCRY2 split at the base of the protostome-deuterostome split was a CRY2 protein that had an affinity to bind with both TIM1 as well as PER. The parallel evolution of CRY proteins with TIM1 proteins was guided by natural selection as TIM1 evolved to have a specific role in the protostome clock. CRY1 resulted from

the duplication of CRY2 so that the CRY2's affinity to bind to PER was retained in deuterostomes and CRY1's affinity to bind to TIM1 is retained in protostomes.

### *The Deuterostome Clock*

The results of the TIM tree indicate that there is a bifurcation at the base of the protostomes where the negative regulator shifted to a PER:TIM1 heterodimer. Because TIM1 evolved at the base of the protostome-deuterostome split (Figure 17), echinoderms have TIM1, but the higher deuterostomes have lost TIM1. Although the negative regulator in echinoderms is presently unknown, it is clear that the negative regulator shifted to a CRY:PER heterodimer in higher deuterostomes (Rubin *et al.* 2006; Engelen *et al.* 2013). It is possible that the pattern of PER expression in deuterostomes could be similar to the expression patterns in the primitive clock (Shearman *et al.* 2000; Akhtar *et al.* 2002; Gillette and Tyan 2009). It can be expected that *I. pulchra* PER expression patterns are similar to those of the higher deuterostome clock. The same is likely true for CRY expression. Although more data are needed, the absence of a change in PER expression in this study indicates that the PER expression patterns do not resemble the PER expression patterns of higher protostomes. It is still possible that with more time points, the data could show that this hypothesis is correct.

There are likely two different CRYs involved in the core-clock mechanism; one indirectly involved in the higher Lophotrochozoa and the Ecdysozoa clock mechanisms and one directly involved in the primitive clock and deuterostome clock mechanisms. In this case, it would be expected that the *I. pulchra* CRY

mRNA sequence would have more in common with both cnidarians and deuterostomes, than it would with the lophotrochozoans and ecdysozoans.

The results of the TIM tree indicate the same pattern found in the Oliveri and others' (2014) CRY Tree. CRY1 is only found in species that have a TIM1 orthologue (Figure 18), and supports the hypothesis that CRY function in the primitive clock and higher deuterostome clock is likely similar. Both the expression of CRY1 and CRY2 are light-driven and is conserved among all animals studied to date (Oliveri *et al.* 2014). The function of CRY1 and CRY2 as negative regulators of the circadian clock may have originated from the synchronization of photoperiod to DNA repair events, linking the two together (Oliveri *et al.* 2014).

Additional data are needed to further support the following three hypotheses: 1) The deuterostome clock carries the plesiomorphic features of the primitive core-clock and the higher lophotrochozoan/ecdysozoan clock is divergent. 2) The evolution of the circadian clock mechanism is directed by changes in negative regulation. 3) The CRY orthologue involved in a given mechanism and how it functions as a negative regulator is dependent on the binding affinity for the co-negative regulator.

The first hypothesis is supported by the current study and may benefit from the inclusion of a CRY study. Additionally the incomplete sequences of IPUL1\_26016 and IPUL1\_17690 need to be completed using a RACE kit. These results also support the second hypothesis. An additional study examining the binding site differences between the negative regulators (PER and CRY) could

help in strengthening this hypothesis. This study would map out specific differences and similarities in the binding site for each protein after they are compared to other metazoan data in the literature. A co-immunoprecipitation study would also confirm that *I. pulchra* PER binds to *I. pulchra* CRY, which would confirm that the negative regulatory loop in the *I. pulchra* core-clock is a PER:CRY heterodimer. The third hypothesis may be elucidated by the additional studies mentioned above, and may also be supported by the findings of a comparison of CRY and PER binding domains in metazoan species used for this study.

## 6.6 Conclusion

The results of this study combined with the previous work in metazoans supports the hypothesis that core-clock protein diversity likely arose from three ancestral proteins (ARNT, CLK, and AHR) through duplication events (Simionato *et al.* 2007). Furthermore, it was correctly hypothesized that PER evolved at the base of the Bilateria. However the hypothesis that PER cycles in a similar expression pattern as *Drosophila* was not supported. Based on indirect evidence, it is possible that PER expression still cycles, but follows an expression pattern more similar to *Mus*. Based on the data presented in this thesis, it is likely that the evolution of the negative regulator(s) shaped the circadian clock. This is indicated by the evolution of PER and TIM1, as well as the different expression patterns of PER between the deuterostomes and the divergent higher protostomes. Because homeostatic needs are dependent on the environment, it

is likely that the change in the negative regulator is also a result of the changing functions of the circadian clock to meet an animal's homeostatic requirement to survive in a specific environment. The future study of *I. pulchra* CRY protein(s) and the evolution of CRY could help validate this hypothesis as well as the hypotheses presented above. The evolution of the circadian clock becomes clearer as we begin to reveal information about how it evolved in the lower Metazoa, and *I. pulchra* could become a useful model to fill the information gap between cnidarians and the higher lophotrochozoans.



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## 9. Appendices

### 9.1 Appendix 1: *Isodiametra pulchra* Collection Site

#### Collection site for Wild-Type *Isodiametra pulchra*



The two circles in the photo above shows the two spots where I collected sand for the *I. pulchra* extractions. These samples also contained a variety of different meiofauna species. Worms extracted from collection point 0 and 0.5 were cultured for 6AM and 6PM RNA extractions.

## 9.2 Appendix 2: Julian Smith's Lab Protocols – modified from De Mulder 2009

### 2A Total RNA Isolation by use of Tri™ reagent (Sigma-Aldrich T9424) – (After De Mulder 2009)

1. Starve worms for > 36 h, in order to prevent diatom RNA contamination
2. Gradually relax animals in 7,14% MgCl<sub>2</sub> (10-15 minutes for *I. pulchra*)
3. Centrifuge specimen down (45 sec, 11.000g), remove all supernatant using a pulled glass pipette
4. Add 500µl TRI™ Reagent, dissolve tissue thoroughly by pipetting up and down
5. Add another 500µl Tri™ reagent and mix by pipetting
6. Add 200µl Chloroform and vortex 15 sec at half speed
7. Incubate tube for 15 min at RT (two different phases should become distinguishable)
8. Centrifuge 20 min at 12.000g (precooled at 4°C)
9. Transfer upper phase into new tube (do not take interphase, as this will contain DNA)
10. Add 500µl isopropanol, turn tube twice up and down and incubate for 10 min at RT
11. Centrifuge 10 min at 12.000g (4°C) and remove supernatant
12. Wash pellet with 1ml precooled EtOH (75%) and centrifuge 5 min at 7.500g (4°C)
13. Remove supernatant and air dry pellet for 10 min
14. Add 20µl of DEPC-treated water to tube. Pipette to dissolve pellet. Keep on ice until frozen-stored.
15. Blank Nano-drop spectrophotometer with DEPC-water, and then measure 1µL of each extraction to assess concentration and purity.

#### Notes:

1. Wash well, so no algae, schmutz from dish, or eggs.
2. Swirl dish to accumulate, transfer to 1.5ml eppendorf tubes
4. Nitrile gloves! Hold tip of pipette on bottom of tube, and pipette up and down to dissociate animals.
5. TRI is added 500µL at a time to keep from accidentally sucking it into P1000
6. Result is creamy pink
8. And all subsequent centrifugations need refrigerated Eppendorf
9. Do this with tube half-immersed in ice/water bath, to keep cold. Leave some upper phase, and avoid the "smear" of interphase on side of tube
10. May need to invert several times to mix completely
11. Remember to orient hinge outside so you know where pellet is
12. 75% EtOH made up from non-denatured 200-proof

13. Tubes on side, cap open in a lid-partly-ajar petri dish. As pellet dries, will become translucent; can still see with M5.

## **2B Reverse-Transcriptase PCR (Phusion)**

### Materials:

Phusion Master mix (New England Biolabs)	12.5 $\mu$ l / 25 $\mu$ l
Template DNA	1 $\mu$ l / 1 $\mu$ l
20 $\mu$ M Fwd Primer	1 $\mu$ l / 1 $\mu$ l
20 $\mu$ M Rev Primer	1 $\mu$ l / 1 $\mu$ l
Nuclease-free water	9.5 $\mu$ l / 22 $\mu$ l

1. Add reagents listed above to PCR tubes. Be sure to change pipet tip each time you add a reagent to the PCR tube. (25 $\mu$ l reaction)
2. Set up the Mastercycler with the settings below

### Mastercycler Settings for Phusion Taq PCR

1. 4°C Hold
2. 4 min. at 98°C
3. 10 sec. at 98°C
4. 30sec. at 55°C
5. 1 min. at 72°C
6. Go to cycle 2 and repeat for 35 cycles
7. 5 min. at 72°C
8. 4°C hold

Run the PCR Product on a gel to verify by band size.



## **2C Reverse-Transcriptase PCR (Standard Taq)**

### Materials:

Standard Taq Buffer	2.5 $\mu$ l
DNTP	0.5 $\mu$ l
Taq DNA Polymerase	0.5 $\mu$ l
Template DNA	1 $\mu$ l
20 $\mu$ M Fwd Primer	1 $\mu$ l
20 $\mu$ M Rev Primer	1 $\mu$ l
Sterile Water	18.5 $\mu$ l (to make a 25 $\mu$ l reaction)

1. Add reagents listed above to PCR tubes. Be sure to change pipet tip each time you add a reagent to the PCR tube.
2. Set up the Master-cycler with the settings below

### Mastercycler Settings for Standard Taq PCR

1. 4°C Hold
2. 4 min. at 98°C
3. 10 sec. at 98°C
4. 30sec. at 55°C
5. 1 min. at 72°C
6. Go to cycle 2 and repeat for 15-35 cycles depending on experiment or for 29 cycle for sqRT-PCR experiments -- see methods section
7. 5 min. at 72°C
8. 4°C hold

Run the PCR Product on a gel to verify by band size.

## **2D Standard Agarose Mini Gel (DNA/RNA) Owl Easy-Cast B1A**

### **Prepare a 1% Gel**

#### *Small Gel*

Agarose (Calbiochem OmniPur #2120) 0.45g  
0.5X TBE 45ml

#### *Large Gel*

Agarose (Calbiochem OmniPur #2120) 0.90g  
0.5X TBE 90ml

1. Put in small Erlenmeyer, and microwave until it just boils. Take out and swirl. Repeat until no granules of Agarose are visible, then microwave and swirl once more. CAUTION—you can superheat this solution and it can boil out of the flask. Be careful.  
Cool gel solution by running cold tap water over the flask. When just cool enough to hold, add 2 $\mu$ L/5 $\mu$ L (small gel /large gel) of Gel Red (Biotium) and swirl to mix.
2. Turn gel tray sideways and push down into box. Pour in gel solution, add comb, and remove bubbles with pipette tip
3. Allow gel to harden for 30 minutes or so.

### **Prepare Samples**

1. Cut a strip of parafilm, and stick to the bench (thumbnail and corners) with surface that was paper-covered (and hence, clean) UP.
2. Put down a 2 $\mu$ L drop of loading dye (we're currently NEB that ships with the ladder) for each sample.
3. Add 6 $\mu$ L of ultrapure water to each drop and mix by pipetting
4. Add 2  $\mu$ L of PCR reaction to each drop of loading dye.
5. Make up a standard with 2 $\mu$ L of loading dye, 2 $\mu$ L of 100bp ladder (Amresco K-180), and 6 $\mu$ L of ultrapure water.

### **Load and Run Gel**

1. Fill Gel box with ~300ml of 0.5X TBE or just enough to cover gel.
2. Use fresh 0.5X TBE every time you run a gel, unless you're doing two gels back-to-back, and after that, empty and wash the box.
3. EC105 power supply is constant-voltage only. Set it to around 140-160 Volts (near maximum on low range) for 0.5x TBE tank buffer (should get 40-45mA); use around 100V for 1x TBE tank buffer.

### 9.3 Appendix 3: Primer Design

#### IPUL1\_3616

TGGGAGTT**TCCTCCACACAGTTAATGGTGGCTC**GAGTGGCTCAATTTATGG  
AAATTGGAGTGTGTGGTGAATACTAATCAAGCGGGGCTTGCCTTGGACAAC  
AACTCCCCGATGTATGCTCGTTAATGTACGGGGGCATCTGTGCTGCTGCTG  
CTGCTGTGGTTGCCGTGGCTGCTGTCTCCTCTGCTCGAAATGCTCCAAGAG  
CCTCCTCCTCCTCCCGAGAAGCAGCCGCATGTCTCATCCTCCGTCTCGCCC  
AGCGCTTCCGCCATGCCAGCCCATCCT**TCCATATCCACTATCAACGCCG**CA  
GGCAGCAGCAGTCGGGAGAACCACTCGGAGATCGAGCGGAGGCGGGCGCA  
ACAAGATGTCCACTTACATCCAGGAGCTGTCCGACATCATCCCCTCCTGCTC  
CAACCTGACCCGCCGCCCGACCAGCTCACCGTCCTCCGCATGGCCGTCC  
AGTATCTCAAATCCCTCCGCGCAGTGGGCAGCAGTCCGGGGGAGGCGGTG  
GACCCGCCGGGGTTCGTACAAGCCGGCGTTCTCACGGACCAGGAGCTGAA  
GCACATCATCATGGAGGCGGCCGACGGCTTC**CTCTTTGTCGTCTCCTGTGA**  
**CA**CCGGCCGCATCCTCTACGTCTCCGACACCATGTCTCCCATCCTCAACCT  
CCCTCAGAACGCGTGGCTGGGCCGGAGTATGTACGAGCTGATCCACCCGG  
ACGACTCGCCCAAGCTGCGAGAACACC**TGTCCATCGGGGACA**ACTTCGGC  
ACCGGCCGCATCCTCGACCTCAAGAGCG**GCACCATCAAGAAGGAGTCATC**  
GCCTTCCACTTGCAACCGCAACACGTCGGGTTCTCGGCGCAGCTTCGTCAT  
CCGACTCAAAGTCGGCTCCGACTCCTCATTGGCCAACGACTCGCACCAGCC  
GAATGGCGGGCGGGCGGTCTCTCGGCCGCCGCCGCGAGGTTCCGCCGAGGCG  
CGTCGCAAAACTTGCCTTCCACTCGCGGGCTCCAATGATGAGCCCCATTGT  
ATCACACATATCACCGGCTATATCAAACAGTGGGCTCAGAACTCCCCAAC  
GGACAACCTCTCGGCGTCGCATTTTCGATGGATCATGCTCATTGGTGGGCCTG  
GCTCGTCTCCAGTCCAGCTCTGCGCCAATGGCAGCCGACCTGGAGGAAAG  
CGCGCCCTGCGAGTTCATCCTCAGACTTAAACTTCCAGATAACCAGCAGTGA  
GACTCTGGTGACCTTCGTTCGACGAGAGAGTGACCAAGGTGCTCGGGTTGG  
CAACATCACAAGTCTCAATAAATGTGTGTTTCGATTTCTGTGCTCCGGAGGA  
CGTTCCAGTATTCCAGCGCCTCGTGTGAGAAGCTGTCGCGGGTAAGGGAG  
CAGAGGTTAGCGCTCTGATTCGCTGGCTCGACTCAGAGGGTGACGACATC  
GCGCTCAATGTTTCTTGTTCATGCTTTTCGTTAACCCGTTTTTACACAAGACGG  
AGTTCCTCGTTGCTACTTGTTCCTTTCACG**TCTCCCCAAAATTTCGATGTACA**  
**CGGAGCTTTGAAAGTGTCA**TATCTGGAATGCCACACAACCTTGCCAGGATCT  
CCCACCCAGTCATCATCGACTCAGTTCTTGATCGCCAATGAAGTTCAGTCGC  
AGTGCTCGCTTCCCCAGCCGCCATTGGCTGGTCAGGGCTTGAATCTGATTG  
GCCATCACCAGGTCTTCTCAAACCTTCGACTGGAGGATTGGCCAATCACAGG  
GCTCCCTCTCCCCACATTTCGTCCCCACTCGAGAACCAGAGTCAGTATCTGG  
GGACTCCTCGATCGGCGGAGGGGACGAGCGGGTCTCTCCTAACCAGCTA  
AGTCCGTCCACCGTGTCTCCACAAACCAGGGGATGGCCGCCGCCGCCGT  
TCCACCTCGCCCTACTCTGACATGGGCAGCCAGTTCTTACCTAGCTCCGA  
ATCTGTACTCAGAAATCAACTACTCAATTATTCCACCCAAATTGAAATAGCCA  
ATAGAATCGGGACGTTTGTGCCCGCCGCGGGCTCACCCAACGAGCTGTATT  
CCTATTCGCAGCCGCTGTTTGCCAGACGTCTCCTCAGCCAGGGTTGCCAT

GGAACTCCTATTGGATCCGGAACGAACCGCAGGGGCTGCCGCCACGGTGC  
TTCCTACCCGGGAATGACTCGCCCGGCTCCAACGGCCTGCTCATCCAGCAG  
ATTCCACACGAATTCACCCAGATGGAAA **GCCAGAGTTCTACCTCTACTGCC**  
**AATTCATATTTCC**AGATAACAATAAGTTTATTGCCACATTATGTTATCAATGTAT  
ATTTTTCGTAMAACCT

#### Primers:

**IP3616F FWD - TCCTCCACACAGTTAATGGTGG** Pred. Size – 2226  
**IP3616F REV – GCAGTAGAGGTAGAACTCTGGC**

IP3616fwd1: **CCACACAGTTAATGGTGGCTC** Pred. size 797  
Ip3616RV1: **GATGACTCCTTCTTGATGGTGC**  
IP3616fwd2: **TGTCCATCGGGGACAACCTTC** Pred. size 805  
Ip3616RV2: **ATGACACTTTCAAAGCTCCGTG**  
IP3616fwd3: **TCTCCCCCAAAAATTTCGATG** Pred. size 751  
Ip3616RV3: **GGAAATATGAATTGGCAGTAGAGG**

#### sqRT-PCR Primers

IPARNTA FWD: **TCCATATCCACTATCAACGCCG** Pred size: 328  
IPARNTA RV: **TGTCACAGGAGACGACAAAGAG**  
IPARNTB FWD: **GTGTGTTTCGATTTCTGTGCTCC** Pred size: 335  
IPARNTB RV: **CGACTGAACTTCATTGGCGATC**

## IPUL1\_2961

GCTTTTAGCCTTGTTGTGATATTACTTTTAAATAG **GTGCTATGTGCTATATAT**  
**CTTTCG**TTAGTTACTCAGAATCTTCATAAAA **TGTCCGTACAGACATCAATTGC**  
TTCTCGCGGCTCGGATTTCTGAACGCGTGATTGAAGGGAGCCTTTGTTCTTGA  
AATAGACTGTGGAACGGGCACAATTCTATCGTCCTCGAGTGGCATTAAAGA  
CATTCTGGGCTGGAAGCAGGACTCCATTACGGAAAAATGTGTCTCTTGTCTG  
GTGCATCCTGACGATGTGGACGACTTCCTTCTCTACTGCCGATGCCCTTC  
GACCAGCTCTATGAACTCCCTCAGTTAACCAATGGAAGCGTTGAGCTCCGC  
GTTGTCAATAATACCGACACGGTGTCTGTTCTTACTCGCATGAAGTACAAAA  
ATGAAGATGATGACGGTACTGCAGTTACTCGATCGTCAGGGTTGTGGGAA  
AACTTCTTCCAATCATCCATCTGTCCAGAACGTCTTCATGTCCACAGTCAC  
GGCTCCTCCGATTTCTGTCTGCTACGAGGTAGCGCC **TCCCTCAACTTGGA**  
**TCTCGTG****GAATATATGATCACAACC**GACCACAA **CACCAGAGTCAATTATGTC**  
**G**ACCCGAGAGTGCAGCGCATTCTGGGCTACTCACAGAGTGATGTGATCGG  
CAAGCCAGGGTACAACCTCGTCCTCAGCGATGACATTCTTTCATCGTCGC  
CGCACAGTTTGACACTGTGATGCGGAAACGCGAGCCACGACCGGT **GTTG**  
**CTCGACTGAAGACTAGG**AATGGGCGGATGGTGCTCAGCAGAACTCGCTGC  
TACATCAACAAGGACCCCTGGACCAAGCAGGTCAAGAGCTACGTCAACGTC  
ACTTCTATCATCAGTAA **GCGATCGGCATATCTTCACTTC**CAACCAGCAGATCA  
GAACGGTGGAGCGACTGCGACGCAACCGCAAGACGTGGAAGGACATTCTG  
AAGAAGCTGGA **CCTGACTGAAGAGCAGAAGGAG**ACCCTCACCAACATGGA  
CCTGCGCAGTTACCTGCGCTACGTGCGCGAGCTCGAGCACGGGGATGAGG  
ATGAGTCGCCGCCGCACCTTTTCCCTCCCTCCTCCCCCTGCACAATCACCG  
AGCTGCCAGACGAATATGACAACAAGGAAATATCGCACCCGTCTCTCCAGCG  
AGCCGTCACTGCTTCTCCTCCCTCTACGG **GAGATGAGCACTCTGGTTCTC**  
**G**GGATCCAGTCACTGAAGCGCCCGCTTTGGAGGAATACGAGGAGTTGGAC  
CGAGACGGAGTCGATCCGTCCC **AATTCTACCCTTCTATCTCACCAG**AGAG  
**TTCAAATATCCAGAATGCC**CTCGGCGATTCTCACACTTTTCTCATCGAGA  
GCAAACCTAATATCCCCAGGCGGGACCTGAGGAATGACTATG **GATGCTACG**  
ACAGCAACTCTACTTGCGCCAGCTACTCAGTCTAGCCACACCAGTACAGA  
ATGCCAGAGTCTCGAGAAGTCTGT **CCTACAGGATGCACTTGAGTC**ACCTCT  
CAGCTCTGCGGACCAGCAGCTGTATGGCATTGGCTTCCGTTAGAATCCCGG  
AAGACCCCCCGGCATGTAAGACCCCCACTTCCCGCAAAGACCCCAGGAAGA  
ACTCGCAATTCGCTGTCTACCACAGTCGGCTCTCTCAGCCCAGGAAGC  
GCGGGAGGCCCTTCTCGCATTGCTCTAAATGAACTAATTGATGTTTCCG  
CGTAGATTACTCGATGGCCTGTAGATCTAGCGATCCCCCGCGGTTTCGTG  
CTCTTCTCATTCTATAACGAATGGAACCTCGATGGAAGCGGTTTTAAATCAA  
CTGAAGTGTAATTTACGGACTGCTATGAACTGCCAATTGGATTGGAAGTAA  
TATCA **GCAGCAACAATCCGGACAAAAA** **TAA**ATTTTGTATAGCTGTGTTAACT  
TTAATTTGTGTATAATAGTATTTTAA

### Primers:

IP2961F FWD - **TGTCCGTACAGACATCAATTGC**

Product Size – 1836

**IP2961F REV – TTTTGTCCGGATTGTTGCTGC**

IP2961FWD1:	<b>GTGCTATGTGCTATATATCTTTCG</b>	Pred. Size: 587
IP2961RV1:	<b>TCGACATAATTGACTCTGGTG</b>	
IP2961FWD2:	<b>CTCGTGGAATATATGATCACAACC</b>	Pred. Size: 780
IP2961RV2:	<b>GGCATTCTGGATATTTTGAAC</b>	
IP2961FWD3:	<b>AATTCTACCCTTCTATCCTCACCAG</b>	Pred. Size: 624
IP2961RV3:	<b>TTATTTTGTCCGGATTGTTGC</b>	

**sqRT-PCR Primers**

IPPERA FWD:	<b>TCCCTCAACTTGGATCTCGTG</b>	Pred. Size: 358
IPPERA REV:	<b>GAAGTGAAGATATGCCGATCGC</b>	
IPPERB FWD:	<b>GAGATGAGCACTCTGGTTCTCC</b>	Pred. Size: 322
IPPERB RV:	<b>TGACTCAAGTGCATCCTGTAGG</b>	

IPUL1\_6832

AGCCTTGTTGTGATATTACTTTTAAATAGGCATGGATCACTTAGCAGAGCAG  
CAACTTCATGCTGCTGCCAGTTCTTTTGGATACCTGAATCAGGACAATGAAT  
ACATTCGTGAGCCCGACTGTGTGCGAAACGTGCAAAGAACTTATTTATTTTCT  
AAAAAGAGAAGACGAGACGAGGCAAATAAGGCGGAAACTCGGATATCTTAA  
AGTTTTCGAAAATGATCTGGTTAACCTAATTCTACACTACTCAGAGGATGTTG  
AGCTGTTTGATATTGTGATACGACTGATGCAGAATCTCAGTACACCTGCCGT  
ACTCTGTTATGGCAAGATACCAGATGATAGAACTTCAGGAGTTATTATATC  
GGCCTTGAAAAATATCTGAGGAGCTACAAGCGTAGCTTTACCAACGAGGAT  
TTGGTGTACAACGTGCGCCTCAGAATGAAAGCGCTTCTTGAGAGCTCCAAG  
GAAGATGACGAGCATGAGCAAGAGGTCCTTGAGCGAATTCTGATCCTCTTC  
CGTAACATGCTACATGCTCCAGCTGACCCTTCAGAGGAGGTGAGGACCGAT  
GATGATGCTTCTCTACATGATCAATTGCTTTATGTGATCAGCAAACTGGTTT  
GGATGCAATTCTAAAGTTTTTGGCCACCAGTGACGCGCACTCGAAGTGGTC  
TCTTCATGTTCTTGAAGTAATTTCCACATGATGCGCGATCAGAGACCCGAG  
CTGCTTGCTGCCACGGGAGCTGAGAAGAGCCGGAAAGAGTTTGAGATAGA  
AGGCGAAGAGCTCACTGAGCTGCTGAACAAACAAAAACAAGACCGAAGAGC  
AAAAATGATGATGACTGGTAGCCGTCAATCTCGTTTCGGCGGGACCTATGTT  
GTCGCAAATCTGAAGTCACTGAACAAAGAAAAGGATCTGATATTCCATGGAA  
CCCAGAACAGAGCGAAACATATTTCTTCGACCAGCTGAAAGAGACGAGGC  
GTAACCAAAGAGGCTTGTGGCTGCTGTTGACCGAGAACTTCCCAGGAGGT  
CGACCAGAGCTGTCAGGCTTTTCTCAAAGAGTTCGCAGTGGACATGCTCG  
ATAATTCATTCAACAGTCTCATGCATTCTGTTATCGATGCGATCAGCAGGGA  
GAAAACCCAACAGCACGATGAGTCTTACTATCTGTGGGCGATTGGGTTCTT  
CTTGGAGTTCAATAGATTCTACAAGTTCAGGACTGATGTCGTCTCTGAAGTC  
CTCAACTCGGAAGCATTTCCTACTATATTTATGTACAGTCAGGACATTACTACG  
AAATGATGCTGCAAGAAAAGAAGGCGAAGTCAGAAGCCGAGAGCTGGGCG  
CGAAGAATGCATCTCGCGCTGAAGGCCTTTGGTGAGCTGCTGAAAACATC  
AATACAATGGGCAAGAGCGAAGACCCCGAAGTAAAGAGAGCTGCCGATATT  
CTGAAGCGTAATGTGTTTTATAACTCTGAATTCCGTGAGCTCTTCTCTTCAT  
GATTAAGCACTTTGACATCACTAAGATGAGTAAATCATACTTGAGAGACCTC  
GTCGAGGCGAATCACATTTTTTTGTAAGTATAGCTCGAAGAGTTTGCCAATA  
ACAAGATGGTCATAGTGAAGAAAGCGAAGAAGAAAAGAAAACCTGCCAAGA  
AATCATCAAAATCACATGGTCAAGGTAATCTGGAGTGCCGAGGGGGTCCG  
AGGACCCTGAAGCAGATCTACAGGCTCAGCTGGAGGAGAAATGGGAAGAA  
TTGGCGCCCATCTTGTGAGCACTTCTTCAGAGACGCATACCATTAACAGAG  
GAGGCAGTCCCTTTCGACCCTATTTAGAACTCCTATTGAGGAACAACAGT  
CCTCCGCCATGTGTGATGTCCAACTGCTCTCAGATCTGGCCAGGCGGCCA  
GAGCCCTGGCTCTCCTTCGGGCATCAAGGGAGGTCTGGCCGGATGCTGAT  
TTTTTCGGGTTCTGCCATGCCCGCCAGAGGATGAGTTCAACACTTTGCGG  
TCGATCCATTTACCAAGAACTCTAGCGTCCGAATAAACCTGACTTCGACAG  
TGAATCCAGCTATCCCGGAAAATGAGGGTGCAGATGCGAACTTGGAAGTTG  
CAGATGATGAACGAACAATTCGGATGCTGCCAGGGAAGAGATTGCTGAAT  
TCGAAAGTGAATCAGAAGGGGAGAGGTCTGAACCAGATGCCGATCGAAAC

GAACAAATTTT**GATTCGGCAGTTTATGGAGC**TTTTTGTCCCGGCCTGAG  
GTCGTCA**GCGCTTATTCTCTTATCCTGCG**GAGATTACAAAACGAATTCACCTT  
TTATGAATCACGTAGCGGTTAAGATGCTCCACCGGATAGCAGTCGATTTAG  
GCGTTCCTGGATTGCTTTTCCAAGCGTCAATTTTCAGGATCTTCCAGGACAT  
TTTCAATACCTTCCCTGGAGCGGCTGCTACTGAGTCGCAGAAGGAACTGTT  
CAATTTCGCCAAATTCATCATGAGAACTTCTTTAAGTCCGTGGTGACCAAT  
AAGTACATCTTCGTTGAGACTCTGTTCTGGAAGGATCTGGCAACGAGCTAC  
GAAGTT**GTGGAAGGCTATGGATCCT**ATGTCAAGAGTTCTGGGGGTGCTGGT  
CATGTGACGTGGAGCGCCGAGGACAATGAGGAGCTTGAGAGACTCTACCG  
TCAGTACCAGGCGTCGGAGATGATGGAAGACGACGGTGGAGTCGTTGAGT  
TCATCATGGAGAACTTCTCGCGGGAATAAGTCGCCCTCAGAATGTCGTCA  
ACCAGTTGGTGAGTCTTGGCATCGTCGCAG**ACAAGACTGAGCTAAGGATGA**  
**CTTC**TCGACGAGAGTTCGCCGGCGTCTGGAGACCTGAGCATGTGAGGGAG  
CTTGAGATTCTTTGGGAGCAGTTCAAGGACTCTGAGAACCCACTA**SATGAA**  
**GTTTGGAAATGCACTGA**CTCATGATCGTCCGAAGAACCGCGTGATGCAGAAG  
CTTCTACAACCTCGGTTTGGCAACCAGTCAAAAAGATCTCCTAAGAAAAGACT  
TCTGGCCTCCG**GAGTTGGAACAGGAAGTGAGGG**ATCTGTATGAAATCCACA  
AGGAAAATGCCGATCCCTGCAGAGGTATTCTGGACAATATCAGTGTTCCGA  
AATCAAGGCGGCAGATCATGAACAACTGAAAGATCTTAACGTGATAGCTGA  
CGTGGCTGAACTGAAGCGACAGAAGCCCAAAGCCGTCAGACAAGAACGTG  
TATGGACAACCGAAGAGAATGACGAACTGATAGAACTATTCCGCAAACACAA  
GACCATCAAACCTAATCCTGCAGAATTGCACATTTTCTGCGAGCTCATCTCAG  
GTTTCGAAGCAATTAAGCATCTT**GGTCTCCGAAATAAGAGCGGGT**CAAAAA  
GAAGCACTGTCTCGAACTTCTTGAAAGCCAATCATATGGACAACCTATGAGGA  
TTCGGAAGAGGAAAGACTTACCAAAAA**ACCACCGAAGATTTCAAATGAG**CA  
GATATTCTCGTGCATTCGGAGCGTCGCCAACAGCTCGTATGCAGAAGCTCT  
TGATTGGCTGAAGTCGAGCCTT**CTGAAAGTTGCGAATGATCGA**GCTGAAGA  
TGAGGAAGATGACGACGATTCCGACCCTGTTTCCGTTCCCTCTAGTTCCTTAC  
AAGGACTCGGCTGAGGAAGCGGTGCTGCCGAGGACTTCAAGAAGTTGTT  
GACTAAACTTGGCATGTGCGCTCCAGAGGAACTGAAGTCTTCTGGAGGGT  
CCCTGCATCGTTGTCAGTGCAATTTCTCAAGGAGAAAGCTGAACTGCTTGAC  
CGTGATTCTGTTCCCTGCTCCTGAGGCTGAGCCGGATGATGCCAAATCC  
TCGGATGGAGAAGTCAACCTTGCTTCTATGAGA**TCTCAGAAGAGACTTCCTA**  
**ATCCTG**CAGCTGAAGATGGGAGTGCATCGGAACCCGAGAATGCACCTCCTA  
CGTCCGAAGTACCCGACCTCGCAAGTGATGGAGAGTCCCCGAGGAAAAAG  
CCCCAGAAGTATGCAGCACTTCTCAAGAAAAGAGCCGAGGAGAAGGCGAG  
AAGGGATTCAAAGAAGCGTCTCCATGATGATGGAAGTGATTTGTCTGATTCCG  
GAGAGGCCGGCAGCTAAACGCCCGAGAGCTACTGAAGCGAAAACAAAGAC  
GTTCAAGTCAAGA**CTCAATAGGTTTGACTCATCTGACA**GCGAGCCTGAGCTT  
CCCATAGGCTCAACCAAGCCTGTTGACGAGGAGGGAGAATCGGACGCGAA  
TGCTGATACGAGTATCTCCAAAAGAAGAAAGTCCCTCGATTCAGAGTCTGAA  
TCGGAACATGAGAAATCCACCAAAAAGAAGGCATTCATATCAGACGGAGAG  
TCTGAAGGAGAACCTGAGGCTGATTCATCGAGCAGTCACGACGATGATGAC  
GTGGTTGCTGGTCCCTGAAGTGAGTGCGCCGACGAAGAAATCCCGGACAGT  
TGTGGAATCGGACAGTGACGATGATATTGTTGAGCCAGAATCAAATCCGTTT  
CCGTTCCCTTGAATCACCTCTGAAATCAAGTGCAATTAATAACCAGAATCAGG



TGCTAAATAATAGTTCCGATGAGAACT **GAGTGTGCTGAATTCGTGCAAT** **AAC**  
**TGCTTCAATGT** GTTTTCTGTCAATTTAGATTATAGAATTCCTGACTTTTGTGC  
AYWAAKTT

**Primers:**

**IPUL6832A FWD – AGGCATGGATCACTTAGCAGAG** **Product Size - 2260**  
**IPUL1\_6832A REV – CGCAGGATAAGAGAATAAGCGC**  
**IPUL1\_6832B FWD - GATTTCCGGCAGTTTATGGAGC** **Product Size - 2390**  
**IPUL1\_6832B REV – ATTGCACGAATTCAGCACACTC**

IP6832FWD1: **AAATAGGCATGGATCACTTAGCA** Pred. size: 749  
IP6832RV1: **TATCTCAAACCTTTCCGGCTCT**  
IP6832 FWD 2: **CTTGAAGTAATTTCCACATGATGC** Pred. size: 802  
IP6832RV2: **AGAGCTCACGGAATTCAGAGTTA**  
IP6832 FWD 3: **TAAAGAGAGCTGCCGATATTCTG** Pred. size: 798  
IP6832RV3: **AAATTTGTTTCGTTTCGATCGGC**  
IP6832 FWD 4: **ATTCGAAAGTGAATCAGAAGGG** Pred. size: 782  
IP6832RV4: **TCAGTGCATTCCAACTTCATC**  
IP6832 FWD 5: **ACAAGACTGAGCTAAGGATGACTTC** Pred. size: 782  
IP6832RV5: **TCGATCATTTCGCAACTTTCAG**  
IP6832FWD 6: **ACCACCGAAGATTTCAAATGAG** Pred. size: 725  
IP6832RV6: **TGTCAGATGAGTCAAACCTATTGAG**  
IP6832 FWD 7: **TCTCAGAAGAGACTTCCTAATCCTG** Pred. size: 745  
IP6832RV7: **ACATTGAAGCAGTTATTGCACG**

**sqRT-PCR Primers:**

**IPTIMA FWD: GCGCTTATTCTCTTATCCTGCG** Pred. size: 327  
**IPTIMA RV: AGGATCCATAGCCTTCCACAAC**  
**IPTIMB FWD: GAGTTGGAACAGGAAGTGAGGG** Pred. size: 250  
**IPTIMB RV: GACCCGCTCTTATTTCCGAGAC**

## IPUL1\_26016

TCCACCCTC **TGGATGCAGAGAAACTGAAGCAAAGCCACAAAAT** GCTACTGA  
GGAAGCAGCAGAGCGAGACGGTTTACTACCGGCACGTGCCCCAGTGCGGA  
TCGTATGTCTGGCTCTACAGCCAGATGACCGTGCTGTCTCATGGCCGACAG  
AGGATCAGCGGTGAAGACAACACCAATTGTACCACAGATTCAAGTCCCGGCC  
ATTCTCGTCATCAACTACATCATCGGCACCGAAGAAGCTGGTCGCAGCGGA  
AACACCGATCAAGTCGACGGTGCAACAACCGACAGAGATTCTCGATGCAKA  
AAAGCCGCGAGCTGCAAGAGATCCTCACAGACGCAAGAAGAGTCGAGAGC  
**CGAACTTGTAAGCAATCA** AAATTTTAGATAATAACGATTCATAATGAACGAT  
TTATTAGTAATTCATAATTGGAACCAATTT

### Primers:

IPUL26016F FWD - **CTGGATGCAGAGAAACTGAAGC** Product Size – 367

IPUL26016F REV – **TTGATTGCTTCACAAGTTCGGC**

IP26016FWD1: **TGAAGCAAAGCCACAAAATG** Pred. Size: 350  
IP26016RV1: **TGATTGCTTCACAAGTTCGG**

### sqRT-PCR Primers:

IP26016FWD1: GCTACTGAGGAAGCAGCAGAG Pred. Size: 305  
IP26016RV1: ACTCTTCTTGCGTCTGTGAGG

## IPUL1\_17690

AAACTAAATAACAAATTGACACAAATGACATCTAGTCGTTCCAGAATGCTCT  
ACGCCGAGACAGCAATCAAATCGGCTATCACTATGATGACTCATCAACAGT  
GGTTACCTTCAGATCCCGGTCGGCCGAGAAGCAATGATGGTGGGCTGCAT  
GAAGAAATCGAACGAAACCCAATACGCGGCATCTGCGGATCCACTCTGGGC  
TACATTTCTGGAATCGACACTCATGGACACCTCCACACAAACGAGGTCCAT  
CCAACAGCCCTCAAGGAGAGGATAAGACCTCTCTCGAAAAGTGCGGCCAAG  
ACGAGAAGAGAGCGGGAGAACA TAGAGTTCATCAACCTGGCCAG GCTGCTT  
CCGGTGCCTCAGGCCATCACAGCGCAACTTGATAAAGCCTCAGTCATTTCG  
CTGACGACCAGCTTGTTGAAATTGCGTGAGCTTCTCCACAACCAGGGGCG  
TATCAAGAAAATACGGACACGTGGCAACTCGAAATCCAGGAGAGGTCCTTT  
TTCTCCTCACATTCTACAGACTCTGGACGGACTGGTTTTCGAGTATCAGCA  
ATGCAAACTCCTGTACA

### Primers:

IP17690IC FWD1: AGAATGCTCTACGCCGAGACAG Pred. Size 539  
IP17690IC RV1: GTACAGGAGTTTTGCATTGCTG

### sqRT-PCR Primers:

IPSIM FWD: GGGCTGCATGAAGAAATCGAAC Pred. Size 332  
IPSIM RV: CCGTATTTTCTTGATACGCCCC

## IPUL1\_923

ACGGTTGCCGGCGAGAGATTCTCTGTTGAGAAAACCTTATTTA **TCCCTGAGTG**  
**AAAATGGTGAAG** GAACTGAAGCAT **GTCAGTATTGTCGTCATTGGCC**ACGTC  
GACAGTGGCAAGTCTACTACCACTGGCCATCTCATCTACAAGTGCGGTGGC  
ATTGACAAGCGAACCAATTGAAAAGTTGAGAAAGAAGCCGCTGAGATGGGA  
AAAGGTTCTTCAAATACGCTTGGGTTCTGGATAAGCTGAAGGCTGAGCGT  
GAGCGTGGTATCACTATTGATATCGCTCTGTGGAAGTTCCAAACAGAGAAGT  
ACTACGTCCTGTCATCGATGCGCCTGGACATCGTGATTTCATCAAAAACAT  
GATCACTGGTACCTCCAAGCGGATTGTGCAGTGTTAGTTGTGCCTGCTGG  
CAAAGGTGAATTCGAGGCTGGTATCTCAAAGAAGGACAAACTAGAGAGCA  
**TGCCCTCCTTGCATTTACTCTCGGTGTGAAGCAGATGATTGTTGCCGTAAAC**  
AAGATGGATGCTTCAGAACCGCCTTACAGTCAATCAAGATACGAGGAAATCT  
GCAAAGAAATCAGCGCCTACTTGAAGAAGGTCGGATACAACCCGAAGACCG  
TTGCTATGGTCCCATCTCTGGTTGGGTGGGAGACAACATGATTGAGGAAT  
CAGCCAACATGCCCTGGTTAAAGAATGGAGCATAG **AGCGAAAGAATGCTG**  
**ATGG**TAAAGAAGAGAAA **GTGTCTGGAAAGACCTTGTTC**AATGCCCTCGATG  
CGATTGTACCGCCTAGCAGACCCACGGATAGACCCCTCAGACTTCTCTCC  
AAGACGTCTACAAGATTGGTGGCATTGGAACAGTGCCAGTCGGCAGAATTG  
AACTGGAATCTTGAAGCCAGCGATGGTGGTTACCTTTGCTCCAGCTAATCT  
GACTACTGAGTGCAAATCAGTTGAGATGCATCACGAGCAGCTGCAAGAAGC  
TGTGCCTGGTGACAACGTTGGTTTTAACGTGAAGAACGTGTGAGTGAAGGA  
TATCAAGAGAGGCATGGTGTGTGGCGATAGCAAGAATGATCCTCCTAAGGA  
AGCGAAGGATTTCACTGCTCAAGTCATCGTTCTTAACCAACCCCGGTGAGAT  
CCACGCCGGTTACTCCCCAGTGCTGGATTGTCACACTGCTCACATTGCCTG  
CAAGTTCGCTGAACTGAAAGAGAAGATCGACAGAAGGTCGGGTAAGAACT  
TGAGGATAACCCAAAGATGGTCAAGTCAGGGGATGCTGCTATTGTTACTAT  
GATTCATCCAAGCCCATGTGTGTTGAGAAGTTCTCTGAGTATGCTCCACTT  
GGCCGTTTCGCTGTTTCGTGACATGAAGCAGACAGTCGCTGTTGGTATCATC  
AAGGACGTTACAAAGGGCGAGTCAGCTGGCAAAGTCACAAAGGCAGCACA  
GAAGGC **CGGTGCGGGAAAGAAGAAATAA**ATTTTATTGAGAATTTTGA **CATAA**  
**TTGAGTCTTCTCGCTGGT**

### Primers:

IP923F FWD: **GTCAGTATTGTCGTCATTGGCC** Pred. Size 1386  
IP923F RV: **TTATTTCTTCTTTCCCGCACCG**  
IP923FWD1: **TCCCTGAGTGAAAATGGTGAAG** Pred. Size: 716  
IP923RV1: **TGAACAAGGTCTTTCCAGACAC**  
IP923FWD2: **AGCGAAAGAATGCTGATGG** Pred. size: 799  
IP923RV2: **CCAGCGAGAAGACTCAATTATG**

### sqRT-PCR Primers

IPEF1a FWD: **ATCTCATCTACAAGTGCGGTGG** Pred. size: 337  
IPEF1a REV: **AGGGCATGCTCTCTAGTTTGTC**

## IPUL1\_11977

ACACAGCTTAGTCACATTCTC **CATTTTGTAAACGTGGTGCGGTTG**CTTTTCAG  
AGATTGTCTTGACTTGATTCAACATGAGCTGCGAAGACGATGTTGCTGCGCT  
TGTCATCGACAATGGCTCCGGAATGTGCAAGGCGGGTTTCGCGGGAGATG  
ACGCGCCTAGGGCTGTCTTCCCATCCATCGTCGGCCGTCCAGACATCAGG  
GAGTGATGGTTGGCATGGGTCAGAAGGACTCCTACGTCGGGGATGAGGCT  
CAGTCCAAGAGAGGAATCCTCACCTCAAGTACCCGATCGAGCACGGAATC  
GTCACCAACTGGGACGACATGGAGAAGATCTGGCATCACACCTTCTACAAC  
GAGCTGCGTGTGGCTCCCGAAGAGCACCCGGTTCTCCTTACTGAGGCCCC  
TCTCAACCCGAAGGCYAACAGGGAGAAGATGACCCAGATCATGTTTCGAGAC  
ATTCAACACTCCGGCCATGTACGTGGCGATCCAGGCCGTGCTGTCGCTGTA  
CGCCTCTGGCCGTACCACTGG **AATAGTCATGGACTCCGGAGATGG**AGTGTC  
CCACACAGTCCCGATCTACGAGGGATACGCCCTGCCTCACGCCATCCTGC  
GTCTGGACCTGGCTGGGCGGG **CCTGACCGACTACCTGATGAAGATCCTG**  
ACTGAGCGCGGATACAGCTTACCACGACGGCGGAGAGGGAAATCGTGCG  
TGACATCAAGGAGAAGCTGTGCTACGTGGCTCTCGACTTCGAGCAGGAGAT  
GCAGACCGCGGCTTCCAGCTCCTCTCTCGAGAAGAGCTACGAGCTCCCCG  
ACGGACAGGTGATCACGATCGGGAACGAGCGGTTCCGGTGTCCGGAGGCG  
CTGTTCCAGCCGTCGTTCTGGGCATGGAGTCGTCTGGCATCCACGAGACC  
ACCTACCAGAGCATCATGAAGTGTGACGTGGACATCCG **CAAGGACCTCTAC**  
**TCCAACACCG**TCATGTCCGGGGGCACCACCATGTACCCCGGTATCGCAGA  
CCGCATGCAGAAGGAGATCGCAGCTCTCGCCCCGCCACCATGAAGGTCA  
AGATCATCGCGCCGCCCGAGAGGAAGTACTCGGTCTGGATCGGGGGCTCT  
ATCCTGGCTTCGCTCTCCACCTTCCAGCAGATGTGGATCAGCAAGCAGGAG  
TACGACGAGTCTGGACCCTCCATCGTCCACAGGAAGTGCTTCTAGACATCG  
CGACTAGTTGCCAGGACATTGGGAAA **CGAGCGGAGAGTTGCAATTATT**TGT  
ATCCAATATTTCTATTTTT **GKCATAAATTACCTCTTCTCTCG**

### Primers:

IP11977F FWD:	<b>TTTTGTAAACGTGGTGCGGTTG</b>	Pred. Size: 1240
IP11977F RV:	<b>AATAATTGCAACTCTCCGCTCG</b>	
IP11977FWD1:	<b>CATTTTGTAAACGTGGTGCG</b>	Pred. Size: 638
IP11977RV1:	<b>AGGATCTTCATCAGGTAGTCGG</b>	
IP11977FWD2:	<b>AATAGTCATGGACTCCGGAGATGG</b>	Pred. Size: 779
IP11977RV2:	<b>CGAGAAGAAGAGGTAATTTATGMC</b>	

### sqRT-PCR Primers:

IPbACTIN FWD:	<b>CCTGACCGACTACCTGATGAAG</b>	Pred. Size: 340
IPbACTIN REV:	<b>GGTGTGGAGTAGAGGTCCTTG</b>	

**9.4 Appendix 4: Expected Band Size vs Actual Band Size**

NB= NO BAND  
 N/A – NO DATA

IPUL1_3616	<i>I. pulchra</i> ARNT	Reading Frame Shift Corrected by Removing A 431			
Full/Fragment/ sqRT-PCR	Primer Name	Forward Primer	Reverse Primer	Pred. Band Size	Actual Band Size
Full	IP3616F	TCCTCCACACAGTTAATGGTGG	GCAGTAGAGGTAGAACTCTGGC	2226	2148
<b>Sequence Result</b>		<b>N/A</b>	<b>N/A</b>	<b>N/A</b>	
Frag. 1	IP3616F1	CCACACAGTTAATGGTGGCTC	GCACCATCAAGAAGGAGTCATC	809	764
<b>Sequence Result</b>		<b>BAD READ</b>	<b>BAD READ</b>	<b>N/A</b>	
Frag. 2	IP3616F2	TGTCCATCGGGGACAACCTTC	CACGGAGCTTTGAAAGTGTCAT	804	773
<b>Sequence Result</b>		<b>BAD READ</b>	<b>BAD READ</b>	<b>N/A</b>	
Frag. 3	IP3616F3	TCTCCCCCAAAAATTCGATG	CCTCTACTGCCAATTCATATTTCC	751	712
<b>Sequence Result</b>		<b>BAD READ</b>	<b>BAD READ</b>	<b>N/A</b>	
sqRT-PCR1	IPARNTA	TCCATATCCACTATCAACGCCG	TGTCACAGGAGACGACAAAGAG	328	NB
<b>Sequence Result</b>		<b>NO BAND</b>	<b>NO BAND</b>	<b>N/A</b>	
sqRT-PCR2	IPARNTB	GTGTGTTTCGATTTCTGTGCTCC	CGACTGAACTTCATTGGCGATC	335	334
<b>Sequence Result</b>		<b>IPARNTB FWD</b>	<b>IPARNTB RV</b>	<b>Size = 335</b>	

All sequencing results for 3616 resulted in poor reads with the exception of one – sqRT-PCR2.

IPUL1_2961	<i>I. pulchra</i> PERIOD	No Reading Frame Shift			
Full/Fragment/ sqRT-PCR	Primer Name	Forward Primer	Reverse Primer	Pred. Band Size	Actual Band Size
Full	IP2961F	TGTCCGTACAGACATCAATTGC	TTTTTGTCCGGATTGTTGCTGC	1836	1811
<b>Sequence Result</b>		<b>B1FR.AAJ130</b>	<b>N/A</b>	<b>Size = 493</b>	
<b>Sequence Result</b>		<b>B2FR.AAJ131</b>	<b>B2RR.AAJ132</b>	<b>Size = 1228</b>	
Frag. 1	IP2961F1	GTGCTATGTGCTATATATCTTTTCG	TCGACATAATTGACTCTGGTG	587	515
<b>Sequence Result</b>		<b>N/A</b>	<b>N/A</b>	<b>N/A</b>	
Frag. 2	IP2961F2	CTCGTGGAATATATGATCACAACC	GGGCATTCTGGATATTTTGAAC	780	751
<b>Sequence Result</b>		<b>N/A</b>	<b>N/A</b>	<b>N/A</b>	
Frag. 3	IP2961F3	AATTCTACCCTTCTATCCTCACCAG	TTATTTTTGTCCGGATTGTTGC	624	587
<b>Sequence Result</b>		<b>N/A</b>	<b>N/A</b>	<b>N/A</b>	
sqRT-PCR1	IPPERA	TTCCCTCAACTTGGATCTCGTG	GAAGTGAAGATATGCCGATCGC	358	367
<b>Sequence Result</b>		<b>IPPERA</b>	<b>N/A</b>	<b>Size = 356</b>	
sqRT-PCR2	IPPERB	GAGATGAGCACTCTGGTTCTCC	TGACTCAAGTGCATCCTGTAGG	322	N/A
<b>Sequence Result</b>		<b>N/A</b>	<b>N/A</b>	<b>N/A</b>	

IPUL1_6832	<i>I. pulchra</i> TIM2	Reading Frame Shift Corrected by adding A 1982			
Full/ Fragment/ sqRT-PCR	Primer Name	Forward Primer	Reverse Primer	Pred. Band Size	Actual Band Size
Full1	IP6832A	AGGCATGGATCACTTAGCAGAG	CGCAGGATAAGAGAATAAGC GC	2260	2148
<b>Sequence Result</b>		<b>C1A – BAD READ</b>	<b>N/A</b>	<b>N/A</b>	
<b>Sequence Result</b>		<b>C1B – BAD READ</b>	<b>N/A</b>	<b>N/A</b>	
Full2	IP6832B	GATTTCCGGCAGTTTATGGAGC	ATTGCACGAATTCAGCACAC TC	2390	2363
<b>Sequence Result</b>		<b>C2AFR.AAJ133</b>	<b>N/A</b>	<b>Size =</b>	
<b>Sequence Result</b>		<b>C2BFR.AAJ134</b>	<b>N/A</b>	<b>Size =</b>	
Frag. 1	IP6832AF1	AAATAGGCATGGATCACTTAGCA	AGAGCCGGAAAGAGTTTGA GATA	749	NB
<b>Sequence Result</b>		<b>NO BAND</b>	<b>NO BAND</b>	<b>N/A</b>	
Frag. 2	IP6832AF2	CTTGAAGTAATTTCCACATGATGC	TAACTCTGAATTCCGTGAGC TCT	802	786
<b>Sequence Result</b>		<b>C11_IP6832AF2F_046_2013-01-16</b>	<b>D11_IP6832AF2R_045_2013-01-16</b>	<b>Size = 804</b>	
Frag. 3	IP6832AF3	TAAAGAGAGCTGCCGATATTCTG	GCCGATCGAAACGAACAAAT TT	798	848
<b>Sequence Result</b>		<b>BAD READ</b>	<b>BAD READ</b>	<b>N/A</b>	
Frag. 4	IP6832BF1	ATTCGAAAGTGAATCAGAAGGG	GATGAAGTTTGGAAATGCACT GA	782	721
<b>Sequence Result</b>		<b>E11_IP6832BF1F_044_2013-01-16</b>	<b>F11_IP6832BF1R_043_2013-01-16</b>	<b>Size = 720</b>	
Frag. 5	IP6832FBF2	ACAAGACTGAGCTAAGGATGACTTC	TCGATCATTTCGCAACTTTCA G	782	773



<b>Sequence Result</b>		<b>G11_IP6821BF2F_042_2013-01-16</b>	<b>A12_IP6821BF2R_048_2013-01-16</b>	<b>Size = 783</b>	
Frag. 6	IP6832FBF3	ACCACCGAAGATTTCAAATGAG	ACCACCGAAGATTTCAAATGAG	725	729
<b>Sequence Result</b>		<b>BAD READ</b>	<b>C12_IP6821BF3R_046_2013-01-16</b>	<b>Size = 707</b>	
Frag. 7	IP6832F7	TCTCAGAAGAGACTTCCTAATCCTG	CGTGCAATAACTGCTTCAATGT	745	733
<b>Sequence Result</b>		<b>D12_IP6821BF4F_045_2013-01-16</b>	<b>E12_IP6821BF4R_044_2013-01-16</b>	<b>Size = 735</b>	

IPUL1_6832	<i>I. pulchra</i> TIM2	(Continued)			
Full/Fragment/ sqRT-PCR	Primer Name	Forward Primer	Full/Fragment/ sqRT-PCR	Primer Name	Forward Primer
sqRT-PCR	IPTIMA	GCGCTTATTCTCTTATCCTGCG	AGGATCCATAGCCTTCCACAAC	327	300
<b>Sequence Result</b>		<b>N/A</b>	<b>N/A</b>	<b>N/A</b>	
sqRT-PCR	IPTIMB	GAGTTGGAACAGGAAGTGAGGG	GACCCGCTCTTATTTGGGAGAC	344	N/A
<b>Sequence Result</b>		<b>N/A</b>	<b>N/A</b>	<b>N/A</b>	

Sequencing reads were either poor for the first portion of this sequence, likely due to primer design. The second half of the sequence resulted in usable reads.

IPUL1_26016	<i>I. pulchra</i> CLOCK?	No Reading Frame Shift but Incomplete Sequence			
Full/Fragment/ sqRT-PCR	Primer Name	Forward Primer	Reverse Primer	Pred. Band Size	Actual Band Size
Full	IP26016ICF	CTGGATGCAGAGAACTGAAGC	TTGATTGCTTCACAAGTTCGGC	367	402
<b>Sequence Result</b>		<b>N/A</b>	<b>N/A</b>	<b>N/A</b>	
sqRT-PCR	IPCLK	GCTACTGAGGAAGCAGCAGAG	ACTCTTCTTGCGTCTGTGAGG	305	365
<b>Sequence Result</b>		<b>N/A</b>	<b>N/A</b>	<b>N/A</b>	

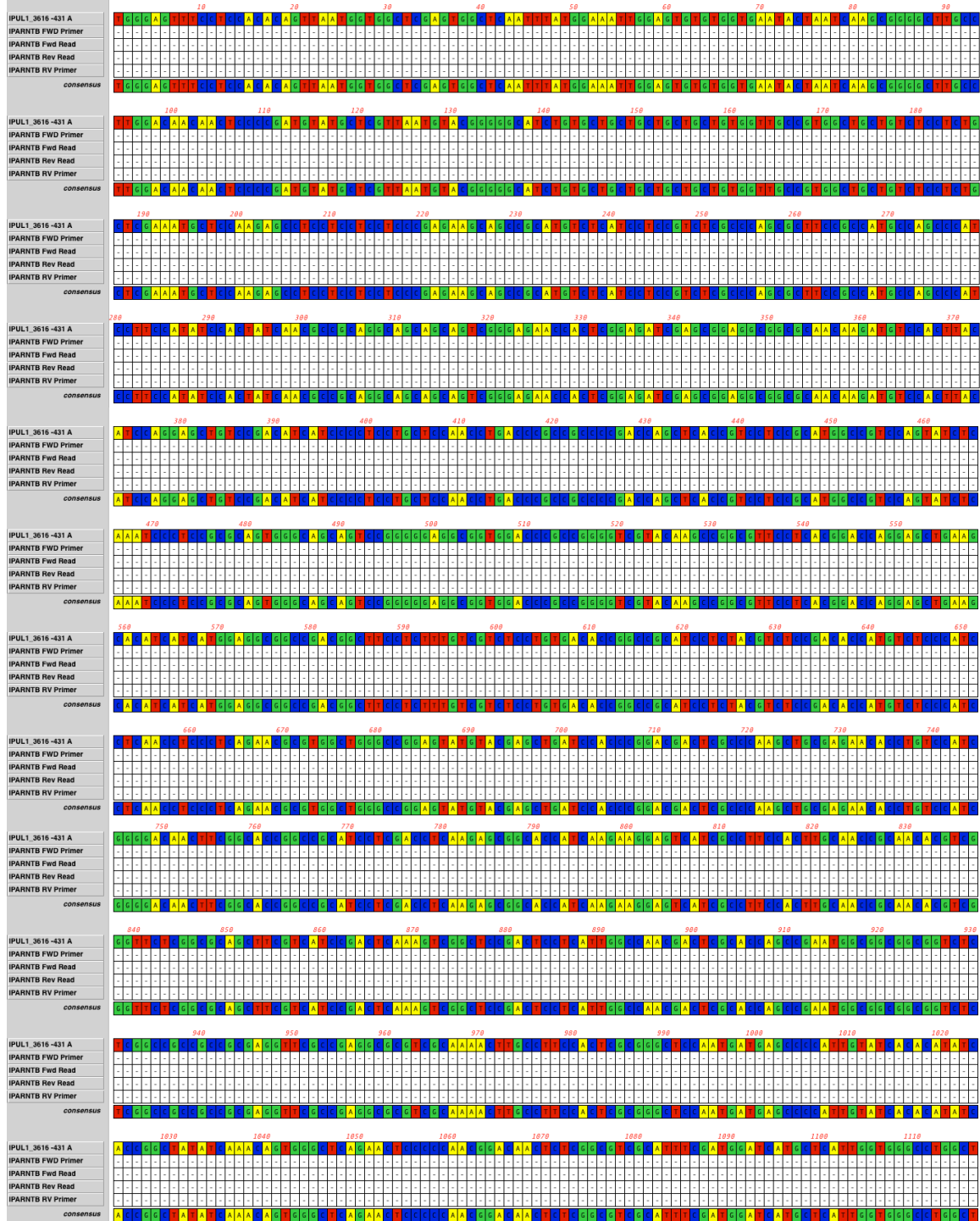
IPUL1_923	<i>I. pulchra</i> EF1a	No Reading Frame Shift			
Full/Fragment/ sqRT-PCR	Primer Name	Forward Primer	Reverse Primer	Pred. Band Size	Actual Band Size
Full	IP923F	GTCAGTATTGTCGTCATTGGCC	TTATTTCTTCTTTCCCGCACCG	1386	N/A
Frag. 1	IP923F1	TCCCTGAGTGAAAATGGTGAAG	TGAACAAGGTCTTTCCAGACAC	716	N/A
Frag. 2	IP923F2	AGCGAAAGAATGCTGATGG	CCAGCGAGAAGACTCAATTATG	799	N/A
sqRT-PCR	IPEF1a	ATCTCATCTACAAGTGCGGTGG	AGGGCATGCTCTCTAGTTTGTC	337	343

IPUL1_11977	<i>I. pulchra</i> bACTIN	No Reading Frame Shift			
Full/Fragment/ sqRT-PCR	Primer Name	Forward Primer	Reverse Primer	Pred. Band Size	Act ual Ban d Siz e
Full	IP11977F	TTTTGTAAACGTGGTGCGGTTG	AATAATTGCAACTCTCCGCTCG	1240	N/A
Frag. 1	IP11977F1	CATTTTGTAAACGTGGTGCG	AGGATCTTCATCAGGTAGTCGG	638	N/A
Frag. 2	IP11977F2	AATAGTCATGGACTCCGGAGATGG	CGAGAAGAAGAGGTAATTTATGMC	779	N/A
sqRT-PCR	IPbACTIN	CCTGACCGACTACCTGATGAAG	GGTGTTGGAGTAGAGGTCCTTG	340	355
IPUL1_17690IC	<i>I. pulchra</i> SIM?	No Reading Frame Shift but Incomplete Sequence			
Full/Fragment/ sqRT-PCR	Primer Name	Forward Primer	Reverse Primer	Pred. Band Size	Actual Band Size
Full	IP17960F	AGAATGCTCTACGCCGAGACAG	GTACAGGAGTTTTGCATTGCTG	539	N/A
sqRT-PCR	IPSIM	GGGCTGCATGAAGAAATCGAAC	CCGTATTTTCTTGATACGCCCC	332	325

Sequencing was not performed for IPUL1\_923 and IPUL1\_11977 because these sequences have been identified – see DeMulder 2009. Additionally IPUL1\_17690 was not sequenced because it was not identified as a core-clock protein and it was incomplete.

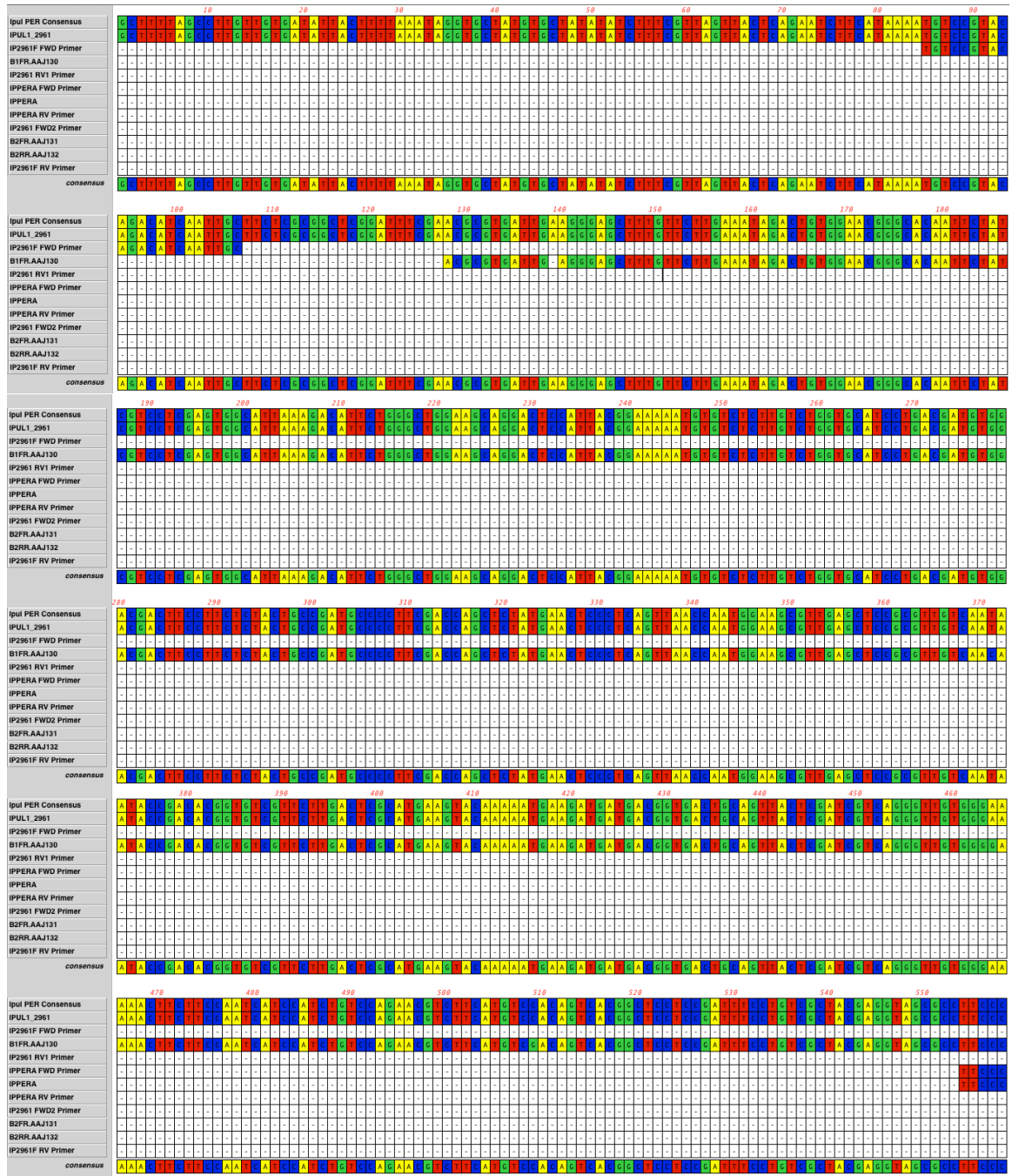
# 9.5 Appendix 5: Sequencing Results and Alignments

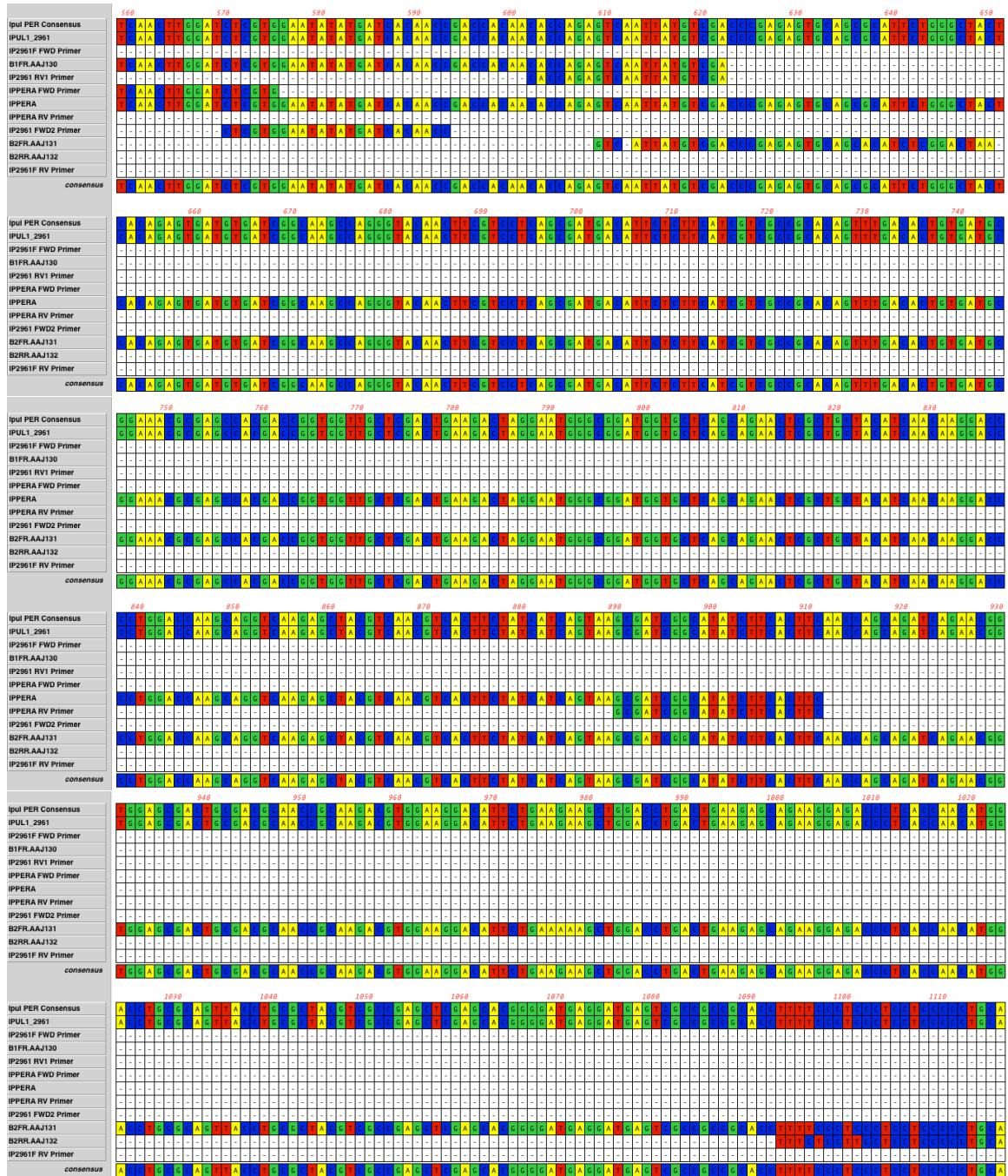
## IPUL1\_3616 (ARNT)





# IPUL1\_2961 (PERIOD)



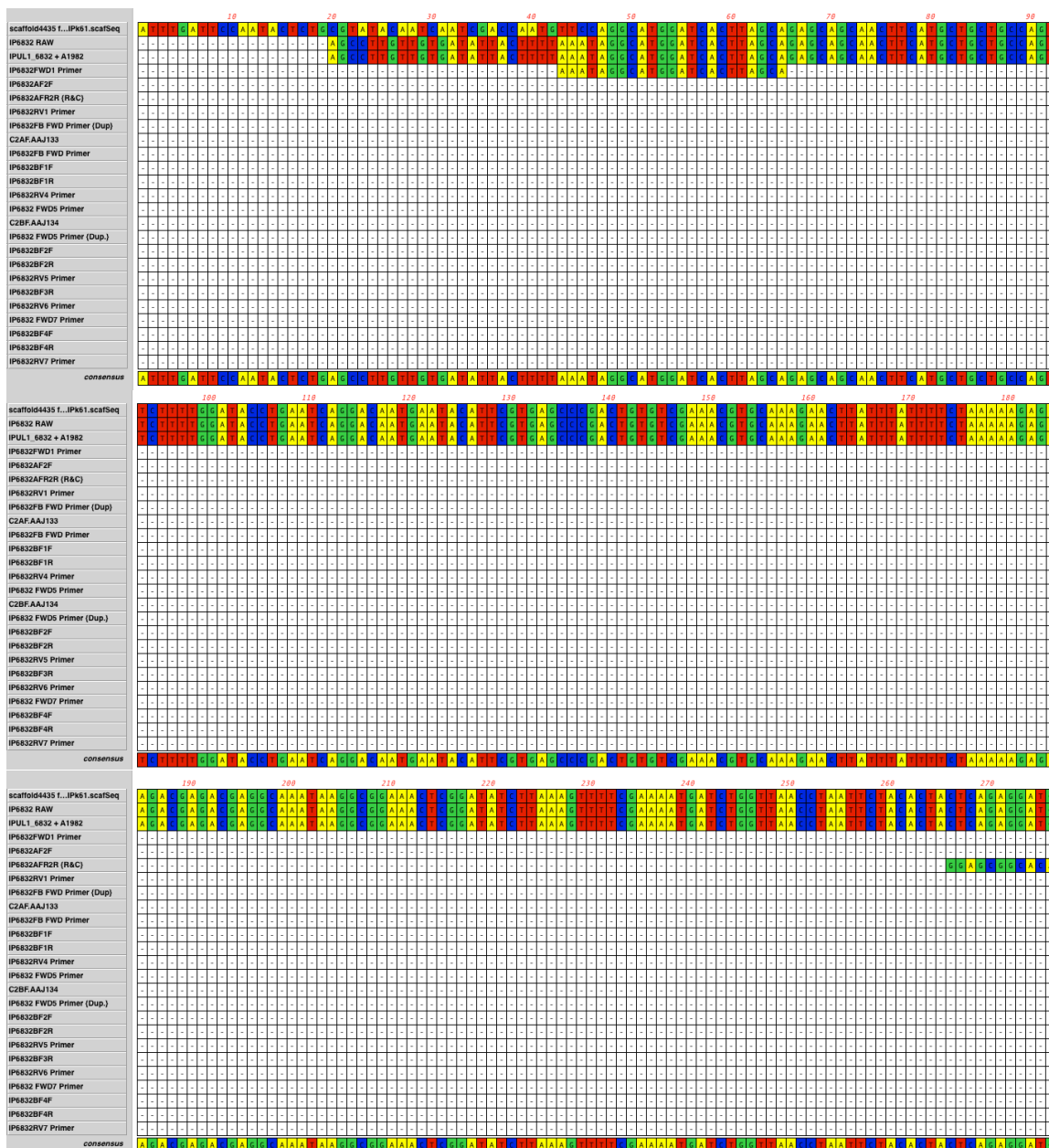


		1120	1130	1140	1150	1160	1170	1180	1190	1200
IpU1 PER Consensus		A	A	A	A	A	A	A	A	A
IPUL1_2961		A	A	A	A	A	A	A	A	A
IP2961F FWD Primer										
B1FR.AAJ130										
IP2961 RV1 Primer										
IPPERA FWD Primer										
IPPERA										
IPPERA RV Primer										
IP2961 FWD2 Primer										
B2FR.AAJ131										
B2RR.AAJ132										
IP2961F RV Primer										
consensus		A	A	A	A	A	A	A	A	A
		1210	1220	1230	1240	1250	1260	1270	1280	1290
IpU1 PER Consensus		A	A	A	A	A	A	A	A	A
IPUL1_2961		A	A	A	A	A	A	A	A	A
IP2961F FWD Primer										
B1FR.AAJ130										
IP2961 RV1 Primer										
IPPERA FWD Primer										
IPPERA										
IPPERA RV Primer										
IP2961 FWD2 Primer										
B2FR.AAJ131										
B2RR.AAJ132										
IP2961F RV Primer										
consensus		A	A	A	A	A	A	A	A	A
		1310	1320	1330	1340	1350	1360	1370	1380	1390
IpU1 PER Consensus		A	A	A	A	A	A	A	A	A
IPUL1_2961		A	A	A	A	A	A	A	A	A
IP2961F FWD Primer										
B1FR.AAJ130										
IP2961 RV1 Primer										
IPPERA FWD Primer										
IPPERA										
IPPERA RV Primer										
IP2961 FWD2 Primer										
B2FR.AAJ131										
B2RR.AAJ132										
IP2961F RV Primer										
consensus		A	A	A	A	A	A	A	A	A
		1400	1410	1420	1430	1440	1450	1460	1470	1480
IpU1 PER Consensus		A	A	A	A	A	A	A	A	A
IPUL1_2961		A	A	A	A	A	A	A	A	A
IP2961F FWD Primer										
B1FR.AAJ130										
IP2961 RV1 Primer										
IPPERA FWD Primer										
IPPERA										
IPPERA RV Primer										
IP2961 FWD2 Primer										
B2FR.AAJ131										
B2RR.AAJ132										
IP2961F RV Primer										
consensus		A	A	A	A	A	A	A	A	A
		1490	1500	1510	1520	1530	1540	1550	1560	1570
IpU1 PER Consensus		A	A	A	A	A	A	A	A	A
IPUL1_2961		A	A	A	A	A	A	A	A	A
IP2961F FWD Primer										
B1FR.AAJ130										
IP2961 RV1 Primer										
IPPERA FWD Primer										
IPPERA										
IPPERA RV Primer										
IP2961 FWD2 Primer										
B2FR.AAJ131										
B2RR.AAJ132										
IP2961F RV Primer										
consensus		A	A	A	A	A	A	A	A	A
		1580	1590	1600	1610	1620	1630	1640	1650	1670
IpU1 PER Consensus		A	A	A	A	A	A	A	A	A
IPUL1_2961		A	A	A	A	A	A	A	A	A
IP2961F FWD Primer										
B1FR.AAJ130										
IP2961 RV1 Primer										
IPPERA FWD Primer										
IPPERA										
IPPERA RV Primer										
IP2961 FWD2 Primer										
B2FR.AAJ131										
B2RR.AAJ132										
IP2961F RV Primer										
consensus		A	A	A	A	A	A	A	A	A





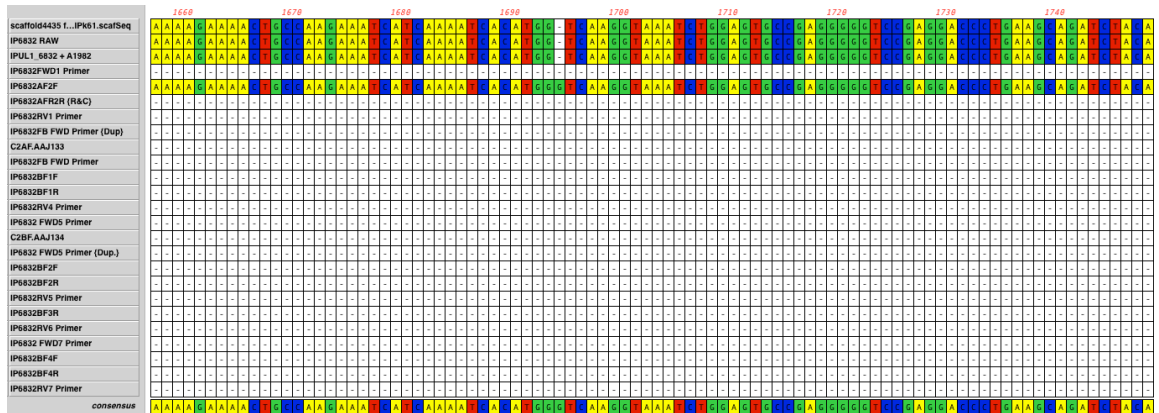
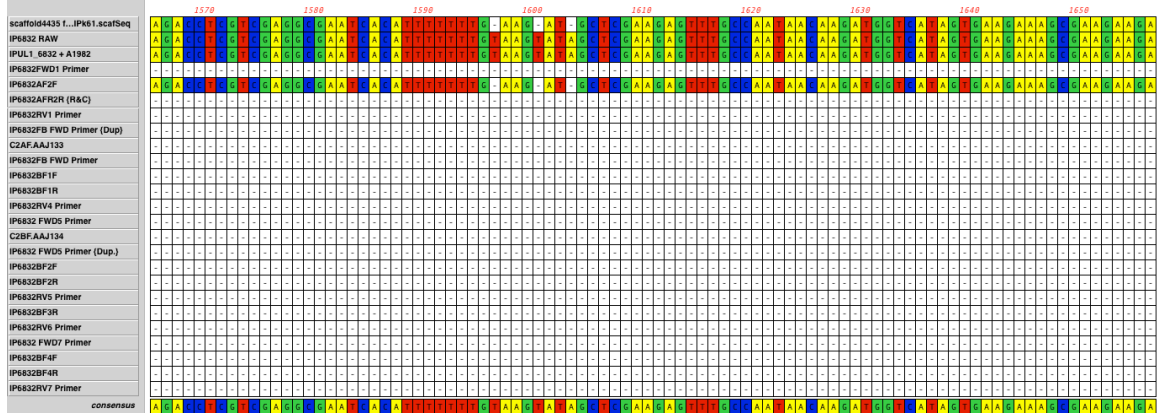
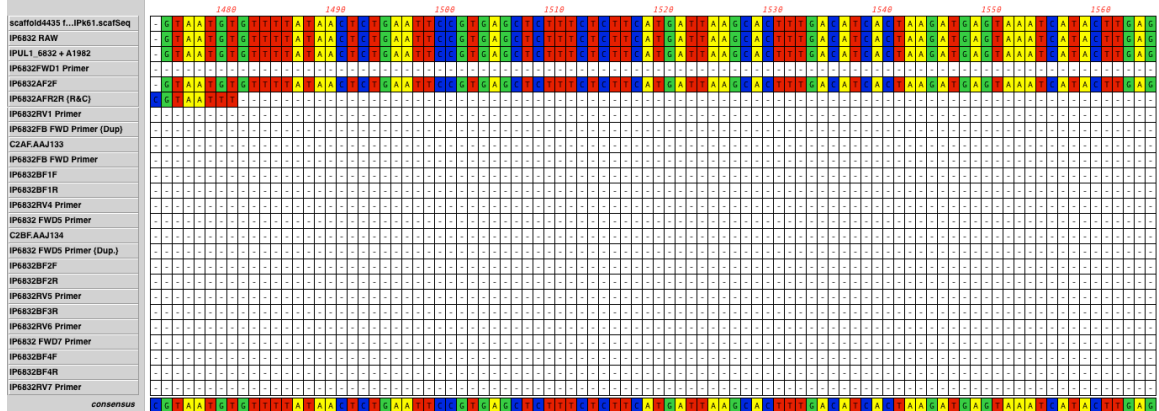
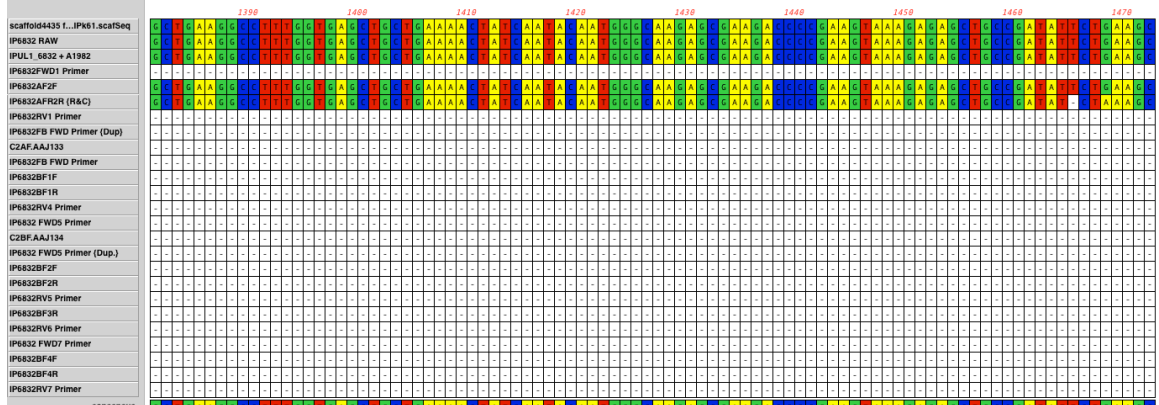
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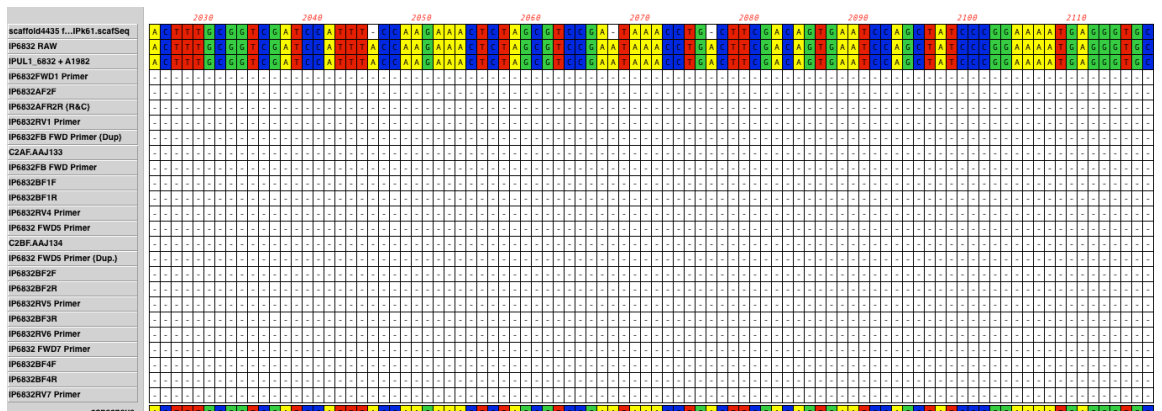
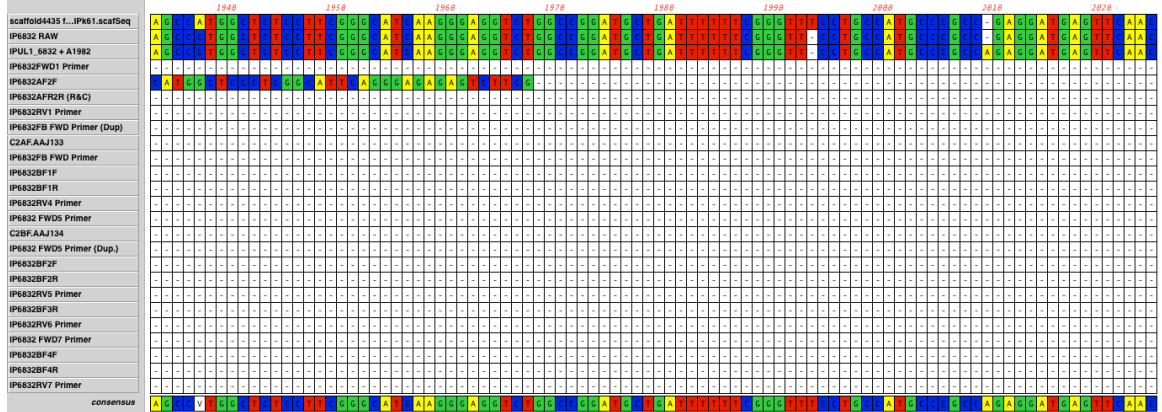
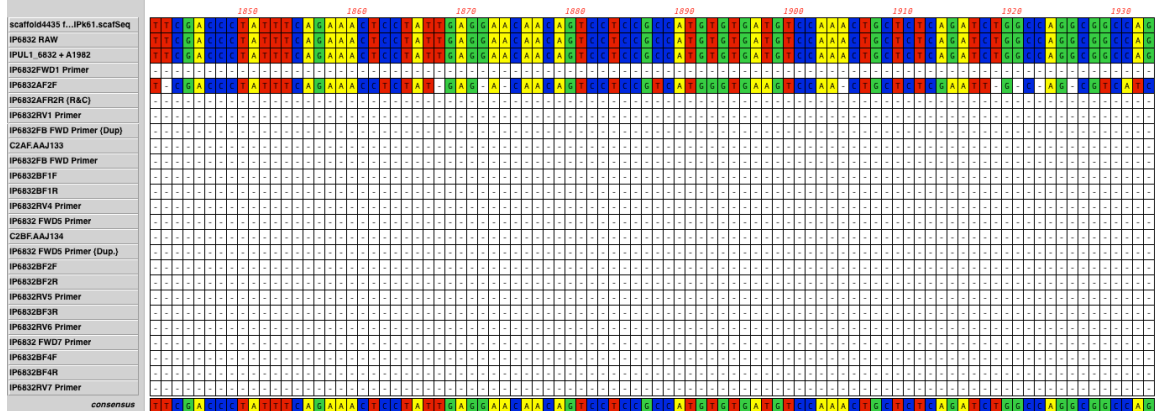
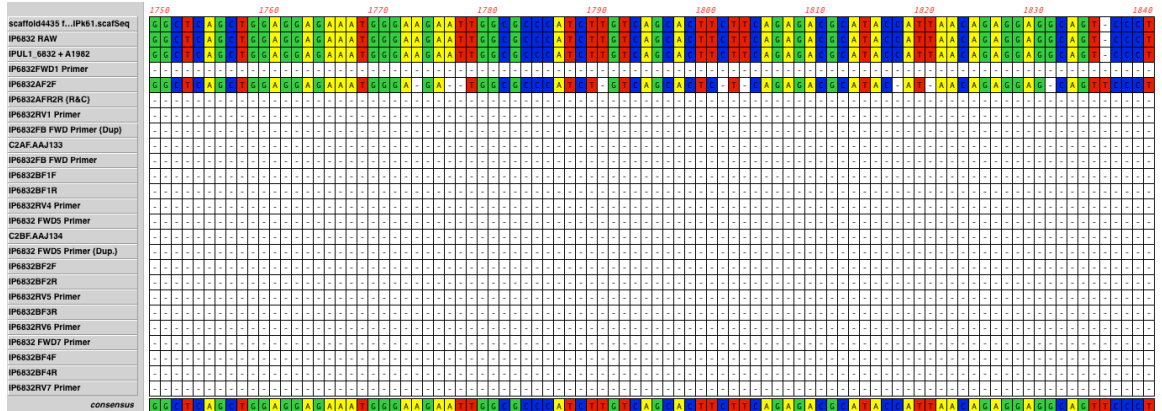


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IP632AF2F	
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IP632RV1 Primer	
IP632FB FWD Primer (Dup)	
C2AF.AAJ133	
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C2BF.AAJ134	
IP632 FWD5 Primer (Dup.)	
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IP632BF2R	
IP632RV5 Primer	
IP632BF3R	
IP632RV6 Primer	
IP632 FWD7 Primer	
IP632BF4F	
IP632BF4R	
IP632RV7 Primer	
consensus	
scf104435 f...IPK61.scaf5eq	
IP632 RAW	370 380 390 400 410 420 430 440 450 460
IPUL1_6832 + A1982	
IP632FWD1 Primer	
IP632AF2F	
IP632AF2R (R&C)	
IP632RV1 Primer	
IP632FB FWD Primer (Dup)	
C2AF.AAJ133	
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IP632BF1F	
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C2BF.AAJ134	
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IP632BF2F	
IP632BF2R	
IP632RV5 Primer	
IP632BF3R	
IP632RV6 Primer	
IP632 FWD7 Primer	
IP632BF4F	
IP632BF4R	
IP632RV7 Primer	
consensus	
scf104435 f...IPK61.scaf5eq	
IP632 RAW	470 480 490 500 510 520 530 540 550
IPUL1_6832 + A1982	
IP632FWD1 Primer	
IP632AF2F	
IP632AF2R (R&C)	
IP632RV1 Primer	
IP632FB FWD Primer (Dup)	
C2AF.AAJ133	
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IP632BF4F	
IP632BF4R	
IP632RV7 Primer	
consensus	
scf104435 f...IPK61.scaf5eq	
IP632 RAW	560 570 580 590 600 610 620 630 640
IPUL1_6832 + A1982	
IP632FWD1 Primer	
IP632AF2F	
IP632AF2R (R&C)	
IP632RV1 Primer	
IP632FB FWD Primer (Dup)	
C2AF.AAJ133	
IP632FB FWD Primer	
IP632BF1F	
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IP632BF2R	
IP632RV5 Primer	
IP632BF3R	
IP632RV6 Primer	
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IP632BF4F	
IP632BF4R	
IP632RV7 Primer	
consensus	

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IP632FWD1 Primer	
IP632AF2F	
IP632AFR2R (R&C)	C C G A C A A A A A A A A A C C C C C C G C A A G C A C G C A C G A A G C G C C A A G C G C C A A G C A A C C C A A C A C G C A A
IP632RV1 Primer	
IP632F B FWD Primer (Dup)	
C2AF.AAJ133	
IP632F1F	
IP632F1R	
IP632RV4 Primer	
IP632 FWD5 Primer	
C2BF.AAJ134	
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IP632BF2R	
IP632RV5 Primer	
IP632BF3R	
IP632RV6 Primer	
IP632 FWD7 Primer	
IP632BF4F	
IP632BF4R	
IP632RV7 Primer	
consensus	C C G A C A A A A A A A A A C C C C C C G C A A G C A C G C A C G A A G C G C C A A G C G C C A A G C A A C C C A A C A C G C A A
scfaffoid4435 f...IPK61.scaf5eq	A A A A A C C C A C G C C C C C A C G C A C G A A A A C G C G A A A A C G C C A A A C A A G C C A A A C A A G C C A A A C A A
IP632 RAW	A A A A A C C C A C G C C C C C A C G C A C G A A A A C G C G A A A A C G C C A A A C A A G C C A A A C A A G C C A A A C A A
IPUL1_6832 + A1982	A A A A A C C C A C G C C C C C A C G C A C G A A A A C G C G A A A A C G C C A A A C A A G C C A A A C A A G C C A A A C A A
IP632FWD1 Primer	
IP632AF2F	
IP632AFR2R (R&C)	A A G G C G A A A A A A G C C C C C A C G G A A G C G A A A A C G C G A A A A C G C C A A A C A A G C C A A A C A A G C C A A A C A A
IP632RV1 Primer	
IP632F B FWD Primer (Dup)	
C2AF.AAJ133	
IP632F B FWD Primer	
IP632BF1F	
IP632BF1R	
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IP632RV5 Primer	
IP632BF3R	
IP632RV6 Primer	
IP632 FWD7 Primer	
IP632BF4F	
IP632BF4R	
IP632RV7 Primer	
consensus	A A A A A C C C A C G C C C C C A C G C A C G A A A A C G C G A A A A C G C C A A A C A A G C C A A A C A A G C C A A A C A A
scfaffoid4435 f...IPK61.scaf5eq	A A A A A A A A A A S A A A C A A A A A S A C A C A C A C G C A C G C A A A C G C C A A A C A A G C C A A A C A A G C C A A A C A A
IP632 RAW	A A A A A A A A A A S A A A C A A A A A S A C A C A C A C G C A C G C A A A C G C C A A A C A A G C C A A A C A A G C C A A A C A A
IPUL1_6832 + A1982	A A A A A A A A A A S A A A C A A A A A S A C A C A C A C G C A C G C A A A C G C C A A A C A A G C C A A A C A A G C C A A A C A A
IP632FWD1 Primer	
IP632AF2F	
IP632AFR2R (R&C)	A A A A A A A A A A S A A A C A A A A A S A C A C A C A C G C A C G C A A A C G C C A A A C A A G C C A A A C A A G C C A A A C A A
IP632RV1 Primer	
IP632F B FWD Primer (Dup)	
C2AF.AAJ133	
IP632F B FWD Primer	
IP632BF1F	
IP632BF1R	
IP632RV4 Primer	
IP632 FWD5 Primer	
C2BF.AAJ134	
IP632 FWD5 Primer (Dup.)	
IP632BF2F	
IP632BF2R	
IP632RV5 Primer	
IP632BF3R	
IP632RV6 Primer	
IP632 FWD7 Primer	
IP632BF4F	
IP632BF4R	
IP632RV7 Primer	
consensus	A A A A A A A A A A S A A A C A A A A A S A C A C A C A C G C A C G C A A A C G C C A A A C A A G C C A A A C A A G C C A A A C A A
scfaffoid4435 f...IPK61.scaf5eq	G A A A A A A A A A C G A
IP632 RAW	G A A A A A A A A A C G A
IPUL1_6832 + A1982	G A A A A A A A A A C G A
IP632FWD1 Primer	
IP632AF2F	
IP632AFR2R (R&C)	G A A A A A A A A A C G A
IP632RV1 Primer	
IP632F B FWD Primer (Dup)	
C2AF.AAJ133	
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IP632BF2R	
IP632RV5 Primer	
IP632BF3R	
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consensus	G A A A A A A A A A C G A

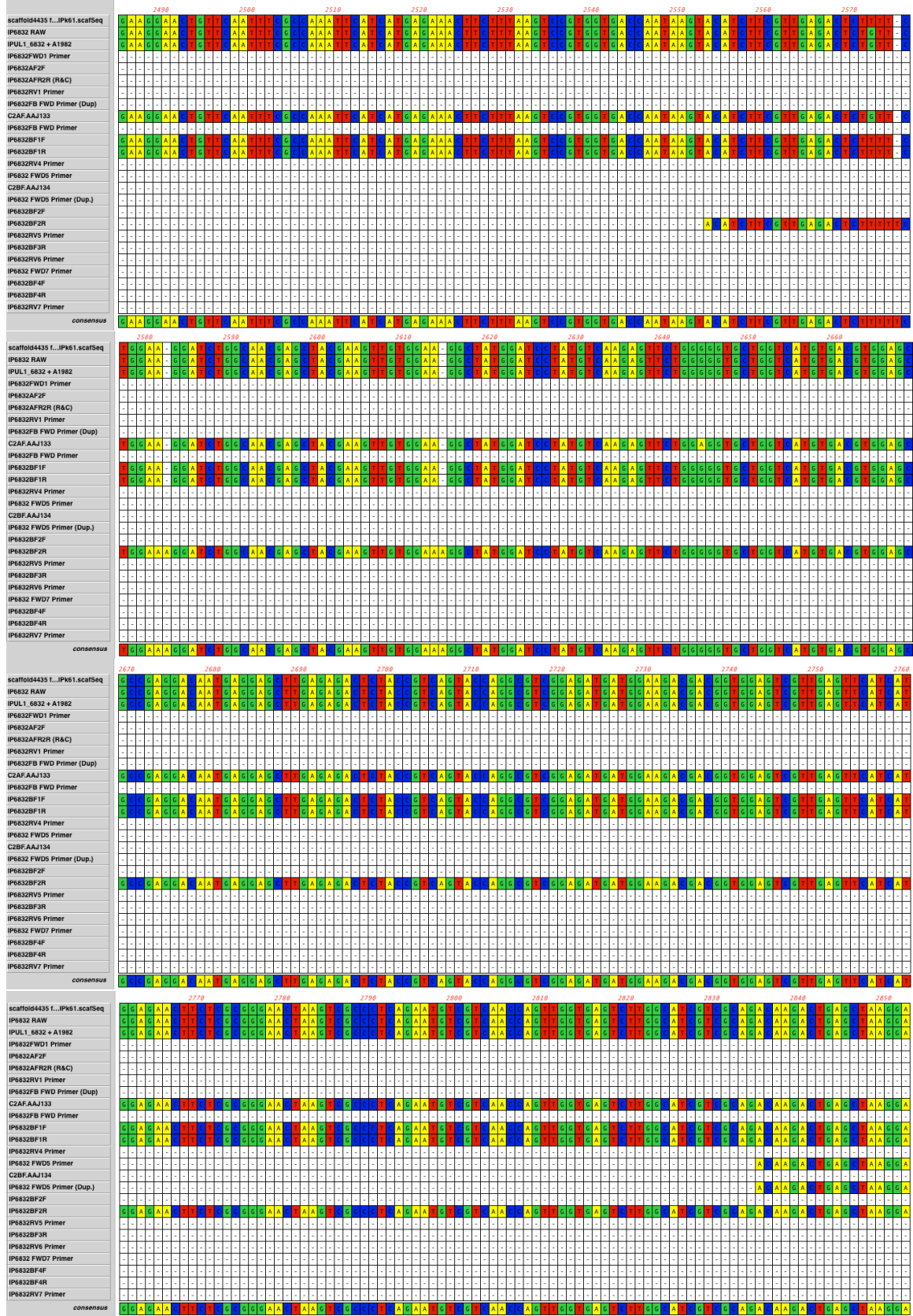
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IP6832 RAW		A A A G G C C G C G C C C G A C A C A A A C A C A G C G A A G G C C A A G C C A A A A G A G C A G C G A G A C C
IPUL1_8832 + A1982		A A A G G C C G C G C C C G A C A C A A A C A C A G C G A A G G C C A A G C C A A A A G A G C A G C G A G A C C
IP6832FWD1 Primer		
IP6832AF2F		A A A G G C C G C G C C C G A C A C A A A C A C A G C G A A G G C C A A G C C A A A A G A G C A G C G A G A C C
IP6832AFR2R (R&C)		A A A G G C C G C G C C C G A C A C A A A C A C A G C G A A G G C C A A G C C A A A A G A G C A G C G A G A C C
IP6832RV1 Primer		
IP6832FB FWD Primer (Dup)		
C2AF.AAJ133		
IP6832FB FWD Primer		
IP6832BF1F		
IP6832BF1R		
IP6832RV4 Primer		
IP6832 FWD5 Primer		
C2BF.AAJ134		
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IP6832 FWD7 Primer		
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IP6832RV7 Primer		
<i>consensus</i>		A A A G G C C G C G C C C G A C A C A A A C A C A G C G A A G G C C A A G C C A A A A G A G C A G C G A G A C C
		1110      1120      1130      1140      1150      1160      1170      1180      1190
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IP6832 RAW		A A A A A A T A T A A A A T A A C A T A G A C G A A A C A C A G C G A A A A A A A A C A A A A A A A A A A A A A A C C G C G
IPUL1_8832 + A1982		A A A A A A T A T A A A A T A A C A T A G A C G A A A C A C A G C G A A A A A A A A C A A A A A A A A A A A A A A C C G C G
IP6832FWD1 Primer		
IP6832AF2F		A A A A A A T A T A A A A T A A C A T A G A C G A A A C A C A G C G A A A A A A A A C A A A A A A A A A A A A A A C C G C G
IP6832AFR2R (R&C)		A A A A A A T A T A A A A T A A C A T A G A C G A A A C A C A G C G A A A A A A A A C A A A A A A A A A A A A A A C C G C G
IP6832RV1 Primer		
IP6832FB FWD Primer (Dup)		
C2AF.AAJ133		
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IP6832BF1F		
IP6832BF1R		
IP6832RV4 Primer		
IP6832 FWD5 Primer		
C2BF.AAJ134		
IP6832 FWD5 Primer (Dup.)		
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IP6832BF4F		
IP6832BF4R		
IP6832RV7 Primer		
<i>consensus</i>		A A A A A A T A T A A A A T A A C A T A G A C G A A A C A C A G C G A A A A A A A A C A A A A A A A A A A A A A A C C G C G
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IPUL1_8832 + A1982		G A T G C G C T T T G G C G A G C T A A A A G A A A A A A A A A G T A A G A G A G A G G C C G G A A G C C C G A A G C C A A A A A A
IP6832FWD1 Primer		
IP6832AF2F		G A T G C G C T T T G G C G A G C T A A A A G A A A A A A A A A G T A A G A G A G A G G C C G G A A G C C C G A A G C C A A A A A A
IP6832AFR2R (R&C)		G A T G C G C T T T G G C G A G C T A A A A G A A A A A A A A A G T A A G A G A G A G G C C G G A A G C C C G A A G C C A A A A A A
IP6832RV1 Primer		
IP6832FB FWD Primer (Dup)		
C2AF.AAJ133		
IP6832FB FWD Primer		
IP6832BF1F		
IP6832BF1R		
IP6832RV4 Primer		
IP6832 FWD5 Primer		
C2BF.AAJ134		
IP6832 FWD5 Primer (Dup.)		
IP6832BF2F		
IP6832BF2R		
IP6832RV5 Primer		
IP6832BF3R		
IP6832RV6 Primer		
IP6832 FWD7 Primer		
IP6832BF4F		
IP6832BF4R		
IP6832RV7 Primer		
<i>consensus</i>		G A T G C G C T T T G G C G A G C T A A A A G A A A A A A A A A G T A A G A G A G A G G C C G G A A G C C C G A A G C C A A A A A A
		1290      1300      1310      1320      1330      1340      1350      1360      1370      1380
scaff0104435 f...IPk61.scaf5eq		A C A A A A G C A G C A A A A A A C A A A A A A A A A A G A A A A A A A A A A G A A G C C A A A A C C A G A G A A C A A A A A
IP6832 RAW		A C A A A A G C A G C A A A A A A C A A A A A A A A A A G A A A A A A A A A A G A A G C C A A A A C C A G A G A A C A A A A A
IPUL1_8832 + A1982		A C A A A A G C A G C A A A A A A C A A A A A A A A A A G A A A A A A A A A A G A A G C C A A A A C C A G A G A A C A A A A A
IP6832FWD1 Primer		
IP6832AF2F		A C A A A A G C A G C A A A A A A C A A A A A A A A A A G A A A A A A A A A A G A A G C C A A A A C C A G A G A A C A A A A A
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IP6832RV1 Primer		
IP6832FB FWD Primer (Dup)		
C2AF.AAJ133		
IP6832FB FWD Primer		
IP6832BF1F		
IP6832BF1R		
IP6832RV4 Primer		
IP6832 FWD5 Primer		
C2BF.AAJ134		
IP6832 FWD5 Primer (Dup.)		
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IP6832RV5 Primer		
IP6832BF3R		
IP6832RV6 Primer		
IP6832 FWD7 Primer		
IP6832BF4F		
IP6832BF4R		
IP6832RV7 Primer		
<i>consensus</i>		A C A A A A G C A G C A A A A A A C A A A A A A A A A A G A A A A A A A A A A G A A G C C A A A A C C A G A G A A C A A A A A

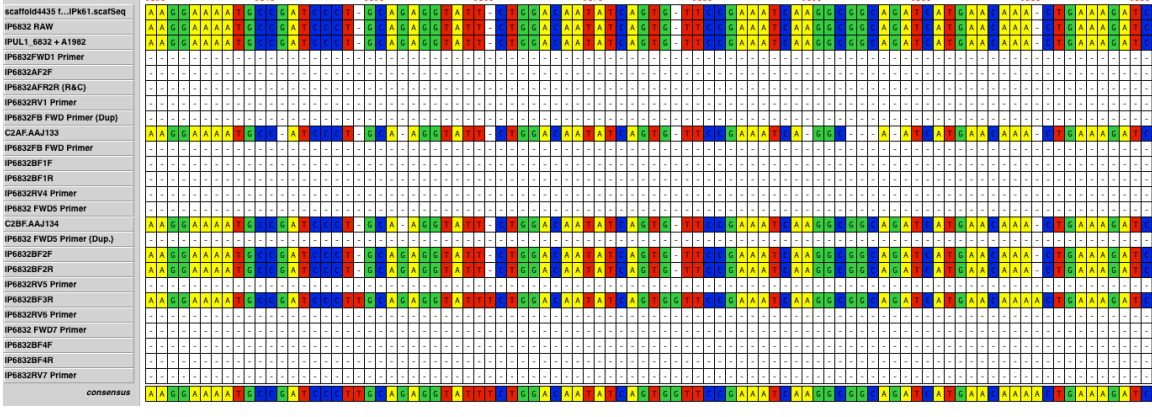
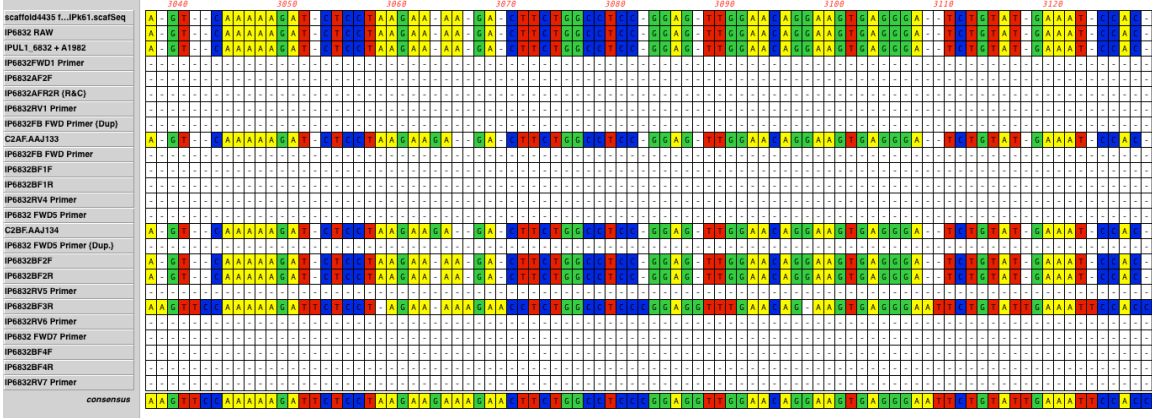
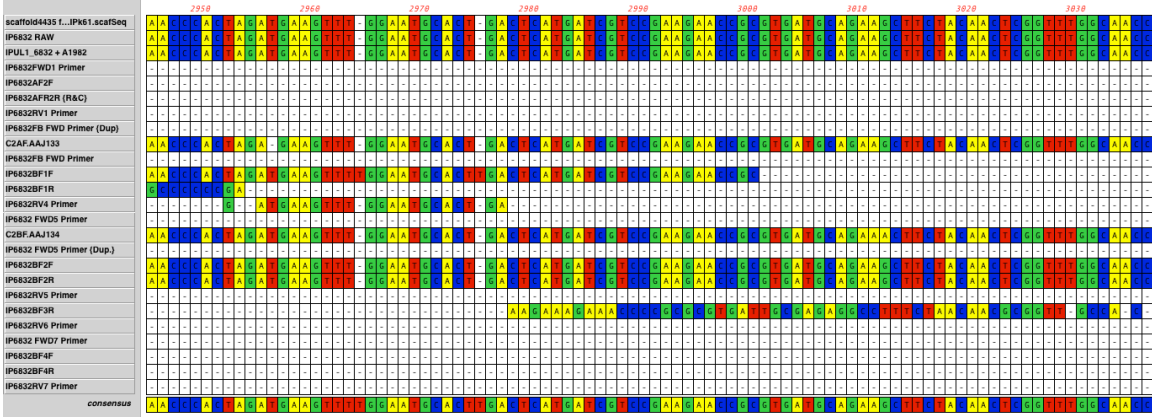
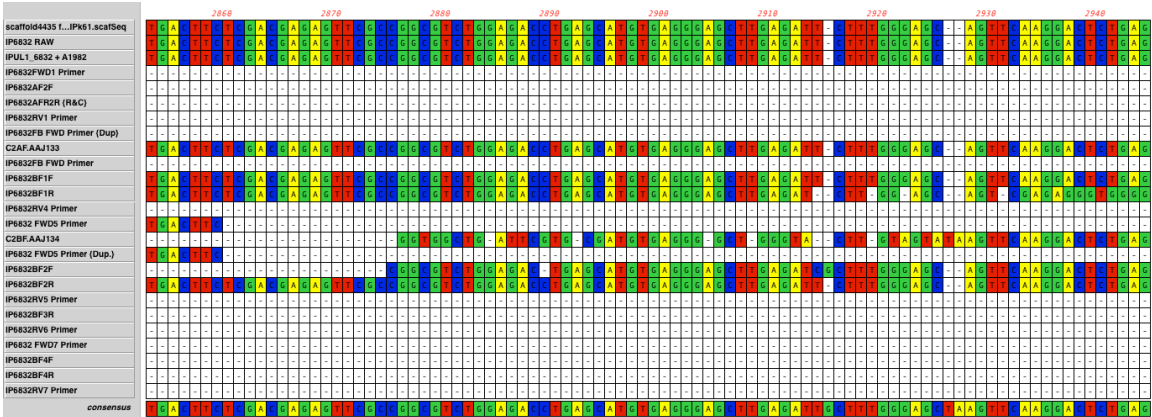




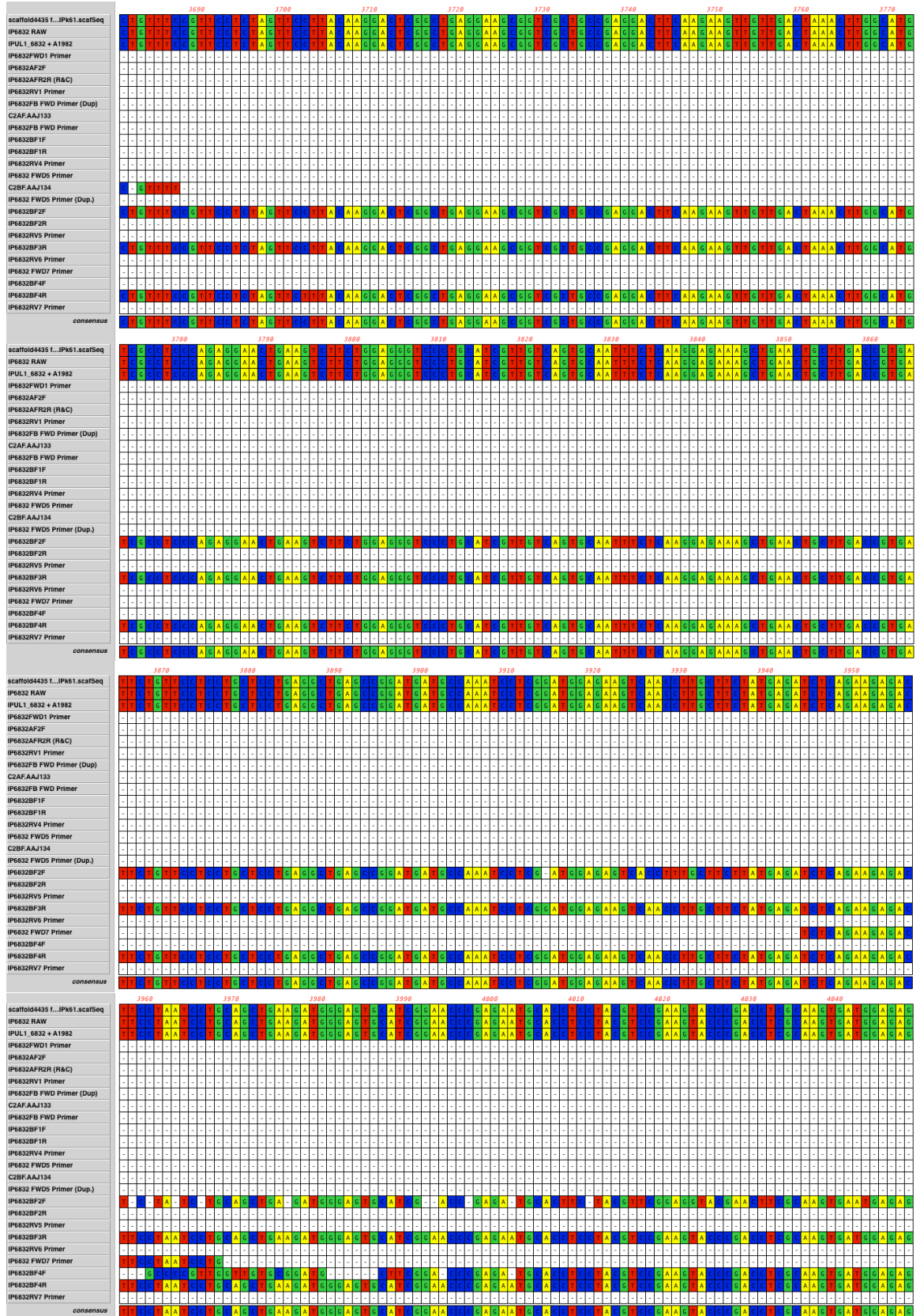








Genomic alignment table for scaffold04435 t...IPK61.acaf5eq. The table displays sequence alignments for various primer pairs (IP632 RAW, IPUL1, IP632FWD1, IP632AF2F, IP632AFR2R, IP632RV1, IP632FB FWD, C2AF.AA133, IP632FB FWD, IP632BF1F, IP632BF1R, IP632RV4, IP632 FWD5, C2BF.AA134, IP632 FWD5, IP632BF2F, IP632BF2R, IP632RV5, IP632BF3R, IP632RV6, IP632 FWD7, IP632BF4F, IP632BF4R, IP632RV7) against a reference sequence. The reference sequence is shown in the 'consensus' row at the bottom of each section. Nucleotides are color-coded: Adenine (A) in blue, Guanine (G) in red, Cytosine (C) in green, and Thymine (T) in yellow. Gaps are represented by dashes (-). The table is divided into three sections with coordinates 3228-3310, 3320-3400, and 3500-3580. Each section includes primer names and their corresponding alignment positions.



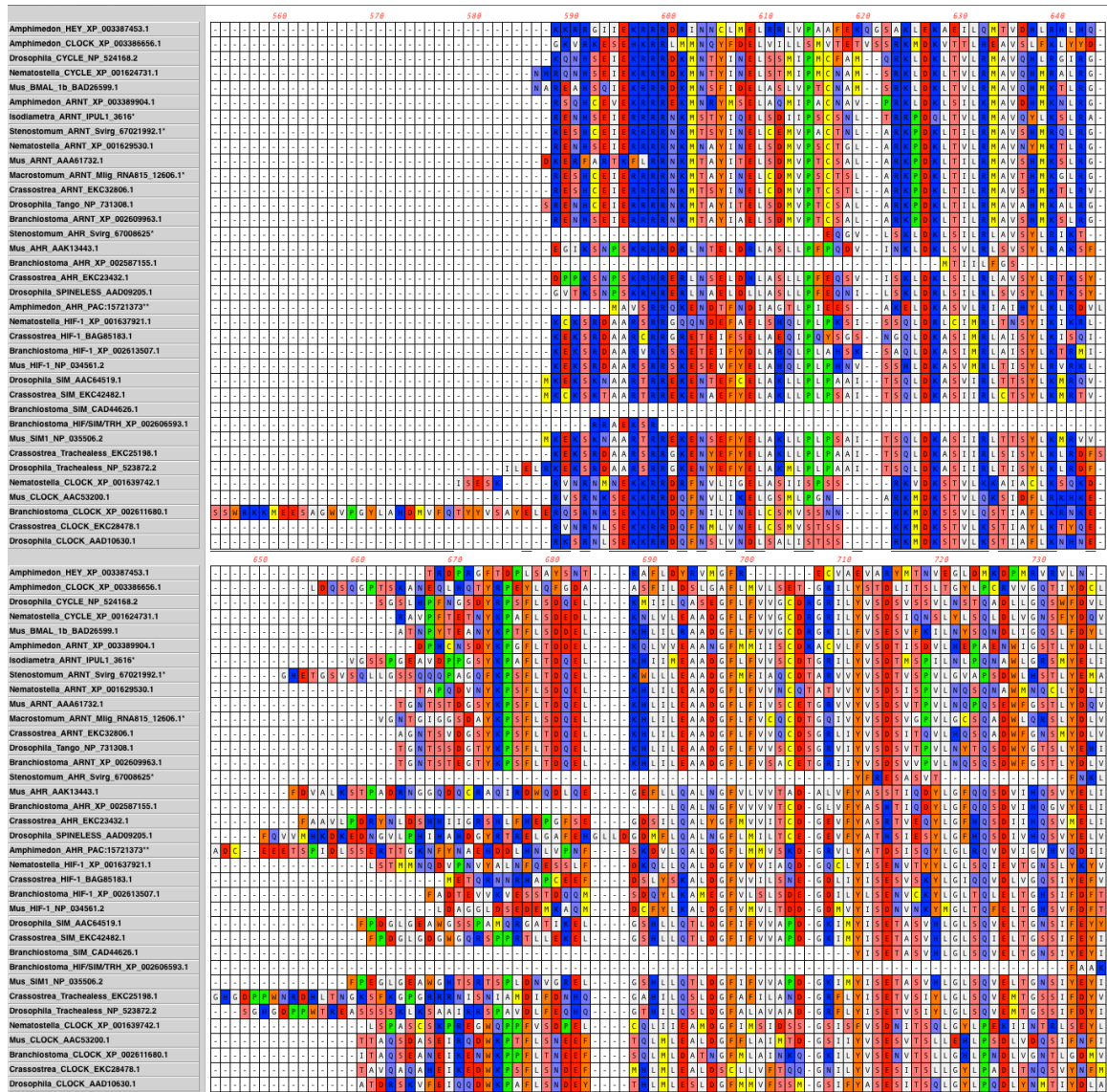
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IP6832FWD1 Primer	
IP6832AF2F	
IP6832AFR2R (R&C)	
IP6832RV1 Primer	
IP6832FB FWD Primer (Dup)	
C2AF_AA133	
IP6832FB FWD Primer	
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IP6832BF4F	
IP6832BF4R	
IP6832RV7 Primer	
consensus	
scaffold04435 f...IPK61.scafSeq	
IP6832 RAW	4150 4160 4170 4180 4190 4200 4210 4220 4230 4240
IPUL1_6832 + A1982	
IP6832FWD1 Primer	
IP6832AF2F	
IP6832AFR2R (R&C)	
IP6832RV1 Primer	
IP6832FB FWD Primer (Dup)	
C2AF_AA133	
IP6832FB FWD Primer	
IP6832BF1F	
IP6832BF1R	
IP6832RV4 Primer	
IP6832 FWD5 Primer	
C2BF_AA134	
IP6832 FWD5 Primer (Dup.)	
IP6832BF2F	
IP6832BF2R	
IP6832RV5 Primer	
IP6832BF3R	
IP6832RV6 Primer	
IP6832 FWD7 Primer	
IP6832BF4F	
IP6832BF4R	
IP6832RV7 Primer	
consensus	
scaffold04435 f...IPK61.scafSeq	
IP6832 RAW	4240 4250 4260 4270 4280 4290 4300 4310 4320
IPUL1_6832 + A1982	
IP6832FWD1 Primer	
IP6832AF2F	
IP6832AFR2R (R&C)	
IP6832RV1 Primer	
IP6832FB FWD Primer (Dup)	
C2AF_AA133	
IP6832FB FWD Primer	
IP6832BF1F	
IP6832BF1R	
IP6832RV4 Primer	
IP6832 FWD5 Primer	
C2BF_AA134	
IP6832 FWD5 Primer (Dup.)	
IP6832BF2F	
IP6832BF2R	
IP6832RV5 Primer	
IP6832BF3R	
IP6832RV6 Primer	
IP6832 FWD7 Primer	
IP6832BF4F	
IP6832BF4R	
IP6832RV7 Primer	
consensus	
scaffold04435 f...IPK61.scafSeq	
IP6832 RAW	4330 4340 4350 4360 4370 4380 4390 4400 4410
IPUL1_6832 + A1982	
IP6832FWD1 Primer	
IP6832AF2F	
IP6832AFR2R (R&C)	
IP6832RV1 Primer	
IP6832FB FWD Primer (Dup)	
C2AF_AA133	
IP6832FB FWD Primer	
IP6832BF1F	
IP6832BF1R	
IP6832RV4 Primer	
IP6832 FWD5 Primer	
C2BF_AA134	
IP6832 FWD5 Primer (Dup.)	
IP6832BF2F	
IP6832BF2R	
IP6832RV5 Primer	
IP6832BF3R	
IP6832RV6 Primer	
IP6832 FWD7 Primer	
IP6832BF4F	
IP6832BF4R	
IP6832RV7 Primer	
consensus	

4420	4430	4440	4450	4460	4470	4480	4490	4500
scaff0104435 f...IPK61.scafSeq								
IP632 RAW								
IPUL1_6832 + A1982								
IP632FWD1 Primer								
IP632AF2F								
IP632AFR2R (R&C)								
IP632RV1 Primer								
IP632FB FWD Primer (Dup)								
C2AF.AAJ133								
IP632FB FWD Primer								
IP632BF1F								
IP632BF1R								
IP632RV4 Primer								
IP632 FWDS Primer								
C2BF.AAJ134								
IP632 FWDS Primer (Dup.)								
IP632BF2F								
IP632BF2R								
IP632RV5 Primer								
IP632BF3R								
IP632RV6 Primer								
IP632FW7 Primer								
IP632BF4F								
IP632BF4R								
IP632RV7 Primer								
consensus								
4510	4520	4530	4540	4550	4560	4570	4580	4590
scaff0104435 f...IPK61.scafSeq								
IP632 RAW								
IPUL1_6832 + A1982								
IP632FWD1 Primer								
IP632AF2F								
IP632AFR2R (R&C)								
IP632RV1 Primer								
IP632FB FWD Primer (Dup)								
C2AF.AAJ133								
IP632FB FWD Primer								
IP632BF1F								
IP632BF1R								
IP632RV4 Primer								
IP632 FWDS Primer								
C2BF.AAJ134								
IP632 FWDS Primer (Dup.)								
IP632BF2F								
IP632BF2R								
IP632RV5 Primer								
IP632BF3R								
IP632RV6 Primer								
IP632FW7 Primer								
IP632BF4F								
IP632BF4R								
IP632RV7 Primer								
consensus								
4610	4620	4630	4640	4650	4660	4670	4680	4690
scaff0104435 f...IPK61.scafSeq								
IP632 RAW								
IPUL1_6832 + A1982								
IP632FWD1 Primer								
IP632AF2F								
IP632AFR2R (R&C)								
IP632RV1 Primer								
IP632FB FWD Primer (Dup)								
C2AF.AAJ133								
IP632FB FWD Primer								
IP632BF1F								
IP632BF1R								
IP632RV4 Primer								
IP632 FWDS Primer								
C2BF.AAJ134								
IP632 FWDS Primer (Dup.)								
IP632BF2F								
IP632BF2R								
IP632RV5 Primer								
IP632BF3R								
IP632RV6 Primer								
IP632FW7 Primer								
IP632BF4F								
IP632BF4R								
IP632RV7 Primer								
consensus								
4700	4710	4720	4730	4740	4750	4760		
scaff0104435 f...IPK61.scafSeq								
IP632 RAW								
IPUL1_6832 + A1982								
IP632FWD1 Primer								
IP632AF2F								
IP632AFR2R (R&C)								
IP632RV1 Primer								
IP632FB FWD Primer (Dup)								
C2AF.AAJ133								
IP632FB FWD Primer								
IP632BF1F								
IP632BF1R								
IP632RV4 Primer								
IP632 FWDS Primer								
C2BF.AAJ134								
IP632 FWDS Primer (Dup.)								
IP632BF2F								
IP632BF2R								
IP632RV5 Primer								
IP632BF3R								
IP632RV6 Primer								
IP632FW7 Primer								
IP632BF4F								
IP632BF4R								
IP632RV7 Primer								
consensus								

## 9.6 Full Alignments and Trees

### 9.6A bHLH-PAS Alignment

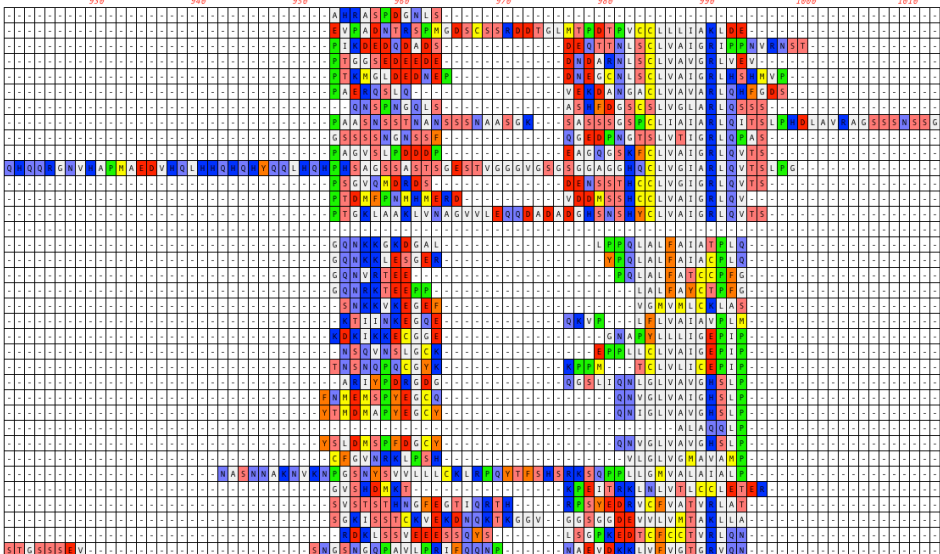
Portion containing the bHLH and PAS domains



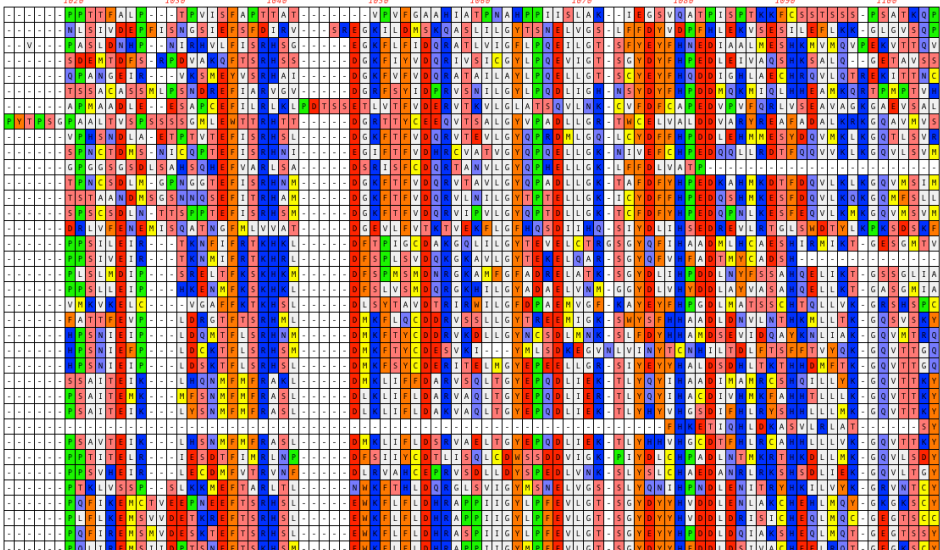
Amphimedon_HEY_XP_003387453.1	748	750	760	770	780	790	800	810	820	
Amphimedon_CLOCK_XP_003386656.1										
Drosophila_CYCLE_NP_524168.2										
Nematostella_CYCLE_XP_001624731.1										
Mus_BMAL1b_BAD26599.1										
Amphimedon_ARNT_XP_003389904.1										
Isoadiametra_ARNT_IPUL1_3616*										
Stenostomum_ARNT_Svirg_67021992.1*										
Nematostella_ARNT_XP_001629530.1										
Mus_ARNT_AA61732.1										
Macrostromum_ARNT_Mlig_RNA815_12606.1*										
Crassostrea_ARNT_EKC2486.1										
Drosophila_Tango_NP_731308.1										
Branchiostoma_ARNT_XP_002609963.1										
Stenostomum_AHR_Svirg_67008625*										
Mus_AHR_AAK13443.1										
Branchiostoma_AHR_XP_002587155.1										
Crassostrea_AHR_EKC23432.1										
Drosophila_SPINELESS_AAD09205.1										
Amphimedon_AHR_PAC:15721373**										
Nematostella_HIF-1_XP_001637921.1										
Crassostrea_HIF-1_BAG85183.1										
Branchiostoma_HIF-1_XP_002613507.1										
Mus_HIF-1_NP_034561.2										
Drosophila_SIM_AAC64519.1										
Crassostrea_SIM_EKC42482.1										
Branchiostoma_SIM_CAD44626.1										
Branchiostoma_HIF/SIM/TRH_XP_002606593.1										
Mus_SIM1_NP_035506.2										
Crassostrea_Tracheales_EKC25198.1										
Drosophila_Tracheales_NP_523872.2										
Nematostella_CLOCK_XP_001639742.1										
Mus_CLOCK_AAC53200.1										
Branchiostoma_CLOCK_XP_002611880.1										
Crassostrea_CLOCK_EKC28476.1										
Drosophila_CLOCK_AAD10830.1										
Amphimedon_HEY_XP_003387453.1	810	840	850	860	870	880	890	900	910	920
Amphimedon_CLOCK_XP_003386656.1										
Drosophila_CYCLE_NP_524168.2										
Nematostella_CYCLE_XP_001624731.1										
Mus_BMAL1b_BAD26599.1										
Amphimedon_ARNT_XP_003389904.1										
Isoadiametra_ARNT_IPUL1_3616*										
Stenostomum_ARNT_Svirg_67021992.1*										
Nematostella_ARNT_XP_001629530.1										
Mus_ARNT_AA61732.1										
Macrostromum_ARNT_Mlig_RNA815_12606.1*										
Crassostrea_ARNT_EKC2486.1										
Drosophila_Tango_NP_731308.1										
Branchiostoma_ARNT_XP_002609963.1										
Stenostomum_AHR_Svirg_67008625*										
Mus_AHR_AAK13443.1										
Branchiostoma_AHR_XP_002587155.1										
Crassostrea_AHR_EKC23432.1										
Drosophila_SPINELESS_AAD09205.1										
Amphimedon_AHR_PAC:15721373**										
Nematostella_HIF-1_XP_001637921.1										
Crassostrea_HIF-1_BAG85183.1										
Branchiostoma_HIF-1_XP_002613507.1										
Mus_HIF-1_NP_034561.2										
Drosophila_SIM_AAC64519.1										
Crassostrea_SIM_EKC42482.1										
Branchiostoma_SIM_CAD44626.1										
Branchiostoma_HIF/SIM/TRH_XP_002606593.1										
Mus_SIM1_NP_035506.2										
Crassostrea_Tracheales_EKC25198.1										
Drosophila_Tracheales_NP_523872.2										
Nematostella_CLOCK_XP_001639742.1										
Mus_CLOCK_AAC53200.1										
Branchiostoma_CLOCK_XP_002611880.1										
Crassostrea_CLOCK_EKC28476.1										
Drosophila_CLOCK_AAD10830.1										



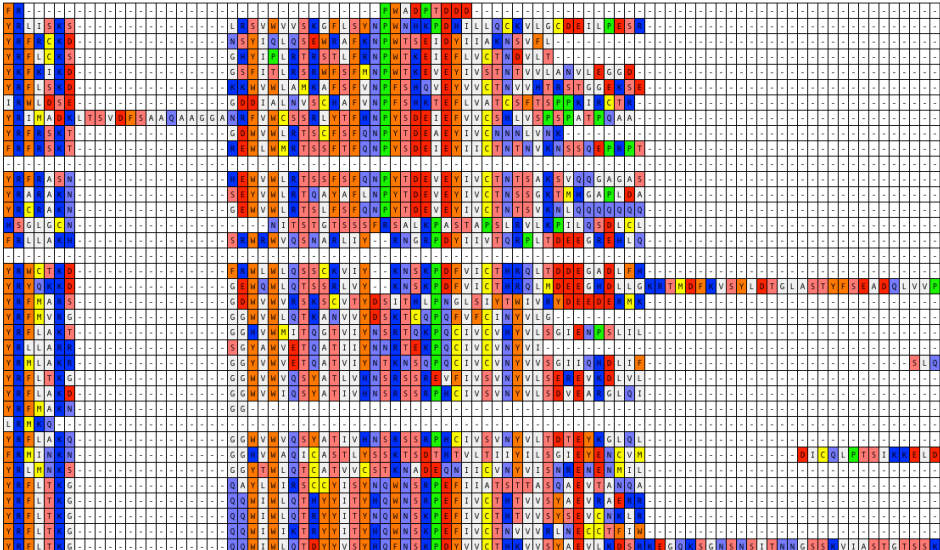
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 Amphimedon\_CLOCK\_XP\_003386656.1  
 Drosophila\_CYCLE\_NP\_524168.2  
 Nematostella\_CYCLE\_XP\_001624731.1  
 Mus\_BMAL1b\_BAD26599.1  
 Amphimedon\_ARNT\_XP\_003389904.1  
 Isodiametra\_ARNT\_IPUL1\_3616\*  
 Stenostomum\_ARNT\_Svrg\_67021992.1\*  
 Nematostella\_ARNT\_XP\_001629530.1  
 Mus\_ARNT\_AAA61732.1  
 Macrostomum\_ARNT\_Mlig\_RNA815\_12606.1\*  
 Crassostrea\_ARNT\_EKC32806.1  
 Drosophila\_Tango\_NP\_731308.1  
 Branchiostoma\_ARNT\_XP\_002609963.1  
 Stenostomum\_AHR\_Svrg\_67008625\*  
 Mus\_AHR\_AAK13443.1  
 Branchiostoma\_AHR\_XP\_002587155.1  
 Crassostrea\_AHR\_EKC23432.1  
 Drosophila\_SPINELESS\_AAD09205.1  
 Amphimedon\_AHR\_PAC:157121373\*\*  
 Nematostella\_HIF-1\_XP\_001637921.1  
 Crassostrea\_HIF-1\_BAG85183.1  
 Branchiostoma\_HIF-1\_XP\_002613507.1  
 Mus\_HIF-1\_NP\_034561.2  
 Drosophila\_SIM\_AAC64519.1  
 Crassostrea\_SIM\_EKC42482.1  
 Branchiostoma\_SIM\_CAD44626.1  
 Branchiostoma\_HIF/SIM/TRH\_XP\_002606593.1  
 Mus\_SIM1\_NP\_035506.2  
 Crassostrea\_Tracheales\_EKC25198.1  
 Drosophila\_Tracheales\_NP\_523872.2  
 Nematostella\_CLOCK\_XP\_001639742.1  
 Mus\_CLOCK\_AAC53200.1  
 Branchiostoma\_CLOCK\_XP\_002611680.1  
 Crassostrea\_CLOCK\_EKC28478.1  
 Drosophila\_CLOCK\_AAD10630.1



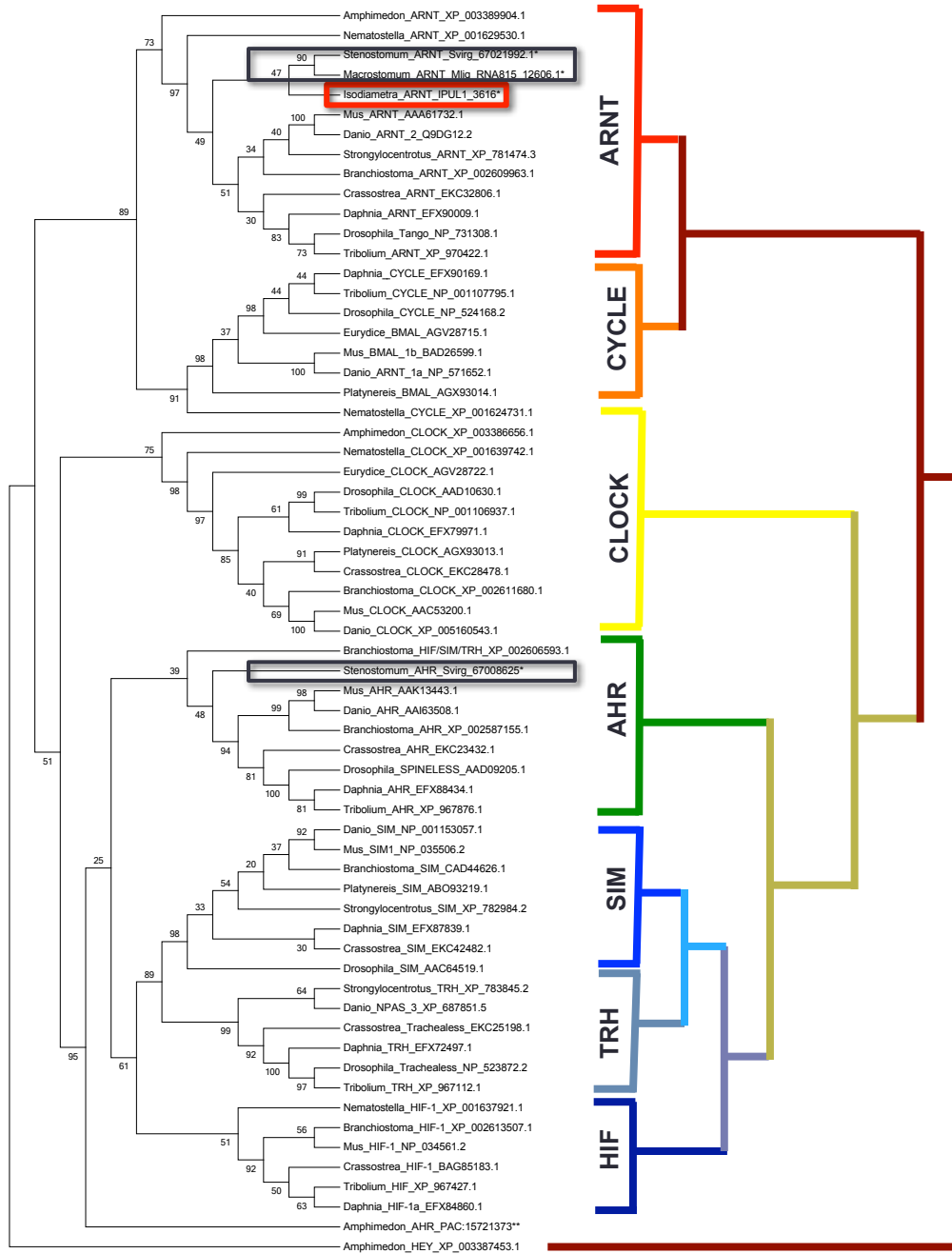
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 Amphimedon\_CLOCK\_XP\_003386656.1  
 Drosophila\_CYCLE\_NP\_524168.2  
 Nematostella\_CYCLE\_XP\_001624731.1  
 Mus\_BMAL1b\_BAD26599.1  
 Amphimedon\_ARNT\_XP\_003389904.1  
 Isodiametra\_ARNT\_IPUL1\_3616\*  
 Stenostomum\_ARNT\_Svrg\_67021992.1\*  
 Nematostella\_ARNT\_XP\_001629530.1  
 Mus\_ARNT\_AAA61732.1  
 Macrostomum\_ARNT\_Mlig\_RNA815\_12606.1\*  
 Crassostrea\_ARNT\_EKC32806.1  
 Drosophila\_Tango\_NP\_731308.1  
 Branchiostoma\_ARNT\_XP\_002609963.1  
 Stenostomum\_AHR\_Svrg\_67008625\*  
 Mus\_AHR\_AAK13443.1  
 Branchiostoma\_AHR\_XP\_002587155.1  
 Crassostrea\_AHR\_EKC23432.1  
 Drosophila\_SPINELESS\_AAD09205.1  
 Amphimedon\_AHR\_PAC:157121373\*\*  
 Nematostella\_HIF-1\_XP\_001637921.1  
 Crassostrea\_HIF-1\_BAG85183.1  
 Branchiostoma\_HIF-1\_XP\_002613507.1  
 Mus\_HIF-1\_NP\_034561.2  
 Drosophila\_SIM\_AAC64519.1  
 Crassostrea\_SIM\_EKC42482.1  
 Branchiostoma\_SIM\_CAD44626.1  
 Branchiostoma\_HIF/SIM/TRH\_XP\_002606593.1  
 Mus\_SIM1\_NP\_035506.2  
 Crassostrea\_Tracheales\_EKC25198.1  
 Drosophila\_Tracheales\_NP\_523872.2  
 Nematostella\_CLOCK\_XP\_001639742.1  
 Mus\_CLOCK\_AAC53200.1  
 Branchiostoma\_CLOCK\_XP\_002611680.1  
 Crassostrea\_CLOCK\_EKC28478.1  
 Drosophila\_CLOCK\_AAD10630.1



Amphimedon\_HEY\_XP\_003387453.1  
 Amphimedon\_CLOCK\_XP\_003386656.1  
 Drosophila\_CYCLE\_NP\_524168.2  
 Nematostella\_CYCLE\_XP\_001624731.1  
 Mus\_BMAL1b\_BAD26599.1  
 Amphimedon\_ARNT\_XP\_003389904.1  
 Isodiametra\_ARNT\_IPUL1\_3616\*  
 Stenostomum\_ARNT\_Svrg\_67021992.1\*  
 Nematostella\_ARNT\_XP\_001629530.1  
 Mus\_ARNT\_AAA61732.1  
 Macrostomum\_ARNT\_Mlig\_RNA815\_12606.1\*  
 Crassostrea\_ARNT\_EKC32806.1  
 Drosophila\_Tango\_NP\_731308.1  
 Branchiostoma\_ARNT\_XP\_002609963.1  
 Stenostomum\_AHR\_Svrg\_67008625\*  
 Mus\_AHR\_AAK13443.1  
 Branchiostoma\_AHR\_XP\_002587155.1  
 Crassostrea\_AHR\_EKC23432.1  
 Drosophila\_SPINELESS\_AAD09205.1  
 Amphimedon\_AHR\_PAC:157121373\*\*  
 Nematostella\_HIF-1\_XP\_001637921.1  
 Crassostrea\_HIF-1\_BAG85183.1  
 Branchiostoma\_HIF-1\_XP\_002613507.1  
 Mus\_HIF-1\_NP\_034561.2  
 Drosophila\_SIM\_AAC64519.1  
 Crassostrea\_SIM\_EKC42482.1  
 Branchiostoma\_SIM\_CAD44626.1  
 Branchiostoma\_HIF/SIM/TRH\_XP\_002606593.1  
 Mus\_SIM1\_NP\_035506.2  
 Crassostrea\_Tracheales\_EKC25198.1  
 Drosophila\_Tracheales\_NP\_523872.2  
 Nematostella\_CLOCK\_XP\_001639742.1  
 Mus\_CLOCK\_AAC53200.1  
 Branchiostoma\_CLOCK\_XP\_002611680.1  
 Crassostrea\_CLOCK\_EKC28478.1  
 Drosophila\_CLOCK\_AAD10630.1

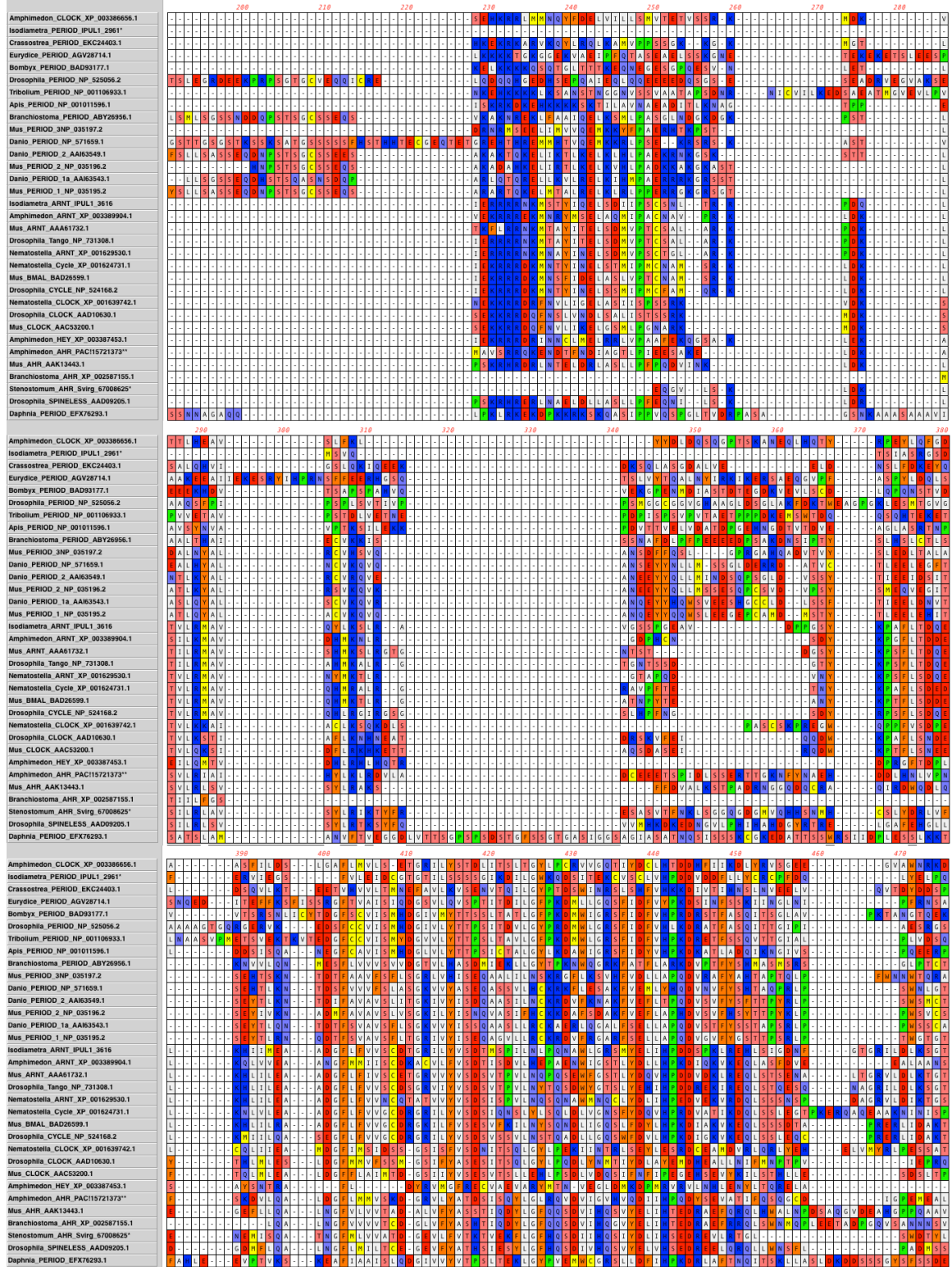


## 9.6B Full bHLH-PAS Tree

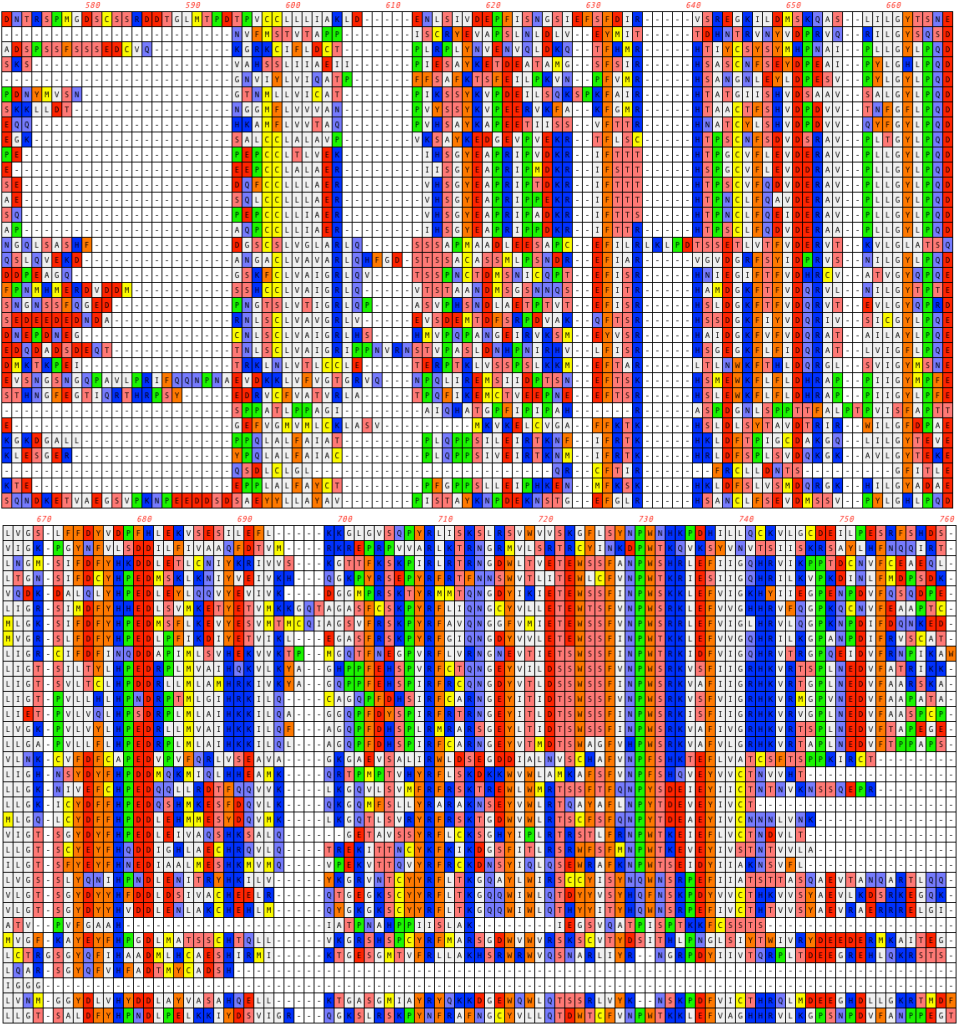


# 9.6C PERIOD Alignment

Portion containing PAS domains

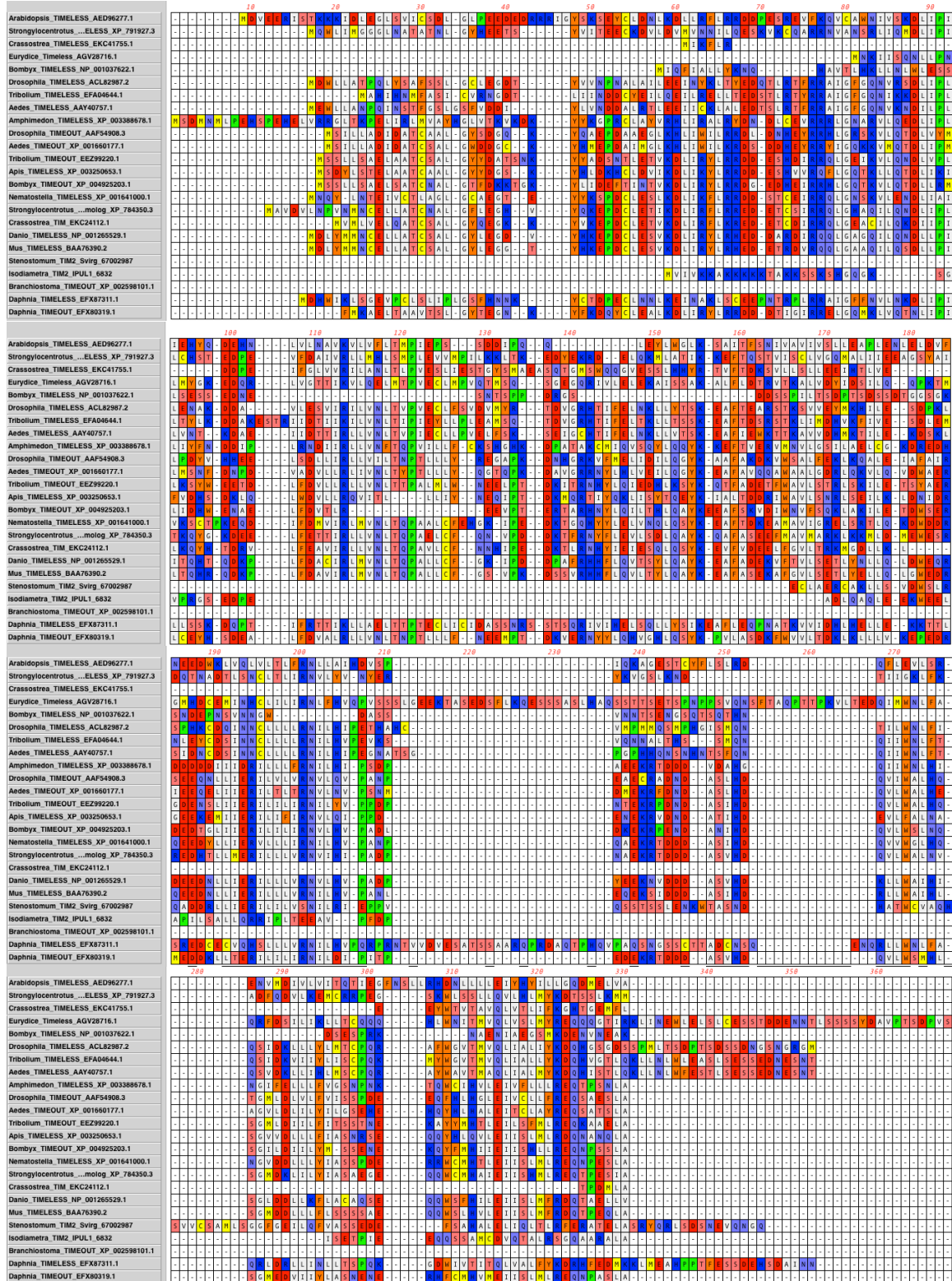


Amphimedon\_CLOCK\_XP\_00386656.1  
 Isodiametra\_PERIOD\_IPUL1\_2961\*  
 Crassostrea\_PERIOD\_EKC24403.1  
 Eurydice\_PERIOD\_AGV28714.1  
 Bombyx\_PERIOD\_BADR3177.1  
 Drosophila\_PERIOD\_NP\_525056.2  
 Tribolium\_PERIOD\_NP\_001106933.1  
 Apis\_PERIOD\_NP\_00111596.1  
 Branchiostoma\_PERIOD\_ABY28956.1  
 Mus\_PERIOD\_3NP\_035197.2  
 Danio\_PERIOD\_NP\_571659.1  
 Danio\_PERIOD\_2\_AA63549.1  
 Mus\_PERIOD\_2\_NP\_035196.2  
 Danio\_PERIOD\_1a\_AA63543.1  
 Mus\_PERIOD\_1\_NP\_035195.2  
 Isodiametra\_ARNT\_IPUL1\_3616  
 Amphimedon\_ARNT\_XP\_00389904.1  
 Mus\_ARNT\_AA61732.1  
 Drosophila\_Tango\_NP\_731308.1  
 Nematostella\_ARNT\_XP\_001624731.1  
 Nematostella\_Cycle\_XP\_001624731.1  
 Mus\_BMAL\_BAD26999.1  
 Drosophila\_CYCLE\_NP\_524168.2  
 Nematostella\_CLOCK\_XP\_001624731.1  
 Drosophila\_CLOCK\_AAD19630.1  
 Mus\_CLOCK\_AACS3200.1  
 Amphimedon\_HEY\_XP\_00387453.1  
 Amphimedon\_AHR\_PAC11572173\*\*  
 Mus\_AHR\_AAK13443.1  
 Branchiostoma\_AHR\_XP\_002587155.1  
 Stenostomum\_AHR\_Svirg\_67008625\*  
 Drosophila\_SPINELESS\_AAD09205.1  
 Daphnia\_PERIOD\_EFX76293.1

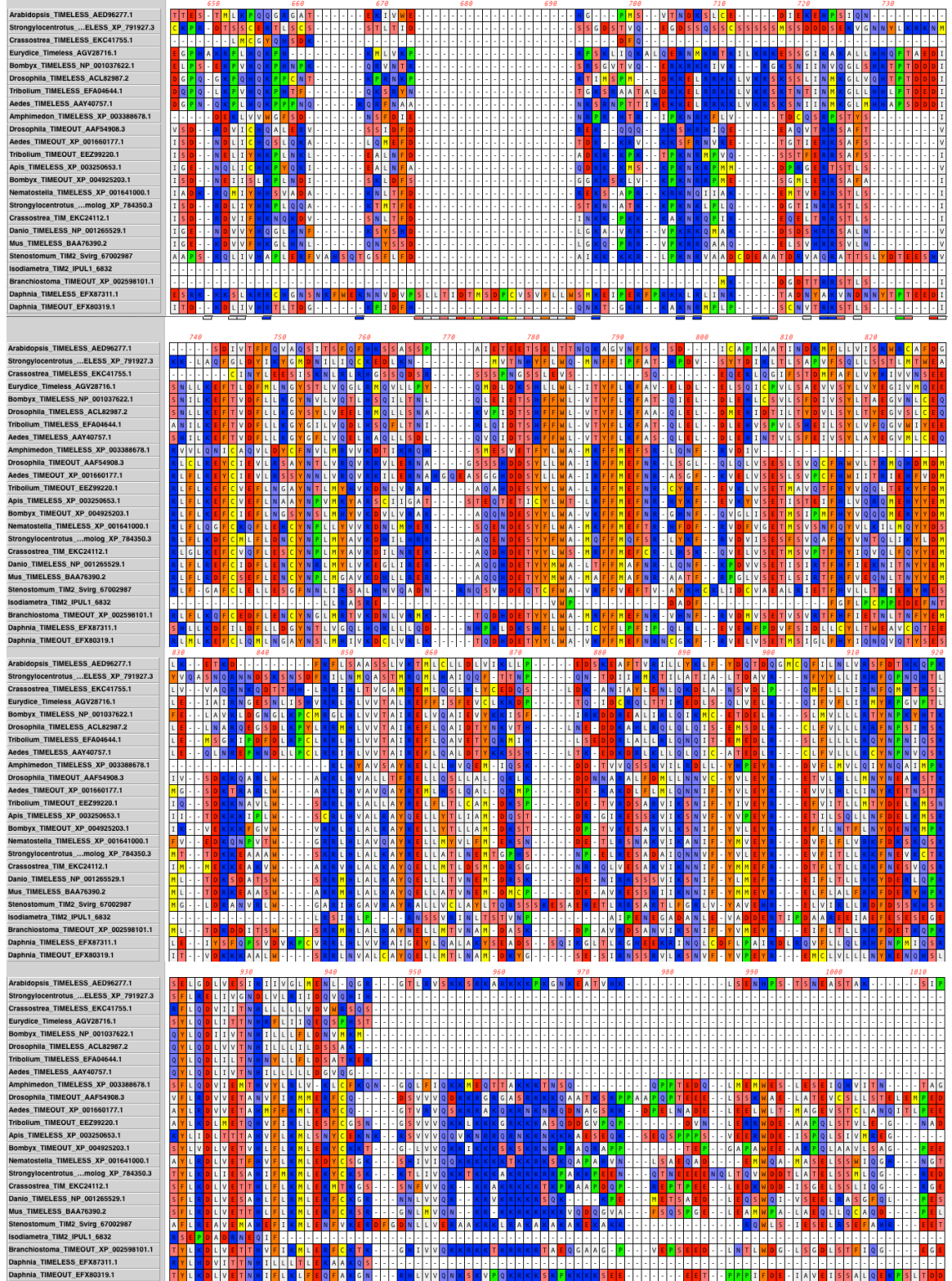


# 9.6D TIM Alignment

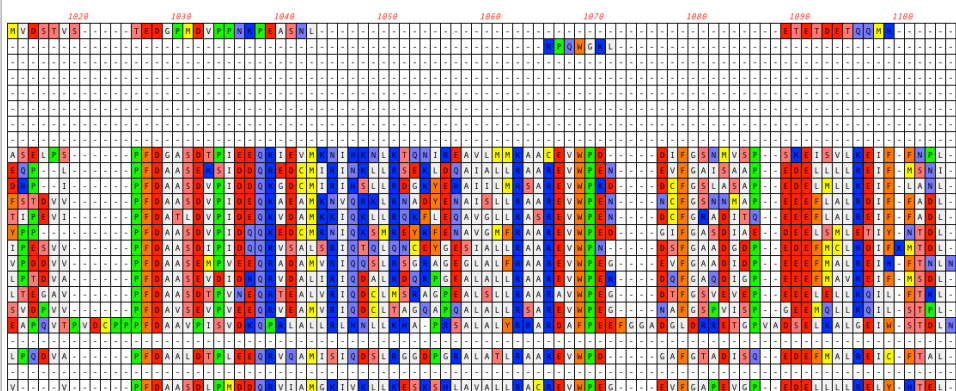
## Portion Containing TIM Domain



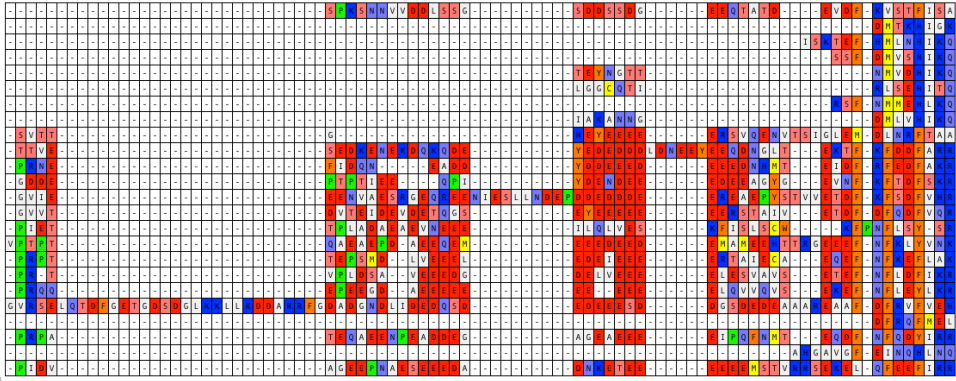
# Portion containing the TIM-C Domain



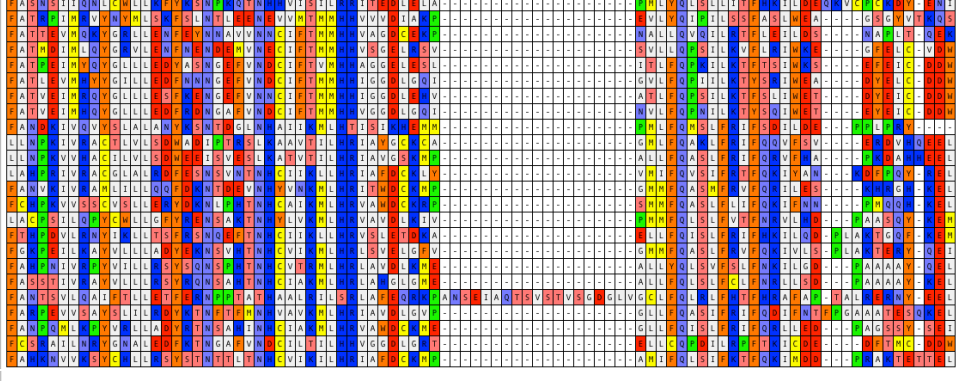
Arabidopsis TIMELESS\_AED96277.1  
 Strongylocentrotus...ELESS\_XP\_791927.3  
 Crassostrea TIMELESS\_EKC41755.1  
 Eurydice TIMELESS\_AGV28716.1  
 Bombyx TIMELESS\_NP\_001037622.1  
 Drosophila TIMELESS\_ACL82987.2  
 Tribolium TIMELESS\_EFA04644.1  
 Aedes TIMELESS\_AAY40757.1  
 Amphimedon TIMELESS\_XP\_003386678.1  
 Drosophila TIMEOUT\_AAF54908.3  
 Aedes TIMEOUT\_XP\_001660177.1  
 Tribolium TIMEOUT\_EEZ9220.1  
 Apis TIMELESS\_XP\_003250553.1  
 Bombyx TIMEOUT\_XP\_004925203.1  
 Nematostella TIMELESS\_XP\_001641000.1  
 Strongylocentrotus...molog\_XP\_784350.3  
 Crassostrea TIM\_EKC24112.1  
 Danio TIMELESS\_NP\_001265529.1  
 Mus TIMELESS\_BAA76390.2  
 Stenostomum TIM2\_Svirg\_67002987  
 Isodiametra TIM2\_IPUL1\_6832  
 Branchiostoma TIMEOUT\_XP\_002598101.1  
 Daphnia TIMELESS\_EFX87311.1  
 Daphnia TIMEOUT\_EFX80319.1



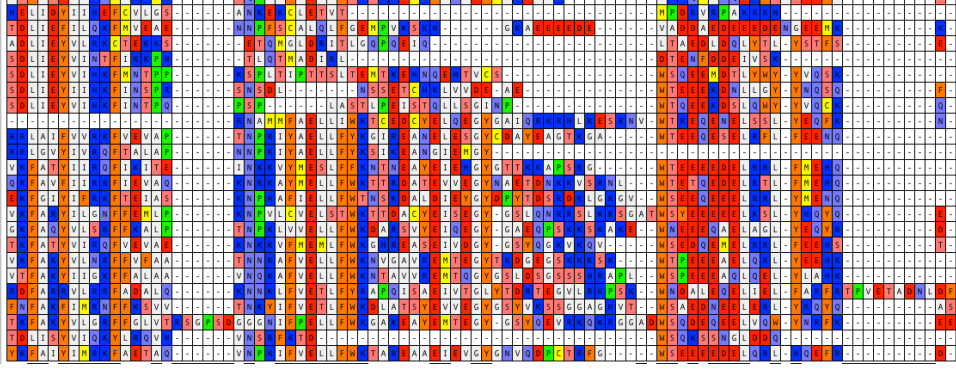
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 Strongylocentrotus...ELESS\_XP\_791927.3  
 Crassostrea TIMELESS\_EKC41755.1  
 Eurydice TIMELESS\_AGV28716.1  
 Bombyx TIMELESS\_NP\_001037622.1  
 Drosophila TIMELESS\_ACL82987.2  
 Tribolium TIMELESS\_EFA04644.1  
 Aedes TIMELESS\_AAY40757.1  
 Amphimedon TIMELESS\_XP\_003386678.1  
 Drosophila TIMEOUT\_AAF54908.3  
 Aedes TIMEOUT\_XP\_001660177.1  
 Tribolium TIMEOUT\_EEZ9220.1  
 Apis TIMELESS\_XP\_003250553.1  
 Bombyx TIMEOUT\_XP\_004925203.1  
 Nematostella TIMELESS\_XP\_001641000.1  
 Strongylocentrotus...molog\_XP\_784350.3  
 Crassostrea TIM\_EKC24112.1  
 Danio TIMELESS\_NP\_001265529.1  
 Mus TIMELESS\_BAA76390.2  
 Stenostomum TIM2\_Svirg\_67002987  
 Isodiametra TIM2\_IPUL1\_6832  
 Branchiostoma TIMEOUT\_XP\_002598101.1  
 Daphnia TIMELESS\_EFX87311.1  
 Daphnia TIMEOUT\_EFX80319.1



Arabidopsis TIMELESS\_AED96277.1  
 Strongylocentrotus...ELESS\_XP\_791927.3  
 Crassostrea TIMELESS\_EKC41755.1  
 Eurydice TIMELESS\_AGV28716.1  
 Bombyx TIMELESS\_NP\_001037622.1  
 Drosophila TIMELESS\_ACL82987.2  
 Tribolium TIMELESS\_EFA04644.1  
 Aedes TIMELESS\_AAY40757.1  
 Amphimedon TIMELESS\_XP\_003386678.1  
 Drosophila TIMEOUT\_AAF54908.3  
 Aedes TIMEOUT\_XP\_001660177.1  
 Tribolium TIMEOUT\_EEZ9220.1  
 Apis TIMELESS\_XP\_003250553.1  
 Bombyx TIMEOUT\_XP\_004925203.1  
 Nematostella TIMELESS\_XP\_001641000.1  
 Strongylocentrotus...molog\_XP\_784350.3  
 Crassostrea TIM\_EKC24112.1  
 Danio TIMELESS\_NP\_001265529.1  
 Mus TIMELESS\_BAA76390.2  
 Stenostomum TIM2\_Svirg\_67002987  
 Isodiametra TIM2\_IPUL1\_6832  
 Branchiostoma TIMEOUT\_XP\_002598101.1  
 Daphnia TIMELESS\_EFX87311.1  
 Daphnia TIMEOUT\_EFX80319.1



Arabidopsis TIMELESS\_AED96277.1  
 Strongylocentrotus...ELESS\_XP\_791927.3  
 Crassostrea TIMELESS\_EKC41755.1  
 Eurydice TIMELESS\_AGV28716.1  
 Bombyx TIMELESS\_NP\_001037622.1  
 Drosophila TIMELESS\_ACL82987.2  
 Tribolium TIMELESS\_EFA04644.1  
 Aedes TIMELESS\_AAY40757.1  
 Amphimedon TIMELESS\_XP\_003386678.1  
 Drosophila TIMEOUT\_AAF54908.3  
 Aedes TIMEOUT\_XP\_001660177.1  
 Tribolium TIMEOUT\_EEZ9220.1  
 Apis TIMELESS\_XP\_003250553.1  
 Bombyx TIMEOUT\_XP\_004925203.1  
 Nematostella TIMELESS\_XP\_001641000.1  
 Strongylocentrotus...molog\_XP\_784350.3  
 Crassostrea TIM\_EKC24112.1  
 Danio TIMELESS\_NP\_001265529.1  
 Mus TIMELESS\_BAA76390.2  
 Stenostomum TIM2\_Svirg\_67002987  
 Isodiametra TIM2\_IPUL1\_6832  
 Branchiostoma TIMEOUT\_XP\_002598101.1  
 Daphnia TIMELESS\_EFX87311.1  
 Daphnia TIMEOUT\_EFX80319.1



	1398	1408	1418	1428	1438	1448	1458	1468	1478
Arabidopsis.TIMELESS.AED96277.1	-	-	-	-	-	-	-	-	-
Strongylocentrotus....LESS.XP.791927.3	-	-	-	-	-	-	-	-	-
Crassostrea.TIMELESS.EKC41755.1	-	-	-	-	-	-	-	-	-
Eurydice.TIMELESS.AGV28716.1	-	-	-	-	-	-	-	-	-
Bombyx.TIMELESS.NP.001037622.1	-	-	-	-	-	-	-	-	-
Drosophila.TIMELESS.ACL82987.2	-	-	-	-	-	-	-	-	-
Tribolium.TIMELESS.EF404644.1	-	-	-	-	-	-	-	-	-
Aedes.TIMELESS.AAY40757.1	-	-	-	-	-	-	-	-	-
Amphimedon.TIMELESS.XP.00338678.1	-	-	-	-	-	-	-	-	-
Drosophila.TIMEOUT.AAF54908.3	-	-	-	-	-	-	-	-	-
Aedes.TIMEOUT.XP.001660177.1	-	-	-	-	-	-	-	-	-
Tribolium.TIMEOUT.EEZ99220.1	-	-	-	-	-	-	-	-	-
Apis.TIMELESS.XP.003250653.1	-	-	-	-	-	-	-	-	-
Bombyx.TIMEOUT.XP.004925203.1	-	-	-	-	-	-	-	-	-
Nematostella.TIMELESS.XP.001641000.1	-	-	-	-	-	-	-	-	-
Strongylocentrotus....molog.XP.784350.3	-	-	-	-	-	-	-	-	-
Crassostrea.TIM.EKC24112.1	-	-	-	-	-	-	-	-	-
Danio.TIMELESS.NP.001265529.1	-	-	-	-	-	-	-	-	-
Mus.TIMELESS.BAA76399.2	-	-	-	-	-	-	-	-	-
Stenostomum.TIM2.Svirg.67002987	-	-	-	-	-	-	-	-	-
Isodiametra.TIM2.IPUL1.6832	-	-	-	-	-	-	-	-	-
Branchiostoma.TIMEOUT.XP.002598101.1	-	-	-	-	-	-	-	-	-
Daphnia.TIMELESS.EFX87311.1	-	-	-	-	-	-	-	-	-
Daphnia.TIMEOUT.EFX80319.1	-	-	-	-	-	-	-	-	-

	1478	1488	1498	1508	1518	1528	1538	1548	1558	1568
Arabidopsis.TIMELESS.AED96277.1	-	-	-	-	-	-	-	-	-	-
Strongylocentrotus....LESS.XP.791927.3	-	-	-	-	-	-	-	-	-	-
Crassostrea.TIMELESS.EKC41755.1	-	-	-	-	-	-	-	-	-	-
Eurydice.TIMELESS.AGV28716.1	-	-	-	-	-	-	-	-	-	-
Bombyx.TIMELESS.NP.001037622.1	-	-	-	-	-	-	-	-	-	-
Drosophila.TIMELESS.ACL82987.2	-	-	-	-	-	-	-	-	-	-
Tribolium.TIMELESS.EF404644.1	-	-	-	-	-	-	-	-	-	-
Aedes.TIMELESS.AAY40757.1	-	-	-	-	-	-	-	-	-	-
Amphimedon.TIMELESS.XP.00338678.1	-	-	-	-	-	-	-	-	-	-
Drosophila.TIMEOUT.AAF54908.3	-	-	-	-	-	-	-	-	-	-
Aedes.TIMEOUT.XP.001660177.1	-	-	-	-	-	-	-	-	-	-
Tribolium.TIMEOUT.EEZ99220.1	-	-	-	-	-	-	-	-	-	-
Apis.TIMELESS.XP.003250653.1	-	-	-	-	-	-	-	-	-	-
Bombyx.TIMEOUT.XP.004925203.1	-	-	-	-	-	-	-	-	-	-
Nematostella.TIMELESS.XP.001641000.1	-	-	-	-	-	-	-	-	-	-
Strongylocentrotus....molog.XP.784350.3	-	-	-	-	-	-	-	-	-	-
Crassostrea.TIM.EKC24112.1	-	-	-	-	-	-	-	-	-	-
Danio.TIMELESS.NP.001265529.1	-	-	-	-	-	-	-	-	-	-
Mus.TIMELESS.BAA76399.2	-	-	-	-	-	-	-	-	-	-
Stenostomum.TIM2.Svirg.67002987	-	-	-	-	-	-	-	-	-	-
Isodiametra.TIM2.IPUL1.6832	-	-	-	-	-	-	-	-	-	-
Branchiostoma.TIMEOUT.XP.002598101.1	-	-	-	-	-	-	-	-	-	-
Daphnia.TIMELESS.EFX87311.1	-	-	-	-	-	-	-	-	-	-
Daphnia.TIMEOUT.EFX80319.1	-	-	-	-	-	-	-	-	-	-