# *Period* and *Clock* as Candidate Genes for *Pdd*, a Major Factor of Voltinism Differences in the European corn borer, *Ostrinia nubilalis*

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# Abstract

Closely related races, strains, or species form the foundation of speciation research. In the European corn borer moth (*Ostrinia nubilalis*), closely related strains are characterized by differences in sex pheromone communication and voltinism (generations per year), both of which contribute to reproductive isolation and the speciation process. Previous studies found that a molecular marker on the sex (Z) chromosome, *Tpi,* exhibits significant allelic differentiation between strains. Evolutionary theory predicts such patterns at genome regions harboring loci for reproductive isolation. Indeed, *Tpi* is closely linked to *Pdd*, a gene or set of genes that affect postdiapause development time (PDD), a major factor of voltinism. Based on the genome of *Bombyx mori* (silkmoth), two genes, *Period* and *Clock*, were identified as potential candidates for the *Pdd* gene. To determine the molecular basis for postdiapause development time, the inheritance patterns of PDD and the genotypes of *Period* and *Clock* were determined. Genotyping results showed that both *Period* and *Clock* genes are candidates for differences in the PDD phenotype, as very few recombinants were discovered. However, a genealogical analysis of *Clock* revealed no allelic differentiation between different voltine populations, suggesting that the causal locus for PDD is found elsewhere on the Z chromosome. Nevertheless, genetic variation at both *Clock* and *Period* may have important consequences for the extent of gene flow between populations.

# Introduction

The European corn borer (ECB), *Ostrinia nubilalis*, is a major agricultural pest in North America, affecting crops such as corn, potatoes, and cotton. ECB outbreaks in the United States cost approximately $2 billion each year in crop losses and control (USDA). Although the European corn borer is native to Europe, North Africa, and Western Asia (Beck 1987), the species is believed to be introduced into North America between 1909 and 1914 on broom corn imported from Hungary or Italy (Vinal 1917). The ECB has now spread throughout North America to most corn-producing areas east of the Rocky Mountains including north into Canada and south into Florida and Mexico (Mason et al. 1996). It is likely that multiple introductions occurred due to various ecotypes found within European corn borer populations, exhibiting different phenotypes (Showers 1993). For example, there are three European corn borer voltine ecotypes found in the United States: the northern univoltine (1 generation/year), central bivoltine (2 generations/year), and southern multivoltine (3-4 generations/year) (Showers 1993).

In addition to voltinism differences, the ECB consists of two behaviorally isolated strains that differ in the sex pheromone communication system. The two strains, named E and Z after their respective isomers, refer to the relative amount of (E) or (Z)-11-tetradecenyhl acetate found in the sex pheromone mixture (Klun et al. 1973). Isolation results from differential male response to the sex pheromone blend produced by females (Roelofs et al. 1987, Glover et al. 1990). Male pheromone response has a genetic factor, *Resp*, found on the ECB Z (sex) chromosome (Roelofs et al. 1987). In New York, there are three distinct races found sympatrically: univoltine Z, bivoltine E, and bivoltine Z (Roelofs et al. 1985).

Both temporal (voltinism) and behavioral (sex pheromone production and response) premating barriers are important in ECB populations, as they account for 75% of the total cumulative reproductive isolation (Dopman et al. 2010). However, these prezygotic barriers are

incomplete, and thus various strains or races of ECB have been difficult to distinguish. At the beginning stages of speciation, the genomes of such strains are variable in genealogies of different gene regions. Yet for speciation to occur, there must be reproductive isolation barriers that limit or prevent gene exchange eventually forming monophyletic or exclusive genetic groups (Coyne and Orr 2004). As a result, speciation genes (or markers closely linked to those barrier genes) are more likely to exhibit an exclusive relationship between recently diverged strains due to selective pressures.

Genetic surveys of allozymes (Harrison & Vawter 1977, Cianchi 1980), mitochondrial DNA (Marcon 1999) and a nuclear gene (Willett and Harrison 1999) have similarly found extensive shared genetic variation, but little differentiation. However, significant allele frequency differentiation has been found at one locus, a sex-linked gene that encodes the enzyme Triose Phosphate Isomerase (*Tpi*) (Glover et al. 1992, Dopman et al. 2005). Out of genealogies for three sex-linked loci (*Tpi*, *Ldh*, and *Kettin*), two autosomal loci, and one mitochondrial locus, only the genealogy for the *Tpi* locus showed the two pheromone strains to be nearly exclusive phylogenetic groups (Dopman et al. 2005). This pattern of exclusivity in *Tpi* demonstrates the absence of gene flow at this locus. In addition, *Tpi* maps to a position on the sex chromosome indistinguishable from the *Pdd* gene (postdiapause development) that is implicated in voltinism.

Voltinism (generations per year) is a premating barrier, as differences in flight periods temporally isolate adults from bivoltine and univoltine populations (Reolofs et al. 1985, Dopman et al. 2010). Although voltine differences are dependent on factors such as photoperiod and temperature, there is also a genetic component. More specifically, variation in voltinism in the ECB derives from the timing of larval diapause – a genetically determined state of suppressed development that can be induced by environmental factors (Beck, 1982). Like many insects,

ECB larvae diapause over winter and then pupate and emerge as adults in the spring and summer. The time to pupate under temperature and photoperiod conditions conducive to breaking diapause is termed postdiapause development time (PDD). Overwintering larvae of multivoltine populations break diapause earlier (shorter PDD) than larvae of univoltine populations (longer PDD) (McLeod 1976). In addition, ECB larvae undergo greater numbers of generations per year in the southern United States, where climatic conditions and cues such as greater photoperiod and light intensity allow for a longer breeding season (Palmer et al. 1985). However under controlled laboratory conditions, differences in voltinism exist between strains, suggesting an important genetic component in voltinism due to postdiapause development (PDD). Postdiapause development time has been found to be primarily controlled by a major gene or set of genes (*Pdd)* that is linked to *Tpi* on the sex chromosome (Glover et al. 1992).

As genetic differences affecting voltinism can reduce gene flow between sympatric populations of bivoltine and univoltine moths, the *Pdd* region of the ECB sex chromosome was further examined. Postdiapause development time is affected by photoperiod and circadian rhythm, thus we looked at two potential candidate genes for voltinism: *Period* and *Clock*. These two "clock" genes were identified in the *Bombyx mori* (silkworm) genome which likely has broad patterns of conserved synteny with ECB (d'Alencon et al. 2010). *Period* and *Clock* have been studied in other insect species including *Drosophila melanogaster* where it has been found that the *Cycle*/*Clock* heterodimer acts as a positive regulator of the transcription of *Period, Timeless,* and other clock genes (Ikeno et al. 2010).

The purpose of this study was to determine the linkage of *Period* and *Clock* genes to the unknown *Pdd* locus on the sex (Z) chromosome. Currently, an ECB genetic linkage map of the Z chromosome has already been constructed with *Tpi* and *Pdd* mapped (Dopman *et al.* 2004,

2005). In addition, the genetic differentiation of *Clock* was analyzed in a panel of ECB moths collected from various regions of the United States and Europe. Evolutionary patterns of descent and diversification were inferred from the DNA sequence data by using phylogenies based on a *Clock* intron sequence.

# Methods

## Insect Populations

ECB insects used to initiate mapping families came from colonies maintained by the New York State Agricultural Experiment Station (NYSAS) in Geneva, NY. UZ and BE colonies were initiated using ~ 500 male and ~500 female ECB and were mass reared using ~100 males and ~100 females each generation (Dopman et al. 2004).The original UZ-strain insects were collected from Bouckville, NY in April 1994, and the BE-strain insects were collected from Geneva, NY in May 1996 (Dopman et al 2010).

Field-collected insects from various regions of the US and Europe were also used for population genetic analysis. US ECB were collected from New York and North Carolina. At Geneva, NY, BE and UZ female larvae and pupae were collected in May 2000 and 2004. The insects were then reared under diapause-breaking conditions to determine PDD time. Meanwhile, female UZ ECB insects were also collected from Madison, NY in May 2000, while BZ individuals were collected from Eden, NY in October 2000. In North Carolina, multivoltine Z and E corn borers were collected in May 2002. E strain insects were collected from Weeksville, NC, while Z strain insects were collected from Fletcher, NC. The Asian corn borer, ACB, (*Ostrinia furnicalis*) was used as an out group species. European ECB were collected from

Piacenza, Italy and Kety, Hungary from maize in 2003. Italian-collected corn borers were bivoltine and either E/Z, while all ECB from Hungary were BZ.

Genetic Mapping Approach

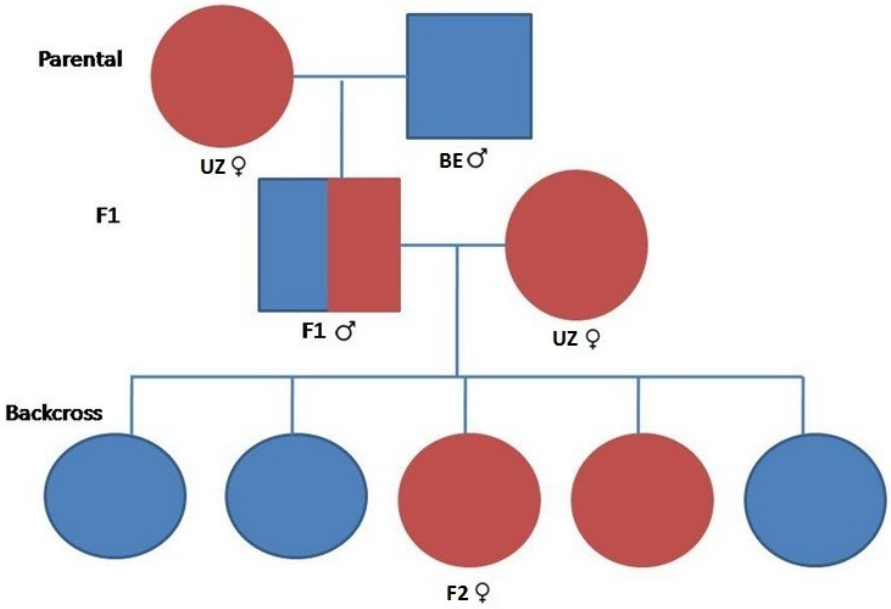


Figure 1. ECB UZ X BE cross mapping family procedure.
Colors: Red refers to UZ individuals, and blue refers to BE individuals. Shape: Circles are females, while squares are males.

Families were initiated by mating ECB adults with different PDD (Figure 1). A single univoltine Z female and bivoltine E male (parental generation) 1-4 days old were placed in a mating cage lined with wax paper. This particular cross (UZ female x BE male) was used, as it has a greater mating success than the reverse cross (BE female x UZ male). Cages were placed in a rearing room (incubator) on a 16:8 light/ dark cycle at a constant temperature of 25 ºC and relative humidity of 60%. After 2-3 days of the initial mating, mating cages were checked for egg masses. Fertilized eggs were noted by the presence of darkened larval head capsules. Wax paper containing fertilized egg masses were then cut and placed into rearing containers with

artificial diet[1]. The F1 males were then crossed with univoltine Z females (parental) to generate backcross offspring. Diapause was induced by a 12:12 light: dark cycle and a 23 ºC temperature and terminated under different conditions (16: 8 light: dark cycle and 26 ºC). Individuals in diapause were placed in individual plastic cups with moist cotton wicking, and the time to pupation (postdiapause development time) was recorded every two days. The pupated individuals were stored in individual cups until eclosion. Only F2 females were used for the genotyping experiment, as female lepidoptera are heterogametic (ZW). Each cross was replicated 14 times to generate 14 families.

DNA for the initial genetic analysis of *Period* and *Clock* came from the backcross mapping family used in Dopman et al. 2005 following a similar procedure. The additional 14 families were generated to increase the sample size of backcross offspring.

Candidate Gene Approach

As postdiapause development is known to be correlated to environmental cues such as photoperiod and temperature, several "clock" genes (genes involved with circadian patterns and photoperiod) were chosen as candidates for the *Pdd* gene (Figure 2). However as the ECB does not have a fully sequenced genome, these “clock” genes were first identified in the genetic model organism, *Drosophila melanogaster* fruit fly. Using FlyBase, a database of *Drosophila* genes, hundreds of clock genes were identified including *Period*, *Clock*, *Cycle*, and *Timeless*. These candidate genes were then narrowed by searching for homologous “clock” genes found on the sex (Z) chromosome of *Bombyx mori*, the silkworm. *B. mori* has a fully sequenced genome as seen by silkworm genome databases such as SilkDB and KAIKOBASE. *Bombyx mori* also has conserved synteny with Lepidoptera (d’Alencon et al. 2010). Two genes: *Period* and *Clock* were

[1] European Corn Borer Diet from Ankeney, Iowa

found to be near the *Tpi* marker and the projected *Pdd* locus on the *Bombyx mori* Z (sex) chromosome. *Period* is 3.94 Mb away from *Tpi*, while *Clock* is 3.2 Mb from *Tpi*. As a result, the two primary candidates of the *Pdd* gene(s) in ECB were *Period* and *Clock*.

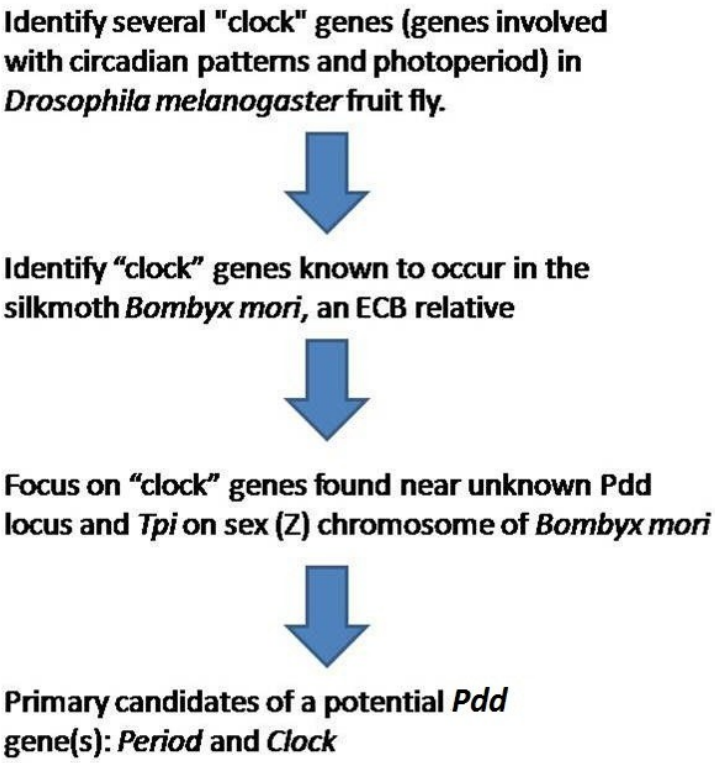


Figure 2: Candidate gene approach schematic

To amplify the *Period* and *Clock* genes, degenerate primers were designed from *B. mori* sequence. *Period* primers F1 and R2 and numerous clock primer pairs were used in PCR under a touchdown 65 ºC thermal cycling profile: (94 ºC for 30 sec, 65 ºC for 30 sec with a decrement of -0.4 ºC per cycle and 72 ºC for 1:30 min with a increment of +2 sec per cycle for 24 cycles) followed by (94 ºC 30 sec, 55 ºC for 30 sec, and 72 ºC for 1:30 min with an increment of +3 sec per cycle) for 12 cycles (Table 3). Only ECB female DNA was used, as female lepidoptera are

heterogametic (ZW) and thus only one copy of the gene is amplified, while males are homogametic (ZZ).

For the *Period* gene, genomic DNA was used for amplification and a 1.2 kb fragment with codons and introns was found. *Period* in ECB has been previously studied, and thus *Period* primers from a collaborator were used. Meanwhile for the *Clock* gene, RNA was used, as *Clock* had not been previously amplified in ECB. RT-PCR[2] was used to generate cDNA from RNA isolated from ECB head tissue. Four different reverse primers were used in RT-PCR including D-10R, D-11R, D-16R, and D-18R (Table 3). A multiple species alignment of the *Clock* gene was used to estimate sequence lengths of different primer pair products.

All possible combinations of *Clock* forward primers and reverse primers (6 forward and 4 reverse) were tested to determine if the primer combination gave a band that was consistent with the proposed sequence size. However, only the combinations of 3 forward primers and 3 reverse primers listed were successful (Table 3). Each PCR was repeated once more in order to obtain hard-to-amplify sequences.

Lastly, *Clock* PCR products with multiple bands (non-specific binding) were visualized using gel electrophoresis. Based on the multiple species *Clock* alignment, the correctly-sized fragment was gel extracted. The resulting products were then purified and sequenced in both directions. Using the PCR sequences, a 900 bp *Clock* cDNA sequence (exons only) was obtained.

Genotyping

Using sequence data from backcross individuals in the Dopman 2005 mapping family, the sequences for *Period* and *Clock* were aligned. New ECB- specific genotyping primers were

[2] Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR

designed that mapped closely to the location of polymorphisms: SNPs (single nucleotide polymorphisms) and indels (insertions-deletions). These primers were used to identify different alleles of *Period* and *Clock*. *Period* F381/R2 genotyping primers were used as a presence/absence assay (Table 3). Thus *Per-1* allele corresponded with a particular genotype and a band in electrophoresis while the *Per-2* allele corresponded with a different genotype and an absence of a band. Meanwhile, *Clock* primers Clk_525_764F2 and Clk_764_911R amplified different sized bands for the *Clk-1* allele (650 bp) and *Clk-2* allele (1 kb).

The genotyping results were then correlated with the actual postdiapause development time (PDD) observed in the mapping family. The same procedure was used for 587 individuals from 6 of the 14 families. DNA from the legs of each individual was extracted, and PCR was run using *Period* and *Clock* genotyping primers.

Population Genetics

A panel of moths totaling 40 samples collected from various US midwestern and eastern states as well as Europe was used for population analysis. The panel individuals also differed in voltinism and pheromone production (Table 1).

*Clock* primers that amplified a variable region (intron) were used: Clk_525_764 F2 and Clk_764_911R (Table 3). The sequences were then aligned, and polymorphisms within the *Clock* intron were identified for population analysis. The first statistical test was a maximum parsimony analysis using a bootstrap statistical resampling procedure. In the analysis, 500 replicates were used to determine which individuals were clustered together. In addition, Tajima's D statistical test was used to detect whether or not patterns of diversity within populations were consistent with neutrality.

| Group | Location | Voltinism | Pheromone |
|---|---|---|---|
| ACB | | | |
| CAS | Italy | B | E/Z |
| ECB (1-20) | New York | B | E |
| ECB (200-300) | New York | B/U | Z |
| ENC | North Carolina | multivoltine | E |
| FNC | North Carolina | multivoltine | Z |
| G | New York | B | E |
| GAZ | Italy | B | E/Z |
| HUN | Hungary | B | Z |
| IOWA | Iowa | B | Z |
| POD | Italy | B | Z |

Table 1: Panel moth description: field collection location, voltinism, sex pheromone

# Results

Both candidate genes, *Period* and *Clock,* showed allelic differentiation based on postdiapause development time (phenotype). Alleles *Per-1* and *Clk-1* were consistent with short PDD, while *Per-2*, *Clk-2* corresponded with long PDD in this mapping family.

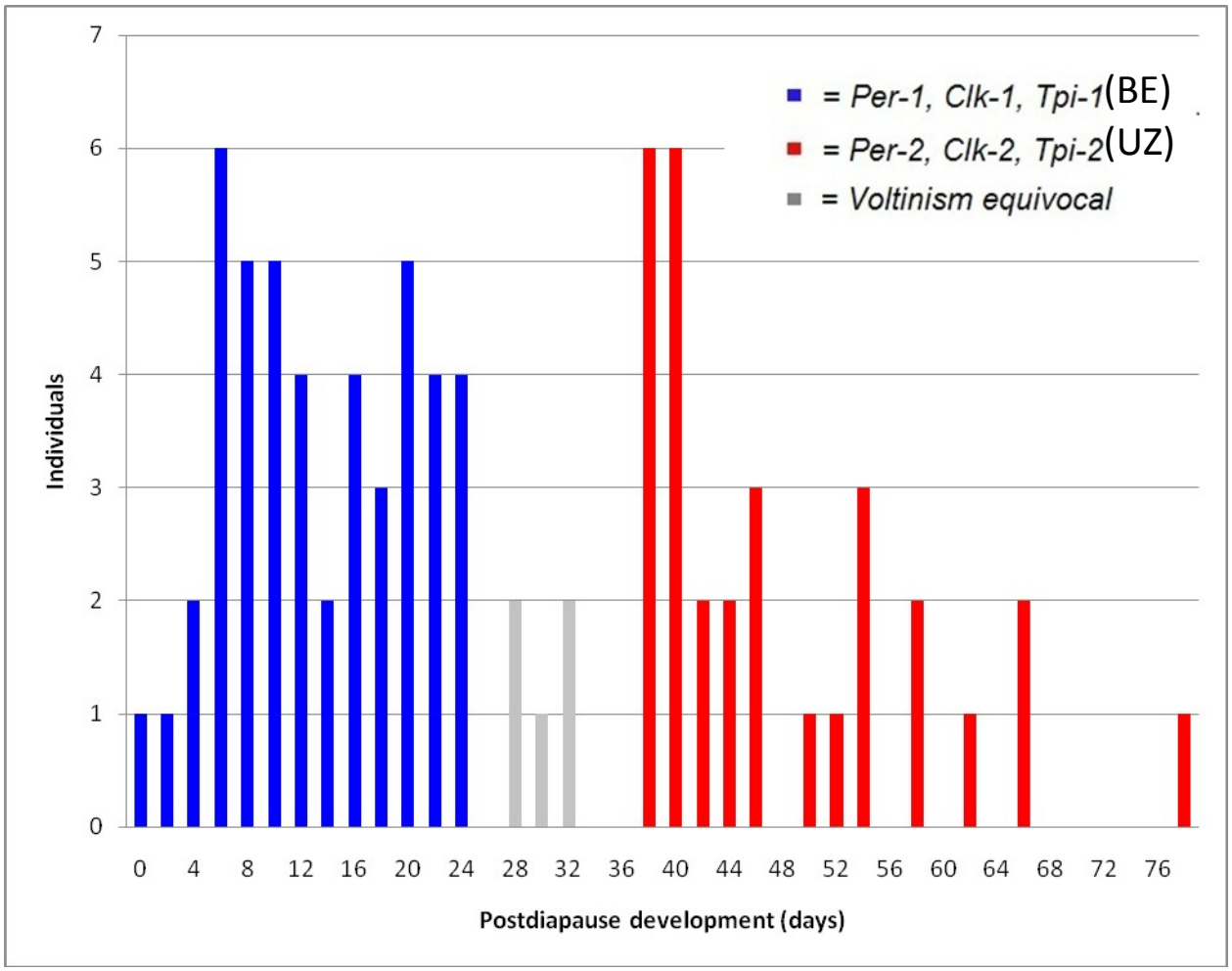


Figure 3. Frequency histogram showing bimodal PDD time for female BC offspring from initial mapping family in Dopman et al. 2005 genotyped for *Period* and *Clock*. The 12-day period starting on day 25 and ending on day 36 divides females into bivoltine (short PDD, n = 41) and univoltine (long PDD, n = 30) groups that correspond with parental phenotypes. Genotyping results for *Per* and *Clk* are shown.

Six additional mapping families that were genotyped displayed a bimodal PDD time distribution in which the female BC offspring were divided into bivoltine (short PDD) and univoltine (long PDD) populations. Allelic differentiation of *Period* and *Clock* was found to be generally consistent with PDD time except for 3 individuals found in Family 9 and 11. In these families, a few ECB with a long PDD phenotype were genotyped for Clk-1(normally associated with short PDD) and Per-2, Tpi-2 (Table 2).

| Family | Long PDD | Per-2, | Tpi-2 | Clk-2 | Clk-1 |
|---|---|---|---|---|---|
| 9 | 2 | 2 | 2 | 0 | 2 |
| 11 | 1 | 1 | 1 | 0 | 1 |

Table 2: Phenotypes and genotypes of recombinants.

Each recombinant is listed under a phenotype and genotype. Phenotypes include short PDD and long PDD. Genotypes consist of alleles -1 or alleles -2.

In the ECB genetic linkage map, both *Period* and *Clock* were found to be tightly linked to *Tpi* and *Pdd* (Figure 4). Additional markers and their respective distances for the ECB genetic linkage map were obtained from Dopman et al. 2005. However, the *Bombyx mori* physical map showed that the distance between *Tpi* and *Period* was 3.94 Mb and that the distance between *Tpi* and *Clock* was 3.2 Mb (Figure 4). *Bombyx mori* genes and their ECB orthologs were listed on the Z chromosome using data from KAIKOBASE, a genomic database for *B. mori*.

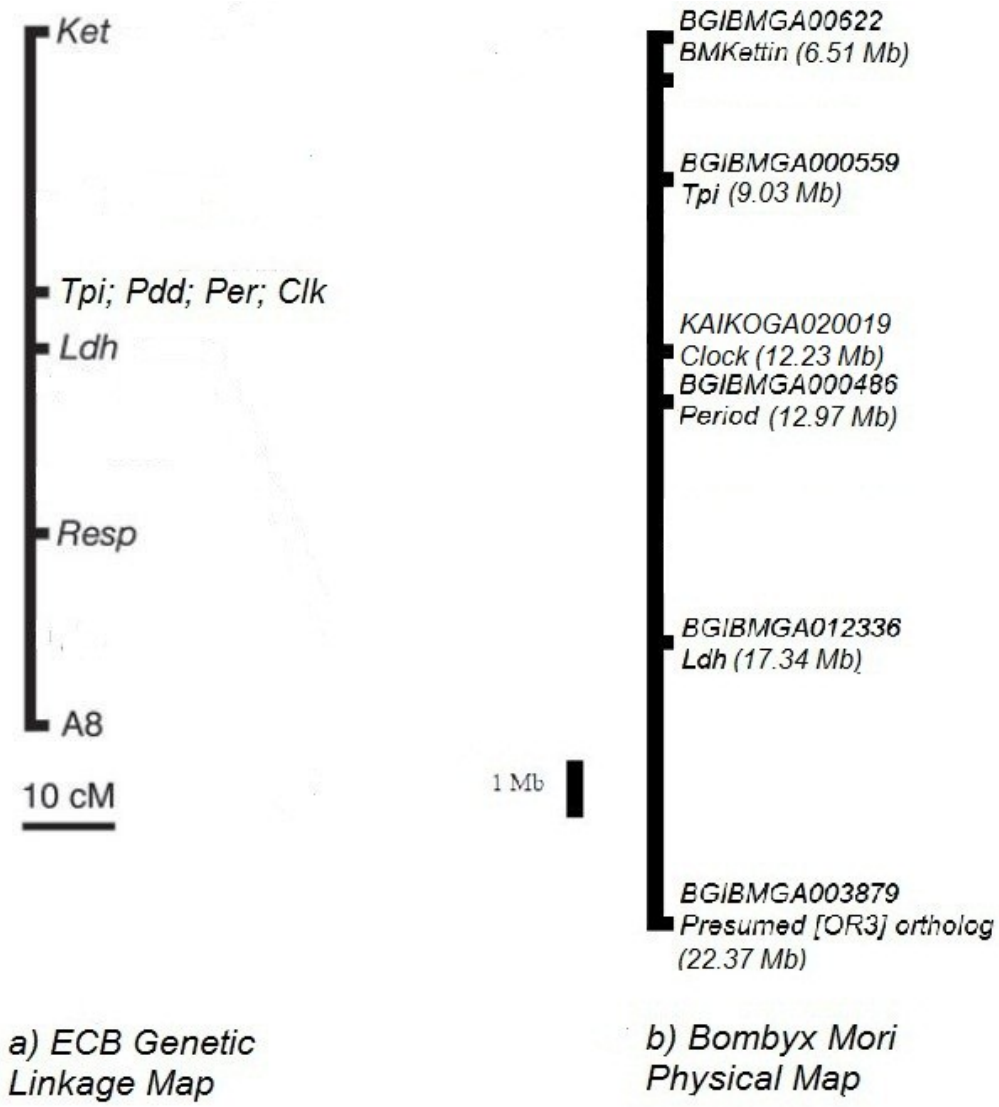


Figure 4. Comparison of *Tpi* region on sex (Z) chromosome of the ECB and *Bombyx mori* (silkmoth) using a) a ECB genetic linkage map and b) *B. mori* physical map

Differences in *Clock* genealogy was found in both the Asian Corn Borer - outgroup (support: 99%) and several Italy-collected ECB (support: 91%) (Figure 5). No sign of exclusivity was

found for Z/E strains or bivoltine/univoltine strains in the US ECB. Tajima's D was used to distinguish whether the Clock sequence evolved through a neutral (random) or nonrandom process. Tajima's D was found to be -0.46478, $P > 0.10$, which shows an excess of low frequency polymorphisms usually found after selective pressure.

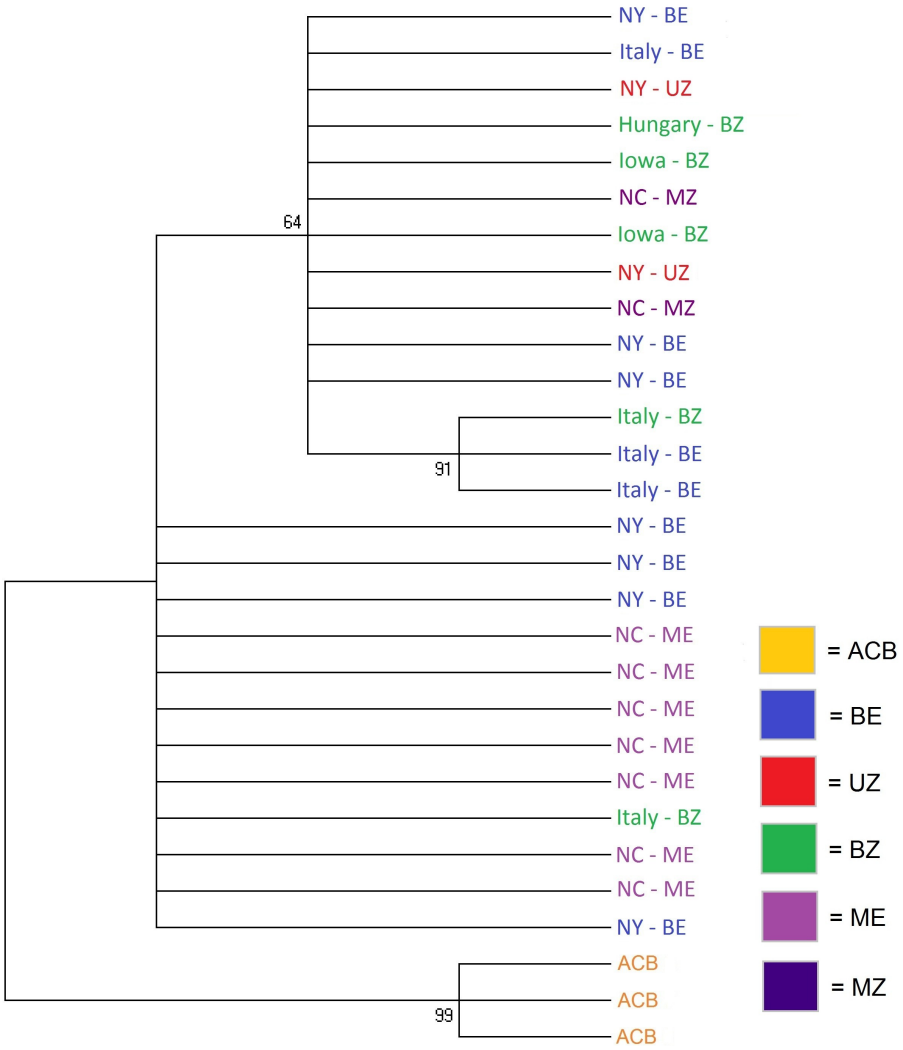


Figure 5. Evolutionary relationships of 29 ECB individuals

The evolutionary history was inferred using the maximum parsimony method on a variable *Clock* intron sequence. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the individuals analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated individuals clustered together in the bootstrap test (500 replicates) is shown next to the branches. All alignment gaps were treated as missing data. There were a total of 1153 positions in the final dataset, out of which 9 were parsimony informative.

# Discussion

Previous research has found that the *Tpi* (triose phosphate isomerase) marker on the ECB Z chromosome exhibits significant allelic frequency differences between E and Z strains (Glover et al.1992, Dopman et al. 2005). As the unknown *Pdd* locus is a major factor of postdiapause time (PDD) and is tightly linked to *Tpi*, it has been suggested that *Pdd* is the target gene for selection that has generated this pattern of exclusivity between pheromone strains. However, the *Pdd* gene has not been yet identified or sequenced. Using *Clock* and *Period* as candidate genes for *Pdd*, genotyping results showed that both genes were generally consistent with the PDD phenotype. Short PDD individuals had *Per-1* and *Clk-1* alelles, while long PDD individuals had *Per-2* and *Clk-2* alleles (Figure 3). Out of 587 female backcross individuals, no recombinants for *Period* were found. However, 3 recombinants for *Clock* were found, in which 3 insects with long PDD phenotype were genotyped for *Clock-1* alleles. Based on these results, *Clock* is less likely to be a potential candidate gene for *Pdd* compared to *Period*. Nevertheless, both genes show relatively tight linkage to the *Tpi* marker and the *Pdd* locus.

In addition, a panel of moths was previously used in a population genetic analysis to determine genealogical differences at *Tpi* (Dopman et al. 2005). A similar genealogical analysis was done for *Clock* in this study. As *Clock* is tightly linked to *Tpi* and *Pdd*, it was hypothesized that the candidate gate would display allelic differentiation in Z and E strains (*Tpi*) or bivoltine and univoltine strains (*Pdd*). Although *Clock* sequences were found for 40 field-collected individuals, only 29 were used in the statistical analysis due to the high divergence found in the amplified intron region. Out of the 10 unused individuals, 8 individuals had analogous sequences, while remaining two had different analogous sequences. Although the Asian Corn Borer and some Italian ECB were clearly differentiated from the rest of panel, US ECB showed little exclusivity between pheromone or voltinism strains (Figure 5). Based on these preliminary

results, there is a considerable variability in gene genealogies at *Clock* for the individuals collected in the United States. In addition, the Italian ECB were also found to be highly discordant as the individuals are found on all branches except for the outgroup (Figure 5). This result is unusual based on the tight linkage between *Tpi* and *Clock*. However, there are some reservations for these results including the fact that the maximum parsimony analysis ignored gaps in the sequence alignment. Yet these alignment gaps are important sources of polymorphisms and likely provide information about selection and exclusivity. Lastly due to the relatively low number of univoltine individuals, a more balanced field-collected sample would be informative in analysis.

Similarly, the Tajima's D test was used to determine whether *Clock* is evolving neutrally or is under selection. The value for the *Clock* genealogies was -0.46478, $P > 0.10$. Although this value was not found to be significant, the negative value represents an excess of low frequency polymorphisms usually found after a selective sweep or a population bottleneck. Thus, it is evident that the Clock gene may not have evolved neutrally but rather has been shaped by a non-random selective process.

Although we have identified *Period* and *Clock* as genes tightly linked to the *Pdd* locus, we still do not know the casual factor for postdiapause development such as an important SNP (single nucleotide polymorphism) in the gene sequence. Certainly, both Period and Clock proteins underlie the important biochemical mechanism of diapause and are likely affected by this unknown causal factor. However, more information is needed about the ECB genetic map and as well as the gene genealogy differences in *Period* and *Clock* to identify the *Pdd* gene.

Altogether, the genotyping and population genetic results suggest a high selective pressure at the *Tpi/Pdd* region. In addition, the genotyping results also show that the

recombination rate in the Z chromosome region containing *Tpi*, *Pdd*, *Clock*, and *Period* is low. There are several potential explanations for the absence of the gene flow at the *Tpi* area. Hypotheses include a wide regional selective sweep or a fixed chromosomal inversion. As *Tpi* is linked to genes involved in pre-mating barriers such as *Pdd* (voltinism) and *Resp* (male sex pheromone response), it is possible that those genes have increased selective pressure and that the genetic variation linked to such advantageous alleles can "hitchhike" to fixation along with the selected allele. However, there has been preliminary evidence suggesting chromosomal inversion in this region, as *Tpi* and another sex-linked marker (*ECB_Z_SodCalcA)* were found to be reversed in gene order on the Z chromosome between ECB and *Bombyx mori* (Du 2010). Furthermore unlike the mapping families produced here, Z-strain cross offspring were found to have a recombinant rate of 4/105, approximately 4% (Harrison – unpublished data). Based on the 1 cM/Mb recombinant rate (1 % recombinant = 1 cM = 1 Mb) found in *Drosophila melanogaster*, this recombinant rate correlates with the 4 Mb distance found in *Bombyx mori*. Previous research has found that there is a high recombinant rate between ECB pheromone races in other sex-linked markers: *Ket* and *Ldh*. These two markers are found at opposite ends of the *Tpi* region and span a 10 cM region. Thus the proposed inverted chromosomal region is between 4 Mb and 10 Mb.

The major importance of chromosomal rearrangements is that they disrupt meiosis and cause at least partial sterility in hybrids (Feder 2009). In addition, fixed chromosomal inversions lower recombination rates by protecting favorable genotyping combinations within regions from being separated. However, the role of chromosomal inversion has not been widely studied in speciation, and its relative importance is controversial. There is no evidence of selection for inversions, and genes affecting divergence are widely distributed throughout genome, not only in

inversions (Feder 2009). If an inversion is found to exist in the *Tpi* region, classical genetic mapping cannot be used for *Period* and *Clock*, as the number of recombinants would not accurately reflect mapping distance. A chromosomal inversion would cause a fitness difference in which hybrids (single recombinants) do not survive and only double recombinants with the restored parental configuration survive. As a result, the *Clock* recombinants are hypothesized to be double recombinants. A clear answer of whether a chromosomal inversion exists at the *Tpi* locus can be found through the comparison of UZ, BE, and BZ ECB Z chromosome genomic sequence. Another way to examine the proposed chromosomal inversion is through fluorescence in situ hybridization (FISH). In FISH, fluorescent probes that bind to *Period*, *Clock*, *Tpi* could be used to detect and locate these specific DNA sequences on the sex chromosome. This could in turn elucidate the order of these genes.

An important premating barrier, sex pheromone response in males, is linked to the *Resp* gene that maps onto the Z chromosome to a region 20-30 cM away from *Tpi*. This adds another layer of complexity to the genetics of speciation. Although *Tpi* is tightly linked to the *Pdd* gene, *Tpi* also shows differentiation between sex pheromone strains: E and Z. The connection between postdiapause development and sex pheromone communication is currently unknown. However, there are several hypotheses about why *Tpi* shows and is linked to both sex pheromone and voltinism differences. One possibility is multiple speciation events throughout evolutionary time, in which there was earlier divergent selection on Z-strain insects for short and long PDD and later selection on E-strains for short PDD. Another explanation is that the previous genetic map created for the *Tpi* region (Dopman et al. 2005) is not accurate in light of the potential chromosomal inversion, as recombinants observed were actually double recombinants. Thus, it is possible that the gene responsible for male sex pheromone response is closer to Tpi than

previously assumed. As a result, a future direction is to look at BZ individuals, as they unlink the voltinism and sex pheromone variables as compared to BE and UZ insects. By creating a new genetic mapping family with UZ and BZ individuals (both thought to be syntenic in the Z chromosome region), it may be possible to avoid the confounding factor of sex pheromone differences.

Both voltinism and sex pheromone differences are important barriers in the ECB. These two premating traits account for 75% of the total reproductive isolation (Dopman et al. 2010). However, there are other isolating barriers including premating, prezygotic, and postzygotic barriers. A major focus in speciation research is to determine the relative contribution of each isolating barrier to the total isolation (Coyne and Orr 1989, Coyne and Orr 2004, Schemske 2010, Dopman et al. 2010). As such isolating barriers act sequentially in individuals, each additional barrier reduces the gene flow not stopped by previous barriers. As a result, early-acting barriers such as premating barriers are believed to be more important in the speciation process than later-acting barriers (Schemske 2010). This is true even if later barriers are stronger in an absolute sense as compared to earlier-acting barriers (Coyne and Orr 2004). However, current speciation research has largely focused on postzygotic barriers such as hybrid sterility and inviability in *Drosophila* species (Coyne & Orr 1998). Thus, research on premating barriers on non-model organisms is an important avenue of research that provides insight into the speciation process.

Speciation is marked by extensive genetic differentiation between the two species' genomes. As there is still existing gene flow between various ECB strains, complete speciation will require selection acting on many different genes. The accumulation of these 'speciation

genes' will then create divergent gene complexes that lead to reproductively isolated groups or species.

Table 3. Summary of primer pairs used to amplify and genotype *Period* and *Clock*

| | | Forward | Primer | Reverse | Primer | | |
|---|---|---|---|---|---|---|---|
| Gene | Use | ID | Sequence | ID | Sequence | PCR Conditions | Band size |
| *Period* | Amplification | F1 | CGCCAGGTGTATGAGACTATCGTG | R2 | ACCCTGAAGACCTGTGCTATC | TD 65[1] | 1.2 kb |
| *Period* | Genotyping | F381 | ATCTTCACATTGCTTTTTAAATTACCTAG | R2 | ACCCTGAAGACCTGTGCTATC | TD 65 | 1 kb or no band |
| *Clock* | Amplification | A-3F | CCACCAACACCCGGAARATGGAYAA | D-11R | CCTCGAAGGGCAGGTANCCDATDAT | 2x PCR @TD 65 | 670 bp |
| *Clock* | Amplification | D-8F | CACCGGGCCCCTCCNATHATHGG | D-16R | GGGTCTGCAGCCAGATCCAYTGYTG | 2x PCR @TD 65 | 165 bp |
| *Clock* | Amplification | D-8F | CACCGGGCCCCTCCNATHATHGG | D-18R | GGACACCACCCGGTGNGTRCANAC | 2x PCR @TD 65 | 235 bp |
| *Clock* | Amplification | A-5F | CCCGGAACCTGTCCGARAARAARMG | D-11R | CCTCGAAGGGCAGGTANCCDATDAT | 2x PCR @TD 65 | 745 bp |
| *Clock* | Amplification | A-3F | CCACCAACACCCGGAARATGGAYAA | D-16R | GGGTCTGCAGCCAGATCCAYTGYTG | 2x PCR @TD 65 | 820 bp |
| *Clock* | Amplification | A-3F | CCACCAACACCCGGAARATGGAYAA | D-18R | GGACACCACCCGGTGNGTRCANAC | 2x PCR @TD 65 | 890 bp |
| *Clock* | Genotyping/ Pop genetics | Clk_525_764F2 | CACTTCGACGACTTGGAGAA | Clk_764_911R | TAGCACGATGTCAGCTCTCC | TD 65 | variable: 600 bp - 1 kb |

1. Touchdown 65 thermal cycling profile: (94 ºC for 30 sec, 65 ºC for 30 sec with a decrement of -0.4 ºC per cycle and 72 ºC for 1:30 min with a increment of +2 sec per cycle) for 24 cycles followed by (94 ºC 30 sec, 55 ºC for 30 sec, and 72 ºC for 1:30 min with an increment of +3 sec per cycle) for 12 cycles.

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