Genome-Wide Characterization of Adaptation and Speciation in Tiger Swallowtail Butterflies Using De Novo Transcriptome Assemblies

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Accepted: May 26, 2013

Data deposition: This project has been deposited at NCBI SRA under the accession number SRP022555.

Abstract

Hybrid speciation appears to be rare in animals, yet characterization of possible examples offers to shed light on the genomic consequences of this unique phenomenon, as well as more general processes such as the role of adaptation in speciation. Here, we first generate transcriptome assemblies for a putative hybrid butterfly species, Papilio appalachiensis, its parental species, P. glaucus and P. canadensis, and an outgroup, P. polytes. Then, we use these data to infer genome-wide patterns of introgression and genomic mosaicism using both phylogenetic and population genetic approaches. Our results reveal that there is little genetic divergence among all three of the focal species, but the subset of gene trees that strongly support a specific tree topology suggest widespread sharing of genetic variation between P. appalachiensis and both parental species, likely as a result of hybrid speciation. We also find evidence for substantial shared genetic variation between P. glaucus and P. canadensis, which may be due to gene flow or ancestral variation. Consistent with previous work, we show that P. appalachiensis is more similar to P. canadensis at Z-linked genes and more similar to P. glaucus at mitochondrial genes. We also identify a variety of targets of adaptive evolution, which appear to be enriched for traits that are likely to be important in the evolution of this butterfly system, such as pigmentation, hormone sensitivity, developmental processes, and cuticle formation. Overall, our results provide a genome-wide portrait of divergence and introgression associated with adaptation and speciation in an iconic butterfly radiation.

Key words: transcriptome, adaptation, hybrid speciation, introgression, Papilio.

Introduction

Young evolutionary radiations offer a special opportunity to explore the interplay among adaptation, speciation, and hybridization in generating biological diversity (Grant 1999; Seehausen 2006; Mallet 2009). One particular phenomenon that appears to rely on a mix of these evolutionary processes is hybrid speciation, which is the formation of a new species as a result of hybridization between two parental species (Mallet 2007; Abbott and Rieseberg 2012). Hybrid speciation is common in plants, where it frequently occurs via allopolyploidy, or a change in chromosome number between parental species and the hybrid offspring (Rieseberg 1997; Soltis PS and Soltis DE 2009; Abbott and Rieseberg 2012). In animals, hybrid speciation appears to be relatively rare and many of the examples that do exist appear to be homoploid hybrid species, having the same chromosome number as their parental taxa (Mallet 2007; Mavárez and Linares 2008). Although hybrid speciation may only account for a small fraction of the species diversity in animals, careful study of this phenomenon can provide more general insights into the origin of reproductive isolation and the potential role of adaptive evolution in the speciation process. This is because incipient homoploid hybrid species face a variety of challenges that are likely to inhibit their persistence (Abbott and Rieseberg 2012). One of these challenges is reproductive isolation. Unlike allopolyploids, homoploid hybrid species are not immediately reproductively isolated from their parental species, and because they must originate in contact with the parental species, it may be difficult for a new hybrid lineage...
to remain distinct and not simply fuse with a parental species by backcrossing. A second challenge is competitive exclusion. Those hybrid lineages that do manage to remain distinct in the face of potential gene flow with parental species must then secure resources and survive in an environment already occupied by the parental taxa. Given the factors acting against the origin of new hybrid species, examination of those hybrid species that have persisted to the present day may inform us as to how reproductive isolation and niche evolution occur on short time scales.

A recently described hybrid butterfly species, *Papilio appalachiensis*, appears to overcome both challenges by occupying a novel environment in which it has higher fitness than its parental species. The parental species, *P. glaucus* and *P. canadensis*, are sister species with parapatric distributions that share a narrow hybrid zone along the border between the United States and Canada (Hagen et al. 1991; Luebke et al. 1988). Although earlier studies considered *P. canadensis* a subspecies of *P. glaucus*, more recent work has documented pronounced reproductive isolation between them, including intrinsic postzygotic isolation (Sperling 1993; Hagen and Scriber 1995; Scriber et al. 1995). A wide variety of additional differences, including divergent habitat (Lederhouse et al. 1995) and host plant preferences (Scriber et al. 1995; Scriber 1996), larval development (Ritland and Scriber 1985), allozyme allele frequencies (Hagen and Scriber 1991), AFLP markers (Winter and Porter 2010; Kunte et al. 2011), and DNA sequence data (Kunte et al. 2011) further support *P. canadensis* as a separate species. One striking morphological difference between *P. glaucus* and *P. canadensis* involves wing pattern mimicry (fig. 1). *Papilio glaucus* females display two distinct wing patterns; a yellow, nonmimetic phenotype that looks like the males and a melanic phenotype that mimics the chemically defended Pipevine swallowtail *Battus philenor* (Brower 1958). In contrast, *P. canadensis* lacks the mimetic female morph with both males and females displaying a similar yellow wing pattern (Hagen et al. 1991). The color of *P. glaucus* females is controlled by a W-linked Mendelian locus and it is further influenced by a Z-linked enabler/suppressor locus that differs between *P. glaucus* and *P. canadensis* (Scriber and Hainze 1987; Hagen and Scriber 1989; Scriber et al. 1996).

A recent species of *P. appalachiensis* (Pavulaan and Wright 2002) exists at high elevation along the Appalachian Mountains and appears to be a hybrid species (Scriber and Ording 2005). Like *P. canadensis*, it is adapted to a cooler thermal zone and it is univoltine. However, like *P. glaucus*, it displays two female morphs, one of which is a dark, mimetic form (Pavulaan and Wright 2004). This unique combination of traits allows this species to occupy a novel, high elevation habitat that is within the range of the mimicry model *B. philenor*. Using a combination of targeted DNA sequencing and AFLP genotyping, Kunte et al. (2011) recently showed that *P. appalachiensis* is a genomic mixture of *P. glaucus* and *P. canadensis* and that it is significantly differentiated from both. As a whole, these results suggest that historical hybridization between *P. glaucus* and *P. canadensis* produced a stable hybrid lineage well adapted to a novel environment.
which persists today as a reproductively isolated species, *P. appalachiensis*.

The genetic data supporting a mosaic genome in *P. appalachiensis* are still rather limited. Therefore, we have focused on fully characterizing the transcriptomes of *P. appalachiensis*, its putative parental species, *P. glaucus* and *P. canadensis*, and an outgroup species, *P. polytes*. We then use these data to examine genome-wide patterns of divergence and genomic mosaicism among the tiger swallowtails using a variety of analytical approaches. We also infer rates of gene flow among the three taxa and characterize genes that have experienced recent positive selection. Our results lend strong support to the hypothesis that *P. appalachiensis* is a hybrid species and provide important insights into the potential functional genetic changes associated with speciation in this well-studied butterfly group.

**Materials and Methods**

**RNA Isolation and Illumina Sequencing**

We generated RNA-seq data for a total of eight pupal RNA samples; two *P. glaucus*, two *P. canadensis*, two *P. appalachiensis*, and two *P. polytes*. *Papilio* polytes and the ingroup taxa come from different within-*Papilio* subclades that may have diverged from one another approximately 35 Ma (Zakharov et al. 2004). The *P. polytes* samples were selected from a lab colony originating from the Philippines, whereas the other species were field-collected in Louisiana (*P. glaucus*), New Hampshire (*P. canadensis*), and West Virginia (*P. appalachiensis*). For *P. polytes*, RNA was extracted from pupal wing discs only, whereas RNA was extracted from entire pupae for the other samples. RNA was extracted with Trizol according to a standard protocol, poly-A purified, and converted to cDNA and barcoded using the Illumina Tru-Seq protocol. The cDNA libraries were then pooled and sequenced using an Illumina HiSeq 2000 sequencer (100 bp paired-end).

**De Novo Transcriptome Assembly**

Raw reads were demultiplexed according to their barcodes and low quality sequences were removed before assembly. After quality filtering, data were combined by species. Trinity version 2012-06-08 (Grabherr et al. 2011) was used to perform de novo transcriptome assembly and open reading frame extraction under default parameters. These parameters include the following: min_contig_length 200, min_kmer_cov 1, max_reads_per_graph 200000, max_number_of_paths_per_node 10, group_pairs_distance 500, and path_reinforcement_distance 75.

**Clustering and Annotating Conserved Coding Sequences**

To identify clusters of homologous sequences among transcriptomes, predicted coding sequence (CDS) regions of each species were used as queries and targets separately for Blat (Kent 2002) to search against data sets for the other three species (reciprocal best hits). The best Blat hits of the longest isoforms with *E* value lower than 10$^{-15}$ were retrieved and only one-to-one orthologous genes existing in all four species were retained. These are the conserved clusters, or “genes,” used in all further analyses. Note that adjusting the *E* value threshold to 10$^{-15}$ reduced the final data set by only 1%. Clusters that contained two or more sequences from the same species were not analyzed further to eliminate potential issues stemming from paralogs. Conserved mitochondrial genes and rRNAs were identified using each transcriptome data set as query for Blat searches against predicted genes and rRNAs in the mitochondrial genome of *Bombyx mandarina* (NCBI Reference Sequence: AY301620.2) (Pan et al. 2008).

**Multiple Alignments and Phylogenetic Analysis**

**Multiple Alignments**

We performed two separate analyses of our conserved clusters, one based on alignment of nucleotide sequences and another based on alignment of predicted peptide sequences. Multiple sequence alignments for both data sets were performed using MUSCLE 3.8 (Edgar 2004) with default parameters.

**Topological Structure Assignment**

To infer the best tree topology for each conserved cluster, we estimated phylogenetic trees using both maximum-likelihood (ML) and neighbor-joining (NJ) methods. First, we used PhyML 3.0 (Guindon et al. 2010) to generate trees under specified topological constraints with either K2P (for nucleotide) or JTT (for protein) models of evolution. The three constrained trees were defined as ((A,C),G,P), ((A,G),C,P), and ((C,G),A,P) where A, C, G, and P stand for *P. appalachiensis*, *P. canadensis*, *P. glaucus*, and *P. polytes*, respectively. We used CONSEL 0.20 (Shimodaira and Hasegawa 2001) to assess the confidence of selecting the best topological structure for each cluster. CONSEL was used to generate Shimodaira and Hasegawa (SH) test *P* values for each tree topology with *P* values ≥ 0.95 indicating significant support for a particular topology. We further estimated trees for all clusters using NJ method, with 1,000 bootstrap pseudoreplicates, using PHYLIP 3.69 (Felsenstein 1989). We focused our subsequent analyses on conserved clusters for which ML, combined with the SH test, and the NJ tree yielded the same tree topology. Similar methods were used to examine tree topologies for specific clusters, which we inferred to be Z-linked, by comparison with the *Heliconius melpomene* genome sequence (*Heliconius Genome Consortium 2012*), or mitochondrial, by comparison with the *Bombyx* mitochondrial genome. All sequence descriptions are based on results of BLASTX searches against NCBI’s nr protein database. Gene ontology terms were assigned to conserved clusters using Blast2GO (Conesa et al. 2005).
2005) and Fisher’s exact tests were used to test for functional enrichment for clusters yielding each of the three tree topologies. Note, setting a false-discovery rate to correct for multiple testing resulted in no significant enrichment. Huang et al. (2008) suggest that multiple testing corrections may be too conservative to effectively guide initial exploratory analyses so we present the uncorrected P values for all GO term enrichment tests.

Detecting Gene Flow among *P. glaucus*, *P. canadensis*, and *P. appalachiensis*

We calculated Patterson’s D-statistic (Green et al. 2010; Durand et al. 2011) to quantify gene flow among the three ingroup taxa, using *P. polytes* as an outgroup. This test examines the phylogenetic distribution of derived alleles (designated “B”) at loci that display either an ABBA or BABA configuration on a four species phylogeny (fig. 2). Summed

![Diagram of gene flow among three species](image)

**Fig. 2.**—Patterson’s D-statistic suggests widespread introgression between *Papilio appalachiensis* and the putative parental species. We calculated a transcriptome-wide D-statistic value for each of three tree topologies (A–C) and found evidence for significant introgression in comparisons with *P. appalachiensis* (D). Results suggest roughly equal introgression between *appalachiensis/canadensis*, compared with *appalachiensis/glaucus* ($D_1$, $P = 0.715$), but much more introgression between *appalachiensis/canadensis* and *appalachiensis/glaucus*, compared with *glaucus/canadensis* ($D_2$ and $D_3$, $P < 0.01$ for both).
across the genome, no enrichment of ABBA or BABA sites is expected as a result of random sorting of ancestral variation. Interspecific gene flow, however, is expected to result in a systematic bias of allele sharing between the two taxa exchanging alleles. For these tests, single nucleotide polymorphisms (SNPs) were extracted from each cluster using ape-package 3.0-6 (Paradis et al. 2004) and adegenet-package 1.3-5 (Jombart and Ahmed 2011). Then, separate tests were performed to detect gene flow in pairwise comparisons among our three ingroup taxa. The number of shared, derived SNPs supporting either an ABBA or BABA pattern was calculated in three comparisons: $D_1$ (glaucus, canadensis, appalachiensis, and polytes), $D_2$ (glaucus, appalachiensis, canadensis, and polytes), and $D_3$ (canadensis, appalachiensis, glaucus, and polytes). Finally, the leave-one-out jackknife estimate was performed using bootstrap-package 2012-04-0 (Tibshirani and Leisch 2012) to determine the standard error for each $D$ value of each cluster and significant deviations from zero were tested using a two tailed z-test. $D$-statistic values that differ from zero are indicative of gene flow.

**Chromosome Distribution of Conserved Clusters**

Although there is no reference genome sequence for Papilio butterflies, we used the fact that synteny is highly conserved between the butterfly *H. melpomene* and the moth *B. mori* (Heliconius Genome Consortium 2012) to examine the genome-wide distribution of our conserved clusters (fig. 3). We also tested whether clusters with the same tree topology were clustered in the genome. To do this, we used Blat to assign conserved clusters to putative orthologs in the *B. mori* and *H. melpomene* genome sequences. *Bombyx mori* genome data were downloaded from SilkDB (http://www.silkdb.org/silkdb/, last accessed June 17, 2013) (Xia et al. 2004) and *H. melpomene* genome data were downloaded from the Butterfly Genome Database (http://www.butterflygenome.org/, last accessed June 17, 2013). We used Spearman Rank Correlation tests to compare the chromosomal-level distribution of clusters with a particular tree topology to a null distribution based on the distribution of all conserved clusters. This analysis was done twice, once using the *Bombyx* genome as a reference and once using the *Heliconius* genome.

**Calculating $K_a/K_s$ Ratios for Conserved Clusters**

For each conserved cluster, we calculated nonsynonymous ($K_a$) and synonymous ($K_s$) substitution rates for every species pair. $K_a$ and $K_s$ were estimated using the unbiased approximation of Li (1993), implemented in seqinr-package 3.0-6 (Charif and Lobry 2007). We performed separate analyses, looking for evidence of positive selection between ingroup (*P. glaucus, P. canadensis*, and *P. appalachiensis*) and outgroup (*P. polytes*) taxa as well as among ingroup taxa.

Clusters yielding large $K_a/K_s$ ratios were checked manually to eliminate spurious results due to poor alignment. Blast2GO was used to test for functional enrichment of clusters displaying evidence of positive selection between ingroup and outgroup taxa.
McDonald–Kreitman Tests

As an additional test of adaptive protein evolution, we performed McDonald–Kreitman (MK) tests (McDonald and Kreitman 1991) on the subset of clusters for which we could identify orthologous transcripts for each individual. To do this, we reassembled transcriptomes for each individual using Trinity, as opposed to combining data by species. Clustering and multiple alignments were performed as described earlier for the combined analysis. We performed two analyses, first comparing among ingroup taxa using all clusters for which we identified one sequence from each of the six ingroup samples, and then comparing ingroup taxa with the outgroup at the subset of these clusters where we could also identify one sequence from each P. polytes sample. MK tests were done using libsequence and MKtest package (Thornton 2003) and statistical significance was inferred using Fisher’s exact test ($P$ values < 0.05). Clusters were annotated using Blast2GO.

Results

Transcriptome Assembly and Conserved Cluster Characterization

We generated between 45 million and 148 million reads per sample, yielding approximately 4.5–14.8 Gb of RNA-seq data per sample. De novo transcriptome assembly for each species yielded a large number of putative single-copy genes (table 1) and combining data among species yielded 3,961 conserved clusters for which all four species contributed a single sequence (fig. 1). The mean CDS of these conserved clusters was 1,392 bp for P. appalachiensis, 1,376 bp for P. canadensis, 1,378 bp for P. glaucus, and 1,336 bp for P. polytes. For comparison, the mean CDS is 1,258 and 1,248 bp for all genes in the reference genome sequence of H. melpomene and B. mori, respectively. Comparisons using the “ortholog hit ratio” (O’Neil et al. 2010) further suggest that our conserved clusters largely span entire genes (supplementary fig. S1, Supplementary Material online). Note that using a much more stringent threshold for ortholog detection, an $E$ value of $10^{-15}$, altered the final data set very little (3,920 clusters compared with 3,961).

Mosaic Transcriptome of P. appalachiensis

Previously, Kunte et al. (2011) demonstrated that Z-linked genes connected P. appalachiensis to P. canadensis while mitochondrial genes, and presumably W-linked genes (these are linked in butterflies because females are the heterogametic sex), connected P. appalachiensis to P. glaucus. We first verified these findings by surveying our conserved clusters for putatively Z-linked and mitochondrial genes, yielding 18 Z-linked genes and 14 mtDNA genes. We found that many clusters did not yield statistically significant tree topologies based on the SH test, and nucleotide and peptide alignments did not always agree on the best tree topology. However, consistent with previous results, the most frequent tree topology for Z-linked genes was that linking P. appalachiensis and P. canadensis (table 2) while the most frequent tree topology for mitochondrial genes was that linking P. appalachiensis and P. glaucus (table 3).

To examine potential mosaicism across the P. appalachiensis genome as a whole, we performed similar phylogenetic analysis of all 3,961 conserved clusters. Because our ingroup taxa are very closely related, the vast majority of clusters did not yield a highly supported tree topology. Indeed, only 179 clusters yielded well-supported tree topologies (SH test plus NJ tree corroborator) in our analysis of the nucleotide data and 303 clusters in the analysis of peptide data. Interestingly, in analysis of both data sets, a similar number of clusters supported all three topologies (table 4). This result is consistent with the mosaic genome expected for P. appalachiensis but it also suggests extensive sharing between P. glaucus and P. canadensis. This may be a result of long-term hybridization between these two species where their ranges overlap. Conserved clusters belonging to each of the three topologies were enriched for a variety of GO terms (table 5).

Gene Flow among P. glaucus, P. canadensis, and P. appalachiensis

To verify our phylogenetic signatures of shared genetic variation among the three tiger swallowtail species, we investigated potential introgression among species using Patterson’s $D$-statistic (Green et al. 2010; Durand et al. 2011). To do this, we counted derived SNP alleles supporting either “ABBA” or “BABA” patterns among the in-group taxa and then calculated the mean $D$ value across our conserved clusters (fig. 2). These results suggest substantial and nearly equal amounts of gene flow between P. glaucus and P. appalachiensis, compared with P. canadensis and P. appalachiensis ($P < 0.01$ for both comparisons). These results also suggest that the amount of gene flow between P. glaucus and P. canadensis is low compared with between each of these species and P. appalachiensis.

Chromosome Distribution of Conserved Clusters

We mapped clusters with different tree topologies back to the reference genome sequence for H. melpomene
and *B. mori* and then compared chromosomal clustering relative to the null hypothesis based on the chromosomal distribution of all conserved clusters. Of 3,961 conserved clusters, we were able to uniquely map 1,884 to the *Heliconius* genome and 2,101 to the *Bombyx* genome.

The results of this analysis suggest that conserved clusters with the same tree topology are likely to be clustered in the *Papilio* genome (table 6). It is important to note that the results are only suggestive of true clustering because this analysis rests on extrapolating the highly conserved synteny between *Heliconius* and *Bombyx* to *Papilio*, a group for which no genome sequence currently exists.

### Table 2

**Tree Topologies of Z-linked Clusters**

| Cluster ID | Nucleotide Alignment | Peptide Alignment | Annotation |
|------------|----------------------|-------------------|------------|
|            | Topological Structure | P Value | Topological Structure | P Value | |
| 105        | ((A,G),C,P) | 0.94 | ((A,C),G,P) | 1 | ww domain-containing adapter protein with coiled-coil domain |
| 611        | ((A,C),G,P)* | 0.87 | ((A,C),G,P)* | 0.77 | Scabrous protein |
| 930        | NA* | 0.79 | ((A,C),G,P) | 0.83 | Sercein 3 |
| 1294       | ((A,C),G,P)* | 0.83 | ((A,C),G,P)* | 0.96 | Catalase |
| 1617       | NA* | 0.97 | NA | — | Ankyrin repeat domain-containing protein 12 |
| 2021       | ((A,C),G,P)* | 0.79 | ((A,C),G,P)* | 0.75 | Disulfide-isomerase a5 |
| 2055       | (C,G),A,P* | 0.79 | (C,G),A,P) | 0.59 | Hepatic leukemia factor |
| 3130       | ((A,G),C,P) | 0.86 | ((A,C),G,P)* | 0.77 | Serine threonine-protein kinase osr1-like |
| 3347       | (C,G),A,P* | 0.82 | (A,C),G,P)* | 0.63 | Tyrosine hydroxylase |
| 3361       | (A,C),G,P)* | 0.68 | NA | — | Y-box protein |
| 3703       | (A,C),G,P)* | 0.72 | (A,C),G,P)* | 0.9 | Tyrosine-protein kinase abl-like |
| 4566       | (C,G),A,P) | 0.79 | (A,C),G,P)* | 0.78 | Acetyl-synthetase |
| 4569       | (A,C),G,P)* | 0.72 | (A,C),G,P) | 1 | Dipeptidase 1-like |
| 4894       | (A,C),G,P)* | 0.87 | (C,G),A,P) | 0.97 | Serine threonine-protein kinase osr1-like |
| 5837       | (A,C),G,P)* | 0.78 | (C,G),A,P) | 0.83 | Protein daughter of sevenless |
| 6828       | (A,C),G,P)* | 0.81 | (A,C),G,P) | 0.73 | Carboxypeptidase N subunit 2-like |
| 6895       | (A,C),G,P)* | 0.94 | (A,C),G,P) | 0.88 | Kettin |

**NOTE.**—Z-linked conserved clusters were identified by comparison with predicted CDS of Z-linked genes in the *Heliconius melpomene* genome sequence. SH *P* values were calculated based on both nucleotide and peptide alignments.

*NA* indicates no best topology because of the same highest value assigned to more than one topological structure.

*Indicates the tree topology was also supported by NJ method. Most of the tree structures not supported by NJ yielded an (A,C,G,P) structure in the NJ tree.

### Table 3

**Tree Topologies of Mitochondrial Clusters**

| Gene | Nucleotide Alignment | Peptide Alignment |
|------|----------------------|-------------------|
|      | Topological Structure | P Value | Topological Structure | P Value |
| 72s  | (C,G),A,P* | 0.824 | NA* | — |
| 16s  | (A,G),C,P* | 0.744 | NA | — |
| ATP6 | (A,G),C,P* | 0.749 | (A,C),G,P) | 0.498 |
| COI  | (A,G),C,P* | 0.673 | (A,C),G,P) | 0.547 |
| COII | (A,G),C,P* | 0.748 | NA | — |
| COIII| (C,G),A,P) | 0.711 | (A,C),G,P) | 0.844 |
| cytB | (A,G),C,P* | 0.797 | (A,G),C,P)* | 0.779 |
| ND1  | (A,G),C,P* | 0.578 | (C,G),A,P) | 1 |
| ND2  | (A,G),C,P* | 0.986 | (A,G),C,P)* | 1 |
| ND3  | (C,G),A,P) | 0.866 | (C,G),A,P)* | 0.792 |
| ND4  | (A,G),C,P* | 0.617 | (A,G),C,P) | 1 |
| ND4L | (A,G),C,P* | 0.763 | NA | — |
| ND5  | (C,G),A,P) | 0.961 | (A,C),G,P) | 0.818 |
| ND6  | (A,G),C,P* | 0.818 | NA | — |

**NOTE.**—Mitochondrial conserved clusters were identified by comparison with predicted mitochondrial CDS or rRNA. SH *P* values were calculated based on both nucleotide and peptide alignments.

*NA* indicates the tree topology was also supported by NJ. Most of the tree structures not supported by NJ yielded (A,G,C,P) structure in the NJ tree,

*Indicates the tree topology was also supported by NJ. Most of the tree structures not supported by NJ yielded (A,C,G,P) structure in the NJ tree.

*NA* indicates no peptide alignment because untranslated RNA sequence (12s and 16s rRNA) or no best topology because of the same highest value assigned to more than one topological structure.

### Table 4

**Number of Conserved Clusters with Well-Supported Tree Topologies**

| Topological Structure | Nucleotide | Peptide | Shared |
|-----------------------|------------|---------|--------|
| ((A,C),G,P) | 71 | 113 | 27 |
| ((A,G),C,P) | 58 | 93 | 19 |
| ((C,G),A,P) | 50 | 97 | 22 |

**NOTE.**—Counts were calculated based on either peptide or nucleotide alignment with the “shared” counts appearing in both groups.
Genes under Positive Selection

A general method to test for positive selection is based on likelihood ratio tests, but this is not a powerful approach with few sequences (Anisimova et al. 2001). Because we only had four sequences in each cluster, we calculated the $K_{aa}/K_{as}$ ratio for each conserved cluster and considered those with a value more than 1 to be candidates for positive selection (Li 1993).

### Table 5

**Functional Enrichment of Conserved Clusters with Various Topological Structures**

| Topology            | GO Term                                           | Category                              | Type | P Value |
|---------------------|---------------------------------------------------|---------------------------------------|------|---------|
| Nucleotide alignment |                                                   |                                       |      |         |
| $(A,C,G,P)$         | GO:0016301 Kinase activity                        | F                                     | 0.005|         |
|                     | GO:0016772 Transferase activity, transferring phosphorus-containing groups | F   | 0.005|         |
|                     | GO:0004672 Protein kinase activity                | F                                     | 0.006|         |
|                     | GO:0016773 Phosphotransferase activity, alcohol group as acceptor | F | 0.006|         |
|                     | GO:0016740 Transferase activity                   | F                                     | 0.016|         |
|                     | GO:0006091 Generation of precursor metabolites and energy | P | 0.028|         |
|                     | GO:0007049 Cell cycle                             | P                                     | 0.001|         |
|                     | GO:0006996 Organelle organization                 | P                                     | 0.001|         |
|                     | GO:0071842 Cellular component organization at cellular level | P | 0.001|         |
|                     | GO:0071841 Cellular component organization or biogenesis at cellular level | P | 0.001|         |
|                     | GO:0051716 Cellular response to stimulus          | P                                     | 0.002|         |
|                     | GO:0050794 Regulation of cellular process         | P                                     | 0.002|         |
|                     | GO:0007165 Signal transduction                    | P                                     | 0.002|         |
|                     | GO:0050896 Response to stimulus                   | P                                     | 0.005|         |
|                     | GO:0009987 Cellular process                       | P                                     | 0.006|         |
|                     | GO:0007005 Mitochondrion organization             | P                                     | 0.008|         |
|                     | GO:0023052 Signaling                              | P                                     | 0.009|         |
|                     | GO:0065007 Biological regulation                  | P                                     | 0.009|         |
|                     | GO:0006811 Ion transport                          | P                                     | 0.010|         |
|                     | GO:0005215 Transporter activity                   | F                                     | 0.011|         |
|                     | GO:0030234 Enzyme regulator activity              | F                                     | 0.012|         |
|                     | GO:0016043 Cellular component organization        | P                                     | 0.012|         |
|                     | GO:0071840 Cellular component organization or biogenesis | P | 0.012|         |
|                     | GO:0032501 Multicellular organismal process       | P                                     | 0.020|         |
|                     | GO:0007275 Multicellular organismal development   | P                                     | 0.020|         |
|                     | GO:0050789 Regulation of biological process       | P                                     | 0.022|         |
|                     | GO:0032502 Developmental process                  | P                                     | 0.041|         |
|                     | GO:00045182 Translation regulator activity        | F                                     | 0.027|         |
|                     | GO:0035556 Intracellular signal transduction      | P                                     | 0.040|         |
| Peptide alignment   |                                                   |                                       |      |         |
| $(A,C,G,P)$         | GO:0005623 Cell                                   | C                                     | 0.000|         |
|                     | GO:0044464 Cell part                              | C                                     | 0.004|         |
|                     | GO:0005622 Intracellular                         | C                                     | 0.005|         |
|                     | GO:0007267 Cell-cell signaling                    | P                                     | 0.015|         |
|                     | GO:0005811 Lipid particle                         | C                                     | 0.019|         |
|                     | GO:0016209 Antioxidant activity                   | F                                     | 0.028|         |
|                     | GO:0007154 Cell communication                     | P                                     | 0.049|         |
|                     | GO:0008283 Cell proliferation                     | P                                     | 0.011|         |
|                     | GO:0007005 Mitochondrion organization             | P                                     | 0.022|         |
|                     | GO:0004518 Nuclease activity                      | F                                     | 0.023|         |
|                     | GO:0030528 Transcription regulator activity       | F                                     | 0.027|         |
|                     | GO:0016032 Viral reproduction                      | P                                     | 0.043|         |
|                     | GO:0016788 Hydrolase activity, acting on ester bonds | F | 0.046|         |
|                     | GO:0007005 Mitochondrion organization             | P                                     | 0.024|         |

*F, P, and C stand for molecular function, biological process, and cellular component, respectively.*
In comparisons with the outgroup, 275 clusters yielded \( K_a/K_s \) ratios more than 1 in all three pairwise comparisons. The functional enrichment of these clusters yielded a variety of terms related to RNA/DNA modification, ion binding and transportation, cell cycle regulation, pigment metabolism, and hormone regulation (table 7). We further examined clusters for evidence of positive selection among the ingroup taxa, which yielded a small number of candidate genes (table 8). Interestingly, there was considerable overlap in the gene sets that emerged from our analysis comparing ingroup taxa, with those that exhibited high \( K_a/K_s \) ratios in comparisons between ingroup and outgroup species, suggesting some recurrent targets of selection. For those that did not overlap, we were particularly interested in genes showing evidence of selection in two pairwise ingroup comparisons, which would suggest adaptive evolution along a single lineage. This pattern could also result from divergent selection between \( P. glaucus \) and \( P. canadensis \) followed by introgression from one of those species into \( P. appalachianus \). Regardless, this approach yielded a list of candidate genes with some apparent enrichment of functions related to mitosis, eddyteroid-induction, and cuticular proteins.

Transcriptome assembly and clustering at the individual level, for MK tests, yielded 2,551 conserved clusters that included one sequence for each ingroup sample. Of these 2,225 also contained a single sequence for each \( P. polytes \) and so could be used to compare ingroup and outgroup taxa. A total of 56 clusters yielded significant \( (P < 0.05) \) MK tests between one or more ingroup taxa, and another 18 were significant in all ingroup versus outgroup comparisons (supplementary table S1, Supplementary Material online). Interestingly, there was only a single instance of overlap between the \( K_a/K_s \) and MK results, with a gene annotated as protein phosphatase regulatory subunit b gamma appearing in both ingroup versus outgroup comparisons. One factor that may contribute to the low overlap between \( K_a/K_s \) and MK results is the modest overlap in the data sets themselves. For instance, of the 62 clusters yielding significant \( K_a/K_s \) results
Table 8
Annotatiom of Clusters under Positive Selection among Ingroup Taxa

| Ks/Ka Ratio | Cluster ID | Annotation |
|-------------|------------|------------|
| A vs. C > 1, | 869 | Histone h1-like |
| A vs. G > 1, | 1537 | Splicing factor arginine serine-rich 6 |
| C vs. G < 1, | 4251 | Cuticle protein BmOrCPR83 (BmEdg84A) |
|            | 5014 | Polo |
|            | 6025 | Spinophilin-like |
|            | 6201 | Uncharacterized protein KIAA1841-like |
| A vs. C > 1, | 621 | Zinc finger protein on ecdysone puffs |
| A vs. G > 1, | 1165 | Vesicle associated |
| C vs. G < 1, | 1475 | Serine protease 14 |
|            | 3028 | Shaker-like potassium channel |
|            | 3111 | Nuclear hormone receptor |
|            | 4179 | Kinase d-interacting substrate of 220kDa-like |
|            | 5888 | NA* |
|            | 6892 | Hypothetical protein KGM_07109 [Danaus plexippus] |
| A vs. G > 1, | 153 | Pab-dependent poly-specific ribonuclease subunit 3-like |
| C vs. G > 1, | 726 | Tata-binding protein-associated phosphoprotein |
| A vs. C < 1, | 2649 | NA |
|            | 2702 | Ecdysone-induced protein 78c |
|            | 3483 | 40S ribosomal protein s3a |
|            | 3565 | Pdz and lim domain protein 3 |
|            | 4364 | Follistatin |
|            | 4564 | Encore protein |
|            | 4589 | Tyrosine-protein kinase fps85d-like isomorf 1 |
| A vs. C > 1, | 24 | Putative rRNA processing protein RRP7 |
| A vs. G < 1, | 114 | Hypothetical protein KGM_04049 [D. plexippus] |
| C vs. G < 1, | 497 | Cuticular protein 76bd |
|            | 754 | Hexokinase |
|            | 1392 | Inositol-triphosphate 3-kinase a-like |
|            | 1836 | G-protein coupled receptor mth2-like |
|            | 2064 | Cuticle protein BmOrCPR141 |
|            | 4222 | Ankyrin repeat domain-containing protein 57 |
|            | 4286 | Hypothetical protein KGM_21585 [D. plexippus] |
|            | 4640 | Rho guanine nucleotide exchange factor 7-like isomorf 1 |
|            | 4973 | Adipocyte plasma membrane-associated protein |
|            | 5095 | Unknown secreted protein [Papilio xuthus] |
|            | 5340 | Katanin p80 wd40-containing subunit b1 |
|            | 6084 | NEDD4-binding protein 2-like |
| A vs. G > 1, | 22 | Elongation factor 1 delta |
| A vs. C < 1, | 36 | Chondroitin 4-sulfotransferase |
| C vs. G < 1, | 854 | Atp-binding cassette sub-family g member 1-like |
|            | 926 | Naked cuticle-like protein |
|            | 1213 | Serine proteinase-like protein 1 |
|            | 2044 | xpg-like endonuclease |
|            | 2069 | RNA helicase-like protein |
|            | 2478 | upf0712 protein c7orf64-like |

Note—Three pairwise comparisons were made among P. glaucus, P. canaden sis, and P. appalachiensis and clusters with one or two ratios >1 were selected. Highlighted cluster IDs also exhibited evidence of positive selection in comparisons between ingroup and outgroup taxa (Table 5). *NA indicates no BLASTX hit against NCBI’s nr protein database.

Discussion
Our phylogenetic approach to transcriptome analysis is conceptually straightforward in that we simply want to track the evolutionary relationships among our three focal species on a gene-by-gene basis by comparing the fit of each gene among ingroup taxa (Table 8), only 22 were included in the data set used for MK tests. Similarly, 275 clusters yielded significant Ks/Ka results in comparisons between ingroup and outgroup taxa, 137 of which were in the data set used for MK tests. Additional factors could inflate or bias our test statistics, further contributing to low overlap in the results. For instance, the Ks/Ka ratio was developed to compare sequences from divergent species and it is known to perform poorly when applied to intraspecific polymorphism data (Kryazhimskiy and Plotkin 2008). Given that our ingroup taxa are closely related, and appear to be exchanging genes, some of the sequence variation we are applying to the data set used for MK tests is likely to be polymorphism, as opposed to fixed differences between species, which may bias the test. Furthermore, our MK tests are likely to be biased toward significant departures from neutrality because we have relatively little intraspecific data from which to estimate polymorphism information (Andolfatto 2008).
with the three possible evolutionary scenarios. In practice, however, this approach presents a variety of challenges that we worked hard to overcome. First, analysis of transcriptome data in the absence of a reference genome sequence presents a serious obstacle, especially in terms of identifying orthologs. Given potentially high sequence similarity among paralogs, identifying orthologous genes across species based on sequence homology alone is difficult (Pepke et al. 2009). A more powerful method for identifying orthologs is based on comparing the identity and order of genes surrounding putative orthologs (Hulsen et al. 2006), but this information is not available from our transcriptome data. Therefore, we applied very stringent filters to our homology-based pipeline which should remove virtually all sequence clusters in which paralogs might be an issue. In particular, we assembled our conserved clusters based on sequence homology, using a stringent matching threshold for comparisons within and between species, and then we discarded any clusters in which one or more species contributed two or more sequences. Our assembly statistics suggest that this approach was successful. Filtered data sets of individual species yielded approximately 10,000 unique gene sequences, which is a slightly less than the 12,669 predicted genes in *H. melpomene* or the 16,866 predicted genes in *Danaus plexippus*. Furthermore, combining data among species yielded 3,961 conserved clusters for which all four species contributed a single sequence.

A second challenge posed by the lack of a reference genome sequence emerges when trying to infer physical dynamics associated with evolutionary genomic phenomena. In particular, the evolutionary processes giving rise to well-resolved gene trees are likely to act on a scale larger than individual genes. For instance, genomic mixing between *P. glaucus* and *P. canadensis* in the formation of *P. appalachiensis* likely involved exchange of large portions of chromosomes, as has been documented in sunflower hybrid species (Rieseberg et al. 1995; Buerkle and Rieseberg 2007). However, without a genome sequence, we cannot test whether similar tree topologies are shared among linked genes. As a workaround, we used the fact that synteny is highly conserved between *Heliconius* and Bombyx to do a preliminary analysis, first mapping our conserved clusters back to the *Heliconius* genome and then to Bombyx. This approach verified that our conserved clusters really do represent a genome-wide sampling of markers. Subsequently, we tested the hypothesis that genes with the same tree topology were clustered in the genome, at the level of chromosomes. Although the results differed somewhat between the *Heliconius* and Bombyx reference, overall they suggested that particular chromosomes are enriched for specific tree topologies.

A third challenge that emerged from our analysis relates to the information content of nucleotide versus peptide alignments, and the unexpected finding that results from these two data sets were not always concordant. For instance, we found that 178 clusters yielded well-supported tree topologies in our analysis of the nucleotide data and 303 clusters in the analysis of peptide data. Surprisingly, there was relatively little overlap in these data sets, with only 68 clusters appearing in both. Although initially concerning, our follow-up analyses revealed that when nucleotide and peptide alignments for the same cluster both yielded statistical support for a topology, it was always the same topology. The real inconsistency then, was in the fact that a given cluster generally would only yield significant support for a given topology based on one of the two alignments. This issue is perhaps not surprising given the recent origin of all three ingroup species and the large amount of genetic variation shared across the group. Furthermore, despite the low overlap, the results of the nucleotide and peptide analyses were largely concordant, yielding approximately equal proportions of clusters for each of the three tree topologies. This suggests that the underlying biological processes giving rise to distinct tree topologies are being captured by both data sets.

**Hybrid Speciation and Genomic Mosaicism**

Our hypothesis of hybrid speciation with widespread genomic mosaicism in *P. appalachiensis* makes a clear prediction about gene tree topologies; we expect a substantial number of gene trees to support both ((A,C),G,P) and ((A,G),C,P) topologies. Consistent with this hypothesis, we found that Z-linked genes generally supported an ((A,C),G,P) topology while mitochondrial genes generally supported an ((A,G),C,P) topology. This pattern, which is consistent with prior results (Kunte et al. 2011), may help to explain the evolutionary origin of *P. appalachiensis* and its long-term maintenance as a separate species. A rich history of work in this system has revealed that female mimicry phenotype in *P. glaucus* is controlled primarily by a W-linked locus (Hagen and Scriber 1989; Scriber et al. 1996) and many of the thermal adaptations that differ between *P. glaucus* and *P. canadensis* are Z-linked (Hagen and Scriber 1995, 1989). The unique phenotype of *P. appalachiensis* combines the female mimetic polymorphism of *P. glaucus* with the cold-adapted traits of *P. canadensis*. Although we have not specifically traced relationships for W-linked markers (none have been identified in these species), our analysis of mtDNA suggests that the maternally inherited mitochondrion and W chromosome of *P. appalachiensis* are derived from *P. glaucus* while the Z chromosome is derived from *P. canadensis*. This is exactly the scenario predicted based on the mixed phenotype of *P. appalachiensis*.

We followed up on this analysis by examining tree topologies for the remaining, presumably autosomal, clusters. We found that approximately 38% of the conserved clusters that yielded a well-supported topology favored ((A,C),G,P) and approximately 31% favored ((A,G),C,P). However, we...
also found that almost 30% of the tree topologies supported the third topology, ((C,G),A,P), linking the putative parental species, with *P. appalachiensis* as their shared sister group. There are at least two potential explanations for widespread sharing of genetic variation between *P. canadensis* and *P. glaucus*. First, these two species have a well-characterized hybrid zone where their distributions meet near the border between Canada and the United States. It is very likely that there is substantial gene flow between these two species across this hybrid zone and that may contribute to the evidence of shared genetic variation we detected. To test this possibility, we calculated Patterson’s $D$-statistic (Green et al. 2010; Durand et al. 2011), a measure of shared genetic variation, and found that evidence for introgression between *P. glaucus* and *P. canadensis* is low relative to introgression into *P. appalachiensis*. Therefore, contemporary gene flow between *P. glaucus* and *P. canadensis* may not explain the roughly equal number of ((C,G),A,P) trees, compared with ((A,C),G,P) and ((A,G),C,P) trees. A second possible explanation is that the signature of shared variation between *P. glaucus* and *P. canadensis* derives from ancestral variation that predates the three species radiation. This scenario is very plausible given that *P. glaucus* and *P. canadensis* diverged only ≈600,000 years ago (Kunte et al. 2011) and both species probably have a much larger population size than *P. appalachiensis*.

Interestingly, although the number of clusters that support each topology is very similar, there may be a small excess of clusters linking *P. appalachiensis* to *P. canadensis*. This suggests that *P. canadensis* may have contributed slightly more to the *P. appalachiensis* genome than did *P. glaucus*. This scenario is consistent with the fact that prior to being described as a separate species, *P. appalachiensis* was often referred to as “giant *canadensis*” (Pavulaan and Wright 2002).

Adaptive Evolution

In addition to tracing the evolutionary history of genomewide markers, we also used our data to perform a broad survey of adaptive protein evolution. Although this analysis is preliminary, some interesting patterns emerge from our initial lists of candidate genes. For instance, genes that showed evidence of adaptive evolution between ingroup and outgroup species were enriched for biological functions that we might expect to be important in this group, such as pigmentation, hormonal sensitivity, and developmental processes. Furthermore, candidates for adaptive evolution among the ingroup taxa point to characteristics such as cuticle formation, which is likely to play a role in thermal adaptation (Futahashi et al. 2008). Much work remains to be done but this data set provides a first-pass list of potential targets for future functional study, and moreover, it provides an initial survey of loci that may have played an important role in the tiger swallowtail radiation.

Supplementary Material

Supplementary table S1 and figure S1 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

Acknowledgments

The authors thank Matthew Aardema, Harry Pavulaan, and David Wright for providing butterfly pupae for this analysis. They thank Nicholas Crawford and Sean Mullen for advice regarding phylogenetic methods and thank the reviewers for comments on the manuscript. This work was supported by the National Science Foundation grant DEB-1316037 to M.R.K.

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Associate editor: George Zhang