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Molecular Evolution and Developmental Expression of Melanin Pathway Genes in Lepidoptera

*Muktai Kuwalekar†, Riddhi Deshmukh†, Ajay Padvi and Krushnamegh Kunte**

National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bengaluru, India

Pigmentation is involved in a wide array of biological functions across insect orders, including body patterning, thermoregulation, and immunity. The melanin pathway, in particular, has been characterized in several species. However, molecular evolution of the genes involved in this pathway is poorly explored. We traced the molecular evolution of six melanin pathway genes in 53 species of Lepidoptera covering butterflies and moths, and representing over 100 million years of diversification. We compared the rates of synonymous and non-synonymous substitutions within and between these genes to study the signatures of selection at the level of individual sites, genes, and branches of the gene tree. We found that molecular evolution of all six genes was governed by strong purifying selection. Yet, a number of sites showed signs of being under positive selection, including in the highly conserved domain regions of three genes. Further, we traced the expression of these genes across developmental stages, tissues, and sexes in the *Papilio polytes* butterfly using a developmental transcriptome dataset. We observed that the expression patterns of the genes in *P. polytes* largely reflected their known tissue-specific function in other species. The expression of sequentially acting genes in the melanin pathway was correlated. Interestingly, melanin pathway genes also showed a sexually dimorphic pattern of developmental heterochrony, i.e., females showed prominent upregulation of melanin pathway genes in pre-pupal stage compared to males, while males showed prominent upregulation in 9-day pupal wings compared to females. Our evolutionary and developmental analyses suggest that the vast diversity of wing patterning and pigmentation in Lepidoptera may have evolved despite largely constrained sequence evolution, with potential contribution from differential developmental expression of genes in a highly conserved pathway.

Keywords: melanization, pigmentation, developmental heterochrony, butterfly wing coloration, wing patterns

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United Kingdom

***Correspondence:**
Krushnamegh Kunte
krushnamegh@ncbs.res.in

† These authors have contributed equally to this work

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INTRODUCTION

The evolution of color patterns contributes to the striking diversity of lifeforms. Coloration is a critical adaptation that impacts an organism's intra- and inter-specific communication and interactions. In several cases, the emergence of sexual dimorphism and polymorphism is also linked to the evolution and differential expression of pigmentation genes (Wittkopp et al., 2009;

Miyazaki et al., 2014; Yassin et al., 2016). Among Lepidoptera, wing coloration and patterning are remarkably diverse, which have shaped several adaptations such as aposematism, crypsis, mimicry, and thermoregulation (True et al., 2005; Hegna et al., 2013; Olofsson et al., 2013; Kronforst and Papa, 2015; Nadeau, 2016; Deshmukh et al., 2018; van't Hof et al., 2019). In insects, melanins, ommochromes, and pterins are three major biosynthesized pigments deposited in developing wing scales (Wittkopp and Beldade, 2009). Melanin pathway and its components—being involved in a wide range of physiological processes—are conserved across insects. For instance, increased melanization in high-altitude moths aids in thermoregulation, resulting in a trade-off between warning coloration and hindwing melanization (Hegna et al., 2013). Melanization is also associated with immunity, wound healing, and protection from both ultraviolet light and parasitoids (Yassine et al., 2012; Bilandžija et al., 2017). Melanin and related pigments—such as DOPA-melanin, dopamine-melanin, NBAD sclerotin, and NADA sclerotin—contribute to the production of black, brown, and yellow coloration (Wright, 1987; Koch et al., 2000a; Zhang et al., 2017a). The availability of genetic manipulation techniques in non-model systems has allowed elucidation of the role of melanin pathway genes in color adaptations (Zhang and Reed, 2017; Zhang et al., 2017a; Matsuoka and Monteiro, 2018). While the developmental mechanisms underlying pigmentation and patterning are being extensively studied, the evolutionary trajectories of these pigmentation genes remain poorly explored.

We chose six melanin pathway genes—*tan*, *black*, *ebony*, *pale* (*tyrosine hydroxylase*), *arylalkylamine N-acetyltransferase 1* (*aaNAT*), and *DOPA decarboxylase* (*DDC*)—whose developmental function in Lepidoptera coloration is well characterized (Zhang et al., 2017a; Matsuoka and Monteiro, 2018). The functions and phenotypic effects of these genes are summarized in **Figure 1** and **Supplementary Table S1**. Melanin synthesis occurs by a branched pathway with tyrosine and uracil as precursors which are shunted into the pathway by *pale* and *DDC* (Zhang et al., 2017a). *black* and *ebony* are involved in the synthesis of N-β-alanyl dopamine (NBAD), which is responsible for yellow coloration, while *tan* breaks down NBAD to dopamine, thus countering the synthesis of NBAD and the activity of *ebony*. *aaNAT* facilitates the formation of colorless cuticles essential for wing pigmentation (**Figure 1** and **Supplementary Table S1**). These genes are thus crucial for melanin production and its deposition on Lepidoptera wings. The pigments observed in adults start appearing on the wings and body tissues during late pupal stages, and black melanin is usually the last one to be deposited (Koch et al., 1998; Wittkopp and Beldade, 2009; Matsuoka and Monteiro, 2018). In this study, we trace the molecular evolution of these six genes in 53 species across nine superfamilies of Lepidoptera (total over 100 million years of divergence) and additionally characterize their expression patterns across various developmental stages and tissues of *Papilio polytes* to address the following questions: (i) What selection pressures have shaped the evolution of these genes in the Lepidoptera? (ii) Do these genes show differential activity in sexually dimorphic species? and (iii) Do they have similar expression patterns during larval pigmentation and adult wing patterning and pigmentation?

MATERIALS AND METHODS

Gene Sequences and Multiple Sequence Alignment

We downloaded whole genome sequences of 53 species of Lepidoptera from GenBank[1], LepBase[2], and GigaDB[3], which best represented different families. We selected six genes which are central to pigmentation and patterning pathways, namely *tan*, *black*, *ebony*, *pale* (*tyrosine hydroxylase*), *aaNAT* (*Arylalkylamine N-acetyltransferase 1*), and *DDC* (*dopa decarboxylase*) (Wright, 1987; Zhang et al., 2017a; Matsuoka and Monteiro, 2018). We performed exon-wise NCBI local tBLASTn to locate these genes within genomes of the selected species (**Supplementary Table S2**). For all the genes, we chose the longest isoform for performing tBLASTn as it incorporates maximum sequence information for the given gene. Using these gene coordinates, we extracted respective genes from the genome using an in-house python script. We chose the hits with the highest similarity scores to avoid misidentification. The downloaded genome sequences showed no evidence of duplication for any of the six melanin pathway genes. Several genome files (such as those of *Bicyclus anynana, P. polytes, Papilio xuthus*, and *Bombyx mori*) had annotated two copies of *aaNAT* and *black*, but they shared low similarity scores (∼40%) and were therefore not considered as duplicates in our analysis. We performed multiple sequence alignment of each gene with MEGA X MUSCLE aligner with codon alignment option (Kumar et al., 2018). We included homologous gene sequences from Diptera as outgroups for respective genes.

Gene Tree Construction and Phylogenetic Analysis

We constructed a species-level phylogeny with two mitochondrial (*cytochrome c oxidase I*, *acetyl-CoA acetyltransferase*) and ten nuclear genes (*elongation factor 1-alpha*, *wingless*, *ribosomal protein S5*, *ribosomal protein S2*, *isocitrate dehydrogenase*, *glyceraldehyde-3-phosphate dehydrogenase*, *malate dehydrogenase*, *catalase*, *CAD*, *ribosomal protein S27a/hairy cell leukemia*) that are used commonly in phylogenetic reconstruction (Wahlberg and Wheat, 2008). We performed multiple sequence alignment on these 13 markers using the codon aligner PRANK v150803 (Löytynoja and Goldman, 2005). For construction of species and gene trees, we calculated the best partition scheme and corresponding sequence evolution model using Partition Finder 2.1.1 (Lanfear et al., 2016). We chose greedy algorithm and Mr. Bayes model of evolution. We selected Bayesian information criterion to compare and choose from the best-fit models. We used a split

[1]http://www.ncbi.nlm.nih.gov

[2]http://www.lepbase.org

[3]http://www.re3data.org

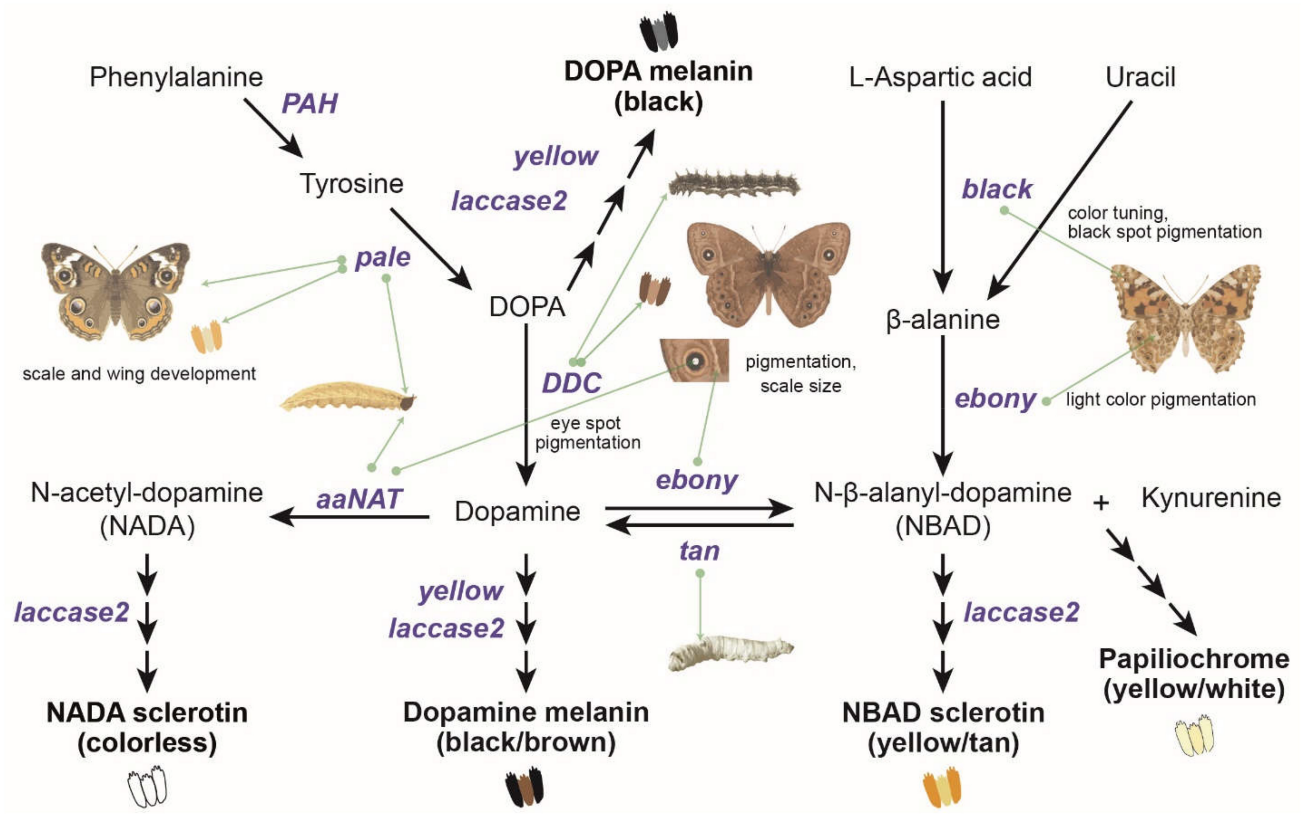

FIGURE 1 | Melanin pathway genes and their phenotypic effects in Lepidoptera. The proposed melanin pathway (Wright, 1987; Zhang et al., 2017a) illustrates the genes/enzymes (colored in purple) that catalyze different steps in the production of four major pigments. The end products of the pathway conjugate with sclerotin to produce differently colored scales (Vavricka et al., 2010), as illustrated. Each gene also regulates individual wing elements in different species of butterflies (green arrows), as deciphered from their knockout phenotypes (summarized in **Supplementary Table S1**).

frequency below 0.01 to assess stationarity and to set the burn-in in Mr. Bayes and then built a consensus tree using the remaining trees (Ronquist et al., 2012).

Determination of Synonymous (dS) and Non-synonymous (dN) Substitution Rates

To calculate site-wise synonymous and non-synonymous substitution rates of each gene, we used their respective MUSCLE alignments and the fixed effect likelihood (FEL) method with a maximum-likelihood (ML) approach (Kosakovsky and Frost, 2005). We plotted and compared codon-wise dN, dS, and dN/dS values for each gene (**Figure 2**) and performed a Kruskal–Wallis test followed by Dunn's test with Bonferroni correction in R (Derek et al., 2020). We also identified sites that have been subjected to pervasive diversification (dN/dS > 1) or purifying selection (dN/dS < 1) within every gene using FEL. We calculated global omega (*R* value, which represents dN/dS for the entire sequence) for respective alignments using AnalyzeCodonData function of HyPhy 2.3.14 (Pond et al., 2005; Kosakovsky et al., 2020; **Table 1**). For comparison, we also calculated *R* for genes used in species-level phylogenetic reconstruction. To identify conserved domains in the given proteins, we used the Conserved Domain Database (CDD) and the CD-Search Tool (Marchler-Bauer et al., 2012). We separately performed FEL analysis and estimated global omega for these domains to compare gene-wide and domain-wide signatures of selection (**Figure 2** and **Table 1**).

Branch-Level, Gene-Wide, and Site-Wise Assessment of Molecular Evolution

We used aBSREL (adaptive Branch-Site Random Effects Likelihood) to test for positive selection along each gene tree (Kosakovsky et al., 2011; Smith et al., 2015). We performed aBSREL analysis in exploratory mode which tests for positive selection along a branch of the gene tree and corrects for multiple testing. aBSREL models site and branch-level omega values but does not test for site-level selection. We used BUSTED to investigate gene-level positive selection. It calculates site-specific evidence ratios and combines this information from sites with weak positive selection to infer selection at the gene level. We carried out MEME (Mixed Effects Model of Evolution) which takes ML approach to test whether individual sites have been subjected to episodic positive or diversifying selection (dN/dS > 1). We performed FEL, MEME,

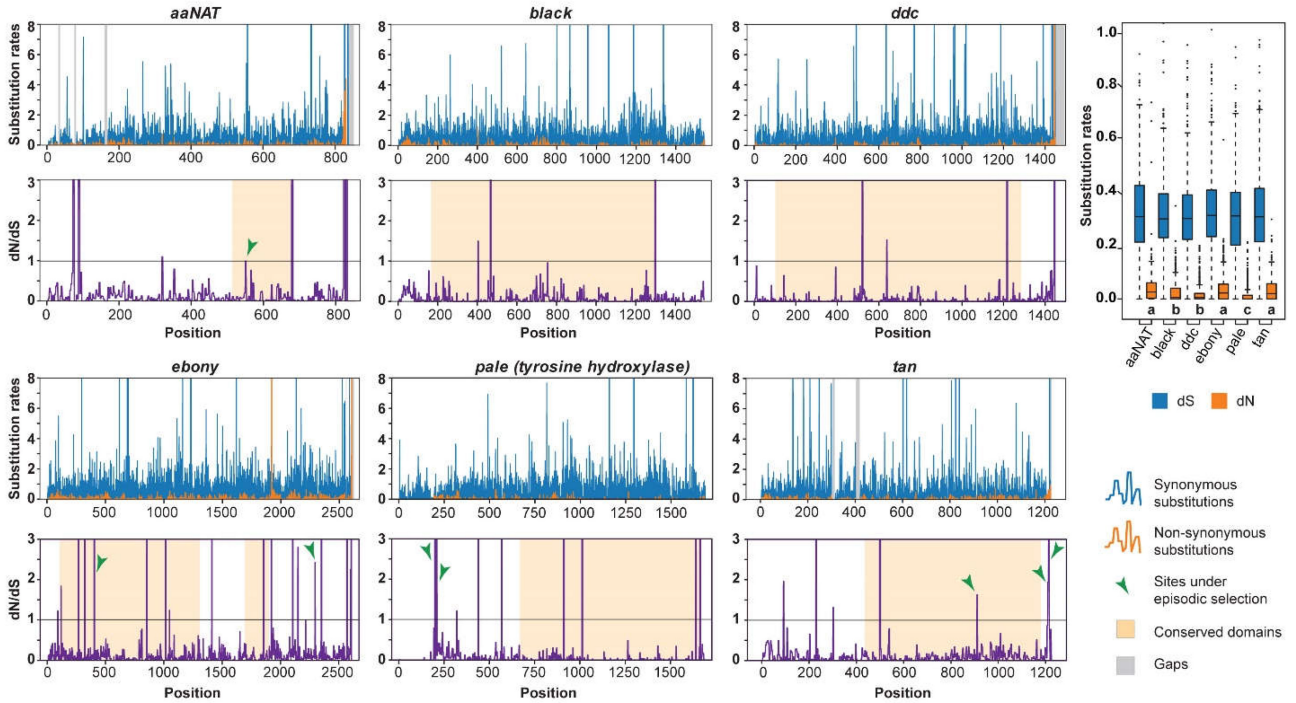

FIGURE 2 | Molecular evolution of selected genes in the melanin pathway. Molecular evolution is shown with individual rates of synonymous (blue) and non-synonymous substitutions (orange) for each codon (top panels) and dN/dS ratios for individual codons (lower panels). Functional domains for each gene are highlighted along with sites under episodic diversifying selection (green arrows, see **Supplementary Table S4**). Rates of substitutions are compared between genes in the boxplot (right) where dN values significantly differ across genes ($p < 0.001$, see **Supplementary Table S3**). Groups that do not differ statistically are represented by the same letter.

BUSTED, and aBSREL on the Datamonkey Adaptive Evolution Server (Weaver et al., 2018), processing the data using in-house scripts.

Sample Collection, RNA Extraction, and Transcriptome Sequencing for Quantification of Expression

We bred greenhouse populations of *P. polytes* from mated wild-caught females. We maintained larvae at 28 ± 4°C on lemon (*Citrus* sp.) and curry plants (*Murraya koenigii*) and adults on Birds Choice^{RM} butterfly nectar. We preserved *P. polytes* at different stages of metamorphosis in TRIzol^{RM}. We collected eggs at 2, 10, and 24 h, and at 3 days after oviposition, and pooled five eggs for each sample to get sufficient amount of RNA. We sampled larvae at 1st, 3rd, and 5th instars. For RNA extraction, we used gutted bodies of 1st and 3rd instar larvae and dissected wing disks from 5th instar larvae and pre-pupae to get three tissue types—forewings, hindwings, and gutted body. We collected pupae at 3, 6, and 9 days after pupation and dissected them to separate the forewings, hindwings, abdomen, thorax, and head. Several pigmentation pathway genes participate in transport and phototransduction in insect eyes (True et al., 2005; Ziegler et al., 2013). To account for altered sensitivity and visual perception due to the mating status of the butterflies, if any, we collected unmated and mated adults separately and further dissected them to separate abdomen, head, eyes, and thorax. We used pure-breeding mimetic lines for this study. We sampled eggs, 1st and 3rd instar larvae in triplicates, 5th instar larvae, pre-pupae, and pupae in duplicates for males and females each, and adults in quadruplicates for each sex.

We extracted RNA from preserved tissues using the chloroform–isopropanol-based extraction method. We prepared libraries using TruSeq® RNA Sample Preparation Kit v2 and used Qubit fluorometric quantification. We also used Bioanalyzer to check library profiles. For transcriptome sequencing, we used 2 × 100 PE runs on Illumina HiSeq 2500 and obtained ∼20 million reads for each sample. We performed quality check on these reads using FastQC, trimmed them using Trimmomatic, and aligned them to *P. polytes* reference genome (Nishikawa et al., 2015) using STAR and HISAT2 aligners. We chose the alignments showing higher mapping percentages. We used HTSeq for obtaining raw counts and edgeR pipeline for analyzing and plotting gene expression for all the genes. To determine the effect of sex on expression, we performed a principal component analysis (PCA) in R using the normalized counts for five melanin pathway genes. We excluded *tan* as it did not show sexual dimorphism in its expression profile. We also performed pairwise correlation tests in R (Pearson correlation) to test similarities of expression profiles for all six melanin pathway genes.

TABLE 1 | Molecular evolution of melanin pathway genes in Lepidoptera.

A.

Gene	*aaNAT*	*black*	*DDC*	*ebony*	*pale*	*tan*
Number of species	37	44	41	39	38	36
Sequence length in nucleotides	852	1545	1503	2622	1689	1277
Percentage of sites with pervasive purifying selection	79.9	89.1	88.02	88.1	88.45	86.06
Percentage of sites with pervasive diversifying selection	0	0	0	0	0	0
Number of branches under episodic diversifying selection	0	2	0	11	2	3
Evidence for gene-wide episodic diversifying selection	Yes	Yes	No	Yes	Yes	No
Number of sites under episodic selection	1	0	0	2	2	3
Global omega value for the gene	0.09	0.06	0.04	0.08	0.04	0.08
Omega value for conserved domain 1	0.07	0.05	0.03	0.07	0.02	0.07
Omega value for conserved domain 2	–	–	–	0.05	–	–

B.

Gene	Global omega value for the gene
Cytochrome c oxidase I	0.03
Acetyl-CoA acetyltransferase	0.03
Elongation factor 1 – alpha	0.017
Wingless	0.03
Ribosomal Protein S5	0.02
Ribosomal protein S2	0.03
Isocitrate dehydrogenase	0.02
Glyceraldehyde-3-phosphate dehydrogenase	0.03
Malate dehydrogenase	0.03
Catalase	0.04
CAD	0.02
Ribosomal protein S27a/hairy cell leukemia	0.006

(A) *A summary of tests of selection at the levels of individual sites, genes and branches. All the genes studied contained a single conserved domain, except for two domains in ebony.* ***(B)*** *Global omega values for genes used in reconstruction of the species tree for taxa used in this study.*

RESULTS

Melanin Pathway Genes Are Single-Copy Conserved Genes Under Strong Purifying Selection in Lepidoptera

We reconstructed gene trees for each of the six genes (**Supplementary Figure S1**). Each gene tree was well supported, and it essentially mirrored the species tree in terms of species relationships and divergence, except *aaNAT*, which had poor resolution with a high degree of polytomy. Absence of long branches and broad similarity with the species tree suggest that these genes have evolved with species divergence and do not seem to have undergone rapid evolution that would be incongruent with the species tree in any lineage.

We plotted dN/dS values for each site in each gene (**Figure 2**). The molecular evolution of these genes was characterized by predominantly synonymous substitutions, suggesting constrained evolution (dN/dS values for most sites were well below 1, with mean dN/dS value for each gene ranging from 0.04 to 0.10). Rates for gene-wide synonymous substitutions were similar, but non-synonymous rates were significantly different between three groups of genes: *black* and *DDC*; *ebony*, *tan* and *aaNAT;* and *pale* (Dunn's test, $p < 0.001$) (**Supplementary Table S3**). Overall, >80% sites showed signatures of pervasive purifying selection ($p < 0.05$), but no sites showed signatures of pervasive diversifying selection in any melanin pathway genes (**Table 1**).

All the genes had a single functional domain except for *ebony*, which contained two (**Figure 2**). *R* values for the six genes ranged between 0.04 and 0.09 and were found to be consistently lower for functional domains compared to the gene-wide values (**Table 1A**). Similarly, *R* values for genes used in species-level phylogenetic reconstruction were considerably lower and ranged between 0.006 and 0.04. We observed that the non-synonymous substitution rate was lowest in *pale*, followed by *black* and *DDC* (**Figure 2**, $p < 0.05$). Since *DDC*, *black,* and *pale* are essential for the synthesis of dopamine—a precursor for multiple pathways including neuromodulation, immune functions, and the circadian cycle (Basu and Dasgupta, 2000; Allen et al., 2011; Shang et al., 2011)—the low dN/dS as well as global *R* values were expected for these genes. On the other hand, *tan*, *aaNAT,* and *ebony* yielded high *R* values, indicating that they may have evolved under relatively relaxed selection (**Figure 2** and **Table 1**).

Melanin Pathway Genes Show Signs of Episodic Positive Selection

In spite of the overall constrained molecular evolution of the six genes, eight sites showed signatures of episodic diversifying selection ($p < 0.05$), including some in the otherwise highly conserved domain regions (**Figure 2**, **Table 1**, and **Supplementary Table S4**). Interestingly, although *pale* generally shows very constrained molecular evolution similar to *DDC* and *black*, it also contained two of the eight sites under episodic positive selection. While BUSTED could not detect gene-wide episodic diversifying selection in *DDC* and *tan*, aBSREL found evidence for it in all the genes except *DDC*.

Melanin Pathway Genes Show Stage-Specific, Often Sexually Dimorphic Expression

We traced the expression of the six genes across different developmental stages, tissues, and sexes in the developmental transcriptome of *P. polytes*. Broadly, we identified two groups with similar expression patterns across stages and tissues: *aaNAT,*

DDC, and *pale* formed one group, while *black* and *ebony* formed another group (**Figure 3**). Since *pale, DDC,* and *aaNAT* convert tyrosine to N-acetyl-dopamine in successive reactions with no intermediates (**Figure 1**), we expected similar activity patterns of these genes. *aaNAT, DDC,* and *pale* showed similar activity across stages with a peak in the pre-pupal stage (**Figure 3** and **Supplementary Figure S2**), suggesting a more general function than melanization-specific activity. Although *ebony* combines the products of *black* and *DDC* to produce NBAD (**Figure 1**), its expression pattern was similar to that of *black,* not *DDC.* This suggested that *ebony* activity was more dependent on *black* than on *DDC.* We tested this possibility with an analysis of co-expression between these genes, which indeed showed that the expression profiles of *ebony* and *black* were most strongly correlated compared to any other pairs among these six genes (**Figure 3C**). Interestingly, *DDC* showed a strong positive correlation with all the other genes except *tan*, which can be attributed to the central function of *DDC* in the melanin pathway (**Figure 3C**), whose products feed into several branches. These correlations need to be experimentally verified.

Unlike any of the other genes, *tan* had a remarkably distinct pattern of expression across development. It showed little to no expression in wings but it was expressed in the 1st and 3rd instar larvae, suggesting possible involvement in larval melanization. Expression of *tan*, *black,* and *ebony* in adult eyes and head may have resulted from their role in photo-transduction. For example, *tan* is involved in metabolism of neurotransmitters in photoreceptors, while *ebony* and *black* play a role in signal transduction in the optic lobe (True et al., 2005; Ziegler et al., 2013). Because of this, we further tested whether mated and unmated females show differential expression since female visual systems may switch from mate choice to host plant search after mating. However, expression of these genes was similar in unmated and mated females as well as in males (**Figure 3B** and **Supplementary Table S5**).

Finally, to test for sexual dimorphism in the expression patterns of the melanin pathway genes, we performed a PCA using normalized counts of five genes excluding *tan.* Most stages and tissues had low expression of melanin pathway genes, which caused most of the points to cluster around zero. Two stages that stood out—pre-pupal tissues and 9-day pupal wings—showed sexual dimorphism. Melanin pathway genes were upregulated in female pre-pupae and male

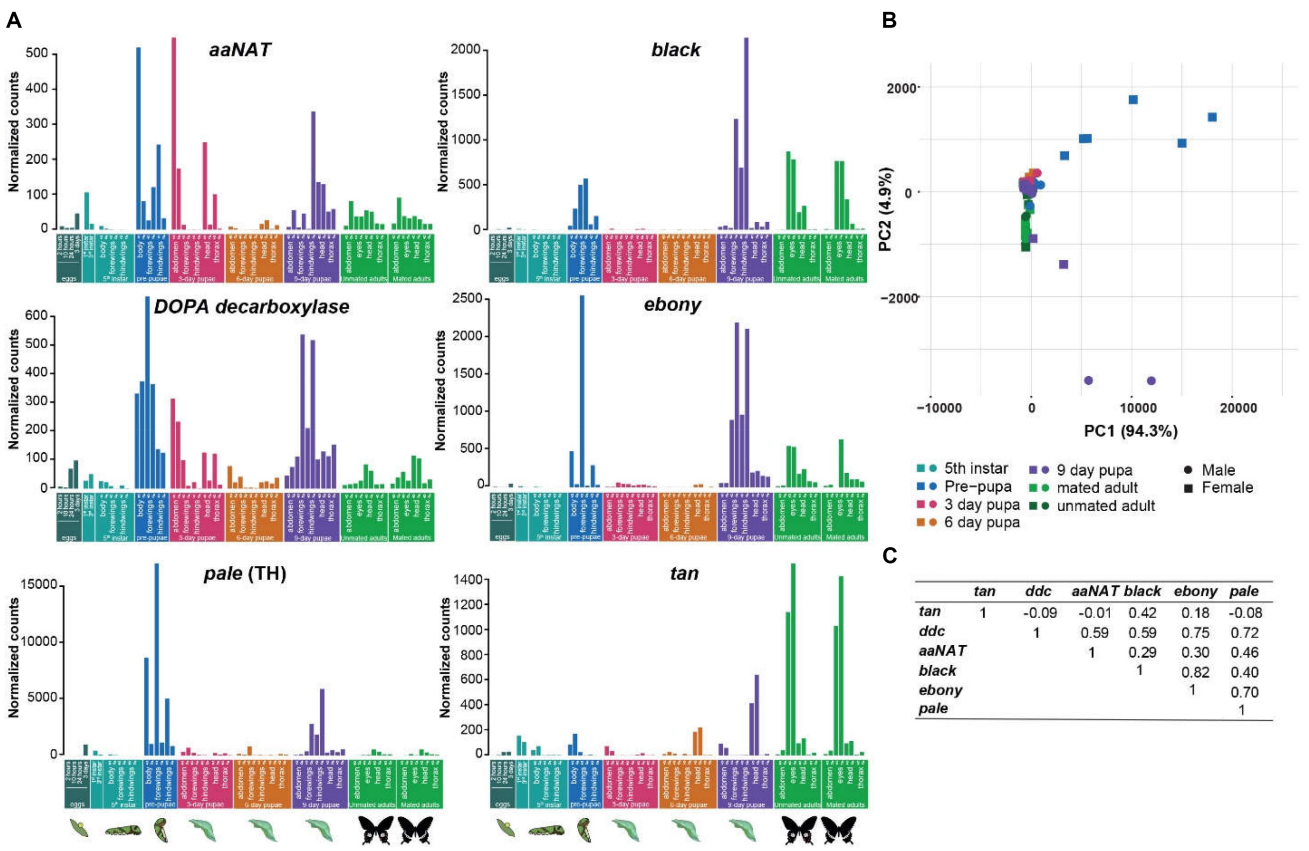

FIGURE 3 | (A) Expression of the six melanin pathway genes across developmental stages of *Papilio polytes*. Means of normalized counts for each stage, tissue and sex (beyond 5th instar larvae) obtained from transcriptome data are plotted. The developmental stages are color-coded and pictorially represented at the bottom (instar indicates larval instars). **(B)** Principal component analysis (PCA) of normalized expression data across developmental stages, tissues and sexes for five genes. **(C)** Pairwise Pearson correlation coefficients for co-expression profiles of the six genes.

wings of 9-day pupae, indicating a sex-specific developmental heterochrony in activity of these genes in developing pupae of *P. polytes.*

DISCUSSION

The melanin pathway is complex and versatile, with products of intermediately placed genes feeding into cross-pathways, performing diverse biological functions (**Figure 1**). The feasibility of genetic manipulation in non-model organisms has made it possible to understand the extent of role played by each gene in pigment production in a wide range of organisms (Mazo-Vargas et al., 2017; Zhang et al., 2017a,b; Connahs et al., 2019). We supplement this understanding by studying the molecular evolution of six genes (*tan*, *black*, *ebony*, *pale*, *aaNAT,* and *DDC*) in the melanin pathway that have prominent roles in color production in butterflies and moths (Koch, 1995; Koch et al., 2000a,b; Futahashi and Fujiwara, 2005; Futahashi et al., 2010; Zhang et al., 2017a; Matsuoka and Monteiro, 2018). With each gene governing a generalized or specialized step, the pathway experiences varied constraints at different levels. We estimated synonymous and non-synonymous substitution rates for each gene using sequence data and tested for signatures of selection. Our study shows that these six genes are highly conserved, despite 100 million years of evolutionary divergence across the sampled Lepidopteran superfamilies (Misof et al., 2014). However, the degree of conservation varies across genes, and most of them even show some signatures of episodic and positive selection.

Our selection analysis revealed that all the melanin pathway genes studied here show signatures of strong purifying selection. The dearth of non-synonymous substitutions across these genes indicates a lack of pervasive diversifying selection at a sequence level, although the developmental regulation of these genes produces dissimilar color patterns across species (Wittkopp et al., 2009; Miyazaki et al., 2014; Yassin et al., 2016). While testing for episodic diversifying selection at the levels of individuals sites, branches and genes, *aaNAT*, *black*, *ebony*, *pale,* and *tan*, but not *DDC*, showed evidence of diversifying selection. Similarly, we did not find any site-level evidence for diversifying selection in *black*. These differential signatures of selection across the genes are consistent with their estimated non-synonymous substitutions, which shows that *black* and *DDC* have followed more constrained evolutionary trajectories compared to the other four genes. The slight difference between domain-wide and gene-wide *R* values suggests that these six genes are nearly as conserved as their functional domains, and in some cases, this may be a result of the domain spanning a large portion of the gene. The melanin pathway genes showed slightly higher *R* values compared to the genes used for phylogenetic reconstruction, reiterating their high degree of conservation. Our study does not offer insights into the functional roles of these sites under diversifying selection. However, it does point out potential developmental genetic targets for future manipulative experiments. The functional roles of these sites may also become clear using protein structural analysis.

We obtained a few contradictory results in our selection analysis with the three methods used. For example, gene-level analysis using BUSTED inferred the presence of episodic diversifying selection in *black*, but MEME could not detect individual sites under selection. This may be because BUSTED is capable of combining information from sites with weak signals of positive selection to infer presence of selection at the whole-gene level, unlike MEME, which detects individual sites and might therefore underestimate sites under selection at the gene level. Similarly, we performed branch-level tests of diversifying selection with aBSREL, whose exploratory module is less likely to detect selection compared to targeted testing (Kosakovsky et al., 2011; Smith et al., 2015). This may have resulted in *aaNAT* not showing branch-level selection despite showing site-level and gene-level diversifying selection using other methods.

We explored the spatio-temporal activity of melanin pathway genes in *P. polytes* using a developmental transcriptome dataset. The first four instars of *P. polytes* larvae mimic bird droppings with black, brown, and white coloration on their bodies. We expected to detect expression of melanin pathway genes in the eggs and 1st and 3rd larval instars, similar to what we see in 9-day old pupae. However, we did not find substantial larval expression of any of the six genes (except *tan*) even though knockout phenotypes in previous studies have reported a lack of larval pigmentation (Zhang et al., 2017a; Matsuoka and Monteiro, 2018). It is possible that the low expression that we detected suffices in triggering melanization, or we may have missed the transient stage where melanin pathway genes are upregulated in early development. Exploring the expression patterns of other genes in the pathway, such as *laccase2* and *yellow*, may help corroborate either hypothesis. The expression patterns of genes also showed signs of sex-specific developmental heterochrony in melanin production, with expression during pupal development peaking early in females (pre-pupae) and late in males (9-day pupae), as corroborated by PCA. While this could be an artifact of sampling, in some cases (especially between male and female pre-pupae and 9-day old pupae), the difference is quite stark and observed across five of the six genes to be attributed to sampling error. It is possible that males and females differentially invest in melanin production and immunity (or other functions that require melanin pathway intermediates), which would also explain the heterochrony we observed. However, this needs further investigation. With the additional complexity of mimicry in this species, and the use of only mimetic females in this study, the effect of mimicry-related wing-pattern reorganization on melanin pathway genes remains to be explored and could perhaps be linked to the developmental heterochrony.

Melanin pathway genes such as *tan, ebony,* and *yellow* are involved in the evolution of color pattern-related sexual dimorphism and polymorphism (Wittkopp et al., 2009; Miyazaki et al., 2014; Yassin et al., 2016), suggesting that these genes have the potential to evolve novel functions despite evolutionarily constrained sequences. Many butterflies and moths exhibit

sexual dimorphism and/or female polymorphism, so they are good system to study signatures of selection in such clades. This would also require information regarding functional allelic variation and duplicated copies of these genes, which we did not encounter in the genes and species that we studied. Alternatively, production of certain color patterns is associated with changes in proximal and/or distal *cis* regulatory regions which govern gene expression. For instance, gain of novel transcription factor binding sites in the *cis*-regulatory region of *yellow* has influenced wing pattern evolution in *Drosophila biarmipes* (Gompel et al., 2005). Examining the regulatory regions of such highly conserved genes and tracing the molecular evolution of upstream regulators might help detect sites that influence color pattern-related sexual dimorphism or polymorphism. Furthermore, selective manipulation of protein-coding versus *cis*-regulatory regions of melanin pathway genes might also help dissect the relative contributions of these genetic elements in pigment production. We have excluded *yellow* gene family from our study even though substantial work has been done on this in flies and butterflies (Arnoult et al., 2013; Miyazaki et al., 2014; Camino et al., 2015; Zhang et al., 2017a; Matsuoka and Monteiro, 2018). We were unable to identify sequences of this gene family from the downloaded genomes with sufficient confidence due to issues with sequence similarity scores. However, the evolution of this gene family has been explored previously (Ferguson et al., 2011).

Our work demonstrates that molecular sequences of the six melanin pathway genes are highly conserved across the vastly diverse Lepidoptera, yet they show some signatures of positive selection. The pattern of conservation and divergence of these genes may be a direct outcome of their functions. Such studies on molecular evolution of genes associated with ecological adaptations (Ferguson et al., 2011; Hughes, 2011; Baral et al., 2019) can help establish baselines against which evolutionary patterns of other genes could be compared. We also explored the developmental expression of these genes across tissues and sexes, which recapitulates what we observe in adult stages, but does not provide insights into larval pigmentation. Our work provides a framework in which broad comparisons can be made across genes and species to understand the genetics and evo-devo of complex pathways that produce the remarkable color patterns displayed by insects.

DATA AVAILABILITY STATEMENT

The raw RNA seq data are deposited in the NCBI SRA database (BioProject PRJNA634605, Accession nos. SAMN15001929 to SAMN15002046; https://dataview.ncbi.nlm.nih.gov/object/PRJNA634605?reviewer=mf4q72fcfvq67spjf5gl1sduop). The accession numbers for genomes used in the molecular evolutionary analysis are provided in **Supplementary Table S2**.

AUTHOR CONTRIBUTIONS

RD and KK designed the study. MK and AP downloaded the sequence data and performed the molecular evolution analysis. RD performed the developmental transcriptome sequencing and analysis. MK and RD prepared the figures and wrote the manuscript. KK conceived and directed the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00226/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.