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Phylogenetic incongruence and the evolutionary origins of cardenolide-resistant forms of Na^+,K^+-ATPase in *Danaus* butterflies

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Abstract

Many distantly-related insect species are specialized feeders of cardenolide-containing host plants such as milkweed (*Asclepias* spp.). Studies have revealed frequent, parallel substitution of a functionally important amino acid substitution (N122H) in the alpha subunit of Na^+,K^+-ATPase (N122H) in many of these species. This substitution facilitates the ability of these insects to feed on their toxic hosts. Among milkweed butterflies of the genus *Danaus*, the previously established phylogeny for this group suggests that N122H arose independently and fixed in two distinct lineages. We re-evaluate this conclusion by examining *Danaus* phylogenetic relationships using >400 orthologous gene sequences assembled from transcriptome data. Our results indicate that the three *Danaus* species known to harbor the N122H substitution are more closely related than previously thought, consistent with a single, common origin for N122H. However, we also find evidence of both incomplete lineage sorting and post-speciation genetic exchange among these butterfly species, raising the possibility of collateral evolution of cardenolide-insensitivity in this species group.

Keywords

Danaus; Danaidae; milkweed butterfly; incomplete lineage sorting; genetic introgression; Na^+,K^+-ATPase

Introduction

Studies of convergent evolution have improved our understanding of the genetic architecture underlying adaptive traits and yielded insights into the extent of constraint on the evolution of novel phenotypes (Stern 2013). One example of this is insects that feed on plants that produce toxic secondary metabolites called cardenolides (Dobler et al. 2011; Agrawal et al.

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Data Accessibility

The raw RNAseq data used in this study can be found at: http://genomics-pubs.princeton.edu/insect_genomics/data.shtml. All other data is available on GenBank as indicated in the text and supplementary information.

2012). These toxins provide defense against herbivory, yet many specialist insect species have nonetheless evolved the ability to feed on cardenolide-containing plants. Some of these specialists also actively sequester cardenolides for use in their own defense against predators (Dobler et al. 2011; Agrawal et al. 2012). Among these insects are milkweed butterflies in the genus *Danaus*, which as larvae feed almost exclusively on *Asclepias* host plants that often contain cardenolides (Ackery and Vane-Wright 1984; Petschenka and Agrawal, 2015). Three *Danaus* species (*D. plexippus*, *D. erippus* and *D. eresimus*) have been shown to harbor a specific, functionally important asparagine to histidine amino acid substitution (N122H) in the alpha subunit of Na^+K^+-ATPase (ATPα, see Fig. 1). This particular substitution has evolved in parallel numerous times in cardenolide-sequestering insects belonging to different orders (Zhen et al. 2012; Dobler et al. 2012).

Despite sharing the N122H substitution, *D. eresimus* is considered a distant relative of *D. plexippus* and *D. erippus* within the *Danaus* genus (Fig 1; Ackery and Vane-Wright 1984; Lushai et al. 2003; Smith et al. 2005; Brower et al. 2010). Given these relationships, a straightforward interpretation is that N122H is phylogenetically incongruent and arose in parallel within the *D. plexippus* and *D. eresimus* lineages (Zhen et al. 2012). However, several other possibilities exist. For example, it is also possible that N122H fixed early on in the evolution of the *Danaus* genus and subsequently reverted back to asparagine in the *D. gilippus/D. chrysippus* lineage. Such a reversion is hypothesized to have occurred in a similar context involving varanid lizards (Ujvari et al. 2015). Another possibility is that of "collateral evolution" (Stern 2013). For example, both asparagine (N) and histidine (H) may have been segregating in a common ancestor of these species and histidine subsequently fixed independently in the *D. plexippus* and *D. eresimus* lineages, while being lost in other *Danaus* species. Another form of collateral evolution is adaptive introgression, which may also explain the sharing of N122H among *Danaus* species. While previously thought to be rare or unimportant, post-speciation genetic exchange has become increasingly recognized as an important contributor to the evolutionary history of many species (Mallet et al. 2015),

In addition to N122H substitution of ATPα, other phylogenetic incongruences in *Danaus* have also been noted. These include differences in chromosome number (Brown et al. 2004), allozyme phenotypes (Kitching 1985) and morphological traits (Ackery and Vane-Wright 1984). In this study, we evaluate the most likely evolutionary scenario underlying the evolution of the N122H substitution by examining species relationships among the North American species of *Danaus* butterfly using ATPα sequences and a large set of protein coding sequences derived from comparative transcriptomic data. We also use this dataset to evaluate the extent to which incomplete lineage sorting (ILS) and genetic introgression may have contributed broadly to phylogenetic incongruences between these species. Both ILS and genetic introgression have previously been proposed to explain observed morphological and genetic incongruences in the *Danaus* genus (Lushai et al. 2003; Smith et al. 2005), but to date neither hypothesis has been examined explicitly.

Materials and Methods

Species phylogeny based on ATPα

We first examined phylogenic relationships among *Danaus* species using available genomic and transcriptomic data for the alpha subunit of Na^+K^+-ATPase (ATPα). Specific information for all *Danaus* samples used is given in Table S1. With the previously reported ATPα coding sequence for *D. plexippus* (Zhen et al. 2012, GenBank Accession No: JQ771507), we employed a nucleotide Blast ('blastn', Altschul et al. 1990) to locate the exons of this gene on scaffold DPSCF300050 of the *D. plexippus* genome assembly (Zhan et al. 2011). We next mapped quality trimmed genomic reads for each sample (Phred QV ≥ 20, contiguous length ≥ 30 nucleotides) to this scaffold using Stampy (v. 1.0.17; Lunter and Goodson 2011) with default parameters except substitution rate, which we set to 0.01. We used SAMtools (v. 0.1.4) to filter the mapped reads (MAPQ > 20, Li et al. 2009), and the MarkDuplicates utility in Picard tools (v. 1.77; http://broadinstitute.github.io/picard/) to remove PCR duplicates. We called genetic variants using the HaplotypeCaller utility in GATK (v. 3.3; McKenna et al. 2010). Finally, for each sample we pulled the full exons of this gene and assembled the coding sequence of ATPα.

This data was combined with the previously reported ATPα sequences for *D. plexippus, D. gilippus* and *D. eresimus*, plus the outgroup milkweed butterfly *Lycorea halia* (Table S1, Zhen et al. 2012). Using jModelTest 2 (v. 2.1.7, Guindon and Gascuel 2003; Darriba et al. 2012), we determined the best-fitting mutation model for this dataset (based on AIC score) was the generalized time reversible model (Tavaré 1986) with a gamma distribution of rate heterogeneity (i.e. GTR + Γ model). We used RAxML (v. 8.1.7, Stamatakis 2014) to generate 1000 bootstrap replicate phylogenies to assess support for each branch.

We used PhyloNetHMM (Liu et al. 2014) to evaluate the level of support across the ATPα coding sequence for alternative phylogenetic relationships among *Danaus* species (specifically [[*D. eresimus*, *D. gilippus*], *D. plexippus*] versus [[*D. eresimus*, *D. plexippus*], *D. gilippus*]). To do this, we used a four-sequence alignment of the coding region of ATPα covering amino acid residues 22 through 996. Included in this alignment were one sequence each from *D. plexippus*, *D. eresimus*, *D. gilippus* and the outgroup species, *L. halia* (from Zhen et al. 2012). We specified priors on substitution rates using RAxML-based estimates of these parameters. Since PhyloNetHMM explicitly models ILS given a particular species tree, variation in support for a species tree is interpreted as evidence for gene flow (introgression) between species.

De novo transcriptome assembly

To examine hypotheses of shared ancestry or adaptive introgression to explain the origins of the N122H substitution in *Danaus* butterflies, it was necessary to have high confidence in species relationships within this genus. Therefore, to broadly examine phylogenetic relationships within *Danaus*, we took advantage of previously produced RNAseq data (Zhen et al. 2012) for the three North American *Danaus* species (*D. plexippus*, *D. eresimus* and *D. gilippus*) and an outgroup (*L. halia*). Using three focal species and an outgroup allowed us to compare the major topology to two possible minor topologies in a series of four-taxa trees,

and assess whether these minor topologies occur at similar frequencies to one another. Such assessments are a common way to investigate the origins of phylogenetic incongruence (e.g. Green et al. 2010).

Mapping sequence reads to a reference can lead to biases in phylogenetic analyses that become more serious as divergence from the reference increases (Hornett and Wheat 2012). Therefore, we instead produced independent *de novo* transcriptome assemblies for each species using the programs Velvet (v. 1.2.10) and Oases (v. 0.2.08) with a kmer length of 31 and a minimum read depth of 10 (Zerbino and Birney 2008; Schulz et al. 2012). In cases where multiple isoforms were assembled, we retained the longest one. To locate orthologous nuclear sequences, the four transcriptome assemblies were compared to amino acid sequences of predicted proteins for the *D. plexippus* reference genome (Dp_geneset_OGS2_pep.fasta, http://monarchbase.umassmed.edu/resource.html), using Blast with a translated nucleotide query ('blastx') and a minimum e-value of 1e-50. Only Blast hits that were at least 100 nucleotides long were retained for subsequent ortholog comparison between the four species. This was intended to reduce the number of regions with limited phylogenetic information and improve alignment accuracy (Talavera and Castresana 2007). We discarded gene regions for which any species had more than one overlapping transcript matching the *D. plexippus* reference protein set (http://monarchbase.umassmed.edu/resource.html) to avoid potential duplicates. Generally, low levels of polymorphism do not affect contig assembly in Velvet, and only a single allele is retained at polymorphic sites (Zerbino and Birney 2008). However, it is possible that highly polymorphic regions will assemble into more than one distinct transcript. In our pipeline, such regions would resemble gene duplicates and subsequently be removed.

We aligned orthologous regions based on amino acid similarity with MUSCLE (Edgar 2004) as implemented in SeaView (v. 4.5.4; Gouy et al. 2010). We then checked each alignment by eye and manually trimmed them to the length of the shortest region observed among the four species. Additional information on these assemblies is given in Table S2.

Species trees

We concatenated all loci into single alignments (i.e. no partitioning between genes) independently for our *de novo*-assembled nuclear gene datasets, and used jModelTest 2 (v. 2.1.7, Guindon and Gascuel 2003; Darriba et al. 2012) to determine the best-fitting mutation model as above. The best fit was the generalized time reversible model (Tavaré 1986) with a proportion of invariable sites and a gamma distribution of rate heterogeneity (i.e. GTR + I + Γ model). We next produced maximum likelihood phylogenies in RAxML (v. 8.1.7, Stamatakis 2014) with 1000 bootstrap replicates for each of the three possible topologies among the *Danaus* species. The site log likelihoods from RAxML were used to perform the approximately unbiased (AU) test implemented in CONSEL 0.2 (Shimodaira and Hasegawa 2001). Two trees were considered statistically different from one another if $p \leq 0.05$.

Discordance among nuclear gene trees

To determine what proportion of genes concur with the primary species tree, and what proportions support the two alternative phylogenetic relationships, we employed a Bayesian

concordance analysis (BCA), as implemented in BUCKy (v. 1.4.3, Larget et al. 2010; Ané et al. 2007). We first produced individual trees for each genetic region in our dataset using MrBayes (v. 3.2.2, Ronquist and Huelsenbeck 2003), with two independent runs of 10^7 generations and a "burn in" period of 10^5 generations. Rather than *a priori* selecting a mutation model for each gene, we used the reversible-jump Markov chain Monte Carlo approach to examine the parameter space for each locus and find the best set of parameters for that particular dataset (Huelsenbeck et al. 2004).

We combined the two independent MrBayes runs for each gene using the mbsum command in BUCKy (v. 1.4.3, Larget et al. 2010). We then examined the extent of discordance as measured by the sample-wide concordance factor (CF) using an α prior of 1. α is an *a priori* discordance parameter that indicates the expected level of discordance among the different genes being analyzed (ranging from 0 to ∞; Larget et al. 2010). When α=0, this indicates that there is no expected discordant topologies among gene trees, whereas when $\alpha=\infty$, this indicates that all gene trees are expected to have independent topologies. In our analysis, using other values of α (0.1 & 10) did not greatly alter inferred levels of incongruence (Table S3).

Distinguishing incomplete lineage sorting and introgression

While BUCKy can reveal the extent of nuclear discordance among taxa, it does not indicate whether it is more likely to be due to ILS or introgression after species splitting (although these are not mutually exclusive). To examine these two hypotheses we employed the ABBA/BABA test to calculate Patterson's D statistic for our dataset (Green et al. 2010). This statistic uses comparisons between three focal samples and an outgroup to determine whether phylogenetically-informative sites are in agreement with the primary phylogeny (AABB sites), or support one of the two possible alternative relationships (ABBA or BABA, respectively). If incomplete lineage sorting is the primary contributor to observed phylogenetic incongruence, then ABBA and BABA sites should be present in approximately equal frequencies (D statistic ≈ 0). However, genetic exchange that occurs after splitting may result in an excess of either ABBA or BABA sites (D statistic $\neq 0$). It should be noted that population structure in the ancestral population could also produce an asymmetry in ABBA/BABA sites, whereas post-splitting, symmetrical genetic exchange could produce equal frequencies of sites (Durand et al. 2011).

The ABBA/BABA test was performed with all informative sites, 4-fold synonymous sites and 0-fold replacement sites from our 478 gene set. The standard deviation of D was determined by block jackknife sampling (Kunsch 1989) with a block size of 40 genes. 95% confidence intervals based on this jackknifed dataset were used to assess if D differed significantly from 0.

Genetic diversity and divergence times

Changes in the effective population size of a species (N_e) over time can influence divergence estimates and patterns of evolution (Charlesworth 2009). Therefore, we examined relative differences in contemporary N_e for each species by calculating 4-fold synonymous genetic diversity (π_{4f}, Nei and Li 1979) within each of the four individual, wild-caught samples. To

do this, we first mapped trimmed reads to the species specific, *de novo*-assembled transcriptome for each of the four butterflies. Trimming, filtering and variant calling were performed as described above. Likely coding sequences were determined by independently comparing each of the four species' *de novo* assembled transcriptomes to the set of *D. plexippus* genome assembly predicted proteins (http://monarchbase.umassmed.edu/resource.html), using Blast with a translated nucleotide query ('blastx'; Altschul et al. 1990) and a minimum e value of 1e-50. Table S4 gives summary information for this read mapping.

We quantified species diversity levels as the length-weighted mean number of nucleotide differences per 4-fold degenerate synonymous site (i.e. π_{4f}). 95% confidence intervals on π_{4f} were estimated by bootstrap sampling each gene set 1,000 times with replacement. We employed a Bayesian method to examine approximate species' splitting times. As there are no known fossils that would allow us to independently calibrate any nodes in our phylogeny, we employed a rate-based estimate of evolution at neutral sites following methods described by Obbard and colleagues (Obbard et al. 2012). For evaluation of neutral evolution, we utilized all 4-fold synonymous sites from the concatenated dataset of our *de novo* assemblies. While mutations at these sites may not be entirely neutral due to codon usage bias (Hershberg and Petrov 2008) and other types of direct or indirect selection (Lawrie et al. 2013; Sella et al. 2009), they are the best available option in this dataset.

To account for variation in evolution rates among genes, we modeled a lognormal distribution of rate variation around the estimated mean mutation rate per year for *H. melpomene* (2.9×10^{-9}, 95% CIs: 6.5×10^{-9} to 2.8×10^{-8}), assuming five generations per year (Malcolm et al. 1987). This allowed us to partially capture potential variation in rates among branches and may have reduced overestimating the length of shorter branches (Schwartz and Mueller 2010).

We ran two MCMC chains of 10^8 iterations in the program BEAST (v. 1.8.1, Drummond and Rambaut 2007), with a log-normal relaxed molecular clock. We used the Hasegawa, Kishino and Yano (HKY, Hasegawa et al. 1985) substitution model with a proportion of invariant sites and a gamma distribution of rate heterogeneity and a starting tree based on our previous analysis of nuclear genes (Fig. 2C), with a birth-death process (Gernhard 2008). The "burn in" period was the initial 10% of states, and parameters were logged every 1,000 iterations. LogCombiner was used to merge our two separate runs (v. 1.8.1, included with the BEAST package). Log files were checked using Tracer (v. 1.6; Rambaut et al. 2014) to ensure that an effective sampling size (ESS) greater than 200 were achieved for each parameter. Divergence times were estimated based on the 95% highest posterior density (HPD) interval.

Results

Phylogenic analysis based on ATPα

The maximum likelihood phylogeny for *Danaus* species based on ATPα sequences is shown in Figure 2A. Contrary to previous findings, this phylogeny groups together *D. eresimus* and *D. plexippus* as sister lineages relative to *D. gilippus*, albeit with limited bootstrap support

(75% on the branch leading to *D. eresimus*/*D. plexippus*). Removing the single non-synonymous substitution encoding N122H results in a phylogeny with the same topology, though bootstrap support for the branch is even lower at 65%. To better establish the cause of this lower level of branch support, we carried out a spatial evaluation of the phylogenetic signature across the ATPα gene using the program PhyloNetHMM (Liu et al. 2014). This analysis reveals that ATPα exhibits conflicting phylogenetic signature across its length with only the middle third of the protein exhibiting strong support for sister-species status for *D. eresimus* and *D. gilippus* (Fig 2B). These results imply either that *D. eresimus* is more closely related to *D. plexippus* than previously thought, or that there has been introgression of at least part of the ATPα gene between the *D. eresimus* and *D. plexippus* lineages.

Phylogenic analysis based on transcriptome data

To distinguish among possible causes for the apparent discordant pattern found in ATPα, we carried out a phylogenetic analysis using transcriptome data. Our *de novo* assembled nuclear gene dataset contained 478 sequences, totaling 220,188 nucleotides, with an average sequence length of 461 nucleotides. This dataset was limited in the total number of genes analyzed as overlapping regions in each gene had to be assembled in all four species for inclusion. The best-supported topology placed *D. plexippus* and *D. eresimus* as sister taxa, with *D. gilippus* a more distant relative (Fig. 1C). The two alternative topologies were significantly worse fits than the primary tree, but they were not significantly different from each other (Table 1). There was 100% bootstrap support for the best tree topology. This relationship is also supported by a dataset of genes mapped to predicted *D. plexippus* coding sequences and also the complete sequences of the mitochondrial genomes for numerous *Danaus* species (see Figs. S1 and S2 for details).

Phylogenetic discordance

Our BUCKy analysis reveals that the primary concordance tree is the same as that determined in the maximum-likelihood analyses (Fig. 2D). However, it also reveals significant discordance (>5%) among individual gene trees and the inferred species tree. A higher proportion of genes supported a closer relationship between *D. gilippus* and *D. plexippus*, than between *D. gilippus* and *D. eresimus*.

The full dataset contained 5,735 informative sites for the ABBA/BABA test. The percentages of sites supporting each of the three possible relationships are given in Figure 3, along with the number of 4-fold synonymous and 0-fold replacement sites. The D statistic for the full dataset was −0.118 (95% CIs: −0.121 to −0.114), for 4-fold synonymous sites it was− −0.134 (95% CIs: −0.138 to −0.130), and for 0-fold, non-synonymous sites it was −0.025 (95% CIs: −0.031 to −0.019). For all classes of sites, the D statistic was significantly less than 0, indicating that ABBA and BABA sites were not equally represented. This was always due to a larger number of BABA sites than ABBA sites, which suggests that a greater amount of gene flow has occurred between *D. plexippus* and *D. gilippus* after splitting than between *D. eresimus* and *D. gilippus*. It is also possible that this result is due to structure in the ancestral populations of these species (Green et al. 2010).

Genetic diversity and divergence time estimates

To assess the relative contemporary effective population sizes (N_e) of these species, we compared levels of 4-fold synonymous site diversity (π_{4f}, Table S5). Of the four species, *D. eresimus* had the lowest synonymous diversity (π_{4f} = 0.010, 95% CIs: 0.009 – 0.011). The other three species all had similar levels of diversity (*L. halia*: π_{4f} = 0.022, 95% CIs: 0.021 – 0.023; *D. gilippus*: π_{4f} = 0.021, 95% CIs: 0.018 – 0.023; *D. plexippus*: π_{4f} = 0.021, 95% CIs: 0.021 – 0.022). The similarities in genetic diversity levels exhibited in *D. plexippus* and *D. gilippus* suggest that they have comparable contemporary N_e, while *D. eresimus* likely has a somewhat smaller N_e.

Differences in N_e between these species could affect estimates of divergence times. Specifically, *D. eresimus* may have had a higher rate of evolution over time than the other three species. However, Tajima's relative rate test (Tajima 1993), using all sites from the concatenated *de novo* dataset suggests that *D. eresimus* has had a similar rate of evolution to *D. gilippus* (χ^2 = 2.46, p=0.074), whereas the rate of evolution in *D. plexippus* was higher than both *D. gilippus* (χ^2 = 31.11, p<0.001) and *D. eresimus* (χ^2 = 20.25, p<0.001). Thus, we do not infer any evolutionary patterns in relation to contemporary N_e in these species.

Bayesian estimates of divergence times suggest that *D. plexippus* and *D. eresimus* diverged 7.2 MYA (95% highest posterior density [HPD]: 6.9 – 7.5), whereas *D. gilippus* diverged from the common ancestor of *D. plexippus* and *D. eresimus* 11.0 MYA (95% HPD: 10.7 – 11.4). These estimates of splitting time are similar to simple estimates based on synonymous divergence and a fixed mutation rate (Table S6), and suggest that the three species diverged relatively rapidly from one another (within 3 – 4 million years). The close timing of speciation events in this clade may have increased the likelihood that ILS occurred.

We explicitly examined the extent of ILS given our estimated divergence times by simulating the coalescent of 10,000 genes in *ms* (Hudson 2002). We used 0.02 for our estimate of θ ($4N_e\mu$) and converted our estimated divergence times to units of $4N_e$ generations (command line: ms 3 10000 -t 0.02 -I 3111 -ej 2.412-ej 3.623 –T). From these simulations, we infer that ~8% of genetic regions may exhibit an alternative topology to that of the true species relationship given the divergence times calculated here. This is less than the ~18% phylogenetic discordance observed, and is consistent with the inference that genetic exchange after these species diverged contributed to some of the observed phylogenetic incongruence observed between these species.

Discussion

The close phylogenetic relationship of *D. eresimus* and *D. plexippus/D. erippus* suggests either that the N122H substitution of ATPα is shared due to common descent or that it introgressed from one lineage into the other. The best-supported phylogeny from whole nuclear gene datasets of the three North American species of *Danaus* supports the former hypothesis (Fig. 2C.). Therefore, the presence of the N122H mutation in both the *D. plexippus/D. erippus* lineage and *D. eresimus* is most parsimoniously explained by a scenario in which it arose (and possibly fixed) in their shared common ancestor (Fig. S3A). However, it is also possible that the N122H mutation arose in a more ancestral *Danaus*

species, and was subsequently lost in other species (Fig. S3B). We cannot confidently differentiate between this and the previous hypothesis with the data currently available, although this second explanation is less parsimonious.

One curious finding of this study is that there is a phylogenetically discordant signal across the length of ATPα and evidence for gene flow between the *D. plexippus* and *D. gilippus* lineages as well as the *D. eresimus* and *D. gilippus* lineages. Thus, while we currently lack the ability to directly test for on-going gene flow between *D. plexippus* and *D. eresimus*, it seems plausible given the evidence for gene flow in the comparisons we can make. The implication is that introgression of ATPα, and thus the N122H mutation, is not substantially less parsimonious than shared ancestry, particularly given the adaptive significance of this substitution. A crude way to evaluate evidence for introgression between *D. plexippus* and *D. eresimus* is to examine levels of sequence divergence. The estimated synonymous divergence (dS) between *D. plexippus* and *D. eresimus* for ATPα (0.15 per site) is somewhat lower than the genome-wide average (0.233). However, the large number of fixed differences between *D. plexippus* and *D. eresimus* implies that if introgression of the ATPα gene occurred, it did not happen recently.

It is becoming increasingly recognized that species divergence is often accompanied by post-splitting genetic exchange and other factors that obscure our ability to make evolutionary inferences. This may be especially true when divergence occurs rapidly. Among North American *Danaus* butterflies, we observe significant phylogenetic discordance between species, which may be attributed to several factors including incomplete lineage sorting (ILS) and post-speciation gene flow. Using coalescent simulations, we infer that ILS is likely to explain some of the observed phylogenetic incongruence. However, we also detect significant evidence for (asymmetrical) gene flow between species, implicating post-speciation gene flow as a contributor to phylogenetic discordance. Similar explanations have been proposed to explain phylogenetic discordance in many other species groups (e.g. Moody and Rieseberg 2012; Cui et al. 2013; Liu et al 2015), including butterflies (Kozak et al. 2015).

This study raises questions about the origins of the extensive phenotypic divergence observed in *D. plexippus* such as changes in wing size and mating strategy (Ackery and Vane-Wright 1984). Interestingly, recent work suggests all contemporary *D. plexippus* populations (including non-migratory ones) originated from a single North American migratory population (Brower et al. 2007; Zhan et al. 2014). The uniquely derived traits of *D. plexippus* may have obscured phylogenetic relationships between *Danaus* species in previous studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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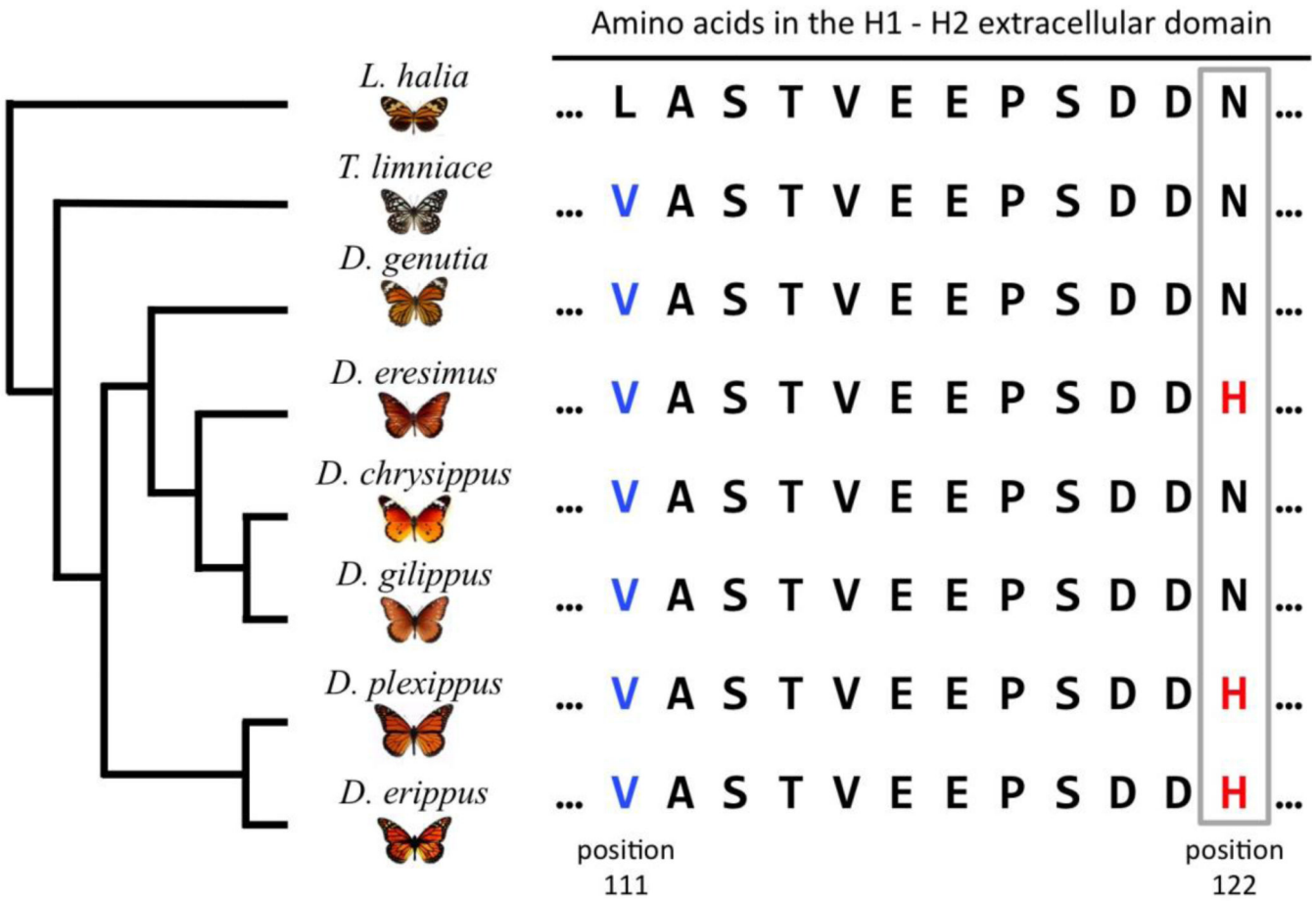


Figure 1.

Previously inferred phylogeny of select *Danaus* species and additional milkweed butterflies (Danainae subfamily; based on: Ackery and Vane-Wright 1984; Lushai et al. 2003; Smith et al. 2005; Brower et al. 2010). Note: Brower et al. (2010) placed *D. eresimus* and *D. gilippus* as sister species, with the *D. chrysippus* group their sister lineage. Also shown are amino acid sequences of the H1–H2 extracellular domain of the ATPα in these species. The presence of a valine at position 111 (blue letters) likely enhances these butterflies' ability to feed on milkweed and possibly sequester cardenolides (Petschenka et al. 2013). The N122H mutation (red letters), observed in *D. plexippus*, *D. erippus* and *D. eresimus*, likely further contributes to increased cardenolide feeding and sequestration abilities (Zhen et al. 2012; Petschenka et al. 2013).

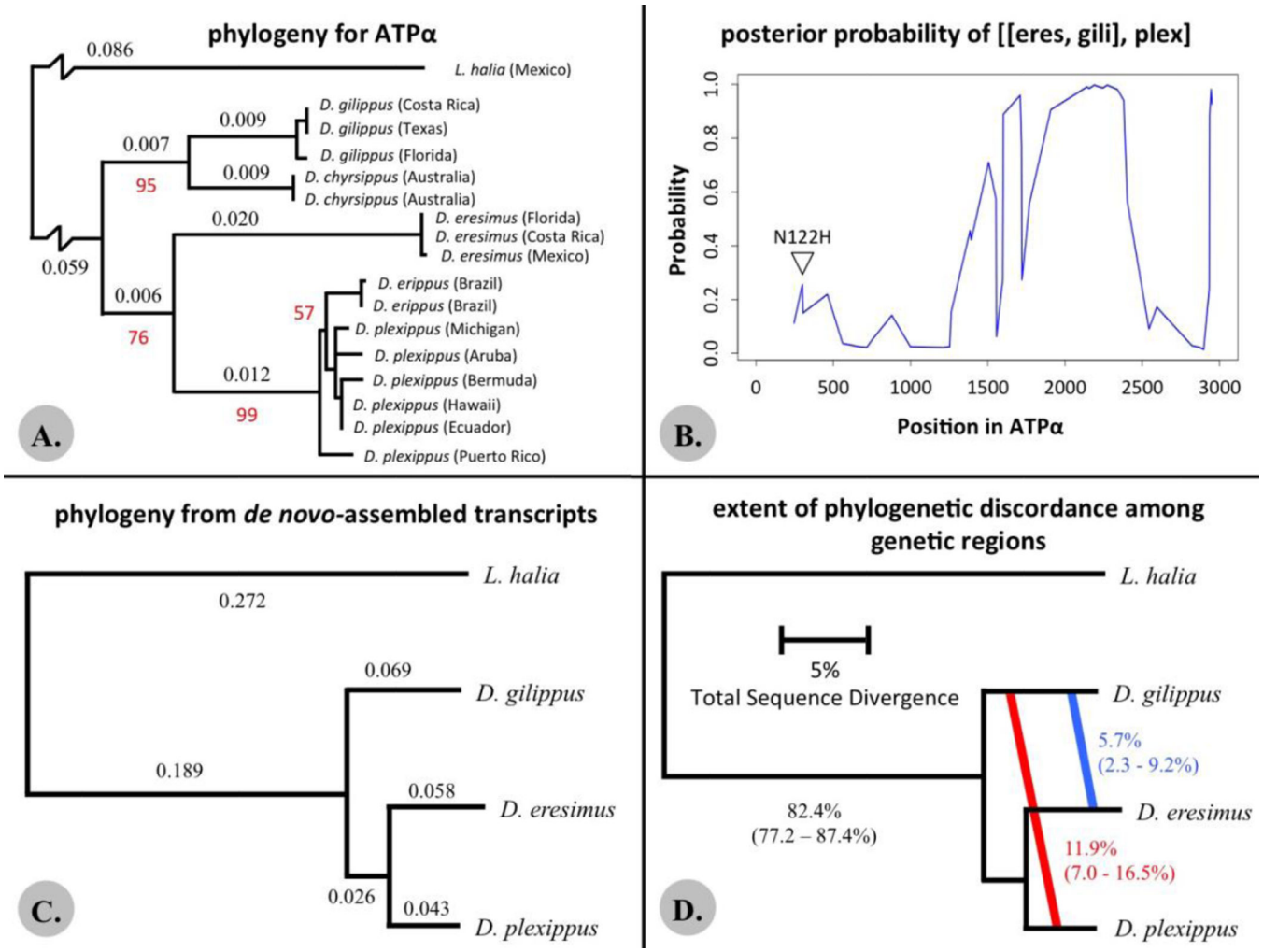


Figure 2.

A) Phylogenetic relationships among *Danaus* butterflies based on ATPα sequences. Black numbers indicate branch lengths for species-level branches. Red numbers indicate bootstrap support for *between* species branches when less than 100%. B) The level of support across ATPα for alternative phylogenetic relationships among *Danaus* species from PhyloNetHMM, using a four-sequence alignment of the coding region of ATPα. C) The best supported phylogenetic relationship as determined using data from 478 *de novo*-assembled nuclear gene sequences. Numbers indicate branch lengths. This tree topology had 100% bootstrap support at all branches. D) Levels of phylogenetic concordance/discordance between the best-supported species tree and individual gene trees. The red text and diagonal line indicates the level of discordance (with credibility intervals) that supports a closer relationship between *D. plexippus* and *D. gilippus* (with α = 1.0). The blue text and diagonal line indicated the level of discordance (with credibility intervals) that supports a closer relationship between *D. gilippus* and *D. eresimus* (with α = 1.0). NOTE: Branch lengths in these trees (A, C & D) are not drawn to the same scale *between* trees.

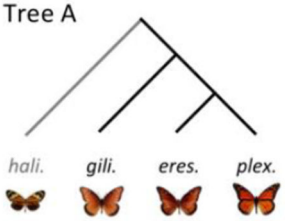
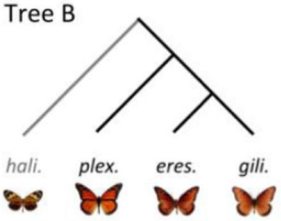
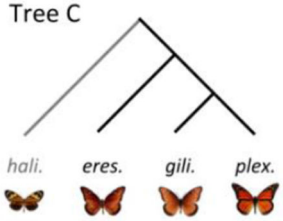
Data type	Total informative sites	Tree A  **AABB**	Tree B  **ABBA**	Tree C  **ABAB** (BABA)
ABBA/BABA (all)	5735	2818 (41.7%)	1287 (22.4%)	630 (28.4%)
ABBA/BABA (syn.)	4869	2389 (49.1%)	1074 (22.1%)	1406 (28.9%)
ABBA/BABA (rep.)	866	2389 (49.1%)	213 (24.6%)	224 (25.9%)

Figure 3.

The percentages of ABBA/BABA informative sites that match the best-supported species tree (Tree A) and the two alternative trees for the three North American *Danaus* species in this study for either all 4-fold/0-fold sites (all), just 4-fold synonymous sites (syn.) or just 0-fold replacement sites (rep.). ('gili.' = *D. gilippus*, 'eres.' = *D. eresimus*, 'plex' = *D. plexippus*, 'hali' = *L. halia*).

Table 1

Comparison of the three possible phylogenetic relationships among the butterflies in this study for our *de novo*-assembled nuclear gene dataset. Significance between trees was determined using the AU test. See Materials and Methods for more details.

Tree Comparison (see Fig. 3)	$\|\Delta \ln L\|$	P-value of AU test
Tree A – Tree B	282.9	<0.001
Tree A – Tree C	297.6	<0.001
Tree B – Tree C	22.6	0.057