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# A molecular phylogeny of the neotropical butterfly genus *Anartia* (Lepidoptera: Nymphalidae)

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## Abstract

While *Anartia* butterflies have served as model organisms for research on the genetics of speciation, no phylogeny has been published to describe interspecific relationships. Here, we present a molecular phylogenetic analysis of *Anartia* species relationships, using both mitochondrial and nuclear genes. Analyses of both data sets confirm earlier predictions of sister species pairings based primarily on genital morphology. Yet both the mitochondrial and nuclear gene phylogenies demonstrate that *Anartia jatrophae* is not sister to all other *Anartia* species, but rather that it is sister to the *Anartia fatima*–*Anartia amathea* lineage. Traditional biogeographic explanations for speciation across the genus relied on *A. jatrophae* being sister to its congeners. These explanations invoked allopatric divergence of sister species pairs and multiple sympatric speciation events to explain why *A. jatrophae* flies alongside all its congeners. The molecular phylogenies are more consistent with lineage divergence due to vicariance, and range expansion of *A. jatrophae* to explain its sympatry with congeners. Further interpretations of the tree topologies also suggest how morphological evolution and eco-geographic adaptation may have set species range boundaries.

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## 1. Introduction

Species within the genus *Anartia* Hubner (1819) are familiar and well-studied Neotropical Nymphalid butterflies. They are among the most commonly encountered butterflies in the Neotropics, partially because of their preference for open and disturbed habitat. They have also proven to be popular research subjects, supporting a wide range of work including studies on the genetics of reproductive isolation between hybridizing species (Davies et al., 1997; Silberglied et al., 1979). For example, experiments have shown that *Anartia amathea* and *Anartia fatima* assortatively mate, and that hybrid breakdown occurs among F2 offspring (Davies et al., 1997; Silberglied and Aiello, unpublished data). Crosses between *A. fatima* and *A. amathea* also demonstrate a form of “Haldane’s Rule,” where the heterogametic female F1 offspring have a markedly reduced tendency to

mate (Davies et al., 1997). These investigations and our recent study on the *A. fatima*–*A. amathea* hybrid zone (Dasmahapatra et al., 2002) lack a phylogenetic context that would permit research on *Anartia* speciation to be directly compared to other studies on the maintenance of species boundaries (Coyne and Orr, 1989). Thus, a principle objective of this study is to produce an inter-specific phylogeny of the genus *Anartia* to provide an evolutionary context for the research conducted on its members.

Although no formal phylogenetic hypothesis has been presented to describe species level relationships within the genus *Anartia*, Silberglied et al. (1979) reviewed past treatments of *Anartia* systematics, and suggested plausible sister species groupings based primarily on genital morphology, wing color patterns and natural history. These data could not resolve branching order among the species, but Silberglied et al. (1979) recognized three phyletic lineages, with *A. fatima* and *A. amathea* forming one lineage, *A. chrysopelea* and *Anartia lytrea* forming a second, and *Anartia jatrophae* forming the third lineage. Fig. 1, depicting *Anartia* geographic range

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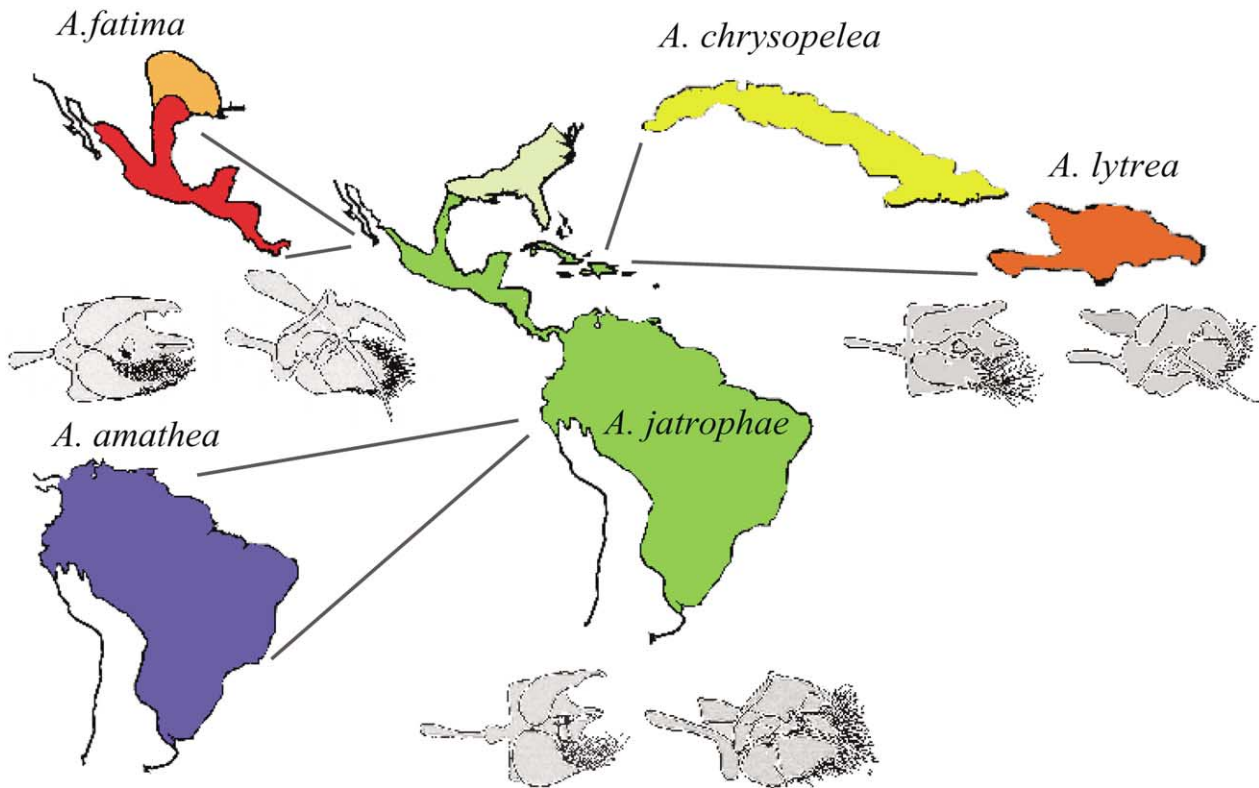


Fig. 1. Geographic ranges and male genital structure representing the three *Anartia* evolutionary lineages proposed by Silberglied et al. (1979). The lightened portions of *A. fatima*'s and *A. jatrophae*'s ranges represent sightings of vagrant or seasonal migrants. The left image of the genital structure is a ventral view; the right is a lateral view with left valve removed. Genitalia figures redrawn from Silberglied et al. (1979).

and genital morphology, highlights the factors that led to the recognition of the three lineages. *A. fatima* and *A. amathea* have allopatric ranges in Central and South America, and share nearly identical genital morphology. The second sister pair, *A. chrysopelea* and *A. lytrea*, also share lineage-specific genital morphology and are restricted to Cuba and Hispaniola respectively. *A. jatrophae* is characterized by distinctive genitalia and a geographic range that overlaps all four other species.

Here, we present gene genealogies based on the mitochondrial cytochrome oxidase subunit I (COI) gene, the leucine tRNA gene, and the cytochrome oxidase subunit II gene (COII), as well as the nuclear-encoded triose-phosphate isomerase (*Tpi*) gene. We examine these nuclear and mitochondrial loci at the interspecific and intraspecific level to provide a formal molecular systematic hypothesis of relationship among *Anartia* species. In turn, we evaluate Silberglied et al.'s (1979) three-lineage hypothesis and the history and biogeography of *Anartia* speciation.

## 2. Materials and methods

Our taxon sampling includes the five currently recognized *Anartia* species as well as *Siproeta stelenes* and

*Junonia evarete* as outgroup genera. We sequenced at least two individuals for each *Anartia* species, but only one individual from each outgroup. *Siproeta* and *Junonia* were chosen in accordance with previous work recognizing their morphological affinities to the *Anartia* species (Silberglied et al., 1979; Young and Stein, 1976). Higher-level systematic relationships among these Nymphalid genera are currently unclear, but morphological evidence suggests that either *Siproeta* or *Junonia* should serve as a near optimal outgroup for phylogenetic analysis of *Anartia* species relationships. We also included *Heliconius erato* and *Dryas iulia* as additional outgroups to assess the stability of basal relationships among ingroup lineages.

Specimens were collected as adults from sites distributed through the Caribbean, Central and South America. Table 1 lists the collection localities for the *Anartia* species and the various outgroups. The specimens were either transported live from the field and frozen at  $-70^{\circ}\text{C}$  in laboratory facilities, or preserved in a DMSO solution (Seutin et al., 1991) after removal of fore and hindwings. Specimens preserved in DMSO were then stored at  $4^{\circ}\text{C}$  to help prevent further DNA degradation. The sets of wings were placed in dry storage for future reference and are available upon request. Genomic DNA was then extracted from the thorax tis-

Table 1  
Number of individuals of *Anartia* and outgroup species and the geographic locations used in this study

Taxon	Number	Locality	Data collected (No. of specimens)
<i>Anartia amathea</i>	1	Trinidad	COI, tRNA, COII, <i>Tpi</i> (1)
	1	Grenada	COI, tRNA, COII, <i>Tpi</i> (1)
	15	Panama	COI, tRNA, COII, <i>Tpi</i> (1); only COI(11); only <i>Tpi</i> (4)
<i>Anartia chrysopelea</i>	2	Cuba	COI, tRNA, COII, <i>Tpi</i> (2)
<i>Anartia fatima</i>	32	Panama	COI, tRNA, COII, <i>Tpi</i> (2); COI, <i>Tpi</i> (1), only COI (28); only <i>Tpi</i> (2)
<i>Anartia jatrophae</i>	2	Trinidad	COI, tRNA, COII, <i>Tpi</i> (1); COI, tRNA, COII (1)
	1	Panama	COI, tRNA, COII, <i>Tpi</i> (1)
<i>Anartia lytrea</i>	2	Hispaniola	COI, tRNA, COII, <i>Tpi</i> (2)
<i>Siproeta stelenes</i>	1	Panama	COI, tRNA, COII (1)
<i>Junonia evarete</i>	1	Panama	COI, tRNA, COII, <i>Tpi</i> (1)
<i>Heliconius erato</i>	1	Panama	COI, tRNA, COII (1)
<i>Dryas iulia</i>	1	Panama	COI, tRNA, COII (1)

Column 4 notes the DNA sequence data collected for each individual, with the number of individuals per data type in parentheses.

sue of individual butterflies following Brower's (www.ent.orst.edu/browera) recommended phenol–chloroform protocol for extracting DNA.

### 2.1. PCR and sequencing

We used both published and original primers for the mtDNA PCR amplifications and sequencing. The published primers are described in Simon et al. (1994), and are highly conserved primer sites in the COI gene, the leucine tRNA gene, and the COII gene. Table 2 lists both the published and newly designed primer positions and directionality along the cytochrome oxidase region. The set of seven primers allowed amplification and sequencing of a 1600 bp fragment. An MJ-100 thermal cycler was used for PCR amplifications, with a cycling profile of 94°C/30 s–48°C/30 s–72°C/1 min—for 4 cycles followed by 29 cycles of 94°C/30 s–52°C/30 s–72°C/1 min, with a final extension phase of 72°C/5 min. PCR products were subsequently run out on a 1% low-melting temperature agarose gel. The PCR products were cut from the gel, gelated for 3 h, and stored at –20°C.

The nuclear marker we used is intron 3 and flanking sections of exons 3 and 4 of the triose-phosphate isomerase (*Tpi*) gene. *Tpi* is an important enzyme for carbohydrate catabolism and has been found to be sex linked in some butterflies (Jiggins et al., 2001). This gene

also appears to be involved in hybrid sterility in crosses between *Heliconius* sister species (Jiggins et al., 2001). *Tpi* has previously been used in studies of *Drosophila* evolution and population genetics as well as the molecular evolution of introns (Hasson et al., 1998; Kwiatowski et al., 1995; Logsdon et al., 1995). The *Tpi* primers we used were designed by Owen McMillan and Dave Heckel for *Heliothis* (Beltrán et al., in press; Jiggins et al., 2001). The primers are situated in exons 3 and 4 of *Heliothis* (GenBank Accession No. U23080), and amplify a fragment of approximately 400–700 bp in the *Anartia* species and outgroups included in this study. The length of the amplified fragment is highly variable owing to species-specific differences in the length of the intron. We used the MJ-100 thermal cycler for the PCR reactions, with the following cycling profile: 94°C/45 s–58°C/45 s to –0.5°C per cycle–72°C/1:45 min for 10 cycles followed by 25 cycles of 94°C/45 s–53°C/45 s–72°C/1:45 min, with a final extension phase of 72°C/10 min. The PCR products were run out on a 1% low melting temperature agarose gel. Corresponding bands were excised from the gel, gelated for 3 h, and stored at –20°C.

The gelated PCR products were used as the DNA template in D-rhodamine (ABI) based dideoxy-chain termination reactions. An MG-100 thermal cycler was used for the sequencing reactions with a profile of

Table 2  
MtDNA oligonucleotide primers used for amplification and sequencing of *Anartia* species and outgroups

Name	Application	Position	Sequence
Jerry (F) <sup>a</sup>	COI	2183	CAACATTTATTTTGATTTTTTGG
Dick (F) <sup>a</sup>	COI	2442	CCAACAGGAATTAATAATTTTAGATGATTAGC
Pat (R) <sup>a</sup>	COI, tRNA	3014	TCCAATGCACTAATCTGCCATATTA
George (F) <sup>b</sup>	COI, tRNA, COII	2783	TAGGATTAGCTGGAATACC
Romeo (F) <sup>b</sup>	COII	3039	TAATATGACAGATTATATGTAATGGA
Strom (F) <sup>b</sup>	COII	3297	TGAACTATTTTACCAGGC
Eva (R) <sup>b</sup>	COII	3772	GAGACCATTACTTGCTTTTCAGTCATCT

F, forward; R, reverse. Sequences are listed from the 5' to 3' end; positions are given relative to the *Drosophila yakuba* mitochondria sequence.

<sup>a</sup> Published in Simon et al. (1994).

<sup>b</sup> Listed in Davies and Bermingham, 2002.

94°C/15 s—cycle to 50°C at –1°C/s—50°C/1 min—60°C/4 min—for 25 cycles. The reactions were then centrifuged through Sephadex columns, and dried down with a speed-vac. Samples were resuspended in loading dye and sequenced in both directions to obtain bidirectional overlap using an ABI 377 automatic sequencer. MtDNA and *Tpi* sequences were read with Sequencing Analysis 3.0 (ABI) software.

Further editing and alignment of the mtDNA sequences was completed with Sequencher 3.1 (Gene Codes) software. Editing and alignment of the *Tpi* sequences was first done with Sequencher 3.1 software to align putative sister species pairs, but we used Clustal X1.8 (Jeanmougin et al., 1998) software to align more distantly related species. We based the Clustal X1.8 alignment parameters on a weighting scheme where gap insertions were weighted five times heavier than gap expansions. The alignment produced by Clustal X1.8 was then reviewed by eye in Seq-Align 1.0 (Rambaut, 1996).

## 2.2. Phylogenetic analysis

For both the mtDNA and *Tpi* data sets, Nexus files were created and exported from Sequencher 3.1 or Seq-Align 1.0, and analyzed with PAUP version 4.0b8 (Swofford, 1994). For both data sets, we first produced partially optimized neighbor-joining (NJ) trees with the log-determinant (Log-Det) distance model (Lockhart et al., 1994) to control for unequal base composition between lineages. We then compared the NJ trees to trees generated from parsimony analysis with branch and bound searches, equal weighting of all character states and a Goloboff fit criterion of  $K = 2$ . Gap states were treated as missing data rather than being assigned as a fifth character state. We also compared the NJ trees to trees generated from maximum likelihood methods with branch and bound searches. The mtDNA maximum likelihood tree was developed from the General Time Reversible substitution model (Rodriguez et al., 1990) with a Gamma distribution (GTR-G). The *Tpi* maximum likelihood tree was based on the HKY-G (Hasegawa et al., 1985) substitution model. Both models were the best-fit assigned by ModelTest 3.0 (Posada and Crandall, 1998). All trees were bootstrapped 1000 times to generate support statistics for branching events.

To determine whether rates of mtDNA molecular evolution are equivalent among taxa, we compared the mtDNA maximum likelihood tree to a maximum likelihood tree assuming a molecular clock. We used both the Kishino–Hasegawa (K–H) test (Kishino and Hasegawa, 1989) and a log-likelihood ratio test (Huelsenbeck and Rennala, 1997) for comparing the alternative tree topologies. Following Goldman et al.'s (2000) recommendations, we interpreted the results of the K–H test using a one-tailed rather than a two-tailed statistic.

The conversion of the significance level (from  $p$  to  $p/2 < 0.05$ ) provides a more conservative statistical interpretation of the K–H test that is consistent with Shimodaira and Hasegawa's (1999) corrected nonparametric test (Goldman et al., 2000).

## 3. Results

### 3.1. Molecular characterization: MtDNA

A 1600 bp mtDNA fragment was sequenced for each species, but we only used 1493 bp in the study to minimize ambiguous calls at the ends of the sequences. The 1493 bp region analyzed for this study begins at mitochondrial nucleotide position 2183 of the *Drosophila* cytochrome oxidase region and ends at position 3676, which lies in the COII gene (Table 2). We obtained complete sequences for 16 individuals (GenBank Accession Nos. AYO38659–AYO38672, AF413684, and AF413706), and generated 801 bp (nucleotides 2183–2983) COI/tRNA fragments for another 11 Panamanian *A. amathea* and 29 *A. fatima* (Table 1; GenBank Accession Nos. AYO38638–AYO38658, AYO10929–AYO10941, and AYO10950–AYO10955).

In the 1493 bp used to generate mtDNA trees, a total of 223 nucleotide sites (15%) were phylogenetically informative. The informative variation was not equally distributed across the three genes: 59% was located in COI, 0.4% in the tRNA, and 40.6% was in COII. In the protein coding areas, the base pair substitutions were more common in third position sites (77%), then first position sites (18.4%), and least common at second position sites (4.6%). No indels were observed that provided synapomorphies among ingroup species, however one 3 bp deletion in the COI subunit distinguished all *Anartia* species plus *J. evarete* from *Siproeta stelenes* and the more distantly related species, *Dryas iulia* and *Heliconius erato*. This synapomorphic indel provides support for a sister relationship between *Anartia* and *Junonia* that is not evident in genetic distance analyses.

### 3.2. Molecular characterization: *Tpi*

We were unable to amplify a *Tpi* fragment for *Siproeta stelenes*, and thus could not include this species in the phylogenetic analyses of the *Tpi* locus. The *Anartia* and *Junonia Tpi* PCR fragment sizes ranged between 398 and 754 bp (GenBank Accession Nos. AYO38673–AYO38691), but the Clustal X1.8 alignment parameters effectively increased the overall length of the locus to 1048 bp (alignment available at <http://nmg.si.edu/bermlab/index.htm>). Our preferred strategy may have introduced minor artifacts into the data set, but it enabled us to utilize all available characters for intraspecific and interspecific comparisons. Alternative strategies such as

reducing the data set to uninterrupted blocks of homologous characters or restricting the analyses to the exon region either destabilized the topology or did not provide sufficient resolution for branching events.

All sequences included 155 bp of the flanking exons, corresponding to positions 416–455 bp of exon 3 and 536–660 of exon 4 in *Heliothis*. The *Tpi* exon sequences had 107 invariant sites, and 34 phylogenetically informative sites. Comparisons among taxa showed that the exon sequence length was invariable. However, the *Tpi* intron region harbored length-variable indels that complicated the alignment of internal portions of the locus. Aligning individuals of the same species as well as putative sister species was simple and could be done by eye, but alignment across lineages was difficult without the aid of Clustal X1.8 software. Nonetheless, no indels were found among conspecific individuals of *A. jatrophae*, *A. lytrea*, or *A. fatima*. The two *Anartia chrysopelea* individuals differed by 2 indels and indel variation among *A. amathea* individuals was high, owing in part to the extent of geographic sampling in this species. While no indel variation was observed among sympatric individuals, allopatric populations of *A. amathea* were distinguished by 5 *Tpi* indels. Generally speaking shared indels could not be identified across species, although Panamanian populations of *A. amathea* and *A. fatima* shared several short *Tpi* indels relative to the Trinidad/Grenada population of *A. amathea*. Because the Panama *A. amathea* and *A. fatima* specimens had species-specific mtDNA profiles, this result suggests broader nuclear introgression between these hybridizing species.

The length differences of the *Tpi* locus among species representing different lineages hampered the overall alignment. For example, *A. amathea*–*A. fatima* *Tpi* introns were 15–132 bp shorter than those of *A. chrysopelea*–*A. lytrea*. The *A. jatrophae* *Tpi* locus was 224 bp shorter than *A. amathea*, 289 bp shorter than *A. fatima*, and over 300 bp shorter than either *A. chrysopelea* or *A. lytrea*. Some of the *A. jatrophae* deletions could be easily identified in comparison to one or more of the other *Anartia* species, whereas others were more ambiguous. While we were able to anchor the overall intron alignment with large homologous blocks and the exon region, indel variability generally lowered our confidence in the reliability of *Tpi* for resolving interspecific relatedness above sister species pairs. Somewhat surprisingly, indel variation did provide useful information on intraspecific population structure in *A. amathea*, the one species for which we sampled multiple populations.

### 3.3. Tree topologies

#### 3.3.1. mtDNA

Pairwise sequence divergence was 0–0.13% between conspecific mtDNA haplotypes, while interspecific di-

vergence was 3–5% between sister species, and 5–8% between species in separate lineages (Fig. 2). Sequence divergence was 8–13% between ingroup species and the four outgroup taxa. Identical tree topologies were obtained from NJ, parsimony and maximum likelihood analyses, with all branching events supported by equivalent bootstrap values (96–100%). The mtDNA maximum likelihood tree presented in Fig. 2 represents the relationships among *Anartia* species that resulted from all analyses: *A. amathea* and *A. fatima* are sister species, *A. jatrophae* falls sister to this clade, and *A. chrysopelea* and *A. lytrea* are sister species, forming a separate third lineage. Trees built from partitioned data sets (COII and tRNA versus COII) had identical topologies, but lower bootstrap values. Analyses based on either data set resolved the topology with bootstrap values ranging between approximately 85–100% (results not shown).

To investigate outgroup choice on *Anartia* tree stability, we sequentially substituted putatively closer outgroups in all analyses (Milinkovitch et al., 1996) and found that the support for deeper nodes declined when we assigned more distantly related outgroups. This was especially true with the trees built from partitioned data sets. We determined that outgroups such as *Siproeta stelenes* (8–9% sequence divergence) and *J. evarete* (9–10%) stabilized the topology and increased bootstrap values of deep *Anartia* nodes better than other Nymphalid taxa such as *Dryas iulia* and *Heliconius erato* (12 and 13% divergence). For example, support for the sister group relationship of *A. jatrophae* to *A. amathea*/*A. fatima* fell from 97% to 90% when the outgroup *Siproeta stelenes* was replaced by *Dryas iulia* in the ML analyses of the complete mtDNA data.

#### 3.3.2. *Tpi*

The *Tpi*-based parsimony tree in Fig. 3 is completely congruent with the *Anartia* mtDNA phylogeny, but with lower or equal bootstrap values. This tree was also recovered in NJ and maximum likelihood analyses. Of particular note is that *A. amathea* intraspecific branching events were better resolved on the *Tpi* tree than on the mtDNA-based phylogeny. Pairwise sequence divergence based on the total alignment of the *Tpi* haplotypes was generally higher than between mtDNA haplotypes representing the same pairwise comparisons (Figs. 2 and 3). This is principally because the introns were included in the *Tpi* divergence calculations. Intraspecific divergence ranged from a low of 1% between individuals of *A. lytrea*, to a high of 17% between allopatric populations of *A. amathea* (but divergence is only 0.7% between *A. amathea* individuals from Trinidad and Grenada). Sequence divergence among sister species pairs ranged from 5% between *A. lytrea* and *A. chrysopelea* to 15–19% for *A. fatima* and *A. amathea*. Distances between lineages were significantly higher than the mtDNA comparisons, ranging between 26%

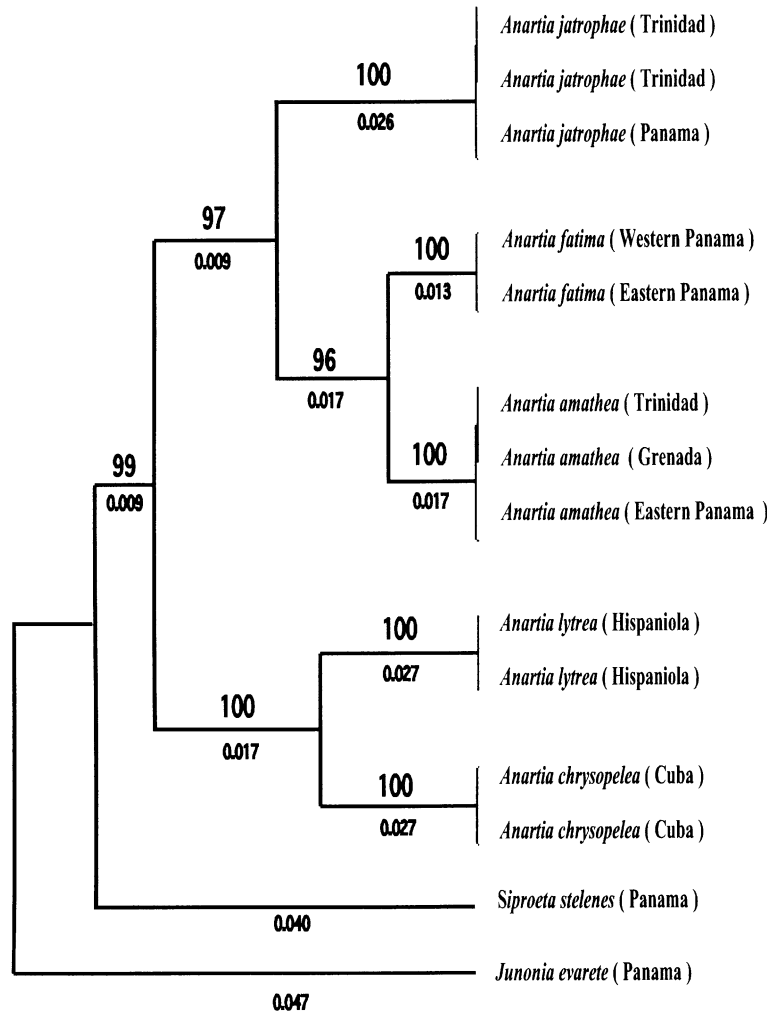


Fig. 2. The mtDNA maximum likelihood hypothesis for *Anartia* species relationships, using the GTR-G model and enforcing a molecular clock. Bootstrap values are above the branches, and are based on 1000 replications. NJ genetic distance (Log-Det) values are given below the branches.

(*A. amathea* to *A. jatrophae*) to 44% (*A. chrysopelea* to *A. amathea*).

Just as well, estimates of divergence between sister species pairs were inconsistent between the *Tpi* and mtDNA data sets. For example, *A. chrysopelea* and *A. lytrea* divergence was 5–6% for both the *Tpi* and mtDNA data, but the *Tpi* genetic distance of 15–19% between *A. amathea*–*A. fatima* was much higher than the mtDNA value of 3%. Indel variation differed significantly between the pairings: no indel variation occurs between *A. chrysopelea* and *A. lytrea* whereas *A. fatima* and *A. amathea* differ by 9 indels. This observation suggests that single nucleotide substitutions occur at equivalent rates for *Tpi* and mtDNA COI–COII sequences, and that indels significantly elevate *Tpi* intron divergence estimates. This result is likely an artifact of genetic distance models unequally reducing the locus size by excluding sites within indels when generating pairwise divergence estimates. Therefore, divergence

estimates were likely to increase relative to the number of excluded sites across the *Tpi* locus.

### 3.4. Relative rates of mtDNA evolution

Recent work questions the use of the K–H test to compare competing trees built from the same data using maximum likelihood methods (Goldman et al., 2000). While we used the K–H test to compare trees built with and without enforcing a molecular clock, we interpreted the results under the more restricted conditions of the one-tailed test recommended by Goldman et al., (2000). The test found no significant difference between the mtDNA maximum likelihood trees ( $p/2 > 0.05$ ). We also found no significant difference when comparing the trees with a log-likelihood ratio test ( $p > 0.05$ ;  $df = 11$ ). This leads us to suggest that the rate of mtDNA nucleotide substitution between taxa has proceeded under clock-like conditions,

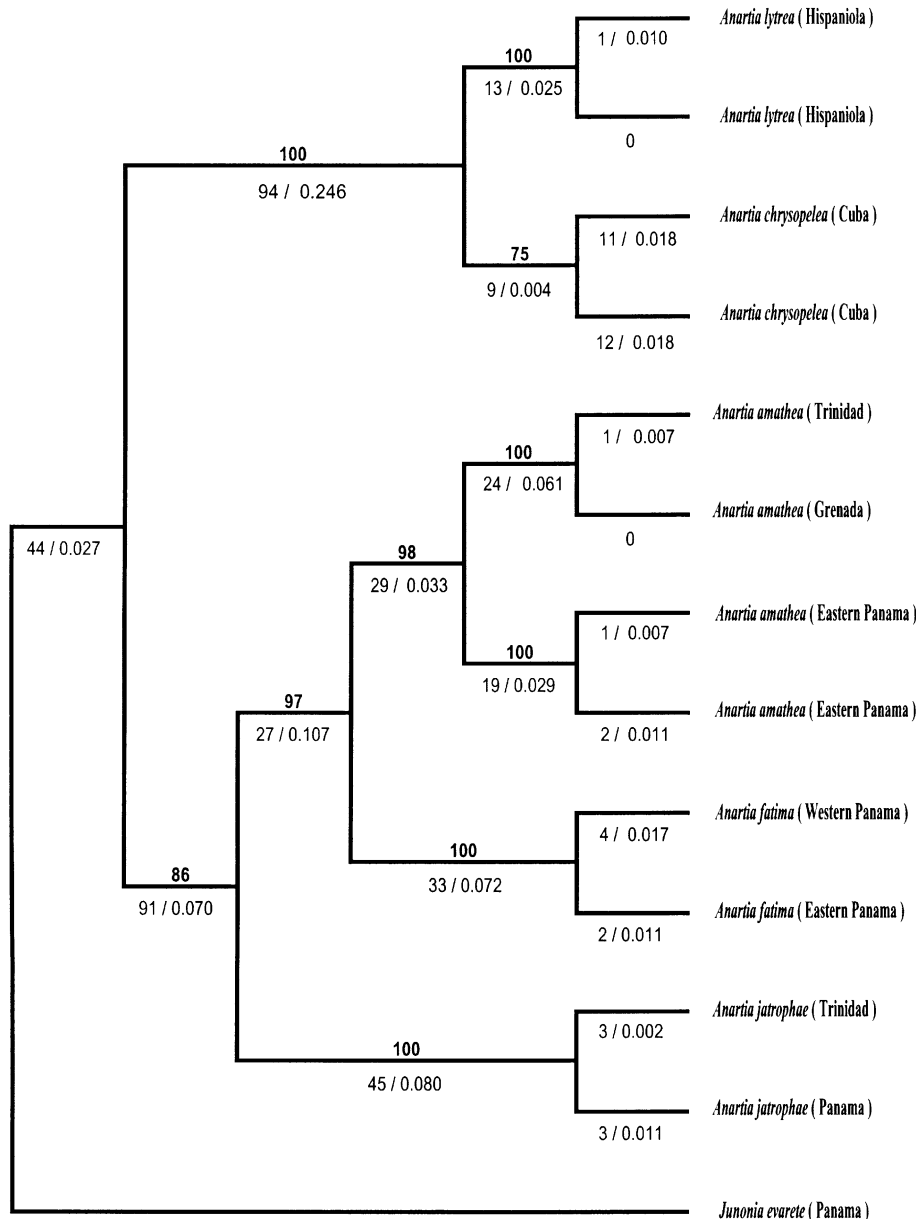


Fig. 3. The *Tpi* maximum parsimony hypothesis for *Anartia* species relationships. Bootstrap values are above the branches, and are based on 1000 replications. The number of character changes and NJ genetic distance (Log-Det) values are given below the branches.

which supports the conversion of pairwise divergence estimates into units of time.

#### 4. Discussion

##### 4.1. Topological congruence between mtDNA, nuclear DNA, allozymes, and morphology

The traditional view of *Anartia* relationships is well supported by the mtDNA and *Tpi* data. All the topologies clearly support splitting *Anartia* into three phylogenetically distinct lineages. In both the mtDNA

and TPI tree topologies (Figs. 2 and 3), *A. jatrophae* is the sister species to the *A. amathea*–*A. fatima* clade, while *A. chrysopelea* pairs with *A. lytrea*. Considering genital and morphological characters, it is not surprising that *A. amathea* forms a clade with *A. fatima*, and *A. lytrea* and *A. chrysopelea* pair together. The surprise lies with *A. jatrophae* because it was predicted to be the outlying sister species to the other four taxa due to its distinct genitalia and a range that sympatrically overlaps all the other *Anartia* species (Silberglied et al., 1979). Yet *A. jatrophae*'s phylogenetic placement as sister to the *A. amathea*–*A. fatima* clade is well supported.

A recent study of *Anartia* based on allozyme frequency distributions also demonstrates the genetic distinctiveness of the three lineages, but the allozyme data do not clearly resolve the phylogenetic relationships among the clades (Dasmahapatra et al., 2002). Both the *Tpi* and the allozyme data also resolve population differentiation that is not found with the mtDNA data. The *Tpi* and allozyme data suggest that Caribbean populations of *A. amathea* are genetically distinct from Central American populations, perhaps representing a *cis/trans*-Andean split. The clear phylogenetic separation of *A. amathea* populations at a regional scale prompted us to undertake a preliminary examination of population structure at a more local scale using *Tpi*. Thus we sequenced the *Tpi* locus for another 3 *A. fatima* (total  $n = 5$ ) and 3 *A. amathea* (total  $n = 7$ ) from Panama. Analysis of this small data set indicated that *Tpi* provides more information than mtDNA for distinguishing fine-scale population genetic structure among *Anartia* populations. For example, *A. fatima* individuals separated by as little as 30 km were distinguished by their *Tpi* genotypes but not by their mtDNA haplotypes. Thus it appears that *Tpi* intron variation may provide useful genetic markers in the study of wing color pattern polymorphism as well as geographic population substructure and historical demography within the different *Anartia* species.

Considering the genitalia of the three lineages (Fig. 1), it appears that genitalic divergence has not been an important feature of the reproductive isolation between the two *Anartia* species pairs. Although it is tempting to speculate that genitalic divergence might have reinforced reproductive isolation between *A. jatrophae* and its sympatric congeners, our data set is not sufficient to address this hypothesis. Nonetheless, if such was the case we might anticipate population genetic evidence indicating that the *A. jatrophae* range expansion(s) are relatively old. Yet, if the range expansion of *A. jatrophae*, and thus sympatry with congeners, is relatively recent as might be tentatively argued from our limited mtDNA and *Tpi* data for Trinidad and Panama individuals, morphological drift would seem the more likely explanation for the genitalic differences among the three lineages.

## 5. Biogeography

The ring structure of the *Anartia* species distributions consistently draws attention to the group. The traditional explanation of the species' biogeography relies on the assumption that the extant form of *A. jatrophae* represents the ancestral *Anartia* species. Because the remaining four species form two sets of allopatric sister species pairs, it would follow that *Anartia* species formation would have involved at least two sympatric speciation events (where Antillean and Continental

lineages split from a widespread ancestor) followed by allopatric differentiation of the derived lineages. This scenario, while possible, reflects incomplete knowledge of the species phylogenetic relationships. Our molecule-based analyses indicate that the extant form of *A. jatrophae* does not represent the ancestor of all *Anartia* species. While the phylogeny does not preclude a *jatrophae*-like widespread ancestor for the genus, it does support a vicariance model of *Anartia* speciation where co-distribution of *A. jatrophae* with its four congeners resulted from range expansion leading to secondary sympatry.

A vicariance model of *Anartia* speciation may be overly simplistic, but it provides a useful null hypothesis because the contemporary biogeography of the group can be explained through a series of allopatric events. Accordingly, the initial allopatric split among *Anartia* lineages separated the Cuba-Hispaniola lineage from the ancestor of the *A. jatrophae/A. amathea/A. fatima* clade. Given the contemporary distributions of the latter three species it is not possible to determine whether this early diversification involved colonization of the Greater Antilles from a Lesser Antilles or continental form. Nevertheless, if we adopt Brower's (1994) inferred molecular clock for arthropod mtDNA sequence divergence (1.1–1.2% per 1 million years of separation), the mtDNA genetic distances observed in this study suggest that the initial diversification of *Anartia* dates to the late Miocene. *Anartia* geographic expansion would thus likely have been aided by the low sea level stand at the end of the Miocene (Haq et al., 1987) that likely caused reductions in the over-water distances between the mainland and Antillean islands and among islands.

Considering that the separation of the Eastern Cuba-Northern Hispaniola block is thought to have occurred during the mid Tertiary it is unlikely that the *A. lytrea-A. chrysopelea* species pair formed as a result of classic vicariance (Buskirk, 1985). It is more likely that speciation followed oceanic dispersal of butterflies from one island to the other. Following a late Miocene dispersal event, the Pliocene high sea level stand (Haq et al., 1987) would have greatly increased over-water distances, and low or nonexistent migration could then have permitted allopatric adaptation to local conditions (i.e., *A. lytrea*'s use of aroid marshland) and the island-specific genetic structure that currently characterizes *A. lytrea* and *A. chrysopelea*.

Formation of the youngest *Anartia* species pair, *A. amathea* and *A. fatima*, would have occurred in the late Pliocene or early Pleistocene according to the Brower (1994) mtDNA clock. This time period corresponds to the completion of the Panamanian isthmus (Coates and Obando, 1996), which provided a terrestrial corridor linking South America to nuclear Central America and could have permitted the continental expansion of the *A. amathea-A. fatima* ancestor. Subsequent allopatric



separation between Central and South American *Anartia* is consistent with a colonization model put forward for Central American freshwater fishes (Bermingham and Martin, 1998), which provides evidence of several colonization pulses followed by allopatric diversification. This model posits that the eustatic sea level rise at the end of the Pliocene (Haq et al., 1987) would likely have inundated parts of the nascent isthmus, thus extirpating the terrestrial fauna in the region of Central Panama. Allopatric vicars would have then had the opportunity to come into secondary contact following the continued rise and development of the Panamanian isthmus, a scenario supported by the position of the *A. fatima*–*A. amathea* hybrid zone in eastern Panama (Dasmahapatra et al., 2002).

Untangling the evolutionary history of *A. jatrophae* and its history of sympatry alongside its four congeners stands as an attractive challenge. Smith et al. (1994) contend that Greater Antillean *A. jatrophae* populations may have originated from Central America or southern North America sources, whereas southern Lesser Antillean and South American *A. jatrophae* have had a distinct history. Smith et al. (1994) invoke *A. jatrophae* subspecific morphological variation across the Antillean islands as evidence for multidirectional colonization. Such a pincer-like colonization of the Antilles was postulated and rejected for *Dryas iulia*, another widely occurring butterfly species with continental and Caribbean island populations (Davies and Bermingham, 2002). Davies and Bermingham's (2002) mtDNA and allozyme data were more consistent with south to north colonization and low gene flow between Lesser and Greater Antillean populations. Most phylogeographic studies of Caribbean birds and reptiles do not support multidirectional colonization (Bermingham et al., 1996; Ricklefs and Bermingham, 2001; Roughgarden, 1995). However, Klein and Brown's (1994) study of yellow warblers and recently acquired data on Caribbean birds (Bermingham and Ricklefs, unpublished data) do provide some examples of multidirectional colonization of the Greater and Lesser Antilles. We nevertheless predict that colonization occurred from a single source given the low level of *Tpi* sequence divergence observed between Panama and Trinidad individuals as compared to the divergence observed between *A. amathea* individuals from the same locations. We also predict that *A. jatrophae*'s expansion is likely to have been quite recent, perhaps owing to Pleistocene reductions in sea level. Clearly, more work on the expansion history of *A. jatrophae* will be required to test these hypotheses.

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### References

- Beltrán, M., Jiggins, C.D., Bull, V., Linares, M., McMillan, W.O., Mallet, J., Bermingham, E., 2002. Phylogenetic discordance at the species boundary: gene genealogies in *Heliconius* butterflies. *Mol. Biol. Evol.* (in press).
- Bermingham, E., Seutin, G., Ricklefs, R., 1996. Regional approaches to conservation biology: RFLPs, DNA sequence, and Caribbean birds. In: Smith, T.B., Wayne, R.K. (Eds.), *Molecular Genetic Approaches in Conservation*. Oxford University Press, New York, pp. 104–124.
- Bermingham, E., Martin, A., 1998. Comparative mtDNA phylogeography of neotropical freshwater fishes: testing shared history to infer the evolutionary landscape of lower Central America. *Mol. Ecol.* 7, 499–517.
- Brower, A.V.Z., 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proc. Natl. Acad. Sci. USA* 91, 6491–6495.
- Buskirk, R., 1985. Zoogeographic patterns and tectonic history of Jamaica and the northern Caribbean. *J. Biogeogr.* 12, 445–461.
- Coates, A.G., Obando, J., 1996. The geologic evolution of the Central American Isthmus. In: Jackson, J., Coates, A.G., Budd, A. (Eds.), *Evolution and Environment in Tropical America*. University of Chicago Press, Chicago, pp. 21–56.
- Coyne, J.A., Orr, H.A., 1989. Patterns of speciation in *Drosophila*. *Evolution* 43, 362–381.
- Dasmahapatra, K., Blum, M.J., Aiello, A., Hackwell, S., Davies, N., Bermingham, E., Mallet, J., 2002. Inferences from a moving hybrid zone. *Evolution* 56 (4), 741–753.
- Davies, N., Aiello, A., Mallet, J., Pomianski, A., Silberglied, R., 1997. Speciation in two neotropical butterflies: extending Haldane's rule. *Proc. R. Soc. Lond. B* 264, 845–851.
- Davies, N., Bermingham, E., 2002. The historical biogeography of two Caribbean butterflies (Lepidoptera: Heliconiidae) as inferred from genetic variation at multiple loci. *Evolution* 56 (3), 573–589.
- Goldman, N., Anderson, J., Rodrigo, A., 2000. Likelihood-based tests of topologies in phylogenetics. *Syst. Biol.* 49 (4), 652–670.
- Haq, B.U., Hardenbol, J., Vail, P.R., 1987. Chronology of fluctuating sea levels since the Triassic. *Science* 235 (4793), 1156–1167.
- Hasegawa, M., Kishino, H., Yano, T., 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* 12, 160–174.
- Hasson, E., Wang, I., Zeng, L., Kreitman, M., Eanes, W., 1998. Nucleotide variation in the triosephosphate isomerase (*Tpi*) locus of *Drosophila melanogaster* and *Drosophila simulans*. *Mol. Biol. Evol.* 15, 756–769.
- Huelsenbeck, J., Rennala, B., 1997. Phylogenetic methods come of age: testing hypotheses in an evolutionary context. *Science* 276, 227–232.
- Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins, D.G., Gibson, T.J., 1998. Multiple sequence alignment with Clustal X. *Trends Biochem. Sci.* 23, 403–405.

- Jiggins, C.D., Linares, M., Naisbit, R.E., Salazar, C., Yang, Z., Mallet, J., 2001. Sex linked hybrid sterility in a butterfly. *Evolution* 55 (8), 1631–1638.
- Kishino, H., Hasegawa, M., 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequences data, and the branching order in the Hominoidea. *J. Mol. Evol.* 29, 170–179.
- Klein, N.K., Brown, W.M., 1994. Intraspecific molecular phylogeny in the yellow warbler (*Dendroica-Petechia*), and implications for avian biogeography in the West-Indies. *Evolution* 48 (6), 1914–1932.
- Kwiatowski, J., Krawczyk, M., Kornacki, M., Bailey, K., Ayala, F., 1995. Evidence against the exon theory of genes derived from the triose-phosphate isomerase gene. *Proc. Natl. Acad. Sci. USA* 92, 8503–8506.
- Lockhart, P.J., Steel, M.A., Hendy, M., Penny, D., 1994. Recovering evolutionary trees under a more realistic model of sequence evolution. *Mol. Biol. Evol.* 11, 605–612.
- Logsdon Jr., J.M., Tyshenko, M.G., Dixon, C., D-Jafari, J., Walker, V.K., Palmer, J.D., 1995. Seven newly discovered intron positions in the triose-phosphate isomerase gene: evidence for the intron-late theory. *Proc. Natl. Acad. Sci. USA* 92, 8507–8511.
- Milinkovitch, M., Leduc, R., Adachi, J., Farnir, F., Georges, M., Hasegawa, M., 1996. Effects of character weighting and species sampling on phylogeny reconstruction: a case study based on DNA sequence data in cetaceans. *Genetics* 144, 1817–1833.
- Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14 (9), 817–818.
- Rambaut, A., 1996. Sequence Alignment Editor 1.0., <http://evolve.zoo.ox.ac.uk/Se-Align/Se-Align.html>.
- Ricklefs, R.E., Bermingham, E., 2001. Nonequilibrium diversity dynamics of the Lesser Antillean avifauna. *Science* 294 (5546), 1522–1524.
- Rodriguez, F.J., Oliver, J.L., Marin, A., Medina, J.R., 1990. The general stochastic model of nucleotide substitution. *J. Theor. Biol.* 142, 485–501.
- Roughgarden, J., 1995. *Anolis Lizards of the Caribbean*. Oxford University Press, Oxford.
- Seutin, G., White, B., Boag, P., 1991. Preservation of avian blood and tissue samples for DNA analyses. *Can. J. Zool.* 69, 82–90.
- Silberglie, R., Aiello, A., Lamas, G., 1979. Neotropical butterflies of the genus *Anartia*: systematics, life histories and general biology. *Psyche* 89, 219–260.
- Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am* 87 (6), 651–701.
- Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol. Biol. Evol.* 16, 1114–1116.
- Smith, D.S., Miller, L.D., Miller, J.Y., 1994. *The Butterflies of the West Indies and South Florida*. Oxford University Press, Oxford.
- Swofford, D.L., 2001. PAUP: phylogenetic analysis using parsimony (\* and other methods), version 4.0., Sinauer, Sunderland, MA.
- Young, A., Stein, D., 1976. Studies on the evolutionary biology of the neotropical butterfly *Anartia fatima* in Costa Rica. *Contr. Biol. Geol. Milwaukee Publ. Mus.* No. 8, March 29, 1976. 29 p. + col. frontispiece.