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A tale of two haplotype groups: Evaluating the New World *Junonia* ring species hypothesis using the distribution of divergent *COI* haplotypes

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Abstract

The New World *Junonia* butterflies are a possible ring species with a circum-Caribbean distribution. Previous reports suggest a steady transition between North and South American forms in Mesoamerica, but in Cuba the forms were thought to co-exist without interbreeding representing the overlapping ends of the ring. Three criteria establish the existence of a ring species: a ring-shaped geographic distribution, gene flow among intervening forms, and genetic isolation in the region of range overlap. We evaluated mitochondrial *cytochrome oxidase I* haplotypes in *Junonia* from 9 species in the Western Hemisphere to test the *Junonia* ring species hypothesis. *Junonia* species are generally not monophyletic with respect to *COI* haplotypes, which are shared across species. However, two major *COI* haplotype groups exist. Group A predominates in South America, and Group B predominates in North and Central America. Therefore, *COI* haplotypes can be used to assess the degree of genetic influence a population receives from each continent. *Junonia* shows a ring-shaped distribution around the Caribbean, and evidence is consistent with gene flow among forms of *Junonia*, including those from Mesoamerica. However, we detected no discontinuity in gene flow in Cuba or elsewhere in the Caribbean consistent with genetic isolation in the region of overlap. Though sampling is still very limited in the critical region, the only remaining possibility for a circum-Caribbean discontinuity in gene flow is at the Isthmus of Panama, where there may be a transition from 98% Group B haplotypes in Costa Rica to 85–100% Group A haplotypes in South America.

Keywords

Junonia; ring species; adaptive radiation; *cytochrome oxidase I*; phylogeography

Introduction

Ring species are groups of species or subspecies with ring-like geographic distributions around regions of unsuitable habitat such that the extreme ends of the range overlap (Mayr 1942). Gene flow occurs through intermediate forms around the ring circumference, but in the overlapping region forms do not interbreed. Ring species have been of particular interest to evolutionary biologists because they may represent a transitional state in species

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formation by geographic vicariance (Irwin *et al.* 2001). Many organisms have been identified as possible ring species, some of which have been studied intensively. Among these, some species have interruptions in gene flow at various points around the supposed ring (e.g. herring gulls, genus *Larus* (Liebers *et al.* 2004)), and are thus not ring species. Other species (e.g. the Asian *Phylloscopus* warblers (Irwin *et al.* 2005; Irwin *et al.* 2008) and the Californian *Ensatina* salamanders (Wake 2001; Pereira & Wake 2009)) appear to show gene flow around the ring, but genetic isolation where the ends of the range overlap, thereby fulfilling the criteria of a ring species. Other proposed ring species have not been studied sufficiently to determine whether they meet the criteria of a ring species: ring-shaped geographic distribution, genetic isolation in the region of range overlap, and gene flow among the intervening forms (Irwin *et al.* 2001).

The New World *Junonia* butterflies are one example of an understudied possible ring species (Table 1). The ring-shaped Caribbean distribution of *Junonia* was identified by Forbes (1928) who noted that there was a steady phenotypic transition in Mexico and Central America from North American color pattern phenotypes (similar to *J. coenia*), to phenotypes typical of South American forms (similar to *J. evarete* and *J. genoveva*). The two most prominent of these phenotypes that increase from North to South are the degree of orange suffusion within the white apical patch on the dorsal forewings and the appearance of blue iridescent scales as a component of the ground coloration on both the dorsal forewings and hindwings (Forbes 1928). However, two apparently separate colonization events of Cuba and the nearby Isle of Pines (one originating from North America and one from South America) and the descendants of these two *Junonia* colonizations were thought to co-exist without interbreeding. New World *Junonia* was popularized as a possible ring species by Mayr (1942, 1963), but no additional information was added beyond what was reported by Forbes (1928). Brown and Heineman (1972) suggested that North American *Junonia* penetration into the Caribbean may not be limited to Cuba, with specimens with North American-like color patterns known from Jamaica and the Cayman Islands. Remington (1985) revisited the *Junonia* ring species hypothesis and provided a map, supplemented with photographs of specimens, showing geographic variation in the genus. Finally, Irwin *et al.* (2001) examined the Caribbean *Junonia* as part of a review of many proposed ring species and speculated that gene flow between forms around the Caribbean was unlikely.

Other studies have shown that forms of New World *Junonia* share identical karyotypes (N=31)(Maeki & Remington 1960; Turner & Parnell 1985), can hybridize and produce fertile offspring in the lab (Hafernik 1982; Paulsen 1994; Paulsen 1996), and engage in interspecific courtship (Minno & Emmel 1993) and possible hybridization (Scott 1986) in the field. Mitochondrial markers show historic gene flow between *Junonia* populations in Mexico (Pfeiler *et al.* 2012a). More recently, molecular genetic work using both mitochondrial and nuclear markers shows hybridization between *Junonia* species appears to take place in the wild and individuals that show genetic evidence of hybrid ancestry are common in some populations (Borchers & Marcus 2014; Gemmell *et al.* 2014).

Barcoding studies of the mitochondrial *cytochrome oxidase I (COI)* gene in *Junonia* show that there are 2 New World mitochondrial haplotype groups (A and B), with 3.9% sequence divergence between them (Pfeiler *et al.* 2012b). However, most mitochondrial genotypes are

shared across several *Junonia* species and most New World *Junonia* species contain individuals with mitochondria of both haplotype groups (Brévignon & Brévignon 2012; Borchers & Marcus 2014; Gemmell *et al.* 2014). Regardless of species, *COI* haplotype group A is most abundant in South America (85% frequency or higher) while haplotype group B predominates in North America (nearly 100% frequency) (Pfeiler *et al.* 2012b; Gemmell *et al.* 2014). Haplotype group frequencies on Martinique and Guadeloupe are intermediate between those in North and South America (Gemmell *et al.* 2014). This suggests that while *COI* barcode haplotypes are not diagnostic for *Junonia* species, they are good markers for measuring the genetic influence from continental North and South America on *Junonia* populations.

The *Junonia* ring species hypothesis (Forbes 1928), predicts that the island of Cuba will show strong genetic influences from both North and South America, but the rest of the Caribbean will show primarily South American influences. It is also expected that there will be a continuous transition in allele frequencies between *COI* haplotype groups A and B in Mexico and Central America to parallel the transition in color pattern phenotypes reported from this region. Finally, the ring species hypothesis predicts a discontinuity in *COI* haplotype group frequencies in the Caribbean in association with the overlapping region where supposedly non-interbreeding forms of *Junonia* co-occur in Cuba at the termini of the ring. We use a combination of phylogenetic analysis, haplotype networks, and biogeographic approaches to test these predictions of the *Junonia* ring species hypothesis. To place the possible New World *Junonia* ring species in a phylogenetic context we also consider prospective Old World sister taxa and the potential origins of haplotype groups A and B.

Materials & Methods

Specimens and DNA Preparation

Junonia specimens from all 9 currently recognized New World species (Borchers & Marcus 2014) were collected from the wild using hand-held butterfly nets and frozen at -20°C or obtained from other collectors (Supporting Information Table S1). Specimens were identified on the basis of morphological characters (Turner & Parnell 1985; Neild 2008; Brévignon & Brévignon 2012), however, it should be noted that *Junonia* taxonomy is in flux due to recent genetic comparisons (Pfeiler *et al.* 2012b; Borchers & Marcus 2014; Gemmell *et al.* 2014). Thorough molecular comparisons between Florida, Texas, and Caribbean *Junonia* are incomplete making it unclear which forms are conspecific (Gemmell *et al.* 2014). We provisionally refer to the *Junonia* from Florida as *J. coenia* (the common buckeye), *J. "evarete"* (the mangrove buckeye), and *J. "genoveva1"* (the tropical buckeye, all sensu Turner and Parnell, 1985) because this is the current common usage of scientific names in Florida (Glassberg *et al.* 2000; Cech & Tudor 2005; Mitter 2013) and is the taxonomy used for our collection and importation permits. For the same reasons, we refer to *Junonia* from Texas as *J. coenia* (the common buckeye), *J. sp. nigrosuffusa* (the dark buckeye), and *J. "genoveva2"* (the mangrove buckeye) (Pfeiler 2011; Pfeiler *et al.* 2012b). We recognize that alternate taxonomic hypotheses for the taxa exist (Neild 2008; Brévignon & Brévignon 2012), but prefer to avoid causing further confusion by withholding taxonomic

judgment until appropriate tests using nuclear markers are conducted. *Junonia* “*genoveva1*” from Florida and *Junonia* “*genoveva2*” from Texas are not conspecific with each other, and it is yet to be determined whether either of these forms is conspecific with *J. genoveva* from South America.

Individual legs were removed from each specimen and DNA was isolated using the DNEasy Blood and Tissue kit (Qiagen, Düsseldorf, Germany). Legs were ground with a ceramic mortar and pestle in 180 µl of tissue lysis buffer ATL. Once homogenized, 20 µl of proteinase K (Qiagen, 600 mU/mL) was added and the mixture was incubated for one hour in a 55°C water bath until tissue was lysed. Samples were then processed manually as previously described (Borchers & Marcus 2014) or loaded into a Qiagen QIAcube extraction robot and processed using the standard instrument protocol for purification of total DNA from animal tissue (Gemmell *et al.* 2014). Samples were evaluated for DNA concentration using a Nanodrop 2000 spectrophotometer (Nanodrop, Wilmington, Delaware, USA) and stored at –20°C until needed.

PCR and Sequencing

Cytochrome oxidase I (COI) PCR products were generated by amplification with LCO1490 and HCO2198 primers (Table 2) (Folmer *et al.* 1994). Quick-Load Taq 2× Mastermix (New England Biolabs, Ipswich, MA, USA) was used in PCR reactions with total volumes of 25 µl. Amplification protocols were run on MyCycler or S1000 Thermal Cyclers (BioRad, Hercules, California, USA) for these and all other PCR amplifications. PCR reaction conditions were: 95°C for 5 minutes; 35 cycles of 94°C for 1 minute, 46°C for 1 minute, and 72°C for 1.5 minutes; and a final 5 minute extension at 72°C before being placed on a 4°C hold. PCR products were loaded into a 1% agarose gel in TAE buffer, run for 1 hour at 78 V, and visualized using ethidium bromide under ultraviolet light.

PCR products were sequenced using the Sanger dideoxy method as previously described (Borchers & Marcus 2014). PCR products were sequenced in both directions with the primers used to generate the products. Sequencing reactions were analyzed on ABI 3130 or ABI 3730×1 automated sequencers (Applied Biosystems, Carlsbad, California, USA) and edited using Sequencher 4.6 software (Sequencher 2005). Primer sequences were removed leaving sequenced amplified products of 658 bp, which were then aligned in CLUSTAL W version 2.1 (Thompson *et al.* 1994; Larkin *et al.* 2007).

Phylogenetic Analysis

In addition to the sequences generated by this study, additional sequences from our earlier work (Borchers & Marcus 2014; Gemmell *et al.* 2014) and previously published Old World *Junonia COI* sequences (Kodandaramaiah & Wahlberg 2007; Kodandaramaiah 2009; Hebert *et al.* 2013; Shi *et al.* 2013) were included in a phylogenetic analysis. *COI* sequences from each New World *Junonia* specimen were compared with each other and duplicate sequences were removed from the analysis (identical sequences are listed in Supporting Information Table S2). Therefore, only one exemplar of each genotype was included in the phylogenetic analysis, minimizing computation time. CLUSTAL W *COI* sequence alignments were converted to NEXUS format and analyzed phylogenetically using several

reconstruction methods (distance, parsimony, likelihood) that rely on vastly different assumptions about sequence evolution, each of which recovered nearly identical trees. For the sake of brevity, we only present the maximum likelihood analysis (HKY model, 10 replicate heuristic searches with random number seeds, tree bisection and reconnection branch swapping algorithm) of PAUP version 4.0b10 (Swofford 1998). In addition, a maximum likelihood bootstrap analysis of the dataset (500 fast addition replicates, retaining nodes with frequency less than 50%) was conducted to assess the degree of support for each node.

Haplotype Network Construction

The aligned *COI* sequences generated by this study, along with sequences from previously published studies from our laboratory (Borchers & Marcus 2014; Gemmell *et al.* 2014) and exemplar *COI* sequences for Old World outgroup species *J. orithya* and *J. villida* (Kodandaramaiah & Wahlberg 2007; Hebert *et al.* 2013; Shi *et al.* 2013) (each previously identified as possible sister taxa to the New World *Junonia* based on morphology and wing color patterns (Seitz 1914; Corbet 1948; Vane-Wright & Tennent 2011)), were formatted for input into Arlequin 3.5 (Schneider *et al.* 2000). No *J. villida* sequences in Genbank contain the complete *COI* barcode, so we used the consensus sequence between a published sequence (NW99-1) (Kodandaramaiah & Wahlberg 2007) and a short *J. villida* *COI* sequence (AUS1, collected Sydney, Australia, 15 Dec.1999) that we generated ourselves (DNA Database of Japan (DDBJ) Accession Number AB981649) for network analysis. An AMOVA analysis was run using the following settings: 1000 permutations, determining the minimum spanning network (MSN) among haplotypes, computing distance matrix, and pair-wise difference with a gamma value of 0. The minimum spanning tree output from the AMOVA analysis was used as input for HapSTAR-0.7 (Teacher & Griffiths 2011), which displays the haplotype network in graphical form. This analysis, as implemented in Arlequin, requires all sequences to be the same length. Therefore, only full-length *COI* barcode fragments were used. Additional graphical adjustments to this network were made using Canvas X (ACD Systems, Seattle, Washington, USA) which included scaling the size of population circles to reflect the number of individuals with a specific *COI* haplotype, and the addition of divisions and colors to reflect geographical regions of a specific haplotype.

Restriction Fragment Length Polymorphism Genotyping

As a supplement to *Junonia* *COI* sequences, we evaluated *COI* PCR products from additional New World *Junonia* specimens by restriction digest to detect cut sites diagnostic for haplotype groups A and B. The quantity and quality of the PCR products amplified from the DNA of some specimens was insufficient for successful DNA sequencing, but was suitable for genotyping by restriction digest. PCR products were generated using a two-step amplification with LCO1490 and HCO2198 primers (as described above) followed by re-amplification using miniCOIF2 and HCO2198 primers (Table 1) (re-amplification conditions were: 95°C for 2 minutes; 5 cycles of 95°C for 1 minute, 46°C for 1 minute, 72°C for 30 seconds; 35 cycles of 95°C for 1 minute, 53°C for 1 minute, and 72°C for 30 seconds; and a final 5 minute extension at 72°C before being placed on a 4°C hold). PCR reactions were prepared using reaction volumes of 25 µl and Qiagen TopTaq Mastermix for these and all other PCR re-amplifications described below.

PCR products of the correct size were evaluated by a triple digest using BseYI, AflIII, and BamHI restriction endonucleases (New England Biolabs, Ipswich, MA, USA) (Fig. 1). A 10 µL aliquot of the PCR product was incubated with 10 µL of the triple digest cocktail (2 µL of NEB Buffer3, 2 µL of BSA (10×, 1 mg/mL), 4 µL of ddH₂O, 0.5 µL of BseYI, 0.5 µL of AflIII and 1 µL of BamHI) for 1 hour in a 37°C water bath. Samples were then heated in a 70°C water bath for 10 minutes to deactivate the enzymes. Samples were stored on ice until they were run on a QIAxcel Advanced instrument (Qiagen) fitted with a DNA Screening Cartridge with QX Size Markers (25–500 bp v. 2.0) and QX Alignment Markers (15 bp–1 kb) using the AL320 electrophoresis method. Most genetic variants (~85%) within haplotype group A contain a BamHI cut site (GGATCC) at 256 bp producing 2 bands of sizes 256 bp and 313 bp. Haplotype group B genotypes lack this BamHI cut site, but instead, contain an AflIII cut site (ACR(G/A)Y(T/C)GT) at 351 bp and a BseYI cut site (CCCAGC) at 501 bp, thereby producing 3 bands of sizes 351 bp, 150 bp and 68 bp. A small minority of haplotype group B genotypes (~5%) lack the BseYI cut site, resulting in 2 bands of sizes 218 bp and 351 bp, which are still easily distinguished from digests of group A haplotypes. Assessing a New World *Junonia* *COI* haplotype at these 3 restriction sites allows for a completely unambiguous assignment to one of the 2 major haplotype groups (Fig. 1).

Samples that failed to amplify with miniCOIF2 and HCO2198 primers were re-amplified from the original LCO1490/HCO2198 PCR products with miniCOIF2 and miniCOIR2 primers (Table 1) using the same re-amplification thermocycler protocol followed by a restriction digest using BamHI. The other two restriction cut sites lie outside the 339 bp PCR amplification product produced by these primers (Fig. 1). Samples were also re-amplified from the LCO1490/HCO2198 PCR products with miniCOIF3 and HCO2198 or miniCOIF3 and miniCOIR3 primers (Table 1) using the same re-amplification protocol and digested with AflIII and BseYI before being assigned to a *COI* haplotype group based on bands produced by all digests (Fig. 1).

Phylogeographic Mapping of *COI* Haplotype Groups

To maximize the geographic representation of *Junonia* beyond our own sampling, we evaluated additional New World *Junonia* *COI* sequences from previously published studies (Kodandaramaiah & Wahlberg 2007; Janzen & Hajibabaei 2009; Marcus *et al.* 2009; Hebert *et al.* 2010; Escobedo 2011; Brévignon & Brévignon 2012; Janzen 2012; Pfeiler *et al.* 2012b; Mitter 2013; Borchers & Marcus 2014; Gemmell *et al.* 2014) and the *Junonia* haplotype group assignments determined by restriction digest in our laboratory, to calculate the proportion of A and B haplotype groups for each species in various geographic regions. This was done to evaluate differences in haplotype group allele frequencies between species, using samples of the same species from different countries (or in some cases in sub-regions within a country (e.g. Texas and Florida, USA)) as replicates to estimate within-region variation. Since in most instances, *COI* haplotypes are not confined to particular species in *Junonia* and occur at very similar frequencies in all species found in a given country (or sub-region), we combined specimens from all species together to calculate and map the haplotype group frequencies geographically. For comparison, species and selected subspecies ranges were determined from collection localities of specimens in our research collection and from published reports, and were plotted onto the same map (Forbes 1928;

Turner & Parnell 1985; Brown *et al.* 1992; Elster *et al.* 1999; Neild 2008; Menezes & Peixoto 2009; Pfeiler 2011; Brévignon & Brévignon 2012; Gernaat *et al.* 2012; Pfeiler *et al.* 2012a; Pfeiler *et al.* 2012b).

To check for discontinuities in the distribution of group A and B haplotypes across the Caribbean we plotted the proportion of group A alleles (as determined above) in each Caribbean population versus the distance from the Southern tip of mainland Florida, USA. We used the “As the Crow Flies” distance calculator (<http://tjpeiffer.com/crowflies.html>) to determine the straight-line distance from Everglades National Park in South Florida to the closest landfall in Cuba, Jamaica, Hispaniola, Puerto Rico, Antigua, Guadeloupe, Dominica, Martinique, and French Guiana. Plotted haplotype group frequency and distance data were then analyzed by logarithmic regression because the expected genetic influence of North American populations on West Indian populations is expected to be inversely proportional to geographic distance.

Results

Phylogenetic Analysis

New *COI* sequences generated by this project were deposited in Genbank, accession numbers KM287911 - KM288274 (364 accessions). Maximum likelihood analysis of these *COI* sequences, plus those from our prior studies (Borchers & Marcus 2014; Gemmell *et al.* 2014), produced 3 phylogenetic trees of likelihood score 3281.34255 (Fig. 2). As previously reported (Pfeiler *et al.* 2012b; Borchers & Marcus 2014; Gemmell *et al.* 2014), there are two distinct monophyletic lineages of mitochondrial haplotypes found in the New World *Junonia*. Haplotype group A is primarily found in South American and Caribbean specimens, but is also present in a few North and Central American *Junonia*. Haplotype group B includes many North American, Central American, and Caribbean specimens, but also includes some South American *Junonia*. Only two named forms of New World *Junonia* can be identified unambiguously on the basis of *COI* barcode haplotype alone: *J. vestina* from the South American Andes (Haplotype A₁ of Pfeiler *et al.* (2012b)) and *J. coenia grisea* from California. Of the two major sublineages identified by Pfeiler *et al.* (2012b) within Haplotype group A, Haplotype A₁ (which includes only *J. vestina*) is monophyletic, while Haplotype A₂ (which includes individuals from 7 *Junonia* species) is unresolved in our analysis (Fig. 2).

The maximum likelihood trees with the best scores place a clade of Old World taxa (bootstrap support 75%) that includes African, Asian, and Indo-Australian species as the sister group to the New World *Junonia* (bootstrap support of 87%). Substantially similar trees were recovered by phylogenetic analysis using Parsimony and Distance reconstruction methods (data not shown). All of these trees also place haplotype groups A and B as sister to each other, making the New World *Junonia* monophyletic, but in the maximum likelihood analysis, this relationship is only supported by a bootstrap value of 36%. A minority of alternate maximum likelihood bootstrap tree topologies maintain the monophyly of the New World *Junonia* as a whole and the monophyly of haplotype group B, but haplotype group A is paraphyletic with respect to haplotype group B (not shown). These alternative bootstrap trees differ from each other with respect to which lineages within haplotype group A are

most closely related to haplotype group B. Finally, a small final group of maximum likelihood bootstrap tree topologies do not support the monophyly of the New World *Junonia* (not shown), which is based on a very small number of informative sites (Borchers & Marcus 2014).

COI Haplotype Network

The minimum spanning haplotype network of New World *Junonia* COI sequences is in concordance with the results of the phylogenetic analysis, reinforcing the conclusion that haplotype groups A and B are distinct lineages (Fig. 3). The two most similar sequences between the two haplotype groups (LCB320 and LCB164) are 11 mutational steps apart. Haplotype group A shows substantially greater within-group genetic structural diversity and geographic structuring of its component genotypes. Group A is 17 mutational steps away from the COI sequence of the nearest outgroup species (*J. villida*, which has an Indo-Pacific distribution). Haplotype group B has lower internal genetic diversity and less geographic structuring with the majority of sequences falling into a single component genotype (LCB166) and is 18 mutational steps away from the *J. villida* outgroup sequence. As in the phylogenetic analysis, the COI sequences from *J. vestina* and *J. coenia grisea* are represented by distinct branches within the haplotype network.

Phylogeography of *Junonia* COI Sequences

We determined the mitochondrial haplotype group assignments of 718 New World *Junonia* specimens for phylogeographic analysis. In most cases, species that co-occur in the same region show statistically indistinguishable haplotype group frequencies (Table 3). However, in different regions, even the same *Junonia* species can show dramatically different haplotype group frequencies. Thus, while there is only a weak species-specific signal in the COI data, there is a strong geographic signal in the distribution of group A and B COI haplotypes in the New World (Fig. 4). North and Central American *Junonia* carry COI alleles that are almost exclusively from haplotype group B. Of the 434 specimens evaluated from this region, only 8 specimens with group A haplotypes were identified (1 each from Panama, Costa Rica, Belize, and Mexico, plus 4 from southern Florida). In South America haplotype group A predominates with the frequency of haplotype group B never exceeding about 15% frequency and is often much lower. The continental transition between predominantly group A haplotypes and predominantly group B haplotypes occurs approximately at the isthmus of Panama, but this transition does not coincide with the range limit of any *Junonia* species (Fig. 4).

In the Caribbean, the frequency of the 2 haplotype groups varies considerably with haplotype group A ranging from 35% (Cuba) to 87% (Dominican Republic). Plotting the abundance of group A haplotypes in each locality versus the linear distance from the North American mainland and fitting a trend line yields a highly significant logarithmic regression ($R^2=0.640$, $p=0.005$) across the entire Caribbean, with no evidence of discontinuity in gene flow in Cuba or elsewhere in the West Indies (Fig. 5).

Discussion

Evaluating the Ring Species Hypothesis

In order to recognize the New World *Junonia* as a ring species, it must meet three criteria: a ring-shaped distribution, gene flow among intervening forms, and reduced or absent gene flow at the extreme ends of the distribution where forms overlap (Irwin *et al.* 2001). The ring-shaped distribution of *Junonia* around the circumference of the Caribbean and on the islands of the Antilles has long been known and was likely a key factor leading to the proposal of the *Junonia* ring species hypothesis (Forbes 1928).

Gene flow among intervening forms in *Junonia* is well-supported by laboratory studies of hybridization (Hafern timer 1982; Paulsen 1994; Paulsen 1996), gradual geographic transitions in color pattern phenotypes around the circumference of the Caribbean (Forbes 1928; Remington 1985), and studies of nuclear and mitochondrial markers showing evidence of ongoing genetic exchange in Argentina, Mexico, and among populations in French Guiana and the Antilles (Pfeiler *et al.* 2012a; Borchers & Marcus 2014; Gemmell *et al.* 2014). In this study, it is clear that mitochondrial haplotypes are shared between nominal *Junonia* species and that the frequency of different *COI* haplotype groups in the Caribbean is a function of the distance of a population from the North or South American mainland, which is also consistent with gene flow.

The final prediction of the *Junonia* ring species hypothesis, as formulated previously in the literature (Forbes 1928; Remington 1985; Irwin *et al.* 2001) would be a discontinuity in gene flow in association with the supposed divergent, overlapping, and non-interbreeding ends of the *Junonia* distribution in Cuba. We would expect to see evidence of this discontinuity with abundant North American mitochondrial haplotypes (group B) present in Cuba, but rare elsewhere in the Caribbean. A logarithmic regression fits the *COI* haplotype group frequency data very well, and no discontinuity is apparent anywhere in the Caribbean (Fig. 4). Instead there appears to be a gradual change in haplotype group frequencies across the archipelago, suggesting genetic admixture (Fig. 5). We therefore reject the ring species hypothesis as originally articulated by Forbes (1928) because gene flow in *COI* is apparently not interrupted in Cuba or elsewhere in the Caribbean. However, it would be highly desirable to evaluate the population genetic structure of the New World ring species hypothesis using nuclear markers to see if the patterns of gene flow observed using mitochondrial *COI* are also evident in other parts of the *Junonia* genome.

While no discontinuity has been detected in Cuba, there may be a discontinuity in the distribution of mitochondrial haplotype groups elsewhere in the range of New World *Junonia*. In particular, the transition between North and South American haplotypes in the region around the Isthmus of Panama might be seen as abrupt (Fig. 4) with few group A alleles penetrating northwards into Central America, and an unknown number of group B alleles found in the northern parts of South America. However, our sampling in this region so far is relatively weak. If the well-sampled populations of *Junonia* from French Guiana are representative of populations from elsewhere on the Guiana Shield, the allele frequency of group B haplotypes would be expected to reach about 15% (Brévignon & Brévignon 2012; Gemmell *et al.* 2014).

An alternative to the ring species hypothesis of Forbes (1928) still requires evaluation: if a circum-Caribbean buckeye ring species exists, it may be that the terminal ends of the ring are not in Cuba as originally hypothesized, but at the transition between Central and South America. The formation of the land bridge making up the Isthmus of Panama was completed approximately 3 million years ago (Knowlton & Weigt 1998). In butterflies, *COI* evolves at a rate of about 1.8% per million years (Brower & DeSalle 1998), putting the divergence of haplotype groups A and B, which differ from each other by 3.9% (Borchers & Marcus 2014), at approximately 2.2 million years ago. The rough congruency of these two estimates is curious, but may be completely coincidental because *Junonia* have been documented to fly across substantial bodies of water during migration events (Williams 1930; Harris 1988). Unfortunately, our sampling in Panama, Columbia, Venezuela, and Ecuador is currently very limited, so we cannot fully evaluate whether this apparent steep discontinuity in mitochondrial genotypes is real or whether it is an artifact of our limited sampling. *Junonia* is abundant in this region (Neild 2008; Gernaat *et al.* 2012) and it should be possible to evaluate this part of the circum-Caribbean distribution of *Junonia* in more detail and test this alternative ring species hypothesis in a future study.

Other Biogeographic Points of Interest in *Junonia*

There are several additional geographic regions within the New World in which the study of population genetic structure of *Junonia* will be very interesting. First, it appears that California populations of *J. coenia grisea* (Austin & Emmel 1998) have *COI* haplotypes that are distinct genetically from populations of *J. coenia coenia* from elsewhere in North America (Pfeiler *et al.* 2012b), whereas most species of *Junonia* cannot be distinguished from one another on the basis of *COI* haplotypes (Brévignon & Brévignon 2012; Borchers & Marcus 2014; Gemmell *et al.* 2014). The ranges of the two subspecies of *J. coenia* are largely separated by the Rocky Mountains, but apparently meet in Arizona and New Mexico, USA and in Northern Mexico (Brock & Kaufman 2003). Different populations of *Junonia coenia* have markedly different host plant preferences (Camara 1997; Gemmell *et al.* 2014), which could contribute to temporal or habitat partitioning, but it is not known to what extent hybridization between subspecies might occur in this region. Given the distinctive *J. coenia grisea* *COI* haplotype (Pfeiler *et al.* 2012b) and the phenotypic differences between this form and nominate *J. coenia* (Austin & Emmel 1998), if gene flow between them is limited or absent, it may be appropriate to elevate *J. coenia grisea* to full species status.

Junonia vestina also has a distinctive *COI* haplotype (A_1) that is not shared with other *Junonia* species (Pfeiler *et al.* 2012b). This species occurs at mid-elevations of the Andes Mountains in Ecuador, Peru, and Bolivia (Neild 2008), but thus far only material from Peru has been studied with molecular tools. Whether the range of *J. vestina* abuts or overlaps with the ranges of lower elevation forms of *Junonia* (both to the East and West of the Andes, which almost exclusively carry *COI* alleles from haplotype group A_2) is unknown, as is whether there is any genetic exchange between them. Based on the limited sampling so far with mitochondrial markers (Pfeiler *et al.*, 2012a; this study), there is no apparent gene flow. Additional fieldwork, sampling, and molecular analysis of *Junonia* specimens of all species

from this region would clarify the degree to which *J. vestina* is unique among New World *Junonia* in its lack of hybridization with other forms.

Finally, Florida, USA also represents a region of particular interest with respect to *Junonia* population genetics. Unlike the regions described above, *Junonia* populations in Florida carry almost exclusively mitochondrial haplotype B. The only exceptions to this are 2 mangrove buckeye specimens (*J. "evarete"* sensu (Turner & Parnell 1985)) in the lower Florida Keys in Monroe County and 2 tropical buckeye specimens (*J. "genoveval"* sensu (Turner & Parnell 1985)) from the Everglades Greenway in Dade County. However, the colonization history of *Junonia* in Florida is better known than in any other geographic region. *Junonia coenia* and *J. "evarete"* are resident in Florida, but *J. "genoveval"* was first recorded from Key Largo in 1961 and became abundant both there and in locations on the Florida mainland by 1978 (Minno & Emmel 1993). *J. "genoveval"* is phenotypically similar to *J. zonalis* from the Caribbean, but the two forms should be compared using nuclear genetic markers before they are determined to be conspecific. Due to variations in climate (the larval host plant is very sensitive to frost) and land development by humans, there have been large fluctuations in the abundance of *J. "genoveval"* (Glassberg *et al.* 2000). Currently, *J. "genoveval"* is difficult to find in the Florida Keys, and all but one of the specimens included here come from a single locality on the Florida mainland. Museum specimens exist covering the entire history and geographic range of *J. "genoveval"* in Florida. Examining the genetic changes that occurred in *J. "genoveval"* as it invaded Florida, dispersed, and possibly hybridized with the resident *Junonia* species would be an informative case study in invasion biology.

Origins of the New World Buckeyes and COI Haplotype Groups

Phylogenetic approaches have yet to establish the sister taxon to the New World *Junonia* or even determine the continent from which the New World was colonized (Kodandaramaiah & Wahlberg 2007). In our minimum spanning haplotype network analysis we included sequences from specimens from the two species that are most morphologically similar to the New World forms: *J. villida* and *J. orithya* (Seitz 1914; Vane-Wright & Tennent 2011). *Junonia villida* has a range extending from the Chagos Islands in the West Indian Ocean to Tonga and the Tuamotu Archipelago in the South Pacific, while *J. orithya* has a range that extends from sub-Saharan Africa to New Guinea. Minimum spanning haplotype networks are a useful supplement to phylogenetic analysis, particularly in cases where complete coalescence of haplotypes in the various daughter lineages has not yet gone to completion and ancestral genotypes are still extant and present among the sequences being analyzed (Posada & Crandall 2001), as is often the case in determining intraspecific phylogenies, or ascertaining relationships among very closely related taxa (Bandelt *et al.* 1999; Jeratthitikul *et al.* 2013).

Our network analysis suggests that the *COI* haplotypes present in the New World *Junonia* are much more similar to that found in *J. villida* than to the haplotypes from *J. orithya* (Fig. 3). It also shows that while haplotypes from *J. orithya* originating in Asia and Australia are similar to one another, *J. orithya COI* from Africa is highly divergent, perhaps suggesting that the two forms are not conspecific. The association between *J. villida* (which has an

Indo-Pacific distribution) and the New World *Junonia COI* haplotypes, supports an old hypothesis that *J. villida* is the sister taxon to New World *Junonia* species (Seitz 1914; Vane-Wright & Tennent 2011).

Junonia villida is marginally closer (17 vs. 18 mutational steps) to genotypes in haplotype group A, but the genetic diversity and the geographical structure of haplotype group A is also much greater than that of haplotype group B. Typically, in the absence of selection, a lack of substantial genetic structure is attributed to high rates of gene flow among populations or to recent colonization (Sotka & Palumbi 2006; Guzmán *et al.* 2011). Since haplotype groups A and B co-occur in the same *Junonia* species, we do not anticipate large differences in gene flow between the 2 haplotype groups, suggesting that the diversification of haplotype group B may be more recent than that of haplotype group A. This could be interpreted as evidence against a hypothesis proposed by Borchers and Marcus (2014) that the co-existence of the two haplotype groups may be the result of an ancient mitochondrial polymorphism present in the founding population of *Junonia* in the New World. Two remaining explanations for the origins of haplotype groups A and B are that mitochondrial haplotype group B evolved within North America from group A genotypes or that there were 2 separate *Junonia* invasions of the New World followed by hybridization events between the descendants of the 2 invasions. *Junonia villida* is extremely widespread, inhabiting islands from the Indian Ocean to the mid-Pacific Ocean (Vane-Wright & Tennent 2011), and it is possible that two invasions of the Western Hemisphere from different source populations of this taxon could contribute to the patterns of haplotype diversity that we observe among the New World *Junonia*. While there has been a recent review of the morphological diversity found in *J. villida* (Vane-Wright & Tennent 2011), no comparable survey of molecular diversity in this species has been conducted to date, representing an opportunity for further study. It is also possible that selection is acting on *Junonia* mitochondrial haplotypes (perhaps on *COI* sequences, but possibly on other mitochondrial sequences linked to *COI*), but this has not been demonstrated and is another opportunity for additional research. If selection is demonstrated, possible scenarios for New World *Junonia COI* haplotype diversification should be revisited (Irwin 2012).

Future Directions

The New World *Junonia* represent an exciting system in which to study both morphological and molecular diversification. The rapid radiation of the New World *Junonia* to occupy diverse habitats, utilize different larval host plants, and display variation in color pattern phenotypes is reminiscent of some other well-known radiations such as the haplochromine cichlids of Lake Victoria (Seehausen 2002), the North American sunflowers (Rieseberg *et al.* 2007), and the swordtails and platyfish of Central America (Marcus & McCune 1999). These radiations share with *Junonia* the propensity for interspecific hybridization in the wild (Cui *et al.* 2013; Keller *et al.* 2013; Bock *et al.* 2014) making them particularly amenable for studying the roles of reticulate evolution in the generation of novel phenotypes. These biological characteristics, the ease of *Junonia* laboratory culture (Marcus 2005), and the diverse tools available for experimentally manipulating *Junonia* butterflies (Marcus 2005; Nijhout 2010; Dhungel *et al.* 2013; Beaudette *et al.* 2014) provide much potential for

identifying and assigning function to genetic variation associated with ecologically relevant traits, linking genotype and phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Map of PCR primer binding sites and restriction enzyme cut sizes for the barcode fragment of *COI*. BamHI restriction sites are only found in haplotype group A alleles while AflIII and BseYI restriction sites are only found in haplotype group B alleles.

Fig. 2. Consensus of 3 maximum likelihood trees depicting the two distinct mitochondrial *COI* haplotype groups found among the New World *Junonia*. The single node that differs between these 3 trees is indicated by an asterisk. Numbers adjacent to nodes indicate bootstrap support. Bootstrap values of less than 30% are omitted. Group A haplotypes occur primarily in South America and the Caribbean. Group B haplotypes occur throughout the Western Hemisphere, but are most common in North and Central America. Most *Junonia* species include individuals with both haplotypes. *Junonia vestina* is unusual in that it is

characterized by the unique haplotype group A₁. Only one exemplar of each haplotype was included in the phylogenetic analysis, but the relative abundance of each haplotype is indicated by the vertical width of each triangle. Taxonomic abbreviations: *J.c.* (*J. coneia*), *J.d.* (*J. divaricata*), *J.e.* (*J. evarete*), *J."e."* (*J. "evarete"*), *J.f.* (*J. sp. flirtea*), *J.g.* (*J. genoveva*), *J."g."* (*J. "genoveva"*), *J.h.* (*J. sp. hilaris*), *J.n.* (*J. nigrosuffusa*), *J.ne.* (*J. neildi*), *J.sp.a.h.* (*J. sp. affin hilaris*), *J.w.* (*J. wahlbergi*) *J.z.* (*J. zonalis*). Geographic abbreviations: AR (Argentina), BO (Bolivia), CR (Costa Rica), CU (Cuba), FG (French Guiana), FL (Florida, USA), GD (Guadeloupe), JA (Jamaica), KY (Kentucky, USA), MA (Martinique), TX (Texas, USA).

Fig. 3.

Haplotype networks generated using complete barcode fragment mitochondrial *COI* haplotypes. Circle areas are scaled to represent the number of individuals that contain a specific *COI* haplotype with the exception of the specimens carrying the LCB166 allele (40% of full scale). Divisions and colors of circles reflect geographic locality. *COI* haplotypes are generally shared across *Junonia* species with the exception of those associated with *J. coenia grisea* and *J. vestina*.

Fig. 5.

Plot of the proportion of haplotype group A genotypes found in *Junonia* populations on the Caribbean Islands and in French Guiana versus the distance of each island from the Southernmost point in mainland Florida, USA. Logarithmic regression analysis of the data yields a highly significant result, with an apparent asymptote at a Group A haplotype frequency of approximately 85%, consistent with the frequency of that haplotype group in much of mainland South America.

Table 1

Described *Junonia* species from the Western Hemisphere with selected subspecies. Also included are some forms with ambiguous species assignments.

Species
<i>Junonia coenia</i> Hübner, 1822 <i>J. coenia grisea</i> Austin & Emmel, 1998
<i>Junonia divaricata</i> C. & R. Felder, 1867
<i>Junonia evarete</i> (Cramer, 1779)
<i>Junonia sp. flirtea</i> (Fabricius, 1793)
<i>Junonia genoveva</i> (Cramer, 1780)
<i>J. sp. hilaris</i> C. & R. Felder, 1867
<i>J. sp. affin. hilaris</i> Borchers and Marcus, 2014
<i>Junonia litoralis</i> Brévignon, 2009
<i>Junonia neildi</i> Brévignon, 2004
<i>J. sp. nigrosuffusa</i> Barnes & McDunnough, 1916
<i>J. sp. pallens</i> C. & R. Felder, 1867
<i>Junonia vestina</i> C. & R. Felder, 1867
<i>Junonia wahlbergi</i> Brévignon, 2008
<i>Junonia zonalis</i> C. & R. Felder, 1867

Table 2

Primer sequences used for *cytochrome oxidase I (COI)* PCR reactions.

Primer	Sequence	Source
LCO1490	GGTCAACAAATCATAAAGATATTGG	(Folmer <i>et al.</i> 1994)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	(Folmer <i>et al.</i> 1994)
miniCOIF2	ATACTATTGTTACAGCCTCATGC	(Gemmell <i>et al.</i> 2014)
miniCOIR2	TGTTGTAATAAAATTAATAGCTCC	(Gemmell <i>et al.</i> 2014)
miniCOIF3	CCCCACTTTCATCTAATATTGC	(Gemmell <i>et al.</i> 2014)
miniCOIR3	TATTTCGATCTGTTAAAAGTATAG	This study

Table 3

Haplotype group assignments for taxa in different regions of the Western Hemisphere. # Areas sampled refers to the number of countries or major regions within countries for each species. Haplotype group proportions are reported as % abundance in the region $\pm$ 1 standard error. Within each region, taxa generally show statistically indistinguishable haplotype group frequencies and are clustered in the table. Exceptional taxa with distinct haplotype group frequencies from other taxa in the same region are listed separately in the table.

Taxon	# Areas Sampled	Total # Specimens	% Haplotype Group A	% Haplotype Group B
North America				
<i>J. coenia</i>	5	193	0 $\pm$ 0%	100 $\pm$ 0%
<i>J. "evarete"</i>	1	60	3.3%	96.7%
<i>J. "genoveva1"</i>	1	22	9.1%	90.9%
<i>J. "genoveva2"</i>	2	20	0 $\pm$ 0%	100 $\pm$ 0%
<i>J. sp. nigrosuffusa</i>	2	47	2.1 $\pm$ 5.9%	97.9 $\pm$ 5.9%
<i>Junonia coenia grisea</i>	1	22	0%	100% $\pm$ 0% [‡]
South America				
<i>J. sp. hilaris</i> *	2	21	90.5 $\pm$ 8.8%	9.5 $\pm$ 8.8%
<i>J. sp. flirtea</i>	4	23	95.7 $\pm$ 2.9%	4.3 $\pm$ 2.9%
<i>J. evarete</i>	4	52	98.1 $\pm$ 1.7%	1.9 $\pm$ 1.7%
<i>J. genoveva</i>	5	35	94.3 $\pm$ 3.6%	5.7 $\pm$ 3.6%
<i>J. litoralis</i>	2	7	71.42 $\pm$ 23.6%	28.6 $\pm$ 23.6%
<i>J. wahlbergi</i>	1	10	80.0%	20.0%
<i>J. divaricata</i>	1	5	80.0%	20.0%
<i>J. sp. affin. hilaris</i> *	1	7	57.1%	42.8%
<i>J. vestina</i>	1	7	100% [†]	0%
Central America				
<i>J. evarete</i>	2	2	50.0 $\pm$ 70.7%	50.0 $\pm$ 70.7%
<i>J. genoveva</i>	2	56	3.6 $\pm$ 69.4%	96.4 $\pm$ 69.4%
Caribbean				
<i>J. neildi</i>	3	13	53.8 $\pm$ 38.1%	46.2 $\pm$ 38.2%
<i>J. zonalis</i>	7	103	67.0 $\pm$ 29.9%	33.0 $\pm$ 29.9%

* *J. sp. hilaris* and *J. sp. affin. hilaris* are extremely difficult to distinguish based on morphological characters without supplemental genotyping with nuclear markers. Some specimens that have not undergone nuclear genotyping have been tentatively assigned to *J. sp. hilaris*.

[†] Exclusively *Junonia vestina* alleles in haplotype Group A1.

[‡] Exclusively California-specific alleles in haplotype Group B.