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DNA BARCODES AND INSIGHTS INTO THE RELATIONSHIPS AND SYSTEMATICS OF BUCKEYE BUTTERFLIES (NYMPHALIDAE: NYMPHALINAE: *JUNONIA*) FROM THE AMERICAS

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ABSTRACT. Nucleotide sequence data from a segment of the mitochondrial cytochrome c oxidase subunit I (COI) gene, known as the barcode segment, were used to examine phylogenetic relationships and systematics of buckeye butterflies (Nymphalidae: Nymphalinae: *Junonia*) from the New World, with emphasis on taxa from western North America. Three nominal species have been recognized for North America, *J. evarete* (Cramer), *J. genoveva* (Cramer), and *J. coenia* Hübner, with additional species recently proposed for the West Indies and northern South America. The distinctive Andean buckeye, *J. vestina* C. Felder & R. Felder, along with *J. evarete* and *J. genoveva*, are also components of the South American fauna. With the exception of *J. vestina*, butterflies comprising the New World *Junonia* have had a confused taxonomic history, and species assignments are often problematic. Our results show that the barcode segment resolves the two major clades of New World *Junonia*, referred to here as clades A and B, with similar high support seen in an earlier phylogenetic study using both mitochondrial and nuclear genes. Within clade A, *J. vestina* resolved in a basal position to *J. evarete* from South America and the Caribbean. The data further suggest that species assignments in some populations of New World *Junonia* clustering in clade B (*J. coenia* + *J. genoveva*) need to be reevaluated. DNA barcodes, although failing to resolve all recognized species and subspecies level taxa of New World *Junonia*, probably owing to relatively recent divergences, can provide valuable tools for identifying the two major lineages, and when used in conjunction with morphological, ecological, behavioral and life history information can provide insights into the taxonomy and evolution of this difficult group.

Additional key words: cytochrome c oxidase subunit I, dispersal, genetic distance, population structure, speciation.

Butterflies commonly known as buckeyes (Nymphalidae: Nymphalinae: *Junonia*) are widely distributed in the Americas, being found from southern Canada to South America. In an early treatment of the genus, Forbes (1928) recognized two species of *Junonia* in the New World, *J. vestina* C. Felder & R. Felder, a high altitude form found throughout the Andes of South America (Fig. 1), and *J. lavinia* (Cramer) [= *J. evarete* (Cramer)] in which he grouped all others forms that were morphologically similar and distinct from *J. vestina*. In the present paper we refer to buckeyes included in *J. lavinia* as the *J. evarete* complex [*Junonia lavinia* is now recognized as a permanently invalid synonym of *J. evarete* (Comstock 1942)]. The genus *Precis* also has been used for the New World buckeyes, but butterflies belonging to this genus are now known to be restricted to Africa (Wahlberg et al. 2005). Recently, Pelham (2008) recognized three nominal species of *Junonia* belonging to the *J. evarete* complex as defined here: *J. evarete* (Cramer), *J. genoveva* (Cramer) and *J. coenia* Hübner, as well as three subspecies: *J. evarete nigrosuffusa* W. Barnes & McDunnough, *J. evarete zonalis* C. Felder & R. Felder and *J. coenia grisea* Austin & J. Emmel. In addition, ongoing taxonomic studies on *Junonia* from the West Indies and northern South America suggest that additional species level taxa

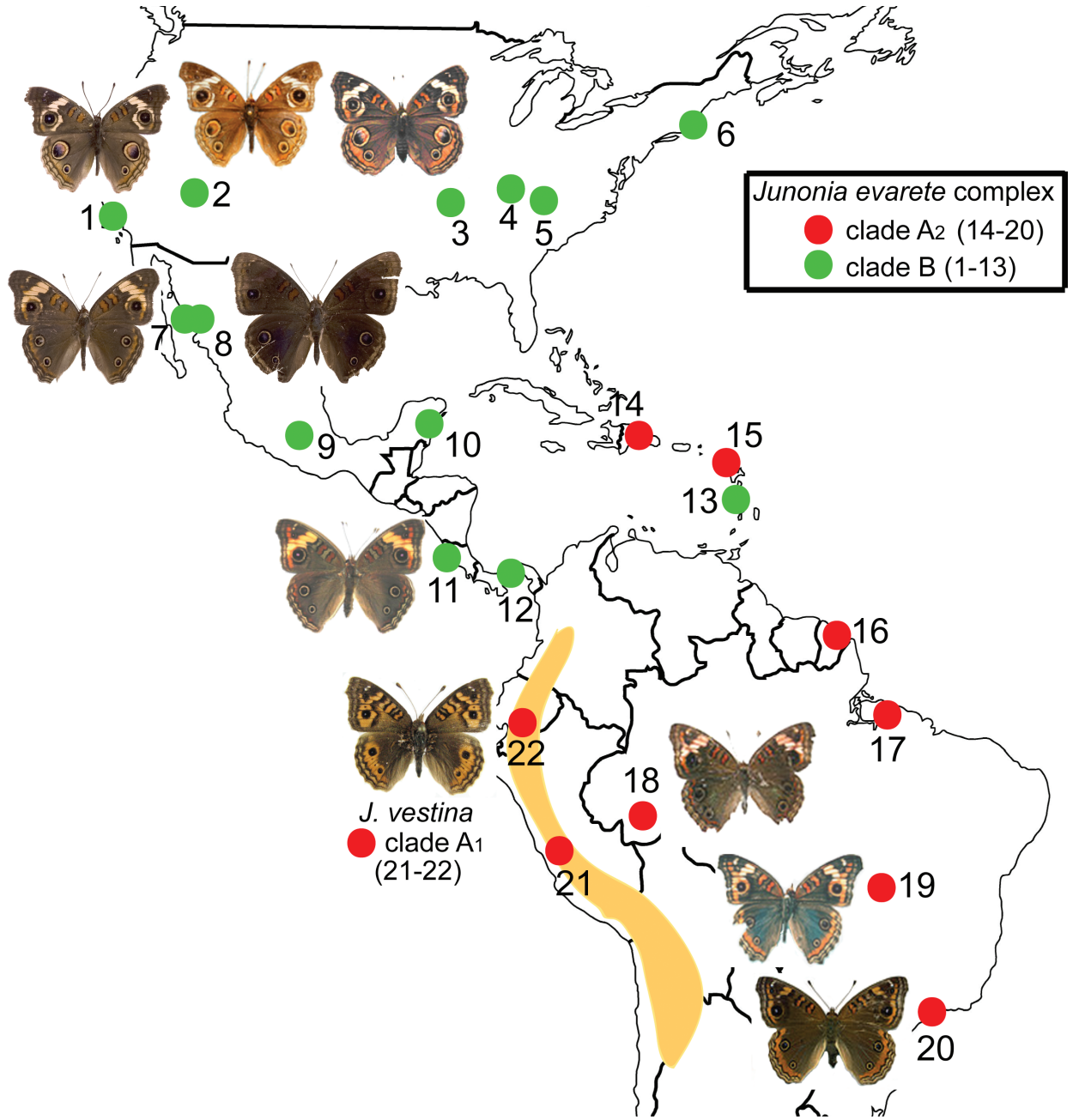

FIG. 1. Map of North and South America showing collection localities and phenotypic variability of *Junonia* spp. at selected localities where dorsal images were available. Red and green dots correspond to the two main clades (A and B, respectively) of New World *Junonia* (see Fig. 2). The shaded area represents the approximate geographic distribution of the Andean buckeye, *J. vestina*. Voucher codes for each species are given below (see Table 1 for details). Scientific names in parentheses are suggested changes in assignment based on data presented here (see Discussion regarding the assignment of *J. nigrosuffusa*) or unpublished data (C. Brévignon, pers. com.). **1,** *J. coenia grisea* (CIAD 10–B03); **2,** *J. coenia coenia* (NW38–18); **3,** *J. coenia coenia* (female) (NW85–13); **4,** *J. coenia coenia* (no image) (Bio175–17); **5,** *J. coenia coenia* (no image) (DNA–ATBI–0802 and –0816); **6,** *J. coenia coenia* (no image) (TDWG–0126); **7,** *J. evarete* (= *J. genoveva*; CIAD 10–B19; Estero del Soldado); **8,** *J. evarete nigrosuffusa* (= *J. nigrosuffusa*; CIAD 10–B24); **9,** *J. evarete* (no image) (= *J. genoveva*; JM6–10); **10,** *J. evarete* (no image) (= *J. genoveva*; MAL–02877); **11,** *J. evarete* (= *J. genoveva*; 05–SRNP–58293); **12,** *J. evarete* (no image) (= *J. genoveva*; YB–BCI12765); **13,** *J. genoveva* (no image) (= *J. neildi* Brévignon [C. Brévignon, pers com.]; NW136–16); **14,** *Junonia* sp. (no image) (= *J. evarete*; NW153–12); **15,** *J. evarete* (no image) (NW136–17); **16,** *Junonia* spp. (no images) (UK4–14, –15, –16); **17,** *J. evarete* (no image) (NW151–3); **18,** *J. evarete* (NW126–20); **19,** *J. evarete* (NW84–15); **20,** *J. genoveva*? (= *J. evarete*; NW155–2); **21,** *J. vestina* (no image) (NN07); **22,** *J. vestina* (Las Culebrillas, Cañar, Ecuador; DNA not extracted). Photograph credits: **2, 3, 18–20,** Nymphalidae Systematics Group (2009); **1, 7, 8,** Wain Evans; **11,** Janzen and Hallwachs (2009); **22,** Jean-Claude Petit.

are also present (Brévignon 2008, 2009).

Although the buckeyes are a well-known and much studied group of nymphalid butterflies, especially with respect to genetic factors involved in evolution and development of eyespots and color patterns (Nijhout 1980; Reed et al. 2007; Monteiro 2008; Kodandaramaiah 2009; Monteiro & Prudic 2010), and the chemical ecology and evolution of hostplant preferences (Bowers 1984; Bowers & Puttick 1989; Bowers & Stamp 1997), the systematics of the *J. evarete* complex has been plagued with uncertainty, with species assignments often questionable and unreliable. The confusion can be traced, at least in part, to the pronounced phenotypic variability in wing maculation and coloration within the genus *Junonia* (Tilden 1971; Hafernik 1982). The apparent loss of type specimens, vague or unknown type localities, and non-standardized use of common names have also added to the taxonomic confusion. Molecular phylogenetic evidence suggests that the ancestor of the *J. evarete* complex probably colonized the New World from Africa or Asia relatively recently, ~2–4 million years ago (Ma) (Kodandaramaiah & Wahlberg 2007), implying that subsequent speciation in this group also is relatively recent. Thus, the possibility for incomplete lineage sorting among diversifying taxa may be high. The many observations of hybridization among phenotypic variants of *Junonia* (Rutkowski 1971; Hafernik 1982) are consistent with this possibility.

In the only comprehensive (worldwide) molecular phylogenetic study conducted to date on *Junonia*, based on 3090 base pairs (bp) from both mitochondrial (cytochrome c oxidase subunit I; COI) and nuclear genes (*wingless* and elongation factor-1α), the three nominal species of the *J. evarete* complex partitioned into two well-supported clades, one comprised of *J. evarete* (Brazil and Guadeloupe) and the other consisting of *J. coenia coenia* (Utah and Tennessee, USA) + *J. genoveva* (Martinique) (Kodandaramaiah & Wahlberg 2007). Because total sample size from the two New World clades was low ($N = 8$), and did not include any populations from western North America (with the exception of a single individual from Utah), the relationships of these previously studied taxa to western populations of *Junonia* remain unclear. We also wished to assess whether molecular data from western populations would provide any additional insights into the results of the hybridization studies of Hafernik (1982) who found high genetic similarity among western taxa.

Given the increase in available COI sequence data for *Junonia* from the DNA barcode initiative (Ratnasingham & Hebert 2007), and the fact that most (633 bp) of the 658 bp barcode region was sequenced by Kodandaramaiah and Wahlberg (2007), we were particularly interested in determining if the barcode segment alone could provide informative characters for inferring phylogenetic relationships and addressing taxonomic uncertainties in *Junonia* from the Americas. DNA barcodes, although sometimes of limited usefulness (Elias et al. 2007; Yassin et al. 2010), have been shown to be highly reliable at species-level identifications within the Lepidoptera in the eastern USA and northwestern Costa Rica, with a success rate of >97% for ~2000 morphologically-defined taxa (Hebert et al. 2003, 2010; Janzen et al. 2005; Hajibabaei et al. 2006). In the present study, we analyzed both new and previously published COI sequences from a total of 85 individuals of New World *Junonia*.

Materials and Methods

Sampling. The new taxa of *Junonia* treated here include (1) *J. coenia grisea* from far western USA (California and southern Oregon) and the Baja California Peninsula, Mexico (type locality: South Pasadena, Los Angeles County, California) (Austin & Emmel 1998); specimens for the present study were collected at a residential development site in Santa Barbara, California, USA, (2) a population from northwestern Mexico that feeds on black mangrove *Avicennia germinans* (L.) L. (Acanthaceae) (Pfeiler 2011). This population is listed as *J. evarete* by Brown et al. (1992) (an assignment initially followed here) and referred to as an intermediate between *J. evarete zonalis* and *J. coenia* by Hafernik (1982); our samples were collected at a mangrove estuary (Estero del Soldado) near San Carlos, Sonora, Mexico, (3) the taxon currently recognized as *J. evarete nigrosuffusa* (Luna-Reyes et al. 2008; Pelham 2008), a large, dark subspecies inhabiting southwestern USA and Mexico, generally inland from the immediate coast (type locality: southeastern Arizona); our specimens were collected in the coastal foothills of the Sierra El Aguaje at San Carlos, Sonora, Mexico. GenBank sequences were available for a population of *Junonia* from the Area de Conservación Guanacaste (ACG), Guanacaste Province in northwestern Costa Rica assigned to *J. evarete*, whose foodplants include *Dyschoriste valeriana* Leonard (Acanthaceae) and *Stachytarpheta jamaicensis* (L.) Vahl (Verbenaceae) (DeVries 1987; D.H. Janzen & M. Hajibabaei unpublished). Additional GenBank sequences were obtained for specimens collected in southern Mexico, Panama, Brazil, Peru, French Guiana, central and eastern USA, and the Caribbean. Details on taxa analyzed, collection data and GenBank accession numbers for the complete data set are given in Table 1.

Table 1. List of species of *Junonia* analyzed for COI, with collection data and GenBank accession numbers.

Species	Voucher code	Locality	Geographic Coordinates	Altitude (m)	Collection date	GenBank access. no.	Ref [a]
J. coenia coenia (USA)	NW38–18	Washington Co. UT			29 May 1998	AY248777	1
(*N* = 6)	NW85–13	Shelby Co., TN			1992 (reared)	AY788643	1
	DNA–ATBI–0802	Swain Co., NC	35.520 N, 83.304 W	607	20 July 2004	GU089962	2
	DNA–ATBI–0816	"	35.550 N, 83.308 W		19 July 2004	GU089963	2
	Bio175–17	Hart Co., KY				EU271674	
	TDWG-0126	Barnstable Co., MA	41.642 N, 70.558 W	36	29 Sept. 2010	HQ964540	6
J. coenia grisea (USA)	CIAD 10–B03	Santa Barbara, CA	34.459 N, 119.759 W	130	19 June 2010	JQ430685	3
(*N* = 7)	CIAD 10–B04	"	"	"	"	JQ430686	3
	CIAD 10–B05	"	"	"	"	JQ430687	3
	CIAD 10–B06	"	"	"	"	JQ430688	3
	CIAD 10–B07	"	"	"	"	JQ430689	3
	CIAD 10–B08	"	"	"	"	JQ430690	3
	CIAD 10–B09	"	"	"	"	JQ430691	3
J. evarete (Brazil)	NW36–2	Minaçu, Goiás			1 Dec. 1998	EU053298	1
(*N* = 5)	NW84–15	Minaçu, Goiás			"	EU053299	1
	NW126–20	Acre				EU053295	1
	NW129–30	Acre				EU053296	1
	NW151–3	Vigia, Pará	0.858 S, 48.142 W		19 July 2006	JQ430732	5
J. evarete (Guadeloupe)	NW136–17	Guadeloupe			16 Dec. 2003	EU053297	1
J. evarete (Mexico)	CIAD 10–B01	San Carlos, Sonora	27.959 N, 110.981 W	<2	8 Jan. 2010	JQ430692	3
(*N* = 22)	CIAD 10–B10	"	"	"	21 July 2010	JQ430693	3
	CIAD 10–B11	"	"	"	16 Aug. 2010	JQ430694	3
	CIAD 10–B12	"	"	"	"	JQ430695	3
	CIAD 10–B13	"	"	"	20 Aug. 2010	JQ430696	3
	CIAD 10–B14	"	"	"	"	JQ430697	3
	CIAD 10–B15	"	"	"	"	JQ430698	3
	CIAD 10–B16	"	"	"	25 Sept. 2010	JQ430699	3
	CIAD 10–B17	"	"	"	"	JQ430700	3
	CIAD 10–B18	"	"	"	"	JQ430701	3
	CIAD 10–B19	"	"	"	"	JQ430702	3

Table 1. (continued)

	CIAD 10–B20	"	"	"	"	JQ430703	3
	CIAD 10–B21	"	"	"	"	JQ430704	3
	CIAD 10–B22	"	"	"	"	JQ430705	3
	CIAD 10–B23	"	"	"	"	JQ430706	3
	CIAD 10–B27	"	"	"	11 Oct. 2010	JQ430707	3
	CIAD 10–B28	"	"	"	"	JQ430708	3
	CIAD 10–B29	"	27.983 N, 111.043 W	42	1 Nov. 2010	JQ430709	3
	CIAD 10–B37	"	27.959 N, 110.981 W	<2	9 Nov. 2010	JQ430710	3
	JM6–10	El Limón, Morelos		1200	29 June 2007	JQ430733	5
	NW162–7	Cerro Frio, Morelos		1600	13 July 2007	JQ430731	5
	MAL–02877	Tulum Pueblo, QR	20.146 N, 87.575 W		7 Feb. 1993	JN201293	7
J. e. nigrosuffusa (Mexico)	CIAD 10–B24	San Carlos, Sonora	27.997 N, 111.047 W	70	1 Oct. 2010	JQ430711	3
(N = 10)	CIAD 10–B25	"	"	"	8 Oct. 2010	JQ430712	3
	CIAD 10–B26	"	"	"	11 Oct. 2010	JQ430713	3
	CIAD 10–B30	"	27.983 N, 111.043 W	42	1 Nov. 2010	JQ430714	3
	CIAD 10–B31	"	"	"	"	JQ430715	3
	CIAD 10–B32	"	"	"	"	JQ430716	3
	CIAD 10–B33	"	"	"	"	JQ430717	3
	CIAD 10–B34	"	27.997 N, 111.047 W	70	2 Nov. 2010	JQ430718	3
	CIAD 10–B35	"	27.968 N, 111.045 W	30	5 Nov. 2010	JQ430719	3
	CIAD 10–B36	"	"	"	"	JQ430720	3
J. evarete (Costa Rica)	03–SRNP–28069	ACG, Guanacaste	11.018 N, 85.450 W	410	9 Sept. 2003	GU334034	4
(N = 22)	03–SRNP–28076	"	"	"	5 Sept. 2003	GU334037	4
	04–SRNP–15110	"	10.838 N, 85.619 W	295	31 Oct. 2004	GU157294	4
	05–SRNP–31184	"	11.019 N, 85.410 W	440	23 April 2005	GU157298	4
	05–SRNP–31186	"	"	"	13 April 2005	GU157295	4
	05–SRNP–31187	"	"	"	26 April 2005	GU157296	4
	05–SRNP–31188	"	"	"	27 April 2005	GU157297	4

Table 1. (continued)

J. evarete (Costa Rica) (continued)	05–SRNP–31215	"	"	"	7 April 2005	GU157299	4
	05–SRNP–57995	"	10.767 N, 85.433 W	325	24 July 2005	GU157288	4
	05–SRNP–57996	"	"	"	"	GU157285	4
	05–SRNP–57998	"	"	"	22 July 2005	GU157287	4
	05–SRNP–57999	"	"	"	23 July 2005	GU157286	4
	05–SRNP–58001	"	"	"	"	GU157290	4
	05–SRNP–58009	"	"	"	"	GU157291	4
	05–SRNP–58011	"	"	"	22 July 2005	GU157280	4
	05–SRNP–58208	"	10.768 N, 85.426 W	480	28 July 2005	GU157292	4
	05–SRNP–58220	"	"	"	6 Aug. 2005	GU157284	4
	05–SRNP–58221	"	"	"	29 July 2005	GU157300	4
	05–SRNP–58241	"	"	"	"	GU157283	4
	05–SRNP–58257	"	"	"	28 July 2005	GU157282	4
	05–SRNP–58293	"	"	"	"	GU157289	4
	05–SRNP–32756	"	11.019 N, 85.410 W	440	16 Aug. 2005	GU157281	4
J. evarete (Panama)	YB-BCI12765	Barro Colorado Is.	9.155 N, 79.848 W	150	24 May 2009	HM416470	6
J. evarete (French Guiana)	UK4–15	Montsinéry				JQ430730	5
Junonia sp. (Fr. Guiana)	UK4–16	Macouria				JQ430722	5
J. genoveva (Fr. Guiana)	UK4–14	Kaw-Roura				JQ430728	5
J. genoveva (Martinique)	NW136–16	Les Salines, Sainte-Anne			15 Feb. 2004	EU053300	1
J. genoveva (Guadeloupe)	UK4–18	Port-Louis				JQ430727	5
(*N* = 2)	UK4–19	Port-Louis				JQ430726	5
J. genoveva? (Brazil)	NW155–2	Ubatuba, São Paulo			21 May 2005	JQ430729	5
Junonia sp. (Dominican	NW153–11	Pedernales			8 Nov. 2006	JQ430725	5
(Republic) (*N* = 3)	NW153–12	Pedernales			"	JQ430724	5
	NW153–14	Hatillo, Azua			9 Nov. 2006	JQ430723	5
J. vestina (Peru)	NN07	near Laguna Huacracocha		4000	26 Oct. 2009	JQ430721	5

[a]References: 1, Kodandaramaiah and Wahlberg (2007); 2, Hebert et al. (2010); 3, this study; 4, Janzen and Hallwachs (2009); 5, Nymphalidae Systematics Group (2009); 6, Ratnasingham and Hebert (2007); Barcode of Life Data System (http://www.barcodinglife.org); 7, Prado et al. (2011).

Molecular protocol and data analysis. Total genomic DNA was extracted from two legs of each butterfly using the DNeasy™ (QIAGEN Inc., Valencia, CA) protocol. The polymerase chain reaction (PCR) was used to amplify the barcode segment of the COI gene with primers LCO1490f and HCO2198r using standard PCR conditions (Folmer et al. 1994). Sequencing reactions were performed on an Applied Biosystems (Foster City, CA) ABI 3730XL DNA sequencer at the DNA Sequencing Facility, University of Arizona, Tucson using the amplifying primers. Sequences were proofread and aligned in ClustalX 1.81 (Thompson et al. 1997) followed by manual editing.

Calculations of Kimura (1980) 2–parameter (K2P) genetic distances (*d*) among sequences were carried out in MEGA version 4.0 (Tamura et al. 2007). Calculations of genetic diversity indices and Tajima's (1989) *D* were performed in DnaSP version 5.00.04 (Librado & Rozas 2009). Relative rate tests (Tajima 1993) of sequence evolution were carried out in MEGA using *J. orithya* as the outgroup. Analysis of molecular variance (AMOVA, Excoffier et al. 1992) performed in ARLEQUIN version 3.5.1.3 (Excoffier & Lischer 2010) was used to test for structure among selected populations of *Junonia.* The calculation of significance ($\alpha = 0.05$) of the fixation index Φ_{ST} was based on 10,000 permutations of the data matrix. Estimates of the number of migrants per generation (N_m) among populations were also calculated in ARLEQUIN.

Phylogenetic analyses. For phylogenetic analyses all COI sequences were trimmed to 633 bp to correspond to the barcode region reported in Kodandaramaiah and Wahlberg (2007). Relationships among haplotypes were assessed with the neighbor-joining (NJ) algorithm of Saitou and Nei (1987) carried out in MEGA using a matrix of K2P distances. We used two African species of *Junonia* as outgroups, *J. orithya* (GenBank EU053315) and *J. westermanni* (GenBank EU053319). Both African species show a close relationship with the New World *Junonia* (Kodandaramaiah & Wahlberg 2007). *Junonia orithya*, in particular, shares similarities in both wing pattern and morphology of male genitalia with New World *Junonia* (Corbet 1948; Tilden 1971). Statistical support for nodes was obtained by bootstrap analyses using 1000 pseudoreplicates (Felsenstein 1985). Confirmation of clades identified from NJ analysis was obtained by constructing phylogenetic trees with (a) Bayesian inference implemented in MrBayes version 3.1 (Huelsenbeck & Ronquist 2001), sampling 4000 trees and using both HKY and GTR nucleotide substitution models, and (b) maximum parsimony (MP) carried out in MEGA using the CNI heuristic search option and 100 random additions of sequences. Clade support for Bayesian trees was estimated utilizing a Markov chain Monte Carlo (MCMC) algorithm and expressed as posterior probabilities; relative support for MP tree topology was obtained by bootstrapping using 500 pseudoreplicates.

RESULTS

Sequence data and genetic diversity. A complete barcode segment (658 bp) was available for 68 of the 85 individuals of *Junonia* shown in Table 1. No stop codons or indels were found in any of the sequences. There were 53 variable sites. Nucleotide composition was nearly identical in the 68 sequences (mean values: 38.7% T, 14.6% C, 31.4% A and 15.2% G). There was a strong bias against G at the third codon position (mean G content 1.4%; range 0.5–2.7%). Inspection of the 658 bp segment in the 68 samples revealed that of the 53 variable sites, none were present in the first 25 bases that were deleted for phylogenetic analyses.

Genetic diversity indices for *Junonia* are shown in Table 2. Two different patterns were observed. Haplotype diversity (h) and nucleotide diversity (π) were relatively high ($h \geq 0.900$; $\pi > 0.003$) in *J. evarete* from Costa Rica and South America (including the Caribbean) and in *J. coenia coenia* from the USA, but were lower ($h < 0.700$; $\pi < 0.002$) in *J. evarete* and *J. evarete nigrosuffusa* from Sonora, Mexico and in *J. coenia grisea* from southern California, USA. The differences in h and π seen in *J. evarete* from Sonora, Mexico and Costa Rica are notable given that sample sizes from the two localities were similar. Tajima's *D* was not significant in any of the taxa. None of the relative rates tests (Tajima 1993) were significant, indicating that a molecular clock could not be rejected for *Junonia.* The AMOVA revealed significant structure among populations of *J. evarete* from Costa Rica ($N = 22$) and Estero del Soldado, Mexico ($N = 19$) ($\Phi_{ST} = 0.398$; $P < 0.0001$). The estimated number of individuals migrating between the two regions per generation (N_m) was 0.756. The AMOVA also showed significant structure between the subspecies *J. coenia coenia* ($N = 6$) from eastern USA and *J. coenia grisea* ($N = 7$) from California ($\Phi_{ST} = 0.787$; $P < 0.001$; $N_m = 0.135$).

Phylogenetic relationships. Preliminary phylogenetic analyses of the three New World taxa (*J. evarete*, *J. genoveva* and *J. coenia coenia*) from Kodandaramaiah and Wahlberg (2007), using only the 633 bp COI barcode segment and *J. orithya* and *J. westermanni* as outgroups, resolved the *J. evarete* and (*J. coenia* + *J. genoveva*) clades (referred to below as clades A and B, respectively) in NJ, MP and Bayesian trees (not shown) with similar (MP) or identical (Bayesian) clade support

values reported by those workers from the combined mitochondrial and nuclear data set of 3090 bp.

The NJ tree of New World *Junonia* based on barcodes, and representing both new and previously published data, is shown in Fig. 2. The NJ tree again resolved clades A and B with high statistical support. In addition to the single *J. genoveva* and two *J. coenia coenia* from Kodandaramaiah and Wahlberg (2007), all sequences of *Junonia* from the USA, Mexico, Costa Rica and Panama clustered in clade B, including those from taxa currently assigned to *J. evarete* and *J. evarete nigrosuffusa*. A short COI sequence (290 bp) assigned to *J. evarete* from Quintana Roo, Mexico (Prado et al. 2011) also clustered in clade B (not shown). Within clade B, a weakly-supported subclade consisting of *J. coenia grisea* from southern California was found. All other populations within clade B were unresolved. The same topology, with similar support values, was obtained on a representative subset of sequences from all taxa using MP and Bayesian analyses (not shown).

All populations of *Junonia* from South America and the Caribbean, with the exception of a single *J. genoveva* (= *J. neildi* Brévignon) from Martinique (NW136–16), clustered in Clade A, including individuals identified as *J. evarete* and *J. genoveva*. The resolution of *J. vestina* in a basal position in clade A (referred to here as clade A_1) was highly supported. The remaining clade A individuals were all closely related (see below) and are grouped into clade A_2.

Pairwise comparisons of K2P genetic distances (d) among New World *Junonia* are shown in Table 3. Mean genetic distances were low ($d \leq 1.1\%$) for all comparisons between taxa within clade B. Genetic distance between the subspecies *J. coenia coenia* and *J. coenia grisea* was $d = 1.0\%$. Mean values in all pairwise comparisons between clades A and B, including comparisons with individuals assigned to *J. evarete* which appear in both clades, ranged from d = 4.0–4.5%. These values are higher than the genetic distances found between the two species from Africa used as outgroups, *J. orithya* and *J. westermanni* (d = 3.4%; not shown in Table 3), and also are higher than the value found between *J. orithya* and clade A_2 (d = 3.9%); the values are slightly lower than d = 5.0 % found between *J. orithya* and clade B. Within clade A, the genetic distance between the distinctive *J. vestina* (clade A_1) and *J. evarete* (clade A_2) was d = 2.1 %. The mean value between *J. evarete* from Costa Rica and Sonora, Mexico, localities separated by ~3250 km, was d = 0.5%. Within population d values for *J. evarete* were 0.0–0.5% (mean d = 0.1%) for Sonora and 0.0–1.4% (mean d = 0.5%) for Costa Rica. One individual of *J. evarete* from Sonora shared the same haplotype with an individual from Costa Rica (see Fig. 3).

DISCUSSION

We have shown that phylogenetic analysis of a 633 bp segment of the mitochondrial COI gene, comprising most of the barcode segment, resolves the two main clades of New World *Junonia* reported previously using a larger data set of both mitochondrial and nuclear genes (Kodandaramaiah & Wahlberg 2007). Barcodes thus provide an informative and relatively inexpensive tool for phylogenetic studies of this group. Assigning individuals of the *J. evarete* complex to their respective clade using morphological characters alone is unreliable and has probably contributed much to the taxonomic

Table 2. Summary of genetic diversity indices and results of neutrality tests (Tajima's D) in theCOI gene segment in *Junonia*.

Species	N	L	k	K	h (± SD)	π (± SD)	Tajima's D
J. evarete (clade A_2)*	12	633	20	10	0.970 ± 0.044	0.00824 ± 0.00105	–0.97
J. evarete (Costa Rica)	22	658	18	14	0.926 ± 0.039	0.00455 ± 0.00075	–1.45
J. evarete (Sonora, Mexico)	19	658	3	5	0.696 ± 0.077	0.00133 ± 0.00023	0.06
J. e. nigrosuffusa (Mexico)	9	658	3	3	0.639 ± 0.126	0.00186 ± 0.00042	0.41
J. coenia coenia (USA)	5	633	4	4	0.900 ± 0.161	0.00253 ± 0.00076	–1.09
J. coenia grisea (USA)	7	658	2	3	0.667 ± 0.160	0.00116 ± 0.00035	–0.27

N, number of sequences; L = sequence length (number of bases); k = number of variable sites; K, number of haplotypes; h, haplotype diversity; π, nucleotide diversity. Values for Tajima's D were not significant for any species at the 0.05 level. *All species from South America and the Caribbean clustering in clade A_2, including those originally assigned to *J. evarete*, *J. genoveva* or *Junonia* sp. (see Fig. 2), were combined under *J. evarete* in Tables 2 and 3. Five shorter sequences (≤600 bp) from *J. evarete* (clade A_2) (NW36–2, UK4–15, UK4–18), *J. e. nigrosuffusa* (CIAD 10–B25) and *J. coenia coenia* (NW38–18) were omitted from Tables 2 and 3.

TABLE 3. Mean K2P genetic distances (*d*) among taxa and geographic populations of New World *Junonia* based on the COI gene (633 bp). Values for *d* within taxa are shown along the diagonal. Shaded area shows taxa included in clade A, *J. vestina* (clade A_1) and *J. evarete* (clade A_2) from South America and the Caribbean (see footnote to Table 2). The remaining taxa all cluster in clade B from North America and the Caribbean.

		1	2	3	4	5	6	7	8
1	*J. coenia coenia* (USA) (*N* = 5)	0.003							
2	*J. coenia grisea* (USA) (*N* = 7)	0.010	0.001						
3	*J. e. nigrosuffusa* (MX) (*N* = 9)	0.003	0.010	0.002					
4	*J. genoveva* (Martinique) (*N = 1*)	0.004	0.009	0.003	-----				
5	*J. evarete* (Sonora, MX) (*N* = 19)	0.004	0.008	0.003	0.003	0.001			
6	*J. evarete* (Costa Rica) (*N = 22*)	0.007	0.011	0.006	0.006	0.005	0.005		
7	*J. evarete* (S. Amer.) (*N* = 12)	0.043	0.043	0.042	0.045	0.044	0.042	0.008	
8	*J. vestina* (Peru) (*N* = 1)	0.040	0.044	0.040	0.043	0.041	0.042	0.021	-----

confusion. Because of evidence for relatively recent divergences in the New World *Junonia*, however, barcodes alone may be of limited usefulness for inferring intra-clade relationships and species identifications, especially within clade B. All new barcode sequences from populations from western North America, comprising several recognized taxa, clustered in clade B and most showed low genetic divergences ($d < 1\%$). The western *J. coenia grisea*, however, resolved as a weakly-supported subclade within clade B, supporting its designation as a subspecies of *J. coenia* (Austin & Emmel 1998). The AMOVA showed significant population structure among *J. coenia grisea* and *J. coenia coenia*, also consistent with subspecies status. Additionally, our analyses revealed that none of the North American *Junonia* from Mexico and Central America currently recognized as *J. evarete*, including *J. evarete nigrosuffusa* from Mexico, clustered with *J. evarete* from South America and the Caribbean (clade A_2). These results suggest that either the taxon currently recognized as *J. evarete* is paraphyletic, or taxonomic assignments of the western populations need to be reconsidered (see below).

Assuming a molecular clock rate of ~2% pairwise sequence divergence per million years for insect COI (Brower 1994; Craft et al. 2010; Pfeiler et al. 2010) we estimate that clades A and B began to diverge ~2.2 Ma. Based on fossil evidence, *Junonia* is thought to have colonized the New World about 2–4 Ma (Kodandaramaiah & Wahlberg 2007). Mean genetic distances between clade A and the outgroup taxa from Africa were 3.9 and 4.3% for *J. orithya* and *J. westermanni*, respectively, suggesting the ancestor of the clade A lineage began to diverge from the African taxa ~2 Ma. Thus, molecular clock considerations and fossil evidence provide estimated dates which are in relatively close agreement, implying that clades A and B began to diverge shortly after colonization of the New World. Because we found no evidence that nucleotide substitution rate in the COI gene in *Junonia* is different from that typically seen in many insects, the low genetic divergences within clade B likely indicate a relatively recent (late Pleistocene or Holocene) radiation and speciation within this group. The low genetic divergences also could result from incomplete lineage sorting and extensive hybridization among diversifying taxa, possibly suggesting just a single, polytypic species. There is evidence, however, apart from the pronounced intra-clade phenotypic variability (Fig. 3), to support recognizing distinct species level taxa within clade B that barcodes are unable to detect.

The low genetic divergences and presumed recent speciation among recognized taxa of *Junonia* comprising clade B are consistent with the conclusions of laboratory hybridization studies showing a high degree of genetic similarity among North and Central American *Junonia* (Hafernik 1982). The taxa used in the hybridization experiments and phenetic analyses of Hafernik (1982) included *J. coenia* (populations from both Texas and California representing what are now recognized as subspecies *J. coenia coenia* and *J. coenia grisea*, respectively), *J. evarete nigrosuffusa* (southern Texas and southeastern Arizona; treated as a full species by Hafernik) and *J. evarete zonalis* (southern Guatemala and northwestern Costa Rica). Caribbean populations, including *J. genoveva*, were excluded from the study [*J. genoveva* is currently listed for southern Texas (Opler et al. 2011; Warren et al. 2011)]. Several lines of circumstantial evidence, however, suggest that the reference populations of *Junonia* from Central America used by Hafernik (1982) may have been from

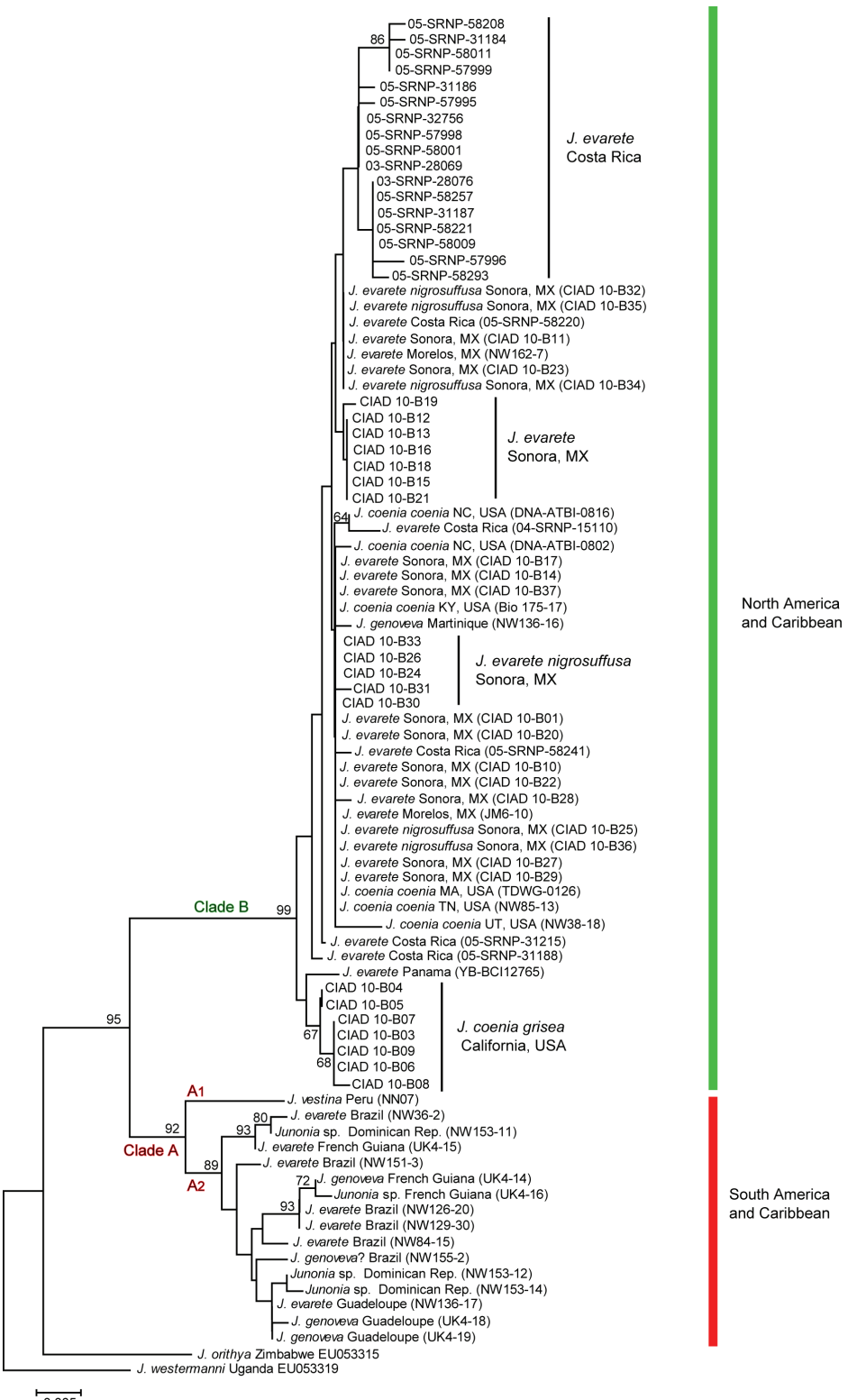

FIG. 2. Neighbor-joining (NJ) tree showing relationships among New World *Junonia* based on COI barcode sequences. Voucher codes are listed for each of the ingroup species (see Table 1 for details). GenBank accession numbers are shown for the outgroups, *J. orithya* and *J. westermanni* from Africa. Red and green bars represent the two main clades (A and B, respectively) of New World *Junonia*. Clade A_1 is comprised of *J. vestina*; clades A_2 and B contain the members of the *J. evarete* complex. Bootstrap support values are shown on branches; values <60% were omitted. Scale bar indicates sequence divergence.

the clade B lineage, most probably from the taxon *J. genoveva*. Specimens from these reference populations were taken at Escuintla, Guatemala and Cañas, Costa Rica, both from the Pacific slope and ~700 and ~75 km, respectively, from the Area de Conservación Guanacaste (ACG). Although multiple species of *Junonia* occur in certain regions, no COI genotypes similar to those found in South American and Caribbean populations of *J. evarete* have thus far been detected in the 45 barcode sequences obtained for *Junonia* from the ACG (D.H. Janzen & M. Hajibabaei, unpublished). *Junonia evarete* genotypes also were not present in the two samples from Morelos, Mexico, or in the samples from Quintana Roo, Mexico (Prado et al. 2011) and Panama (Fig. 1). Because of the genetic similarities and lack of reproductive isolation, Hafernik (1982) concluded that *J. evarete nigrosuffusa* and *J. evarete zonalis* represented a cline from Central America to southern Texas and should be considered conspecific. We have shown, however, that *J. evarete nigrosuffusa* from Mexico and *J. evarete* from South America and the Caribbean show a mean genetic divergence (d = 4.2%; Table 3) well within the range of values seen for species level taxa in Lepidoptera based on barcodes (Hajibabaei et al. 2006; Hebert et al. 2010). Finding high genetic identity in hybridization studies between individuals of clades A_2 and B would not be expected in two distinct taxa with relatively high genetic divergences. For example, in Jamaica where *J. evarete* and *J. genoveva* both occur, no evidence was found for natural hybridization among the two taxa (Turner & Parnell 1985). However, the conclusions of Hafernik (1982) are consistent with our findings if the Central America taxon used in that study was from the *J. genoveva* lineage and not a subspecies of *J. evarete*. Our argument assumes that *J. evarete* was correctly identified in the earlier molecular study of Kodandaramaiah and Wahlberg (2007). Photographs of *J. evarete* studied by those authors (see Fig. 1) match closely the phenotype of the recently assigned neotype of *J. e. evarete* from Suriname, South America (Neild 2008), suggesting that the identification was correct.

Based on the above arguments we propose that the population of *Junonia* from Mexico that utilizes black mangrove (*Avicennia germinans*) as a larval host, as well as the specimens shown in Table 1 from Morelos

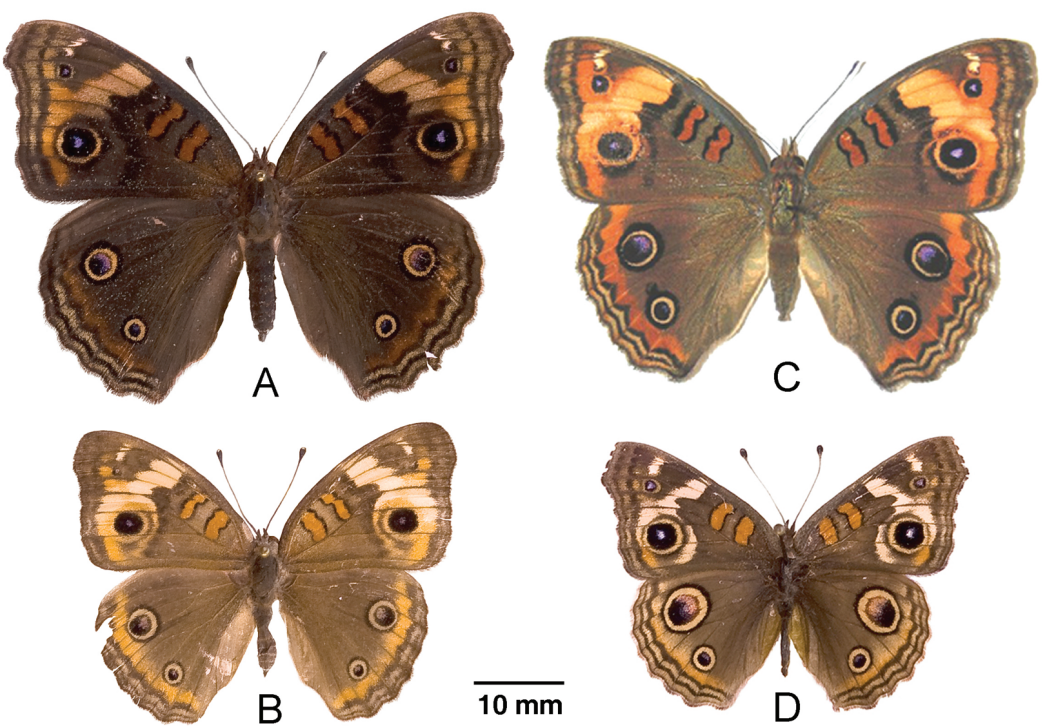

FIG. 3. Comparison of adult females of *Junonia* from western North America showing phenotypic variability. **(A)** *J. evarete nigrosuffusa* (= *J. nigrosuffusa*; see Discussion) (San Carlos, Sonora, Mexico; CIAD 10–B32); **(B)** *J. evarete* (= *J. genoveva*) (Estero del Soldado, near San Carlos, Sonora, Mexico; CIAD 10–B11); **(C)** *J. evarete* (= *J. genoveva*) (Area de Conservación Guanacaste, Guanacaste Province, Costa Rica; 05–SRNP–58220); (D) *J. coenia grisea* (Santa Barbara, California, USA; CIAD 10–B04). Haplotypes for COI were identical for specimens A, B and C; specimen D differs by 5 nucleotide substitutions. Specimens A, B and D are wild-caught; specimen C was reared. Specimen B from Estero del Soldado is a worn individual; ground color of recently eclosed specimens is deep brown (Pfeiler 2011). Scientific names in parentheses are suggested changes in assignment based on data presented here. Photograph credits: **(A), (B)** and **(D),** Wain Evans; **(C),** Janzen and Hallwachs (2009).

(JM6–10 and NW162–7) and Quintana Roo, Mexico (MAL–02877), Panama (YB-BCI12765), and the population from Costa Rica that utilizes *Dyschoriste valeriana* and *Stachytarpheta jamaicensis*, be removed from *J. evarete* and provisionally reassigned to *J. genoveva*. These new assignments agree with an earlier observation that a possible subspecies of *J. genoveva* occurs in coastal regions of western Mexico (Vargas et al. 1996). Ongoing research on *Junonia* from the Caribbean, however, suggests that the mangrove buckeye probably consists of more than one species, including the recently-named *J. litoralis* Brévignon and *J. neildi* Brévignon (Brévignon 2009). In addition, the clade A_2 individual from São Paulo Brazil (Fig. 1, locality 20), was reared on *Avicennia* sp. indicating that representatives of both clades A and B have adapted to feeding on black mangrove. A more thorough examination of relationships among taxa of *Junonia* in the Americas that utilize black mangrove and other host plants may ultimately require revision of our provisional assignment.

Although significant structure was found between the populations of *Junonia* from Estero del Soldado, Mexico and Costa Rica, the low mean genetic distance between the two populations (d = 0.5%) agrees well with intraspecific divergences in Lepidoptera based on barcodes (Hajibabaei et al. 2006; Hebert et al. 2010). Phenotypic differences of adults, however, together with the different host plants utilized by larvae, suggest that these two populations may warrant recognition as distinct subspecies. Also, the higher haplotype and nucleotide diversities of the Costa Rica population compared with the Sonora population (Table 2) suggest that dispersal and colonization proceeded from a southern source population northward along the Pacific slope of North America (Pfeiler et al. 2012). Haplotype and nucleotide diversities were also relatively low in *J. evarete nigrosuffusa* and *J. coenia grisea* (Table 2), but low sample sizes did not allow for unambiguous interpretations of demographic patterns.

The barcode data also suggest that *J. evarete nigrosuffusa* be removed as a subspecies of the *J. evarete* lineage, as it clearly nests within clade B rather than clade A_2 (Fig. 2). Two possible alternative assignments, previously proposed by others, are consistent with the genetic data. These include recognizing *nigrosuffusa* as a subspecies of *J. genoveva* (Vargas et al. 1996; Warren et al. 1998; Glassberg 2001), or as a subspecies of *J. coenia* [as originally described by Barnes and McDunnough (1916)]. A third possibility, also previously proposed but supported only by morphological and ecological data, is to recognize the taxon as a full species (Tilden 1971; Emmel & Emmel 1973; Miller & Brown 1981; Bailowitz & Brock 1991; Brown et al. 1992; Brown 2004). In northwestern Mexico, *J. genoveva* and *J. nigrosuffusa* are generally ecologically isolated and morphologically distinct (Fig. 3), with larvae of the two species utilizing different host plants (Tilden 1971; Hafernik 1982; Bailowitz & Brock 1991; Brown et al. 1992; Vargas et al. 1996; Warren et al. 1998; Pfeiler 2011). Our field observations in the San Carlos region of Sonora have revealed no evidence for hybridization, although adults of both lineages are occasionally encountered feeding together (Pfeiler 2011). There are reports, however, of intermediates between the coastal *J. genoveva* and *J. nigrosuffusa* in other regions of western Mexico (Vargas et al. 1996), as well as intermediates between *J. coenia* and *J. nigrosuffusa* from southeastern Arizona (K. Hansen pers. com.). We suggest that, at least for northwestern Mexico, *J. nigrosuffusa* and *J. genoveva* meet the two basic criteria consistent with ecological speciation, i.e. evidence for ecologically-based divergent selection and assortative mating (Chamberlain et al. 2009). Strong adult dispersal capability (Adler & Dudley 1994), together with the ability of larvae to adapt to a variety of host plants from different families, are traits that would favor survival and potentially lead to ecological speciation during the radiation of the New World *Junonia*.

In summary, we have shown that COI barcodes can distinguish *J. vestina* from members of the *J. evarete* complex, and can resolve the two subspecies of *J. coenia*, but overall are of limited usefulness in species identifications within the complex itself. Nonetheless, barcodes are a valuable tool in taxonomic studies of this group for their ability to easily identify the two major clades of the *J. evarete* complex found in the New World, which is difficult, if not impossible, by morphological analysis alone. The ability to unambiguously identify clades A and B will contribute to our understanding of the degree of phenotypic variability and larval host plant preferences within each lineage. More extensive sampling will be required to determine the complete distribution of the two clades in the New World [e.g., records of *J. evarete zonalis* in southern Florida (Warren et al. 2011) suggest the presence of clade A in the USA, and clade B probably occurs South America], but given the widely separated geographic localities in the Americas sampled to date (Fig. 1), it seems unlikely that barcodes will demonstrate additional deep divergences within the *J. evarete* complex. Other molecular markers, however, such as amplified fragment length polymorphisms (AFLPs), show promise of being able to reveal recent divergences that barcodes fail to detect (Dasmahapatra et al. 2010).

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