Geographical Populations and "Subspecies" of New World Monarch Butterflies (Nymphalidae) Share a Recent Origin and Are Not Phylogenetically Distinct

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ABSTRACT To test prior results with a more sensitive technique and larger sample sizes, we assessed genetic diversity among far-flung monarch butterfly, *Danaus plexippus* (L.) (Lepidoptera: Nymphalidae), populations from North and South America by using mitochondrial DNA sequences. Our new data corroborate the previously documented close genetic similarity among individuals and reveal no phylogenetic structure among populations throughout the species' New World range in North and South America. Despite this intraspecific homogeneity, the monarch is clearly distinct from its sister taxon *Danaus erippus* (Cramer). The evidence suggests that the monarch has colonized its current distribution in relatively recent evolutionary time. Implications for conservation and regulatory policy over interregional transfer are discussed.

KEY WORDS mtDNA, *Danaus plexippus*, Nymphalidae, biogeography, genetic diversity

THE MONARCH BUTTERFLY, *Danaus plexippus* (L.), is perhaps the world's most charismatic butterfly species, famed for its milkweed-derived chemical defense, its involvement in mimicry, and most notably its spectacular annual migratory cycle and overwintering aggregations of the North American populations on the California coast and in montane central Mexico. The distribution of the monarch ranges from southern Canada to central South America and throughout the Antilles. Central American, South American, and Antillean populations are referred to the subspecies *D. plexippus megalippe* Hübner, which is morphologically distinct from *D. plexippus plexippus* and does not exhibit migratory behavior (Ackery and Vane-Wright 1984, Smith et al. 1994). The monarch has also colonized Australia, Hawaii, the Philippines, and several other Pacific islands, presumably after the dispersal of milkweed host plants (*Asclepias*) in historical time (Vane-Wright 1993). Disregarding recent human-assisted dispersal, the subgenus *Danaus* is endemic to the New World and contains two other species, the Hispaniolan *Danaus cleophile* (Godart) and the monarch's sister species *Danaus erippus* (Cramer). *D. erippus* replaces the monarch in south temperate South America and is reported to exhibit migratory behavior, but to a less spectacular degree (Ackery and Vane-Wright 1984, Kitching et al. 1993). The monarch's migratory abilities may have evolved as a strategy to allow exploitation of larval food plant resources in temperate North America during the summer (Brower 1977).

As noted, migratory monarch populations overwinter in two separate regions of North America, and there has been considerable discussion in the literature as to whether there has been mixing of populations east and west of the Rocky Mountains (reviewed in Brower 1995). This hypothesis has been tested by mark–release–recapture studies, which show that butterflies from the Great Plains eastward tend to migrate toward Mexico, whereas butterflies from Washington, Oregon, and California tend to migrate to the California coast (Urquhart and Urquhart 1977). Direct evidence for contemporary inter-regional dispersal is scant, stemming from a few suggestive mark–release–recapture vectors (Monarch Watch) and anecdotal accounts such as Pyle (1999). Brower and Boyce (1991) examined population structure between eastern and western monarch populations by using mtDNA restriction fragment-length polymorphism data. They found remarkably low levels of inferred nucleotide divergence both within and between the two populations, with most individuals sharing a single common haplotype, resulting in an estimated nucleotide diversity of 0.00016. Shephard et al. (2002) conducted a phenetic study of allozyme frequencies among North American and Australian monarch populations. They, too, found little geographical structure within North America and extensive shared polymorphism between eastern and western samples. Of course, patterns of shared allozyme polymorphism are not necessarily reliable indicators of population history (Turner et al. 1979, Barbadilla et al. 1996).

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Table 1. Samples included in this study (single specimens per locality except as noted)

Identity	Code(s)	Locality	Voucher
D. plexippus (eastern)	Mex1-Mex10	Michoacan, Mexico	OSAC
D. plexippus (western)	Cal1-Cal20	Santa Cruz, CA	OSAC
D. plexippus (nonmigrant)	C-3-8	Villavicencio, Colombia	AMNH
	E-4-2b	Tinalandia, Ecuador	AMNH
	PE-10-1	Cuzco, Peru	AMNH
	PE-15-1	Apurimac, Peru	AMNH
	PE-17-1	Lima, Peru	AMNH
	PE-19-8	Tingo Maria, Peru	AMNH
	TT1	Tobago, West Indies	OSAC
	TT2	Trinidad, West Indies	OSAC
D. erippus	DE1	Buenos Aires, Argentina	CUIC
Tirumala hamata	NSW1	Brisbane, Australia	AMNH

OSAC, Oregon State Arthropod Collection; AMNH, American Museum of Natural History; CUIC, Cornell University Insect Collection.

Here, we expand upon prior results by conducting an explicit character-based test of phylogenetic distinctness of monarch populations, based on mtDNA sequences from the samples analyzed by Brower and Boyce (1991), including additional representatives from the original populations studied, as well as Neotropical monarchs and *D. erippus.* The current results are the first to explore the biogeographical origins of the monarch in a cladistic context, and they strengthen the hypothesis that all New World monarch populations are closely related and recently derived from a common ancestor.

Materials and Methods

Individual butterflies were collected in the field (see Table 1 for locality data) and transported to the laboratory either frozen or in vials of 100% ethanol. Genomic DNA was isolated from individual butterfly thorax or thorax + abdomen following a standard phenol-chloroform extraction procedure (Brower 1994a). DNA was resuspended in purified water and stored at −20°C. A region of the mtDNA COI, tRNA leu, and COII genes corresponding to positions 2,217–3,772 in the *Drosophila yakuba* Burla mitochondrial genome (Clary and Wolstenholme 1985) was amplified via direct polymerase chain reaction (PCR) of genomic templates. The PCR products were cleaned by silica bead precipitation (Bio 101, Vista, CA) and sequenced using flanking and internal primers (Table 2) with an ABI 377 (Applied Biosystems, Foster City, CA).

Sequences were read from electropherograms and edited and aligned directly in PAUP* (Swofford 2002), which was used for subsequent data analyses. Sequences were generated in both directions for partial but not complete overlap of the entire region (some sequences were not complete, but excluding them did not change the resolution of the analysis). A sequence from *Tirumala hamata* (Macleay), a representative of the sister genus to *Danaus* (Ackery and Vane-Wright 1984), was included as the outgroup. Base proportions were calculated using MacClade 3.07 (Maddison and Maddison 1999). Distance matrices were calculated based on uncorrected pairwise comparisons between individuals. Cladistic analysis was conducted on a 1.0-Ghz G4 Macintosh computer, with heuristic searches with characters weighted equally, and character states unordered. Successive approximations weighting (SAW; Farris 1969) based on maximum rescaled consistency indices of characters was applied to the results of the parsimony analysis. Branch support for the *plexippus-erippus* node was calculated by anticonstraints (Bremer 1994). The hypothesis of historical distinctness among geographical populations of monarchs from eastern and western North America and South America was tested using cladistic haplotype analysis (CHA; Brower 1999). Under the CHA test criterion, a single falsifying instance results in rejection of a hypothesis of historical distinctness, so the limited samples from South America are sufficient to provide a critical test in this case.

Unique sequences are deposited with GenBank (accession no. AY569130–AY569159), and the aligned data matrix is available at http://www.science.oregonstate.edu/systematics/browera/research/.

Results

Sequences were generated for 38 monarchs representing eastern and western North American and widespread South American populations, one *D. erippus*, and one *T. hamata.* Among the 38 monarchs, 11

Table 2. PCR and sequencing primers used in this study

Name	Strand	3′ Position[a]	Sequence (5′-3′)
RUDY	S	2217 (COI)	GAA GTT TAT ATT TTA ATT TTA CCG GG
GEORGE I	S	2792 (COI)	ATA CCT CGA CGT TAT TCA GA
GEORGE III	S	2783 (COI)	TAG GTI TAG CIG GAA TAC CTC G
LIDDY	A	2965 (COI)	CAT TAT AAG AAT GTT CAG CAG G
JESSE	S	2994 (COI)	GAA CAT TCI TAT AAT GAA CTC/T CCT
PAT	A	3014 (tRNAleu)	AAT GCA CTA ATC TGC CAT ATT A
MAMIE	A	3113 (COII)	TGT TCT/C ATT AAT GGA/G GAA GC/TT CTA T
IKE	S	3126 (COII)	ATT AC/TC AAA ATA GAA/G CTT CIC C
PHYLLIS	A	3275 (COII)	GTA ATA GCI GGT AAA/G ATA GTT CA
STROM	S	3291 (CO II)	TAA TTT GAA CTA TC/TT TAC CIG C
BARBARA I	A	3661 (CO II)	CCA CAA ATT TCT GAA CAT TGA CCA
EVA	A	3772 (tRNA lys)	GAG ACC ATT ACT TGC TTT CAG TCA TCT

[a] Primer positions based on *D. yakuba* sequence (Clary and Wolstenholme 1985).

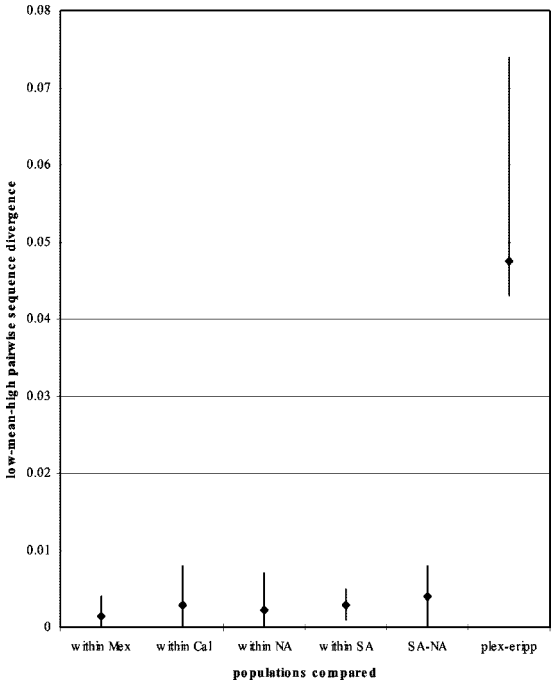

Fig. 1. Uncorrected pairwise sequence divergence of mtDNA among various populations of *D. plexippus* and between *D. plexippus* and *D. erippus.* Dots represent means; whiskers represent high and low values within each sample. No data point is scored more than once; thus, only east-west differences are scored for "within NA."

possessed sequences identical to that of another individual, and an additional seven differed from others only by uninformative autapomorphies. Three mtDNA haplotypes were shared between groups of Californian and Mexican individuals. The degree of sequence divergence among monarchs within and between regions sampled, including North versus South America, ranges from 0 to 0.8%, with an overall intraspecific pairwise mean of 0.29% (Fig. 1). By contrast, the mean divergence between *D. plexippus* and the *D. erippus* individual is 4.75%, a fivefold increase over the maximum *plexippus-plexippus* divergence, suggesting that *D. erippus* is a distinct species and not merely an austral geographical race of *D. plexippus* (Kitching et al. 1993). These degrees of sequence divergence are well below the typical insect mtDNA saturation point of 25–30% (DeSalle et al. 1987, Brower and DeSalle 1998). Average nucleotide composition of the region sequenced is A + T rich, particularly at third codon positions in the coding regions (Table 3),

Table 3. Average nucleotide composition of the sequenced region among all samples

Percent	A	G	C	T
1st Position	35.4	15.0	19.7	29.9
2nd Position	24.2	21.1	13.3	41.4
3rd Position	37.5	4.0	0.5	58.0
Overall	32.3	13.4	11.2	43.1

as is typical of insect mtDNA (Liu and Beckenbach 1992).

Of 1,532 bases in the data matrix, 1,303 are constant among all taxa, 175 are variable but uninformative, and 54 are parsimony-informative. If the matrix is restricted to the *D. plexippus* samples only, the number of uninformative variable sites drops to 65, and the parsimony-informative sites to 16. Due to this lack of informative variation, the cladistic analysis terminated prematurely in numerous analytical runs when the computer ran out of memory after storing 73,800 trees of 194 steps. Elimination of redundant identical sequences did not alleviate this problem. Efforts to obtain greater resolution via SAW were unsuccessful, again due to this lack of informative character variation.

The results of these various terminated searches were consistent in length and in their failure to provide unambiguous resolution to any geographically exclusive clades within *D. plexippus.* Given this consistency, it is plausible to infer that the results reflect the underlying pattern of the data. Figure 2A shows a strict consensus of 73,800 fundamental cladograms of 194 steps. None of the regional populations is distinct under the test criterion of CHA (Brower 1999). Figure 2B shows a phylogram representing one from among the thousands of equally parsimonious fundamental cladograms to illustrate the extreme similarity among the *D. plexippus* sequences (branch lengths correspond to inferred numbers of character state transformations). In this case, the South American individuals seem to represent a clade nested among the North American samples, but this pattern of grouping is contradicted by alternate, equally parsimonious trees from the same analysis. It is not possible to infer a geographical point of origin for *D. plexippus* based on this pattern of intraspecific relationships, but parsimonious optimization based on the distributions of the sister taxa implies a Neotropical origin.

Discussion

These data provide phylogenetic support for the hypothesis that all New World monarch butterflies are extremely closely related. They offer explicit character evidence corroborating the phenetic results of mtDNA restriction fragment-length polymorphism analysis of Brower and Boyce (1991) and the allozyme frequency study of Shephard et al. (2002). The use of sequence data permits several analytical advances over the similarity-based restriction fragment-length polymorphism approach, including assessment of a greater number of characters, better inference of homology, and cladistic analysis of the results (Brower 1999). The expanded geographical and taxonomic sampling included here allows many interpretations that strengthen and refine hypotheses advanced in the earlier work regarding Pleistocene population bottlenecks in *D. plexippus.*

Applying the 1.2% per million years per lineage mtDNA divergence rate hypothesized for closely re-

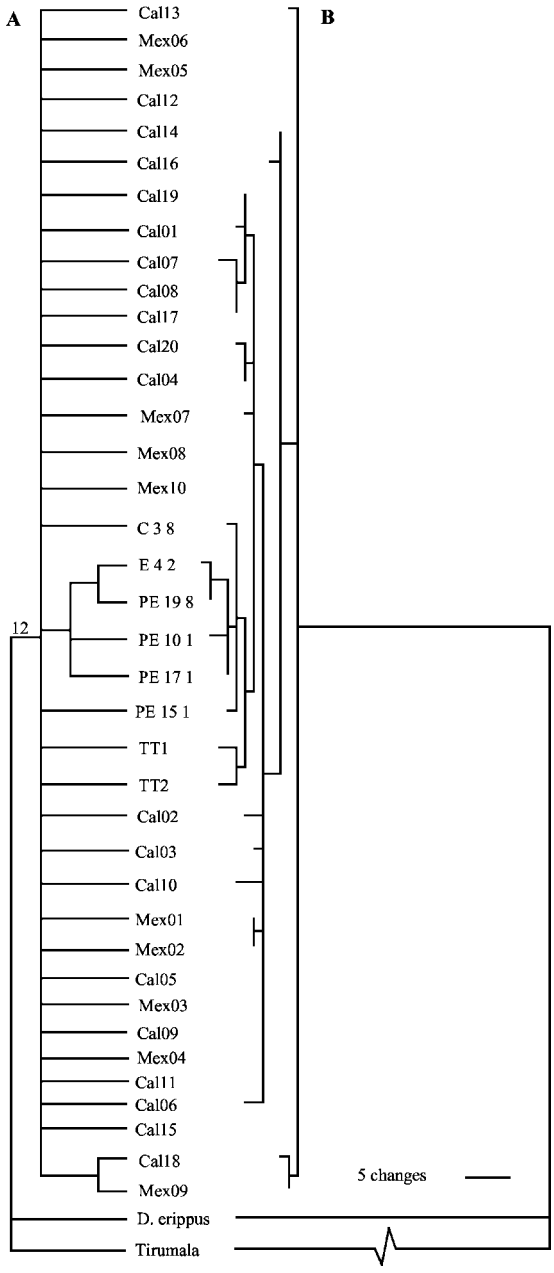

Fig. 2. Cladistic relationships among the individual butterflies studied. (A) Strict consensus of 73,800 cladograms of 194 steps (CI = 0.6604; RI = 0.7805). The branch support value for the node separating the monarchs and *D. erippus* is shown. (B) One of these trees represented as a phylogram with branch lengths proportional to number of steps implied under ACCTRAN, to show the extreme lack of differentiation among *D. plexippus* individuals from across the species' range.

lated insect taxa by Brower (1994b), the separation of *D. erippus* and *D. plexippus* can be estimated to have occurred some 2 million years ago, at the base of the Pleistocene. This degree of divergence and implied age are comparable to that between deep divisions among geographical races of *Heliconius erato* L. and *Heliconius melpomene* L. (Brower 1994b, 1996). Applying the same clock estimate to the maximum pairwise difference observed among the *D. plexippus* populations sampled (0.8%, an order of magnitude less than the *plexippus-erippus* distance) yields a divergence time estimate of ≈300,000 yr, suggesting that monarchs have spread throughout their current North and South American range in relatively recent geological history. Although these dates are only rough point estimates, they highlight the likely recency of the monarch's colonization of North America. This time line is further supported by the presence of shared haplotypes among eastern and western populations, and the lack of cladistic distinctness among any of the populations sampled. The pattern of low mtDNA divergence exhibited by the monarchs is similar to that discovered among populations of *Heliconius charithonia* (L.) ranging from Florida to Ecuador (Davies and Bermingham 2002) and that of *Battus philenor* (L.) from Mexico and the United States (Fordyce and Nice 2003), suggesting that a lack of mtDNA differentiation accompanying post-Pleistocene northward range expansion may be a common pattern among North American butterflies. Comparable patterns have also been reported in several European species (Joyce and Pullin 2001, Aagaard et al. 2002).

Although they seem to be very genetically homogeneous across their range in both mtDNA and allozyme allele frequencies (Eanes and Koehn 1978, Shephard et al. 2002), temperate and tropical monarchs exhibit morphological and behavioral differences, suggesting recent evolutionary responses to differing environmental conditions across different parts of the species' distribution. *D. plexippus megalippe* exhibits shorter wings and generally darker coloration with reduction of orange spots in the apex of the forewing (Smith et al. 1994). In addition, migratory and nonmigratory populations of *D. plexippus plexippus* within North America exhibit different loads of and susceptibilities to the protozoan parasite *Ophryocystis elektroscirrha* (Altizer et al. 2000). Thus, the arguments presented in Brower et al. (1995, 1996) regarding the dangers of transferring monarchs among populations are still relevant, and the U.S. Department of Agriculture's rules (USDA Web page) prohibiting transfer of monarchs across the North American continental divide still seem prudent. Indeed, the apparent genetic homogeneity of monarchs may make them particularly susceptible to pandemic infection by pathogens or parasites. And, as R. M. Pyle (personal communication) stated, "if monarch populations become homogenized by human activity (via either scientific release of marked individuals in reciprocal transfer studies, commercial release at social functions, or by school programs), then the opportunity to study biogeographical patterns among them with more sensitive techniques in the future will be spoiled."

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