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MITOCHONDRIAL DNA VARIATION IN MONARCH BUTTERFLIES

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The natural history and ecology of the monarch butterfly (*Danaus plexippus* L.) have been studied extensively (Ackery and Vane-Wright, 1984). The nearly cosmopolitan monarch is best known for the massive migration undertaken each autumn by North American populations. This event culminates with the butterflies' aggregation into huge overwintering colonies in the Transvolcanic Range of central Mexico and along the coast of California. The monarch is the only temperate representative of an otherwise exclusively tropical subfamily of the Nymphalidae, and although it shares the danaine susceptibility to freezing temperatures in all life stages (Calvert et al., 1983; Anderson and Brower, 1988; Masters et al., 1988), it has been able to exploit the extensive food plant resources of temperate North America through the evolution of this migratory behavior. The selective advantage of seasonal range expansion to utilize large populations of temperate milkweeds (*Asclepias* species) is regarded as the primary driving force of this unique strategy (Brower, 1977; Young, 1982).

The range of the monarch in North America is divided by the Rocky Mountains into eastern and western populations, which retreat to separate refugia each autumn. Monarchs east of the Rocky Mountains migrate to Mexico, while those west of the Rockies migrate to the Pacific coast (Brower, 1985). Marked eastern butterflies released in Idaho were recovered in California, demonstrating environmental, rather than genetic, control over the migratory behavior (Urquhart, 1987). Current gene flow between eastern and western populations has been assumed from the apparent lack of wing-length differentiation and purported range contact in the northeastern Rocky Mountains (Urquhart and Urquhart, 1977; Urquhart, 1987). However, data demonstrating such gene flow have not been published, and the paucity of records of substantial monarch populations in the Great Basin or the northern Rockies suggests that mixing between the eastern and western migratory populations occurs rarely if at all.

Whether or not, and to what degree genetic differentiation may have accumulated between these populations, is particularly relevant to understanding the evolution of the migratory phenomenon, and the monarch's current distribution. The as yet undetermined environmental cues or barriers that guide the butterflies to their respective overwintering grounds provide broad biogeographical separation between the two migrating populations. Is the species genetically differentiated between the overwintering localities? Have the two populations been separate for an evolutionarily significant period of time?

The analysis of intraspecific phylogeny of populations via molecular markers (e.g., mitochondrial DNA sequence variation) is a particularly useful method for elucidating historical and phylogenetic relationships underlying population differentiation (Avise, 1989; Avise et al., 1987). In addition, the study of evolutionary history through molecular data in organisms that are ecologically well understood may yield surprising insights into population genetics, challenging current paradigms and extending the limits of our knowledge of evolution at the molecular level.

We have used 13 restriction endonucleases to examine mitochondrial DNA sequence variation in both eastern and western populations of the monarch, as well as in individuals from morphologically divergent populations in Trinidad and Tobago. Surprisingly, all the monarchs show virtually identical mtDNA restriction fragment patterns. The sole variant mtDNA genotype discovered is present in a single individual in each of the eastern and western population samples.

MATERIALS AND METHODS

Monarch butterflies were collected from four field sites in the U.S., Mexico, and the West Indies (Table 1). Living butterflies (*D. plexippus plexippus*) were transported to the laboratory in glassine envelopes from overwintering colonies at Sierra Chincua and Natural Bridges State Park. Live specimens from Trinidad and Tobago (*D. plexippus megalippe*) were preserved in 70% ethanol in the field, after their wings had been removed. DNA was prepared from individual butterflies after a slightly modified version of the protocol described in Harrison et al. (1987). Living butterflies were frozen and stored in a −80°C freezer. Head, thorax, and abdomen, or head and thorax only were used in all preparations (antennae, wings, and legs were trimmed off and discarded). Live-frozen butterflies were kept on ice between removal from the freezer and grinding, while ethanol-preserved samples were vacuum dried at room temperature for five minutes before grinding. Precipitation of DNA was achieved in some

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TABLE 1. Sampling localities with approximate geographical coordinates and numbers of individuals analyzed.

Site	*N*	Date of collection
Sierra Chincua, Michoacan, Mexico (19°37′ N, 100°18′ W)	12	20 January 1988
Natural Bridges State Park, Santa Cruz, California (36°58′ N, 122°01′ W)	12	12 February 1988
Crown Point, Tobago, West Indies (11°11′ N, 60°46′ W)	1	29 May 1988
Central Trinidad, West Indies (10°18′ N, 61°11′ W)	3	31 May 1988

samples by substitution of one equal volume of isopropanol for the two volumes of ethanol described in Harrison et al. (1987), with no observed detrimental effect on the quality or quantity of the DNA yield.

Thirteen restriction enzymes (New England Biolabs) were used to assess mtDNA fragment patterns following the standard procedures given by Maniatis et al. (1982) and in manufacturer's recommendations. Eleven enzymes (*Ase*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Sca*I, *Ssp*I, and *Xba*I) recognize specific six-base nucleotide sequences. *Bst*NI recognizes the sequence CC(A or T)GG, and *Sau*3AI recognizes the four-base sequence GATC. Digests of individual butterflies were electrophoresed in 0.8% to 2.0% agarose gels and transferred to nylon membranes with 0.4 N NaOH. Purified mtDNA for probe was isolated using the protocol of Harrison et al. (1987). Intact mitochondria were isolated from 25 freshly killed monarch butterfly thoraces by differential centrifugation. These were lysed in 2% SDS, and purified by equilibrium centrifugation on a CsCl-propidium iodide gradient. Purified mtDNA was labeled with ^{32}P by random priming (Feinberg and Vogelstein, 1983). The labeled probe was annealed to the individual mtDNA fragments on the nylon membranes in 7.5% SDS/525 mM $NaPO_4$/1 mM EDTA/1.0% BSA(w:v) at 55°C. Resulting restriction fragment patterns were visualized through autoradiographs.

RESULTS

The mitochondrial DNA of 28 monarch butterflies (12 individuals from each North American collection, 3 from Trinidad, and 1 from Tobago) was analyzed with all 13 restriction enzymes. Table 2 contains the recognition sequences of these enzymes and the resulting fragments from each digest. Cleavage at one site by *Sca*I and lack of cleavage by *Bam*HI were confirmed through double digests with other restriction enzymes. Three enzymes, *Ssp*I, *Dra*I, and *Ase*I, cleaved the molecule so frequently that all digestion products could not be observed. These enzymes recognize sequences composed entirely of adenine and thymine, and thus the high frequency with which they cut indicates that *Danaus plexippus* mtDNA is A+T rich, as has been demonstrated in other insect taxa (Moritz et al., 1987; Harrison, 1989). The mean size of the mtDNA molecule, based on summation of restriction fragments (excluding patterns from the enzymes discussed above) and restriction site map data is 14.8 Kb (SD 0.2 Kb). Thus, the mtDNA of *D. plexippus* appears somewhat smaller than many animal mitochondrial genomes (Moritz et al., 1987), but typical of other nymphalid butterflies (A. Brower, unpubl. data). The minimum size of coding regions in human, mouse, cow, and *Drosophila yakuba* is 14415 bp, not including the control region (calculated from Brown, 1985), suggesting that

TABLE 2. Recognition sequences and the sizes of fragments discerned for thirteen restriction enzymes from all individuals sampled.

Enzyme	Rec. seq.	Number of fragments	Fragment sizes (Kb)	Σ
*Ase*I[a]	ATTAAT	>16[b]	0.71 0.68 (0.63) 0.59 0.52 0.50 0.43 0.40 0.31 0.275 0.23 0.197 0.167 0.14 0.105 0.08	—
*Bam*HI	GGATCC	0	—	—
*Bgl*II	AGATCT	2	9.2 5.4	14.6
*Bst*NI	CCA/TGG	5	8.3 4.05 1.35 0.60 0.60	14.9
*Dra*I	TTTAAA	>10	—	—
*Eco*RI	GAATCC	2	10.4 4.3	14.7
*Eco*RV	GATATC	3	8.5 5.65 0.35	14.5
*Hin*dIII	AAGTCC	5	6.3 4.7 1.7 0.8 0.45	14.75
*Pst*I	CTGCAG	3	7.15 5.7 2.0	14.85
*Sau*3AI	GATC	15	2.04 1.83 1.65 1.30 1.27 1.13 1.04 0.96 0.90 0.86 0.55 0.38 0.32 0.31 0.30	14.85
*Sca*I	AGTACT	1	15.0	15.0
*Ssp*I	AATATT	>6	—	
*Xba*I	TCTAGA	4	5.9 5.3 2.0 1.95	15.15

[a] Fragment generated by the *Ase*I polymorphism given in parentheses.

[b] In cases where many small fragments were generated number and sizes of fragments are given for those that could be reliably scored.

TABLE 3. Mitochondrial nucleotide diversity (π), haplotypic diversity (h) and mean allozyme heterozygosity per locus (H) for several insect species listed in order of increasing nucleotide diversity values. Both *Danaus* and *Lymantria* are Lepidoptera. *Drosophila* is a member of the Diptera, *Pissodes* a member of the Coleoptera, and *Magicicada* a member of the Homoptera.

	Mitochondrial DNA				Allozymes		
Taxon	π	h	N	Reference[1]	H	N	Reference
Lymantria dispar	0.000	0.000	24	A	0.00164	359	B
Danaus plexippus	0.00016	0.133	28	—	0.0965	77–91	C
Drosophila melanogaster							
Worldwide	0.00134	0.655	92	D	0.102	323	E
D. simulans							
Seychelles	0.029[2]	0.121	16	F	0.086	26	G
South Africa	0.000	0.000	25	F	0.085	32	G
America	0.000	0.000	10	F	—	—	—
(Worldwide)	0.0187	0.512	144	F	0.094	210	G
D. albomicans	0.010	0.758	16	H	—	—	—
D. subobscura	0.008	0.674	261	I	—	—	—
Pissodes strobi	0.0125	0.97	106	J	0.172	30	K
					0.246	28	L
P. nemorensis	0.0045	0.74	70	J	0.189	28	M
P. terminalis	0.0011	0.49	42	J	0.145	38	M
Magicicada species[3]	—	—	—		0.61, 0.71	97–1,095	N
Lineage A	0.0001	0.052	78	O	—	—	
Lineage B	0.0012	0.472	40	O	—	—	

[1] References: A, Harrison and ODell (1989); B, Harrison et al. (1983); C, Eanes and Koehn (1978); D, Hale and Singh (1987); E, Singh and Rhomberg (1987); F, Baba-Aïssa et al. (1988); G, Choudhary and Singh (1987); H, Chang et al. (1989); I, Alfonso et al. (1990); J, Boyce (1990); K, Phillips and Lanier (1985); L, Chilcote (1985); M, Phillips (1984); N, Martin and Simon (1990); O, Simon (1979).

[2] All estimates of π in *D. simulans* and *Pissodes* species based on coding region variation, excluding size variation.

[3] *Magicicada* species limits are not concordant with mitochondrial DNA lineages (Martin and Simon, 1990). Thus, mtDNA variation and allozyme variation are presented separately.

monarch mtDNA does not contain much noncoding sequence, or possibly that some gene(s) has (have) moved out of the mitochondrial DNA, as in *Ascaris* (Wolstenholme et al., 1987).

No size variation in the mitochondrial genome was observed either among or within individuals. Only a single polymorphism was resolved among all monarchs in the entire study: the loss of a recognition site for *Ase*I. This variant was observed in one individual in both of the North American samples. All other restriction fragment patterns were monomorphic among all individuals, including those from Trinidad and Tobago. We thus infer that the single *Ase*I polymorphism is the result of nucleotide substitution and not the result of an insertion or deletion. The estimated number of nucleotide differences per nucleotide site of the two haplotypes (p of Nei, 1987) is 0.00123. Estimated nucleotide diversity, π (Nei, 1987), for all sites examined across all individuals is 0.00016. Haplotype or clonal diversity (h of Nei, 1987) is estimated to be 0.133.

DISCUSSION

The lack of divergence between and polymorphism within these populations of *D. plexippus* is a dramatic departure from predictions based on the results of similar surveys in many other taxa. For example, mtDNA studies of vertebrates (Avise, 1986; Moritz et al., 1987) generally reflect a degree of differentiation at the population and intraspecific level at least 10 times as high as we have observed. The few available analyses of other insect populations also reveal mtDNA variability within and between populations greater than that found in monarchs. Bark weevils (*Pissodes* species) (Boyce, 1990), whirligig beetles (*Dinutes assimilis*) (B. Nuernberger, pers. comm.), honeybees (*Apis mellifera* sspp.) (Smith and Brown, 1990) and several *Drosophila* species (DeSalle et al., 1986; Latorre et al., 1986) all show substantial polymorphism within and among regional populations (Table 3). Levels of geographical variation in mtDNA lower than in the monarchs have been reported only in introduced North American populations of the gypsy moth (*Lymantria dispar*) (Harrison and ODell, 1989), within some local populations of *Drosophila simulans* (Baba-Aïssa et al., 1988), and in northern populations of the periodical cicada, *Magicicada* (Martin and Simon, 1990) (see below).

This striking homogeneity in mtDNA genotype among widely dispersed and geographically isolated populations of *D. plexippus* may be explained as a product of low mutation rates, natural selection and/or stochastic processes. Low rates of mitochondrial DNA sequence evolution would preclude the accumulation of variation. A survey of mtDNA variation in other *Danaus* species is necessary to test for a significant reduction in the evolutionary rate of mtDNA in the *D. plexippus* lineage. If the rate of mtDNA evolution in *D. plexippus* is not substantially lower than has been found in other insects, selection and/or drift must be reducing variation to the observed level. Strong purifying selection against mtDNA sequence variation would tend to eliminate new variants, maintaining low levels of polymorphism. Recent studies have documented apparent nonneutral behavior of alternative mitochondrial genotypes (MacRae and Anderson, 1988; Fos et al., 1990), but abundant haplotypic diversity of mtDNA in a wide variety of taxa suggests that the bulk of mtDNA variation is neutral (Avise, 1986; Moritz et al., 1987). Moreover, strong selection would not be expected to act on nucleotide substitutions that do not change amino acid sequence (so called silent substitutions). Thus, the maintenance of virtual monomorphism in the mtDNA of monarch butterflies under ecologically heterogeneous conditions by purifying selection would be unprecedented.

Stochastic population processes provide a more plausible explanation for the observed low levels of variation. Small amounts of mtDNA polymorphism in other insect populations [*Magicicada* (Martin and Simon, 1990), North American and South African *Drosophila simulans* (Baba-Aïssa et al., 1988) and North American gypsy moths (Harrison and ODell, 1989)], are attributed to population bottlenecks and rapid rates of maternal lineage extinction. Tajima (1989) has analyzed the effects of changing population size on levels of haplotype diversity and average number of nucleotide differences among haplotypes. Historical population bottlenecks influence mean number of nucleotide differences between haplotypes more strongly than overall levels of haplotype diversity. By contrast, current population size has a greater influence over haplotype diversity. Both nucleotide polymorphism and haplotype diversity are relatively low in *D. plexippus* (Table 3), suggesting a reduction in female effective population size in the relatively recent past.

Loss of mtDNA variation is also dependent on many parameters of population demography. Avise et al. (1984) have modeled the mtDNA lineage extinction process within a species. Their results indicate that under certain demographic regimes lineage sorting, leading to the loss of mtDNA variability, can be quite rapid, even when population sizes are relatively large. In particular, Avise et al. (1984) have shown that a high variance of number of progeny per female can significantly increase the rate of mtDNA lineage loss from a species.

The monarchs' annual cycle of migration to localized, climatically buffered overwintering aggregations and subsequent population expansion back into temperate regions suggest that the variance in effective fecundity per female may be relatively high. Such a pattern may accelerate the loss of mtDNA variation in the species. However, this effect may in part be countered by rapid expansion of the population over many generations or a very large, stable population size through time (Avise et al., 1984).

Current *D. plexippus* population sizes are very large. Typical densities at Mexican overwintering sites are almost 10 million butterflies per hectare (Brower et al., 1977) with colonies ranging in size from 0.1 to over 5 hectares in size (Brower and Calvert, 1985). Moreover, within recent geological history, monarch populations have probably expanded rapidly in response to the extension of the range of *Asclepias* over North America. Thus, while a high variance in female reproductive success may accelerate loss of mtDNA variability in this species, a reduction in historical population sizes seems necessary to explain the lack of variation observed in the monarch.

While the lack of polymorphism in mtDNA suggests that the monarch has experienced a significant reduction in female effective population sizes in the past, levels of allozyme heterozygosity indicate that the effective population size of the entire species has remained large enough to retain nuclear genetic variation. This contrast is not unexpected, because at a given population size, allozyme markers are less prone to stochastic loss of variability (Nei, 1987). Levels of mtDNA variation are particularly susceptible to reductions in population size because mitochondrial effective population sizes (*Ne*) are one fourth that of nuclear genes (4*Ne*). In addition, female monarch butterflies frequently carry several spermatophores (Brower, 1985), indicating the possibility of multiple paternal contributions, which would boost the effective population size of nuclear genes without changing that of mtDNA.

We feel that a historical population bottleneck is the most likely explanation for the low levels of nucleotide polymorphism and haplotype diversity in *D. plexippus* mtDNA. Our results may be the result of a post-Pleistocene recolonization similar to the scenario developed by Martin and Simon (1990) to explain the unusually low levels of mtDNA variation in populations of periodical cicadas. That the restriction fragment patterns in morphologically distinct monarch populations from Trinidad and Tobago are identical to those found in North America argues that the bottleneck must also have predated the divergence of migratory *D. plexippus plexippus* and nonmigratory *D. plexippus megalippe.* Whether the dramatic North American migratory phenomenon predated the genetic bottleneck or not is not addressed by our data. A propensity to migrate is present in a number of other danaine species (Ackery and Vane-Wright, 1984). If the monarch's exceptional migratory behavior had evolved before or during the Pleistocene, migrations may have merely been reduced, or shifted southwards during periods of glacial advance. Such shifts might have homogenized the migratory population with ancestors of the current South American and Antillean populations.

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