Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8649–8653, November 1986
Evolution

Evolution of mitochondrial DNA in *Drosophila subobscura*

(population genetics/colonization/human population size/founder effects/Mother Eve hypothesis)

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ABSTRACT **The colonization of the New World by the Palearctic species *Drosophila subobscura* was first detected in 1978 in South America and around 1982 in western North America. The ensuing dramatic expansion of the species, in territory as well as numbers, provides an opportunity for studying evolution in a scale rarely possible. We have used 10 restriction endonucleases to analyze the mitochondrial DNA (mtDNA) of individuals from 23 widely dispersed localities. Only two mtDNA composite morphs have been detected in the Americas. None of the two morphs has been found in Africa, and only one in the Atlantic islands; but both are widespread in Europe, which provides no clue of the precise geographic origin of the colonizers. The amount of nucleotide-substitution polymorphism detected in *D. subobscura* is typical for animals, but it is greater in the Old than in the New World, presumably due to the recent colonization by a limited number of colonizers. Assuming standard evolutionary rates of mtDNA base substitution, the mtDNA morphs found in *D. subobscura* can be traced to a single one that existed no less than one million years ago. We argue against the inference that the *D. subobscura* flies now living descend from only one or a few females that lived at that time. This type of inference, which we call the "Mother Eve hypothesis," has been made to conclude that the human population went through a severe constriction about 200,000 years ago, so that all living humans descend from only one or a few women who lived at that time. The Mother Eve hypothesis is fallacious.**

Grand natural experiments in evolution are treasured by scientists. The evolutionist seeks knowledge in the comparative study of organisms, in the investigation of processes under the conditions that prevail in nature, and in laboratory experiments necessarily limited in time and in scope, from which he daringly extrapolates to the lofty scale of the evolutionary process. Rarely, a natural catastrophe or some other unplanned occurrence triggers a sequence of events that provide a precious opportunity for the study of evolution in action. The colonization of the western Americas by the Old World species *Drosophila subobscura*, and its rapid expansion there, which started less than a decade ago, is that sort of opportunity.

D. subobscura is a Palearctic species distributed all over Europe except central and northern Scandinavia, as well as in Northwest Africa and in the Atlantic islands, the Azores, Madeira, and the Canaries. The population genetics and ecology of this species have been intensively investigated by numerous, mostly European, scientists for more than four decades, placing *D. subobscura* among the best known species in such respects (1).

In February 1978, *D. subobscura*, never before found in the Americas, was discovered in Puerto Montt, in southern Chile, where numerous collections had been made over many years (2). Subsequently, the species has spread in Chile to include a region from 29° to 53° latitude and eastward into Argentina (3). In many localities throughout that range, *D. subobscura* has become the most abundant *Drosophila* species. Starting around 1982, *D. subobscura* has appeared in collections made in the Pacific Northwest from central California to southern British Columbia (4). Members of our laboratory at the University of California, Davis, collected and identified species of the *obscura* group of *Drosophila* in nearby locations at monthly intervals from 1971 to 1975 amounting to several hundred thousand individuals, many of which were genetically studied by electrophoresis, chromosomally, and otherwise. No *D. subobscura* flies were ever found. In October 1983, *D. subobscura* represented about 10% of the *obscura* flies collected in the area; in May 1985, their frequency had increased to 20–70%, depending on the precise site of the collection.

Mitochondrial DNA (mtDNA) genotypes are clones transmitted from female to progeny and are not recombined during sexual reproduction. They may, therefore, provide definitive information about the female lineage from which an individual descends. The possibility of achieving such discrimination depends, of course, on the extent and distribution of the mtDNA intraspecific polymorphisms, which are extensive in most organisms studied (5). We present here a study of the *D. subobscura* mtDNA, largely motivated by the goal of identifying the Old World population(s) from where the New World colonizers have originated. Investigation of the chromosomal and enzymatic polymorphisms have so far failed to achieve such identification (2, 3, 6).

MATERIALS AND METHODS

Samples of *D. subobscura* were obtained from 23 localities: 14 in the Old World, 8 in North America, and 1 in South America (Fig. 1). The experimental strains ("isofemale strains") were derived each from a single gravid female collected in the wild. Thirty-two isofemale strains were studied, two strains from each of 9 localities and one from each of the other 14 sites.

Extraction of mtDNA was according to the method of Chang (7), which was modified from ref. 8. Fifteen young flies were gently homogenized in a 1.5-ml microcentrifuge tube containing 320 μl of 10 mM Tris/60 mM NaCl/5% (wt/vol) sucrose/10 mM EDTA, pH 7.8. Four hundred microliters of 1.25% $NaDodSO_4$/300 mM Tris/5% sucrose/10 mM EDTA/0.8% diethyl pyrocarbonate (freshly mixed), pH 9, was then added. The mixture was incubated at 65°C for 30 min, after which 120 μl of 3 M sodium acetate was added and the mixture was kept in ice for 45 min. After centrifugation for 10 min in an Eppendorf centrifuge, the supernatant was added to 1 volume of 2-propanol and left standing at room temperature for 5 min, which was followed by a 5-min Eppendorf centrifugation. The supernatant was discarded, and the pellet was resuspended in 250 μl of distilled water with 0.25% diethyl pyrocarbonate (freshly mixed) and left at room

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Abbreviations: mtDNA, mitochondrial DNA; kb, kilobase(s).

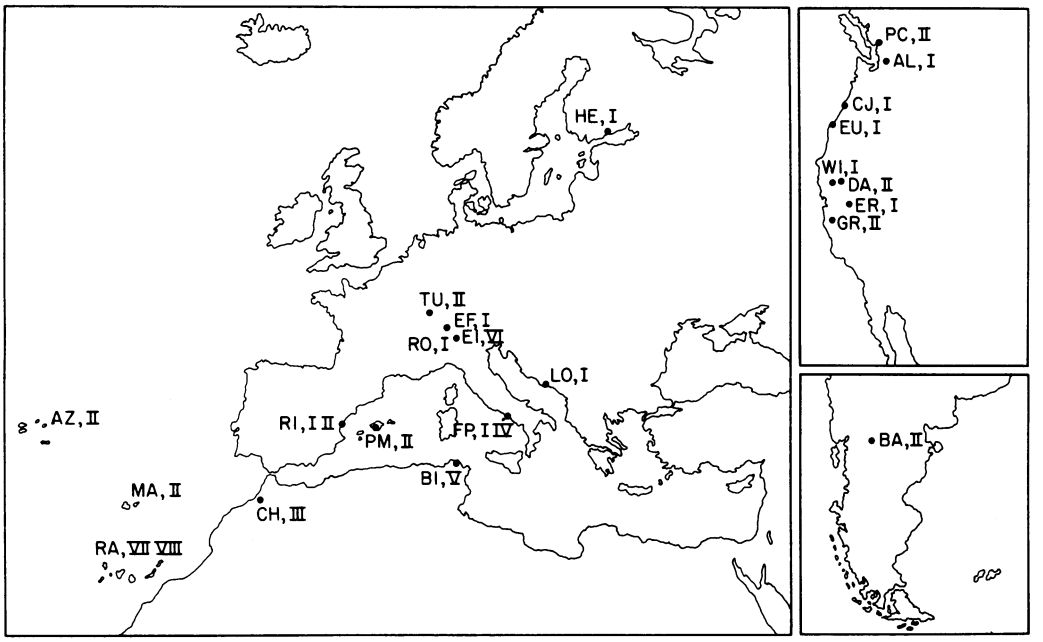

FIG. 1. Geographic origin of the *D. subobscura* strains and their mtDNA patterns (I–VIII, see Table 1). Fourteen localities (19 strains) are from the Old World. In approximate north-to-south and east-to-west order, they are Helsinki (HE), Finland; Tübingen (TU), West Germany; Eierbrecht (EI, two strains), Effretikon (EF), and Rochefort (RO), Switzerland; Lokrum (LO), Yugoslavia; Formia-Ponza (FP, two strains), Italy; Palma de Mallorca (PM) and Ribarroja (RI, two strains), Spain; Bizerte (BI), Tunisia; Chechaouen (CH), Morocco; Azores (AZ, two strains); Madeira (MA); and Raíces (RA, two strains), Canary Islands. The nine localities (13 strains) from the New World are Port Coquitlam (PC), British Columbia; Alta Loma State Park (AL, two strains), Washington; Cave Junction (CJ), Oregon; Eureka (EU), Davis (DA), Winters (WI, two strains), El Río Vineyard (ER, two strains), and Gilroy (GR, two strains), California; and Bariloche (BA), Argentina.

temperature for 30 min. Then 250 μl of distilled water, 0.1 volume of 3 M sodium acetate, and two volumes of ethanol were added, and the mixture was left on ice for 10 min. The DNA was then spun down for 5 min in the Eppendorf centrifuge and washed with 70% ethanol. Residual ethanol was removed by drying the precipitate in a desiccator for 30 min, after which the DNA was dissolved in an appropriate amount of 10 mM Tris/10 mM EDTA, pH 8. An enriched fraction of mtDNA was obtained with this extraction procedure, which gave well-resolved bands after the following digestion procedures.

Six of the 10 restriction endonucleases used recognize 6-base-pair sequences: *Eco*RI, *Hin*dIII, *Hpa* I, *Bam*HI, *Pst* I, and *Xba* I. The other four recognize 4-base-pair sequences: *Hae* III, *Hpa* II, *Hha* I, and *Tha* I. The endonucleases were purchased from Boehringer Mannheim, New England Biolabs, and Pharmacia.

The DNA was digested with the endonucleases in a final volume of 20 μl, following the supplier's recommendations. RNase (2 μg/ml) was added to the digestion mixture. The digestion fragments were separated in 0.7, 1.0, or 1.8% agarose by gel electrophoresis using a Tris/acetate/EDTA buffer. Bacteriophage λ DNA digested with *Hin*dIII provided size markers. After electrophoresis, the gel was stained with ethidium bromide (0.1 μg/ml).

RESULTS

We estimate the length of the *D. subobscura* mtDNA at 16.5 kilobases (kb); the same length was found with all restriction enzymes, except *Eco*RI, which gives an apparent length of 16.0 kb. (We did not score segments that may have been less than 0.5 kb and were repeatedly unclear in our gels.) No length polymorphisms were detected among the 32 strains. Fig. 2 illustrates some typical digests.

The 10 endonucleases used yield a total of 42 mtDNA restriction sites in the 32 isofemale strains of *D. subobscura*; 18 sites (43%) are the same for all strains, the other 24 (57%) are polymorphic. Five enzymes produce only one (*Bam*HI) or two (*Pst* I, *Xba* I, *Hha* I, and *Tha* I) restriction fragments, all the same ones in every strain. The other five enzymes have from five to nine restriction sites each. Fig. 3 diagrams the patterns obtained with the five endonucleases that yield site polymorphisms. The fragments are consecutively numbered from largest to smallest, starting with 1. No more than three patterns are discovered with any one enzyme among the 32 strains.

Composite patterns ("morphs") of the mtDNA can be obtained by combining the patterns obtained with the 10 endonucleases. Eight morphs emerge, specified in Table 1. (The five monomorphic enzymes are not listed). Two mtDNA morphs, I and II, are common and one or both are present throughout Europe and the New World (Fig. 1). They are the only two patterns found in the Americas. Morph I is present in 14 (44%) and morph II in 11 (34%) of the 32 *D. subobscura* strains. The other six morphs, III–VIII, appear to be narrow

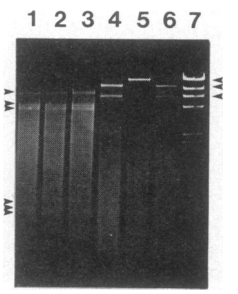

FIG. 2. *D. subobscura* mtDNA digests obtained with endonuclease *Hpa* II and *Hae* III. Lanes 1–3: mtDNA from strains ER, PM, and HE (see Fig. 1), respectively, all of which exhibit *Hpa* II pattern A (see Fig. 3). Lanes 4–6: mtDNA from the same strains, digested with *Hae* III: ER and HE (lanes 4 and 6) exhibit pattern A; PM exhibits pattern C. Lane 7: λ DNA digested with *Hin*dIII, which provides size standards. Migration is from the top; arrowheads indicate the positions of experimental bands.

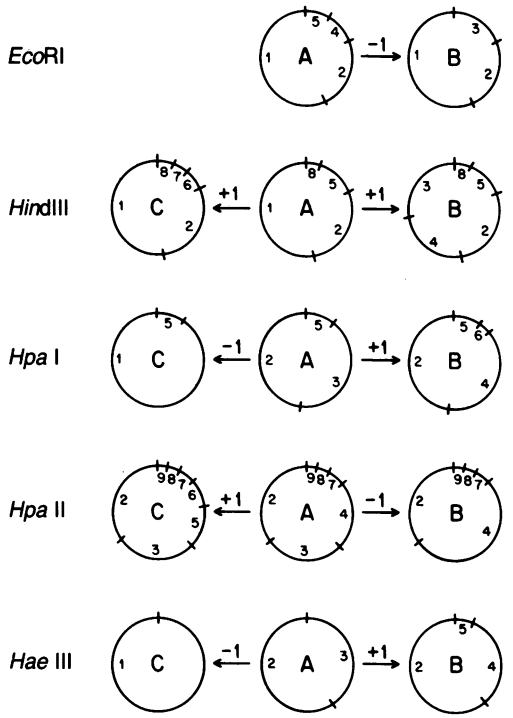

FIG. 3. Diagram of the restriction patterns obtained with five endonucleases in *D. subobscura* mtDNA. The most common pattern is designated A, from which the gain or loss of a single restriction site yields patterns B and C. Fragments are numbered consecutively, starting with 1, according to their size. Locations shown for the restriction sites are arbitrary.

endemics, each found in only one locality. The two common morphs differ from each other in that morph I has one more *Hae* III restriction site than morph II (see Table 1 and Fig. 3).

The eight mtDNA morphs can be connected in a "most parsimonious" network, which gives the minimum number of mutational steps required to connect all the morphs (Fig. 4). There is no evidence of homoplasy; i.e., that the same mutation may have occurred more than once. The network represents a possible evolutionary pathway in the evolution of *D. subobscura* mtDNA. The network does not, however, have direction and hence does not tell us which one is the most ancestral morph. But it is apparent in Fig. 4 that morph I is the ancestral one for at least five of the other seven. (This inference depends on the reasonable assumption that a given morph does not arise independently more than once.) The central position of morph I makes it necessary to pass through it in order to go from any one pattern to all the others

Table 1. Restriction pattern for the eight mtDNA morphs found in *D. subobscura*

	Restriction enzyme pattern					Strains*	
Morph	*Eco*RI	*Hin*dIII	*Hpa* I	*Hpa* II	*Hae* III	Number	Percent
I	A	A	A	A	A	14	43.7
II	A	A	A	A	C	11	34.4
III	A	C	A	A	C	1	3.1
IV	A	A	A	C	A	1	3.1
V	A	A	B	A	A	1	3.1
VI	B	A	C	A	A	2†	6.3
VII	A	B	A	A	B	1	3.1
VIII	A	B	A	B	B	1	3.1

A, B, and C refer to the patterns shown in Fig. 3 for the five endonucleases revealing restriction polymorphisms.
*Geographic locations of the strains are shown in Fig. 1.
†These two strains are from the same locality: Eierbrecht, Switzerland.

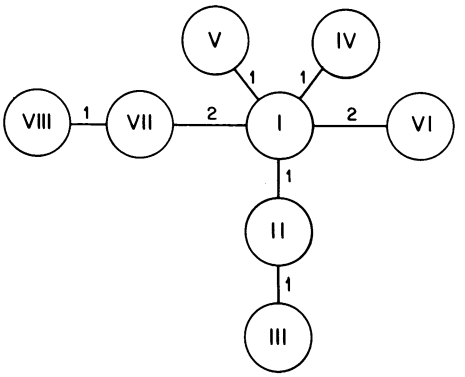

FIG. 4. Network of the eight composite morphs of *D. subobscura* mtDNA. The morphs are connected in a way that minimizes the total number of restriction-site changes required. Numbers with the connecting lines refer to the minimum number of restriction-site changes required to go from one morph to the next.

(except that if either III or VIII is the most ancestral one, one additional morph precedes the derivation of morph I). Morph I is also the most common one, with a frequency of 44% in the sample.

The genetic similarity between the mtDNAs of any two strains may be estimated by the proportion, F, of the digestion fragments they share (9, 10). If it is assumed that changes in fragment size are due to nucleotide substitutions, rather than to insertions or deletions, the frequency, p, of nucleotide differences per nucleotide site can also be estimated (11–13). Table 2 gives the values of F (above the diagonal) and p (below the diagonal) for all pairwise comparisons between the eight composite patterns. The mean number of fragments shared by any two patterns is $F = 0.860$, with a 0.727–0.949 range. The proportion of nucleotide substitutions between patterns ranges from 1% (between patterns I and V or between II and III) to more than 5% (between III or VI and VIII). The two most common patterns, I and II, differ in 1.6% of their nucleotides.

More meaningful than F and p for the purpose of evaluating the mtDNA polymorphism of the species is π, a measure of nucleotide diversity that takes into account the frequency of the various mtDNA morphs among the strains (13). The value of π is 0.83% for all 32 strains, 1.10% for the 19 European strains, and 0.45% for the 13 American strains. π is a measure of variation analogous to the "heterozygosity" (average frequency of heterozygous loci) used for diploid genomes, except that it measures variation per nucleotide site rather than per locus. In addition to the variation in the whole species measured by π, there is in *D. subobscura* considerable variation within local populations, as suggested by the existence of two different mtDNA morphs in each of three

Table 2. Genetic differentiation between the eight mtDNA morphs of *D. subobscura*

Composite morph	I	II	III	IV	V	VI	VII	VIII
I	—	0.946	0.893	0.947	0.947	0.890	0.897	0.842
II	0.016	—	0.946	0.893	0.893	0.830	0.877	0.821
III	0.024	0.010	—	0.842	0.842	0.778	0.828	0.772
IV	0.015	0.035	0.042	—	0.897	0.836	0.848	0.828
V	0.009	0.024	0.033	0.023	—	0.873	0.848	0.793
VI	0.022	0.036	0.049	0.035	0.026	—	0.786	0.727
VII	0.023	0.029	0.038	0.040	0.032	0.047	—	0.949
VIII	0.042	0.050	0.058	0.047	0.050	0.064	0.015	—

Above the diagonal: F, proportion of restriction fragments shared. Below the diagonal: p, estimated proportion of base differences between the morphs.

localities, one-third of the nine from which two strains were sampled.

Fig. 5 is a dendrogram based on the matrix of p, the proportion of base differences per site between the morphs (Table 2, below the diagonal). This dendrogram reflects the close similarities between morphs I and V, II and III, and VII and VIII, apparent in Fig. 4. It is, however, misleading in other respects; for example, morphs I and II appear relatively different, although they are separated by p values only slightly higher than between II and III or between I and V. The qualitative relationships reflected in Fig. 4 are in this case more accurate and informative than the quantitative cluster analysis employed for Fig. 5. The network method does not depend on the statistical and other tenuous assumptions required for the dendrogram (9).

DISCUSSION

The size of the *D. subobscura* mtDNA is estimated at 16.5 kb, which is about average for *Drosophila* as well as for other animals. (The range for most animals studied is 15.7 to 19.5 kb, ref. 14.) We have found no evidence of size variation within the species. No length variation has been detected in most other animals studied, although small size polymorphisms do occur in some species, particularly in the control region where both replication and transcription of the mtDNA are initiated (14–16).

The nucleotide diversity for the species, on the basis of all 32 strains sampled is $\pi = 0.0083$ per nucleotide site. The diversity is greater in the Old than in the New World, 0.0110 vs. 0.0045, presumably reflecting the recent colonization of the Americas by *D. subobscura*. Indeed, the American polymorphism is totally due to the apparently even distribution of the two common morphs, I and II, throughout the American samples.

The *D. subobscura* mtDNA levels of polymorphism are similar to those reported for many animal populations, which are often between 0.01 and 0.02 (reviews in refs. 5, 14, and 17). But the values reported in most cases are for variation between morphs and thus are overestimates of variation between individuals or strains, because they do not take into account the presence of a given morph in more than one sample. The average diversity among the *D. subobscura* mtDNA morphs is $\bar{p} = 0.034$, apparently higher than the typical values for most animal species. The average mtDNA diversity among human *individuals*, not morphs, is 0.004 (14, 18, 19), similar to the value observed among the New World *D. subobscura* isofemale strains.

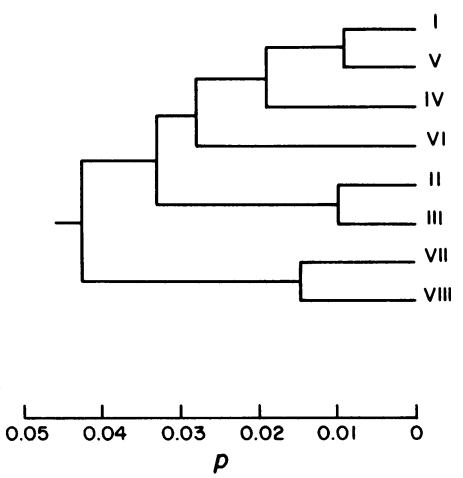

FIG. 5. Dendrogram of the eight composite morphs, obtained by the unweighted pair-group method of cluster analysis, starting from the matrix of p values given in Table 2.

The rate of mtDNA evolution has been studied extensively (5, 9, 14, 16, 20–22). Rates are estimated by assuming that they are constant; i.e., that there is a molecular evolutionary clock for mtDNA. Alternatively, the estimated rates of mtDNA evolution may be seen simply as average values over certain time spans and groups of organisms, in which case extrapolation to other organisms or time spans would represent little more than an educated guess. There is at present no convincing evidence that mtRNA evolves like a molecular clock, at rates that would persist from one group of organisms to another or over long evolutionary spans. Rather, the diversity of rates estimated on the basis of different data sets suggests considerable variation in the rate of mtDNA evolution, although a decision must be withheld until more extensive data sets become available.

The estimated rates of nucleotide substitution in the mtDNA of mammals range from 2.5×10^{-9} to 25.4×10^{-9} per site per year (21–23), with 10×10^{-9} commonly accepted as a standard value. Estimates for the rate of evolution of *Drosophila* mtDNA, which are primarily based on comparisons between *D. melanogaster* and its close relatives, range from 2.4×10^{-9} to 62.5×10^{-9} per site per year (16). If we use the lowest rate, the time of divergence between the *D. subobscura* mtDNA morphs would be on the average about 14 million years (range, 4–27 Myr). The higher rate gives an average divergence time for the morphs of 540,000 years (range, 0.16–1.0 Myr). The uncertainties are enormous, yet the available estimates of mtDNA evolutionary rates indicate that the *D. subobscura* morphs are old, some having originated no later than the early Pleistocene.

Backwards extrapolation in time does, therefore, indicate that the evolution of the eight *D. subobscura* morphs can be traced to a single morph that existed no less than 1.0 Myr ago (if the slower rate of evolution is accepted, the date would be 27 Myr ago). It does not follow, however, that all *D. subobscura* now living descended from a single female that lived at that time. Yet, this kind of inference, which we shall refer to as the "Mother Eve hypothesis," has been made repeatedly (9, 18, 24, 25). Consider, for example, the average sequence heterogeneity among 21 humans, which is 0.18% (18). If the rate of nucleotide substitution is 10 per 10^{-9} years, the observed mtDNA heterogeneity observed in today's human population could have been generated from a single female who lived 180,000 years ago. This suggests, according to Brown (18), that present-day humans have evolved from a single mating pair—or from a small, mitochondrially monomorphic population—that existed at that time (18, 25). Similar arguments have also been advanced for other organisms, such as *Drosophila* (24); the conclusion that mankind descends from one or few women who lived around 180,000 years ago has reached the daily press and the popular scientific publications (26).

The Mother Eve hypothesis is, however, fallacious. Consider any human individual living today, whom we shall call Ego. Maternal transmission guarantees that Ego's mitochondria are all derived from his maternal grandmother but that does not mean that Ego descends from only one of his grandmothers, or only one of his great-grandmothers, and so on. The mitochondria of all living humans may very well have derived from a single ancestral female living some 200,000 years ago, but that does not support the Mother Eve hypothesis that "all living humans may have evolved from a small, mitochondrially monomorphic population that existed as recently as 200,000 years ago" (ref. 25, p. 154).

Today's world population of *D. subobscura* consists of many millions of individuals. It might very well be the case that, a few hundred thousand years hence, all *D. subobscura* flies have mtDNAs derived from morph I. That would not

mean that the mtDNA of the descendants derives from only one *D. subobscura* currently living—morph I is found in 44% of the living population. More importantly, the individuals living in that remote generation would count among their ancestors not only those females from which they inherited their mitochondria but also innumerable other females and males from which they inherited their nuclear hereditary material. Relevant calculations can be obtained from ref. 27. For example, a population of 15,000 unrelated females has a probability of 0.5 that the mitochondria of all individuals living 18,000 generations later (about 360,000 years for humans) will have derived from a single female. That female might be considered a Mother Eve with respect to the mitochondria, but no population constriction is thereby required in her generation. She coexisted with thousands of individuals who contributed nuclear hereditary material to the descendants.

It has been suggested that restriction analysis of mtDNA may be "the most sensitive technique available for reconstructing evolutionary relationships among conspecific populations" (ref. 9, p. 293). It was with this conviction that we undertook to analyze the mtDNA of *D. subobscura* populations, seeking whether the origin of the New World colonizers could be ascertained. Other genetic techniques, such as chromosomal inversion polymorphisms, have failed to give significant clues (2, 3). The presence of morphs I and II in the North American populations demands that the colonizers of the New World comprise at least two gravid females, although there could be many more. But the extensive distribution of morphs I and II in Europe makes it impossible to identify the population(s) whence the colonizers may have originated.

The minimum-mutation network of the *D. subobscura* morphs (Fig. 4) shows that, whichever one may have been the ancestral one, all other morphs but one or two at most have derived from morph I. Only morphs I and II are widely distributed, whereas all others are found in only a single locality. It seems likely, therefore, that most or all morphs III–VIII arose locally and were never extensively distributed. If they were ever widespread in the past, they would be likely to persist now in more than one locality, something not supported by the evidence available.

We have estimated above that the time of divergence of the morphs goes back at least hundreds of thousands of years, if not millions. If the conjecture of local origination and endemism is correct for most morphs, the conclusion would follow that the mtDNA morphs persist locally for millions of generations. The rate of evolution of mtDNA is known to be high, about 5 to 10 times greater than the rate of evolution of nuclear DNA (14, 28). Thus, new mtDNA morphs must arise continuously in natural populations of *D. subobscura*. (If the average rate of mtDNA evolution is $10–20 \times 10^{-9}$ per site per year, about one in 3000 mitochrondria would add one new base substitution each year.) The hypothesis of neutral genetic drift, generally assumed for the evolution of mtDNA, would find it difficult to account for a pattern of local persistence for millions of generations of mtDNA morphs, in the face of the continuous origination of new ones. An alternative interpretation of the situation is that restriction analysis estimates of mtDNA sequence divergence are considerably in error. But the evidence that exists suggests the contrary: namely, that mtDNA restriction analysis estimates agree well with the values obtained when the nucleotide sequences become known (5). It may not be warranted at present to conclude that mtDNA evolution is not neutral. But the erroneous divergence estimates obtained when constant rates are assumed, as well as the points just made, bring at least this matter into question.

We thank Drs. A. Beckenbach, V. Cabrera, H. Jungen, D. Marinkovic, A. Prevosti, and A. Saura for providing *D. subobscura* strains; C. Zimmerman for technical advice; and J. Avise, A. Prevosti, and M. Tibayrenc for valuable comments on the manuscript. This work was supported by grants from the Comité Conjunto Hispano-Norteamericano Para La Cooperación Científica y Técnica, Spain, and from the U.S. Department of Energy.

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