Mitochondrial DNA Haplotype Frequencies in Natural and Experimental Populations of *Drosophila subobscura*

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ABSTRACT

The evolution of *Drosophila subobscura* mitochondrial DNA has been studied in experimental populations, founded with flies from a natural population from Esporles (Majorca, Balearic Islands, Spain). This population, like other European ones, is characterized by the presence of two very common (>96%) mitochondrial haplotypes (called I and II) and rare and endemic haplotypes that appear at very low frequencies. There is no statistical evidence of positive Darwinian selection acting on the mitochondrial DNA variants according to Tajima's neutrality test. Two experimental populations, with one replicate each, were established with flies having a heterogeneous nuclear genetic background, which was representative of the composition of the natural population. Both populations were started with the two most frequent mitochondrial haplotypes, but at different initial frequencies. After 13 to 16 generations, haplotype II reached fixation in three cages and its frequency was 0.89 by generation 25 in the fourth cage. Random drift can be rejected as the force responsible for the observed changes in haplotype frequencies. There is not only statistical evidence of a linear trend favoring a mtDNA (haploid) fitness effect, but also of a significant nonlinear deviation that could be due to a nuclear component.

A recurrent observation in studies of mitochondrial DNA evolution in Old and New World populations of *Drosophila subobscura* during more than 10 yr is the high prevalence of two haplotypes and the sporadic appearance of low-frequency endemic, *i.e.*, rare, ones (Latorre *et al.* 1986, 1992; Afonso *et al.* 1990; Rozas *et al.* 1990; Moya *et al.* 1993; González *et al.* 1994). It is yet an unresolved question which evolutionary forces account for this distribution. Recent studies based on mtDNA nucleotide sequences of *D. melanogaster* and related species (Kaneko *et al.* 1993; Ballard and Kreitman 1994; Rand *et al.* 1994), of Mus species (Nachman *et al.* 1994) and of humans and chimpanzees (Nachman *et al.* 1996) have shown deviations from a neutral model, which have been interpreted as caused by a large class of mildly deleterious mutations. On the contrary, fitness components studies in the evolution of natural and experimental populations, carried out with Drosophila, have shown a diversity of results with very different interpretations. On the basis of changes in the mtDNA haplotype frequencies in experimental populations and fitness estimates, MacRae and Anderson (1988, 1990) and Jenkins *et al.* (1996) have suggested that mtDNA variants of *Drosophila pseudoobscura* evolve in a nonneutral fashion. Singh and Hale (1990) argued that these results can be interpreted as caused by an incompatibility system, similar to the one promoted by Wolbachia endosymbionts in *Drosophila simulans* (Nigro and Prout 1990). Fos *et al.* (1990) showed that natural selection acts on mitochondrial DNA haplotypes in *D. subobscura*, but they also attributed an important effect to the nuclear genetic background. Their results and conclusions have been criticized by both Hutter and Rand (1995) and Kilpatrick and Rand (1995) who argued that population cages were not replicated. Kilpatrick and Rand (1995) did not obtain evidence of selection in competition experiments between mitochondrial haplotypes in *Drosophila melanogaster*, and their results were interpreted as caused by a temporary hitchhiking of nuclear background on mtDNA (Babcock and Asmussen 1996). On the contrary, similar experiments between *D. pseudoobscura* and *Drosophila persimilis* (Hutter and Rand 1995) identified mtDNA as a source of fitness variation and also found evidence of cyto-nuclear interactions affecting fitness.

The main goal of the present work is to test the hypothesis of neutral evolution in the mtDNA of *D. subobscura*. For this purpose, we take advantage of previous studies on experimental evolution and temporal and geographic mtDNA haplotype distribution of *D. subobscura* (see above), to study haplotype frequency changes in two replicated experimental populations. These popula-

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tions were derived from a representative set of isofemale lines from a natural population whose mtDNA haplotype frequencies have also been studied.

MATERIALS AND METHODS

The natural population: *D. subobscura* flies were captured in a pine forest near Esporles (Majorca Island, Spain) with conventional traps of fermented banana. Once in the lab, 204 females were placed individually in a vial with food and kept in an incubator at 19°. When the F_1 larvae appeared, females were used to determine their maternal mitochondrial haplotypes (see below).

Extraction and digestion of mtDNA isofemale lines: An enriched fraction of mtDNA was obtained as previously described (Latorre *et al.* 1986). Routinely, enough DNA could be extracted from 15 individuals for digestion with at least five restriction enzymes. The following restriction enzymes were used: *Eco*RI, *Eco*RV and *Hin*dIII (recognition sequence of 6 bp), and *Hpa*II and *Hae*III (recognition sequence of 4 bp). Previous studies have shown that these restriction enzymes reveal a rich mtDNA polymorphism in *D. subobscura* (Afonso *et al.* 1990; Latorre *et al.* 1992).

Digested DNAs were separated on horizontal 0.6–1.2% agarose gels in 0.5× TBE containing 0.1 μg/ml EtBr. Lambda phage DNA digested with *Hin*dIII and double digested with *Hin*dIII-*Eco*RI was used as a size standard.

A mtDNA restriction map was obtained by means of all possible single and double digestions. The different restriction patterns and haplotypes are named according to the notation given by Latorre *et al.* (1986, 1992).

Presence of Wolbachia: To exclude an incompatibility system in *D. subobscura* promoted by the presence of Wolbachia, a PCR assay using 16S *rDNA* Wolbachia-specific primers was carried out. Primers were designed according to O'Neill *et al.* (1992). These primers amplify a fragment of 869 bp. Two different strains of *Sitophilus oryzae* containing Wolbachia were used as positive controls. In addition, two *D. subobscura* regions were used as positive control for DNA amplification: the V4-18S *rDNA* (the V4 variable region of the 18S *rDNA*) and the ND5 mtDNA (the subunit 5 of the NADH dehydrogenase). The sequences of the primers are as follows: 18SV4up-5′-ACC TGCAGCAGCCGCGGTAATTCCAGC-3′ and 18SV4down-5′-GCTCTAGACGTACTTGGCAAATGCTTTCGC-3′ (that amplify a fragment of 427 bp) and ND5up-5′-TGACCAGCTAGC TATTCTGATC-3′ and ND5down-5′-GCTATAGCTAGCCCC TACAC-3′ (that amplify a fragment of 984 bp). PCR conditions were as follows: an initial denaturation for 5 min at 94° and 30 cycles of denaturation at 94° for 1 min, annealing at 55° for 1 min and extension at 72° for 2 min. Total DNA was extracted as in Martínez *et al.* (1992) but excluding the alkaline treatment.

Figure 1 shows a sample PCR obtained with the three sets of primers from DNA belonging to *D. subobscura* individuals from Mallorca (Esporles, this study), Menorca and Valencia populations, as well as DNA from the two strains of *S. oryzae*. As it can be observed there was no amplification in *D. subobscura* with the Wolbachia-specific primers.

Discrete generation experiments: Population cages were initiated with flies derived from 51 *D. subobscura* isofemale strains from Esporles (Majorca, Spain). A total of 20 isofemale strains bore mitochondrial haplotype I and the other 31 haplotype II.

Two experimental populations, both replicated (denoted as A1 and A2, and B1 and B2, respectively) were initiated at the same time with 1000 individuals (500 males and 500 females) representative of the genetic composition of the natural population from Esporles. The starting frequencies in cages A1 and A2 were 30% for haplotype I (300 individuals, 15 individuals from each isofemale strain) and 70% for haplotype II (700 individuals, 22 or 23 individuals from each isofemale strain). The starting frequencies of mitochondrial haplotypes in cages B1 and B2 were 70% for haplotype I (700 individuals, 35 individuals from each isofemale strain) and 30% for haplotype II (300 individuals, 9 or 10 individuals from each isofemale strain).

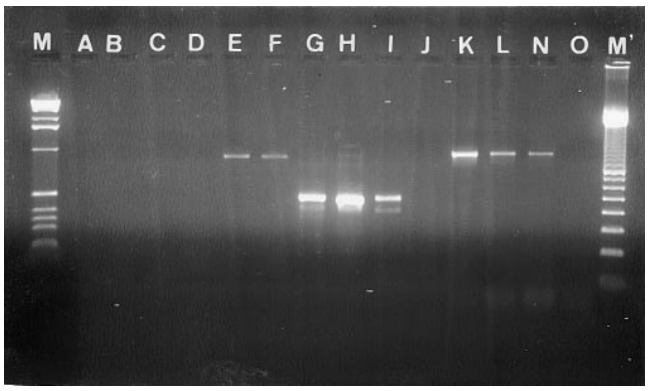

Figure 1.—PCR amplifications. Lanes A, B, and C correspond to total *D. subobscura* DNA from one isoline from Mallorca (Esporles, this study), Menorca and Valencia (Spain), respectively, amplified with Wolbachia 16S *rDNA*-specific primers. Lanes G, H, and I correspond to the same DNAs amplified with V4-18S *rDNA* primers, and lanes K, L, and N with ND5 primers. Lanes D, J, and O correspond to negative controls from each set of primers. Lanes E and F correspond to Wolbachia DNA from two strains of *S. oryzae* amplified with 16S *rDNA*-specific primers. M is the 100-bp ladder molecular marker (Pharmacia, Piscataway, NJ) and M′ is the 1-kb ladder molecular marker (GIBCO BRL, Gaithersburg, MD).

Cages were started with 13 food cups and kept at 19°. Egg laying lasted for 6 days, after which the food cups with eggs and larvae were moved to a new cage. When the F_1 appeared, 13 more food cups were added and left 6 days for egg laying; this set of food cups was then moved to a new population cage. All cages followed a similar cycle of discrete generations.

Estimation of mtDNA haplotypes frequencies in experimental populations: About 100 individuals (see results) were sampled each generation and used for extraction of mtDNA and estimation of haplotype frequencies. mtDNA was again extracted according to Latorre *et al.* (1986) with the following modifications. Two flies were homogenized in an Eppendorf tube with 50 μl of buffer I (10 mm Tris-HCl, 60 mm NaCl, 5% sucrose, 10 mm EDTA, pH 7.8). After homogenization, 62.5 μl of buffer II (300 mm Tris-HCl, 1.25 % SDS, 5 % sucrose, 10 mm EDTA, pH 9.0) were added and tubes were incubated at 65° for 30 min. After incubation, 18 μl of 3 m potassium acetate (pH 4.8) were added and tubes were kept at −20° for 15 min. Tubes were centrifuged in a microfuge at maximum speed for 15 min. The supernatant was transferred to a fresh tube, and an equal volume of isopropanol was added, mixed and incubated at room temperature for 10 min. DNA was precipitated by centrifugation at maximum speed for 15 min, washed with 70% ethanol, vacuum dried, and resuspended in 11.5 μl of TE buffer.

DNA was digested with restriction endonuclease *Hae*III in a final volume of 15 μl. This enzyme cuts genomic DNA frequently but has only a few recognition sites in the mitochondrial genome of *D. subobscura* (a single restriction site in haplotype II and two sites in haplotype I). In agarose gels, mtDNA bands are, therefore, easily distinguishable against a background of genomic DNA by visual inspection (one 15.8-kb

TABLE 1

mtDNA restriction sites and frequencies of the corresponding haplotypes of *D. subobscura* from Esporles population

Haplotype	Restriction enzyme					Frequency	Percentage
	*Eco*RI	*Eco*RV	*Hae*III	*Hin*dIII	*Hpa*II		
I	1 1 1 1	0 1	1 1 0	1 0 1 0 0 1	1 0 1 0 1 1	80	39.2
II	1 1 1 1	0 1	1 0 0	1 0 1 0 0 1	1 0 1 0 1 1	116	56.9
III	1 1 1 1	0 1	1 1 0	1 0 1 0 0 1	1 0 1 1 1 1	1	0.5
IV	1 1 1 1	0 1	1 0 0	1 0 1 0 0 1	1 0 1 1 1 1	1	0.5
V	1 1 1 1	0 1	1 0 0	1 0 1 1 0 1	1 0 1 0 1 1	1	0.5
VI	1 1 1 1	0 1	1 1 0	1 0 1 0 1 1	1 0 1 0 1 1	1	0.5
VII	1 1 1 1	1 1	1 0 0	1 0 1 0 0 1	1 0 1 0 1 1	1	0.5
VIII	1 1 1 1	0 1	1 0 0	1 1 1 0 0 1	1 0 1 0 1 1	1	0.5
IX	1 1 1 1	0 1	1 0 0	1 0 1 0 0 1	1 1 1 0 1 1	1	0.5
X	1 1 1 1	0 1	1 0 1	1 0 1 0 0 1	1 0 1 0 1 1	1	0.5

band for haplotype II and two, 9.0- and 6.8-kb, bands for haplotype I). Because DNA was extracted from two flies simultaneously, three different restriction fragment patterns could be distinguished: a single 15.8-kb band (indicating that the two flies carried haplotype II); two bands of 9.0 and 6.8 kb (indicating that the two flies carried haplotype I) and three bands of 15.8, 9.0 and 6.8 kb (indicating that each fly had a different mtDNA haplotype). Partial digests can be easily distinguished because of both the intensity of the bands and the presence of undigested molecular forms, *i.e.*, supercoiled and relaxed.

Statistical analyses: The intensity of selection acting on mtDNA haplotypes was analyzed following two methods. The first method (Fisher and Ford 1947; Schaffer *et al.* 1977) tests the null hypothesis that changes observed in haplotype frequencies can be explained as the result of random drift. Fisher and Ford (1947) showed that the effective size of a population in each generation and the sample size taken from it can be used to generate a matrix of covariances between the haplotype frequencies observed in the various generations. By means of a χ^2 statistic this covariance matrix can be used to test the hypothesis that random drift alone accounts for the observed changes in haplotype frequency. This method is sensitive only to fairly large differences in selective values, *i.e.*, of the magnitude $s = 0.05$ (Schaffer *et al.* 1977). The second method is based on a model of linear change in frequency due to selection, which may be used to detect selection coefficients as small as 0.01 (Schaffer *et al.* 1977). The magnitude of the selection differential per generation is measured by a linear parameter and the significance for such a linear trend as well as nonlinear deviations thereof are evaluated by χ^2 tests with 1 and $g - 2$ degrees of freedom respectively, where g is the number of generations elapsed between the samples. Both tests use haplotype frequencies which are arcsin squared transformed and given in radians. It should be noted that curvilinearity is substantially decreased by this transformation, which has the effect of expanding the scale at the ends of the haplotype frequency range. Additionally, the test can respond to the linear component in a trend, even if the underlying phenomenon is nonlinear.

RESULTS

mtDNA restriction enzyme analysis carried out on 204 flies from the natural population of Esporles yielded a total of 10 haplotypes (Table 1). Haplotype I (39.2%) and haplotype II (56.9%) accounted for more than 95% of all haplotypes. These two haplotypes differ by a single *Hae*III site. Haplotypes IV, V, VII, VIII, IX and X differ by a single site from haplotype II, whereas haplotype III and VI differ by a single site from haplotype I. Tajima's (1989) D statistic was used to test the deviation from neutrality and yielded a D value of -1.29, which is not statistically significant, at the 0.05 level, for a sample size of 204 individuals.

Flies belonging to the natural population of Esporles that had a heterogeneous, representative nuclear background (see materials and methods) and bore either mtDNA haplotype I or II were placed in experimental population cages and allowed to evolve for a number of generations (Table 2). Two replicates for each initial starting frequency of both haplotypes were used. The experimental protocol was designed to minimize the potential effects of random drift by starting each generation with about a thousand eggs, larvae and pupae competing for resources in food cups. Monitoring the frequency change of haplotype II versus I showed that replicates gave similar results. When haplotype II was started at a higher frequency (70%) it reached fixation at generations 14–15. The same tendency toward fixation of haplotype II was observed when its starting frequency was 0.3. A decrease of haplotype II in population cage B2 was observed in the first three generations, which may be attributed to some uncontrolled factor. By generations 4–5 it regained the starting frequency and, from that moment, it increased until the last checked generation (25), where its frequency was 88.7%. The B1 population cage reached fixation at generation 16.

Results from χ^2 tests for the presence of a selective pressure favoring haplotype II are given in Table 3. The tests have been carried out assuming two different effective numbers of females, *i.e.*, $N_f = 250$ or 1500. Irrespective of these numbers, the χ^2 values for testing random genetic drift were always highly significant in

TABLE 2

Frequency changes, sample size for mtDNA determination and percentage of haplotype II in the evolution of the four experimental populations of *D. subobscura*

II:I (%)	Population cage		Generation																					
			1	2	3	4	5	6	7	8	9	12	13	14	15	16	17	18	19	22	25			
70:30	A1	Frequency	87	86	81	95	92	94	91	89			98	104										
		Sample size	106	108	102	108	108	108	106	102			98	104										
		Percentage	82.1	79.6	79.4	88.0	85.2	87.0	85.8	87.3			100	100										
	A2	Frequency	77	54	66	67	88	75	88			113	55	74	92									
		Sample size	106	108	108	98	118	104	112			114	56	74	92									
		Percentage	72.6	50.0	61.1	68.4	74.6	72.1	78.6			99.1	98.2	100.0	100.0									
30:70	B1	Frequency	52	65	167	96	94	74	63	62				98		104		102	90					
		Sample size	100	110	190	106	112	108	92	98				106		104		102	90					
		Percentage	52.0	59.1	87.9	90.6	83.9	68.5	68.5	63.3				92.5		100.0		100.0	100.0					
	B2	Frequency	24	16	16	32	31	26	37		37		33		39		43		41	58	94			
		Sample size	106	110	108	106	104	86	110		106		108		106		98		84	94	106			
		Percentage	22.6	14.5	14.8	30.2	29.8	30.2	33.6		34.9		30.6		36.8		43.9		48.8	61.7	88.7			

all cages ($P < 0.01$), clearly disallowing the null hypothesis of random genetic drift. In all cases, linear selection was statistically significant ($P < 0.01$). Additionally, in spite of the linearizing effects of the arcsin squared transformation (see materials and methods), a nonlinear deviation was also statistically significant ($P < 0.01$).

DISCUSSION

In the survey of mtDNA haplotype frequencies in the Esporles population (Table 1) haplotype II was found at higher frequency (56.9%) than haplotype I (39.2%). A similar frequency distribution was observed in a study of mtDNA from a different *D. subobscura* population in which extensive and recurrent seasonal sampling was carried out (González *et al.* 1994). In fact, the higher frequency of haplotype II than that of haplotype I seems to be the pattern in this species, at least during the last 12 years. From 1985 to 1997, the mtDNA restriction analysis of 1684 flies, thoroughly sampled over the distribution range of the species (Latorre *et al.* 1986; Afonso *et al.* 1990; Moya *et al.* 1993; González *et al.* 1994; and other unpublished results from our laboratory), show the following overall distribution: 673 with haplotype I (40%), 860 with haplotype II (51%), and 151 with rare haplotypes (9%). A possible explanation for the stable frequencies of the two haplotypes is balancing selection (Clark 1984). In that case, however, Tajima's *D* would be positive, rather than negative. A negative value is compatible with either a purifying selection or a population bottleneck. However, it is too risky to conclude that mtDNA of *D. subobscura* is evolving neutrally based only on a test that has been performed with eight polymorphic sites. To perform appropriate neutrality tests at the nucleotide level, we are currently sequencing 984 bp of the ND5 gene from more than 45 mtDNA haplotypes of *D. subobscura* from different locations, *i.e.*, haplotypes I and II as well as some rare ones, and *Drosophila guanche*, which is closely related to *D. subobscura*. Tajima's *D*, and Fu and Li's (1993) *D* and *F* do not show significant deviation from neutrality or, when significant, they are negative, in agreement with the results obtained here. Moreover, McDonald and Kreitman's (1991) test, does not show departure from neutrality (C. Martínez, R. Bueno, A. González, E. Barrio, A. Moya, unpublished results). Taking all these results together, we have not found evidence of positive selection in the evolution of mtDNA of *D. subobscura*. On the contrary, the stability of the frequencies of haplotypes I and II over more than twelve years may be accounted for by their drifting in a population of very large effective size.

If haplotypes I and II are neutral in nature, the fixation of haplotype II in the population cage experiments deserves an explanation. A point to be mentioned is that population cages do not constitute a natural envi-

TABLE 3

χ^2 values under either random drift (selective neutrality) or linear directional change for the four *D. subobscura* experimental populations

Population cage	N_f = 250						N_f = 1500					
	Drift model		Linear model		χ^2 deviation	d.f.	Drift model		Linear model		χ^2 deviation	d.f.
	χ^2	d.f.	χ^2	d.f.			χ^2	d.f.	C^2	d.f.		
A1	43.87	9	16.52	1	27.35	8	81.79	9	43.30	1	38.48	8
A2	86.08	10	37.00	1	49.08	9	192.09	10	125.00	1	67.09	9
B2	139.59	11	36.67	1	102.93	10	247.86	11	98.67	1	149.18	10
B2	66.86	13	28.20	1	38.65	12	142.75	13	86.65	1	56.10	12

Two different effective population sizes, N_f, have been assumed: 250 and 1500 females, respectively. All values are statistically significant ($P < 0.01$).

ronment. Consequently, factors promoting positive selection of haplotype II in population cages could be counterbalanced by other factors under natural conditions. Regarding experimental populations, balancing selection, frequency-dependent selection, some sort of habitat-specific selection or genetic hitchhiking of mtDNA by a nuclear target are *ad hoc* explanations because we have no clear physiological/ecological evidence for a mechanism. Fos *et al.* (1990) showed that when two mtDNA haplotypes of *D. subobscura* compete in experimental populations one or the other approaches fixation depending on the nuclear background with which they are associated. The two populations used in that report were from very different geographic origins (one from Canary Islands, Spain, bearing the endemic mtDNA haplotype VIII, and the other from Helsinki, Finland, with mtDNA haplotype I), and different lines, homozygous for all chromosomal inversions, were synthesized. The nuclear background effect was manifested because the mtDNA haplotype that reached fixation in each case was the one placed within its own nuclear background.

The population cage experiments were set up with different lines of wild flies belonging to the Esporles population evolving under relatively high effective population sizes (see materials and methods). Thus, in addition to the segregating mtDNA variation, there is considerable nuclear variation segregating as well. For example, the rich and adaptive inversion polymorphism of *D. subobscura* in Mediterranean populations is well known (Krimbas and Loukas 1980; Orengo and Prevosti 1996). This raises the possibility that the shift in mtDNA haplotype frequencies could be caused by not only to selection acting on mtDNA variants, but also by temporary hitchhiking of mtDNA haplotype II by the selected nuclear genetic variation (Kilpatrick and Rand 1995; Babcock and Asmussen 1996) or a functional coadaptation between nuclear and mitochondrial genomes (Clark and Lickegaard 1988; MacRae and Anderson 1988; Fos *et al.* 1990; Hutter and Rand 1995). It must be remarked (see Table 3) that there is statistical evidence not only of a linear trend favoring a mtDNA (haploid) fitness effect, but also of a significant nonlinear deviation that could be due to a fitness effect of a nuclear component on the haplotype trajectories.

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