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Linkage disequilibria between mtDNA haplotypes and chromosomal arrangements in a natural population of *Drosophila subobscura*

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The association between mtDNA haplotypes and chromosomal arrangements in a natural population of *Drosophila subobscura* from Calvia (Balearic Islands, Spain) was studied in order to search for linkage disequilibria, in an attempt to explain the populational dynamics of the mtDNA haplotypes of this species in nature. The presence of *Wolbachia* was not detected. Two main haplotypes (I and II) were found, as well as a series of less common ones. The Tajima *D*-test seemed to indicate some kind of seasonal population bottleneck. An analysis of linkage disequilibrium and factorial analysis of correspondences detected an association between haplotype I and the J_{ST} inversion and haplotype II and the J_1 inversion.
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Introduction

Drosophila subobscura is a Palaearctic species of the obscura subgroup of *Drosophila*, and is distributed all over Europe (except central and northern Scandinavia), the Middle East, northern Africa and the Atlantic islands of the Azores, Madeira, and the Canaries. In addition, it has recently colonized the American continent (Ayala *et al*, 1989, and references therein).

A consistent observation in studies of mitochondrial DNA evolution in Old and New World populations of this species for more than 15 years is the high prevalence of two widespread and almost equally frequent haplotypes (I and II) and the sporadic appearance of rare ones at a low frequency (Latorre *et al*, 1986; García-Martínez *et al*, 1998; Castro *et al*, 1999). The exception is the Canary Islands, where an endemic haplotype is the most frequent on some islands (Pinto *et al*, 1997). Moya *et al* (1993) studied the degree of differentiation at the nucleotide level for the two main haplotypes. Only three differences were found and they proved to be silent changes at the protein level; one of which corresponded to the *Hae*III restriction site that distinguishes both haplotypes which is located in the ND5 gene.

It is still an unresolved question which evolutionary forces account for the coexistence of the main haplotypes I and II in nature. In the literature, studies concerning the population genetic dynamics of mtDNA have often, although not always, shown the rejection of neutral patterns, mainly in humans and their associated commensal taxa (flies and mice) (see Gerber *et al* (2001) for a recent revision). In this way, Nachman *et al* (1994) in *Mus* species and Nachman *et al* (1996) in humans and chimpanzees, and Kennedy and Nachman (1998) in rats, have shown that mtDNA nucleotide sequences deviate from a neutral model, and this has been interpreted as being caused by a large class of mildly deleterious mutations. Taking into account the changes in the mtDNA haplotype frequencies in experimental populations and fitness estimates in *Drosophila*, MacRae and Anderson (1988, 1990) and Jenkins *et al* (1996) suggested that mtDNA evolves in a non-neutral fashion in *D. pseudoobscura*. Fos *et al* (1990) discuss the importance of positive natural selection in *D. subobscura* mtDNA haplotypes, but also consider the effect of the nuclear genetic background to be very important and, in conjunction with random drift, this dictates the final outcome where there are competing haplotypes. Kilpatrick and Rand (1995) failed to obtain evidence of selection in competition experiments between mitochondrial haplotypes in *D. melanogaster*, but their results were interpreted as being caused by a temporary hitchhiking of nuclear background on mtDNA. In an extensive report, Rand and Kann (1998) enlarged the study to non-commensal species (fishes, birds, bears) and they found evidence for both neutral and nonneutral patterns of diversity. In an experiment with the *D. subobscura* haplotypes I and II, García-Martínez *et al* (1998) observed the fixation of haplotype II over haplotype I in population cages. They rejected random drift as the force responsible for the observed changes in haplotype frequencies in these population cages, which contrasted with the stability found in nature. They also pointed out

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that the shift in mtDNA haplotype frequencies would not necessarily be caused by selection acting on mtDNA variants themselves, but also possibly by temporary hitchhiking of mtDNA haplotypes with selected nuclear genetic variation, or a functional co-adaptation between the nuclear and mitochondrial genomes. Castro *et al* (1999) studied the genetic structure of *D. subobscura* from different locations of Majorca and Minorca (Balearic Islands, Spain) by means of allozymes and mitochondrial DNA. In a cytonuclear disequilibrium analyses, they showed the difficulty of detecting significant cytonuclear associations in natural populations because these turned out mainly to be transient.

Chromosomal polymorphism for inversions of *D. subobscura* has been extensively studied both in Europe and in the recently colonized areas of North and South America (Krimbas, 1993; Prevosti *et al*, 1988). The geographical pattern of latitudinal variation for most of the gene arrangements is the same in these three areas, which shows the adaptive nature of this polymorphism, which is mainly mediated by temperature. This adaptation to climatic conditions has also been detected by temporal changes in inversion frequencies (Orengo and Prevosti, 1996). Therefore, it is interesting to consider the possible relation between the mtDNA haplotypes and chromosomal arrangements in terms of hitchhiking, because if an association exists and is selectively maintained, it could help to understand the dynamics of the different mtDNA haplotypes in nature.

For these reasons, the main purpose of the current study was to test any association between inversion polymorphism and the mtDNA haplotypes in a natural population of *D. subobscura*.

Materials and methods

Two hundred and one isofemale lines of *D. subobscura* were sampled from a pine forest near Calvia (Island of Majorca) in the spring of 1997. The flies were collected with conventional traps using fermented bananas. Once in the laboratory, the females were placed individually in vials with food and kept in an incubator at 19°C. When the F_1 larvae appeared, the offspring were used to determine the maternal mitochondrial haplotypes. Males from the F_1 larvae were chosen to analyse the chromosomal arrangements. In this way, from each isofemale line, information was obtained about the mtDNA haplotype and the inversions of one chromosome set.

Extraction and digestion of mtDNA
An enriched fraction of mtDNA was obtained using the method described by Latorre *et al* (1986). This fraction was digested with five restriction enzymes. Three of the enzymes (*Eco*RI, *Eco*RV and *Hin*dIII) recognize sequences of 6 bp, whereas the other two (*Hpa*II and *Hae*III) sequences of 4 bp. These enzymes were selected for their ability to detect mtDNA polymorphisms (Castro *et al*, 1999, and references therein). The fragments obtained by digestion were separated on horizontal 1–2% agarose gels. To determine fragment size, DNA molecular weight markers (II, IV and VI) (Roche) were used as size standards. After electrophoresis, gels were stained with 0.1 μg ethidium bromide mL^{-1} and visualized with a 260 nm UV light transilluminator. A mtDNA restriction map was obtained by means of all possible single and double digestions of the mtDNA. The different restriction patterns obtained, using a given enzyme and the haplotypes, were named according to the notation of Latorre *et al* (1986, 1992) and Castro *et al* (1999).

Inversion polymorphism analysis
A male of each isofemale line was individually crossed with females from the laboratory strain *chcu*, which is homokaryotypic for all the chromosomes (A_{ST}, J_{ST}, E_{ST}, O_{3+4}, U_{ST}). The polytene chromosomes of one third instar female larvae were stained and squashed in lacto-aceto-orcein solution.

Statistical analyses
Tajima's *D*-test (Tajima, 1989) was used to test for any departure from neutrality for the mtDNA haplotype distribution in the population. The rationale of the test is that in a panmictic population, under the neutral mutation model, no difference would be expected between the average number of nucleotide differences (ie nucleotide heterozygosity) and the number of segregating sites, when the latter is adjusted by dividing by $\Sigma_{i=1}^{i=n-1} 1/i$, where *i* is the *i*th sequence and *n* the sample size.

To test for linkage disequilibrium between the chromosomal arrangements and the mtDNA haplotypes, the *D* and *D'* (Lewontin, 1964) parameters were used.

$$D_{ij} = p_{ij} - p_i p_j$$

where D_{ij} is the disequilibrium value between the *i* arrangement and the *j* haplotype; p_{ij} is the frequency of the combination *i* arrangement-*j* haplotype in the sample; p_i and p_j are the frequencies of the *i* arrangement and *j* haplotype in the sample, respectively.

$$D' = D/|D_{max}, \text{ where}$$

$$D_{max} = min\ [(1 - p_i)\ p_j,\ p_i\ (1 - p_j)] \text{ if } D > 0$$

$$D_{max} = max\ [-p_i\ p_j, -\ (1 - p_i)\ (1 - p_j)] \text{ if } D < 0$$

The statistical significance of the disequilibria was calculated by means of the Fisher's exact test for independence in a 2×2 contingency table (Sokal and Rohlf, 1995). To diminish any statistical artefacts in the significance that could arise when multiple comparisons are made the following were applied: (i) a sequential Bonferroni test (Rice, 1989) to correct the probabilities when many tests are carried out simultaneously; (ii) only those combinations giving expected numbers ≥5 in all cells were considered for the analysis. Moreover, the least frequent arrangement of each chromosome was omitted to allow for independence among the tests; (iii) only haplotype I was analysed individually, because the results for haplotype II are the same as for haplotype I, but with a change in sign.

The factorial analysis of correspondences (Arenas *et al*, 1997) was also used to test for any association between haplotypes I and II and the chromosomal arrangements.

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 following the methodology of García-Martínez *et al* (1998). In this method, 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. Moreover, two *D. subobscura* regions were used as positive controls for DNA amplifications: the V4–18S rDNA (a 427 bp fragment from the V4 variable region of the 18 S rDNA) and the ND5 mtDNA (a 984 bp fragment from subunit 5 of NADH dehydrogenase). Six lines (three of haplotype I and three of haplotype II) were used at random from the Calvia population. Since the determination was negative, the presence of *Wolbachia* in the population was excluded. Only a few lines of *D. subobscura* were used for two reasons: (i) this determination was the second one made on the island of Majorca. The first one was in a different population (Esporles) a few years ago, and was also negative (García Martínez *et al*, 1998); (ii) we have not detected any kind of cytoplasmic incompatibility in the population, such as embryo mortality or production of all-male progeny in crosses with different strains.

Results

The cleavage map of the restriction enzymes, based on the physical mtDNA map of *D. yakuba* (Clary and Wolstenholme, 1985) is shown at the bottom of Figure 1. As can be seen, the NADH and CO complex genes included most of the polymorphic sites. Table 1 shows the haplotypes found in the Calvia population. As in other populations, haplotypes I and II were the most frequent (95.5%), with haplotype II being more common than haplotype I. The less common haplotypes accounted for only 4.5%. The relationship between haplotypes I and II and the rarer ones is also indicated in Figure 1, which represents an unrooted phylogenetic tree of the 10 haplotypes of *D. subobscura* mtDNA. Three haplotypes were derived from haplotype I and five from haplotype II, which were caused by one or two mutations in the mtDNA. In the resolution of this tree, we followed Wagner's parsimony method of the MIX program included in the PHYLIP package (Felsenstein, 1993).

Tajima's *D*-test was used to detect departures of the mtDNA haplotypic distribution from the neutrality hypothesis. The *D* value was −1.629, which was not significant at the 5% level, but was at 10%, indicating an almost significant excess of rare haplotypes. Table 2 shows the chromosomal arrangement frequencies within haplotypes. Interestingly, the X haplotype had a rare arrangement in the E chromosome (E_{1+2+22}). The *D* and *D'* values, which detect linkage disequilibrium between the chromosomal arrangements and haplotype I, are presented in Table 3. As can be seen, a significant disequilibrium of haplotype I and inversion J_{ST} was detected. This result was confirmed and complemented by means of a factorial analysis of correspondences. This is a type of analysis based on principal components with χ^2 distance, which is appropriate for the representation of the contingency tables and, in our case, a simultaneous representation of the inversions and haplotypes was made in conjunction with the statistical significance of this association. As before, the only significant difference was found with the J chromosome inversions. A statistical association of haplotype I with the J_{ST} inversion and haplotype II with J_1 ($\chi^2_{(2)} = 10.3$, $P < 0.01$) was found.

Discussion

The same pattern as reported in previous papers was found with respect to the mtDNA haplotype distribution in the Calvia population (García-Martínez *et al*, 1998; Castro *et al*, 1999, and references therein). That is, we detected a high prevalence of haplotypes I and II and a series of less common ones derived from them both. Tajima's *D* value can help us to understand, at least partly, this result. The negative *D* obtained is compatible with certain kinds of selection, such as recent directional selection, and also with population bottlenecks. Although purifying selection might also generate a negative *D* value, it would be expected to be more intense. Therefore, another explanation, based on a population bottleneck seems to be more acceptable. It is possible that the population has not completely reached equilibrium as a consequence of the periodic seasonal bottlenecks (winter and summer), which reduce the effective population size, followed by an expansion and, as a result, an excess of the less common haplotype polymorphism during this expansion. This result, together with previous ones (eg, Fos *et al*, 1990; Castro *et al*, 1999), seem to indicate the important effect of random drift on mtDNA in experimental and natural populations. Thus, a possible explanation to account for the coexistent distribution of haplotypes I and II in nature would be that the dynamics of the frequencies of these haplotypes are determined by genetic drift in a population of large effective size. This fact would be reinforced because of the synonymous changes that differentiate the two haplotypes and make them phenotypically equivalent. However, the dynamics are not simple, and other evolutionary forces must be taken into account. For example, in population cages we have also found a clear selective advantage of haplotype II over haplotype I when they are competing together (García-Martínez *et al*, 1998). This could be a consequence not only of the selection acting directly on the haplotypes, but also of the hitchhiking of mtDNA haplotypes with some nuclear markers. To confirm this possible hitchhiking, a previous cytonuclear association experiment was carried out between nuclear markers (isozymes) and mtDNA haplotypes (Castro *et al*, 1998, 1999). In that experiment, 13 systems were studied and a disequilibrium associated with *Acph-2* was detected, although this could represent a transient disequilibrium established at the moment the samples were taken. In addition, it is possible that the choice of the nuclear markers was not the most appropriate one. The chromosomal polymorphism for inversions of this species is a much better candidate to use in this type of experiment due to its adaptive value (Prevosti *et al*, 1988; Orengo and Prevosti, 1996). The present study detected a significant association between haplotype I and the J_{ST} inversion and between haplotype II and the J_1 inversion. This association can hardly be attributed to a random event because of the large number of isofemale lines analysed and the accurate statistics used. Consequently, some kind of epistatic interaction could be considered to explain the equilibrium of haplotypes I and II found in nature. Nevertheless, some resampling should be carried out to confirm this association, since it is possible that it could seasonally disappear and reappear because it could be transient. If this epistatic interaction is real, it could explain the superiority of haplotype II over haplotype I detected by
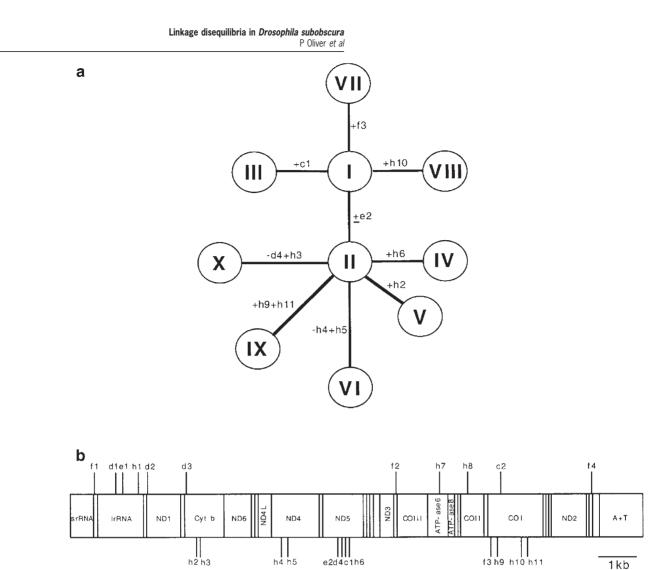
a

b

Figure 1 (**a**) Unrooted phylogenetic tree for the 10 mtDNA haplotypes of the Calvia population of *Drosophila subobscura*. The haplotypes are connected in a way that minimizes the total number of necessary site changes. The number of mutational steps is indicated on each branch. (**b**) *D. subobscura* mtDNA organization based on the genetic map of *D. yakuba* given by Clary and Wolstenholme (1985). Conserved sites are shown above and polymorphic sites below the map. Abbreviations for the genes are as follows: srRNA and lrRNA, small and large subunits of ribosomal RNA, respectively; ND1–6, subunits of the NADH dehydrogenase complex; Cytb, cytochrome *b*; COI-III, subunits of cytochrome oxidase; A+T, regulatory noncoding region. Each site is named with a letter, for each one of the restriction endonucleases, followed by a number corresponding to a specific restriction site. (d) *Eco*RI; (c) *Eco*RV; (e) *Hae*III; (h) *Hpa*II and (f) *Hind*III.

Table 1 Different haplotypes defined by the polymorphic sites in the sample of *Drosophila subobscura* from Calvia. Each site is named as in the restriction map. Presence or absence of a given polymorphic site is indicated by a plus (+) or a minus (−) sign, respectively

Haplotype	EcoRI d4	EcoRV c1	HaeIII e2	HindIII f3	HpaII h2	h3	h4	h5	h6	h9	h10	h11	No.	%
I	+	−	+	−	−	−	+	−	−	−	−	−	79	39.3
II	+	−	−	−	−	−	+	−	−	−	−	−	113	56.2
III	+	+	+	−	−	−	+	−	−	−	−	−	1	0.5
IV	+	−	−	−	−	−	+	−	+	−	−	−	2	1.0
V	+	−	−	−	+	−	+	−	−	−	−	−	1	0.5
VI	+	−	−	−	−	−	−	+	−	−	−	−	1	0.5
VII	+	−	+	+	−	−	+	−	−	−	−	−	1	0.5
VIII	+	−	+	−	−	−	+	−	−	−	+	−	1	0.5
IX	+	−	−	−	−	−	+	−	−	+	−	+	1	0.5
X	−	−	−	−	−	+	+	−	−	−	−	−	1	0.5
Total													201	100

No. = number of isofemale lines showing a given haplotype.

Table 2 Chromosomal arrangement frequencies within haplotypes of the *Drosophila subobscura* population. The sample size is 79 for haplotype I, 113 for haplotype II and nine for haplotypes III to X combined. The X haplotype had a rare arrangement in the E chromosome

Arrangement	Haplotype		
	I	*II*	*III-X*
A_{ST}	0.2911	0.3097	0.3333
A_1	0.0380	0.0354	0.1111
A_2	0.6709	0.6549	0.5556
J_{ST}	0.2911	0.1150	0.2222
J_1	0.5823	0.7699	0.4444
J_{3+4}	0.1266	0.1150	0.3333
U_{ST}	0	0.0088	0
U_2	0.0380	0.0531	0.1111
U_{1+2}	0.4684	0.4690	0.4444
U_{1+2+8}	0.4557	0.4159	0.4444
U_{2+13}	0.0380	0.0531	0
E_{ST}	0.1038	0.1239	0
E_{1+2}	0.1429	0.2124	0.1111
E_{1+2+9}	0.1169	0.0973	0
E_{1+2+22}	0	0	0.1111
$E_{1+2+9+3}$	0.0130	0.0088	0.1111
$E_{1+2+9+4}$	0.5584	0.4513	0.6667
$E_{1+2+9+12}$	0.0650	0.1062	0
O_{ST}	0.0641	0.0265	0.1111
O_{3+4}	0.2051	0.2301	0.1111
O_{3+4+1}	0.0256	0.0885	0
O_{3+4+2}	0.0128	0.0088	0.1111
O_{3+4+7}	0.0513	0.0354	0
O_{3+4+8}	0.4231	0.4248	0.4444
O_{3+4+17}	0.0513	0.0088	0
O_{3+4+22}	0.0128	0.0177	0.1111
$O_{3+4+23+6}$	0.1538	0.1593	0.1111

Table 3 Values of D and D' between chromosomal arrangements and haplotype I

	D	D'	P	P_{corr}
A_{ST}	−0.0045	−0.0362	0.8734	1.0000
A_2	0.0039	0.0278	0.8774	1.0000
J_{ST}	0.0426	0.3864	0.0026	0.0362
J_1	−0.0454	−0.2512	0.0069	0.0862
U_{1+2}	−0.0002	−0.0008	1.0000	1.0000
U_{1+2+8}	0.0096	0.0412	0.6574	0.9999
E_{ST}	−0.0048	−0.1027	0.8183	1.0000
E_{1+2}	−0.0168	−0.2245	0.2567	0.9617
E_{1+2+9}	0.0047	0.0752	0.8103	1.0000
$E_{1+2+9+4}$	0.0258	0.1261	0.1835	0.9122
$E_{1+2+9+12}$	−0.0099	−0.2743	0.4399	0.9970
O_{3+4}	−0.0060	−0.0672	0.7252	1.0000
O_{3+4+8}	−0.0004	−0.0024	1.0000	1.0000
$O_{3+4+23+6}$	−0.0013	−0.0205	1.0000	1.0000

P_{corr} = Probability corrected by the sequential Bonferroni test.

García-Martínez *et al* (1998) in population cages, because in this situation, it is possible that some kind of mtDNA-inversion interaction (with the same or different arrangements) could be established at the moment the experiments were carried out. Moreover, studies of the

fitness components for the haplotypes (I, II and the less common ones) could provide valuable information about their relative selective characteristics.

The association found between the haplotypes and the chromosome J arrangements might be related to the different physiological substrates. The J chromosome contains, among others, the allozyme loci *Est-3*, *Est-7* and *Aph* (Krimbas, 1993), and the enzymes coded by these loci can be included in a group established by Gillespie and Langley (1974) based on the capacity to make use of different physiological substrates. Differences between the two haplotypes might indicate a differential use of the physiological substrates for each haplotype and differences in the availability of these substrates according to seasonal variation.

Future work should include the study of the fitness components and life-history characters (ie viability and developmental time, longevity, resistance to desiccation, competition, etc) for all the haplotypes, as well as experiments with population cages, but controlling the arrangements, and the variation of the mtDNA at the nucleotide sequence level using additional I, II and rare haplotypes.

Acknowledgements

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