Dynamics of the mtDNA Haplotype Variability in a *Drosophila subobscura* Population Over a Two-Year Period

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Abstract: Restriction site analysis of mitochondrial DNA (mtDNA) was carried out on 607 isofemale lines, corresponding to monthly samples obtained over a two-year period, from a single geographic population of *Drosophila subobscura* to evaluate the possible changes in the action of the evolutionary forces with respect to the variation of the seasonal enviromental conditions. The haplotype distribution pattern was: (1) two highly frequent haplotypes and (2) sporadic haplotypes that were almost never found again after a population bottleneck; similar to that observed for the entire range of this species on the European continent. We detected significant negative *D*-values for the entire population, reflecting an excess of rare haplotypes. The results confirm that the coexistence of the main haplotypes in nature, and the rare ones, could be mainly caused by seasonal population expansions. However, the fact that the frequencies of the two major haplotypes are exchanged in some months, could indicate that the action of selection cannot be discarded, possibly acting upon gene arrangement associated with mtDNA haplotypes.

Keywords: mtDNA, haplotype variability, *Drosophila subobscura*.

INTRODUCTION

Drosophila subobscura is a Palaearctic species of the obscura subgroup of *Drosophila*. It is distributed over most of Europe, 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 [1, 2]. A consistent observation in studies of mitochondrial DNA (mtDNA) evolution in Old and New World populations of this species is the high prevalence of two widespread and almost equally frequent haplotypes (I and II) and the sporadic appearance of rare haplotypes that are generally present in not more than a single locality [3-8]. The exception is the Canary Islands, where an endemic haplotype (VIII) is the most frequent on some of the islands [9]. Formally, this variability distribution can be classified as a classical polymorphism, with a number of rare haplotypes within each population [10].

In recent years, different studies have tried to identify the evolutionary forces accounting for the widespread equidistribution of the two main haplotypes as well as the low frequency of the remaining haplotypes, and the relative roles played by random genetic drift and natural selection [5, 11]. Many studies concerning the genetic dynamics of mtDNA in *D. subobscura* indicate the existence of cytonuclear interactions between mtDNA and nuclear markers. Oliver *et al.* [8] found linkage disequilibria between mtDNA haplotypes and chromosomal arrangements, with haplotype I associated with the J_{ST} inversion and haplotype II with the J_1 inversion. When haplotypes I and VIII were allowed to compete in experimental populations, fixation depended on the nuclear genetic background with which these haplotypes were associated [12]. Similar experiments with haplotypes I and II (with heterogeneous nuclear genetic backgrounds) were not conclusive. Haplotype II prevailed in the study of García-Martínez *et al.* [11], but not that of Oliver *et al.* [13], in which both haplotypes coexisted after 33 generations. However, when the experiments aimed to examine the fitness and life-history traits of laboratory populations, haplotype II proved to have a selective superiority over haplotype I with respect to longevity, resistance to desiccation, and larval development time [14], whereas females mated to haplotype I males had more offspring [15]. In the same study, the mating pattern indicated assortative mating, with couples of the same haplotype, mainly those of haplotype I, mating more often. However, the significant differences detected under experimental conditions disappeared in wild populations, where random mating was the rule.

The results of other studies on *D. subobscura* populations indicate that the equidistribution of haplotypes I and II can be explained by random drift of selectively neutral mutants. Partial mtDNA sequencing (15%) only identified three silent substitutions, one of which corresponded to the *Hae*III restriction site that distinguishes both haplotypes. Moya *et al.* [5] considered the two haplotypes to be phenotypically equivalent, although they recognized that further sequencing could reveal certain differences between the two mtDNAs. González *et al.* [6] failed to detect significant seasonal changes in mtDNA variability in a *D. subobscura* popula-

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tion, contrary to what was found by DeSalle *et al.* [16] in a similar experiment with *Drosophila mercatorum*.

The main purpose of the present work was to evaluate the possible changes in the action of the evolutionary forces that could be acting on the population dynamics of the mtDNA haplotypes in the wild along the seasons and weather conditions. For this reason, we have studied the genetic structure of a natural Mediterranean population of *D. subobscura* in relation to mtDNA and the resolution of the dilemma regarding the temporal and geographical homogeneity of frequencies of the two most common haplotypes in this species, by studying their monthly variation over a two-year period. At the same time, the subsistence of the rare haplotypes was studied, comparing the results to those from previous studies on the same population.

MATERIALS AND METHODOLOGY

Drosophila Stocks

Mitochondrial DNA restriction site polymorphisms were analyzed in 607 isofemale lines of *Drosophila subobscura* from a wild population in Calvià (Balearic Islands, Spain). The isofemale lines were obtained during monthly captures in 2001 and 2002. Sampling was done at the beginning of each month and lasted from one day (when flies were abundant) to two weeks (when flies were scarce). The samples were collected at the time of day when conditions, such as temperature, were most favourable for *D. subobscura*. Each monthly capture resulted in a maximum of 50 female flies. The flies were collected with conventional traps containing fermented bananas. Once in the laboratory, females were placed individually into tubes with food and two male flies and maintained in an incubator at 19ºC.

mtDNA Analysis

Enriched fractions of mtDNA from each isofemale line were obtained by the methods described in Latorre *et al.* [17]. The mtDNA was digested with five restriction enzymes (*Hae*III, *Eco*RI, *Eco*RV, *Hin*dIII, and *Hpa*II). These enzymes were selected for their ability to detect mtDNA polymorphisms [17]. Digestion fragments were separated on horizontal slab 1–2% agarose gels containing 0.5 μg of ethidium bromide per ml, visualized with a 260-nm UV light transilluminator, and photographed when necessary. Fragment sizes were determined with DNA molecular mass markers (II, IV, and VI; Roche). An mtDNA restriction map was created based on all possible single and double digestions of the mtDNA. The different composite restriction patterns or haplotypes are named in accordance with Latorre *et al.* [17] and Castro *et al.* [7].

Statistical Analyses

The degree of mtDNA differentiation within and between populations (*Vw* and *Vb*), and the degree of population subdivision (N_{ST}) were estimated following the method of Lynch and Crease [18], with a computer program kindly supplied by the authors. The degree of mtDNA differentiation depends on the number of substitutions per nucleotide site between pairs of random haplotypes and is estimated according to the maximum likelihood estimator developed by Nei [19] (p. 104). The existence of 4- and 6-bp-specific enzymes was taken into account. To test the homogeneity of haplotype temporal distribution, the *V* statistic [16] (equation 2) was applied to the arcsin-transformed frequency of each haplotype. Under the null hypothesis of no temporal heterogeneity, the *V* statistic is distributed as a chi-square with *n-1* degrees of freedom, *n* being the number of populations sampled. Tajima's *D*-test [20] was used to test for any departure from neutrality for the mtDNA haplotype distribution in the different monthly and seasonal samples, as well as in the entire population. The rationale of the test is that in a panmictic population, under the neutral mutation model, no difference is expected between the average number of nucleotide differences and the number of segregating sites. Any departure of the frequency spectrum of variants from the neutral prediction will affect estimates of the latter, but not the former.

RESULTS

The cleavage map of the restriction enzymes, based on the physical mtDNA map of *D. yakuba* [21], is shown at the bottom of Fig. (**1**). Seventeen polymorphic sites and eleven conserved regions were found. As seen in the figure, genes encoding the NADH and CO complexes account for most of the polymorphic sites. One endonuclease, *Eco*RI, yielded the same restriction pattern in all the isofemale lines analyzed, while the restriction patterns obtained with the other four enzymes were polymorphic, ranging between 2 (*Eco*RV) and 12 (*Hpa*II). Table **1** shows the different polymorphic sites used to define each haplotype identified in this study. Based on the polymorphic sites distinguished by the four restriction enzymes, 23 composite restriction patterns or haplotypes were obtained. The relationship between haplotypes I and II and the rare haplotypes is depicted in Fig. (**1**), which shows an unrooted phylogenetic tree of the 23 detected haplotypes. Ten of the haplotypes were derived from haplotype I and 11 from haplotype II. All were caused by unique mutational events in the mtDNA; the exception was haplotype XVI, with two.

Table **2** shows, on a monthly, seasonal, and yearly basis, the number of isofemale lines of each haplotype between the years 2001 and 2002 (months in which no flies were captured are not represented), and Fig. (**2**) is a climograph with the superimposed absolute frequencies of the haplotypes (the rare haplotypes being grouped)**.** Few or no isolines were obtained in some months due to adverse weather conditions for *D. subobscura*. Flies were captured in summer 2002 due to an unusual wet season, but not in summer 2001 due to the hot and dry weather that is typical of the Mediterranean climate during this season. Of the 607 analyzed isolines, 272 (44.81%) proved to be of haplotype I, 311 (51.23%) were haplotype II, and only 24 (3.95%) could be assigned to the other 21 haplotypes. Rare haplotypes were usually detected in a single isofemale line, with the exceptions being haplotypes XII (with two) and XVII (with three, and detected in both years). As found in other populations [4, 8], haplotypes I and II were highly predominant and the remaining haplotypes were derived from them, appearing at low frequencies. It is interesting to note that none of the rare haplotypes identified in this study were found in the first sample at the same location in 1997 by Oliver *et al.* [8].

No evidence of temporal heterogeneity in haplotype frequency was found when the *V* statistic [16] was applied to

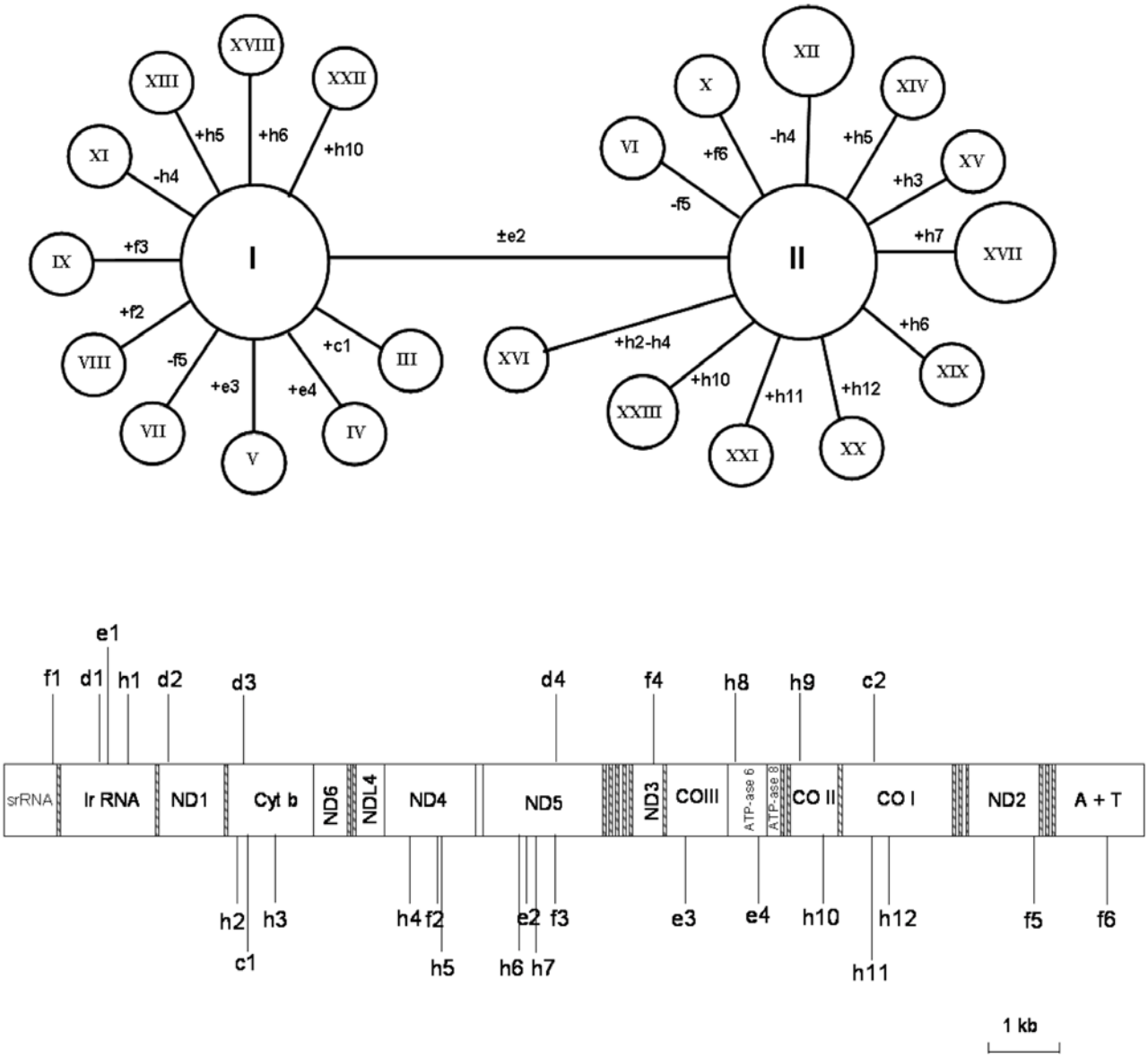

Fig. (1). *Top*: Unrooted phylogenetic tree for the 23 haplotypes of *D. subobscura* identified between 2001 and 2002 in Calvià (see Table **1**). The haplotypes are connected in a way that minimizes the number of site changes. The number of mutational steps is indicated on each branch. *Bottom*: *D. subobscura* mtDNA organization based on the genetic map of *D. yakuba* reported by Clary and Wolstenholme [21]. 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; 8, ATPase 8; A+T, regulatory noncoding region. Each site is named with a letter, for each 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.

the two-year period. *V* was 17.3 for haplotype I and 14.7 for haplotype II. As the remaining haplotypes were sporadic, they were pooled into one group, with a *V*= 12.8 (23 degrees of freedom). Similar and non-significant results were recorded for 2001 and 2002.

Table **3** shows the mtDNA differences between samples pooled by seasons. The total amount of mtDNA polymorphism can be estimated by the sum of the average number (V_w) of substitutions per nucleotide site for random pairs of haplotypes from the same population and the average number (V_b) between populations. Most of the observed variation in our data was concentrated within the seasonal samples (V_w = 0.00431 ± 0.00384), whereas the between-samples variation was very low (V_b = –0.00002 ± 0.00007). Additionally, the fraction of the nucleotide variation between samples, N_{ST}, was 0.006 ±0.081, which was not significantly different from zero, indicating between-samples homogeneity. The monthly and yearly data gave very similar results and can be obtained from the senior author upon request.

Table **4** gives the estimates of Tajima's *D*-test [20] for the monthly samples and for samples pooled by seasons and years. Positive *D*-values were recorded for the spring months in both years and in the autumn months of 2001, while in the remaining months the *D*-values were negative. With respect to samples pooled by seasons and years, the *D*-values were always negative except in autumn 2001. Only when all the

Table 1. The Different Haplotypes, Defined by the Polymorphic Sites, and their Corresponding Number of Isofemale Lines from the *D. subobscura* Calvià Population Between 2001 and 2002. Each Site is Named as in the Restriction Map (Fig. 1). The Presence or Absence of a Given Polymorphic Site is Indicated by a Plus (+) or Minus (–) Sign, Respectively

	*Eco*RV	*Hae*III			*Hind*III				*Hpa*II									
Haplotype	**c1**	**e2**	**e3**	**e4**	**f2**	**f3**	**f5**	**f6**	**h2**	**h3**	**h4**	**h5**	**h6**	**h7**	**h10**	**h11**	**h12**	**Total**
I	–	+	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	272
II	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	311
III	+	+	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	1
IV	–	+	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	1
V	–	+	+	–	–	–	+	–	–	–	+	–	–	–	–	–	–	1
VI	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	1
VII	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	1
VIII	–	+	–	–	+	–	+	–	–	–	+	–	–	–	–	–	–	1
IX	–	+	–	–	–	+	+	–	–	–	+	–	–	–	–	–	–	1
X	–	–	–	–	–	–	+	+	–	–	+	–	–	–	–	–	–	1
XI	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	1
XII	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	2
XIII	–	+	–	–	–	–	+	–	–	–	+	+	–	–	–	–	–	1
XIV	–	–	–	–	–	–	+	–	–	–	+	+	–	–	–	–	–	1
XV	–	–	–	–	–	–	+	–	–	+	+	–	–	–	–	–	–	1
XVI	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	1
XVII	–	–	–	–	–	–	+	–	–	–	+	–	–	+	–	–	–	3
XVIII	–	+	–	–	–	–	+	–	–	–	+	–	+	–	–	–	–	1
XIX	–	–	–	–	–	–	+	–	–	–	+	–	+	–	–	–	–	1
XX	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–	+	1
XXI	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–	+	–	1
XXII	–	+	–	–	–	–	+	–	–	–	+	–	–	–	+	–	–	1
XXIII	–	–	–	–	–	–	+	–	–	–	+	–	–	–	+	–	–	1

flies were pooled the data allowed to reject the hypothesis of neutrality.

DISCUSSION

In the present work, the mtDNA structure diversity of a *D. subobscura* population was determined and the information applied with the goal of deepening in the evolutionary forces implicated to solve the dilemma of the temporal and geographical homogeneity in frequencies of the two main haplotypes in nature, and the distribution of the rare ones. Although sampling proved arduous or non-productive in most winter and summer months due to adverse weather conditions for *D. subobscura*, the results indicate at least one important seasonal bottleneck in summer 2001 (Fig. **2**). There are references to population bottlenecks due to cold winters [4, 6], but our experience suggests that dry summers are the main reason for the depletion of Mediterranean populations. *D. subobscura* is found as far north as Scandinavia, and in the coldest months we observed the flies on the bait (at 10–13ºC they would hop and walk, but not fly); however, when the weather was hot and dry they were completely absent.

The degree of mtDNA differentiation within and between monthly samples (*Vw* and *Vb*) as well as the degree of population subdivision (N_{ST}) gave no significant results, indicating population homogeneity. Furthermore, the use of the *V* statistic did not yield evidence of temporal heterogeneity in haplotype frequency. These results, jointly with the negative *D*-values for the entire population, support an excess of low-frequency polymorphisms in the population. This excess of rare haplotypes could arise due to recovery from a recent selective sweep (the sweep of a single allele followed by the recovery of single mutations), but this explanation seems unlikely due to the presence of the two main equally frequent

Table 2. The Number of Isofemale Lines Showing a Given Haplotype as Determined in the *D. subobscura* Population in Calvià Between 2001 and 2002. Results are Given by Month, Season, and Year. Observe that Few or no Isolines were Obtained in Some Months Due to Adverse Weather Conditions for *D. subobscura*. Months in which no Flies were Captured are not Represented

Haplotype	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI	XXII	XXIII	Total
Group																								
Feb. 01	20	30				1		1					1											53
Mar. 01	25	28	1								1					1								56
Winter 2001	**45**	**58**	**1**			**1**		**1**			**1**		**1**			**1**								**109**
Apr. 01	20	32																		1				53
May 01	21	29																				1	1	52
June 01	17	8															1							26
Spring 2001	**58**	**69**															**1**			**1**		**1**	**1**	**131**
Summer 01																								
Nov. 01	4	9																						13
Dec. 01	5	5																						10
Autumn 01	**9**	**14**																						**23**
Total 2001	**112**	**141**	**1**			**1**		**1**			**1**		**1**			**1**	**1**			**1**		**1**	**1**	**263**
Jan. 02	2	0																						2
Feb. 02	24	26			1				1															52
Mar. 02	23	24										2		1										50
Winter 2002	**49**	**50**			**1**				**1**			**2**		**1**										**104**
Apr. 02	21	30																						51
May 02	28	23															1							52
June 02	27	23																1						51
Spring 2002	**76**	**76**															**1**	**1**						**154**
July 02	0	1															1							2
Aug. 02	19	28		1						1					1						1			51
Summer 02	**19**	**29**		**1**						**1**					**1**		**1**				**1**			**53**
Oct. 02	0	1																						1
Nov. 02	16	14					1												1					32
Autumn 02	**16**	**15**					**1**												**1**					**33**
Total 2002	**160**	**170**		**1**	**1**		**1**		**1**	**1**		**2**		**1**	**1**		**2**	**1**	**1**		**1**			**344**
Total	**272**	**311**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**1**	**1**	**1**	**607**

haplotypes in the population; or from population expansions after a bottleneck in summer and winter; process repeated over time. This last explanation seems to be the most plausible to explain the distribution of haplotypes I and II and the rare ones.

Can we discard the action of selection? In both 2001 and 2002, in general haplotype II was more numerous than I, but II was clearly surpassed in number by haplotype I in June 01 and May and June 02 (Table **2**, Fig. **2**). González *et al.* [6] did not find temporal heterogeneity either but, in their study, although haplotype I was always less frequent than haplotype II, the difference between them was lowest in the spring samples. Our work has similarities with González *et al.* [6], but we have improved it mainly in the fine dissection of the temporal samples (monthly), the sample size (607) and the knowledge of climatic conditions. In spite of the lack of statistical significance our results could reflect selection due to environmental heterogeneity. For example, in spring, there might be a factor favouring haplotype I. Note that in the spring months there is a conjunction of humidity and optimum temperature for the species (19ºC, Fig. **2**). That the superiority of one haplotype could be counterbalanced by the other in different situations, which would explain the haplotype proportions found in nature, was pointed out by Castro *et al.* [15] and Christie *et al.* [14]. Both studies noted that one haplotype proved more efficient than the other depending on the fitness or life-history trait studied. These authors also suggested that differences in adaptation could be promoted by the differential effects of selection acting on the two haplotypes, either directly on the mtDNA or by selective co-adaptation between the nuclear and mtDNA genomes (epistatic selection and/or hitchhiking on mtDNA haplotypes). Seasonal changes in gene arrangement frequencies have been extensively reported in the literature [22-24] and transient linkage disequilibria between mtDNA haplotypes and chromosomal arrangements were recently described [8, 13]; therefore, the most plausible explanation would not be direct selection on mtDNA but epistatic selection. Furthermore, the demographic structural characteristics of *D. subobscura* have pointed to significant seasonal differences [25]. Since these differ remarkably throughout the year, the monthly analysis has been very important to characterize the demographic parameters of each haplotype, as one haplotype could effectively be favoured momentarily to the detriment of the other.

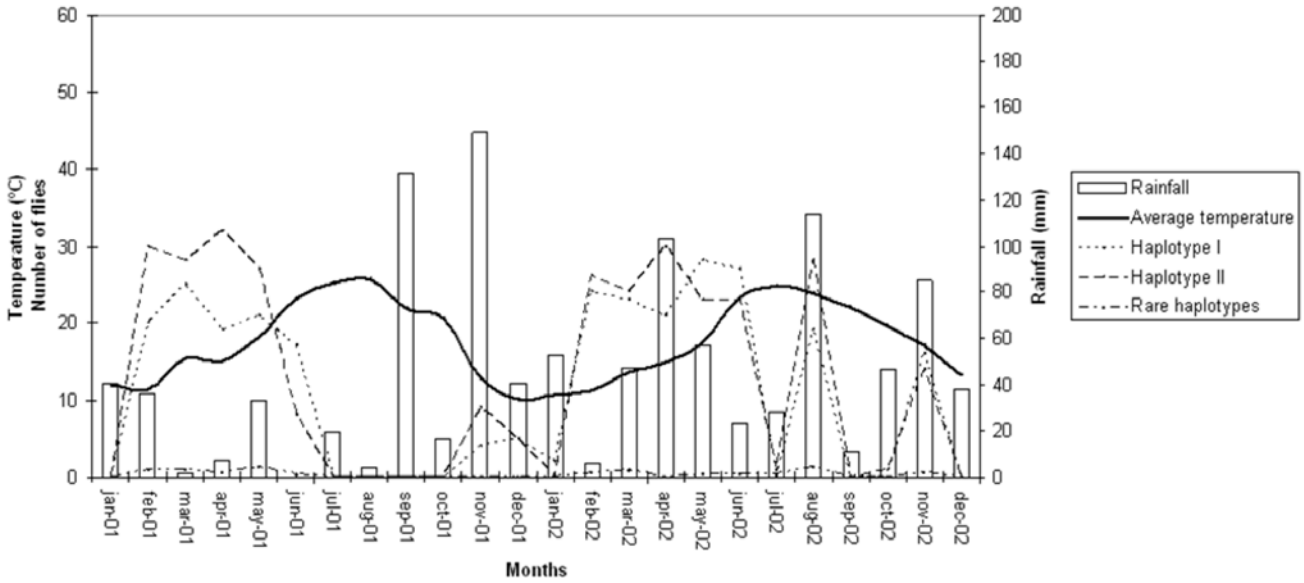

Fig. (2). Climograph corresponding to Calvià between 2001 and 2002, with the superimposed absolute frequencies of the haplotypes (the rare haplotypes are grouped)**.** Few or no isolines were obtained in some months due to adverse weather conditions for *D. subobscura*.

Table 3. mtDNA Differentiation ($\times 10^{-5}$) in *D. subobscura* within and Between Seasons (Standard Errors in Parentheses). Values on the Main Diagonal are the Within-Population Differentiation (*Vw*), and those Above the Diagonal the Between-Populations Differentiation (*Vb*)

	Winter 01	Spring 01	Autumn01	Winter 02	Spring 02	Summer 02	Autumn 02
Winter 01	462 (384)	-3 (389)	-7 (400)	-3 (380)	-1 (412)	-1 (325)	-4 (367)
Spring 01		409 (378)	-7 (428)	-2 (402)	-1 (434)	-1 (354)	-4 (389)
Autumn 01			365 (376)	-2 (421)	0 (455)	-11 (359)	-2 (411)
Winter 02				441 (392)	-3 (418)	+4 (350)	-7 (372)
Spring 02					387 (377)	+6 (382)	-7 (402)
Summer 02						487 (390)	+5 (341)
Autumn 02							469 (404)

What happens to the less common haplotypes? None of our rare haplotypes correspond to those reported in the study by Oliver *et al.* [8] (done at the same location in 1997), even though our study is the most extensive to date and is based on the largest number of *D. subobscura* isolines analyzed thus far. Nonetheless, we must admit that unsuccessful re-sampling of those rare haplotypes reported by Oliver *et al*. [8] could also have been due to their extremely low frequencies. Furthermore, with respect to the rare haplotypes described herein, it is interesting to note that while the five restriction enzymes yielded 17 polymorphic sites, a total of 21 haplotypes were obtained, 10 derived from I and 11 derived from II (Table **1**, Fig. **1**). This is because some of the haplotypes shared the same rare polymorphic site for one restriction enzyme, i.e., *Hin*d III or *Hpa*II, but differed in the polymorphic site for *Hae*III that distinguishes haplotype I from haplotype II. This is not a new observation, because while in this study there were five such cases (Table **1**), other cases are documented in the literature. There is at least one such case in Afonso *et al.* [3], one in García-Martinez *et al.* [11], another in González *et al*. [6], and five in Castro *et al.* [7], four of which were detected within the same neighbouring populations.

CONCLUSION

We can say that the most parsimonious explanation for the populational dynamics of the major mtDNA haplotypes over time in natural populations is by means of population size fluctuations due to periodic seasonal bottlenecks followed by expansions. However, the fact that the frequencies of the two major haplotypes are exchanged in some months could indicate that the action of selection cannot be discarded. Epistatic selection due to environmental heterogeneity (seasonal changes) could act upon the gene arrangement frequencies associated with the mtDNA haplotypes. These results confirm what was previously established in others papers. In addition, rare haplotypes that appear locally and are expected to accumulate with time instead mainly become extinct due to bottlenecks, but also to selection when non-silent changes at the protein level are involved.

Table 4. Estimates of *D* Values and their Significance, According to Tajima [20], for the *D. subobscura* Population in Calvià Between 2001 and 2002. Note that a Minimum of 3 Individuals Must be Used; Accordingly, Samples with Fewer Individuals are not Represented

Sample	Sample size	*D*
Feb 01	53	-0.695
Mar 01	56	-0.561
Apr 01	53	0.303
May 01	52	0.521
Jun 01	26	0.096
Nov 01	13	0.950
Dec 01	10	1.464
Feb 02	52	-0.238
Mar 02	50	-0.137
Apr 02	51	1.635
May 02	52	0.410
Jun 02	51	0.398
Aug 02	51	-1.017
Nov 02	32	-0.328
Winter 2001	109	-1.221
Spring 2001	131	-0.450
Autumn 2001	23	1.433
Winter 2002	104	-0.790
Spring 2002	154	-0.017
Summer 2002	53	-1.246
Autumn 2002	33	-0.319
Year 2001	263	-1.455
Year 2002	344	-1.580*
2001+2002	607	-2.252**

*: $p<0.10$.
**: $p<0.01$.

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