

Divergent evolution of molecular markers during laboratory adaptation in *Drosophila subobscura*

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Abstract The impact of genetic drift in population divergence can be elucidated using replicated laboratory experiments. In the present study we used microsatellite loci to study the genetic variability and differentiation of laboratory populations of *Drosophila subobscura* derived from a common ancestral natural population after 49 generations in the laboratory. We found substantial genetic variability in all our populations. The high levels of genetic variability, similar across replicated populations, suggest that careful maintenance procedures can efficiently reduce the loss of genetic variability in captive populations undergoing adaptation, even without applying active management procedures with conservation purposes, in organisms that generate a high number of offspring such as *Drosophila*. Nevertheless, there was a significant genetic differentiation between replicated populations. This shows the importance of genetic drift, acting through changes in allele frequencies among populations, even when major changes in the degree of genetic diversity in each population are not involved.

Keywords Domestication · Genetic drift · Effective population size · Captivity · Microsatellites · Divergent evolution

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Introduction

Random genetic drift is a powerful mechanism that produces evolutionary changes at the population level (Crow and Kimura 1970). It is a stochastic process associated with the sampling of a finite number of gametes during reproduction of the individuals in a population. This causes random allele frequency changes between generations, at a rate that is dependent on the number of effective breeders (i.e. effective population size) (Falconer and Mackay 1996). As a consequence of these sampling effects across generations, some alleles will eventually be lost while others may become fixed leading thus to a loss of genetic variability within populations. Also, the fate of these alleles will vary among populations leading to progressive genetic differentiation between them (Hartl and Clark 1989).

The loss of genetic variability is particularly important in small and confined populations, because the effects of genetic drift are not effectively counteracted by the input of new alleles due to either mutation or migration. Several conservation studies have tried to deal with this issue, developing strategies to maximize the genetic diversity in captive populations (e.g. see Caballero and Toro 2000; Frankham 2005; Rodríguez-Ramilo et al. 2006). These include, for example, procedures aiming to equalise family contributions (i.e. to increase the effective population size) or managing these contributions to minimize global coancestry between parents (when pedigree information is available).

Laboratory studies can be useful to analyse an evolutionary process in action and, through the study of the changes in the genetic composition of different populations in a temporal perspective, allow addressing the impact of genetic drift in both genetic diversity and population

differentiation. Also, through previous knowledge of the history of the populations under analysis it is possible to disentangle the effects of different evolutionary mechanisms (genetic drift, founder effects, population size, selection, migration etc.), contrary to most studies in natural populations (Chippindale 2006; Morgan et al. 2005).

Several recent empirical studies in laboratory populations have focused on how different population sizes affect the rates of genetic diversity decline (e.g. see England et al. 2003; Montgomery et al. 2000; Rodríguez-Ramilo et al. 2006). Fewer studies in laboratory populations have analysed the long-term effects of genetic drift upon population differentiation as populations adapt to a common environment (but see Morgan et al. 2003; Simões et al. 2008). In spite of this lack of studies, it is a known fact that genetic drift should be taken into account in experimental evolutionary studies, shown by the need of having replicate populations in all studies (Rose et al. 1996). But how much genetic differentiation actually occurs between these populations? This is a relevant question both at a scientific and methodological level, and it is surprising that so few studies have addressed it, given the huge amount of biological material available to tackle this issue (e.g. for reviews of experimental evolution studies see Chippindale 2006; Prasad and Joshi 2003).

Analysis of laboratory-controlled populations of known history also allows estimating the effective population size (N_e). The effective population size is a key parameter in both population and quantitative genetics that determines the strength of genetic drift and all the genetic consequences associated with it (Falconer and Mackay 1996; Frankham et al. 2002; Wang and Caballero 1999; Wang 2005). Therefore, the maintenance of a high effective population size is a priority in conservation programmes of captive populations (Caballero and Toro 2000; Frankham et al. 2002). Often there is no knowledge on the effective size of populations, and the use of census size as proxy may be quite misleading. Most studies have reported low ratios of effective sizes relative to census sizes for both laboratory and natural populations (e.g. Briscoe et al. 1992; Frankham 1995). This is due to the fact that N_e can be reduced by diverse aspects such as fluctuations in population size over generations, variation in family size, differences in sex ratio, mating system, selection, etc... (Caballero 1994; Frankham 1995; Wang 2005). Frankham (1995) reported an average of 0.11 for N_e/N ratios in natural populations, with a wide range of values (from nearly 0 to 0.6), probably due both to the diverse influence of these factors and to lack of precision of the estimates, particularly in natural populations (see Frankham et al. 2002). Laboratory studies give the possibility to determine both effective and census sizes more accurately than in natural populations and thus can be a valuable tool to

empirically estimate N_e/N ratios and evaluate the effects of different factors that might affect them.

In the present study we quantify the levels of genetic variability and differentiation of laboratory populations derived from a common ancestral natural population after prolonged evolution in the laboratory environment. We also search for a possible signature of selection in the molecular genetic patterns presented by our laboratory adapting populations. Specifically, we characterize 9 microsatellite loci in three laboratory replicate populations of *Drosophila subobscura* after 49 generations of adaptation to the laboratory (NW populations). We compare the data obtained in these populations with another set of populations (TW populations) recently introduced in the same environment and collected from the same natural location (see Matos et al. 2004), using the same set of microsatellite loci. These recently introduced populations are used as a proxy measure of initial genetic variability close to laboratory foundation.

The specific aims of our study are: (1) to analyse the genetic variability in populations evolving for 49 generations in a constant and common environment, as measured by molecular markers; (2) to study the genetic differentiation in these laboratory populations; (3) to estimate the effective population sizes as well as their ratio relative to census sizes; (4) to analyse past demographic history, testing for possible evidences of demographic reduction in these laboratory adapting populations.

Materials and methods

Foundation and maintenance of the laboratory populations

In March 1998 the NW laboratory population of *Drosophila subobscura* was founded with 300 females and 280 males collected from a pinewood near Sintra, Portugal. Once in the laboratory, groups of females and males were put together in single vials and all eggs were collected to ensure the first laboratory generation (F1). Collections of eggs laid by the first generation females were split, giving rise to three replicate populations from the second generation on, referred to as NW₁₋₃ (Matos et al. 2002). In October 2001, an additional foundation (called TW) was carried out, from the same place, with 110 females and 44 males. Laboratory establishment and subsequent replication procedures for the TW populations were similar to those described above for the NW populations. From the beginning all populations were maintained in similar conditions, e.g. discrete generations of 28 days, reproduction at a young age (around 7–10 days after emergence) with controlled temperature (18°C). Flies were kept in vials of

31.4 cm³ controlling adult densities at around 50 individuals per vial and larval densities around 80 per vial. At each generation, emergences from the 24 vials within each replicate population were randomised using CO₂ anaesthesia. Adult population sizes ranged, in general, between 600 and 1,200 individuals (see Matos et al. 2002; Simões et al. 2007 for further details).

Microsatellite analysis and genotyping methods

The three recently introduced TW replicate populations (TW_{1–3}) were analysed after three generations of laboratory culture (corresponding to the first generation of reproductive isolation after the derivation of the three replicates). The three NW replicate populations (NW_{1–3}) were assayed at their 49th generation in the lab (47 generations of reproductive isolation after the derivation of the three replicates at the second generation). Approximately 50 randomly chosen females were analysed for each of the six populations mentioned above.

Nine microsatellite loci (*dsub01*, *dsub02*, *dsub05*, *dsub10*, *dsub19*, *dsub20*, *dsub21*, *dsub23* and *dsub27*) of known chromosomal location (Santos et al. 2010) were analysed in this study. These markers were first identified and characterized by Pascual et al. (2000, 2001) and were chosen for this study due to their high polymorphism (result obtained after performing an initial survey with 30 individuals) and coverage of the genome (all chromosomes were represented; see chromosomal location in Table 3). Highly polymorphic loci were chosen as they are likely to be more informative considering the objectives of our study, i.e. they allow the analysis of the molecular genetic changes of a given population during long-term laboratory evolution and also facilitate detecting the possible differences between the populations studied. Loci *dsub05*, *dsub19* and *dsub21* are X-linked.

Single fly genomic DNA was obtained using the extraction protocol described by Gloor et al. (1993). The forward primer of each locus was end-labelled with 6-Fam, Hex or Ned. All 9 loci were amplified using four different PCR reactions: *dsub02* + *dsub05*; *dsub10*; *dsub20* + *dsub21* + *dsub27*; *dsub01* + *dsub19* + *dsub23*. The amplification reactions were conducted in a total volume of 25 µl with 2.5 pmol of each primer, 3 µl dNTP's (1 mM), 2.5 µl 10× buffer, 1 U *Taq* polymerase and 1 µl of DNA solution.

All reactions were performed on an ABI GeneAmp PCR System 2700 thermocycler using the following program: 5 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 54°C and 30 s at 72°C; a final step of 5 min at 72°C. The alleles were sized on an ABI PRISM 310 sequencer (Perkin-Elmer) using ABI GeneScan-500 ROX as an internal standard.

Microsatellite data analysis

The genetic variability in each locus and population was measured as the number of alleles, expected heterozygosity and observed heterozygosity using MSA software, version 4.05 (Dieringer and Schlötterer 2003). Tests for differences among replicate populations from the same foundation were carried out on these genetic parameters using Friedman ANOVA. To test for differences between NW and TW populations, Wilcoxon matched pairs tests were performed using as paired data the average estimate of each variability measurement, for each locus and set of replicate populations. These analyses were performed using Statistica 8.0.

To determine genetic differentiation between all population pairs, values of F_{st} (Weir and Cockerham 1984) were calculated using FSTAT v2.9.3.2 (Goudet 2001). The significance of pairwise F_{st} values was estimated by permuting genotypes among populations 10,000 times. To account for multiple testing, the sequential Bonferroni correction was applied (Rice 1989). We also tested the hypothesis that genetic differentiation, as measured by global F_{st} values, was higher among the NW than among the TW set of populations using a permutation test (10,000 iterations) in the FSTAT software. In addition to the estimation of F_{st} values, another measure of genetic differentiation— G'_{st} —was also used. G'_{st} is a standardized measure of differentiation proposed by Hedrick (2005) in order to remove the problematic effects of the dependence of F_{st} values relative to the levels of genetic variation. G'_{st} pairwise values between populations were estimated using the SMOGD (Crawford 2009).

A hierarchical analysis of molecular variance (locus by locus AMOVA) was performed to analyse the distribution of the total genetic variance due to three different sources of variation: between groups (NW and TW), between populations/within groups and within populations. These analyses were carried out using ARLEQUIN, version 3.11 (Excoffier et al. 2005).

Effective population sizes (N_e) for each NW population were estimated from microsatellite data using two methodologies: the loss of heterozygosity method (see Crow and Kimura 1970) and a pseudo-likelihood approach (Wang 2001). Using the loss of heterozygosity method, the effective population sizes (N_e) for each NW population were estimated using the formula

$$\frac{H_t}{H_0} = \left(1 - \frac{1}{2N_e}\right)^t$$

(Crow and Kimura 1970). The data used were the expected heterozygosity of NW populations at generation 49 (H_t) and the expected heterozygosity of TW populations at generation 3 (H_0). N_e was calculated substituting t by 46,

i.e. the number of generations elapsed between the two sets of populations (considering the TWs as a surrogate of the NWs at the third laboratory generation). For each NW population we estimated N_e using as initial heterozygosity the pooled estimate of the three TW populations. The estimation of effective population sizes using a pseudo-likelihood approach was obtained with the MLNE software (Wang 2001) using the same body of data mentioned above. The pseudo-likelihood approach uses the changes in allele frequencies in temporal samples to estimate the average effective population size in the interval considered assuming genetic drift as the only factor causing these changes (Williamson and Slatkin 1999). All analyses were performed allowing a maximum N_e value of 1,000. N_e was calculated for each NW population assuming that TW populations represent, at an acceptable degree, the allele frequencies that NW populations had at their generation 3.

We also analysed the demographic history of our laboratory populations specifically searching for signs of possible past bottlenecks. We used two different approaches: the BOTTLENECK test (Cornuet and Luikart 1996) and the M ratio (Garza and Williamson 2001). The first approach compares the observed heterozygosity with the heterozygosity expected given the observed number of alleles in each population. Since the number of alleles is more affected than the heterozygosity during a bottleneck, populations that have suffered a reduction in population size are expected to present an excess of heterozygosity (relative to that expected considering their number of alleles). We assumed a two-phase model of microsatellite mutation (TPM) for our microsatellite data, which seems more adequate to explain the evolution of microsatellite loci in *Drosophila subobscura* (see Pascual et al. 2001). The model parameters included 20% of multistep changes (80% single-step mutations), and 5,000 iterations. Setting the frequency of multi-step mutations at either 15 or 30% provided similar results.

The M ratio, as described by Garza and Williamson (2001) was also estimated. M is the ratio of the total number of alleles for a given locus relative to its range in allele size. M was thus calculated for each locus and then averaged across loci for a given population. Significance of the M value was then tested relative to a critical value M_c estimated such that 95% of the simulations of an equilibrium population had $M > M_c$. We used here as critical M_c the value of 0.68 which corresponds to the simulated value for an equilibrium population with the following parameters: $P_s = 90\%$ (proportion of one-step mutations), $\Delta_g = 3.5$ (average size of multi-step mutations), $\theta = 10$ and sample size of 50 individuals (see Figs. 7 and 8 of Garza and Williamson 2001). In our study, a θ value ($=4N_e\mu$) of 10 can be considered as a good proxy of the variability of an equilibrium population. We considered

the range of μ estimates in *D. melanogaster* (9.3×10^{-6} , according to Schug et al. (1998); and 5.1×10^{-6} , according to Vazquez et al. (2000)) and the historical N_e estimated for a population of *D. subobscura* sampled in Barcelona ($N_e = 310.913$; see Pascual et al. 2001).

To test for possible signs of selection two different statistical approaches were applied to our microsatellite dataset. First we computed the LnRH test (Schlötterer 2002; Kauer et al. 2003) based on the ratio of expected heterozygosities between two populations:

$$\text{LnRH} = \text{Ln} \left[\frac{\left(\frac{1}{1-H_{\text{pop1}}} \right)^2 - 1}{\left(\frac{1}{1-H_{\text{pop2}}} \right)^2 - 1} \right]$$

The rationale of this test is that microsatellite loci located in regions under positive selection will present decreased variation relative to neutral expectations (e.g. Kauer et al. 2003). To apply this test, ratios of expected heterozygosities per locus were calculated for each NW replicate population using the TW data (averaged across populations) as a measure of the initial expected heterozygosity in our laboratory populations (e.g. H_e NW₁/ H_e TW for the NW₁ population). To account for the different effective population sizes of X chromosomes, a correction was introduced for the X chromosomal loci heterozygosities (Kauer et al. 2002):

$$H_{\text{corr}} = 1 - \frac{1}{\sqrt{1 + k \left[\left(\frac{1}{1-H_{\text{obs}}} \right)^2 - 1 \right]}}$$

the correction factor k was 1.33 assuming a balanced sex ratio (Pascual et al. 2004). Since LnRH values are expected to follow a normal distribution for neutrally evolving microsatellite loci (Schlötterer 2002), significant deviations of standardized LnRH values from the Z distribution indicate a putative selective sweep (Kauer et al. 2003). This test was applied for each NW replicate population.

The second test performed to detect selection was based on the approach described in Beaumont and Nichols (1996) and Beaumont and Balding (2004). This approach aims to identify outlier loci based on the comparison of observed F_{st} values in the data with a neutral distribution of expected F_{st} values obtained from a coalescent simulation model. This test is based on the fact that the distribution of F_{st} is strongly related to the heterozygosity at a given locus (Beaumont and Nichols 1996). The outliers are then identified in a plot of F_{st} versus heterozygosity with each locus generating a datapoint. We applied this test to our NW populations using the FDIST2 software (Beaumont and Nichols 1996), and generating a neutral distribution based on a 100 island model of population structure and an expected mean F_{st} of 0.105. We performed two separate

analyses: one assuming a Stepwise Mutation Model (SMM) and the other assuming an Infinite Allele Model (IAM), with 50,000 paired values of F_{st} and heterozygosity being computed in order to generate the distribution. Although this approach is based on the infinite island model, it seems to be quite robust for other demographic scenarios (Beaumont and Balding 2004).

Results

Genetic variability in NW and TW populations

Table 1 summarizes the genetic variability detected in the longer established NW and the recently introduced TW laboratory populations. Overall, the data showed a high genetic variability in these laboratory populations. Significant differences in allele number (n_A) were detected among the NW populations (Friedman ANOVA, $df = 2$, $P < 0.04$), with NW₂ presenting the highest number of alleles. TW populations showed no significant differences in allele number (Friedman ANOVA, $df = 2$, $P > 0.05$). No significant differences in observed (H_{obs}) or expected (H_{exp}) heterozygosities were obtained among NW replicate populations or among TW replicate populations (Friedman ANOVA, $df = 2$, $P > 0.05$). However, there were clear differences in genetic variability between NW and TW populations, being the NW populations less variable. The mean number of alleles per locus was significantly lower in NW populations when compared with TW (Wilcoxon matched pairs test; $Z = 2.666$, $df = 18$, $P < 0.008$). Expected heterozygosity and observed heterozygosities were also significantly lower in NW populations (Wilcoxon matched pairs test; $Z = 2.666$, $P < 0.008$; and $Z = 2.073$, $P < 0.039$, respectively).

We calculated a decline in expected heterozygosity of about 12.8% for NW₁, 8.9% for NW₂ and 10.7% for NW₃, during the 49 generations of laboratory evolution, assuming that the pooled TW genetic variability reflects the initial NW variability.

Table 1 Genetic variability in NW and TW laboratory populations

Population	n	n_A	H_{obs}	H_{exp}
NW ₁	44.7	8.3	0.737	0.780
NW ₂	45.6	10.7	0.765	0.815
NW ₃	47.6	9.3	0.804	0.798
TW ₁	46.2	16.7	0.832	0.890
TW ₂	46.7	17.4	0.864	0.893
TW ₃	47.0	17.6	0.819	0.899

n Mean number of individuals per locus, n_A mean allele number per locus, H_{obs} mean observed heterozygosity, H_{exp} mean expected heterozygosity

Genetic differentiation in NW and TW populations

Significant genetic differentiation as measured by F_{st} was observed for all NW population pairs and also for all NW versus TW pairwise comparisons (Table 2). Interestingly, pairwise F_{st} values between NW population pairs were consistently higher than those obtained for NW versus TW pairwise comparisons. On the other hand, pairwise F_{st} values between TW populations were much smaller, showing non-significant genetic differentiation. Similar results were obtained when all loci or only autosomal loci were considered (data not shown). Pairwise G'_{st} comparisons for the six populations studied provided quite similar results to those described above, with generally higher differentiation values between the NW populations (average of 0.479), intermediate differentiation between NW versus TW populations (average of 0.397) and very low differentiation between TW populations (average of 0.044). Also, the relative magnitude of population differentiation considering the ratio of the average NW's pairwise values relative to the average NW versus TW pairwise values was quite similar for both G'_{st} and F_{st} .

The global F_{st} value among NW populations was significantly higher than the global F_{st} obtained among TW populations, as assessed over 10 000 permutations ($P = 0.049$; with F_{st} NW = 0.105, 95% CI = 0.075; 0.136; and F_{st} TW = 0.004, 95% CI = 0.002; 0.007).

A hierarchical analysis of variance (AMOVA) performed on the NW and TW groups of populations assigned the highest percentage of variation (93.42%) to the within population (between individuals) level. The between groups (NW versus TW) and the between populations/within groups source of variation explained 1.14 and 5.44% of the total variation, respectively. All variance components were significant.

Table 3 shows the percentage of variation attributed to each variance component for each locus independently. As expected from the results obtained in the global AMOVA, the V_c (variation within populations) component includes the majority of variation obtained. However, it is interesting

Table 2 Pairwise F_{st} (above diagonal) and G'_{st} (below diagonal) values for NW and TW populations

	NW ₁	NW ₂	NW ₃	TW ₁	TW ₂	TW ₃
NW ₁	–	0.1036	0.1344	0.0803	0.0853	0.0820
NW ₂	0.4805	–	0.0755	0.0537	0.0455	0.0483
NW ₃	0.5831	0.3789	–	0.0621	0.0615	0.0575
TW ₁	0.4418	0.3624	0.3892	–	0.0056	0.0041
TW ₂	0.4992	0.3234	0.3752	0.0576	–	0.0027
TW ₃	0.4741	0.3354	0.3697	0.0371	0.0375	–

Bold values represent significant Pairwise F_{st} comparisons after Bonferroni correction ($P < 0.05$)

Table 3 Percentage of variation of AMOVA variance components per locus

Locus	Chrom.	V _a %	V _b %	V _c %
dsub05	A (08E)	1.506	7.959	90.535
dsub19	A (15B-C)	-0.064	6.463	93.601
dsub21	A (11A)	0.630	8.649	90.722
dsub23	J (19D)	0.826	4.354	94.821
dsub27	J (33A)	1.064	4.118	94.818
dsub10	U (36A)	-0.302	6.947	93.355
dsub20	E (62B)	2.127	3.736	94.137
dsub01	O (90A)	2.314	3.054	94.632
dsub02	O (78A)	2.062	3.627	94.311
Total	-	1.140	5.440	93.420

Chrom. Chromosome and cytological band location in brackets (Santos et al. 2010)

V_a % between groups variance component (NW vs. TW)

V_b % between population/within groups variance component

V_c % within population variance component

All positive values are significant ($P < 0.01$)

to note that the percentage of variation associated with the V_b (between populations/within groups variance) component was consistently higher for the microsatellite loci located in the A (sexual) chromosome. For the V_a (between groups) component, it is worth mentioning that two (*dsub01* and *dsub02*) of the three loci with the highest percentage of variation are located in the same autosomal (O) chromosome.

Demographic history of NW populations

Table 4 shows the effective population size (N_e) and N_e/N ratios for NW populations using different estimators, with N being the average census population size estimated per generation. Based on the loss of expected heterozygosity through time, NW populations presented effective population sizes ranging from 168 (in NW₁) to 248 (in NW₂), with ratios of effective population size relative to the census size between 0.173 and 0.262.

Table 4 Estimates of effective population size (N_e) and N_e/N ratios for NW populations

Population	$N_{e,h}$	$N_{e,pl}$	N	$N_{e,h}/N$	$N_{e,pl}/N$
NW ₁	168	151.9 (121.4–189.5)	968.8	0.173	0.157
NW ₂	248	289.4 (228.0–368.9)	947.5	0.262	0.305
NW ₃	204	212.9 (168.7–269.3)	916.7	0.223	0.232

$N_{e,h}$ Effective population size estimated by the loss of heterozygosity method, $N_{e,pl}$ effective population size estimated according to Wang (2001), N census population size

The effective population sizes estimated using a pseudo-likelihood approach according to Wang (2001) were quite similar to those obtained through the loss of expected heterozygosity except for the NW₂ population, which presented a higher effective population size under the former method.

Our laboratorial populations were also tested for a possible historical demographic reduction—see Table 5. Using the Cornuet and Luikart (1996) approach, most populations showed no indication of either historical demographic decline or expansion, except NW₁ for which a signal of demographic decline was observed. This result is consistent with our previous variability analysis which shows that this population presented the lowest values of genetic variability (see Table 1) and also a lower N_e (see Table 4). On the other hand, using the M ratio we obtained indications of significant population decline in the three NW populations, while no such indication was observed for any of the TW populations.

Multilocus tests of selection

Figure 1 shows the results obtained for the two multilocus tests applied to detect selection in our microsatellite dataset. No microsatellite locus presented any consistent indication of positive selection. No values fell outside the 95% limits of the distribution of standardized LnRH values, with the exception of locus *dsub05* in the NW₁ population. Similar F_{st} expected values were obtained with the two mutation models (IAM and SMM) with the FDIST2 test. No clear signature of selection was found, with the only exception being locus *dsub20* suggesting uniform selection among NW populations only when the Infinite Allele Model was assumed.

Table 5 Tests of past bottlenecks in NW and TW populations

Population	Bottleneck		M ratio
	Wilcoxon P -value	H	
NW ₁	0.014	Excess	0.661*
NW ₂	0.500	No deviation	0.644*
NW ₃	0.125	No deviation	0.665*
TW ₁	0.410	No deviation	0.847 n.s.
TW ₂	0.367	No deviation	0.915 n.s.
TW ₃	0.213	No deviation	0.902 n.s.

Wilcoxon P -value one tailed for population excess, Wilcoxon rank sign test, H Heterozygosity excess indicates a Bottleneck

M Observed M ratio is considered significant ($* P < 0.05$) for $M < M_c$ (M_c = critical M value of 0.680, see “Material and methods”)

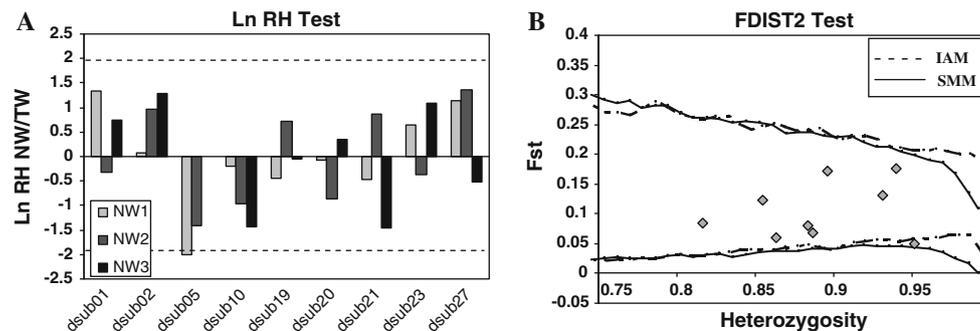


Fig. 1 Multilocus tests for the detection of selection. **A** LnRH test—heterozygosity ratios (NW/TW) were performed for each of the three NW replicate populations relative to the average TW heterozygosity (e.g. H_e NW₁/TW average H_e). LnRH ratios are presented for all the 9 loci analysed. Dashed lines represent the 95% confidence interval of the standardized normal distribution. **B** Fdist2 test, with the F_{st}

versus Heterozygosity plot for the 9 loci in the NW populations. The upper and lower quantiles (0.975 and 0.025, respectively) of the estimated neutral distribution are shown for each of the mutation models assumed: Infinite Allele Model (IAM)—dashed line; and Stepwise Mutation Model (SMM)—solid line (see [Material and methods](#) for further details)

Discussion

Genetic variability in laboratory populations

Our laboratory populations showed an overall high genetic variability in the 9 microsatellite loci analysed (see [Table 1](#)). Nevertheless, the genetic variability of our longer established populations (NW) was significantly lower than the recently established populations (TW). We attribute this difference primarily to the effects of genetic drift (see next section).

The results obtained for our recently established TW populations resemble those of European wild populations of *Drosophila subobscura* reported by Pascual et al. (2001), with similar levels of genetic variability both in terms of expected heterozygosity (0.895 and 0.874, respectively) and average allele number (17.2 and 15.6, respectively). This suggests that the foundation process and the three generations in laboratory culture produced little impact in the genetic diversity of TW populations relative to wild populations. Our a priori assumption that TW genetic variability reflects that presented by NW populations in the first generations of laboratory culture seems thus highly probable. Furthermore, the difference in the number of founders between the two sets of populations does not seem to have a high impact given that according to Gregorius (1980) a sample of 117 (similar to TW founding females) is enough to detect alleles with a frequency equal or higher than 0.05 with a probability of 95%, while a sample of 341 (slightly higher than the number of NW founding females) is needed to detect alleles with a 0.02 frequency. In that sense, a number around 100 inseminated females collected from the field will most likely provide a good measure of the genetic variability of a given wild population (at least in this species). Another relevant point concerning our aforementioned assumption

is that it depends on a general stability of allelic frequencies in a given natural population across years. Although few data exists on this point, our expectation is reinforced by a study by Noor et al. (2000) showing general similarity of allele frequencies in microsatellite loci in a colonizing natural population of *Drosophila subobscura* between samples taken 5 years apart.

It is interesting to note that the expected heterozygosity of our longer established NW populations (0.798) is still higher than the values obtained for American introduced natural populations (0.727), which present lower genetic variability than European native populations as a result of a strong founder effect associated with the colonization of America (Pascual et al. 2007). Also, NW populations present a considerably higher average allele number than the American populations (9.4 and 5.5, respectively). This is probably due to the mild bottleneck in our populations as a result of laboratory culture in contrast to the severe bottleneck associated to the colonization event (Pascual et al. 2007).

The percentage of decline in expected heterozygosity was very similar between all NW populations considering TW as the initial diversity (9–13% from the initial diversity). This corresponds to an average decline in expected heterozygosity of 0.0025 (with a standard error of 0.0003) per generation in our populations. It is interesting to note the similarity of these values with those observed for the TW populations between generations 3 and 40—a decline of around 10% (Simões et al. 2008). This leads to an average decline of expected heterozygosity of 0.0028 (with a standard error of 0.0003) per generation. Thus, there is a high concordance between the decay in heterozygosity estimated by real-time evolution analysis (the TW data; see Simões et al. 2008) and the comparative approach used here (TW data as a surrogate of the initial NW data). This indicates that comparisons across extant populations may

lead to valid inferences of the evolutionary dynamics of molecular markers, in particular changes in genetic variability, presented by a given population through time.

Loss of genetic variability and adaptation to captivity are two major problems in ex-situ conservation programmes (Frankham et al. 2002). In these programmes, maintenance of captive populations focuses on maximizing effective population sizes while reducing the effects of adaptation to non-natural conditions (see Caballero and Toro 2000; Frankham et al. 2000; Rodríguez-Ramilo et al. 2006; Woodworth et al. 2002). The most common strategy to satisfy both requirements is to manage these populations by equalising parental contributions, i.e. where each individual contributes equally to the next generation (e.g. see Wang 1997). However, few studies have compared the performance of long-term captive populations under managed versus non managed conditions (Rodríguez-Ramilo et al. 2006).

In managed populations, in which there is equalisation of parental contributions and thus with a null variance in family size, it is expected that the effective population size would almost double the census size (see Frankham et al. 2002). According to this, the expected rate of decline in heterozygosity for a population with an N_e of 200—which would correspond to a census size N of around 100—is 0.0025 per generation. This expectation was tested in the work of Rodríguez-Ramilo et al. (2006), using data from the first 38 generations of captivity in *Drosophila melanogaster*. In managed populations with a census size of 100 individuals the aforementioned authors found a rate of heterozygosity loss per generation of 0.0009, below the expected rate of decline for this N_e (0.0025, as reported above). On the other hand, the observed decline of 0.005 in non-managed populations of $N = 100$ matched the expected decline assuming that $N_e = N$.

The decline of heterozygosity in our NW populations (0.0025 per generation) lies between those of Rodríguez-Ramilo et al. (2006) for managed and non-managed populations, although our populations were non-managed and clearly adapting to the laboratory conditions. This is probably due to the high population census sizes (of around 950), with our estimates using microsatellite information suggesting an effective size of around 200 individuals. Variance in reproductive success is known to contribute to a decrease in the effective number of breeders (Falconer and Mackay 1996). This is a highly plausible explanation in the present study since our populations presented a clear adaptive process to captive conditions during this period and thus a probable differential contribution of certain individuals to the next generation (Matos et al. 2002).

Nevertheless, the relatively high levels of genetic diversity (heterozygosity and allele number) observed in our NW populations, even after 49 generations of

adaptation to a confined environment and the relatively high number of alleles in comparison to colonizing *D. subobscura* populations (Pascual et al. 2007), suggests that no major bottlenecks occurred during this period.

Assessing the role of drift in causing genetic differentiation between populations

We presented evidence supporting a clear role of genetic drift in promoting genetic differentiation during the 49 generations of laboratory evolution in our laboratory populations. As expected, genetic drift led to highly significant genetic differentiation between NW populations during evolution in the laboratory environment, with a global F_{st} of 0.105 and a G'_{st} of 0.479. This differentiation is explained by fluctuations in allele frequencies and the differential loss of alleles in NW populations, leading to a different subset of alleles in each NW population. The lower F_{st} values observed between NW and TW populations may be caused by the larger number of alleles present in TW populations including those also found in NW populations or by the inverse relationship reported between locus polymorphism and F_{st} values (Carreras-Carbonell et al. 2006). However given that F_{st} , G'_{st} and also D_{est} (according to Jost 2008; data not shown) pairwise estimates revealed a similar pattern of genetic differentiation in our laboratory populations, i.e. high differentiation between NW's and lower differentiation when considering NW's versus TW's, this lower differentiation observed is more likely due to the first explanation.

Few studies have analysed the genetic differentiation between laboratory populations evolving under a common regime. Morgan et al. (2003) analysed the dynamics of molecular markers in several laboratory lines of house mice under artificial selection for locomotor behavior. Their hierarchical design included two different treatments (selection vs. control groups) and four lines within each group, allowing to disentangle the effects of selection—responsible for the divergence between the two groups—from the stochastic effects such as genetic drift and founder events—leading to divergence between lines within groups. We have used a similar approach considering the NW populations as the selection group and the TW populations as the “control” group (surrogate of the ancestral state of the NW populations previous to lab adaptation). Significant genetic differentiation between lines (within groups) was observed by Morgan et al. (2003) after 14 generations of selection, with an average value of 0.149, using microsatellite data. This value is of the same order of magnitude as the observed in the present study among NW populations ($F_{st} = 0.105$), which have been submitted to nearly 50 generations of laboratory adaptation. Given the discrepancy in the number of generations analysed between the two studies, a higher

divergence between lines could be expected in our study. However, the higher effective population sizes in our populations relative to the house mice populations analysed by Morgan et al. (2003) ($N_e = 20$) is likely to have counteracted the effect of the generation interval.

On the other hand, Morgan et al. (2003) found no differentiation between selection groups. Overall, the authors concluded that genetic divergence between selection groups, as well as among replicate lines within a selection group were not different from expectations of neutral evolution. Similarly, we found that differences between NW and TW groups are small accounting for a low, albeit significant, percentage of the global variance (1.14%), in spite of detecting significant pairwise differences between NW and TW individual populations (as assessed by F_{st}). This observation is rather interesting since it suggests that genetic drift was the main evolutionary mechanism involved in the molecular genetic differentiation in our NW populations despite nearly 50 generations of laboratory evolution and adaptation to captive conditions. In fact, no signature of either directional or balancing selection was obtained in the multilocus tests applied to our microsatellite dataset. This provides further evidence for the overall neutral evolution of the microsatellite loci analysed in this study even though adaptation at the phenotypic level is clearly occurring. Higher marker density coverage of the whole genome should be used to identify specific genes selected during laboratory adaptation.

However, the impact of genetic drift in causing population differentiation can also differ between loci. Locus by locus AMOVA presented higher variance for the between populations/within groups component for all loci located in the A chromosome. The higher effect of genetic drift due to the lower effective population size of this (sexual) chromosome is likely to account for this result. Accordingly, it has been shown that population size changes lead to particularly lower X-linked diversity (Pool and Nielsen 2007). On the other hand, a high percentage of variation between groups was observed in the two microsatellite loci localized in the O chromosome. This might suggest that, in this chromosome, our microsatellite loci could be in linkage disequilibrium with genes involved in laboratory adaptation, thus generating higher differences between the longer term laboratory populations and the recently introduced ones. In fact, another microsatellite localized in this chromosome—*dsub 14*—not used in this study, suggested sign of positive selection (Simões et al. 2008, 2009), though more data are needed to reinforce this result.

Inferring demographic history in laboratory populations

N_e estimates based on the decline of expected heterozygosity and pseudo-likelihood method were fairly close, with

an average estimate among replicate populations of around 21.9–23.1% of the actual census population size (N), respectively (see Table 4). It is remarkable the similarity of these values with the direct estimates obtained in the TW populations—an average N_e/N of 17 to 25% (Simões et al. 2008). In fact the values estimated by the comparative approach—NW and TW data—and the direct temporal data—TW generation 3 and 40—overlap (data not shown). This reinforces our previous observation that the molecular dynamics during laboratory adaptation may be acceptably inferred from a comparative method, at least when populations founded from the same natural population are involved provided it has not undergone major demographic changes.

Since no strong fluctuation of the census size was registered in our NW populations (data not shown), it is likely that the low effective size relative to the census size is a consequence of the high heterogeneity in the parental contributions to the next generation (variance in family sizes). In fact, previous studies of laboratory adaptation support an unequal contribution of genotypes for the next generation, as indicated by a clear selective response in several life-history traits (Gilligan and Frankham 2003; Matos et al. 2002; Simões et al. 2007). Nevertheless, the N_e/N values that we found are in general higher than previously reported for either captive or wild populations (Briscoe et al. 1992; Frankham et al. 2002). As stated above, the laboratory conditions under which our populations are maintained may allow retaining larger effective population sizes and therefore higher variability levels.

The patterns of genetic variability described above suggest the occurrence of a mild bottleneck in the laboratory history of our NW populations (see first section of the “Discussion”). The tests we have applied to detect past demographic fluctuations in population demography also support this hypothesis. When considering the M ratio, signs of a past bottleneck were obtained for all NW populations, while using the Bottleneck algorithm, only the NW₁ population presented signs of past reduction. It appears thus that the M ratio is the most sensitive approach to detect past bottlenecks under the specific demographical conditions experienced by our laboratory populations. This might be due to the fact that the Bottleneck test is more suited to detect strong and recent reductions in population size (see Pascual et al. 2001) while M ratio appears to be a method more suitable to correctly detect bottlenecks lasting several generations (see Williamson-Natesan 2005). In fact, the range of M values obtained for our populations (around 0.66) also corroborates the hypothesis of a mild bottleneck, particularly when compared to the M values of 0.5 obtained for two North American populations of *D. subobscura* (Fort Bragg and Bellingham; using data from Pascual et al. (2001)), which suffered a severe bottleneck associated with the colonization of the American continent.

In general, we can conclude that our laboratory evolving NW populations present signs of a non-severe bottleneck effect, most likely as a result of the continuous evolution under relatively moderate population sizes during its nearly 50 generations in the lab.

General conclusions

Our data indicate that genetic drift is the main evolutionary mechanism leading to significant molecular genetic divergence between populations during laboratory adaptation at least when using microsatellite loci. We have additionally shown that neither directional nor balancing selection seem to have had any direct impact in the molecular genetic patterns observed in our populations, despite undergoing an adaptive process to the laboratory environment. Nevertheless, genetic drift did not lead to a major loss of genetic variability within populations after 49 generations in a confined environment. The low decline in genetic variability of our populations throughout captivity, even though adaptation is occurring, suggests that careful maintenance procedures (e.g. fairly constant census sizes, randomisation of individuals in vials to avoid non-random mating) can efficiently reduce the loss of genetic variability in captive populations, without necessarily applying active management procedures with conservation purposes. This might be an important strategy in organisms that generate a high number of offspring such as *Drosophila* although less applicable in other cases (such as mammals for example). Finally, our data give support to inferences of the evolutionary molecular dynamics of a population by a comparative approach, at least when the populations compared derive from the same natural population and no dramatic genetic changes have occurred.

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References

- Beaumont MA, Balding DJ (2004) Identifying adaptive genetic divergence among populations from genome scans. *Mol Ecol* 13:969–980
- Beaumont MA, Nichols RA (1996) Evaluating loci for use in the genetic analysis of population structure. *Proc R Soc Lond B* 263:1619–1626. Fdist2 software available from <http://www.rubic.rdg.ac.uk/~mab/software.html>
- Briscoe DA, Malpica JM, Robertson A et al (1992) Rapid loss of genetic variation in large captive populations of *Drosophila* flies: implications for the genetic management of captive populations. *Conserv Biol* 6:416–425
- Caballero A (1994) Developments in the prediction of effective population size. *Heredity* 73:657–679
- Caballero A, Toro MA (2000) Interrelations between effective population size and other pedigree tools for the management of conserved populations. *Genet Res* 75:331–343
- Carreras-Carbonell J, Macpherson E, Pascual M (2006) Population structure within and between subspecies of the Mediterranean triplefin fish *Tripterygion delaisi* revealed by highly polymorphic microsatellite loci. *Mol Ecol* 15:3527–3539
- Chippindale AK (2006) Experimental Evolution. In: Fox C, Wolf J (eds) *Evolutionary genetics: concepts and case studies*. Oxford University Press, London, pp 482–501
- Cornuet JM, Luikart G (1996) Description and power analysis of two tests for inferring recent population bottlenecks from allele frequency data. *Genetics* 144:2001–2014
- Crawford NG (2009) SMOGD: Software for the measurement of genetic diversity. *Mol Ecol Resou* 10:556–557. Available from <http://www.ngcrawford.com/django/jost/>
- Crow JF, Kimura M (1970) *An introduction to population genetic theory*. Harper & Row, New York
- Dieringer D, Schlötterer C (2003) Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. *Mol Ecol Notes* 3:167–169
- England PR, Osler GHR, Woodworth LM et al (2003) Effects of intense versus diffuse population bottlenecks on microsatellite genetic diversity and evolutionary potential. *Conserv Genet* 4:595–604
- Excoffier L, Laval G, and Schneider S (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* 1:47–50. Available at <http://cmpg.unibe.ch/software/arlequin3/>
- Falconer DS, Mackay TFC (1996) *Introduction to quantitative genetics*. Longman, Harlow
- Frankham R (1995) Effective population size/adult population size ratios in wildlife: a review. *Genet Res* 66:95–107
- Frankham R (2005) Stress and adaptation in conservation genetics. *J Evol Biol* 18:750–755
- Frankham R, Manning H, Margan SH, Briscoe DA (2000) Does equalisation of family sizes reduce genetic adaptation to captivity? *Anim Conserv* 3:357–363
- Frankham R, Ballou JD, Briscoe DA (2002) *Introduction to conservation genetics*. Cambridge University Press, Cambridge
- Garza JC, Williamson E (2001) Detection of reduction in population size using data from microsatellite DNA. *Mol Ecol* 10:305–318
- Gilligan DM, Frankham R (2003) Dynamics of genetic adaptation to captivity. *Conserv Genet* 4:189–197
- Gloor GB, Preston CR, Johnson-Schlitz DM et al (1993) Type I repressors of P element mobility. *Genetics* 135:81–95
- Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from <http://www.unil.ch/izea/software/fstat.html>
- Gregorius H-R (1980) The probability of losing an allele when diploid genotypes are sampled. *Biometrics* 36:643–652
- Hartl DL, Clark AG (1989) *Principles of population genetics*. Sinauer Associates, Massachusetts
- Hedrick PW (2005) A standardized genetic differentiation measure. *Evolution* 59:1633–1638
- Jost L (2008) G_{ST} and its relatives do not measure differentiation. *Mol Ecol* 17(18):4015–4026
- Kauer M, Zangerl B, Dieringer D, Schlötterer C (2002) Chromosomal patterns of microsatellite variability contrast sharply in African and non-African populations of *Drosophila melanogaster*. *Genetics* 160:247–256

- Kauer MO, Dieringer D, Schlötterer C (2003) A multilocus variability screen for positive selection associated with the “out of Africa” habitat expansion of *Drosophila melanogaster*. *Genetics* 165: 1137–1148
- Matos M, Avelar T, Rose MR (2002) Variation in the rate of convergent evolution: adaptation to a laboratory environment in *Drosophila subobscura*. *J Evol Biol* 15:673–682
- Matos M, Simões P, Duarte A et al (2004) Convergence to a novel environment: comparative method versus experimental evolution. *Evolution* 58:1503–1510
- Montgomery ME, Woodworth LM, Nurthen RK et al (2000) Relationships between population size and loss of genetic diversity: comparisons of experimental results with theoretical predictions. *Conserv Genet* 1:33–43
- Morgan TJ, Garland T Jr, Irwin BL et al (2003) The mode of evolution of molecular markers in populations of house mice under artificial selection for locomotor behavior. *J Heredity* 94: 236–242
- Morgan TJ, Evans MA, Garland T Jr et al (2005) Molecular and quantitative genetic divergence among populations of house mice with known evolutionary histories. *Heredity* 94:518–525
- Noor MAF, Pascual M, Smith KR (2000) Genetic variation in the spread of *Drosophila subobscura* from a nonequilibrium population. *Evolution* 54:696–703
- Pascual M, Schug MD, Aquadro CF (2000) High density of long dinucleotide microsatellites in *Drosophila subobscura*. *Mol Biol Evol* 17:1259–1267
- Pascual M, Aquadro CF, Soto V, Serra L (2001) Microsatellite variation in colonizing and paleartic populations of *Drosophila subobscura*. *Mol Biol Evol* 18:731–740
- Pascual M, Mestres F, Serra L (2004) Sex-ratio in natural and experimental populations of *Drosophila subobscura* from North America. *Journal of Zoological Systematics and Evolutionary Research* 42:33–37
- Pascual M, Chapuis MP, Mestres F et al (2007) Introduction history of *Drosophila subobscura* in the New World: a microsatellite based survey using ABC methods. *Mol Ecol* 16:3069–3083
- Pool JE, Nielsen R (2007) Population size changes reshape genomic patterns of diversity. *Evolution* 61:3001–3006
- Prasad NG, Joshi A (2003) What have two decades of laboratory life-history evolution studies on *Drosophila melanogaster* taught us? *J Genet* 82:45–75
- Rice WR (1989) Analyzing tables of statistical tests. *Evolution* 43:223–225
- Rodríguez-Ramilo ST, Moran P, Caballero A (2006) Relaxation of selection with equalization of parental contributions in conservation programs: An experimental test with *Drosophila melanogaster*. *Genetics* 172:1043–1054
- Rose MR, Nusbaum TJ, Chippindale AK (1996) Laboratory evolution: the experimental wonderland and the cheshire cat syndrome. In: Rose MR, Lauder GV (eds) *Adaptation*. Academic Press, San Diego, pp 221–241
- Santos J, Serra L, Sole E, Pascual M (2010) FISH mapping of microsatellite loci from *Drosophila subobscura* and its comparison to related species. *Chromosome Res* 18:213–226
- Schlötterer C (2002) A microsatellite-based multilocus screen for the identification of local selective sweeps. *Genetics* 160:753–763
- Schug MD, Hutter CH, Wetterstrand KA et al (1998) The mutation rates of di-, tri- and tetranucleotide repeats in *D. melanogaster*. *Mol Biol Evol* 15:1751–1760
- Simões P, Rose MR, Duarte A et al (2007) Evolutionary domestication in *Drosophila subobscura*. *J Evol Biol* 20:758–766
- Simões P, Pascual M, Santos J et al. (2008) Evolutionary dynamics of molecular markers during local adaptation: a case study in *Drosophila subobscura*. *BMC Evol Biol* 8:66 (<http://www.biomedcentral.com/1471-2148/8/66>)
- Simões P, Pascual M, Santos J et al. (2009) Correction: evolutionary dynamics of molecular markers during local adaptation: a case study in *Drosophila subobscura*. *BMC Evol Biol* 9:133. (<http://www.biomedcentral.com/1471-2148/9/133>)
- Vazquez F, Perez T, Albornoz J et al (2000) Estimation of the mutation rates in *Drosophila melanogaster*. *Genet Res* 76:323–326
- Wang J (1997) More efficient breeding systems for controlling inbreeding effective size in animal populations. *Heredity* 79: 591–599
- Wang J (2001) A pseudo-likelihood method for estimating effective population size from temporally spaced samples. *Genet Res* 78: 243–257
- Wang J (2005) Estimation of effective population sizes from data on genetic markers. *Phil Trans R Soc B* 360:1395–1409
- Wang J, Caballero A (1999) Developments in predicting the effective size of subdivided populations. *Heredity* 82:212–226
- Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution* 38:1358–1370
- Williamson EG, Slatkin M (1999) Using maximum likelihood to estimate population size from temporal changes in allele frequencies. *Genetics* 152:755–761
- Williamson-Natesan EG (2005) Comparison of methods for detecting bottlenecks from microsatellite loci. *Conserv Genet* 6:551–562
- Woodworth LM, Montgomery ME, Briscoe DA, Frankham R (2002) Rapid genetic deterioration in captive populations: Causes and conservation implications. *Conserv Genet* 3:277–288