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How much can history constrain adaptive evolution? A real-time evolutionary approach of inversion polymorphisms in *Drosophila subobscura*

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Abstract

Chromosomal inversions are present in a wide range of animals and plants, having an important role in adaptation and speciation. Although empirical evidence of their adaptive value is abundant, the role of different processes underlying evolution of chromosomal polymorphisms is not fully understood. History and selection are likely to shape inversion polymorphism variation to an extent yet largely unknown. Here, we perform a real-time evolution study addressing the role of historical constraints and selection in the evolution of these polymorphisms. We founded laboratory populations of *Drosophila subobscura* derived from three locations along the European cline and followed the evolutionary dynamics of inversion polymorphisms throughout the first 40 generations. At the beginning, populations were highly differentiated and remained so throughout generations. We report evidence of positive selection for some inversions, variable between foundations. Signs of negative selection were more frequent, in particular for most cold-climate standard inversions across the three foundations. We found that previously observed convergence at the phenotypic level in these populations was not associated with convergence in inversion frequencies. In conclusion, our study shows that selection has shaped the evolutionary dynamics of inversion frequencies, but doing so within the constraints imposed by previous history. Both history and selection are therefore fundamental to predict the evolutionary potential of different populations to respond to global environmental changes.

Introduction

Polymorphic chromosomal rearrangements, such as inversions, are frequently found to be associated with climatic gradients and are known to play an important role in local adaptation and speciation (Dobzhansky, 1970; Krimbas, 1992; Noor *et al.*, 2001; Balanyà *et al.*, 2003, 2006; Hoffmann *et al.*, 2004; Umina *et al.*, 2005; Hoffmann & Rieseberg, 2008; Kirkpatrick, 2010; Lowry & Willis, 2010; Ayala *et al.*, 2012, 2014). A key effect of inversions is to reduce and redistribute recombination, which impinges on patterns of nucleotide diversity in those genomic regions close to, or included in, the inverted fragments (Hasson & Eanes, 1996; Navarro *et al.*, 2000; Andolfatto *et al.*, 2001; Laayouni *et al.*, 2003; Munté *et al.*, 2005). The suppression of meiotic recombination in heterokaryotypes may facilitate capture of locally adaptive alleles across multiple linked loci and allow inversions to spread through a local population (Dobzhansky, 1970; Kirkpatrick & Barton, 2006; Kirkpatrick, 2010). Moreover, chromosomal inversions have been shown to track environmental changes, such as seasonal variation (Rodríguez-Trelles

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et al., 1996) and global warming (Balanyà *et al.*, 2006, 2009; Hoffmann & Rieseberg, 2008). Chromosomal inversions are thus likely candidates to have an important role in adaptation to new environments or to respond to short-term environmental shifts.

Drosophila subobscura provides one of the clearest examples of adaptive inversion polymorphism. In earlier studies, the geographical distribution of polymorphism of gene arrangements of *D. subobscura* was interpreted as being mainly shaped by historical events (Krimbas & Loukas, 1980). More recent empirical data supports that natural selection is a key force acting on the inversion polymorphism of this species. Indeed, *D. subobscura* shows repeatable latitudinal clinal variation for inversions in three continents (Prevosti *et al.*, 1988; Balanyà *et al.*, 2003, 2006), as well as remarkable seasonal variation in inversion frequencies (Rodríguez-Trelles *et al.*, 1996, 2013). Furthermore, its inversion polymorphism also presents a clear response both to global warming (Balanyà *et al.*, 2006, 2009) and to extreme thermal events in nature (Rodríguez-Trelles *et al.*, 2013). Finally, laboratory experiments have successfully linked these polymorphisms with physiological traits such as thermal preference and tolerance (Dolgova *et al.*, 2010; Rego *et al.*, 2010; Calabria *et al.*, 2012), life-history traits (Santos *et al.*, 1986; Santos, 2009) and morphological traits (wing size and shape; see Orengo & Prevosti, 2002; Fragata *et al.*, 2010).

Some classical studies in *Drosophila* have analysed the evolution of inversion frequencies in controlled laboratory environments to clarify the selective mechanisms acting on these polymorphisms and have obtained a variety of results (Wright & Dobzhansky, 1946; Lewontin, 1958; Krimbas, 1967; Anderson *et al.*, 1972; Watanabe & Watanabe, 1977; Inoue, 1979). Lack of proper replication was, however, an important caveat in some of these experiments (e.g. Krimbas, 1967; Watanabe & Watanabe, 1977; Inoue, 1979). A synchronous evolutionary analysis of replicated sets of populations initially differentiated in nature is clearly lacking. Moreover, few studies have analysed how historical differences in inversion polymorphisms affect the ability of populations to respond to new environmental conditions. This is particularly important considering the homogenizing effect of global warming and evidence of its impact on inversions.

Recently, we have studied the impact of prior history, selection and drift in phenotypic traits during adaptation to a new laboratory environment, using *D. subobscura* populations derived from three distinct latitudes in the European cline (Fragata *et al.*, 2014). Historical constraints did not play a major role during adaptation, as complete convergence between populations occurred for life-history, physiological and morphological (body size) traits after only 14 generations since laboratory introduction. Nevertheless, phenotypic convergence does not necessary imply genetic convergence. It is thus an open question whether convergence is also observed for inversion polymorphisms.

In this study, we analyse the evolutionary patterns of inversion frequency changes in these populations and study the impact of evolutionary forces (namely history, genetic drift and selection) in this variation. Ultimately, this study will shed light on the role of historical constraints vs. selection in the evolution of inversion frequencies in a new environment, and their importance in the ability to adapt to environmental changes in general.

Materials and methods

Founding and maintenance of laboratory populations

Flies were collected in August 2010 from three locations across Europe: Adraga (Portugal), Montpellier (France) and Groningen (the Netherlands). The number of founding females was 234 for Adraga, 171 for Montpellier and 160 for Groningen. These were used to establish three foundations in the laboratory: 'Ad' (Adraga), 'Mo' (Montpellier) and 'Gro' (Groningen). Each foundation was maintained in separate families, each derived from one wild female. Inbreeding was avoided by crossing females with males from different lines at the 1st generation and with a random sample of males from all lines in the 2nd generation. At generation 3, a large outbred population was generated with the contribution of all families of a given foundation (see details in Fragata *et al.*, 2014). All foundations were threefold-replicated at generation 4.

Populations were maintained under standard laboratory conditions (Matos *et al.*, 2002; Simões *et al.*, 2007, 2008; Fragata *et al.*, 2014), involving synchronous discrete generations of 28 days, reproduction close to peak fecundity, photoperiod of 12h of light: 12h of dark at 18 °C and 1200 individuals. Flies were kept in vials with controlled density both for eggs (around 70 per vial) and adults (around 50 per vial). At each generation, flies emerging from the several vials of a given population were thoroughly randomized 4–5 days after emergence, using CO_2 anaesthesia. Egg collection for the next generation was done 1 week later.

Chromosomal inversions

Chromosomal inversions were scored at generations 2, 6, 15, 25 and 40 in all populations (three replicate populations per foundation, except at generation 2, not yet replicated). They were determined by scoring one 3rd instar female larva originated from individual crosses of males from the populations with virgin females from the *chcu* marker strain (Balanyà *et al.*, 2004; Simões *et al.*, 2012). These males were obtained directly from the population at all generations, except at generation

2, where males were originated from a brother–sister cross performed at generation 1. The number of individuals analysed per population and generation was as follows: generation 2 – 159 individuals for Adraga, 115 for Montpellier and 127 for Groningen; generations 15 and 40 – around 60 individuals per replicate population; and generations 6 and 25 – around 100 males per replicate population. Here, we follow the convention in Krimbas & Loukas (1980), and overlapping inversions on a given chromosome are indicated by a continuous line running below the numbers designating the inversions. Inversions on the same chromosome but not overlapping are indicated by a broken line running below the numbers.

Statistical methods

Diversity and differentiation in chromosomal polymorphism

Chromosomal diversity for each chromosome, foundation and generation was estimated as expected heterozygosity (H_e) and allelic richness. H_e was measured for each population and generation as $1 - \sum_{i=1}^{k} p_i^2$, with p_i being the relative frequency of chromosomal arrangement *i*. Allelic richness was estimated as number of segregating arrangements weighted by minimum sample size ($n = 60$). These parameters were estimated using FSTAT v2.9.3.2 (Goudet, 2001). Differences in polymorphism were assessed with ANCOVAs using the following model:

$$Y = \mu + Found + Gen + Chr + Found * Gen + Chr * Gen + Found * Chr + Found * Chr * Ger + \varepsilon, \quad (1)$$

where *Y* refers to H_e or allelic richness, *Found* corresponds to the three foundations, *Chr* refers to the five chromosomes, and *Gen* corresponds to the several generations assayed, as covariate (generations 2–40).

Differentiation in chromosomal inversions was estimated between foundations within generations and for each foundation between generations 6 and 40, using a hierarchical AMOVA, with foundations and replicate populations nested within foundations (Theta-f). Theta-f values were obtained through GDA (Lewis & Zaykin, 2001).

Temporal changes in inversions frequencies

All analyses of temporal changes of frequencies of inversions were performed excluding data from generation 2 due to lack of replication. This allowed to analyse the temporal dynamics based in three fully independent data sets for each foundation.

A principal component analysis (PCA) using a correlation matrix was applied to transformed inversion frequency data (2*square root of inversion frequency, following Balanyà *et al.* (2006)) of all chromosomal arrangements, across populations and generations. To analyse temporal changes in inversion frequencies, an ANCOVA model was applied on the PC1 coordinates (which explained 36.96% of total variation) as follows:

$$Y = \mu + Found + Rep\{Found\} + Gen + Gen * Found + \varepsilon, \quad (2)$$

where *Y* refers to the PC1 coordinates, *Found* corresponds to the three foundations, *Rep* (Replicate) nested within foundation corresponds to the three replicate populations of each foundation (random factor), and *Gen* corresponds to the generations assayed (as covariate).

To test for differences in temporal dynamics of specific inversions, we applied an ANCOVA model similar to the one described above on arcsine-transformed frequencies of each inversion. Only inversions that presented an average frequency of at least 5% across generations and foundations were analysed this way.

We further analysed the multivariate trajectories for inversion frequencies using the method described in Adams & Collyer (2009). We thus estimated differences between pairs of foundations in magnitude (differences between first and the last generations), direction (standardized differences between angles of the first axis of the PCA) and shape (deviations of corresponding generations between two scaled and aligned trajectories). To estimate statistical significance of these differences, 9999 residual randomization permutations were performed. We used the rgl package (Adler & Murdoch, 2012) in R (R Core Development Team, 2008) for multivariate analyses. To estimate the Euclidean distance between each pair of foundations at generations 6 and 40, we used average scores across replicate populations per foundation for each principal component (a total of 29). To calculate the Euclidean distance significance a null distribution was created using 9999 permutations of replicate populations between foundations at each generation.

Significance of changes in frequency of specific inversions between generation 6 and 40 was assessed by Cochran–Mantel–Haenszel (CMH statistic) chi-squared test, which allows testing for differences in replicated systems (Landis *et al.*, 1978). Adjustment for multiple testing followed the false discovery rate (FDR) procedure of Benjamini & Yekutieli (2001, theorem 1.3). This correction was applied to 11 tests performed for Adraga and Groningen and 13 tests for Montpellier.

To test whether changes in inversion frequencies obtained from the CMH statistic could be explained solely by genetic drift, 9999 simulations were performed for each arrangement and replicate. Using generation 6 as the starting point, we simulated, using the multinomial distribution (under a Wright–Fisher model, assuming only drift and no mutation or migration), the evolution of allele frequencies up to generation 40. *P*-values were defined as the proportion of simulations that provided equal or higher values than the observed data (equal or lower in case of decreasing inversion fre-

quencies) – see details in Additional Material and Methods (Data S1).

For inversions with evolutionary dynamics suggesting selection, we tested different models (dominance, additivity and recessiveness; see Table 1) following Rodríguez-Trelles *et al.* (2013) and a range of selective coefficients from 0.01 to 0.5, in 0.01 increments. The range of selection coefficients chosen for further analysis was defined considering the best fit between expected and observed data using the Kolmogorov–Smirnov Statistics (*D*) (see example for $O_{\underline{3+4}+\underline{7}}$ in Ad_3 – Fig. S1). For each selective coefficient inside the chosen range, we simulated 1000 times the evolution of inversion frequencies with both drift and selection. We then used two different approaches to measure the fit of the output: average of mean residuals of each simulation to the real inversion frequency and average of the *D* statistic. The selective coefficient that provided the best fitted model in each case was the one with lower mean residual or *D* statistic (see example for $O_{\underline{3+4}+\underline{7}}$ in Ad_3 – Fig. S2). To define the best model, we plotted the 95% confidence interval of expected frequencies at each generation and used information from the mean residuals, *D* statistic and visual inspection (see details in Additional Material and Methods [Data S1]).

Results

Diversity and differentiation in chromosomal polymorphism

Analysis of inversion frequencies in the initial generations (G2 and G6) reflects previously described latitudinal clinal patterns found in nature (Balanyà *et al.*, 2003), most markedly for A_2, A_{ST}, E_{ST}, $E_{\underline{1+2+9}}$, $E_{\underline{1+2+9+3}}$, $E_{\underline{1+2+9+12}}$, O_{ST}, $O_{\underline{3+4}}$, U_{ST} and $U_{\underline{1+8}+\underline{2}}$ (Table S1; Fig. 1). Some of these inversions presented a significant evolutionary dynamics across generations.

Overall, variation in levels of polymorphism (both H_e and allelic richness) was observed across chromosomes (ANCOVA, H_e: $F_{(4,162)} = 5.41$, $P = 0.0004$; allelic richness: $F_{(4,165)} = 74.85$, $P < 0.0001$), with consistently lower values for A and J chromosomes across foundations and generations.

In general, inversion heterozygosity did not differ between foundations, with an overall significant decrease throughout generations (ANCOVA, $F_{(1,162)} = 103.96$, $P < 0.0001$; see Fig. S3). Differences in these temporal dynamics were observed across chromosomes ($F_{(4,162)} = 15.17$, $P < 0.0001$) and foundations ($F_{(2,162)} = 6.43$, $P = 0.0021$), in this last case due to more stable heterozygosity of Groningen foundation (pairwise ANCOVAs: Ad-Gro: $F_{(1,107)} = 14.663$, $P = 0.00022$; Mo-Gro: $F_{(1,110)} = 4.815$, $P = 0.030$; Ad-Mo: $F_{(1,107)} = 1.929$, $P = 0.168$; see Fig. S3 and Table S2). Allelic richness was different between foundations ($F_{(2,165)} = 5.24$, $P = 0.0062$), with Montpellier presenting higher values. As found for H_e, an overall decrease was observed throughout generations ($F_{(1,165)} = 40.82$, $P < 0.0001$) and differences in this decrease were found across chromosomes ($F_{(4,165)} = 10.11$, $P < 0.0001$) and also across foundations ($F_{(2,165)} = 3.72$, $P = 0.0263$), with Montpellier showing a steeper decline than Groningen (pairwise ANCOVA, Mo-Gro: $F_{(1,110)} = 8.873$, $P = 0.0036$).

Foundations differed significantly in inversion frequencies in all generations, but the overall level of differentiation between them did not generally increase through time (Table 2). Pairwise tests between foundations only detected nonsignificant results for Montpellier vs. Groningen comparison at generation 15, and Adraga vs. Montpellier at generation 40 (Table 2). Between generations 6 and 40, a significant increase in differentiation was only found between Montpellier and Groningen (Table 2). All foundations showed a significant differentiation in inversion frequencies between generations 6 and 40, with Montpellier presenting higher Theta-f values: Ad – 0.199 (CI: 0.117–0.242); Mo – 0.268 (CI: 0.123–0.352); Gro – 0.138 (CI: 0.070–0.213).

Multivariate analysis of chromosomal polymorphisms

A principal component analysis was performed on transformed inversion frequencies, using data from generations 6 to 40, for all replicate populations of the

Table 1 Selection models for inversion frequency changes.

Selection models	Relative fitness of karyotypes (e.g. for A_2)			p_{t+1} (positive selection – A_2, $E_{\underline{1+2+9+12}}$)	$1\text{-}p_{t+1}$ (negative selection – A_{ST}, $O_{\underline{3+4}+\underline{7}}$)
	A_2/A_2	A_2/A_{Other}	A_{Other}/A_{Other}		
Dominance (*h = 0*)	1	1	1-*s*	$\frac{s\times q_t^2\times p_t}{1-(s\times q_t^2)}+p_t$	$\frac{s\times q_t\times p_t^2}{1-(2s\times q_t)+s\times q_t^2}+p_t$
Additivity (*h = 1/2*)	1	1-0.5*s*	1-*s*	$\frac{\frac{1}{2}s\times q_t\times p_t}{1-(s\times q_t)}+p_t$	$\frac{\frac{1}{2}s\times q_t\times p_t}{1-(s\times q_t)}+p_t$
Recessiveness (*h = 1*)	1	1-*s*	1-*s*	$\frac{s\times q_t\times p_t^2}{1-(2s\times q_t)+s\times q_t^2}+p_t$	$\frac{s\times q_t^2\times p_t}{1-(s\times q_t^2)}+p_t$

p_{t+1} (positive selection) and $1\text{-}p_{t+1}$ (negative selection) correspond to the frequency of the studied inversion in the next generation ($t + 1$) and *s* to the selective coefficient (positive constant). As in Rodríguez-Trelles *et al.* (2013), these models have several assumptions: random mating population; selection acting solely on egg to adult viability; diallelic locus (in the example, A_{others} includes all the arrangements present in chromosome A, except the A_2) – see also Material and Methods for further details.

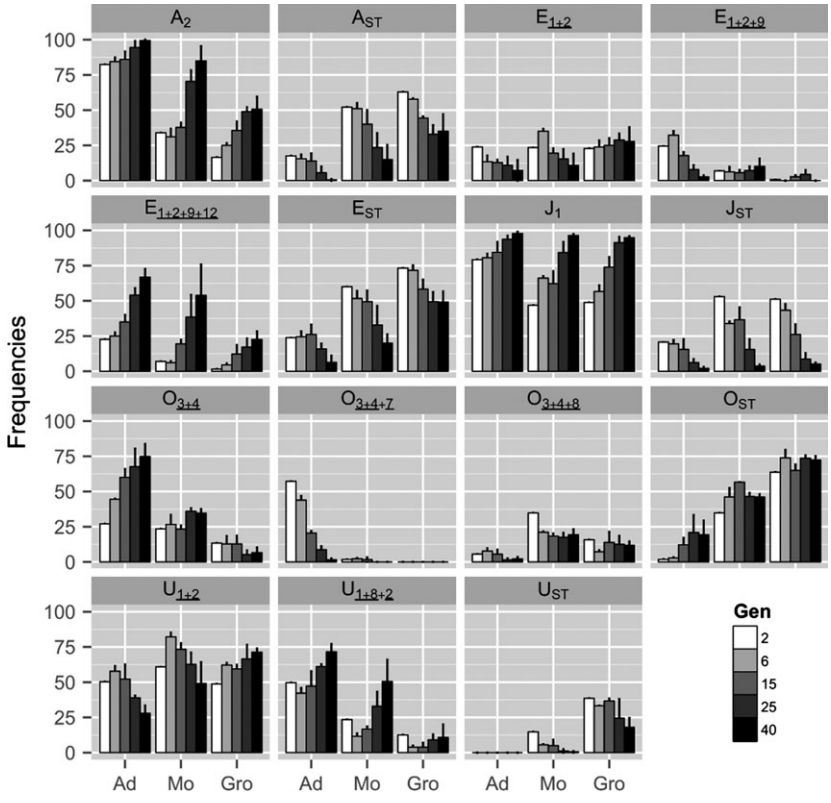

Fig. 1 Frequency of chromosomal arrangements across generations and foundations. Ad – Adraga; Mo– Montpellier; Gro – Groningen. Error bars correspond to the standard error calculated from the differences among the three replicate populations of each foundation.

Table 2 Hierarchical analyses of the genetic differentiation (Theta-f) between foundations and generations for inversion frequencies. Upper and lower CI intervals (95%) were obtained after 5000 bootstrap iterations across chromosomes.

Theta-f	Gen 6	Gen 15	Gen 25	Gen 40
Overall	0.187	0.133	0.136	0.199
Lower CI	0.114	0.069	0.069	0.133
Upper CI	0.247	0.188	0.201	0.254
Ad vs. Mo	0.199	0.152	0.063	0.067
Lower CI	0.110	0.069	0.007	−0.013
Upper CI	0.276	0.227	0.109	0.161
Ad vs. Gro	0.289	0.214	0.269	0.357
Lower CI	0.178	0.113	0.168	0.263
Upper CI	0.381	0.295	0.357	0.428
Mo vs. Gro	0.045	0.019	0.052	0.118
Lower CI	0.013	−0.015	0.015	0.092
Upper CI	0.085	0.072	0.159	0.138

three foundations. The first axis explained 36.96% and the second axis explained 12.74% of the total variation. Whereas the first axis reflected predominantly historical differentiation in the wild, the second axis accounted for changes during laboratory evolution. Inversions with a higher contribution in the first axis were A_2, $E_{\underline{1+2+9+12}}$, $O_{\underline{3+4}}$ and $U_{\underline{1+8}+\underline{2}}$ vs. A_1, A_{ST}, E_{ST}, $E_{\underline{1+2}}$, O_{ST} and U_{ST}. For the second axis, the inversions with a higher weight were J_1, $O_{\underline{3+4}+\underline{12}}$ and $O_{\underline{3+4+13}+\underline{12}}$ vs. $E_{\underline{1+2+9}}$, J_{ST} and $O_{\underline{3+4}+\underline{7}}$ (see Fig. 2).

Using the overall PCA data, we performed a multivariate evolutionary trajectory analysis (Adams & Collyer, 2009). We analysed three different parameters: magnitude (rate of response), direction (convergence vs. divergence) and shape (evolutionary path) of the trajectories. Pairwise comparisons between foundations showed differences in magnitude between Groningen and Montpellier, differences in direction between all foundations, and no differences in the shape of the trajectory (Table 3, Fig. 2). To determine whether foundations were differentiated in multivariate space at generations 6 and 40, we calculated Euclidean distances between pairs of foundations at each generation. For all comparisons, distances were significant, without any clear tendency to decrease (see Table 3c).

An ANCOVA using the PC1 coordinates showed overall differences between foundations ($F_{(2,24)} = 40.09$, $P < 0.0001$) and temporal changes across generations ($F_{(1,24)} = 100.56$, $P < 0.0001$). These temporal changes were different across foundations (foundation*generation term $F_{(2,24)} = 7.20$, $P = 0.0036$). Altogether, this indicates historical differentiation between foundations, overall evolutionary dynamics in the laboratory and impact of genetic background on the dynamics of different foundations.

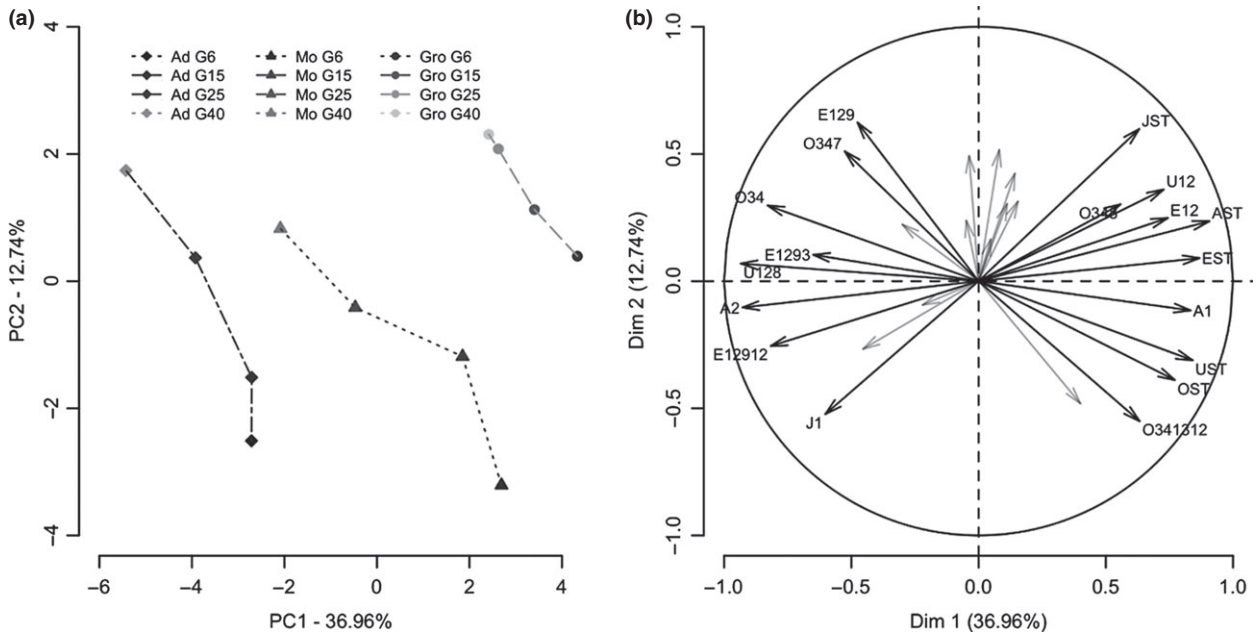

Fig. 2 Multivariate evolutionary trajectories using principal component analysis (a) and plot of explanatory variables (b) for 1st and 2nd axis. Diamonds represent Adraga, triangles represent Montpellier, and circles represent Groningen. Darker to lighter tone indicates increasing generations. Inversions with a lower weight are plotted in grey. The convention used throughout the text to designate arrangements could not be used here due to limitations of the software package used.

The ANCOVA on specific inversions indicated significant response across foundations for inversions A_1, A_2, A_{ST}, $E_{\underline{1+2}}$, $E_{\underline{1+2+9+12}}$, E_{ST}, J_1, $U_{\underline{1+8}+\underline{2}}$ and $U_{\underline{1+2}}$. Also, differences in evolutionary dynamics between foundations were found for A_1, $O_{\underline{3+4}}$, $U_{\underline{1+2}}$ and $U_{\underline{1+8}+\underline{2}}$ (Table S3, Fig. 1). We further focus on processes that might explain temporal changes in inversion frequencies.

Evolutionary dynamics of specific inversions

The Cochran–Mantel–Haenszel chi-squared test (Table 4) was applied to inversions with frequencies higher than 5% in all populations, and also to those that presented more than 5% change in frequency between generations 6 and 40, at least for one foundation. Significant changes between these two generations were obtained for several of the most frequent inversions (see Table 4), with the exception of inversions O_{ST} in both Groningen and Montpellier, A_1 and $U_{\underline{1+8}+\underline{2}}$ in Groningen and $O_{\underline{3+4+8}}$ in Montpellier.

To test whether changes in inversions frequencies were significantly different from neutral expectations, we performed simulations on data of specific inversions presenting a significant CMH statistic. Significant departure from neutral expectations in at least two of the three replicate populations (after FDR correction) was obtained for A_2 (also A_{ST} because Adraga only has two inversions in this chromosome) and $O_{\underline{3+4}+\underline{7}}$ for Adraga; and A_2, A_{ST} and $E_{\underline{1+2+9+12}}$ for Montpellier (see Table 4).

For these inversions different selective models (dominance, additivity and recessiveness; see Table 1) and a range of selective coefficients were tested. In the case of $O_{\underline{3+4}+\underline{7}}$ in Adraga, we obtained the best fit with an additive model for all replicate populations, considering both lower residuals and *D*, and also after visual inspection of various models (Table 5, Fig. 3). The range of the best selective coefficients was between 0.18 and 0.27 (Table 5). On the other hand, frequency changes for A_2 in Adraga could not be attributed to any particular model in two tested populations (Ad_2 and Ad_3). In fact, A_2 changes could be explained by a recessive model with a low selective coefficient or by an additive model with a higher selective coefficient. As for A_2 changes in the Montpellier populations (Mo_1 and Mo_3), the model that best fitted the data was a recessive model with a selective coefficient between 0.14 and 0.16 (Table 5). Conversely, for A_{ST}, there was inconsistency between replicate populations: although an additive model seemed to fit best Mo_3 populations, no model was particularly suited to explain changes in the Mo_1. The same happened with $E_{\underline{1+2+9+12}}$ where there was inconsistency between replicate populations, with the additive model being the best fit for Mo_3, whereas the dominance model was best suited for Mo_1.

Table 3 Principal component analysis.

a) Eigenvectors for the first two axes of the principal component analysis, using all arrangements, foundations and generations

Arrangement	PC 1	PC 2
A_1	0.255	0.060
A_2	−0.283	0.054
A_{ST}	0.277	−0.122
E_{1+2}	0.227	−0.129
E_{1+2+9}	−0.145	−0.324
$E_{1+2+9+12}$	−0.250	0.133
E_{ST}	0.265	−0.047
J_1	−0.184	0.272
O_{3+4}	−0.253	−0.155
O_{ST}	0.236	0.202
U_{1+8+2}	−0.285	−0.036
U_{1+2}	0.222	−0.187

b) Pairwise differences and significance levels with permutation tests using multivariate trajectory analysis (see Material and Methods)

Gen	Foundations	Statistic
Magnitude	Ad-Mo	3.384n.s.
	Ad-Gro	12.191m.s.
	Gro-Mo	15.574*
Direction	Ad-Mo	49.493**
	Ad-Gro	62.847***
	Gro-Mo	40.928*
Shape	Ad-Mo	0.101n.s.
	Ad-Gro	0.246n.s.
	Gro-Mo	0.253n.s.

c) Pairwise Euclidean distances between foundations at each generation analysed

Comparison	Gen 6	Gen 15	Gen 25	Gen 40
Ad vs. Mo	6.929	5.760	4.847	5.586
Ad vs. Gro	8.528	6.998	7.132	8.754
Mo vs. Gro	5.433	4.202	5.164	5.394

(a) PC 1 explains 36.96% of the total variation and PC2 explains 12.74%; only arrangements that had an average frequency of at least 5% across generations and foundations are presented.
(b) Significance levels: $P < 0.001$***; $0.01 > P > 0.001$**; $0.05 > P > 0.01$ *; $0.1 > P > 0.05$ m.s.; $P > 0.1$ n.s.

Discussion

Impact of genetic background on the evolution of inversions under uniform selection

Our populations presented clear initial differentiation in chromosomal inversion frequencies for all 5 chromosomes, according to expectations from the European cline (Krimbas & Loukas, 1980; Prevosti *et al.*, 1988; Balanyà *et al.*, 2003; Simões *et al.*, 2012). Throughout adaptation to laboratory environment, historical differentiation remained high, despite a significant role of selection in some inversions. The sole exception was found in the last generation (40), where indications of convergence were observed between Adraga and Montpellier populations.

As expected, we found an overall decrease in inversion polymorphism (e.g. Santos *et al.*, 2005), although it was not pervasive across chromosomes or foundations, with a lower decrease of heterozygosity in Groningen populations, partly due to a smaller drop of frequencies of standard inversions. In general, these differences suggest the effect of distinct genetic backgrounds between populations.

Some consistent evolutionary patterns emerged in our study such as the decline in standard inversion frequencies for all foundations and chromosomes, except in O_{ST}. In a thermal selection study using *D. subobscura* populations from Puerto Montt (South America), Santos *et al.* (2005) found a decrease in standard inversions frequencies of populations maintained at 18 °C for A, J and O chromosomes and an increase for E_{ST}. There are obvious contrasts between these results and ours, despite similar environmental temperature (18 °C) and number of generations in the laboratory. Contrasting backgrounds of founding populations may have contributed to these differences because bottlenecks effects considerably reduced chromosomal diversity in South American colonizing populations (Prevosti *et al.*, 1985).

The pattern of decline of standard inversions in the new environment may be due to the fact that these arrangements increase in frequency at higher latitudes and can be characterized as cold climate (Krimbas, 1992; Rego *et al.*, 2010). In fact, Rego *et al.* (2010) found that, in general, *D. subobscura* flies carrying these arrangements preferred colder temperatures (e.g. between 15 and 16 °C), below those experienced by populations in our study (18 °C). However, as mentioned above, the magnitude of the decline differed between foundations, with Groningen populations (which had initial higher standard frequencies) presenting a smaller decline than both Adraga and Montpellier. It appears thus that, although selection shaped the evolutionary dynamics of inversion frequencies, it has done so within historical constraints of each population. This suggests that, extrapolating to nature, evolutionary forces responsible for the observed clinal shifts due to global warming (e.g. Balanyà *et al.*, 2006, 2009) are strong, as they have been able to override to some extent constraints associated with historical variation.

Do changes in inversion frequencies explain phenotypic convergence?

We have previously shown a clear and quick convergence after only 14 generations in laboratory for fecundity, starvation resistance and body size (Fragata *et al.*, 2014). However, in the present study, no general convergence was found in inversion frequencies. Although some inversions showed a similar selective response

Table 4 Cochran–Mantel–Haenszel (CMH) statistic and simulations (for each replicate population) applied to the most frequent arrangements.

Arrangement	Ad_1	Ad_2	Ad_3	Ad (CMH)	Mo_1	Mo_2	Mo_3	Mo (CMH)	Gro_1	Gro_2	Gro_3	Gro (CMH)
A_2	n.s.	***	***	28.733***	***	n.s.	**	140.082***	*	n.s.	n.s.	47.354***
A_{ST}	–	–	–	–	**	n.s.	*	71.258***	n.s.	n.s.	n.s.	28.101***
A_1					n.s.	n.s.	n.s.	36.003***				2.268 n.s.
E_{ST}	m.s.	n.s.	n.s.	35.120***	n.s.	n.s.	m.s.	49.562***	n.s.	n.s.	n.s.	23.195***
$E_{\underline{1+2+9+12}}$	m.s.	**	n.s.	100.355***	**	n.s.	***	157.572***	*	n.s.	n.s.	43.951***
$E_{\underline{1+2}}$	n.s.	n.s.	n.s.	8.091*	n.s.	n.s.	n.s.	34.471***				
$E_{\underline{1+2+9}}$	n.s.	n.s.	n.s.	53.858***								
$E_{\underline{1+2+9+3}}$	n.s.	n.s.	m.s.	19.584***								
J_1	n.s.	***	n.s.	30.197***	n.s.	n.s.	m.s.	69.773***	m.s.	m.s.	*	84.452***
O_{ST}	*	m.s.	n.s.	48.140***				0.022 n.s.				1.066 n.s.
$O_{\underline{3+4}}$	n.s.	n.s.	*	46.408***	n.s.	n.s.	n.s.	6.002*	n.s.	n.s.	n.s.	7.799*
$O_{\underline{3+4+7}}$	*	*	**	105.712***								
$O_{\underline{3+4+8}}$								0.171 n.s.				
$U_{\underline{1+2}}$	–	–	–	–	n.s.	n.s.	**	63.029***	n.s.	n.s.	n.s.	7.223*
$U_{\underline{1+8+2}}$	n.s.	n.s.	n.s.	31.916***	n.s.	n.s.	**	96.177***				3.873 n.s.
U_{ST}					n.s.	n.s.	n.s.	10.220**	n.s.	n.s.	n.s.	16.462***

CMH statistic and simulations use data from generations 6 to 40. Significance levels for CMH after FDR correction ($n = 11$) for Adraga and Groningen: ***$P < 0.00033$ ($\alpha = 0.001$); **$0.00033 < P < 0.0033$ ($\alpha = 0.01$); *$0.0033 < P < 0.017$ ($\alpha = 0.05$); m.s. $0.017 < P < 0.033$ ($\alpha = 0.1$); n.s. $P > 0.033$. Significance levels for CMH after FDR correction ($n = 13$) for Montpellier: ***$P < 0.00031$ ($\alpha = 0.001$); **$0.00031 < P < 0.0031$ ($\alpha = 0.01$); *$0.0031 < P < 0.016$ ($\alpha = 0.05$); m.s. $0.016 < P < 0.031$ ($\alpha = 0.1$); n.s. $P > 0.031$. Significance levels for simulations were also assessed after FDR correction – $n = 11$ for Adraga and Montpellier – see above; $n = 8$ for Groningen: ***$P < 0.00037$ ($\alpha = 0.001$); **$0.00037 < P < 0.0037$ ($\alpha = 0.01$); *$0.0037 < P < 0.018$ ($\alpha = 0.05$); m.s. $0.018 < P < 0.037$ ($\alpha = 0.1$); n.s. $P > 0.037$. CMH statistic (pooled ratio) and significance levels are presented for each foundation. Significance of the simulations applied (see also Material and Methods) is shown for each replicate population. Dashes indicate cases where only one alternative arrangement was present, thus its analysis was redundant.

across foundations, the overall pattern that emerges does not support a clear association between selection at the inversion frequency level and phenotypic selection. We have previously discussed (see Fragata *et al.* (2014) and references therein) that phenotypic convergence does not imply underlying genetic convergence.

There can be several explanations for why the evolutionary dynamics of inversion frequencies did not mirror the convergence patterns at phenotypic level. A simple explanation might be that inversions are not associated with relevant phenotypic traits. However, whenever they have been carefully looked at, associations between inversions and morphological, physiological and life-history traits in *D. subobscura* were found, for instance body size (Orengo & Prevosti, 2002; Fragata *et al.*, 2010), thermotolerance (Dolgova *et al.*, 2010; Rego *et al.*, 2010; Calabria *et al.*, 2012), thermal preference (Rego *et al.*, 2010), mating success (Santos *et al.*, 1986) and viability (Santos, 2009). So it is unlikely that the contrasting patterns between inversions and the phenotypic traits for which we saw convergence are due to a lack of association between them. Another possible explanation is that, despite ample evidence of adaptive value of inversions (Hoffmann *et al.*, 2004; Hoffmann & Rieseberg, 2008), many do not confer to their carriers any particular selective value in our laboratory environment. However, the consistent pattern observed in the increase (in the case of A_2, J_1) or decrease (in the case of A_{ST}, J_{ST}, E_{ST} and U_{ST}) of some inversions in all three foundations points to a possible common adaptive value of these inversions, a finding that is not evident when we consider each population independently. Even in the presence of selection, historical differentiation may still play an important role in the absence of convergence at the inversion frequency level: our populations showed different starting inversion frequencies as expected, but not all inversions analysed here were simultaneously segregating in natural populations, which can constrain convergent evolution.

History can also affect the genetic content of a given inversion producing different targets for selection between populations (Dobzhansky, 1970; Kirkpatrick & Barton, 2006; Kirkpatrick, 2010). According to the Dobzhansky's co-adaptation hypothesis (Dobzhansky, 1970), it is expected that inversions vary in genetic content across geographically distant populations. Nevertheless, most studies did not find genetic differentiation within inversions (but see Kennington & Hoffmann, 2013). In *D. subobscura*, several studies suggest low within-inversion genetic differentiation across the European cline (Rozas *et al.*, 1995; Simões *et al.*, 2012; Pegueroles *et al.*, 2013), although they have used a limited number of genetic markers. In contrast with those results, Santos (2009) detected recombination load in several crosses of homokaryotypic lines for several inversions of the O chromosome, in a *D. subobscura* pop-

Table 5 Initial range of the selection coefficients and best selective coefficient.

				Mean Resid.		D Stat.	
Arrang	Rep	Heritability	Sel. Coef. Range	Sel. Coef	Avg	Sel. Coef.	Avg
A_2	Ad_1	h0	0.03–0.32	0.22	0.005	0.18	0.469
	Ad_1	h05		0.07	0.005	0.06	0.492
	Ad_1	h1		0.05	0.006	0.03	0.496
	Ad_2	h0	0.08–0.39	0.39	0.004	0.38	0.498
	Ad_2	h05		0.14	0.004	0.25	0.465
	Ad_2	h1		0.08	0.003	0.13	0.462
	Ad_3	h0	0.14–0.42	0.41	0.004	0.42	0.699
	Ad_3	h05		0.32	0.000	0.33	0.295
	Ad_3	h1		0.18	0.000	0.18	0.292
O_{3+4+7}	Ad_1	h0	0.23–0.5	0.23	0.006	0.23	0.361
	Ad_1	h05	0.21–0.41	0.27	0.003	0.22	0.279
	Ad_1	h1	0.12–0.41	0.40	0.002	0.40	0.323
	Ad_2	h0	0.11–0.31	0.17	0.004	0.14	0.291
	Ad_2	h05		0.19	0.003	0.18	0.273
	Ad_2	h1		0.28	0.003	0.31	0.294
	Ad_3	h0	0.18–0.38	0.20	0.003	0.18	0.305
	Ad_3	h05		0.24	0.003	0.23	0.274
	Ad_3	h1		0.32	0.007	0.38	0.440
A_2	Mo_1	h0	0.1–0.35	0.14	0.026	0.14	0.375
	Mo_1	h05		0.13	0.022	0.14	0.356
	Mo_1	h1		0.14	0.024	0.15	0.348
	Mo_2	h0	0.01–0.25	0.08	0.011	0.09	0.426
	Mo_2	h05		0.08	0.015	0.09	0.451
	Mo_2	h1		0.08	0.023	0.10	0.498
	Mo_3	h0	0.08–0.28	0.16	0.038	0.18	0.499
	Mo_3	h05		0.15	0.030	0.16	0.493
	Mo_3	h1		0.16	0.029	0.16	0.486
A_{ST}	Mo_1	h0	0.02–0.32	0.08	0.022	0.08	0.444
	Mo_1	h05		0.10	0.021	0.08	0.446
	Mo_1	h1		0.11	0.022	0.11	0.449
	Mo_2	h0	0.02–0.32	0.03	0.015	0.04	0.589
	Mo_2	h05		0.04	0.013	0.05	0.561
	Mo_2	h1		0.05	0.010	0.05	0.547
	Mo_3	h0	0.1–0.33	0.17	0.003	0.14	0.361
	Mo_3	h05		0.22	0.003	0.22	0.363
	Mo_3	h1		0.33	0.006	0.33	0.476
$E_{1+2+9+12}$	Mo_1	h0	0.07–0.26	0.12	0.016	0.12	0.412
	Mo_1	h05		0.16	0.022	0.19	0.415
	Mo_1	h1		0.24	0.070	0.24	0.702
	Mo_2	h0	0.03–0.32	0.03	0.010	0.07	0.531
	Mo_2	h05		0.03	0.011	0.11	0.543
	Mo_2	h1		0.04	0.011	0.32	0.610
	Mo_3	h0	0.1–0.32	0.18	0.019	0.21	0.349
	Mo_3	h05		0.24	0.018	0.24	0.320
	Mo_3	h1		0.32	0.143	0.32	0.557

The best selective coefficients were those associated with lower mean residuals and *D* statistic. Average (Avg) of the mean residuals and *D* statistic are presented for the best selective coefficient. For the heritability models: h0 corresponds to dominance, h05 to additivity and h1 to recessiveness. The selective coefficient range selected for further analysis was obtained by simulating once each selection coefficient for each heritability model (from 0.01 to 0.5 in increments of 0.01 per replicate population). When the range values were very close between models, we used the same interval for all models in each replicate population.

ulation. Thus, this study shows that the genetic content of a given inversion can change even inside the same population. A deep genomic coverage within and outside inverted fragments across populations is essential to give further insight into this issue (e.g. see Corbett-Detig & Hartl, 2012; Kennington & Hoffmann, 2013).

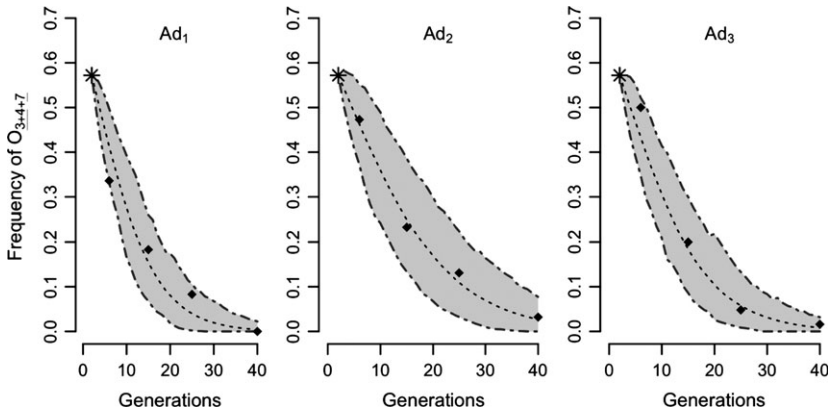

Fig. 3 Plot of observed and expected O_{3+4+7} frequency changes and associated 95% boundaries for the three Adraga populations. The model with the best fit (additive model) is presented for each replicate population. Diamonds represent the observed frequencies at each generation assayed. Asterisk represents the initial frequency from which all simulations were computed.

Do inversions present signs of adaptive response?

We found significant changes through time due to selection, both positive (A_2, in Adraga and Montpellier and $E_{1+2+9+12}$ only in Montpellier) and negative (A_{ST} for Adraga and Montpellier and O_{3+4+7} only for Adraga). It is interesting that, despite the pervasive pattern of frequency decline for all standard arrangements (with the exception of O_{ST}), we could only detect selection for the A_{ST} inversion for Adraga and Montpellier. It might be the case that we lacked statistical power to detect a selective pattern due to low initial standard inversion frequencies in these populations. On the other hand, Groningen populations presented a consistent (across chromosomes and populations), mild decline from initially high standard inversion frequencies. Overall, negative selection may have been implicated across foundations.

Contrary to Inoue (1979), where negative selection was pervasive, we found contrasting selection patterns, with both positive and negative selection apparently playing a role in the evolution of the different inversions. Kapun *et al.* (2014) also found, in *D. melanogaster* populations, indications of positive selection in some inversions. In our study, the most consistent pattern was the negative selection for the O_{3+4+7} arrangement for Adraga. We found that the best model to explain the changes in frequencies was an additive model. In fact, this arrangement presented a striking decline from an initial frequency of around 60% to almost 0, for which we estimated an overall selective coefficient (s) of 0.18 to 0.27. This suggests a rather strong negative selection for O_{3+4+7}, three times higher than that reported for the decline of inversions in *D. melanogaster* cage populations (s = 0.06; see Inoue, 1979).

This arrangement presents an interesting pattern in natural populations. It shows repeatable clinal variation, across three continents, with higher frequencies at lower latitudes (Prevosti *et al.*, 1988; Balanyà *et al.*, 2003). Moreover, this arrangement shows clear seasonal variation in southern *D. subobscura* populations in Europe (Rodríguez-Trelles *et al.*, 1996, 2013), rising in frequency in late spring (corresponding to a decline of other inversions, particularly O_{ST}), early summer, with temperatures above 15 °C, and declining in colder seasons (Rodríguez-Trelles *et al.*, 1996, 2013). It seems that fluctuating temperature is the environmental cue driving these chromosomal inversions changes, something that was prevented here. Nevertheless, our populations were kept at 18 °C, so it is unlikely that the decline in O_{3+4+7} frequencies could be directly related to a thermal response. Another possible explanation for the decrease in frequency of this particular arrangement might be found in the presence of lethal genes. In fact, in *D. subobscura* North American populations, Mestres *et al.* (2001) found an association between lethal genes and the O_{3+4+7} arrangement, and an indication of a heterotic effect in heterokaryotypes. Araúz *et al.* (2009) also found lethal genes in O_{3+4+7} arrangements in a Barcelona population. However, the slow rate of decline of this arrangement in our populations, with a selective coefficient between 0.18 and 0.27, does not favour this hypothesis.

To sum up, we found that a strong initial historical signature is still maintained in the pattern of inversion frequencies despite 40 generations of evolution in a common environment. Therefore, observed convergence at the phenotypic level (Fragata *et al.*, 2014) was not associated with a similar evolutionary dynamics in inversion frequencies. Nevertheless, some common patterns arose between populations, namely decrease in frequency of most cold-climate standard inversions that were initially present in all populations at various frequencies according to the latitudinal cline. We found that selection acted within the limits imposed by high historical constraints to shape evolutionary dynamics of chromosomal inversions. This study shows that to predict the evolutionary potential of populations, it is

important to take into account the role of history and selection. This issue is even more vital when populations are facing the loss of biodiversity due to the effects of global environmental changes.

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Data accessibility

Raw data of inversions available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.n0mv4.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Simulated $O_{\underline{3+4}+\underline{7}}$ frequency changes.

Figure S2 Mean residuals (a) and *D* Statistic (b) for the simulated $O_{\underline{3+4}+\underline{7}}$ frequency changes.

Figure S3 Evolutionary changes in inversion heterozygosity across generations and foundations.

Table S1 Frequencies and number of individuals analyzed per arrangement, population and generation.

Table S2 Allelic richness (A) and Expected heterozygosity (H_e) for the several chromosomes, populations and generations analyzed.

Table S3 ANCOVA model on specific arrangements.

Data S1 Additional Material and Methods.

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