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Tracking changes in chromosomal arrangements and their genetic content during adaptation

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Abstract

There is considerable evidence for an adaptive role of inversions, but how their genetic content evolves and affects the subsequent evolution of chromosomal polymorphism remains controversial. Here, we track how life-history traits, chromosomal arrangements and 22 microsatellites, within and outside inversions, change in three replicated populations of *Drosophila subobscura* for 30 generations of laboratory evolution since founding from the wild. The dynamics of fitness-related traits indicated adaptation to the new environment concomitant with directional evolution of chromosomal polymorphism. Evidence of selective changes in frequency of inversions was obtained for seven of 23 chromosomal arrangements, corroborating a role for inversions in adaptation. The evolution of linkage disequilibrium between some microsatellites and chromosomes suggested that adaptive changes in arrangements involved changes in their genetic content. Several microsatellite alleles increased in frequency more than expected by drift in targeted inversions in all replicate populations. In particular, there were signs of selection in the O_{3+4} arrangement favouring a combination of alleles in two loci linked to the inversion and changing along with it, although the lack of linkage disequilibrium between these loci precludes epistatic selection. Seven other alleles increased in frequency within inversions more than expected by drift, but were not in linkage disequilibrium with them. Possibly these alleles were hitchhiking along with alleles under selection that were not specific to those inversions. Overall, the selection detected on the genetic content of inversions, despite limited coverage of the genome, suggests that genetic changes within inversions play an important role in adaptation.

Introduction

There has long been evidence for an adaptive role of chromosomal inversions (Dobzhansky, 1950; Hoffmann *et al.*, 2004; Balanyà *et al.*, 2006), but the specific roles that they play in adaptation are poorly understood. Chromosomal inversions have been known in the genus *Drosophila* for almost a century (vid. Sturtevant, 1921), but they also occur in a large number of taxa including insects, plants and vertebrates, presenting the patterns of local adaptation in multiple species (Hoffmann & Rieseberg, 2008). For example, chromosomal inversions seem to play an important role in the repeated adaptation of stickleback fish populations to fresh water from marine ancestors (Jones *et al.*, 2012). In *Anopheles*, evidence has been found for the involvement of a single inversion in adaptation to savannah

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and highland habitats (Ayala *et al.*, 2013). In humans, inversions have mostly been associated with reproductive sterility, although a common inversion in Europeans has been shown to increase female fertility and thus is likely to be under positive selection (Stefansson *et al.*, 2005). In plants, polymorphic inversions contribute to adaptation of inland and coastal ecotypes (Lowry & Willis, 2010) and play a role during the domestication of cultivars (Yang *et al.*, 2012). Many studies have suggested an adaptive role of inversions in *Drosophila* species. For example, the frequencies of inversions follow latitudinal and altitudinal gradients (Dobzhansky, 1948; Prevosti *et al.*, 1988; Kapun *et al.*, 2014) as well as seasonal cycles (Rodríguez-Trelles *et al.*, 2013). In *Drosophila subobscura*, evidence for an adaptive role for inversions comes from repeated clinal gradients in chromosomal inversion frequencies that evolved fast from standing variation in colonizing events (Prevosti *et al.*, 1988). Fruit fly inversion frequencies even change in parallel with climate change (Umina *et al.*, 2005; Balanyà *et al.*, 2006).

Several hypotheses have been proposed to explain how inversions arise and spread through populations (for a review, see Hoffmann & Rieseberg, 2008). There is general agreement that they have monophyletic origins (Aquadro *et al.*, 1991; Rozas *et al.*, 1999; but see Aguado *et al.*, 2014). The main theories for their subsequent evolution focus on (i) the direct selective value of chromosomal arrangements, ii) heterokaryotype advantage or (iii) the reduction in recombination within the inverted segments (Hoffmann & Rieseberg, 2008; Pegueroles *et al.*, 2010a). In his classic model of co-adaptation, Dobzhansky (1943, 1970) proposed that alleles at different loci within an inversion are 'co-adapted' due to synergistic epistatic effects on the fitness of a local population, resulting in higher fitness value than that expected from their disjoint effects. As different chromosomal arrangements show reduced recombination rates among them, inversions carrying a favourable combination of co-adapted alleles can spread to fixation if their spread is not counteracted by migration. Kirkpatrick & Barton (2006) proposed an alternative 'local adaptation' model, in which inversions are favoured if they capture locally adapted alleles, without the necessity of epistatic effects on fitness.

Disentangling these two hypotheses is troublesome. Several studies have analysed the genetic composition of chromosomal arrangements trying to elucidate which model better explains their adaptive value. In *Drosophila*, high linkage disequilibrium patterns have been detected among genes within inversions, particularly near their breakpoints (Laayouni *et al.*, 2003; Schaeffer *et al.*, 2003; Nóbrega *et al.*, 2008; but see Munté *et al.*, 2005). This result is in agreement with the expectation that inverted segments reduce recombination, particularly near breakpoints in heterokaryotypes (Navarro *et al.*, 2000; Simões *et al.*, 2012). However, as both hypotheses lead to the expectation that selection favours certain combinations of genes within inverted regions, such finding does not allow discrimination between the two models. On the other hand, Kennington *et al.* (2006) found a strong linkage between genes within chromosomal arrangements, away from breakpoints, interspersed with genes with low association, indicative of epistatic selection, as expected with Dobzhansky's model. Another expectation of the co-adaptation hypothesis is that the same inversion will show genetic differentiation across populations, which does not seem to be the case according to most of the studies (Rozas *et al.*, 1995; Schaeffer *et al.*, 2003; Simões *et al.*, 2012; Pegueroles *et al.*, 2013; but see Kennington & Hoffmann, 2013).

One approach to the identification of epistatic selection is the analysis of consistency in linkage disequilibrium patterns between populations under similar selective pressures, specifically through the partitioning of linkage disequilibrium into within- and between-population components (Ohta, 1982; also see Whittam *et al.*, 1983). Loci that are not subject to selection are most likely to show a large between-population component for linkage disequilibrium (Ohta, 1982; Black & Krafsur, 1985). On the other hand, systematic associations among alleles in multiple populations isolated from each other may be taken as circumstantial evidence of the direct action of natural selection on the loci involved (Lewontin, 1964). In such cases, a relatively large within-population component and a relatively small between-population component are expected, because disequilibrium is in the same direction in all populations. This outcome may indicate epistatic selection on the loci involved, although caution is needed in this interpretation. If rates of recombination are low, natural selection acting on variation at one locus can indirectly affect the associations between neutral alleles at closely linked loci through 'hitchhiking'. Moreover, nonselective factors such as founder effects and genetic drift can generate a transient linkage disequilibrium that may persist for many generations (Whittam *et al.*, 1983), although in this latter case it is less likely that systematic associations will be sustained across multiple populations long isolated from each other.

Real-time adaptive evolution in fitness-related traits has been repeatedly observed in *D. subobscura* laboratory populations studied by our group, whether derived from nearby locations (Simões *et al.*, 2008b; Santos *et al.*, 2012) or from latitudinally widely separated locations (Fragata *et al.*, 2014b). We have also shown that founder effects lead to fast genetic differentiation in neutral markers among colonizing populations derived from the same source population, with a detectable impact on later adaptation (Santos *et al.*, 2012, 2013). In addition, Fragata *et al.* (2014a) found temporal changes in the frequencies of several inversions during

laboratory adaptation across populations derived from contrasting latitudes, although convergence was not observed.

However, no experimental evolution study has up to now tracked chromosomal arrangements together with their genetic content during adaptation to a novel environment. The study of the temporal changes in both inversion frequencies and their genetic content should shed light on the selective processes acting on standing variation for chromosomal arrangements. Parallel evolution in the frequencies of chromosomal inversions across replicates, as well as of specific alleles in linkage disequilibrium within them, may reveal the mechanisms of adaptive evolution involving chromosomal inversions.

With this goal in mind, here we present a study of the evolution of life-history traits, chromosomal inversion polymorphisms and their genetic content for about 30 generations of adaptation to the laboratory, involving three replicate populations of *D. subobscura*. We characterize (i) the evolutionary dynamics of life-history traits among these populations in comparison with those of long-established populations; (ii) changes in the frequency of chromosomal arrangements over the same period; and (iii) changes in the genetic content of these arrangements using microsatellite loci localized across all chromosomes, inside and outside inversions.

Materials and methods

Sampling and experimental design

In March 2008, 151 females and several males of *D. subobscura* were collected from a pinewood in Sintra (Adraga, Portugal) to establish a laboratory population, 'SW'. Although most females were already fertilized, flies were initially maintained in vials with 3–5 females and 2–3 males, from which approximately 1300 descendants were obtained. The eggs laid by the flies of the first laboratory-reared generation were then split to give rise to three replicate populations, SW_1, SW_2 and SW_3. From this point on, these three replicates were maintained separately, adapting in parallel with the novel environment (laboratory). This environment included discrete generations of 28 days, reproduction close to the point of peak fecundity, photoperiod of 12 h of light and 12 h of darkness, a temperature of 18 °C and controlled densities of 50 adult flies or 80 eggs per vial. Each population was cultured using numerous vials, with adult population census sizes of 600–1200 individuals (Simões *et al.*, 2008b; Santos *et al.*, 2012). At each generation, adults were mixed and redistributed over several vials to lay eggs for the subsequent generation.

At the time of founding of the three replicate SW populations, three TW populations, derived in the same manner from the same location in October 2001 (Simões *et al.*, 2007), had already been adapting to the same laboratory conditions for 83 generations. These populations were used as control for life-history traits assays and as reference for the chromosomal inversion polymorphism analyses.

Assays of life-history traits

Phenotypic assays were performed at generations 4, 7, 11, 14, 20 and 33 of the $SW_{1–3}$ populations. Because life-history traits were measured at different points in time and could be subjected to environmental fluctuations between assayed generations, the TW populations, already adapted to laboratory conditions, were assayed in synchrony (at their generations 87, 90, 94, 97, 103 and 116, respectively) and used as control in these assays. For each population, 16–18 pairs of flies were assayed. Single virgin females were crossed with single virgin males and then transferred daily to fresh medium vials over 12 days. The eggs laid by females were counted every 24 h. After 12 days, each pair was transferred to a vial containing plain agar medium and survival under these starvation conditions was recorded at 6-h intervals. Four life-history traits were analysed: age of first reproduction (A1R), the number of days between emergence and the first egg laying; early fecundity (F1–7), corresponding to the number of eggs laid between days 1 and 7 after emergence; peak fecundity (F8–12), the number of eggs laid between days 8 and 12; and female starvation resistance (RF), the number of hours until death after the transfer to non-nutri tive agar medium following the procedure in Simões *et al.* (2008b). As a proxy for fitness, a composite phenotype (CPhen) was estimated from these traits by performing a discriminant analysis between two generations, following the procedure given in Santos *et al.* (2012). The values of generations 4 and 14 were used because it is the period with the faster evolutionary rate, as expected from previous studies (Matos *et al.*, 2004; Simões *et al.*, 2007) and also observed in the present work (see Results). The CPhen value in each generation for each SW population was obtained as $\mathrm{CPhen} = \Sigma_{i=1}^{4} p_i l_i$, in which p_i is the ith trait mean value at that generation and l_i refers to the linear discriminant coefficient for the ith trait. The linear standardized coefficient for each trait was as follows: A1R = −3.06; F1–7 = −5.60; F8–12 = 4.05; and RF = −0.45. The evolutionary trajectory of the CPhen was then obtained.

Chromosomal polymorphism

Chromosomal polymorphism was scored using 51–90 larvae per population at generations 4, 14 and 28 of the $SW_{1–3}$ populations and at generations 83 and 118 of the $TW_{1–3}$. TW populations were used as reference to determine whether laboratory evolution of chromoso-

mal polymorphisms among SW populations converged on the frequencies of the long-established TW populations. A total of 660 larvae were scored for the three SW populations: 254 larvae at generation 4, 243 at generation 14 and 163 at generation 28. For the TW, 420 larvae were scored for chromosomal inversions: 241 larvae at generation 83 and 179 at generation 118. For each population, randomly picked males were mated with 2–3 virgin females of the cherry-curled (*chcu*) strain of *D. subobscura*. This strain is homozygous for the recessive markers *cherry eyes* and *curled wings* and homokaryotypic for the five chromosomes, featuring the arrangements A_{ST}, J_{ST}, U_{ST}, E_{ST} and $O_{\underline{3+4}}$ (Fig. 1). For each cross, the salivary glands of one female third-instar larva were extracted and stained with 2% orcein in 60% acetic acid mixed 50 : 50 with lactic acid. The remaining larval material was stored in absolute ethanol for later molecular analyses. Analysis of the orcein-stained slides allowed us to determine half of the parent-male chromosomal arrangements from the known chromosome patterns expected from a cross with *chcu* chromosomes. Male chromosomal arrangements were classified following Kunze-Mühl & Müller (1958) and Krimbas & Loukas (1980). Following the convention established in the latter study, overlapping inversions in the same chromosomal arrangement are underlined together.

Microsatellite loci scoring

For each SW population, between 43 and 59 larvae scored for their inversion polymorphisms were genotyped at generations 4 and 28. Clean genomic DNA was extracted from the remaining biological material of each larva following the protocol of Pascual *et al.* (1997). The DNA was resuspended in 50 μL of deionized sterile water. Each larva was genotyped at 22 microsatellite loci (Pascual *et al.*, 2000) selected according to their cytological locations (Santos *et al.*, 2010) – loci *dsub5*, *dsub11*, *dsub21*, *dsub70* and *dsub76* are located in chromosome A; *dsub20*, *dsub28*, *dsub53* and *dsub79* are localized in chromosome E; *dsub59*, *dsub69* and *dsub74* are in chromosome J; *dsub1*, *dsub2*, *dsub4*, *dsub12*, *dsub14*, *dsub29*, *dsub34* and *dsub47* are in chro-

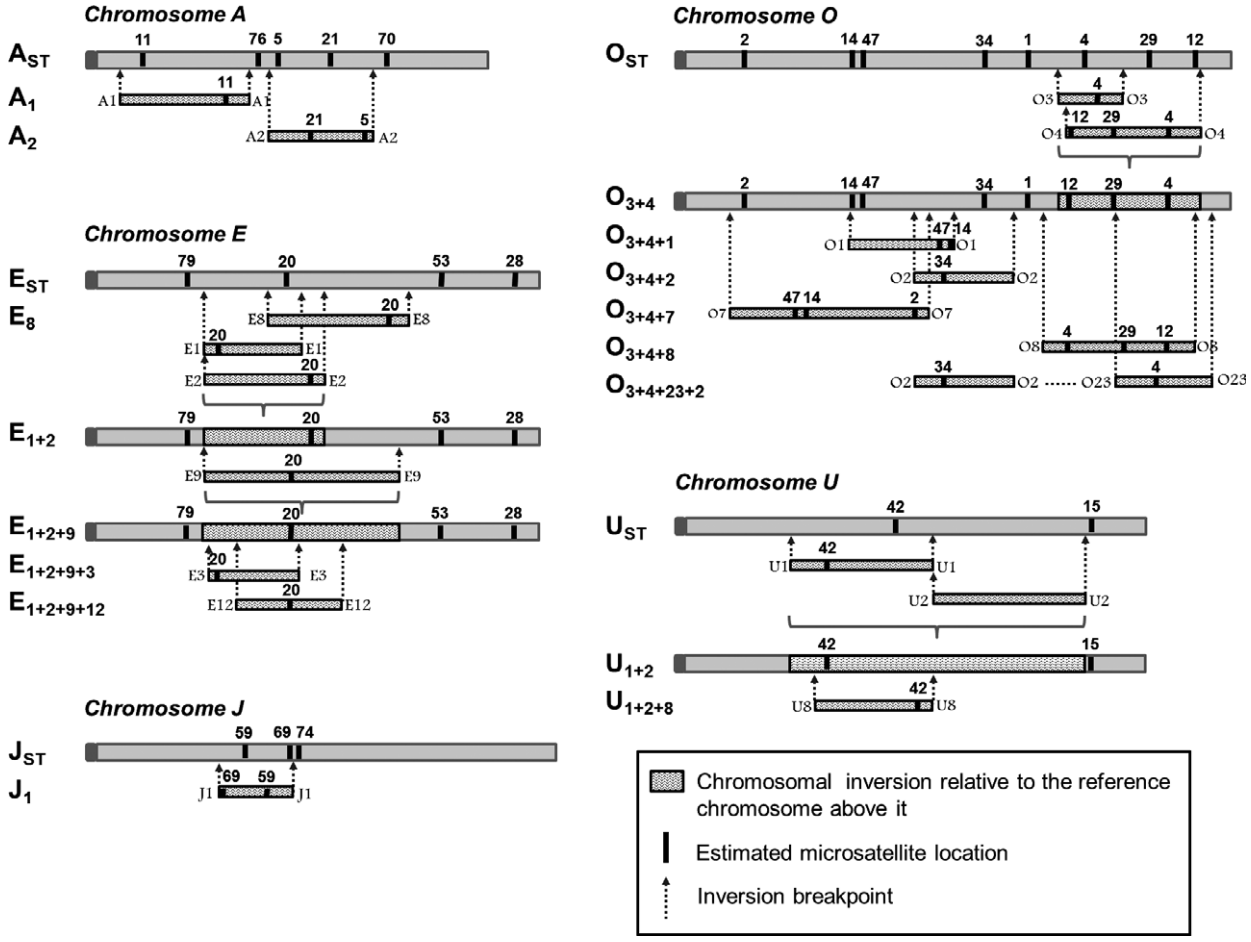

Fig. 1 Microsatellite chromosomal locations relative to inversion breakpoints.

mosome O; *dsub15* and *dsub42* are in chromosome U (see Fig. 1 for cytological location relative to inversions). Microsatellite sequences are available at GenBank with the accession numbers GU732209–80 (Santos *et al.*, 2010). Multiple simultaneous amplification reactions were conducted for each larva in a total volume of 15 μL using 1 μL of genomic DNA, 2 μM of each primer (see Table S1) and the QIAGEN® Multiplex PCR Kit (NASDAQ, Hilden, Germany). The *chcu* strain is highly inbred and homozygous for all assayed microsatellites, allowing to easily assigning the microsatellite alleles to the respective parental chromosome. Consequently for each larva, the chromosomes of the parental males were characterized for their chromosomal inversions and for the alleles at the amplified microsatellite loci. Raw data are available at Dryad repository.

Data analysis

Life-history traits parameters

Life-history evolution of the three SW populations was inferred from the phenotypic data of the synchronous assays of $SW_{1–3}$ and $TW_{1–3}$ populations. The evolutionary trajectory was estimated from the difference, at each generation, between the average trait value of a given SW population and the average value of the synchronously assayed TW replicate with the same randomly ascribed number (Matos *et al.*, 2002). This was carried out to diminish the confounding effects of environmental heterogeneity between temporally spaced assays. Pairing in the analysis helped mitigate block effects, because the SW and TW populations with the same number were distributed in the same racks, kept in the same area of the incubator and manipulated at the same time. The evolutionary trajectories of each population for each trait were fit to a linear-log regression model to obtain the evolutionary rate from the regression coefficient on ln-generation number, as we have performed in previous studies (Simões *et al.*, 2007). To test whether SW populations significantly evolved in the laboratory environment, an ANCOVA model was used for each life-history trait, using as data at each generation the difference between SW and TW populations described above, considering the replicate populations ($SW_{1–3}$) as random factor and the ln-transformed generation number as a covariate. Significance of the generation term was taken to indicate evolutionary changes in SW populations arising from laboratory culture. The differentiation of each trait between the SW and TW populations at the beginning of the experiment was also tested using the same ANCOVA and defined by the intercept of the trajectory.

Chromosomal polymorphism analysis

Changes in the frequency of inversions between generations were tested using the package *lawstat* (Gastwirth *et al.*, 2015) to run the Cochran–Mantel–Haenszel chi-squared test (CMH statistic) in R (R Development Core Team, 2008), which allows testing for differences in replicated systems (Landis *et al.*, 1978). Two-by-two matrices were run testing for repeatability of changes in frequencies between generations (with one degree of freedom). Only inversions present in the three replicates at generation four were tested. Correction for multiple inference hypothesis tests was made following Benjamini & Yekutieli (2001), using the false discovery rate (FDR) method. Whenever multiple tests were involved in this study, the same correction was applied.

Variability in chromosomal polymorphism was assessed by estimating 'allelic' richness (A) and expected heterozygosity (H_E) in FSTAT 2.9.3.2 (Goudet, 1995) at generations 4, 14 and 28. For these estimates, chromosomal arrangements were considered as alleles and each chromosome as a locus. Expected heterozygosity for chromosome A (the sex chromosome) was adjusted as in Kauer *et al.* (2002), assuming a 1 : 1 sex ratio (Pascual *et al.*, 2004). The test of each variability estimate (A or H_E) was carried out with a mixed ANCOVA model, with the effect of the covariate generation (4, 14 and 28), the random factor population ($SW_{1–3}$), the fixed effect of chromosome (the five chromosomes) and their interactions. The genetic differentiation in chromosomal polymorphism between populations was estimated by F_{ST} (Weir & Cockerham, 1984), using Arlequin 3.5.1.3 (Excoffier & Lischer, 2010).

Testing the role of selection vs. drift on the frequency changes in chromosomal arrangements

To test whether the frequency change in chromosomal arrangements between generations four and 28 was a likely outcome of genetic drift alone, simulations were run using R (R Development Core Team, 2008). The R script that we prepared is available in the Dryad repository. The observed frequency at generation 4 in each population and the estimated effective population size along the generation range were used to perform the simulations. Effective population size (N_e) was estimated by the Bayesian method (Berthier *et al.*, 2002) considering the changes in microsatellite allele frequencies between generations 4 and 28, using the 22 microsatellite data of each population. As N_e is known to increase through time in the laboratory (Simões *et al.*, 2008a; Santos *et al.*, 2012), the lower 95% confidence interval limit was used as a starting point and considered the N_e at generation 4 (Table S2). At each generation, an increase of 1/24 of the difference in N_e between the two 95% confidence intervals was implemented, to achieve the higher limit of the 95% confidence interval after 24 generations, the number of generations elapsed between the two assayed laboratory populations. The frequencies of inversions obtained at each simulated generation, under the expectation of drift, were used to simulate the expected frequencies

on the subsequent generation. This was reiterated until generation 28 of the simulation. A multinomial distribution was generated using a Wright–Fisher model, with only drift and no mutation. A total of 5000 simulations were run to obtain a final distribution of frequencies of the targeted inversion at the final generation. *P*-values were calculated as two times the proportion of simulations giving equal or higher values than the observed data (equal or lower in the case of decreasing inversion frequencies). Whenever that proportion was higher than 50%, the *P*-value considered was 1.

Linkage disequilibrium analysis

To screen for an association at each generation between each chromosome and their microsatellite loci, as well as between loci, the multiallelic linkage disequilibrium statistic $D'm$ (Hedrick, 1987) was estimated with PowerMarker version 3.25 (Liu & Muse, 2005). The association was tested by *G*-test in the same software and FDR was applied. Moreover, for each chromosome, the $D'm$ between chromosome and locus was tested by mixed ANOVA with the effect of generation (fixed factor, generations 4 and 28), the random factor population, locus (fixed factor) and their interactions.

In both generations, we estimated two variance components – D'^2_{IS} and D^2_{IT} – of linkage disequilibrium between particular chromosomes and their microsatellite loci following Ohta (1982) and using the program linkDos (Garnier-Gere & Dillmann, 1992). D'^2_{IS} is the variance in the frequency of combinations of alleles in the three SW replicated populations and D^2_{IT} is the variance of disequilibrium considering the frequency of alleles in the total population. We estimated Ohta's ratio of variances in linkage disequilibrium as D'^2_{IS}/D^2_{IT}, which may indicate selection for values lower than 0.5 (Black & Krafsur, 1985). This is because $D'^2_{IS} + D'^2_{ST} = D^2_{IT}$ and under selection $D'^2_{IS} < D'^2_{ST}$ is expected (Ohta, 1982). In particular, we were interested in identifying haplotypes responding to selection in the new environment and, at the same time, wished to avoid the deflation effect that possibly low recombination rates together with a shared recent founder event of our populations may *per se* have on this ratio (cf. Whittam *et al.*, 1983). Thus, we targeted specific cases in which this ratio started at high values and decayed to values below 0.5. Ohta's ratio was also estimated between microsatellites located in the same chromosome.

To determine whether LD is due to a particular allele associated with a given chromosomal arrangement, at generations 4 and 28 the 'interallelic' linkage disequilibrium statistics D' (Lewontin, 1964) was estimated with MIDAS (Gaunt *et al.*, 2006). For microsatellite loci showing a significant linkage disequilibrium with the same chromosomal arrangement in more than one population, D' between particular alleles and arrangements was estimated. The conservative Yates correction was used to prevent the overestimation of statistical significance for small data sets.

Changes in the allelic composition of chromosomal arrangements through time

One specific aim of this study was to detect changes in chromosomal polymorphism alongside with changes in the genetic content of arrangements due to positive selection. Several criteria were used to define the set of inversions to analyse in this context (see Results). The differentiation in the allelic composition of specific chromosomal arrangements through time was analysed using individuals with the same chromosomal arrangement in each population. F_{CT} for each chromosomal arrangement between generations was estimated by a hierarchical AMOVA, considering the three replicates at each generation, using Arlequin 3.5.1.2 (Excoffier & Lischer, 2010).

Microsatellite alleles showing a significant temporal change in frequency within a chromosomal arrangement by the CMH statistic were further analysed for the probability of that change being caused by genetic drift alone. Following a approach similar to the one used for chromosomal arrangements, 5000 simulations were run to obtain a final distribution of frequencies of the targeted alleles at generation 28 expected by genetic drift. Each simulation started with the frequency of the targeted allele at generation 4. The effective population size used for simulations in each population was obtained similarly to the method used for chromosomal arrangements, but using only the data of individuals with the same chromosomal arrangement, for the 22 microsatellites (Table S3).

Results

Evolutionary trajectories of life-history traits

SW populations showed a significant initial differentiation from the control TW populations for age of first reproduction ($F_{1,15.9} = 5.07$, $P = 0.04$), early fecundity ($F_{1,15.7} = 10.57$, $P = 0.005$) and the composite phenotype ($F_{1,15.8} = 10.61$, $P = 0.005$) (Fig. 2). Significant evolutionary change was found for early fecundity and for the composite phenotype ($F_{1,14} = 7.90$ $P = 0.014$ and $F_{1,14} = 8.11$, $P = 0.013$, respectively), with SW populations converging towards the TW values. A marginally significant trajectory was observed for age of first reproduction ($F_{1,14} = 4.20$, $P = 0.060$), whereas neither peak fecundity nor female starvation resistance showed a significant trend through time (Fig. 2).

Changes in frequencies of chromosomal arrangements through time

A total of 23 chromosomal arrangements were identified in SW populations at generation 4 (Table S4).

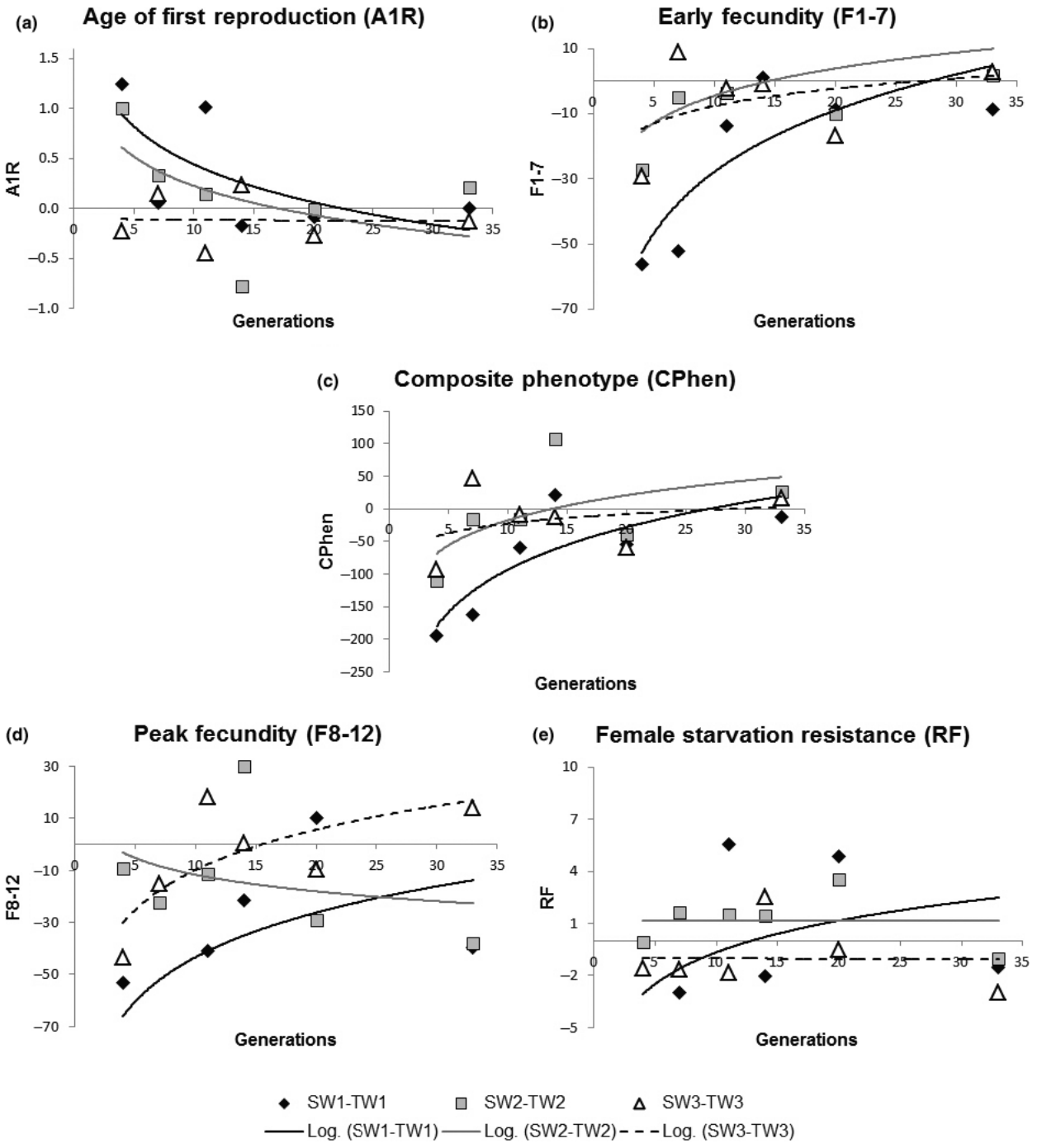

Fig. 2 Evolutionary response of SW populations in five life-history traits for 33 generations of laboratory adaptation. The evolutionary trajectories were calculated using as data for each generation the difference between the mean of the SW populations and the mean of the TW (control) populations under a linear-log regression model.

There was a significant differentiation in chromosomal arrangement frequencies between SW populations at generation 4 ($F_{ST(G4)} = 0.021$, $P < 0.001$), differentiation which had decreased by generation 14 ($F_{ST(G14)} = 0.016$, $P = 0.007$), and finally, this was not significant by generation 28 ($F_{ST(G28)} = 0.001$, $P > 0.05$). ANCOVA tests on chromosomal arrangement variability showed a significant decline between generations for both H_E and A

(Tables S5 and S6). Highly significant differences in H_E and A were found among chromosomes (Table S5), with E and O chromosomes being the most polymorphic (Table S6). Moreover, there was a significant generation*chromosome interaction for both H_E and A, with a higher loss of arrangements for chromosome O and a higher decline of inversion diversity for chromosomes A and U (Tables S5 and S6).

Changes in inversion frequency between generation 4 and generation 28 were tested using the CMH test, which estimates significance in replicated designs, regardless of cause – genetic drift or selection (Table 1). CMH tests were applied only to the 16 inversions present in the three replicate populations at generation 4 (Table S4). All arrangements with an initial frequency higher than 50% showed significant increases in frequency, with the exception of J_1 (Fig. 3; Table 1). Six of the 11 lower-frequency arrangements decreased significantly in frequency over generations (Fig. 3; Table 1). Interestingly, O_{ST} significantly increased in frequency in the three SW populations despite its low initial frequency, changing from 10.67 ± 0.98% at generation 4 to 31.47 ± 2.92% at generation 28 (Fig. 3). This result counterbalances the decrease in O_{3+4+7}, which was the second most frequent arrangement (after O_{3+4}) at generation 4 in chromosome O, falling from 25.27 ± 2.45% to 3.63 ± 0.93% over the same period. The seven inversions that were not present in all replicates at generation 4 were lost in subsequent generations (Fig. 3; Table S4). For TW populations, only 13 chromosomal arrangements were identified. The chromosomal arrangements increasing in frequency across generations in SW populations were the same as those that had high frequencies in the long-established TW populations (Table S4).

Frequency distributions were simulated to test whether the change in inversion frequencies between generations 4 and 28 for each SW population was a likely outcome of genetic drift alone for the 16 chromosomal arrangements tested by the CMH statistic (Table 1). Of the eleven arrangements with significant changes in frequency between generations as assessed with the CMH test, seven significantly deviated from genetic drift expectations in at least two replicates after FDR correction (Table 1). Four of these significantly deviated across all the three SW populations: A_{ST} and O_{3+4+7} consistently decreased in frequency in all three replicates, whereas A_2 and O_{ST} showed a consistent increase, which could not be explained by drift alone given their initial frequencies (Fig. 3; Table 1).

Linkage disequilibrium between chromosomes and microsatellite loci

We detected several significant linkage disequilibria ($D'm$) between chromosomal arrangements and microsatellites for all chromosomes, except for chromosome U (Fig. S1). There was a general tendency for linkage disequilibrium to increase over generations, although this effect was statistically significant only for those loci located in chromosome A (Table S7). The degree of LD between microsatellites and chromosomes

Chromosome	Inversion	CMH	SW_1	SW_2	SW_3
Chr A	A_{ST}	30.228**,††	**0.0004††**	**0.0058††**	**0.0080†**
	A_2	31.919**,††	**0.0004††**	**0.0050††**	**0.0056††**
Chr E	E_{ST}	2.203	0.3494	1	0.2924
	E_{1+2}	10.652**,††	**0.0008††**	0.8174	0.7902
	E_{1+2+9}	12.445**,††	**0.0168†**	0.2680	**0.0412**
	$E_{1+2+9+3}$	0.162	0.5404	1	0.1872
	$E_{1+2+9+12}$	31.612**,††	**0.0002††**	0.3146	**0.0014††**
Chr J	J_{ST}	0.694	0.3506	0.4970	0.2338
	J_1	0.694	0.3378	0.5094	0.2338
Chr O	O_{ST}	27.584**,††	**0.0092†**	**0.0128†**	**0.0022††**
	O_{3+4}	5.573*,†	0.0514	0.4360	0.3660
	O_{3+4+1}	6.940**,†	**0.0172†**	0.9136	0.7996
	O_{3+4+7}	32.774**,††	**0.0024††**	**0.0028††**	**0.0004††**
	$O_{3+4+23+2}$	3.186	1	1	0.3026
Chr U	U_{1+2}	20.162**,††	**0.0002††**	0.3384	**0.0168†**
	U_{1+8+2}	21.442**,††	**0.0002††**	0.2258	**0.0168†**

Table 1 Cochran–Mantel–Haenszel (CMH) statistic and probability of obtaining the observed frequency for each inversion at generation 28 under the effect of genetic drift alone, considering its frequency at generation 4. Simulations were run using the estimated effective population size along the generation range. CMH tests were carried out for inversions detected at generation four in the three replicates.

P-values significantly deviating from the neutral expectation are in boldface.

*Significant ($P < 0.05$) or **highly significant ($P < 0.01$) tests.

†Tests that hold significant or ††highly significant after false discovery rate correction for multiple tests: for chromosomes A, J and U (two tests) significant for $0.0067 < P < 0.0333$ (for $\alpha < 0.05$) or highly significant for $P < 0.0067$ (for $\alpha < 0.01$); For chromosomes E and O (five tests) significant for $0.0044 < P < 0.0219$ (for $\alpha < 0.05$) or highly significant for $P < 0.0044$ (for $\alpha < 0.01$).

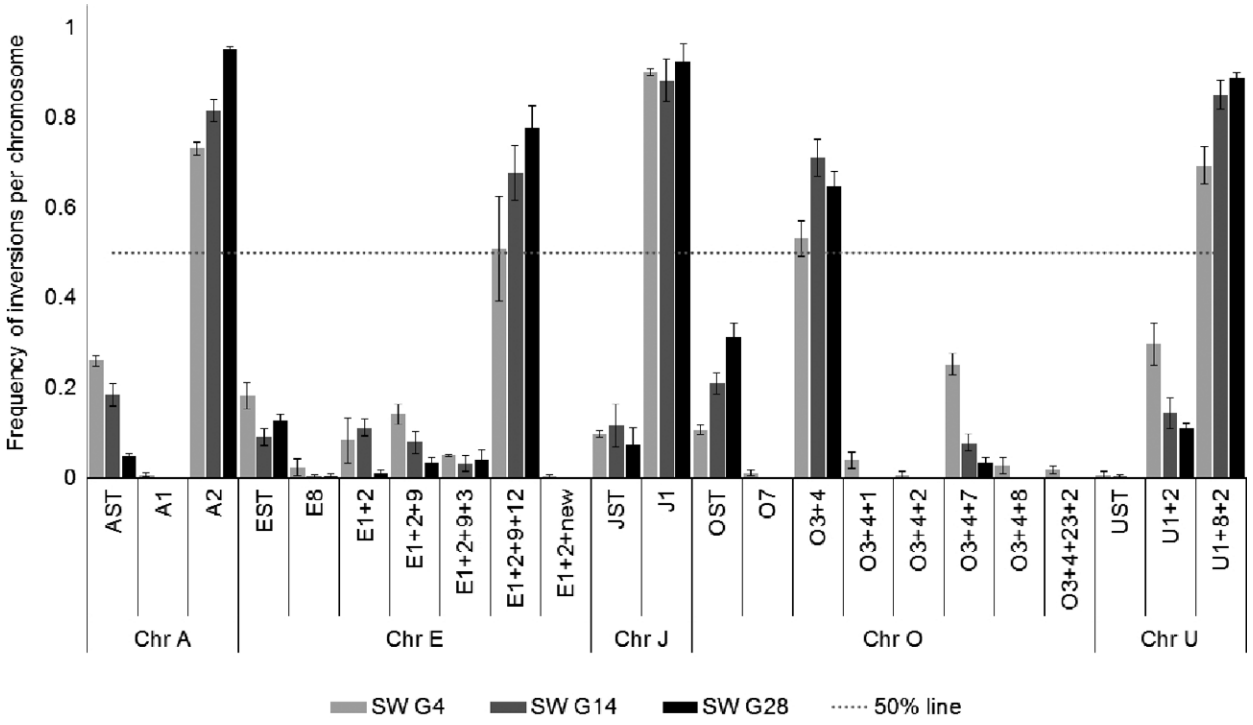

Fig. 3 Mean chromosomal inversion frequencies in SW populations at generations 4, 14 and 28 since establishment in the laboratory. Bars indicate the standard error calculated from the differences among the three replicated populations. The dotted line corresponds to the 50% frequency. The underlying of overlapped inversions according to the convention used throughout the text could not be used here due to the restriction of the software used.

varied significantly between microsatellite loci on the A and O chromosomes (Table S7). Interestingly, in chromosome O, there was a significant generation by locus interaction. Loci within the O_{3+4} region (*dsub4*, *dsub29*, *dsub12*), together with the neighbouring loci *dsub34* and *dsub1* (Fig. 1), increased in linkage disequilibrium with chromosomal arrangements over generations, whereas loci within the O_7 inversion decreased in linkage disequilibrium (Fig. S1).

In accordance with the expectation of genetic drift, there was an increase in Ohta's ratio between chromosomes and microsatellites across generations (Fig. 4). However, Ohta's ratio between chromosome E and *dsub79* and between chromosome O and microsatellites in the region of inversion O_{3+4} together with the neighbouring loci *dsub34* and *dsub1* decreased to values below 0.5 in that period, deviating from drift expectation (Fig. 4). Thus, the pattern of associations of loci with chromosome O obtained from Ohta's ratio is in agreement with the increase in $D'm$ across generations for the same microsatellite loci (Fig. S1).

When linkage disequilibrium between pairs of microsatellites was inferred, there was a broad tendency for it to decrease from generation 4 to generation 28 (Fig. S2). The exception was for the microsatellite loci located in or close to the O_{3+4} region (seen to increase $D'm$ with the O chromosome, see above), which maintained linkage disequilibrium at generation 28 (Fig. S2). In general, Ohta's ratio between microsatellite loci increased between generations, with high values at generation 28, as expected by genetic drift (Fig. S3). Some microsatellites in the region of O_{3+4} or close to it were again an exception to the general pattern, showing a decline to values below 0.5, which was not expected to occur by drift.

Genetic content of specific inversions

To tackle how the genetic content of particular chromosomal arrangements changes during adaptation, we analysed microsatellite allele frequencies within arrangements and their linkage disequilibrium across generations. These analyses were restricted to chromosomes showing a significant linkage disequilibrium with at least one microsatellite locus ($D'm$; see Fig. S1), which excludes chromosome U. Furthermore, we focused the analysis on those arrangements whose changes in frequency could not be explained by genetic drift alone at least in two replicate populations (Fig. 3; Table 1), which led to the inclusion of inversions A_{ST}, A_2; E_{1+2+9}, $E_{1+2+9+12}$, O_{ST}, O_{3+4+7}. We also included in the analysis all inversions of chromosomes that showed

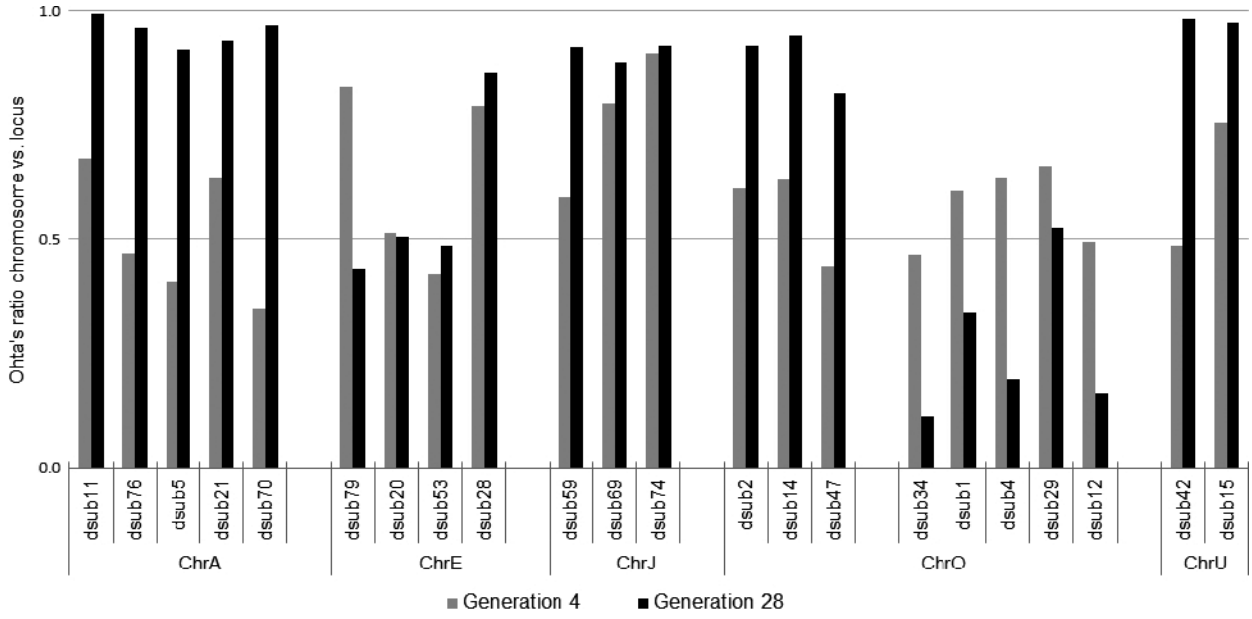

Fig. 4 Ohta's ratio between each of the five chromosomes and each locus located in it, both at generation 4 (grey) and at 28 (black). A ratio below 0.5 (dashed line) is suggestive of selective forces affecting the linkage association between chromosomes and loci.

conspicuous drops to values below 0.50 in Ohta's ratio for at least one microsatellite locus. Using this criterion too led to the inclusion of all other inversions in chromosomes E and O (see Fig. 4). With these steps, the two inversions of chromosome J were excluded. Next, we only analysed those arrangements present across generations in more than 4 larvae in each replicated population, and thus, we excluded $O_{\underline{3+4+7}}$, A_{ST} and other low-frequency inversions in the E and O chromosomes (Fig. 3; Table S4). Following these criteria, we analysed genetic differentiation between generations (F_{CT}) for individuals bearing the A_2, $E_{\underline{1+2+9+12}}$, E_{ST}, $O_{\underline{3+4}}$ and O_{ST} arrangements. All differed significantly in their genetic content between generations with the exception of O_{ST} that was excluded at this point (Table S8).

All alleles showing an increase in frequency between generations at the three replicates (Fig. S4) were tested by the CMH chi-squared test. Significant increase was detected for alleles *193* (*dsub5*), *173* (*dsub21*) and *177* (*dsub76*) in the A_2 arrangement, for allele *169* (*dsub20*) in E_{ST}, for *190* (*dsub20*) in $E_{\underline{1+2+9+12}}$ and for alleles *231* (*dsub2*), *197* (*dsub4*), *265* (*dsub12*), *115* (*dsub14*), *252* (*dsub29*) and *116* (*dsub34*) in $O_{\underline{3+4}}$ arrangement (CMH statistic > 6.5, $P < 0.02$). Simulations were performed to assess the effect of drift on the increase in the above-mentioned alleles. Most of the changes could not be explained by drift alone (Table 2) and, except for arrangement A_2, were very consistent across replicates. Nevertheless, only alleles *197* (*dsub4*) and *116* (*dsub34*) showed a significant linkage disequilibrium with inversion $O_{\underline{3+4}}$ at generation 28 in all populations, suggesting hitchhiking with genes involved in the selective change in that arrangement across generations (Table 3). The combination of these two alleles signifi-

Table 2 Cochran–Mantel–Haenszel (CMH) statistic and probability of obtaining the observed frequency for specific alleles at generation 28, at a given inversion, under the effect of genetic drift alone considering its frequency at generation 4. Simulations were run using the estimated effective population size along the generation range considering only individuals with the specific inversion. See details in text.

Chromosome	Locus	Allele	CMH	SW1	SW2	SW3
A_2	*dsub5*	193	38.342**	0.0780	**0.0014**	**0.0002**
	dsub21	173	11.887**	0.7490	0.0900	**0.0002**
	dsub76	177	15.192**	0.0660	**0.0002**	0.0570
E_{ST}	*dsub20*	169	6.571*	**0.0002**	**0.0002**	**0.0002**
$E_{\underline{1+2+9+12}}$	*dsub20*	190	11.750**	**0.0002**	**0.0260**	**0.0004**
$O_{\underline{3+4}}$	*dsub2*	231	15.871**	**0.0002**	**0.0050**	**0.0048**
	dsub4	197	13.284**	**0.0194**	0.0812	**0.0002**
	dsub12	265	7.446**	**0.0060**	0.3774	**0.0010**
	dsub14	115	19.866**	**0.0002**	**0.0004**	**0.0238**†
	dsub29	252	15.343**	**0.0394**†	**0.0014**	**0.0002**
	dsub34	116	25.846**	**0.0002**	**0.0002**	**0.0004**

P-values significantly deviating from the neutral expectation are in boldface.

*Significant ($P < 0.05$) or **highly significant ($P < 0.01$) tests.

†Test that did not hold the significance after false discovery rate correction for multiple tests: three tests for A_2, with $P < 0.027$ (for $\alpha < 0.05$) and six tests for $O_{\underline{3+4}}$ with $P < 0.020$ (for $\alpha < 0.05$).

Table 3 Alleles in linkage disequilibrium with specific chromosomal arrangements at generation 4 and generation 28 for SW populations, measured by D'. Microsatellites for which there was linkage disequilibrium in at least one population/generation are only presented.

Chromosomal arrangement	Locus	Generation 4			Generation 28		
		SW_1	SW_2	SW_3	SW_1	SW_2	SW_3
A_2	*dsub5*	193*					
	dsub70	304**/308*	304*	304**		304*	
	dsub76		177*				
$E_{\underline{1+2+9+12}}$	*dsub20*	184**/190*			190*	190*	
	dsub28						
	dsub53	302***		302***			302**
	dsub79			149*		149**	149***
$O_{\underline{3+4}}$	*dsub1*	269*/271*			271**		258**
	dsub4				197*	197*	197**
	dsub12				265*/270*		270*
	dsub14			104**			
	dsub29						255**
	dsub34		116*		116**	116***	116***
	dsub47		142*				
O_{ST}	*dsub1*	275***			275***	275***	275***
	dsub4	195**		195*	195***	195***	195***
	dsub12	264***	264***	264***	264***	264***	264***
	dsub14			115*			
	dsub29	252*	225*	252**	252***		252**
	dsub34	110*		110*	110***	110***	110***
	dsub47			154**		152*	

Asterisks correspond to the significance of D' values after Yates correction: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

cantly increased from generation 4 to generation 28 in the chromosomal arrangement $O_{\underline{3+4}}$, from a mean frequency of 5.89 ± 1.52% to 24.15 ± 3.19% across the three replicates (CMH statistic = 12.217, $P = 0.000$). Nevertheless, no significant linkage disequilibrium was detected between these two alleles (measured with D') when considering only the individuals with the arrangement $O_{\underline{3+4}}$. Thus, the two alleles seem to have increased in frequency independently from each other, within each population, to a frequency above that expected under drift alone (Table 2). The increase in frequency of allele *116* was particularly strong (from 26.24 ± 4.55% to 63.32 ± 5.55%; CMH statistic = 25.846, $P = 0.000$), corresponding to a general drop in frequency of almost all other alleles at this locus and, more noticeably, to a considerable drop in the frequency of allele *110* – one of the more frequent alleles at generation 4 (from 35.45 ± 3.60% to 3.99 ± 0.92%; CMH statistic = 29.409, $P = 0.000$). Effects of both positive selection on allele 116 and negative selection on allele *110* due to hitchhiking might thus have been involved. Allele *197* did not show such an extreme change in frequency (from 14.07 ± 3.65% to 39.07 ± 6.89%; CMH statistic = 13.284, $P = 0.000$), suggesting weaker selection.

Curiously in O_{ST}, there was also a consistent increase in frequency of a combination involving alleles in the same two microsatellites – allele *110* in *dsub34* (that decreased frequency in $O_{\underline{3+4}}$) and allele *195* in *dsub4* (both in linkage disequilibrium with the inversion, Table 3). This led to a change in frequency from 76.67 ± 14.53% to 86.77 ± 6.73% of this combination. As expected given their already high frequency from the start of the experiment, this change was not statistically significant (CMH statistic = 0.832, $P = 0.362$). Moreover, these two alleles did not exhibit linkage disequilibrium, which might be due to their high frequency in both generations. On the other hand, these alleles were predominant and already in linkage disequilibrium with O_{ST} at generation 4 in two replicates, which might explain their evolution.

It is worth mentioning that *dsub79*, the only microsatellite on the E chromosome that showed a drop in Ohta's ratio, did not show a consistent increase in frequency for any particular allele across inversions. Nevertheless, there was a significant drop in frequency for allele *149* in E_{ST} (CMH statistic = 6.102, $P = 0.014$), the predominant allele for all inversions (Fig. S4C). This together with negative linkage disequilibrium at generation 28 in all replicates ($-1 < D' < -0.523$, $0.00002 < P < 0.03$) suggests that negative selection might have been involved. This may explain the observed change in Ohta's ratio.

Discussion

In this study, we tracked the evolutionary dynamics of three replicated populations of *D. subobscura* adapting to

a novel environment (the laboratory), for about 30 generations, studying five life-history traits, 23 chromosomal arrangements and 22 microsatellite loci. The three populations showed clear improvements in life-historical components of fitness across the initial 33 generations after colonizing the new environment, particularly for early fecundity and a composite phenotype. Of the initial 23 chromosomal arrangements, seven of them changed frequency through time consistently across replicates, to a degree greater than expected from genetic drift alone. Selective changes in the genetic content of some chromosomal arrangements were also detected. Nevertheless, observed patterns of linkage disequilibrium between specific microsatellite alleles within arrangements did not support an evolutionary scenario involving consistent epistasis.

The role of selective pressures in inversions after a colonization event

The adaptive value of inversions has long been studied in wild populations of *Drosophila*. In *D. subobscura*, chromosomal polymorphism has been shown to vary seasonally (Rodríguez-Trelles *et al.*, 1996, 2013) and latitudinally (Prevosti *et al.*, 1988; Balanyà *et al.*, 2003, 2006), even in response to global warming (Balanyà *et al.*, 2006, 2009; Rezende *et al.*, 2010). Moreover, several phenotypic traits have been linked to inversion polymorphism in *D. subobscura*, including wing size and shape (Orengo & Prevosti, 2002; Fragata *et al.*, 2010), thermal preference and thermal tolerance (Dolgova *et al.*, 2010; Rego *et al.*, 2010; Calabria *et al.*, 2012), mating success (Santos *et al.*, 1986) and viability (Santos, 2009). However, few *D. subobscura* studies have analysed real-time evolution of inversion frequencies during adaptation to novel environments (but see Santos *et al.*, 2005; Fragata *et al.*, 2014a), and as far as we know, none have assayed whether changes in frequency are due to particular inversion haplotypes increasing in frequency.

In our populations, the frequencies of chromosomal inversions significantly changed in a directional way across replicates between generations 4 and 28. The significant genetic differentiation between replicates at generation 4, indicating a role of founder effects at their establishment, was followed by a drop in differentiation between populations through time probably due to losses of chromosomal polymorphisms which might have been caused by both selection and genetic drift. Nevertheless, if the reduction in genetic variability had been only due to sampling effects, we would expect an increase in differentiation between replicate populations through time (Frankham, 2012; Santos *et al.*, 2013), which was not the case, suggesting an underlying selective effect. It could be claimed that the reduction in differentiation between generations might be due to the consistent loss across populations of initially low-frequency chromosomal arrangements, together with an increase in the most frequent arrangement. Nevertheless, this hypothesis would not explain either the consistent increase in frequency across replicates of the arrangement O_{ST}, which had a low frequency at generation 4, or the maintenance of other initial low-frequency arrangements, as was observed for chromosome E.

In general, the chromosomal arrangement frequency changes observed here were consistent and significant across replicates. Moreover, these inversion frequencies converged to those of populations that had long been established in the same environment and derived from the same natural location. The fact that changes in several arrangements significantly deviated from the expectations of genetic drift in a consistent manner across replicates provides a substantial evidence for a nonrandom adaptive role of chromosomal inversions in populations adapting to laboratory conditions.

In another work in our laboratory, populations of *D. subobscura* sampled from distant localities, which were highly initially differentiated in chromosomal inversion polymorphism, showed quick convergence to the same life-history fitness traits when adapting to the same laboratory conditions, although that convergence was achieved along different routes and with different rates (Fragata *et al.*, 2014b). Nevertheless, the temporal changes in inversion polymorphism did not feature overall convergence across populations (Fragata *et al.*, 2014a). It is an open question whether heterogeneous genetic content of inversions due to different evolutionary histories among those populations led to a decoupling of convergence at the phenotypic and inversion levels. Interestingly, there was a striking similarity in the evolution of inversion frequencies when comparing the data in the present study with data from other populations derived from the same natural location in different periods (Fragata *et al.*, 2014a), reinforcing a role for selection in the changes observed here.

The two arrangements that clearly exhibited positive selection responses were A_2 and O_{ST}. A_2 is considered a 'warm-adapted' arrangement and O_{ST} a 'cold-adapted' arrangement, based on their association with maximum temperature along the European cline (Menozzi & Krimbas, 1992; Rego *et al.*, 2010). These two arrangements have shown predictable responses in laboratory populations subjected to a high-temperature regime, with A_2 increasing and O_{ST} decreasing in frequency after 1 year of adaptation (Santos *et al.*, 2005). The fact that both A_2 and O_{ST} increased in our populations, together with a decline in $O_{\underline{3+4}+\underline{7}}$ (more frequent in the south and summer; see Rodríguez-Trelles *et al.*, 1996), suggests that there is more to the evolutionary dynamics of inversions than temperature *per se*. A conspicuous finding is that a similar indication of selection acting on both A_2 and $O_{\underline{3+4}+\underline{7}}$ was obtained in different populations derived from the same natural location, founded

in a different year and season (Fragata *et al.*, 2014a). This occurred despite contrasting initial frequencies, particularly for O_{3+4+Z}, which further reinforces a role of selection in the changes observed.

In the O chromosome, the O_{3+4} arrangement also tended to increase over the course of adaptation. Nevertheless, we were not able to statistically distinguish between directional selection or other mechanisms such as balancing selection – due to heterokaryotype advantages of O_{3+4}/O_{ST} – or drift (Hoffmann & Rieseberg, 2008; see also Hedrick, 2012). Against the balancing selection hypothesis, Santos (2009) did not find heterokaryotypic advantage in *D. subobscura* crosses between homokaryotypic lines, including O_{3+4} and O_{ST} arrangements. Whichever specific mechanisms are involved, both the data in our SW populations at the end of this study and data from the TW populations, more than 115 generations after laboratory founding, indicate the active maintenance of considerable chromosomal polymorphism in most chromosomes.

Evolution of genetic content of particular inversions: is there evidence of selection favouring specific haplotypes?

It has been suggested that selection drives inversion frequencies because their recombination reduction might promote hitchhiking along with adaptive genes captured inside them (Hoffmann & Rieseberg, 2008) whether independently locally adapted (Kirkpatrick & Barton, 2006) or epistatically interacting (Dobzhansky, 1970). Several studies in *Drosophila* have tackled the evolutionary mechanisms underlying inversion evolution with contrasting results. Santos (2009) detected recombination load when comparing the fitness of flies with different patterns of recombination across the O chromosome, supporting the hypothesis that gene arrangements protect favourable combinations of alleles interacting epistatically. Similarly, regions of high linkage among loci within inversions interspersed with regions of low linkage (Kennington *et al.*, 2006) and linkage levels between loci not decreasing away from breakpoints (Munté *et al.*, 2005) have been interpreted as indicating selection acting against recombinants to maintain sets of co-adapted genes. Nevertheless, several studies analysing the genetic differentiation of the same chromosomal arrangement between populations reported very low differentiation values, not expected under the co-adaptation hypothesis (Rozas *et al.*, 1995; Schaeffer *et al.*, 2003; Simões *et al.*, 2012; Pegueroles *et al.*, 2013; but see Kennington & Hoffmann, 2013).

Our study supplies rare data for temporal changes in the genetic composition of inversions, data that are suggestive with respect to the question whether epistatic selection is in play. Evidence for the reproducible evolution of linkage disequilibrium between specific alleles within the same inversion would support the involvement of such epistasis. On the other hand, consistent temporal changes in linkage disequilibrium between some alleles and a given chromosomal arrangement, but not among those alleles within the arrangement, would suggest a mode of evolution of inversions without interaction among the adaptive genes involved.

In our populations, the temporal dynamics of linkage disequilibrium between chromosomes and loci located in them were mostly in accordance with expectations of drift. An exception was the striking reduction in the variance ratio of linkage disequilibrium between the structural genetic arrangements of the O chromosome and the loci included in inversion O_{3+4} together with the neighbouring loci *dsub34* and *dsub1*. We detected a conspicuous suggestion of positive selection in the O_{3+4} arrangement favouring a particular combination of alleles at two loci, one located inside the inverted region (*dsub4*) and the other one outside it (*dsub34*). For both alleles, linkage disequilibrium evolved together with the inversion, suggesting that a particular haplotype linked to these markers and to the inversion might be under selection due to epistatic interaction. Nevertheless, the absence of linkage disequilibrium between the two alleles within this arrangement indicates instead that they increased in frequency independently, without interaction between them.

Locus *dsub34* is located approximately 3.7 Mb from the closer breakpoint outside the O_{3+4} inversion (estimated as in Pegueroles *et al.*, 2010a), thus in a region presenting reduction, but not complete inhibition of recombination in heterokaryotypes (Pegueroles *et al.*, 2010b). Lack of genetic differentiation between O_{ST} and O_{3+4} has been reported for *Atpα* (Pegueroles *et al.*, 2013), a gene close to our assayed locus *dsub34* (0.2 Mb), indicating ongoing recombination in this area in heterokaryotypes. In our study, we did not find LD between *dsub34* and the O chromosome at generation 4, although it had arisen at generation 28. LD thus built up during the adaptive process, specifically LD involving allele *116* and the O_{3+4} inversion. Importantly, in our analysis of O_{3+4} individuals, we did not find indications of responses to selection involving any allele of *dsub01*, a microsatellite much closer to the breakpoint of the inversion (1.2 Mb). Altogether, these results could be explained by a transient association between allele *116* (*dsub34*) and a selected allele within O_{3+4}, polymorphic for alleles in dsub01. But an alternative explanation is epistatic selection involving one gene inside the inversion (but not close to *dsub04*) and one outside, closer to *dsub34* than to *dsub01*. Epistatic selection involving *Atpα* has been proposed as an explanation of its linkage disequilibrium with the O_7 and O_1 inversions, despite being located outside them (Pegueroles *et al.*, 2016). Similarly, we cannot rule out the hypothesis that the LD we have detected between *dsub34* and the O_{3+4} arrangement might involve epistatic selection. More localized markers would be

needed to test for the involvement of epistasis in the evolution of the O chromosomal arrangements.

We also detected increases in the frequency of seven other alleles located in particular inversions, beyond those expected to occur by drift alone. Nevertheless, these alleles were generally not in linkage disequilibrium with their inversions and may thus have changed in frequency by hitchhiking alongside with allelic variants of genes under selection that were not specific to a given inversion. These data suggest that it is possible that evolution and maintenance of inversions is not necessarily an outcome of synergistic epistatic effects of favourable combinations of genes captured within inversions.

However, the case of O_{ST} showed a different evolutionary picture. In particular, there was a consistent increase in O_{ST} of a different set of alleles in the same two microsatellite loci that exhibited the patterns suggestive of selection in $O_{\underline{3+4}}$ (*dsub4* and *dsub34*), both in strong linkage disequilibrium with the O_{ST} inversion. The high frequency of these alleles from the start might have prevented a statistical detection of significant increases in allele frequency and in linkage disequilibrium between them, even though this combination might be spreading to fixation due to co-adapted epistatic genes. On the other hand, the maintenance of that unbroken combination might be simply due to reduced recombination in heterokaryotypes (Pegueroles *et al.*, 2010b).

Many previous studies support the hypothesis that the adaptive role of inversions is due to reduction in recombination (e.g. Schaeffer *et al.*, 2003; Munté *et al.*, 2005; Kennington *et al.*, 2006; Pegueroles *et al.*, 2010a, 2013; Kennington & Hoffmann, 2013), whether due to co-adaptation or local adaptation. Our study did not produce any clear evidence for epistatic selection. However, this does not necessarily imply that our data favour the local adaptation model as we cannot preclude additional evolutionary mechanisms, such as direct adaptive value or heterokaryotype advantage (Hoffmann & Rieseberg, 2008; Kennington & Hoffmann, 2013). Nevertheless, there is little evidence in the literature for the action of these other mechanisms.

It is important to note that more extensive sequencing of the genome would allow better detection of differences in the genetic content of inversions and as such contribute to a more effective test of alternative hypotheses for the evolution of chromosomal polymorphisms. Some genome-wide studies have started addressing the evolution of inversions (Franssen *et al.*, 2014; Kapun *et al.*, 2014; Tobler *et al.*, 2014). Nevertheless, until now there have not been conclusive data targeting the genetic content within the same inversion across populations. A powerful approach might be to combine a real-time evolution study, as we did here, with a wide number of markers, using next-generation sequencing. This would be an important next step to take in *D. subobscura*, once there is a fully assembled reference genome.

In summary, we show that populations undergoing adaptation to a new environment also show directional changes in the frequencies of chromosomal arrangements, some of which are demonstrably due to selection. This study also suggests that the genetic content of chromosomal arrangements responds to selection. The fact that this was detected despite a lack of extensive coverage of the genome suggests that genetic changes within inversions have an important role in adaptation.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Linkage disequilibrium estimate ($D'm$; Hedrick, 1987) in SW populations between each locus and chromosome at generation 4 (grey) and 28 (black).

Figure S2 Linkage disequilibrium estimate ($D'm$; Hedrick, 1987) between pairs of loci located in the same chromosome, both at generation 4 (grey) and 28 (black).

Figure S3 Ohta's ratio between loci at each chromosome, both at generation 4 (grey) and 28 (black).

Figure S4 Microsatellite allele frequency changes between generations 4 and 28 at arrangements indicating differentiation in genetic content: A_2 (a), $E_{\underline{1+2+9+12}}$ (b), E_{ST} (c) and $O_{\underline{3+4}}$ (d, e) in the three SW populations.

Table S1 Multiplexing and Primers used for PCR amplification of the 22 microsatellites.

Table S2 Effective population size (N_e) and 95% confidence interval (CI) estimated by the Bayesian method (Berthier *et al.*, 2002) considering changes in microsatellite allele frequencies between generations 4 and 28.

Table S3 Effective population size (N_e) and 95% confidence interval (CI) estimated by the Bayesian method (Berthier *et al.*, 2002) considering changes in microsatellite allele frequencies between generations 4 and 28 for the targeted inversions A_2, $E_{\underline{1+2+9+12}}$, E_{ST} and $O_{\underline{3+4}}$ (see Results).

Table S4 Relative frequency of chromosomal arrangements and number of larvae analyzed at each population and generation *per* chromosome.

Table S5 ANCOVA testing several factors and their interactions on the variability estimates for chromosomal arrangements.

Table S6 Mean chromosomal arrangement variability (± standard error, calculated from the differences among the three replicated populations) *per* chromosome and generation and percentage of decline of each estimate between generations 4 and 28.

Table S7 ANOVA testing the effect of generation (Gen), Population (Pop), Locus and their interactions on the linkage disequilibrium estimate ($D'm$; Hedrick, 1987) between chromosomal inversions and microsatellite loci for each chromosome.

Table S8 Differentiation in genetic content between generations 4 and 28 assessed by a locus by locus hierarchical AMOVA for chromosomal arrangements whose changes cannot be explained by drift alone (see text).

Data deposited at Dryad: doi: 10.5061/dryad.c5bf7

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