Monitoring chromosomal polymorphism in *Drosophila subobscura* over forty years

Dorcas J. ORENGO, Eva PUERMA and Montserrat AGUADÉ

Departament de Genètica, Facultat de Biologia, and Institut de Recerca de la Biodiversitat (IRBio), Universitat de Barcelona, Barcelona, Spain

Correspondence: Dorcas J. Orengo, Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain.

Email: dorcasorengo@ub.edu

Abstract

The inversion chromosomal polymorphism of *Drosophila subobscura* is considered to be adaptive due to its responses at different time scales to temperature changes. This work reports the longest-term study of chromosomal polymorphism for a single population of *D. subobscura* with climatic data from the collecting site itself. The chromosomal analysis of *D. subobscura* samples collected six times over a 40-year period at the same location and in the same seasonal interval has revealed the continuous presence of 16 common and six moderately rare chromosomal arrangements through the period. This analysis also corroborates the previously detected negative relationship between the frequencies of the standard (cold-climate) arrangement on each of its five chromosomes and temperature, as well as between a comprehensive measure of cold adaptation (the total autosomal proportion of standard arrangement) and temperature. These and previous results would support that species harboring cold and warm adapted arrangements in chromosomal polymorphism, like *D. subobscura*, can rapidly respond to environmental changes.

Key words: Diptera, Drosophilidae, inversion polymorphism, population genetics, temperature, temporal variation.

INTRODUCTION

The study of the relationship between geographic, or temporal, variation at any genetically based observational level, and variation either in environmental variables or in the repertoire of interacting species constitutes a powerful approach to uncover adaptations as well as to point to the putative abiotic and biotic driving factors. The analysis of chromosomal variation in natural populations of different *Drosophila* species has revealed that chromosomal polymorphism can respond to environmental cues. Indeed, the extensive studies performed in multiple species have revealed, among others, changes through environmental gradients (e.g. latitudinal clines: Mettler *et al*. 1977; Krimbas & Loukas 1980; Knibb *et al*. 1981; Hasson *et al*. 1995; McAllister 2002; Etges & Levitan 2004; Stocker *et al*. 2004), and cyclical changes (e.g. seasonal fluctuations: Dobzhansky 1956; Dobzhansky & Ayala 1973; Stalker 1980; Etges 1991).

The present study focuses on *Drosophila subobscura* Collin, a species of the *obscura* group that exhibits a rich chromosomal polymorphism across its distribution (i.e. both in its native Palearctic range and in the newly colonized Pacific coast of North and South America). Its chromosomal polymorphism has proved not only to have responded to environmental cues in the past but also to have the capacity to rapidly adapt to more recent challenges by changing the frequencies of particular chromosomal arrangements that carry alleles favored in the new environment. Firstly, new latitudinal clines that parallel the existing European cline (Prevosti 1966; Prevosti *et al*. 1984; Krimbas 1992) were established in both North and South America soon after its colonization to these

new habitats (Prevosti *et al*. 1985, 1988, 1990; see however Castañeda *et al*. 2013). Secondly, two of the three known *D. subobscura* populations that exhibit cyclical seasonal changes (Burla & Götz 1965; Fontdevila *et al*. 1983; Rodríguez-Trelles *et al*. 1996; Rodríguez-Trelles 2003; Rodríguez-Trelles *et al*. 2013) rapidly responded to a sudden increase in temperature (Rodríguez-Trelles *et al*. 2013). Thirdly, the pairwise comparison of older and more recent samples along the latitudinal clines generally revealed an increase in the frequencies of warm-climate arrangements (Solé *et al*. 2002; Balanyà *et al*. 2004). Finally, the comparative analysis of samples from the Barcelona area collected over a 29-year period revealed a significant correlation between chromosomal arrangement frequencies and temperature (Orengo & Prevosti 1996). All these observations point to temperature as one of the environmental factors driving adaptation in this species through changes in chromosomal polymorphism.

Here, we have analyzed chromosomal polymorphism over a 40-year period in a population of *D. subobscura* from the Barcelona area (Observatori Fabra). Our data set consists of six samples collected at Observatori Fabra in early November, which is one of its population explosion periods in this area. Five samples collected in 1971, 1972, 1987, 1988 and 1989 are a subset of those previously analyzed by Orengo and Prevosti (1996), whereas the sixth sample was collected in 2011. This constitutes to our knowledge the longest-term study on the species chromosomal polymorphism with climatic data from the collecting site itself. It should be also noted that according to the data registered at Observatori Fabra during the last 100 years (1914-2013) the 30-year mean

temperature preceding the previous last sampling year (1989) was quite constant and oscillated around 14.5ºC whereas since 1989 this mean temperature measure has been increasing steadily and achieved ~15.5ºC in 2011. Thus, the analysis including a sample collected 22 years after 1989 (i.e. at the temperature turning point) should allow us to test whether the chromosomal polymorphism traces the global warming tendency or whether it rather responds in a fine-scale way to temporal erratic changes in temperature.

MATERIALS AND METHODS

Wild-caught males from the 2011 sample were individually crossed with virgin females of the *ch cu* laboratory strain that is homokaryotypic for all chromosomes (A_{st}, J_{st}, U_{st}, E_{st} and $O_{\underline{3+4}}$). Then, one male from each F_1 progeny was backcrossed with virgin females from the *ch cu* strain. Polytene chromosome preparations were obtained from salivary glands of F_1 and/or F_2 third-instar larvae that were dissected, stained and squashed in lacto-aceto-orcein solution. Whenever possible, eight F_1 third-instar larvae were analyzed, which allowed us to obtain the wild male complete karyotype (two sets of autosomes and one X chromosome) with a high probability (P = 0.992). Otherwise, eight F_2 third-instar larvae were karyotyped, which only allowed us to obtain one set of autosomes from the wild-caught male also with a high probability (P = 0.992).

Genetic heterogeneity of gene arrangement frequencies (q) among samples was tested using a G-test (Sokal & Rohlf 1995). Gene arrangement frequencies were normalized by the arcsin ($q^{1/2}$) transformation prior to the analysis of their

temporal relationship with environmental variables. The total rainfall recorded daily, the humidity recorded daily at 07:00 h, and the daily mean as well as maximum and minimum temperatures were obtained from Observatori Fabra. In each case, the period spanning from May to November (i.e. six months prior to collection time) was considered as it spans the two population explosion periods of *D. subobscura* in this area (de Frutos & Prevosti 1984; Orengo & Prevosti 2002), and it is therefore the period in which the environmental variables might have affected the population genetic composition.

RESULTS AND DISCUSSION

A total of 164 autosomal chromosomes and 47 X (A) chromosomes could be characterized from the sample of *D. subobscura* collected in 2011. All the arrangements commonly found in the Barcelona area were found in this sample (Table 1). In fact, only five chromosomal arrangements (U_1, $E_{\underline{1+2+9+4}}$, $O_{\underline{3+4}+17}$, $O_{\underline{3+4+16}}$, and $O_{\underline{3+4+1+25}}$) previously found at Observatori Fabra at very low frequencies (Table 1) were absent in this sample. It should be added that the 2011 sample included one male carrying the $J_{\underline{3+4}}$ arrangement (see Fig. S1), which had never been reported before in the Iberian Peninsula (Krimbas & Loukas 1980; Prevosti *et al*. 1984; Solé *et al*. 2002).

Our analysis of *D. subobscura* chromosomal polymorphism over a 40-year period allowed us to test for long-term changes in inversion frequencies. The Observatori Fabra population chromosomal polymorphism includes 16 common chromosomal arrangements, with average frequency higher than 5%, and six moderately rare chromosomal arrangements, with average frequency between

1.5 and 5% (Table 1). Their frequencies are, however, highly heterogeneous among samples, with apparently erratic changes (Table 1, and summarized for the standard arrangements in Fig.1), as indicated by the highly significant G-tests (Table 1). In general, the arrangement frequencies in the 2011 sample fall within the range of observed variation in the previous samples, with only the frequency of A_1 standing out as higher than ever. The presence over the 40-year period of most chromosomal arrangements at similar frequency levels in the study population would support the involvement of natural selection in their maintenance.

The repeated sampling of this population revealed the occasional absence of various low-frequency arrangements (e.g. U_1, $E_{\underline{1+2+9+4}}$, and $O_{\underline{3+4}+17}$), which probably reflected sampling error. In contrast, the sporadic presence of $O_{\underline{3+4+16}}$ that is normally associated with inversion O_2 (as $O_{\underline{3+4+16}+2}$), and that of $O_{\underline{3+4+8}+7}$ would rather suggest that they might repeatedly arise *de novo*, through recombination. The same mechanism has been proposed for the origin of O_7 from $O_{\underline{3+4}+7}$ and O_{st} chromosomes. O_7 is steadily present, though at rather low frequencies, in the study population across the analyzed period, possibly reflecting the higher frequencies of the originating chromosomal arrangements (Table 1). In addition, we detected novel arrangements in *D. subobscura* and previously undetected arrangements in the study area. The $O_{\underline{3+4+1+25}}$ arrangement that was described from one wild caught male in the 1989 sample (Orengo & Prevosti 1992) presumably corresponds to a new inversion (O_{25}) arisen in the study population on an $O_{\underline{3+4}+1}$ arrangement. In contrast, the $J_{\underline{3+4}}$ arrangement that was detected in one male of the 2011 sample (see Fig. S1)

occurs at more or less high frequencies in Iran, Asia Minor, South Balkans, and the eastern and southern part of the Mediterranean (Krimbas 1993) and at very low frequencies in Sardinia and the Balearic Islands (Prevosti *et al*. 1984; Solé *et al*. 2002). It is rather unlikely that the $J_{\underline{3+4}}$ arrangement has recently originated *de novo* in the study population given that it is a complex arrangement differing from J_{st} by two overlapping inversions. Most possibly some flies have recently immigrated from eastern populations into the Barcelona area. As a matter of fact, the involuntary human action has recently introduced several exotic Diptera species in the area, such as the Tiger mosquito (*Aedes albopictus* Skuse) first detected in 2004 and *Drosophila suzukii* Matsumura detected in Catalonia in 2008 and near Barcelona in 2009 (Calabria *et al*. 2012). The colonization of Western Europe by the two above-mentioned invasive species is being intensively studied because they represent sanitary and/or economic threats. The introduction of individuals of native species from distant populations is more difficult to monitor but it might also occurs with some frequency.

In order to study the putative relationship between changes in chromosomal polymorphism and changes in environmental variables, only frequencies of the standard arrangement, typical of colder regions, of each chromosome were used as a previous study had shown that their latitudinal changes in frequency are complementary to those of all other gene arrangements in Europe (Prevosti *et al*. 1988). Figure 1 and Table 1 show that the frequencies of the standard arrangements tend to decrease along the studied 40-year period, despite that they exhibit a low to moderate increase (0.4-9.44%) between 1989 and 2011. A

similar trend is observed also when the proportions of standard arrangements are averaged across autosomes (Table 1).

Correlation coefficients were calculated between the normalized frequencies, arcsin ($q^{1/2}$), of the standard arrangements and different environmental variables (rainfall, humidity and temperature). As previously reported (Orengo & Prevosti 1996), only temperature exhibited a significant correlation with chromosomal polymorphism (Table 2; results for rainfall and humidity not shown). The coefficients obtained with T_{M-N}, mean temperature from May to November, for the standard arrangements in the Observatori Fabra population were all negative; the probability that all the five coefficients are negative is very low (P = 0.031). However, the correlations were significant only for two of the five chromosomes, U and O. It should be noted that the results were not changed when using other temperature measures and time periods (data not shown). The total percentage of the autosomal length of standard arrangement (%St) estimated according to Kunze-Mühl and Sperlich (1962), which is a more comprehensive and meaningful measure of cold-climate chromosomal content in this species, exhibited a negative correlation with temperature. For comparative purposes, the correlation coefficients between the standard arrangement frequencies and latitudes were calculated for the same European populations as in Prevosti *et al*. (1985). The coefficients for the five autosomes and %St were all positive as expected, given that temperature decreases with increasing latitude, and highly significant (Table 2).

Previous studies on temporal changes in chromosomal polymorphism of *D. subobscura* detected a general decrease in the frequencies of standard

arrangements through time (de Frutos & Prevosti 1984; Gosteli 1990; Orengo & Prevosti 1996; Rodríguez-Trelles *et al*. 1996; Solé *et al*. 2002; Balanyà *et al*. 2004; Zivanovic & Mestres 2011). This trend was considered an adaptive response to the global warming undergone in the last decades (Balanyà *et al*. 2006, 2009). Our 40-year period analysis of the relationship between chromosomal polymorphism in a single population of *D. subobscura* and temperature, more specifically between the frequencies of the standard arrangement on each of five acrocentric chromosomes and temperature, has revealed a negative relationship. The sign of this relationship is concordant with that expected from previous observations that have pointed to temperature as one of the major factors driving past and recent adaptations to changing environments in this species (Rodríguez-Trelles *et al*. 2013).

Although the standard arrangements tended to decrease their frequencies along the 40-year period, it is worth noting that the opposite trend was observed between the last two samples collected in 1989 and 2011 (Fig. 1, Table 1). This observation should not lead, however, to the conclusion that changes in chromosomal polymorphism do not parallel the global climatic change, but rather that chromosomal polymorphism is more plastic than so far considered in tracing temperature changes (see also Rodríguez-Trelles *et al*. 2013). Indeed, any changes detected between only two temporal (or geographical) samples have to be taken with caution. In the case here studied, fluctuations in annual temperature may result in some colder years embedded within the long-term trend of increasing temperature. If variation in chromosomal polymorphism truly responds to climatic factors, frequencies of chromosomal arrangements should

oscillate following fluctuation of climatic factors. The correlations here detected between the frequencies of chromosomal arrangements and temperature clearly point to the influence of changing climatic factors on chromosomal polymorphism in this species.

It has been argued that difference in collecting times of multiple samples in studies at large spatio-temporal scales might be a confounding factor (Rodríguez-Trelles & Rodríguez 2010), especially in the case of flexible chromosomal polymorphism (i.e. with seasonal variation). There is, however, no evidence for seasonal frequency fluctuations of the *D. subobscura* standard chromosomal arrangements in the Barcelona area (de Frutos & Prevosti 1984). In consideration of this confounding factor, our samples were all collected in the same seasonal interval, i.e. at the beginning of the fall population explosion of *D. subobscura*.

Our results, like those of Rodríguez-Trelles *et al*. (2013), highlight that *D. subobscura* can promptly adapt itself in front of environmental challenges, more specifically responding to increasing or decreasing temperatures, given that its chromosomes are polymorphic in cold-adapted or warm-adapted arrangements. It is this rapid response that probably accounts for the magnitude and direction of the changes not being steady through time.

ACKNOWLEDGMENTS

We thank Alfons Puertas from Observatori Fabra for facilitating our access to the Observatori Fabra fields. We also thank David Salguero and Juan Manuel Calvo for helping with fly collection, and Gema Blasco for technical assistance. This work was supported by grants BFU2007-63228 and BFU2012-35168 from Ministerio de Economía y Competitividad, Spain, and 2009SGR-1287 from Comissió Interdepartamental de Recerca i Innovació Tecnològica, Generalitat de Catalunya, Spain to M.A.

REFERENCES

Balanyà J, Solé E, Oller JM, Sperlich D, Serra L (2004) Long-term changes in the chromosomal inversion polymorphism of *Drososphila subobscura*. II. European populations. *Journal of Zoological Systematics and Evolutionary Research* **42,** 191–201.

Balanyà J, Oller JM, Huey RB, Gilchrist GW, Serra L (2006) Global genetic change tracks global climate warming in *Drosophila subobscura*. *Science* **313,** 1773–1775.

Balanyà J, Huey RB, Gilchrist GW, Serra L (2009) The chromosomal polymorphism of *Drosophila subobscura*: a microevolutionary weapon to monitor global change. *Heredity* **103,** 364–367.

Burla H, Götz W (1965) Veranderlichkeit des chromosomalen Polymorphismus bei *Drosophila subobscura*. *Genetica* **36,** 83–104.

Calabria G, Máca J, Bächli G, Serra L, Pascual M, (2012) First records of the potential pest species *Drosophila suzukii* (Diptera: Drosophilidae) in Europe. *Journal of Applied Entomology* **136,** 139–147.

Castañeda LE, Balanyà J, Rezende EL, Santos M (2013) Vanishing chromosomal inversion clines in *Drosophila subobscura* from Chile: Is behavioral thermoregulation to blame? *The American Naturalist* **182,** 249–259.

de Frutos R, Prevosti A (1984) Temporal changes of chromosomal polymorphism in natural populations of *D. subobscura*. *Genetica* **63,** 181–187.

Dobzhansky Th (1956) Genetics of Natural Populations XXV. Genetic changes in populations of *Drosophila pseudoobscura* and *Drosophila persimilis* in some localities in California. *Evolution* **10,** 82–92.

Dobzhansky Th, Ayala F (1973) Temporal frequency changes of enzyme and chromosomal polymorphisms in natural populations of *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America* **70,** 680–683.

Etges WJ (1991) Seasonal variation among gene arrangements in *Drosophila robusta*. *Drosophila Information Service* **70,** 65–66.

Etges WJ, Levitan M (2004) Paleoclimatic variation, adaptation and biogeography of inversion polymorphism in natural populations of *Drososphila robusta*. *Biological Journal of the Linnean Society* **81,** 395–411.

Fontdevila A, Zapata C, Alvarez G, Sanchez L, Méndez J, Enriquez I (1983) Genetic coadaptation in the chromosomal polymorphism of *Drosophila subobscura*. I. Seasonal changes of gametic disequilibrium in a natural population. *Genetics* **105,** 935–955.

Gosteli M (1990) Chromosomal polymorphism in natural populations of *Drosophila subobscura* near Zürich, Switzerland: A contribution to long-term comparisons. *Genetica* **81,** 199–204.

Hasson E, Rodríguez C, Fanara JJ, Naveira H, Reig OA, Fontdevila A (1995) The evolutionary history of *Drosophila buzzatii*. XXVI. Macrogeographic patterns of inversion polymorphism in New World populations. *Journal of Evolutionary Biology* **8,** 369–384.

Knibb WR, Oakeshott JG, Gibson JB (1981) Chromosome inversion polymorphisms in *Drosophila melanogaster*. I. Latitudinal clines and associations between inversions in Australasian populations. *Genetics* **98,** 833–847.

Krimbas CB (1992) The inversion polymorphism of *Drosophila subobscura*. In: Krimbas CB, Powell JR (eds) *Drosophila Inversion Polymorphism*, pp 127–220. CRC Press, Boca Ratón.

Krimbas CB (1993) *Drosophila subobscura: Biology, Genetics and Inversion Polymorphism*. Verlag Dr Kovac, Hamburg.

Krimbas CB, Loukas M (1980) The inversion polymorphism of *Drosophila subobscura*. In: Hecht H, Steere WC, Wallace B (eds) *Evolutionary Biology* Vol. 12**,** pp 163–234. Plenum Press, New York and London.

Kunze-Mühl E, Sperlich D (1962) Vergleichende Untersuchungen über den chromosomalen Strukturpolymorphismus in Insel- und Festlandspopulationen von *Drosophila subobscura. Zeitschrift für induktive Abstammungs- und Vererbungslehre* **93,** 237–248.

McAllister BF (2002) Chromosomal and allelic variation in *Drosophila americana*: selective maintenance of a chromosomal cline. *Genome* **45,** 13–21.

Mettler LE, Voelker RA, Mukai T (1977) Inversion clines in populations of *Drosophila melanogaster*. *Genetics* **87,** 169–176.

Orengo DJ, Prevosti A (1992) A new spontaneous inversion in chromosome O of *Drosophila subobscura*. *Drosophila Information Service* **71**,159–160.

Orengo DJ, Prevosti A (1996) Temporal changes in chromosomal polymorphism of *Drosophila subobscura* related to climatic changes. *Evolution* **50,** 1346–13350.

Orengo DJ, Prevosti A (2002) Relationship between chromosomal polymorphism and wing size in a natural population of *Drosophila subobscura*. *Genetica* **115,** 311–318.

Prevosti A (1966) Chromosomal polymorphism in western Mediterranean populations of *Drosophila subobscura*. *Genetical Research* **7,** 149–158.

Prevosti A, de Frutos R, Alonso G, Latorre A, Monclús M, Martínez MJ (1984) Genetic differentiation between natural populations of *Drosophila subobscura* in the Western Mediterranean Area with respect to chromosomal variation. *Genetics Selection Evolution* **16,** 143–156.

Prevosti A, Serra L, Ribó G *et al*. (1985) The colonization of *Drosophila subobscura* in Chile. II. Clines in the chromosomal arrangements. *Evolution* **39,** 838–844.

Prevosti A, Ribó G, Serra L *et al*. (1988) Colonization of America by *Drosophila subobscura*: experiment in natural populations that supports the adaptive role of chromosomal inversion polymorphism. *Proceedings of the National Academy of Sciences of the United States of America* **85,** 5597–5600.

Prevosti A, Serra, L Segarra C, Aguadé M, Ribó G, Monclús M (1990) Clines of chromosomal arrangements of *Drosophila subobscura* in South America evolve closer to Old World patterns. *Evolution* **44,** 218–221.

Rodríguez-Trelles F (2003) Seasonal cycles of allozyme-by-chromosomal-inversion gametic disequilibrium in *Drosophila subobscura*. *Evolution* **57,** 839–848.

Rodríguez-Trelles F, Rodríguez MA (2010) Measuring evolutionary responses to global warming: cautionary lessons from *Drosophila*. *Insect Conservation and Diversity* **3,** 44–50.

Rodríguez-Trelles F, Alvarez G, Zapata C (1996) Time-series analysis of seasonal changes of the O inversion polymorphism of *Drosophila subobscura*. *Genetics* **142,** 179–187.

Rodríguez-Trelles F, Tarrío R, Santos M (2013) Genome-wide evolutionary response to a heat wave in *Drosophila*. *Biology Letters* **9,** Article ID 20130228. DOI: 10.1098/rsbl.2013.0228

Sokal RR, Rohlf FJ (1995) *Biometry*. Freeman WH and Company. New York.

Solé E, Balanyà J, Sperlich D, Serra L (2002) Long-term changes in the chromosomal inversion polymorphism of *Drososphila subobscura*. I. Mediterranean populations from southwestern Europe. *Evolution* **56,** 830–835.

Stalker HD (1980) Chromosome studies in wild populations of *Drosophila melanogaster*. II. Relationship of Inversion frequencies to latitude, season, wing-loading and flight activity. *Genetics* **95,** 211–223.

Stocker AJ, Foley B, Hoffman A (2004) Inversion frequencies in *Drosophila serrata* along an eastern Australian transect. *Genome* **47,** 1144–1153.

Zivanovic G, Mestres F (2011) Changes in chromosomal polymorphism and global warming: The case of *Drosophila subobscura* from Apatin (Serbia). *Genetics and Molecular Biology* **34,** 489–495.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Polytene J chromosomes of a heterokaryotype for the J_{st} and $J_{\underline{3+4}}$ arrangements.

Table 1 Gene arrangement frequencies (%) in six samples collected at Observatori Fabra through a 40-year period

Arrangement	1971 *n* = 40	1972 *n*= 137	1987 *n* = 97	1988 *n* = 724	1989 *n* = 979	2011 *n* = 164	G-test	
A_{st}	42.86	43.50	23.50	36.41	29.50	34.04	10.42 [a]	
A_1	4.76	–	5.90	4.89	3.37	19.15	–	
A_2	52.38	56.50	70.60	58.69	67.12	46.81	14.60 [a]	*
Jst	35.00	32.80	26.00	20.72	19.10	26.83	20.53 [a]	***
J_1	65.00	67.20	74.00	79.28	80.90	72.56	21.12 [a]	***
J_{3+4}	–	–	–	–	–	0.61	–	
U_{st}	2.50	4.50	2.00	2.35	1.43	1.83	–	
U_1	2.50	–	–	0.55	0.61	–	–	
U_{1+2}	45.00	55.60	35.70	54.56	48.72	61.59	23.90 [a]	***
U_{1+2+6}	–	–	–	–	0.51	0.61	–	
U_{1+2+8}	50.00	39.90	62.20	42.54	48.72	35.98	25.68 [a]	***
E_{st}	30.00	38.50	29.90	26.24	23.29	32.73	18.31 [a]	***
E_8	2.50	3.10	2.10	2.62	2.96	1.21	–	
E_{1+2}	20.00	37.70	22.70	13.67	8.78	16.97	29.78 [a]	***
E_{1+2+9}	35.00	8.50	25.80	10.63	16.95	18.79	38.99 [a]	***
$E_{1+2+9+3}$	2.50	3.80	1.00	7.87	6.23	5.45	11.72 [b]	*
$E_{1+2+9+4}$	–	–	7.20	0.41	0.92	–	–	
$E_{1+2+9+12}$	10.00	28.50	11.30	38.53	40.76	24.85	69.35 [a]	***
O_{st}	32.50	28.90	12.10	12.84	10.52	12.80	39.76 [a]	***
O_7	2.50	1.50	1.00	1.38	1.43	1.83	–	
O_{3+4}	17.50	28.90	28.30	23.06	26.76	34.15	11.43 [a]	*
O_{3+4+1}	10.00	3.70	3.00	5.38	8.99	5.49	14.94 [b]	**
O_{3+4+2}		0.70	3.00	4.14	5.11	2.44	–	
O_{3+4+7}	32.50	28.90	33.30	38.53	33.30	24.39	15.53 [a]	**
O_{3+4+8}	5.00	4.40	8.10	8.29	6.74	14.02	11.71 [b]	*
O_{3+4+17}	–	–	2.00	1.79	2.66	–	–	
O_{3+4+22}	–	3.00	9.10	3.45	3.78	1.83	–	
O_{3+4+16}	–	–	–	–	0.20	–	–	
$O_{3+4+16+2}$	–	–	–	0.97	0.41	2.44	–	
$O_{3+4++8+7}$	–	–	–	0.14	–	0.61	–	
$O_{3+4+1+25}$	–	–	–	–	0.10	–	–	
%St	67.11	68.10	64.55	63.24	62.66	65.58	–	
T_{M-N}	19.42	18.26	20.23	20.17	20.68	20.85	–	

n, number of autosomes studied; T_{M-N}, mean temperature recorded from May to November; %St, total percentage of autosomal length with standard arrangement. G-tests were only conducted for arrangements recorded in all six samples.

[a] G-tests with five degrees of freedom because all six expected absolute frequencies were higher than five.

[b] G-tests with four degrees of freedom because the expected absolute frequencies of one of the samples was lower than five and this sample was therefore excluded.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; without correction for multiple testing.

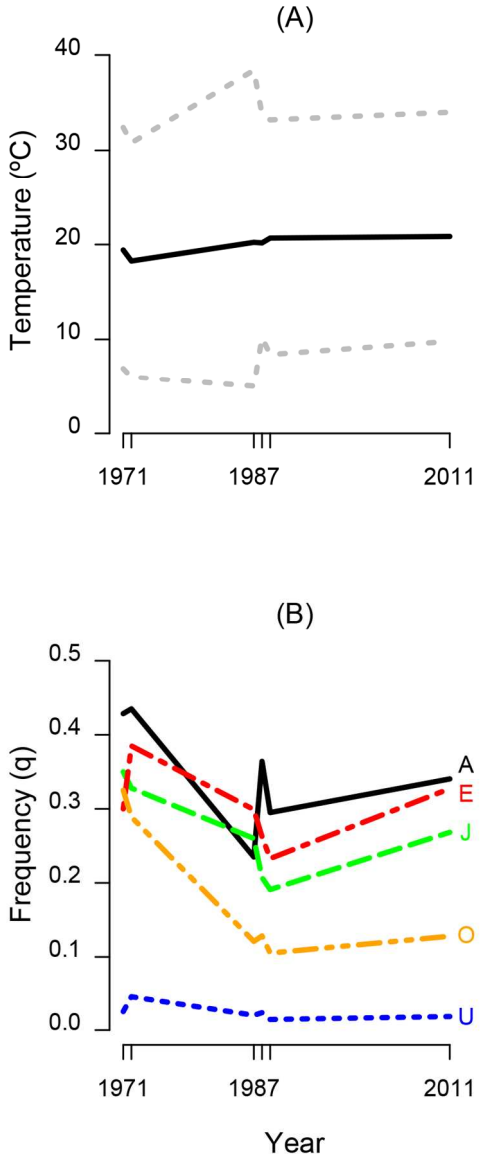
Table 2 Correlation coefficient between normalized arrangement frequencies or total percentage of the autosomal length with standard arrangement (%St) and mean temperature from May to November (T_{M-N}) or latitude

	Correlation coefficient *r*			
Arrangement	T_{M-N}		Latitude[a]	
A_{st}	-0.696		0.880	***
J_{st}	-0.707		0.972	***
U_{st}	-0.955	**	0.975	***
E_{st}	-0.691		0.973	***
O_{st}	-0.844	*	0.870	***
%St	-0.785	¶	0.947	***

[a] from Prevosti *et al*. 1985, except for the %St value
¶ 0.05 < *P* <0.1; * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001; without correction for multiple testing.

Figure Legend

Figure 1 Temporal changes in temperature and chromosomal arrangement frequencies. (A) Solid black line: mean temperature recorded from May to November (T_{M-N}) at the collecting site (Observatori Fabra); dotted gray lines: mean maximum and minimum temperatures for the same period, (B) frequency (*q*) of the standard arrangement on each major chromosome of *D. subobscura*.



(A): solid black line mean, temperature recorded from May to November (T_{MN}) at the collecting site (Observatori Fabra); dotted gray lines, mean maximum and minimum temperatures for the same period. (B): frequency (*q*) of the standard arrangement of each major chromosome of *D. subobscura*.
184x423mm (600 x 600 DPI)