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ORIGINAL ARTICLE

Global warming and chromosomal inversion adaptation in isolated islands: *Drosophila subobscura* populations from Madeira

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Abstract

Global warming is an environmental phenomenon to which species must adapt to survive. *Drosophila subobscura* presents an adaptive capacity due to its chromosomal inversion polymorphism. Until now, the impact of global warming on this polymorphism has been studied in *D. subobscura* populations located either on a continental mainland or on islands not far from a continent. In this context, gene flow could be a relevant mechanism allowing the movement of thermally adapted inversions between populations. Our aim was to sample and study the chromosomal polymorphism on Madeira, a small isolated island in the Atlantic Ocean. We compared our findings with those reported in the same location approximately four and five decades ago. Moreover, we studied whether global warming has occurred on this island by analyzing mean, maximum and minimum temperatures over a 55-year period. All atmospheric parameters have increased significantly, consistent with climate change expectations. Frequencies and chromosomal thermal index values of thermal adapted inversions remained quite stable over years. Furthermore, J, U and O chromosomes are almost fixed for "warm" adapted inversions. Thus, if there is little genetic variability remaining and temperatures continue increasing, island populations of *D. subobscura* might be on the threshold of endangerment. However, apart from selection, genetic drift and inbreeding, other processes, such as phenotypic plasticity or thermoregulatory behavior, could be involved in the survival of the species' populations. Finally, although in danger, *D. subobscura* is a generalist that lives in humanized environments, and this fact could favor its persistence on Madeira Island.

Key words: chromosomal inversion, climate change, selection, temperature, thermoregulatory behavior.

INTRODUCTION

Global warming has a profound impact on living organisms, providing an excellent opportunity to study the genetic components of adaptation (Hoffmann & Sgrò 2011; Franks & Hoffmann 2012). For instance, in a given species, a possible experimental approach is to collect samples either over time or in distinct localities of the distribution area with different climatic conditions and to study differences in genetic composition (Solé *et al*. 2002; Hoffmann *et al*. 2004; van Heerwaarden & Hoffmann 2007). However, a large number of species could adapt to global warming by migrating to other places. In this sense, it could be interesting to study the effects of climatic changes on species located in small oceanic islands. Compared with continental populations, they usually have reduced gene flow, while genetic drift and inbreeding are important evolutionary factors (for recent examples see Hoeck *et al*. 2010; Rogell *et al*. 2010; Furlan *et al*. 2012;

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Mattila *et al.* 2012; Wang *et al.* 2014; Fountain *et al.* 2016; Funk *et al.* 2016).

Chromosomal inversion polymorphism is considered one of the most useful genetic markers in studies of adaptation to global warming. Inversions were discovered by Sturtevant (1921) and their adaptive value was widely recognized by Dobzhansky (see Lewontin *et al.* 1981). These kind of chromosomal mutations are considered cornerstones in the adaptation or speciation of a large number of organisms (see, for instance, Feuk *et al.* 2005; Kirkpatrick & Barton 2006; Hoffmann & Rieseberg 2008; McAllister *et al.* 2008; Kirkpatrick 2010; Nie *et al.* 2012; Ayala *et al.* 2014; Fuller *et al.* 2018). In particular, the role of inversions with regard to thermal adaptation was studied in depth using different species of the *Drosophila* genus (for example, Dahlgaard *et al.* 2001; Anderson *et al.* 2003, 2005; Levitan 2003; Hoffmann *et al.* 2004; Levitan & Etges 2005; Umina *et al.* 2005; Rane *et al.* 2015; Kapun *et al.* 2016). In this context, *D. subobscura* can be considered a global warming model species. It presents a rich chromosomal polymorphism, that is, a large number of different inversions and combinations of inversions (overlapped or not) called arrangements (Krimbas 1992, 1993; Balanyà *et al.* 2009). This polymorphism is adaptive to many environmental conditions and many inversions (and arrangements) have changed in frequency according to global warming expectations (Orengo & Prevosti 1996; Rodríguez-Trelles & Rodríguez 1998; Solé *et al.* 2002; Balanyà *et al.* 2004, 2006, 2009; Rezende *et al.* 2010; Zivanovic & Mestres 2011; Zivanovic *et al.* 2012, 2015; Orengo *et al.* 2016). All these studies have been carried out either in populations located on the continent or on islands close to the mainland. That is why we considered it would be interesting to study the impact of global warming on an oceanic island population.

The chosen island was Madeira, located in the Atlantic Ocean at 978 km south of Portugal, 700 km west of Africa and 450 km north of the Canary Islands. Its total area is 796 km^2. It enjoys a mild climate with temperature variations through the year and differences between the regions facing north and those facing south. These conditions favor agricultural activity based on Mediterranean and tropical species. The island is considered a hotspot of biodiversity with a large number of endemic species (Borges *et al.* 2008). *Drosophila subobscura* was reported for the first time on Madeira by Prevosti (1972), although previous studies of drosophilid fauna could have mistakenly classified this species as *D. obscura* (Monclús 1984; Bächli & Báez 2002). Furthermore, within the laurisilva environment, *D. subobscura* coexists with its closely related species of the *obscura* group, *D. madeirensis*, which is endemic to the island (Monclús 1984). *Drosophila subobscura* from Madeira has been used to study distinct evolutionary problems by means of diverse genetic markers (Pinto *et al.* 1997; Khadem *et al.* 1998, 2001; Brehm *et al.* 2004; Herrig *et al.* 2014), but only two previous analyses of chromosomal inversion polymorphism have been carried out (Prevosti 1972; Larruga *et al.* 1983).

Our main aim was to compare the current composition and frequencies of *D. subobscura* chromosomal inversion polymorphism on Madeira with that reported earlier. Also, we wanted to ascertain whether predicted climatic effects of global warming could be observed on the island analyzing rainfall and minimum, maximum and mean temperatures, over years. Finally, if variation both in inversions and climatic change were observed, the final objective was to assess a possible relation between the adaptive inversions and global warming.

MATERIALS AND METHODS

Fly samples

Drosophila subobscura individuals were collected at Camacha (32°40′N 16°50′W), Curral das Freiras (32°43′N 16°58′W) and Prazeres (32°45′N 17°12′W) during November and December 2016 (Fig. 1). Flies were collected using open banana baits, sampled by netting in the morning and late afternoon. The captured flies were put in bottles together with some local vegetation and kept in darkness. At the end of the day, the samples were transferred to the laboratory where they were separated and classified. Males were put together (20–30 individuals) in large plastic vials with Carolina Instant (Carolina Biological Supply Co., Burlington, North Carolina, USA) *Drosophila* Medium (17-3200), whereas females were placed in individual vials and allowed to lay eggs. All vials were sent to Barcelona for the chromosomal analysis. Individual wild

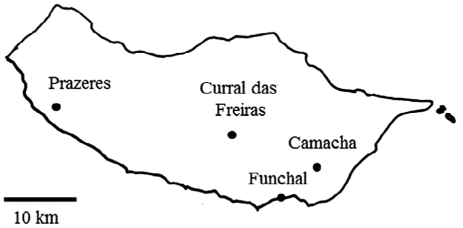

Figure 1 Map of Madeira showing locations of the *Drosophila subobscura* populations studied (Prazeres, Curral das Freiras and Camacha). The capital of the island, Funchal, is also shown, as a reference.

males (or sons of isofemale lines) were crossed with two virgin females of the *chcu* homokaryotypic strain that carries the recessive mutations *ch* (cherry eyes) and *cu* (curled wings), both located in the O chromosome. With regard to chromosomal inversions, the strain is homokaryotypic A_{st}, J_{st}, U_{st}, E_{st} and $O_{\underline{3+4}}$. Third instar F_1 larvae were dissected to obtain the polytene chromosomes of the salivary glands, which were stained and squashed in aceto-orcein solution. For the study of inversions we have followed the classification and nomenclature of Kunze-Mühl and Müller (1958) and Krimbas (1993). The chromosomal inversion polymorphisms from our Madeira collections were compared with those obtained previously in Terreiro da Luta (32°41′N 16°53′W) and Curral das Freiras (32°43′N 16°58′W) in 1970 by Prevosti (1972) and in Poiso (32°43′N 16°58′W) and Ribeiro (32°46′N 16°51′W) in 1978 by Larruga *et al.* (1983).

Statistical analyses and meteorological data

All computations were carried out using R language (R Development Core Team 2014). To compare the chromosomal inversion polymorphism between different locations and/or years, Fisher's exact test was used and the corresponding *P*-values were obtained using the bootstrap procedure (100,000 runs; statistically significant at $P < 0.05$). All analyses were carried out using the *fisher.test* function of the package *stats*. As multiple testing was computed, results were corrected by means of the false discovery rate method (Benjamini & Hochberg 1995) using the function *p.adjust* (package *stats*).

To estimate the probability of appearance or disappearance of several inversions in our Madeira collection (2016), the following permutation procedure was used. Using Prevosti's collection as a base, we generated B = 50,000 samples with replacement with our Madeira sample size and we estimated the probability that infrequent inversions were included in the 2016 collection. Also, we fixed the 2016 collection as a base and Prevosti's plus Larruga's sample size, and then we generated B = 50,000 samples with replacement to estimate the probability that the new inversions were included in the previous samples.

Finally, to place the Madeira polymorphism with regard to other Palearctic and American *D. subobscura* populations, comparisons between O chromosome inversion polymorphisms (the most studied chromosome in this species) were carried out (Table S1). The Bhattacharyya distance (Bhattacharyya 1946) was used and a principal coordinate analysis was carried out with this set of populations (Balanyà *et al.* 2006; Mestres *et al.* 2009). The computations were carried out using the *mds* function from *vegan* package and graphically displayed with the *eqscplot* function of *MASS* package. Finally, GEVA-Ward was chosen as cluster method, because it is considered to be the optimal method for analyzing chromosomal inversion data (Irigoien *et al.* 2010; Zivanovic *et al.* 2016). The cluster was obtained using the *hclust* function of *stats* package of R. To measure how reliable the cluster preserved the pairwise distances between the original data, the Pearson cophenetic correlation was computed (*cophenetic* function of *stats* package).

Values of minimum (Tmin), maximum (Tmax) and mean (Tmean) temperatures and rainfall were obtained from Funchal Meteorological Station. Although this is not one of our trapping sites, it allows for the tracking of meteorological variables on the island. To analyze the possible effect of global warming in Madeira, we used data from each October (the month before trapping the flies) between 1961 and 2016. For each of the three temperatures (Tmin, Tmax and Tmean), a temporal series was computed using Statgraphics software (Statgraphics Technologies, The Plains, Virginia, USA). As rainfall was appreciably erratic, a temporal series was not computed. Finally, the chromosomal thermal index (CTI; Arenas *et al.* 2018) was computed to measure the thermal adaptation of the whole karyotype. The index varies from 0 to 1, where 1 means that only "warm" adapted inversions are present. Chromosomes were classified as "warm" or "cold" using the well-established criterion of Menozzi and Krimbas (1992) and Rego *et al.* (2010). Thus, in Madeira, the following inversions (or arrangements) were considered "warm": A_2, J_1, $U_{\underline{1+2}}$, $U_{\underline{1+8}+2}$, $E_{\underline{1+2+9}}$, $E_{\underline{1+2+9+12}}$ and $O_{\underline{3+4}}$; whereas A_{st}, J_{st}, U_{st}, E_{st} and O_{st} were classified as "cold". The remaining inversions were considered unrelated to thermal adaptation.

RESULTS

Chromosomal inversion polymorphism

It is interesting to compare the chromosomal polymorphism from this study (Table 1) with data from previous studies (Prevosti 1972; Larruga *et al.* 1983). From a qualitative point of view, the chromosomal composition is moderately similar. However, the inversions $A_{\underline{2+6}}$, $E_{\underline{1+2+9+3}}$ and O_{st}, previously detected by Prevosti (1972), are absent in both Larruga *et al.* (1983) and the present research. The probabilities that these inversions were included in our 2016 sample were estimated as 0.6501, 0.8558 and 0.9997, respectively. Therefore, the most likely scenario is that they

Table 1 Chromosomal inversion polymorphism frequencies (Freq.) of three populations of *Drosophila subobscura* analyzed from Madeira (Camacha, Curral das Freiras and Prazeres)

	Camacha		Curral das Freiras		Prazeres		Total Madeira†	
Chromosomal inversion	*n*	Freq.	*n*	Freq.	*n*	Freq.	*n*	Freq.
A_{st}	14	0.230	3	0.130	11	0.282	28	0.228
A_2	47	0.770	20	0.870	28	0.718	95	0.772
Total	61		23		39		123	
J_{st}	9	0.123	3	0.100	3	0.059	15	0.097
J_1	64	0.877	27	0.900	48	0.941	139	0.903
Total	73		30		51		154	
U_{st}	–	–	–	–	1	0.019	1	0.006
U_1	1	0.014	–	–	–	–	1	0.006
$U_{\underline{1}+\underline{2}}$	50	0.685	23	0.767	38	0.731	111	0.716
$U_{\underline{1+8}+\underline{2}}$	22	0.301	7	0.233	13	0.250	42	0.271
Total	73		30		52		155	
E_{st}	13	0.183	9	0.300	17	0.333	39	0.257
$E_{\underline{1+2}}$	31	0.437	11	0.367	18	0.353	60	0.395
$E_{\underline{1+2+9}}$	1	0.014	–	–	1	0.020	2	0.013
$E_{\underline{1+2+9+12}}$	26	0.366	10	0.333	14	0.275	50	0.329
$E_{\underline{1+2+9+new}}$	–	–	–	–	1	0.020	1	0.007
Total	71		30		51		152	
$O_{\underline{3+4}}$	72	0.986	30	1	50	0.980	152	0.987
$O_{\underline{3+4}+\underline{2}}$	1	0.014	0	0	1	0.020	2	0.013
Total	73		30		51		154	

†Joint analysis of the three populations.
–, not applicable; *n*, number of chromosomes.

Table 2 *P*-values of Fisher's exact test in comparisons between studies of the chromosomal inversion polymorphism of *Drosophila subobscura* undertaken in Madeira

Chromosome	This research *vs.* Prevosti (1972)	This research *vs.* Larruga *et al.* (1983)	Prevosti (1972) *vs.* Larruga *et al.* (1983)
A	**0.0300**	**0.0308**	0.7334
J	0.8058	0.6362	0.0998
U	0.1469	0.1210	0.8975
E	0.8058	0.1133	**0.0308**
O	**0.0308**	1.0000	**0.0180**
All	**0.0308**	**0.0405**	**0.0300**

False discovery rate correction was applied and significant values ($P < 0.05$) appear in bold.

disappeared from the Madeira populations. Also, in the present study, inversions U_{st} and U_1 were detected for the first time and a new inversion, never before reported, was observed in the species ($E_{\underline{1+2+9+new}}$). The probabilities that these inversions were present in the previous Madeira collections were estimated as 0.9536, 0.9538 and 0.9576, respectively. Thus our hypothesis is that they appeared recently. The detection, for the first time, of common inversions in the species distribution area (albeit with low frequencies) seems to indicate a certain level of gene flow from the continent. The observation of new inversions in *D. subobscura* is a recurrent phenomenon previously reported (Orengo & Prevosti 1996; Zivanovic & Sperlich 2000; Solé *et al.* 2002; Balanyà *et al.* 2003, 2004), but their fate is usually extinction (Sperlich & Pfriem 1986; Powell 1997). Finally, it is worth pointing out that, although the O chromosome is highly polymorphic for inversions (Krimbas 1992, 1993), our Madeira collections are almost monomorphic for $O_{\underline{3+4}}$ (Table 1).

We compared the chromosomal polymorphism composition in Curral das Freiras between our sample and Prevosti's (1972) and no significant differences were obtained for any chromosome (A, P = 0.725; J, P = 1; U, P = 0.358; E, P = 0.801 and O, P = 1). In our collections, as no significant differences were observed between Camacha, Curral das Freiras and Prazeres, it is possible to consider all the *D. subobscura* samples from Madeira as a unique population (Table S2). We observed significant differences in chromosomal compositions between the three studies [Prevosti (1972), Larruga *et al.* (1983) and the present research] when all chromosomes were considered together (Table 2). Moreover, we detected significant differences in E and O chromosomes between the two former samples. This

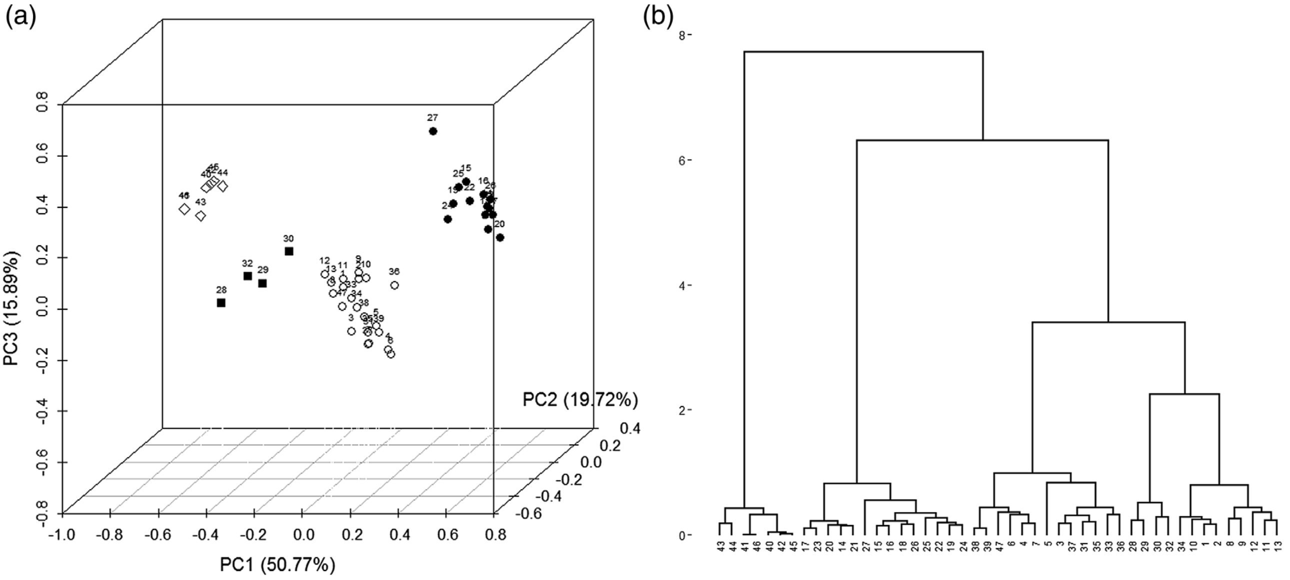

Figure 2 Multivariate analysis of the O chromosome inversion polymorphism in natural populations of *Drosophila subobscura*. (a) Principal coordinate (PC) analysis. Four groups of populations were clearly determined, from left to right: Madeira samples (open diamond, ◇), Balkan populations (filled square, ■), remaining European samples (open circle, ○) and American populations (filled circle, ●). (b) GEVA-Ward cluster study. The first partition separated Madeira collections from other *D. subobscura* collections. In both analyses, the populations analyzed were: 1. Montpellier (France); 2. Lagrasse (France); 3. Queralbs (Catalonia, Iberian Peninsula); 4. Riba-roja (Iberian Peninsula); 5. Calvià (Majorca Island); 6. Punta Umbría (Iberian Peninsula); 7. Málaga (Iberian Peninsula); 8. Groningen (The Netherlands); 9. Louvaine-la-Neuve (Belgium); 10. Villars (France); 11. Tübingen (Germany); 12. Vienna (Austria); 13. Leuk (Switzerland); 14. Santiago de Chile (Chile); 15. Chillán (Chile); 16. Laja (Chile); 17. Valdivia (Chile); 18. Puerto Montt (Chile); 19. Coyhaique (Chile); 20. Gilroy (USA); 21. Davis (USA); 22. Eureka (USA); 23. Medford (USA); 24. Salem (USA); 25. Centralia (USA); 26. Bellingham (USA); 27. Port Hardy (Canada); 28. Kamariste (Serbia); 29. Petnica (Serbia); 30. Zanjic (Montenegro); 31. Font Groga 2004 (Catalonia, Iberian Peninsula); 32. Mt. Parnes (Greece); 33. Observatori Fabra (Catalonia, Iberian Peninsula); 34. Font Groga 2007 (Catalonia, Iberian Peninsula); 35. Font Groga 2011 (Catalonia, Iberian Peninsula); 36. Font Groga 2012 (Catalonia, Iberian Peninsula); 37. Font Groga 2013 (Catalonia, Iberian Peninsula); 38. Font Groga 2014 (Catalonia, Iberian Peninsula); 39. Font Groga 2015 (Catalonia, Iberian Peninsula); 40. Camacha (Madeira); 41. Curral das Freiras 2016 (Madeira); 42. Prazeres (Madeira); 43. Terreiro da Luta (Madeira); 44. Curral das Freiras 1970 (Madeira); 45. Poiso (Madeira); 46. Ribeiro (Madeira); 47. Adraga (continental Portugal) (Prevosti 1972; Larruga *et al.* 1983; Mestres *et al.* 1998; Zivanovic *et al.* 2000; Solé *et al.* 2002; Balanyà *et al.* 2003, 2004; Araúz *et al.* 2009; Fragata *et al.* 2010; Calabria 2012; Galludo *et al.* 2018).

could be a product of an increase in frequency of E_{st}, $E_{\underline{1+2}}$ and $E_{\underline{1+2+9}}$ and a decrease of $E_{\underline{1+2+9+12}}$ ($E_{\underline{1+2+9+3}}$ disappeared in the second study). Furthermore, in the second study, O_{st} was absent and $O_{\underline{3+4}}$ increased. The loss of O_{st} and the increase of $E_{\underline{1+2+9}}$ are in accordance with global warming expectations, although this loss could equally be due to genetic drift. The increase of E_{st} (considered a "cold" inversion) is most probably attributable to genetic drift. In the comparison between the second study and the present research only the A chromosome was significant, due to an increase of A_{st} and a decrease of A_2. This result is at odds with the global warming hypothesis, because A_{st} and A_2 are considered "cold" and "warm", respectively. Finally, when comparing both extreme samples [Prevosti (1972) and the present study], A and O chromosomes showed significant variations: A_{st} increased and A_2 decreased, and we saw an increase in $O_{\underline{3+4}}$, whereas O_{st} was not detected in the present analysis. Therefore, these results could be interpreted in the same way as those previously explained.

The multivariate analysis using the O chromosome frequencies in natural populations showed that collections from the island of Madeira were clearly differentiated from other populations (Fig. 2). In the principal coordinate analysis, first, second and third axes explained the 50.77%, 19.72% and 15.89%, respectively (Fig. 2a). Four groups are manifestly differentiated: from left to right it is possible to observe the collections from Madeira, the group of Balkan populations, the remaining European collections and the American populations. European populations are distributed along the second axis and follow a clinal distribution, with Mediterranean samples at the bottom, central and north European at the top. American populations are separated, due to the strong founder event, and are sorted along latitude. However, although they belong to different hemispheres, North and South American populations are mixed in the graphic, following a climatic pattern. According to weather, Atlantic climate populations are at the top of the group, regardless of their American origin and the same happens with Mediterranean climate populations, which appear at the bottom of the group. Equivalent results were obtained from the cluster analysis (Fig. 2b). The cophenetic correlation coefficient was 0.813, indicating that the tree properly describes the genetic distance between populations. According to the O chromosome inversion polymorphism, the first partition separated off the Madeira collection. The second divergence was between American and European populations. Inside the first group, samples with a Mediterranean climate were clustered together (including samples from North and South America), whereas the second group contained those belonging to an Atlantic climate (also with North and South American populations). Analyzing the European cluster, two groups are clearly differentiated: one containing the populations with a Mediterranean climate, the other Atlantic. In the Mediterranean group it is possible to detect the populations from Majorca (Calvià), Catalonia and other Iberian Peninsula samples. In the other large European group, two clusters can be observed: one with Balkan populations and the other with the remaining European collections. In this last group, the first cluster contained the French populations and one Catalan sample and the central and north European samples are grouped in the second cluster.

Climatic change and chromosomal inversions

Temperatures showed a significant increase, according with global warming expectations. For Tmin, Tmax and Tmean, the *P*-values were lower than 0.0001 in all cases. Graphical displays and lineal trends for these temperatures are presented in Figure S1. The rainfall pattern was irregular, in accordance with global warming expectations. These results are in agreement with those reported in Santos *et al.* (2004), Cropper and Hanna (2014) and Tomé *et al.* (2014).

We analyzed the chromosomal polymorphism for all available Madeiran collections but considered only those inversions classified as "warm" or "cold", using the CTI index (Table 3). These values ranged between 0.714 and 0.781 and can be considered high, being surpassed only by the sample from Etna on Sicily (0.958), analyzed by Prevosti *et al.* (1984). No significant differences were detected in the comparisons between the CTI values of our whole Madeira sample and the two earlier samples (Table S3), which could indicate that thermal adapted chromosomal inversions have not changed in frequency over time.

Table 3 Chromosomal thermal index (CTI) values from studies of the chromosomal inversion polymorphisms of *Drosophila subobscura* undertaken in Madeira

Population	CTI	Reference
Camacha	0.774	Present research
Curral das Freiras	0.773	Present research
Prazeres	0.714	Present research
Total Madeira	0.754	Present research
Poiso	0.754	Larruga *et al.* (1983)
Ribeiro	0.781	Larruga *et al.* (1983)
Total Madeira	0.767	Larruga *et al.* (1983)
Terreiro da Luta	0.754	Prevosti (1972)
Curral das Freiras	0.781	Prevosti (1972)
Total Madeira	0.761	Prevosti (1972)

DISCUSSION

There is widespread concern about the different phenomena that can damage our planet, and one of the most urgent is climate change (Ripple *et al.* 2017). The way in which living organisms are able to adapt to survive this environmental change is an essential issue in evolutionary biology. Studies of the *Drosophila* genus have proved it to be an excellent biological model (Levitan 2003; Levitan & Etges 2005; Umina *et al.* 2005; Overgaard *et al.* 2014; Tobler *et al.* 2015). The *D. subobscura* species has been especially interesting due to its rich chromosomal inversion polymorphism and the accumulation of a large number of studies carried out in different biogeographic areas, over time (Orengo & Prevosti 1996; Rodríguez-Trelles & Rodríguez 1998; Solé *et al.* 2002; Balanyà *et al.* 2004, 2006, 2009; Rezende *et al.* 2010; Zivanovic & Mestres 2011; Zivanovic *et al.* 2012, 2015; Orengo *et al.* 2016). However, it was scientifically valuable to study the chromosomal inversion polymorphism of this species in an isolated place with low gene flow, such as the island of Madeira. As this is a small island and its *D. subobscura* populations have suffered many bottlenecks (due, for instance, to forest fires or floods), their effective population sizes (N_e) are expected to be small. In these conditions, genetic drift and inbreeding could be evolutionary mechanisms with relatively dramatic effects. In natural *D. subobscura* populations, the number of generations per year has been estimated at four to six (Begon 1976; Mestres *et al.* 2001). Therefore, selection and/or genetic drift plus inbreeding could have been active through 32–48 and 152–228 generations (representing the time lapses between first and second studies and second and third, respectively).

From our study, it seems that all populations, at least in the southern half of the island, were moderately uniform. For this reason, we analyzed the inversion polymorphism, considering Madeira as a single population. Although this island is distant from the continent, the tourist industry means large amounts of food are imported, mainly from continental Portugal. Fruits and other vegetables could act as carriers for *D. subobscura* individuals, most likely in the larvae or pupa stages. However, the composition of the chromosomal inversions was stable over time and, in the present study, only two Palearctic inversions were detected for the first time on Madeira. There are two competing hypotheses for their presence: either they were already present on the island but remained undetected, or they are newly arrived from the continent. The first is improbable due to the large sample sizes analyzed by Prevosti (1972) and Larruga *et al.* (1983). We think that continental inversions are able to reach Madeira by way of unintentional human transport, but would soon disappear due to genetic drift or selection, because they are non-adaptive to the particular island environment. Although significant differences were observed for several particular chromosome comparisons between the three collections from Madeira (Prevosti 1972; Larruga *et al.* 1983; present study), the chromosomal composition over time is fairly constant in the type of inversions and their relative abundance, even though eight (32–48 generations) and 38 years (152–228 generations) have elapsed between these three collections. Different reasons could explain some of the particular differences detected. For instance, Larruga *et al.* (1983), like us, collected the samples in the autumn, but Prevosti (1972) trapped flies in the summer. Furthermore, we sampled *D. subobscura* individuals just after extensive forest fires in August and floods in October had ravaged the island, and these events could have produced population bottlenecks altering the inversion frequencies.

If we focus only on the chromosomal inversions considered thermally adapted (Menozzi & Krimbas 1992; Rego *et al.* 2010; Arenas *et al.* 2018), although small fluctuations have been observed, in general their composition has not changed over time. The consistency of the CTI values is very similar in the three collections available and no significant differences were detected. The similarity of the CTI values over time contrasted greatly with the dramatic global warming observed when analyzing Tmin, Tmax and Tmean over a 55-year period (an increase of approximately 2°C). Also, the erratic pattern of rainfall during these years was in accordance with the expectations of climate change. The main evolutionary question is: why has thermally adapted chromosomal polymorphism on Madeira not responded to global warming? It was found that the chromosomal polymorphism of the island is poor and particular when compared to closer mainland populations: a product of a historic founder event and adaptations to the special island environment. This situation was demonstrated beyond doubt when comparing the O chromosome inversion composition on Madeira with other *D. subobscura* populations from the whole distribution area, using multivariate analyses (principal coordinate analysis and cluster). Moreover, *D. subobscura* on the island seems to be close to the thermal adaptation limit, because "warm" inversions appear in dramatically high frequencies for three of the chromosomes (0.903, 0.994 and 0.987, for J, U and O, respectively). However, "cold" inversions are present in non-negligible frequencies for the A (0.228) and E (0.257) chromosomes. Two considerations must be taken into account: the inversions considered "warm" or "cold" probably do not imply a direct adaptive effect of temperature (Santos *et al.* 2005) and other karyotypic regions not covered by inversions could contain thermal adaptation genes (Arenas *et al.* 2018). For instance, the A_{st} frequency (considered as a "cold" inversion) that we detected on Madeira (0.228, collected in autumn/winter) was significantly higher than that found by Prevosti (1972) (0.110, but trapped in summer) and Larruga *et al.* (1983) (0.115, also collected in autumn), which could indicate that this inversion might be a response to something other than temperature, because it has changed over the years in the opposite direction to that expected. However, the different trapping seasons and/or genetic drift could explain these differences. Additionally, although thermal adapted genes in *D. subobscura* could be distributed throughout the whole karyotype, a larger than expected number of these genes were located inside inverted regions (Laayouni *et al.* 2007).

The particular chromosomal polymorphism composition seems to be properly adapted to the environmental and biotic conditions of the island, and gene flow appears irrelevant. In this situation, other possibilities must be considered in front of the increase of temperature. In continental populations, in addition to selection of the available genetic variability, other explanations are possible. Thus, *D. subobscura* individuals can migrate to areas of their thermal preference, because it is a species with a high capacity for dispersion (Greuter 1963; Loukas & Krimbas 1979; Serra *et al.* 1987) and reacts according to its thermal preference (Dolgova *et al.* 2010; Rego *et al.* 2010). Consequently, variations in the continental latitudinal clines according to global warming expectations could also be attributed to a greater or lesser extent to migration (Santos 2007). As this explanation is not applicable to an isolated island, other hypotheses have to be explored, such as, for example, thermal plasticity (Bonamour *et al.* 2019; Kelly 2019). In this case, the direct influence of environmental factors (including temperature) on the development of individual phenotypes is considered a key element in the phenotypic change of populations and their persistence (Chevin *et al.* 2010; Fragata *et al.* 2016). However, phenotypic plasticity could involve many fitness costs for the individuals, regardless of the phenotype expressed (Dewitt *et al.* 1998). Furthermore, there is a limit to the effectiveness of plastic response (Mitchell *et al.* 2011). Another possibility is that selection on thermal-related traits is compensated by fly behavior (Huey & Pascual 2009). In this sense, the Bogert effect is defined as the ectoderm thermoregulatory behavior that compensates for environmental temperature variation (Huey *et al.* 2003; Castañeda *et al.* 2013). It was reported that behavior is important in the *Drosophila* genus to maintain temperature within a physiological permissive range (Dillon *et al.* 2009). For instance, these thermoregulatory behaviors could manifest as variations in seasonal and daily activity patterns or displacements to beneficial microclimates (Stevenson 1985).

Global warming is altering both the terrestrial and marine ecosystems of Madeira (Cruz *et al.* 2009; Clemente *et al.* 2014). Moreover, some extinctions and invasions have been reported (see, for instance, Gardiner 2003; Wirtz 2005; Ribeiro *et al.* 2009). These changes could even be harmful for the human population on the island, producing economic losses and health problems (Carvalho *et al.* 2014; Liu-Helmersson *et al.* 2016). Given the present situation, we are unable to predict the future of *D. subobscura* populations on Madeira. The particular ecosystem alterations inflicted by global warming might not be a serious problem for the species, because it is a generalist species closely associated with human or humanized environments (Krimbas 1992, 1993). However, the direct impact of temperature on *D. subobscura* could pose a difficult problem to overcome, due to its limited genetic variability, plasticity and mobility in such a small and isolated island as Madeira. For all these reasons, *D. subobscura* could be an excellent model species for studying the effects of global warming on the evolution of organisms inhabiting isolated oceanic islands.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1. Populations of *Drosophila subobscura* from Madeira, Europe and America used in comparisons of O chromosome inversion polymorphisms.

Table S2. *P*-values of Fisher's exact test in all comparisons between the chromosomal inversion polymorphisms of three populations of *Drosophila subobscura* analyzed on Madeira.

Table S3. Results of the chromosomal thermal index (CTI) comparisons of three collections of *Drosophila subobscura* analyzed on Madeira.

Figure S1. Lineal trends and graphical displays for minimum (Tmin), maximum (Tmax) and mean (Tmean) temperatures and rainfall in Madeira (1961–2016).