



RESEARCH ARTICLE

# Long-term changes in the inversion chromosomal polymorphism: *Drosophila subobscura* population from Rasht (north of Iran)

MANHAZ KHADEM<sup>1</sup>, CONCEPCIÓ ARENAS<sup>2</sup>, JOAN BALANYÀ<sup>3,4</sup> and FRANCESC MESTRES<sup>3,4\*</sup> 

<sup>1</sup>Faculdade de Ciências da Vida, Banco de Germoplasma, Universidade da Madeira, Campus Penteada, 9020-150 Funchal, Portugal

<sup>2</sup>Departament de Genètica, Microbiologia i Estadística. Secció Estadística, Universitat de Barcelona, Barcelona (Spain), Av. Diagonal, 643, 08028 Barcelona, Spain

<sup>3</sup>Departament de Genètica, Microbiologia i Estadística. Secció Genètica Biomèdica, Evolució i Desenvolupament, Universitat de Barcelona, Barcelona (Spain), Av. Diagonal, 643, 08028 Barcelona, Spain

<sup>4</sup>IRBio – Institut de Recerca de la Biodiversitat, Universitat de Barcelona. Barcelona (Spain), Av. Diagonal, 643, 08028 Barcelona, Spain

\*For correspondence. E-mail: fmestres@ub.edu.

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**Abstract.** *Drosophila subobscura* is characterized by a rich chromosomal polymorphism for inversions. Many inversions are adaptive to global warming and can be classified as ‘warm’ or ‘cold’ adapted. However, most studies were carried out from European populations located in the central area of the species distribution or from American colonizing populations. For this reason, we aimed to analyse the isolated and marginal Rasht population, located in the Hyrcanian forests area (Iran). The chromosomal polymorphism for inversions was compared with the previous Rasht samples (Rasht I and II) obtained 57 years ago. This polymorphism has changed based on the inversion composition and frequencies. Interestingly, the polymorphism for inversions was scarce and similar to that of Madeira, an isolated Atlantic island. Likely, this similarity is a consequence of the marginal location and isolation of the Rasht population. Also, the chromosomal thermal index (CTI) was 0.445, showing a significant increase over those from Rasht I (0.184) and II (0.210). All these observations were in agreement with the global warming expectations. Moreover, the CTI was also computed for Russian Caucasus and Turkish populations collected more than 40 years ago to better understand the adaptive potential of *D. subobscura* and to study the similarity between populations of different geographic areas. In summary, the inversions of *D. subobscura* also changed in marginal and isolated populations in agreement with the global warming expectations, and an open question is to know where is the threshold for this evolutionary change.

**Keywords.** chromosomal inversion; adaptation; global warming; hyrcanian ecosystem; *D. subobscura*.

## Introduction

The evolutionary relevance of chromosomal inversions was showed up by Dobzhansky using *Drosophila pseudoobscura* as model organism (a complete review of this topic can be found in Lewontin *et al.* 1981). For evolutionary research, the Palearctic species pretty similar to *D. pseudoobscura* is *D. subobscura*. This later species is distributed all over Europe with the exception of northern Scandinavia and Iceland (Krimbas 1992, 1993). It is found in the republics of the former Soviet Union, although its precise distribution in this area is not known. *D. subobscura* is also present in all eastern and southern Mediterranean coasts, and in the

Atlantic coast of Morocco. In the Eastern Mediterranean, it is present throughout Turkey until the Caucasus and the northern region of Iran. The species inhabits the Mediterranean islands and is also found in Madeira and Canary Is.

*D. subobscura* presents a karyotype constituted by one dot and five acrocentric chromosomes: A (X chromosome), E, J, O and U. All chromosomes, with the exception of the dot, present a rich polymorphism for inversions (Krimbas 1992, 1993). Although there were previous studies (Dubinin *et al.* 1937; Frizzi 1941), it is considered that the first systematic analyses of the inversion chromosomal polymorphism using *D. subobscura* populations were initiated by the German and Swiss groups almost at the same time in the

early 50s of the previous century (Mainx 1950; Mainx *et al.* 1953; Stumm-Zollinger 1953). After these pioneering researchers, other European evolutionary geneticists soon began to focus their evolutionary studies on the inversion polymorphism of this species (for a revision see Krimbas 1993). A review of *D. subobscura* populations analysed with regard to this polymorphism can be found in Krimbas (1992, 1993) and Sillero *et al.* (2020). It was discovered that many inversions (or their combinations, the so-called arrangements), presented frequency variations according to latitudinal clines (Krimbas and Loukas 1980). Two possible hypotheses could explain this observation: the clinal distribution would be a consequence of historical processes or due to an adaptive value of the inversions at different environmental conditions related to the latitude (for instance, temperature). The answer came by chance with the colonization of America by *D. subobscura*. The species was detected for the first time in Puerto Montt (Chile) in 1978 (Brncic *et al.* 1981) and in Port Townsend (Washington) in 1982 (Beckenbach and Prevosti 1986). It spreads very fast along the west coasts of South and North America, and frequencies of the same inversions were distributed according to latitudinal clines in both continents (Prevosti *et al.* 1988, 1989). Both clinal distributions were equivalent and also showed the same direction as those found in the Old World. Thus, this natural experiment with two replicates supported the adaptive role of *D. subobscura* inversions.

Likely, the clinal distribution of inversion frequencies could be the effect of natural selection favouring the adaptation to temperature. In a complete study, Menozzi and Krimbas (1992) defined those inversions considered ‘warm’ and ‘cold’ adapted. According to these ideas, an increase of ‘warm’ and a decrease of ‘cold’ adapted inversions would be expected under the global warming model. The first result confirming this hypothesis was the research carried out in the *D. subobscura* population of Barcelona (Orengo and Prevosti 1996). Later, a large number of chromosomal inversions polymorphism studies in different populations of this species validated the effect of natural selection on ‘warm’ and ‘cold’ inversions due to global warming (for instance, Rodriguez-Trelles and Rodriguez 1998; Solé *et al.* 2002; Balanyà *et al.* 2004, 2006, 2009; Rego *et al.* 2010; Dolgova *et al.* 2010; Zivanovic and Mestres 2011; Orengo *et al.* 2016; Galludo *et al.* 2018). Nevertheless, it is valuable to analyse the isolated or marginal populations to measure changes of inversion frequencies according to temperature and other environmental factors (Zivanovic *et al.* 2012, 2015, 2016, 2021; Madrenas *et al.* 2020). Further, it would be relevant to study the changes in this polymorphism in some regions of *D. subobscura* distribution with particular climatic and environmental conditions.

This was the main aim of this research, to collect *D. subobscura* samples in the Iranian population of Rasht and to study its chromosomal inversion polymorphism. This population belongs to the Hyrcanian ecosystem, with particular conditions of climate and vegetation. The obtained

data was compared to those from that population collected 57 years before by Götz (1967). Also, as more information on the distribution of inversions all around the Palearctic and American populations is now available, we reanalysed the data from Turkey, Russian Caucasus and Iran obtained many years ago to better understand the adaptive value of inversions in these particular regions.

## Material and methods

### *Description of the trapping area and D. subobscura collection*

The sampling area of the present study was the University of Guilan (UG) campus, 16 km south of the city of Rasht in the north of Iran (37°16'50"N, 49°34'59"E), mainly around the faculty of Agricultural Science. The most abundant vegetation was pines, with ornamental and fruit trees. The university campus is located in an agriculture area with some patchy continuation of Hyrcanian forests (Saravan forest). These forests form a unique vegetation cover massif (Aliakbari *et al.* 2021) that stretches 850 km along the southern coast of the Caspian Sea and the northern slopes of the Alborz mountains. They are composed of 80 different woody species (Kiapasha *et al.* 2017) and a large animal biodiversity (Tohidifar *et al.* 2016; Amiri *et al.* 2021). In 2019, this ecosystem was included in the World Heritage List of UNESCO (<https://whc.unesco.org/en/list/1584/>). The regional climate is classified as warm Mediterranean in the east, and Mediterranean in the west (Sagheb Talebi *et al.* 2014). The average yearly precipitation is about 1000 mm, the mean yearly temperature is 20.5°C and the mean humidity is about 76% (IRIMO, I.R. of Iran Meteorological Organization).

The presence of *D. subobscura* in the sampling area was confirmed by using wet traps (apple vinegar). Once confirmed, then banana traps were used to capture the individuals alive. The samplings were carried out during November 2019. Flies were collected in the morning and late afternoon by netting with open banana baits. The captured flies were transferred to the laboratory for separation and classification. To establish isofemale lines, each *D. subobscura* female was placed individually in vials containing Carolina Instant Drosophila Medium food (ref. 17-3200, Carolina Biological Supply, Burlington, USA). When larvae were observed in the vials, they were sent to Barcelona for chromosome analyses.

### *Genetic crosses and polytene chromosomes preparation*

Males of isofemale lines were crossed with two virgin females of the *chcu* homokaryotypic strain which carries the recessive mutations *ch* (cherry eyes) and *cu* (curled wings), both located in the O chromosome. The strain is

homokaryotypic for the inversions:  $A_{st}$ ,  $J_{st}$ ,  $U_{st}$ ,  $E_{st}$  and  $O_{3+4}$ . All genetic crosses were carried out in individual vials containing 25 mL of standard corn–meal–sugar–agar–yeast medium and were kept at 17°C, 60% relative humidity and 12 h / 12 h light/dark cycle. Third instar  $F_1$  larvae were dissected in saline solution (NaCl 0.9%) to obtain the polytene chromosomes of the salivary glands, which were stained and squashed in acetic/lactic orcein solution (2% (w/v) orcein in glacial acetic acid / lactic acid in 1:1 rate). For each isofemale line, at least eight  $F_1$  third-instar larvae were analysed to obtain the complete karyotype for the autosomes (J, U, E and O). The slides were observed using phase-contrast under an Olympus BX41 microscope. For the cytological analysis of chromosomal inversions, the Kunze-Mühl and Müller (1958) and Krimbas (1993) chromosome maps were used.

### Statistical analysis

R language (R Development Core Team 2014) was used to carry out all statistical computations. To compare the chromosomal inversion composition of the Rasht population between its three available samples (Rasht I, Rasht II and Rasht 19) and the possible departure of observed frequencies of chromosomal karyotypes from Hardy–Weinberg expectations for Rasht 19, Fisher’s exact test was used (statistically significant  $P < 0.05$ ). This test was selected because it is considered more precise than the chi-square when the expected frequencies are small. Using a bootstrap procedure (100,000 runs) the corresponding  $P$  values were obtained. The FDR correction (Benjamini and Hochberg 1995) was applied in all cases of multiple comparisons and it was reported as significant for  $P < 0.05$ . The  $CTI$  index was used to measure the thermal adaptation of the whole karyotype (Arenas *et al.* 2018), and was computed for the Iranian populations of Rasht (I, II and 19), Shahi (currently Ghaemshahr) and Chalus (Götz 1967), the Russian population of Goriatchy Klutch (Russian name: Gorjatschi Kljutsch; located in Krasnodar region at the northern side of the Caucasus range) (Sperlich *et al.* 1981) and the Turkish populations of Antalya, Silifke, Tarsus, Bursa, Zonguldak, Samsun and Trabzon (Götz 1967). Finally, the Köppen climate classification of populations (Kottek *et al.* 2006; Rubel and Kottek 2011) was obtained from <https://en.climate-data.org/>.

The similarity between Iranian, Turkish, Goriatchy Klutch (Russia) and other previously studied *D. subobscura* populations regarding their composition of inversions for the O chromosome were analysed as follows. 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). Moreover, GEVA-Ward was chosen as the cluster method, because it is considered the most appropriate for chromosomal inversion data (Irigoién *et al.* 2010; Zivanovic *et al.* 2016). Further, the

Pearson cophenetic correlation was computed to quantify how reliably the cluster preserved the pairwise distances between the original data. High values of this correlation indicate that the obtained tree preserves the pairwise distances between the original populations (Sokal and Rohlf 1962). The *D. subobscura* populations selected for this study, which reflect the O chromosomal composition of different regions of the species geographic distribution area were: Observatori Fabra (Spain, 1995) (Mestres *et al.* 1998); Petnica (Serbia, 1995), Kamariste (Serbia, 1996) and Zanjic (Montenegro, 1997) (Zivanovic *et al.* 2000); Lagrasse (France, 1997), Queralbs (Spain, 1997), Calvià (Spain, 1997), Montpellier (France, 1998), Riba-roja (Spain, 1998), Punta Umbria (Spain, 1998), Málaga (Spain, 1998) (Sole *et al.* 2002); Gilroy (USA, 1994), Davis (USA, 1994), Eureka (USA, 1994), Medford (USA, 1994), Salem (USA, 1994), Centralia (USA, 1994), Bellingham (USA, 1994), Port Hardy (Canada, 1994), Santiago de Chile (Chile, 1999), Chillán (Chile, 1999), Laja (Chile, 1999), Valdivia (Chile, 1999), Puerto Montt (Chile, 1999), Coyhaique (Chile, 1999) (Balanyà *et al.* 2003); Groningen (The Netherlands, 1999), Louvaine-la-Neuve (Belgium, 1999), Tübingen (Germany, 2000), Leuk (Switzerland, 2000), Villars (France, 2001), Vienna (Austria, 2001) (Balanyà *et al.* 2004); Font Gropa (Spain, 2004), Mt. Parnes (Greece, 2006) (Araúz *et al.* 2009); Adraga (Portugal, 2008) (Fragata *et al.* 2010); Font Gropa (Spain, 2007) (Calabria, 2012); Font Gropa (Spain, 2011, 2012, 2013, 2014 and 2015) (Galludo *et al.* 2018); Terreiro and Curral (Madeira 1970) (Prevosti 1972); Poiso and Riberio (Madeira, 1978) (Larruga *et al.* 1983); Camacha, Curral and Prazeres (Madeira, 2016) (Madrenas *et al.* 2020).

### Results

The chromosomal inversion frequencies from the Rasht population sampled in 2019 (Rasht 19) are presented in table 1. The polymorphism is poor when compared to that from the central area of the species distribution (Krimbas 1992, 1993), presenting few different arrangements (only three for the A, J, U and E chromosomes, and four for the O). However, a new inversion of *D. subobscura* was observed for the first time and named  $O_{26}$  (figure 1 a&b). This inversion is located in the segment SI of the O chromosome, is overlapped on the  $O_{3+4}$  arrangement ( $O_{3+4+26}$ ) and its approximate breakpoints are in sections 96A and 93B of the Kunze-Mühl and Müller map (1958). It is interesting to compare the polymorphism detected in our sample (November 2019) with those obtained by Götz in Rasht I and Rasht II in June 1962 (Götz 1967), that is 57 years ago. Assuming that the mean number of generations per year is 5 in *D. subobscura* (Begon 1976; Mestres *et al.* 2001), 285 generations have elapsed from the original Rasht samples to ours. Considering the whole karyotype, there are not significant differences between Rasht I and II (adjusted  $P = 0.9730$ ). Thus, both populations do not differ.

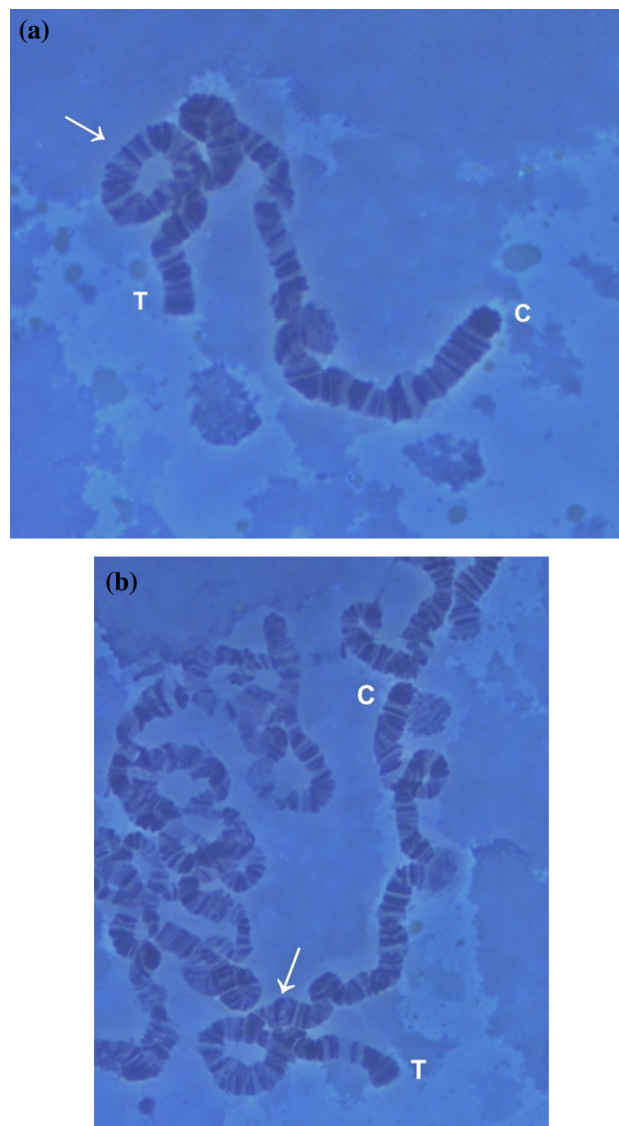
**Table 1.** Frequencies of *D. subobscura* chromosomal inversions from Rasht (Iran) in the 2019 sample (abbreviated in the text as Rasht 19).

Chrom. inversions	Thermal adapt.	N	%
A <sub>st</sub>	C	1	5.3
A <sub>1</sub>	C	16	84.2
A <sub>2</sub>	W	2	10.5
Total		19	
J <sub>st</sub>	C	8	22.2
J <sub>1</sub>	W	25	69.4
J <sub>3+4</sub>	N	3	8.3
Total		36	
U <sub>1+2</sub>	W	34	94.4
U <sub>1+2+7</sub>	N	1	2.8
U <sub>1+8+2</sub>	W	1	2.8
Total		36	
E <sub>st</sub>	C	12	33.3
E <sub>1+2</sub>	N	19	52.8
E <sub>1+2+9</sub>	W	5	13.9
Total		36	
O <sub>st</sub>	C	1	2.8
O <sub>3+4</sub>	W	32	88.9
O <sub>3+4+2</sub>	N	1	2.8
O <sub>3+4+26</sub>	N	2	5.6
Total		36	

N, number of chromosomes; %, percentage of each inversion.

With regard to thermal adaptation 'C', 'W' and 'N' stand for 'cold', 'warm' and 'nonthermal' adapted, respectively. This classification was obtained following the criterion of Menozzi and Krimbas (1992) and Rego *et al.* (2010).

However, significant differences were detected between Rasht 19 and Rasht I (adjusted  $P = 0.0015$ ) and between Rasht 19 and Rasht II (adjusted  $P = 0.0015$ ), indicating that the polymorphism has changed over time. The comparisons of the frequencies for each individual chromosome of the karyotype were carried out and are presented in table 2. Only the comparisons between Rasht 19 and Rasht I, and between Rasht 19 and Rasht II were significant for the J chromosome (adjusted  $P = 0.0015$  and  $0.0015$ , respectively) and for the O chromosome (adjusted  $P = 0.0240$  and  $0.0150$ , respectively). The frequencies of chromosomal karyotypes are shown in table 3, and they did not deviate for the Hardy–Weinberg equilibrium expectations (J chromosome,  $P = 1.0000$ ; U chromosome,  $P = 0.3333$ ; E chromosome  $P = 1.0000$ ; O chromosome,  $P = 0.5075$ ). Thus, it seems that inversions combine at random to generate the karyotypes. *CTI* values, with their interval of confidence, for the Iranian (Rasht 19, Rasht I, Rasht II, Chalus and Shahi), Turkish (Antalya, Silifke, Tarsus, Bursa, Zonguldak, Samsun and Trabzon) and Russian (Goriatchy Klutch) populations with their Köppen climate classification are presented in table 4. With regard to this index, there are significant differences between Rasht 19 and Rasht I (test = 2.725, adjusted  $P = 0.0192$ ) and between Rasht 19 and Rasht II (test = 2.463, adjusted  $P = 0.0207$ ). Thus, this index has increased its value significantly over

**Figure 1.** Two images at 400× of heterokaryotypes carrying the O chromosome new inversion ( $O_{3+4+26}/O_{3+4}$ ). C and T indicate the centromere and telomere tips, respectively. (a) Arrow shows the inversions loop. (b) Arrow indicates the breakpoints.

time in accordance with global warming expectations. However, no significant differences were observed between Rasht I and II (test =  $-0.377$ , adjusted  $P = 0.7064$ ).

**Table 2.** Comparisons between the chromosomal inversion frequencies between Rasht 19 (R19), Rasht I (RI) and Rasht II (RII).

Chromosome	R19 vs. RI	R19 vs. RII	RI vs. RII
A	0.1947	0.2537	0.2537
J	<b>0.0015</b>	<b>0.0015</b>	1.0000
U	0.2532	0.1797	1.0000
E	0.5080	0.5080	0.8452
O	<b>0.0240</b>	<b>0.0150</b>	1.0000

The adjusted  $P$  values are presented. Those significant values appear in bold.



**Table 3.** Frequencies of *D. subobscura* chromosomal karyotypes from Rasht (Iran) in the 2019 sample (abbreviated in the text as Rasht 19).

Karyotypes	<i>N</i>	%
$J_{st}/J_{st}$	1	5.9
$J_{st}/J_1$	4	23.5
$J_{st}/J_{3+4}$	2	11.8
$J_1/J_1$	9	52.9
$J_1/J_{3+4}$	1	5.9
Total	17	
$U_{1+2}/U_{1+2}$	15	88.2
$U_{1+2}/U_{1+8+2}$	1	5.9
$U_{1+2}/U_{1+2+7}$	1	5.9
Total	17	
$E_{st}/E_{st}$	3	17.6
$E_{st}/E_{1+2}$	4	23.5
$E_{st}/E_{1+2+9}$	1	5.9
$E_{1+2}/E_{1+2}$	6	35.3
$E_{1+2}/E_{1+2+9}$	2	11.8
$E_{1+2+9}/E_{1+2+9}$	1	5.9
Total	17	
$O_{st}/O_{3+4}$	1	5.9
$O_{3+4}/O_{3+4}$	14	82.4
$O_{3+4}/O_{3+4+2}$	1	5.9
$O_{3+4+26}/O_{3+4+26}$	1	5.9
Total	17	

*N*, number of individuals; %, percentage of each karyotype.

The general distribution of *D. subobscura* populations with regard to the O chromosome inversion polymorphism using the PCoA is shown in figure 2, and a detailed image involving the Iranian populations is presented in figure 3. The first, second and third axes explain the 42.54%, 24.90% and 14.35% of the variability, respectively. Only the first and second axes are presented in

figures 2 and 3, as the inclusion of the third axis in the graphical representation does not provide additional information. This statistical approach differentiates in one group the American and in another the European population. The Turkish populations are grouped and close to the Europeans. The Iranian and Madeiran samples are clustered together, but this is likely due to the poor O chromosomal polymorphism of both geographic areas. This group is rather close to the Balkan populations, as it is also the sample from Goriatchy Klutch (Russian Caucasus). The tree obtained by the GEVA-Ward cluster method is shown in figure 4. Pearson cophenetic correlation is 0.807, indicating that the dendrogram properly describes the original pairwise distances. The first partition separates the Balkan, Russian Caucasus, Madeiran and Iranian samples from the rest. The Madeiran and Iranian are clustered together, and the Russian population is closer to them than to the Balkan. In the other part of the tree, the next partition separates all American populations. The third partition differentiates the Turkish from the European populations. Finally, a phylogenetic tree analysing only the Iranian populations is presented in figure 5. This tree was supported by a high value of Pearson cophenetic correlation (0.948), indicating that the obtained tree properly represents how populations are grouped. In this dendrogram, the first partition separates Rasht 19 from the all the other Iranian populations.

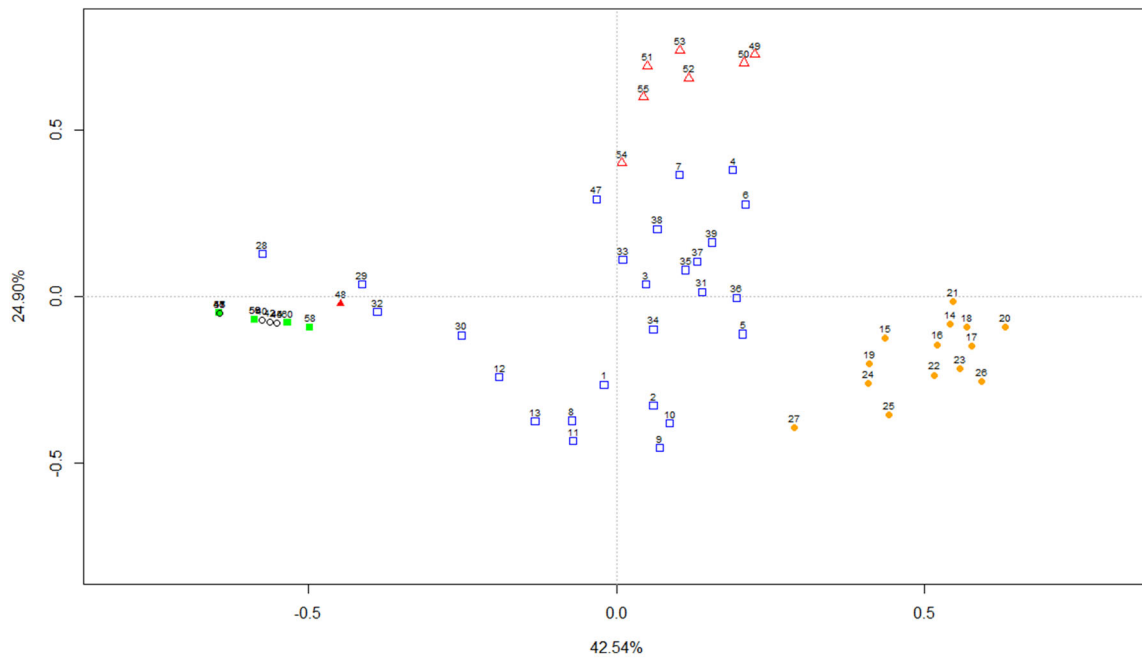
**Discussion**

After the pioneering work of Götz (1967), *D. subobscura* was described once more from Iran, in the Lake Urmia National Park (Parchami-Araghi *et al.* 2016). Thus, the new sample obtained in the present research is very valuable to

**Table 4.** CTI values, with their interval of confidence, for the Iranian (Rasht 19, Rasht I, Rasht II, Chalus and Shahi), Turkish (Antalya, Silifke, Tarsus, Bursa, Zonguldak, Samsun and Trabzon) and Russian (Goriatchy Klutch) *D. subobscura* populations. Climate is presented according to the Köppen classification.

Population	Country	Year	CTI	Interval of confidence	Climate
Rasht 19	Iran	2019	0.445	[0.279, 0.591]	Cfa
Rasht I	Iran	1962	0.184	[0.086, 0.278]	Cfa
Rasht II	Iran	1962	0.210	[0.112, 0.304]	Cfa
Chalus	Iran	1962	0.298	[0.140, 0.441]	Cfa
Shahi	Iran	1962	0.309	[0.210, 0.401]	Csa
Antalya	Turkey	1962	0.640	[0.539, 0.723]	Csa
Silifke	Turkey	1962	0.560	[0.456, 0.649]	Csa
Tarsus	Turkey	1962	0.473	[0.364, 0.569]	Csa
Bursa	Turkey	1962	0.472	[0.347, 0.581]	Csa
Zonguldak	Turkey	1962	0.520	[0.418, 0.609]	Cfa
Samsun	Turkey	1962	0.255	[0.141, 0.362]	Cfa
Trabzon	Turkey	1962	0.357	[0.244, 0.460]	Cfb
Goriatchy Klutch	Russia	1979	-0.402	[-0.574, -0.196]	Cfa

Cfa, humid subtropical climate; Csa, hot-summer Mediterranean climate; Cfb, oceanic climate.

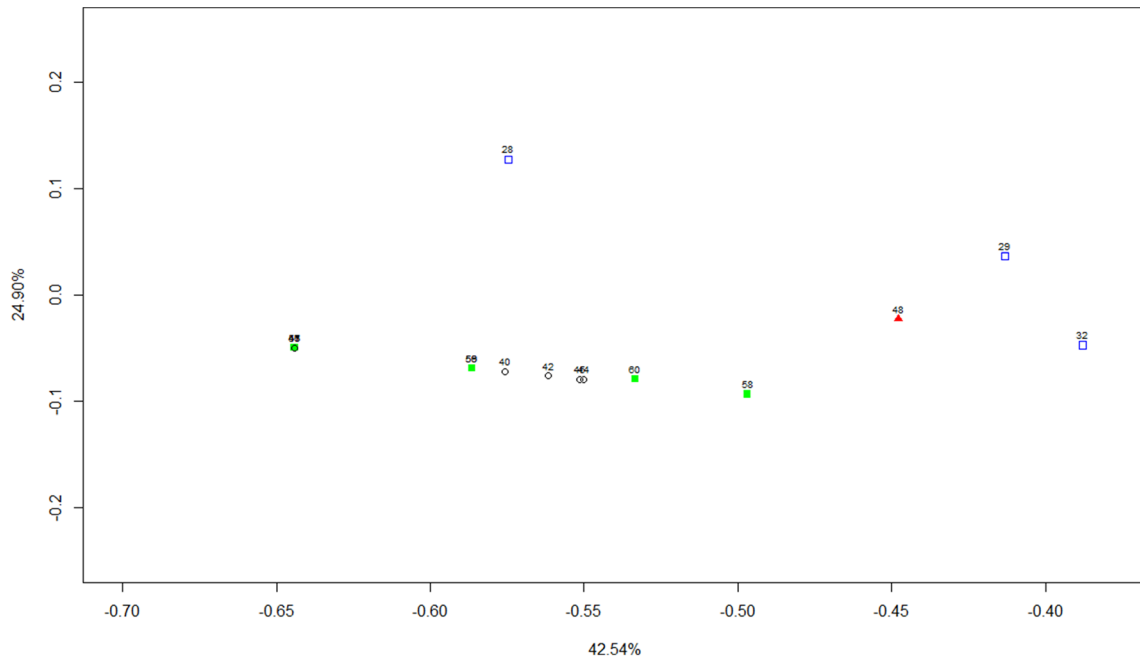


**Figure 2.** PCoA analysis with the O chromosome inversion polymorphism from different *D. subobscura* populations. Populations are identified by symbols: open blue squares indicate European, orange full circles stand for American, red open triangle indicate Turkish, red full triangle stands for Russian, green full squares indicate Iranian, open black circle stand for Madeiran. The populations used were: 1. Montpellier (France), 2. Lagrasse (France), 3. Queralbs (Spain), 4. Riba-roja (Spain), 5. Calvià (Spain), 6. Punta Umbría (Spain), 7. Málaga (Spain), 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 (USA.), 28. Kamariste (Serbia), 29. Petnica (Serbia), 30. Zanjic (Montenegro), 31. Font Gropa 2004 (Spain), 32. Mt. Parnes (Greece), 33. Observatori Fabra (Spain), 34. Font Gropa 2007 (Spain), 35. Font Gropa 2011 (Spain), 36. Font Gropa 2012 (Spain), 37. Font Gropa 2013 (Spain), 38. Font Gropa 2014 (Spain), 39. Font Gropa 2015 (Spain), 40. Camacha (Portugal), 41. Curral 2016 (Portugal), 42. Prazeres (Portugal), 43. Terreiro (Portugal), 44. Curral 1970 (Portugal), 45. Poiso (Portugal), 46. Ribeiro (Portugal), 47. Adraga (Portugal), 48. Goriatchy Klutch (Russia), 49. Antalya (Turkey), 50. Silifke (Turkey), 51. Tarsus (Turkey), 52. Bursa (Turkey), 53. Zonguldak (Turkey), 54. Samsun (Turkey), 55. Trabzon (Turkey), 56. Rasht I (Iran), 57. Rasht II (Iran), 58. Chalus (Iran), 59. Shahi (Iran), 60. Rasht 2019.

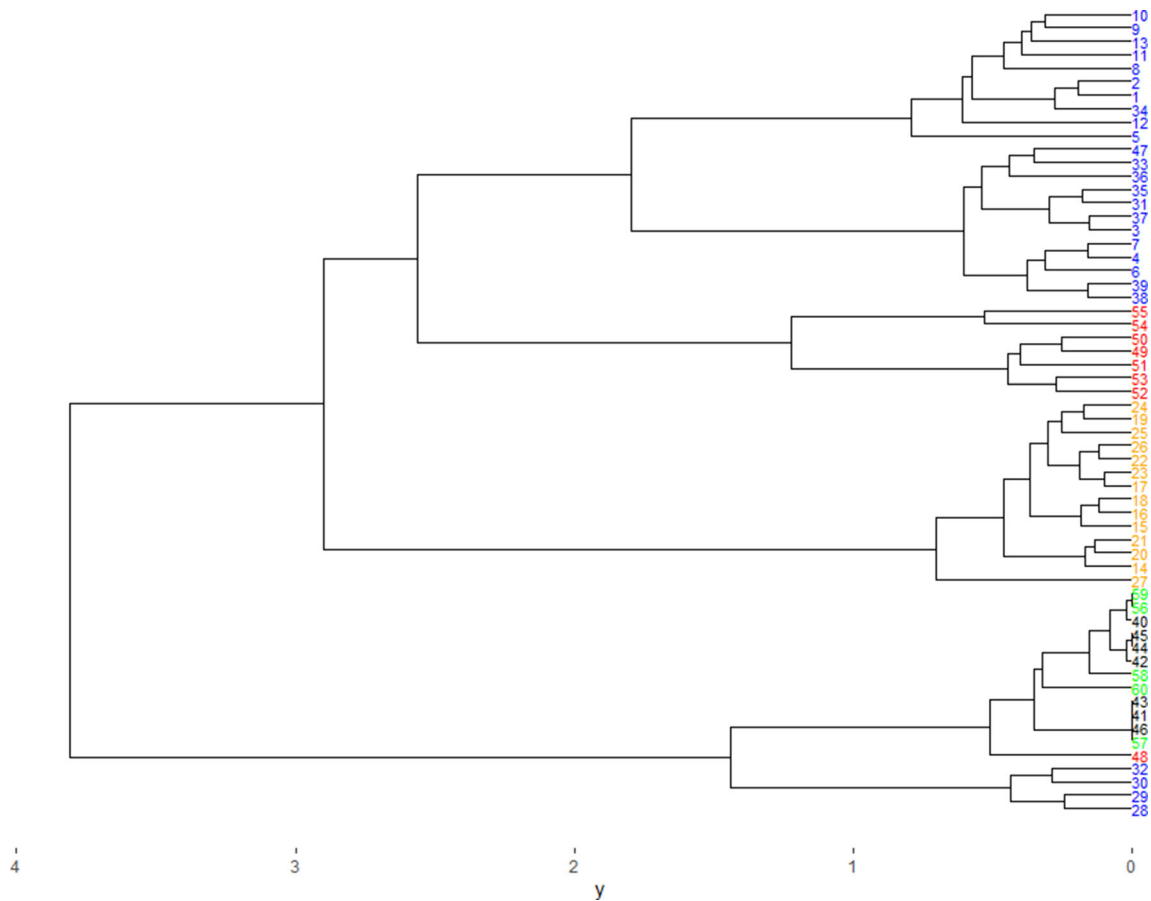
confirm the presence of this species in that geographic region. The *D. subobscura* inversion chromosomal polymorphism of Rasht 19 population is poor, presenting a few different inversions (table 1), as expected for a marginal isolated population (Krimbas 1992, 1993), as were those from Iran previously studied by Götz (1967). All these sites (Rasht, Chalus and Shahi) are located in the Hyrcanian ecosystem, considered a refugium during the Quaternary glaciations (Röhrig 1991; Saberi-Pirooz et al. 2018; Amiri et al. 2021). Likely, the Hyrcanian ecosystem presents favourable conditions for *D. subobscura*, although it is a marginal and isolated area with regard to the species distribution (Krimbas 1992, 1993). The marginality of Madeiran Is. and Iranian populations could be the reason for their close association both in PCoA and cluster analyses. Furthermore, they are clearly separated from other American or Palearctic populations of *D. subobscura*.

However, in the Rasht population, the inversion chromosomal composition has changed over time, with an increase of several ‘warm’ adapted inversions ( $J_1$ ,  $U_{1+8+2}$  and  $E_{1+2+9}$ ) and a decrease of ‘cold’ adapted ( $J_{st}$  and  $E_{st}$ ).

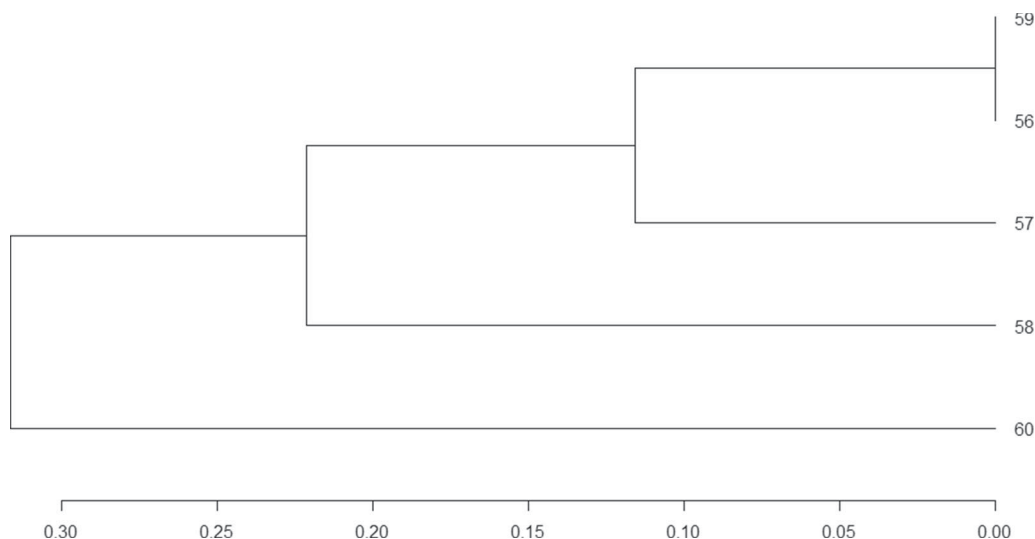
Several inversions of the species were reported for the first time in Rasht, although in low frequencies ( $U_{1+2+7}$ ,  $U_{1+8+2}$ ,  $O_{st}$  and  $O_{3+4+26}$ ). These arrangements could either have remained undetected in previous samples due to their low frequencies or some degree of gene flow would be possible from other populations. Further, Rasht 19 was in the first partition in the cluster analysis of Iranian populations, indicating that it presented a differentiation with regard to the O chromosomal inversions (figure 5). Interestingly, Rasht 19 showed a significant increase of *CTI* values with regard to Rasht I and II, in accordance with the global warming expectations. It has been described that the Hyrcanian ecosystem has become warmer and drier during the last 50 years. Data from the Rasht station indicate an increase in the mean annual temperature by about 1.28°C and an increase of 2.45°C in the minimum temperature. For the Baboulsar station, these values were 1.44°C and 1.80°C, respectively. Moreover, there has been a reduction in the annual precipitation of 55.6 mm in Gorgan and 409.4 mm in Anzali (Tohidifar et al. 2016). The Hyrcanian forests are considered highly vulnerable to climate change (Naqinezhad



**Figure 3.** Detail of PCoA analysis for better view of the differentiation between Iranian and Madeiran populations. Several populations completely overlap due to their high similarity in O chromosomal inversions polymorphism. Numbers stand for the same populations described in figure 2.



**Figure 4.** GEVA-Ward dendrogram showing the clusters of *D. subobscura* populations based on their O chromosome inversion polymorphism. Numbers stand for the same populations described in figure 2.



**Figure 5.** GEVA-Ward tree showing the relations between the Iranian *D. subobscura* populations based on their O chromosome inversion polymorphism. The populations used were: 56. Rasht I (Iran), 57. Rasht II (Iran), 58. Chalus (Iran), 59. Shahi (Iran), 60. Rasht 2019.

*et al.* 2022) and for this reason, drastic changes in biodiversity could be produced. It seems that *D. subobscura* from marginal and isolated populations are capable of reacting to global warming increasing the frequency of ‘warm’ adapted and decreasing the ‘cold’ adapted inversions, as has been observed in Rasht and also in the Atlantic oceanic island of Madeira (Madrenas *et al.* 2020). However, the threshold of this kind of adaptation is unknown. Likely once this limit is reached, the fate of those populations would be extinction. In Rasht 19, there are still rather abundant frequencies of inversions considered ‘cold’ adapted (table 1), thus the frequencies of  $A_1$ ,  $J_{st}$  and  $E_{st}$  are 84.2%, 22.2% and 33.3%, respectively. Although it seems that there is still a margin for thermal adaptation, it could be that the directional selection of these chromosomal inversions would reduce some fitness components due to the linkage disequilibrium with other genes (Lynch and Walsh 1998; Sgrò and Hoffmann 2004; Zivanovic *et al.* 2021). Also, inversions not only allow to adapt to the temperature but to other environmental and biological factors, that could be directly related to temperature or not (Galludo *et al.* 2018; Kapun and Flatt 2019). Different species of *Drosophila* genus are adapted to distinct environmental conditions (Atkinson and Shorrocks 1977; Avelar *et al.* 1987; Holt 1990; Krimbas 1993; Powell 1997; Tidon 2006; Sisodia and Singh 2010), and *D. subobscura* seem to be adapted to a low minimum temperature, not a very high maximum temperature (but with a large difference between both temperatures), a certain degree of humidity and scarce rainfall (Galludo *et al.* 2020). The evolution of the climatic conditions in the Hyrcanian ecosystems would determine the persistence or not of their *D. subobscura* populations.

The thermal adaptation of the Russian (Sperlich *et al.* 1981) and Turkish (Götz 1967) *D. subobscura* populations are also very valuable from an evolutionary genetics point of

view. The *CTI* value for Goriatchy Klutch is low ( $-0.402$ , table 4). It is true that the sample was small, but it contained rather high frequencies of ‘cold’ adapted inversions:  $A_{st}$  (31.3%),  $A_1$  (68.7%),  $J_{st}$  (45.2%),  $U_{st}$  (32.3%),  $E_{st}$  (35.5%) and  $O_{st}$  (64.6%). The climate is described as Cfa, being humid subtropical with cold winters, with an average annual temperature of  $11.8^{\circ}\text{C}$  and abundant rainfall during the year, but mainly in December (<https://en.climate-data.org/>). The *CTI* values are similar to those reported for *D. subobscura* central and northern Europe populations (Arenas *et al.* 2018). Interestingly, the Turkish populations studied by Götz (1967) belong to two groups: those from northern Turkey, facing the Black Sea (Bursa, Zonguldak, Samsun and Trabzon) and those in the south, in the coast of the Mediterranean sea (Antalya, Silifke and Tarsus). In general, those from this latter group present higher *CTI* values, although all Turkish populations presented similar *CTI* values to those reported in the central and western Mediterranean region (Arenas *et al.* 2018). The Antalya, Silifke and Tarsus populations all presented the same type of climate, Csa (Mediterranean hot summer climates). However, in the Black Sea group, populations differed according to the climate: Bursa, Csa (Mediterranean hot summer climates); Zonguldak and Samsun, Cfa (Humid subtropical climate); Trabzon, Cfb (Oceanic climate). Further analyses of inversion chromosomal polymorphism in *D. subobscura* populations from the Eastern Mediterranean could be relevant to properly understand the specie’s adaptation to global warming.

In summary, the isolated and marginal populations of *D. subobscura* seem to adaptively respond to global warming by increasing the frequencies of their ‘warm’ adapted inversions and decreasing their ‘cold’ adapted. In general, this species can be considered an excellent model organism to track adaptive changes related to global warming



(Balanyà *et al.* 2006, 2009; Orengo *et al.* 2016; Galludo *et al.* 2018; Zivanovic *et al.* 2021).

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