Chromosomal Thermal Index: a comprehensive way to integrate the thermal adaptation of *Drosophila subobscura* whole karyotype

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Abstract: *Drosophila* has demonstrated to be an excellent model to study the adaptation of organisms to global warming, with inversion chromosomal polymorphism having a key role in this adaptation. Here, we introduce a new index (Chromosomal Thermal Index or *CTI*) to quantify the thermal adaptation of a population according to its composition of 'warm' and 'cold' adapted inversions. This index is intuitive, has good statistical properties and can be used to hypothesis on the effect of global warming on natural populations. We show the usefulness of *CTI* using data from European populations of *D. subobscura*, sampled in different years. Out of 15 comparisons over time, 9 showed significant increase of *CTI*, in accordance with global warming expectations. Although large regions of the genome outside inversions contain thermal adaptation genes, our results show that the total amount of 'warm' or 'cold' inversions in populations seems to be directly involved in thermal adaptation, whereas the interactions between the inversions content of homologous and non-homologous chromosomes are not relevant.

K*ey words*: *Drosophila*; thermal adaptation; chromosomes; inversions; global warming

Introduction

Almost a century ago, Sturtevant (1917, 1921) described chromosomal inversions for the first time in *Drosophila melanogaster*. He observed that their main genetic effect was recombination reduction. According to this author, inversions would be inherited as single Mendelian units, but we now know that this interpretation is not exact. Inversions have been studied in different organisms (Hoffmann and Rieseberg 2008), but *Drosophila* remains to be the most widely studied genus (Dobzhansky 1970; Powell 1997; Krimbas and Powell 1992, 2000; Hoffmann et al. 2004). In this genus, the adaptive value of inversions has been repeatedly reported. One exciting result has been the observation that inversions respond to global warming (Orengo and Prevosti 1996; Rodriguez-Trelles and Rodriguez 1998; Solé et al. 2002; Levitan 2003; Balanyà et al. 2004, 2006, 2009; Levitan and Etges 2005; Umina et al. 2005; Zivanovic and Mestres 2010, 2011; Rezende et al. 2010; Zivanovic et al. 2012, 2014a; Rodriguez-Trelles et al. 2013). Balanyà et al. (2006), using principal component analyses, developed one index for temperature and another for chromosomal polymorphism (for the whole karyotype) to study the relationships between both. Although this is a useful approach, we introduce here a new measure of the whole karyotype response to temperature that allows comparisons between populations by means of a robust test with good statistical properties. The integration provided by the whole karyotype is extremely valuable, because we have previously detected an additive effect of inversions located in different chromosomes in Balkan populations (Zivanovic et al. 2014b, 2016).

Thus, our main aim is to define a new index, called Chromosomal Thermal Index (*CTI*) and to use confidence intervals for hypothesis testing. We apply and test the usefulness of *CTI* by analyzing chromosomal inversion polymorphism data from natural populations of *D. subobscura*. We also discuss the results obtained with this index in the context of chromosomal adaptation to global warming.

Materials and methods

The CTI index

To define the CTI index, it is essential to properly identify which chromosomal inversions (or arrangements, that is combinations of inversions) are considered as ‘warm’ or ‘cold’, that is, thermally adapted. Usually, there are many experimental criteria that allow the precise classification of chromosomes in these categories.

Let W, C and TA represent the total number of warm, cold and thermally adapted chromosomes ($TA = W + C$). Thus, the CTI index is defined by:

$$CTI = \frac{W - C}{W + C} = \frac{W - C}{TA}$$

which takes values in [-1, 1] and measures an excess of ‘warm’ chromosomes (positive sign) or cold chromosomes (negative sign) relative to the total number of thermally adapted chromosomes. It is easy to see that CTI can be written as:

$$CTI = \frac{2W - TA}{TA} = 2\frac{W}{TA} - 1 = 2p - 1$$

where, $p = \frac{W}{TA}$ is the probability, for an adapted chromosome, of being a warm chromosome. It may be of interest to check, for a population whether *CTI* index matches a certain value or to compare *CTI* index between populations. For this reason, exact and asymptotic confidence intervals and hypothesis tests were constructed.

Assume a sample of size n of thermally adapted chromosomes in which w_0 and c_0 are ‘warm’ and ‘cold’, respectively $(n = w_0 + c_0)$ and that we are interested in testing whether the *CTI* index matches a certain value I_0 (null hypothesis $H_0\colon CTI = I_0$). The Score test statistics

$$Z = \frac{\frac{w_0}{n} - \frac{I_0 + 1}{2}}{\sqrt{\left(\frac{I_0 + 1}{2} \times \frac{1 - I_0}{2}\right)/n}}$$

Under the null hypothesis, Z follows approximately a standard normal $N(0,1)$ distribution. Thus, for a fixed significance level α the critical region for a two sided or one sided test will be $|Z| > Z_{1-\alpha/2}$; $Z > Z_{1-\alpha}$ or $Z < Z_{1-\alpha}$, respectively. Furthermore, confidence intervals based on Z can be approximately constructed,

$$\frac{2w_0}{n}\left(\frac{n}{n + Z^2_{1-\alpha/2}}\right) + \left(\frac{Z^2_{1-\alpha/2}}{n + Z^2_{1-\alpha/2}}\right) - 1$$

$$\pm\, 2 * Z_{1-\alpha/2}\sqrt{\frac{1}{n + Z^2_{1-\alpha/2}}\left[\frac{w_0}{n} * \left(1 - \frac{w_0}{n}\right) * \frac{n}{n + Z^2_{1-\alpha/2}} + \frac{1}{4} * \frac{Z^2_{1-\alpha/2}}{n + Z^2_{1-\alpha/2}}\right]}\,.$$

If the sample is small, it is better to use exact test statistics and confidence intervals. The corresponding p-values are:

$P = \sum_{j=w_0}^{n} \binom{n}{j} \left(\frac{I+1}{2}\right)^{j} \left(\frac{1-I_0}{2}\right)^{n-j}$ for the alternative $H_1\colon CTI > I_0$;

$P = \sum_{j=0}^{w_0} \binom{n}{j} \left(\frac{I_0+1}{2}\right)^{j} \left(\frac{1-I_0}{2}\right)^{n-j}$ for the alternative $H_1\colon CTI < I_0$;

$P = \sum_{j=0}^{n} \pi_j I(\pi_j \leq \pi)$ for the alternative $H_1: CTI \neq I_0$, where $I(\pi_j \leq \pi) = 1$ if $\pi_j \leq \pi$ and $I(\pi_j \leq \pi) = 0$ if $\pi_j > \pi$ being $\pi = \binom{n}{w_0}\left(\frac{I_0+1}{2}\right)^{w_0}\left(\frac{1-I_0}{2}\right)^{n-w_0}$ and $\pi_j = \binom{n}{j}\left(\frac{I_0+1}{2}\right)^{j}\left(\frac{1-I_0}{2}\right)^{n-j}$ for $j = 0, \cdots, n$.

An exact confidence interval for CTI has as inferior (*IL*) and superior (*SL*) limits:

$$IL = 2\frac{w_0}{w_0 + (n - w_0 + 1) \cdot F_{2(n-w_0+1), 2w_0, 1-\alpha/2}} - 1,$$

and $SL = 2\frac{(w_0+1) \cdot F_{2(w_0+1), 2(n-w_0), 1-\alpha/2}}{(n-w_0) + (w_0+1) \cdot F_{2(w_0+1), 2(n-w_0), 1-\alpha/2}} - 1$, where F is the Fisher-Snedecor distribution.

To test the hypothesis of equality in CTI indexes, let w_1 and w_2 be the number of 'warm' thermally adapted chromosomes for samples with sizes n_1 and n_2, respectively. Then the Score statistic

$$Z = \frac{\frac{w_1}{n_1} - \frac{w_2}{n_2}}{\sqrt{\left(\frac{w_1 + w_2}{n_1 + n_2}\right) * \left(1 - \frac{w_1 + w_2}{n_1 + n_2}\right) * \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

is normally distributed for large n_1 and n_2. As before, for a fixed significance level α the critical region for a two sided ($CTI_1 \neq CTI_2$) or one sided ($CTI_1 > CTI_2$ or $CTI_1 < CTI_2$) test will be $|Z| > Z_{1-\alpha/2}$; $Z > Z_{1-\alpha}$ or $Z < Z_{1-\alpha}$, respectively.

The Score interval is obtained by inverting the Score test statistic. For small samples size, it is better to use exact test statistics, however the corresponding expressions are not included for simplicity.

To facilitate the computations, functions in R code were written and are available from the authors upon request.

Statistical analyses

The confidence intervals and hypothesis tests developed in the above Section were used to compare *CTI* values from natural populations of *D. subobscura* and $P < 0.05$ was used for significance. We compute the *CTI* index for the whole karyotype and also for only one chromosome. As multiple testing was carried out, results were corrected using false discovery rates (FDR) method (Benjamini and Hochberg 1995).

Latitudinal variation of *CTI* index was studied from European populations which were collected twice in an interval of many years (Solé et al. 2002; Balanyà et al. 2004; Zivanovic et al. 2012, 2015). Regression lines of *CTI* values on latitude were obtained for both, 'old' and 'new' samples and statistical differences in slope and elevation were computed.

Results

To demonstrate the usefulness of the index, we computed it in populations of *Drosophila subobscura*, a species considered to be particularly sensitive to global warming (Balanyà et al. 2009). This species has a karyotype with five acrocentric and one dot chromosomes. These chromosomes, except for the dot, are polymorphic for inversions (or arrangements). Many of them are adapted to thermal variation (Orengo and Prevosti 1996; Rodríguez-Trelles and Rodríguez 1998; Solé et al. 2002; Balanyà et al. 2004; Rezende et al. 2010; Zivanovic and Mestres 2011; Zivanovic et al. 2012, 2015). To classify the inversions (or arrangements) as thermally adaptated to 'warm' or 'cold', we followed the well-established criterion of Menozzi and Krimbas (1992), ratified by Rego et al. (2010). This criterion is based on the presence of significant correlation coefficients between variables related to temperature and chromosomal inversions. The following inversions (or arrangements) are considered ‘warm’: A_2, J_1, U_{1+2}, U_{1+8+2}, E_{1+2+9}, $E_{1+2+9+12}$, O_{3+4}, O_{3+4+1} and O_{3+4+8}, whereas A_{st}, A_1, J_{st}, U_{st}, E_{st} and O_{st} are classified as ‘cold’. The remaining chromosomal inversions (or arrangements) of the species (more than 70), which didn’t fit the thermal adaptation criterion, were not considered for the analyses. The *CTI* and confidence interval values obtained from different natural populations of *D. subobscura* are compiled in Table 1. Some interesting patterns can be noticed in terms of latitude and year of collection. For example *CTI*, for the collections trapped in similar years, increases with decreasing latitude (Table 1). One population (Zürich) was sampled for more than 2 years (Gosteli, 1990). The *CTI* values increased over years and were significantly different for most (8 out of 10) pairwise comparisons (Table S1[1]). It is also interesting to analyze the European populations which were collected twice in an interval of many years (Solé et al. 2002; Balanyà et al. 2004; Zivanovic and Mestres 2011; Zivanovic et al. 2012, 2015). For the same population, when comparing ‘old’ with ‘new’ samples, the *CTI* index always increases. Out of 15 comparisons the differences were significant in 9 cases: Groningen (test = –7.914, $P = 0.0$), Louvain-la-Neuve (test = –4.328, $P = 0.0$), Tübingen (test = –4.034, $P = 0.0$), Vienna (test = –2.841, $P = 0.0135$), Leuk (test = –1.36, $P = 0.1876$), Apatin (test = –3.199, $P = 0.0052$), Villars (test = –2.567, $P = 0.0191$), Avala (test = –0.666, $P = 0.5060$), Petnica (test = –2.673, $P = 0.0187$), Montpellier (test = –2.060, $P = 0.0591$), Lagrasse (test = –1.356, $P = 0.1876$), Queralbs (test = –1.400, $P = 0.1876$), Riba-roja (test = –2.014, $P = 0.0600$), Punta Umbría (test = –2.410, $P = 0.0265$), Málaga (test –2.611, $P = 0.0191$). Another valuable observation is

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[1] Supplementary data are available with the article through the journal Web site at http://nresearchpress.com/doi/suppl/gen-2017-0124.

the behavior of *CTI* in the twelve populations aligned from Holland to Spain (Groningen, Louvain-la-Neuve, Tübingen, Vienna, Leuk, Villars, Montpellier, Lagrasse, Queralbs, Riba-roja, Punta Umbría and Málaga) following a latitudinal cline (Solé et al. 2002; Balanyà et al. 2004). In the 'old' samples and following latitude, negative values of the index were observed for the first seven populations, from Groningen to Montpellier. The five remaining 'old' collections, from Lagrasse to Málaga, showed positive values. However, in an equivalent study but using the 'new' samples, *CTI* values were negative only for the first six populations, from Groningen to Villars. Thus, the geographical location where the *CTI* changes from negative to positive values was displaced northward as expected by global warming. The corresponding confidence intervals associated with the index values of these neighbor populations were also in agreement with this northward movement (Table1).

Finally, we computed the regressions of *CTI* on latitude for 'old' and 'new' samples (Fig. 1). In both cases, the elevation and slope were significant (in all cases $P < 0.001$), indicating that latitude explains changes in *CTI*. When comparing both line regressions, the slopes were not significant different (F = 1.12; $P = 0.3034$), but the elevations were significant different (F = 6.99; $P = 0.0156$). This result indicates that the line for the 'new' populations is located above than that of 'old' populations, in accordance with global warming expectations.

It is also possible to apply the *CTI* index for individual chromosomes. We selected the O chromosome because this is the largest and most polymorphic for inversions in *D. subobscura* (Kunze-Mühl and Müller 1958; Krimbas 1992, 1993). We have computed *CTI* for the O chromosome using the same collection of 15 populations distributed in a latitudinal cline which were collected twice in an interval of many years (Solé et al. 2002; Balanyà et al. 2004; Zivanovic and Mestres 2011; Zivanovic et al.

2012, 2015) (Table S2). Almost all populations show an increase in *CTI* over years but out of 15 comparisons the differences were significant in only 6 cases (Table S3). For example, the Avala population shows no differences in *CTI* between the older (2004) and the newer collection (2011). Interestingly, four populations (Apatin, Villars, Petnica and Punta Umbria) showed to have an increase in *CTI* values based on whole karyotype were missed by the index calculation based on the O chromosome. Another interesting observation is that there is not a continuous series of populations with negative *CTI* values followed by another group showing positive values. For instance, Montpellier in 1972 had a negative value, and Lovaine-la-Neuve in 1999 showed a positive value (Tables S2 and S3). We also analyzed the *CTI* behavior in a pair of latitudinal studies for the O chromosome of *D. subobscura* (Pinsker and Sperlich 1981; Mestres et al. 1994). The results obtained showed that with only one chromosome the index is as informative as the *CTI* based on the entire karyotype (Table 2). However, the three Swedish populations cannot be differentiated and although the values tend to increase with lower latitudes, there are several exceptions (Fort Augustus or Tübingen). Similar results were obtained by analyzing the E chromosome individually (Tables S4 and S5). It seems that the new index is very valuable when integrating the information provided by the whole chromosomal set, but loses power if applied only to individual chromosomes.

Discussion

Global warming is a current and serious problem and more observations in nature and experimental research are needed. However, novel quantification approaches of biological events related with this topic are also of interest. Thus, this new introduced index is very valuable because it allows integrating the information of the whole karyotype in a single value and has good statistical properties. However, the *CTI* can be useful only if chromosomes with ‘warm’ and ‘cold’ inversions are properly defined. This is not an inherent problem with the index, but a wrong classification of inversions could yield anomalous results. In our examples, the inversions of European *D. subobscura* populations were sorted out in ‘warm’ or ‘cold’ according to the well-established criterion of presenting a significant correlation with environmental variables and also with latitude, as described for the first time in Menozzi and Krimbas (1992).

Although the adaptive value of inversions to climatic conditions has been established in many species (Feder et al. 2003; Ayala et al. 2011; Cassone et al. 2011), *Drosophila* in general (for instance see Overgaard et al. 2014 or Tobler et al. 2015) and *D. subobscura* in particular (Balanyà et al. 2009 or Dolgova et al. 2010) are considered excellent organisms to study thermal adaptations. We have demonstrated the *CTI* usefulness in a series of examples from *D. subobscura* populations, and their analyses have yielded a series of conclusions. When comparing population samples over years, *CTI* can quantify the changes over time of chromosomal inversions according to global warming expectations. Also, the index (and the confident interval associated) changes the sign northward according to latitude as expected. Finally, the index can be applied to particular chromosomes, but loses power to detect global warming effects on some populations. Thus *CTI* effectively integrates information from the whole karyotype. This index can be also used in other *Drosophila* species where adaptive inversions are or could be related to temperature as in the case of *D. robusta* (Levitan 1992, 2003; Levitan and Etges 2005), *D. melanogaster* (Lemeunier and Aulard 1992), *D. guarani* group (Brncic 1953) *D. repleta* group (Wasserman 1992), Hawaiian *Drosophila* species (Carson 1992), *D. ananassae* and other Indian species (Singh 2015). Even in the future, thermal adaptive inversions could be detected due to the intensive research on chromosomal inversions in different organisms (Pennisi 2017).

From the *CTI* results obtained in our examples, information can be also inferred on the nature of the karyotypic thermal adaptation. As defined, the index is computed as a balance between ‘warm’ and ‘cold’ inversions (or arrangements). It is the total amount of chromosomes with ‘warm’ and ‘cold’ inversions present in the population which defines its thermal adaptation. It seems that interactions between inversions located in different chromosomes are not especially relevant. This is in agreement with recent studies that did not detect any interaction between the inversion composition of homologous and non-homologous chromosomes (Zivanovic et al. 2014b, 2016). Thus, at the cytological level, the total amount of individual inversions rather than their specific combinations seemed to be the main targets of selection.

When studying *CTI* index in European populations of *D. subobscura*, thermally adapted inversions show, for the most part, additive effects. This is seen in the enrichment of Southern populations with ‘warm’ inversions and thermal adaptation being dependent on the total number of ‘warm’ or ‘cold’ adapted inversions (or arrangements) rather than particular inversions. While for the most part, *D. subobscura* seems to have no major (and minor) inversions in thermal adaptation, caution should be exercised in that some population might deviate from the pattern. In our analysis, Montpellier was the only population to deviate, with no whole karyotype *CTI* effect, but significant roles of chromosomes O and E. Despite this one population, for most thermal adaptation seems more broadly distributed throughout the whole karyotype and not concentrated in particular chromosomes. This is in agreement with the location of thermal adaptation QTL mapped in *D. melanogaster* (Norry et al. 2004, 2008; Morgan and Mackay 2006; Takahashi et al. 2011).

In summary, *CTI* is able to gather together the adaptive information of the whole karyotype in a useful way that allows easy and reliable statistic comparisons between different populations. For these reasons, it is a valuable tool to quantify the thermal adaptation in many species. Furthermore, from the analysis of *D. subobscura* populations, *CTI* values suggest that thermal inversions act in an additive way, without particular inversions being major contributors to global warming adaptations.

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Fig. 1. Regression lines of *CTI* on latitude. Open squares and dashed line correspond to 'old' samples, whereas solid squares and solid line indicate 'new' samples.

Table 1. *CTI* values (with their confidence interval) for the whole karyotype in European populations of *D. subobscura*. Populations have been sorted according to their latitude.

Population	Country	Year	Latitude	Longitude	*CTI*	Confidence interval	Reference
Drobak	Norway	1962	59º40'N	10º40'E	-0.915	[-0.950, -0.858]	Sperlich (1964)
Groningen	Netherlands	1964	53º13'N	6º34'E	-0.731	[-0.781, -0.673]	Balanyà et al. (2004)
Groningen	Netherlands	1999	53º13'N	6º34'E	-0.335	[-0.420, -0.246]	Balanyà et al. (2004)
Louvain-la-Neuve	Belgium	1976	50º40'N	4º36'E	-0.509	[-0.593, -0.416]	Balanyà et al. (2004)
Louvain-la-Neuve	Belgium	1999	50º40'N	4º36'E	-0.235	[-0.319, -0.148]	Balanyà et al. (2004)
Tübingen	Germany	1975	48º31'N	9º3'E	-0.614	[-0.668, -0.556]	Balanyà et al. (2004)
Tübingen	Germany	2000	48º31'N	9º3'E	-0.415	[-0.497, -0.327]	Balanyà et al. (2004)
Vienna	Austria	1955	48º13'N	16º22'E	-0.600	[-0.664, -0.529]	Balanyà et al. (2004)
Vienna	Austria	2001	48º13'N	16º22'E	-0.469	[-0.527, -0.407]	Balanyà et al. (2004)
Zürich	Switzerland	1963	47º23'N	8º33'E	-0.622	[-0.647, -0.596]	Gosteli (1990)
Zürich	Switzerland	1964	47º23'N	8º33'E	-0.575	[-0.601, -0.546]	Gosteli (1990)

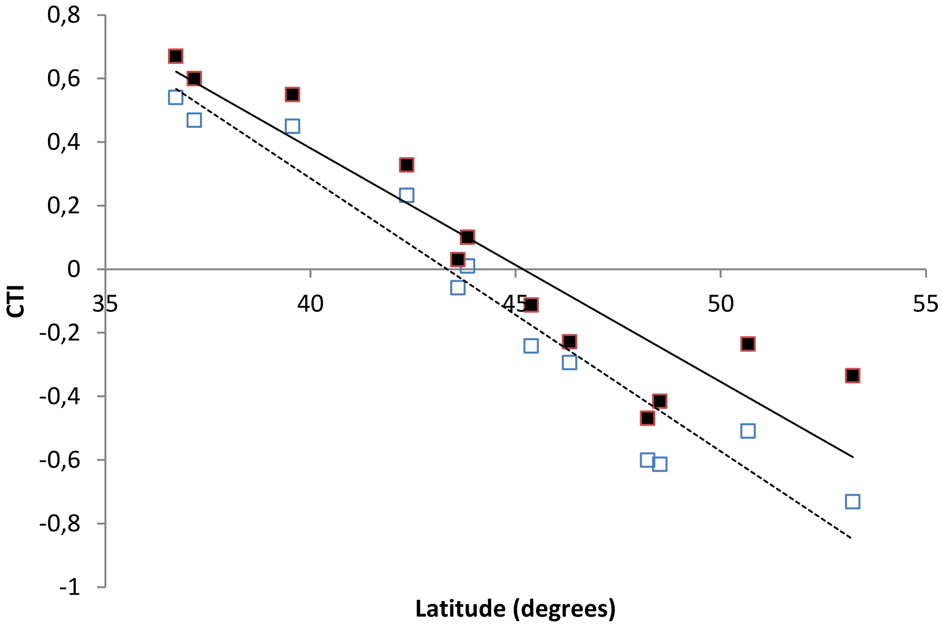
Zürich	Switzerland	1984	47º23’N	8º33’E	-0.527	[-0.573, -0.478]	Gosteli (1990)
Zürich	Switzerland	1986	47º23’N	8º33’E	-0.522	[-0.546, -0.497]	Gosteli (1990)
Zürich	Switzerland	1987	47º23’N	8º33’E	-0.468	[-0.487, -0.448]	Gosteli (1990)
Leuk	Switzerland	1977	46º19’N	7º38’E	-0.294	[-0.349, -0.237]	Balanyà et al. (2004)
Leuk	Switzerland	2000	46º19’N	7º38’E	-0.228	[-0.306, -0.147]	Balanyà et al. (2004)
Apatin	Serbia	1994	45°40’N	19°0’E	-0.347	[-0.439, -0.250]	Zivanovic and Mestres (2011)
Apatin	Serbia	2009	45°40’N	19°0’E	-0.025	[-0.211, 0.161]	Zivanovic and Mestres (2011)
Villars	France	1976	45º23’N	0º42’E	-0.241	[-0.306, -0.173]	Balanyà et al. (2004)
Villars	France	2001	45º23’N	0º42’E	-0.113	[-0.186, -0.040]	Balanyà et al. (2004)
Avala	Serbia	2004	44º48’N	20º30’E	0.374	[0.245, 0.494]	Zivanovic et al. (2015)
Avala	Serbia	2011	44º48’N	20°30’E	0.426	[0.328, 0.516]	Zivanovic et al. (2015)
Carasco	Italy	<1975	44º21’N	9º21’E	-0.023	[-0.129, 0.084]	Prevosti et al. (1984)
Petnica	Serbia	1995	44º14′N	9º55′E	0.122	[0.021, 0.222]	Zivanovic et al. (2012)
Petnica	Serbia	2010	44º14′N	9º55′E	0.354	[0.211, 0.487]	Zivanovic et al. (2012)
Montpellier	France	1972	43º36’N	3º52’E	-0.058	[-0.127, 0.011]	Solé et al. (2002)

Montpellier	France	1998	43º36’N	3º52’E	0.030	[-0.019, 0.080]	Solé et al. (2002)
Lagrasse	France	1962	43º5’N	2º37’E	0.010	[-0.104, 0.123]	Solé et al. (2002)
Lagrasse	France	1997	43º5’N	2º37’E	0.101	[0.028, 0.173]	Solé et al. (2002)
Queralbs	Spain	1966	42º21’N	2º91E	0.233	[0.113, 0.348]	Solé et al. (2002)
Queralbs	Spain	1997	42º21’N	2º91E	0.328	[0.253, 0.400]	Solé et al. (2002)
Alfano	Italy	<1975	40º11’N	15º25’E	0.525	[0.406, 0.631]	Prevosti et al. (1984)
Riba-roja	Spain	1963	39º34’N	0º34’W	0.450	[0.358, 0.535]	Solé et al. (2002)
Riba-roja	Spain	1998	39º34’N	0º34’W	0.550	[0.497, 0.600]	Solé et al. (2002)
Etna	Italy	<1975	37º45’N	14º59’E	0.958	[0.910, 0.985]	Prevosti et al. (1984)
Punta Umbría	Spain	1970	37º10’N	6º57’W	0.469	[0.390, 0.540]	Solé et al. (2002)
Punta Umbría	Spain	1998	37º10’N	6º57’W	0.600	[0.520, 0.676]	Solé et al. (2002)
Málaga	Spain	1963	36º43’N	4º25’W	0.540	[0.457, 0.613]	Solé et al. (2002)
Málaga	Spain	1998	36º43’N	4º25’W	0.670	[0.604, 0.728]	Solé et al. (2002)

Table 2. *CTI* values for O chromosome in European populations of *D. subobscura*. Populations have been sorted according to their latitude.

Population	Country	Year	Latitude	Longitude	*CTI*	Confidence interval	Reference
Gävle	Sweden	1987	60º40’N	17º8’E	-1	[-1, -0.610]	Mestres et al. (1994)
Sunne	Sweden	1977	59º50’N	13º8’E	-1	[-1, -0.899]	Pinsker and Sperlich (1981)
Lilla-Edet	Sweden	1988	58º8’N	12º08’E	-1	[-1, -0.846]	Mestres et al. (1994)
Fort Augustus	Scotland	1979	57º8’N	4º40’W	-0.420	[-0.572, -0.248]	Pinsker and Sperlich (1981)
Gesten	Denmark	1988	55º31’N	9º12’E	-0.871	[-0.903, -0.591]	Mestres et al. (1994)
Ter-Apel	Netherlands	1988	52º52’N	7º3’E	-0.469	[-0.617, -0.298]	Mestres et al. (1994)
Crécy	France	1988	50º06’N	1º53’E	-0.122	[-0.341, 0.106]	Mestres et al. (1994)
Taulé	France	1988	48º36’N	3º53’W	0.014	[-0.232, 0.260]	Mestres et al. (1994)
Tübingen	Germany	1978	48º31’N	9º3’E	-0.693	[-0.793, -0.572]	Pinsker and Sperlich (1981)
Aizenay	France	1988	46º44’N	1º36’W	0.189	[-0.052, 0.415]	Mestres et al. (1994)
Formia	Italy	1980	41º15’N	13º26’E	0.743	[0.588, 0.856]	Pinsker and Sperlich (1981)
Ponza	Italy	1977	40º45’N	12º57’E	0.899	[0.772, 0.967]	Pinsker and Sperlich (1981)

Cinisi	Italy	1978	38º10’N	13º6’E	0.982	[0.900, 1]	Pinsker and Sperlich (1981)


0,8
0,6
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-0,6
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CTI
35
40
45
50
55
Latitude (degrees)

Supplementary Table S1. Results of the *CTI* index comparisons in samples of different years from Zürich (Switzerland). The value of the test is presented above with the corresponding value of *P* below. Significant values ($P < 0.05$) are in bold.

	Zürich 1964	Zürich 1984	Zürich 1986	Zürich 1987
Zürich 1963	–2.473	–3.647	–5.417	–8.920
	0.0191	**0.0008**	**0.0000**	**0.0000**
Zürich 1964	-	–1.769	–2.768	–6.032
		0.0854	**0.0093**	**0.0000**
Zürich 1984	-	-	–0.169	–2.220
			0.8660	**0.0330**
Zürich 1986	-	-	-	-3.348
				0.0016

Supplementary Table S2. *CTI* values (with their confidence interval) for the O chromosome in fifteen European populations of *D. subobscura* collected in different years. Populations have been sorted according to their latitude.

Population	Country	Year	Latitude	Longitude	*CTI*	Confidence interval	Reference
Groningen	Netherlands	1964	53º13'N	6º34'E	-0.910	[-0.967, -0.810]	Balanyà et al. (2004)
Groningen	Netherlands	1999	53º13'N	6º34'E	-0.093	[-0.296, 0.116]	Balanyà et al. (2004)
Louvain-la-Neuve	Belgium	1976	50º40'N	4º36'E	-0.462	[-0.650, -0.237]	Balanyà et al. (2004)
Louvain-la-Neuve	Belgium	1999	50º40'N	4º36'E	0.162	[-0.039, 0.353]	Balanyà et al. (2004)
Tübingen	Germany	1975	48º31'N	9º3'E	-0.795	[-0.878, -0.682]	Balanyà et al. (2004)
Tübingen	Germany	2000	48º31'N	9º3'E	-0.415	[-0.497, -0.327]	Balanyà et al. (2004)
Vienna	Austria	1955	48º13'N	16º22'E	-0.760	[-0.863, -0.620]	Balanyà et al. (2004)
Vienna	Austria	2001	48º13'N	16º22'E	-0.372	[-0.509, -0.222]	Balanyà et al. (2004)
Leuk	Switzerland	1977	46º19'N	7º38'E	-0.341	[-0.458 -0.216]	Balanyà et al. (2004)
Leuk	Switzerland	2000	46º19'N	7º38'E	-0.230	[-0.430, -0.045]	Balanyà et al. (2004)
Apatin	Serbia	1994	45°40'N	19°0'E	-0.326	[-0.517, -0.114]	Zivanovic and Mestres (2011)

Apatin	Serbia	2009	45°40’N	19°0’E	-0.025	[-0.211, 0.161]	Zivanovic and Mestres (2011)
Villars	France	1976	45º23’N	0º42’E	0.033	[-0.117, 0.181]	Balanyà et al. (2004)
Villars	France	2001	45º23’N	0º42’E	0.202	[0.043, 0.354]	Balanyà et al. (2004)
Avala	Serbia	2004	44º48’N	20º30’E	0.564	[0.300, 0.764]	Zivanovic et al. (2015)
Avala	Serbia	2011	44º48’N	20º30’E	0.550	[0.336, 0.722]	Zivanovic et al. (2015)
Petnica	Serbia	1995	44º14′N	9º55′E	0.200	[-0.011, 0.398]	Zivanovic et al. (2012)
Petnica	Serbia	2010	44º14′N	9º55′E	0.467	[0.161, 0.708]	Zivanovic et al. (2012)
Montpellier	France	1972	43º36’N	3º52’E	-0.257	[-0.404, -0.101]	Solé et al. (2002)
Montpellier	France	1998	43º36’N	3º52’E	0.229	[0.117, 0.336]	Solé et al. (2002)
Lagrasse	France	1962	43º5’N	2º37’E	0.151	[-0.092, 0.381]	Solé et al. (2002)
Lagrasse	France	1997	43º5’N	2º37’E	0.307	[0.145, 0.457]	Solé et al. (2002)
Queralbs	Spain	1966	42º21’N	2º91E	0.069	[-0.203, 0.333]	Solé et al. (2002)
Queralbs	Spain	1997	42º21’N	2º91E	0.317	[0.132, 0.485]	Solé et al. (2002)
Riba-roja	Spain	1963	39º34’N	0º34’W	0.396	[0.113, 0.633]	Solé et al. (2002)
Riba-roja	Spain	1998	39º34’N	0º34’W	0.452	[0.285, 0.598]	Solé et al. (2002)

Punta Umbría	Spain	1970	37º10’N	6º57’W	0.588	[0.242, 0.826]	Solé et al. (2002)
Punta Umbría	Spain	1998	37º10’N	6º57’W	0.750	[0.537, 0.889]	Solé et al. (2002)
Málaga	Spain	1963	36º43’N	4º25’W	0.182	[-0.273, 0.586]	Solé et al. (2002)
Málaga	Spain	1998	36º43’N	4º25’W	0.667	[0.454, 0.822]	Solé et al. (2002)

Supplementary Table S3. For the O chromosome of *D. subobscura*, values of *CTI*, statistical test between 'old' and 'new' samples and corresponding values of *P* in the comparison of 15 natural populations collected in a large interval of years. Significant values ($P < 0.05$) are in bold.

Population	*CTI* 'Old' sample	*CTI* 'New' Sample	Test value	*P*
Groningen	-0.910	-0.093	-7.447	**0.0000**
Louvain-la-Neuve	-0.462	0.162	-4.193	**0.0000**
Tübingen	-0.795	-0.415	-4.901	**0.0000**
Vienna	-0.760	-0.372	-3.908	**0.0003**
Leuk	-0.341	-0.230	-1.061	0.3343
Apatin	-0.326	-0.025	-2.194	0.0604
Villars	0.033	0.202	-1.589	0.1861
Avala	0.564	0.550	0.094	0.9254
Petnica	0.200	0.467	-1.538	0.1861
Montpellier	-0.257	0.229	-5.101	**0.0000**

Lagrasse	0.151	0.307	-1.138	0.3344
Queralbs	0.069	0.317	-1.594	0.1862
Riba-roja	0.396	0.452	-0.381	0.7531
Punta Umbría	0.588	0.750	-1.059	0.3344
Málaga	0.182	0.667	-2.389	**0.0422**

Supplementary Table S4. *CTI* values (with their confidence interval) for the E chromosome in fifteen European populations of *D. subobscura* collected in different years. Populations have been sorted according to their latitude.

Population	Country	Year	Latitude	Longitude	*CTI*	Confidence interval	Reference
Groningen	Netherlands	1964	53º13’N	6º34’E	-0.972	[-0.992, -0.902]	Balanyà *et al.* (2004)
Groningen	Netherlands	1999	53º13’N	6º34’E	-0.933	[0.977, -0.813]	Balanyà *et al.* (2004)
Louvain-la-Neuve	Belgium	1976	50º40’N	4º36’E	-0.912	[-0.970, -0.756]	Balanyà *et al.* (2004)
Louvain-la-Neuve	Belgium	1999	50º40’N	4º36’E	-0.750	[-0.857, -0.580]	Balanyà *et al.* (2004)
Tübingen	Germany	1975	48º31’N	9º3’E	-0.919	[-0.960, -0.837]	Balanyà *et al.* (2004)
Tübingen	Germany	2000	48º31’N	9º3’E	-0.888	[-0.952, -0.750]	Balanyà *et al.* (2004)
Vienna	Austria	1955	48º13’N	16º22’E	-0.840	[-0.912, -0.718]	Balanyà *et al.* (2004)
Vienna	Austria	2001	48º13’N	16º22’E	-0.838	[-0.904, -0.732]	Balanyà *et al.* (2004)
Leuk	Switzerland	1977	46º19’N	7º38’E	-0.848	[-0.906, -0.761]	Balanyà *et al.* (2004)
Leuk	Switzerland	2000	46º19’N	7º38’E	-0.838	[-0.914, -0.706]	Balanyà *et al.* (2004)
Apatin	Serbia	1994	45°40’N	19°0’E	-0.728	[-0.845, -0,546]	Zivanovic & Mestres (2011)

Apatin	Serbia	2009	45°40’N	19°0’E	-0.385	[-0.670, -0.0]	Zivanovic & Mestres (2011)
Villars	France	1976	45º23’N	0º42’E	-0.831	[-0.902, -0.716]	Balanyà *et al.* (2004)
Villars	France	2001	45º23’N	0º42’E	-0.694	[-0.799, -0.546]	Balanyà *et al.* (2004)
Avala	Serbia	2004	44º48’N	20º30’E	0.384	[0.059, 0,583]	Zivanovic *et al.* (2015)
Avala	Serbia	2011	44º48’N	20º30’E	0.380	[0.150, 0.571]	Zivanovic *et al.* (2015)
Petnica	Serbia	1995	44º14′N	9º55′E	-0.024	[-0.232, 0.186]	Zivanovic *et al.* (2012)
Petnica	Serbia	2010	44º14′N	9º55′E	0.143	[-0.183, 0.440]	Zivanovic *et al.* (2012)
Montpellier	France	1972	43º36’N	3º52’E	-0.064	[-0.193, 0.068]	Solé *et al.* (2002)
Montpellier	France	1998	43º36’N	3º52’E	-0.585	[-0.674, -0.478]	Solé *et al.* (2002)
Lagrasse	France	1962	43º5’N	2º37’E	-0.630	[-0.792, -0.383]	Solé *et al.* (2002)
Lagrasse	France	1997	43º5’N	2º37’E	-0.496	[-0.637, -0.323]	Solé *et al.* (2002)
Queralbs	Spain	1966	42º21’N	2º91E	-0.447	[-0.661, -0.165]	Solé *et al.* (2002)
Queralbs	Spain	1997	42º21’N	2º91E	-0.186	[-0.358, -0.001]	Solé *et al.* (2002)
Riba-roja	Spain	1963	39º34’N	0º34’W	0.050	[-0.166, 0.262]	Solé *et al.* (2002)
Riba-roja	Spain	1998	39º34’N	0º34’W	0.271	[0.126, 0,404]	Solé *et al.* (2002)

Punta Umbría	Spain	1970	37º10’N	6º57’W	0.440	[0.250, 0.597]	Solé *et al.* (2002)
Punta Umbría	Spain	1998	37º10’N	6º57’W	0.172	[-0.038, 0.368]	Solé *et al.* (2002)
Málaga	Spain	1963	36º43’N	4º25’W	0.013	[-0.208, 0.233]	Solé *et al.* (2002)
Málaga	Spain	1998	36º43’N	4º25’W	0.608	[0.433, 0,739]	Solé *et al.* (2002)

Supplementary Table S5. For the E chromosome of *D. subobscura*, values of *CTI*, statistical test between 'old' and 'new' samples and corresponding values of *P* in the comparison of 15 natural populations collected in a large interval of years. Significant values ($P < 0.05$) are in bold.

Population	*CTI* 'Old' sample	*CTI* 'New' Sample	Test value	*P*
Groningen	-0.972	-0.933	-1.001	0.5026
Louvain-la-Neuve	-0.912	-0.750	-1.753	0.1997
Tübingen	-0.919	-0.888	-0.566	0.7141
Vienna	-0.840	-0.838	-0.038	0.9699
Leuk	-0.848	-0.838	-1.167	0.9290
Apatin	-0.728	-0.385	-1.996	0.1997
Villars	-0.831	-0.694	-1.742	0.1997
Avala	0.384	0.380	-0.184	0.9290
Petnica	-0.024	0.143	-0.829	0.5554
Montpellier	-0.064	-0.585	6.061	**0.0000**

Lagrasse	-0.630	-0.496	-0.964	0.5026
Queralbs	-0.447	-0.186	-1.558	0.2235
Riba-roja	0.050	0.271	-1.679	0.0932
Punta Umbría	0.440	0.172	1.923	0.0544
Málaga	0.013	0.608	-4.182	**0.0000**