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Project: Thermal tolerance of chromosomal inversion polymorphisms in *Drosophila subobscura*

Background:

Clinal patterns occur when there are continuous phenotypic or genetic variations over space. It is widely considered that these changes arise from a balance between local selection and migration, although there can also occur clinal variation without selection.

One of the most studied examples of clinal differentiation is *Drosophila subobscura* that spans more than 30º latitude in the Old World: from North Africa to Scandinavia – and has a well-known cline of chromosomal polymorphisms and body size. This cline was recently repeated in South and North America, due to two recent colonization episodes[5], representing a worldwide replicate experiment of the role of latitude in the evolution of natural populations

One of the main questions arising from this natural experiment was if there was a repeatability of the inversion polymorphisms present in the Old World *vs.* New World populations. The scoring of the New World populations showed that those inversions had the same correlation sign of frequency with latitude as in the original Old World populations, although further studies showed that the inversion clines did not continue to converge to the native Old World baseline[2].

These results strongly suggest that there is a global selective agent that is responsible for the similar clinal patterns obtained in the three continents. Naturally temperature seemed to be the most likely candidate to test for the

variation observed. To test this hypothesis, Mauro Santos and collaborators [6-8] took a large and genetically heterogeneous stock collected from Puerto Montt (the epicentre of the American invasion) and set up three replicate lines at each of three different (constant) temperatures: 13º, 18º (the presumed optimum), and 22ºC. The lines were let to evolve by laboratory natural selection for several years under carefully controlled density conditions and were scored for inversion frequencies[8]. If the temperature was indeed the key selective agent that ensured the clinal differentiation observed, it was to be expected that flies evolving at high temperatures would have the characteristics of the low latitude populations.

Although there was a shift of chromosome frequencies, this variation was inconsistent with expectations based on clinal patterns[7, 8]. The difference between these results and the natural variation may derive from the fact that temperature is not the main environmental factor responsible for the variation between clinal populations. Although at this time this we cannot rule out this hypothesis, it is also possible that the experimental conditions of the previous work do not adequately mimic the surrounding environment causing the discrepancy between results.

Aims of the visit

The main aim of this project was to analyse a possible correlation between thermal resistance, inversion polymorphism and body size in populations of *Drosophila subobscura* founded from a natural population near Sintra, Portugal.

Therefore during this visit our main goals were:

1) Test the resistance of individual flies to thermal stress;

2) Characterize the chromosomal inversions of the individuals subjected to thermal stress;

3) Measure wing size of the individuals subjected to thermal stress.

Material and methods

Population stocks

The *Drosophila subobscura* population analyzed in this study was established using a large number of founders (210 females) collected in a pinewood near Adraga, Portugal (38º 47' 57.48" N; 09º 28' 52.27 W). When this population was in its third laboratorial generation it was split into three replicate lines (Ad_i, i = 1,2,3). All lines were maintained at 18ºC, with a 12h: 12h light/dark cycle in a regime of discrete generations with controlled adult and larval densities. Adults were kept in Plexiglas cages (27 x 21 x 16 cm), 1500 individuals per cage, supplied with liberal amounts of food, larval development took place in 130ml bottles containing 50ml of drosophila medium placing in each bottle 200-250 eggs. When this study was carried out the flies had been in the lab for 9 generations.

Gene arrangements

In *D. subobscura* it is difficult to recognize the gene arrangements in all possible homozygous/heterozygous combinations. Therefore, virgin males and females from the replicated lines were individually crossed to 3-4 flies from the *ch-cu* marker strain in order to determine their chromosome arrangements. This strain is homozygous for the morphological recessive markers on the O chromosome cherry eyes (*ch*) and curled wings (*cu* [9]), and its genetic background is highly homogeneous and fixed for the standard gene arrangements in all major acrocentric chromosomes but chromosome O, where it is fixed for gene arrangement O_{3+4} ([4]). Whenever feasible, one F1 female third-instar larva derived from each cross with the homozygous *ch-cu* stock was examined for its inversion loops in polytene chromosomes to determine the gene arrangements of one set of the chromosomes from the wild-type fly. Salivary glands were stained with 2% orcein in 60% acetic acid mixed 50:50 with lactic acid. The chromosomal arrangements were designated according to Kunze-Mühl and Müller ([3]). All crosses were maintained at 18ºC until the assays for thermal preference (7-8 days) after which they were placed at 13ºC to slow development and allow bigger larvae.

Due to the large sample size of this experiment, the flies were assayed in 4 batches one batch per week (each batch comprising 360 flies, 60 females and 60 males of each replicate line, with the exception of the last batch that only comprised 240 flies). To guarantee that all the flies are the same age at the time they are assayed, four different egg collections were made between December 2008 and January 2009 (each one week apart from the previous).

Thermal resistance

The thermal resistance assay was done exposing the flies to an increasing temperature gradient in water-baths. Water-baths provided with a heater allowed us to gently increase the temperature from 24ºC to 38ºC (the temperature were the last fly in the water bath was knocked-down). Flies were individually placed in sealed empty vials and immersed in water-baths at 24ºC (60 flies per water-bath), at 10 minute intervals they were individually scored for mobility (fly active or knocked-down) and the temperature of the water was increased by 1ºC. This procedure was repeated every 10 minutes until the last fly was knocked down (38ºC). After this assay the flies were individually placed in eppendorfs containing a fixing solution (3:1, alcohol: glycerol) and stored at 4ºC for later wing measurements.

Wing measurements

Wing size and shape of each experimental fly was measured as described by [8]. Briefly, wings were removed from each fly and fixed in DPX under cover slips on microscope slides. Bitmap images of the wings were be captured with a Sony CCDIris video camera connected to a PC with MGI VideoWave software and mounted on a Zeiss Axioskop compound microscope, using a 2.5 objective. The images were stored on a computer and then used to record the x and y coordinates of 13 morphological landmarks using the Fly Wing 15Lmk plug-in (kindly provided by Chris P. Klingenberg) implemented in ImageJ software (http://rsb.info.nih.gov/ij/).

Analysis of wing size and shape was performed using the methods provided by geometric morphometrics, which precisely separates morphological variation (i.e., variation in form) into size and shape components [8]. Size is a one-dimensional trait and the measure most widely used in geometric morphometrics is centroid size (CS) that can be computed in a normalized form as the square root of the sum of squared Euclidian distances between each landmark to the centroid (centre of gravity) of all landmarks divided by the square root of the number of landmarks. Individual size is therefore represented by a scalar. The shape of an original configuration of landmarks is the geometrical information that is invariant to uniform scaling (variation in size), translation (differences in position), and rotation (differences in orientation). In contrast to size, shape is an inherently multidimensional space and we will use Procrustes superimposition to characterize shape variation. This method allows comparing configurations of landmarks by optimally superimposing (according to a least-squares criterion) homologous landmarks in two or more specimens to achieve an overall best fit.

Main Results

Because the chromosomal inversions have to be scored for each individual and only Professor Joan Balanyà can read them, there was no time to finish the collection of all the inversion data until the writing of this report. Also due to the short time I have been in Barcelona the data collection for the wings is still in progress. Therefore I will only present here an exploratory analysis, with the data obtained so far, for the frequency of chromosomal inversions of male and female flies.

As expected from other studies [1, 8] there is a significant difference between the number of inversions observed in each chromosome (table 1). The A and J chromosomes present a high frequency for the A_2 and J_{st} (fig. 1). While in chromosomes U, E and O the difference in frequencies is not as high as observed for the other two chromosomes, although there is a more common inversion (U_{1+2+8}, $E_{1+2+9+12}$ and O_{3+4}, fig. 1),.

To search for possible differences in inversion frequencies between replicates and/or males and females, the frequencies of each inversion for males and females of each replicate were calculated. These frequencies were transformed by $2\sqrt{\text{frequency}}$, which provides a very good Euclidean approximation to Bhattacharyyas's distance, similarly to what was done by Balanyà *et al.* (2006) [1]. A Principal Component Analysis was carried out with the transformed data.

The distribution of both females and males of each replicate on the first axis (see fig. 1) suggests that there are differences between replicate.

It is expected that, when populations are inserted in a new environment, they will suffer the combined action of natural selection and genetic drift. It is also expected that during adaptation, in characters closely related to fitness, natural selection will lead to a more homogeneous performance in all the replicate of the same population, diminishing differences derived from genetic drift. However, due to the short time (9 generations) that these populations have been in the laboratory, genetic drift and replicate founder effect may have the predominance over selection and so the observed divergence between replicate may be a product of the combination of these effects.

Besides the differences between replicate there is also differences between males and females in the second axis (fig.1). It is known that females and males present differences at several characteristics (e.g. body size), but until now there hasn't been reported differences in chromosomal inversion frequencies between the two sexes. Therefore it will be of extreme interest to test if the differences in the inversion frequencies are related to different temperature resistance or size and shape of the wing, or are simply sex specific.

Table 1 – List of inversion observed (until the writing of this report) for each of the 5 chromosomes present in *D. subobscura*.

A	J	U	E	O
Ast	Jst	Ust	Est	Ost
A1	J1	U1+2	E8	O7
A2		U1+2+8	E1+2	O3+4
			E1+2+9	O3+4+1
			E1+2+9+3	O3+4+2
			E1+2+9+12	O3+4+6
				O3+4+7
				O3+4+8
				O3+4+12
				O3+4+23+2

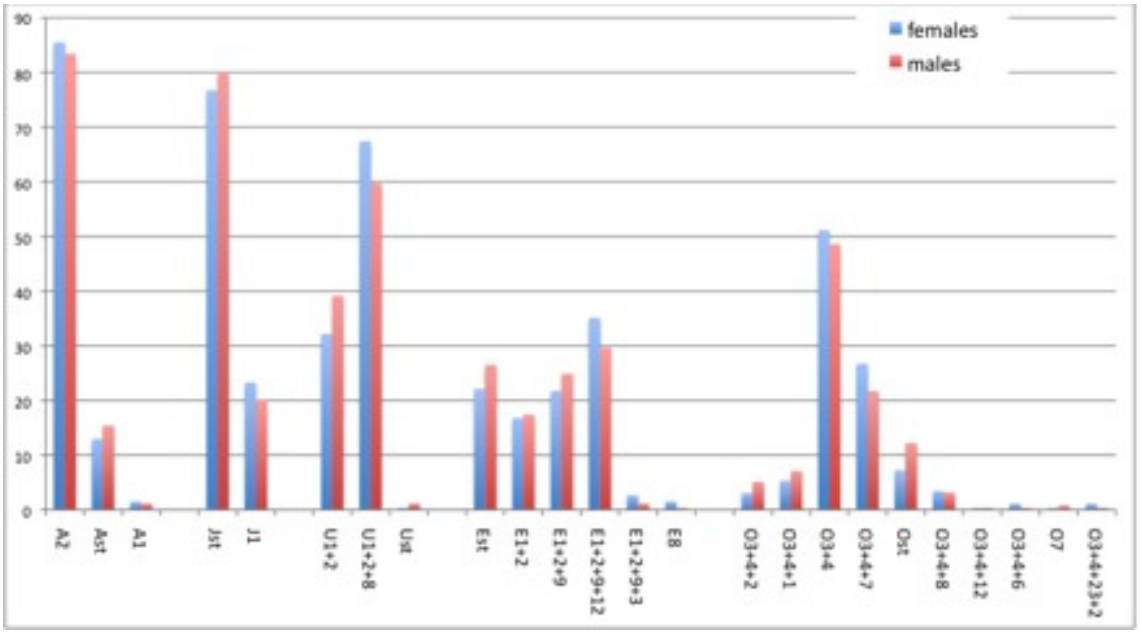

Fig. 1 – Frequency of chromosomal inversions of the 5 chromosomes (A, J, U, E, O) for males and females. The sample size corresponds to 262 for females and 253 for males.

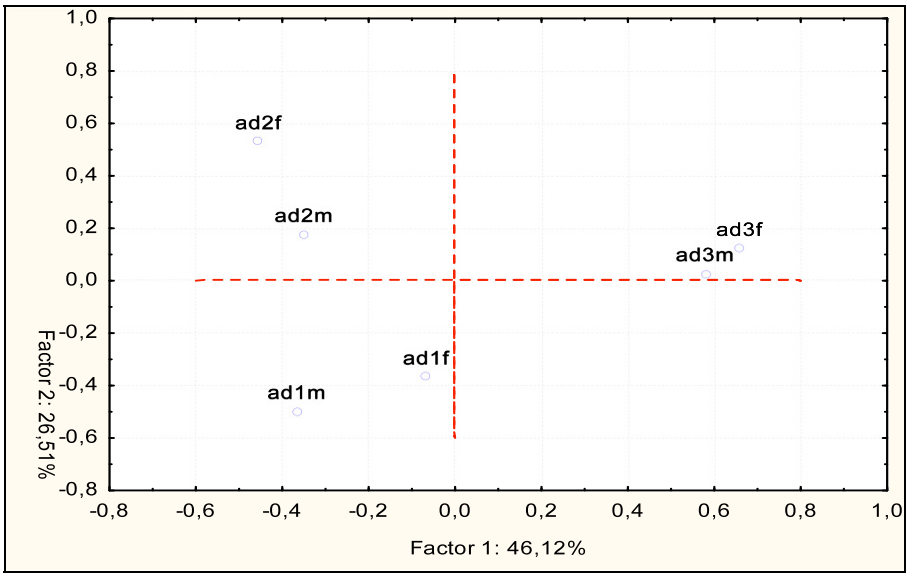

Fig. 2 – Principal component analysis for the males and females of the three replicate populations using the transformed data for all the chromosomes.

Expected Publications

It is expected at least one publication resulting from this collaboration.

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