Parallel clines of chromosomal inversion in seaweed flies associated with thermal variation

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Abstract:

Chromosomal inversions which form blocks of linked genes are increasingly recognized for their role in maintaining intra-specific diversity. They are predicted to be relevant genetic architectures for local adaptation in the face of gene flow. However, pinpointing the underlying traits and functional mechanisms under selection remains challenging. The seaweed fly *Coelopa frigida* harbors several large polymorphic inversions, one of which (*Cf-Inv(4.1)*) displays a latitudinal cline of frequencies along the North American Atlantic Coast. This distribution suggests a putative role in adaptation along the eco-climatic gradient. To further investigate this hypothesis, we designed a molecular marker to identify the different karyotypes and studied natural and experimental populations from America and Europe. We confirmed that this inversion is also present and polymorphic in Europe and displays parallel latitudinal clines across continents, providing strong indirect support that *Cf-Inv(4.1)* is under natural selection along similar environmental gradients. We found that *Cf-Inv(4.1)* had no effect on cold tolerance but a significant impact on egg-to-adult survival and fecundity under different thermal conditions. We speculate that fitness associated with the inversion may be shaped by subtle life-history differences whose relative advantage depend on climate. While our experimental approaches provided insights into genotype-phenotype associations, it is worth noting that natural selection acts on the overall fitness, involving complex sets of traits. This is especially relevant for inversions linking hundreds of genes. This multi-gene property also explains why inversions are particularly likely to be involved in repeated parallel adaptation to environmental gradients, as demonstrated here in the seaweed fly.

Keywords:

Parallel adaptation; Thermal gradient; Diptera; Functional mechanisms; Fitness; Experimental evolution

Running title: Parallel clinal inversions along thermal gradients

Introduction

From the early discovery of chromosomal rearrangements by cytogenetics to the recent advances in whole genome sequencing, an increasing body of evidence has demonstrated the importance of structural genomic variation in adaptation and diversification. A chromosomal inversion is a structural variant defined as a segment of a chromosome in which two sequences in reversed orientations segregate in the population (Allendorf et al., 2012). This different orientation translates into reduced gene flow between the two arrangements (the standard and the inverted sequence), as recombination is limited in heterokaryotypes (Tigano & Friesen, 2016). Therefore, the two arrangements tend to diverge through time and, due to high linkage disequilibrium, can behave as two alleles of one "supergene". This type of polymorphism is frequently involved in speciation, specialization of ecotypes, and local adaptation (Wellenreuther & Bernatchez, 2018) because chromosomal inversions can preserve combinations of locally-adapted alleles and thus limit the outbreeding depression effect in a context of high gene flow (Kirkpatrick & Barton, 2006; Schaal et al., 2022, Yeaman, 2013). Thanks to this property, and because inversions polymorphism can be retained over large spatial and temporal scales, chromosomal inversions are also predicted to be repeatedly involved in parallel adaptation to similar ecological gradients (Westram et al., 2022).

The importance of chromosomal inversions in local adaptation and intra-specific diversification is supported by empirical cases in which adaptive ecotypes and morphotypes have been associated to inversion polymorphisms in plants, insects, birds, and mammals (Hager et al., 2022; Jay et al., 2022; Lindtke et al., 2017; Purcell & Brelsford, 2025; Wellenreuther & Bernatchez, 2018). Beside the charismatic cases in which the inversion-phenotype association has been well-described, more and more studies are detecting large polymorphic inversions by scanning the genomes (Fang & Edwards, 2024; Li et al., 2023; Liang et al., 2024). However, understanding the functional and putatively adaptive effects of those inversions remains a challenge. Clues about the selective forces acting on inversions can be inferred from the distribution of polymorphism in natural populations, particularly when parallel patterns are observed across similar environmental gradients. For example, a sharp frequency change in multiple inversions in *Littorina* species, repeatedly observed across a habitat transition, supports their role in local adaptation (Faria et al., 2019). Similarly, chromosomal inversions in *Drosophila melanogaster* form parallel frequency clines across continents and seasons, suggesting that the inversion polymorphisms are under selection by an environment that varies in space and time (Kapun & Flatt, 2019). Such observational data benefits from further experiments. For instance, in *Drosophila subobscura*, inversions were associated with different responses to thermal stress in the laboratory, partially explaining the observed latitudinal clines in frequencies (Rego et al., 2010). The clinal inversion polymorphism In(3R)P in *Drosophila melanogaster* contributes to latitudinal variation in multiple fitness-related traits, acting as a 'supergene' likely maintained by fitness trade-offs and balancing selection across geographic regions (Durmaz et al., 2018). In the seaweed fly *Coelopa frigida*, quantifying life-history trait variation associated with the inversion *Cf-Inv(1)* and with environmental heterogeneity explained the persistence of a long-term balanced polymorphism (Mérot et al., 2020). Overall, field-based and lab-based studies of inversion polymorphisms thus remain critical to understand the functional impact of the multiple inversions detectable by genomic tools. Only by elucidating the relationship between inversion, fitness, and environment can we understand the spatial and temporal dynamics of those large variants and the evolution of their genomic content.

In the seaweed fly *Coelopa frigida*, three large polymorphic inversions have been detected on chromosome 4 (*Cf-Inv(4.1), Cf-Inv(4.2), Cf-Inv(4.3)*) in North American populations (Mérot et al., 2021). The phenotypes associated to those inversions are still unknown, although their frequencies form a strong latitudinal cline along the North American East Coast (Mérot et al., 2021), suggesting that they may play a role in climate adaptation, possibly in thermal adaptation. Indeed, temperature is one of the main environmental factors varying with latitude and is expected to be a strong selective force for ectotherm insects (Denlinger & Lee Jr, 2010), and especially for *C. frigida*, whose distribution extends from temperate to polar regions (Dobson, 1974). The frequency cline is particularly sharp for the 6.9 Mb-long inversion *Cf-Inv(4.1)* with a frequency that varies from 0.27 to 0.75 across a range of 10° of latitude. Cytogenetic studies (Aziz, 1975) have suggested that the inversion *Cf-Inv(4.1)* may also be carried by European populations, providing an ideal system to assess the contribution of chromosomal inversions to parallel adaptation along ecogeographic gradients, as well as natural replicates to investigate the putative functional impact of the inversion. However, the karyotype frequency differences along the latitudinal gradient in Europe have not yet been investigated.

We assessed whether the cytogenetically identified European polymorphism on chromosome 4 corresponds to the genomically identified North American inversion *Cf-Inv(4.1)*. After validating a genetic marker to genotype the inversion on both continents, we investigated the geographic distribution of frequencies along a North-South cline in Europe. The demonstration of inter-continental parallelism would provide indirect evidence that the latitudinal clines of inversion frequency are the result of divergent selection based on latitude, and not the simple effect of neutral processes such as genetic drift and isolation by distance (Westram et al., 2022). Through an experimental approach, we then tested the hypothesis that the cline of inversion frequencies reflects the role of *Cf-Inv(4.1)* in adaptation to the latitudinal thermal gradient. We tested the effect of *Cf-Inv(4.1)* on chill tolerance (supercooling point, chill coma recovery time) and measured fitness components (fecundity, longevity, development time, egg-to-adult survival) under different thermal conditions for both inversion homokaryotypes.

Methods

Study organism and field samplings

Ceolopa frigida is an acalyprate fly (Diptera: Coelopidae) that lives and feeds in the banks of decomposing seaweeds on the shores of the Atlantic Ocean. It is distributed from Massachussets (USA) to the Canadian Arctic in North America and from Brittany (France) to Svalbard (Norway) in Europe.

Adult flies were collected along the coasts of the Atlantic Ocean in six North American locations (Supplementary Table 1) during summer 2022 and ten European locations during summer 2023 (Fig. 1A, Tab. 1). Between 100 and 500 individuals per location were either stored in ethanol 96%, stored at -80°, or kept alive in tubes with a piece of cotton soaked in a solution of mannitol (0.5%) to start laboratory populations. Flies from six locations per continent were maintained in the laboratory in plastic boxes of 1.7L (13 × 8.5 × 18 cm) containing approximately 50% of Fucacea *(Fucus serratus* and/or *Fucus vesiculous*) and 50% of Laminariaceae (*Saccharina latissima* and/or *Laminaria digitata*). At each generation, emerging adults were captured over a few days using an insect vacuum and presented with fresh seaweed to stimulate egg-laying. A random subsample of eggs was then collected (to control for density) and spread on the same natural seaweed substrate in two to three boxes per population. Each population was started with 100-300 individuals to ensure enough genetic diversity, and each generation usually produced 100-300 adults. The laboratory populations were kept for 7 to 14 generations before starting the experiments described below.

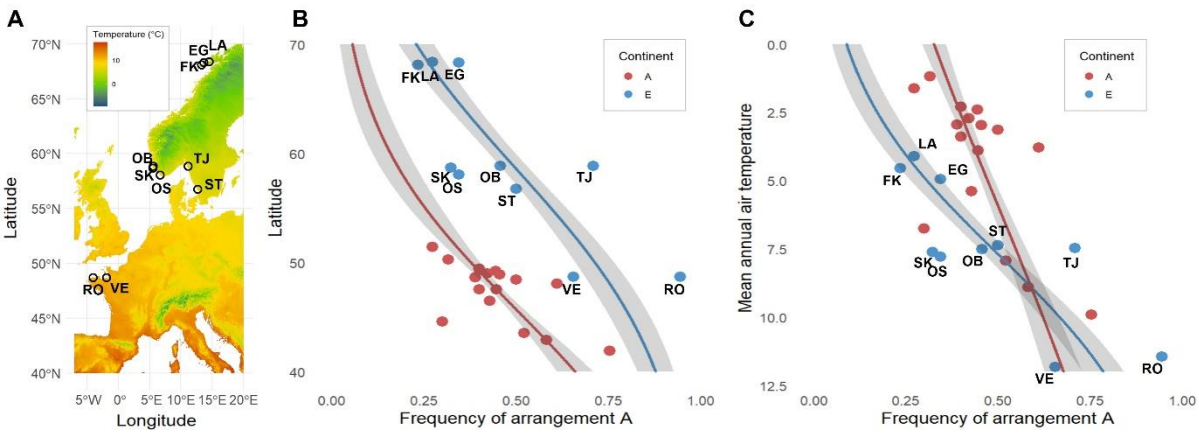

Fig. 1. *Coelopa frigida* sampling and inversion frequency clines along the latitude gradients. (A) Map of sampling locations in Europe. The colour gradient represents mean annual air temperatures. (B) Frequency of *Cf-Inv(4.1)* arrangement A as a function of latitude (C). Frequency of *Cf-Inv(4.1)* arrangement A as a function of mean annual air temperature. Red dots represent the American data provided by Mérot *et al.* (2021), from 16 locations sampled in September/October 2016. Blue dots represent the European data, from 10 locations sampled in March/April 2023. Red line is the binomial regression adjusted on American data. Blue line is the binomial regression adjusted on European data. Grey ribbon is the confident interval 95% of the average predictions of the binomial model, which takes into account sample sizes.

Tab 1. Table of European locations coordinates, mean annual air temperature, karyotypes counts, frequency of arrangement A and p-values of Chi-squared test of Hardy-Weinberg equilibrium.

Location	Latitude	Longitude	Annual mean temperature (°C)	N AA	N AB	N BB	Frequence A	p-value HW
FK	68.09899	13.24322	4.5	2	18	27	0,23	0.64
LA	68.39371	14.47901	4.1	3	19	24	0,27	0.77
EG	68.30302	13.63961	4.9	4	23	18	0,34	0.38
OB	58.86962	5.56719	7.5	10	11	13	0,46	**0.04**
SK	58.69299	5.54701	7.6	3	14	14	0,32	0.85
OS	58.07161	6.64781	7.7	6	21	21	0,34	0.83
TJ	58.87095	11.11980	7.4	23	22	3	0,71	0.45
ST	56.76591	12.62948	7.3	8	27	8	0,50	0.09
RO	48.72543	-4.00535	11.4	32	4	0	0,94	0.72
VE	48.69565	-1.87943	11.8	24	7	11	0,65	**4.28 x 10^5**

Detecting inversion *Cf-Inv(4.1)* in Europe

The inversion *Cf-Inv(4.1)* was inferred by principal component analysis (PCA) on whole genome sequences from 1,146 North American *C. frigida* (Mérot et al., 2021). To test whether the inversion was also present and polymorphic in Europe, we re-used a dataset of 56 South Scandinavian *C. frigida* that were collected and sequenced by Emma Berdan and Maren Wellenreuther in 2015. Those 56 samples were whole-genome sequenced with paired-end short-reads 150 bp in an Illumina Hi-Seq machine at a coverage of ~30X at SciLifeLab (Sweden). Reads were trimmed and aligned with bwa-mem v 0.7.15. SNPs were called with bcftools mpileup v1.15 and stored as a variant calling format (VCF). This dataset was restricted to the putative inversion region (LG4 from position 1,088,816 to position 7,995,568) and filtered for a minor allele frequency of ≥10%. We summarized the genetic variation in this genomic region with a PCA (prcomp function in R) to search for a putative signature of three clusters, as typically observed in non-recombining inversions. Putative karyotypes for the inversion *Cf-Inv(4.1)* were assigned visually based on the three observed clusters.

To investigate whether the inversion sequence is conserved across continents, amplified fragments of 667 bp, corresponding to the gene DN9360 (positions 1,636,902 to 1,638,332) were sequenced at the Plate-forme d'Analyses Génomiques (Université Laval, Québec, Canada) for 8 North American samples and at Macrogen Europe for 13 Scandinavian samples. The forward and reverse sequences were processed using Geneious 2023.0.4, aligned by pairs, trimmed at 488 bp, corrected for sequencing ambiguities, and realigned altogether. A haplotype network was built using the Popart software (http://popart.otago.ac.nz) through the Minimum spanning network method (Bandelt et al., 1999) with $\varepsilon = 0$.

Developing a diagnostic marker to genotype the inversion

We aimed to develop a SNP marker which would allow genotyping with a simple PCR and digestion assay. The marker was first developed based on the North American WGS dataset. Several SNPs were selected according to the following conditions: (1) being located in coding regions, (2) being fixed between the two arrangements (at least a difference of allele frequency of 99% between the two homokaryotypic groups), (3) being alternatively cut or not cut by a common restriction enzyme. After several assays, we selected two linked SNPs (located on chromosome 4 at position 1,637,841 and 1,637,843) in the previously mentioned 667 bp-long coding region which amplified consistently well (see primers in Supplementary Table 2) and where the restriction enzyme EcoRI cut the arrangement A but not the arrangement B. The whole protocol of the diagnostic assay is available in supplementary materials (Supplementary Methods 2 and 3; Supplementary Figure 1). We performed the diagnostic assay using the DNA of a subset of 104 North American individuals (see DNA extraction method in Mérot et al., 2021) and calculated the concordance between karyotypes determined with the marker and those given by the whole-genome PCA. We used the same approach to validate the marker in Europe, using the DNA extracts

provided by Emma Berdan (see Supplementary Methods 1 for extraction protocol) for a subset of 38 samples out of the 56 used for the PCA (no DNA could be recovered from the remaining samples).

Investigating the distribution of inversion frequencies in Europe

In the 10 European locations spanning a latitude from 48.69565° to 69.39371° (Fig. 1A), we used the diagnostic marker to genotype 31 to 48 flies per location, using flies that were stored in ethanol 96% or at -80°C since the sampling.

All statistical analyses, described in this section and further, were performed on R version 4.4.3. The frequencies of the two arrangements were calculated for each location, as well as the proportion of the three karyotypes (AA, AB, BB). Hardy-Weinberg equilibrium in the karyotypes proportion was tested for using the function HWAlltests from the package *Hardy-Weinberg*. To assess the correlation between inversion frequencies and a putative climatic cline, annual mean temperature data were extracted from the database 'Worldclim' (Fick & Hijmans, 2017) and the associations between frequencies and latitude/temperature were tested with a binomial regression using the glm function with the link "logit".

Experimentally testing the role of inversion *Cf-Inv(4.1)* in response to cold stress

To test the predictions that cold tolerance varies across locations along the latitudinal cline and is associated with inversion karyotypes, we experimentally measured two traits reflecting cold stress tolerance: the supercooling point (SCP) and the chill coma recovery time (CCRT). Experiments were performed on 288 and 320 individuals respectively, from five North American locations (see Supplementary Table 1) raised in lab for three to eight generations. Detailed methods are available in supplementary materials (Supplementary Methods 4 and 5). Briefly, the supercooling point was defined as the temperature at the onset of the freezing exotherm peak for each fly exposed to a gradually decreasing temperature at a rate of -0.5°C/min (from 5 to -25°C). Chill coma recovery time was defined as the time before the insect spontaneously recovers coordinated neuromuscular function and stand on legs following removal from a cold exposure at -5°C for 16h (equivalent to a cold winter night). Experimental individuals were genotyped for inversion *Cf-Inv(4.1)* and *Cf-Inv(1)* using the same protocol described above (Supplementary Methods 2 and 3). Supercooling point was normally distributed and analyzed using a Linear Model (function lm). Chill coma recovery time was analyzed with a Generalized Linear Model (GLM) with Gamma family, which is appropriate for a time-to-event variable. Both models included mass, sex, location, and karyotypes for both inversions as explanatory variables, and were tested with the function Anova from package *car* (Fox & Weisberg, 2018), which performs a F test on LMs and a Likelihood Ratio Test on GLMs. When necessary, pairwise permutation t-tests or log-rank tests were performed to identify the modalities that differed from others.

Experimentally testing the combined effects of inversion *Cf-Inv(4.1)* and temperature on fitness

Creating lineages of *C. frigida* homokaryotes for *Cf-Inv(4.1)*

To perform fitness experiments, we made four experimental lines (North American AA and BB, European AA and BB) which were homokaryotypic for the inversion *Cf-Inv(4.1)* but highly variable in the rest of the genome. To start the 1st generation, 700 virgin individuals from the laboratory populations were genotyped using a piece of leg cut under CO_2 anesthesia. A total of 314 homokaryote individuals were selected for egg laying (N=43 for AA-America, N=92 for BB-America, N=24 for AA-Europe, N=155 for BB-Europe). Those lines were maintained at 20°C (reference temperature) using the raising method described earlier for six generations before starting the experiments. The karyotype of 12 randomly-chosen individuals per lineage was checked every two generations.

Measuring fitness during larval development

Larval fitness was assessed by two traits: development time and viability (i.e., survival from egg to adult). For each of the four homokaryote lines, we collected a large number of eggs. Those were divided, using a paint brush soaked in sea water, into 30 batches of approximately 100 eggs, which were then spread on a piece of *Saccharina latissima* and photographed to be counted *a posteriori* using ImageJ 1.54b (Supplementary Figure 8). The 30 batches were placed in a plastic box of dimension 10.5 × 7.5 × 11 cm filled with 90 g of seaweeds (50% *Fucus*

sp., 50% *Saccharina sp.*) and distributed randomly across five thermal conditions: 10, 15, 20, 25 and 30°C (six batches per temperature per lineage). An amount of 45 g of algae was added at half of the development time, i.e., on day 3, 5, 7, 14 and 31 for temperatures 30, 25, 20, 15 and 10°C respectively. During the emergence phase, new adults were removed from the boxes every day, counted and sexed. Data at 10°C were not statistically analyzed because of a very low viability, which resulted in a very low sample size (n = 4).

Measuring fitness at adult stage

Adult fitness was assessed by measuring two life-history traits: adult longevity (*i.e.*, time between adult emergence and death) and female fecundity. For each of the four homokaryote lines, we used 36 virgin individuals to create 18 couples, that were split across three temperatures: 10, 20 and 30°C (six couples per temperature per line). To ensure virginity, individuals were collected at the pupa stage, stored individually at 20°C, and sexed upon emergence. Because females generally emerge a few days before males, they were first placed alone in plastic boxes of dimensions 8 × 8 × 5 cm with a piece of *Saccharina latissima* and a piece of *Fucus vesiculosus* of approximately 3 × 3 cm, and split across the three experimental conditions. A male from the same line was added one to two days later. From the following day, the boxes were checked on a regular basis (every day at 20° and 30°, every other day at 10°) to count clutches of eggs, replace the pieces of seaweed, and check for dead individuals. The death of a female meant the end of the fecundity measurement but the box was kept at the experimental temperature to continue measuring the remaining male's longevity. Dead males were replaced by new males of the same lineage (whose longevity was not measured). To estimate female fecundity, we measured: (i) the size of the first clutch, (ii) the mean clutch size, (iii) the total number of eggs laid, and (iv) the number of clutches laid during the entire life.

Disentangling possible confounding factor effects from *Cf-Inv(1)*

Besides *Cf-Inv(4.1)*, *Coelopa frigida* carries another polymorphic inversion on chromosome 1, *Cf-Inv(1)*, which affect life-history traits such as development time, survival, and fecundity. We controlled for the frequency of *Cf-Inv(1)* in the experimental lines by genotyping a subsample of 35 to 48 individuals per line (see methods in Mérot et al., 2018). The frequency of *Cf-Inv(1)* was analyzed with a Chi-square test of homogeneity.

Statistical analyses

Variation in each response variable (longevity, female fecundity, development time, viability) was analyzed with GLMs including the following explanatory variables: inversion karyotype, temperature and continent. For longevity and development time, we also tested the effect of sex.

Development time and longevity were analyzed using a GLM based on a Gamma distribution, being time-to-event variables. Viability data were analyzed using binomial GLM with a logit link function for binary outcome (dead vs. alive). Number of clutches and total fecundity, expected to follow a Poisson distribution, were analyzed with a GLM based on a Poisson, or a Quasi-Poisson law to address the overdispersion of residuals. First clutch size and mean clutch size, expected to follow a normal distribution, were analyzed with a GLM with quasi family (link = identity) because of overdispersion of the residuals with a simple linear regression. Accounting for the relatively small sample size, especially for viability and female fecundity, we decided to remove interactions of more than two factors.

After validating independence of the residuals, we investigated the explanatory contribution of each variable with deviance analyses (function Anova from the package *car* (Fox & Weisberg, 2018)). When necessary, multiple comparisons were performed using the method of Estimated Marginal Means (function emmeans from the functi *emmeans* in version 1.8.8 (Lenth *et al*., 2023)), to test the difference between modalities of significant factors and between combinations of modalities in the case of interactions. R^2 was estimated based on model deviance, as 1 – (model deviance / null model deviance).

We modelized the thermal performance curves for viability with a simple quadratic model ($viability = a \times temperature^2 + b \times temperature + c$), using R packages *rTPC* and *nls_multstart* (Padfield et al., 2021). We extracted the estimated values of maximal performance (rmax), optimal temperature (Topt), critical thermal

limits (CTmin and CTmax), thermal tolerance (amplitude of temperature for which viability is above zero), and breadth (amplitude of temperature for which viability is above 80% of rmax).

Results

The inversion *Cf-Inv(4.1)* is shared across continents

Whole genome sequencing of European *C. frigida* supported the presence of a polymorphic inversion in the same genomic region as *Cf-Inv(4.1)*. The first principal component decomposing genetic variation on Chromosome 4 within positions 1-8Mb explained 8.3% of variation and displayed, as predicted, three distinct groups of individuals (Fig. 2A).

The network constructed from the 488 bp-fragment sequenced within the gene DN9360 showed that genetic differentiation was much higher between the two arrangements than between samples coming from the two continents (Fig. 2B). The sequences of arrangements A and B differed by a minimum of 16 nucleotides (3.3% divergence), while the difference between North American and European sequences of the same arrangement was either one or two nucleotides (0.4% of divergence).

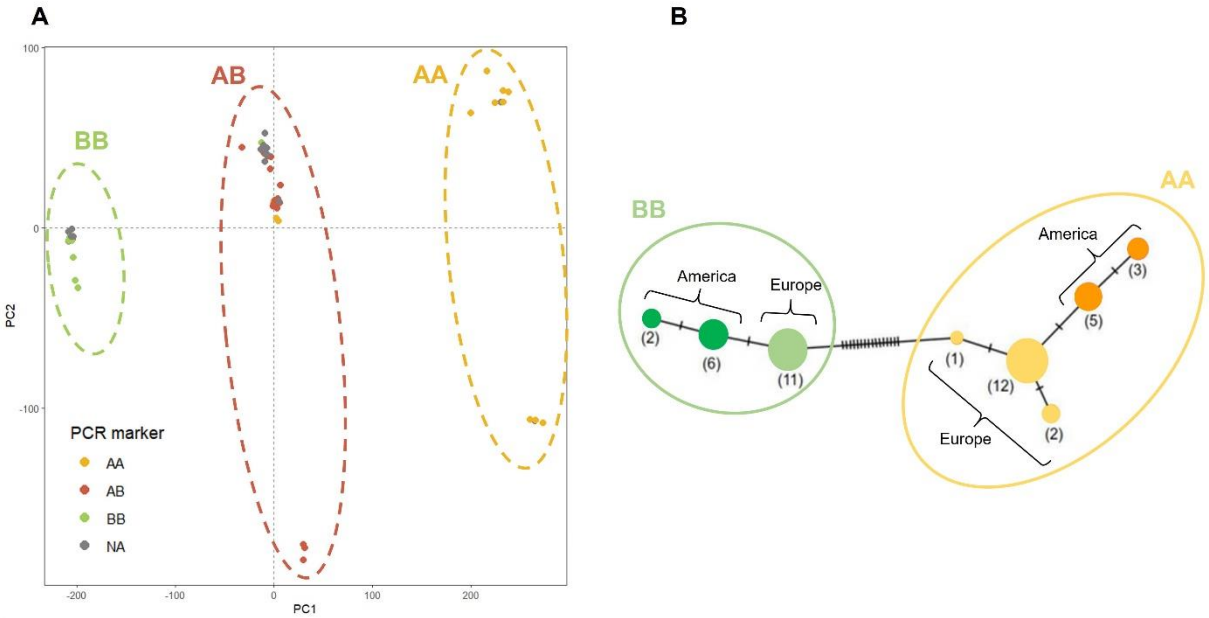

Fig. 2. Inversion *Cf-inv(4.1)* is shared across continents. (A) PCA decomposing genetic variation captured by SNPs between positions 1,636,902 and 1,638,332, corresponding to the location of inversion *Cf-inv(4.1)* detected in America. PC1 explains 8.3% of the variance and displays 3 clusters, characteristic of a non-recombining region. PC2 explains 3.7% of variance. Points are colored according to the karyotypes given by the designed diagnostic marker. Grey points represent the samples for which no DNA was available for amplification. (B) Haplotype network representing similarities and differences between haplotypes from 8 American and 13 Scandinavian individuals at a region of 488 pb around the diagnostic marker. Circle areas are proportional to the number of haplotypes with identical sequences. Sticks represent the number of substitutions between two circles. Two main haplotype groups were found corresponding to the A and the B arrangements, as labelled. The network was built with the Minimum spanning network method.

The diagnostic marker shows good accuracy for *Cf-Inv(4.1)* genotyping in America and in Europe

In North America, the diagnostic marker showed a very good accuracy with 99% concordance with inversion karyotypes determined by PCA (Mérot et al., 2021). Out of 103 individuals genotyped with the marker, 102 were consistent with the karyotype obtained from the PCA on whole genome sequences. One individual genotyped as AA by the PCA was assigned as AB by the marker (Supplementary Table 3). In Europe, 32 out of 38 individuals were consistent, representing a concordance rate of 84%. Most of the remaining individuals (5) were assigned as heterokaryotes by the PCA but as homokaryotes by the diagnostic marker (4 AA and 1 BB). One individual was also assigned BB by the PCA and heterokaryote by the diagnostic marker (Supplementary Table 3).

Inversion *Cf-Inv(4.1)* forms two parallel latitudinal clines

The inversion *Cf-Inv(4.1)* showed a cline of frequencies co-varying with latitude and annual mean air temperature in Europe (Fig. 1), as in North America (Mérot et al., 2021). The relationship between latitude and the frequency of the arrangement A is significant on both continents with a slope of -0.12 (binomial regression, $\chi^2 = 67.66$, df = 1, p-value < 0.001, $R^2 = 0.82$) for America and a slope of -0.11 (binomial regression, $\chi^2 = 100.44$, df = 1, p-value < 0.001, $R^2 = 0.93$) for Europe. It is worth noting that the association between latitude and inversion frequency in Europe holds true when using a possibly higher-confidence marker, *i.e.* only individuals genotyped by PCA (binomial regression, $\chi^2 = 8.18$, df = 1, p-value = 0.004, Supplementary Figure 3A). We also found a significant relationship between the inversion frequency and the annual mean temperature in both North America, with a slope of 0.12 (Fig. 1C, binomial regression, $\chi^2 = 69.93$, df = 1, p-value < 0.001, $R^2 = 0.83$) and in Europe, with a slope of 0.30 (Fig. 1C, binomial regression, $\chi^2 = 101.51$, df = 1, p-value < 0.001, $R^2 = 0.93$). The association was also tested with minimal winter temperature, with consistent results (see Supplementary Figure 4). All European locations were at the Hardy-Weinberg equilibrium (Table 1, Chi-squared test, p-value > 0.05), except OB (58.9°N, 5.6°E), in which heterokaryotypes were slightly under-represented ($\chi^2 = 4.11$, p-value = 0.043); and VE (48.7°N, 1.9°W), in which heterokaryotypes were strongly under-represented ($\chi^2 = 16.74$, p-value < 0.001). In America, only one location (KA, *cf.* Mérot et al., 2021) was not at the Hardy-Weinberg equilibrium ($\chi^2 = 4.55$, p-value = 0.033), with heterokaryotypes being slightly over-represented.

Coelopa frigida exhibits a high chill tolerance, not associated with *Cf-Inv(4.1)*

Cold tolerance experiments demonstrated low freezing points, with a mean SCP of -16°C (CI95% = [-16.4; -15.6]), typical of freeze-avoidant species. The CCRT experiment suggested a high tolerance of chilling as after 16h at -5°C, only 2.8% of *C. frigida* were dead while 97.2% recovered. Recovery was also relatively fast with a recovery time ranging from 4.5 minutes to 26.5 minutes, and a mean value of 12.2 minutes (CI95% = [-11.8; 12.6]). Neither the SCP nor the CCRT were significantly affected by the karyotype at inversion *Cf-Inv(4.1)* despite testing 65 AA, 104 AB and 97 BB for SCP (F = 0.98, df = 2, p-value = 0.38) and 41 AA, 113 AB and 89 BB for CCRT ($\chi^2 = 4.67$, df = 2, p = 0.097). Small effects associated with mass and sex, as well as location and karyotype at inversion *Cf-Inv(1)* were detected but remain marginal in explaining the total variance (see Supplementary Results 1).

Egg-to-adult viability is impacted by temperature and *Cf-Inv(4.1)*

Temperature had a significant impact on the viability of the developing individuals ($\chi^2 = 68.48$, df = 3, p-value < 0.001, Fig. 3). Maximum viability was at 20°C (0.26 ± 0.14), and decreased as temperature moved away from the optimum both towards lower or higher temperatures. At 10°C, viability was very low and adult flies were recovered in only two replicates out of 24 replicates but always in BB (one European BB with 2.5% of viability and one American BB with 4% of viability). Karyotype ($\chi^2 = 12.60$, df = 1, p-value < 0.001), continent ($\chi^2 = 14.07$, df = 1, p-value < 0.001), and their interaction ($\chi^2 = 174.05$, df = 1, p-value < 0.001) significantly impacted viability (Fig. 3). When considering all thermal conditions together AA karyotypes had a higher viability than BB karyotypes ($\chi^2 = 12.6$, df = 1, p-value = < 0.001) although this result hides differences across continents. Viability was significantly higher for AA than BB in North America (z = 10.80, p-value < 0.001), whereas the opposite effect was observed in Europe (z = 6.97, p-value < 0.001). We also detected a significant effect of the interaction between inversion karyotype and temperature ($\chi^2 = 35.27$, df = 3, p-value < 0.001), reflected in the slightly different shape and width of thermal performance curves (Supplementary Figure 5). Thermal curves revealed higher maximal viability and a higher amplitude of tolerated temperatures for the AA karyotype over BB in America, but the opposite effect in Europe (Supplementary Table 4).

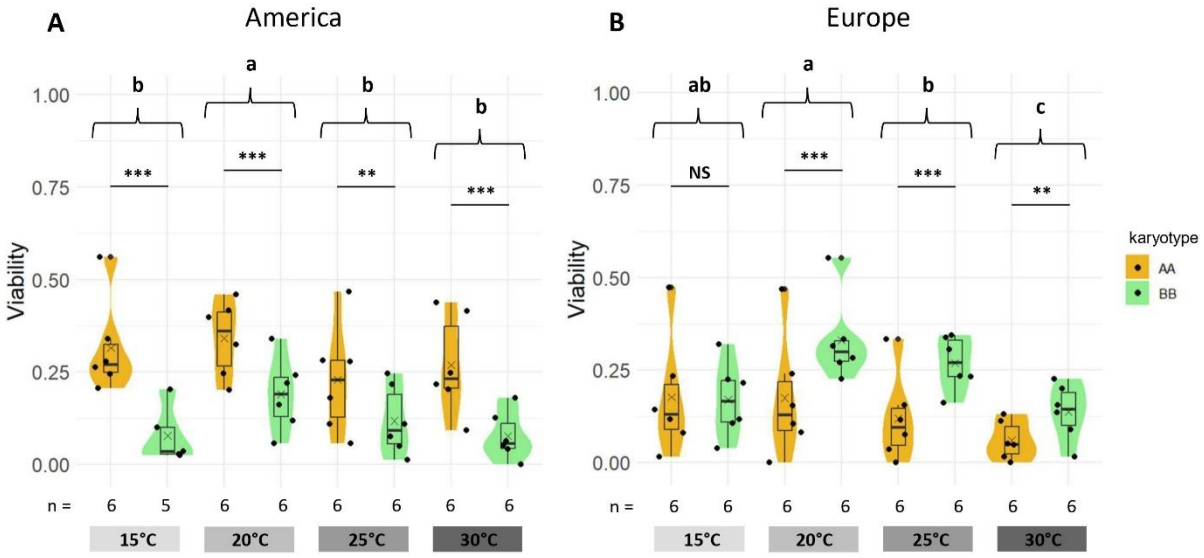

Fig. 3. Egg-to adult viability. Viability for each combination of temperature and inversion karyotype in America (A) and in Europe (B). Viability was quantified as the percentage of eggs developing until emerging adults under controlled temperature (10, 15, 20, 25 and 30°C). Data are not shown for 10°C because only two replicates produced adults with a very low survival rate (<5%).

Development time is mostly determined by sex and temperature

Development time was mostly influenced by temperature (χ^2 = 49 566, df = 3, p-value < 0.001), as the model including temperature alone explained 96.6% of the variance. Development time decreased significantly when the temperature increased (8.1 ± 0.9 days at 30°C; 9.9 ± 1.1 days at 25°C; 15.2 ± 1.2 days at 20°C; 28.1 ± 2.1 days at 15°C; 87 ± 10.4 days at 10°C; Supplementary Figure 6). Across all thermal conditions, females developed faster on average than males (χ^2 between 97 and 250, df = 1, p < 0.001 at all temperatures), with males emerging on average 1, 1.1, 1.5 and 2.2 days after the females at 30, 25, 20 and 15°C respectively. Generally, the inversion did not have any significant effect on development time (χ^2 = 0, df = 1, p-value = 0.55) although we detected a very small effect size at 15°C interacting with sex (χ^2 = 11.01, df = 1, p < 0.001) and continent (χ^2 = 10.65, df = 1, p < 0.01).

Adult longevity is mostly determined by temperature

Analysis of adult longevity revealed no effect of *Cf-Inv(4.1)* but a strong effect of temperature, (χ^2 = 799, df = 2, p-value < 0.001, Fig 4C). The longevity at 10°C (34 ± 14 days) was higher than the longevity at 20°C ((11 ± 4 days, t = 14.02, df = 108, p-value < 0.001), which was itself higher than at 30°C (5 ± 1 days, t = 9.51, df = 108, p-value < 0.001). Continent and sex also significantly affected longevity (continent: χ^2 = 16.46, df = 1, p-value < 0.001; sex: χ^2 = 8.06, df = 1, p-value < 0.01), but post-hoc pairwise comparisons suggest a very marginal effect (with females living slightly longer than males, and North American flies living slightly longer than European flies). Adult longevity did not differ significantly between inversion karyotypes (χ^2 = 0.7, df = 1, p-value = 0.4, Fig 4C), even when comparing karyotypes within temperature modalities. Global model explained 87% of variance in longevity.

Female fecundity is higher in *Cf-Inv(4.1)* BB homokaryotypes

Female fecundity varied significantly between inversion karyotypes, with an advantage to BB, regardless of temperature (Fig. 4A).

The number of eggs in the first clutch was mostly affected by the inversion karyotype (χ^2 = 19.31, df = 1, p-value < 0.001). The BB females laid on average 144 ± 44 eggs in their first clutch compared to 97 ± 37 for AA females. The effect of the inversion interacted with continent (χ^2 = 7.87, df = 1, p-value = 0.005). Indeed, the size of the

first clutch for BB was significantly larger in North America (z = 5.26, p-value < 0.001) but not in Europe (z = 1.002, p-value = 0.75) although the same tendency was observed. Temperature did not have a significant impact on this trait (Fig. 4A, $\chi^2 = 1.41$, df = 2, p-value = 0.50). Mean clutch size followed the same trend, with significantly larger clutches for BB females without any interaction with temperature (see Supplementary Results 2, Supplementary Figure 7A).

The number of clutches laid by females during their life was not significantly impacted by the inversion karyotype (Fig. 4B, $\chi^2 = 0.20$, df = 1, p-value = 0.66). It was impacted by temperature ($\chi^2 = 40.85$, df = 2, p-value < 0.001), continent ($\chi^2 = 16.63$, df = 1, p-value < 0.001), and the interaction between the two ($\chi^2 = 7.01$, df = 2, p-value = 0.03), so that this global model explained 62.81% of the variance. American females laid more clutches than European females (z = 3.54, p-value < 0.001), with an average of 2.83 ± 1.79 clutches in America and 1.53 ± 1.52 in Europe. The number of clutches followed a thermal curve with an optimum at 20°C and a significant decrease at 30°C (Fig. 4B, z = 4.91, p-value < 0.001), and at 10°C (z = 4.48, p-value < 0.001). However, no significant effect of the interaction between inversion karyotype and temperature was detected ($\chi^2 = 1.30$, df = 2, p-value = 0.52, Fig 6B).

The total fecundity of a female is the product of the two traits previously analyzed: the number of clutches laid by the female and the size of these clutches. Therefore, total fecundity was significantly impacted by all the variables that had an effect on the number of clutches or the clutch size: temperature, karyotype, continent and their interactions (see Supplementary Results 3, Supplementary Figure 7B).

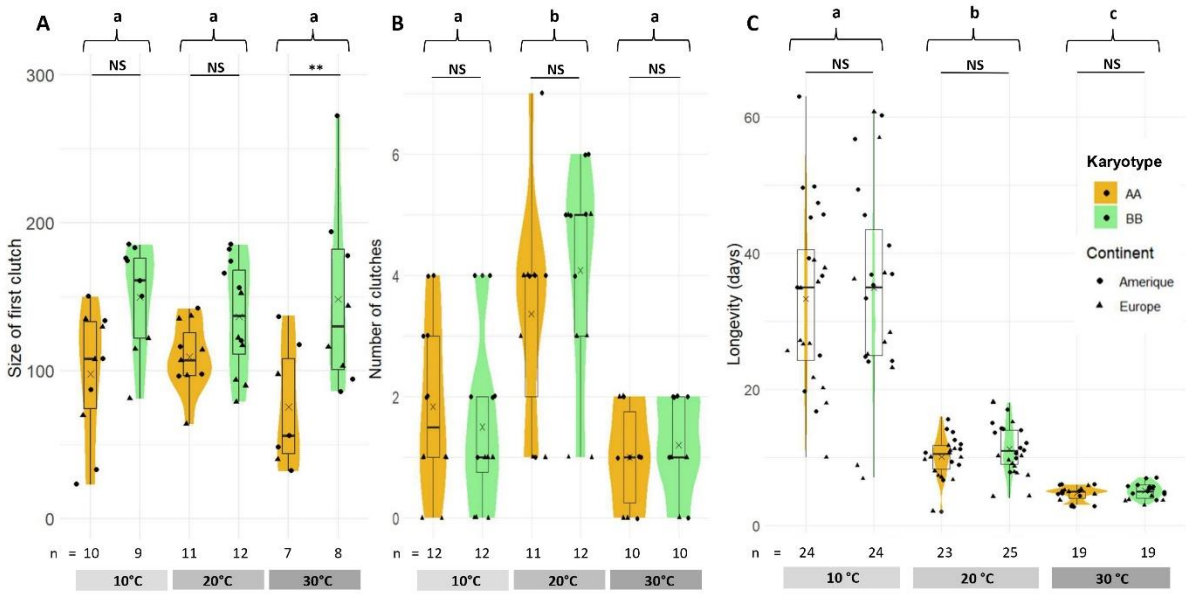

Fig. 4. Fitness at adult stage. (A) Number of eggs laid in the first clutch of females for each combination of continent and karyotype. (B) Number of clutches laid over the life of females for each combination of temperature and karyotype. (C) Longevity of both sexes in days for each combination of temperature and karyotype.

Disentangling possible confounding factors

Besides the different genetic background across continents, which may explain part of the fitness variability between Europe and America, we controlled for the frequency of the chromosome I supergene, *Cf-Inv(1)*. For the American continent, *Cf-Inv(1)* was unlikely to affect our results since its frequency did not significantly differ across American experimental populations (Chi-squared test, $\chi^2 = 0.02$, df = 1, p-value = 0.878). However, the European experimental lineage AA displayed a considerably higher frequency of the arrangement α for *Cf-Inv(1)* than the BB lineage (Chi-squared test, $\chi^2 = 26.54$, df = 1, p-value < 0.001). Because the arrangement α induces reduced survival and an increased fecundity (Mérot et al., 2020), it may confound some of the effects of *Cf-Inv(4)* identified in America, and partially explain why no significant difference in fecundity is observed in Europe and why the reverse pattern is observed for viability.

Discussion

Polymorphic inversions are increasingly detected by population genomics and attract a lot of attention in evolutionary biology due to their putative key role in adaptation and diversification (Berdan et al., 2023). However, understanding the evolution of inversions requires not only genomic characterization but also knowledge about their distribution in time and space, as well as their functional impact on phenotypes (Lowry & Willis, 2010). Focusing on an inversion previously detected from genomic data in *C. frigida* (Mérot et al., 2021), our study provides indirect evidence that this inversion is conserved across continents and involved in parallel clines along latitudinal eco-climatic gradients. Results from experiments revealed an effect of *Cf-Inv(4.1)* on some life-history traits, partially interacting with temperature, but did not fully explain the latitudinal cline. While the interaction between fitness, inversion, and temperature is complex, we speculate that the relative fitness advantage of the different karyotypes may vary with temperature, or other factors co-varying with latitude, and determine the local balance of inversion frequency.

A conserved inversion polymorphism

Several lines of evidence show that the polymorphic inversion *Cf-Inv(4.1)*, previously described in America (Mérot et al., 2021), is also polymorphic and conserved in Europe. First, genetic variation within the region of *Cf-Inv(4.1)* show a high genetic structure in three clusters as observed on the PCA. This confirms that the SNPs presents in the region are highly linked, as expected in the absence of recombination between the two arrangements, and that there is genetic differentiation between the two inversion arrangements. Second, a 488 bp coding locus supports a high sequence similarity between continents (within each arrangement) vs. a strong divergence between arrangements (on both continents). In other words, the absence of recombination at the inversion level keeps the genetic divergence between the two arrangements far higher than the divergence caused by isolation by distance. While the age of the inversion is at least of 61,000 – 134,000 years (Mérot et al., 2021), the geographic origin of the inversion and the history of continent colonization remain unknown. However, our results support the hypothesis that the inversion *Cf-Inv(4.1)* has a single origin and was either formed before the geographic expansion of the species or spread across the range of the species. Such a worldwide distribution is not unusual, with inversion polymorphisms being sometimes even shared across species boundaries (Knief et al., 2024; Tuttle et al., 2016). For example, Kapun et al. (2023) demonstrated that the inversion In(3R)Payne in *Drosophila melanogaster* originated in sub-Saharan tropical regions and subsequentially spread around the world, being maintained by selection on four continents. Several cosmopolitan inversions are also described in stick-insects (Lindtke et al., 2017), in ruffs (Küpper et al., 2016). In most cases, inversions that remain polymorphic for a long time and across all populations are arguably under a form of balancing selection (Wellenreuther & Bernatchez, 2018). This balancing selection can result from different mechanisms including frequency-dependent selection, antagonistic pleiotropy, overdominance, disassortative mating and spatially/temporally variable selection (Cheng et al., 2012; Chouteau et al., 2017; Kim et al., 2017; Tuttle et al., 2016).

In *C. frigida*, the high divergence between arrangements led us to develop a marker with high concordance in America (99%) but a slightly lower accuracy in Europe (84%). The discordance could be explained either by the fact that a high number of missing data in whole genome sequences tends to place an individual among heterokaryotypes on the PCA, or by the fact that the SNPs targeted by the diagnostic assay may not be completely fixed between arrangements in Europe, possibly due to gene conversion (Chen et al., 2007). Further analyses including comparable whole-genome data would be necessary to better quantify the degree of similarity between continents along all the inverted regions. In any case, the large sample size provide confidence in the inferences from the intercontinental comparative analyses of inversion distribution.

Parallel clines of inversion frequencies along similar gradients

Because chromosomal inversions favor the long-term co-existence of ecotypes and can be under strong selection (Faria, Johannesson, et al., 2019), they are predicted to be frequently involved in repeated local adaptation across distant locations (Westram et al., 2022). For example, in the East African honeybees *(Apis mellifera)*, two genomic regions are highly divergent between highland and lowland populations of three distinct pairs, suggesting that these putative inversions are repeatedly implicated in adaptation to altitude (Christmas et al., 2019; Wallberg et al., 2017). This pattern can also be observed at larger geographical scale. An important role of inversions in parallel

adaptation is supported by theoretical simulations (Westram et al., 2022) as well as by numerous empirical studies across patchily distributed habitats like in stick insects and Atlantic cod (Lindtke et al., 2017; Matschiner et al., 2022), or along continuous environmental gradients as in sticklebacks and periwinkles (Jones et al., 2012; Morales et al., 2019).

Studying the frequency of inversion *Cf-Inv(4.1)* in *C. frigida* at different locations along the European latitudinal gradient highlighted a cline that is parallel to the one identified on the Eastern coastline of North America by Mérot et al. (2021). On both continents, the latitudinal cline was in the same direction with the B arrangement more frequent in the North and the A arrangement more frequent in the South. This parallelism provides indirect evidence that the inversion is involved in adaptation to the same environmental gradient across continents (Westram et al., 2022).

Because several eco-climatic variables - such as photoperiod, temperature seasonality, desiccation, risk of frost, but also often competition, diseases and parasitism prevalence (Cuevas et al., 2019) - co-vary with latitude, it is difficult to disentangle which factor may exert natural selection on the inversion. Temperature is a good candidate because it is one of the most stressful and influential abiotic factors for the survival all of living organisms, and especially for ectotherms, such as insects (Denlinger & Lee Jr, 2010). Testing this hypothesis, we reported in fact a significant relationship between inversion frequencies and annual mean air temperature on both continents. Such parallel association between inversion and temperature gradients is also observed in several other species, particularly Drosophila. For example, in *D. melanogaster*, four inversions form worldwide parallel latitudinal clines (Kapun & Flatt, 2019), suggesting that inversions may be an important genetic architecture for repeated adaptation along thermal gradients.

Phenotypic impact of *Cf-Inv(4.1)* and temperature

Although the implication of inversions in adaptation to different environments is increasingly suspected or demonstrated in a large number of species, pinpointing the underlying traits and functional mechanisms remain challenging and requires a direct, experimental approach. Adaptation along a climatic gradient may in fact involve a wide range of traits. Insects are particularly sensitive to temperature, which affects their survival at all stages and fecundity (Régnière et al., 2012). Typically, each species has an optimal range of temperature and higher temperature induces lower survival while lower temperature delays development (Hoffmann et al., 2003). Although thermal tolerance, often assessed through critical thermal limits, has traditionally been used to predict climate adaptation, and species persistence with climate change, recent studies suggest that reproductive thermal limits may provide a more accurate predictor of vulnerability. These limits could offer a finer measure of species thermal susceptibility than survival (Parratt et al., 2021; van Heerwaarden & Sgrò, 2021). Our results in *C. frigida* confirm that temperature plays an important role in several life-history traits. The life cycle duration of *C. frigida* increased when temperature decreased, with a longer development time but also a longer longevity. Viability and fecundity (total fecundity and number of clutches) were also strongly affected by temperature, forming classical thermal performance curves. At the thermal limits, insects may display different responses including reversible loss of motor function (hot/cold coma). Interestingly, some of those traits are associated with inversions in other species, hence explaining variation in inversion frequencies along climatic gradients. For example, Ma et al. (2024) experimentally demonstrated the role of three inversions in range expansion across a climate gradient mediated by an effect on the maximal critical temperature (CTmax). In the mosquito *Anopheles gambiae*, the chromosomal inversion 2La, which form aridity clines in Africa, is associated with increased survival to heat stress following heat hardening (Rocca et al., 2009).

In *C. frigida*, we did not observe any effect of *Cf-Inv(4.1)* on cold tolerance, with our selected metrics, but we did report significant tolerance to prolonged exposure to subzero temperatures (16h at -5°C) and deep supercooling ability (ranging from -7.8°C to -23.9°C). This resistance suggests that *C. frigida* follows a freeze-avoidance strategy, specifically classified as "chill-tolerant" according to Bale's classification (Bale, 1993). Chill-tolerant species can endure extended exposure to sub-zero temperatures by remaining in a supercooled state, but they risk mortality when temperatures exceed their supercooling point (SCP). In those species, measuring SCP alone may not be the most relevant method for assessing cold tolerance, as they will likely be more affected by prolonged chilling rather than by freezing. To fully characterize thermal tolerance in *C. frigida*, a more comprehensive

approach is likely needed, such as the Thermal Death Time (TDT) methods, which combines many intensities of stress at many durations (Jørgensen et al., 2022; Rezende et al., 2014).

Cf-Inv(4.1) had direct and significant impacts on some life-history traits, in interaction with thermal conditions. Egg-to-adult viability varied with temperature and inversion karyotypes, forming classical performance curves, with overall broader amplitude and higher survival in AA karyotypes than BB. Female fecundity, on the other hand is higher for BB than AA, suggesting that the inversion *Cf-Inv(4.1)* may strongly influence fitness. Parallelism in inversion-phenotype relationship is nevertheless not confirmed in our dataset. For fecundity, the signal is in the same direction on both continents but not significant in Europe while viability shows a pattern opposite to that observed in America. Beyond a different genetic background between the two continents, we also noticed that the inversion *Cf-Inv(1)*, which also impacts fecundity and viability (Mérot et al., 2020), was in heterogeneous frequencies in European lines and may have acted as a confounding factor. Overall, it is interesting to note that the inversion *Cf-Inv(4.1)* tend to have opposing effects on different fitness traits, possibly placing the two karyotypes at different levels in the trade-off between surviving and reproducing. This seems to be a common property of large inversions/supergenes, possibly due to the high number of genes linked together or to the accumulation of deleterious mutations, and it is supported by numerous examples in the literature, as in zebra finches (Pei et al., 2023), in *Heliconius numata* butterflies (Jay & Joron, 2022) and in stick-insects (Nosil et al., 2023). Indeed, if the inversion links an additive beneficial allele with a recessive deleterious allele, polymorphism can be maintained through antagonistic pleiotropy of the entire inversion haplotype (Pei, 2021).

The challenge of understanding the clinal pattern in the light of functional impacts

While the parallel clines in inversion frequency support a role for spatially variable selection along the eco-climatic gradient, laboratory experiments highlight antagonistic fitness effects for broad conditions of temperature. This may reflect the fact that inversion evolution is likely driven by multiple interacting processes occurring at the same time because large inversions encompass hundreds of genes and impact a broad range of phenotypes (Berdan et al., 2023). For instance, Nosil et al. (2023) demonstrated that an inversion polymorphism in *Timema* stick insects is maintained by a combination of processes, including life-history trade-offs, heterozygote advantage, local adaptation to different hosts, and gene flow.

The cline of inversion frequencies observed in the wild let us expect the southern arrangement to be favored under high temperatures and the northern arrangement to be favored under low temperatures. Although experiments revealed an effect of *Cf-Inv(4.1)* on some life-history traits, we didn't detect sufficient interaction with temperature to explain the cline. It is worth to note that beside constant exposures, the effect of temperature could act through diverse variables such as temperature seasonality, daily fluctuations, or even act in combination with other climatic variables (precipitations, season duration, day length). For instance, Xing & Zhao (2022) demonstrated the effect of daily temperature amplitudes on longevity, fecundity and the thermal tolerance of adult diamondback moths. Furthermore, the cline could be explained by inversion-latitude effects on fitness traits that were not measured, like mating or foraging activity under varying conditions of light and temperature (Rivas et al., 2018). The inversion contains in fact relevant genes possibly underlying phenotypic variation along a latitudinal gradient in circadian rhythm in interaction with temperature. For exemple, *Cf-Inv(4.1)* includes *regucalcin*, a gene paralogous to Drosophila cold acclimatation gene (*Dca*), which is involved in the adaptive response to low temperatures (Arboleda-Bustos & Segarra, 2011) and *bric à brac*, whose expression level varies with temperature in *Drosophila melanogaster* (De Castro et al., 2018), and which is a key regulator of sex pheromone choice in European corn borer moth males, strongly influencing mating behaviors (Unbehend et al., 2021). It is also worth noting the proximity of genes involved in the circadian rhythm such *period* and *tilB*, which also mediate the interaction between temperature and the biological clock (Kaiser et al., 2016; Sehadova et al., 2009) with *period* showing a latitudinal cline in *Drosopila* (Sawyer et al., 1997). The gene *Asterix*, also located within *Cf-Inv(4.1)* is an essential gametogenesis factor that is conserved from insects to humans (Ipsaro & Joshua-Tor, 2022), and could be linked to the effect of the inversion on fecundity observed in *C. frigida*.

Classically a cline of frequencies is thought to result from equilibrium between selection and migration. However, (Barton & Gale, 1993) mentioned an alternative model according to which a cline is formed by balancing selection whose equilibrium varies between populations according to the environment. According to Westram et al. (2022), this scenario is particularly relevant for stable, large-scale clines, as it allows for the combination of strong

selection with subtle frequency gradients. For instance, this model is strongly suspected to underlie inversion *In(3R)Payne* parallel clines in *Drosophila melanogaster*, which displays gradual frequency changes across a broad geographic range (Kapun & Flatt, 2019; Westram et al., 2022). We therefore speculate that, in the context of a trade-off between fecundity (higher in BB) and thermal stress tolerance (higher in AA), the relative fitness value of both arrangements of *Cf-Inv(4.1)* may differ along the latitudinal thermal gradient, so that frequencies at equilibrium vary clinally. It is possible that high latitude conditions, such as low temperature but also shorter days or a shorter season, select for a higher investment in fecundity which would increase fitness value of arrangement B. Conversely, environmental conditions at low latitude, such as higher temperature or stronger fluctuations of temperature due to a thinner and less-buffered wrack bed habitat, in combination with competition and desiccation stress, could select for an enhanced survival during larval phase, which would increase the fitness value of arrangement A. Overall, such as scenario of a balanced cline (Barton & Gale, 1993) is more plausible for a polymorphic cline, where neither allele reaches fixation at either end of the cline, as we observed for *Cf-Inv(4.1).* As this kind of inversion under balancing selection is expected to be polymorphic over a wide range, it is also more probable that the colonization of a new environmental gradient, even by a small funding group, result in the appearance of a parallel large-scale cline (Westram et al., 2022).

Conclusion and prospects

With this study, we highlight a shared polymorphic inversion in *C. frigida* between Europe and America, which exhibits conservation of its sequence and parallel latitudinal clines. This provides empirical support for the prediction that inversions are often involved in repeated adaptation to parallel environmental gradients (Westram et al., 2022). By integrating field-based and lab-based data, we enhanced the understanding of the functional implications of chromosomal inversions. We demonstrated that *Cf-Inv(4.1)* affects fecundity and the thermal curve for larval survival. We conjecture that the overall fitness resulting from a trade-off between those traits may be differently balanced by temperature. Such a scenario would explain the variation of inversion frequency equilibrium along the North-South gradient, a phenomenon which seems to be partially reproduced under controlled conditions of experimental evolution.

To further address the role of chromosomal inversions in parallel adaptive processes, it is critical to explore all levels of variability. For example, it is yet unclear to what extent parallelism extends to the whole inversion sequence. Sequencing over the entire inversion is now required to trace the evolutionary and demographic history of rearrangements and to understand intra-inversion diversity (*cf.* Kim et al., 2022; Knief et al., 2024; Lundberg et al., 2023). Additionally, a deeper understanding of functional effects, and their conservatism or novelty across continents, could be inferred by investigating the extent of parallelism in gene expression (*e.g.* Fuller et al., 2016; Said et al., 2018). In fact, while our genetic data tend to support repeated patterns of inversion distribution across continents, experimental data suggest that associated phenotypes not be so consistent, underlining the notion of parallelism may depend on the biological level considered (Bolnick et al., 2018; Fischer et al., 2021; Therkildsen et al., 2019).

Data Availability

Whole genome sequences are available on ENA (PRJEB86057).

Data supporting this study are available in supplementary materials.

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Author Contribution Statements

LN: Conceptualization, Formal analyses, Investigation, Project administration, Visualization, Writing – original draft.

EB: Conceptualization, Formal analyses, reviewing the draft.

PDW: Conceptualization, reviewing the draft.

HC: Conceptualization, reviewing the draft.

AC: Formal analyses, Investigation, Visualization.

SG: Conceptualization, co-supervision, reviewing the draft

CM: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft.

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Conflict of Interest

The authors declare no conflict of interest.

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