Ancestrally polymorphic chromosomal inversions as potential drivers of speciation

Running title: Inversions and speciation in *Drosophila*

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Abstract

Chromosomal inversions may play a central role in speciation through their ability to reduce recombination and therefore genetic exchange at inverted parts of the genome between diverging populations. We analysed long- and short-read whole-genome data from sympatric and allopatric populations of two *Drosophila virilis* group species, *D. montana* and *D. flavomontana*, to understand how inversions have contributed to the divergence of these sister species. Using highly contiguous genome assemblies for both species, we identified three large alternatively fixed inversions on the X chromosome and one in each of the autosomes 2L, 4 and 5. A comparison of demographic models estimated for inverted and non-inverted (colinear) chromosomal regions suggest that these inversions most likely arose before or around the time of the species split. We also detected low rate of interspecific gene flow (introgression) from *D. montana* to *D. flavomontana*, which was further reduced inside inversions and was lower in allopatric than in sympatric populations. Together these findings suggest that the inversions have reduced gene exchange between these sister taxa both before and after the onset of species divergence and that low levels of recent introgression may have led to the reinforcement of reproductive barriers previously detected between these species. Our results suggest that ancestrally polymorphic inversions may act as drivers of speciation by allowing the accumulation of divergence inside inversions, while gene flow at colinear regions continues until the evolving reproductive barriers complete speciation.

Key words: chromosomal inversion, coalescence, *Drosophila flavomontana, Drosophila montana*, genetic divergence, introgression, speciation

Introduction

Chromosomal inversions, genomic regions with reversed gene order relative to the standard form, have been detected in many species of insects, fish, birds, mammals and plants, but their frequency varies widely between and even within taxa (Stone et al. 1960; Wellenreuther and Bernatchez 2018). Inversions are suggested to be important drivers of adaptation and speciation with gene flow, as they can suppress recombination between alternate rearrangements and create associations between sets of alleles conferring local adaptation, mate choice and genetic incompatibilities (Sturtevant 1921; Butlin 2005; Hoffmann and Rieseberg 2008). While experimental evidence of inversions' effects on speciation is still scarce, genomic data from new species pairs, together with proper inference of the history of inversions and interspecific gene flow, offer a promising way to increase our understanding on the role of inversions in speciation (Faria and Navarro 2010; Faria et al. 2018).

Inversions have attracted scientists for decades, which has given rise to a wide range of theories on the role of inversions in adaptation and speciation with gene flow (reviewed in (Hoffmann and Rieseberg 2008; Faria and Navarro 2010)). A newly established inversion may be favoured by selection if it protects locally adapted allele complexes within inverted region by reducing recombination between populations with alternate rearrangements (Kirkpatrick and Barton 2006). Also, inversion breakpoints may be direct targets of selection, if they change the reading frame and/or expression patterns of genes located on these regions (Matzkin et al. 2005; Guerrero et al. 2012). Once an inversion becomes fixed between diverging lineages, it may contribute to their speciation in different ways depending on whether the populations evolve in isolation or in contact with each other. In allopatric speciation, long-term geographic isolation between populations will inevitably lead to a build-up of genetic incompatibilities as an incidental by-product of drift and selection (Seehausen et al. 2014). In secondary contact between these populations, the existing genetic incompatibilities residing within inversions will be protected, while the ones located outside of them are more susceptible to the homogenizing effects of gene flow (Noor et al. 2001). On the other hand, populations that diverge in the presence gene flow may accumulate genetic divergence and genetic incompatibilities within inversions over generations of reduced recombination on the inverted gene regions (Navarro and Barton 2003). In both scenarios, inversions harbouring incompatibilities delay species' fusion and provide time for additional barriers to emerge. For example, prezygotic reproductive barriers are expected to be reinforced in response to genetic incompatibilities and maladaptive hybridization (reinforcement) (Servedio and Noor 2003), particularly if the loci responsible for prezygotic isolation and genetic incompatibilities are located within inversions (Trickett and Butlin 1994; Butlin 2005). Two kinds of empirical observations give indirect support for these theories. First, genes maintaining local adaptation, as well as premating barriers and genetic incompatibilities between species, have often found to be located within fixed inversion differences (Noor et al. 2001; Lowry and Willis 2010; Fishman et al. 2013). And second, fixed inversions generally have elevated genetic divergence compared to colinear chromosomal regions (Noor et al. 2007; Kulathinal et al. 2009; Lohse et al. 2015).

Speciation via suppressed recombination may occur in nature if (1) closely related species carry alternatively fixed inversions, (2) inversions suppress recombination and (3) the suppression of recombination plays an important role in reproductive isolation (Faria and Navarro 2010). So far only a few studies have dissected the evolutionary history of inversions to explore their role in speciation (e.g. (Lohse et al. 2015; Fuller et al. 2018)). Demographic models can quantify the age of inversions in relation to the species' divergence time of the colinear background, and the amount of long-term effective post-divergence gene flow (introgression) inside and outside inversions (Noor and Bennett 2009). Similarly, recent or ongoing introgression can be diagnosed by comparing estimated gene flow between sympatric and allopatric population pairs (Noor and Bennett 2009). Inversions, which have fixed around or after the onset of species divergence can be favoured by selection to protect existing genetic incompatibilities from the homogenising effects of introgression, if the species live in contact with each other at some stage. On the other hand, inversions whose divergence time predates the species' split must have been in a polymorphic state in the common ancestral populations, with different frequencies

in subpopulations/lineages. These inversions may play a major role in establishing patterns of elevated genetic divergence within inversions, even without extensive gene flow occurring after the species' split (Noor and Bennett 2009; Fuller et al. 2018).

Two *Drosophila virilis* group species, *Drosophila montana* and *Drosophila flavomontana*, offer a great opportunity to investigate the role of inversions in the formation of species boundaries. Based on the polytene chromosome studies, these species have several alternatively fixed inversions (Stone et al. 1960; Throckmorton 1982), but studies on the genomic divergence of the inversions are still lacking. Species of the *montana* complex (*D. montana*, D. *flavomontana*, *D. lacicola* and *D. borealis*) have diverged ~3.8 Mya in the Rocky Mountains area (Patterson 1952; Throckmorton 1982; Hoikkala and Poikela 2022; Yusuf et al. 2022), and presently *D. montana* and *D. flavomontana* inhabit variable climates in the Rocky Mountains and the western coast of North America (Hoikkala and Poikela 2022). In the mountains, *D. montana* has spread to higher altitudes than *D. flavomontana*, while on the western coast, where *D. flavomontana* has spread relatively recently, both species live at low altitudes (Fig. 1; (Patterson 1952; Poikela et al. 2019; Hoikkala and Poikela 2022)). In both sites, populations of the two species can be regarded as sympatric or parapatric. Moreover, *D. montana* has allopatric populations on high latitudes, e.g. in Alaska, where *D. flavomontana* does not exist (Fig. 1; (Poikela et al. 2019)). *D. montana* and *D. flavomontana* have strong pre- and postzygotic barriers across their distribution range, and *D. flavomontana* also show signs of reinforcement in prezygotic barriers in sympatric populations (Poikela et al. 2019). Regardless of the strong reproductive isolation, some interspecific hybrids have been reported to be found in nature (Patterson 1952; Throckmorton 1982), and they can also be produced in the laboratory (Poikela et al. 2019).

Here, we explored whether and how inversions can contribute to speciation process, using *D. montana* and *D. flavomontana* as a model system. Long- and short-read sequencing data from allopatric and sympatric populations of the species enabled us to accurately identify the presence of alternatively fixed inversions between the species. Demographic modelling allowed us to estimate the age of these inversions and estimate their effect on the long-term rate of introgression. We asked the following questions:

(1) How many alternatively fixed inversions do *D. montana* and *D. flavomontana* carry?
(2) What are the likely ages of these inversion differences and how do they compare to the species divergence time?
(3) Have inversions contributed to the speciation process by protecting existing genetic incompatibilities in secondary contact and/or by accumulating them in the presence of gene flow?

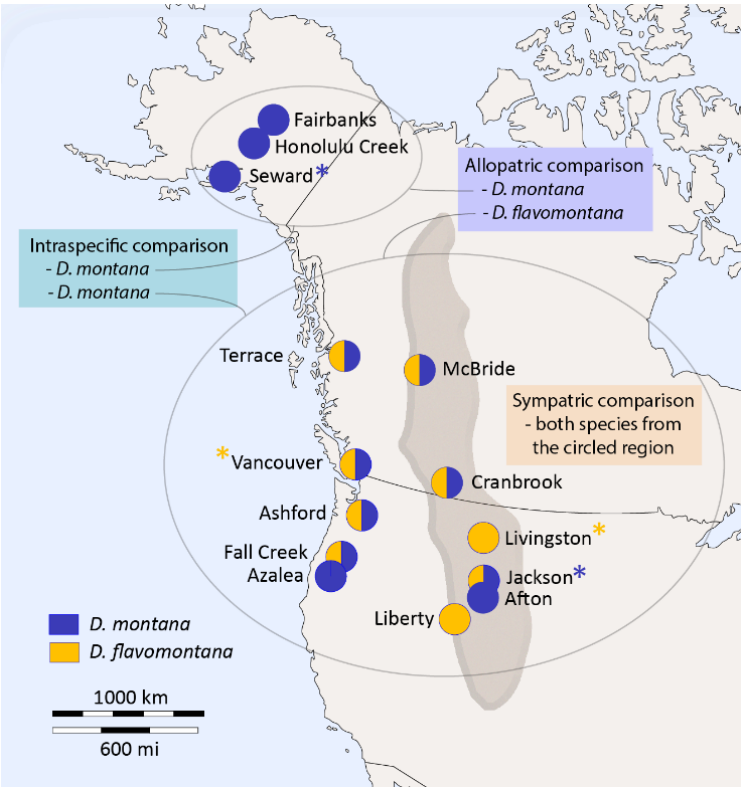

Figure 1. Sampling sites of sympatric (or parapatric) and allopatric *D. montana* and *D. flavomontana* populations in North America. Pie charts indicate the sampling sites for one or both species. PacBio data was obtained from the pools of females from one isofemale strain originating from the sample sites indicated with asterisks. Illumina data was obtained for single wild-caught females for all sites with and without asterisks. Detailed information on sequenced samples is given in Table S1.

Results and Discussion

We generated PacBio (Pacific Biosciences) sequencing data for females from two *D. montana* and two *D. flavomontana* isofemale strains originating from allopatric and sympatric populations (Fig. 1; Table S1). We also generated Illumina re-sequencing data for 12 *D. montana* and 9 *D. flavomontana* wild-caught females (one female per population per species). These data enabled us to generate contiguous high-quality genome assemblies for both species and accurately identify alternatively fixed inversions between the species, as well as to examine species' evolutionary history and the role of inversions and introgression therein. In species comparisons, *D. montana* and *D. flavomontana* samples from the Rocky Mountains and western coast are referred as "sympatry", and the comparisons between *D. montana* from Alaska and *D. flavomontana* from the mountains and the coast as "allopatry" (Fig. 1; Table S1).

Construction and annotation of species genomes

Two contiguous genome assemblies for each species were generated using the PacBio data of two *D. montana* and *D. flavomontana* isofemale strains and the Illumina data for the respective founder females collected from the wild (Table S1, Table S2). The assembled genomes had a total length of 181-194 Mb (Table 1), which resemble those of previously assembled *D. montana*, *D. flavomontana* and several other *Drosophila* species (128-198 Mb) (Miller et al. 2018; Parker et al. 2018; Yusuf et al. 2022). A small proportion of each assembly (0-18 contigs; span = 0.0-9.9 Mb) was excluded as contaminant sequences, mainly bacteria, based on the coverage, GC% and taxonomic annotation of contigs (Fig. S1-S4). From the 3285 BUSCO groups, 96.9-98.0% were complete single-copy BUSCOs, while 0.4-0.5% could not be identified (Table 1). The BUSCO values were similar to the ones in other *Drosophila* assemblies (Miller et al. 2018). Repetitive sequences comprised 25.5-29.9% of our assemblies (Table

1), which is close to the repeat content reported for other *Drosophila* species (e.g. 26.5% in *D. virilis*, 28.6% in *D. melanogaster*, 22.9% in *D. mojavensis*, and 19.9% in *D. elegans*; NCBI Annotation Report). Finally, we annotated 15,696-16,047 genes in each assembly, which falls within the range of 14,699-17,730 identified genes for other *Drosophila* species (e.g. (Yang et al. 2018)). Our combination of long- and short-reads resulted in more contiguous and complete assemblies for both species compared to the previously published *D. montana* and *D. flavomontana* genomes generated only from short-read data (Parker et al. 2018; Yusuf et al. 2022). For example, the previous *D. montana* genome had an N50 of 41 kb compared to the N50 values of 1.3-11.0 Mb in our assemblies (Table 1).

We built a chromosome-level reference genome for *D. montana* by scaffolding with *Drosophila lummei* genome, and for *D. flavomontana* by first scaffolding one assembly with the other (within species) and then with the *Drosophila lummei* genome (details in Methods). For both chromosome-level genomes, the total genome size, BUSCO values and the number of repeats and genes slightly decreased compared to the original, non-scaffolded assemblies (Table 1). Given the longer genome and higher BUSCO values of the *D. montana* chromosome-level reference genome, subsequent analyses were performed using the *D. montana* genome as a reference by default. However, to quantify the effect of reference bias we repeated the demographic inference using *D. flavomontana* as a reference.

Table 1. Genome contiguity, completeness and annotation of assemblies. N50 = half of the genome is in contigs larger than or equal to the N50 contig size. N50 count = half of the genome consists of the number indicated in N50 count.

		D. montana Seward, USA monSE13F37	*D. montana* Jackson, USA monJX13F48	*D. flavomontana* Livingston, USA flaMT13F11	*D. flavomontana* Vancouver, Canada flaVAN14F20	*D. montana* chromosome-level reference genome	*D. flavomontana* chromosome-level reference genome
Genome contiguity							
	Genome size (Mb)	184.3	181.0	192.6	193.5	145.5	142.3
	Total contigs	324	796	376	296	6	6
	Longest contig (Mb)	29.1	15.2	29.0	26.8	32.5	30.7
	N50 (Mb)	11.0	1.3	9.8	7.8	26.5	27.2
	N50 count	5	20	6	6	3	3
Genome completeness							
	Total complete BUSCOs (%; n: 3285)	98.1	98.5	97.5	97.3	91.9	91.5
	Single copy BUSCOs (%)	97.6	98.0	97.1	96.9	91.7	91.2
	Duplicated BUSCOs (%)	0.5	0.5	0.4	0.4	0.2	0.3
Genome annotation							
Repeat elements	**GC level (%)**	40.2	40.2	40.2	40.1	40.3	40.3
	Masked repeats (%)	26.6	25.5	29.9	28.2	16.8	15.1
Gene annotation	**Genes (N)**	15,696	15,819	15,940	16,047	15,446	14,901
	Mean gene length (bp)	1,640	1,637	1,568	1,564	1,571	1,557
	Total complete BUSCOs (%; n: 3285)	94.2	94.7	92.5	92.9	88.5	87.3
	Single copy BUSCOs (%)	84.4	84.5	84.1	84.5	79.9	79.3
	Duplicated BUSCOs (%)	9.8	10.2	8.4	8.4	8.8	8.0

Genetic differentiation of *D. montana* and *D. flavomontana* populations from climatically diverse regions with different species compositions

We visualised the variation in the Illumina re-sequence data of wild-caught *D. montana* and *D. flavomontana* females from different parts of North America with different species abundancies (Table S1, S2) to trace the species' evolutionary history. We first summarised genetic differentiation of the samples in a principal component analysis (PCA) using 9,064,171 filtered SNPs (from coding, intronic and intergenic regions). The first principal component (PC1) explained 50% of the total genetic variance and clearly separated *D. montana* and *D. flavomontana* samples from each other (Fig. 2A, Table S4). PC2 explained 4% of the total variance and captured mainly variation in *D. montana*, while variation in *D. flavomontana* was lower (Fig. 2A; Table S4). PC2 separated Alaskan (allopatric) *D. montana* populations (Honolulu Creek, Seward, Fairbanks) from the other *D. montana* populations, but also showed some variation among non-Alaskan populations (Fig. 2A).

Next, we explored climatic patterns of fly sampling sites to determine the extent to which climatic conditions may have affected the genetic differentiation of the samples. We performed a PCA on 19 bioclimatic variables of each fly sampling site (Table S5, Table S6) to reduce correlations between the variables and summarized climatic patterns prevailing in the sites. This PCA revealed three principal

components (PCs) with eigenvalues >1, of which the first two PCs explained 80% of the climatic variation (Fig. 2B; Table S7). The first PC clearly separated inland and coastal populations and suggested that populations from the mountainous inland experience cold winters and high seasonal temperature variation, while coastal populations experience milder temperatures and high precipitation throughout the year (Fig. 2B; Table S8). The second PC separated populations based on latitude and summer temperatures and distinguished Alaskan populations (Honolulu Creek, Seward, Fairbanks) from the other populations (Fig. 2B; Table S8).

Together these results show that *D. montana* and *D. flavomontana* populations are genetically diverged regardless of their climatic origin and species coexistence. Genetic differentiation was greater among *D. montana* populations than among *D. flavomontana* populations, which is likely due to *D. montana*'s larger distribution area and the fact that *D. flavomontana* has spread across North America more recently than *D. montana* (Hoikkala and Poikela 2022). Finally, the genetic differentiation between allopatric (from Alaska) and sympatric (from the Rocky Mountains and the western coast) *D. montana* populations likely reflects a demographic history of intraspecific divergence, local adaptation to climatic conditions, or both.

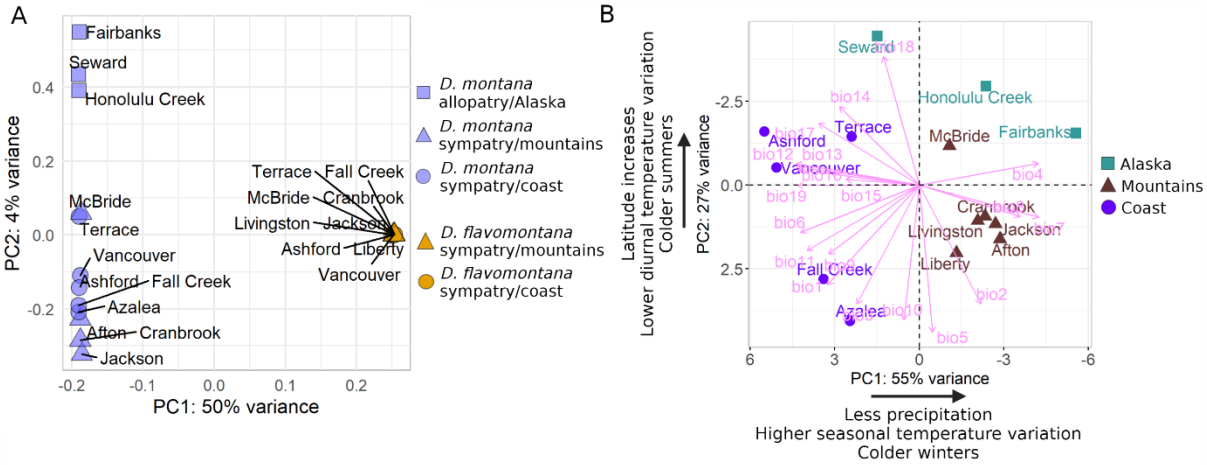

Figure 2. A principal component analysis (PCA) (A) on whole-genome SNP data of *D. montana* (*D. mon*) and *D. flavomontana* (*D. fla*) samples originating from different sites of North America and (B) on 19 bioclimatic variables (detailed explanations in Table S5) of each fly sampling site.

D. montana and *D. flavomontana* chromosomes differ by several large inversions

We combined long- and short-read genomic data to characterize inversion breakpoints in *D. montana* and *D. flavomontana*. We identified several large (>0.5kb) inversions that were alternatively fixed between North American *D. montana* or *D. flavomontana* samples (Fig. 3; Table S9). The X chromosome contained three partly overlapping inversions, two of which were fixed in *D. montana* (7.2Mb and 14.2Mb) and one in *D. flavomontana* (3.2Mb) (Fig. 3; Table S9). The left arm of the 2nd chromosome (2L) contained one inversion fixed in *D. montana* (3.9Mb), and chromosomes 4 and 5 each contained one inversion fixed in *D. flavomontana* (13.8Mb and 9.2Mb, respectively) (Fig. 3; Table S9). The sizes of *D. montana* and *D. flavomontana* inversions were obtained from the reference genomes of each species (Table S9). All inversions were homozygous in the studied Illumina samples (Table S9), except the 2L inversion, which was heterozygous in all *D. montana* samples. The right arm of the 2nd chromosome (2R) and the 3rd chromosome were not found to contain any inversions (Fig. 3). Based on the old polytene chromosome studies, where rearrangements were observed from salivary gland chromosomes using microscopy, the three alternatively fixed inversions between *D. montana* and *D. flavomontana* on the X chromosome were named E, F and G (Stone et al. 1960). These inversions could not be distinguished in more detail, and in contrast to our results, Stone et al. (1960) suggest that all of them are fixed in *D. flavomontana*. The inversions on the 4th and 5th chromosome have been named J

and E, respectively, in karyotype studies, but the 2L inversion could not be assigned to its previous name (Stone et al. 1960)

The average size of the inversions fixed in *D. montana* was 8.4Mb and those in *D. flavomontana* 9.4Mb. The X chromosome contained a higher number of inversions between these species than the autosomes (Fig. 3, Table S9). The average size of the reported inversions is 8.4 Mb both in animals and plants (Wellenreuther and Bernatchez 2018). Inversion frequencies are known to vary greatly between closely related *Drosophila* species: for example, *D. virilis* has no inversions while both its close relatives *D. americana* and *D. novamexicana* and more distant relatives including *D. montana* and *D. flavomontana* have several (Stone et al. 1960). It is not clear whether large inversions are common because of an observational bias, or because they suppress recombination between a larger set of locally adapted genes relative to shorter inversions and are thus under stronger positive selection (Wellenreuther et al. 2019). Our finding of a larger number of inversions on the X is consistent with theory showing that the fixation probability of X chromosomal inversions is higher than that of autosomal inversions, because selection can more effectively favour beneficial and purify deleterious recessive X-linked alleles than autosomal ones (Charlesworth et al. 1987; Vicoso and Charlesworth 2006; Charlesworth et al. 2018; Connallon et al. 2018).

The polytene chromosomes studies by Stone et al. (1960) and Throckmorton (1982) suggest that *D. montana* and *D. flavomontana* carry additional inversions that were not identified in this study. In particular, *D. flavomontana* may harbour one fixed inversion of unknown size on the 3rd chromosome (inversion E; (Stone et al. 1960)), which we might have missed due to the higher fragmentation of this chromosome compared to the other chromosome contigs (Table S3). Given our limited sample size and the explicit focus on fixed inversion differences, polymorphic inversions previously found in these species (Stone et al. 1960), which may be associated with local adaptation or other evolutionary processes within species (Fang et al. 2012; Kapun et al. 2016; Wallberg et al. 2017), were not identified here.

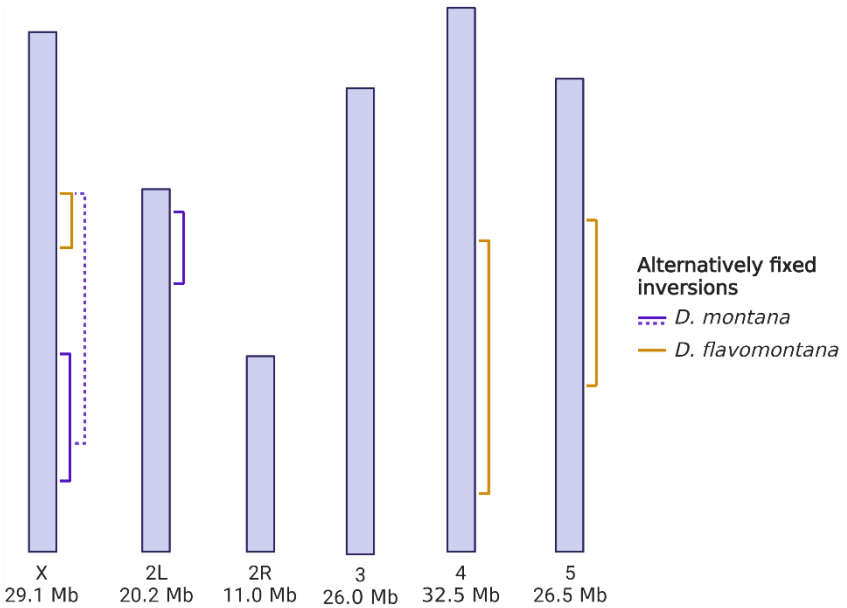

Figure 3. Chromosomes and large alternatively fixed inversions of *D. montana* (blue solid and dashed lines) and *D. flavomontana* (orange solid lines) (Table S9). The inversions are illustrated against *D. montana* chromosome-level genome coordinates and the numbers below each chromosome refer to its length in the assembly. Chromosome 2 has left (2L) and right (2R) arms that are separated by a submetacentric centromere.

Genetic divergence between *D. montana* and *D. flavomontana* is greater inside than outside inversions

We analysed the mean genetic divergence (d_{XY}) to test whether inversions have reduced recombination and introgression between *D. montana* and *D. flavomontana*, and if so, whether it is ancient or recent. In the latter case, d_{XY} should be lower in sympatry compared to allopatry (Harrison and Larson 2014). We estimated d_{XY} separately for coding, intronic and intergenic sequences and inverted and colinear regions of the genome. These analyses were carried out for allopatric and sympatric comparisons of the species as well as for intraspecific comparisons of *D. montana* populations (allopatric and sympatric *D. montana*). Given the potentially different evolutionary history of the X chromosome and autosomes (Vicoso and Charlesworth 2006; Charlesworth et al. 2018), analyses for the X (both colinear and inverted regions) were performed separately.

Mean d_{XY} values (between *D. montana* and *D. flavomontana*, and between the two *D. montana* populations) were remarkably similar for intergenic and intronic sequences but much lower in coding sequences (Fig. 4), as expected given the stronger selective constraint on coding sequence (Halligan and Keightley 2006).

d_{XY} was slightly, but consistently lower in sympatric compared to allopatric comparisons of the species across all chromosomal regions (Fig. 4; Table S10). Moreover, genetic divergence at non-coding (i.e. intergenic and intronic) sequences was consistently higher in inverted compared to colinear regions (Fig. 4), although the result was non-significant for the intergenic sequences of the 5th and the X inversion in sympatric populations (Table S10). We also found increased d_{XY} at coding sites for the X inversion compared to the colinear regions in allopatry and sympatry, and for the 4th chromosomal inversion compared to the colinear regions in allopatry (Fig. 4; Table S10). In contrast, the 2L inversion had lower d_{XY} at coding sites compared to the colinear regions in allopatry and sympatry (Fig. 4). The comparisons of d_{XY} at inverted and colinear regions were less clear within *D. montana* (Fig. 4). d_{XY} was significantly higher for the regions containing the 4th and the X inversions (intergenic, intronic and coding sequences) and significantly lower in the regions containing the 2L inversion (intergenic, intronic and coding sequences) compared to the colinear regions (Fig. 4; Table S10). Plotting d_{XY} in sliding windows showed an increase in genetic divergence, especially around the inversion breakpoints and for X chromosomal inversions, for interspecific, but not intraspecific comparisons (Fig. 5).

Overall, our finding of consistently higher genetic divergence of the non-coding sequences inside compared to outside of inversions supports the idea that inversions suppress gene exchange and facilitate the accumulation of genetic differences (Fig. 4; (Navarro and Barton 2003; Kirkpatrick and Barton 2006)). We also find that genetic divergence was highest around inversion breakpoints and in the series of overlapping inversions on the X (Fig. 5), where recombination is most suppressed (Hoffmann and Rieseberg 2008). Similar signatures of elevated genetic divergence between closely related species inside and around inversion breakpoints have been detected in other *Drosophila* species pairs (Noor et al. 2007; Kulathinal et al. 2009; Lohse et al. 2015), *Helianthus* sunflowers (Barb et al. 2014), *Sorex* shrews (Basset et al. 2006), and *Anopheles* mosquito (Michel et al. 2006). Furthermore, our finding of lower genetic divergence in sympatry compared to allopatry (Fig. 4) is consistent with low levels of recent introgression in sympatry (Noor and Bennett 2009).

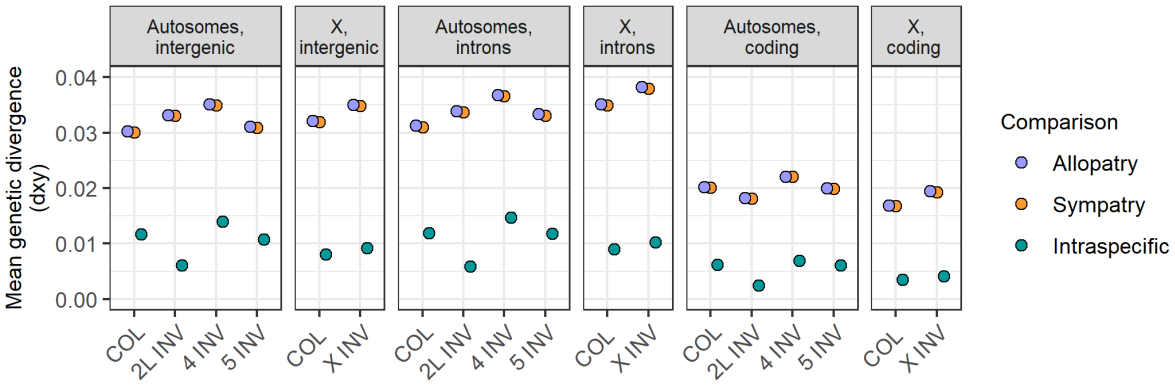

Figure 4. Mean genetic divergence (measured as d_{XY}) at intergenic, intronic and coding sequences, for autosomal and X chromosomal colinear (COL, background) and inverted (INV) chromosomal regions. Divergence is shown for allopatric and sympatric comparisons of *D. montana* and *D. flavomontana* and for intraspecific comparisons of *D. montana.* P-values are shown in Table S10.

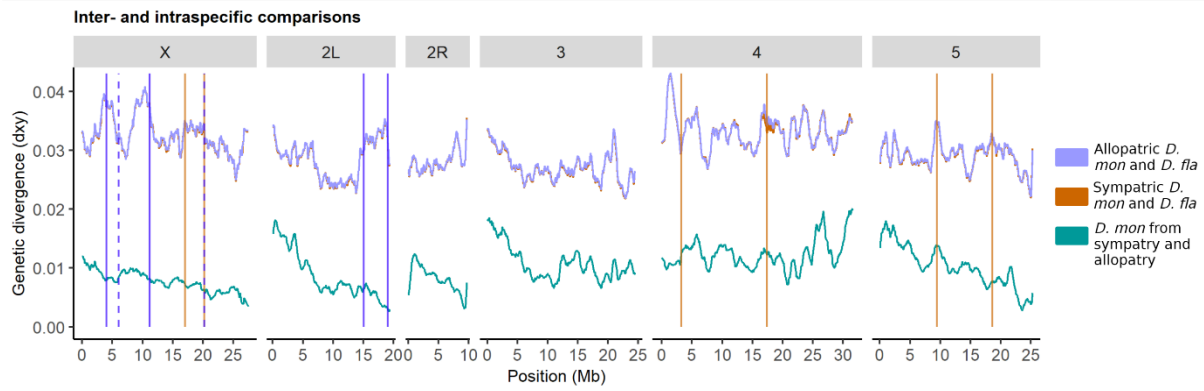

Figure 5. Genetic divergence across the genome (coding, intronic and intergenic regions) in sliding windows for allopatric and sympatric comparisons of *D. montana* and *D. flavomontana* (interspecific), and for *D. montana* originating from allopatry and sympatry (intraspecific). Inversion breakpoints of *D. montana* (blue) and *D. flavomontana* (orange) are marked with vertical lines (solid and dashed lines represent breakpoints of different inversions; see Fig. 3).

Hierarchical model comparison suggests species have diverged with a very low levels of post-divergence gene flow from *D. montana* to *D. flavomontana*

We used an analytic likelihood method to fit a series of demographic models of species divergence with and without long-term post-divergence gene flow, i.e. isolation with migration (IM) and strict divergence (DIV) models (Fig. 7) to the data summarised in terms of the bSFS (see Methods). The evolutionary history of the X chromosome (Vicoso and Charlesworth 2006; Charlesworth et al. 2018) and inversions may differ from other regions and these genomic partitions were therefore analysed separately from colinear, autosomal regions. Thus, our initial model comparison was based on only intergenic sequences of colinear autosomal regions (repetitive regions were excluded). We performed separate analyses for sympatric and allopatric comparisons of *D. montana* and *D. flavomontana* and for intraspecific comparison of *D. montana.* We carried out this initial model comparison twice, using both *D. montana* and *D. flavomontana* as a reference genome, to evaluate the potential effects of reference bias. Parameter estimates and support values (lnCL) under all demographic models are shown in Table 2 for 64 bp blocks and using *D. montana* as a reference (analogous analyses for all four combinations of block length and reference genomes are given in Table S11).

For both allopatric and sympatric comparisons (and for three of the four combinations of reference genomes and block sizes used), the best-fitting demographic scenario was an IM model assuming introgression from *D. montana* into *D. flavomontana* (Table 2, S11). Our parametric bootstrap analyses

showed that the improvement in fit of this IM model compared to the DIV model was significant suggesting a low but genuine signal of introgression (Fig. S5, S6). The only exception was the analysis using *D. flavomontana* as a reference genome and shorter blocks (64bp). In this case, the DIV model could not be rejected (Fig. S5, S6). However, estimates for all parameters (T and the three N_e parameters) were extremely similar regardless of the model (DIV and IM), block size (64bp and 128bp) and reference genome (*D. montana* and *D. flavomontana*) used (Table S11). Given the overall support for post-divergence gene flow and inherent bias of methods such as gIMble to underestimate migration rates (M), we present the IM model as the best fitting/most plausible scenario throughout. Accordingly, the subsequent analyses were conducted assuming an IM model with migration from *D. montana* into *D. flavomontana* (Table 2). Yusuf et al. (2022) also recently found signatures of introgression between *D. montana* and *D. flavomontana* using a different approach, which gives further support for our introgression signal.

For the intraspecific comparison within *D. montana*, the DIV model could not be rejected in any analysis. When using 64bp blocks, DIV and IM models had equal support, irrespective of which species was used as a reference (Table 2, S11). Analyses based on longer blocks (128bp) estimated slightly higher support for an IM model assuming post-divergence gene flow from allopatric (Alaskan) *D. montana* to sympatric (coastal/mountain) *D. montana* (Table 2, S11). However, our parametric bootstrap analyses showed that the associated improvement in fit compared to the simpler DIV model was non-significant. Thus, the DIV model could not be rejected (Table S5, S6). It is possible that allopatric *D. montana* populations exchange genetic material with the closest sympatric *D. montana* populations (e.g. Terrace and McBride) but not with the more distant ones, and thus the genuine post-divergence gene flow signal was not detected. Consequently, the subsequent intraspecific analyses were conducted using the DIV model (Table 2).

Table 2. Support (measured as ΔlnCL) and parameter estimates for divergence time (T), migration rate (M) and effective population sizes (N_e) for studied populations and their common ancestral population under strict divergence (DIV, M=0) and isolation with migration (IM) models with both gene flow directions. The model comparison is based on the *D. montana* reference genome and block size 64 bp, and was performed for intergenic autosomal colinear regions to minimize the effects of selection. Grey shading indicates the best-fit model for each comparison.

Comparison	Model	*D. mon* Ne	*D. fla* Ne	Ancestral Ne	T	M	lnCL	ΔlnCL
Allopatric comparison	DIV	714914	400196	1460808	2405724	-	-43059297	12929
(*D. mon* vs *D. fla*)	IM *D. mon --> D. fla*	728215	386120	1395634	2573631	1.13E-08	-43046368	0
	IM *D. fla --> D. mon*	714135	400661	1453429	2426511	1.27E-09	-43059034	12666
Sympatric comparison	DIV	746171	397288	1453485	2370833	-	-129149797	48447
(*D. mon* vs *D. fla*)	IM *D. mon --> D. fla*	762494	380755	1378191	2564214	1.34E-08	-129101350	0
	IM *D. fla --> D. mon*	744781	398093	1441519	2405381	2.21E-09	-129147830	46480
	Model	***D. mon allop* Ne**	***D. mon symp* Ne**	**Ancestral Ne**	**T**	**M**	**lnCL**	**ΔlnCL**
Intraspecific comparison	DIV	1150743	1688437	876904	227573	-	-38019871	0
(*D. mon* vs *D. mon*)	IM *D. mon symp. --> D. mon allop.*	1150745	1688438	876904	227573	3.38E-16	-38019871	0
	IM *D. mon allop. --> D. mon symp.*	1150695	1687674	876901	227611	1.65E-09	-38019871	0

Species-specific inversions were fixed earlier or around the species' split and introgression was lower inside compared to outside of inversions and in allopatry compared to sympatry

We used the best-fit isolation with migration (IM) model (Table 2) to examine the speciation history of *D. montana* and *D. flavomontana* and test the potential role of inversions and introgression in their speciation process. As before, the analysis was limited to intergenic regions to minimize the effects of selection, and separate analyses were carried out for the X chromosome and autosomes and for inverted and colinear regions. To estimate the timing of potential recent introgression, we also analysed the split time for *D. montana* living in contact (sympatry) or in isolation (allopatry) with *D. flavomontana* using the simpler strict divergence (DIV) model (intraspecific comparison; Table 2).

Taking the estimates for the colinear autosomal background as face value, *D. montana* and *D. flavomontana* have diverged ca 3.4 Mya (Fig. 6A, 6C). The divergence time estimates of the inversions

differ from each other, and the inversions on the 4th, 5th and the X chromosome predate the divergence time estimated for the colinear background (ca 3.6-4.0 Mya) (Fig. 6A, 6C; Table S12). In contrast, the 2L inversion has fixed after the onset of species divergence (ca 3.1 Mya) (Fig. 6A, 6C; Table S12). For all chromosome partitions, genetic diversity (Pi) and effective population size (N_e) of *D. montana* were approximately two times as large as those of *D. flavomontana* (Fig. S7, S8). *D. montana* populations living in contact (sympatry) and in isolation (allopatry) with *D. flavomontana* have diverged much more recently than *D. montana* and *D. flavomontana*, approximately 350 000 years ago (Fig. 6C).

Introgression from *D. montana* to *D. flavomontana* was lower inside than outside inversions both for the autosomes and for the X chromosome (Fig. 6B; Table S13), which is in accordance with the finding that genetic divergence of non-coding (intergenic and intronic) sequences was consistently higher inside than outside of inversions (Fig. 4; Table S10). Moreover, the migration rate estimates were significantly higher in sympatry compared to allopatry (Fig. 6B; Table S14), which agrees with the slightly, but consistently lower genetic divergence in sympatric compared to allopatric comparisons of the species (Fig. 4; Table S10).

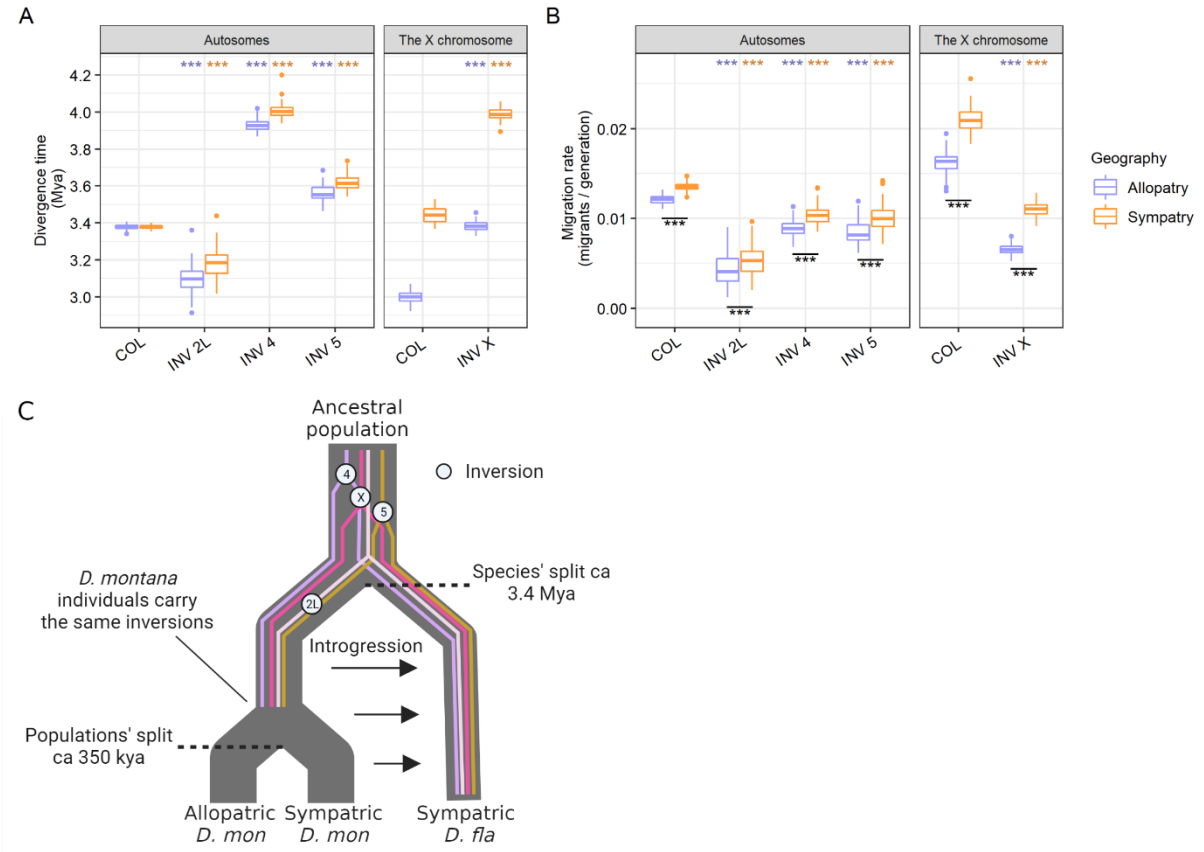

Figure 6. (A) The split times and (B) migration rate for different chromosomal regions and for *D. montana* and *D. flavomontana* originating from allopatric and sympatric populations. Confidence intervals were inferred using simulations under the best-fit IM model (simulated 100 times). Significance levels were obtained from generalized linear models (*** $P < 0.001$), where i) colinear region (COL, background) was compared to inverted (INV) regions separately for autosomes and the X chromosome and for allopatric and sympatric comparisons (significances above boxplots), and where ii) differences in migration rate between allopatric and sympatric populations were compared within each chromosomal region (significances below boxplots). Whiskers represent ±1.5×IQR. (C) Illustration of the likely evolutionary history of *D. montana* and *D. flavomontana* (split times are rough mean estimates across allopatric and sympatric comparisons).

Taken together, our analyses suggest that *D. montana* and *D. flavomontana* diverged ca 3-4 Mya (depending on the analysed regions), which is close to the recent estimate of 3.8 Mya based on 1336 single-copy orthologs and 100 randomly sampled small introns (Yusuf et al. 2022). All fixed inversions

in *D. montana* and *D. flavomontana*, except the inversion on 2L, predate the species split, and so must have existed already in the common ancestral population. According to Noor and Bennett (2009) and Fuller et al. (2018), the elevated genetic divergence observed within inversions predating species divergence is likely a consequence of reduced recombination and gene flow between locally adapted populations in the common ancestor rather than from a reduction in post-divergence gene flow or ongoing introgression. This is further supported by the fact that the estimated long-term rate of migration from *D. montana* to *D. flavomontana* was extremely small and several orders of magnitude lower than e.g. a similar estimate for *Drosophila mojavensis* and *Drosophila arizonae* (Lohse et al. 2015). *D. mojavensis* and *D. arizonae* have approximately one migrant per generation, while our estimate for *D. montana* and *D. flavomontana* is roughly 1 migrant in 80 generations. Thus, even the total probability of a lineage sampled in *D. flavomontana* to trace back to *D. montana* via migration (1-e^(-T M)) is only 3.4%.

Fuller et al. (2018) have recently proposed that ancestrally segregating inversions may act as slowly progressing drivers of speciation, by accumulating positively selected genetic differences conferring early ecological divergence, prezygotic barriers and an incidental build-up of genetic incompatibilities. Colinear regions, on the other hand, can keep mixing until the formation of strong reproductive isolation. For example, selection against introgressed ancestry in the new genetic background may generate reinforcement via prezygotic barriers to prevent maladaptive hybridization, and eventually complete the speciation process (Servedio and Noor 2003). Our results support this idea, and in fact, we have recently shown with experimental approach that introgression from *D. montana* to *D. flavomontana* across the X chromosome is prevented by a dominant incompatibility locus residing within the overlapping X chromosomal inversions (Poikela et al. 2022). However, we cannot verify whether the incompatibility allele on the X has evolved before or around the species' split, or afterwards (post-speciation event), and whether the lower migration rate inside compared to outside of inversions is a consequence of direct selection against introgression or simply the reduced recombination between alternate rearrangements. In contrast to the other inversions, the 2L inversion has fixed after the onset of species' divergence and shows lower levels of introgression than the other inversions, which may indicate strong selection against introgression. This could potentially imply causality between the fixation of the 2L inversion and the completion of speciation. With a similar approach, Lohse et al. (2015) showed that introgression between *D. mojavensis* and *D. arizonae* had ceased around the time when multiple overlapping inversions had fixed around the species split.

The low levels of introgression between *D. montana* and *D. flavomontana* observed here agree well with our previous evidence for strong pre- and postzygotic barriers between the species (Poikela et al. 2019). Also, species' differences in the usage of host trees and their ability to tolerate cold, accompanied with altitudinal and latitudinal variation in their distribution (Throckmorton 1982; Poikela et al. 2021), might have contributed to species' ecological isolation and reduced their encounters in nature. Intriguingly, we found higher levels of introgression in sympatry compared to allopatry, which suggests at least some gene flow from *D. montana* to *D. flavomontana* over the past ~350 000 years, i.e. after the allopatric (Alaskan) *D. montana* population diverged from *D. montana* coexisting with *D. flavomontana*. This is consistent with our previous finding that *D. flavomontana* has developed stronger prezygotic barriers against *D. montana* in sympatry compared to allopatry, presumably as a result of reinforcement (Poikela et al. 2019). Even low levels of introgression can facilitate strengthening of prezygotic barriers to prevent maladaptive hybridization between species (Servedio and Noor 2003; Cruickshank and Hahn 2014).

Even though many species pairs differ from each other by multiple inversions, the majority of inversion differences must have arisen after speciation (Faria and Navarro 2010). Performing pairwise comparisons for younger and older species would offer a more holistic view of the role of inversions in speciation events. In our case, characterizing inversions and investigating divergence times and introgression across all species of the *montana* phylad of the *virilis* group (*D. montana*, *D. flavomontana*, *D. borealis* and *D. lacicola*) (Hoikkala and Poikela 2022) could provide valuable additional information.

436 In general, investigating millions of years old events by fitting necessarily drastically simplified scenarios
437 of reality involve uncertainties. Experimental approaches, potentially with the help of CRISPR/Cas9 gene
438 editing technique, could allow the creation of artificial inversions or reversion of existing ones, and
439 direct tests of the role of inversions in speciation (Faria and Navarro 2010; Faria et al. 2018; Hopkins et
440 al. 2020; White et al. 2020).

441 Conclusions

442 It has proven extremely difficult to test if and how inversions facilitate speciation, and empirical
443 evidence on the role of inversions in speciation is largely lacking (Faria and Navarro 2010; Fuller et al.
444 2019). We explored these questions in two sister species of the *D. virilis* group, *D. montana* and *D.*
445 *flavomontana*. Our main goals were (1) to characterize alternatively fixed chromosomal inversions of
446 *D. montana* and *D. flavomontana*, (2) to investigate the age of the inversions, and (3) to identify
447 whether the inversions have restricted recombination between *D. montana* and *D. flavomontana*, and
448 either protected existing genetic incompatibilities in secondary contact, or have facilitated the
449 accumulation of incompatibilities in the presence of gene flow.

450 Taking advantage of long- and short-read genome sequencing technologies, we were able to generate
451 the high-quality contiguous reference assemblies for *D. montana* and *D. flavomontana*. These genomes
452 enabled us to accurately characterize inversions that are alternatively fixed between these sister
453 species across their distribution area in North America. We were able to assign the majority of these to
454 inversions that were described for the species already in the 1950s and 1960s based on polytene
455 chromosome studies (Stone et al. 1960). Our analyses show clearly that the inversions on the 2L, 4th,
456 5th and the X chromosome arose either before or at the onset of species divergence. Thus, the elevated
457 genetic divergence inside compared to outside of inversions results most likely from restricted
458 recombination and gene flow between locally adapted populations of the ancestral form. Accordingly,
459 our results support the idea that ancestrally polymorphic inversions can drive the speciation process by
460 aiding the accumulation of genetic incompatibilities (Fuller et al. 2018), while colinear regions keep
461 exchanging genetic material until strong reproductive isolation has formed. This is also supported by
462 our recent finding that the X chromosomal inversions contain a strong incompatibility locus that
463 effectively restricts introgression between *D. montana* and *D. flavomontana* in the first few backcross
464 generations (Poikela et al. 2022).

465 Overall, our study suggests that inversions may indeed be early triggers in the speciation process and
466 highlights the value of interpreting the evolutionary effects of inversions through the lens of
467 demographic models. However, in doing so, we have ignored much of the mechanistic and selective
468 detail of inversion evolution. Regions with repetitive sequences, such as transposable elements, tRNAs,
469 ribosomal genes or segmental duplications, are prone to breakage and are often the initial source of an
470 inversion (Kapun and Flatt 2018). Investigating repetitive sequences or small structural variations
471 around the inversion breakpoints would increase our understanding on how the inversions originated
472 in the first place. Moreover, inversions are not static through their lifetime, but evolve in response to
473 changes in selection, genetic drift, new mutations and gene flux (occurring via double cross-overs and
474 gene conversion), as well as by interactions with colinear genome or with other inversions (Faria et al.
475 2018). Given the many, sometimes entangled processes affecting the origin and the fixation of
476 inversions, models that can extract information about both demography and the selective forces acting
477 on inversions in the early stages of speciation are the next obvious step in understanding how
478 inversions facilitate the origin of species (Faria et al. 2018).

Materials and Methods

Sample collections and maintenance

D. montana and *D. flavomontana* females were collected from several sites in the Rocky Mountains and the western coast of North America, and Alaska between 2013 and 2015 (Fig. 1; Table S1). Sites in the Rocky Mountains and the western coast of North America are either inhabited by both species (sympatric sites: Jackson, Cranbrook, McBride, Terrace, Vancouver, Ashford, Fall Creek), or by one of the species with nearby sites inhabited by both species (parapatric sites: Liberty, Afton, Livingston, Azalea) (Fig. 1, Table S1). *D. montana* also has stable populations in high latitudes in Alaska, where *D. flavomontana* does not exist. We refer to the comparisons between *D. montana* and *D. flavomontana* from the Rocky Mountains and from the western coast as "sympatry", and those between *D. montana* from Alaska and *D. flavomontana* from the Rocky Mountains or the western coast as "allopatry" (Fig. 1).

The newly collected females were brought to the fly laboratory, with a constant light, 19±1 ˚C and ~60% humidity, in the University of Jyväskylä, Finland. Females that had mated with one or several males in nature were allowed to lay eggs in malt vials for several days, after which they were stored in 70% EtOH at -20 ˚C. The emerged F_1 progeny of each female were kept together to produce the next generation and to establish isofemale strains. After that also the F_1 females were stored in 70% EtOH at -20 ˚C.

DNA extractions and sequencing

We performed PacBio long-read sequencing and generated four genome assemblies from two *D. montana* and two *D. flavomontana* isofemale strains that had been kept in the fly laboratory since their establishment (Fig. 1; Table S1). DNA of the Seward *D. montana* and both *D. flavomontana* samples were extracted from a pool of 60 three days old females per isofemale strain using cetyltrimethylammonium bromide (CTAB) solution with RNAse treatment, Phenol-Chloroform-Isoamyl alcohol (25:24:1) and Chloroform-Isoamyl alcohol (24:1) washing steps, and ethanol precipitation at the University of Jyväskylä, Finland. DNA of the Jackson *D. montana* sample was extracted with the "DNA Extraction SOP For Animal Tissue" protocol and purified with Ampure beads at BGI (Beijing Genomics Institute). Quality-checked DNA extractions of the Seward *D. montana* sample and both *D. flavomontana* samples were used to generate >15 kb PacBio libraries, which were all sequenced on two SMRT cells within a PacBio Sequel system (Pacific Biosciences, United States) at the Norwegian Sequencing Centre. DNA of the Jackson *D. montana* sample was used to generate >20 kb PacBio libraries and was sequenced on one SMRT cell using the PacBio Sequel system at BGI. Average PacBio raw read coverage was 27-35X per sample, except for the Jackson *D. montana* sample which was sequenced at 63X coverage. Detailed information on the PacBio raw reads of each sample is provided in Table S2.

We generated Illumina re-sequencing data for 12 *D. montana* and 9 *D. flavomontana* single wild-caught females or their F_1 daughters from several locations in North America (Fig. 1; Table S1). DNA extractions were carried out at the University of Jyväskylä, Finland, as described above. Quality checked DNA was used to produce an Illumina library for each sample in three batches. First, Nextera libraries were used to generate 150 bp paired-end (PE) reads on two lanes using HiSeq4000 Illumina instrument by Edinburgh Genomics. Second, one Truseq library was used to generate 150 bp PE reads on one lane of a HiSeq4000 Illumina instrument at the Norwegian Sequencing Centre. Third, Truseq libraries were used to generate 150 bp PE reads on one lane of a HiSeq X-Ten Illumina instrument at BGI. We generated on average 46-95X coverage per sample, except for the *D. montana* sample from Seward, which was sequenced to 371X coverage. Detailed information on Illumina raw reads is provided in Table S2.

525 Genome assemblies

526 We generated initial *de novo* assemblies for each PacBio dataset and the respective Illumina reads using
527 the wtdbg2 pipeline v2.5 (RedBean; Ruan & Li, 2020) and MaSuRCA hybrid assembler v3.3.9 (Zimin et
528 al., 2017). To improve assembly contiguity, we used quickmerge for both assemblies of each sample
529 (Chakraborty et al. 2016). The initial assembly statistics are given in Table S15. We polished the resulting
530 assemblies with the respective Illumina reads using Pilon v1.23 (Walker et al. 2014) and removed
531 uncollapsed heterozygous regions using purge_dups (Guan et al. 2020).

532 We identified genomic contaminants in the assemblies with BlobTools v1.1 (Laetsch and Blaxter 2017).
533 PacBio and Illumina reads were first mapped back to each assembly with minimap2 (Li, 2018) and BWA
534 mem (Burrows-Wheeler Aligner) v0.7.17 (Li & Durbin, 2009), respectively. Contigs in the assemblies
535 were then partitioned into taxonomic groups based on similarity search against the NCBI nucleotide
536 database (BLASTn 2.9.0+; Camacho et al., 2009) and Uniref90 (Diamond v0.9.17; Buchfink, Xie, & Huson,
537 2015). Finally, contigs were visualized on a scatter plot and coloured by putative taxonomic groups (Fig.
538 S1-S5). Non-Diptera contigs were removed manually from the assemblies based on sequence GC
539 content, read coverage and taxonomy assignment. We estimated the completeness of the assemblies
540 with the BUSCO pipeline v5.1.2 using the Diptera database "diptera_odb10" (Seppey, Manni &
541 Zdobnov, 2019), which searches for the presence of 3285 conserved single copy Diptera orthologues.

542 We constructed chromosome-level reference genomes for both species by scaffolding contigs of the
543 original assemblies with a reference-genome -guided scaffolding tool RagTag v2.1.0 (Alonge et al.,
544 2019), which orients and orders the input contigs based on a reference using minimap2 (Li 2018). We
545 used default settings except for the increased grouping confidence score (-i) which was increased to
546 0.6. We performed two rounds of RagTag. We first scaffolded the Seward *D. montana* assembly with
547 the Jackson *D. montana* assembly (which did not improve the assembly due to higher fragmentation of
548 the latter). For the second round of RagTag, we used the *Drosophila lummei* genome, which was
549 constructed using PacBio and Illumina reads and assigned to chromosomes using the published
550 *Drosophila virilis* chromosome map (Schäfer et al. 2010) and *D. virilis* assembly dvir_r1.03_FB2015_02
551 obtained from Flybase (Poikela et al. in prep.). Respectively, we scaffolded the Livingston *D.*
552 *flavomontana* assembly with the Vancouver *D. flavomontana* assembly and then with *D. lummei*. In *D.*
553 *montana* and *D. flavomontana*, chromosome 2 has right (2R) and left (2L) arms, separated by a
554 (sub)metacentric centromere, whereas in other *virilis* group species the centromere is located near one
555 end of the chromosome 2 (Stone et al. 1960). Therefore, scaffolding of chromosomes 2L and 2R was
556 not feasible with the *D. lummei* genome.

557 For the *D. montana* chromosome-level reference genome, the X chromosomes (29.1Mb), 2L (20.2Mb)
558 and 2R (11.0Mb) could not be further scaffolded and were kept as they are, while the lengths of
559 chromosomes 3, 4 and 5 were increased substantially by scaffolding. The longest contig of chromosome
560 3 increased from 5.8Mb to 26.0Mb (constructed from 37 contigs), that of chromosome 4 from 12.3Mb
561 to 32.5Mb (28 contigs), and that of chromosome 5 from 19.5Mb to 26.5Mb (11 contigs; Table S3). For
562 the *D. flavomontana* chromosome-level reference genome, the X chromosome (29.0Mb) could not be
563 further scaffolded, while the lengths of all other chromosomes increased due to scaffolding.
564 Chromosome 2L increased from 10.2Mb to 20.4Mb in length (3 contigs), 2R from 10.4Mb to 10.6Mb (3
565 contigs), the 3rd chromosome from 7.8Mb to 24.5Mb (33 contigs), the 4th chromosome from 20.0Mb
566 to 30.7Mb (14 contigs), and the 5th chromosome from 23.5Mb to 27.2Mb (4 contigs; Table S3).

567 Genome annotations

568 All genome assemblies were annotated for repetitive regions and genes. *De novo* libraries of repeat
569 sequences were built for each assembly using RepeatModeler v2.0.1 (Flynn et al. 2019), and repetitive
570 regions were softmasked – together with *Drosophila*-specific repeats (Dfam_3.1 (Hubley et al. 2016)
571 and RepBase-20181026 (Bao et al. 2015) – using RepeatMasker v4.1.0 (Smit *et al*. 2013-2015). Gene
572 models were predicted on the softmasked assemblies of *D. montana* using the BRAKER2 pipeline. For

573 gene annotation, we used RNA-seq data (Illumina Truseq 150bp PE) from whole-body female and male
574 *D. montana* adult flies collected in Finland (Parker et al. 2021). RNA-seq reads were trimmed for adapter
575 contamination and read quality using fastp v0.20.0 (Chen et al. 2018), and mapped to both softmasked
576 *D. montana* assemblies using STAR v2.7.0 (Dobin et al. 2013). Finally, *D. montana* gene annotations
577 were carried out with BRAKER2s ab initio gene prediction pipeline with RNA-seq and protein evidence
578 using Augustus v3.3.3 and GeneMark-ET v4.48 (Stanke et al. 2006; Stanke et al. 2008; Li et al. 2009;
579 Barnett et al. 2011; Lomsadze et al. 2014; Hoff et al. 2016; Hoff et al. 2019). Protein predictions of the
580 Jackson *D. montana* assembly with the best BUSCO values (see the Results) were used to annotate all
581 *D. flavomontana* genomes using the BRAKER2s ab initio gene prediction pipeline with GenomeThreader
582 and AUGUSTUS (Stanke et al. 2006; Stanke et al. 2008; Gremme 2013; Buchfink et al. 2015; Hoff et al.
583 2016; Hoff et al. 2019). Annotation completeness was assessed using BUSCO v5.1.2 against the
584 'diptera_odb10' database (Seppey *et al.* 2019).

585 Mapping, variant calling and variant filtering

586 To investigate genome-wide variation in sympatric and allopatric populations of the species, we
587 mapped all Illumina samples to the chromosome-level *D. montana* assembly. We quality-checked
588 Illumina PE reads of each sample with FastQC v0.11.8 (Andrews 2010) and trimmed for adapter
589 contamination and low-quality bases using fastp v0.20.0 (Chen et al., 2018). We mapped each trimmed
590 Illumina sample against the genome using BWA mem v0.7.17 with read group information (Li and
591 Durbin 2009), sorted alignments with SAMtools v1.10 (Li et al. 2009) and marked PCR duplicates with
592 sambamba v0.7.0 (Tarasov et al. 2015). The resulting BAM-files were used for variant calling with
593 freebayes v1.3.1-dirty (Garrison and Marth 2012). Raw variants were processed with gIMble preprocess
594 (genome-wide IM blockwise likelihood estimation toolkit; (Laetsch et al. 2022)). In brief, non-SNP
595 variants were deconstructed into allelic primitives, where remaining non-SNPs were removed in
596 addition to any SNP variant within two bp of a non-SNP. Genotype calls of remaining SNP variants were
597 set to missing if any of the following assumptions was violated: i) sample depth (FMT/DP) between 8
598 and 2SD from the mean coverage, ii) read directionality placement balance (RPL>=1, RPR>=1), or iii)
599 read strand placement balance (SAF>=1, SAR>=1).

600 Principal component analysis of whole-genome and climatic data

601 To group Illumina samples according to their species and population type, we performed a principal
602 component analysis (PCA) on the filtered VCF-file, including all samples and chromosomes as well as
603 coding, intronic and intergenic SNPs, using PLINK v1.9 package ((Chang et al. 2015), www.cog-
604 genomics.org/plink/1.9/). The VCF-file was converted to PLINK's BED/BIM format and the PCA was run
605 with PLINK's --pca function.

606 We performed another PCA on the climatic factors at fly sampling sites to visualize the climatic variation
607 amongst sites. First, we downloaded climatic information from WorldClim database v2.1 2.5min spatial
608 resolution, dataset 1970-2000, http://www.worldclim.org, (Fick and Hijmans 2017)) using latitudinal
609 and longitudinal coordinates of each site (Table S1) and extracted 19 bioclimatic variables using the
610 "raster" package v2.8-19 ((Hijmans and Etten 2020), Table S5, Table S6). We then performed PCA on
611 the bioclimatic variables, describing temperature and humidity conditions in each site. We performed
612 the PCA using the "FactoMineR" package (Lê et al. 2008) in R v3.4.3 (R Core Team 2017) and R studio
613 v1.1.383.

614 Characterization of chromosomal inversions

615 We identified large (>500kb) alternatively fixed inversions between *D. montana* and *D. flavomontana*
616 using both long- and short-read data. First, we mapped PacBio reads of each sample against each of
617 the four assemblies using ngmlr v0.2.7 (Sedlazeck et al. 2018) and obtained structural variant (SV)
618 candidates from the SV identification software, Sniffles v1.0.12 (Sedlazeck et al. 2018). Second, we
619 mapped Illumina read pairs against each of the four assemblies as explained in the "Mapping, variant

calling and variant filtering" paragraph. The resulting BAM-files were given to Delly v0.8.1, which identifies structural variants based on paired-end read orientation and split-read evidence (Rausch et al. 2012). Third, we used SURVIVOR (Jeffares et al. 2017) to identify species-specific, widespread inversions that were shared by Sniffles and Delly outputs and that are found in at least in 9 *D. montana* (out of 12) and 6 *D. flavomontana* (out of 9) samples. Putative breakpoints of each inversion were located within a single contig, except for the 4th chromosome inversion where breakpoints were located in two different contigs (Table S9). This inversion was therefore verified by mapping long- and short-read data against the *D. lummei* genome which has a more contiguous chromosome 4 (Table S9). The putative breakpoints of all inversions were confirmed visually with Integrative Genomics Viewer (IGV) v2.8.0 (Thorvaldsdóttir et al. 2012) using both long- and short-read data (Example of the IGV view shown in Fig. S9).

Modelling divergence and post-divergence gene flow

We analysed mean genetic divergence (d_{XY}) and fitted models of species' divergence with and without long-term interspecific gene flow between and within species using gIMble (Laetsch et al. 2022). This analytic likelihood method uses the joint distribution of mutation types in short sequence blocks, the blockwise site frequency spectrum (bSFS) across subsamples of pairs of individual genomes to fit a series of models of speciation history. We summarised data by the bSFS for two block lengths 64 bp and 128 bp.

To investigate the role of inversions in speciation, we fitted separate demographic models to inverted and colinear regions of the genome and to sympatric and allopatric comparisons of *D. montana* and *D. flavomontana*. Given the potentially different evolutionary history of the X chromosome and the autosomes (Vicoso and Charlesworth 2006; Charlesworth et al. 2018), we ran separate analyses for the X chromosome and autosomes. Colinear regions were combined across autosomes as these regions are expected to share the same evolutionary history, while inversions from different chromosomes may differ in age and were analysed separately. The overlapping inversions of the X chromosome were analysed together following Counterman and Noor (2006) and Lohse et al. (2015). We also analysed the split time of *D. montana* populations living in isolation (allopatry) and in contact (sympatry) with *D. flavomontana* to evaluate the timing of potential recent introgression between the two species. The intraspecific divergence time was inferred from the colinear autosomal regions, i.e. the same data partition we used to infer the interspecific background history.

We calculated d_{XY} for different genomic regions (i.e. colinear and inverted autosomes, and colinear and inverted X chromosome) and for allopatric and sympatric populations to evaluate the role of inversions in suppressing gene exchange. For a comparison, d_{XY} was also analysed for the intraspecific comparisons of *D. montana* (Fig. 1). These analyses were carried out separately for coding, intronic and intergenic regions (repetitive regions were excluded from all data partitions). To test whether genetic divergence was reduced within inversions, we simulated data sets corresponding in size to the data sampled for each inversion under the background demography (inferred from colinear autosomal regions) and compared the observed d_{XY} to the distribution. We simulated inversion datasets under a minimal, conservative model of recombination, which allows for gene conversion but no cross-over. We assumed a rate of (initiation of) gene conversion of 3.59×10^{-8} per bp per generation. This corresponds to recent estimates for *Drosophila pseudoobscura* and *Drosophila persimilis* (1.4×10^{-5} converted sites per bp per generation; mean GC tract length of 390 bp) (Korunes and Noor 2018) and simulated sequences of length 10kb, two orders of magnitude shorter than total length of intergenic sequence per inversion.

To investigate the speciation process of *D. flavomontana* and *D. montana*, we compared the fit of different demographic models. We limited the initial model selection of allopatric, sympatric and intraspecific comparisons to intergenic data of colinear autosomal regions (repetitive regions excluded) to minimize the effects of selection. The simplest, strict divergence (DIV) model considers isolation at time *T* without interspecific gene flow, i.e. isolation in allopatry (Fig. 7A). The isolation with migration (IM) model allows unidirectional migration rate at a constant rate *M* (Fig. 7B). The IM model was fitted

for both gene flow directions (i.e. from *D. montana* to *D. flavomontana* and from *D. flavomontana* to *D. montana*, and from allopatric to sympatric *D. montana* and from sympatric to allopatric *D. montana*). The DIV and IM models allow asymmetric effective population size (N_e) between the descendent populations, and the possibility of a separate N_e for the ancestral population. Analyses based on the bSFS assume a constant mutation rate (μ) across blocks and no recombination within them. We assumed a mutation rate (μ) of 2.8×10^{-9} per site per generation, based on an estimate of the spontaneous mutation rate in *Drosophila melanogaster* (Keightley et al. 2014). The estimates of *T* are converted into absolute time using $t = T \times 2N_e \times g$, where $N_e = \theta/(4\mu)$ and g is generation time. We assumed one generation per year, the generation time of Alaskan *D. montana* populations and most likely that of the ancestral population of the species, even though other *D. flavomontana* and *D. montana* populations presently have two generations per year (Tyukmaeva et al. 2020). To consider the potential effects of reference bias, we performed model fitting and selection twice using both *D. montana* and *D. flavomontana* chromosome-level assemblies as reference genomes.

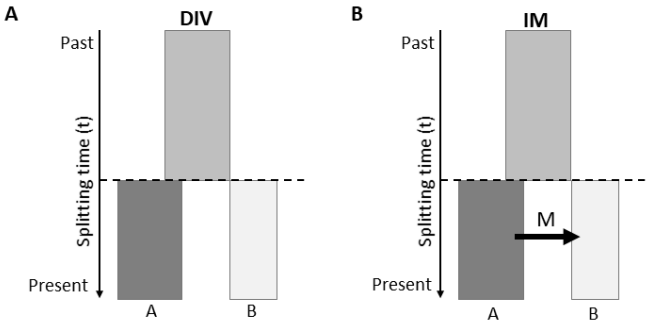

Figure 7. Sketches of the demographic models. The block widths indicate relative effective sizes (N_e) of the ancestral population and its two descendants A and B. (A) This model assumes no gene flow, i.e. strictly allopatric speciation (Div). (B) The isolation with migration IM) model allows unidirectional gene flow at rate *M* migrants per generation in this case from population A to B.

To estimate the uncertainty in parameter estimates, i.e. the difference in support (ΔlnCL) between different demographic scenarios, we performed a parametric bootstrap. We used gIMble simulate to simulate 100 replicate datasets (of the same size as the real data in terms of numbers of blocks). To include the effect of linkage between blocks, we simulated data in 1000 chunks assuming a recombination rate of 8.9×10^{-9} calculated from the total map length (i.e. 1.76×10^{-8} divided by two given the absence of recombination in males). Specifically, we simulated data under the best fitting DIV model and fitted that model and an IM model to each replicate to obtain a null distribution of ΔlnCL between models.

Finally, we performed demographic analyses under the best-fit models separately for different genomic partitions (i.e. colinear and inverted autosomes, and colinear and inverted X chromosome) and for allopatric and sympatric comparisons. The uncertainty in estimates of T and M for each data partition were inferred from 100 parametric bootstrap replicates/simulations. Parameter estimates from simulated datasets were analysed using a generalized linear model with Gamma (T, M) or Gaussian (M) distribution, depending on the normality of the data, in R. Here, colinear regions were compared to the inverted regions separately for autosomes and the X chromosome and for allopatric and sympatric populations, and estimates of M were compared between allopatric and sympatric populations within each chromosomal region.

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Author notes

K.L., M.K. and N.P. designed the study. N.P. carried out the DNA extractions and conducted the genome analyses with input from K.L. and D.R.L. K.L. designed and coded the simulations for d_{XY} analyses. M.K. supervised the laboratory work and funded the study. N.P. drafted the manuscript and all the authors helped in finalizing it.

Data accessibility

Raw reads of PacBio and Illumina samples as well as genome assemblies generated from the raw reads will be deposited on SRA at the time of publication.

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