Inversions shape the divergence of *Drosophila pseudoobscura* and *D. persimilis* on multiple timescales

Abstract

By shaping meiotic recombination, chromosomal inversions can influence genetic exchange between hybridizing species. Despite the recognized importance of inversions in evolutionary processes such as divergence and speciation, teasing apart the effects of inversions over time remains challenging. For example, are their effects on sequence divergence primarily generated through creating blocks of linkage-disequilibrium pre-speciation or through preventing gene flux after speciation? We provide a comprehensive look into the influence of inversions on gene flow throughout the evolutionary history of a classic system: *Drosophila pseudoobscura* and *D. persimilis*. We use extensive whole-genome sequence data to report patterns of introgression and divergence with respect to chromosomal arrangements. Overall, we find evidence that inversions have contributed to divergence patterns between *Drosophila pseudoobscura* and *D. persimilis* over three distinct timescales: 1) segregation of ancestral polymorphism early in the speciation process, 2) gene flow after the split of *D. pseudoobscura and D. persimilis*, but prior to the split of *D. pseudoobscura* subspecies, and 3) recent gene flow between sympatric *D. pseudoobscura* and *D. persimilis*, after the split of *D. pseudoobscura* subspecies. We discuss these results in terms of our understanding of evolution in this classic system and provide cautions for interpreting divergence measures in other systems.

Keywords: inversions, introgression, divergence, recombination, speciation

Introduction

Divergence and speciation sometimes occur in the presence of gene exchange between taxa. Estimates suggest that over 10% of animal species hybridize and exchange genes with related species (Mallet, 2005). Analyses in the genomic era have provided further evidence of the widespread prevalence of hybridization and revealed many previously unanticipated instances of hybridization (Payseur & Rieseberg, 2016; Taylor & Larson, 2019). Understanding genetic exchange between species gives us insights into the genetic processes underlying later stages of the speciation continuum. Many approaches can examine evidence for introgression, including comparing sympatric vs. allopatric populations to test for differences in nucleotide divergence. Other available methods for characterizing gene flow include model-based frameworks and examinations of differences in divergence reflected in coalescence times. Differences in coalescence times are often observed between species in regions where recombination is limited in hybrids, such as fixed chromosomal inversion differences (Guerrero, Rousset, & Kirkpatrick, 2012). When species differing by inversions hybridize, the collinear genomic regions can freely recombine, while inverted regions experience severely limited genetic exchange in hybrids and often accumulate greater sequence differentiation over generations. This process can lead to locally adapted traits and reproductive isolating barriers mapping disproportionately to inverted regions (reviewed in Ayala & Coluzzi 2005; Butlin 2005; Jackson 2011).

Many studies examine the timing and frequency of gene exchange between hybridizing species, with emphasis on the implications of patterns of divergence in allopatric vs sympatric pairs and in regions of reduced recombination in hybrids. However, different approaches sometimes yield distinct interpretations regarding the presence or extent of introgression. Model-based approaches yield important insights but are also limited in the scenarios that they consider and the

assumptions they make about population histories and evolutionary rates (reviewed in Payseur & Rieseberg 2016). Further, shared patterns of variation are often interpreted as evidence of ongoing gene flow, but segregating ancestral polymorphism could also be the primary, or even the sole, driver of these patterns (Fuller, Leonard, Young, Schaeffer, & Phadnis, 2018). In the ancestral population of two species, segregating chromosomal inversions may shield inverted regions of the genome from recombination, thus facilitating the divergence of sympatric ecotypes or populations. Heightened within-species differentiation in inverted regions has been observed in many systems, including *Rhagoletis pomonella* (Michel et al., 2010), *Anopheles gambiae* (Manoukis et al., 2008), and *Mimulus guttatus* (Lowry & Willis, 2010). Such heightened differentiation between karyotypes may persist along the speciation continuum, making it difficult to disentangle the effects of inversions reducing recombination in the ancestral population vs reducing introgression upon secondary contact. Fuller *et al.* (2018) recently discussed the possibility that ancestrally segregating inversions that sort between species may provide a "head-start" in molecular divergence, possibly predisposing them to harbor a disproportionate fraction of alleles associated with species differences. Unlike models assuming homogenization of collinear regions via post-speciation gene flow, this model predicts that young species that diverged in allopatry may also exhibit higher divergence in inverted regions than collinear regions. These models are not mutually exclusive: dynamics of the ancestral population as well as post-speciation gene flow can shape patterns of variation between species.

Disentangling the effects of ancestral polymorphism from the effects of post-speciation gene flow is a fundamental puzzle in understanding speciation. To achieve a cohesive picture of how hybridization influences divergence and speciation, we need to consider the approaches outlined above in a model system with extensive whole-genome sequence data to assess models and reconcile interpretations of possible signals of introgression. The sister species pair *Drosophila pseudoobscura* and *D. persimilis* present an ideal opportunity to dissect an evolutionary history of divergence nuanced by

multiple inversions, lineage sorting, and gene flow. Despite the rich history of work on understanding speciation and divergence in *D. pseudoobscura* and *D. persimilis*, there are unresolved questions about the rates and timing of introgression between these species. A few F_1 hybrids of these species have been collected in the wild (Powell 1983) and many previous studies have documented molecular evidence of introgression, detectable in both nuclear and mitochondrial loci (e.g., Machado *et al.* 2002; Machado & Hey 2003; Hey & Nielsen 2004; Fuller *et al.* 2018). Inverted regions between these species exhibit greater sequence differences than collinear regions, and this pattern was previously inferred to result from introgression post-speciation. McGaugh and Noor (2012) used multiple genome sequences of both species and an outgroup, and reinforced previous studies (e.g., Noor *et al.* 2007) showing that the three chromosomal inversions differ in divergence time. They inferred a "mixed mode geographic model" (Feder, Gejji, Powell, & Nosil, 2011) with sporadic periods of introgression during and after the times that the inversions spread. However, in addition to confirming evidence for gene flow between *D. pseudoobscura* and *D. persimilis* after speciation, Fuller *et al.* (2018) recently argued the inversions arose within a single ancestor species, differentially sorted in the descendant species, and this sorting of ancestral polymorphisms may explain observed patterns of nucleotide variation. To fully understand the role of hybridization in the speciation process, the contrasting models must be reconciled.

We acquired extensive whole-genome sequence data to re-explore patterns of introgression and divergence in the *Drosophila pseudoobscura* / *D. persimilis* system. We leverage the allopatric *D. pseudoobscura* subspecies, *D. pseudoobscura bogotana* (*D. p. bogotana)* and two outgroup species (*D. miranda* and *D. lowei*) to distinguish recent from ancient effects of inversions on gene flow. Note that we use *D. pseudoobscura* to refer to both *D. pseudoobscura* subspecies (*D. pseudoobscura pseudoobscura* and *D. pseudoobscura bogotana*), and we specify *D. p. pseudoobscura* or *D. p. bogotana* when we are specifically referring to only one of the two. Much of the previous support for post-speciation gene flow

between these species has focused on comparisons of *D. persimilis* and *D. p. pseudoobscura* (Fuller et al., 2018; Hey & Nielsen, 2004; Kulathinal, Stevison, & Noor, 2009; Machado et al., 2002; R. L. Wang, Wakeley, & Hey, 1997). In addition to analyzing *D. persimilis* and *D. p. pseudoobscura* genomes, we sequenced multiple strains of *D. p. bogotana* to provide a comparative dataset that allows us to clarify the role of inversions over the evolutionary history of these species by considering three distinct time scales: 1) pre-speciation segregation of ancestral polymorphism, 2) post-speciation ancient gene flow, and 3) recent introgression (Figure 1). In this context, we use pre- and post-speciation to refer to estimated divergence times of *D. persimilis* and *D. pseudoobscura*, though we note that speciation is a continuum and the present study does not address the emergence of reproductive isolation or the degree of species barriers. Patterns of divergence between *D. persimilis* and allopatric *D. p. bogotana* can be explained by the effects of segregating ancestral polymorphism and by gene flow prior to the split of *D. p. bogotana* (Figure 1, green and blue regions). In comparing the sympatric species, *D. persimilis* and *D. p. pseudoobscura,* the same forces factor into patterns of divergence, with the added effects of recent or ongoing gene flow (Figure 1, orange arrows). We leverage these two comparisons to weigh the relative contributions of recent genetic exchange.

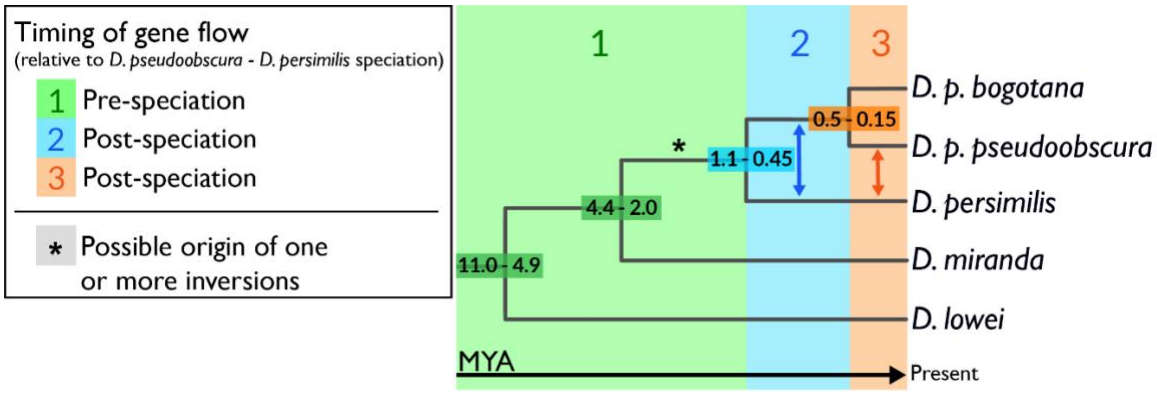


Figure 1 | Gene flow in the context of the evolutionary history of *D. pseudoobscura* and *D. persimilis*. We consider how inversions differing between *D. pseudoobscura* and *D. persimilis* might shape patterns of divergence by affecting recombination at 3 timescales: 1) prior to the estimated

split of *D. pseudoobscura* and *D. persimilis,* by shaping recombination in populations with segregating inversion polymorphisms, 2) after the split of *D. pseudoobscura and D. persimilis*, but prior to the split of subspecies *D. p. pseudoobscura* and *D. p. bogotana,* and 3) during recent introgression between *D. p. pseudoobscura* and *D. persimilis*. Here, we show the evolutionary context and approximate divergence times of the taxa considered in the present study, with arrows indicating gene flow between *D. pseudoobscura* and *D. persimilis.* Node ages are summarized from the literature for the divergence of subspecies *D. p. pseudoobscura* and *D. p. bogotana* (S W Schaeffer & Miller, 1991; R. L. Wang & Hey, 1996), the divergence of *D. pseudoobscura* and *D. persimilis* (Fuller et al., 2018; Hey & Nielsen, 2004; R. L. Wang & Hey, 1996), the divergence of *D. miranda* from the clade that contains *D. pseudoobscura and D. persimilis* (Beckenbach, Wei, & Liu, 1993; R. L. Wang & Hey, 1996), and the divergence of *D. lowei* from the rest of the group (Beckenbach et al., 1993).

We first examine patterns of divergence in inverted regions compared to collinear regions, and we discuss evidence for segregation of inversion polymorphisms early in the speciation continuum. We next examine evidence of post-speciation gene flow to test whether some of this genetic exchange predates the split of *D. p. bogotana,* and we discuss signals of possible introgression in the past 150,000 years since the split of the allopatric *D. p. bogotana,* from North American *D. pseudoobscura* (*D. p. pseudoobscura*) (S W Schaeffer & Miller, 1991). Kulathinal *et al.* (2009) previously argued that recent post-speciation gene flow contributes to the difference in coalescence time between inverted and collinear regions, observable in the higher genetic similarity in collinear regions between *D. persimilis* and sympatric *D. p. pseudoobscura* compared to similarity between *D. persimilis* and allopatric *D. p. bogotana*. That study also tested for an excess of shared, derived bases between *D. persimilis* and *D. p. pseudoobscura* compared to *D. persimilis* and allopatric *D. p. bogotana.* Their application of this D-statistic precursor suggested a borderline statistically significant signature of gene flow, but this test was limited by low sequencing coverage. Using recent statistical approaches applied to high-coverage resequencing, we compare the sympatric (*D. p. pseudoobscura*) and allopatric (*D. p. bogotana)* subspecies in their similarity to *D. persimilis*. Our estimation of Patterson's D-statistic and f_d (Martin, Davey, & Jiggins, 2015) indicates very recent gene exchange in collinear regions. Perhaps surprisingly, divergence measures to *D. persimilis* are also higher for

allopatric than sympatric *D. pseudoobscura* subspecies in *both* inverted and collinear regions. We discuss several possible explanations for this pattern, including 1) extensive recent gene exchange throughout much of the genome, even in inverted regions, 2) the role of segregating inversions in ancestral populations, and 3) differences in evolutionary rates across taxa. We discuss these results in the context of the extensive past work towards understanding divergence and speciation in this classic system, and we provide cautions for interpreting divergence measures in other systems.

Methods

Genomic datasets

Whole-genome short-read sequence data were analyzed from 19 *D. p. pseudoobscura* and 8 *D. persimilis* strains, along with 4 *D. p. bogotana* strains as an allopatric point of comparison. Both males and females were sequenced, all from inbred strains listed in Supplementary Table 1 with SRA accessions (Korunes, Myers, Hardy, & Noor, 2020; McGaugh et al., 2012; Samuk, Manzano-Winkler, Ritz, & Noor, 2020). We used *D. lowei* as an outgroup. *D. lowei* likely diverged from the rest of the *D. pseudoobscura* subgroup 5-11 MYA (Beckenbach et al., 1993), and hybrids between these two species are sterile (Heed, Crumpacker, & Ehrman, 1969). Scripts used for genome alignment, SNP calling, and analyses are available on GitHub (https://github.com/kkorunes/Dpseudoobscura_Introgression). To avoid biasing identification of variants towards *D. pseudoobscura*, the *D. miranda* reference genome assembly (DroMir2.2; GenBank assembly accession GCA_000269505.2) was chosen as the reference for all ~~subsequent~~ alignments. *D. miranda* diverged from *D. pseudoobscura* only within the past 2-4 million years (Beckenbach et al., 1993; R. L. Wang & Hey, 1996), facilitating alignment of *D. pseudoobscura* and *D. persimilis* genomes to the *D. miranda* genome assembly. Further, the arrangement of the assembled *D. miranda* chromosomes matches the published contig order and arrangement of *D. pseudoobscura* (Stephen W.

Schaeffer et al., 2008), but with the advantage of being assembled into 6 continuous chromosome arms: chromosomes XL, XR, 2, 3, 4, and 5. Here, we analyze the majority (83%) of the assembled genome. We exclude only regions where we cannot reasonably examine introgression and divergence: chromosome 3, which presents confounding factors from its inversion polymorphisms within species, including over 30 inversion polymorphisms known to segregate within *D. pseudoobscura* and *D. persimilis* (Dobzhansky & Epling, 1944; Jeffrey R Powell, 1992), and the very small (<2 Mb) portion of the genome found on the largely nonrecombining "dot" chromosome (chromosome 5).

Alignments and variant calling

To confirm the arrangement of *D. pseudoobscura* contigs with respect to the *D. miranda* reference, each *D. pseudoobscura* chromosome was split into lengths of 1 Mb, and these segments were aligned to the *D. miranda* reference using BWA-0.7.5a (Li & Durbin, 2009). We then extracted the 2 kb regions surrounding published inversion breakpoints to obtain the breakpoint locations in the coordinates of the *D. miranda* reference (see Supplementary Table 2). After confirming that the arrangement of the assembled *D. miranda* chromosomes matched the arrangement of the *D. pseudoobscura* contig order and arrangement described by Schaeffer *et al.* (2008), all sequencing data were aligned to the reference genome of *D. miranda* using BWA-0.7.5a (Li & Durbin, 2009), and Picard command line tools were used to mark adapters and duplicates (http://broadinstitute.github.io/picard). Unphased SNPs were called used GATK v4 and filtered based GATK's hard filtering recommendations (McKenna et al., 2010; Van der Auwera et al., 2013), excluding sites with QualByDepth (QD) < 2.0, FisherStrand (FS) > 60, StrandOddsRatio (SOR) > 3.0, MQ < 40, MQRankSum < -12.5, ReadPosRankSum < -8.

Patterns of divergence

The resulting VCF files were then processed using PLINK (Purcell et al., 2007). VCFs were converted to PLINK's bed/bim format, keeping only sites that passed the filters described above. SNPs were pruned for linkage disequilibrium using the --indep-pairwise function of PLINK ("--indep-pairwise 50 50 0.5") before performing principal components analysis (PCA) using PLINK's --pca function to confirm the grouping of individuals within their respective species (Figure 1; Supplementary Figure 1). Admixtools was used to estimate Patterson's D-statistic (Patterson et al., 2012) using *D. lowei* as on outgroup to polarize ancestral vs derived alleles. For input into Admixtools, we used the *convertf* program of Admixtools to convert each PLINK ped file to Eigenstrat format which includes a *genotype* file, a *snp* file, and an *indiv* file. Per recommendations in the Admixtools documentation, we defined the physical positions of each SNP in the *snp* file to be 10 kb apart from each adjacent SNP to allow Admixtools to interpret every 100 SNPs as 1 Mb or 1cM, since this software uses centiMorgans as the unit for block size during jackknifing and assumes that 1 Mb = 1 cM. We set the block size parameter to 0.01 cM, which in this case is interpreted as blocks of 100 SNPs. Next, *qpDstat* was used to obtain D-statistics for each chromosome. These four-population tests were of the form (((A,B), C), D), where A = *D. p. bogotana,* B = *D. p. pseudoobscura,* C = *D. persimilis,* and D = *D. lowei.* To study signatures of introgression along the genome, we applied f_d (Martin et al., 2015) in genomic intervals that presented an excess of ABBA over BABA sites. Using non-overlapping windows of 100 SNPs, we calculated f_d using the Dinvestigate program from Dsuite (Malinsky, Matschiner, & Svardal, 2020). Absolute divergence, D_{xy}, was calculated using custom scripts over fixed window sizes of 50 kb. D_{xy} was calculated from variant and invariant sites after subjecting SNPs to the filters described above and filtering invariant

sites based on depth (depth >= 10). Per-site depths for all sites were acquired from BAM files using Samtools ("samtools depths -a <in>") (Li et al., 2009).

Models of gene flow

To test for evidence of gene flow after the split of *D. pseudoobscura* and *D. persimilis,* but before the split of *D. p. bogotana,* we used the maximum-likelihood methods derived by Costa & Wilkinson-Herbots (2017) to compare scenarios of divergence between *D. persimilis* and *D. p. bogotana* (Figure 2). We first considered models of divergence in isolation without gene flow following the split of an ancestral population (Figure 2A) either with constant population size (Iso) or allowing changes in population size (Iso2). We then considered a model of divergence in isolation-with-migration (IM) with constant (but potentially asymmetric) gene flow since the split of an ancestral population until the present (Figure 2B), and finally we considered four scenarios of divergence in isolation-with-initial-migration (IIM) with gene flow until some timepoint in the past and divergence in isolation since that timepoint (Figure 2C).

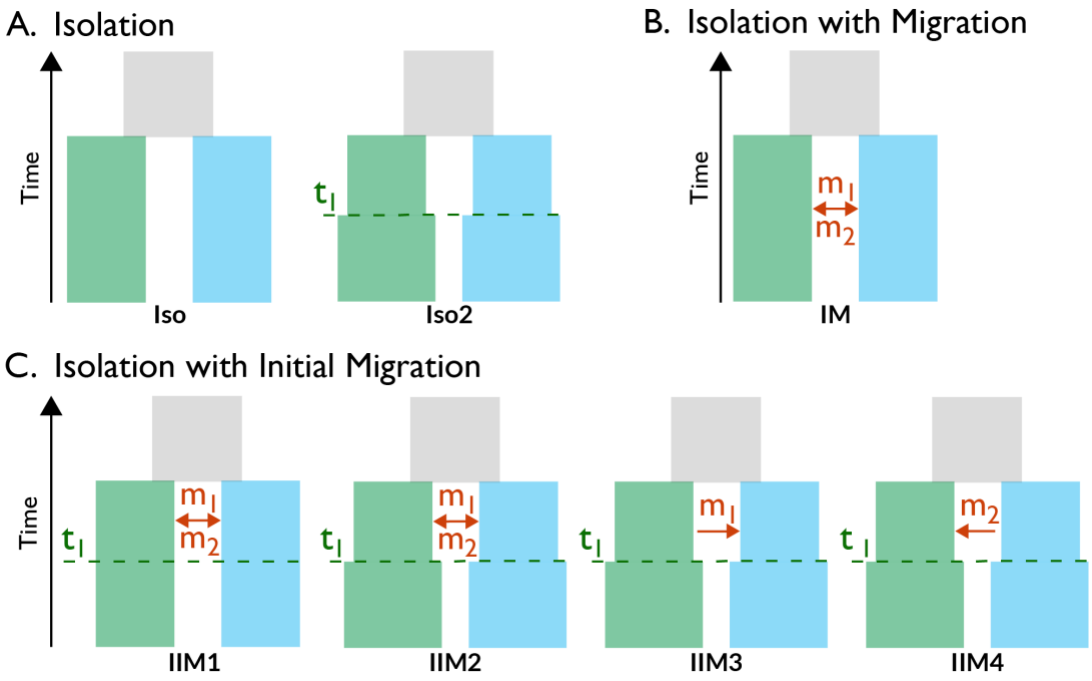


Figure 2 | Models of Divergence. We considered the following coalescent models described by Costa & Wilkinson-Herbots (2017) to consider scenarios of divergence of *D. persimilis* and *D. p. bogotana* (represented by the left and right lineages, respectively) since the split of the ancestral population (gray box): (A) divergence in isolation without gene flow, with either constant population sizes (Iso) or allowing changes in population sizes (Iso2); (B) divergence in isolation-with-migration (IM) with constant (but potentially asymmetric) gene flow; and (C) divergence in isolation-with-initial-migration (IIM) with gene flow until some timepoint (t_1) in the past. Under the IIM model, we tested the four scenarios shown from left to right: the first scenario (IIM2) assumes constant population sizes, the second (IIM2) allows for changes in population sizes, and the third (IIM3) and fourth (IIM4) allow for changes in population size but assume unidirectional gene flow.

We computed the likelihood of our *D. persimilis* and *D. p. bogotana* sequence data under each of the seven scenarios described above and in Figure 2 (Iso, Iso2, IM, IIM1, IIM2, IIM3, and IIM4). To reduce potential effects of selection, we used intergenic loci spaced at least 2 kb apart, similar to the strategy of Wang & Hey (2010), which similarly utilized diploid genome sequences from inbred lines of another *Drosophila* species pair and served as the empirical dataset used to illustrate the methods in Costa & Wilkinson-Herbots (2017). Linkage-disequilibrium decays within tens to hundreds of bases in *Drosophila* (Langley, Lazzaro, Phillips, Heikkinen, & Braverman, 2000), so we expect that avoiding genic regions will minimize the effects of linked selection. To identify intergenic

regions in the *D. miranda* genome, we used the set of all *D. pseudoobscura* gene annotations published by Flybase (http://flybase.org, Full Annotation Release 3.04), and we used BLAST to identify genomic regions with significant similarly to the *D. pseudoobscura* gene annotations, using cutoffs of evalue = 10^{-6} and percent identity = 80 (Altschul, Gish, Miller, Myers, & Lipman, 1990). From the remaining regions, we then randomly sampled 500 bp segments separated by at least 2 kb to create a set of ~15,000 intergenic loci. To ensure that our results were robust against the effects of linkage within inverted regions, we sampled regions expected to be freely-recombining throughout the timescales examined: i.e., we excluded any loci from the inverted regions, leaving ~11,000 intergenic, collinear loci. We then randomly divided these loci into three nonoverlapping subsets to satisfy the models' requirement of independent estimates of pairwise differences and mutation rates in loci (1) within *D. persimilis*, (2) within *D. p. bogotana*, and (3) between *D. persimilis* and *D. p. bogotana*. Costa & Wilkinson-Herbots (2017) recommends using per-locus relative mutation rates, which we calculated using the average distance to the outgroup *D. lowei*, following the equation from Yang (2002), which gives the relative mutation rate at a locus as the outgroup distance at that locus divided by the average outgroup distance along all loci. To select the model that best fits the data, we then tested the relative support among nested divergence models using likelihood-ratio tests following the sequence of pairwise comparisons shown in Table 1, where the degrees of freedom in each test is the difference in the number of parameters between alternative models (Costa & Wilkinson-Herbots, 2017).

Tests for differences in evolutionary rate

Finally, to test for differences in evolutionary rate that might influence observed patterns of divergence and gene flow, differences in substitution rates among the lineages were assessed with Tajima's relative rate test, using *D. lowei* as the outgroup (Tajima 1993; scripts available on GitHub

repository linked above). Tajima's relative rate test was applied to the combined set of SNPs from chromosomes 2, 4, XL, and XR—excluding sites where the outgroup *D. lowei* was heterozygous or missing data. We next inferred relative clock rates within the tree using coalescent phylogenetic inference in StarBEAST2, a method specifically designed for multilocus genomic datasets (Ogilvie, Bouckaert, & Drummond, 2017). To choose a dataset similar to the empirical dataset shown to perform well in Ogilvie et al. (2017), we took a subset of 20 autosomal loci from the 500 bp collinear, intergenic loci used to test the coalescent models above. To enable estimation of per-species clock rates, we used an uncorrelated log-normal clock model (UCLN). The site models were set to the HKY substitution model, and the phylogenetic relationship was reconstructed under the default Yule process. StarBEAST2 was run using a chain length of 100 million, sampled every 1,000 generations, yielding an effective sample size >200 for the posterior of each parameter. The TreeAnnotator program provided with BEAST was used to calculate the posterior expectation and 95% credibility intervals of per-species clock rates, and a summary tree of the posterior distribution was visualized in FigTree v1.4.4 (Rambaut, 2018). For alignments used and all StarBEAST2 parameters, the xml is provided in the GitHub repository linked above.

Results

Patterns of divergence in inverted vs collinear regions

The suppression of crossing over within inversions leads to distinct signatures of nucleotide divergence within and near inversions. One of the advantages of this system for studying the evolutionary effects of chromosomal inversions is the existence of *D. p. bogotana:* a clear allopatric point of comparison for the North American *D. persimilis* and *D. p. pseudoobscura*. By including 4 *D. p. bogotana* genomes, we were able to compare patterns of divergence for both *D. persimilis* vs *D. p. pseudoobscura* and *D. persimilis* vs. *D. p. bogotana.* Figure 3 presents windowed divergence between *D.*

persimilis and *D. p. bogotana* and divergence between *D. persimilis* vs *D. p. pseudoobscura* for the three chromosome arms that contain fixed (chromosome 2, XL) or nearly-fixed (chromosome XR) inversion differences between *D. persimilis* and *D. pseudoobscura*. Here, we do not include the highly polymorphic inversions of chromosome 3, where a complex series of overlapping inversions pre-dates the estimated speciation timing of *D. persimilis* and *D. pseudoobscura*, resulting in large segments of high, long-term LD (Aquadro, Weaver, Schaeffer, & Anderson, 1991; Fuller, Haynes, Richards, & Schaeffer, 2017; Wallace, Detweiler, & Schaeffer, 2011). To consider the effects of inversions on divergence, we contrast observed patterns within inversions to regions outside the inversions (collinear) and to the subset of collinear regions that can be predicted to be reasonably freely-recombining (denoted as collinear$_{FR}$). Collinear$_{FR}$ excludes the 5 Mb windows adjacent to telomeric and centromeric chromosome ends, which undergo very little crossing over (Andolfatto & Wall, 2003; Kulathinal, Bennett, Fitzpatrick, & Noor, 2008; Stevison & Noor, 2010). Similarly, collinear$_{FR}$ excludes regions within 2.5 Mb outside of inversion breakpoints, based on previous reports of crossover suppression 1-2 Mb beyond inversion breakpoints (Kulathinal et al., 2009; Machado, Haselkorn, & Noor, 2007; Stevison, Hoehn, & Noor, 2011).

First, confirming ~~many~~ previous studies (Kulathinal et al., 2009; Machado et al., 2007; McGaugh & Noor, 2012; M. A. F. Noor, Garfield, Schaeffer, & Machado, 2007; Stevison et al., 2011), we observed that *D. persimilis* vs *D. p. pseudoobscura* divergence is significantly higher in inverted than collinear windows, regardless of whether the inverted regions are compared to all collinear windows or to the collinear$_{FR}$ subset (Supplementary Table 3). Second, our inclusion of the allopatric *D. persimilis* vs *D. p. bogotana* comparison reveals several interesting new patterns. Estimates of divergence between *D. persimilis* and *D. p. bogotana* are consistently higher than estimates of divergence between *D. persimilis* vs *D. p. pseudoobscura*, even in breakpoint-adjacent regions, as we discuss further below. We also note that *D. persimilis* vs allopatric *D. p. bogotana* divergence is higher

in inverted than collinear windows on chromosomes 2 and XL (the difference is nonsignificant on chromosome XR unless the comparison is restricted to collinear$_{FR}$; Supplementary Table 3).

Higher divergence in inverted vs collinear regions could be due to pre-speciation segregation of inversion polymorphisms in the ancestral population or to interspecies gene flow homogenizing collinear regions. Here, we focus on testing the latter, since previous work already provides evidence for exchange between karyotypes due to inversion polymorphisms segregating in the ancestral population. Briefly, the ages of the inversions examined here have been consistently inferred as pre-dating the estimated divergence time of *D. pseudoobscura* and *D. persimilis* (Fuller et al. , 2018; Hey & Nielsen, 2004; R. L. Wang & Hey, 1996). While we do not repeat these analyses in full, we note that our estimates of average divergence within the inversions (Supplementary Table 3) are consistent with previous accounts of the relative divergence and ages of the inversions (Fuller et al., 2018; McGaugh & Noor, 2012; M. A. F. Noor et al., 2007). For both *D. persimilis* vs *D. p. pseudoobscura* and *D. persimilis* vs *D. p. bogotana*, we compared measures of windowed divergence among the inverted regions. Each pairwise comparison between the inversions yielded a significant difference wherein XL > 2 > XR ($p < 0.0001$, Mann-Whitney U test; Supplementary Table 3). Given the evidence that inversions were segregating in the ancestral population of these species, we test for evidence of post-speciation gene flow and attempt to disentangle the timing of such gene flow.

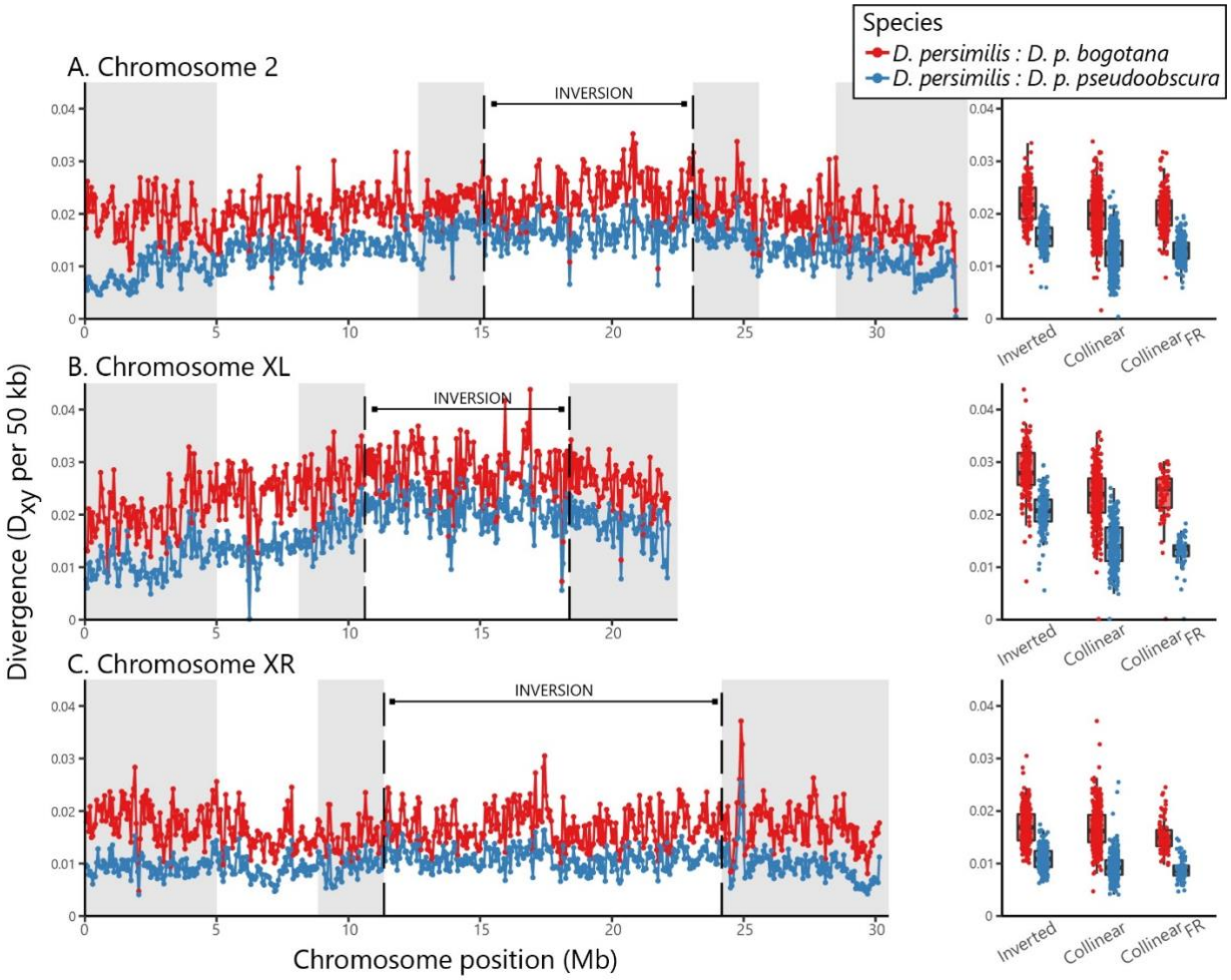


Figure 3 | Genome-wide divergence between species. On the left, each of the 3 inversion-bearing chromosome arms are plotted from centromere (0) to telomere, inversion boundaries are shown with vertical black lines. D_{xy} per 50 kb window is plotted to show absolute divergence between *D. persimilis* and *D. p. bogotana* (red) and absolute divergence between *D. persimilis* vs *D. p. pseudoobscura* (blue). Boxplots (right) summarize these divergence estimates by region: Inverted, Collinear, and $Collinear_{FR}$. $Collinear_{FR}$ is the subset of collinear positions predicted to be freely recombining (excludes the grayed-out positions near inversion breakpoints or chromosome ends).

Evidence for early post-speciation exchange

To test for evidence of gene flow after speciation but before the split of *D. p. pseudoobscura* and *D. p. bogotana* (Figure 1), we fit observed patterns of collinear region intergenic nucleotide variation in *D. persimilis* and *D. p. bogotana* to models of divergence in isolation, isolation-with-

338 migration (IM), and isolation-with-initial-migration (IIM) using maximum-likelihood estimation of
339 parameters under these models (Figure 2; Costa & Wilkinson-Herbots 2017). In traditional IM
340 models applied to infer gene flow, parameter estimates can be biased by the underlying assumption
341 that gene flow is constant. IIM specifically addresses this assumption by operating on the premise of
342 an initial period of gene flow followed by isolation. An IIM framework is appropriate for the *D.*
343 *persimilis* and *D. p. bogotana* comparison, given our knowledge that these taxa have been evolving in
344 allopatry for the past 150,000 years (S W Schaeffer & Miller, 1991). Indeed, a nested model
345 comparison to test the relative support among the models rejects the null hypothesis of divergence
346 in isolation and suggests that IIM models best fit the data (Table 1 and Supplementary Table 4). All
347 models allowing for migration and population size change gave a significantly better fit than a model
348 of strict divergence in isolation, and the log-likelihood of the data under the tested models was
349 maximized in the IIM2 scenario (Table 1 and Supplementary Table 4). The IIM2 model estimates
350 parameters under divergence with potentially asymmetric bidirectional gene flow until some
351 timepoint in the past and, unlike the IIM1 model, does not assume constant population sizes (Figure
352 2). We also considered models similar to IIM2, but assuming unidirectional gene flow between *D.*
353 *persimilis* and *D. p. bogotana* (IIM3 and IIM4). Nested model comparison supports the choice of any
354 of the three models with varying population sizes (IIM2, IIM3, or IIM4) over IIM1, and the
355 likelihood of IIM2 supports bidirectional gene flow (Table 1). An application of the Costa &
356 Wilkinson-Herbots (2017) framework to the sympatric *D. persimilis* and *D. p. pseudoobscura*
357 comparison can be found in Fuller et al. (2018), which also found the best fit to be an IIM model,
358 compatible with our results suggesting gene flow between the *D. persimilis* and *D. pseudoobscura*
359 lineages after speciation. Importantly, our application of this framework to the allopatric *D. persimilis*
360 and *D. p. bogotana* comparison demonstrates that a significant amount of this exchange likely
361 occurred prior to the split of *D. p. bogotana* (region 2 in Figure 1).

Table 1 | Forward selection of the best model† of *D. persimilis - D. p. bogotana* divergence using the maximized log-likelihood (LogL) under each model in likelihood-ratio tests.

H_0	H_1	Deg. of Freedom	LogL H_0	LogL H_1	LRT Statistic	P-value
Iso	IM	2	-25511.23	-25492.84	36.78	1.031e-08
Iso	Iso2	2	-25511.23	-25467.66	87.14	1.196e-19
IM	IIM1	1	-25492.84	-25492.84	0	-
IM	IIM2	3	-25492.84	-25442.42	100.84	1.025e-21
Iso2	IIM2	3	-25467.66	-25442.42	50.48	6.313e-11
IIM1	IIM2	2	-25492.84	-25442.42	100.84	1.267e-22
IIM1	IIM3	1	-25492.84	-25447.73	90.22	2.131e-21
IIM1	IIM4	1	-25492.84	-25444.03	97.62	5.069e-23

† *See Figure 2 for illustration of the different models.*

Patterson's D-statistic and f_d suggest recent introgression

Given the evidence for gene flow between *D. persimilis* and *D. pseudoobscura*, we next examined whether some of this gene flow was very recent (within the past 150,000 years). To contrast sympatric and allopatric subspecies of *D. pseudoobscura* in their similarity to *D. persimilis*, we applied Patterson's D-statistic and f_d (Martin et al., 2015) using the tree: (((*D. p. bogotana*, *D. p. pseudoobscura), D. persimilis), D. lowei))).* Patterson's D-statistic is an implementation of ABBA-BABA, which uses parsimony informative sites to test whether derived alleles ("B") in *D. persimilis* are shared with *D. p. bogotana* or with *D. p. pseudoobscura* at equal frequencies. Derived alleles in *D. persimilis* may be shared with *D. p. pseudoobscura* due to ancestral polymorphism, ancient gene flow (prior to the split of the two *D. pseudoobscura* subspecies), recent gene flow (since the split of the two *D. pseudoobscura* subspecies), or a combination of these factors. The null expectation is that the two phylogeny-discordant patterns, ABBA and BABA, should be present equally if ancestral polymorphism and ancient gene flow are the sole drivers of patterns of divergence. Gene flow between *D. p. pseudoobscura* and *D. persimilis* since the split of the two *D. pseudoobscura* subspecies (estimated at 150,000 years ago: Schaeffer & Miller 1991) would promote an excess of ABBA over BABA patterns, particularly on freely recombining chromosomes. Indeed, ABBA sites exceed BABA sites

on all chromosomes (Supplementary Table 5), and chromosome 4 shows an unambiguously significant excess of ABBA (|Z-score| >= 5), suggesting that the phylogenetic relationship between these four taxa does not fully explain the observed patterns of divergence and some very recent gene exchange has occurred between the North American species. Furthermore, the genome wide z-score for collinear regions is significant (|Z-score| = 7.215), and none of the z-scores for inverted regions are significant (Supplementary Table 5).

Given the observed excess of ABBA over BABA sites throughout the genome, we next applied f_d to quantify this excess in smaller genomic intervals. In comparison to D-statistics, f_d is less affected by differences in effective population size and is better suited to identifying introgression regions (Martin et al., 2015). The genome-wide patterns of f_d support the evidence of gene flow between *D. p. pseudoobscura* and *D. persimilis*, particularly in the collinear regions of the genome (**Error! Reference source not found.**). Inverted regions exhibit markedly lower f_d compared to collinear regions (**Error! Reference source not found.**A). This difference is statistically significant on all inversion-bearing chromosomes, regardless of whether the inverted regions are compared to all collinear regions or just the conservative subset contained in $collinear_{FR}$ (**Error! Reference source not found.**B; $p < 0.01$ for all comparisons, Mann-Whitney U).

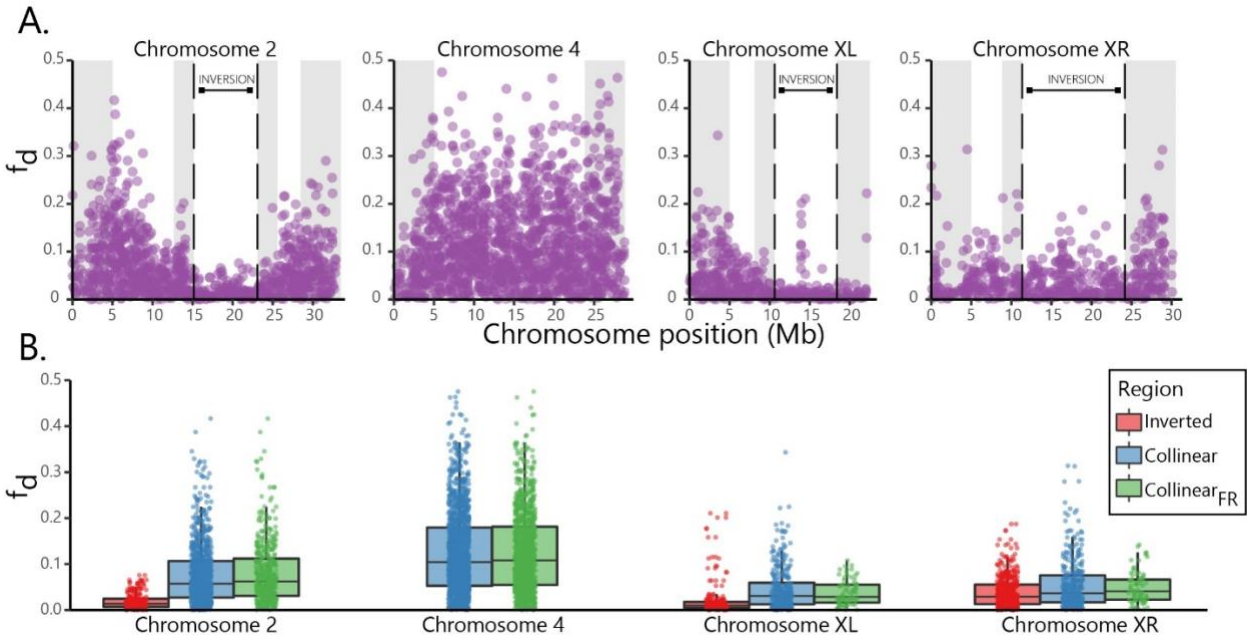
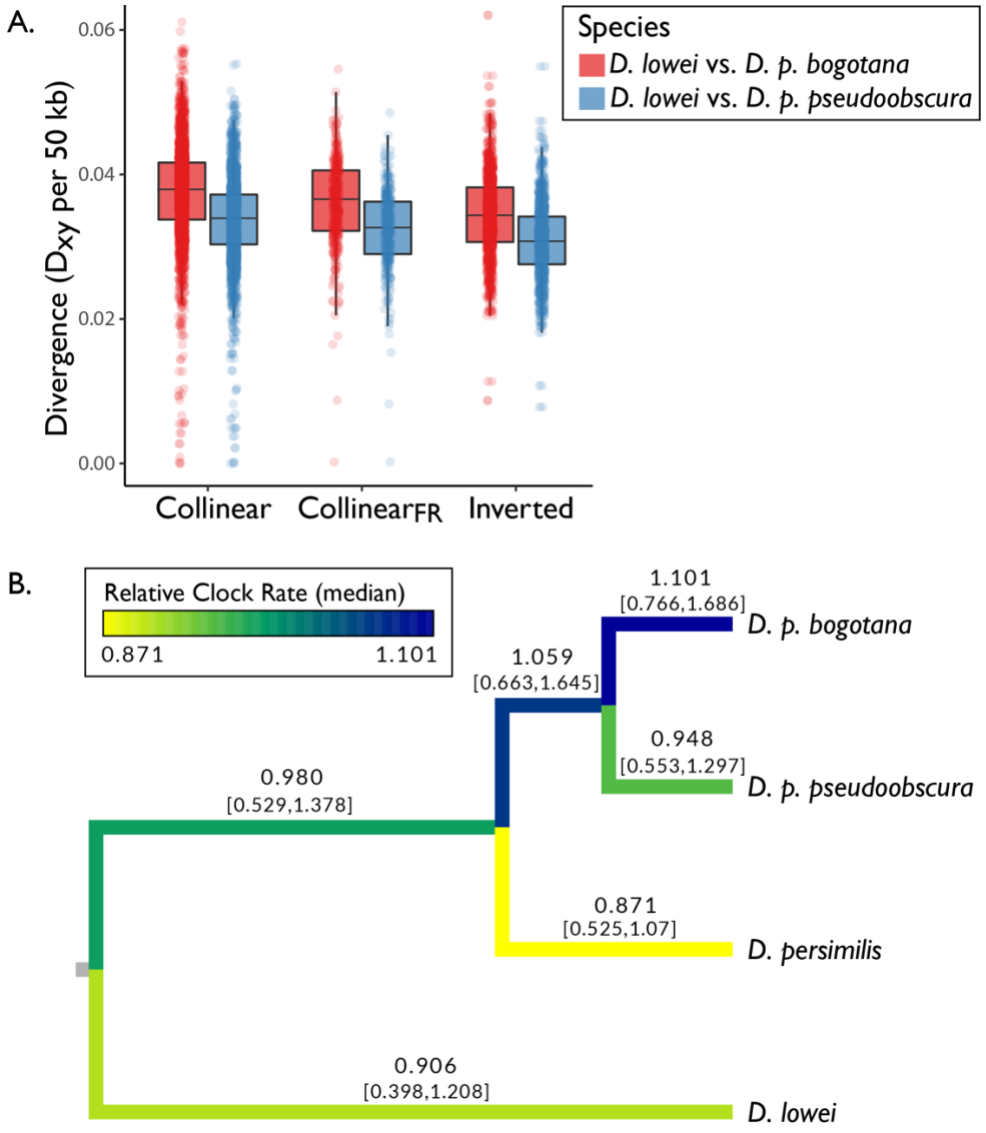


Figure 4 | Signals of introgression along the genome. (A) The estimated proportion of introgression (f_d) between *D. p. pseudoobscura* and *D. persimilis* is shown in non-overlapping 100 SNP windows along chromosomes 2, 4, XL, and XR. Inversion boundaries are shown with dashed black lines, and collinear regions are grayed-out where they approach inversion breakpoints or chromosome ends (windows excluded from Collinear$_{FR}$). (B) A summary of the introgression estimates by region: Inverted, Collinear, and Collinear$_{FR}$.

Gene flow may act alongside other evolutionary forces that contribute to patterns of higher divergence in allopatry vs sympatry

All three inversion-bearing chromosomes exhibit lower divergence in the *D. persimilis:D. p. pseudoobscura* comparison vs the *D. persimilis:D. p. bogotana* comparison (Figure 3). Notably, this lower divergence in the sympatric comparison is statistically significant for both the collinear and inverted regions ($p < 0.001$ on each chromosome, Mann-Whitney U test). Divergence between the species pairs in both inverted and collinear regions shows the magnitude of this difference (Figure 3). This pattern is consistent across all strains: differentiation in the inverted regions between *D. persimilis:D. p. bogotana* is higher than differentiation between *D. persimilis* and any of the North American *D. p. pseudoobscura* genomes (Supplementary Figure 2).

The observation that divergence is lower in sympatry compared to allopatry even in the inverted regions was not anticipated. A possible explanation for the lower divergence in the sympatric species pair is that gene flow is homogenizing these species even in inverted regions. Estimates of divergence between *D. persimilis* and *D. p. bogotana* are consistently higher than estimates of divergence between *D. persimilis* vs *D. p. pseudoobscura*, even in breakpoint-adjacent regions (Figure 3). Though there is evidence that double crossovers and gene conversions occur within inversions, and gene conversion may occur in regions adjacent to inversion breakpoints (Crown, Miller, Sekelsky, & Hawley, 2018; Korunes & Noor, 2017, 2019; Stephen W Schaeffer & Anderson, 2005; Stevison et al., 2011), an alternative explanation is that *D. p. bogotana* has experienced more substitutions per site. Comparing each subspecies of *D. pseudoobscura* to the outgroup, *D. lowei,* we note that divergence between *D. lowei* and *D. p. bogotana* is significantly greater than divergence between *D. lowei* and *D. p. pseudoobscura* in both collinear and inverted regions (Figure 5A; $p < 1 \times 10^{-8}$ for each of the three examined regions, Mann-Whitney U tests). This difference in divergence in unlikely to be explained by gene flow, since *D. lowei* does not produce hybrids with either subspecies of *D. p. pseudoobscura.* Genome-wide comparison of the relative substitution rates (Tajima, 1993) between the lineages reveals that *D. p. bogotana* has experienced significantly more substitutions per site than *D. p. pseudoobscura* relative to the outgroup species, *D. lowei* (Supplementary Table 6). To further explore the possibility of variable evolutionary rates among the lineages, we estimated relative clock rates using a Bayesian multispecies coalescent method, StarBEAST2 (Ogilvie et al., 2017). We find that the median estimated relative clock rate is highest in *D. p. bogotana* (Figure 5B). While these results suggest that variable clock rates may explain some of the divergence patterns among these lineages, we use caution in interpreting these results, as there is substantial uncertainty inherent in clock estimation.



438

439 **Figure 5 | Divergence from the outgroup *D. lowei*.** (A) The distribution of windowed D_{xy} in
440 Inverted, Collinear, and $Collinear_{FR}$ regions (summarized together from chromosomes 2, 4, XL, and
441 XR) showing divergence between *D. lowei* and each *D. pseudoobscura* subspecies: *D. p. bogotana* (red)
442 and *D. p. pseudoobscura* (blue). For each of the three examined regions, *D. lowei* and *D. p. bogotana* is
443 significantly greater than divergence between *D. lowei* and *D. p. pseudoobscura* ($p < 1 \times 10^{-8}$ for each
444 comparison, Mann-Whitney U test). (B) Relative clock rates per species obtained from the posterior
445 distribution from StarBEAST2. Median clock rates are displayed for each branch, with confidence
446 intervals in brackets representing the 95% highest posterior density.

447

Discussion

Our model-based examination of gene flow and statistical tests for excess shared variation found between *D. persimilis* and sympatric and allopatric subspecies of *D. pseudoobscura* indicates post-speciation gene exchange, including both early post-speciation gene flow and gene flow within the past ~150,000 years. Specifically, we interpret the excess of ABBA > BABA sites as evidence that *D. p. pseudoobscura* and *D. persimilis* have exchanged genes in collinear regions since the split of *D. p. bogotana*. The ABBA–BABA test is well-suited for considering the net effect of gene flow over large genomic regions, since it leverages patterns from many loci and thus accounts for variance among loci, while the related statistic, f_d, is better suited for application to genomic windows.

The observed patterns in f_d show reduced signals of introgression in inverted vs collinear regions and raise interesting questions about why f_d varies along each inversion and between the inversions. We expect that the amount of introgression within each inversion reflects the interplay of the inversion's age, size, genic content, X vs autosomal differences, and other genomic features. For example, the smaller length (~7 Mb) of the inversion on chromosome XL may result in less gene flux due to the reduced possibility for double crossover compared to the longer inversion (~12 Mb) on chromosome XR. Windows with high f_d values may also provide candidates for regions experiencing adaptive introgression or other functionally important evolutionary processes. For example, within the inversion of chromosome XL there are 4 windows with f_d greater than two standard deviations from the chromosome wide mean of 0.04 (Supplementary Table 7). Though we do not have a clear hypothesis about specific genetic variation that might be driving this pattern, we note that our BLAST results suggest that there are numerous genes within the approximately 538 kb region containing these windows, including several BLAST hits within the 4 high f_d windows (Supplementary Table 7). These genes, or nearby genes, may be interesting candidates for future

471 work. Overall, patterns in f_d highlight several potential areas for future work and provide the key
472 finding of significantly lower f_d in inverted compared to collinear regions (**Error! Reference source**
473 **not found.**), supporting the idea that inversions have acted as barriers to gene flow.
474 This evidence that introgression is driving patterns of divergence between *D. pseudoobscura*
475 and *D. persimilis* is in agreement with previous reports of ongoing hybridization in these species
476 (Dobzhansky, 1973; Hey & Nielsen, 2004; Machado & Hey, 2003; J R Powell, 1983). Despite our
477 evidence for recent gene exchange, it appears that introgression is not the sole driver of patterns of
478 divergence between these species overall. While D-statistics and f_d suggest an excess of shared,
479 derived alleles across the genomes of *D. pseudoobscura* and *D. persimilis*, these statistics may be biased
480 by factors such as ancestral population structure and differences in effective population size (He,
481 Liang, & Zhang, 2020; Martin et al., 2015; Slatkin & Pollack, 2008). In comparison to Patterson's D-
482 statistic, f_d is less sensitive to local variation in recombination rate and divergence. However, it can
483 still be biased by regions of reduced interspecies divergence, which may distort tests for recent
484 introgression (Martin et al., 2015), so the conclusion of recent introgression would be tentative
485 based on these results alone. Here, we explore other important factors that might underlie the
486 observed patterns of divergence, with particular consideration of how these factors might confound
487 signals of recent introgression.
488 As seen in Figure 3 and in previous studies (Kulathinal et al., 2009), divergence is higher in
489 inverted vs collinear regions in this system. This difference holds for divergence between *D. persimilis*
490 and either *D. p. bogotana* or *D. p. pseudoobscura*. The lower observed divergence in sympatry compared
491 to allopatry even in the inverted regions is somewhat surprising given the expectation that
492 recombination in hybrids will be restricted in these inverted regions. This observation led us to
493 consider the possibility that the allopatric subspecies, *D. p. bogotana*, might have experienced more
494 nucleotide substitutions per site than the other taxa. Thus, we considered four non-mutually

exclusive factors that might contribute to the observed patterns of divergence with respect to chromosomal arrangement: 1) the segregation of ancestral polymorphism (as advocated by Fuller *et al.* (2018)), 2) increased ~~branch length~~ substitution rate (branch length) in the allopatric *D. p. bogotana,* 3) gene flow prior to the split of *D. p. bogotana*, and 4) recent/ongoing gene flow (the latter two discussed in Powell 1983; Wang & Hey 1996; Wang *et al.* 1997; Noor *et al.* 2001, 2007; Machado & Hey 2003; Hey & Nielsen 2004; Machado *et al.* 2007; Kulathinal *et al.* 2009; McGaugh & Noor 2012). Achieving a cohesive view of the role of inversions in species divergence relies on considering the combined effects of these factors.

While it is challenging to disentangle these factors, we suggest that an important area for future work will be developing statistical approaches to summarize patterns of divergence while adjusting for other evolutionary dynamics. For example, D_{xy} could potentially be leveraged to provide insight into the effects of introgression on divergence after adjusting for differences in branch lengths. For the sympatric pair *D. persimilis* vs *D. p. pseudoobscura,* any difference in D_{xy} in inverted regions compared to collinear regions could be due to the segregation of ancestral inversion polymorphism or to post-speciation genetic exchange. In contrast, any difference in D_{xy} in inverted regions compared to collinear regions in the allopatric pair *D. persimilis* vs *D. p. bogotana* could be driven by the segregation of ancestral inversion polymorphism or by post-speciation gene flow prior to the split of *D. p. bogotana.* This comparison will not reflect any recent gene flow, since *D. p. bogotana* has evolved in allopatry for the past 150,000 years.

To explore how this contrast might be used to isolate the effects of recent introgression on divergence, we compared the divergence of each *D. pseudoobscura* subspecies from *D. persimilis* to the divergence of each *D. pseudoobscura* subspecies from *D. lowei* using the following equation to define the "introgression effect": (D_{xy} [*D.persimilis*:*D.p.bogotana*] - D_{xy} [*D.persimilis*:*D.p.pseudo.*]) – (D_{xy} [*D.lowei*:*D.p.bogotana*] - D_{xy} [*D.lowei*:*D.p.pseudo.*]). The first half of this equation should include the

519 effects of branch length in *D. p. bogotana* and the effects of any introgression between *D. pseudoobscura*
520 and *D. persimilis* (Supplementary Figure 3A). Since *D. lowei* does not hybridize with any of these
521 species, the second half of the equation should reflect only the effects of branch length in *D. p.*
522 *bogotana*. Thus, we propose that the difference between these terms should subtract effects of
523 evolutionary rate, leaving effects of recent introgression.

524 Such a proposed "introgression effect" statistic may be one potential strategy for examining
525 the relative influence of recent introgression on the reduction in *D. persimilis* vs *D. pseudoobscura*
526 divergence in sympatry vs allopatry. Applying this strategy to the present data, we note that inverted
527 vs. collinear regions differ significantly, suggesting that introgression has influenced divergence in
528 the collinear regions more so than the inverted regions (Supplementary Figure 3B; $p < 1 \times 10^{-8}$, all
529 inverted vs collinear comparisons, Mann-Whitney U test) and providing evidence that branch-length
530 differences alone are insufficient to fully explain the patterns of divergence. As we are interested in
531 whether, when, and how chromosomal inversions are contributing to patterns of divergence by
532 suppressing gene flow, the observed difference suggests that this strategy may provide a useful way
533 to consider the relative contribution of recent introgression compared to ancestral polymorphism
534 and branch length in species groups where similar allopatric-sympatric contrasts could be conducted.
535 However, we emphasize that the behavior of this kind of statistic remains to be explored. While any
536 conclusions based on this strategy are tentative at best, we hope that Supplementary Figure 3
537 stimulates discussion on future approaches.

538 Taken together, our results suggest that contributions from recent gene flow only partially
539 explain observed divergence patterns. Patterns of divergence between *D. persimilis* and *D.*
540 *pseudoobscura* may be explained by a combination of segregating ancestral polymorphism and post-
541 speciation gene flow. We applied a model-based approach to investigate the timing of gene flow
542 between *D. persimilis* and *D. pseudoobscura*. Our results suggest that an isolation-with-initial-migration

543 model best explains the divergence of *D. persimilis* and *D. p. bogotana* when compared to a model of
544 strict isolation. This result provides further evidence for gene flow between *D. persimilis* and *D.*
545 *pseudoobscura*, and it suggests that some of this gene flow occurred prior to the split of *D. p. bogotana*
546 and remains detectable in observed genetic patterns.

547 Our results question interpretations from earlier studies of this system. Given that *D. p.*
548 *bogotana* can be reasonably assumed to not be currently exchanging genes with either *D. persimilis* or
549 *D. p. pseudoobscura* (S W Schaeffer & Miller, 1991; R. L. Wang et al., 1997), *D. persimilis*: *D. p. bogotana*
550 divergence was argued to be a suitable "negative control" for examining the effect of recent
551 hybridization between *D. persimilis* and *D. p. pseudoobscura* (Brown et al., 2004). By this argument, the
552 effect of recent gene flow can be estimated by an allopatric vs sympatric comparison of the
553 difference in divergence (whether in DNA sequence or in phenotype) in inverted regions to
554 divergence in collinear regions. Specifically, Brown *et al.* (2004) and Chang and Noor (2007) inferred
555 multiple hybrid sterility factors between *D. p. bogotana* and *D. persimilis* that did not distinguish North
556 American *D. p. pseudoobscura* and *D. persimilis* (Brown et al., 2004; Chang & Noor, 2007). Similarly,
557 Kulathinal *et al.* (2009) observed significantly greater sequence difference between *D. p. bogotana* and
558 *D. persimilis* than between *D. p. pseudoobscura* and *D. persimilis*. In both cases, the authors interpreted
559 the difference to result from recent homogenization of the collinear regions in the latter pair. Based
560 on our findings, we suggest this difference may result at least in part from the accelerated rate of
561 divergence in *D. p. bogotana* (Figure 5, Supplementary Table 6).

562 Notably, there are also significant differences in demographic history and environmental
563 factors among the lineages. *D. p. bogotana* may have experienced a population bottleneck upon
564 colonization of South America leading to a subsequently small effective population size (Machado et
565 al., 2002; S W Schaeffer & Miller, 1991; R. L. Wang & Hey, 1996). These past reports of smaller
566 effective population size in *D. p. bogotana* are corroborated by our maximum-likelihood estimates

under all considered models of divergence (Supplementary Table 4, where θ_B reflects the relative population size of *D. p. bogotana* during the stage with genetic exchange between subpopulations, and θ_{C2} reflects the relative population size of *D. p. bogotana* during the isolation stage.) Additionally, the process of genetic divergence that shapes alleles responsible for local adaptation and hybrid incompatibility can extend deep into the history of the species. In fact, the influence of inversions on the divergence of a species pair can predate the split of the species. Inversion polymorphisms in the ancestral population of a species pair can contribute to patterns of higher sequence differentiation between species in those inverted regions (Fuller et al., 2018). Separating these effects requires an understanding of the timing and extent of introgression, which can only be understood with an appreciation for the evolutionary processes occurring in each of the taxa at hand. Overall, we caution that simple allopatry-sympatry comparisons can easily be misleading, and the population histories and rates of evolution of the examined species should be carefully considered. We present evidence of gene flow between *D. pseudoobscura* and *D. persimilis* both before and after the split of *D. p. bogotana* from North American *D. p. pseudoobscura*. Though there are many remaining questions about how inversions shape divergence, our findings build on the large body of work in this classic system to provide evidence that inversions have contributed to the divergence of *D. pseudoobscura* and *D. persimilis* over multiple distinct periods during their speciation: 1) early in the speciation continuum of *D. pseudoobscura* and *D. persimilis*, due to segregation of inversions in the ancestral population, 2) post-speciation gene flow prior to the split of *D. p. bogotana*, and 3) recent gene flow.

AUTHOR CONTRIBUTIONS

KLK and MAFN were responsible for the project's conception and design, in consultation with CAM. KLK performed the analyses and prepared the manuscript with essential feedback and revisions from MAFN and CAM.

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