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

Running Title: Inversions shape divergence over time

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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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All raw sequence data will be provided on NCBI's Short Read Archive by the time of publication, with all sequence accession numbers in Supplementary Table 1. Scripts used for data analysis are available on GitHub: https://github.com/kkorunes/Dpseudoobscura_Introgression

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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 chromosomal 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) pre-speciation segregation of ancestral polymorphism, 2) post-speciation ancient gene flow, and 3) recent gene flow. We discuss these results in terms of our understanding of evolution in this classic system and provide cautions for interpreting divergence measures in similar datasets 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 *et al.* 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

42 assumptions they make about population histories and evolutionary rates (reviewed in Payseur &
43 Rieseberg 2016). Further, shared patterns of variation are often interpreted as evidence of ongoing
44 gene flow, but segregating ancestral polymorphism could also be the primary, or even the sole,
45 driver of these patterns (Fuller *et al.* 2018). In the ancestral population of two species, segregating
46 chromosomal inversions may shield inverted regions of the genome from recombination, thus
47 facilitating the divergence of sympatric ecotypes or populations. Heightened within-species
48 differentiation in inverted regions has been observed in many systems, including *Rhagoletis pomonella*
49 (Michel *et al.* 2010), *Anopheles gambiae* (Manoukis *et al.* 2008), and *Mimulus guttatus* (Lowry & Willis
50 2010). Such heightened differentiation between karyotypes may persist along the speciation
51 continuum, making it difficult to disentangle the effects of inversions reducing recombination in the
52 ancestral population vs reducing introgression upon secondary contact. Fuller *et al.* (2018) recently
53 discussed the possibility that ancestrally segregating inversions that sort between species may
54 provide a "head-start" in molecular divergence, possibly predisposing them to harbor a
55 disproportionate fraction of alleles associated with species differences. Unlike models assuming
56 homogenization of collinear regions via post-speciation gene flow, this model predicts that young
57 species that diverged in allopatry may also exhibit higher divergence in inverted regions than
58 collinear regions. These models are not mutually exclusive: dynamics of the ancestral population as
59 well as post-speciation gene flow can shape patterns of variation between species.
60 Disentangling the effects of ancestral polymorphism from the effects of post-speciation gene
61 flow is a fundamental puzzle in understanding speciation. To achieve a cohesive picture of how
62 hybridization influences divergence and speciation, we need to consider the approaches outlined
63 above in a model system with extensive whole-genome sequence data to assess models and reconcile
64 interpretations of possible signals of introgression. The sister species pair *Drosophila pseudoobscura* and
65 *D. persimilis* present an ideal opportunity to dissect an evolutionary history of divergence nuanced by

66 multiple inversions, lineage sorting, and gene flow. Despite the rich history of work on
67 understanding speciation and divergence in *D. pseudoobscura* and *D. persimilis*, there are unresolved
68 questions about the rates and timing of introgression between these species. A few F_1 hybrids of
69 these species have been collected in the wild (Powell 1983) and many previous studies have
70 documented molecular evidence of introgression, detectable in both nuclear and mitochondrial loci
71 (e.g., Machado *et al.* 2002; Machado & Hey 2003; Hey & Nielsen 2004; Fuller *et al.* 2018). Inverted
72 regions between these species exhibit greater sequence differences than collinear regions, and this
73 pattern was previously inferred to result from introgression post-speciation. In the largest scale
74 study, McGaugh and Noor (2012) used multiple genome sequences of both species and an
75 outgroup, and reinforced previous studies (e.g., Noor *et al.* 2007) showing that the three
76 chromosomal inversions differ in divergence time. They inferred a "mixed mode geographic model"
77 (Feder *et al.* 2011) with sporadic periods of introgression during and after the times that the
78 inversions spread. However, in addition to confirming evidence for gene flow between *D.*
79 *pseudoobscura* and *D. persimilis* after speciation, Fuller *et al.* (2018) recently argued the inversions arose
80 within a single ancestor species, differentially sorted in the descendant species, and this sorting of
81 ancestral polymorphisms may explain observed patterns of nucleotide variation. To fully understand
82 the role of hybridization in the speciation process, the contrasting models must be reconciled.
83 We acquired extensive whole-genome sequence data to re-explore patterns of introgression
84 and divergence in the *Drosophila pseudoobscura* / *D. persimilis* system. We leverage the allopatric *D.*
85 *pseudoobscura* subspecies, *D. pseudoobscura bogotana* (*D. p. bogotana)* and two outgroup species (*D. miranda*
86 and *D. lowei*) to distinguish recent from ancient effects of inversions on gene flow. To help clarify
87 the role of inversions over the evolutionary history of these species, we consider three distinct time
88 scales: 1) pre-speciation segregation of ancestral polymorphism, 2) post-speciation ancient gene
89 flow, and 3) recent introgression (Figure 1). 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.

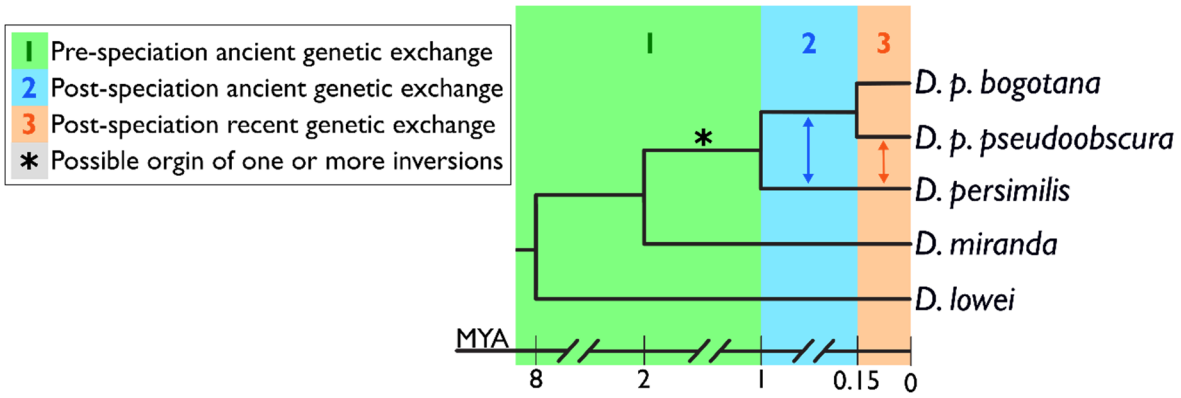


Figure 1 | Gene flow in the context of the evolutionary history of *D. pseudoobscura* and *D. persimilis*. We consider how inversions differing between *D. pseudoobscura* and *D. persimilis* might shape patterns of divergence by affecting gene flow at 3 timescales: 1) pre-speciation recombination in ancestral populations with segregating inversion polymorphisms, 2) post-speciation ancient gene flow, and 3) recent introgression. Here, we show the evolutionary context and approximate divergence times of the taxa considered in the present study, with arrows indicating post-speciation gene flow between *D. pseudoobscura* and *D. persimilis.*

We first examine patterns of divergence in inverted regions compared to collinear regions, and we discuss evidence for early, pre-speciation exchange. 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*) (Schaeffer & Miller 1991). We implement Patterson's D-statistic to contrast the sympatric (*D. p. pseudoobscura*) and allopatric (*D. p. bogotana)* subspecies in their similarity to *D. persimilis*. Kulathinal *et al.* (2009) previously argued that recent post-speciation gene flow contributes to the difference in coalescence

111 time between inverted and collinear regions, observable in the higher genetic similarity in collinear
112 regions between *D. persimilis* and sympatric *D. p. pseudoobscura* compared to similarity between *D.*
113 *persimilis* and allopatric *D. p. bogotana*. That study also tested for an excess of shared, derived bases
114 between *D. persimilis* and *D. p. pseudoobscura* compared to *D. persimilis* and allopatric *D. p. bogotana.*
115 Their application of this D-statistic precursor suggested a borderline statistically significant signature
116 of gene flow, but this test was limited by low sequencing coverage. Nonetheless, consistent with that
117 and other previous studies, we observe higher divergence between these species in inverted than
118 collinear regions, and our implementation of Patterson's D-statistic indicates very recent gene
119 exchange in collinear regions. Notably, divergence measures to *D. persimilis* are also higher for
120 allopatric than sympatric *D. pseudoobscura* subspecies in *both* inverted and collinear regions. One
121 possible explanation for this pattern is extensive recent gene exchange throughout much of the
122 genome, even in inverted regions. To determine the relative effect of introgression, we correct for
123 different evolutionary rates across taxa, and we consider the role of segregating inversions in
124 ancestral populations. We discuss these results in the context of the extensive past work towards
125 understanding divergence and speciation in this classic system, and we provide cautions for
126 interpreting divergence measures in similar datasets in other systems.

127 ## *Methods*

128 ### Genomic datasets

129 Whole-genome short-read sequence data was analyzed from 19 *D. p. pseudoobscura* and 8 *D.*
130 *persimilis* strains, along with 4 *D. p. bogotana* strains as an allopatric point of comparison (both males
131 and females were sequenced, all from inbred strains listed in Supplementary Table 1 with SRA
132 accessions). We used *D. lowei* as an outgroup. *D. lowei* and *D. pseudoobscura* likely diverged 5-11 MYA
133 (Beckenbach *et al.* 1993), and hybrids between these two species are sterile (Heed *et al.* 1969). Scripts

134 used for genome alignment, SNP calling, and analyses are available on GitHub
135 (https://github.com/kkorunes/Dpseudoobscura_Introgression). To avoid biasing identification of
136 variants towards *D. pseudoobscura*, the *D. miranda* reference genome assembly (DroMir2.2; GenBank
137 assembly accession GCA_000269505.2) was chosen as the reference for all subsequent alignments.
138 *D. miranda* diverged from *D. pseudoobscura* only within the past ~2 million years (Wang & Hey 1996),
139 facilitating alignment of *D. pseudoobscura* and *D. persimilis* genomes to the *D. miranda* genome
140 assembly. Further, the arrangement of the assembled *D. miranda* chromosomes matches the
141 published contig order and arrangement of *D. pseudoobscura* (Schaeffer *et al.* 2008), but with the
142 advantage of being assembled into 6 continuous chromosome arms: chromosomes XL, XR, 2, 3, 4,
143 and 5. Here, we analyze the majority (83%) of the assembled genome. We exclude only regions
144 where we cannot reasonably examine introgression and divergence: chromosome 3, which presents
145 confounding factors from its inversion polymorphisms within species (Dobzhansky & Epling 1944;
146 Powell 1992), and the very small (<2 Mb) portion of the genome found on the largely
147 nonrecombining "dot" chromosome (chromosome 5).

148 **Alignments and variant calling**

149 To confirm the arrangement of *D. pseudoobscura* contigs with respect to the *D. miranda*
150 reference, each *D. pseudoobscura* chromosome was split into lengths of 1 Mb, and these segments
151 were aligned to the *D. miranda* reference using BWA-0.7.5a (Li & Durbin 2009). We then extracted
152 the 2 kb regions surrounding published inversion breakpoints to obtain the breakpoint locations in
153 the coordinates of the *D. miranda* reference (see Supplementary Table 2). After confirming that the
154 arrangement of the assembled *D. miranda* chromosomes matched the arrangement of the *D.*
155 *pseudoobscura* contig order and arrangement described by Schaeffer *et al.* (2008), all sequencing data
156 were aligned to the reference genome of *D. miranda* using BWA-0.7.5a (Li & Durbin 2009), and

157 Picard was used to mark adapters and duplicates (http://broadinstitute.github.io/picard). Variants
158 were called used GATK v4 and filtered based GATK's hard filtering recommendations (McKenna *et*
159 *al.* 2010; Van der Auwera *et al.* 2013), excluding sites with QualByDepth (QD) < 2.0, FisherStrand
160 (FS) > 60, StrandOddsRatio (SOR) > 3.0, MQ < 40, MQRankSum < -12.5, ReadPosRankSum < -8.

161 **Patterns of divergence**

162 The resulting VCF files were then processed using PLINK (Purcell *et al.* 2007). VCFs were
163 converted to PLINK's bed/bim format, keeping only sites that passed the filters described above.
164 SNPs were pruned for linkage disequilibrium using the --indep-pairwise function of PLINK ("--
165 indep-pairwise 50 50 0.5") before performing principal components analysis (PCA) using PLINK's -
166 -pca function to confirm the grouping of individuals within their respective species (Figure 1;
167 Supplementary Figure 1). Admixtools was used to implement Patterson's D-statistic (Patterson *et al.*
168 2012) using *D. lowei* as on outgroup to polarize ancestral vs derived alleles. For input into
169 Admixtools, we used the *convertf* program of Admixtools to convert each PLINK ped file to
170 Eigenstrat format which includes a *genotype* file, a *snp* file, and an *indiv* file. Per recommendations in
171 the Admixtools documentation, we defined the physical positions of each SNP in the *snp* file to be
172 10 kb apart from each adjacent SNP to allow Admixtools to interpret every 100 SNPs as 1 Mb or
173 1cM, since this software uses centiMorgans as the unit for block size during jackknifing and assumes
174 that 1 Mb = 1 cM. We set the block size parameter to 0.01 cM, which in this case is interpreted as
175 blocks of 100 SNPs. Next, *qpDstat* was used to obtain D-statistics for each chromosome. These
176 four-population tests were of the form (((A,B), C), D), where A = *D. p. bogotana,* B = *D. p.*
177 *pseudoobscura,* C = *D. persimilis,* and D = *D. lowei.* To study signatures of introgression along the
178 genome, we applied f_d (Martin *et al.* 2015) in genomic intervals that presented an excess of ABBA
179 over BABA sites. Using non-overlapping windows of 100 SNPs, we calculated f_d using the

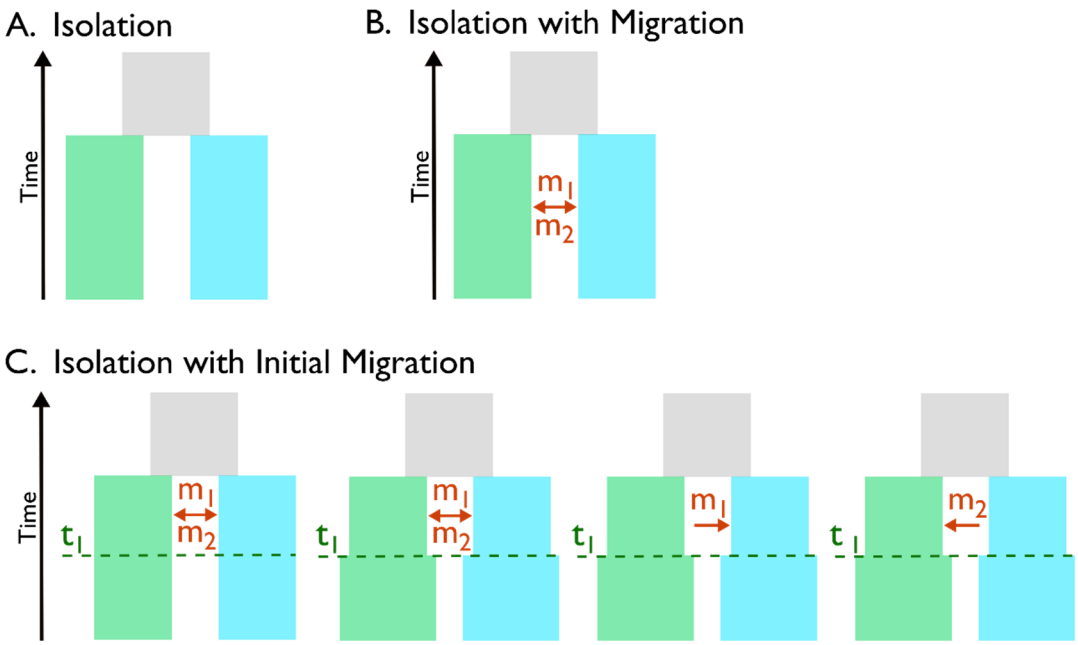
180 Dinvestigate program from Dsuite (Malinsky *et al.* 2020). Absolute divergence, D_{xy}, was calculated
181 using custom scripts over fixed window sizes of 50 kb. D_{xy} was calculated from variant and invariant
182 sites after subjecting SNPs to the filters described above and filtering invariant sites based on depth
183 (depth >= 10). Per-site depths for all sites were acquired from BAM files using Samtools ("samtools
184 depths -a <in>") (Li *et al.* 2009). Finally, to test for differences in evolutionary rate that might
185 influence observed patterns of divergence and gene flow, differences in substitution rates among the
186 lineages were assessed with Tajima's relative rate test, using *D. lowei* as the outgroup (Tajima 1993;
187 scripts for implementation available on GitHub repository linked above). Tajima's relative rate test
188 was applied to the combined set of SNPs from chromosomes 2, 4, XL, and XR—excluding sites
189 where the outgroup *D. lowei* was heterozygous or missing data.

190 **Models of gene flow**

191 To test for evidence of gene flow after the split of *D. pseudoobscura* and *D. persimilis,* but
192 before the split of *D. p. bogotana,* we used the maximum-likelihood methods derived by Costa &
193 Wilkinson-Herbots (2017) to compare three scenarios of divergence between *D. persimilis* and *D. p.*
194 *bogotana* (Figure 2): (A) divergence in isolation (Iso) without gene flow following the split of an
195 ancestral population; (B) divergence in isolation-with-migration (IM) with constant (but potentially
196 asymmetric) gene flow since the split of an ancestral population until the present; and (C) divergence
197 in isolation-with-initial-migration (IIM) with gene flow until some timepoint in the past and
198 divergence in isolation since that timepoint. Under the IIM model, we tested four scenarios: IIM1
199 estimates parameters under divergence with potentially asymmetric bidirectional gene flow until
200 some timepoint in the past, assuming constant population sizes; IIM2 is the same scenario as IIM1,
201 but allows for changes in population sizes; IIM3 and IIM4 are the same as IIM2, but assume

202 unidirectional gene flow from population 2 to population 1 (IIM3) or from population 1 to

203 population 2 (IIM4).



204

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

214 We computed the likelihood of our *D. persimilis* and *D. p. bogotana* sequence data under each

215 of the six scenarios described above and in Figure 2 (Iso, IM, IIM1, IIM2, IIM3, and IIM4). To

216 reduce potential effects of selection, we used intergenic loci spaced at least 2 kb apart, similar to the

217 strategy of Wang & Hey (2010). Linkage-disequilibrium decays within tens to hundreds of bases in

218 *Drosophila* (Langley *et al.* 2000), so we expect that avoiding genic regions will minimize the effects of

219 linked selection. To identify intergenic regions in the *D. miranda* genome, we used the set of all *D.*

220 *pseudoobscura* gene annotations published by Flybase (http://flybase.org, Full Annotation Release
221 3.04), and we used BLAST to identify genomic regions with significant similarly to the *D.*
222 *pseudoobscura* gene annotations, using cutoffs of evalue = 10^{-6} and percent identity = 80 (Altschul *et al.*
223 1990). From the remaining regions, we then randomly sampled 500 bp segments separated by at
224 least 2 kb to create a set of ~15,000 intergenic loci. We then randomly divided these loci into three
225 nonoverlapping subsets to satisfy the models' requirement of independent estimates of pairwise
226 differences and mutation rates in loci (1) within *D. persimilis*, (2) within *D. p. bogotana*, and (3)
227 between *D. persimilis* and *D. p. bogotana.* Costa & Wilkinson-Herbots (2017) recommends using per-
228 locus relative mutation rates, which we calculated using the average distance to the outgroup *D. lowei*,
229 following the equation from Yang (2002), which gives the relative mutation rate at a locus as the
230 outgroup distance at that locus divided by the average outgroup distance along all loci. To select the
231 model that best fits the data, we then tested the relative support among the divergence models using
232 likelihood-ratio tests following the sequence of pairwise comparisons shown in Table 1, where the
233 degrees of freedom in each test is the difference in the dimensions of parameter space (Costa &
234 Wilkinson-Herbots 2017). To ensure that our results were robust against the effects of linkage
235 within inverted regions, we next sampled regions expected to be freely-recombining throughout the
236 timescales examined: i.e., we repeated these analyses excluding any loci from the inverted regions,
237 leaving ~11,000 intergenic, collinear loci.

238 *Results*

239 Patterns of divergence in inverted vs collinear regions

240 The suppression of crossing over within inversions leads to distinct signatures of nucleotide
241 divergence within and near inversions. Figure 3 presents windowed (50 kb windows) estimates of
242 divergence between *D. persimilis* and *D. p. bogotana* and divergence between *D. persimilis* vs *D. p.*

243 *pseudoobscura* for the three chromosome arms that contain fixed (chromosome 2, XL) or nearly-fixed
244 (chromosome XR) inversion differences between *D. persimilis* and *D. pseudoobscura*. As previously
245 observed, divergence is low in regions near the centromere (Noor *et al.* 2007; Kulathinal *et al.* 2009).
246 To consider the effects of inversions on divergence, we contrast observed patterns within inversions
247 to regions outside the inversions (collinear) and to the subset of collinear regions that can be
248 predicted to be reasonably freely-recombining (denoted as collinear_{FR}). Collinear_{FR} excludes the 5 Mb
249 windows adjacent to telomeric and centromeric ends of chromosome assemblies, which undergo
250 very little crossing over and harbor reduced sequence diversity (Andolfatto & Wall 2003; Kulathinal
251 *et al.* 2008; Stevison & Noor 2010). Similarly, collinear_{FR} excludes regions within 2.5 Mb outside of
252 inversion breakpoints, based on previous studies which have reported suppression of crossing over
253 extending 1-2 Mb beyond inversion breakpoints (Machado *et al.* 2007; Kulathinal *et al.* 2009;
254 Stevison *et al.* 2011).

255 Confirming many previous studies (Machado *et al.* 2007; Noor *et al.* 2007; Kulathinal *et al.*
256 2009; Stevison *et al.* 2011; McGaugh & Noor 2012), we observed that *D. persimilis* vs *D. p.*
257 *pseudoobscura* divergence is significantly higher in inverted than collinear windows, regardless of
258 whether the inverted regions are compared to all collinear windows or to the collinear_{FR} subset (see
259 Supplementary Table 3 for average divergence estimates and statistical comparisons). *D. persimilis* vs
260 allopatric *D. p. bogotana* divergence is also higher in inverted than collinear windows on
261 chromosomes 2 and XL, with a nonsignificant difference on chromosome XR (though the
262 difference is significant if the comparison is restricted to collinear_{FR}; Supplementary Table 3). The
263 higher divergence in inverted vs collinear regions could be due to pre-speciation segregation of
264 inversion polymorphisms in the ancestral population or to interspecies gene flow homogenizing
265 collinear regions. We next examined evidence of pre-speciation genetic exchange.

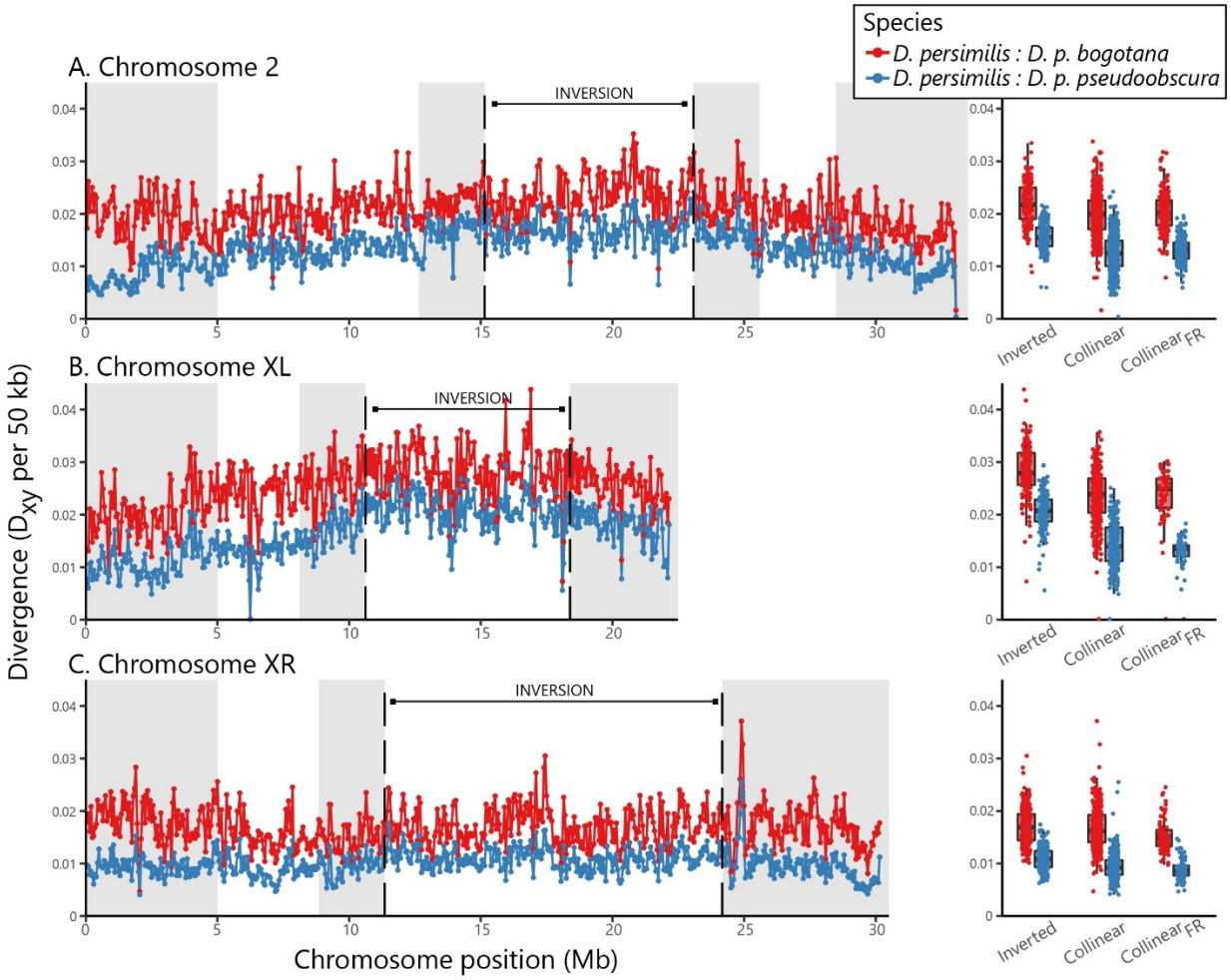


Figure 3 | Genome-wide divergence between species. On the left, each of the 3 inversion-bearing chromosome arms are plotted from centromere (0) to telomere, inversion boundaries are shown with vertical black lines. D_{xy} per 50 kb window is plotted to show divergence between *D. persimilis* and *D. p. bogotana* (red) and 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).

Confirming evidence for exchange between karyotypes early in the speciation continuum

Previous studies identified differences in sequence divergence between *D. persimilis* and *D. p. pseudoobscura* among the three inverted regions (Noor *et al.* 2007; McGaugh & Noor 2012; Fuller *et al.* 2018). They interpreted this finding as evidence that the derived inversions arose during, or interspersed by, periods where gene exchange was occurring in other regions of the genome. For

280 both *D. persimilis* vs *D. p. pseudoobscura* and *D. persimilis* vs *D. p. bogotana*, we compared measures of
281 windowed divergence among the inverted regions. Each pairwise comparison between the
282 inversions yielded a significant difference wherein XL > 2 > XR ($p < 0.0001$, Mann-Whitney U test;
283 Supplementary Table 3). Our observed average divergence within the inversions confirms previous
284 accounts of the relative divergence and apparent relative age of the inversions (Noor *et al.* 2007;
285 McGaugh & Noor 2012; Fuller *et al.* 2018), supporting the previous suggestions of ancestral
286 exchange prior to the completion of speciation. We next explored the possibility of exchange after
287 speciation but before the split of *D. p. pseudoobscura* and *D. p. bogotana* (region 2 in Figure 1).

288 **Evidence for early post-speciation exchange**

289 Much of the previous support for post-speciation gene flow between these species has
290 focused on comparisons of *D. persimilis* and *D. pseudoobscura* (Wang *et al.* 1997; Machado *et al.* 2002;
291 Hey & Nielsen 2004; Kulathinal *et al.* 2009; Fuller *et al.* 2018). To distinguish between post-
292 speciation ancient gene flow and recent introgression (Figure 1), we leveraged the allopatric *D. p.*
293 *bogotana*. Some past studies suggesting evidence of introgression between *D. pseudoobscura* and *D.*
294 *persimilis* also leveraged this allopatric subspecies (Machado & Hey 2003; Brown *et al.* 2004; Chang &
295 Noor 2007; Kulathinal *et al.* 2009). We sampled intergenic loci from *D. persimilis* and *D. p. bogotana,*
296 and we fit the observed patterns of nucleotide variation to models of divergence in isolation,
297 isolation-with-migration (IM), and isolation-with-initial-migration (IIM) using maximum-likelihood
298 estimation of parameters under these models (Figure 2; Costa & Wilkinson-Herbots 2017). In
299 traditional IM models applied to infer gene flow, parameter estimates can be skewed by the
300 underlying assumption that gene flow is constant. IIM specifically addresses this assumption by
301 operating on the premise of an initial period of gene flow followed by isolation. Maximizing
302 parameter likelihoods under an IIM framework is appropriate for the *D. persimilis* and *D. p. bogotana*

303 comparison, given our knowledge that these taxa have been evolving in allopatry for the past
304 150,000 years (Schaeffer & Miller 1991). Indeed, nested model comparison to test the relative
305 support among the models rejects the null hypothesis of divergence in isolation and suggest that IIM
306 models best fit the data. These results were consistent between the full set of loci and the subset
307 sampled from only collinear regions (Table 1 and Supplementary Table 4). All models allowing for
308 migration gave a significantly better fit than a model of strict divergence in isolation, and the log-
309 likelihood of the data under the tested models was maximized in the IIM2 scenario (Table 1 and
310 Supplementary Table 4, 5). The IIM2 model estimates parameters under divergence with potentially
311 asymmetric bidirectional gene flow until some timepoint in the past and, unlike the IIM1 model,
312 does not assume constant population sizes (Figure 2). We also considered models similar to IIM2,
313 but assuming unidirectional gene flow from *D. p. bogotana* to *D. persimilis* (IIM3) or from *D. persimilis*
314 to *D. p. bogotana* (IIM4). Nested model comparison supports the choice of any of the three models
315 with varying population sizes (IIM2, IIM3, or IIM4) over IIM1, and the likelihood of IIM2 supports
316 bidirectional gene flow (Table 1). These results provide additional support for gene flow between
317 the *D. persimilis* and *D. pseudoobscura* lineages after speciation and demonstrate that a significant
318 amount of this exchange was relatively ancient (region 2 in Figure 1), occurring prior to the split of
319 *D. p. bogotana.*

320 **Table 1** | Forward selection of the best model† of *D. persimilis - D. p. bogotana* divergence using the
321 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	-33702.73	-33659.39	86.68	1.505e-19
IM	IIM1	1	-33659.39	-33659.39	0	-
IM	IIM2	3	-33659.39	-33538.92	240.94	5.960e-52
IIM1	IIM2	2	-33659.39	-33538.92	240.94	4.792e-53
IIM1	IIM3	1	-33659.39	-33543.76	231.26	3.166e-52
IIM1	IIM4	1	-33659.39	-33542.89	233	1.322e-52

322 † *See Figure 3 for illustration of the different models.*

323 **D-statistics suggest recent genetic exchange**

324 Given the evidence for gene flow between *D. persimilis* and *D. pseudoobscura*, we next
325 examined the timing of this gene flow. To contrast sympatric and allopatric subspecies of *D.*
326 *pseudoobscura* in their similarity to *D. persimilis*, we implemented Patterson's D-statistic using the tree:
327 (((*D. p. bogotana*, *D. p. pseudoobscura), D. persimilis), D. lowei))).* Patterson's D-statistic is an
328 implementation of ABBA-BABA, which uses parsimony informative sites to test whether derived
329 alleles ("B") in *D. persimilis* are shared with *D. p. bogotana* or with *D. p. pseudoobscura* at equal
330 frequencies. Derived alleles in *D. persimilis* may be shared with *D. p. pseudoobscura* due to ancestral
331 polymorphism, ancient gene flow (prior to the split of the two *D. pseudoobscura* subspecies), recent
332 gene flow (since the split of the two *D. pseudoobscura* subspecies), or a combination of these factors.
333 The null expectation is that the two phylogeny-discordant patterns, ABBA and BABA, should be
334 present equally if ancestral polymorphism and ancient gene flow are the sole drivers of patterns of
335 divergence. Gene flow between *D. p. pseudoobscura* and *D. persimilis* since the split of the two *D.*
336 *pseudoobscura* subspecies (estimated at 150,000 years ago: Schaeffer & Miller 1991) would promote an
337 excess of ABBA over BABA patterns, particularly on freely recombining chromosomes. Indeed,
338 ABBA sites exceed BABA sites on all chromosomes (Table 2), and chromosome 4 shows an
339 unambiguously significant excess of ABBA (|Z-score| >= 5), suggesting that the phylogenetic
340 relationship between these four taxa does not fully explain the observed patterns of divergence and
341 some very recent gene exchange has occurred between the North American species. Furthermore,
342 the genome wide z-score for collinear regions is significant (|Z-score| = 7.215).
343

344 **Table 2** | D-statistics. For each region†, the number of sites with BABA and ABBA patterns are
345 provided along with the total number of SNPs at sites where all four populations have data.

Region	D-Statistic	Standard Error	Z-score	BABA Sites	ABBA Sites	SNPs
Whole-Genome						
Inverted Regions	-0.0111	0.0046	-2.401	10,269	10,500	2,063,794
Collinear Regions	-0.0179	0.0020	-9.154**	44,162	45,776	6,180,121
Collinear_{FR} Regions	-0.0170	0.0024	-7.215**	30,749	31,813	3,750,444
Chromosome 4						
Whole Chromosome	-0.0142	0.0027	-5.335**	22,606	23,256	2,526,132
Chromosome 2						
Whole Chromosome	-0.0149	0.0032	-4.713*	18,654	19,220	2,833,597
Inverted Region	-0.0044	0.0084	-0.531	3,381	3,411	695,574
Collinear Regions	-0.0172	0.0033	-5.099**	15,273	15,809	2,138,123
Collinear_{FR} Region	-0.0176	0.0048	-3.686*	7,061	7,314	927,665
Chromosome XL						
Whole Chromosome	-0.0161	0.0053	-3.041*	7,860	8,117	1,659,395
Inverted Region	-0.0036	0.0099	-0.364	2,323	2,340	606,742
Collinear Regions	-0.0213	0.0061	-3.476*	5,537	5,778	1,052,653
Collinear_{FR} Region	-0.0388	0.0125	-3.114*	1,513	1,635	245,552
Chromosome XR						
Whole Chromosome	-0.0174	0.0045	-3.881*	11,086	11,479	1,978,707
Inverted Region	-0.0173	0.0066	-2.633	5,236	5,420	893,006
Collinear Region	-0.0176	0.0061	-2.875	5,849	6,059	1,085,701
Collinear_{FR} Region	-0.0224	0.0133	-1.688	1,577	1,649	265,259

346 † The presented chromosomal regions include entire chromosomes, inverted regions only, collinear regions only, and collinear regions
347 excluding sequence within 5 mb of chromosome ends or within 2.5 mb of inversion breakpoints (Collinear_{FR}).
348 * Significant at |z| >= 3.
349 ** Significant at |z| >= 5.

350 Given the observed excess of ABBA over BABA sites throughout the genome, we next
351 applied f_d to quantify this excess in smaller genomic intervals. In comparison to D-statistics, f_d is less
352 affected by differences in effective population size and is better suited to identifying introgression
353 regions (Martin *et al.* 2015). The genome-wide patterns of f_d support the evidence of gene flow
354 between *D. p. pseudoobscura* and *D. persimilis*, particularly in the collinear regions of the genome
355 (Figure 4). Inverted regions exhibit markedly lower f_d compared to collinear regions (Figure 4A).
356 This difference is statistically significant on all inversion-bearing chromosomes, regardless of
357 whether the inverted regions are compared to all collinear regions or just the conservative subset
358 contained in collinear_{FR} (Figure 4B; $p < 0.01$ for all comparisons, Mann-Whitney U).

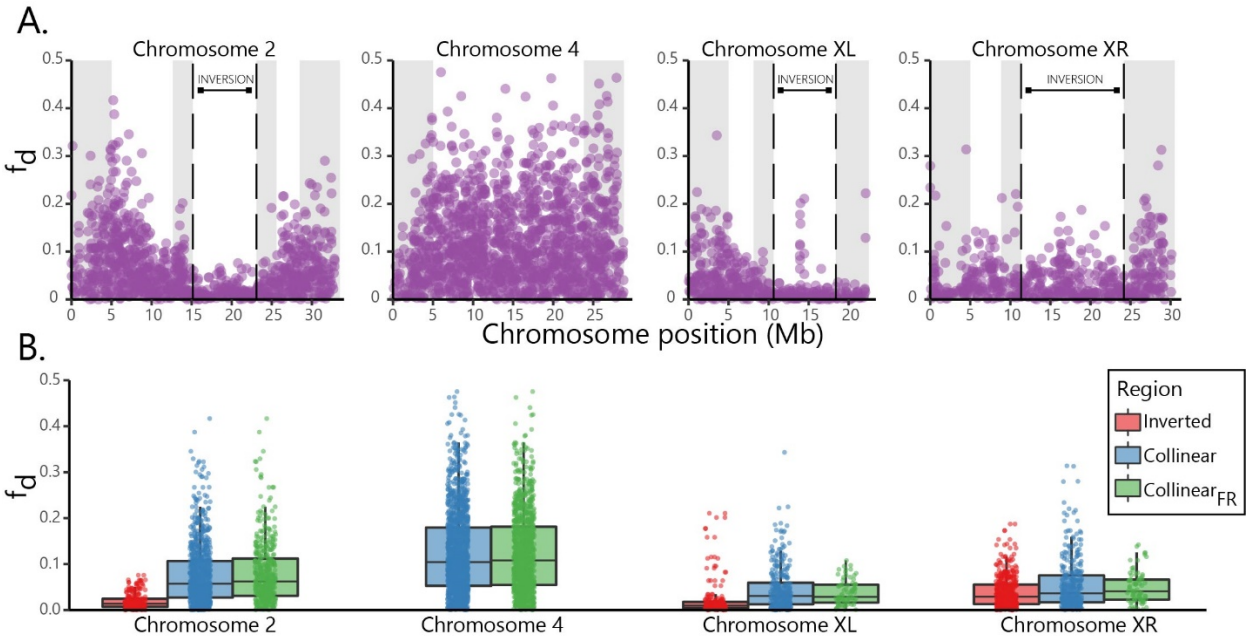


Figure 4 | Signals of introgression along the genome. (A) The estimated proportion of introgression (f_d) between *D. pseudoobscura* and *D. persimilis* is shown in non-overlapping 100 SNP windows along chromosomes 2, 4, XL, and XR. Inversion boundaries are shown with dashed black lines, and collinear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

Is recent gene flow responsible for 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).

376 The observation that divergence is lower in sympatry compared to allopatry even in the
377 inverted regions is somewhat surprising. A possible explanation for the lower divergence in the
378 sympatric species pair is that gene flow is homogenizing these species even in inverted regions.
379 Though there is evidence that double crossovers and gene conversions occur within inversions
380 (Schaeffer & Anderson 2005; Stevison *et al.* 2011; Crown *et al.* 2018; Korunes & Noor 2019), an
381 alternative explanation is that *D. p. bogotana* has experienced more substitutions per site, possibly due
382 to differences in demographic history among the lineages. *D. p. bogotana* may have experienced a
383 population bottleneck upon colonization of South America leading to a subsequently small effective
384 population size (Schaeffer & Miller 1991; Wang & Hey 1996; Machado *et al.* 2002), which might
385 allow drift to result in a higher fixation rate of slightly deleterious mutations (Whitlock 2000;
386 Charlesworth 2009). Indeed, genome-wide comparison of the relative substitution rates (Tajima
387 1993) between the lineages reveals that *D. p. bogotana* has experienced significantly more substitutions
388 per site than *D. p. pseudoobscura* relative to the outgroup species, *D. lowei* (Supplementary Table 6)
389 To correct for differences in evolutionary rates in these populations, we considered the
390 effects of introgression on divergence after adjusting for an elevated rate of fixation leading to a
391 longer branch length in *D. p. bogotana*. We compared the divergence of each *D. pseudoobscura*
392 subspecies from *D. persimilis* to the divergence of each *D. pseudoobscura* subspecies from *D. lowei* using
393 the following equation to define the "introgression effect": (D_{xy} [*D.persimilis*:*D.p.bogotana*] - D_{xy}
394 [*D.persimilis*:*D.p.pseudo.*]) – (D_{xy} [*D.lowei*:*D.p.bogotana*] - D_{xy} [*D.lowei*:*D.p.pseudo.*]). The first half of this
395 equation should include the effects of branch length in *D. p. bogotana* and the effects of any
396 introgression between *D. pseudoobscura* and *D. persimilis* (Figure 5A). Since *D. lowei* does not hybridize
397 with any of these species, the second half of the equation should reflect only the effects of branch
398 length in *D. p. bogotana*. Thus, the difference between these terms should subtract the effects of
399 evolutionary rate, leaving the effects of recent introgression. As we are interested in whether, when,

400 and how chromosomal inversions are contributing to patterns of divergence by suppressing gene
401 flow, we compared inverted vs. collinear regions in their introgression effects. After subtracting the
402 effects of branch length in *D. p. bogotana*, there is still evidence that differential gene flow in inverted
403 vs collinear regions has statistically significantly influenced patterns of divergence between *D.*
404 *pseudoobscura* and *D. persimilis*. The distributions of the introgression effect values in each of the
405 sampled *D. p. pseudoobscura* genomes differs significantly between inverted and collinear regions ($p <$
406 0.001, Mann-Whitney U test; Figure 5B). Notably, the introgression effect is positive, albeit small, in
407 the inverted regions, potentially due to double crossovers and gene conversions occurring within
408 inversions (Schaeffer & Anderson 2005; Stevison *et al.* 2011; Crown *et al.* 2018; Korunes & Noor
409 2019).

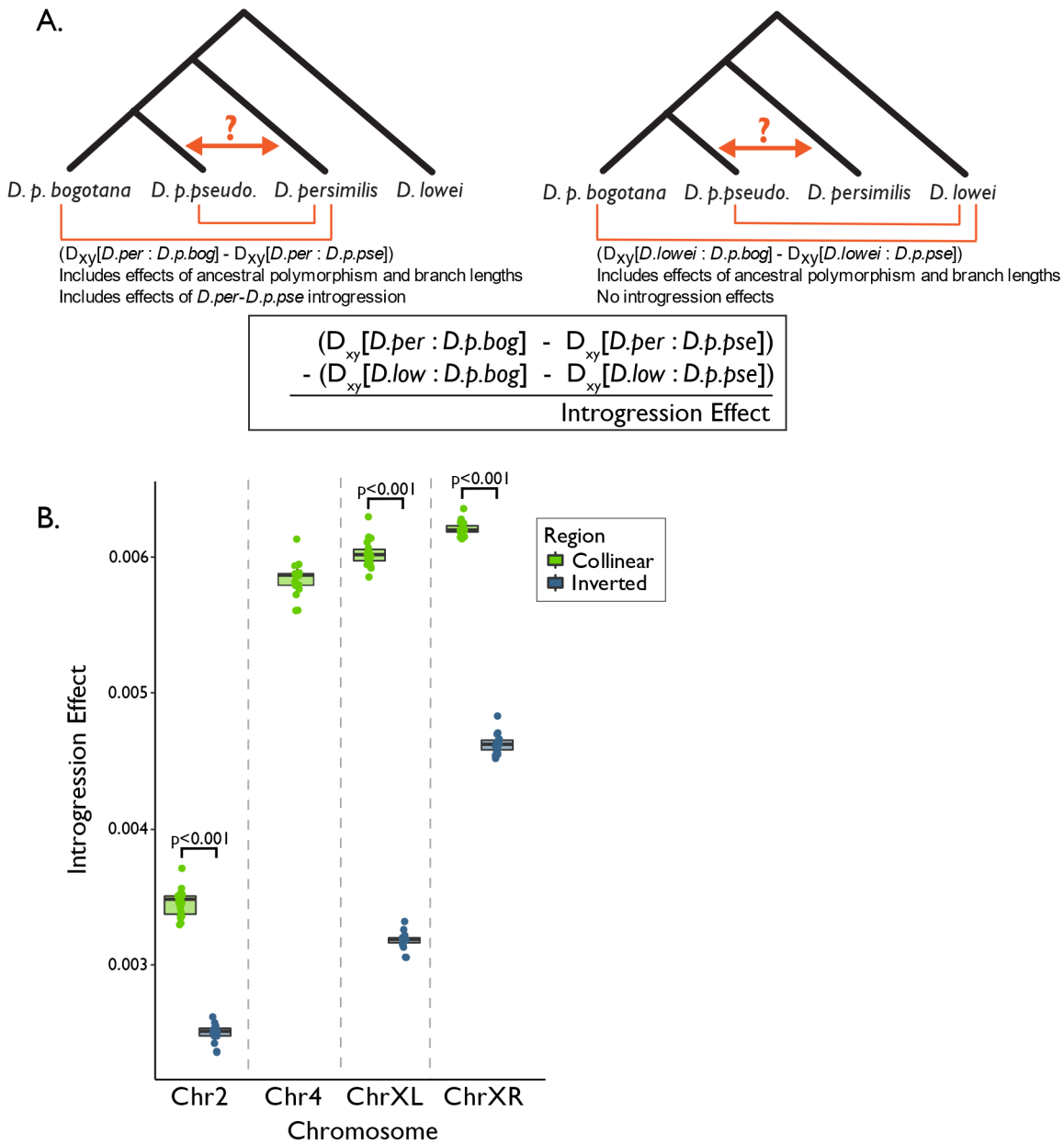


Figure 5 | The effects of introgression on divergence after correcting for evolutionary rate. (A) Shows the strategy for examining the "introgression effect" of gene flow on divergence. The first half of the equation compares the divergence of each *D. pseudoobscura* subspecies from *D. persimilis,* and the second half compares the divergence of each *D. pseudoobscura* subspecies from *D. lowei.* The difference between these terms, plotted in (B) for each of the sampled *D. p. pseudoobscura* genomes, should reflect the effects of introgression after correcting for branch length.

417 ## ***Discussion***

418 Our model-based examination of gene flow and our application of Patterson's D-statistic to
419 examine shared variation found between *D. persimilis* and sympatric and allopatric subspecies of *D.*
420 *pseudoobscura* indicates post-speciation gene exchange, including both early post-speciation gene flow
421 and gene flow within the past ~150,000 years. Specifically, we interpret the excess of ABBA >
422 BABA sites as evidence that *D. pseudoobscura* and *D. persimilis* have exchanged genes in collinear
423 regions since the split of *D. p. bogotana*. Our analyses span the majority of the assembled genome,
424 excluding only the ~17% of the genome where we cannot reasonably apply these methods to
425 examine introgression and divergence (see *Methods*).

426 This evidence that introgression is driving patterns of divergence between *D. pseudoobscura*
427 and *D. persimilis* is in agreement with previous reports of ongoing hybridization in these species
428 (Dobzhansky 1973; Powell 1983; Machado & Hey 2003; Hey & Nielsen 2004). Despite our evidence
429 for recent gene exchange, it appears that introgression is not the sole driver of patterns of
430 divergence between these species overall. While D-statistics and f_d suggest an excess of shared,
431 derived alleles across the genomes of *D. pseudoobscura* and *D. persimilis*, these statistics may be biased
432 by factors such as ancestral population structure and differences in effective population size (Slatkin
433 & Pollack 2008; Martin *et al.* 2015; He *et al.* 2020). In comparison to Patterson's D-statistic, f_d is less
434 sensitive to local variation in recombination rate and divergence. However, it can still be biased by
435 regions of reduced interspecies divergence, which may distort tests for recent introgression (Martin
436 *et al.* 2015), so the conclusion of recent introgression would be tentative based on these results alone.
437 Here, we explore other important factors that might underlie the observed patterns of divergence,
438 with particular consideration of how these factors might confound signals of recent introgression.

439 As seen in Figure 3 and in previous studies (Kulathinal *et al.* 2009), divergence is higher in
440 inverted vs collinear regions in this system. This difference holds for divergence between *D. persimilis*
441 and either *D. p. bogotana* or *D. p. pseudoobscura*. The lower observed divergence in sympatry compared
442 to allopatry even in the inverted regions is somewhat surprising given the expectation that
443 recombination in hybrids will be restricted in these inverted regions. This observation led us to
444 consider the possibility that the allopatric subspecies, *D. p. bogotana*, might have experienced more
445 nucleotide substitutions per site than the other taxa. Thus, we considered four non-mutually
446 exclusive factors that might contribute to the observed patterns of divergence with respect to
447 chromosomal arrangement: 1) the segregation of ancestral polymorphism (as advocated by Fuller *et*
448 *al.* (2018)), 2) increased branch length in the allopatric *D. p. bogotana,* 3) gene flow prior to the split of
449 *D. p. bogotana*, and 4) recent/ongoing gene flow (the latter two discussed in Powell 1983; Wang &
450 Hey 1996; Wang *et al.* 1997; Noor *et al.* 2001, 2007; Machado & Hey 2003; Hey & Nielsen 2004;
451 Machado *et al.* 2007; Kulathinal *et al.* 2009; McGaugh & Noor 2012). Achieving a cohesive view of
452 the role of inversions in species divergence relies on considering the combined effects of these
453 factors.
454 For the sympatric pair *D. persimilis* vs *D. p. pseudoobscura,* any difference in D_{xy} in inverted
455 regions compared to collinear regions could be due to the segregation of ancestral inversion
456 polymorphism or to post-speciation genetic exchange. In contrast, any difference in D_{xy} in inverted
457 regions compared to collinear regions in the allopatric pair *D. persimilis* vs *D. p. bogotana* could be
458 driven by the segregation of ancestral inversion polymorphism or by post-speciation gene flow prior
459 to the split of *D. p. bogotana*. This comparison will not reflect any recent gene flow, since *D. p.*
460 *bogotana* has evolved in allopatry for the past 150,000 years. We leveraged this contrast to isolate the
461 effects of recent introgression on divergence, and our findings here suggest that contributions from
462 recent gene flow only partially explain observed divergence patterns.

463 The observed "introgression effect" can be thought of as an indicator of the relative
464 influence of recent introgression on the reduction in *D. persimilis* vs *D. pseudoobscura* divergence in
465 sympatry vs allopatry. Our method does not account for all evolutionary forces shaping nucleotide
466 divergence, so we do not consider it a quantitative measurement of introgression. However, it
467 provides a useful way to consider the relative contribution of recent introgression compared to
468 ancestral polymorphism and branch length. The second half of the introgression effect equation
469 (Figure 5A; D_{xy}[*D.lowei*:*D.p.bogotana*] - D_{xy}[*D.lowei*:*D.p.pseudo.*]) includes the effects of branch length
470 in *D. p. bogotana* and the effects of ancestral polymorphism without including any effects of recent
471 introgression. We can subtract this term from D_{xy}[*D.persimilis:D.p.bogotana*] to obtain a hypothetical
472 D_{xy} reflecting what divergence between *D. persimilis* and *D. p. pseudoobscura* might look like without
473 the effects of recent introgression, ancestral polymorphism, or longer branch length in *D. p. bogotana.*
474 Dividing the introgression effect by this hypothetical D_{xy} gives the hypothetical change in D_{xy} in
475 allopatry vs sympatry due specifically to recent introgression. Given the observed average
476 introgression effect (0.0045) and the hypothetical D_{xy}[*D. persimilis*:*D. p. pseudoobscura*] of 0.01, recent
477 introgression might explain roughly half of the reduction in D_{xy} observed in sympatry vs allopatry.
478 As such, the homogenization effect of recent introgression on sequence divergence appears far from
479 trivial.

480 These results suggest that patterns of divergence between *D. persimilis* and *D. pseudoobscura* are
481 explained by a combination of segregating ancestral polymorphism and post-speciation gene flow.
482 We applied a model-based approach to investigate the timing of gene flow between *D. persimilis* and
483 *D. pseudoobscura.* Our results suggest that an isolation-with-initial-migration model best explains the
484 divergence of *D. persimilis* and *D. p. bogotana* when compared to a model of strict isolation. This result
485 provides further evidence for gene flow between *D. persimilis* and *D. pseudoobscura*, and it suggests that

486 some of this gene flow occurred prior to the split of *D. p. bogotana* and remains detectable in
487 observed genetic patterns.

488 Our results question interpretations from earlier studies of this system. Given that *D. p.*
489 *bogotana* can be reasonably assumed to not be currently exchanging genes with either *D. persimilis* or
490 *D. p. pseudoobscura* (Schaeffer & Miller 1991; Wang *et al.* 1997), *D. persimilis*:*D. p. bogotana* divergence
491 was argued to be a suitable "negative control" for examining the effect of recent hybridization
492 between *D. persimilis* and *D. p. pseudoobscura* (Brown *et al.* 2004). By this argument, the effect of recent
493 gene flow can be estimated by an allopatric vs sympatric comparison of the difference in divergence
494 (whether in DNA sequence or in phenotype) in inverted regions to divergence in collinear regions.
495 Specifically, Brown *et al.* (2004) and Chang and Noor (2007) inferred multiple hybrid sterility factors
496 between *D. p. bogotana* and *D. persimilis* that did not distinguish North American *D. p. pseudoobscura*
497 and *D. persimilis* (Brown *et al.* 2004; Chang & Noor 2007). Similarly, Kulathinal *et al.* (2009) observed
498 significantly greater sequence difference between *D. p. bogotana* and *D. persimilis* than between *D. p.*
499 *pseudoobscura* and *D. persimilis*. In both cases, the authors interpreted the difference to result from
500 recent homogenization of the collinear regions in the latter pair. Based on our findings, we suggest
501 this difference may result at least in part from the accelerated rate of divergence in *D. p. bogotana*.

502 Overall, we caution that simple allopatry-sympatry comparisons can easily be misleading, and
503 the population histories and rates of evolution of the examined species should be carefully
504 considered. Variation in N_e due to events such as recent population bottlenecks in one of the taxa
505 can dramatically influence evolutionary rates (reviewed in Charlesworth 2009; Lanfear *et al.* 2014).
506 Additionally, the process of genetic divergence that shapes alleles responsible for local adaptation
507 and hybrid incompatibility can extend deep into the history of the species. In fact, the influence of
508 inversions on the divergence of a species pair can predate the split of the species. Inversion
509 polymorphisms in the ancestral population of a species pair can contribute to patterns of higher

510 sequence differentiation between species in those inverted regions (Fuller *et al.* 2018). Separating
511 these effects requires an understanding of the timing and extent of introgression, which can only be
512 understood with an appreciation for the evolutionary processes occurring in each of the taxa at
513 hand. Though there are many remaining questions about how inversions shape divergence, we
514 present evidence that inversions have contributed to the divergence of *D. pseudoobscura* and *D.*
515 *persimilis* over multiple distinct periods during their speciation: 1) pre-speciation segregation of
516 inversions in the ancestral population, 2) post-speciation gene flow prior to the split of *D. p. bogotana*,
517 and 3) recent gene flow.

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