

1 Experimental introgression in *Drosophila*: asymmetric postzygotic isolation associated with
2 chromosomal inversions and an incompatibility locus on the X chromosome

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5 Running title: Experimental introgression in *Drosophila*

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51 **Abstract**

52 Interspecific gene flow (introgression) is an important source of new genetic variation, but selection
53 against it can reinforce reproductive barriers between interbreeding species. We used an experimental
54 approach to trace the role of chromosomal inversions and incompatibility genes in preventing
55 introgression between two partly sympatric *Drosophila virilis* group species, *D. flavomontana* and *D.*
56 *montana*. We backcrossed F₁ hybrid females from a cross between *D. flavomontana* female and *D.*
57 *montana* male with the males of the parental species for two generations and sequenced pools of
58 parental strains and their reciprocal 2nd generation backcross (BC₂mon and BC₂fla) females. Contrasting
59 the observed amount of introgression (mean hybrid index, HI) in BC₂ female pools along the genome to
60 simulations under different scenarios allowed us to identify chromosomal regions of restricted and
61 increased introgression. We find no deviation from the HI expected under a neutral null model for any
62 chromosome for the BC₂mon pool, suggesting no evidence for genetic incompatibilities in backcrosses
63 towards *D. montana*. In contrast, the BC₂fla pool showed high variation in the observed HI between
64 different chromosomes, and massive reduction of introgression on the X chromosome (large X-effect).
65 We find that this observation is compatible with reduced recombination combined with at least one
66 dominant incompatibility locus residing within the X inversion(s). Overall, our study suggests that
67 genetic incompatibilities arising in chromosomal inversions can play an important role in speciation.

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69 **Keywords:** chromosomal inversions, experimental evolution, genetic incompatibilities, hybridization,
70 introgression, X-effect

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89 Introduction

90 Interspecific gene flow (introgression) is an important source of genetic variation for adaptation to new
91 environments (Abbott et al., 2013; Anderson & Hubricht, 1938; Lewontin & Birch, 1966). At the same
92 time, selection against introgression at certain loci acts to maintain barrier loci and protect species'
93 integrity from the negative effects of hybridization (Barton & Bengtsson, 1986; Ravinet et al., 2017;
94 Servedio & Noor, 2003; Wu, 2001). The patterns of genomic divergence and the permeability of species
95 boundaries in certain genomic regions provides valuable insights into the genomic regions that
96 contribute to speciation (Harrison & Larson, 2014). However, we still lack a good understanding of how
97 barrier genes are arrayed within the genome, how effectively and in what generation they restrict
98 introgression, and what kind of role chromosomal inversions and sex chromosomes play in maintaining
99 genetic barriers (Butlin, 2005; Coughlan & Matute, 2020; Coyne & Orr, 2004; Faria & Navarro, 2010;
100 Gompert, Lucas, Nice, & Buerkle, 2012; Nosil & Feder, 2012).

101 Speciation in isolation (allopatry), occurring via drift or indirect effects of selection, can lead to the
102 “incidental” establishment of intrinsic genetic incompatibilities (Coyne & Orr, 2004; Tang & Presgraves,
103 2009). These incompatibilities generally involve negative epistatic interactions between two or more
104 loci, where new alleles arising in one or both of the interacting lineages function well in their own genetic
105 background, but interact negatively with the alleles of other species in hybrids (Bateson-Dobzhansky-
106 Muller incompatibilities, BDMIs or DMIs; Coyne & Orr, 2004; Orr, 1995; Presgraves, 2010b). Lack of gene
107 flow may also increase the fixation probability of meiotic drive loci (loci that manipulate meiotic process
108 to favour their own transmission) and their suppressor loci within each population and drive the
109 genomic divergence of these populations (Crespi & Nosil, 2013). Compared to allopatric speciation,
110 where both BDMIs and neutral differences between species are expected to build up randomly along
111 the genome, divergence with gene flow leads to clusters of species- or population-specific loci that are
112 sheltered from recombination (Abbott et al., 2013; Butlin, 2005; Felsenstein, 1981). Accordingly, an
113 accumulation of BDMIs between species may be drastically different with and without gene flow.
114 Importantly, in the presence of gene flow BDMIs can only accumulate if they are favoured by selection
115 (Bank, Bürger, & Hermisson, 2012).

116 Chromosomal inversions are a major factor rearranging the genome and inducing changes in gene
117 interactions and expression patterns (Hoffmann & Rieseberg, 2008; Kirkpatrick & Barton, 2006;
118 Sturtevant, 1921; Dobzhansky, 1940). Inversions may gain a fitness advantage and spread through
119 conspecific populations if they reduce recombination between co-adapted genes (Kirkpatrick & Barton,
120 2006; Navarro & Barton, 2003). Once inversions have become fixed between species, they can generate
121 postzygotic isolation in several ways. They can prevent interspecific gene flow directly by inducing
122 problems in chromosome pairing during meiosis, which can lead to malformed gametes and reduced
123 hybrid fertility and viability (Coyne & Orr, 2004; Hoffmann & Rieseberg, 2008; Rieseberg, 2001).
124 However, these problems are partially avoided in *Drosophila*, since malformed gametes remain in the
125 polar nuclei and do not enter the developing gametes (Hoffmann & Rieseberg, 2008; Sturtevant &
126 Beadle, 1936). Perhaps more importantly, the limited recombination across inverted regions,
127 particularly near inversion breakpoints and within overlapping inversions, facilitates the build-up of
128 BDMIs via divergent selection and/or drift (Fishman, Stathos, Beardsley, Williams, & Hill, 2013; Khadem,
129 Camacho, & Nóbrega, 2011; Mcgaugh & Noor, 2012; Navarro & Barton, 2003; Noor, Grams, Bertucci, &
130 Reiland, 2001). Thus, species-specific inversions harbouring BDMIs may act as strong barriers to gene
131 flow (Hoffmann & Rieseberg, 2008; Noor et al., 2001).

132 The disproportionate involvement of sex chromosomes in reproductive isolation in many systems is
133 captured by two general observations: Haldane’s rule – the increased F_1 inviability and sterility of the
134 heterogametic sex compared to the homogametic sex (Haldane, 1922; Orr, 1997; Turelli & Orr, 2000) –
135 and the large X-effect – the fact that the X chromosome shows a disproportionately large effect on the
136 sterility and inviability of backcross hybrids (Masly & Presgraves, 2007; Turelli & Orr, 2000). Explanation

137 for both observations often presume recessivity of X-linked alleles, which can lead to more pronounced
138 effects in hemizygous than in heterozygous hybrids ("Dominance theory"; Coyne & Orr, 2004; Turelli &
139 Orr, 1995, 2000) and/or rapid evolution of X-linked alleles facilitating BDMLs as a byproduct ("Faster X
140 evolution"; Charlesworth, Campos, & Jackson, 2018; Charlesworth, Coyne, & Barton, 1987). The X
141 chromosome has also been suggested to be enriched for genes that create postzygotic isolation in
142 hybrids compared to autosomes (Coyne, 2018). In particular, meiotic drive loci are more frequent on
143 the X than on autosomes, and incompatibilities between drivers and their suppressors in hybrids may
144 generate problems in hybrid development (Courret, Chang, Wei, Montchamp-Moreau, & Larracuente,
145 2019; Crespi & Nosil, 2013; Crown, Miller, Sekelsky, & Hawley, 2018).

146 Pairwise BDMLs may involve substitutions in both diverging lineages, or derived substitutions in one
147 lineage and preserved ancestral alleles in another lineage (Barbash, Awadalla, & Tarone, 2004; Cattani
148 & Presgraves, 2009; Coyne & Orr, 2004). BDMLs can also result from cumulative effects of many small
149 incompatibilities or from a single incompatibility between two complementary genes, and the
150 complexity of the incompatibility interaction does not reflect the severity of the barrier (Orr, 1995;
151 Presgraves, 2010a). Importantly, and in contrast to interactions within a locus where a dominant allele
152 masks a recessive allele, in epistatic interactions between different loci a dominant allele at one locus
153 may interact with dominant or recessive alleles at other loci. Epistatic interactions involving dominant
154 alleles are of special interest in the context of BDMLs but have received less attention than BDMLs
155 involving recessive alleles.

156 Two closely-related species of the *Drosophila virilis* group, *D. montana* and *D. flavomontana*, provide an
157 excellent test case for studying the evolution of BDMLs. The species originate from the Rocky Mountains
158 of North America, where the divergence of the *montana* complex species (*D. flavomontana*, *D. lacicola*
159 and *D. borealis*) most likely occurred (Hoikkala & Poikela, 2022; Patterson, 1952; Throckmorton, 1982).
160 *D. montana* has expanded around the northern hemisphere, whereas *D. flavomontana* has remained in
161 North America (Hoikkala & Poikela, 2022). *D. montana* lives generally in colder environments and uses
162 different host trees than *D. flavomontana* (Patterson, 1952; Throckmorton, 1982). Reproductive
163 barriers between *D. montana* females and *D. flavomontana* males are nearly complete, while in the
164 reciprocal cross strong postzygotic isolation is accompanied by prezygotic barriers of variable strength
165 (Poikela et al., 2019). Regardless of these barriers, the two species can be crossed to obtain backcross
166 progenies in both parental directions (Poikela et al., 2019), and interspecific hybrids have reportedly
167 been found in nature (Patterson, 1952; Throckmorton, 1982). Our recent demographic modelling shows
168 that the species have diverged ~3 Mya, with low levels of postdivergence gene flow from *D. montana*
169 to *D. flavomontana* (Poikela et al., in prep.). Moreover, we found in these species several alternatively
170 fixed inversions, which were already present in their common ancestor, and which may have
171 contributed to the build-up and maintenance of adaptive traits and reproductive barriers by restricting
172 gene flow between the evolving lineages (Poikela et al., in prep.).

173 The goal of this study was to determine which genomic regions are likely to accommodate dominant
174 BDMLs in hybrids between *D. montana* and *D. flavomontana*, paying special attention to fixed inversions
175 and the X chromosome. We investigated BDMLs between these species experimentally by sequencing
176 pools of *D. montana* females from an allopatric population and *D. flavomontana* females from a
177 (presently) parapatric population, as well as pools of 2nd backcross generation (BC₂) females in both
178 directions (Fig. 1). We identified chromosomal regions with decreased and increased introgression by
179 quantifying the amount of introgressed genetic material (mean hybrid index, HI) along the genome in
180 both backcross pools. We then compared the observed HI to the distribution of chromosome-wide HI
181 in *in silico* replicates of this "introgress-and-resequence" experiment under contrasting assumptions
182 about the presence and location of BDMLs. Since this experimental design involved only backcross
183 females, only BDMLs involving a dominant allele could affect allele frequencies in the pool while
184 recessive-recessive BDMLs were masked (Table 1). Our main questions were:

- 185 (i) Does the strength and genomic distribution of genetic incompatibilities between *D.*
186 *montana* and *D. flavomontana* differ between the reciprocal crosses?
187 (ii) Do the species show increased genetic divergence and decreased introgression within
188 chromosomal inversions, and could this be caused by inversions' propensity to suppress
189 recombination and harbour genetic incompatibilities?
190 (iii) Does the X chromosome show less introgression than autosomes (large X-effect)? And if
191 yes, why?

192 2 Materials and methods

193 2.1 Fly material

194 We collected fertilised *D. montana* females from Seward, Alaska, USA (60°09'N; 149°27'W) and *D.*
195 *flavomontana* females from Livingston, Montana, USA (45°20'N; 110°36'W) in 2013. The distance
196 between the sites is ~3000 km. Alaskan *D. montana* can be regarded as an allopatric population, as *D.*
197 *flavomontana* has not been found above 54°N (Poikela et al., 2019). In contrast, the *D. flavomontana*
198 population from Montana can be regarded as a parapatric, as the two species are known to coexist in
199 the Rocky Mountains, even though we found only *D. flavomontana* on the collecting site (Poikela et al.,
200 2019).

201 Progenies of wild-caught *D. montana* and *D. flavomontana* females were used to establish isofemale
202 strains, which were maintained in continuous light and 19 °C for about 23 generations (~3 years) in the
203 University of Jyväskylä (Finland) prior to their use in the present study. We performed the crossing
204 experiment using flies of *D. montana* strain SE13F37 and *D. flavomontana* strain MT13F11. For the
205 crosses, the flies were sexed under light CO₂ anaesthesia within three days after emergence, when they
206 were still virgins. Males and females were transferred into fresh malt-vials once a week and used in the
207 crossing experiments at age 20 ± 2 days when they were sexually mature (Salminen & Hoikkala, 2013).

208 2.2 Crossing experiment

209 We started the crossing experiment by performing a single-pair cross between *D. flavomontana* female
210 and *D. montana* male, as reciprocal cross is not successful. Our crossing design (outlined in Fig. 1) only
211 involved hybrid females because F₁ males are largely sterile (Päällysaho, Aspi, Liimatainen, & Hoikkala,
212 2003; Poikela et al., 2019), and because *Drosophila* males lack recombination (crossing-over) in meiosis
213 The initial cross produced seven F₁ females, which were backcrossed towards both parental species:
214 four were mated to *D. montana* males and three to *D. flavomontana* males. The 1st backcross generation
215 females (BC₁mon and BC₁fla females) were backcrossed to the same paternal species as in the previous
216 generation to obtain BC₂mon and BC₂fla females (82 females in both directions). BC₂ females were
217 collected within three days after their emergence and stored in -20 °C for DNA extractions.

218 2.3 Fertility of BC₁ females

219 We defined the fertility of BC₁ females (BC₁mon and BC₁fla females) by checking whether they produced
220 progeny after mating with a *D. montana* or *D. flavomontana* male (Fig. 1). BC₁ females that produced
221 no progeny were considered sterile. We used a one-sample Student's t-test (*t-test* function) to test
222 whether the BC₁ females from the reciprocal crosses showed reduced fertility, when the expected
223 fertility is 1. We also compared the fertility of BC₁ females between the reciprocal crosses to define
224 possible asymmetries (BC₁mon vs. BC₁fla), using a generalised linear model (GLM) with Binomial
225 distribution (1=fertile, 0=sterile) (*glm* function). All analyses were conducted in base R v1.2.1335-1 and
226 R studio v3.6.1.

227 2.4 Pool-sequencing, mapping, and variant calling

228 We made DNA extractions from four pools, one pool of each parental strain (*D. montana* SE13F37 and
229 *D. flavomontana* MT13F11) and pools for the two 2nd generation backcrosses (BC₂mon and BC₂fla). Each

230 pool consisted of 82 females. We used cetyltrimethylammonium bromide (CTAB) solution with RNase
231 treatment, Phenol-Chloroform-Isoamyl alcohol (25:24:1) and Chloroform-Isoamyl alcohol (24:1)
232 washing steps and ethanol precipitation. Nextera library preparation and 150 bp Illumina paired-end
233 sequencing were performed at Edinburgh Genomics, UK. Illumina paired-end reads of all four samples
234 were quality-checked with FastQC v0.11.8 (Andrews 2010) and trimmed for adapter contamination and
235 low-quality bases using fastp v0.20.0 (Chen, Zhou, Chen, & Gu, 2018). After filtering, the total number
236 of reads per pool varied from 152 to 174 million, and the mean length and insert size peak being 141-
237 143bp and 150bp, respectively (Table S1). The mean coverage of the pools varied from 170 to 220 (Table
238 S1).

239 All analyses were based on reads mapped to a *D. montana* chromosome-level genome assembly with
240 full gene annotation (Poikela et al. in prep.). Filtered Illumina reads of each sample were mapped to the
241 unmasked reference genome using BWA mem (Burrows-Wheeler Aligner) v0.7.17 with read group
242 information (Li & Durbin, 2009). The alignments were sorted with SAMtools v1.10 (Li et al., 2009) and
243 PCR duplicates marked with sambamba v0.7.0 (Tarasov, Vilella, Cuppen, Nijman, & Prins, 2015). The
244 obtained BAM-files were used for variant calling with the softmasked version of the reference genome
245 using freebayes parallel v1.3.1-dirty (Garrison & Marth, 2012) with --no-population-priors --hwe-priors-
246 off --use-mapping-quality --ploidy 2 --theta 0.02 --haplotype-length -1).

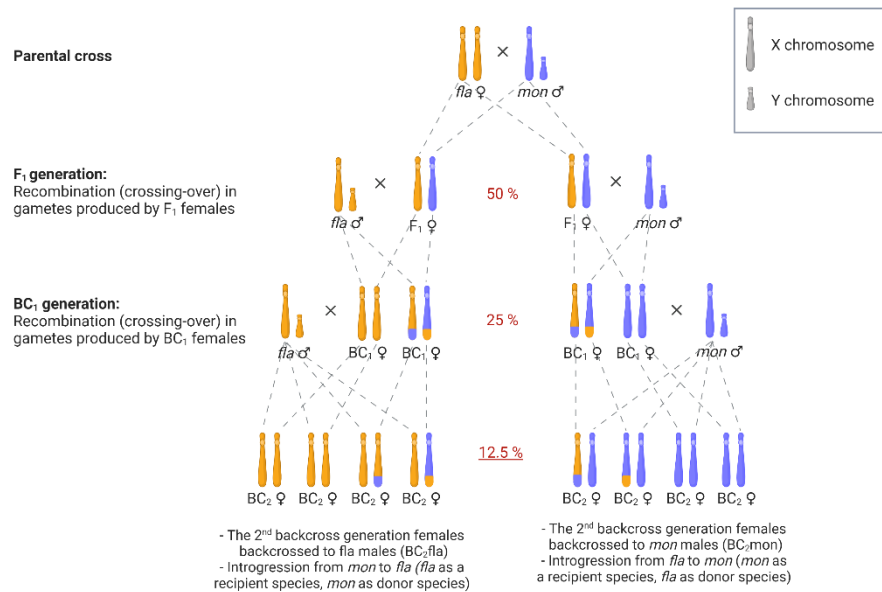
247 Variant calling detected a total of 8,876,483 variants. To normalise the representation of variants, the
248 resulting VCF-file was processed with vt normalize (Tan, Abecasis, & Kang, 2015). The variants were
249 filtered for quality and SNPs (single nucleotide polymorphism) using bcftools filter and view v1.9 (Li,
250 2011). We chose only biallelic SNPs with a minimum depth of 80 to reliably calculate allele frequencies
251 and to minimize potential reference bias. These quality filtering steps resulted in a total of 5,047,746
252 SNPs.

253 2.5 Inversion breakpoints

254 The breakpoints of alternatively fixed inversions of *D. montana* and *D. flavomontana* on the X
255 chromosome and chromosomes 2L, 4 and 5 were obtained from Poikela et al. (in prep.). The presence
256 of the inversions in Illumina samples of parental pools was verified by passing the respective BAM-files
257 to Delly v0.8.1 (Rausch et al., 2012), which identifies structural variants based on paired-end read
258 orientation and split-read evidence. The inversion breakpoints were also confirmed visually by checking
259 the orientation and insert size around each breakpoint in the Interactive Genomics Viewer
260 (Thorvaldsdóttir, Robinson, & Mesirov, 2012) (Example plot shown in Fig. S1). Inversion breakpoints are
261 shown in Fig. 3, Fig. 4 and Table S2.

262 2.6 Genetic differentiation, hybrid index and the types of genetic incompatibilities

263 The expected amount of genetic material transferred from one species into the other (hybrid index, HI)
264 halves with every backcross generation (Fig. 1). Thus, in the pool of 2nd backcross generation hybrid
265 females, the genome-wide HI is expected to be 12.5% in the absence of BDMIs (Fig. 1). However, given
266 the random inheritance of chromatids in gametes and the randomness of cross-over locations, we
267 expect substantial variation around the expected mean HI, even in the absence of BDMIs.



268

269 Figure 1. Illustration of the crossing experiment showing the inheritance of sex chromosomes (inheritance of
 270 autosomes is similar to that of female X chromosomes). F₁ females, produced in a single-pair cross between *D.*
 271 *flavomontana* (*fla*) female and *D. montana* (*mon*) male, were backcrossed to either *D. flavomontana* or *D.*
 272 *montana* male. In the next generation, each BC₁ female was mated with a male of its paternal species. In every
 273 generation, the expected amount of genetic material that is transferred from the gene pool of one species into
 274 the gene pool of another one (introgression) is halved (red percentages). Thus, under a null neutral model, we
 275 expect a mean HI of 12.5 % for the BC₂ pools that were sequenced. Note that recombination occurring in the
 276 gametes produced by F₁ and BC₁ females creates variation in the expected amount of HI. For simplicity, the figure
 277 shows products of only one cross-over event that has occurred in each backcross direction.

278 To estimate the amount of introgression in the BC₂ pools, we computed the HI in both pools along the
 279 genome based on species-diagnostic SNPs (variants that are differentially fixed between the parental
 280 pools). Allele frequencies for each SNP in all four pools were calculated by dividing “Alternate allele
 281 observation count (AO)” by “the total read depth (DP)” (relative to *D. montana* reference genome).
 282 Diagnostic variants were defined as SNPs with allele frequency 1 in one parental pool and 0 in the other
 283 one (1 = all reads supporting the alternate allele, 0 = all reads supporting the reference allele). The total
 284 number of SNPs that were differentially fixed between the parental species was 1,109,701.

285 We compared collinear and inverted parts of each chromosome in terms of the density of diagnostic
 286 SNPs. Each chromosome was divided into 200kb non-overlapping windows (55-147 windows per
 287 chromosome depending on the chromosome length), and the number of diagnostic SNPs in each
 288 window was counted using a custom script
 289 (https://github.com/vihoikka/SNP_mapper/blob/main/snp_binner.py). The data was analysed using a
 290 generalised linear model (*glm* function) with a Poisson distribution, where the number of window-wise
 291 SNPs was used as a response variable, and different chromosomes and different chromosomal partitions
 292 (collinear, inverted) as explanatory variables. The analyses were performed in base R using R v1.2.1335-
 293 1 and R studio v3.6.1.

294 Using the diagnostic SNPs, we calculated the mean hybrid index (HI) separately for different
 295 chromosomes for BC₂*fla* and BC₂*mon* pools. We also estimated the fraction of sequence without any
 296 introgressed material (HI = 0%) separately for each chromosome for both pools. Finally, we plotted HI
 297 in non-overlapping windows of 400 SNPs for each chromosome and BC₂ pool using a custom script
 298 (https://github.com/vihoikka/SNP_mapper/blob/main/datasmoother.py). In principle, recombination
 299 breakpoints involving the two ancestral backgrounds (Fisher junctions; Fisher, 1954) should be visible

300 as step changes in the HI of each pool. Assuming on average one cross-over per chromosome and female
301 meiosis, the expected number of recombination breakpoints per chromosome generated during the
302 experiment is given by the total number of females ($n_{BC_1} + n_{BC_2}$; Table S3) contributing to each pool
303 (96 and 104 for BC_2 mon and BC_2 fla pools, respectively). Note that the number of junctions between *D.*
304 *montana* and *D. flavomontana* ancestral material is lower since not all cross-over events in BC_1 females
305 are junctions. In practice, however, the resolution especially for breakpoints that are unique to a single
306 BC_2 individual (which correspond to a change in allele frequency of $1/82$) is limited by the randomness
307 in sequencing coverage of the pool.

308 Given that this experiment was started with a single-pair cross between the parental species and
309 continued with repeated backcrosses between hybrid females and parental males, all backcross
310 individuals inherited a maximum of one allele per locus from the donor species (Fig. 1.). Thus, the
311 genomes of BC individuals are a mosaic of two types of tracts: i) homozygous for the genetic background
312 of the recipient species or ii) heterozygous between species. This limits the types of BDMs that can be
313 expressed (Table 1). Dominant-dominant pairwise BDMs arise already in the F_1 generation and, if
314 severe, can cause sterility/inviability in both sexes. Recessive-recessive pairwise BDMs are not possible
315 in our experiment even if they were X-linked since i) all BC individuals involved in the experiment were
316 females (no hemizyosity), and ii) the expression of these incompatibilities would require homozygous
317 tracts for both species (Fig. 1). Hence, dominant-recessive BDMs are the only strong postzygotic
318 barriers that we expect to detect in this study.

319 Table 1. BDM model for incompatibilities (see Coyne & Orr, 2004). Here gene A_1 of one species interacts negatively
320 with gene B_2 of another species. Underscore represents any allele, and it does not change the outcome. Note that
321 dominance refers to an allele's effect on fitness on a hybrid genetic background, and it does not necessarily assume
322 dominance of alleles on their normal background within species.

dominant-dominant incompatibility (both loci act dominantly):

$A_1_B_2_$ hybrids are affected in the F_1 generation

recessive-recessive incompatibility (both loci act recessively):

$A_1A_1B_2B_2$ hybrids are affected in the F_2 generation

dominant-recessive incompatibility (A_1 acts dominantly, B_2 recessively):

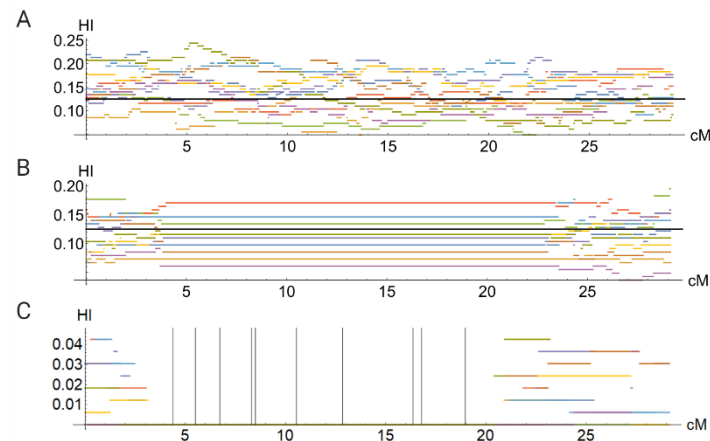
$A_1_B_2B_2$ hybrids are affected in backcross generations

323 2.7 Simulating the backcross and re-sequence experiment

324 Given the stochastic nature of inheritance of chromatids in gametes and the randomness of cross-over
325 locations in meiosis, we expected substantial variation in the HI around the expectation of 12.5% (Fig.
326 1). To evaluate whether the observed mean HI of each chromosome deviates significantly from that
327 expected under simple models of introgression with or without inversions and/or extreme BDMs, we
328 simulated the crossing experiment under three different scenarios using Mathematica (Wolfram
329 Research, Inc., version 11.02 Champaign, IL). All simulations were conditioned on the number of BC_2
330 females each BC_1 female contributes to the pool (Table S3). We also assumed one cross-over per female
331 per chromosome in meiosis (a map length of 50cM). Given that the experiment involves two generations
332 of crosses between hybrid females and pure parental males, our simulation only tracks the haplotype
333 of female gametes contributing to BC_1 and BC_2 individuals. All *in silico* backcross experiments were
334 simulated, separately for each chromosome, 10,000 times to obtain 5% and 95% quantiles for the mean
335 HI.

336 First, we simulated the experiment under a simple null model of neutral introgression, i.e. assuming no
337 BDMs and no cross-over suppression due to inversions (SIM1, Fig. 2A). Second, we simulated the

338 experiment similarly under neutrality, but including the breakpoint locations of inversions that are
339 alternately fixed between *D. montana* and *D. flavomontana*. This was done simply by disallowing cross-
340 over events within inverted regions (inversions breakpoints in Table S2), i.e. we did not attempt to
341 include interchromosomal effects (SIM2, Fig. 2B). Third, we simulated the experiment under a model
342 that assumes a single BDMI at a random position within the inverted part of the chromosome (SIM3,
343 Fig. 2C). This single locus cannot be introgressed beyond the F_1 generation, i.e. BC_1 and BC_2 females that
344 are heterozygous for this locus are not produced. Note that while we refer to this as a BDMI for
345 simplicity, we did not explicitly simulate pairwise incompatibilities. Thus, this locus can be regarded as
346 a BDMI involving a dominant allele on the introgressing background (donor species) that is incompatible
347 with one or more recessive alleles in the recipient background.



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349 Figure 2. Introgression experiment was simulated under different scenarios. Example plots of simulated hybrid
350 indices (HI) (A) under neutrality, (B) in the presence of neutral inversions, and (C) in the presence of inversions
351 with a single dominant BDMI. For simplicity, here simulations were run 10 times.

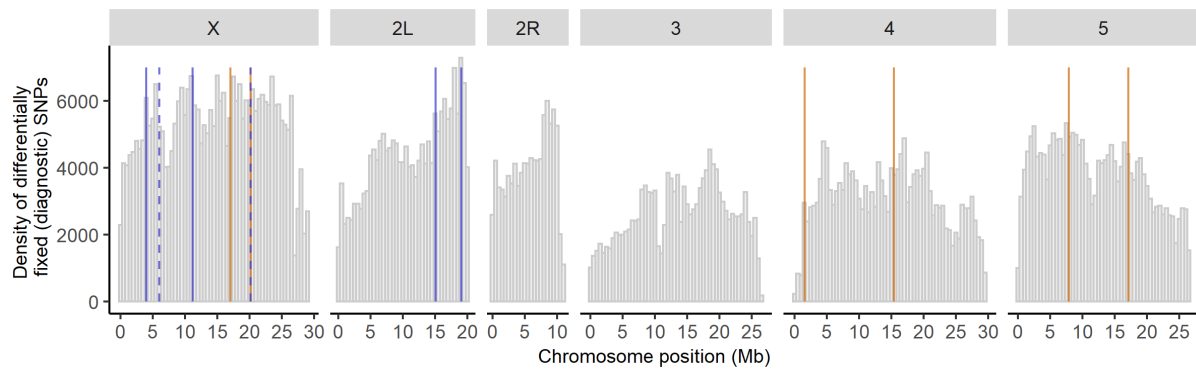
352 3 Results

353 3.1 BC_1 females from the backcrosses towards *D. flavomontana* showed stronger genetic 354 incompatibilities / postzygotic isolation than the ones from the backcrosses towards *D. montana*

355 The proportion of fertile females in the BC_1 generation was 75% and 42% for the BC_1 mon and BC_1 fla
356 hybrids, respectively, and significantly reduced in both reciprocal crosses when comparing to the
357 expected fertility of 1 (BC_1 mon: $t_{19} = -2.52$, $P = 0.021$; BC_1 fla: $t_{54} = -8.67$, $P = 8.371e^{-12}$). Furthermore, the
358 proportion of fertile BC_1 mon females (75%) was significantly higher than that of BC_1 fla females (42%)
359 (GLM, $z_{1,73} = -2.45$, $P = 0.015$; Fig. S2). These findings show that while both crosses suffer from BDMIs
360 affecting female fertility, these incompatibilities are more pronounced in backcrosses towards *D.*
361 *flavomontana* than towards *D. montana* (asymmetric postzygotic isolation, or unidirectional
362 incompatibilities in the sense of Turelli & Moyle, 2007).

363 3.2 Genetic divergence between *D. montana* and *D. flavomontana* has accumulated within inverted 364 chromosome regions especially on the X chromosome

365 The density of SNPs (SNPs divided into equal-sized bins) that were differentially fixed between *D.*
366 *montana* and *D. flavomontana* parental pools was higher on the X chromosome than on any of the
367 autosomes ($P < 0.001$; Fig. 3; Table S4). For each chromosome containing inversions, the density of fixed
368 differences was higher in inverted than in collinear regions of the genome ($P < 0.001$; Fig. 3; Table S5),
369 as expected due to the reduction in recombination within inverted regions (note that chromosomes 2R
370 and 3 have no inversions).



371

372 Figure 3. Density of differentially fixed SNPs (in 200kb windows) between parental species across each
373 chromosome. Orange and blue vertical lines represent species-specific *D. flavomontana* and *D. montana*
374 chromosomal inversions, respectively. Solid and dashed vertical lines describe breakpoints of different inversions.
375 Chromosome 2 involves left (2L) and right (2R) arms separated by a submetacentric centromere.

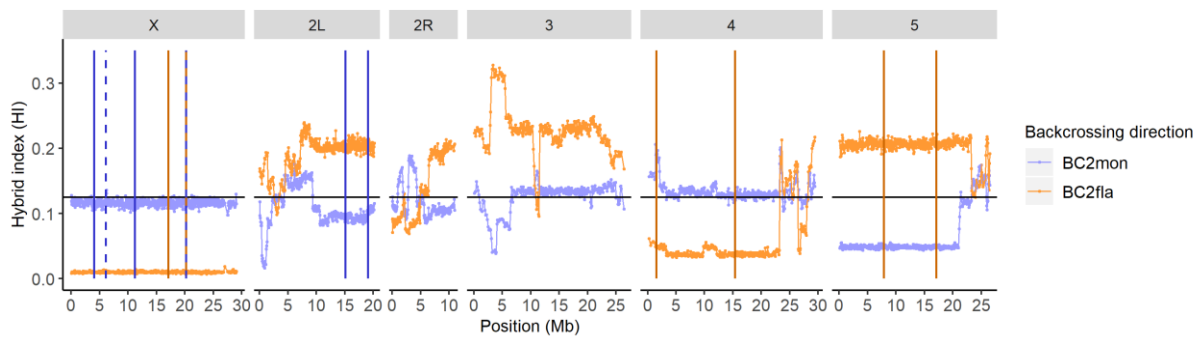
376 3.3 Large differences in HI between chromosomes – evidence for BDMIs located within X chromosomal 377 inversions

378 The mean amount of introgression (hybrid index, HI) of hybrids backcrossed to *D. montana* (BC₂mon)
379 did not deviate significantly from the neutral expectation of 12.5% for any chromosome (SIM1) (Fig. 4A;
380 Fig. 5). Moreover, the number of SNPs that showed no introgression was low, 0.03-0.22%, across the
381 entire genome (Table S6).

382 In contrast, BC₂f₁a hybrids showed a significant deviation in mean HI from the neutral scenario (SIM1)
383 for all chromosomes, except chromosome arms 2L and 2R (Fig. 4; Fig. 5B; Table S6). The mean HI was
384 significantly decreased on the X and 4th chromosome and significantly increased on the 3rd and 5th
385 chromosome compared to the neutral expectation (SIM1) (Fig. 4B; Fig. 5; Table S6). Interestingly, the
386 reduced introgression on the 4th chromosome and the increased introgression on the 5th chromosome
387 could be explained by the reduction in cross-over due to inversions present on these chromosomes,
388 without invoking any selection acting on incompatibilities (SIM2) (Fig. 4, 5E, 5F). Under this scenario,
389 the mean HI showed no deviation from the expectation of 12.5% under neutrality but had an increased
390 variance across simulation replicates (Fig. 5E, 5F). Also, the number of SNPs that showed no
391 introgression varied from 0.12% to 1.22% for chromosomes 2L, 2R, 4 and 5 (Table S6).

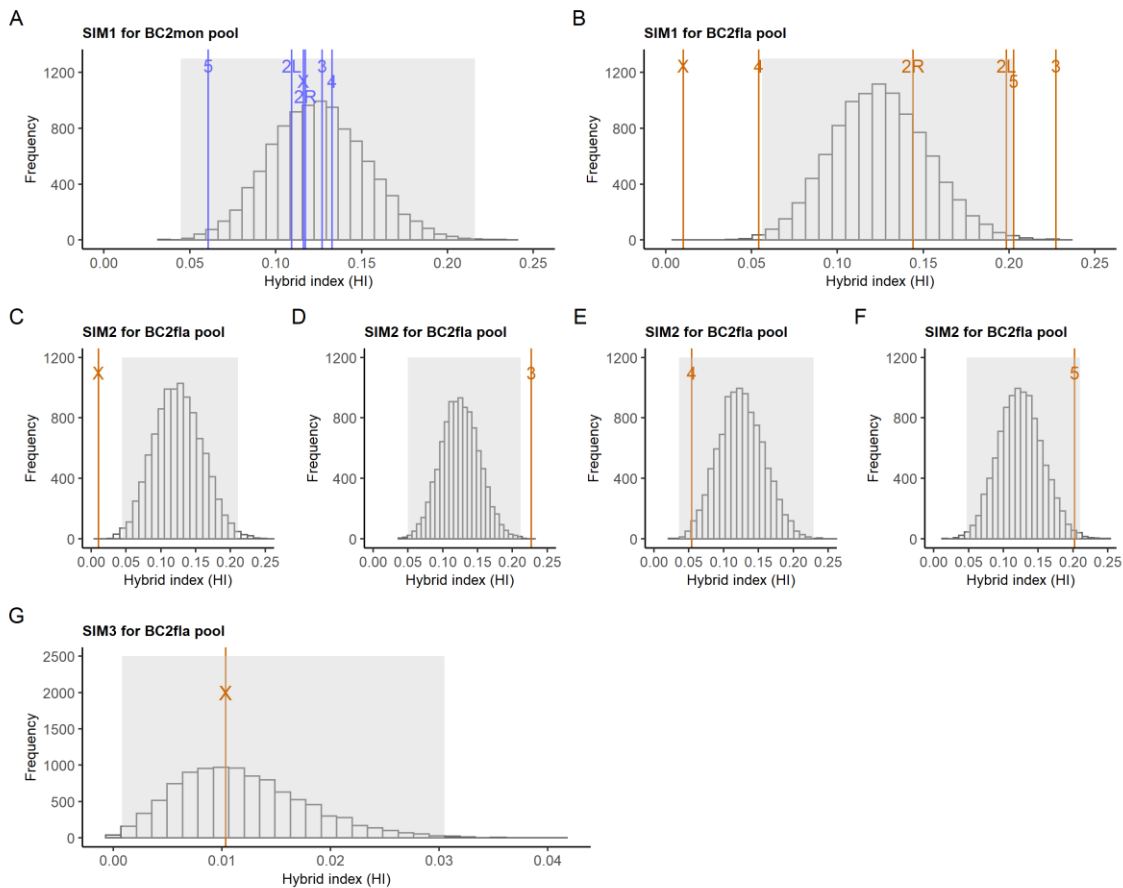
392 The observed decrease in the mean HI of BC₂f₁a hybrids on the X chromosome could not be explained
393 solely by a reduction in cross-over rate due to inversions (Table S6, Fig. 4, 5C). Instead, our simulations
394 show that the drastic reduction in mean HI on the X chromosome is compatible with a single dominant
395 incompatibility locus residing within the X inversions (SIM3) (Fig. 4, 5G). In other words, the data are
396 consistent with a dominant X chromosomal *D. montana* allele that interacts negatively with autosomal
397 homozygous recessive *D. flavomontana* alleles. Intriguingly, 30 % of the differentially fixed SNPs
398 between the species on the X chromosome showed no introgression, emphasising the strength of the
399 X-effect (Table S6).

400 Finally, we conclude that the increase in mean HI observed in BC₂f₁a hybrids on the 3rd chromosome
401 cannot be explained by any of the simple scenarios we simulated. Given that we either assumed
402 neutrality or a single dominant incompatibility locus, which is maximally deleterious in the BC₁ and BC₂
403 females, this is not surprising. The number of SNPs showing no introgression was only 0.18% for the 3rd
404 chromosome.



405

406 Figure 4. Observed hybrid index (HI) of 2nd backcross generation female pools towards *D. montana* (BC₂mon) and
 407 *D. flavomontana* (BC₂fla) in windows of 400 non-overlapping SNPs along the genome. For chromosome 2 the left
 408 (2L) and right (2R) arms are separated by a metacentric centromere. The black horizontal line represents the
 409 expected amount of introgression, HI = 12.5 %, under neutrality. Vertical lines represent species-specific *D.*
 410 *flavomontana* (yellow) and *D. montana* (blue) chromosomal inversions. Solid and dashed vertical lines show
 411 breakpoints of different inversions.



412

413 Figure 5. Hierarchical representation of the most meaningful simulations (10,000 replicates/simulation) of the 2nd
 414 generation backcross experiments towards *D. montana* (BC₂mon) and *D. flavomontana* (BC₂fla). The grey area of
 415 each figure represents Bonferroni corrected 5% and 95% quantiles and the space between them (regions beyond
 416 the area are statistically significant). Simulations under neutrality (SIM1) and the observed mean hybrid index (HI)
 417 of each chromosome for (A) BC₂mon pool and (B) BC₂fla pool. Simulations under neutral inversions (SIM2) and
 418 observed mean HI of BC₂fla pool for (C) the X chromosome, (D) chromosome 3, (E) chromosome 4, and (F)

419 chromosome 5. (G) Simulations involving inversions with a single locus against introgression (SIM3) and observed
420 mean HI for the X chromosome of BC₂fla pool.

421

422 **4 Discussion**

423 A major theme in speciation research is to understand how the loci inducing genetic incompatibilities
424 (BDMIs) in interspecific crosses are distributed across the genome, what role chromosomal inversions
425 and the X chromosome may play in their distribution and what types of epistatic interactions matter for
426 BDMIs (reviewed in (Coughlan & Matute, 2020; Coyne, 2018; Faria, Johannesson, Butlin, & Westram,
427 2018; Hoffmann & Rieseberg, 2008)). To shed light on these questions, we performed reciprocal
428 backcrosses between *D. montana* and *D. flavomontana* and traced the regions of reduced introgression
429 in 2nd backcross generation (BC₂) females.

430 **4.1 Postzygotic barriers between *D. montana* and *D. flavomontana* show asymmetry in their strength**

431 We have previously shown that pre- and postzygotic barriers between *D. montana* females and *D.*
432 *flavomontana* males are practically complete, while both types of barriers between *D. flavomontana*
433 females and *D. montana* males are weaker (Poikela et al., 2019). In crosses between *D. flavomontana*
434 females and *D. montana* males, F₁ hybrid males are sterile, but roughly half of the F₁ females are fertile
435 (Poikela et al., 2019). Accordingly, here we backcrossed fertile F₁ females with males of both parental
436 species, and observed a clear asymmetry in the strength of postzygotic barriers between the two
437 backcross directions. BC₁ hybrid females born from the backcrosses between F₁ females and *D. montana*
438 males showed rather high fertility, and the genetic incompatibilities observed in BC₂ females had no
439 detectable effect. In contrast, when backcrossing F₁ hybrid females with *D. flavomontana* males, more
440 half of the BC₁ females were sterile, and BC₂ females showed signs of strong BDMIs. This asymmetry
441 could be a consequence of a history of unidirectional introgression from *D. montana* into *D.*
442 *flavomontana* in nature (Poikela et al., in prep.), if it had induced selection against introgression at
443 certain loci especially within the X chromosomal inversions, but homogenised genetic divergence on
444 collinear regions. This kind of pattern in the permeability of species boundaries have been found to
445 contribute to speciation also in other species (Harrison & Larson, 2014).

446 It is surprising that introgression has not occurred from *D. flavomontana* to *D. montana* in nature, given
447 that backcrossing towards *D. montana* (BC₂mon) was relatively successful in this study. The most
448 obvious reason for this discrepancy is that laboratory experiments may not reveal all reproductive
449 barriers relevant in wild populations. For example, hybrids may have problems in mate choice in the
450 wild, or they may face challenges to feed or reproduce on species-specific host trees. Moreover, also
451 the male hybrids regain fertility in backcross generations (data not shown), which may contribute to
452 introgression in nature. Finally, in this study we used a *D. montana* population that is allopatric to *D.*
453 *flavomontana*, while BDMIs may well be stronger between *D. montana* and *D. flavomontana*
454 populations living in close contact.

455 **4.2 The role of inversions and the X chromosome in reducing recombination and introgression from *D.*** 456 ***montana* to *D. flavomontana* (BC₂fla pool)**

457 Inversions have suggested to contribute to speciation, if three criteria are met: closely related species
458 must carry alternatively fixed inversions, the inversions must suppress recombination, and this
459 suppression of recombination facilitates reproductive isolation (Faria & Navarro, 2010). We have
460 recently identified several alternatively fixed inversions in *D. montana* and *D. flavomontana*, and shown
461 that these inversions have increased genetic divergence and lower historical introgression compared to
462 colinear chromosome regions (Poikela et al., in prep.). In the present study, we show that these
463 inversions have an increased number of alternatively fixed SNPs compared to colinear regions, which is
464 in agreement with their increased genetic divergence shown in Poikela et al. (in prep.). We also show
465 that inversions effectively suppress recombination in hybrid individuals across a large swathe of the

466 genome (Fig. 4). Finally, we find that the drastic reduction in introgression on the X chromosome can be
467 explained by inversions that are associated with at least one dominant X chromosomal *D. montana*
468 incompatibility allele interacting negatively with recessive autosomal *D. flavomontana* alleles. This
469 negative epistatic interaction could cause the observed low hybrid fertility, and supports the idea that
470 inversions act as strong barriers to gene flow by facilitating the establishment of BDMLs (Hoffmann &
471 Rieseberg, 2008; Navarro & Barton, 2003; Noor et al., 2001).

472 While the involvement of the X chromosome in hybrid problems may not be surprising (see e.g. Masly
473 & Presgraves, 2007; Tao, Chen, Hartl, & Laurie, 2003), the fact that it involves a dominant incompatibility
474 locus is. The “dominance theory” (e.g. Turelli & Orr, 1995, 2000), which aims to explain the
475 disproportionate role of the X chromosome in hybrid incompatibilities, relies on the presence of
476 recessive incompatibilities on the X and therefore cannot explain our result. However, the “dominance
477 theory”, as well as the “faster-male theory” and dosage compensation (reviewed in Coyne, 2018;
478 Presgraves, 2008), can still explain the hybrid male sterility previously observed in crosses between *D.*
479 *flavomontana* and *D. montana* (Poikela et al., 2019). Accumulation of meiotic drive elements on the X
480 chromosome could be another plausible explanation for the large X-effect in general (reviewed in
481 Patten, 2018), but is unlikely in our system for two reasons. First, meiotic drivers should increase their
482 own transmission in both backcross directions, leading to decreased introgression in the BC₂fla pool and
483 increased introgression in the BC₂mon pool, which we did not see. Second, meiotic drive systems
484 described in *Drosophila* are typically involved in sperm killing and not in female sterility (Courret et al.,
485 2019). Although cytoplasmic incompatibilities have been detected in other *montana* complex species
486 of the *Drosophila virilis* group (Patterson, 1952; Throckmorton, 1982), they are not likely to play a major
487 role in these crosses since all hybrids had *D. flavomontana* cytoplasm (and crosses were more
488 unsuccessful in this direction). Finally, the large effect of the X we find could potentially be explained by
489 “faster X evolution”, based on the idea that selection increases the frequency of advantageous recessive
490 alleles more effectively on the X chromosome than on autosomes, irrespectively of whether the
491 incompatibilities themselves are recessive (Charlesworth et al. 1987, 2018). Also, the X chromosome
492 could simply contain more genes that are prone to create postzygotic isolation than those on the
493 autosomes (Coyne, 2018).

494 Several autosomes showed deviations from the expected hybrid indices in the BC₂fla pool in the present
495 study. Based on our simulations, the reduced and increased introgression on the 4th and 5th
496 chromosomes, respectively, could be explained by inversions’ ability to restrict recombination which
497 increases the variance in chromosome-wide HI. However, if we calculate the expected allele frequencies
498 for a dominant–recessive BDML by hand for the first two backcross generations, the allele frequencies
499 (i.e. HI) after selection would be 1/22 (4.5%) for the dominant and 2/11 (18.2%) for the recessive *D.*
500 *montana* allele in the BC₂fla pool (see Fig. S2). These frequencies are close to the observed frequencies
501 on chromosomes 4 (5.4%) and 2L (19.8%), respectively. It is therefore tempting to speculate that a
502 pairwise BDML loci exist on these chromosomes. Finally, the 3rd chromosome, which lacks species-
503 specific inversions, showed a drastic increase in introgression, and was not explained by any of our
504 simulations. We note that our simulations did not consider an interchromosomal effect, where
505 inversions may trigger an increase in recombination on other freely recombining chromosomes (Crown
506 et al., 2018; Stevison, Hoehn, & Noor, 2011). However, this would only decrease the variance in HI on
507 chromosomes lacking fixed inversions and so cannot explain the increase in HI for chromosome 3 in the
508 BC₂fla pool.

509 In future research, combing the crosses with quantitative trait loci (QTL) analyses might help to link
510 BDMLs to e.g. specific genes (Johnson, 2010) or gene duplicates or transpositions (Bikard et al., 2009;
511 Masly, Jones, Noor, Locke, & Orr, 2006). BDML genes could also be searched by tracing the expression-
512 phenotypes in interspecific hybrids in these genes (Landry et al., 2005). However, recombination
513 suppression of inversions presents a challenge for mapping BDMLs, and would potentially require a

514 complex reversion of the X chromosomal inversions with genome editing tools, and repeating the
515 current experiment to narrow down the regions of reduced introgression (Hopkins, Tyukmaeva,
516 Gompert, Feder, & Nosil, 2020). Overall, finding the exact loci driving species' isolation may be difficult,
517 as BDMIs are often complex and co-evolve with rapidly evolving heterochromatic DNA (Satyaki et al.,
518 2014).

519 **5 Conclusions**

520 “Introgress-and-resequence” studies that combine interspecific backcrosses with genome-wide
521 analyses and simulations are an effective approach for identifying BDMIs, in particular those involving
522 dominant alleles. Our study supports the idea that inversions aid the accumulation of BDMIs due to
523 reduced recombination, and shows that strong BDMIs coupled with suppressed recombination
524 effectively restrict introgression beyond the inverted part of the genome in the first two backcross
525 generations. We conclude that the large X-effect we observed in our experiment may result from at
526 least one dominant incompatibility locus residing within several overlapping inversions. If the design
527 were extended to study interspecific F₂ hybrids, assuming that the F₁ female and male hybrids are viable
528 and fertile, one could investigate recessive-recessive BDMIs in the same way. Overall, we provide a novel
529 framework for investigating the role of inversions and the X chromosome as genetic barriers to
530 introgression, which we hope will encourage similar studies on a larger number of species and strains.

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536 **Data Accessibility**

537 Raw reads will be made publicly available in the SRA (BioProject XXX), and phenotypic data and
538 Mathematica notebook including simulations in Dryad at the time of publication.

539 **Author Contributions**

540 KL, AH and NP designed the study. NP performed the hybrid backcrosses and analysed the genomic data
541 with input from KL and DRL. KL performed the simulations. AH and MK supervised and funded the
542 research. NP, AH and KL drafted the manuscript and all authors finalised it.

543 **Conflict of interest**

544 The authors declare no conflict of interest.

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551 **Ethics declaration**

552 Neither species is endangered, and the flies were collected along watersides on public lands outside
553 National and State parks, where insect collecting does not require permits in the USA (The Wilderness
554 Act of 1964, section 6302.15).

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