1 **Title:** Direct gamete sequencing reveals no evidence for segregation distortion in house

2 mouse hybrids

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16   **Abstract:**

17   Understanding the molecular basis of species formation is an important goal in

18   evolutionary genetics, and Dobzhansky-Muller incompatibilities are thought to be a

19   common source of postzygotic reproductive isolation between closely related lineages.

20   However, the evolutionary forces that lead to the accumulation of such incompatibilities

21   between diverging taxa are poorly understood. Segregation distorters are believed to be an

22   important source of Dobzhansky-Muller incompatibilities between *Drosophila* species and

23   crop plants, but it remains unclear if these selfish genetic elements contribute to

24   reproductive isolation in other species. Here, we collected viable sperm from first-

25   generation hybrid male progeny of *Mus musculus castaneus* and *M. m. domesticus*, two

26   subspecies of rodent in the earliest stages of speciation. We then genotyped millions of

27   single nucleotide polymorphisms in these gamete pools and tested for a skew in the

28   frequency of parental alleles across the genome. We show that segregation distorters are

29   not measurable contributors to observed infertility in these hybrid males, despite sufficient

30   statistical power to detect even weak segregation distortion with our novel method. Thus,

31   reduced hybrid male fertility in crosses between these nascent species is attributable to

32   other evolutionary forces.

33

34   **Introduction**:

35   The Dobzhansky-Muller model [1,2] is widely accepted among evolutionary

36   biologists as a primary explanation for the accumulation of intrinsic reproductive

37   incompatibilities between diverging lineages [3,4]. Briefly, this model posits that genes

38   operating normally in their native genetic background can be dysfunctional in a hybrid

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39   background due to epistatic interactions with alleles from a divergent lineage. Although

40   elucidating the molecular basis of speciation has been a central focus for decades, loci

41   contributing to Dobzhansky-Muller incompatibilities (DMIs) have proved challenging to

42   identify primarily because they are, by definition, incompatible in combination (review by

43   [3-6]). As a result, the specific genetic changes responsible for the onset of reproductive

44   isolation between lineages remain largely obscure.

45   The rapid evolution of selfish genetic elements within lineages is thought to be a

46   potent source of DMIs between diverging taxa. Segregation distorters are one such selfish

47   element that increase their transmission through heterozygotes by either disabling or

48   destroying gametes that failed to inherit the distorting allele [7,8]. Because males

49   heterozygous for a distorter produce fewer viable sperm, segregation distorters can

50   decrease the fitness of carriers. In this case, other loci in the genome are expected to evolve

51   to suppress distortion [9]. This coevolution of drivers and suppressors has been suggested

52   to be a widespread source of DMIs between diverging lineages: hybrids of isolated

53   populations in which such coevolutionary cycles have occurred may suffer lower fertility as

54   drivers become uncoupled from their suppressors in a mixed genome [10-12]. Indeed,

55   there is evidence that SDs contribute to hybrid male sterility in several *Drosophila* species

56   pairs (*e.g.* [13-15], reviewed by [4,12]) as well as in many crop species (*e.g.* [16-20]).

57   However, comparatively little is known about genetics of speciation aside from these

58   groups, and it remains unclear if distorters contribute to hybrid sterility in other taxa more

59   generally.

60   Analyses aimed at identifying the genetic targets of positive selection suggest that

61   segregation distorters may be an important source of DMIs in mammalian lineages. One

62   particularly intriguing finding shows a substantial overrepresentation of loci associated

63   with spermatogenesis and apoptosis within the set of genes with the strongest evidence for

64   recurrent positive selection in mammals (*e.g.* [21,22]). These functions in turn are

65   potentially driven, at least in part, by segregation distorters, which are expected to leave

66   just such a mark of selection as they sweep through a population. Therefore, mammals are

67   an appealing group in which to test for segregation distortion and its role in speciation.

68   In particular, *Mus musculus domesticus* and *M. m. castaneus* are two subspecies of

69   house mice in the earliest stages of evolving reproductive isolation [23,24]. Indeed, these

70   subspecies are estimated to be approximately 500,000 years diverged from one another

71   [24]. Hybrid males suffer from reproductive deficiencies [25]; specifically, the vas deferens

72   of first-generation hybrid (F_1) males contain more apoptotic sperm cells than either pure

73   strain, and numerous loci affecting fertility in hybrid males have been reported,

74   particularly in F_2 individuals [26]. Finally, Wager [27] identified eight genomic regions that

75   exhibited significant deviations from Mendelian segregation in an F_2 mapping population

76   derived from these two subspecies, which may be consistent with the action of segregation

77   distorters in their hybrids (but see below). In combination with the comparative genomic

78   evidence and phenotypic observations described above, these data suggest that

79   coevolution of SDs and their suppressors may contribute to DMIs in *M. musculus*.

80   The conventional approach to identifying SD relies on detecting a skew in the allele

81   frequencies of second-generation hybrids in a large genetic cross. However, methods that

82   rely on genotyping progeny unavoidably conflate segregation distortion, female effects on

83   sperm function, and differential viability. Additionally, practical issues limit the power of

84   these experiments—specifically, the ability to produce and genotype hundreds to

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85   thousands of individuals in order to detect distorters of small effect—particularly in

86   vertebrates. Therefore, as a result of modest sample sizes, many experiments designed to

87   detect SD using genetic crosses are underpowered and unable to detect even moderate

88   distortion.

89   Here, we explore a novel approach to surveying the genome for SD by directly

90   sequencing viable gametes from F_1 hybrid *M. m. domesticus*/*M. m. castaneus* males. Briefly,

91   we enriched for viable sperm in hybrids and then sequenced these sperm in bulk, along

92   with a control tissue, to identify any skew in the representation of either parental

93   chromosome in the viable sperm relative to the control (Figure 1). While we demonstrate

94   via simulation that our experimental design has excellent power, we find no evidence of SD

95   in this cross, suggesting that segregation distorters are not a primary contributor to male

96   infertility in *M. m. castaneus* and *M. m. domesticus* hybrids. Nonetheless, this approach can

97   be applied to a wide range of species, and we therefore expect that it will be a useful means

98   to study the frequency and impact of segregation distortion more generally.

99

100   **Materials and Methods**:

101   <u>Reference Genome Assembly:</u> To generate robust genome assemblies for each of the two

102   strains of interest, we aligned all short read data for *M. m. castaneus* strain (CAST/EiJ) and

103   *M. m. domesticus* strain (WSB/EiJ) from a recent large-scale resequencing project [28] to

104   the MM9 genome assembly using BWA v0.7.1 [29] for initial mapping. For reads that failed

105   to map with high confidence, we remapped using stampy v1.0.17 [30]. We realigned reads

106   that overlap indels, and called SNPs and indels for each strain using the Genome Analysis

107   Tool Kit (GATK, [31]). For each program, we used default parameters, except that during

108   variant calling we used the option '––sample_ploidy 1,' because the strains are extremely

109   inbred.

110   We generated a consensus sequence for each strain at sites where both assemblies

111   have high quality data. That is, if both CAST and WSB assemblies had a q30 minimum

112   quality genotype (either indels or SNPs) that site was added to both consensus sequences.

113   Otherwise, if either or both assemblies were below this quality threshold at a given site, we

114   used the MM9 reference allele for both.

115

116   Alignment Simulation: Our goal was to align short read data to a single diploid reference

117   genome, comprised of assemblies from the two parental strains. The mapping quality,

118   which indicates the probability that a read is incorrectly mapped in the position indicated

119   by the aligner, should then provide a reliable means of distinguishing whether a read can

120   be confidently assigned to one of the parental genomes. To confirm the accuracy of this

121   approach and to identify suitable quality thresholds, we performed simulations using

122   SimSeq (https://github.com/jstjohn/SimSeq). We used the sequencing error profiles

123   derived from our mapped data (below) and found qualitatively similar error rates using the

124   default error profile included with the SimSeq software package (data not shown). For both

125   the CAST and WSB genomes, we simulated 10,000,000 pairs of 94-bp paired-end reads,

126   whose size distribution was set to match that of our libraries (below). We then mapped

127   these reads back to the single reference genome containing both CAST and WSB consensus

128   sequences. We scored reads as 'mapping correctly' if they mapped to within 10 bp of their

129   expected location measured by their left-most coordinate and on the correct subspecies'

130   chromosome. If the pair mapped, we required that the insert length be less than 500 bp,

131   which is well within three standard deviations of the mean insert size of our data and

132   should therefore encompass the vast majority of read pairs. If both reads in a pair mapped

133   and met our criteria above, we used the higher mapping quality of the two, and discarded

134   the other read. This filter is important, here and below, as it avoids counting pairs as

135   though their provenance is independent of their pair.

136

137   <u>Experimental Crosses and Swim-Up Assay:</u> To create first-generation (F_1) hybrids of *Mus*

138   subspecies, we crossed 2 *M. m. castaneus* males to 3 *M. m. domesticus* females and 2 *M. m.*

139   *domesticus* males to 5 *M. m. castaneus* females in a harem-mating scheme. In total, we

140   produced 8 male F_1s in each direction of the cross. F_1 males whose sire was *M. m. castaneus*

141   (CAST genome) are referred to as CW, and those whose sire was *M. m. domesticus* (WSB

142   genome) as WC. All males were housed individually for a minimum of two weeks prior to

143   sacrifice between 90 and 120 days of age.

144   To enrich for viable sperm from each F_1 male, we performed a standard swim up

145   assay [32]. First, immediately following sacrifice, we collected and flash-froze liver and tail

146   control tissues (liver samples, $N = 16$; tail samples $N = 8$). Then, we removed and lacerated

147   the epididymides of each male, placed this tissue in 1.5 ml of human tubal fluid

148   (Embryomax® HTF, Millipore), and maintained the sample at a constant 37 °C for 10

149   minutes. Next, we isolated the supernatant, containing sperm that swam out of the

150   epididymides, and spun this sample for 10 minutes at 250 g. We then discarded the

151   supernatant, repeated the wash, and this time allowed sperm to swim up into the solution

152   for an hour to select the most robust cells. Finally, we removed the solution, transferred

153   them to new vial, pelleted these sperm by centrifugation, and froze them at -80 °C.

154

155 <u>Library Preparation and Sequencing:</u> For each F_1 hybrid male, we first extracted DNA from

156 sperm, liver, and tail tissues identically using a protocol designed to overcome the difficulty

157 of lysing the tightly packed DNA within sperm nuclei (Qiagen *Purification of total DNA from*

158 *animal sperm using the DNeasy Blood & Tissue Kit; protocol 2*). We sheared this DNA by

159 sonication to a target insert size of 300 bp using a Covaris S220, then performed blunt-end

160 repair, adenylation, and adapter ligation following the manufacturer protocol (New

161 England BioLabs). Following ligation, libraries were pooled into two groups of 16 and one

162 group of 8 based on the adapter barcodes. Prior to PCR, each pool was subject to automated

163 size selection for 450-500 bp to account for the addition of 175 bp adapter sequences,

164 using a Pippen Prep (Sage Science) on a 2.0% agarose gel cassette. PCR was performed

165 using six amplification cycles, and then we re-ran the size selection protocol to eliminate

166 adapter dimer prior to sequencing. Finally, we pooled the three libraries and sequenced

167 them on two lanes of a HiSeq 2500. Each sequencing run consisted of 100 bp paired-end

168 reads, of which the first 6 bp are the adapter barcode sequence, and the remaining 94 bp

169 are derived from randomly-sheared gDNA.

170 <u>Alignment and Read Counting:</u> We aligned read data to the combined reference genome

171 using 'BWA mem' as described above in the alignment simulation. We removed potential

172 PCR duplicates using Picard v1.73. We then filtered reads based on the alignment filtering

173 criteria described above for the simulated data. Because copy number variations may pose

174 problems for our analysis, we attempted to identify and exclude these regions. Specifically,

175 we broke the genome into non-overlapping 10 kb windows. Then, within each library, we

176 searched for 10 kb regions that had a sequencing depth greater than two standard

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177   deviations above the mean for that library. All aberrantly high-depth windows identified

178   were excluded in downstream analyses in all libraries. These regions, representing

179   approximately 7% of the windows in the genome, are reported in Supplemental Table S1.

180   Although deletions in one parental strain relative to the MM9 genome could also skew the

181   parental allele frequencies for sequenced tissues, these copy number variable regions

182   would affect both somatic tissues and gametes equivalently, and we therefore do not expect

183   copy number variable regions to yield false positive results.

184   Next, to identify regions showing evidence of segregation distortion, we conducted

185   windowed analyses with 1 Mb between the centers of adjacent windows. We counted reads

186   in each window as a decreasing function of their distance from the center of the window,

187   and included no reads at distances greater than 20 cM, thereby placing the most weight in a

188   window on the center of the window. We then analyzed each window in two mixed-effects

189   generalized linear models. Both models included random effects for the libraries and

190   individuals. The first model includes no additional factors. The second had fixed effects for

191   tissue, direction of cross, and an interaction term based on tissue by direction of cross

192   effects, and thus has five fewer degrees of freedom than the first model. Hence, for each

193   window, we assessed the fit of the second model relative to the first using a likelihood ratio

194   test, wherein the log likelihood ratio should be chi-square distributed with 5 degrees of

195   freedom. Afterwards, we applied a false-discovery rate multiple testing correction to the

196   data [33]. We performed all statistical analyses in R [34].

197

198   Power Simulations: To estimate the power of our method, we simulated distortion data. We

199   began by selecting sites randomly distributed across the genome, and for each site drew a

200   distortion coefficient from a uniform distribution between -0.05 and 0.05. Each read on the

201   parental genome that was susceptible to distortion was counted on the distorting genome

202   with probability equal to the distortion coefficient multiplied by the probability that no

203   recombination events occurred between the distorted locus and the read. We also did the

204   alternative (*i.e.* switching reads from the distorted against genome to the distorting

205   genome) by multiplying by the probably that a recombination event was expected to occur

206   in the genomic interval between the distorter and the read. We determined recombination

207   probabilities using the genetic map reported in [35]. We performed the simulation for both

208   parental genomes, and then again for each parental genome but with the distortion limited

209   to one direction of the cross (*e.g.* only sperm from CW males experienced distortion). A

210   direction-specific effect could occur if, for example, suppressing alleles are present on the Y

211   chromosome of one subspecies and therefore are only present in CW or WC males.

212

213   **Results**

214   After addressing the possibility of contamination, labeling, and quality issues (see

215   Supplemental Text S1, Supplemental Table S2), we ran our analysis of the data across all

216   autosomes, excluding regions with evidence for copy-number variations (described in

217   Methods). With the exception of windows on chromosome 16 (see below), we found no

218   windows with a statistically significant signature of segregation distortion. The lowest

219   uncorrected *p*-value for any window (aside from those on chromosome 16) was 0.0224,

220   which is not significant when we corrected for multiple tests. Thus, we did not find

221   evidence for segregation distortion in any of the genomic regions considered (Figure 2).

222   By contrast, on chromosome 16, we identified 15 contiguous windows with

223   significantly skewed allele frequencies following correction for multiple comparisons

224   (minimum p = 5.026E-4; Figure 3). However, upon closer examination, it appears that this

225   signal is driven almost entirely by a single liver sample, that of individual CW10. If this

226   sample is removed from the dataset, this chromosome no longer shows significant

227   deviation from expectations. When comparing the relative read depths across

228   chromosomes 16 and 1, CW10's liver sample also appears to have disproportionately lower

229   depth on this chromosome relative to CW10's sperm sample (p = 3.02E-5; X^2-test). These

230   results suggest that this pattern is likely driven by a somatic aneuploidy event in CW10's

231   liver that occurred relatively early in liver development and is not the result of distortion in

232   the sperm library.

233   Through simulation, we ensured that we have sufficient statistical power, given our

234   experimental design and data quality, to detect segregation distortion if it is indeed

235   occurring in hybrid males. We found that we have 50% power to detect SD to

236   approximately 0.014, or 1.4% (this number reflects the positive or negative deviation from

237   the null expectation, 0.5, at α = 0.001) if distortion affects CW and WC males equally (Figure

238   4). In other words, we have 50% power to detect distortion that is greater than 51.5% or

239   less than 48.5%. If there is directionality to the distortion effect (*i.e.* only CW or only WC

240   males experience SD), we have 50% power to detect distortion of 0.016 for CW males and

241   0.018 for WC males (at α = 0.001). This slight difference in power based on cross direction

242   likely reflects differences in sequencing depth between WC and CW sperm and liver

243   samples. It is also important to note that because read mapping and sequencing, as well as

244   divergence between the CAST and WSB strains and their divergence from the reference

245   genome, are non-uniform across the genome, different regions of the genome will differ

246   slightly in power to detect distortion.

247

248   **Discussion**

249   Elucidating the genetic mechanisms underlying species formation is a central goal of

250   evolutionary biology. Although there has been progress in identifying the genetic basis of

251   reproductive isolation in a few elegant instances (*e.g.* [36-38]), including several in

252   *Drosophila* (*e.g.* [39,40]), it is unclear how general these results are. For example, SDs

253   contribute to reproductive isolation in some young Drosophila species pairs ([13-15]) but

254   here, to our surprise, we find no evidence for segregation distortion between two nascent

255   species of mouse, *M. m. castaneus/M. m. domesticus,* despite strong experimental power.

256   Our conclusion must be qualified to some degree. SDs are generally classified as

257   either gamete disablers or gamete killers depending on their mode of action (reviewed in

258   [7,8]). We expect to detect gamete killers with our approach since their victims may not be

259   present in the epididymides, or, if present, these sperm would not be captured in our

260   stringent swim up assay. Our ability to detect gamete- disablers, however, depends on the

261   specific mechanism by which these genetic elements act. If the motility or longevity of a

262   sperm cell is sufficiently impaired, it is likely that this sperm would fail to swim into

263   solution and remain motile over the course of the assay, but if the distortion effect has a

264   very subtle effect on motility or impairs function later in the sperm life cycle (*e.g.* by

265   causing a premature acrosome reaction), it is unlikely that our method could detect these

266   effects. Thus, although gamete killers are not prevalent sources of DMIs in these subspecies,

267   we cannot completely exclude the possibility that gamete disablers contribute to *M.*

268   *musculus* species formation. However, it is worth nothing that disablers cannot explain the

269   reported observation of increased apoptosis of sperm cells in hybrid males [26].

270   Conventional methods of detecting SDs (*i.e.* genotyping progeny) are usually

271   statistically underpowered and thus unable to detect even modest distortion effects.

272   Moreover, requiring the presence of offspring from F_1 hybrids unavoidably conflates

273   viability, gamete competition, and segregation distortion effects. By contrast, our

274   simulations demonstrate that by sequencing high quality gametes from individual hybrid

275   males and comparing allele ratios in these gametes to those of somatic tissues, we have

276   excellent power to detect even relatively weak SDs, of less than two percent. In support of

277   this point, we successfully detected an aneuploidy event that resulted in a four percent

278   difference in allele frequencies relative to expectations within only a single biological

279   replicate. Nonetheless, we found little evidence that SDs are active in F_1 hybrid males,

280   which indicates that segregation distortion (*i.e.* gamete killing) is not a primary contributor

281   to reduced F_1 male fertility in these subspecies.

282   Because our method of determining the allele ratios in bulk preparations of viable

283   gametes relative to somatic tissues is very general, we expect that it will be useful in a wide

284   variety of systems for an array of questions. Provided one can accurately phase the diploid

285   genome of an individual, by *e.g.* using complete parental genotype data when inbred strains

286   are not available, it is straightforward to apply this method to assay segregation distortion

287   in a wide variety of taxa (including humans). Thus, we are now well positioned to survey

288   the prevalence of segregation distortion both within and between a diversity of species.

289   This approach also allows segregation distortion to be weighed against other possible

290   sources of DMIs that may occur during spermatogenesis, oogenesis, fertilization, or

13

291   embryogenesis, but that leaves an identical signature to SD in conventional cross-based

292   experiments. Furthermore, because SDs can increase in frequency in populations despite

293   deleterious consequences for the host, these selfish genetic elements may also be an

294   important source of disease alleles. For example, it has been suggested that SDs contribute

295   to the perpetuation of split-hand/split-foot disease [41], retinal dystrophy [42] and

296   Machado-Joseph disease [43] in humans. Hence, the method introduced here has the

297   potential to improve our understanding of disease evolution in addition to the contribution

298   of SDs to the evolution of reproductive isolation between diverging lineages.

299   While segregation distorters may be an important mechanism of speciation in

300   *Drosophila* and crop plants, efforts to detect SD in other diverging lineages—especially

301   studies with high statistical power—have been limited. We find that at least in *M. m.*

302   *castaneus*/*M. m. domesticus* hybrids, segregation distorters are not measurable

303   contributors to observed infertility in F1 hybrid males, despite strong statistical power to

304   detect them, suggesting that reduced hybrid male fertility in these nascent species is

305   attributable to other underlying genetic causes. Further studies, using the novel approach

306   developed here will provide a powerful way to gain more comprehensive understanding of

307   the role of SDs within and between populations.

308

309   **Acknowledgements**

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434

Author Contributions

436   RCD, EJP, DH, and HEH conceived and designed experiments. RCD, EJP, and HEH wrote the

437   manuscript. RCD and EJP performed experiments. RCD analyzed the data.

438

Figure and Table Legends

440   Figure 1. Schematic of experimental cross scheme. Inbred parental strains were crossed,

441   and individual F1 males (purple) sacrificed at between 3 and 6 months, when their sperm

442   were subjected to a swim up assay. Libraries were prepared from liver or tail (control; left)

443   and sperm (experiment; right) samples, sequenced, and then aligned to a diploid reference

444   genome; subspecies of origin were determined for as many sequences as possible.

445

446   <u>Figure 2.</u> Average proportion CAST reads in sperm libraries versus liver libraries, using all

447   males (A), using only CW males (B), and using only WC males (C). Lines indicate the

448   approximate threshold at which we would have 50% power to detect distortion at the

449   alpha = 0.0001 level (see Methods for how this threshold was calculated).

450

451   <u>Figure 3.</u> Proportion of informative reads that are derived from the CAST genome across

452   chromosome 16. CW10's liver sample is shown in red, and CW10's sperm sample is shown

453   in green. All other CW libraries are represented in black for liver and in blue for sperm.

454

455   <u>Figure 4.</u> Minimum level of distortion that could be detected given a specified significance

456   threshold (α, y-axis), and desired power (x-axis).

457

458   <u>Supplemental Text S1.</u> Supplemental methods describing quality control steps to ensure

459   samples are not contaminated or mislabeled.

460

461   <u>Supplemental Table S1.</u> List of genomic windows excluded from all downstream analyses

462   due to detection of individual libraries with unusually high depth.

463

464   <u>Supplemental Table S2.</u> Quality control results for the quantity of reads in each library

465   derived from the Y chromosome, X chromosome, and mtDNA.

466

467   <u>Supplemental Table S3.</u> Alignment simulation results showing the relationship between

468   the reported mapping quality for a read and its probability of correct assignment to the

469   genomic location from which it was derived.

470