Title: Segregation distorters are not a primary source of Dobzhansky-Muller incompatibilities in house mouse hybrids

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Abstract:

Understanding the molecular basis of species formation is an important goal in evolutionary genetics, and Dobzhansky-Muller incompatibilities are thought to be a common source of postzygotic reproductive isolation between closely related lineages. However, the evolutionary forces that lead to the accumulation of such incompatibilities between diverging taxa are poorly understood. Segregation distorters are an important source of Dobzhansky-Muller incompatibilities between *Drosophila* species and crop plants, but it remains unclear if the contribution of these selfish genetic elements to reproductive isolation is prevalent in other species. Here, we genotype millions of single nucleotide polymorphisms across the genome from viable sperm of first-generation hybrid male progeny in a cross between *Mus musculus castaneus* and *M. m. domesticus*, two subspecies of rodent in the earliest stages of speciation. We then search for a skew in the allele frequencies of the gametes and show that segregation distorters are not measurable contributors to observed infertility in these hybrid males, despite sufficient statistical power to detect even weak segregation distortion with our novel method. Thus, reduced hybrid male fertility in crosses between these nascent species is attributable to other evolutionary forces.

Introduction:

The Dobzhansky-Muller model (Dobzhansky 1937, Muller 1942) is widely accepted among evolutionary biologists as the primary explanation of the accumulation of intrinsic reproductive incompatibilities between diverging lineages (Coyne and Orr 2004, Presgraves 2010). Briefly, this model posits that genes operating normally in their native

genetic background can be dysfunctional in a hybrid background due to epistatic interactions with alleles from a divergent lineage. Although elucidating the molecular basis of speciation has been a central focus for decades, Dobzhansky-Muller incompatibilities (DMIs) have proved challenging to study because of their powerful effects on hybrid fitness (review by Coyne and Orr 2004, Noor and Feder 2006, Presgraves 2010, Wu and Ting 2004). As a result, the specific genetic changes responsible for the onset of reproductive isolation between lineages remain largely obscure.

The rapid evolution of selfish genetic elements is thought to be a potent source of DMIs between diverging lineages. In particular, segregation distorters are selfish elements that increase their transmission through heterozygous males by either disabling or destroying sperm that did not inherit the distorting allele (Lyttle 1991, Taylor and Ingvarsson 2003). Because males heterozygous for a distorter produce fewer viable sperm, segregation distorters can decrease the fitness of carriers. In this case, other loci in the genome are expected to evolve to suppress distortion (Hartl 1975). This coevolution of drivers and suppressors has been suggested to be a widespread source of DMIs between diverging lineages, and thus likely a contributor to reproductive isolation (Hurst and Pomiankowski 1991, Frank 1991, McDermott and Noor 2010). Indeed, there is strong evidence that segregation distorters are a primary cause of hybrid male sterility in several *Drosophila* species pairs (*e.g.* Tao *et al.* 2007ab, Phadnis *et al.* 2010, reviewed by McDermott and Noor 2010, Presgraves 2010) as well as in many crop species (*e.g.* Bohn and Tucker 1940, Cameron and Moav 1957, Sano *et al.* 1979, Loegering and Sears 1963). However, comparatively little is known about genetics of speciation in natural populations

aside from these taxa, and it remains unclear if distorters contribute to hybrid sterility in other taxa more generally.

Comparative analyses aimed at identifying the genetic targets of positive selection suggest that segregation distorters may be an important source of DMIs in mammalian lineages. One particularly intriguing finding shows a substantial overrepresentation of loci associated with spermatogenesis and apoptosis within the set of genes with the strongest evidence for recurrent positive selection (*e.g.* Nielsen *et al.* 2005, Kosiol *et al.* 2008). These functions in turn are potentially driven at least in part by segregation distorters, which are expected to leave just such a mark of selection as they sweep through a population. Therefore, mammals are an appealing group in which to test for segregation distortion and its role in speciation.

In particular, *Mus musculus domesticus* and *M. m. castaneus* are two subspecies of house mice in the earliest stages of the evolution of reproductive isolation (Boursot *et al.* 1996, Geraldes *et al.* 2008). Hybrid males suffer from many reproductive deficiencies (Davis *et al.* 2007); specifically, they are known to have decreased testis size and to produce fewer sperm than either parental subspecies (White *et al.* 2012). Moreover, it has been reported that there are numerous loci that affect fertility in hybrid males and also that the vas deferens of first-generation hybrid (F_1) males contain more apoptotic sperm cells than either pure strain (White *et al.* 2012). In combination with comparative genomic evidence, these phenotypic observations suggest that coevolution of segregation distorters and their suppressors may contribute to DMIs in *M. musculus*.

The conventional method of identifying segregation distortion relies on detecting a skew in the allele frequencies of the offspring of a heterozygous individual. However,

methods that rely on genotyping progeny unavoidably conflate segregation distortion, female effects on sperm function, and differential viability. Moreover, practical issues limit the power of these experiments—specifically, the ability to produce and genotype hundreds to thousands of offspring to detect distorters of small effect, particularly in vertebrates. As a result of modest sample sizes, many experiments designed to detect distortion based on segregation in genetic crosses are underpowered and unable to detect even moderate distortion. Hence, it is challenging to study segregation distorters through a conventional crossing scheme.

Here, we explore a novel approach to surveying the genome for segregation distortion by directly sequencing viable gametes from F_1 hybrid *M. m. domesticus*/*M. m. castaneus* males. Briefly, we enriched for viable sperm in hybrids and then sequenced these sperm in bulk, along with control tissues, to identify any skew in the representation of either parental chromosome in the viable sperm relative to the control. While we demonstrated via simulation that our experimental design has excellent power to detect segregation distorters, we found no evidence of segregation distortion in this cross, suggesting that segregation distorters are not a primary contributor to male infertility in *M. m. castaneus* and *M. m. domesticus* hybrids. Nonetheless, this approach can be applied to a wide range of species, and we therefore expect that it will be a useful means to study the frequency and impact of segregation distortion more generally.

Methods:

Reference Genome Assembly: To generate robust genome assemblies for each of the two strains of interest, we aligned all short read data for *M. m. castaneus* strain (CAST/EiJ) and

106 *M. m. domesticus* strain (WSB/EiJ) from a recent large-scale resequencing project (Keane et
107 al 2011) to the MM9 genome assembly using BWA mem v0.7.1 (Li and Durbin 2009) for the
108 initial mapping. For reads that failed to map with high confidence, we remapped using
109 stampy v1.0.17 (Lunter and Goodson 2011). We realigned reads that overlap indels, and
110 called SNPs and indels for each strain using the Genome Analysis Tool Kit (GATK, DePristo
111 et al 2011). For each program, we used default parameters, except that during variant
112 calling we used the option '--sample_ploidy 1,' because the strains are extremely inbred.
113 We called the consensus sequence for each strain at sites where both assemblies
114 have high quality data. That is, if both CAST and WSB assemblies had a q30 minimum
115 quality genotype (either indels or SNPs) that site was added to both consensus sequences.
116 Otherwise, if either or both assemblies were below this quality threshold at a given site, we
117 recorded for each consensus the MM9 reference allele.
118
119 Alignment Simulation: Our goal was to align short read data to a single diploid reference
120 genome, comprised of assemblies from the two parental strains. The mapping quality,
121 which indicates the probability that a read is incorrectly mapped in the position indicated
122 by the aligner, should then provide a reliable means of distinguishing whether a read can
123 be confidently assigned to one of the parental genomes. To confirm the accuracy of this
124 approach and to identify suitable quality thresholds, we performed simulations using
125 SimSeq (https://github.com/jstjohn/SimSeq). We used the sequencing error profiles
126 derived from our mapped data (below) and found qualitatively similar error rates using the
127 default error profile included with the SimSeq software package (data not shown). For both
128 the CAST and WSB genomes, we simulated 10,000,000 pairs of 94-bp paired-end reads,

whose size distribution was set to match that of our libraries (below). We then mapped these reads back to the single reference genome containing both CAST and WSB consensus sequences. We scored reads as 'mapping correctly' if they mapped to within 10 bp of their expected location measured by their left-most coordinate and on the correct subspecies' chromosome. If the pair mapped, we required that the insert length be less than 500 bp, which is well within three standard deviations of the mean insert size of our data and should therefore encompass the vast majority of read pairs. If both reads in a pair mapped and met our criteria above, we used the higher mapping quality of the two, and discarded the other read. This filter is important, here and below, as it avoids counting pairs as though their provenance is independent of their pair.

Experimental Crosses and Swim-Up Assay: To create first-generation (F_1) hybrids of *Mus* subspecies, we crossed 2 *M. m. castaneus* males to 3 *M. m. domesticus* females and 2 *M. m. domesticus* males to 5 *M. m. castaneus* females in a harem-mating scheme. In total, we produced 8 male F_1s in each direction of the cross. F_1 males whose sire was *M. m. castaneus* (CAST genome) are referred to as CW, and those whose sire was *M. m. domesticus* (WSB genome) as WC. All males were housed individually for a minimum of two weeks prior to sacrifice between 90 and 120 days of age.

To enrich for viable sperm from each F_1 male, we performed a standard swim up assay (Holt et al. 2010). First, immediately following sacrifice, we collected and flash-froze liver and tail control tissues (liver samples, $N = 16$; tail samples $N = 8$). We then removed and lacerated the epididymides of each male, placed this tissue in 1.5 ml of human tubal fluid (Embryomax® HTF, Millipore), and maintained the sample at a constant 37 °C for 10

minutes. Next, we isolated the supernatant, containing sperm that swam out of the epididymides, and spun this sample for 10 minutes at 250 g. We then discarded the supernatant, repeated the wash, and this time allowed sperm to swim up into the solution for an hour to select the most robust cells. Finally, we removed the solution, transferred them to new vial, pelleted these sperm by centrifugation, and froze them at -80 °C.

Library Preparation and Sequencing: For each F_1 hybrid male, we first extracted DNA from sperm, liver, and tail tissues identically using a protocol designed to overcome the difficulty of lysing the tightly packed DNA within sperm nuclei (Qiagen *Purification of total DNA from animal sperm using the DNeasy Blood & Tissue Kit; protocol 2*). We sheared this DNA by sonication to a target insert size of 300 bp using a Covaris S220, then performed blunt-end repair, adenylation, and adapter ligation following the manufacturer protocol (New England BioLabs). Following ligation, libraries were pooled into two groups of 16 and one group of 8 based on the adapter barcodes. Prior to PCR, each pool was subject to automated size selection for 450-500 bp to account for the addition of 175 bp adapter sequences, using a Pippen Prep (Sage Science) on a 2.0% agarose gel cassette. PCR was performed using six amplification cycles, and then we re-ran the size selection protocol to eliminate adapter dimer prior to sequencing. Finally, we pooled the three libraries and sequenced them on two lanes of a HiSeq 2500. Each sequencing run consisted of 100 bp paired-end reads, of which the first 6 bp are the adapter barcode sequence, and the remaining 94 bp are derived from randomly-sheared gDNA.

Alignment and Read Counting: We aligned read data to the combined reference genome using 'BWA mem' as described above in the alignment simulation. We removed potential

PCR duplicates using Picard v1.73. We then filtered reads based on the alignment filtering criteria described above for the simulated data. Because copy number variations may pose problems for our analysis, we attempted to identify and exclude these regions. Specifically, we broke the genome into non-overlapping 10 kb windows. Then, within each library, we searched for 10 kb regions that had a sequencing depth greater than two standard deviations above the mean for that library. All aberrantly high-depth windows identified were excluded in downstream analyses in all libraries. These regions, representing approximately 7% of the windows in the genome, are reported in Supplemental Table S1.

Next, to identify regions showing evidence of segregation distortion, we conducted windowed analyses with 1 Mb between the centers of adjacent windows. We counted reads in each window as a decreasing function of their distance from the center of the window, and included no reads at distances greater than 20 cM, thereby placing the most weight in a window on the center of the window. We then analyzed each window in two mixed-effects generalized linear models. Both models included random effects for the libraries and individuals. The first model includes no additional factors. The second had fixed effects for tissue, direction of cross, and an interaction term based on tissue by direction of cross effects, and thus has five fewer degrees of freedom than the first model. Hence, for each window, we assessed the fit of the second model relative to the first using a likelihood ratio test, wherein the log likelihood ratio should be chi-square distributed with 5 degrees of freedom. Afterwards, we applied a false-discovery rate multiple testing correction to the data (Benjamini and Hochberg 1995). We performed all statistical analyses in R (R Development Core Team 2011).

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 We determined recombination probabilities using the genetic map reported in Cox *et al.*
207 (2009). We performed the simulation for both parental genomes, and then again for each
208 parental genome but with the distortion limited to one direction of the cross (*e.g.* only
209 sperm from CW males experienced distortion). A direction-specific effect could occur if, for
210 example, suppressing alleles are present on the Y chromosome of one subspecies and
211 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 (Figure 1), which is not significant when we corrected for multiple tests.

By contrast, on chromosome 16, we identified 15 contiguous windows with significantly skewed allele frequencies following correction for multiple comparisons (minimum p = 5.026E-4; Figure 2). However, upon closer examination, it appears that this signal is driven almost entirely by a single liver sample, that of individual CW10. If this sample is removed from the dataset, this chromosome no longer shows significant deviation from expectations. When comparing the relative read depths across chromosomes 16 and 1, CW10's liver sample also appears to have disproportionately lower depth on this chromosome relative to CW10's sperm sample (p = 3.02E-5; X^2-test). These results suggest that this pattern is likely driven by a somatic aneuploidy event in CW10's liver that occurred relatively early in liver development and are not the result of distortion in the sperm library.

One concern for the interpretation of our results is whether we have sufficient statistical power, given our experimental design, to detect segregation distortion if it is indeed occurring in hybrid males. We addressed this issue through simulation. First, for the purpose of assessing power, we selected an *ad hoc* significance level of α = 0.001. Given that this cutoff is substantially lower than we observed in most genomic windows, it is likely a conservative measure for assessing power. Based on our simulations, we found that we have 50% power to detect segregation distortion to approximately 0.015 (this number reflects the positive or negative deviation from the null expectation, 0.5) if distortion affects CW and WC males equally. In other words, we have 50% power to detect distortion that is greater than 51.5% or less than 48.5%. If there is directionality to the distortion effect (*i.e.* only CW or only WC males experience SD), we have 50% power to detect distortion of 0.017 for CW males and 0.019 for WC males. This slight difference in power

244 based on cross direction likely reflects differences in sequencing depth between WC and
245 CW sperm and liver samples. It is also important to note that because read mapping and
246 sequencing, as well as divergence between the CAST and WSB strains and their divergence
247 from the reference genome, are non-uniform across the genome, different regions of the
248 genome will differ slightly in power to detect distortion.
249
250 **Discussion**
251 Elucidating the genetic mechanisms underlying species formation is a central goal of
252 evolutionary biology. Although there has been progress in identifying genes that contribute
253 to reproductive isolation with a few elegant examples (*e.g.* Bradshaw and Schemske 2003,
254 Lassance *et al.* 2010, Mihola *et al.* 2009), several from *Drosophila* species (*e.g.* Ting *et al.*
255 1998, Masly *et al.* 2006, Bayes and Malik 2009), it is unclear how generalizable these
256 results are. For example, segregation distorters contribute to reproductive isolation in
257 some young Drosophila species pairs (Phadnis and Orr 2010; Tao et al. 2007ab) but here,
258 to our surprise, we find no evidence for segregation distortion between two nascent
259 species *M. m. castaneus*/*M. m. domesticus,* despite strong experimental power.
260 This conclusion however must be qualified to some degree. Segregation distorters
261 are generally classified as either gamete disablers or gamete killers depending on their
262 mode of action (reviewed in Lyttle 1991, Taylor and Ingvarsson 2003). We expect that
263 gamete killers would be detected by our approach since their competitors may not be
264 present in the epididymides. If present, these sperm would not be captured in our stringent
265 swim up assay. Our ability to detect gamete- disablers, however, depends on the specific
266 mechanism by which these genetic elements disable their competitors. If the motility or

longevity of a sperm cell is sufficiently impaired, it is likely that this sperm would fail to swim into solution, but if the distortion effect has a very subtle effect on motility or impairs function later in the sperm life cycle (*e.g.* by causing a premature acrosome reaction), it is unlikely that our method could detect these effects. Thus, although gamete killers are not prevalent sources of DMIs in these subspecies, we cannot completely exclude the possibility that gamete disablers are important in *M. musculus* species formation. However, it is worth nothing that disablers cannot explain the reported observation of increased apoptosis of sperm cells in hybrid males (White *et al.* 2012).

Conventional methods of detecting segregation distortion (*i.e.* genotyping progeny) are usually statistically underpowered and thus unable to detect even modest distortion effects. Moreover, requiring the presence of viable progeny unavoidably conflates viability, gamete competition, and segregation distortion effects. By contrast, sequencing high quality gametes from individual males and comparing allele ratios in these gametes to those of somatic tissues, we have excellent power to detect fairly modest segregation distorters. For example, we could detect an aneuploidy event that resulted in a 4% difference in the allele frequencies of a single individual relative to expectations. Nonetheless, we found little evidence that segregation distorters are active in F_1 hybrid males, which indicates that segregation distortion (*i.e.* gamete killing) is not a primary contributor to reduced F_1 male fertility in these subspecies.

Because our method of determining the allele ratios in bulk preparations of viable gametes relative to somatic tissues is very general, we expect that it will be useful in a wide variety of systems for a diversity of questions. Provided one can accurately phase the diploid genome of an individual, by *e.g.* using complete parental genotype data when

inbred strains are not available, it is straightforward to apply this method to assay segregation distortion in a wide variety of taxa (including humans) and thus more easily survey the prevalence of segregation distortion as an isolating barrier both within and between species. This approach allows segregation distortion to be weighed against other possible sources of DMIs that may occur during spermatogenesis, oogenesis, fertilization, or embryogenesis, but that leaves an identical signature to SD in conventional cross-based experiments (*e.g.* White *et al*. 2012). Furthermore, extensions of our method may help to increase the generality of this approach. For example, if suitable fluorescent probes specific to cell states of interest are available, it would be straightforward to divide these cell populations using fluorescence-assisted sorting techniques, and determine the differences in allele frequencies between states. Importantly, this need not be limited to gamete cells, thus our method may have applications to a variety of other fields (*e.g.* cancer biology).

While segregation distorters appear to be an important mechanism of speciation in *Drosophila* and crop plants, efforts to detect SD in other diverging lineages—especially those with high statistical power—have been limited. We find that at least in *M. m. castaneus*/*M. m. domesticus* hybrids, segregation distorters are not measurable contributors to observed infertility in F1 hybrid males, despite strong statistical to detect them, suggesting that reduced hybrid male fertility in these nascent species is attributable to other underlying genetic causes. Further studies, using the novel approach developed here, will provide a powerful way to gain more comprehensive understanding of the role of SD in the evolution of reproductive isolation between diverging lineages.

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Author Contributions

RCD, EJP, DH, and HEH conceived and designed experiments. RCD, EJP, and HEH wrote the manuscript. RCD and EJP performed experiments. RCD analyzed the data.

Figure and Table Legends

Figure 1. Average proportion CAST reads in sperm libraries versus liver libraries. Using all males (A), using only CW males (B), and using only WC males (C). Lines indicate the approximate threshold at which we would have 50% power to detect distortion at the alpha = 0.001 level (see Methods for how this threshold was calculated).

Figure 2. Proportion of informative reads that are derived from the CAST genome across chromosome 16. CW4's liver sample is shown in red, and CW4's sperm sample is shown in green. All other CW libraries are represented in black for liver and in blue for sperm.

Figure 3. Probability of detecting segregation distortion loci based on simulations wherein distortion has no polarity (A), is in CW males only (B), or is in WC males only (C). For

visualization, all simulations are normalized to a 50:50 null expectations to account for differences in the idiosyncratic mapping properties of regions of the genome that may not conform to 50:50 expectations (see Figure 1).

Supplemental Figure S1. Cartoon of experimental cross scheme. Inbred parental strains are crossed, and individual F1 males sacrificed at 4 months, when their sperm are subjected to a swim up assay. Libraries were prepared from liver, tail and sperm samples, sequenced, and then aligned to a reference genome and subspecies of origin is determined.

Supplemental Text S1. Supplemental methods describing quality control steps to ensure samples are not contaminated or mislabeled.

Supplemental Table S1. List of genomic windows excluded from all downstream analyses due to detection of individual libraries with unusually high depth.

Supplemental Table S2. Quality control results for the quantity of reads in each library derived from the *Y* chromosome, *X* chromosome, and mtDNA.

Supplemental Table S3. Alignment simulation results showing the relationship between the reported mapping quality for a read and its probability of correct assignment to the genomic location from which it was derived.

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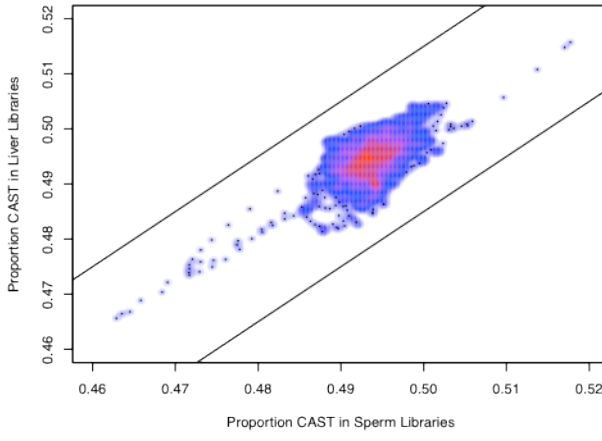
Proportion CAST in Liver Libraries
0.46
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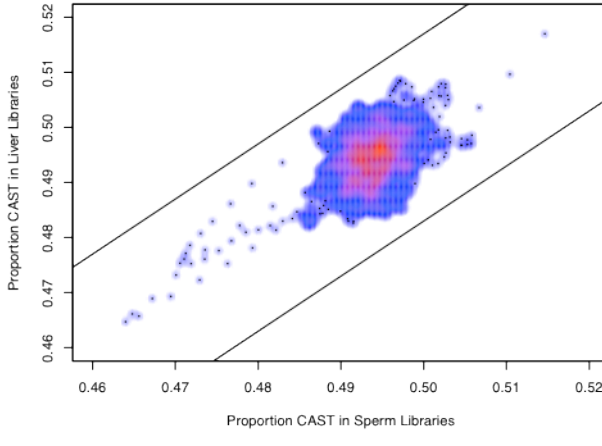
0.50
0.51
0.52
Proportion CAST in Sperm Libraries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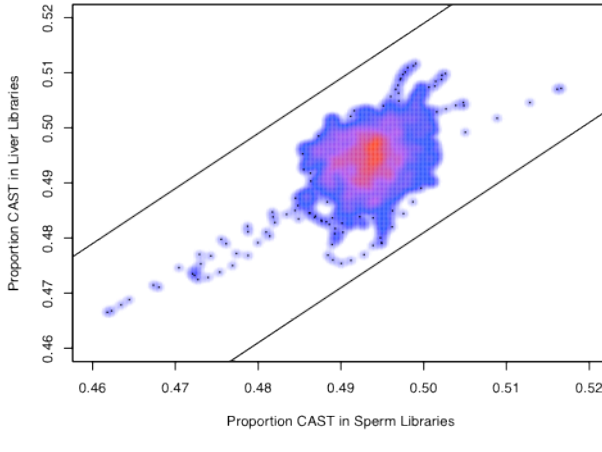
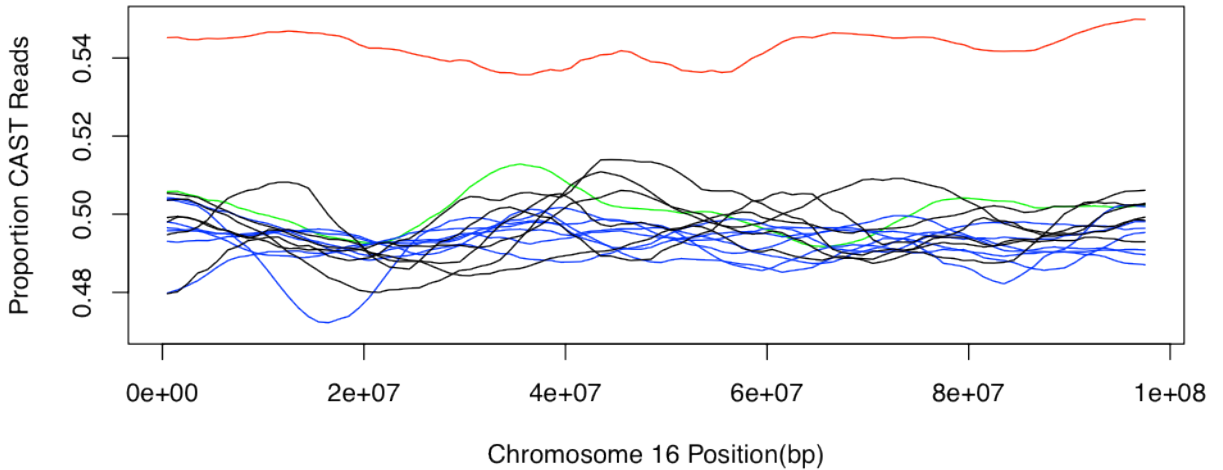
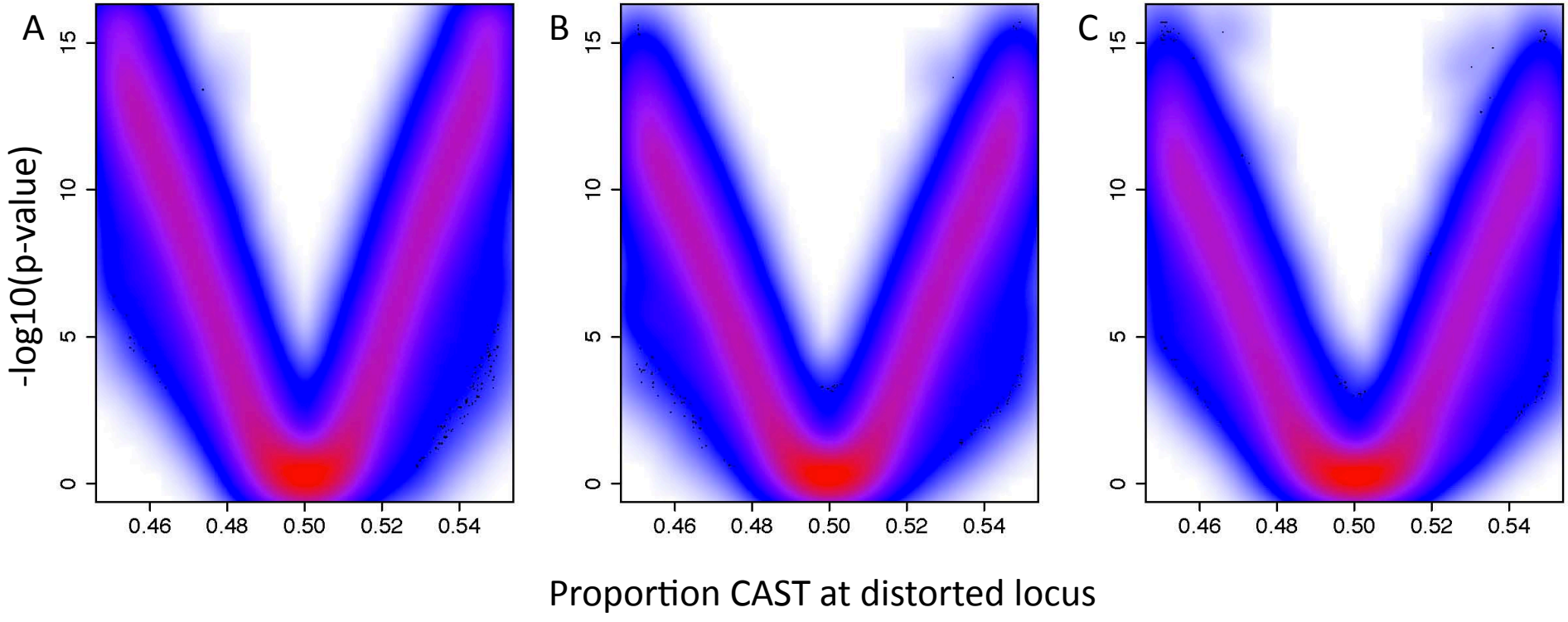
0.52
Proportion CAST in Sperm Libraries


Proportion CAST in Liver Libraries
0.46
0.47
0.48
0.49
0.50
0.51
0.52
Proportion CAST in Sperm Libraries


Proportion CAST Reads
0.48
0.50
0.52
0.54
0e+00
2e+07
4e+07
6e+07
8e+07
1e+08
Chromosome 16 Position(bp)


A
B
C
-log10(p-value)
0
5
10
15
0.46
0.48
0.50
0.52
0.54
Proportion CAST at distorted locus