Genetic Architecture of Hybrid Male Sterility in Drosophila: Analysis of Intraspecies Variation for Interspecies Isolation

Laura K. Reed¹,², Brooke A. LaFlamme¹,³, Therese A. Markow¹,⁴

¹ Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona, United States of America, ² Department of Genetics, North Carolina State University, Raleigh, North Carolina, United States of America, ³ Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York, United States of America, ⁴ Division of Biological Sciences, University of California San Diego, La Jolla, California, United States of America

Abstract

Background: The genetic basis of postzygotic isolation is a central puzzle in evolutionary biology. Evolutionary forces causing hybrid sterility or inviability act on the responsible genes while they still are polymorphic, thus we have to study these traits as they arise, before isolation is complete.

Methodology/Principal Findings: Isofemale strains of D. mojavensis vary significantly in their production of sterile F₁ sons when females are crossed to D. arizonae males. We took advantage of the intraspecific polymorphism, in a novel design, to perform quantitative trait locus (QTL) mapping analyses directly on F₁ hybrid male sterility itself. We found that the genetic architecture of the polymorphism for hybrid male sterility (HMS) in the F₁ is complex, involving multiple QTL, epistasis, and cytoplasmic effects.

Conclusions/Significance: The role of extensive intraspecific polymorphism, multiple QTL, and epistatic interactions in HMS in this young species pair shows that HMS is arising as a complex trait in this system. Directional selection alone would be unlikely to maintain polymorphism at multiple loci, thus we hypothesize that directional selection is unlikely to be the only evolutionary force influencing postzygotic isolation.

Introduction

During the modern synthesis, Dobzhansky and Mayr proposed the Biological Species Concept that defines species by their ability to exchange genetic material within their group while being prevented from exchanging genetic material between groups [1,2]. Reproductive isolating mechanisms subsequently have been the focus of extensive research such that a great deal now is understood about mechanisms of both pre and postzygotic isolation. Capturing the process of speciation early enough to determine the initial genetic causes of reproductive isolation, however, is challenging. Consequently a gap still remains in our understanding of the mechanisms underlying the very first stages of postzygotic isolation.

Postzygotic isolation exists when a hybrid, or its progeny, experience a reduction in fitness relative to the parental types. Whether the genetic variation needed for postzygotic isolation is segregating within species in the form of epistatic variation [3,4] or instead arises as de novo mutations in allopatric populations [5] remains controversial. Thus, there is need for further empirical study to determine the roles of segregating and de novo polymorphism in speciation, and the underlying architecture of such variation [3].

Three general patterns of postzygotic isolation are well supported. First, Haldane’s rule [6] states that decreases in hybrid fitness are more common in the heterogametic sex; second, hybrid sterility often appears earlier in species divergence than hybrid inviability [7,8]; and third, heterogametic isolation generally appears earlier in speciation than homogametic isolation [8,9]. Taken together, these patterns suggest that the earliest manifestation of postzygotic isolation likely will be hybrid sterility in the heterogametic sex. These patterns hold across diverse taxa in which the male is the heterogametic sex, such as mammals and Lepidoptera [10]. Various theories exist about the basis for Haldane’s rule [11–13]. The explanations with the most consistent empirical support revolve around the X (Z)-chromosome [14], leading to the expectation that the X-chromosome plays a substantial genetic role in the occurrence of postzygotic isolation in any given system.
The problem of how a trait that decreases the fitness of hybrids could evolve between two populations sharing a common ancestral genome without either population passing through a state of reduced fitness is solved by the Dobzhansky-Müller (DM) model of reproductive isolation [1,15–17]. For example, if derived alleles of two loci arise independently in two populations then, upon meeting in a hybrid, they may be incompatible, leading to decreased fitness [18]. Several known interacting genomic regions have been implicated in hybrid incompatibilities [19–23]. In addition, the incompatible alleles of the Îµ and Hmr genes in the Drosophila melanogaster – D. simulans pair have been demonstrated functionally to cause hybrid male lethality [24].

Expanding the Dobzhansky-Müller model, Orr [18] proposed that the number of two locus incompatibilities between two populations will increase as a function of the square of their divergence times. If more complex genetic scenarios are considered, the accumulation of incompatibilities is even faster. Those genetic incompatibilities that initially defined the two species therefore, can rapidly become obscured by subsequent genetic differentiation. Efforts to elucidate the origin of isolation can be facilitated by identifying the architecture of postzygotic isolating mechanisms in incipient species, where the number of postzygotic incompatibilities are still few and, ideally, not yet fixed within the populations.

A major lingering question in speciation genetics concerns the actual genetic architecture of postzygotic isolation as it first emerges. Does it have a simple or a complex genetic basis at the earliest stages of the speciation process? A burdensome disadvantage to genetic studies of postzygotic isolation is that the phenotype of interest, sterility or inviability, precludes direct genetic crossing schemes. Many successful studies of species pairs, in which the factors underlying isolation are effectively fixed, utilized the common occurrence of asymmetry [25] and/or Haldane’s Rule. While the genetic dissections of incompatibility phenotypes by backcrosses and introgression [10,24,26,27] have contributed significantly to our current understanding of the genetics of postzygotic isolation, it is not without limitations. Since the crossing schemes employed potentially disrupted the very co-adapted gene complexes that might be critical to the F1 phenotype we cannot be certain that the genetic architecture for the incompatibility observed in the F1 hybrid is the same as in the F1 phenotype contributing to postzygotic isolation has been demonstrated in Tribolium by joint-scaling analysis [28,29] showing that co-adapted gene complexes may indeed be important in the architecture of hybrid incompatibilities.

In the present study, we circumvented the limitations of the hybrid phenotype by exploiting within-species polymorphism for between-species postzygotic isolation to determine the genetic architecture of segregating variation for F1, hybrid male sterility directly. We used Drosophila mojavensis and D. arizonae, a recently diverged species pair (0.66–1.2 my [30,31]) and a model system for the study of speciation [32]. Crosses between D. mojavensis females and D. arizonae males exhibit widespread between-population [33,34] and within-population variation for the HMS phenotype suggesting polymorphism at multiple loci within D. mojavensis [33]. The polymorphism for HMS shows that this trait is not yet fixed in D. mojavensis. Since intraspecific evolution is the process by which gene frequencies change, only a trait exhibiting genetic variation is capable of evolving. Thus HMS in this system is still capable of experiencing the forces of evolution (selection and/or drift) and can give insight into how HMS evolves. In a novel design, we have utilized the within-population variation for production of F1 HMS to determine the genomic regions and epistatic interactions that shape the architecture of postzygotic isolation within the F1 hybrid males. Below we address the following questions: What is the nature and number of polymorphic loci within D. mojavensis contributing to the HMS phenotype? And what can that tell us, in conjunction with other information about this species pair, about the evolution of HMS?

### Materials and Methods

#### Lines used for mapping

Genetic lines were derived from lines used in the HMS polymorphism study by Reed and Markow [33]. Isofemale D. mojavensis lines were collected from Santa Catalina Island (CI) in April 2001. The Low (CI-10) line exhibiting low levels of HMS was inbred through full-sib mating for six generations and the High (CI-12) line exhibiting high levels of HMS was inbred for eight generations. The lines had minimum inbreeding coefficients of 0.734 and 0.826 respectively [35]. They showed significant differences from each other for HMS (presence of motile sperm) observed when females from those lines were crossed to D. arizonae males from an inbred line (minimum inbreeding coefficient of 0.859) collected at Peralta Canyon Trail Head, east of Phoenix, Arizona (AZ), in April 1997. All lines show normal male fertility when the males are genetically pure D. mojavensis or D. arizonae [33]. The sterility phenotype is only manifested in the species hybrid state. Homozygosity of the High line, used in the D. mojavensis genome sequencing project, had been confirmed by sequencing five marker loci in 10 females (L. Matzkin, unpublished) and an additional seven loci in 10 individuals by Agencourt Biosciences, and none showed sequence variation. The two D. mojavensis QTL lines were homokaryotypic, lacking inversion polymorphism. The lines used showed extreme contrasting HMS (sperm motility) phenotypes in heterospecific crosses (Table 1) as well as strong viability.

#### Identifying Markers and Genotyping

We designed fluorescently-tagged PCR primers for flanking sequences of microsatellite loci identified by Ross et al. [36]. Additional candidate microsatellite sequences were drawn from Staten et al. [37]. Potential loci were genotyped in a panel of four individuals from each D. mojavensis line to confirm that the lines contained different alleles for the locus. We genotyped 132 potential loci but only found 25 that distinguished the High and Low HMS genotypes. Six loci were identified that had a consistent high degree of polymorphism among the four lines. These loci were then genotyped in a panel of 132 individuals from each of the four lines.

#### Table 1. Hybrid Sperm Motility in sons of mothers from the High and Low D. mojavensis lines and in the intercross populations of the High and Low lines, when females crossed to D. arizonae fathers.

| Cross | No Motility | Motility$^2$ | Standard Error |
|-------|-------------|--------------|----------------|
| Low   | 0.54 (35)   | 0.46 (30)    | 0.062          |
| F1    | 0.07 (11)   | 0.93 (154)   | 0.019          |
| F2    | 0.15 (62)   | 0.85 (353)   | 0.017          |
| RF1   | 0.07 (13)   | 0.93 (177)   | 0.018          |
| RF2   | 0.13 (75)   | 0.87 (483)   | 0.014          |
| High  | 0.09 (13)   | 0.91 (131)   | 0.024          |

$^1$Cross is Genotype of the D. mojavensis mother.

$^2$Motility is defined as a male having one or more motile sperm.

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Low lines (primer sequences not already described in Staten et al. [37] shown in Table S1). Polymorphic marker discovery was inhibited by the general deficit of neutral microsatellite variation in the wild population from which the mapping lines were derived [38]. We had markers on the X-chromosome and the four major autosomes but failed to find a marker on the 6th dot chromosome. Markers were assigned to chromosome using one or more of the following methods: 1) BLASTing [39] the flanking sequence against the D. melanogaster genome sequence to identify which Muller element and thereby to what chromosome in D. mojavensis it belonged; 2) linkage mapping to allozyme markers assigned to chromosome by Zouros [40,41]; 3) looking for close linkage between assigned markers and unassigned makers. Informative loci were multiplexed and PCR amplified using HotStarTaq DNA Polymerase (Qiagen Inc.). Genotyping was performed by the Genomic Analysis and Technology Core (GATC) at the University of Arizona using an ABI 3730 DNA Analyzer. Genotypes were read using Genotyper ver. 1.1 from Applied Biosystems.

Crossing Design

The crossing design used was an innovative modification of a standard F2 line cross (Figure 1). Five virgin F2 females, at six days post-eclosion, were paired with five sexually mature virgin male D. arizonae (AZ) for 48 hours. After 48 hours, the females were placed individually into vials, allowed to oviposit for four days, then removed and frozen for later genotyping. Virgin hybrid males were collected from those vials producing viable adults. Hybrid males were allowed to reach sexual maturity at 9–12 days post-eclosion before phenotyping. Use of the F2 mothers’ genotypes allowed for a largest possible sample of recombinant genotypes (given limited resources for genotyping) while the use of the sons’ phenotypes allowed for a good estimate of their mother’s average phenotype, maximizing the quality and quantity of the data. A reciprocal design was also performed; instead of using Low females crossed to High males, a large number of attempted matings between F2 and RF2 females and D. arizonae males failed to produce offspring as was expected considering the significant prezygotic isolation between the species. Seven percent of the F2 (224 of 3214) and the 12% of the RF2 (278 of 2339) mothers produced larvae after being mated to D. arizonae males.

Mating and/or oviposition took place on standard opuntia-banana food (http://flyfood.arl.arizona.edu/opuntia.php3?g2=2) while virgins were maintained on standard cornmeal food (http://flyfood.arl.arizona.edu/cornmeal.php3). Cultures were maintained at 24+/−1 degree Celsius and under a strict 12h:12h light:dark cycle.

Phenotyping

Male Drosophila store mature sperm in a seminal vesicle, a structure at the base of each testis. To assess sperm motility, males were anesthetized with ether just prior to dissection. Testes and seminal vesicles were dissected from the male in sperm buffer (0.05 M Tris, 1.1% NaCl, 0.1% Glucose, 0.01% L-arginine, 0.01% L-lysine, pH 8.7). Reproductive organs were transferred to a fresh slide with an 11 microliter droplet of sperm buffer, stretched with forceps to be linear, and then placed under a coverslip. Pressing the coverslip down on the sample forced all sperm out of the seminal vesicle, allowing motility of the sperm to be observed under dark-field microscopy. The entire area where potentially motile sperm could be found was visually scanned and each seminal vesicle assigned an integer score from 0 to 6 on a sperm motility scale.

Unlike mammalian systems that have short sperm, Drosophila sperm are extremely long, forming a tangled wiggling knot, in which the movement of any individual sperm cannot be tracked. The zero-to-six scale is a reasonable means to approximate the amount of motility exhibited by Drosophila sperm, and is a refinement of the presence/absence of motility scheme used in many previous studies of sperm motility in Drosophila [33,42,43]. A quantitative measurement of sperm motility more suitable for quantitative genetic analysis results from the zero-to-six scale. A score of “0” meant there was no motility observed, “1” was one or two motile sperm, “2” being several motile sperm, “3” being several motile sperm in two or more areas of the field, “4” is three to four areas of moderate motility, “5” being several large areas of high motility, and “6” being wild-type where most of the field showed high levels of sperm motility (as is observed in non-hybrid males of these species). A male’s phenotype was the average score of his two seminal vesicles. We phenotyped up to ten sons for each mother (mean family size of 3.4) and found no trends of decreasing motility scores with time (first verses second testis scored). The genotype of the male being scored was not known during phenotyping. A subset of samples were also scored and rescored in a randomized order while blind to sample identity. Replicate scores were highly correlated (R^2 = 0.79, p<0.0001), validating this method for estimating Drosophila sperm motility suggesting its utility for future studies. Sperm motility is one phenotypic component of sterility. We have shown in previous work that motile sperm are required though not always sufficient for males to be able to reproduce in this system due to additional genetic polymorphism for other phenotypic components of sterility [33]. Studies of other phenotypic components of sterility would be interesting though not required to assess the importance of natural genetic variation for HMS as estimated by sperm motility in this system.

Linkage Mapping

F2 or RF2 females that successfully produced hybrid male offspring were genotyped for the 25 microsatellite markers described above. Maximum likelihood marker (Figure S1) order was calculated for each chromosome and the Haldane mapping...
function was used to assign linkage distances between markers in centiMorgans (cM) using Mapmaker 3.0 [44].

Autosomal genotypes were pooled from the F2 and RF2 crosses for linkage map calculation while they are separated for the X-chromosome due to differences in X-chromosome inheritance patterns in the two crosses. Marker density was an average of 27.6 cM.

**QTL Mapping**

QTL mapping was conducted using Windows QTL Cartographer 2.5 [45,46]. We conducted composite interval mapping [47,48] on the F2, RF2, and combined data sets using a common linkage maps for the autosomes and their respective linkage maps for the X-chromosome. Composite interval mapping (CIM) uses cofactors from other regions to control for genome-wide influences when regression analysis at any given genomic location is being tested [47,48]. For our study a multiple regression analysis at each 2 cM segment of each chromosome was performed using five randomly selected markers from elsewhere in the genome as cofactors to control for genetic effects in other genomic regions (Model 6 in QTL Cartographer). A 10 cM region around the test location was excluded for selection of the five control markers. A likelihood ratio (LR) test was performed for each position comparing the null hypothesis that no QTL is present at that position to the alternative that there is a QTL present. To control for any violations of normality, multiple testing, or the possible disruption of the asymptotic distribution of the chi-squared test statistic by idiosyncrasies of the study (due to factors such as multiple QTL per chromosome or distorted segregation ratios), QTL peak significance was assessed by 1000 permutations [49].

Two datasets were used in mapping. In the one called sons, sons of each mother were analyzed with their mother’s genotype and their individual motility phenotype to account for the within mother variation in her sons’ motility scores. Analyses were also conducted on the second dataset using the mother’s Best Linear Unbiased Prediction (BLUP) for her sons’ motility scores. Sperm motility in the parental, F1, RF1, F2, and RF2 lines were presented in Table 1. High and Low lines showed significant differences in the proportion of inter-species hybrid sons with motile sperm (91% and 46% respectively), and motility clearly was dominant in all hybrids (Table 1). Sperm motility scores were normally distributed (Shapiro-Wilks W = 0.99, p = 0.38), with a mean of 2.35+/−0.08.

Female F2s (149 F2 and 155 RF2), of the species *D. mojavensis*, were crossed to male *D. arizonae* for QTL analysis. Sperm motility was scored, from 0 to 6, in the interspecific hybrid males. 304 F2 mothers were genotyped at 25 microsatellite markers across the genome and used to generate the linkage map. Phenotypes were mapped relative to genotype in two ways. One was to use each son’s motility score, hereafter referred to as sons, as a sample of the mother’s phenotype and the other was to map a mother’s Best Linear Unbiased Prediction (BLUP) for her sons’ motility scores. Sons better captures non-additive effects, while BLUP highlights the additive effects in the architecture.

**Epistatic Interactions**

We assessed epistatic interactions using methods modified from Moehring and Mackay [52] and Morgan and Mackay [53]. We tested for interaction effects between all pairs of markers using a repeated measures model of the form:

\[
Y = \mu + F + C + M_1 + M_2 + M_1^*M_2 + \epsilon
\]

where F is the effect of mother, C is the effect of the direction of the cross (F2 vs. RF2), M1 is the effect of genotype at marker 1 and M2 is the effect of genotype at marker 2. All 300 possible two-way interactions were tested and significance of the interaction term (M1*M2) was assessed by Bonferroni correction and False Discovery Rate (FDR). All calculations were implemented using the SAS Mixed procedure (SAS 9.1.3, SAS Institute Inc., Cary, NC 2000-2004). The BLUP data is well suited to determine ‘breeding quality’ for hybrid male sperm motility calculated in mean and variance in offspring scores, as a summary of her additive genetic variation among sons. Maternal effects were identified as a significant cross by main effect interaction. Hybrid male phenotype (n = 304) was regressed against the interaction between marker genotypes as the four main effect loci and the direction of the cross (F2 vs. RF2) in the multiple regression model of the form:

\[
Y = \mu + C + M_1 + M_1^*C + \epsilon
\]

Where C in the direction of the cross and M1 is the genotype at the marker associated with one of the main effect QTL.

**Results**

Intbred *D. mojavensis* lines showing High and Low phenotypic levels of HMS when crossed to *D. arizonae* were used in a standard F2 (and reciprocal) intercross QTL mapping design (Figure 1). Sperm motility in the parental, F1, RF1, F2, and RF2 lines are presented in Table 1. High and Low lines showed significant differences in the proportion of inter-species hybrid sons with motile sperm (91% and 46% respectively), and motility clearly was dominant in all hybrids (Table 1). Sperm motility scores were normally distributed (Shapiro-Wilks W = 0.99, p = 0.38), with a mean of 2.35+/−0.08.

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**Main effects**

There were two striking outcomes of the main effects analysis; first that the F2 and RF2 mapping populations did not match more closely, and second, no main effects were detected on the X-chromosome. Main effect QTL were detected, however, on the 2nd, 3rd, and 5th chromosomes (Table 2, Figure 2). Note that estimated effect sizes (percent of phenotypic variation explained) of all detected QTL are likely to be overestimates due to the Beavis effect [54,55], thus, there are likely to be additional genetic factors contributing to observed differences between lines that were not detected in this study. Also the actual genotype of the hybrid males at a given marker can only fall into two possible genotypic classes, High or Low for the *D. mojavensis* half of their genome: they will be monomorphic with respect to the *D. arizonae* half of their genome. Thus, the variance due to non-additive effects identified in the QTL mapping corresponds to intra-genome epistasis rather than intra-locus dominance. Finally, remember that these two lines are only a sample from a larger population. There may be additional polymorphic factors on the X-chromosome or autosomes segregating in the population as a whole that were not captured by this biparental cross. One QTL with estimated effect size of 10.9–22.1% occurred on the 3rd chromosome at about 60 cM and was observed in both datasets (sons and BLUP) and across all population samples (F2, RF2, and combined). The RF2 population sample did not reveal any other significant QTL.

QTL were also identified on the 2nd chromosome. Analysis of the sons dataset in the F2 and combined populations found two and one QTL on the 2nd chromosome respectively (Table 2, Figure 2). The combined population sons QTL at 58 cM has a large non-
additive effect of 2.23 motility units and estimated effect size is 55.3%. The two QTL found in the F2 population sons dataset (at 32 and 66 cM) also had large (and opposite) non-additive effects of –2.17 and 2.16 motility units, respectively. The RF2 sons dataset had an elevated likelihood ratio on the 2nd chromosome but it did not reach significance. The BLUP datasets exposed a significant QTL on 2nd chromosome for the combined population (60 cM) and the RF2 population (50 cM) with a non-additive effect of 0.92

Table 2. Maximum Likelihood Position and Effect Size for individual sons and BLUP QTL found in Composite Interval Mapping.

| Data Type | Cross Type | Chromosome | Position (cM) | LR² | Additive Effect | Non-Additive Effect | R² |
|-----------|------------|------------|---------------|-----|----------------|---------------------|----|
| IS        | F₂        | 2          | 32            | 23.68 | 0.24           | –2.165              | 0.488 |
| IS        | F₂        | 2          | 66            | 27.12 | –0.381         | 2.164               | 0.506 |
| IS        | F₂        | 3          | 61.2          | 41.64 | –0.902         | –0.137              | 0.157 |
| IS        | F₂        | 5          | 54.5          | 28.39 | –0.802         | –0.427              | 0.096 |
| IS        | RF₂       | 3          | 59.2          | 46.24 | –0.79          | 0.357               | 0.127 |
| IS        | Combined  | 2          | 58            | 56.82 | –0.392         | 2.225               | 0.553 |
| IS        | Combined  | 3          | 61.2          | 73.24 | –0.739         | 0.132               | 0.109 |
| BLUP      | F₂        | 2          | 69.2          | 18.98 | –0.344         | –0.046              | 0.122 |
| BLUP      | RF₂       | 2          | 50            | 12.07 | –0.255         | 1.101               | 0.593 |
| BLUP      | RF₂       | 3          | 59.2          | 23.64 | –0.476         | 0.294               | 0.221 |
| BLUP      | Combined  | 2          | 60            | 13.8  | –0.197         | 0.921               | 0.443 |
| BLUP      | Combined  | 3          | 65.2          | 37.95 | –0.406         | 0.138               | 0.153 |

1 Individual Son (IS) or the Best Linear Unbiased Prediction (BLUP) values.
2 cM is centiMorgans.
3 LR is likelihood ratio.

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Figure 2. Composite interval mapping results for intraspecific variation for interspecific hybrid male sterility. Likelihood ratio values for the F₂ (dashed red), RF₂ (dashed blue), and combined (solid black) data sets are given for the X through 5th chromosome. The X-chromosome scale is normalized to “combined” map length for the F₂ and RF₂ maps. The threshold for significance is the horizontal line (red- F₂, blue- RF₂, and black- combined). (a) Mapping results considering each son as measure of the mother’s phenotype. Black asterisks indicate the location of markers with significant pairwise epistatic effects and yellow asterisks indicate two additional markers showing a significant deviation from the additive expectation. There is clear evidence for a QTL of major effect on the 3rd chromosome for all data sets, on the 2nd chromosome for the F₂ and combined data sets, and on the 5th for the F₂ dataset. (b) Mapping results of the BLUP values for each mother. There is evidence for a QTL on major effect on the 3rd chromosome in all three datasets and on the 2nd chromosome in the combined dataset.

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to 1.10 motility units, with estimated effect size of 44.3 to 59.3%.

There are several possible explanations for the discrepancy between F2 and RF2 populations. First, we may have failed to detect QTL due to simple sampling variance between the F2 and RF2 populations. Second, the different compositions of the X-chromosomes in the reciprocal populations could lead to an X-by-autosome interaction. Finally, an interaction between the nature of the cross (e.g., cytoplasm) and the QTLs could account for (or underlie) the observed differences between reciprocal crosses.

We address each of these below.

Sampling Variance. To test whether the inevitable force of sampling variance was driving the differences observed between the F2 and RF2 populations, we subsampled from the datasets to test for consistency of the signal. From each population 100 datasets were created by randomly selecting 80% of the data points from the original dataset. Complete mapping analyses, including permutations for significance thresholds, were then performed on the subsampled datasets. Despite the reduced power of the smaller datasets, the QTLs found on the full dataset remained detectable in between 27% and 70% of the subsampled datasets (Figure S2), while spurious QTL were only detected in a maximum of 24% (e.g. see left portion of chromosome 5 in the F2 dataset). Subsampling indicates that QTL identified in the complete dataset remain detectable in a large portion of the subsets despite variation in sampling and spurious QTL (those not detected in the complete dataset but in the subsets) rarely arise due to sampling variance. The consistency between our subsampled datasets and our complete dataset suggests that our findings are fairly robust to variation in sampling and that the differences between the F2 and RF2 are likely biologically real, though some of the differences could still be due in part to sampling variance.

Inheritance of the X-chromosome: If an important X-linked genetic difference between the parental lines interacts with the autosomes, the cross direction will affect the frequency of that factor in the mapping population. This, in turn, would influence the detection and nature of autosomal QTL that interact with the X. The X-linked factor could primarily influence the penetrance or expression of the genetic variation at the autosomal factor and thus have a significant epistatic effect without having a main effect. To test for X-by-autosome interactions, as well as for other epistasis, we looked for significant pairwise interactions between all markers across the combined dataset (n = 304). We found seven interactions with a FDR significance of 0.05 or less, two of which were between the X-chromosome and the autosomes (Table 3, Figure 2). Epistatic interactions between the X-chromosome and the autosomes therefore could be contributing to some of the differences observed between the F2 and RF2 populations. We also

Table 3. Significant Marker-Marker Interactions

| Marker A | Marker B  | Chromosome A | Chromosome B | p-value  | FDR    |
|----------|-----------|--------------|--------------|----------|--------|
| dmoj030  | a3_11_1   | X            | 2            | 0.00112  | 0.0496 |
| m2_18_2  | m2_19_2   | X            | 4            | 0.00072  | 0.0496 |
| a3_11_1  | m2_19_2   | 2            | 4            | 0.0006   | 0.0496 |
| m2_17_15 | m2_19_2   | 3            | 4            | 0.00116  | 0.0496 |
| dmoj4050 | dmoj4060  | 4            | 4            | 0.00116  | 0.0496 |
| m2_19_2  | m4_9_1    | 4            | 5            | 0.00013  | 0.0393 |
| a3_12_6  | m4_9_1    | 5            | 5            | 0.0003   | 0.0453 |

Markers and their corresponding chromosones with interaction effects with a False Discovery Rate (FDR) of 0.05 or less. Interactions significant after a Bonferroni correction are in bold and interactions significant at an FDR of 0.05 are italicized.

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found 5 inter-autosomal epistatic interactions. Notice that markers on the 4th chromosome were involved in five of the seven interactions, thus the 4th chromosome appears to be playing an important epistatic role in the HMS in this system worthy of additional study. Only one of nine markers involved in these pairwise interactions (m4_9_1 on the 5th chromosome) resides in or near a main effect QTL. Two additional markers (a1_2_1 on chromosome 3 and n1_10_11 on chromosome 4) did not appear in the list of significant marker by marker interactions, but exhibited significant deviations from the additive expectation after a Bonferroni correction, indicating that there are at least two other unidentified epistatic interactions. Taken together, not only does the X-chromosome appear to play an epistatic role in shaping the genetic architecture of HMS in this system, but so does within-species epistasis throughout the rest of the genome.

Maternal effects: Cytoplasmic factors may also underlie interactions between the direction of the cross and the QTLs. We tested for marker by cross interactions for the four markers showing main effects and found a significant interaction effect of marker dmoj2210 on the 2nd chromosome with cross (p = 0.0142), after controlling for the main effect of the 3rd chromosome (Figure 3). This marker did not show evidence of interacting with the X-chromosome in the test for epistasis above. Chromosome 2 is where the large and opposing non-additive QTL were found in the test for epistasis above. Chromosome 2 is where the large and opposing non-additive QTL were found in the F2 population but not in the RF2 populations. The significant cross by marker interaction on the 2nd chromosome is a likely contributor to the differences seen in the mapping for the two crosses. The cross effect is not necessarily independent of the epistatic effect of the X-chromosome found above. But since dmoj2210 did not show any evidence of interacting with the X-chromosome, it is likely that the cytoplasm plays a role in HMS. Thus, both the X-chromosome and the cytoplasmic environment should be further explored in future studies of HMS.

Discussion

The first question we proposed to answer in this study was: what is the basic architecture of HMS in the early stages of its evolution? Is HMS caused by only a few factors of large effect or does it have a more complex basis, involving multiple loci and epistatic interactions? An earlier study [33] showed evidence of multiple polymorphic factors for HMS within D. mojavensis and additional data show genetic polymorphisms for HMS segregating in the sister species D. arizonae (Reed, unpublished). Our present QTL study reveals that HMS, even at the earliest stages of speciation, can be surprisingly complex. We found within-population variation not only for several QTL of main effect, but also for significant within-species epistatic interactions. Similar complexity has been seen in other systems, such as with the Lhs-Hmr gene pair, where the incompatibility requires an epistatic interaction with the hybrid genetic background to be expressed [59]. There is likely to be more segregating genetic variation in this species that was not captured by these two particular mapping lines, and fixed genetic factors that could not be detected by this design; potentially both of which further contribute to the complexity of this trait. While previous investigations on more diverged species pairs [10] have revealed a multi-factorial genetic basis for post-zygotic isolation, these studies were unable to determine whether the polygenicity had arisen early or was a byproduct of long divergence time. We show that HMS has a polygenic and epistatic basis even early in the evolutionary process, before the ability to produce HMS is fixed within a species. These findings are consistent with the complex genetic basis of early postzygotic isolation found in Tribolium [28,29].

In addition to the unexpected complexity of HMS in this incipient species pair, we were surprised to discover no main-effect QTL segregating on the X-chromosome, though we did find an epistatic effect. While we may have lacked the statistical power to detect X-linked main effects, main effects were detected on other chromosomes indicating that sample size alone is not the full explanation. There may still be fixed X-linked effects that could not be detected by this design, and fixed effects on the X are more likely due its smaller effective population size. Demuth and Wade did demonstrate the role of epistatic variation on the X-chromosome on Haldane’s Rule both theoretically [13] and empirically [28,29]. We also found such a role for the X-chromosome in our epistasis tests.

Finally, our data show a striking difference between reciprocal crosses, suggesting a potential role for maternal effects and genome-wide epistatic variation on HMS. Cytoplasmic and epistatic effects have been demonstrated in other systems as well [24,28,29,59]. Zouros and colleagues have mapped the genetic basis of HMS resulting from the reciprocal cross (female D. arizonae mated to male D. mojavensis) to the third, fourth and Y-chromosomes [40,41,60–62]. Whether or not the third chromosome effect in the cross using D. arizonae mothers is a function of the same factor or factors as revealed in our study remains unknown.

The second question we proposed to answer was: what can the genetic architecture of HMS tell us, in conjunction with other information, about its evolution? Substantial within-species natural polymorphism for between species postzygotic isolation has been characterized in many independent taxa thus far, including at least nine Drosophila species groups, [33,34,43,63–70], Tribolium beetles [71,72], Chorthippus parallelus grasshoppers [73], Mus musculus [74], Xiphophorus fish [75], Crepis hawksberg weed [76], Gossypium cotton [77] and Mimulus monkey flowers [78]. Thus, intraspecific polymorphism for postzygotic isolation appears to be a common phenomenon. Shuker et al. [73] argue that witnessing substantial within-species polymorphism for hybrid incompatibilities means that loci contributing to the hybrid phenotype are likely to be neutral or nearly-neutral in the parental species’ genetic background. Finding that a trait is polymorphic at multiple loci,
supports the role of drift and/or balancing selection in the genetics of the trait. If directional selection was occurring on the genes related to the trait, no matter what the within-species function of the genes, they would be polymorphic for only the briefest of times [79]. The probability of catching multiple polymorphic loci under directional selection would be minute. Considering that there is evidence of balancing selection on male reproductive proteins in several systems [80–83] it is not unreasonable to hypothesize that it might also be playing a role in HMS.

A handful of genes causing hybrid incompatibilities have been identified in other species and all but one (a gene transposition [84]) show evidence of being under positive selection (OddH in D. mauritiana, [85]; Xiph-2 in Xiphophorus, [86]; Naf96, Hov, Lhr in the D. melanogaster/D. simulans species pair [24,87,88]), leading to the impression that most postzygotic speciation genes experience directional selection. Given our findings with D. mojavensis and in other systems discussed above, however, we believe it is premature to discount the role of drift and balancing selection in speciation.

Empirically, we need to characterize more completely the genetic architecture of incompatibility phenotypes during early stages to find reproductive isolation genes before they are fixed and test those loci for evidence of selection. In the present study we have begun the empirical aspect of this process.

Supporting Information

Table S1 Primers for Microsatellites.

Found at: doi:10.1371/journal.pone.0003076.s001 (0.06 MB DOC)

Figure S1 Linkage map for D. mojavensis. Distance between markers given in centiMorgans.

References

1. Dobzhansky T (1937) Genetics and the origin of species. New York: Columbia University.
2. Mayr E (1942) Systematics and the Origin of Species. New York: Columbia University Press.
3. Goodnight CJ, Wade MJ (2000) The ongoing synthesis: A reply to Coyne, Barton, and Turelli. Evolution 54: 317–324.
4. Wade MJ, Goodnight CJ (1998) Perspective: The theories of Fisher and Wright in the context of metapopulations: When nature does many small experiments. Evolution 52: 1537–1553.
5. Coyne JA, Barton NH, Turelli M (2000) Is Wright's shifting balance process important in evolution? Evolution 54: 306–317.
6. Haldane JBS (1922) Sex ratio and sexual sterility in animal hybrids. Journal of Genetics 12: 101–109.
7. Wu CJ (1992) A note on Haldane's rule: hybrid inviability versus hybrid sterility. Evolution 46: 1504–1507.
8. Coyne JA, Orr HA (1997) “Patterns of speciation in Drosophila” revisited. Evolution 51: 295–303.
9. Coyne JA, Orr HA (1989) Patterns of Speciation in Drosophila. Evolution 43: 362–381.
10. Coyne J, Orr HA (2004) Speciation. Sunderland, MA: Sinauer Associates Inc.
11. Turelli M, Orr HA (1995) The dominance theory of Haldane’s rule. Genetics 140: 389–402.
12. Orr HA, Presgraves DC (2000) Speciation by postzygotic isolation: forces, genes and molecules. Bioessays 22: 1085–1094.
13. Demuth JP, Wade MJ (2005) On the theoretical and empirical framework for studying genetic interactions within and among species. American Naturalist 165: 524–536.
14. Masly JP, Presgraves DC (2007) High-Resolution Genome-Wide Dissection of the Two Ranks of Incompatibility in Drosophila. PLoS Biology 5: e243.
15. Muller HJ (1942) Isolating mechanisms, evolution and temperature. Biological Symposia 6: 71–123.
16. Muller HJ (1949) Boundary of the Drosophila work on systematics. In: Huxley J, ed. The New Systematics. Oxford: Clarendon, pp 185–260.
17. Bateson W (1909) Heredity and variation in modern lights. In: Seward AC, ed. The New Systematics. Oxford: Clarendon. pp 185–268.
18. Orr HA (1995) The Population-Genetics of Speciation - the Evolution of Hybrid Incompatibilities. Genetics 139: 1805–1813.
19. Carvajal AR, Gauduera MR, Naveira HF (1996) A three-locus system of interspecific incompatibility underlies male inviability in hybrids between Drosophila buzzatii and D-koppgeae. Genetics 98: 1–19.
20. Christie P, Macnair MR (1984) Complementary Lethal Factors in 2 North-American Populations of the Yellow Monkey Flower. Journal of Heredity 75: 510–511.
21. Swigart AL, Fishman L, Willis JH (2006) A simple genetic incompatibility causes hybrid male sterility in mimulus. Genetics 172: 2463–2479.
22. Hutchinson JB (1932) The genetics of cotton. Part VII. “Crumpled”: A new dominant in Asiatic cottons produced by complementary factors. Journal of Genetics 25: 281–291.
23. Kazianis S, Gutbrod H, Naim RS, McEntire BB, Della Coletta L, et al. (1998) Localization of a CDK2 gene in Linkage Group V of Xiphophorus fishes defines it as a candidate for the DIFF tumor suppressor. Genes Chromosomes & Cancer 22: 210–220.
24. Brideau NJ, Flores HA, Wang J, Maheshwari S, Wang X, et al. (2006) Two Dohzhansky-Muller genes interact to cause hybrid lethality in Drosophila. Science 314: 1292–1295.
25. Turelli M, Moode LC (2007) Asymmetric postmating isolation: Darwin’s corollary to Haldane’s rule. Genetics 176: 1059–1080.
26. Barbash DA, Ashburner M (2003) A novel system of fertility rescue in Drosophila hybrids reveals a link between hybrid lethality and female sterility. Genetics 163: 217–228.
27. Presgraves DC (2003) A fine-scale genetic analysis of hybrid Incompatibilities in drosophila. Genetics 163: 955–972.
28. Demuth JP, Wade MJ (2007) Population differentiation in the beetle Tribolium castanum. I. Genetic architecture. Evolution 61: 494–509.
29. Demuth JP, Wade MJ (2007) Population differentiation in the beetle Tribolium castanum. II. Haldane’s rule and incipient speciation. Evolution 61: 694–699.
30. Matzkin LM (2004) Population genetics and geographic variation of alcohol dehydrogenase (Adh) paralogs and glucose-6-phosphate dehydrogenase (G6pd) in Drosophila mojavensis. Molecular Biology and Evolution 21: 276–288.
31. Reed LK, Nyboer M, Markov TA (2007) Evolutionary relationships of Drosophila mojavensis geographic host races and their sister species Drosophila arizonae. Molecular Ecology 16: 1007–1022.
32. Markov TA, Bocur GD (1998) Reproductive isolation in Sonoran Desert Drosophila: testing the limits of the rules. In: J. HD. S H. B, eds. Endless Forms: species and speciation. Oxford: Oxford University Press. pp 234–244.
Pantazidis AC, Zouros E (1988) Location of an Autosomal Factor Causing Vigneault G, Zouros E (1986) The Genetics of Asymmetrical Male-Sterility in Lorieux M, Perrier X, Goffinet B, Lanaud C, Deleon DG (1995) Maximum- Fisher RA, Bailey NTJ (1949) The Estimation of Linkage with Differential Xu SZ (2003) Estimating polygenic effects using markers of the entire genome. Beavis W (1994) The power and deceit of QTL experiments: lessons from Churchill GA, Doerge RW (1994) Empirical Threshold Values for Quantitative Zeng ZB (1993) Theoretical Basis for Separation of Multiple Linked Gene Lander E, Abrahamson J, Barlow A, Daly M, Lincoln S, et al. (1987) Mapmaker: Zouros E (1991) Searching for speciation genes in the species pair Drosophila Map of Drosophila mojavensis. Blue Genetics 5. Ross CL, Markow TA (2006) Microsatellite variation among diverging populations of Drosophila mojavensis. Journal of Evolutionary Biology 19: 1691–1700. Zouros E (1991) Searching for speciation genes in the species pair Drosophila mojavensis and D. arizonae. In: Young JWP, ed. Molecular Techniques in Taxonomy. Berlin: Springer-Verlag. pp 233–247. Orr HA, Irving S (2001) Complex epistasis and the genetic basis of hybrid sterility in the Drosophila pseudoobscura Bogota-USA hybridization. Genetics 158: 1089–1100. Kemp A, Frank AK (2005) Speciation in progress: A continuum of reproductive isolation in Drosophila bipunctata. Genetics 125: 55–68. Lander E, Abrahamson J, Barlow A, Daly M, Lincoln S, et al. (1987) Mapmaker a Computer Package for Constructing Genetic-Linkage Maps. Cytogenetics and Cell Genetics 46: 642–642. Bostem CJ, Weir BS, Zeng ZB (1994) Zmapqa QTJ cartographer. In: Smith G, Gasova JS, Benkel B, Chesnais J, Fairchild W, et al., eds. Proceedings of the 5th world congress on genetics applied to livestock production:Computing strategies and software. Guelph, Ontario, Canada: Organizing committee, 5th world congress on genetics applied to livestock production, pp 65–66. Bostem CJ, Weir BS, Zeng ZB (2002) QTJ Cartographer. 1.16 ed. Raleigh, NC: Department of Statistics, North Carolina State University. Zeng ZB (1993) Theoretical Basis for Separation of Multiple Linked Gene Effects in Mapping Quantitative Trait Loci. Proceedings of the National Academy of Sciences of the United States of America 90: 10972–10976. Zeng ZB (1994) Precision Mapping of Quantitative Trait Loci. Genetics 136: 1147–1168. Churchill GA, Doerge RW (1994) Empirical Threshold Values for Quantitative Trait Mapping. Genetics 138: 963–971. Mclean RA, Sanders WL, Strosap WW (1991) A Unified Approach to Mixed Linear-Models. American Statistician 45: 34–64. Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sunderland, MA: Sinauer Associates Inc. Moehring AJ, Mackay TFC (2004) The quantitative genetic basis of male mating behavior in Drosophila melanogaster. Genetics 167: 1249–1260. Morgan TJ, Mackay TFC (2006) Quantitative trait loci for thermostolerance phenotypes in Drosophila melanogaster. Heredity 96: 232–242. Beavis W (1994) The power and deceit of QTJ experiments: lessons from comparative QTJ studies. Report of the 49th annual corn and sorghum research conference. Washington, DC: American Seed Trade Association. pp 252–268. Xu SZ (2003) Estimating polygenic effects using markers of the entire genome. Genetics 163: 789–801. Fisher RA, Bailey NTJ (1949) The Estimation of Linkage with Differential Viability. Heredity 3: 215–228. Lorieux M, Goffinet B, Perrier X, Delcon DG, Lanaud C (1995) Maximum-Likelihood Models for Mapping Genetic-Markers Showing Segregation Distortion 1. Backcross Populations. Theoretical and Applied Genetics 90: 73–80. Lorieux M, Perrier X, Goffinet B, Lanaud C, Delcon DG (1995) Maximum-Likelihood Models for Mapping Genetic-Markers Showing Segregation Distortion 2. F2 Populations. Theoretical and Applied Genetics 90: 81–89. Pontecorvo G (1943) Viability interactions between chromosomes of Drosophila melanogaster and Drosophila simulans. Journal of Genetics 45: 51–66. Vignaud G, Zouros E (1986) The Genetics of Asymmetrical Male-Sterility in Drosophila-Mojavesiens and Drosophila-Arizoniens Hybrids - Interactions between the Y-Chromosome and Autosomes. Evolution 40: 1160–1170. Puntanizas AC, Zouros E (1988) Location of an Autosomal Factor Causing Sterility in Drosophila-Mojavesiens Males Carrying the Drosophila-Arizoniens Y-Chromosome. Heredity 60: 299–304. Zouros E, Lodha K, Martin PA (1988) Male Hybrid Sterility in Drosophila - Interactions between Autosomes and Sex-Chromosomes in Crosses of Drosophila-Mojavesiens and Drosophila-Arizoniens. Evolution 42: 1321–1331. Macknight RH (1939) The sex-determining mechanism of Drosophila miranda. Genetics 24: 180–201. Orr HA, Irving S (2005) Segregation distortion in hybrids between the Bogota and USA subspecies of Drosophila pseudoobscura. Genetics 169: 671–682. Patterson JT, Stone WS (1952) Evolution in the Genus Drosophila. New York: Macmillan. Kimura M (1980) Habitat differentiation and speciation in the Drosophila australis species complex (Diptera: Drosophilidae). Kontyu 55: 429–436. Ayala FJ, Tracey ML, Bart LG, Ehrlenfeldt JG (1974) Genetic and reproductive differentiation of the subspecies Drosophila mauritiana caribaea. Evolution 28: 44–41. Mainland GB (1942) Genetic relationships in the Drosophila furnesi group. University of Texas Publications 4228: 74–112. Nolte V, Weigel D, Schlotterer C (2008) The impact of shared ancestral variation on hybrid male lethality - a 16 codon indel in the Drosophila simulans Lhr gene. Journal of Evolutionary Biology 21: 551–555. Crow JF (1942) Cross fertility and isolating mechanisms in the Drosophila mulleri group. University of Texas Publications 4228: 53–67. Wade MJ, Johnson NA (1994) Reproductive Isolation between 2 Species of Flour Beetles, Tribolium-Castaneum and T-Fremani - Variation within and among Geographical Populations of T-Castaneum. Heredity 72: 155–162. Wade MJ, Johnson NA, Jones R, Siguel V, McNaughton M (1997) Genetic variation segregating in natural populations of Tribolium castaneum affecting traits observed in hybrids with T-Fremani. Genetics 147: 1253–1247. Shuker DM, Underwood K, King TM, Butlin RK (2005) Patterns of male sterility in a grasshopper hybrid zone imply accumulation of hybrid incompatibilities without selection. Proceedings of the Royal Society B: Biological Sciences 272: 2491–2497. Good JM, Handel MA, Nachman MW (2008) Asymmetry and polymorphism of male hybrid sterility during the early stages of speciation in house mice. Evolution 62: 50–65. Nairn RS, Kazanis S, McEntire RB, DellaColetta I, Walter RB, et al. (1996) A CDKN2-like polymorphism in Xiphophorus LG V is associated with UV-B-induced melanoma formation in platyfish-swordtail hybrids. Proceedings of the National Academy of Sciences of the United States of America 93: 13042–13047. Hollingshead L (1930) A lethal factor in crepis effective only in an interspecific hybrid. Genetics 15: 114–140. Stephens SG (1950) The Genetics of Corky. 2. Further Studies on Its Genetic Basis in Relation to the General Problem of Interspecific Isolating Mechanisms. Journal of Genetics 50: 9–20. Swofford AL, Mason AR, Willis JH (2007) Natural variation for a hybrid incomparability between two species of Mimulus. Evolution 61: 141–151. Kimura M, Ohta T (1969) Average Number of generations until Fixation of a Mutant Gene in a Finite Population. Genetics 61: 763-88. Gasper J, Swanson WJ (2006) Molecular population genetics of the gene encoding the human fertilization protein zonadhesin reveals rapid adaptive evolution. American Journal of Human Genetics 79: 820–830. Hamm D, Mauz BS, Wolfner MF, Aquadro CF, Swanson WJ (2007) Evidence of amino acid diversity-enhancing selection within humans and among primates at the candidate sperm-receptor gene PKDREJ. American Journal of Human Genetics 81: 44–52. Begun DJ, Whitley P, Todd BL, Waldrip-Dail HM, Clark AG (2000) Molecular population genetics of male accessory gland proteins in Drosophila. Genetics 156: 1697–1708. Levitan DR, Ferrell DL (2006) Selection on gamete recognition proteins depends on sex, density, and genotype frequency. Science 312: 267–269. Mady JP, Jones CD, Noor MA, Locke J, Orr HA (2006) Gene transposition as a cause of hybrid sterility in Drosophila. Science 313: 1448–1450. Ting CT, Tsaur SC, Wu ML, Wu CI (1998) A rapidly evolving homeobox in the site of a hybrid sterility gene. Science 282: 1501–1504. Scharlt M, Hornung U, Gunther H, Voell FN, Winthrett J (1999) Melanoma loss-of-function mutants in xiphophorus caused by Xmrk-oncogene deletion and gene disruption by a transposable element. Genetics 153: 1385–1394. Barbash DA, Simo DF, Tarone AM, Roote J (2003) A rapidly evolving MYB-related protein causes species isolation in Drosophila. Proceedings of the National Academy of Sciences of the United States of America 100: 5302–5307. Presgraves DC, Balagepalan L, Almarrz SM, Orr HA (2003) Adaptive evolution drives divergence of a hybrid inviability gene between two species of Drosophila. Nature 423: 715–719.