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Genetics and Lineage-Specific Evolution of a Lethal Hybrid Incompatibility Between *Drosophila mauritiana* and Its Sibling Species

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ABSTRACT

The Dobzhansky–Muller model posits that intrinsic postzygotic reproductive isolation—the sterility or lethality of species hybrids—results from the evolution of incompatible epistatic interactions between species: favorable or neutral alleles that become fixed in the genetic background of one species can cause sterility or lethality in the genetic background of another species. The kind of hybrid incompatibility that evolves between two species, however, depends on the particular evolutionary history of the causative substitutions. An allele that is functionally derived in one species can be incompatible with an allele that is functionally derived in the other species (a derived-derived hybrid incompatibility). But an allele that is functionally derived in one species can also be incompatible with an allele that has retained the ancestral state in the other species (a derived-ancestral hybrid incompatibility). The relative abundance of such derived-derived *vs.* derived-ancestral hybrid incompatibilities is unknown. Here, we characterize the genetics and evolutionary history of a lethal hybrid incompatibility between *Drosophila mauritiana* and its two sibling species, *D. sechellia* and *D. simulans*. We show that a hybrid lethality factor(s) in the pericentric heterochromatin of the *D. mauritiana X* chromosome, *hybrid lethal on the X* (*hlx*), is incompatible with a factor(s) in the same small autosomal region from both *D. sechellia* and *D. simulans*, *Suppressor of hlx* [*Su(hlx)*]. By combining genetic and phylogenetic information, we infer that *hlx-Su(hlx)* hybrid lethality is likely caused by a derived-ancestral incompatibility, a hypothesis that can be tested directly when the genes are identified.

SPECIATION often involves the evolution of intrinsic postzygotic reproductive barriers—including the sterility and inviability of hybrids—that limit the potential for genetic exchange between populations or species (Dobzhansky 1937; Coyne and Orr 2004). Hybrid sterility and inviability in animals are usually caused by incompatible gene interactions: often functionally divergent genes from one species are incompatible with interacting genes from another species. Many studies have mapped such hybrid incompatibility genes to small chromosomal regions (Naveira and Fontdevila 1986; Pantazidis *et al.* 1993; Carvajal *et al.* 1996; Hollocher and Wu 1996; True *et al.* 1996; Sawamura and Yamamoto 1997; Naisbit *et al.* 2002; Presgraves 2003; Tao *et al.* 2003; Slotman *et al.* 2004; Moyle and Graham 2005; Sweigart *et al.* 2006; Masly and Presgraves 2007; Good *et al.* 2008) and, in several cases, identified the causative genes. These studies reveal that hybrid incompatibilities can involve functionally divergent protein-coding genes (Ting *et al.* 1998; Barbash *et al.* 2003; Presgraves *et al.* 2003; Brideau *et al.* 2006; Mihola *et al.* 2009; Phadnis and Orr 2009; Tang and Presgraves 2009), chimeric duplicate genes (Wittbrodt *et al.* 1989), repetitive DNA (Sawamura and Yamamoto 1997), and gene movement (Masly *et al.* 2006).

However, none of these individual hybrid incompatibility loci causes sterility or inviability on its own. Rather, as Dobzhansky (1937) and Muller (1940, 1942) first explained, hybrid fitness problems must involve deleterious epistatic interactions that evolve as incidental by-products of divergence (see Orr 1996). In the usual depiction of the so-called Dobzhansky–Muller model, an ancestral population with the two-locus genotype *aabb* splits into two geographically isolated lineages and each fixes new and different substitutions (yielding *AAbb* and *aaBB* lineages, respectively); when brought together in hybrids (*AaBb*), an incompatibility between these substitutions causes hybrid sterility or hybrid inviability (Figure 1A). Recent theory shows that the evolution of hybrid incompatibilities should follow some simple rules. For example, hybrid incompatibilities should be asymmetric (*i.e.*, *A* is incompatible with *B*, but *a* should be compatible with *b*), should often be complex (*i.e.*, involve three or more loci; Cabot *et al.* 1994; Orr 1995), and should snowball with time (*i.e.*, the number of incompatibilities between two populations should increase faster than linearly with divergence; Orr 1995; Orr and Turelli 2001).

In Figure 1A, hybrids suffer from an incompatible epistatic interaction between a derived *A* allele and a

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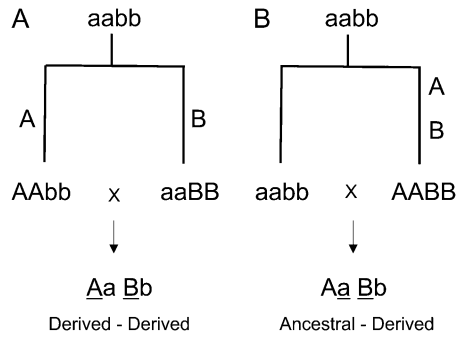

Figure 1.—The Dobzhansky–Muller model for the evolution of postzygotic isolation. An ancestral population *aabb* splits into two independent populations that then accumulate substitutions. Epistatic interactions between underlined substitutions cause hybrid incompatibilities. (A) A derived-derived incompatibility. (B) A derived-ancestral incompatibility.

derived *B* allele (*i.e.*, a derived-derived hybrid incompatibility). But as Muller (1942) pointed out, if both substitutions occur in the same lineage (yielding *AABB* and *aabb* lineages; Figure 1B), then hybrids (*AaBb*) could suffer from an incompatible epistatic interaction between a derived *B* allele and an ancestral *a* allele (*i.e.*, a derived-ancestral hybrid incompatibility). Assuming that all substitutions are independent, so that causative substitutions accumulate in both lineages, theory predicts that derived-derived hybrid incompatibilities should be more common (Orr 1995). The reason is that derived alleles can be incompatible with both derived and ancestral alleles, but ancestral alleles can *only* be incompatible with derived alleles [ancestral alleles must be compatible with one another (Orr 1995)]. If, however, substitutions are not independent, the expected relative frequency of derived-ancestral incompatibilities increases. In the extreme case, in which all substitutions occur in one lineage, *only* derived-ancestral incompatibilities are possible (Orr 1995). There is good reason to believe that the substitutions involved in hybrid incompatibilities are not independent. Imagine, for instance, that two interacting loci coevolve so that substitution of the *A* allele favors the subsequent substitution of the *B* allele at an interacting locus (Presgraves and Stephan 2007; Schlosser and Wagner 2008; Tang and Presgraves 2009). This kind of coevolutionary nonindependence will tend to concentrate substitutions among interacting partner loci in one lineage, enriching for derived-ancestral incompatibilities compared to a scenario of independent substitutions.

Data on the relative abundance of derived-derived *vs.* derived-ancestral hybrid incompatibilities are lacking as few interacting partners have been mapped and characterized. Incompatible partners causing hybrid lethality have been genetically characterized between *Drosophila melanogaster* and *D. simulans* (Hutter *et al.* 1990; Sawamura *et al.* 1993; Sawamura and Yamamoto 1993; Brideau *et al.* 2006), and incompatible partners causing hybrid sterility have been mapped in Drosophila (Pantazidis *et al.* 1993) and in Mimulus (Sweigart *et al.* 2006). However, none has established the species lineage in which the functionally derived alleles at the incompatible partner loci evolved. The hybrid incompatibility identified by Masly *et al.* (2006), who showed that a gene transposition causes male sterility in *D. melanogaster–D. simulans* hybrids, is a special case: *JYalpha*, a gene essential for male fertility, is on the fourth chromosome in *D. melanogaster* but has moved onto the third chromosome in the *D. simulans* lineage. Thus, hybrid males homozygous for the *D. simulans* fourth chromosome and the *D. melanogaster* third are sterile as they lack the *JYalpha* gene. The transposition of *JYalpha* caused a derived-ancestral hybrid incompatibility: a derived change (the absence of *JYalpha* on the fourth in *D. simulans*) is incompatible with the ancestral state (the absence of *JYalpha* on the third in *D. melanogaster*).

Here we characterize the genetics and evolutionary history of a new lethal hybrid incompatibility between *D. mauritiana* and its sibling species, *D. sechellia* and *D. simulans*, three species that diverged nearly simultaneously ~250,000 years ago (Kliman *et al.* 2000; McDermott and Kliman 2008). Our analysis builds on an earlier genomewide screen for hybrid incompatibilities between *D. mauritiana* and *D. sechellia* in which four hybrid lethal regions were identified, including one near the base of the *D. mauritiana X* chromosome (Masly and Presgraves 2007). In this article we refine the mapping of this X-linked factor, which we call *hybrid lethal on the X* (*hlx*), and we map an incompatible partner, *Suppressor of hlx* [*Su(hlx)*], to a small autosomal region. Finally, using comparative mapping, we infer a most-parsimonious history for the evolution of the *hlx-Su(hlx)* hybrid lethality, which appears to result from a derived-ancestral hybrid incompatibility.

MATERIALS AND METHODS

Fly stocks: We used stocks of three Drosophila species: *D. sechellia w*, *D. simulans* w^{XD1}, and *D. simulans* w^{NIG}, kindly provided by Jerry Coyne; and a large collection of *D. mauritiana w* stocks, each bearing single inserts of the *P*[w^+] construct (described in True *et al.* 1996), kindly provided by Yun Tao. We also used 42 stocks of *D. sechellia w* that are homozygous for small *P*[w^+]-marked autosomal introgressions of *D. mauritiana* material. These stocks were produced by selectively introgressing a *D. mauritiana P*[w^+]-marked region into *D. sechellia w* for 15 generations of repeated backcrossing (for details see Masly and Presgraves 2007). All crosses were done at room temperature (23–24°) on standard cornmeal-agarose medium.

Mapping the X-linked hybrid lethal: In previous work, Masly and Presgraves (2007) found that *D. sechellia* lines with small introgressions of cytological region 18DE of the *D. mauritiana X* chromosome suffer recessive hybrid lethality (see results below). To confirm these original findings, we constructed new introgression lines with both *D. sechellia* and

D. simulans. In particular, following the introgression procedure of Masly and Presgraves (2007), we moved the 18DE region of *D. mauritiana*, marked with the $P[w^+]$-insert *2E1*, into *D. sechellia w*, *D. simulans* w^{XD1}, and *D. simulans* w^{NIG}. Briefly, we crossed, *e.g.*, *D. simulans* w^{XD1} females to *D. mauritiana 2E1* $P[w^+]$-insert bearing males. In each subsequent generation, we selected $P[w^+]$-bearing hybrid females and backcrossed them to *D. simulans* w^{XD1} males (for details see Masly and Presgraves 2007). To score hybrid lethality, we transferred parents to fresh vials three times and pooled progeny numbers from the three vials.

Rather than stop our introgressions at generation 15, we produced 259 sublines over 60 generations of continued introgression. We used the *D. simulans* w^{XD1} for these advanced generation sublines as this stock showed the highest fecundity. Of the 259 sublines, 131 ultimately produced viable $P[w^+]$-marked sons and 128 sublines remained hybrid lethal. After 15 generations of introgression, we maintained the sublines by crossing 10 $P[w^+]$-bearing females with 10 *w* brothers, and scored their progeny for sex and eye color each generation. We collected 5–10 $P[w^+]$-female (and, if available, viable $P[w^+]$-male) progeny from 3–4 lethal and 2–3 viable lines every generation for 45 generations and froze them at −20° for genotyping at molecular markers (see below).

Lethal phase: To determine if hybrid lethality was embryonic or postembryonic, we scored egg-hatch rates from two kinds of females: those heterozygous for a $P[w^+]$-marked hybrid lethal factor on the X chromosome (for which half of their sons die) and those lacking the hybrid lethal (as controls). We set up individual females in vials containing a small plastic spoon with fly food colored with grape juice and painted with a live yeast suspension (see Hoffmann *et al.* 1986). All egg collections were done in an incubator at 24°. Every 24 hr, females were transferred to a new vial for three successive transfers, producing four egg-hatch counts per female. After the third transfer, females were transferred to a standard vial with cornmeal-agarose medium and allowed to produce progeny; we then scored the number of w^+ and *w* male and female progeny.

Mapping the autosomal partner: To map possible autosomal suppressors of hybrid lethality, we crossed lethal-introgression bearing females to males from 42 different viable and fertile lines carrying small *D. mauritiana* autosomal segments introgressed into *D. sechellia*. These 42 lines were produced by Masly and Presgraves (2007) and maintained in our lab. For each of the 42 tests, we crossed ∼10 virgin females heterozygous for the lethal X-linked introgression to ∼15 males from each of the 42 autosomal introgressions. Parental adults were transferred every 5–6 days until they ceased to produce progeny. All progeny were scored for sex and eye color.

Molecular markers: The cytological location for the *2E1* $P[w^+]$-insert was originally inferred from salivary gland squashes to be in cytological subdivisions 18DE (True *et al.* 1996). The genomic flanking sequences of the *P*-element (provided by Y. Tao) show that the insert sits in the 5′-UTR of the *jog* locus in cytological bands 18F2–18F4. To map the X-linked hybrid lethal, we used a combination of microsatellite markers and single-nucleotide differences between lines. We used the *D. simulans* genome sequence to identify candidate microsatellite markers using the Tandem Repeats Finder software (Benson 1999) and to design primers flanking the microsatellite loci. Each generation we genotyped a subset of w^+ females and (when available) recombinant w^+ males from selected viable and lethal sublines with three microsatellite and three SNP markers. Microsatellite marker *17.07* is in the *Bx* locus, in cytological subdivision 17C; microsatellite marker *20.06* is located in cytological band 20D1; and microsatellite marker *20.07* is in the *fog* locus in cytological band 20D2. SNP markers were surveyed at three genes in cytological region 20EF: *CG13865* and *CG40485*, and *su(f)*.

Genotyping introgression breakpoints: We isolated genomic DNA following a single fly extraction protocol from Puregene DNA purification kit (Gentra Systems). To genotype microsatellites, we PCR amplified marker regions using standard protocols and visualized species–specific microsatellite array length differences on a 8% polyacrylamide gel stained with ethydium bromide. To genotype SNP differences, we used TILLING (Till *et al.* 2006) following the protocols of the Transgenomic SURVEYOR mutation detection kit (Transgenomic). This kit uses a mismatch-specific DNA endonuclease to scan for mismatches in heteroduplex DNA. Briefly, to genotype individual flies using TILLING, we PCR amplified a marker region, formed heteroduplex DNA, cut the heteroduplex DNA with SURVEYOR endonuclease, and then visualized the digestion products on a 2% agarose gel. As our loci are X linked, we used different heteroduplex formation steps for the two sexes. To form heteroduplex DNA for $P[w^+]$-introgression males, we mixed equal amounts of PCR product from individual $P[w^+]$-introgression males with PCR product from *D. simulans* and, separately, from *D. mauritiana*. For $P[w^+]$ introgression females, no DNA mixing was necessary as these females are heterozygous for the introgressed regions. After heteroduplex formation, we treated 30 μl of the sample with SURVEYOR enhancer and SURVEYOR endonuclease (1.5–2 μl of each) and incubated the mixture for 25–30 min at 42°. We stopped the reaction with 1/10 stop solution and froze the samples at −20° until ready for loading in a 2% agarose gel. All SURVEYOR reactions were performed simultaneously with positive controls involving heteroduplexed DNA between two species and negative controls involving pure-species DNA from a single line.

For $P[w^+]$-introgression females, if digestion of PCR products was detected the line was inferred to be heterozygous (*D. mauritiana*/*D. simulans*) at the assayed marker. Conversely, if no digestion was detected, the line was inferred to be homozygous for *D. simulans* material at the assayed marker. For $P[w^+]$-introgression males, if digestion was detected for heteroduplexed DNA between the introgression line and *D. simulans* but not *D. mauritiana* PCR products, the line was inferred to be hemizygous *D. mauritiana* at the assayed marker. Conversely, if digestion was detected for heteroduplexed DNA between the introgression line and *D. mauritiana* but not *D. simulans* PCR products, the line was inferred to be hemizygous *D. simulans* at the assayed marker.

RESULTS

A locus on the *D. mauritiana X* chromosome causes lethality in *D. sechellia* and *D. simulans* genetic backgrounds: Previous work showed that a genetic factor at the base of the *D. mauritiana X* chromosome causes lethality when introgressed into an otherwise *D. sechellia* genetic background (Masly and Presgraves 2007). To confirm this X-linked hybrid lethality, we first generated new introgression lines: for 6–8 generations we backcrossed females carrying the *D. mauritiana 2E1* $P[w^+]$-insertion to *D. sechellia w* males. We then scored progeny from introgression hybrid females heterozygous for the *D. mauritiana 2E1* $P[w^+]$-insertion crossed to *D. sechellia w* males. From this cross we expect four zygotic genotypes: females with and without the *2E1* introgression and

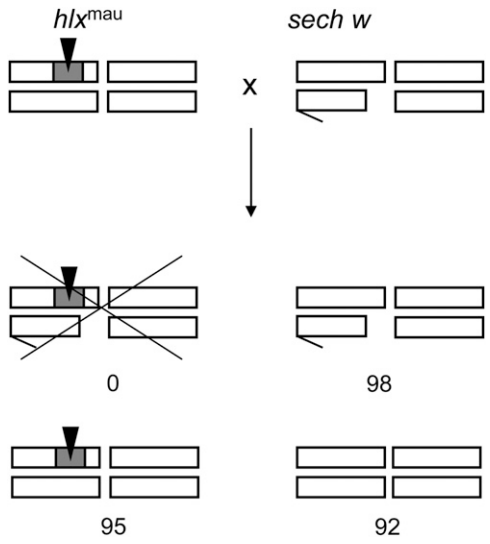

FIGURE 2.—A locus on the *D. mauritiana X* chromosome causes lethality in a *D. sechellia* genetic background. *D. sechellia w* females heterozygous for hlx^{mau} are crossed to *D. sechellia w* males. Male offspring that inherit hlx^{mau} are lethal. Open rectangles represent one pair of sex chromosomes and a representative pair of autosomes in *D. sechellia*; the short, hooked rectangle represents the Y chromosome. The shaded box represents introgressed *D. mauritiana* genetic material marked with a $P[w^+]$-element, shown as an inverted solid triangle. Numbers below the offspring genotypes are representative for the cross (see Table 1).

males with and without the *2E1* introgression (Figure 2). Flies with the *D. mauritiana 2E1* introgression will be red eyed as they carry the $P[w^+]$-insert in an otherwise *D. sechellia w* background. Figure 2 shows that while both red- and white-eyed females appear in roughly equal numbers, only white-eyed males appear and red-eyed males are absent. These findings show that introgression hybrid males inheriting the *2E1* $P[w^+]$-marked introgression from *D. mauritiana* are lethal whereas their siblings are viable. We thus named the responsible genetic factor *hybrid lethal on the X* (*hlx*). The fact that females heterozygous for the introgression are viable suggests that *hlx* acts either as a male-specific hybrid lethal or as a recessive hybrid lethal.

To test if the *D. mauritiana* allele, hlx^{mau}, also causes hybrid lethality in *D. simulans*, we introgressed *2E1* $P[w^+]$-marked fragments from *D. mauritiana* into two different *D. simulans* stocks, w^{XD1} and w^{NIG}. Table 1 shows that males with mostly *D. simulans* genetic backgrounds that inherit hlx^{mau} are lethal whereas their siblings are viable. These results show that the *D. mauritiana* allele of *hlx* is lethal when introgressed into *D. sechellia* and into two lines of *D. simulans*.

In some crosses, a small number of $P[w^+]$-bearing introgression males appeared (Table 1). These males could represent rare escapers of hybrid lethality or rare recombinant males that inherit X chromosomes for which the *2E1* $P[w^+]$-insert and hlx^{mau} have become separated by recombination. (Viable $P[w^+]$-bearing introgression males were invariably sterile; not shown.) To determine if most w^+ males were escapers or recombinants, we set up their w^+ sisters individually in separate vials and scored their progeny for eye color. If $P[w^+]$-bearing males are escapers, then these crosses should produce escapers at a similar rate. If, however, lethality is largely complete and $P[w^+]$-bearing males have inherited recombinant chromosomes, then individual w^+ sisters should fall into two distinct classes: those with nonrecombinant hlx^{mau}-bearing chromosomes should produce almost exclusively white-eyed sons; and those with recombinant hlx^{mau}-free chromosomes should produce a 1:1 ratio of red- and white-eyed sons. Table 2 shows that the presence of $P[w^+]$-bearing males is best explained by recombination. There are few, if any, escapers of hlx^{mau}-based hybrid lethality.

***hlx^{mau}* causes postembryonic hybrid lethality:** We next tested if lethality was embryonic or postembryonic. For these experiments, we used *D. simulans* w^{XD1} introgression females as their fecundity was higher than the *D. sechellia w* and *D. simulans* w^{NIG} lines. We set up 15 hlx^{mau}/hlx^{sim} females from each of three viable and three lethal lines, allowed them to lay eggs for four consecutive 24-hour periods, and scored the number of hatched and unhatched eggs for each after 28 hr. We found no significant difference in the percentage of unhatched eggs between the lethal and viable lines (lethal: 40.7% ± 3.8%; viable: 46.2% ± 5.9%; permutation test $P = 0.417$). Hybrid lethality therefore appears to be postembryonic.

Fine-scale mapping of *hlx*: For fine-scale genetic mapping, we focused on the *D. simulans* w^{XD1} introgression stocks. As recombination occasionally separates the *2E1* $P[w^+]$-insert from hlx^{mau}, we generated many hybrid lethal-bearing sublines and continued the introgression procedure. Over the course of 20–45 additional generations of introgression, we recovered and genotyped 131 viable and 128 lethal $P[w^+]$-bearing sublines for different combinations of six molecular markers on either side of the 18DE region. We tested for a genetic association between hybrid lethality and the species origin of the markers (Table 3). The distal (leftmost) marker shows no association between species origin and hybrid lethality (microsatellite marker *17.07*, Fisher's exact $P = 0.365$). In contrast, the SNP marker in *CG13865* shows a highly significant association (Fisher's exact $P = 7.23 \times 10^{-29}$): introgression males bearing *D. simulans* material at *CG13865* are always viable whereas introgression males bearing *D. mauritiana* material at *CG13865* are nearly always lethal. The association is not, however, perfect. We recovered four viable $P[w^+]$-bearing males that carry *D. mauritiana* material at *CG13865* (we genotyped these exceptional males twice to confirm these results). Assuming that hybrid lethality is complete, these four males suggest that *hlx* is proximal to *CG13865* (20F3–20F4; Figure 3A). *CG13865* is the last, most-proximal gene in the contiguous *D. melanogaster* (v. 5.1) assembly of the X chromosome. The sequence

TABLE 1

D. mauritiana hlx* causes hybrid lethality in both of its sibling species, *D. simulans* and *D. sechellia

Species	Line	Generation[a]	Females			Males		
			w^+	w	w^+/w ratio	w^+	w	w^+/w ratio
D. simulans	*sim* w^{XD1}	14	99	105	0.943	9	110	0.082
	sim w^{XD1}	10	72	71	1.014	5	123	0.04
	sim w^{NIG}	6	26	25	1.04	1	31	0.032
	sim w^{NIG}	9	66	62	1.065	3	49	0.061
D. sechellia	*sech w*	6	95	92	1.033	0	98	0
	sech w	8	101	114	0.886	2	105	0.019

[a] The number of generations for which the *hlx* region of *D. mauritiana* was introgressed into *D. simulans* or *D. sechellia* genetic backgrounds.

scaffold bearing *CG13865* extends to heterochromatin region h26 (heterochromatin regions in the X are designated h26–h34; Figure 3B; Hoskins *et al.* 2007). The *hlx* locus thus resides in the highly repetitive, transposon-rich, and gene-poor pericentric heterochromatin of the X chromosome.

To further refine the position of *hlx* in the heterochromatin, we genotyped 135 introgression chromosomes for SNP differences at the *su(f)* locus. The *su(f)* locus lies in scaffold Xhet (GenBank accession no. CP00208), which maps to cytological regions h26–h27 but is not currently contiguous with the euchromatic assembly (Figure 3B; Hoskins *et al.* 2007). We also found a significant association at this marker (Fisher's exact test $P = 4.22 \times 10^{-34}$). The four viable $P[w^+]$-bearing males carrying *D. mauritiana* material at *CG13865* also carried *D. mauritiana* material at *su(f)*, placing the location of *hlx* proximal to *su(f)* in the centromeric heterochromatin. Only one known protein-coding gene, *ATbp*, lies ~5 kb proximal to *su(f)*. However, our repeated attempts to genotype recombinants at *ATbp* were unsuccessful. Further traditional genetic mapping of the *hlx* locus is, for the moment, not feasible.

Hybrid lethality is caused by an X–autosome incompatibility: The Dobzhansky–Muller model predicts that hlx^{mau} is incompatible with one or more loci from *D. simulans* and *D. sechellia*. In principle, the incompatible partner gene(s) could be located on the autosomes or on the non-*D. mauritiana* part of the $P[w^+]$-bearing introgression X. To distinguish these possibilities, we tested for an X–autosome interaction. In particular, we crossed *D. sechellia* females heterozygous for a lethal introgression of hlx^{mau} to *D. mauritiana* males. All progeny from this cross are heterozygous for the autosomes and, importantly, half of the sons will inherit the hlx^{mau} on an otherwise *D. sechellia X* chromosome (Figure 4A). If these males are viable, it suggests that some factor(s) in the *D. mauritiana* autosomal genome can dominantly suppress hlx^{mau} hybrid lethality. We found that, indeed, sons inheriting hlx^{mau} are completely viable when given a set of *D. mauritiana* autosomes (Table 4, line 1 *vs.* 2). The same result is obtained when we cross *D. simulans* females with lethal introgressions of hlx^{mau} to *D. mauritiana* males (Table 4, line 3 *vs.* 4). These results show that the *D. mauritiana* autosomes possess one or more dominant *Suppressor of hlx* [*Su(hlx)*] loci. Put differently, hlx^{mau} is incompatible with a recessive autosomal factor(s) from *D. sechellia* and *D. simulans*. Importantly, these results also exclude two other explanations. First, hlx^{mau} is not involved in a simple X–X hybrid incompatibility. Second, the lethality of hlx^{mau} introgressions cannot be attributed to linked spontaneous mutations that accumulated during the introgression procedure. If introgression males are lethal because of an X-linked

TABLE 2

Occasional viable w^+ hybrid males are recombinants, not escapers

	Line	Generation[a]	Females			Males			Class
			w^+	w	w^+/w ratio	w^+	w	w^+/w ratio	
1. Original line	*sim* w^{XD1}	55	20	28	0.71	9	23	0.39	
2. Sublines	*sim* w^{XD1}	56	9	15	0.60	0	13	0.00	Lethal
3.	*sim* w^{XD1}	56	15	10	1.50	0	15	0.00	Lethal
4.	*sim* w^{XD1}	56	28	28	1.00	0	36	0.00	Lethal
5.	*sim* w^{XD1}	56	13	12	1.08	9	11	0.82	Recombinant
6.	*sim* w^{XD1}	56	4	7	0.57	2	4	0.50	Recombinant
7.	*sim* w^{XD1}	56	21	17	1.24	15	20	0.75	Recombinant

[a] The number of generations for which the *hlx* region of *D. mauritiana* was introgressed into *D. simulans* or *D. sechellia*.

TABLE 3

Genotype-marker association for six molecular markers around 18DE

Marker	Position[a]	Class	Genotype		*P*-value[b]
			mau	non-*mau*	
17.07	18368602	Lethal	14	18	
		Viable	12	11	0.365
20.06	21952484	Lethal	19	0	
		Viable	4	24	1.27 e^{-9}
20.07	22037065	Lethal	72	0	
		Viable	4	41	2.37 e^{-27}
CG40485	22362444	Lethal	94	0	
		Viable	4	83	2.27 e^{-47}
CGI3865	22415346	Lethal	42	0	
		Viable	4	78	7.23 e^{-29}
su(f)	XHet:69195	Lethal	70	0	
		Viable	4	61	4.22 e^{-34}

[a] *D. melanogaster* R5.11 sequence starting coordinate.
[b] *P*-value for Fisher's exact test.

recessive mutation, there is no reason why they ought to be rescued by the *D. mauritiana* autosomes.

A genetic screen for *Su(hlx)* loci on the *D. mauritiana* autosomes: To map the autosomal *Su(hlx)* partner loci, we crossed heterozygous hlx^{mau}-bearing *D. simulans* w^{XD1} introgression females to males from 42 different lines of *D. sechellia* homozygous for small autosomal $P[w^+]$-marked introgressions from *D. mauritiana* (Figure 4B). [The latter are a subset of the viable and fertile autosomal introgressions (on average ~2 Mb in size) maintained from the original screen by Masly and Presgraves (2007).] We used hlx^{mau}-bearing *D. simulans* w^{XD1} lines for these crosses because their fecundity is substantially higher than that of hlx^{mau}-bearing *D. sechellia w* lines. For each of the 42 crosses, four zygotic genotypes will be produced: daughters will possess either the autosomal introgression only or both hlx^{mau} *and* the autosomal introgression; similarly, sons will possess either the autosomal introgression only or both hlx^{mau} *and* the autosomal introgression (Figure 4B). If the hlx^{mau}-bearing males remain inviable, a *Su(hlx)* partner locus does not reside in the autosomal introgression. But if hlx^{mau}-bearing males are rescued, we can infer that a *Su(hlx)* partner resides in the autosomal introgression (Figure 4B). For most crosses, we were able to distinguish the two kinds of male progeny (those inheriting hlx^{mau}-introgression and those not) by $P[w^+]$ dosage. Males inheriting only the autosomal introgression will be heterozygous for a $P[w^+]$-insert and thus have light red (or orange) eye color. In contrast, males with both an autosomal introgression *and* the hlx^{mau}-introgression are heterozygous for one $P[w^+]$-insert and hemizygous for the other and will thus express, in effect, three doses of $P[w^+]$. These males should therefore have a strong red-eye phenotype. In addition to eye color, the sex ratio among progeny is also informative: in crosses lacking *Su(hlx)* rescue we expect a 1:2 sex ratio as half of the males die; however, if *Su(hlx)* rescue occurs we expect a 1:1 sex ratio.

Only *one* of the 42 autosomal introgressions strongly suppresses the hybrid lethality of hlx^{mau}: hybrid males inheriting hlx^{mau} *and* an autosomal introgression from the 33F–34A region of *D. mauritiana* are completely viable (Table 5, line 1). None of the 41 other regions suppressed hybrid lethality (not shown). It is important to note, however, that this screen tests a relatively small fraction of the *D. mauritiana* genome; more *Su(hlx)* loci could reside in untested regions.

The focal males described above are hybrids with genetic material from three species: a hlx^{mau}-bearing X chromosome from *D. simulans* w^{XD1}, one set of *D. simulans* w^{XD1} autosomes, and one set of *D. sechellia w* autosomes with a small *D. mauritiana* $P[w^+]$-marked introgression. As these complex genotypes are not ideal, we performed two further crosses. First, we crossed heterozygous hlx^{mau}-bearing *D. sechellia* females to *D. sechellia* males with the 33F–34A introgression from *D. mauritiana*. As expected, hybrid males bearing hlx^{mau} and the *D. mauritiana* allele, $Su(hlx)^{mau}$, in an otherwise *D. sechellia w* genetic background are fully viable (Table 5, line 2). Second, we introgressed the 33F–34A $P[w^+]$-insert from *D. mauritiana* into a *D. simulans* w^{XD1} genetic background via eight generations of repeated backcrossing. We then crossed heterozygous hlx^{mau}-bearing *D. simulans* w^{XD1} females to *D. simulans* w^{XD1} males homozygous for a *D. mauritiana* 33F–34A introgression. We found that hybrid males bearing hlx^{mau} *and* the *D. mauritiana* allele, $Su(hlx)^{mau}$, in an otherwise *D. simulans* w^{XD1} genetic background are fully viable (Table 5, line 3). These results show that $Su(hlx)^{mau}$ can rescue lethal hlx^{mau}-bearing hybrid males in both *D. sechellia* and *D. simulans* genetic backgrounds.

Previous work estimated that the 33F–34A $P[w^+]$-marked *D. mauritiana* introgression into *D. sechellia* is ~1.6 Mb long (Masly and Presgraves 2007). To refine this estimate, we genotyped 15 additional molecular markers in the region. The distal breakpoint falls between *CG6405* and *Elf* (33E4), and the proximal breakpoint falls between *CG16848* and *CG16956* (34B11). The introgression is thus 0.975–1.03 Mb long, a region comprising 111–120 predicted genes.

DISCUSSION

The genetic analyses presented here yield two main results. First, we have mapped *hlx*, a locus that causes hybrid lethality, to the pericentric heterochromatin of the X chromosome. The *D. mauritiana* allele, hlx^{mau}, causes complete postembryonic hybrid lethality when hemizygous in an otherwise *D. sechellia* or *D. simulans*

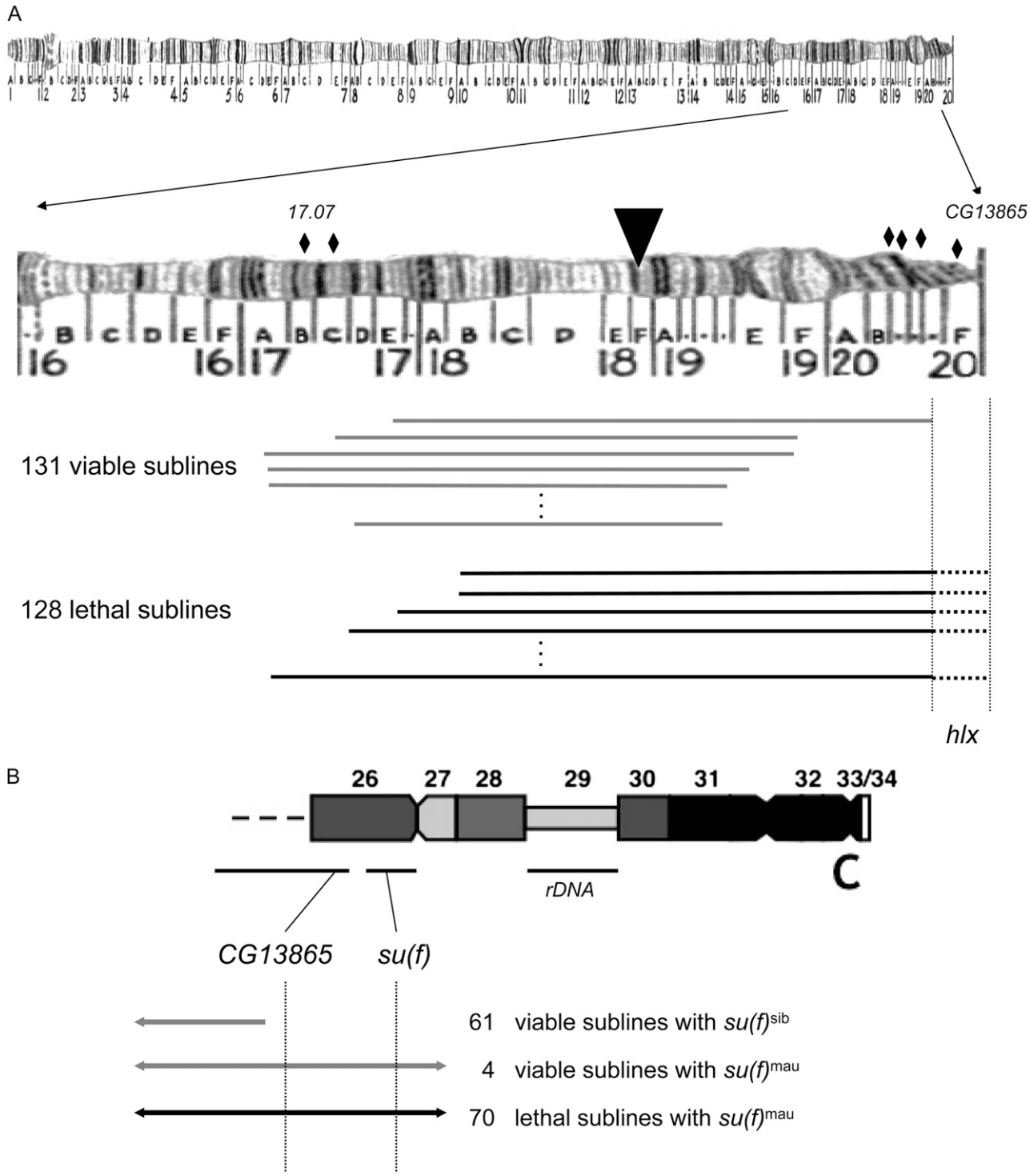

FIGURE 3.—Fine-scale genetic mapping of *hlx*. (A) A collection of 131 viable lines (shaded) and 128 lethal lines (solid) was used to map the location of *hlx* to the bracketed interval proximal to *CG13865*. Diamonds represent position of molecular markers, and the inverted solid triangle represents location of $P[w^+]$-insert. (B) Cytogenetic map of the centromeric heterochromatin of the X chromosome (of *D. melanogaster*) showing heterochromatic regions h26–h34 and the centromere, (C) Solid and shaded lines represent hybrid lethal and viable sublines genotyped at *CG13865* and *su(f)*; mau, *D. mauritiana* and "sib," *D. simulans* or *D. sechellia*. The number of lines genotyped is shown for each class.

genetic background. Second, we have mapped an autosomal partner locus, *Su(hlx)*, that interacts with *hlx*. In particular, the *D. mauritiana* allele, $Su(hlx)^{mau}$, can completely suppress the hybrid lethality of hlx^{mau} in both *D. sechellia* and *D. simulans* genetic backgrounds. These findings show that hlx^{mau} is incompatible with at least one recessive autosomal locus whose functional allelic state is shared by *D. sechellia* and *D. simulans*. Below, we

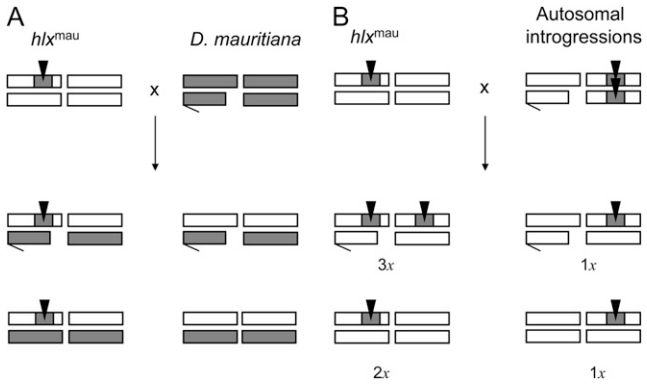

FIGURE 4.—(A) Test for an X–autosome interaction. *D. sechellia* females heterozygous for hlx^{mau} were crossed to *D. mauritiana* males. If hlx^{mau}-bearing hybrid males are viable, *D. mauritiana* possesses one or more dominant, autosomal suppressors of hlx^{mau}. (B) A genetic mapping screen for *Su(hlx)* in *D. mauritiana* autosomal genome. *D. simulans* females heterozygous for hlx^{mau} are crossed to *D. sechellia* males homozygous for small autosomal introgressions from *D. mauritiana*. If hlx^{mau}-bearing males are viable, the *D. mauritiana* introgression possesses a dominant *Su(hlx)*.

infer the evolutionary history of the genetic substitutions leading to the *hlx-Su(hlx)* hybrid incompatibility and then consider the possible genetic basis for hybrid lethality.

Evolutionary history of the *hlx-Su(hlx)* hybrid incompatibility: Combining the genetic mapping results with the phylogenetic history of the three *D. simulans* clade species allows us to make two important inferences about the *hlx-Su(hlx)* incompatibility (see also MOYLE and NAKAZATO 2008). First, our results show that the *D. mauritiana* allele of *hlx* causes hybrid lethality in both *D. sechellia* and *D. simulans* genetic backgrounds. The most parsimonious evolutionary history is one in which the *hlx* substitution(s) causing hybrid lethality is derived in the *D. mauritiana* lineage. Similarly, we found that the *D. mauritiana* allele of *Su(hlx)* suppresses hybrid lethality in both *D. sechellia* and *D. simulans*, implying that the substitution(s) causing suppression of hlx^{mau} hybrid lethality is also derived in the *D. mauritiana* lineage. Taken together, these genetic results imply that the functionally derived hlx^{mau} allele is incompatible with the functionally *ancestral Su(hlx)* alleles of *D. sechellia* and *D. simulans* (Figure 5). The nearly simultaneous splitting of the *D. simulans* clade species has led to extensive lineage sorting: all three possible genealogical relationships can be detected among different loci (Figure 5; HEY and KLIMAN 1993; KLIMAN and HEY 1993; HILTON *et al.* 1994; KLIMAN *et al.* 2000; TING *et al.* 2000; MALIK and HENIKOFF 2005; MCDERMOTT and KLIMAN 2008). However, our inference that the causative substitutions at *hlx* and *Su(hlx)* occurred in *D. mauritiana* should be robust to uncertainty in the genealogical relationships at these loci (Figure 5).

Second, if *hlx* and *Su(hlx)* are derived in the *D. mauritiana* lineage, we can say something about the order in which the relevant substitutions occurred. Of the two possible orderings—*hlx* followed by *Su(hlx)* or *Su(hlx)* followed by *hlx*—only one is allowed by natural selection. The derived hlx^{mau} could not evolve first as it causes lethality in an ancestral *Su(hlx)* genetic background. In contrast, nothing prevents the derived $Su(hlx)^{mau}$ substitution from evolving first, after which the derived hlx^{mau} can evolve in the permissive $Su(hlx)^{mau}$ genetic background. Thus, regardless of which of the three genealogical histories obtains at the *hlx* and *Su(hlx)* loci, the relevant substitutions at both most likely occurred in the *D. mauritiana* lineage. Once the loci have been identified at the molecular level, we will be able to validate this inferred history using molecular population genetics.

Genetic basis of the *hlx-Su(hlx)* hybrid lethality: The localization of *hlx* to the gene-poor pericentric heterochromatin of the X raises the possibility that the *hlx-Su(hlx)* hybrid lethality is caused by something other than an incompatibility between two protein-coding genes. One possibility is that *hlx* is a kind of repetitive satellite DNA. If so, then *Su(hlx)* might be a protein-coding gene that regulates or interacts with heterochromatin. Among the 120 candidate genes in the *Su(hlx)* region, three have known or predicted chromatin-binding functions: *A16*, *Scm-related gene containing four mbt domains* (*Sfmbt*), and *Sir2*. *A16* and *Sfmbt* are relatively uncharacterized, but *Drosophila Sir2* is of special interest as it has roles in heterochromatin silencing (including suppression of position effect variegation on the *X*) and sex determination. A loss-of-function mutation at *Sir2* in *D. melanogaster* causes aberrant expression of *Sex Lethal* in male embryos, disrupting dosage compensation and causing male-specific larval lethality (ROSENBERG and PARKHURST 2002). It is therefore

TABLE 4

***D. mauritiana* autosomes suppress hlx^{mau}-based hybrid lethality**

		Female progeny			Male progeny		
Female parent	Male parent	w^+	w	w^+/w ratio	w^+	w	w^+/w ratio
1. hlx^{mau}/hlx^{sech}; *sech/sech*	*sech w*	103	109	0.945	0	103	0
2. hlx^{mau}/hlx^{sech}; *sech/sech*	*mau w*	105	102	1.029	95	103	0.922
3. hlx^{mau}/hlx^{sim}; *sim/sim*	*sim* w^{XD1}	47	57	0.825	0	60	0
4. hlx^{mau}/hlx^{sim}; *sim/sim*	*mau w*	97	92	1.054	87	95	0.916

TABLE 5

***Su(hlx)* locus maps to *D. mauritiana* region 33F-34A**

Female parent	Male parent	Female progeny			Male progeny		
		2*x*	1*x*	2*x*/1*x* ratio	3*x*	1*x*	3*x*/1*x* ratio
1. hlx^{mau}/hlx^{sim}; *sim/sim*	hlx^{sech}/*Y*; 33*F*-34A^{mau}/33*F*-34A^{mau}	193	178	1.084	180	202	0.891
2. hlx^{mau}/hlx^{sech}; *sech/sech*	hlx^{sech}/*Y*; 33*F*-34A^{mau}/33*F*-34A^{mau}	112	103	1.087	76	98	0.776
3. hlx^{mau}/hlx^{sim}; *sim/sim*	hlx^{sim}/*Y*; 33*F*-34A^{mau}/33*F*-34A^{mau}	105	98	1.071	81	90	0.900

possible that *Su(hlx)* alleles from *D. simulans* and *D. sechellia* act as *Sir2* loss-of-function mutations in hlx^{mau} introgression males. Although the *hlx-Su(hlx)* incompatibility causes postembryonic lethality in males, consistent with the *Sir2* hypothesis, we cannot be certain that hlx^{mau} causes male-specific lethality as there is no straightforward way to test the viability of hlx^{mau}/hlx^{mau} introgression females.

A second possibility is that an essential gene on the ancestral X chromosome moved to the *Su(hlx)* autosomal region in the *D. mauritiana* lineage. In this case, hlx^{mau} introgression males die because they lack an essential gene: the gene is absent from the hlx^{mau} region of the X *and* from the *Su(hlx)* autosomal region of *D. sechellia* and *D. simulans*. Introgression males with hlx^{mau} can then be rescued when supplied with the gene in the *Su(hlx)* autosomal region of *D. mauritiana*. This scenario is similar to the *JYalpha*-mediated hybrid male sterility described by Masly *et al.* (2006). Notably, *JYAlpha* moved from its ancestral position on the heterochromatic dot-fourth chromosome to 3R in the *D. simulans* lineage. An obvious candidate for gene movement in the *hlx-Su(hlx)* incompatibility is the viability-essential

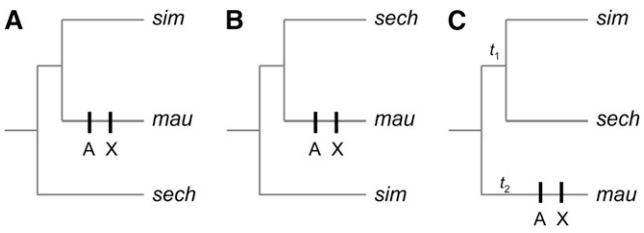

Figure 5.—Inferring the evolutionary history of the substitutions causing the *hlx-Su(hlx)* hybrid incompatibility. (A) Assuming genealogical histories with either *D. sechellia* (*sech*) or (B) *D. simulans* (*sim*) as outgroup species, the most parsimonious histories have the causative substitutions at *hlx* and *Su(hlx)* derived in the *D. mauritiana* (*mau*) lineage. X, X-linked *hlx* substitution; A, autosomal *Su(hlx)* substitution. (C) Assuming a genealogical history with *D. mauritiana* as the outgroup species, the causative substitutions at *hlx* and *Su(hlx)* could be derived in *D. mauritiana* (t_1) or in the common ancestor of the *D. simulans*–*D. sechellia* (t_2); however, given the disparity in branch lengths ($t_2 \ll t_1$), there has been more time for *hlx* and *Su(hlx)* to evolve along the external branch leading to *D. mauritiana* than the very short internal branch of the *D. simulans*–*D. sechellia* common ancestor. The functionally derived hlx^{mau} allele therefore appears to be incompatible with functionally ancestral *Su(hlx)* alleles from *D. sechellia* and *D. simulans*.

ribosomal (rDNA) locus. In *D. melanogaster*, the 18S, 5.8S, and 28S ribosomal RNAs are encoded by a large tandem array of rRNA genes in the pericentric heterochromatin of the X chromosome (h29, Figure 3B). Classical genetic work in *D. melanogaster* showed that the rDNA locus is the only vital locus in heterochromatin proper (Zhimulev 1998). In species of the *D. anannassae* complex, the rDNA locus has moved to the fourth chromosome (Roy *et al.* 2005). We can, however, rule out movement of the rDNA locus in *D. mauritiana* as *in situ* hybridization experiments have shown that the rDNA locus is present near the base of the X chromosome in *D. simulans*, *D. sechellia*, and *D. mauritiana* (Lohe and Roberts 2000; Roy *et al.* 2005). We cannot rule out the possibility that new, viability-essential genes have evolved or moved to the X heterochromatin in the common ancestor of the *D. simulans* clade species and then subsequently moved off of the X in *D. mauritiana*.

Relationship of *hlx* to two other hybrid lethality factors: In crosses between *D. melanogaster* and members of the *D. simulans* species complex, two hybrid lethality factors also map to narrow intervals at the base of the X chromosome. In crosses between *D. simulans* females and *D. melanogaster* males, the X-linked *Zygotic hybrid rescue* (*Zhr*) factor from *D. melanogaster* causes dominant embryonic lethality of hybrid daughters (Sawamura *et al.* 1993; Sawamura and Yamamoto 1993). Zhr^{mel} maps to region h32 of the pericentric heterochromatin of the X (Sawamura *et al.* 1995; Sawamura and Yamamoto 1997; Zhimulev 1998). Another X-linked hybrid lethal was discovered by chromosomal deletion (deficiency, *Df*) mapping in F_1 hybrid females between *D. melanogaster* and the *D. simulans* clade species (Coyne *et al.* 1998). When *D. melanogaster* females heterozygous for deficiencies over dominantly marked balancer chromosomes (*Df/Bal*) are crossed to *D. mauritiana* males, hybrid daughters inheriting deficiencies in cytological region 20C–20F die whereas their balancer-inheriting sisters are viable (Coyne *et al.* 1998). Interestingly, *Df*-bearing hybrid daughters from crosses to *D. simulans* and *D. sechellia* are not lethal, consistent with the evolution of a recessive X-linked lethal in region 20C–20F in *D. mauritiana*. The fact that three hybrid lethals—*hlx*, *Zhr*, and the hybrid lethal of Coyne *et al.* (1998)— map to the same gene-poor pericentric region of the X raises the possibility that the same locus has repeatedly evolved hybrid lethality.

We can exclude the possibility that *hlx* and the hybrid lethal of Coyne *et al.* (1998) are the same locus: our mapping results place *hlx* proximal to 20F3–20F4, whereas new deletion mapping data from our laboratory place the hybrid lethal of Coyne *et al.* (1998) distal to 20F (M. V. Cattani, unpublished results). We cannot, however, exclude the possibility that *hlx* and *Zhr* are the same locus. Zhr^{mel} is thought to be an array of 359-bp repeats belonging to the 1.688 g/cm^3 family of satellite DNA specific to *D. melanogaster* that is incompatible with a maternal factor(s) from its sibling species of the *D. simulans* clade. Zhr^{mel} causes dominant embryonic lethality in F_1 hybrid females from sibling species mothers and *D. melanogaster* fathers (Sawamura *et al.* 1993; Sawamura and Yamamoto 1993). Although hybrid males from *D. simulans* mothers do not normally inherit the X-linked Zhr^{mel}, experimentally introducing Zhr^{mel} kills hybrid males as well (Sawamura and Yamamoto 1997). The hybrid lethality of Zhr^{mel} is thus embryonic, dominant, and independent of sex. These properties contrast with the hybrid lethality of hlx^{mau}, which is postembryonic and either recessive or male specific (see results). The different properties of *Zhr* and *hlx* suggest that they are different loci or, at a minimum, functionally distinct alleles. In either case, the mapping of *hlx* to the pericentric heterochromatin is consistent with an emerging theme: hybrid incompatibilities often involve rapidly evolving heterochromatic elements (Sawamura and Yamamoto 1997; Fishman and Willis 2005) and genes whose products interact with heterochromatin (Barbash *et al.* 2003; Brideau *et al.* 2006). If this trend persists as more hybrid incompatibility factors are identified, it could signal that intrinsic postzygotic isolation typically evolves as a byproduct of genomic conflicts rather than ecology (*e.g.*, Henikoff *et al.* 2001).

Conclusions: We have identified an apparently simple X–autosome hybrid incompatibility in which the two Dobzhansky–Muller partners appear to be functionally derived in the *D. mauritiana* lineage. As *hlx* resides in the unmapped and poorly characterized pericentric heterochromatin of the *D. mauritiana X*, our immediate efforts will focus on the fine-scale mapping and identification of *Su(hlx)*. Once *Su(hlx)* is identified, we will determine if hybrid lethality is caused by gene movement, by a protein–DNA incompatibility, or by protein–protein incompatibility. Population genetic analyses of *Su(hlx)* will then allow us to formally test if the relevant substitutions occurred in the *D. mauritiana* lineage and to determine the evolutionary forces causing its divergence.

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