Adaptive evolution drives divergence of a hybrid inviability gene between two species of *Drosophila*

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Speciation—the splitting of one species into two—occurs by the evolution of any of several forms of reproductive isolation between taxa, including the intrinsic sterility and inviability of hybrids. Abundant evidence shows that these hybrid fitness problems are caused by incompatible interactions between loci: new alleles that become established in one species are sometimes functionally incompatible with alleles at interacting loci from another species. However, almost nothing is known about the genes involved in such hybrid incompatibilities or the evolutionary forces that drive their divergence. Here we identify a gene that causes epistatic inviability in hybrids between two fruitfly species, *Drosophila melanogaster* and *D. simulans*. Our population genetic analysis reveals that this gene—which encodes a nuclear pore protein—evolved by positive natural selection in both species' lineages. These results show that a lethal hybrid incompatibility has evolved as a by-product of adaptive protein evolution.

One of the long-standing goals of speciation research is to establish the molecular identities, functions and evolutionary histories of the genes that cause hybrid sterility and inviability[1,2]. So far, however, only three putative hybrid incompatibility genes have been identified[3–5]. One reason for this paucity of molecular data is that the characters of interest—hybrid sterility and inviability—are by their nature barriers to crossing. These characters are therefore often refractory to many classical genetic approaches[6]. Historically, this problem has proved especially serious in one of our best genetic model organisms, *D. melanogaster*, which is completely reproductively isolated from its closest relatives. All hybrids between *D. melanogaster* and its sibling species are sterile or inviable[7,8] (but see refs 9, 10). Thus, the impressive complement of genetic and molecular tools available in *D. melanogaster* has not been fully brought to bear on the genetics of speciation.

To address this problem, we previously carried out a systematic screen that makes use of the genetic tools of *D. melanogaster*[11]. This screen takes advantage of the fact that most hybrid incompatibilities involve epistatically interacting alleles[12,13] that act as recessives in species hybrids[13,14]. Crosses between *D. melanogaster* females and *D. simulans* males normally produce only hybrid daughters—hybrid males die at the larval–pupal transition[7]. Our crossing scheme involves first rescuing hybrid males from this hybrid incompatibility and then exposing them to other potential hybrid incompatibilities. We cross *D. melanogaster* females heterozygous for an autosomal deficiency (a small chromosomal deletion; *Df*) and a balancer (*Bal*) chromosome to *D. simulans* males carrying *Lethal hybrid rescue* (*Lhr*), a mutation that restores the viability of ordinarily lethal hybrid males[15]. We are interested in the relative viability of *Df* hybrid males that are simultaneously hemizygous for a small region of the *D. simulans* autosomal genome and the *D. melanogaster* X chromosome (Fig. 1a; see Methods). These males are forced to develop using only *D. simulans* (*sim*) alleles at the autosomal loci deleted and only *D. melanogaster* (*mel*) alleles at all X-linked loci (Fig. 1a), allowing us to detect any recessive alleles on the *sim* autosomes that are involved in hybrid lethal interactions with recessive alleles on the *mel* X chromosome. By screening ~70% of the *D. simulans* autosomal genome, we identified 20 small regions, each of which is capable of causing complete hybrid inviability[11].

Here we present the fine-scale genetic, molecular and evolution-

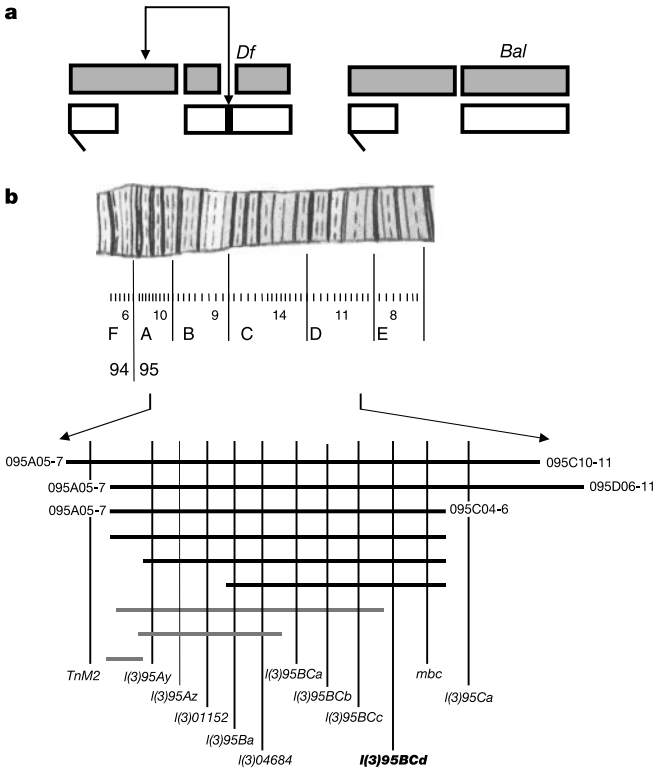

Figure 1 Deficiency mapping hybrid lethality. **a**, Schematic of hybrid male genotypes tested, with the sex chromosomes (left, Y chromosome with hook) and one autosome (right) shown. Grey chromosomes, *D. melanogaster*; white, *D. simulans*. Hybrid males are produced from the cross of *Df/Bal D. melanogaster* females to *Lhr D. simulans* males. Black, autosomal region causing hybrid lethality by its incompatibility with the *D. melanogaster* X chromosome. **b**, Interspecific complementation tests using deficiencies (horizontal lines) and loss-of-function mutations at loci (vertical lines) in cytological region 95 of chromosome arm 3R. Grey, complements hybrid lethality; black, fails to complement hybrid lethality. Deficiencies tested are, in order, from the top: *Df(3R)mbc-30*; *Df(3R)mbc-R1*; *Df(3R)mbc-BG1*; *Df(3R)mbc-15A*; *Df(3R)nau-9*; *Df(3R)mbc-F5.3*; *Df(3R)nau-4a*; *Df(3R)CA*; *Df(3R)nau-11a4* (see refs 45, 46). Only alleles of *l(3)95BCd* fail to complement hybrid lethality.

ary analysis of the first of the hybrid inviability genes that we have been able to identify.

Nup96 causes inviability in species hybrids

We mapped a hybrid inviability gene in hybrid males using nine overlapping deficiencies from *D. melanogaster* to cytological region 95AB on chromosome 3R (Fig. 1b). These crosses narrowed hybrid inviability to just two possible complementation groups: *l(3)95BCd* and *myoblast city* (*mbc*). We nevertheless tested presumed loss-of-function mutations at 12 loci that span the region, singly testing individual *mel* mutations for their ability to uncover hybrid lethality when heterozygous with the *sim* wild-type allele. We found that mutant alleles at only one locus failed to complement the *sim* hybrid lethal factor, *l(3)95BCd* (Fig. 1b). To confirm that the *sim* wild-type allele of *l(3)95BCd* causes hybrid lethality through an epistatic interaction with the *mel* X, we switched the species origin of the X chromosome in hybrids while holding the rest of the genotype constant. Hybrid males that were heterozygous $l(3)95BCd^{E53.1}/sim$ and carried the *mel* X were inviable (*Df/Bal* ratio = 0.05, $n = 101$; Table 1); by contrast, hybrid males that had an identical genotype (including cytoplasm) and carried a *sim* X chromosome were viable (*Df/Bal* ratio = 1.29, $n = 39$). The *sim* allele of *l(3)95BCd* thus interacts with a gene(s) on the *mel* X chromosome to cause hybrid lethality.

We determined that the molecular lesions in *l(3)95BCd* mutant alleles affect the sequence CG10198, which represents the *Drosophila* homologue of a dicistronic gene encoding two functionally distinct nucleoporins (Nups), *Nup98-Nup96* (Fig. 2a). Nup98 and Nup96 are two of ~30 distinct Nups that function in the nuclear pore complex (NPC)[16–19]. NPCs are among the largest macromolecular complexes in eukaryotic cells and function as the sole site of cytonuclear trafficking of RNAs and proteins. The Nup98 and Nup96 proteins are both found on the nucleoplasmic and cytoplasmic sides of the NPC. However, Nup98 is a mobile protein that shuttles on and off the NPC[20–22], whereas Nup96 is stably bound at the NPC, where it seems to have a structural role[23]. Both proteins function in RNA export[21,24,25]. A BLAST search of the entire *Drosophila* genome shows that *Nup98-Nup96* is a single-copy gene in *D. melanogaster*; Southern blot and sequence data indicate that the same is true in *D. simulans* (data not shown). As in other eukaryotes, *Drosophila Nup98-Nup96* is alternatively transcribed, giving rise to a minor 3.5-kilobase (kb) *Nup98* transcript and a major 7.3-kb *Nup98-Nup96* transcript. Northern blot data show that both transcripts are expressed at all developmental stages examined, from embryo to early adult (data not shown). The former encodes the Nup98 protein, but the latter encodes a single, large precursor polyprotein that, in other eukaryotes, and thus presumably in flies, autoproteolytically cleaves itself between residues 1028(F) and 1029(S), yielding separate Nup98 and Nup96 proteins[26–28] (Fig. 2b). The 12 residues surrounding this cleavage site are nearly perfectly conserved in yeast, nematodes, rodents, humans and flies.

Complementation tests show that hybrid lethality is caused by the *D. simulans* (Ds) Nup96 protein and suggest (but do not prove) that hybrid lethality involves its amino terminus. First, mutations that fail to complement hybrid lethality disrupt *D. melanogaster* (Dm) Nup96 but leave DmNup98 intact: two mutations, one with no (F1.13) and one with incomplete (339) complementation, produce truncated DmNup96 and intact DmNup98 (Table 1 and Fig. 2b); similarly, E53.1, which also uncovers hybrid lethality, produces no DmNup96 and a nearly intact DmNup98 (truncated just seven amino acids short of the autoproteolysis cleavage site; Table 1). These findings show that intact DmNup98 is insufficient to rescue hybrid lethality and that disrupted DmNup96 uncovers hybrid lethality. Second, the relevant region of DsNup96 seems to be its N terminus. Hybrid lethality can be rescued by supplying hybrid males with a truncated form of DmNup96 (mutation F1.15, which truncates DmNup96 at residue 1142), indicating that the region between residues 1029 and 1142 is involved in hybrid lethality. Interestingly, this short region contains the only viability-essential portion of the *Nup98-Nup96* yeast homologue, *Nup145* (ref. 24). (That the longer DmNup96-F1.13 mutant allele does not rescue lethality is consistent with the possibility that its rescue is compromised by interfering higher-order structure and/or its intrinsic instability. Note, however, that as increasingly longer mutant alleles of DmNup96 are used—for example, 339, *Df(3R)CA15*—hybrid viability is gradually restored; Table 1.) Together, these findings show that DsNup96 causes hybrid lethality and they are at least consistent with the notion that lethality involves its N terminus.

Adaptive evolution drove the substitutions in *Nup96*

We next studied the evolutionary history of *Nup98-Nup96*. Focusing first on divergence at *Nup98-Nup96* between *D. simulans* and *D. melanogaster*, we used a sliding window method to study rates of non-synonymous (amino-acid changing; K_a) and synonymous (non-amino-acid changing; K_s) divergence across the entire 5.9-kb coding sequence. K_a/K_s ratios greater than 1 represent extremely stringent, but definitive, evidence for positive natural selection[29]. We detect a dramatic peak in divergence with $K_a/K_s > 1$ (Fig. 2c). Although not significantly greater than 1 (the $K_a/K_s > 1$ standard is notoriously conservative), this striking divergence is at least suggestive of a history of positive natural selection in the vicinity of the relevant (incompatible) substitutions. To obtain a more detailed history and to perform a more powerful analysis, we surveyed DNA polymorphisms by sequencing the entire 2.8-kb *Nup96* gene from African populations of *D. melanogaster* and *D. simulans*. We studied alleles sampled from African populations because these are less likely to be confounded by demographic effects seen in recently founded cosmopolitan populations[30]. We analysed 15 alleles of *D. simulans* and 15 of *D. melanogaster* from isofemale lines collected in Zimbabwe. If the *Nup96* gene evolved under strict neutrality, only functionally unconstrained sites should vary within and between species. Thus, the neutral expectation is that the ratio of replacement (*R*) to silent (*S*) polymorphisms within species will be roughly

Table 1 **Mutations disrupting *Nup96* fail to complement hybrid lethality**

Allele	Mutation cDNA position	Nucleotide change	Amino-acid position	Amino-acid change	Affected protein	Hybrid males recovered		*Df/Bal* ratio	Lethal in hybrids?
						Df/sim	*Bal/sim*		
E53.1	**3063**	**TGG to TGA**	**1021**	**W to STOP**	**Nup98**	**5**	**96**	**0.052**	**Yes**
F8.5	3115	GAG to CAG	1039	E to Q	Nup96	83	47	1.766	No
F1.15	3425	TTG to TAG	1142	L to STOP	Nup96	59	35	1.686	No
F1.13	**4600**	**CAG to TAG**	**1534**	**Q to STOP**	**Nup96**	**2**	**35**	**0.057**	**Yes**
339	**5176**	**CAG to TAG**	**1726**	**Q to STOP**	**Nup96**	**19**	**62**	**0.306**	**Partial**
C14.7	–	–	–	–	–	90	26	3.462	No
Df(3R)CA15	≥5074	Deletion	≥1693	Deletion	Nup96	178	162	1.099	No

Mutant alleles of the *D. melanogaster Nup98-Nup96* gene were tested for their ability to uncover hybrid lethality when heterozygous with the *D. simulans* wild-type allele. *D. melanogaster* females heterozygous for a mutation over a dominantly marked balancer were crossed to *D. simulans Lhr* males. The ratio of mutation- to balancer-inheriting hybrid sons was scored (see Fig. 1). Mutations that uncover hybrid lethality are in bold font.

Table 2 **Adaptive evolution caused *Nup96* divergence between species**

		Polymorphic			Divergent				
		R	*S*	*R*/*S* ratio	*R*	*S*	*R*/*S* ratio	*G*-value	*P*-value
1	*D. melanogaster* versus *D. simulans**	27	108	0.250	27	34	0.794	11.888	0.00056
2	*D. melanogaster* versus *D. yakuba*	5	43	0.116	69	152	0.454	9.984	0.00158
3	*D. simulans* versus *D. yakuba*	21	68	0.309	60	139	0.432	1.334	0.24803
4	*D. melanogaster* versus *D. mauritiana*	5	43	0.116	32	51	0.627	13.202	0.00028
5	*D. simulans* versus *D. mauritiana*	22	69	0.319	3	13	0.231	0.233	0.62911
6	*D. melanogaster* lineage†	5	43	0.116	16	21	0.762	12.351	0.0012
7	*D. simulans* lineage	22	69	0.319	10	8	1.250	6.567	0.0104

*Pooled polymorphism data.
†Substitutions that could not be unambiguously assigned to either lineage were excluded.

equal to the *R*/*S* ratio of fixed differences between species[31]. Our data, however, strongly reject neutrality for *Nup96*: there is a highly significant excess of replacement changes fixed between species, indicating a history of adaptive evolution (Table 2, line 1).

After sequencing single alleles from two related species, *D. yakuba* and *D. mauritiana*, we mapped substitutions onto the known phylogeny of the *D. melanogaster* group[32] and, in particular, onto the *D. melanogaster* and *D. simulans* lineages (Fig. 3). Comparing the *R*/*S* ratio of substitutions that occurred in the lineages leading to *D. melanogaster* and *D. simulans* to the *R*/*S* ratio of polymorphisms within each species, respectively, reveals that adaptive evolution drove an excess of replacement substitutions in both species' histories (Table 2, lines 6, 7). Two lines of evidence suggest that these bouts of adaptation occurred in the fairly distant past. First, all substitutions in the *D. simulans* lineage occurred before the split with *D. mauritiana* (that is, >0.26 Myr ago; Fig. 3). Second, there is no evidence of a recent selective sweep in either species. Such an event would leave a signature of reduced nucleotide diversity in the region[33,34] and characteristic patterns in the distribution of allele frequencies—namely, an excess of rare variants[35] and an excess of high-frequency derived variants[36]. However, mean synonymous nucleotide diversities in the coding regions of *Nup96* are typical, if not slightly high, for autosomal loci sampled from African populations[37]: $\pi = 0.0193$ in *D. melanogaster* and $\pi = 0.0285$ in *D. simulans* ($\pi = 4N_e\mu$, where N_e is effective population size and μ is the per-site mutation rate). Moreover, neither species' frequency spectrum deviates significantly from neutral equilibrium expectations using the tests of Tajima[38] and of Fay and Wu[36] ($P \geq 0.335$ in all tests). Therefore, the adaptive substitutions fixed at *Nup96* do not seem to be recent events in either species.

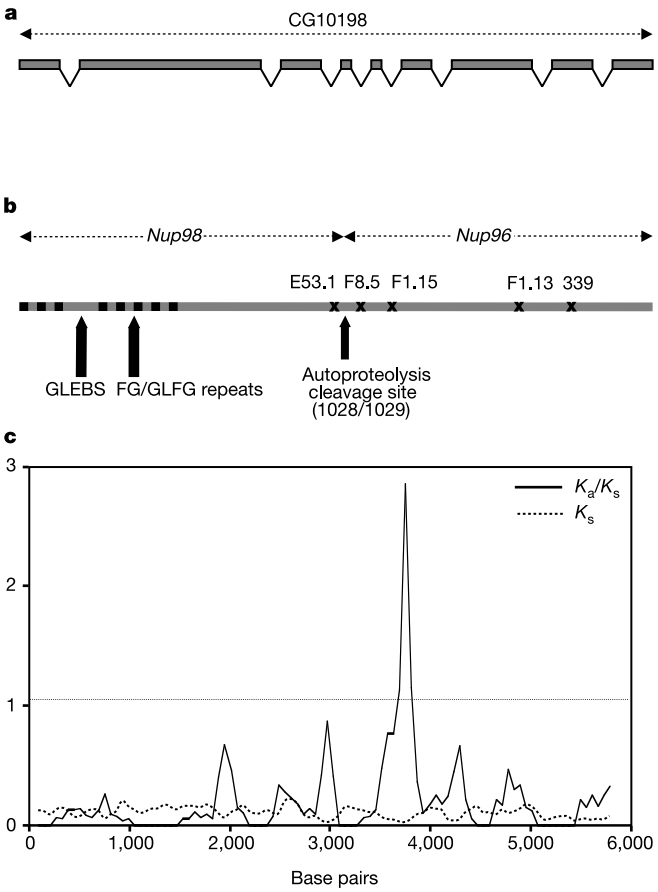

Figure 2 *Nup98-Nup96* structure and evolution. **a**, *Nup98-Nup96* (CG10198) gene structure. **b**, Nup98 and Nup96 protein structure with several conserved motifs indicated: GLE-binding site in Nup98; FG/GLFG repeats in Nup98; and autoproteolysis cleavage site. Locations of molecular lesions in *l(3)95BCd* mutant alleles are indicated by **x**. **c**, Sliding window analysis of the ratio of non-synonymous (K_a) and synonymous (K_s) substitution rates. Window size, 180 base pairs (bp).

Conclusions

Our molecular and population genetic analyses of *Nup96* allow us to address a number of issues in speciation genetics[1,2]. First, a single gene can explain the hybrid lethality of a small chromosomal region identified in our deficiency screen. This finding contrasts with those from studies of hybrid male sterility that have found that several tightly linked incompatibility factors seem to be required to cause complete hybrid sterility[39,40]. Second, *Nup96* is a viability-essential gene within species, performing a fundamental cell-biological function. Thus, this instance of a hybrid incompatibility involves an 'ordinary' gene, not a selfishly propagating genomic parasite (for example, repetitive DNA or transposon) as previous workers have speculated[41]. Third, *Nup96* evolved by positive natural selection in both species' lineages. As an incidental by-product of this adaptation, the *D. simulans* Nup96 protein is no longer compatible with an (unknown) interacting factor(s) encoded by the *D. melanogaster* X chromosome. Fourth, our functional and population genetic data

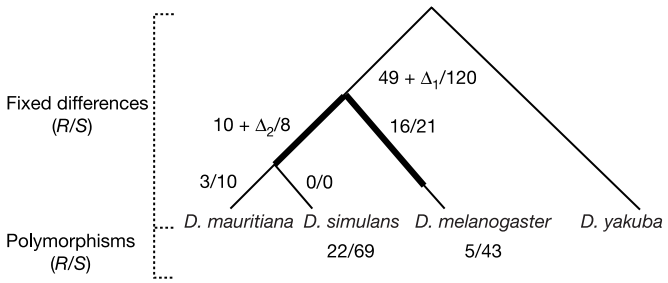

Figure 3 Evolutionary history of *Nup96* with ratios of replacement to silent substitutions mapped onto the known phylogeny of the *D. melanogaster* group species. *R*/*S* ratios of fixed differences are shown on the branches of the phylogeny; polymorphisms in *D. simulans* and *D. melanogaster* are shown at the tips of phylogeny. Bold branches indicate those in which *Nup96* experienced adaptive evolution (for statistics, see Table 2, lines 6, 7). Δ_1, a 12-bp, in-frame indel; Δ_2, a 3-bp, in-frame insertion.

implicate structural divergence in the DsNup96 protein. This case of hybrid inviability does not therefore seem to involve the divergence of *cis*-regulatory sequences[42]. Last, *Nup96* is a single-copy gene. Its role in hybrid inviability could not therefore have evolved in accordance with a recent theory that suggests that hybrid incompatibilities arise as by-products of divergence among duplicate genes[43].

Several questions about our hybrid inviability gene remain. First, as *Nup96* encodes a stable constituent of NPCs, we speculate that nuclear transport is disrupted in inviable hybrid males that are hemizygous for *DsNup96* and that carry an incompatible *D. melanogaster* X chromosome, but not in (viable) hybrid males that carry the co-adapted *D. simulans* X chromosome. It will be of great interest to determine whether the (at present unknown) interacting factor encoded by the X chromosome shows evidence of correlated adaptive evolution. Second, we know nothing about the specific selective forces that caused the adaptive evolution of *Nup96* in each species' history. Indeed, this adaptive evolution is surprising given that NPCs are otherwise remarkably conserved in architecture[44], in the number of nucleoporins[18,19] and in the functions of homologous nucleoporins[16,17] among eukaryotes.

Whether the conclusions drawn from *Nup96* and from the few other hybrid incompatibility genes studied so far will prove general remains to be seen. But the current analysis shows that our screen for recessive hybrid lethals is effective and should yield information on the identities and evolutionary histories of many other hybrid incompatibility genes. □

Methods

Crosses

All crosses were performed at 24 °C and flies were reared on standard cornmeal–yeast–agar medium. Species crosses were made by mass mating 15–20 *D. melanogaster* females to 15–25 *D. simulans* males. *Nup96* was mapped by crossing *D. melanogaster* females heterozygous for a deficiency or a recessive lethal mutation over a dominantly marked balancer (for example, TM3, *Sb*) to *D. simulans* males carrying *Lhr* (Fig. 1). *Lhr* rescues the normally dead hybrid males from this species cross[15], exposing them to other (recessive) hybrid incompatibilities unmasked by the deficiencies or mutations used[11] (Fig. 1). We scored the number of hybrid males inheriting the deficiency or loss-of-function mutation and those inheriting the balancer. Deficiencies were considered lethal when the ratio of deficiency-carrying (or mutation-carrying) hybrid males to balancer-carrying hybrid males was ≤0.10. Deficiency- and balancer-inheriting hybrid females from these crosses were always viable.

We established the genetic breakpoints of *Df(3R)CA15* and *Df(3R)mbc-BG1* by complementation tests within *D. melanogaster*. These deficiencies were produced concurrently with the others described in refs 45, 46. *Df(3R)CA15* fails to complement mutations affecting *l(3)04684* through *l(3)95BCd* (Fig. 1b). We focused on CG10198 as the molecular candidate for *l(3)95BCd* because Southern analysis shows that the distal breakpoint of *Df(3R)CA15* truncates the 3′-end of CG10198 (negative strand) but does not affect the distal-adjacent gene *mbc* (positive strand), thereby splitting these two genes. Sequencing of CG10198 from *l(3)95BCd* mutant lines confirmed the presence of molecular lesions (Table 1).

To test the epistatic basis of the *sim l(3)95BCd* allele's hybrid lethality, the incompatible *mel* X chromosome was replaced with a compatible *sim* X chromosome in hybrid males. This was accomplished using a *mel* attached-X chromosome (two X chromosomes fused to a single centromere) to enforce paternal inheritance of the *sim* X chromosome[11]. (In *Drosophila*, sex is determined by the ratio of the number of Xs to that of each autosome.) Briefly, we crossed *D. melanogaster C(1)M4*, y^2; *l(3)95BCd*$^{E53.1}$/TM3, *Tb Sb* females to *D. simulans Lhr* males. Offspring inheriting the *mel* attached-X chromosome develop as females homozygous for the *D. melanogaster* X, whereas those inheriting their only X from their *sim* father develop as hybrid males hemizygous for the *D. simulans* X chromosome. Note that hybrid sons from this cross inherit their cytoplasm (and all associated maternal factors) from their *D. melanogaster* mothers, so that these hybrid males are genotypically identical to those from the original cross, except at X-linked loci. Hybrids from this cross were scored as above.

Sequencing

Primers designed from the annotated *D. melanogaster* genome sequence, CG10198, were used for polymerase chain reaction (PCR) amplification of genomic DNA, followed by direct sequencing of both strands of the PCR products. Sequencing was done using ABI prism BigDye Chemistry (Perkin Elmer) on an automated ABI sequencer. Lethal mutant alleles of *l(3)95BCd* were balanced over TM3–GFP (green fluorescent protein) chromosomes within *D. melanogaster* and *Nup98-Nup96* was sequenced from genomic DNA extracted from homozygous non-GFP embryos. Wild-type alleles were sequenced from genomic DNA extracted from 15 isofemale lines of *D. melanogaster* collected in Zimbabwe; 15 isofemale lines of *D. simulans* collected in Zimbabwe; *D. simulans Lhr*; *D. mauritiana* 0214-6; and *D. yakuba* Tai 15.

Sequence analysis

All sequences were edited using Sequencher version 3.0 and then manually aligned in SeAl version 1.0. Sliding window estimation of K_a/K_s was performed using *K*-estimator[47]. Fay and Wu's[36] *H*-test was performed using the online program available at http://crimp.lbl.gov/htest.html; all other population genetic analyses were performed using the DnaSP program[48].

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