Lineage-Specific Evolution of the Complex *Nup160* Hybrid Incompatibility Between *Drosophila melanogaster* and Its Sister Species

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ABSTRACT Two genes encoding protein components of the nuclear pore complex *Nup160* and *Nup96* cause lethality in F_2-like hybrid genotypes between *Drosophila simulans* and *Drosophila melanogaster*. In particular, *D. simulans Nup160* and *Nup96* each cause inviability when hemizygous or homozygous in species hybrids that are also hemizygous (or homozygous) for the *D. melanogaster X* chromosome. The hybrid lethality of *Nup160*, however, is genetically complex, depending on one or more unknown additional factors in the autosomal background. Here we study the genetics and evolution of *Nup160*-mediated hybrid lethality in three ways. First, we test for variability in *Nup160*-mediated hybrid lethality within and among the three species of the *D. simulans* clade— *D. simulans*, *D. sechellia*, and *D. mauritiana*. We show that the hybrid lethality of *Nup160* is fixed in *D. simulans* and *D. sechellia* but absent in *D. mauritiana*. Second, we explore how the hybrid lethality of *Nup160* depends on other loci in the autosomal background. We find that *D. simulans Nup160*-mediated hybrid lethality does not depend on the presence of *D. melanogaster Nup96*, and we find that *D. simulans* and *D. mauritiana* are functionally differentiated at *Nup160* as well as at other autosomal factor(s). Finally, we use population genetics data to show that *Nup160* has experienced histories of recurrent positive selection both before and after the split of the three *D. simulans* clade species ~240,000 years ago. Our genetic results suggest that a hybrid lethal *Nup160* allele evolved before the split of the three *D. simulans* clade species, whereas the other autosomal factor(s) evolved more recently.

KEYWORDS speciation; hybrid incompatibility; hybrid inviability; nuclear pore complex

MANY species come to be reproductively isolated through the evolution of genetic incompatibilities that cause intrinsic sterility or inviability in interspecific hybrids (Dobzhansky 1937; Coyne and Orr 2004). Genetic substitutions that are neutral or favorable in the genetic background of one species can be severely deleterious when combined with the genetic background of another species (Dobzhansky 1937; Muller 1940, 1942). Decades of genetic analyses have provided broad support for this so-called Dobzhansky-Muller model—hybrid sterility and inviability typically result from incompatible epistatic interactions between two or more genetic factors (Coyne and Orr 2004). More recently, molecular analyses have turned to identifying the genes involved in hybrid incompatibilities and determining their evolutionary histories, functions within species, and dysfunctions in species hybrids. In *Drosophila*, these analyses have revealed that hybrid incompatibilities are typically genetically complex (involving three or more factors), targets of recurrent positive selection, and involved in genetic conflict with selfish genetic elements (Johnson 2010; Presgraves 2010a, b; Maheshwari and Barbash 2011).

For nearly a century, *Drosophila melanogaster* and *Drosophila simulans* have been important models for the study of hybrid incompatibilities owing to the genetic resources available in *D. melanogaster* (Sturtevant 1920; Provine 1991; Sawamura 2000; Barbash 2010). In crosses between *D. melanogaster* females and *D. simulans* males, the *X*-linked *Hybrid male rescue* (*Hmr*) gene of *D. melanogaster* is incompatible with the autosomal *Lethal hybrid rescue* (*Lhr*) gene of *D. simulans*, killing F_1 hybrid males as late-stage larvae (Brideau *et al.* 2006). Both genes encode DNA-binding proteins that localize to centromeric heterochromatin (Thomae *et al.* 2013), affect expression of transposable elements and satellite DNA (Satyaki *et al.* 2014), and have histories of positive selection (Barbash *et al.* 2004; Brideau *et al.* 2006). The *Hmr-Lhr* hybrid incompatibility is genetically complex, requiring at least one additional

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unknown factor to cause hybrid lethality (Brideau *et al.* 2006). In the reciprocal cross, between *D. simulans* females and *D. melanogaster* males, the *X*-linked Zygotic *hybrid rescue* (*Zhr*) locus of *D. melanogaster* corresponds to a large species-specific pericentric block of 359-bp satellite DNA (Sawamura *et al.* 1995; Ferree and Barbash 2009) that is incompatible with an unidentified maternal factor, known as *maternal hybrid rescue* (*mhr*), present in many *D. simulans* lines (Sawamura *et al.* 1993; Orr 1996; Gerard and Presgraves 2012), killing F_1 hybrid females as embryos (Hadorn 1961). Selfish repetitive DNA is implicated in the evolution of both *Hmr-Lhr* and *mhr-Zhr* hybrid incompatibilities.

At each of these loci, rescue mutations (compatible alleles) have been recovered that can rescue hybrids from lethality and, for some, hybrid female sterility (Watanabe 1979; Hutter and Ashburner 1987; Davis *et al.* 1996; Barbash and Ashburner 2003). These rescue mutations, when combined with other *D. melanogaster* tools (Sawamura *et al.* 2000; Presgraves 2003; Masly *et al.* 2006), have facilitated the mapping and identification of three additional hybrid incompatibility genes that affect F_2-like hybrid genotypes. The male fertility-essential gene *JYalpha* has transposed from chromosome *4* in *D. melanogaster* to chromosome *3* in *D. simulans*, so hybrids homozygous for *D. simulans* chromosome *4* in an otherwise *D. melanogaster* genetic background completely lack *JYalpha* and are male sterile (Masly *et al.* 2006). And two *D. simulans* genes, *Nup160* and *Nup96*, are incompatible with an unidentified factor(s) on the *D. melanogaster X* chromosome, causing hybrid lethality (Presgraves *et al.* 2003; Tang and Presgraves 2009; Sawamura *et al.* 2010).

Nup160 and *Nup96* both encode protein components of the nuclear pore complex (NPC). The NPC mediates all molecular traffic between the cytoplasm and nucleus and interacts with DNA to regulate gene expression and chromatin organization (Capelson *et al.* 2010; Kalverda and Fornerod 2010; Liang and Hetzer 2011; Grossman *et al.* 2012). Its ~30 different protein constituents (termed *nucleoporins*), protein-protein interactions, and overall architecture are largely conserved among eukaryotes (Bapteste *et al.* 2005; Neumann *et al.* 2010). Despite these deeply conserved functions, nucleoporins present some of the strongest evidence for recurrent adaptive protein evolution in the *Drosophila* genome (Begun *et al.* 2007; Presgraves and Stephan 2007; Langley *et al.* 2012; Nolte *et al.* 2013; Garrigan *et al.* 2014). *Nup160* and *Nup96* have histories of recurrent positive natural selection in both the *D. melanogaster* and *D. simulans* lineages (Presgraves *et al.* 2003; Tang and Presgraves 2009), leading to speculation that these and other nucleoporins have engaged in antagonistic co-evolutionary interactions with retroviruses, retrotransposons, or meiotic drive elements (Presgraves 2007; Presgraves and Stephan 2007). *Nup160* is part of a complex genetic incompatibility, with hybrid lethality requiring the appropriate genotype at three or more loci. Hybrids die when homozygous or hemizygous for the *D. melanogaster X* chromosome (hereafter X^{mel}), homozygous or hemizygous for *D. simulans Nup160* (hereafter $Nup160^{sim}$),

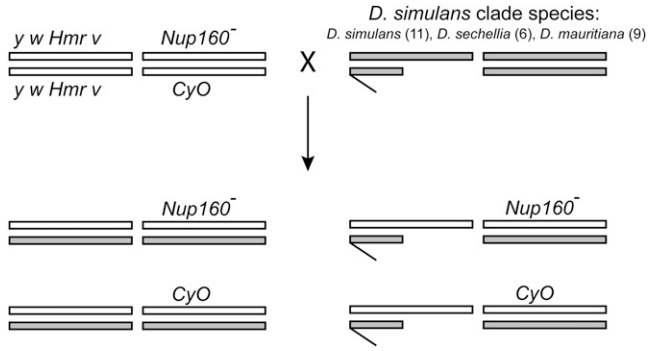

Figure 1 Testing for *Nup160*-mediated lethality in hybrid males between *D. melanogaster* and the three *D. simulans* clade species. *D. melanogaster y w Df(1)Hmr v*; *Nup160*$^{-}$*/CyO* females were crossed to males from multiple isofemale lines of *D. simulans* (*n* = 11), *D. sechellia* (*n* = 6), and *D. mauritiana* (*n* = 9). For each, sex chromosome and second chromosome genotypes are shown (third and fourth chromosomes not shown), with *D. melanogaster* = white and *D. simulans*, *D. sechellia*, or *D. mauritiana* = gray.

and heterozygous for the autosomes. Changing the genotype at any one of these loci—*e.g.*, introducing a $Nup160^{mel}$ transgene, replacing X^{mel} with X^{sim}, or making the autosomal background homozygous *D. melanogaster*—suppresses hybrid lethality (Sawamura *et al.* 2004, 2010; Tang and Presgraves 2009).

In this report we further characterize the genetics and evolutionary history of the *Nup160* hybrid incompatibility. First, we test for variability in *Nup160*-mediated hybrid lethality among the three species of the *D. simulans* clade—*D. simulans*, *D. sechellia*, and *D. mauritiana*—which diverged from one another ~240,000 years ago (Kliman *et al.* 2000; Garrigan *et al.* 2012). We find that *Nup160*-mediated lethality occurs in hybrids from crosses of *D. melanogaster* with *D. simulans* and *D. sechellia* but not *D. mauritiana*. Interestingly, the same holds for *Nup96* (Barbash 2007). Second, we test whether *D. simulans* and *D. mauritiana* have functionally diverged at *Nup160* and/or at a different, genetically unlinked autosomal locus required to kill *D. melanogaster* hybrids. Third, because the NUP96 and NUP160 proteins physically interact at the NPC as part of the NUP107 subcomplex (Belgareh *et al.* 2001; Lutzmann *et al.* 2002), we test whether *Nup160*-mediated lethality depends on the hybrid genotype at *Nup96* (and vice versa). Finally, we investigate the recent molecular evolutionary history of *Nup160* among the three *D. simulans* clade species. Our genetic analyses suggest that a *Nup160* allele with the capacity to cause hybrid lethality evolved before the split of the three *D. simulans* clade species but that only *D. simulans* and *D. sechellia* possess the additional autosomal factor(s) required for hybrid lethality.

Materials and Methods

Stocks, nomenclature, and crosses

The *PiggyBac* transposable element insertion *PBac{RB}RfC38*^{e00704} (obtained from the Exelixis Collection at Harvard Medical School) disrupts the overlapping 3′-untranslated

Table 1 *Nup160*-mediated hybrid lethality is fixed in *D. simulans* and *D. sechellia* but absent in *D. mauritiana*

	Hybrid females			Hybrid males			
Male parent[a]	*Nup160*$^-$	*CyO*	Relative viability	*Nup160*$^-$	*CyO*	Relative viability	Percent rescue
D. simulans							
sim 005	24	47	0.51**	0	20	0.00**	42.6
sim 007	32	50	0.64*	0	21	0.00**	42.0
sun 017	34	35	0.97	0	15	0.00**	42.9
sim 019	50	59	0.85	0	18	0.00**	30.5
sim 026	34	44	0.77	0	14	0.00**	31.8
sim 036	59	49	1.20	0	21	0.00**	42.9
sim 049	14	27	0.52*	0	14	0.00**	51.9
sim 058	21	33	0.64	0	16	0.00**	48.5
sim 065	37	46	0.80	0	20	0.00**	43.5
sim 224	48	77	0.62**	0	32	0.00**	41.6
sim w501	69	83	0.83	0	27	0.00**	32.5
Total	422	550	0.77**	0	218	0.00**	39.6
D. sechellia							
sec Robertson	52	65	0.80	3	31	0.10**	47.7
sec Praslin	44	56	0.79	0	18	0.00**	32.1
sec w	39	33	1.18	1	15	0.07**	45.5
sec sy007	45	57	0.79	2	33	0.06**	57.9
sec sy034	46	88	0.52**	1	30	0.03**	34.1
sec iso81	33	63	0.52**	1	16	0.06**	25.4
Total	259	362	0.72**	8	143	0.06**	39.5
D. mauritiana							
mau iso105	49	50	0.98	17	15	1.13	30.0
mau iso152	58	62	0.94	30	24	1.25	38.7
mau R11	70	53	1.32	22	16	1.38	30.2
mau R23	41	36	1.14	8	19	0.42*	52.8
mau R35	39	43	0.91	13	19	0.68	44.2
mau R45	47	45	1.04	17	11	1.55	24.4
mau R48	53	49	1.08	23	18	1.28	36.7
mau R57	30	30	1.00	13	16	0.81	53.3
mau w	215	205	1.05	82	73	1.12	35.6
Total	602	573	1.05	225	211	1.07	36.8

* $P < 0.05$, ** $P < 0.01$, χ^2-tests.
[a] For all crosses, *D. melanogaster y w Df(1)Hmr v*; *Nup160*$^-$*/CyO* females were crossed to *D. simulans*, *D. sechellia*, or *D. mauritiana* males.

regions of two genes, *Nup160* and *RfC38* (Thibault *et al.* 2004), and causes lethality in *D. melanogaster–D. simulans* hybrids that are hemizygous ($X^{mel}Y^{sim}$) or homozygous for the *D. melanogaster X* chromosome ($X^{mel}X^{mel}Y^{sim}$). The *Nup98*$^{E53.1}$ mutation disrupts the protein-coding sequence corresponding to the NUP96 protein and, similarly, causes lethality in *D. melanogaster–D. simulans* hybrids that are hemizygous ($X^{mel}Y^{sim}$) or homozygous for the *D. melanogaster X* chromosome ($X^{mel}X^{mel}Y^{sim}$). For simplicity, we hereafter refer to *PBac{RB}RfC38*^{e00704} as *Nup160*$^-$ and *Nup98*$^{E53.1}$ as *Nup96*$^-$. To rescue F_1 hybrid males resulting from crosses between *D. melanogaster* females and *D. simulans*, *D. sechellia*, and *D. mauritiana* males, we used the *y w Df(1) Hmr v* chromosome (provided by D. Barbash, Cornell University), which bears a partial deletion of *Hybrid male rescue* (*Hmr*) that removes the first 96 amino acids of the HMR protein (Barbash and Lorigan 2007). To rescue hybrid males in a different cross, we used *D. simulans w*; *Lhr*^{1}, which has a mutation in the *Lethal hybrid rescue* (*Lhr*) gene. To test for variation in *Nup160*-mediated hybrid lethality within and among the *D. simulans* clade species, we used 10 *D. simulans* isofemale lines collected from Zimbabwe (provided by C. Aquadro, Cornell University), 6 *D. sechellia* isofemale lines (provided by J. Coyne, University of Chicago, and from the Tucson Stock Center), and 8 *D. mauritiana* isofemale lines (provided by J. Coyne, University of Chicago, and M. Ramos Womack, Princeton University).

To introgress *Nup160*^{sim} from *D. simulans* into *D. mauritiana white (w)*, we used a *D. simulans* strain with a tightly linked *PiggyBac* transgene bearing the visible dominant marker *eYFP* inserted on chromosome arm *2L* (provided by D. Stern, Janelia Farm Research Campus, Howard Hughes Medical Institute). This *PBac{eYFP}* insert is at genomic sequence coordinate *2L*:11,756,533 (D. Stern, personal communication), and *Nup160* is at *2L*:11,123,777–11,129,335 (on the *D. melanogaster* reference assembly version R6.02), placing the *YFP* marker ~627 kb proximal to *Nup160*. We crossed *mau w* females to *sim YFP* males and collected fertile hybrid *sim YFP/mau* females; we then backcrossed *sim YFP/mau* virgin females to *mau w* males recurrently for six generations. After generation six, the *sim YFP* introgression was maintained by selection through males (without further recombination). To make the complementary introgression of *Nup160*^{mau} from *D. mauritiana* into *D. simulans w*^{501}, we used two *D. mauritiana* strains that

each have a *P*-element transgene bearing the dominant visible mini-w^+ marker on chromosome arm *2L* (True *et al.* 1996). These insertions, *P{w⁺}4G4C* and *P{w⁺}4G5*, are located at coordinates *2L*:12,700,666 and *2L*:12,700,661 (Araripe *et al.* 2006), placing them both ~1.57 Mb proximal to *Nup160*. Briefly, we crossed *sim* w^{501} females to *mau P{w⁺}* males and collected fertile hybrid *mau P{w⁺}/sim* females; we then backcrossed *mau P{w⁺}/sim* virgin females to *sim* w^{501} males recurrently for six generations. After generation six, the *mau P{w⁺}* introgressions were maintained by selection through males (without further recombination). We confirmed the presence of $Nup160^{sim}$ and $Nup160^{mau}$ alleles in the introgression lines by sequencing a 550-bp PCR amplicon from exon 6 of *Nup160* (details later).

All crosses were done at 22–23°. For each cross, at least two replicates were set up with ~20 virgin females and ~25 males. After larvae appeared, parents were transferred to fresh vials every 3 days for as long as they continued to produce progeny. Hybrid progeny were scored for at least 14 days after eclosion of the first fly until no more flies eclosed for 3 consecutive days.

Lethal phase

To determine the lethal phase of *Nup160*-mediated lethality, we crossed *D. melanogaster y w*; *Nup160⁻/CyO* females to *D. simulans Lhr* males. Hybrid male larvae from this cross have yellow mouthparts, whereas hybrid female larvae have black (wild-type) mouthparts. We collected hybrid larvae at the third instar stage, separated them by sex, transferred them to vials with fresh food, and then, following pupation, checked whether the pupae were alive or dead every ~12 hr.

Sequencing of Nup160 and molecular population genetics analysis

To sequence *Nup160* from *D. mauritiana* and *D. sechellia* lines, we first extracted genomic DNA from single females using the PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis, MN). The entire ~5.1-kb coding region of the *Nup160* gene was PCR amplified with the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN). Both strands of the PCR products were sequenced directly with internal primers with ABI Prism BigDye Terminator chemistry (Applied Biosystems, Foster City, CA) on an automated ABI sequencer at the University of Rochester Functional Genomics Core Facility. All sequences were assembled and manually checked using Sequencher v. 4.5 (Genecodes, Ann Arbor, MI) and then manually aligned in Se-Al v. 2.0 (http://tree.bio.ed.ac.uk/software/seal/). Sequences have been deposited in GenBank under accession numbers KR817621–KR817638. *Nup160* sequences from *D. melanogaster* and *D. simulans* are from Tang and Presgraves (2009) (GenBank accession numbers FJ600378–FJ600401). All population genetics analyses were performed with DnaSP v.5 (Librado and Rozas 2009).

Table 2 $X^{mel}Y^{sim}$; *Nup160⁻/sim* hybrid males die as pupae at ~60 hr of development

	Hybrid males (*n* = 29)		Hybrid females (*n* = 36)	
Time (hrs)[a]	Dead	Eclosed	Dead	Eclosed
12–48	0	0	0	0
60	11	0	0	0
72	1	0	0	0
84–108	0	0	0	0
120	0	9	0	34
132	0	8	2	0
Totals	12	17	2	34

[a] Hybrid third instar larvae were collected, separated by sex, and checked every 12 hr for lethality (see *Materials and Methods*).

Results

Effect of Nup160 in hybrids between D. melanogaster and the three D. simulans clade species

We first tested whether *Nup160*-dependent lethality is fixed in *D. simulans*. We crossed *D. melanogaster y w Df(1)Hmr v*; *Nup160⁻/CyO* females to *D. simulans* (*sim*) males from 11 isofemale lines (Figure 1). The resulting *Nup160⁻* hybrids must develop using only wild-type *D. simulans Nup160*. We found that the viability of the resulting *Nup160⁻/sim* hybrid females was lower than that of *CyO/sim* hybrid females in all but one cross (*sim 036*) and significantly lower in four crosses (Table 1). Overall, the relative viability of *Nup160⁻/sim* hybrid females is ~75% that of *CyO/sim* hybrid females. While we recovered abundant *CyO/sim* hybrid males, we failed to recover *any Nup160⁻/sim* hybrid males (Table 1). These results confirm that *Nup160*-mediated lethality occurs in *Hmr*-bearing hybrid males and is not therefore a special property of the *D. simulans Lhr*[1] genotypes used in previous analyses (Tang and Presgraves 2009; Sawamura *et al.* 2010). These results also show that *Nup160*-dependent hybrid lethality appears to be fixed in *D. simulans* (Table 1).

To determine the lethal phase of the *Nup160* incompatibility, we crossed *D. melanogaster y w*; *Nup160⁻/CyO* females to *D. simulans w*; *Lhr*[1] males, collected and separated hybrid male and female larvae based on the color of the larval mouthparts, and then scored viability of pupae every 12 hr. At 60–72 hr after pupa formation, about half the male pupae (12 of 29) were dead, as evidenced by deterioration of morphological structures and darkening of tissue (Table 2). All remaining 17 males eclosed at ~132 hr after pupa formation, and all were *CyO/sim*. Among hybrid females, 34 of 36 eclosed at ~120 hr after pupa formation, and 15 were *CyO/sim*. We checked the two dead female pupae by opening the pupal cases and found that unlike the dead male pupae, they were fully developed but failed to eclose (their wing phenotypes could not be scored). These findings show that *Nup160*-mediated hybrid lethality occurs at an early pupal stage [consistent with the findings of Maehara *et al.* (2012)], prior to the establishment of adult morphological structures. *Nup160*-mediated lethality thus occurs later than *Hmr-Lhr*-mediated hybrid lethality (late-stage larvae) (Sturtevant 1920) but earlier than *Nup96*-mediated

Table 3 *Nup160*-mediated hybrid lethality does not depend on *Nup96* genotype, and vice versa

		Autosomal genotype			
Replicate	Hybrid sex	*CyO/sim*; *Nup96^{-}/sim*	*CyO/sim*; *TM3,Ser/sim*	*Nup160^{-}/sim*; *Nup96^{-}/sim*	*Nup160^{-}/sim*; *TM3,Ser/sim*
1	X^{mel}/Y^{sim}	0	29	0	0
	X^{mel}/X^{sim}	31	40	50	40
2	X^{mel}/Y^{sim}	1	32	0	0
	X^{mel}/X^{sim}	48	44	57	58
3	X^{mel}/Y^{sim}	0	18	0	0
	X^{mel}/X^{sim}	31	47	56	43
Total	X^{mel}/Y^{sim}	1	79	0	0
	X^{mel}/X^{sim}	110	131	163	141

hybrid lethality (late-stage pupae to pharate adult) (Barbash 2007).

We next tested whether *Nup160*-mediated hybrid lethality occurs in *D. melanogaster–D. sechellia* hybrids and *D. melanogaster–D. mauritiana* hybrids. We crossed *D. melanogaster y w Df(1)Hmr v*; *Nup160^{-}/CyO* females to males from six *D. sechellia* isofemale lines and nine *D. mauritiana* isofemale lines. *Nup160^{-}/sech* hybrid females show reduced relative viability in five of six crosses, two significantly so (~72%) (Table 1), and *Nup160^{-}/sech* hybrid males are effectively lethal (relative viability ~6%) (Table 1). These results show that *Nup160*-mediated hybrid lethality is fixed in *D. sechellia* and behaves similarly to *D. simulans*, with moderate effects in hybrid females and lethal effects (or nearly so) in hybrid males. In contrast, *Nup160^{-}/mau* hybrid females and *Nup160^{-}/mau* hybrid males show no reduced viability relative to control *CyO/mau* siblings (Table 1). Interestingly, the effects of *Nup160*— lethal in $X^{mel}Y^{sim}$ and $X^{mel}Y^{sech}$ hybrids but not in $X^{mel}Y^{mau}$ hybrids—parallels that observed for *Nup96* (Barbash 2007). These results together raised the possibility that the *Nup160* and *Nup96* hybrid incompatibilities may not be genetically independent.

The hybrid lethal effect of Nup160^{sim} does not require the presence of Nup96^{mel}

Given that the NUP96 and NUP160 proteins are predicted to physically interact with one another at the NPC (Belgareh *et al.* 2001; Lutzmann *et al.* 2002), we tested the possibility that the lethal effect of *Nup160*^{sim} in hybrid males specifically requires the presence of *Nup96*^{mel}. We constructed *D. melanogaster Nup160^{-}/CyO*; *Nup96^{-}/TM3*, *Ser* double-mutant females and crossed them to *D. simulans w*; *Lhr*^{1} males. This cross produces four hybrid female genotypes and four hybrid male genotypes. All four hybrid female genotypes are viable, occurring in expected ratios (Table 3). And, as expected, hybrid males with both the *Nup160^{-}/sim*; *TM3*, *Ser/sim* and *CyO/sim*; *Nup96^{-}/sim* genotypes are inviable (Table 3) (Presgraves *et al.* 2003; Tang and Presgraves 2009; Sawamura *et al.* 2010; Maehara *et al.* 2012). If *Nup160*^{sim} requires *Nup96*^{mel} to cause hybrid lethality (or vice versa), then double-mutant *Nup160^{-}/sim*; *Nup96^{-}/sim* hybrid males ought to be viable. Instead, we failed to recover any hybrid males with the genotype *Nup160^{-}/sim*; *Nup96^{-}/sim*. We conclude that *Nup160*^{sim} does not require *Nup96*^{mel} (and, similarly, that *Nup96*^{sim} does not require *Nup160*^{mel}) to kill hybrids. These findings are consistent with two possibilities: *Nup160*^{sim} and *Nup96*^{sim} behave as loss-of-function alleles in the hybrid genetic background or *Nup160*^{sim} and *Nup96*^{sim} have neomorphic lethal effects in hybrids, but these do not require the presence of *Nup160*^{mel} and/or *Nup96*^{mel}.

Lineage-specific Nup160-mediated hybrid lethality depends on Nup160 and autosomal background

Previous work established that the *Nup160* hybrid incompatibility is complex, requiring (1) hemizygosity (or homozygosity) for X^{mel}, (2) hemizygosity (or homozygosity) for *Nup160*^{sim}, and (3) at least one unknown dominant factor in the *D. simulans* autosomes (Sawamura *et al.* 2004, 2010, 2014). Our finding that *Nup160*-mediated lethality occurs in *D. simulans–D. melanogaster* hybrid males but not in *D. mauritiana–D. melanogaster* hybrid males therefore raises two nonexclusive possibilities: *D. simulans* and *D. mauritiana* are functionally differentiated at *Nup160* and/or at some other autosomal factor(s) essential for hybrid lethality.

To test these possibilities, we performed reciprocal introgression experiments. First, we introgressed *Nup160*^{mau} (marked with a tightly linked dominant visible marker, *P{w^{+}}*; see *Materials and Methods*) into an otherwise *D. simulans* genetic background, backcrossing through fertile hybrid females for six generations. We then crossed *D. melanogaster y w Df(1)Hmr v*; *Nup160^{-}/CyO* females to *D. simulans* males heterozygous for the introgression *sim w*^{501}*/Y*; *Nup160*^{mau} *P{w^{+}}/Nup160*^{sim}. This cross yields four hybrid female genotypes and four hybrid male genotypes: non-*P{w^{+}}* hybrids are *Nup160^{-}/Nup160*^{sim} and *CyO/Nup160*^{sim}, and *P{w^{+}}* hybrids are *Nup160^{-}/Nup160*^{mau} and *CyO/Nup160*^{mau}. As Table 4 shows, *Nup160^{-}/Nup160*^{mau} and *Nup160^{-}/Nup160*^{sim} hybrid male genotypes are both inviable. This finding shows that *Nup160*^{mau} causes complete hybrid lethality when combined with at least one other dominant autosomal factor from *D. simulans*. Because *Nup160*^{mau} does not cause hybrid lethality when on its own autosomal background, we conclude that at least one autosomal background factor required for hybrid lethality has functionally diverged between *D. mauritiana* and *D. simulans*.

Table 4 *Nup160*-mediated hybrid lethality depends on species-specific allele and genetic background

	Hybrid females			Hybrid males			
	Nup160^{-}	*CyO*	Relative viability	*Nup160*$^{-}$	*CyO*	Relative viability	Percent rescue[b]
Nup160^{mau} *P{w+}4G4C*	46	47	0.98	0	27	0.00*	57.4
Nup160^{sim}	54	40	1.35	0	22	0.00*	55.0
Nup160^{mau} *P{w+}4G5*	111	68	1.63*	0	68	0.00*	100.0
Nup160^{sim}	124	92	1.35	0	82	0.00*	89.1
Nup160$^{sim.YFP}$	282	271	1.04	19	76	0.25*	28.0
Nup160^{mau}	325	297	1.09	155	142	1.09	47.8

[a] Alternative second chromosomes transmitted by *Nup160* introgression males crossed to *D. melanogaster Hmr*; *Nup160*$^{-}$/*CyO* females.
[b] Percent rescue = (number of *CyO* hybrid males/number of *CyO* hybrid females) × 100.
*$P<0.05$, χ^2-test.

Next, we introgressed *Nup160*^{sim} (marked with a tightly linked dominant visible marker, *PBac{eYFP}*; see *Materials and Methods*) into a largely *D. mauritiana* genetic background, backcrossing through fertile hybrid females for six generations. We then crossed *D. melanogaster y w Df(1)Hmr v*; *Nup160*$^{-}$*/CyO* females to *D. mauritiana* males heterozygous for the introgression *mau w/Y*; *Nup160*^{sim} *YFP/Nup160*^{mau}. This cross yields four hybrid female genotypes and four hybrid male genotypes: non-*YFP* hybrids are *Nup160*$^{-}$*/Nup160*^{mau} and *CyO/Nup160*^{mau}, and *YFP* hybrids are *Nup160*$^{-}$*/Nup160*^{sim} and *CyO/Nup160*^{sim}. As Table 4 shows, *Nup160*$^{-}$*/Nup160*^{mau} (non-*YFP*) hybrid males are viable, but *Nup160*$^{-}$*/Nup160*^{sim} (*YFP*) hybrid males are sublethal (relative viability ~25%). These findings show that the *Nup160* alleles of the two species are functionally divergent: *Nup160*^{sim} causes partial lethality in a *D. mauritiana* autosomal background, but *Nup160*^{mau} does not. The fact that *Nup160*^{sim} causes incomplete hybrid lethality in a *D. mauritiana* autosomal background (as opposed to the complete lethality observed in a *D. simulans* autosomal background) (Table 1) further suggests that the autosomal background factor(s) also has functionally diverged between *D. simulans* and *D. mauritiana*. However, we cannot exclude the possibility that an unknown autosomal factor, tightly linked to the *Nup160* locus, was co-introgressed with *Nup160*^{sim}, facilitating its hybrid lethal effect.

Molecular evolution of Nup160 in the D. simulans clade

Since the split of *D. melanogaster* and *D. simulans*, both lineages have experienced recurrent positive selection at *Nup160* (Table 5, line 1) (Tang and Presgraves 2009). Here we extend these analyses by surveying polymorphism and divergence at *Nup160* (~5.1 kb) from 8 *D. sechellia* and 10 *D. mauritiana* lines. McDonald-Kreitman (MK) tests (McDonald and Kreitman 1991) reject the neutral hypothesis because *Nup160* has an excess of fixed nonsynonymous differences among all three pairs of *D. simulans* clade species in pooled analyses (Table 5, lines 2–4). With *D. yakuba Nup160* as a distant outgroup species, we parsimony-mapped nonsynonymous and synonymous substitutions onto five branches of the species phylogeny: the *D. melanogaster* terminal branch, the *D. simulans* clade ancestor internal branch, the *D. simulans* terminal branch, the *D. sechellia* terminal branch, and the *D. mauritiana* terminal branch. Codons experiencing multiple substitutions over the five-species history were excluded from the branch-specific analyses because they cannot be mapped unambiguously to particular branches. Using mapped substitutions, we asked whether recurrent adaptive evolution occurred in all lineages or in a subset. Furthermore, by mapping substitutions to the internal branch of the *D. simulans* clade ancestor, we asked whether *Nup160* experienced positive selection before, after, or before *and* after the split of the three *D. simulans* clade species ~240,000 years ago (Garrigan *et al.* 2012). To perform the MK test for the internal branch, we assumed that the population of the *D. simulans* clade ancestor had the same numbers of nonsynonymous and synonymous polymorphisms as found in the extant *D. simulans* population (Table 5, line 6). These branch-specific MK tests provide strong evidence for recurrent adaptive evolution at *Nup160* in the common ancestor of the *D. simulans* clade (prior to 240,000 years ago) as well as within *D. simulans* (since ~240,000 years ago) (Table 5, lines 6 and 7). The MK tests for *D. mauritiana* and *D. sechellia* lineages did not reject the neutral null hypothesis (Table 5, lines 8 and 9). None of the three *D. simulans* clade species showed evidence of a recent and/or strong selective sweep: mean silent nucleotide diversity at *Nup160* is comparable to that of other autosomal loci in these species (Kliman *et al.* 2000; Legrand *et al.* 2011), and neither Tajima's *D* (Tajima 1989) nor Fay and Wu's *H* (Fay and Wu 2000), two summaries of the site-frequency spectra, revealed significant deviations from standard neutral expectations in any of the three species (Table 6).

Discussion

Our work reveals two main findings. The first is that *Nup160*-mediated lethality in hybrids between *D. melanogaster* and its sibling species is fixed in *D. simulans* ($n = 11$) and in *D. sechellia* ($n = 6$) but absent from *D. mauritiana* ($n = 9$). Previous work established that the lethal hybrid incompatibility between *Nup160*^{sim} and *X*^{mel} requires at least one additional (unknown) dominant autosomal factor from *D. simulans* (Sawamura *et al.* 2004, 2010). Consistent with this result, our second main finding is that *Nup160 and* at least one additional autosomal factor required for hybrid lethality are functionally divergent between *D. simulans* and *D. mauritiana*:

Table 5 Evidence for lineage-specific recurrent adaptive protein evolution at *Nup160*

		Polymorphic			Divergent			
Line		R	S	R/S	R	S	R/S	Fisher's exact *P*-value
1	*D. melanogaster–D. simulans* pooled[a]	27	154	0.175	58	64	0.906	9.3×10^{-10}
2	*D. simulans–D.mauritiana* pooled	34	168	0.202	20	4	5.000	8.2×10^{-11}
3	*D. simulans–D. sechellia* pooled	19	107	0.178	27	20	1.350	1.1×10^{-7}
4	*D. sechellia–D. mauritiana* pooled	19	87	0.218	12	18	0.667	0.015
5	*D. melanogaster* lineage	10	56	0.179	18	32	0.563	0.015
6	*D. simulans* clade ancestral lineage	17[b]	100[b]	0.170	24	15	1.600	4.4×10^{-8}
7	*D. simulans* lineage	17	100	0.170	12	3	4.000	4.4×10^{-7}
8	*D. sechellia* lineage	2	7	0.286	8	6	1.333	0.197
9	*D. mauritiana* lineage	34	168	0.202	1	0	—	0.172

[a] *D. melanogaster* and *D. simulans* data are from Tang and Presgraves (2009).
[b] For this MK test, the *D. simulans* clade ancestral population is assumed to have the same numbers of nonsynonymous and synonymous polymorphisms as the extant *D. simulans* population sample.

Nup160^{mau} kills hybrids when in a *D. simulans* autosomal background but not its own (Tables 1 and 4), showing that the *D. simulans* and *D. mauritiana* autosomal backgrounds are functionally different, and in a *D. mauritiana* autosomal background, *Nup160*^{sim} causes partial hybrid lethality, but *Nup160*^{mau} does not (Tables 1 and 4), showing that the two *Nup160* alleles are functionally different.

Our genetic analyses allow several inferences about the phylogenetic history of the *Nup160* hybrid incompatibility. For *Nup160*, the alleles of all three *D. simulans* clade species can cause hybrid lethality (*Nup160*^{sim} and *Nup160*^{sech} on their respective autosomal backgrounds and *Nup160*^{mau} when introgressed into a *D. simulans* autosomal background). Therefore, the capacity of *Nup160* to cause hybrid lethality almost certainly evolved in the common ancestor of the *D. simulans* clade species. For the autosomal background factor(s), our finding that the *Nup160* hybrid incompatibility kills hybrids from crosses with *D. simulans* and *D. sechellia* but not *D. mauritiana* raises three possibilities. First, the autosomal factor(s) evolved in the common ancestor of all three species but was reversed subsequently in *D. mauritiana*. This scenario seems doubtful, requiring the incidental chance reversal of hybrid incompatibility (there is no selection favoring compatibility of *Nup160*^{mau} with *D. melanogaster*). Second, the autosomal factor(s) is shared in *D. simulans* and *D. sechellia* owing to common ancestry—it either evolved in the common ancestor of these two species after the split from *D. mauritiana* or it evolved in one species and was exported to the other via gene flow (Garrigan *et al.* 2012; Matute and Ayroles 2014). Third, the autosomal factor(s) in *D. simulans* and *D. sechellia* may have converged independently on hybrid lethality. The second and third scenarios both imply that the necessary components of the complex *Nup160* hybrid incompatibility evolved more recently than ~240,000 years ago.

Like *Nup160*, *Nup96*-mediated hybrid lethality is lineage specific, genetically complex, and likely of relatively recent origin. *Nup96*^{sim} *and Nup96*^{sech} cause hybrid lethality when combined with hemizygous (or homozygous) X^{mel}, but *Nup96*^{mau} does not (Presgraves *et al.* 2003; Barbash 2007). However, the fact that *Nup96* in *D. simulans* has experienced no nonsynonymous substitutions since its split from *D. mauritiana* implies that *Nup96*^{sim} and *Nup96*^{mau} are functionally equivalent (Presgraves *et al.* 2003). Therefore, at least one additional unknown autosomal factor must be present in *D. simulans* that is absent in *D. mauritiana* (Barbash 2007). These considerations suggest that some components of the *Nup96* hybrid incompatibility also evolved after the split of the *D. simulans* clade species. It appears, then, that both *Nup160* and *Nup96* hybrid incompatibilities evolved well after the species split of *D. melanogaster* and the *D. simulans* clade ancestor and therefore were inconsequential to any reproductive isolation realized in natural populations.

The *Nup160* and *Nup96* hybrid incompatibilities evolved at similar times, have comparable hybrid lethal effects among the three *D. simulans* clade species, are both part of complex multicomponent hybrid incompatibilities, and produce proteins predicted to interact directly at the NPC. It is therefore tempting to speculate that these two hybrid incompatibilities are not independent. While *Nup160* and *Nup96* hybrid incompatibilities may have evolved for similar, nonindependent reasons—most simply, *e.g.*, as incidental by-products of NPC evolution (see later)—their hybrid lethal effects appear genetically independent in two ways. First, the lethality of our double-mutant hybrid males shows that *Nup160*^{sim} does not require the presence of *Nup96*^{mel}, nor does *Nup96*^{sim} require the presence of *Nup160*^{mel} (Table 3).

Table 6 Summaries of DNA sequence polymorphism at *Nup160* in four *Drosophila* species

Species	n[a]	bp[b]	S[c]	π[d]	π_{silent}[e]	Tajima's *D*	*FWH*[f]
D. melanogaster	12	5037	96	0.0059	0.0151	−0.315	−0.839
D. simulans	12	5009	188	0.0128	0.0328	0.102	0.192
D. sechellia	8	5037	8	0.0007	0.0016	−0.312	0.432
D. mauritiana	10	5024	151	0.0095	0.0244	−0.595	0.221

[a] n = number of chromosomes sampled from each species.
[b] bp = number of nucleotides in the intraspecies alignment.
[c] S = the number of segregating sites.
[d] π = average nucleotide diversity at all sites.
[e] π_{silent} = average nucleotide diversity at silent sites.
[f] *FWH* = normalized Fay & Wu's *H*.

Second, in an otherwise *purely D. melanogaster* genetic background, homozygous (or hemizygous) *Nup160*^{sim} is not lethal in *Nup96*^{sim}/*Nup96*^{mel} heterozygotes, and homozygous (or hemizygous) *Nup96*^{sim} is not lethal in *Nup160*^{sim}/*Nup160*^{mel} heterozygotes (Sawamura *et al.* 2014). The latter findings would seem to rule out the possibility that *Nup96*^{sim} is the dominant autosomal factor required for *Nup160*-mediated hybrid lethality (and vice versa). It is possible that a different autosome-encoded NPC protein, perhaps one of the other NUP107 subcomplex proteins or its interactors, is required for *Nup160*- and *Nup96*-mediated hybrid lethality (see also Sawamura *et al.* 2014).

Previous population genetics analyses showed that *Nup160* experienced parallel bouts of recurrent adaptive protein evolution in *D. melanogaster* and, separately, in *D. simulans* (Tang and Presgraves 2009) (Table 5). The present analyses further suggest that *Nup160* experienced recurrent positive selection in the *D. simulans* clade ancestor (earlier than 240,000 years ago) *and* in the *D. simulans* lineage following the split from *D. mauritiana* and *D. sechellia* (later than 240,000 years ago). There is no evidence for recurrent positive selection in *D. mauritana*, which has evolved very slowly (only a single mappable nonsynonymous substitution), or in *D. sechellia*, which has an order of magnitude less variability than the other species. Why *Nup160*, *Nup96*, and other nucleoporins have evolved rapidly (Begun *et al.* 2007; Presgraves and Stephan 2007; Langley *et al.* 2012; Nolte *et al.* 2013; Garrigan *et al.* 2014) remains unclear. Nucleoporins interact with retroviruses and retrotransposons (Irwin *et al.* 2005; Dennis *et al.* 2012; Le Sage and Mouland 2013; Marini *et al.* 2015), suggesting the opportunity for antagonistic co-evolution with pathogens and/or selfish genetic elements (Presgraves and Stephan 2007). Furthermore, the NPC, along with other nuclear transport proteins, may have evolved in response to segregation distortion in the male germ line (Presgraves 2007; Tracy *et al.* 2010; Phadnis *et al.* 2012). There is, however, reason to doubt earlier suggestions that nucleoporins of the NUP107 subcomplex evolved to suppress or compensate for the meiotic drive of selfish centromeres in the female germ line (Presgraves and Stephan 2007; Sawamura 2012): the NUP107 subcomplex in *Drosophila*, unlike in mammals, does not localize to centromeres or kinetochores (Katsani *et al.* 2008). Whatever the cause of recurrent evolution at *Nup160*, the present data suggest that *D. melanogaster* and the *D. simulans* clade ancestor inherited some unresolved genetic conflict from their common ancestor. In the *D. simulans* lineage but not in the *D. mauritiana* and *D. sechellia* lineages, this conflict involved nonsynonymous substitutions at *Nup160*. The lack of evidence for a hard selective sweep in *D. simulans* may indicate that the conflict (or at least the role of *Nup160* in the conflict) has been quiescent during the recent past or, perhaps more likely, that the sweeps were soft. Given the history of natural introgression between the *D. simulans* clade species (Garrigan *et al.* 2012), we can further surmise either that the agent(s) of conflict was not exported from *D. simulans* into its two sister species via migration or that resolution of the conflict in *D. mauritiana* and *D. sechellia* involved other genes.

The biological basis of *Nup160*-mediated hybrid lethality is still unclear. The hybrid lethality of *Nup160* is not due to haploinsufficiency because hybrids homozygous for *Nup160*^{sim} are inviable (Sawamura *et al.* 2004, 2010). Furthermore, the hybrid lethality of *Nup160* is not due to specific suppression of *Lhr* rescue because *Hmr*-rescued males also die (Table 1). This conclusion is strengthened by a difference in lethal phase: the *Hmr-Lhr* hybrid incompatibility kills late larvae, whereas the *Nup160* hybrid incompatibility kills pupae (see also Maehara *et al.* 2012). Sawamura and colleagues have shown that *Nup160* also causes female sterility and, among escapers of hybrid lethality, developmental delay and morphological defects (Sawamura *et al.* 2010; Maehara *et al.* 2012). This broad range of phenotypes suggests that fundamental cellular functions are compromised by the *Nup160* hybrid incompatibility. It will be of interest to determine whether hybrid lethality results from disruption of an essential nucleoporin-mediated function—*e.g.*, nuclear transport, gene expression, and the regulation of chromatin—or some novel gain-of-function hybrid phenotype.

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