GENETIC VARIATION FOR POSTZYGOTIC REPRODUCTIVE ISOLATION BETWEEN *CAENORHABDITIS BRIGGSAE* AND *CAENORHABDITIS* SP. 9

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The process of speciation is key to the origins of biodiversity, and yet the *Caenorhabditis* nematode model system has contributed little to this topic. Genetic studies of speciation in the genus are now feasible, owing to crosses between the recently discovered *Caenorhabditis* sp. 9 and the well-known *C. briggsae* producing fertile F$_1$ hybrid females. We dissected patterns of postzygotic reproductive isolation between these species by crossing eight isogenic strains of *C. briggsae* reciprocally with six strains of *C. sp. 9*. We determined that overall patterns of reproductive isolation are robust across these genetic backgrounds. However, we also quantified significant heritable variation within each species for interspecific hybrid incompatibilities for total adult progeny, egg-to-adult viability, and the percentage of male progeny. This demonstrates that intraspecific variation for interspecific hybrid incompatibility occurs despite extensive, albeit incomplete, reproductive isolation. Therefore, this emerging general phenomenon of variable reproductive isolation is not restricted to highly interfertile, early-stage incipient species, but also applies to species in the latest stages of the speciation process. Furthermore, we confirm Haldane's rule and demonstrate strongly asymmetric parent-of-origin effects (Darwin's corollary) that consistently manifest more extremely when hermaphroditic *C. briggsae* serves as maternal parent. These findings highlight *Caenorhabditis* as an emerging system for understanding the genetics of general patterns of reproductive isolation.

KEY WORDS: Fecundity, genetic variation, hybridization, reproductive isolation, speciation.

Genetic differentiation between diverging lineages results in the evolution of reproductive barriers that further restrict gene flow between the lineages, ultimately yielding complete speciation. Despite the importance of extrinsic isolating barriers and prezygotic mechanisms of isolation in this process (Coyne and Orr 2004; Schluter 2009), intrinsic postzygotic reproductive barriers, such as hybrid inviability and sterility, are particularly amenable to genetic analysis because they cause developmental phenotypes that are easily observed and quantified under laboratory conditions. The Bateson-Dobzhansky-Muller (BDM) model of genic speciation posits between-locus epistatic incompatibilities

Dobzhansky-Muller incompatibilities (DMIs) as the cause of such hybrid inviability and sterility (Bateson 1909; Dobzhansky 1936; Muller 1942). This model can be illustrated simply with a two-locus example, starting with an ancestral species with diploid genotype *aabb*. Upon separation into two lineages, new alleles will arise by mutation and will eventually become fixed in each lineage, by either drift or selection, resulting in genotypes *AAbb* in one and *aaBB* in the other. The new alleles (*A* and *B*) function well in their own genetic backgrounds, but have never been "tested" together by selection. Upon secondary contact, the *A* and *B* alleles will meet each other in hybrids (*AaBb*) and might be

incompatible (Orr 1995; Turelli and Orr 2000; Orr and Turelli 2001). The BDM model has been formalized theoretically and is considered the main explanation for intrinsic postzygotic isolation, with broad empirical support (Orr et al. 2004; Wu and Ting 2004; Presgraves 2010).

The standard BDM model for postzygotic isolation, however, presumes that DMI alleles are fixed in each incipient species (Orr 1995). Because new mutations that arise must always pass through a polymorphic state before fixation, it is inevitable that incipient species pairs, upon secondary contact, will be polymorphic for at least some DMIs. In fact, genetic variation for postzygotic reproductive isolation is present in numerous plant and animal taxa (Cutter 2012), including common models for speciation research (Wade and Johnson 1994; Rieseberg 2000; Reed and Markow 2004; Kopp and Frank 2005; Sweigart et al. 2007; Good et al. 2008). Inclusion of this phenomenon of variable reproductive isolation in models of speciation is not yet mainstream, but its pervasiveness suggests that theory and empirical research must begin to incorporate it into predictions about the process of speciation. The analysis of such polymorphism for incompatibility loci will help illuminate the forces that are most important for the origin of reproductive isolation between species. For example, both positive selection and genetic drift can lead alleles to fixation, but positive selection does so much quicker. If all incompatibility alleles are subject to positive selection, then polymorphic DMIs should be exceptionally rare (Lopez-Fernandez and Bolnick 2007). And yet, Shuker et al. (2005) conclude that heritable variation for reproductive isolation in a European grasshopper cline is selectively neutral within each species. As more studies demonstrate the existence of polymorphism for incompatibility loci, the more we must take seriously the possibility that genetic drift, balancing selection, and mutation–selection balance, in addition to species-wide directional selection, are important contributing forces acting within species on loci implicated in reproductive isolation (Cutter 2012).

Caenorhabditis nematodes, like *Drosophila* fruit flies, have a wide array of molecular and genetic tools available that would be invaluable in the study of the genetics of speciation (Hodgkin 2005). Unfortunately, the majority of interspecific crosses between known species in the genus yield zygotes that arrest during embryogenesis, although crosses of *Caenorhabditis briggsae* males with *C. brenneri* and *C. remanei* females result in rare occurrences of first-stage larval arrest (Baird et al. 1992; Baird and Yen 2000). Additionally, crosses between *C. remanei* females and *C. briggsae* males result in rare hybrid adults, which are all female and sterile (Hill and L'Hernault 2001; Baird 2002). The absence of male hybrids is consistent with Haldane's rule, where the heterogametic (male) sex is more affected by hybrid inviability and sterility (Haldane 1922; Baird 2002). This conformity with Haldane's rule occurs, in part, as a by-product of

sexual transformation, where some of the phenotypic females are genetically male (hemizygous for the X chromosome) (Baird 2002). Previous work also demonstrated that gametic interactions seem largely concordant in some hetero-specific crosses in that, for example, hetero-specific sperm and sperm-derived proteins can stimulate female ovulation (Hill and L'Hernault 2001; Miller et al. 2001), but that fertilization fails in many species pairs (Baird et al. 1992).

Recent phylogenetic sampling of *Caenorhabditis* has revealed closely related species pairs that are only partially reproductively isolated from each other (Cutter et al. 2010; Woodruff et al. 2010; A. Dey and A. D. Cutter, unpubl. data). Consequently, the *Caenorhabditis* model system may now be applied to the genetics and evolution of reproductive isolation (Woodruff et al. 2010). Crosses between the newly discovered gonochoristic *C.* sp. 9 with the androdioecious *C. briggsae* result in the first demonstration of fertile F_1 interspecific hybrids within the genus (Woodruff et al. 2010). These sister species are estimated to have diverged $\sim 10 \times 10^6$ generations ago, and both species have been isolated from southern India, among other locations (Cutter et al. 2010). Their ability to produce fertile F_1 hybrids, despite generally strong postzygotic isolation, has instigated interest in the genetic basis of two key features: the evolution of hermaphroditism and the evolution of reproductive isolation. Woodruff et al. (2010) characterized the broad patterns observed from crosses between the two species, using a single genetic background (strain) of each, in an attempt to elucidate the genetic origins of selfing hermaphroditism. They showed that crosses between the two species result in fertile F_1 hybrid females and rare sterile hybrid males, consistent with Haldane's rule. They also found that hybrids exhibit parent-of-origin asymmetries for the strength of Haldane's rule and for F_1 fertility, a pattern recently dubbed "Darwin's corollary to Haldane's rule" (Turelli and Moyle 2007).

Here, we extend *Caenorhabditis* as a study system for the evolution and genetics of speciation to address two main objectives. We performed reciprocal crosses between multiple genetically distinct strains of *C. briggsae* and *C.* sp. 9. Genetically differentiated phylogeographic groups of *C. briggsae* raise the potential for local adaptation and drift to generate intraspecific variation for phenotypic traits (Prasad et al. 2011), possibly including variation for hybrid incompatibilities (e.g., Reed and Markow 2004; Sweigart et al. 2007). Thus, our first objective aims to determine whether *C. briggsae* and *C.* sp. 9 harbor intraspecific genetic variation for hybrid incompatibilities. Our second objective aims to further characterize the extent of Haldane's rule and Darwin's corollary to Haldane's rule, that were first demonstrated by Woodruff et al. (2010), and to derive explanations for these patterns of postzygotic reproductive isolation.

Table 1. Strains of *Caenorhabditis briggsae* and *C.* sp. 9 used in this study.

Species	Strain	Geographic origin	Comments
C. briggsae	AF16	India, wild isolate	Tropical phylogeographic group*, low mtDNA heteroplasmy**
C. briggsae	HK104	Okayama, Japan, wild isolate	Temperate phylogeographic group*, low mtDNA heteroplasmy**
C. briggsae	HK105	Sendai, Japan, wild isolate	Temperate phylogeographic group*, high mtDNA heteroplasmy**
C. briggsae	JU726	China, wild isolate	Tropical phylogeographic group*, low mtDNA heteroplasmy**
C. briggsae	ED3083	South Africa, wild isolate	Tropical phylogeographic group*, low mtDNA heteroplasmy**
C. briggsae	VT847	Hawaii, United States, wild isolate	Tropical phylogeographic group*, high mtDNA heteroplasmy**
C. briggsae	ED3092	Kenya, wild isolate	Local phylogeographic group*, no mtDNA heteroplasmy**
C. briggsae	JU1341	Kerala, India, wild isolate	Local phylogeographic group*, no mtDNA heteroplasmy**
C. sp. 9	JU1418	Trivandrum, Kerala, India	Inbred line of JU1325
C. sp. 9	JU1419	Trivandrum, Kerala, India	Inbred line of JU1325
C. sp. 9	JU1420	Trivandrum, Kerala, India	Inbred line of JU1325
C. sp. 9	JU1421	Trivandrum, Kerala, India	Inbred line of JU1325
C. sp. 9	JU1422	Trivandrum, Kerala, India	Inbred line of JU1325
C. sp. 9	EG5268	Democratic Republic of the Congo	"Population strain"

*From Cutter et al. (2010); **From Howe and Denver (2008).

Materials and Methods
STRAIN STOCKS AND MAINTENANCE

To measure variation in reproductive isolation, we performed crosses between eight strains of *C. briggsae* and six strains of *C.* sp. 9 (Table 1). Each *C. briggsae* strain is essentially isogenic, representing distinct phylogeographic groups found in the species (Cutter et al. 2010) as well as a range of reported mitochondrial heteroplasmy frequencies (Howe and Denver 2008). We also used *C. briggsae* strain PS9393 that has a pharyngeally expressed green fluorescent protein (GFP) transgenic marker (*syls804*) introgressed onto the X chromosome of an otherwise AF16 genetic background (courtesy Paul Sternberg). This GFP strain was used to test for sexual transformation of hybrids by mating male PS9393 to *C.* sp. 9 and looking for non-GFP female progeny, which would be indicative of sexual transformation of genetic males into phenotypic females (Baird 2002). All strains are available from the *Caenorhabditis* Genetics Centre (CGC) or from the Felix lab strain collection (http://www.justbio.com/worms).

Caenorhabditis sp. 9 was first isolated from rotting leaves and flowers in southern India, its identity as a new species being confirmed by mating tests with available *Caenorhabditis* species in culture (M.-A. Felix, pers. comm.). This strain (JU1325), termed a "population strain" because it derived from a collection of individuals obtained from the wild, provides the source for five isofemale lines (JU1418–JU1422, created by inbreeding for 25 generations; M.-A. Felix, pers. comm.). These five isofemale lines plus "population strain" EG5268 (a wild isolate found in the Democratic Republic of the Congo, a gift from Michael Ailion) form the basis of genetically distinct strains of *C.* sp. 9 that we used in crosses.

We cultured, maintained, and crossed all strains on petri dishes containing NGM-Lite agar seeded with *Escherichia coli* strain OP50 at 25°C. Crosses used 35-mm dishes with 10-mm diameter bacterial spot; a temperature of 25°C permits high fecundity for both *C. briggsae* and *C.* sp. 9 (Woodruff et al. 2010; Prasad et al. 2011). As *C. briggsae* is androdioecious, with XØ males such as *C. elegans*, males were initially induced by heat shock: L4/young adult stage hermaphrodites were placed at 31°C for 6 h to promote chromosomal nondisjunction, then returned to 25°C. Resulting male progeny were used in maintenance crosses to sustain males in the population. Strains were cleaned of any contaminating bacteria or fungus using a standard bleaching protocol before being used in crosses (Stiernagle 1999).

EXPERIMENTAL PROTOCOL FOR INTERSPECIFIC CROSSES

To quantify reproductive isolation, we performed reciprocal crosses for pairwise strain combinations (Fig. 1). In parallel, we
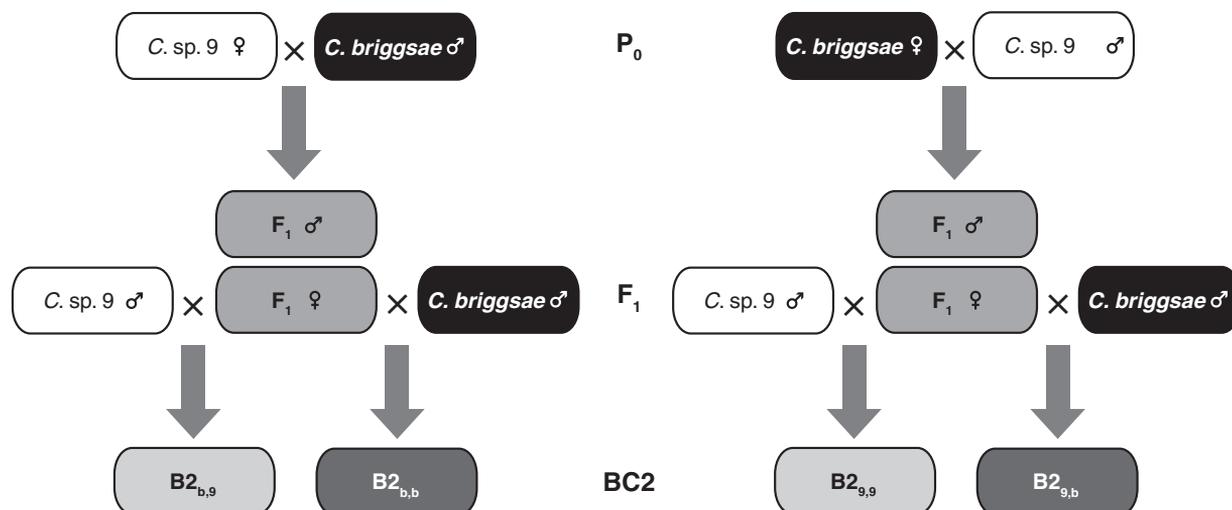
Figure 1. Schematic diagram of the experimental interspecific cross design used in this study. The two parental species, *Caenorhabditis briggsae* and *C.* sp. 9, were used in reciprocal crosses at the P_0 generation (top) to give F_1 hybrids and then backcrossed to parental species (middle) to test for F_1 female fertility. Backcross nomenclature follows Woodruff et al. (2010). Reciprocal crosses and backcrosses were performed in replicate for eight strains of *C. briggsae* and six strains of *C.* sp. 9. Not shown in the diagram are within-strain intraspecific control crosses for each strain of parental species as well as between-strain intraspecific crosses for 59 random strain combinations of *C. briggsae*. As maternal parents in all crosses, *C. briggsae* hermaphrodites were depleted of self-sperm prior to mating with males.

performed within-strain intraspecific control crosses to compare to interspecific F_1 hybrid production. We performed crosses in blocks, where one block consisted of two replicates of each interspecific cross and its reciprocal plus two replicates of each intraspecific cross. In 2009, each block consisted of 140 interspecific crosses between seven strains of *C. briggsae* and five strains of *C.* sp. 9 (Table 1; excluding JU1341, see below). We received *C.* sp. 9 strain EG5268 in the late summer of 2009, thus it was not included in the majority of blocks performed that year. Each block of 140 interspecific crosses was divided into trials that consisted of reciprocal crosses between one or two *C.* sp. 9 strains with eight *C. briggsae* strains. One trial was performed per day.

In 2010, we added recently discovered and genetically distinct *C. briggsae* strain JU1341 (Cutter et al. 2010), and continued using *C.* sp. 9 strains JU1418–JU1422 and strain EG5268. Each trial in 2010, completed in one day, comprised reciprocal crosses between two strains of *C.* sp. 9 and four strains of *C. briggsae*. Each block consisted of two replicates of each cross and its reciprocal for all strain pairs.

Each cross involved one female (or hermaphrodite purged of self-sperm, see below) and six males allowed to mate for 24 h, after which the males were removed. We picked *C.* sp. 9 females as fourth-stage larvae (L4) to ensure that they had not mated with conspecific males. In contrast, *C. briggsae* hermaphrodites are self-fertile and protandrous, and make a limited number of sperm before switching irreversibly to oocyte production. To exclude the effects of self-sperm in crosses involving *C. briggsae*

hermaphrodites, we created sperm-depleted hermaphrodites by transferring hermaphrodites to new plates until they stopped laying self-fertilized eggs and thus had used all the self-sperm in their spermathecae.

From each cross, we counted the number of F_1 eggs, F_1 adult progeny, and the abundance of males among the F_1 adult progeny. F_1 progeny were allowed to grow until they reached adulthood, after which we put the plates at 4°C to stop their growth for counting. We performed an average of eight replicates for each of the 96 reciprocal interspecific strain cross combinations and the 14 intrastrain control crosses, giving a total of 37,636 adult progeny counted from 945 females.

We determined the fertility of the F_1 hybrid progeny during the 2009 trials. Specifically, we separated out two virgin F_1 females from each cross combination and mated one of them to *C. briggsae* males of the parental strain and the other to *C.* sp. 9 males of the parental strain (Fig. 1). After one week, F_1 females were marked as fertile if any backcross progeny were observed on the dish. For each reciprocal maternal genetic background, an average of 34 replicates was included in analysis of F_1 fertility.

To determine egg-to-adult viability of F_1 hybrid embryos, we counted both the number of eggs and, subsequently, the number of adult progeny for crosses in the 2010 trials. We transferred the female daily until she stopped laying eggs, counting the eggs on the plate immediately after the transfer. For each reciprocal maternal genetic background, an average of 15 replicates was included in analysis of egg-to-adult viability.
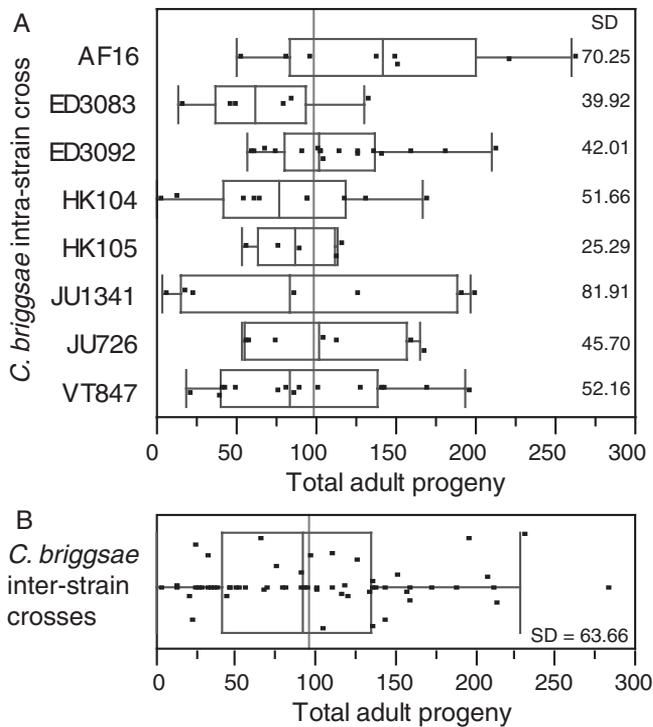
Figure 2. Intraspecific progeny production for *C. briggsae*. (A) Box-plots show the median and interquartile range for intrastrain crosses. Points beyond the whiskers represent potential outlier values. The vertical line is the grand mean across all within-strain crosses; standard deviation (SD) for each strain is indicated to the right. (B) Distribution of progeny production from crosses between random combinations of *C. briggsae* strains (*n* = 59). Vertical line indicates the mean total number of adult progeny produced across interstrain crosses; SD is comparable to intrastrain SD values.

CAENORHABDITIS BRIGGSAE INTRASPECIFIC CONTROL CROSSES

When comparing total adult progeny numbers between interspecific crosses of different *C. briggsae* strains, we need to know whether differences observed between strains reflect intrinsic fitness differences of strains or a measure of reproductive isolation. To address this, we counted the number of adult progeny produced from a random sample of 59 intraspecific interstrain *C. briggsae* crosses and obtained a distribution of the variation for total progeny numbers within the species (Fig. 2). We then compared the variation in total adult progeny in *C. briggsae* intrastrain crosses to this distribution (Fig. 2). We also compared the intrastrain control fecundities to interspecific fecundities on a per-strain basis. As for interspecific crosses, *C. briggsae* hermaphrodites were sperm-depleted prior to mating with males.

DATA ANALYSIS

We developed a nested linear model to test which effects influence the number of adult progeny from interspecific crosses. Data

for total adult progeny were $\log_{10} + 1$ transformed, to better approximate a normal distribution and then used as the response variable. The full model included the following factors: species of female parent, species of male parent, strain of female parent nested within species, strain of male parent nested within species, the interaction between parent species, whether crosses were performed in 2009 or 2010, and whether females were transferred each day. All factors were treated as fixed effects. All data analysis was carried out using JMP version 7 or 9, with data files available as Supporting information.

For F_1 egg-to-adult viability, we constructed a model to explain variation in percent viability as a function of maternal strain and paternal strain. We conducted the analysis separately on each interspecific cross because we detected no significant intraspecific differences in viability. The 18 replicates for which counts of F_1 adults slightly exceeded egg counts were assigned a viability value of 100%. Analyses using arcsin-square-root transformation were qualitatively and quantitatively very similar, so we report only the untransformed analysis to ease interpretation. Finally, for the cross of *C.* sp. 9 females with *C. briggsae* males, we modeled the frequency of males among F_1 progeny [transformed as $\log_{10}(\%$ male $+ 1)$] as a function of maternal strain and paternal strain, with transfer status and year as covariates. We restricted the analysis of male frequency to this cross because so few males were observed in the reciprocal.

Results
PATTERNS OF POSTZYGOTIC ISOLATION
Haldane's rule

Both reciprocal crosses between *C. briggsae* and *C.* sp. 9 exhibit Haldane's rule. Across genetic backgrounds, hybrid males represented 13.8% of hybrid progeny in interspecific crosses with *C.* sp. 9 as the mother, significantly less than the expected 50% (Table 2; $\chi^2 = 3959.64$, df = 1, $P < 0.0001$). In the reciprocal cross, males were even rarer, representing only 0.19% of total F_1 hybrid progeny (Table 2). The asymmetry for the proportion of males produced from the two reciprocal crosses is significant (Welch's ANOVA, $F_{1,217.81} = 227.27, P < 0.0001$), indicating consistency with Darwin's corollary in terms of sex-specific hybrid viability. Baird (2002) showed that Haldane's rule in *Caenorhabditis* could be due in part to sexual transformation: genetic male hybrids (XØ) appear phenotypically female. We tested for this here by crossing *C. briggsae* males having an X-linked GFP transgenic marker (strain PS9393) to *C.* sp. 9 females (JU1421). Genetically transformed hybrid XØ females will not express GFP, because their sole X chromosome derives from the *C.* sp. 9 mother. We found that only 11 of 403 hybrid females (2.7%) lacked GFP expression. Consequently, sexual transformation does not appear to be a sufficient explanation for Haldane's rule in this species pair.

Table 2. Summary of the analysis hybrid F_1 males from reciprocal crosses between *C. briggsae* and *C. sp. 9.*

Maternal species	Number of crosses	Observed number of hybrid F_1 males	Observed number of total hybrid F_1 progeny	Expected number of males (50%)	χ^2-value	df	*P*
C. sp. 9	239	1045	7562	3751	3959.64	1	<0.0001
C. briggsae	252	7	3705	1852.5	3677.06	1	<0.0001

For the cross of *C.* sp. 9 females and *C. briggsae* males, we could explain 22% of the variation in male frequency ($F_{14,201} = 5.38$, $P < 0.0001$). The back-transformed model least-squares mean percent males was 4.9%, somewhat lower than the estimate from a simple count of males in crosses (13.8%; Table 2). This disparity reflects the strongly skewed distribution of male frequency among crosses and the fact that the model accounts for differences in sample size among strains. Differences among maternal ($P < 0.0001$) and among paternal ($P = 0.0004$) genetic background both explain significant variation in the frequency of F_1 males, with up to sixfold differences between strains (Fig. 3), indicating heritable variation for Haldane's rule in both *C.* sp. 9 and *C. briggsae*. We also performed a complementary analysis, with the statistical model constructed to explain variation in the count of males ($\log_{10} + 1$ transformed) as a function of parental strains, counts of hybrid adults ($\log_{10} + 1$ transformed), the interactions of strain and hybrid adults, and year and whether the replicate was transferred daily. This analysis yielded qualitatively equivalent results, except that maternal strain was only marginally significant (effect $P = 0.047$) and the transfer effect also was significant ($P = 0.0044$); only the interaction term between maternal strain and hybrid adult count was significant ($P = 0.0006$). The dominant explanatory variable for the count of males was the number of hybrid adults ($P < 0.0001$), as expected, which accounted for 43% of the variance in the incidence of males (total model $r^2_{adj} = 0.632$, $F_{27,211} = 16.12$, $P < 0.0001$).

Egg to adult survival

To examine whether hybrids suffer a reduced number of progeny that survive to adulthood compared with intraspecific control crosses, for a subset of crosses, we counted both the number of embryos laid as well as the subsequent number of adults that developed. Hybrids in both reciprocal crosses experience significantly lower egg to adult survival compared with *C. briggsae* and *C.* sp. 9 intraspecific controls (post-hoc Tukey's HSD; oneway ANOVA $F_{3,264} = 52.27$, $P < 0.0001$; Fig. 4). Mean ($\pm 1SE$)
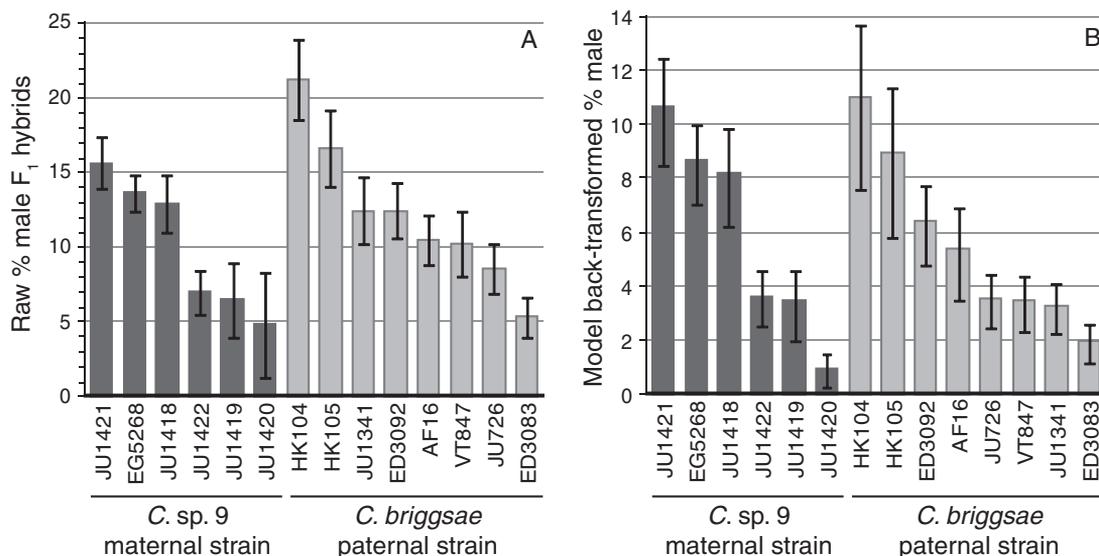

Figure 3. Maternal and paternal genetic backgrounds contribute to variation in the incidence of males among hybrid progeny of *C. sp. 9* females crossed with *C. briggsae* males. (A) The arithmetic mean frequency of males of a given parental strain, averaged across heterospecific genetic backgrounds. (B) Back-transformed model least-squares mean estimates of male frequency for each parental strain. The values in (B) better account for the skewed distribution of male frequencies, sample size differences among genetic backgrounds, and other covariates (see text).
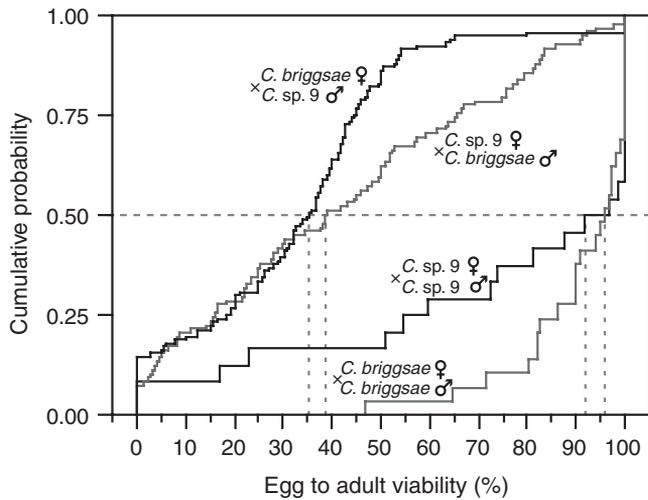
Figure 4. Cumulative probability distribution for egg to adult survival for reciprocal interspecific crosses and within-strain intraspecific crosses of *C. briggsae* and *C.* sp. 9. Horizontal dashed line represents the 50th percentile (median); vertical dashed lines indicate the median value for each of the four cross-types. Median viability is 38.9% and 35.4% when *C.* sp. 9 is maternal and paternal parent, respectively. Conspecific crosses yield median viabilities of 94.1% for *C.* sp. 9 and 95.9% for *C. briggsae*. Average viability for interspecific crosses is significantly lower than intraspecific crosses (see text).

hybrid viability was 41.7% ($\pm$3.0%) and 33.2% ($\pm$2.1%), respectively, for *C.* sp. 9 as maternal and paternal parent. By contrast, intraspecific crosses yielded mean 75.3% ($\pm$6.9%) and 90.7% ($\pm$2.3%) viability for *C.* sp. 9 and *C. briggsae*, respectively. Although F_1 hybrids with *C.* sp. 9 mothers have a nominally higher average egg to adult survival than F_1 hybrids with *C. briggsae* mothers (Fig. 4), this difference is not significant after multiple test correction (Tukey's HSD).

On average, F_1 hybrids with *C.* sp. 9 as maternal parent exhibited a mean 58% mortality between fertilization and adulthood, versus 66% mortality for the reciprocal cross. This degree of embryonic/larval mortality could fully account for Haldane's rule if hybrid female viability (v_f) is 72.4% with relative male viability ($p = v_m/v_f$) 16% with *C.* sp. 9 as maternal parent, and if $v_f = 67.9\%$ and $p = 0.19\%$ for the reciprocal cross. We derive this by assuming a 1:1 sex ratio at fertilization ($z_m = z_f$), according to the following general relations:

$$v = \frac{(v_m z_m + v_f z_f)}{z_m + z_f} \quad \text{and}$$

$$f_m = \frac{v_m z_m}{v_m z_m + v_f z_f},$$

where v is overall viability of hybrids, v_m and v_f are the male- and female-specific viabilities, z_m and z_f are the number of male and

female zygotes, and f_m is the frequency of males among surviving adults. Substituting v_m with pv_f, solving each for p and setting them equal then allows us to derive an expression for v_f as a function of v and f_m and for p as a function of f_m:

$$v_f = 2v(1 - f_m) \quad \text{and}$$

$$p = \frac{f_m}{1 - f_m}.$$

We can then insert our empirical estimates of both v and f_m to infer the sex-specific juvenile mortality necessary to explain the observed severity of Haldane's rule.

Both maternal and paternal genetic backgrounds contributed significantly to variation in hybrid F_1 egg-to-adult viability, for both cross directions (*C.* sp. 9 female $\times$ *C. briggsae* male $F_{12,85} = 7.91$, $P < 0.0001$, maternal effect $P < 0.0001$, paternal effect $P = 0.016$; *C. briggsae* sperm-depleted hermaphrodite $\times$ *C.* sp. 9 male $F_{12,104} = 4.22$, $P < 0.0001$, maternal effect $P = 0.018$, paternal effect $P < 0.0001$). These results were robust to a complementary statistical model that explained variation in the number of hybrid adults ($\log_{10} + 1$ transformed) as a function of maternal and paternal strain, number of hybrid eggs ($\log_{10} + 1$ transformed), and the strain $\times$ egg count interactions (data not shown). Genetic backgrounds of the parents could explain 46% of the variation in viability when *C.* sp. 9 was the maternal parent, and 25% of the variation when *C.* sp. 9 was the paternal parent. When *C.* sp. 9 was maternal parent, JU1418 and JU1421 yielded significantly higher F_1 viability than other strains (Tukey's HSD). In the reciprocal cross, *C.* sp. 9 EG5268 paternal parents yielded significantly lower viability than other strains (except statistically indistinguishable from JU1420; Tukey's HSD).

Fertility of F_1 hybrids

To determine the degree to which F_1 hybrid females are fertile, we backcrossed them (from both reciprocal crosses) to *C. briggsae* and *C.* sp. 9 males. Total fertility is 34% (as measured by the percentage of F_1 hybrids producing at least some viable backcross progeny) across all F_1 females that had *C.* sp. 9 P_0 mothers and both sets of backcrosses. This is significantly higher than the 24% fertility of female F_1 hybrids that had *C. briggsae* mothers (Fig. 5; Fisher's exact test, $P = 0.0138$). Another asymmetry observed is that F_1 hybrid females from both reciprocal crosses are significantly more fertile when backcrossed to *C.* sp. 9 males than to *C. briggsae* males (Fig. 5; parental reciprocal crosses pooled: Fisher's exact test, $P < 0.0001$). In fact, while backcrosses to *C.* sp. 9 males have fertility rates of 62% and 38% for F_1s derived from *C.* sp. 9 P_0 mothers and *C. briggsae* P_0 mothers, respectively, the fertility rates from backcrosses to *C. briggsae* males are only 4.3% and 9.2%, respectively.
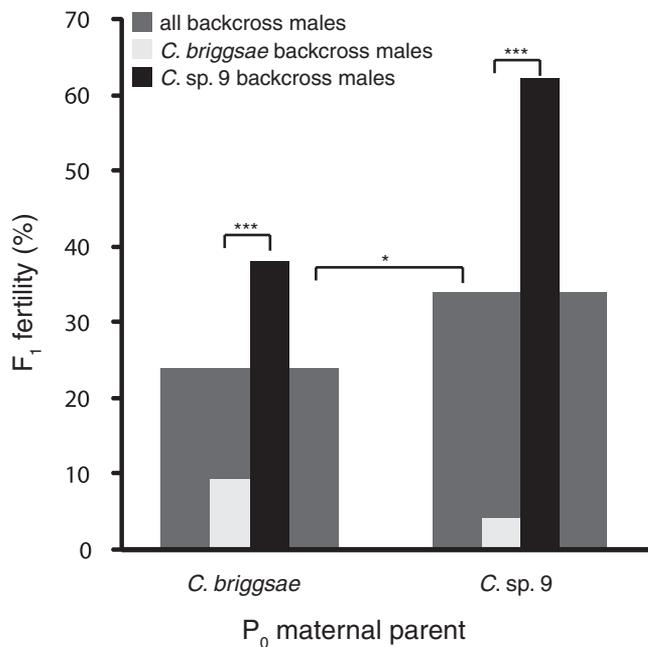
Figure 5. Fertility of F_1 females in backcrosses to *C. briggsae* (light gray bars) and to *C.* sp. 9 (black bars). Overall F_1 female fertility for each reciprocal cross of parental species, averaged across backcross types, is indicated in the dark gray bars. For *C. briggsae* as P_0 maternal parent, $n = 200$; for *C.* sp. 9 as P_0 maternal parent, $n = 241$. Significant differences are indicated above each comparison (*$P < 0.05$, ***$P < 0.0001$).

FACTORS INFLUENCING DIFFERENCES IN COUNTS OF VIABLE F_1 ADULTS

Effects of species and cross-type

We constructed a nested linear model to determine the factors that explain variation in the number of adult progeny across all crosses of *C. briggsae* and *C.* sp. 9. This model can explain 44.9% of the variation that is observed in the number of total adult progeny produced (Table 3; $F_{29,915} = 27.55$, $P < 0.0001$). Of the effects in our model, the effects of father species, mother species × father species, mother strain nested within species, and father strain

nested within species all had highly significant contributions (all $P < 0.0001$; Fig. 6). The effect of mother species did not contribute significantly to the model for log-transformed F_1 progeny counts ($P = 0.0836$). The strong father species effect supports the notion of polymorphic paternal factors that differ between the two species (further supported by post-hoc tests, described below), although the formal possibility remains that prezygotic factors contribute to this effect (e.g., male mating efficiency). Post-hoc tests (Tukey's HSD) on mother species × father species indicate a significant difference between the interactions of *C.* sp. 9 × *C. briggsae* ($9 \times B$) and $B \times 9$, but not between the two intraspecific interactions, 9×9 and $B \times B$ (Fig. 7). This implies Darwin's corollary for Haldane's rule (asymmetry in reciprocal crosses) for total adult progeny ($P < 0.0001$). This effect also is supported by the fact that the least-square mean for $B \times B$ is nominally higher than that for 9×9, while for the interspecific interactions, the $9 \times B$ least-square mean value is significantly higher than that of $B \times 9$.

As described in the Methods, data for this experiment were collected over two years; 2009 and 2010. Also, in 2010, we began tabulating the total number of eggs produced from each cross in addition to the total count of adult progeny, which required transfer of the maternal parent each day to count eggs. Thus, the data for total adult progeny are collected from maternal females that spent their life on one plate, and maternal females that were transferred each day until they stopped laying eggs. We included these two effects, year and transferred, in the model as two-term fixed effects. As they are marginally significant, we left them in the model (Table 3; transferred: $P = 0.0412$, year: $P = 0.0103$).

Caenorhabditis briggsae and *C.* sp. 9 differ from each other by reproductive mode: *C. briggsae* is androdioecious, with hermaphrodites and males, whereas *C.* sp. 9 is gonochoristic, with females and males. This fact impacts the experimental design as well as the implications of our results. To be able to compare the reciprocal crosses between the two species, we needed to ensure that we did not inadvertently count self-progeny as hybrids for matings with *C. briggsae* hermaphrodites. To do so, we

Table 3. Contribution of effects to the nested linear model that explain variation in total adult cross progeny (log-transformed; significant effects in bold).

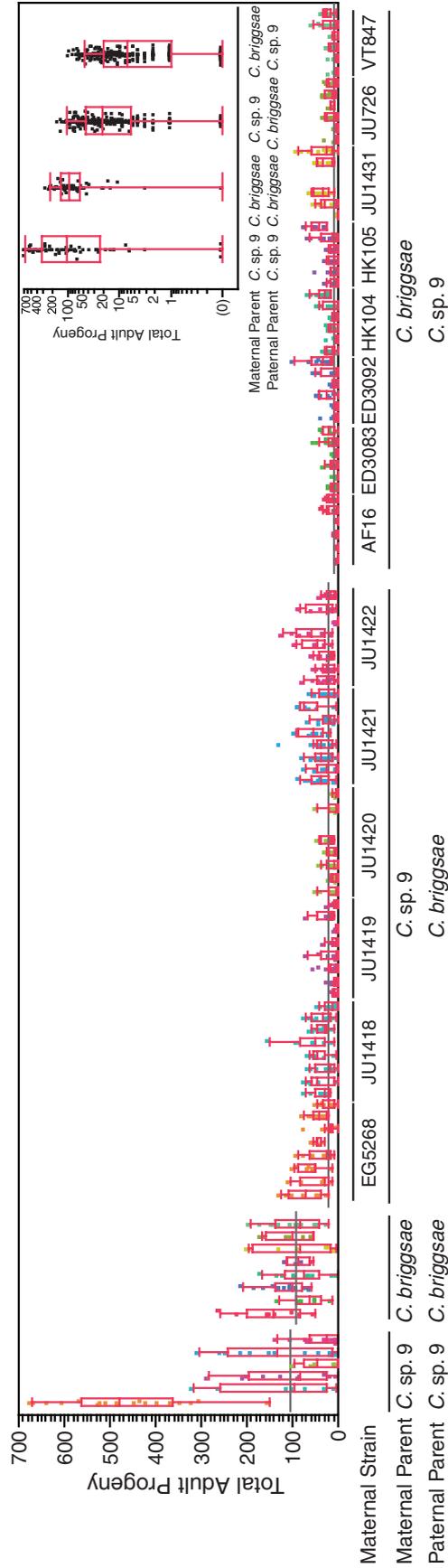| Source	df	Sum of squares	*F*-statistic	*P*
Model ($r^2_{adj} = 0.449$)	Model: 29, Error: 915	$SS_{model} = 208.5$, $SS_{error} = 238.8$	27.55	**<0.0001**
Mother species	1	0.7828	3.000	0.084
Father species	1	10.39	39.82	**<0.0001**
Mother species × father species	1	71.06	272.3	**<0.0001**
(Strain) Mother Species	12	53.04	16.94	**<0.0001**
(Strain) Father Species	12	39.72	12.68	**<0.0001**
Transferred	1	1.091	4.179	**0.041**
Year	1	1.727	6.617	**0.010**

Figure 6. Box-plots of adult progeny counts for each cross-type and strain combination. Cross-type and maternal strain is indicated below the x-axis. Within the set of boxes for a given maternal strain for hybrid crosses, paternal strains are presented in the same order as for the maternal strains of the reciprocal cross. Horizontal gray lines for each cross-type represent the average across genetic backgrounds for that cross-type. Inset shows a box-plot of the adult progeny counts for all genetic backgrounds of a given cross-type, on a log scale. Boxes indicate median and interquartile range; points beyond whiskers are potential outliers. A nested linear model of log-transformed values identifies significant differences among cross-types and among strains for both maternal and paternal parents (see text).
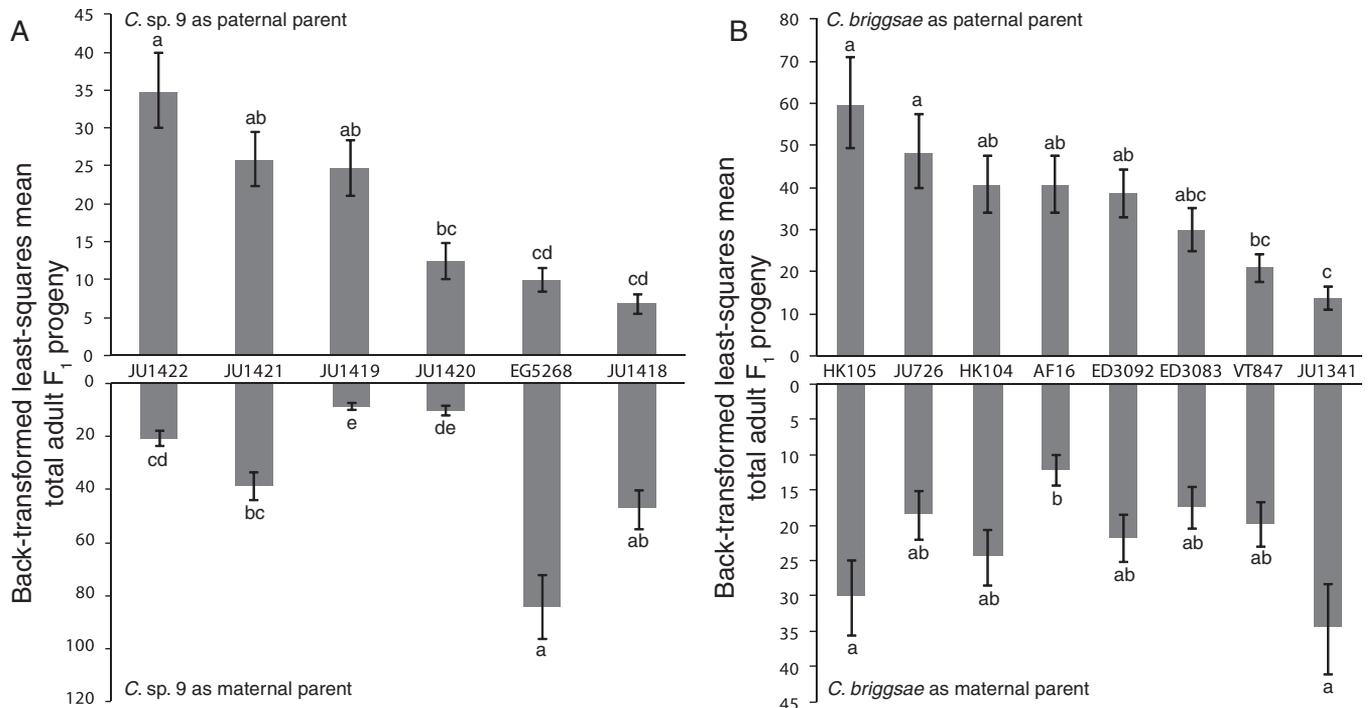
Figure 7. Post-hoc comparisons of back-transformed least-squares mean F$_1$ hybrid adult counts for each reciprocal cross. (A) Averages for strains of *C.* sp. 9 as paternal (top) and maternal parent (bottom). (B) Averages for strains of *C. briggsae* as paternal (top) and maternal parent (bottom). Strains are indicated between the top and bottom panels. Within a panel, values sharing the same letter are statistically indistinguishable (Tukey's HSD). Error bars represent back-transformed standard error.

sperm-depleted all hermaphrodites used in this experiment; a process that takes five days. Consequently, *C. briggsae* sperm-depleted hermaphrodites were five days older than the *C.* sp. 9 females used in the crosses. This age discrepancy could potentially affect the number of eggs laid per species, as *C. briggsae* sperm-depleted hermaphrodites might die before they finish laying all fertilized eggs. However, in the model this did not appear to be the case, as the intraspecific cross information reflected in the "B×B" interaction term had a nominally higher least-squares mean than the interaction term "9×9," signifying that even if the age of *C. briggsae* sperm-depleted hermaphrodites influences the number of eggs they lay, this number is still higher than the number of eggs laid in *C.* sp. 9 intraspecific crosses (under the log-transformed model). The untransformed medians are also very similar to each other (Fig. 6).

Intraspecific variation for interspecific adult hybrid progeny production

The nested effects in the above model also test for differences among strains within each species for the total number of adult progeny produced. There are significant differences among strains in *C. briggsae* as both the maternal and paternal parent, indicating the presence of intraspecific heritable variation for both maternal and paternal factors contributing to reproductive iso-

lation. Statistically conservative post-hoc tests (Student's *t* with Bonferroni correction, $\alpha_{adj} = 0.000549$) show significant differences between the *C. briggsae* strains JU1341 and AF16 ($P < 0.0001$) and HK105 and AF16 ($P = 0.0002$), when *C. briggsae* is the maternal parent (Figs. 6, 7). When *C. briggsae* is the paternal parent, we find significant differences between strain JU1341 and five other strains (for all, $P < 0.0001$) and between strain VT847 and two other strains ($P \leq 0.0003$) (Figs. 6, 7). We were unable to formally test for an effect of *C. briggsae* phylogeographic origin on patterns of reproductive isolation, due to the small number of distinct genetic backgrounds we included from each of *C. briggsae*'s phylogeographic groups. However, there is a visual trend of "temperate clade" strains (HK104, HK105) tending to have higher hybrid progeny production and more males than "tropical clade" strains (AF16, ED3083, JU726, ED3083) (Figs. 3, 7B). Strains of *C.* sp. 9 also show significant differences in the number of adult progeny produced both when it is the maternal and paternal parent (Table 3; Figs. 6, 7).

Within-species variation in adult progeny production

To ensure that the differences observed for the number of total progeny produced within each species in hybrid crosses were due to differences in reproductive compatibility and not simply reflective of intraspecific variation for progeny production, we performed within-strain control crosses for all strains used in

the experiment. There is no significant difference among means for total adult progeny among the different *C. briggsae* strains (ANOVA, $F_{7,67} = 1.59$, $P = 0.1523$), consistent with previous findings for *C. briggsae* reared at intermediate temperatures (Prasad et al. 2011). We also tested for a rank correlation between intrastrain reproductive success and interspecific reproductive success. We found no significant correlation for *C. briggsae* as maternal parent (Spearman rank-sum test, $P = 0.29$) or paternal parent ($P = 0.82$), which is consistent with no strain being consistently a "high quality" parent in both intra- and interspecific crosses.

To further examine variation for progeny production in *C. briggsae*, we performed 59 interstrain intraspecific crosses, using random combinations of the *C. briggsae* strains (Fig. 2). The distribution of progeny counts from these interstrain crosses is comparable to the variation observed for intrastrain crosses (Fig. 2). Progeny of intraspecific crosses of *C. briggsae* strains AF16 and HK104 have been shown previously to exhibit elevated embryonic mortality, delayed development, and segregation distortion that seems specific to this strain combination (Dolgin et al. 2008; Ross et al. 2011). Such effects might explain the greater density of interstrain fecundities near zero than observed for intrastrain crosses, despite similar means and variances for crosses within and between strains of *C. briggsae*.

Intrastrain crosses for *C. sp. 9*, however, did show significant differences in mean number of progeny among strains (ANOVA, $F_{5,66} = 38.6530, P < 0.0001$). Subsequent post-hoc tests (Tukey's HSD) revealed that "population strain" EG5268 produces significantly more progeny than the other inbred iso-female lines of *C. sp. 9*. This likely reflects the manifestation of some degree of inbreeding depression in the inbred isofemale lines, as has been observed in other obligately outcrossing species of *Caenorhabditis* (Dolgin et al. 2007). For *C. sp. 9* as maternal parent, we found no significant correlation between strain rank reproductive output for intraspecific and interspecific crosses ($P = 0.33$). However, we found a negative rank correlation for *C. sp. 9* male reproductive success between intraspecific and interspecific crosses (Spearman's $\rho = -0.89, P = 0.019$). This result implies that males from strains that achieve high intraspecific reproductive success produce fewer interspecific hybrid progeny than other strains.

Discussion

Speciation is a necessary hallmark of the diversification process for all organisms. The genus of nematode roundworms *Caenorhabditis*, like *Drosophila*, boasts extensive genetic and molecular tools, but has not previously contributed much toward understanding the speciation process. This is because fertile F_1 hybrid progeny, which are essential to dissecting the genetics of postzygotic isolation, could not be produced from crosses of

previously known species in the genus (Baird et al. 1992; Baird and Yen 2000; Hill and L'Hernault 2001). The recent discovery of *C. sp. 9* in 2007 by Marie-Anne Felix now allows the first opportunity to study the genetics of reproductive isolation within this model genus. There are two main purposes to this study. First, we quantify the general features of intrinsic postzygotic reproductive isolation, expanding on the observations of Woodruff et al. (2010). Second, we document significant differences among genetic backgrounds of both species for the number of adult hybrid progeny produced in interspecific crosses, the egg-to-adult viability, and the incidence of male hybrid progeny. The presence of such heritable variation for incompatibility loci in nematodes joins an emerging general pattern of variable reproductive isolation between closely related species, and calls for a synthesis of this phenomenon with the standard fixed BDM incompatibility framework in the evolution of intrinsic postzygotic isolation.

HALDANE'S RULE
Here, we demonstrate that Haldane's rule is robust across a broad range of genetic backgrounds of both *C. sp. 9* and *C. briggsae*. When *C. sp. 9* serves as maternal parent, hybrid males occurred at a frequency of 13.8%, whereas *C. briggsae* mothers produced hybrid males at a frequency of just 0.19%. This asymmetry in Haldane's rule between the two reciprocal crosses is discussed below in "Darwin's corollary to Haldane's rule." Our confirmation of Haldane's rule is qualitatively similar to Woodruff et al. (2010), but quantitatively different: Woodruff et al. (2010) found males to comprise 34% of hybrid progeny with maternal *C. sp. 9*, and no hybrid males with *C. briggsae* mothers. Note, however, that Woodruff et al. (2010) conducted crosses using the single *C. briggsae* strain AF16 and the *C. sp. 9* "population strain" JU1325 (derived from a founding stock of many males and females). We also did not observe any male hybrids in crosses with AF16 as the mother, suggesting that heritable variation among the strains for hybrid male viability or incomplete penetrance might explain quantitative variation in Haldane's rule for hybrid male viability (Wade et al. 1999; Lopez-Fernandez and Bolnick 2007). Moreover, we used a much greater sample size for this cross (3705 adults here versus 53 adult hybrids in Woodruff et al. (2010)), which allowed us to detect the low incidence of hybrid males in crosses with *C. briggsae* as the maternal parent. Woodruff et al. (2010) also observed more males than we did in crosses with *C. sp. 9* as the maternal parent (for *C. sp. 9* females × AF16 *C. briggsae* males, we found 11.1% hybrid males vs. 34% by Woodruff et al. (2010)). We conducted all crosses at 25°C, whereas Woodruff et al. (2010) conducted crosses at 20°C. If higher temperature reduces hybrid male viability during development, as has been seen in *Tribolium* (Wade et al. 1999), then the higher temperatures used in our study could contribute to the apparent rarity of hybrid males.

Because JU1325 (like EG5268) was founded from a collection of individuals from the wild, it is probably genetically diverse, having sampled a variety of recessive deleterious mutations segregating at mutation–selection balance in nature. We used inbred derivatives of strain JU1325 in crosses (JU1418–JU1422). It is possible that, as they were inbred, some of these mutations became fixed (yielding the apparent inbreeding depression) and also sensitized their genetic backgrounds to male-specific hybrid lethality. If so, then some of the among-strain variation in hybrid incompatibility that we demonstrate will reflect slightly deleterious polymorphisms within *C*. sp. 9. Although such alleles are not expected to eventually become fixed within *C*. sp. 9 or to contribute to a standard BDM incompatibility, they may nevertheless contribute substantially to overall reproductive isolation.

The level of embryonic/larval mortality (58–66%) is sufficient to account for Haldane's rule in this system. Future work that aims to determine the sex of dead embryos would be valuable to confirm whether this is indeed the case. A potential alternative explanation comes from Baird's (2002) discovery that crosses between *C. remanei* females and *C. briggsae* males yield Haldane's rule, in part, by sexual transformation. In these crosses, both XX and XØ individuals look phenotypically female. We tested for sexual transformation in *C*. sp. 9/*C. briggsae* hybrids using crosses with *C. briggsae* males that express a dominant X-linked GFP marker. We found that sexual transformation is insufficient to explain Haldane's rule in *C*. sp. 9/*C. briggsae* hybrids, as only 2.7% of phenotypic female hybrids lacked GFP expression (which is indicative of sexual transformation). This suggests that sexual transformation might represent a breakdown of sex determination pathways primarily in highly divergent species pairs, such as *C. remanei* and *C. briggsae*. Finally, we note that our attempts to amplify and sequence fragments of Wolbachia-like genomes from these two species failed (data not shown), so Wolbachia-like infections also are an unlikely potential cause of Haldane's rule in this system (cf. Shoemaker et al. 1999). Consequently, the dominance theory (Muller 1942), which posits that X-linked incompatibility factors cause Haldane's rule, provides the best current model for Haldane's rule between *C*. sp. 9 and *C. briggsae*, as in many other taxa (Coyne and Orr 2004).

In *C*. sp. 9/*C. briggsae* hybrids, Haldane's rule holds not only for viability, but also for F₁ fertility (Woodruff et al. 2010). We measured fertility as the proportion of F₁ hybrid females that, when backcrossed to a parental species, produce at least some viable progeny. Depending on the direction of the parental cross, female F₁ hybrids have a fertility between 38% and 62% when backcrossed to *C*. sp. 9 males, and Woodruff et al. (2010) found that all F₁ hybrid males tested were sterile when mated to *C*. sp. 9 females. The dominance theory for Haldane's rule can explain both hybrid sterility and inviability, although the genetic basis differs between these two types of postzygotic isolation.

Studies of *Drosophila* species pairs with different divergence times show that male sterility tends to evolve before inviability and to involve more DMIs (Wu 1992; Wu and Davis 1993; Coyne and Orr 1997). As males still arise from *C*. sp. 9 × *C. briggsae* hybridizations, but are sterile, it is likely that the genetic causes of hybrid male sterility evolved first. Further genetic analysis of this species pair should aim to dissect hybrid inviability and sterility in detail.

DARWIN'S COROLLARY TO HALDANE'S RULE

We detected multiple asymmetries for the reciprocal crosses of *C. briggsae* with *C*. sp. 9, a pattern referred to as Darwin's corollary to Haldane's rule (Turelli and Moyle 2007). A general pattern that emerged is that F₁ hybrids with *C. briggsae* as the maternal parent were more severely affected by each asymmetry than the hybrids with *C*. sp. 9 as the maternal parent. Specifically, crosses in which *C. briggsae* was the maternal parent (1) produced significantly fewer males than the reciprocal, (2) hybrid females had significantly lower fertility than hybrid females from the reciprocal cross, (3) hybrids had a nominally lower egg-to-adult viability (albeit not statistically significant), and (4) produced significantly fewer total adult progeny than the reciprocal.

Asymmetries in reciprocal crosses might occur in *C*. sp. 9/*C. briggsae* hybrids due to the effects of uniparentally inherited DMIs (uDMIs), such as X-linked uDMIs, maternal effects, and cytonuclear interactions. Turelli and Moyle (2007) suggested that differences between two species in the ratio of the rates of mitochondrial to nuclear evolution between two species can result in consistent asymmetries between reciprocal crosses. If the differences in these ratios are due to a systematic bias, then the species with the higher ratio of mitochondrial to nuclear evolution would be predicted to be the "worse" maternal parent, as was found in a clade of Centrarchid fish (Bolnick et al. 2008). Both nuclear and mitochondrial rates of mutation have been found to be higher in *C. briggsae* than in *C. elegans* (Howe and Denver 2008; Phillips et al. 2009), but it remains to be seen whether the ratio of these two rates of evolution is higher than that of *C*. sp. 9.

Martin-Coello et al. (2009) proposed gametic isolation due to sexual selection as a potential explanation for Darwin's corollary. We found a significant negative correlation between *C*. sp. 9 intrastrain ranks and *C*. sp. 9 interspecific paternal ranks ($P = 0.019$), implying that "good" paternal strains in intrastrain crosses are "poor" paternal strains in interspecific crosses. Sexually antagonistic sexual selection is one possible explanation for this pattern. Antagonistic sexual selection can give rise to gametic isolation when there is competition among sperm for access to female gametes and when there is antagonism, such as polyspermy, between the gametes (Gregory and Howard 1994; Vacquier 1998; Price et al. 2001). Martin-Coello et al. (2009) propose that when two species that experience different strengths of sexual

selection hybridize, there will be an asymmetry between the reciprocal crosses for fertilization success.

The breeding system differences between *C. briggsae* and *C.* sp. 9 likely cause very different levels of male–male sperm competition and ovum defensiveness between the two species. Given the rarity of genetically successful mating by *C. briggsae* in nature (Cutter et al. 2006), *C. briggsae* surely has much lower male–male sperm competition than dioecious species such as *C.* sp. 9. If sexual antagonism in the form of ovum protection against polyspermy is a factor in ovum/sperm evolution in *Caenorhabditis*, then we would expect crosses between *C. briggsae* sperm-depleted hermaphrodites and *C.* sp. 9 males to have more progeny than the reciprocal, owing to lower defenses of *C. briggsae* ova against competitive *C.* sp. 9 sperm and to *C.* sp. 9 ova being more protective against polyspermy by the less competitive *C. briggsae* sperm (Martin-Coello et al. 2009). A model of parent–offspring conflict also predicts such a pattern for crosses between selfing and outcrossing species (Brandvain and Haig 2005). Because our results actually show the opposite pattern, these mechanisms of ovum–sperm or parent–offspring antagonism seem unlikely causes of Darwin's corollary for *C. briggsae*/*C.* sp. 9 hybrids.

F$_1$ FERTILITY

The fertility of F$_1$ hybrid females, irrespective of the reciprocal cross from which they derived, was significantly higher when backcrossed to *C.* sp. 9 males than to *C. briggsae* males (see also Woodruff et al. 2010). Successful backcrosses to *C. briggsae* males were quite rare, comprising only 6.5% of backcrosses (vs. 51.3% successful backcrosses to *C.* sp. 9 males). Of the 14 fertile *C. briggsae* backcrosses, five involved strain ED3083 and four involved strain HK105. With so few fertile backcrosses, we cannot determine confidently whether these two strains were more successful because of chance or a systematic difference; however, this should be tested explicitly in future work.

GENETIC EXPLANATIONS FOR ASYMMETRIC PATTERNS OF HYBRID INCOMPATIBILITY

Several explanations involving DMIs could potentially explain the patterns that we documented for asymmetries in F$_1$ hybrid viability and fertility, some of which are not necessarily mutually exclusive. Interactions between dominant factors on *C.* sp. 9 autosomes with recessive factors on *C. briggsae* autosomes can explain the rarity of successful backcrosses to *C. briggsae* males: only in such backcrossed zygotes will *C. briggsae* loci become homozygous and manifest their effects on reproductive isolation. Woodruff et al. (2010) performed further crosses with the progeny of F$_1$ female × *C.* sp. 9 male crosses (F$_1$ females from *C.* sp. 9 maternal P$_0$ crosses). Let this generation of progeny be called B2$_{b,9}$ where the first subscript represents the P$_0$ paternal parent and the second subscript represents the backcross male parent

(b = *C. briggsae*, 9 = *C.* sp. 9; Fig. 1), following the nomenclature of Woodruff et al. (2010). Their results showed that B2$_{b,9}$ females mated to *C. briggsae* males fail to produce viable progeny, whereas crosses between B2$_{b,9}$ males and *C. briggsae* sperm-depleted hermaphrodites produce 30% viable progeny. Because recessive autosomal factors should affect both of those crosses equally, additional or alternate incompatibilities must be at work.

To cause the asymmetry between B2$_{b,9}$ female × *C. briggsae* male and *C. briggsae* sperm-depleted hermaphrodite × B2$_{b,9}$ male crosses, there must be incompatibilities that are uniparentally inherited. Specifically, interactions between *C. briggsae* nuclear recessive factors and *C.* sp. 9 cytoplasmic factors could explain many of the asymmetric parent-of-origin patterns from our study as well as in Woodruff et al. (2010). Any individual that inherits a *C.* sp. 9 cytoplasm and both copies of recessive *C. briggsae* incompatibility loci would have lower fitness than individuals without this combination of incompatibilities. Consequently, this form of incompatibility could explain the lower reproductive success of B2$_{b,9}$ females crossed with *C. briggsae* males than (1) B2$_{b,9}$ males crossed with *C. briggsae* sperm-depleted hermaphrodites and (2) B2$_{b,9}$ females crossed to *C.* sp. 9 males. Additionally, such a nuclear recessive × cytoplasmic incompatibility can explain the observation by Woodruff et al. (2010) that B3$_{b,9,9}$ females (derived from a cross between the above-mentioned B2$_{b,9}$ female and a *C.* sp. 9 male) crossed to *C.* sp. 9 have higher viability than B3$_{b,9,9}$ females crossed to *C. briggsae* males. Further crosses could further discern *C. briggsae* nuclear recessive × *C.* sp. 9 cytoplasmic incompatibilities in causing the observed asymmetries. Specifically, if *C. briggsae* nuclear recessive × *C.* sp. 9 cytoplasmic incompatibilities are the main cause of fitness loss in backcrossed hybrids, then B2$_{9,9}$ and B2$_{9,b}$ females (which have *C. briggsae* cytoplasm) backcrossed to parental males should all have equivalent fitness because they would be unaffected by this class of incompatibility. In any case, we predict an important role of cyto–nuclear interactions in reproductive isolation in this system, and given the unusual mitochondrial evolutionary dynamics in *C. briggsae* (Howe and Denver 2008; Raboin et al. 2010), they may also plausibly underlie variability within *C. briggsae* for hybrid incompatibility with *C.* sp. 9.

Incompatibilities involving paternal factors might also contribute to reproductive isolation between *C. briggsae* and *C.* sp. 9. A recessively acting *C. briggsae* paternal factor can explain the very low levels of successful backcrosses to *C. briggsae* males by F$_1$ hybrid females from both parental reciprocal crosses. Paternal effect genes, although not as well studied as maternal effects, have been found to be crucial to embryogenesis in *C. elegans*, as in the sperm-supplied SPE-11 (Browning and Strome 1996). In *C. elegans*, a paternal effect gene has also been found to be involved in a synthetic lethal interaction with a naturally occurring zygotic deletion allele, leading to partial incompatibility between

two genetic backgrounds (Seidel et al. 2008). The role of paternal factors in reproductive isolation between *C. briggsae* and *C.* sp. 9 is also supported in this study by the number of significant differences between strains of both species in the "paternal strain nested in species" effect term. A conceivable alternative to paternal-effect postzygotic incompatibility is prezygotic mating efficiency differences among male genetic backgrounds, as has been documented within *C. elegans* (Teotonio et al. 2006; Anderson et al. 2010; Murray et al. 2011) and between *Caenorhabditis* species (Chasnov and Chow 2002; Garcia et al. 2007). Males of *C.* sp. 9 certainly are more vigorous than males of *C. briggsae*, but, perhaps counterintuitively, the production of a mating pheromone by virgin females and virgin female mating-facilitation behavior (Chasnov et al. 2007; Garcia et al. 2007) is likely to actually induce more successful heterospecific mating by *C. briggsae* males than conspecific mating to hermaphrodites that do not express such traits. Further studies are needed to determine mechanistically how paternal effects contribute to reproductive isolation between these two species.

INTRASPECIFIC VARIATION FOR REPRODUCTIVE ISOLATION

We demonstrated that heritable variation for hybrid viability exists within both *C. briggsae* and *C.* sp. 9, as both maternal and paternal parents. We conclude that polymorphic incompatibility loci in *C. briggsae* and *C.* sp. 9 occur on maternally inherited factors, such as mitochondria, and potentially that some polymorphic incompatibility loci exert paternal effects, in addition to nuclear-encoded incompatibility loci. This phenomenon of heritable variation for reproductive isolation appears to be a common and underappreciated aspect of speciation, with many examples now known to harbor polymorphism at loci involved in reproductive isolation (Wade and Johnson 1994; Shuker et al. 2005; Sweigart et al. 2007; Good et al. 2008; Reed et al. 2008; Rieseberg and Blackman 2010; Cutter 2012). Standard theory on the BDM model for speciation does not take account of polymorphism for incompatibility loci, yet polymorphism for incompatibility alleles can greatly impact the evolutionary dynamics of speciation (Martin and Willis 2010).

While intuition suggests that polymorphic incompatibility loci will be more common during early stages of speciation, and diminish as reproductive isolation completes, theory does not currently describe the expectations for this dynamic (Orr 1995; Turelli and Orr 2000; Orr and Turelli 2001). In general, we do not know what fraction of overall isolation between incipient species should be due to fixed versus polymorphic factors. We note that the sums of squares are similar for intraspecific versus interspecific contributions to differences in the numbers of hybrid adult progeny (Table 3), suggesting that fixed and polymorphic incompatibilities might contribute roughly equally even for these

species at an advanced stage of reproductive isolation. Current estimates suggest that *C.* sp. 9 and *C. briggsae* are quite divergent at the molecular level (pairwise synonymous-site divergence $K_S \sim$ 15%) and thus have been isolated for a substantial amount of time (Cutter et al. 2010). Our observations of variation for incompatibility between these species highlight the relevance of heritable variation for reproductive isolation between both early stages and late stages of the speciation process. This indicates that variable reproductive isolation must be considered more vigorously by theory and empirical work to derive a complete understanding of the evolution of reproductive isolation, including insight into the evolutionary forces (genetic drift and selection) acting on incompatibility loci within their own species' genetic background.

Having identified heritable variation for reproductive isolation in the *Caenorhabditis* model system, the door is now open to identify the underlying nucleotide changes responsible for generating polymorphic incompatibility loci and better understanding of their evolutionary history. It remains an important unsolved problem whether the evolutionary forces acting on fixed versus polymorphic isolating loci are similar or different and, in particular, how this translates into the relative roles of genetic drift and selection in the evolution of reproductive isolation.

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