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Insights Into Species Divergence and the Evolution of Hermaphroditism From Fertile Interspecies Hybrids of Caenorhabditis Nematodes

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ABSTRACT

The architecture of both phenotypic variation and reproductive isolation are important problems in evolutionary genetics. The nematode genus Caenorhabditis includes both gonochoristic (male/female) and androdioecious (male/hermaprodite) species. However, the natural genetic variants distinguishing reproductive mode remain unknown, and nothing is known about the genetic basis of postzygotic isolation in the genus. Here we describe the hybrid genetics of the first Caenorhabditis species pair capable of producing fertile hybrid progeny, the gonochoristic *Caenorhabditis* sp. 9 and the androdioecious *C. briggsae*. Though many interspecies F_1 arrest during embryogenesis, a viable subset develops into fertile females and sterile males. Reciprocal parental crosses reveal asymmetry in male-specific viability, female fertility, and backcross viability. Selfing and spermatogenesis are extremely rare in XX F_1, and almost all hybrid self-progeny are inviable. Consistent with this, F_1 females do not express male-specific molecular germline markers. We also investigated three approaches to producing hybrid hermaphrodites. A dominant mutagenesis screen for self-fertile F_1 hybrids was unsuccessful. Polyploid F_1 hybrids with increased *C. briggsae* genomic material did show elevated rates of selfing, but selfed progeny were mostly inviable. Finally, the use of backcrosses to render the hybrid genome partial homozygous for *C. briggsae* alleles did not increase the incidence of selfing or spermatogenesis relative to the F_1 generation. These hybrid animals were genotyped at 23 loci, and significant segregation distortion (biased against *C. briggsae*) was detected at 13 loci. This, combined with an absence of productive hybrid selfing, prevents formulation of simple hypotheses about the genetic architecture of hermaphroditism. In the near future, this hybrid system will likely be fruitful for understanding the genetics of reproductive isolation in Caenorhabditis.

THE genetic basis of phenotypic diversity is an important, albeit poorly understood phenomenon. Caenorhabditis nematodes provide a system that can address such an issue. *Caenorhabditis elegans* can act as an excellent point of reference for comparative development studies (Félix 2007; Lin *et al.* 2009; Schulze and Schierenberg 2009), and the variation in reproductive mode within Caenorhabditis is an alluring subject for such investigations (Haag 2005). Some Caenorhabditis species are gonochoristic (male/female), whereas others are androdioecious (male/hermaphrodite; Figure 1). Hermaphrodites and females are somatically similar, but while females only make oocytes, hermaphrodites briefly undergo spermatogenesis before switching to oogenesis (Ellis and Schedl 2006). This striking interspecies difference is not only discrete and easily scored, but is also of great consequence for reproductive strategies and population genetics.

Many studies have addressed the evolution of germline sex determination in Caenorhabditis. Phylogenetic analyses suggest that the trait has evolved convergently in this lineage multiple times (Cho *et al.* 2004; Kiontke *et al.* 2004). Consistent with this, differences in the presence and functions of germline sex determination genes have been uncovered between the convergently evolved *C. elegans* and *C. briggsae* (Nayak *et al.* 2005; Hill *et al.* 2006; Guo *et al.* 2009). Similarities in germline sex determination between gonochoristic and androdioecious Caenorhabditis species have also been found (Haag and Kimble 2000; Chen *et al.* 2001; Haag *et al.* 2002). Remarkably, reverse genetic manipulations can cause a *C. remanei* female to produce activated sperm and lay self-progeny (Baldi *et al.* 2009). However, despite these successes, there has been little progress in identifying the historical causative genetic differences distinguishing hermaphrodites from their female ancestors. Indeed, because the exact cause of the sperm-to-oocyte switch in *C. elegans* remains elusive (Ellis and Schedl 2006) candidate-gene approaches to understanding the evolution of this trait in other Caenorhabditis species are problematic. The female–hermaphrodite species pairs studied thus far have been quite diverged

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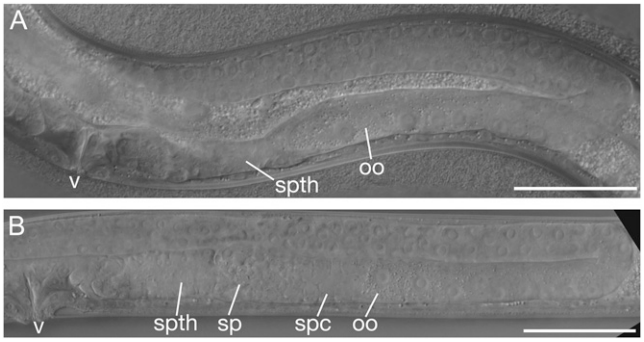

Figure 1.—*C. briggsae* and *C.* sp. 9 differ in reproductive mode. (A) A young *C.* sp. 9 JU1325 adult female. The most proximal germ cell is an oocyte (oo), and no sperm cells are present. (B) A young *C. briggsae* VT847 adult hermaphrodite. Mature sperm (sp) are the most proximal population of germ cells, followed by developing spermatocytes (spc) and oocytes (oo). Bars, 100 μm. spth, spermatheca; v, vulva.

from each other (Haag and Kimble 2000; Cutter 2008). Here, we explore the possibility that a more closely related mixed-mode species pair might open the door to traditional genetic trait mapping via hybrids.

In addition to the evolution of novel forms, another long-standing problem in biology is the genetic basis of postzygotic reproductive isolation. Indeed, the literature on interspecies hybrids is vast in Drosophila (Orr 2005) and other taxa (Presgraves 2010). Recent advances in Drosophila (Presgraves *et al.* 2003; Brideau *et al.* 2006; Ferree and Barbash 2009; Phadnis and Orr 2009; Tang and Presgraves 2009) have provided insights into the genetic bases of postzygotic reproductive isolation. Furthermore, these results are largely consistent with the notion that Dobzshanski–Muller incompatibility factors epistatically interact to promote hybrid inviability and sterility, helping to confirm a theory of how reproductive isolation can evolve (Dobzhansky 1937; Muller 1942). However, the Caenorhabditis system has made very few contributions to this issue (Baird *et al.* 1992; Baird and Yen 2001; Hill and L'Hernault 2001; Baird 2002; Seidel *et al.* 2008). This is somewhat surprising considering the breadth of subjects this system has been used to examine (*e.g.*, de Bono and Bargmann 1998; Griffitts *et al.* 2001; Raizen *et al.* 2008). However, hybrid genetics has largely been impossible in this system due to the inability of any Caenorhabditis interspecies hybridization to successfully produce fertile hybrid progeny (Baird *et al.* 1992).

We have discovered a new gonochoristic Caenorhabditis species, provisionally named *C.* sp. 9, that is capable of producing fertile hybrids with the androdioecious *C. briggsae*. The existence of fertile hybrids between species of different reproductive mode opens up the possibility of using trait mapping approaches to examine the genetic basis of hermaphroditic spermatogenesis. Additionally, it allows the Caenorhabditis system to contribute to the study of the genetics of postzygotic reproductive isolation. Here, experiments conducted in this new hybrid system that pertain to both of these issues are described.

MATERIALS AND METHODS

Nomenclature: To simplify discussion of the numerous hybrid crosses described herein, we developed a shorthand to denote specific hybrid generations (see Figure 2). The prefixes "F" and "B" are used for intercrosses and backcrosses, respectively, and are followed by standard size numbers denoting the *n*th generation since the pure species intercross. In addition, a subscript is used to specify the species identity of each sex in the crossing scheme that produced that generation. The sex of the animal corresponding to the subscript is assumed to be male unless otherwise denoted with an "f" for female or "h" for hermaphrodite. For instance, $F1_b$ denotes the generation resulting from the *C. briggsae* male × *C.* sp. 9 female parental hybrid cross (Figure 2A). Conversely, the generation resulting from the reciprocal parental cross would be the $F1_9$ (Figure 2B). A generation resulting from a scheme where the P0 father was *C. briggsae* and the hybrid female progeny were subsequently backcrossed to *C.* sp. 9 males would be the $B2_{b,9}$ generation (Figure 2D). And, when a $B2_{b,9}$ hybrid male is crossed to a *C. briggsae* hermaphrodite, the $B3_{b,9,bh}$ generation results (Figure 2G). When the directionality of a given cross is of no consequence, the subscript will be omitted.

Maintenance and strains: Animals were maintained according to standard *C. elegans* protocols (Wood 1988), with the exception of increased agar concentration in nematode growth medium (NGM) plates to 2.2%. Cultures were kept at 20° unless otherwise indicated. Inbred lines of *C.* sp. 9 were generated through 25 generations of full-sibling inbreeding. Strains used in this study include *C. briggsae* AF16 (sequenced reference strain), *C. briggsae* VT847 (mapping strain), *C. briggsae* HK104 (mapping strain), *C. briggsae* CP4 [*dpy(nm4)II*] *C. briggsae* CP99 [*Cbr-unc-119* (*nm67*), courtesy of C. G. Thomas], *C. briggsae* CP116 (polyploid strain, this study), *C.* sp. 9 JU1325 (wild isolate from India), *C.* sp. 9 EG5268 (wild isolate from Congo, a gift of Michael Aillon), *C.* sp. 9 JU1422 (inbred derivative of JU1325), and *C.* sp. 9 JU1420 (inbred derivative of JU1325).

C. sp. 9 strain JU1325 was isolated by M.A.F. from rotting flowers and leaves sampled in the Zoo/Botanical Garden of Trivandrum, Kerala, India on December 21, 2007. The sample was kept in a plastic tube for 2 weeks. Nematodes were then isolated on agar plates seeded with *Escherichia coli* OP50 as described in Barrière and Félix (2006). Anatomical observation tentatively assigned JU1325 to the Elegans group of Caenorhabditis. Test crosses to *C. remanei*, *C. briggsae*, *C. brenneri*, *C.* sp. 5, and *C. elegans* indicated JU1325 was sufficiently reproductively isolated from other species of the Elegans group to consider it a new species of Caenorhabditis and that it was likely very closely related to *C. briggsae*. Sequence data are also consistent with this conclusion (Cutter *et al.* 2010).

Determination of viability, sex ratio, and brood size: We define viability as the fraction of laid embryos that develop into adults. To measure viability, three females or hermaphrodites and five males were mated. *C.* sp 9 females were picked at the L4 stage to ensure virginity, and all L4 *C. briggsae* hermaphrodites used for hybrid crosses were purged of all self-sperm by daily plate transfers of solitary animals. After mating overnight, males were removed, the mothers were moved to a fresh plate, and the eggs on the previous plate were counted. This was repeated about every 12 hr until no more embryos were laid. The plates were scored for female and male adults 6 days

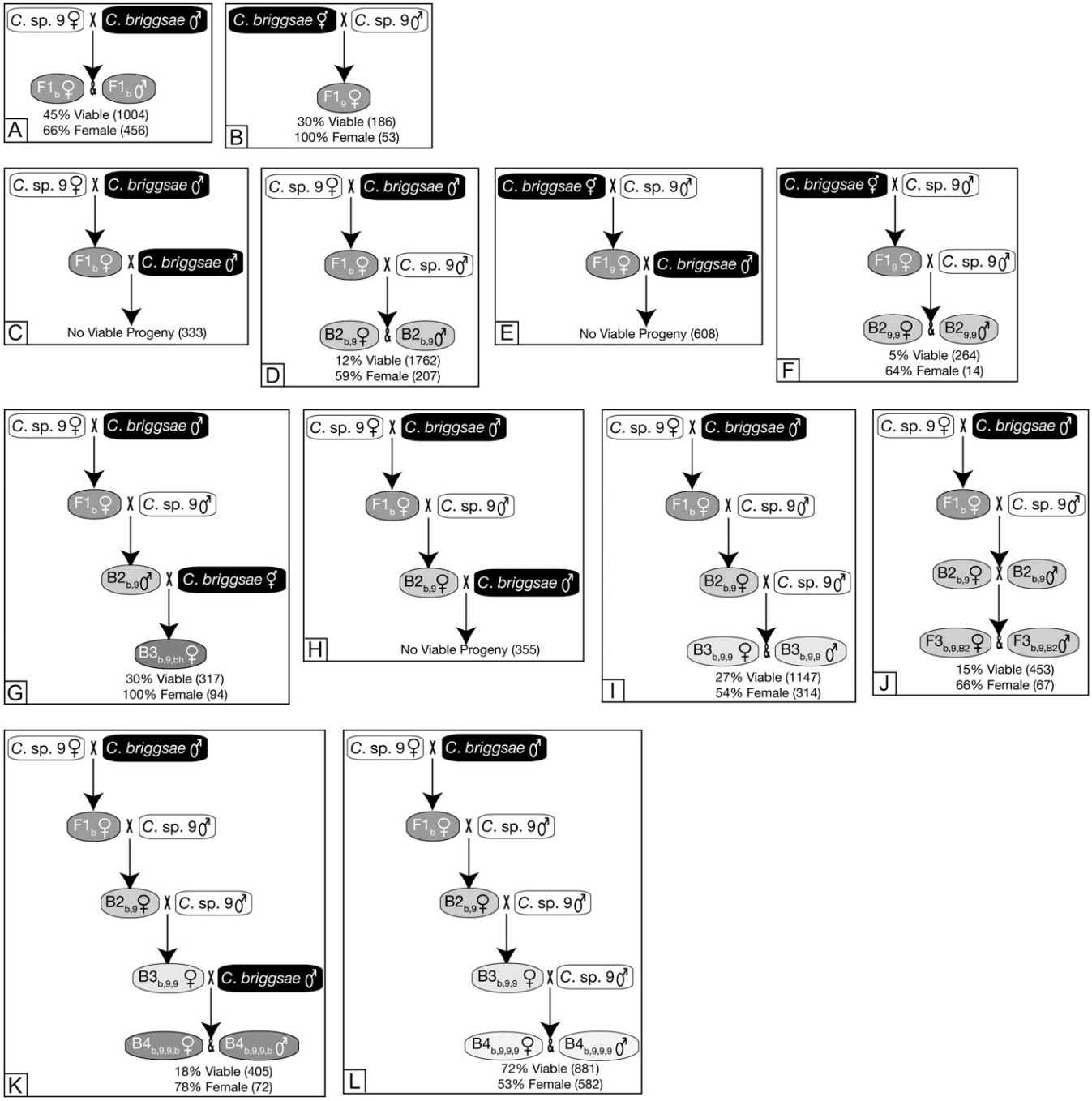

FIGURE 2.—Summary of various hybrid crosses. (A–L) Each panel represents a particular cross, with parents above and realized progeny below. Progeny are numbered via the scheme described in MATERIALS AND METHODS. Numbers in parentheses represent the number of embryos and adult progeny scored to produce percentages of viable and female progeny, respectively. All hybrids derived using the *C. briggsae* strain AF16 and the *C.* sp. 9 strain JU1325, except in panels E and F, where *C.* sp. 9 JU1422 was used.

after laying. The sex ratio is defined as the fraction of total adults that are female. Brood sizes, defined as the number of embryos laid by a given XX animal, were determined via a similar procedure, except matings with individual mothers were used. All brood size, viability, and sex ratio measurements were performed with *C.* sp. 9 JU1325 and *C. briggsae* AF16 unless otherwise indicated.

Fertility was measured on selected hybrid populations by single worm matings. For males, one male was placed with four wild-type *C.* sp. 9 females. If embryos were present on the plate the next day, the worm was marked as fertile. For females, the test was done with one virgin female and five *C.* sp. 9 males. The percentage of fertility is derived from the fraction of single worm matings that yield embryos. Plates where the individual worm being assayed had fled the plate were discarded. The extent of F1 male sterility was also evaluated by determining the fraction of males with abnormal gonad morphology under differential interference contrast (DIC) microscopy.

Determination of selfing and spermatogenesis incidence in hybrids: We define "selfing" as the production of embryos in the absence of mating, which in all known Caenorhabditis species only occurs in the presence of XX spermatogenesis. To measure this in hybrids, XX L4 animals were removed from males and left overnight at 20°. Up to 50 L4 animals were picked to a single plate for the scoring of selfing. If embryos were observed on the plate, the plate was examined for the presence of an animal with embryos in its uterus. Typically, no more than one hybrid selfer was observed per plate. In addition, virgin young adult XX animals (produced as above) were scored for the presence of sperm-like or spermatocyte-like cells via DIC microscopy.

Immunoblotting: Protein samples were prepared by transferring 100 worms into 30 μl of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4), followed by addition of 30 μl 95% Laemmli sample buffer (Bio-Rad) + 5% β-mercaptoethanol. SDS polyacrylamide protein gels were run according to standard methods (Sambrook and Russell 2001). Both anti-MSP mouse monoclonal antibodies (provided by D. Greenstein; Kosinski *et al.* 2005) and anti-α-tubulin mouse antibodies (Sigma) were added to the blocking solution at dilutions of 1:1000. The secondary antibody, a horseradish peroxidase-conjugated anti-mouse conjugate (GE Healthcare), was used at a dilution of 1:1500 for 1.5 hr. Antibody-bound proteins were visualized using the SuperSignal Chemiluminescent substrate (Pierce Technology).

RT–PCR: RNA preparations were made by transferring worms of the appropriate age and sex into RNA-ase free water at a concentration of 4 worms/μl, with ~200 worms per sample. TRI reagent (Molecular Research Center) was added to each preparation, and the samples were frozen at −80°, thawed, pelleted in a microcentrifuge, and then lysed with a plastic pestle in a 1.5-ml microfuge tube. RNA was then purified via phenol/chloroform extraction and precipitation with isopropanol. RNA was reconstituted with RNA-ase free water, using 1 μl for every 4 worms in the initial preparation. Five microliters of an RNA prep was used in a RT–PCR reaction using the AccessQuick kit (Promega). Primers AD115 (5′-TCGACGACTTGGCTGTGCAAC-3′) and AD116 (5′-TTGAC GAGCTGTTTGATGCCCACC-3′) were used to amplify a 245-bp fragment of the *cb-fog-3* transcript, and primers EH37 and EH38 (Hill and Haag 2009) were used to amplify a 250-bp fragment of all *C. briggsae* actin paralogs. Reactions were then run on a 1% agarose ethidium bromide gel to visualize the amplicons.

Mutagenesis and screening: Synchronized cultures of *C.* sp. 9 JU1422 L4 larvae were mutagenized for 4 hr using 50 mM ethyl methanesulfonate (EMS) according to standard methods (Brenner 1974). Animals were washed multiple times in M9 buffer and distributed onto seeded NGM plates. Approximately 10 such mutagenized virgin *C.* sp. 9 females were then mated with ~20 *C. briggsae* AF16 males overnight, after which all parental males were removed. Plates were subsequently scored for the next 7 days for the presence of F2 embryos, which, given complete F1 male sterility, were likely to be due to XX self-fertility.

Construction of a polyploid *C. briggsae* strain: Polyploid Caenorhabditis lines have been used to determine the chromosomal basis of sex determination in this genus (Nigon 1951; Madl and Herman 1979). A modified version of Madl and Herman's (1979) heat-shock protocol was used to generate a similar strain of *C. briggsae.* The wild-type *C. briggsae* AF16 and the dumpy *C. briggsae* CP4 (*nm4*) strains were shifted to 30° overnight. AF16 males were crossed with CP4 hermaphrodites, and 300 F1 L4 wild-type hermaphrodite progeny were singled to separate plates. The F2 self-progeny were scored for large animals, low brood size, and a high proportion of males, all of which are indicative of polyploids (Madl and Herman 1979). One such animal was found, and it was confirmed to sire polyploid progeny (likely 4A:3X, see Figure 8 below). This animal was used to generate the CP116 strain.

DNA preparations for genotyping: For DNA preparations, worms were picked into lysis buffer (50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM MgCl2, 0.45% NP-40, 0.45% Tween 20, 0.01% gelatin) at a concentration of 2 worms/μl with ~200 worms/prep. Proteinase K was added to a concentration of 100 μg/ml, and the preparation was frozen at −80° for at least 15 min. Samples were subsequently incubated for 1 hr at 65° and then at 95° for 30 min, after which they were used directly for PCR and genotyping. Control DNA samples for evaluating *C. briggsae* AF16/*C. briggsae* VT847 intrastrain segregation distortion were produced by using half AF16 worms and half VT847 worms. Samples representing the Mendelian expectations for $B3_{b,9,bh}$ animals were produced by mixing 50% *C.* sp. 9 JU1422 (not a productive template), 40% *C. briggsae* AF16, and 10% *C. briggsae* VT847. For X-linked markers, proportions of 25% *C.* sp. 9 JU1422, 50% *C. briggsae* AF16, and 25% *C. briggsae* VT847 were used for the Mendelian expectation control DNA preparation. Here, the expectation would be different from the autosomes because the hybrid X is donated by the male (Figure 9).

Genotyping: A total of 23 polymorphic molecular markers distinguishing *C. briggsae* AF16 and VT847 were used for genotyping, and all of these markers have been mapped to a physical position on the *C. briggsae* genome sequence (Koboldt *et al.* 2010). A total of 22 of these markers are single nucleotide polymorphisms (SNPs) and one an insertion/deletion (indel) of 18 bp. A total of 13 SNPs were genotyped via pyrosequencing technology and 9 via restriction fragment ("snip-SNP") analysis, and the indel marker was assayed by agarose gel electrophoresis. All genotyping methods required a PCR amplification step. For the snip-SNP and indel assays, primers previously designed for interstrain *C. briggsae* mapping were used (Koboldt *et al.* 2010).

For pyrosequencing reactions, amplification and sequencing primers were designed around the SNPs of interest using software provided by the manufacturer (Qiagen, formerly Biotage; supporting information, Table S1). For the PCR step, 0.5 μl of a DNA preparation (as described above) was used with a 30-μl mixture containing: 0.5 μM of an untailed primer, 0.1 μM of a tailed primer, 0.4 μM of a universal biotinylated primer, 0.25 mM of each dNTP, 1X ThermoPol PCR buffer (New England Biolabs), 1.5 mM Mg^{2+}, and 1 unit of *Taq* DNA polymerase per 10 μl. A single biotinylated primer was used for all pyrosequencing PCR reactions (Aydin *et al.* 2005). The cycling conditions were as follows: 95° denature (2 min), [95° denature (30 sec), 60° annealing (30 sec), 72° extension (30 sec)], 72° extension (5 min) with the bracketed subroutine repeated for 40 total cycles. The same conditions were used for each assay. Assays that only amplified *C. briggsae* DNA, and failed to amplify *C.* sp. 9 DNA, were used for genotyping. A total of 5 μl of all PCR reactions were visualized on an agarose gel to confirm amplification. Single-stranded PCR amplicons were purified with streptavidin sepharose beads (GE Healthcare) according to the manufacturer's instructions. Pyrosequencing reactions were performed using the PyroMarkTM Q96 ID machine (Qiagen) according to the manufacturer's instructions. Resulting data were analyzed using Allele Quantification software provided by the manufacturer. This software estimates allele frequencies of the polymorphic alleles through integration of the pyrogram peaks (Lavebratt *et al.* 2004).

For snip-SNP assays, the PCR conditions are as above, but with untailed, nonbiotinylated primers (0.5 μM each) designed

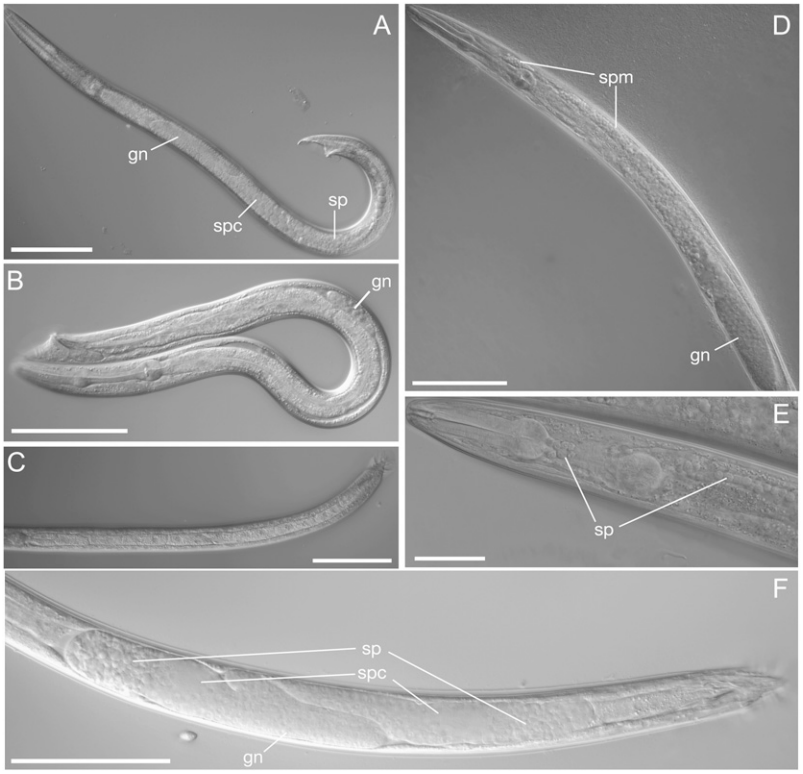

Figure 3.—Hybrid males have abnormal gonads. (A) A wild-type *C.* sp. 9 JU1422 adult male, with a wild-type gonad (gn), spermatocytes (spc), and sperm (sp). (B) A hybrid $F1_9$ male with an underdeveloped gonad (gn) and no mature germ cells. (C) A hybrid $F1_9$ male with no discernible gonad. (D) A hybrid backcross ($B2_{b,9}$) male with sperm (sp) located abnormally in the anterior. (E) A higher magnification image of the head of a hybrid backcross ($B2_{b,9}$) male with mislocalized sperm. (F) $B2_{b,9}$ male with developing spermatocytes (spc) and sperm (sp) oriented to both the anterior and posterior of the animal. Bars, 100 μm for all panels except for E, where it represents 50 μm. Hybrids shown in B and C were generated with the lines *C.* sp. 9 JU1325 and *C. briggsae* AF16, and the hybrids shown in C–F were generated with the lines *C.* sp. 9 1422 and *C. briggsae* AF16.

by Koboldt *et al.* (2010), 25 μl reaction volumes, and a 1-min extension time. After amplification, 1.2 μl of the appropriate restriction endonuclease (NEB) was added to reaction and incubated at the appropriate temperature for 2 hr. To quantify the allele frequencies of the polymorphisms, a standard curve for every assay was generated by performing the assay on DNA preps of known AF16/VT847 allele frequencies of 0.5/0.5, 0.6/0.4, 0.7/0.3, 0.8/0.2, and 0.9/0.1. AF16, VT847, *C.* sp. 9 JU1422, and AF16/VT847 F2 controls were also run. All control and test DNA were run out on the same 1% ethidium bromide agarose gel to visualize the polymorphic bands. Band intensities were quantified using ImageJ software (Abramoff *et al.* 2004). The ratio of the VT847 and AF16 diagnostic band intensities for the control reactions were plotted against their known allele frequencies and a best-fit regression line was then used to estimate the allele frequencies of the test reactions. This same general process was used for the indel marker, but here no restriction digest step was necessary, and the samples were separated on a 2% agarose gel to resolve the bands differing in size by 18 bp. To facilitate the comparison between the Mendelian control and hybrid $B3_{b,9,bh}$ results, raw data were normalized by forcing the average allele frequency of each Mendelian expectation control to its known value.

RESULTS

***C. briggsae* and *C.* sp. 9 produce fertile hybrids:** A summary of the various hybrid crosses and backcrosses we have examined and the naming scheme used to describe them is presented in Figure 2. Prior to this study, the highest reported Caenorhabditis interspecies hybrid viability is 6% for crosses of *C. remanei* females to *C. briggsae* males (Baird *et al.* 1992). In contrast, one-third to one-half of hybrid F1 progeny from reciprocal crosses of *C. briggsae* and *C.* sp. 9 were viable, with the remainder arresting during embryonic development. This viability is dependent on the direction of the parental cross. When *C.* sp. 9 is the mother, the F1 have a viability of 45%, whereas when *C. briggsae* is the mother, the viability is 30% (Figure 2, A and B). This difference between these reciprocal crosses can be accounted for entirely by an extreme difference in male-specific viability. When *C.* sp. 9 is the mother, 34% of the progeny are male, whereas when *C. briggsae* is the mother, no viable F1 male progeny were observed. The average brood size (70 embryos laid, $n = 5$, SE = 11), sex ratio, and viability of the $F1_b$ are all significantly different (*t*-test $P < 0.01$) from the conspecific *C.* sp. 9 cross. Here the average brood size is 259 ($n = 3$, SE = 32), the sex ratio is 53% female ($n = 1890$), and the viability is 82% ($n = 2312$).

$F1_b$ males exhibit delayed development and most are atypically small. No cross ever performed with F1 males was successful, and all males examined under Nomarski microscopy ($n = 94$) had gonadal defects (Figure 3). All lacked obvious spermatocytes or sperm, and 37% had no gonad at all. In contrast, using single-pair mating tests (see materials and methods), the vast majority of F1 females successfully produce embryos when crossed with *C.* sp. 9 males (Table 1). The resulting $B2_9$ progeny are less viable than the F1 (Figure 2, D and F), but surviving females are comparable in fertility to F1 females (data not shown), and viable males, roughly one quarter of which are fertile, are also produced (Table 1 and Figure 2G).

TABLE 1

Incidence of fertility in hybrid animals

Animal	Laid embryos % (*n*)
C. sp. 9 F	93 (98)
C. sp. 9 M	90 (100)
$F1_b$ F	95 (99)
$F1_9$ F	83 (101)
$B2_{b,9}$ M	24 (138)

The incidence of fertility was determined through single worm mating tests. Animals were mated with *C.* sp. 9 males (M) or females (F). All hybrids were derived using the *C. briggsae* strain AF16 and the *C.* sp. 9 strain JU1422.

Multiple asymmetries in postzygotic isolation exist between *C. briggsae* and *C.* sp. 9: In addition to the F1 sex ratio and viability asymmetries described above, other asymmetries were observed in later hybrid generations. F1 females in both reciprocal crosses are completely unable to produce viable progeny with *C. briggsae* males (Figure 2, C and E), with all progeny arresting during embryonic development. In contrast, they produce viable male and female progeny with *C.* sp. 9 males (Figure 2, D and F), with ~24% of $B2_{b,9}$ males being fertile (Table 1). Furthermore, the viability of B2 progeny depends on the identity of the P0 mother; the roughly twofold greater viability of $B2_{b,9}$ progeny than $B2_{9,9}$ progeny (Figure 2, D *vs.* F) is highly significant (χ^2 $P = 0.0019$, d.f. = 1). $B2_{b,9}$ males exhibit a wider range of male germline phenotypes than F1 males (Figure 3, D–F). This included gonads with sperm oriented toward the anterior (Figure 3D), animals with sperm apparently localized outside of the gonad (Figure 3E), and gonads with female-like dual polarity (Figure 3F). A total of 34% ($N = 167$) of $B2_{b,9}$ males have morphologically normal gonads. While fertile $B2_{b,9}$ males can successfully mate with *C. briggsae* hermaphrodites to produce hybrid progeny (Figure 2G), their sisters can never produce viable hybrid progeny with *C. briggsae* males (Figure 2H). Hybrid females can only be backcrossed to *C. briggsae* males to produce viable hybrid progeny after being backcrossed with *C.* sp. 9 for two generations (Figure 2K).

In addition to the above asymmetries, pure species *C.* sp. 9 males greatly reduce the brood size of *C. briggsae* hermaphrodites and prevent them from laying self-progeny (Figure 4). About 50 hr after mating with a conspecific male, *C. briggsae* hermaphrodites are laying an average of 6 embryos per hour. However, after mating with *C.* sp. 9 males, *C. briggsae* hermaphrodites stop laying altogether by this time, despite the presence of both sperm and oocytes in the reproductive tract. *C.* sp. 9 females stop laying embryos ~67 hr after mating (with either conspecifc or *C. briggsae* males). Examination of such postreproductive *C.* sp. 9 under DIC optics revealed that they had consistently run out of sperm.

***C.* sp. 9 has a low viability at temperatures <20°:** It was noticed that *C.* sp. 9 strains grow poorly at 15°, so the viability of *C.* sp. 9 and hybrid F1 animals were examined at a range of temperatures (Figure 5). At 27°, the viability of *C. briggsae* is 95% and the viability of *C.* sp. 9 is 89%. However, at lower temperatures (*i.e.*, <20°), *C. briggsae* has a much higher viability than does *C.* sp. 9 (87 *vs.* 6% at 15°, respectively). Hybrid F1 animals always displayed viability much lower than either *C.* sp. 9 or *C. briggsae* at all temperatures observed, but are similar to *C.* sp. 9 in performing particularly poorly below 20°.

Hermaphroditism is rare in hybrid F1 XX animals: Because *C.* sp. 9 and *C. briggsae* differ in reproductive mode, the germline sex of hybrid XX animals is of considerable interest. Three phenotypes were used to assess the presence of hermaphroditism among hybrid animals: the incidence of selfing (*i.e.*, the fraction of apparent XX animals that laid embryos with eggshells in the absence of males), the incidence of spermatogenesis (the fraction of XX adults that appeared to have sperm-like cells under DIC microscopy), and the presence of sperm-specific molecular markers in XX hybrids.

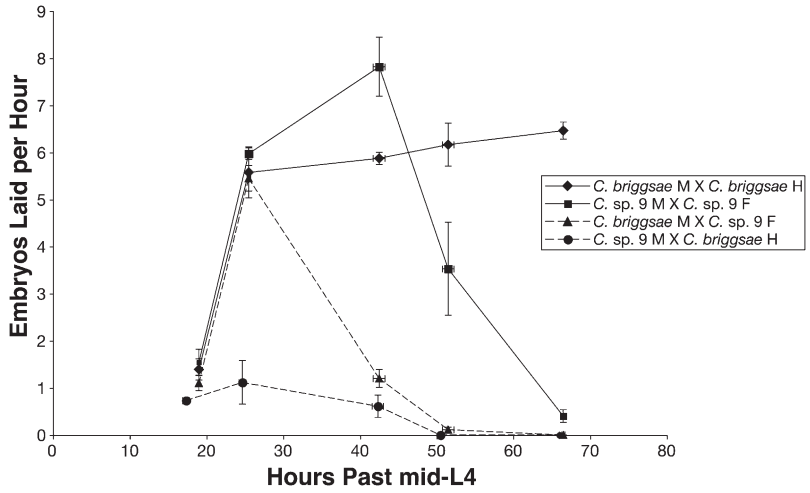

FIGURE 4.—*C.* sp. 9 males reduce the brood size of *C. briggsae* hermaphrodites. Conspecific crosses are solid lines, and hybrid crosses are dashed lines. Individual females or hermaphrodites were mated with four males overnight, after which the females were moved without the males to a new laying window twice a day. At least three replicates were performed for every cross. The error bars represent one standard error. The lines *C.* sp. 9 JU1325 and *C. briggsae* AF16 were used for all observations.

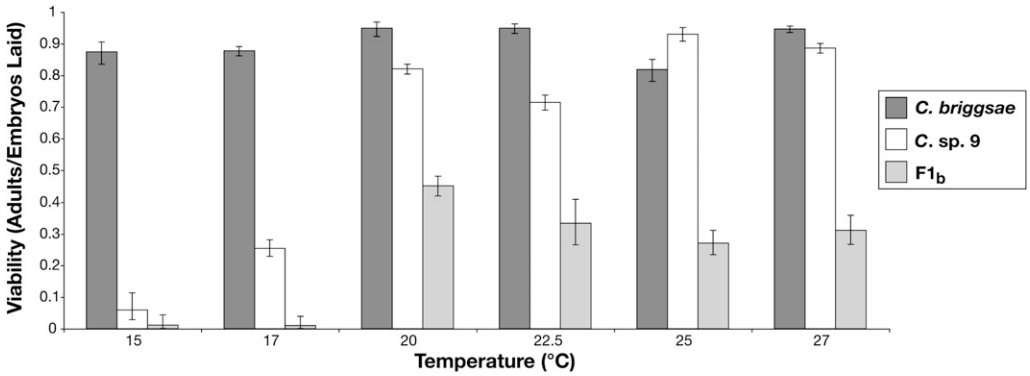

FIGURE 5.—Viabilities of *C. briggsae*, *C.* sp. 9, and $F1_9$ hybrids at different temperatures. Viability was measured as the number of adults resulting from the total number of embryos laid. All hybrid $F1_9$ were generated from crossing *C. briggsae* males to *C.* sp. 9 females. Error bars represent 95% confidence intervals. The lines *C.* sp. 9 JU1325 and *C. briggsae* AF16 were used for all observations. Sample sizes at temperatures 15°, 17°, 20°, 22.5°, 25°, and 27°, respectively: *C. briggsae* AF16 (342, 1883, 377, 867, 472, and 1800); *C.* sp. 9 JU1325 (136, 1078, 2312, 1396, 534, and 1616); and hybrid $F1_b$ (174, 195, 1004, 162, 510, and 383).

Both selfing and spermatogenesis are rare in the F1 (Table 2). Overall, the incidence of any detectable selfing in the F1 is very low, and all selfed embryos observed died prior to hatching. However, the incidence of selfing varies with respect to the strain of *C.* sp. 9 that is used. When the wild isolates JU1325 and EG5862 are crossed with *C. briggsae*, selfing is observed in 1.2% and 0.3% of hybrid F1, respectively. If the inbred line JU1422 is used, then selfing is never seen. The use of other strains of *C. briggsae*, such as HK104 and VT847, does not reveal any significant increase in the incidence of selfing (Table 2). Progeny of the reciprocal parental crosses differ in their incidence of selfing, although not significantly so.

Because selfing requires more than just the generation of sperm and oocytes in a female soma (BALDI *et al.* 2009), XX hybrids were also observed under DIC to investigate the possibility that many hybrids made sperm (and were in fact hermaphrodite) but were unable to produce self-progeny. The incidence of spermatogenesis in young adult XX hybrid F1 animals is higher than that of the incidence of selfing itself, but never exceeds a few percent (Table 2). Also, in most F1 females germline development is delayed with respect to *C.* sp.

TABLE 2

Number of hermaphrodites in hybrid generations

Animal scored	*C. briggsae* strain(s) used	*C.* sp. 9 strain used	Selfers % (*n*)	Spermatogenic % (*n*)
P0	NA	JU1422	0 ($n > 100$)	0 (118)
P0	NA	EG5268	0 (109)	—
$F1_b$	AF16	JU1422	0 (230)	0 (92)
$F1_b$	AF16	JU1325	1.2 (494)	0 (123)
$F1_b$	AF16	EG5268	0.3 (360)	3.5 (114)
$F1_9$	AF16	JU1325	0 (106)	—
$F1_b$	VT847	JU1422	0 (34)	—
$F1_b$	HK104	JU1422	0 (29)	—
$F1_b$	VT847	JU1325	0 (32)	—
$F1_b$	HK104	JU1325	0 (68)	—
$F1_b$	VT847	EG5268	0 (26)	—
$F1_b$	HK104	EG5268	0 (31)	—
$F1_b$	CP116 (polyploid AF16)	JU1325	0 (137)	8.3 (24)
$F1_b$	CP116 (polyploid AF16)	EG5268	2.3 (91)	15 (177)
$B3_{b,9,bh}$	AF16	JU1422	0 (181)	0 (65)
$B3_{b,9,bh}$	AF16	JU1325	0 (41)	—
$B3_{b,9,bh}$[a]	VT847 first AF16 second	JU1325	0 (128)	—
$B4_{b,9,9,b}$	AF16	JU1422	0 (202)	—
$B4_{b,9,9,b}$	AF16	JU1420	0 (396)	—
$B4_{b,9,9,b}$	AF16	JU1325	2.6 (117)	—

Selfer, animal that lays embryos without mating; spermatogenic, female/hermaphrodite has sperm-like cells under DIC microscopy; —, not determined. See Figure 2 for how $B3_{b,9,bh}$ and $B4_{b,9,9,b}$ animals are constructed.

[a] The $B3_{b,9,bh}$ generation used for the genotyping shown in Figure 10 was constructed with three Caenorhabditis strains as in Figure 9.

9 females. In *C.* sp. 9 females, typically at least one mature oocyte is fully developed by young adulthood (Figure 1A). However, in hybrid F1 animals, oftentimes no differentiated germ cells or incomplete oocytes ("ooids") are seen in the proximal germline of young adult XX animals (Figure 6, A and B). However, the stacking oocyte phenotype characteristic of normal unmated females is seen in most older hybrid F1 animals (Figure 6C). This, in tandem with the result that most hybrid F1 females are fertile (above, Table 1), suggests that germline development is delayed but otherwise normal in F1 females. Indeed, young adult F1 females observed to have no differentiated germ cells under DIC microscopy were rescued, and all displayed the stacking oocyte phenotype after ~12 hr at 20° (n = 10). Aside from rare hermaphrodites with clear populations of spermatocytes (Figure 5D), no hybrid F1 XX animals displayed sexually ambiguous populations of germ cells proximal to the oocytes.

The rarity of spermatogenesis and selfing in hybrid F1 XX animals does not entirely exclude the possibility that the hermaphroditism trait is codominant in this system. Despite the lack of morphologically sperm-like cells in the vast majority of F1 XX animals, their germlines could still possess cryptic male characteristics. Indeed, sex-specific germline molecular markers have been used in *C. elegans* to reveal the sexual identity of germ cells in the absence of morphological characteristics (Jones *et al.* 1996). Furthermore, the observed delayed oogenesis in young adult F1 XX animals is suggestive of codominant hermaphroditism. To investigate this possibility, the possible expression of male-specific molecular markers was examined in the $F1_b$ generation. Major sperm protein (MSP) is a crucial sperm cytoskeletal protein that is also implicated in oocyte maturation (Smith 2006). *fog-3* is a TOB-domain protein that is necessary for spermatogenesis in *C. elegans*, *C. briggsae*, and *C. remanei* (Chen *et al.* 2001). Neither MSP nor *fog-3* transcripts are detectably expressed in XX $F1_b$ L4 and young adult animals, though both are detectable in hermaphrodites and males (Figure 7, A and B). These results suggest that most hybrid F1 XX animals do not harbor germ cells with cryptic male character and that with only rare exceptions the female germline state is dominant in XX hybrid F1.

Attempts to produce hybrid hermaphrodites via mutagenesis: The genetic mechanism underlying female dominance may be due to a small number of hyperactive female-promoting genes in XX *C.* sp. 9 germ cells. If this were the case, then mutation of one of these genes could permit hermaphrodite-like levels of spermatogenesis in hybrid F1 XX animals and perhaps thereby provide enough viable F2 progeny to allow establishment of hybrid hermaphrodite lines. To test this possibility, mutagenized *C.* sp. 9 females were mated with *C. briggsae* males and their F1 progeny screened for the ability to produce viable self-progeny. No such F1 progeny were uncovered after screening ~15,000 mutagenized haploid genomes. In *C. elegans*, this treatment would result in an average of 7.5 null mutations per gene (Brenner 1974).

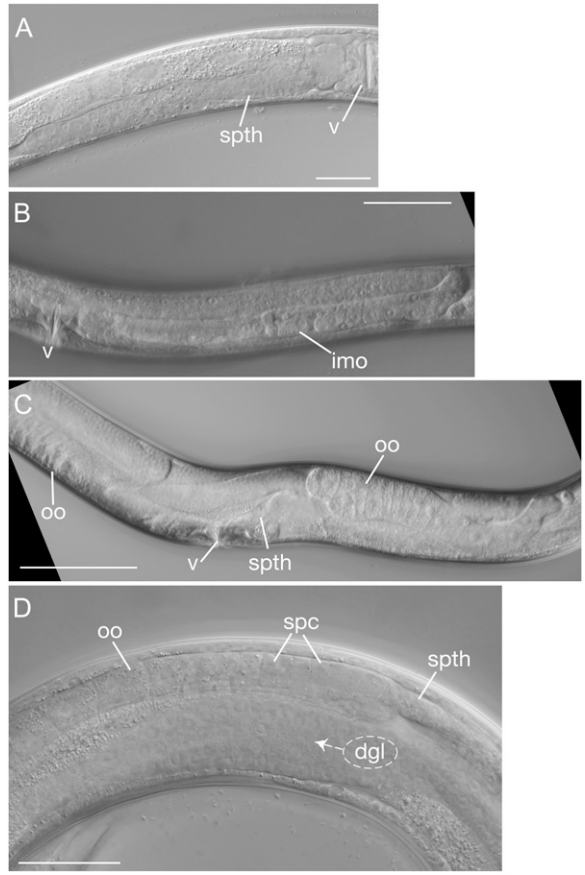

Figure 6.—Hybrid F1 female germlines. (A) A young hybrid $F1_b$ adult female displaying delayed germline development with no discernible differentiated germ cells despite having an adult vulva (v). The empty spermatheca (spth) is noted. (B) A young hybrid $F1_b$ adult female with small, proximal immature oocytes (imo). (C) A hybrid $F1_b$ adult female with stacking oocytes (oo) and an empty spermatheca (spth). (D) A rare hybrid $F1_b$ adult hermaphrodite with clear proximal spermatocytes (spc) and oocytes (oo). This animal was recovered and failed to lay any embryos. dgl, distal germline. Bar, 50 μm for A, B, and D; 100 μm for C. The lines *C.* sp. 9 JU1325 and *C. briggsae* AF16 were used for all panels, with the exception of D, where the *C.* sp. 9 line EG5468 was used.

Extent of F1 XX spermatogenesis is sensitive to the dosage of *C. briggsae* genetic material: As an alternative to the oligogenic hypothesis investigated above, the genetic mechanism underlying the recessivity of hermaphroditism could be due to haploinsufficiency, perhaps at many loci. That is, the hermaphroditism trait may be rarely expressed in the F1 because there is only one copy of the *C. briggsae* genome in the F1 instead of the two copies that exist in the parent. To test this possibility, a tetraploid strain of *C. briggsae*, CP116, was created. Two lines of evidence suggest that hermaphrodites in this strain are 4A:3X tetraploids. First, there is a high

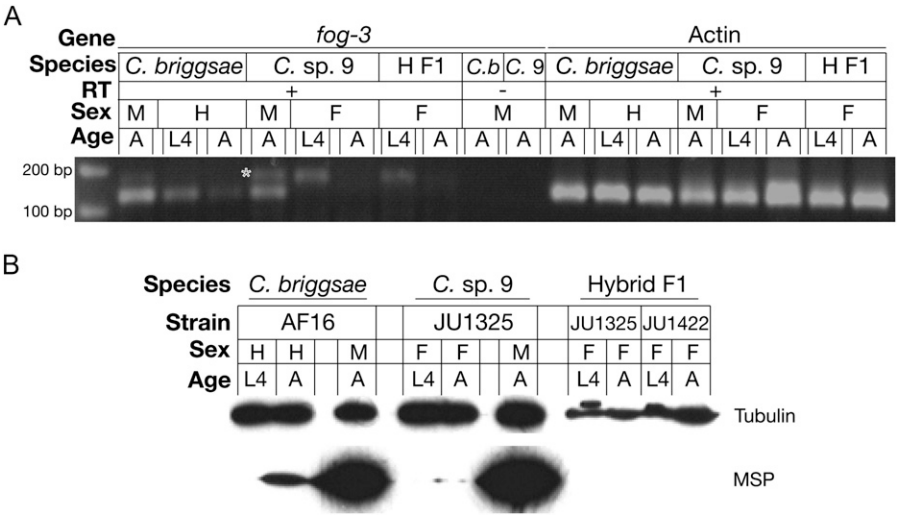

FIGURE 7.—Sperm-specific molecular markers in hybrid F1 females. (A) RT–PCR for the sperm-specific transcript *fog-3*. Primers specific for an actin transcript were used as a positive control. All corresponding actin and *fog-3* reactions used RNA from the same preparations in equal quantities. The asterisk denotes an uncharacterized amplicon that is not sex biased in *C.* sp. 9. H, hermaphrodite; A, adult; H F1, hybrid $F1_b$. (B) Western blot for the sperm-specific protein major sperm protein (MSP). An anti-tubulin antibody was used as a positive control. Asterisks denote nonspecific proteins. All protein preparations were made with 100 worms. Blot was exposed overnight to ensure greatest possibility of protein detection in the hybrids.

incidence of male progeny arising from virgin CP116 hermaphrodites (39.7%, *n* adult progeny = 229). In addition, when CP116 diakinesis-stage oocytes were examined under fluorescent microscopy using Hoescht staining, a majority of them contained 11 or 12 chromosomes (70%, *n* animals = 40; Figure 8B). No such oocyte observed had <9 chromosomes. This is in contrast to wild-type *C. briggsae* oocytes, which all contain 6 chromosomes (Figure 8A).

Tetraploid (likely 4A:2X) *C. briggsae* CP116 males were crossed with diploid *C.* sp. 9 females to generate a triploid hybrid $F1_b$ with a 2:1 ratio of *C. briggsae* to *C.* sp. 9 genetic material. When triploid hybrid $F1_b$ are produced using wild isolates of *C.* sp. 9, the incidence of selfing increases to 2.3%, and the incidence of spermatogenesis increases significantly to 14% when compared to diploid hybrid F1 (χ^2 *P*-value = 0.006, d.f. = 1; Table 2). Surprisingly, a small proportion of *C. briggsae* CP116/*C.* sp. 9 EG5268 self-progeny progress through embryonic development. Most undergo larval arrest, but one adult F2 was observed. It did not lay any embryos, but was observed to have both sperm and oocytes. No viable triploid hybrid F1 are produced when *C. briggsae* CP116 is crossed to the inbred *C.* sp. 9 strain JU1422. The great increase in both overt selfing and spermatogenesis in triploids with excess *C. briggsae* gene content suggests that hybrid F1 XX germline fate is at least somewhat sensitive to the dosage of "hermaphroditizing genes" and that haploinsufficiency can partly account for the dominance of the female germline state in the hybrid F1.

Partial homozygosity of *C. briggsae* loci in the hybrids does not reveal hermaphroditism: To further investigate the potential of this hybrid system for understanding the genetic basis of hermaphroditism, possible segregation of the hermaphroditism trait was examined in recombinant hybrid generations. Segregation of the trait in the traditional F2 intercross and *C. briggsae* backcross populations cannot be examined due to the developmental and reproductive incompatibilities of the hybrid system and to the recessivity of the hermaphroditism (see above). For these reasons, more unconventional crossing designs were used to produce animals with substantial homozygosity for *C. briggsae* alleles, which allows potential segregation of the recessive hermaphroditism trait if it has a simple genetic architecture and key alleles are not linked to hybrid lethality and sterility factors.

Two generations were investigated for the segregation of the hermaphroditism trait. One is the progeny of $B2_{b,9}$ males and *C. briggsae* hermaphrodites ($B3_{b,9,bh}$ animals; Figure 2G). The other results from crossing $B3_{b,9,9}$ hybrid females with *C. briggsae* males to produce $B4_{b,9,9,b}$ animals (Figure 2K). These generations should be homozygous for *C. briggsae* alleles at a nonzero fraction of their genomes if they undergo a Mendelian pattern of segregation. Other crosses potentially yielding hybrids with homozygous *C. briggsae* regions were examined but either yielded no viable progeny ($F1_b$ female × $B2_{b,9}$ male) or no hermaphrodites ($B2_{b,9}$ female × $B2_{b,9}$ male).

The incidence of selfing and spermatogenesis is 0% among $B3_{b,9,bh}$ XX animals (Table 2). In $B4_{b,9,9,b}$ XX animals, the selfing incidence varies, depending upon which *C.* sp. 9 strain is used. A total of 2.6% of XX $B4_{b,9,9,b}$ animals were selfers when the wild isolate strain of *C.* sp. 9, JU1325, was used, but none were seen with JU1420 and JU1422, the inbred strains derived from it (Table 2). This figure is not significantly different from that observed in hybrid F1 produced with JU1325 (1.2%; χ^2 $P = 0.5075$, d.f. = 1). These results suggest that partial homozygosity for *C. briggsae* genes in hybrids does little or nothing to allow reemergence of the hermaphroditism trait.

Hybrid animals show segregation distortion: It is possible that hermaphrodites are absent in the $B3_{b,9,bh}$

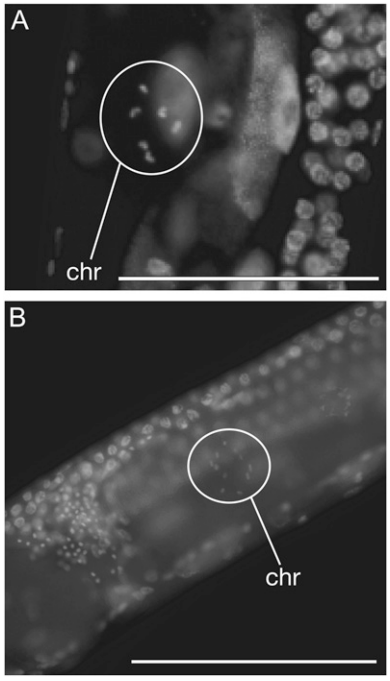

Figure 8.—A polyploid strain of *C. briggsae*. (A) A wild-type *C. briggsae* AF16 hermaphrodite with 6 chromosomes. Bar, 50 μm. (B) A polyploid *C. briggsae* CP116 hermaphrodite with 11 chromosomes. Bar, 100 μm. chr, chromosome. Both of these images are focused upon oocytes arrested in diakinesis stage of meiotic prophase I, with DNA stained with Hoescht 33258.

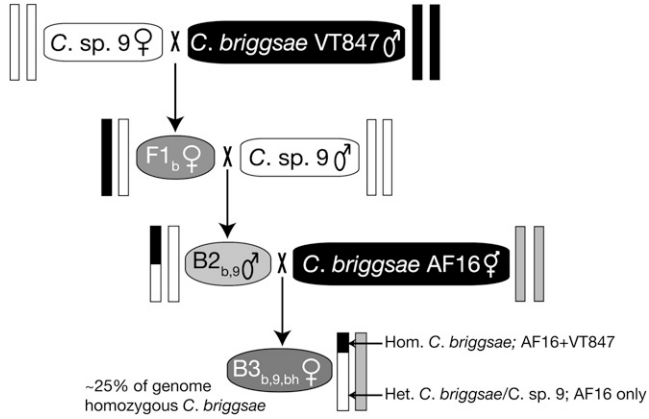

Figure 9.—Scheme for genotyping hybrid animals with partially homozygosity for *C. briggsae* alleles. Existing markers for mapping *C. briggsae* mutations (Koboldt *et al.* 2010) were used to genotype $B3_{b,9,bh}$ hybrid animals. The *C. briggsae* strain VT847 was used for the parental cross, whereas a different *C. briggsae* strain, the AF16-derived *Cbr-unc-119(nm67)* strain CP99, was used for the final cross. The mapping generation was then heterozygous for AF16 and VT847 when homozygous for *C. briggsae* and was homozygous AF16 when heterozgygous *C. briggsae/C.* sp. 9. Following a Mendelian pattern of segregation, a given locus was expected to be heterozygous AF16/VT847 (or homozygous *C. briggsae*) 25% of the time.

and $B4_{b,9,9,b}$ generations because multiple *C. briggsae* alleles must be homozygous for the trait to be observed. However, the patterns of hybrid viability suggest that there may be certain genotypes that promote hybrid lethality. If such hybrid lethal loci were linked to loci essential for hermaphrodite development, key genotypes would become inaccessible. To investigate this possibility, $B3_{b,9,bh}$ animals were genotyped at multiple loci to determine the extent of segregation distortion in this hybrid generation.

The crossing scheme in Figure 9 was used to allow the use of previously generated *C. briggsae* genetic markers (Koboldt *et al.* 2010). One strain of *C. briggsae* (VT847) was used as the P0 *C. briggsae* parent, and another strain of *C. briggsae* (the AF16-derived *Cbr-unc-119(nm67)* strain CP99) was used for the final backcross (Figure 9). This creates nonuncoordinated (non-Unc) $B3_{b,9,bh}$ hybrids that have one entire copy of the *C. briggsae* CP99 genome and one hybrid genome copy expected to contain ~25% *C. briggsae* VT847 DNA. Such hybrids would thus be expected to be homozygous for *C. briggsae* at a given locus 25% of the time. Only assays that amplified *C. briggsae* DNA and failed to amplify *C.* sp. 9 DNA were utilized. Thus, homozygosity at a given *C. briggsae* locus would be revealed by presence of both polymorphic variants for the two *C. briggsae* strains (AF16/VT847; Figure 9). In contrast, heterozygosity at the species level (*C. briggsae/C.* sp. 9) would be revealed through hemizygosity for the *C. briggsae* strain AF16 (Figure 9).

A total of 23 markers were used with an average distance of 3.98 Mb (~12.4 cM) (Hillier *et al.* 2007) between markers. Since the average size of the *C. briggsae* block of the recombinant hybrid chromosome was expected to be 12.5 cM, the assays provide reasonable power to detect segregation distortion if it were present. All 23 markers were confirmed to be dimorphic in *C. briggsae* AF16 and VT847, and no interstrain segregation distortion was seen (Figure S1; J. A. Ross, unpublished results). Control DNA preparation was made with proportions of *C. briggsae* AF16, *C. briggsae* VT847, and *C.* sp. 9 JU1422 animals equal to the proportions that would be expected in the hybrid $B3_{b,9,bh}$ generation if all of the genetic loci behaved in a Mendelian fashion. This facilitated the recognition of segregation distortion when compared with the hybrid $B3_{b,9,bh}$ generation genotypes.

Among the 23 markers genotyped, 13 displayed significant deviation (Mann–Whitney *U* test *P*-value <0.05) from the Mendelian expectation (Figure 10). Three markers showed no difference from expectation, and 7 markers displayed a nonstatistically significant deviation from the Mendelian expectation, all in the same direction. Strikingly, all 20 observed instances of substantial segregation distortion were underrepresentations of *C. briggsae* alleles, which is itself a highly significant deviation from random error model (binomial sign test, $P < 0.0001$). However, at no marker locus were *C. briggsae* alleles completely excluded. For certain

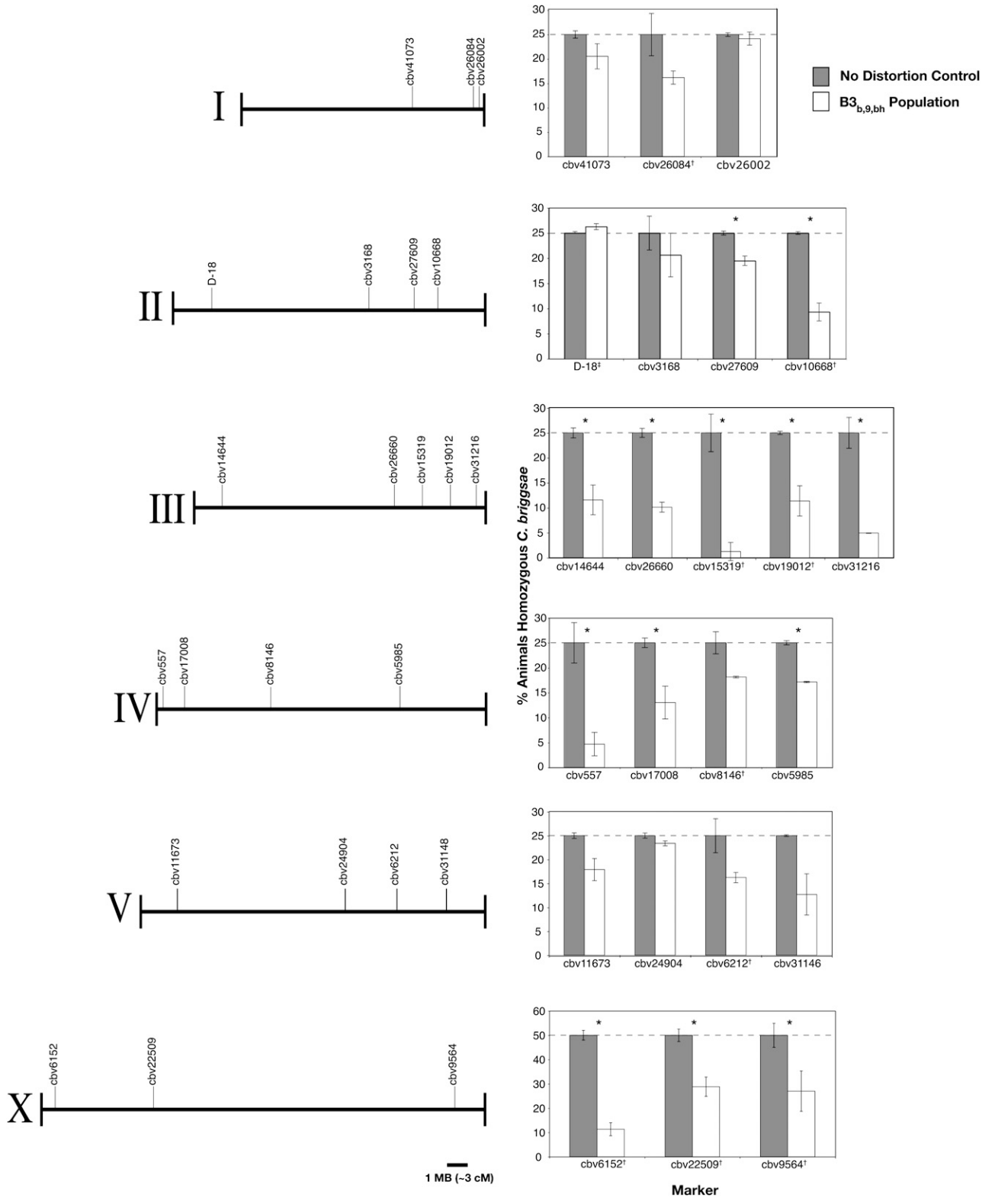

Figure 10.—Patterns of segregation of loci in hybrids with homozygosity for some *C. briggsae* alleles. Animals in the hybrid $B3_{b,9,bh}$ generation (Figure 9), which represents the backcross with the highest potential to produce *C. briggsae* homozygosity, were used for genotyping. On the left are the physical positions of the markers used for the genotyping. On the right is displayed the percentage of $B3_{b,9,bh}$ homozygosity for *C. briggsae* at each locus. Pyrosequencing, RFLP, or indel analysis was used to estimate the frequencies of *C. briggsae* AF16 and *C. briggsae* VT847 alleles for each marker. The control (shaded bars) was a mixture of *C. briggsae* AF16, *C. briggsae* VT847, and *C.* sp. 9 JU1422 worms in the same proportions expected for hybrid alleles under Mendelian segregation. Note that *C.* sp. 9 DNA was included to mimic the hybrid genome composition, but does not support PCR amplification of polymorphic *C. briggsae* sites. The percentage of *C. briggsae* homozygosity measured for the Mendelian controls was

regions of *C. briggsae* chromosomes II, III, IV, and X (Figure 10), it appears that either homozygosity in the $B3_{b,9,bh}$ generation or heterozygosity in the previous (B2) generation adversely affects hybrid fitness.

DISCUSSION

Reproductive isolation in Caenorhabditis: The genetic basis of postzygotic isolation has been an intensely studied problem (Coyne and Orr 2004), and recent advances in fruit flies (Ferree and Barbash 2009), mice (Mihola *et al.* 2009), fish (Kitano *et al.* 2009), and plants (Bikard *et al.* 2009) have provided insights into the mechanisms that promote reproductive barriers. Recent work has also revealed genetic factors responsible for intraspecies postzygotic isolation in *C. elegans* (Seidel *et al.* 2008). However, little is known about the genetics of interspecies postzygotic isolation in Caenorhabditis due to the previous absence of fertile hybrids between Caenorhabditis species (Baird *et al.* 1992). The recently discovered *C.* sp. 9 promises to allow application of the genetic and genomic tools of the Caenorhabditis model genus to the problem.

The types of asymmetric patterns observed in the *C.* sp. 9/*C. briggsae* hybrid generations are not uncommon in hybrid systems. In many such systems, the heterogametic sex (XY/XO males or ZW/ZO females) is the disadvantaged sex with respect to hybrid viability and fertility (Presgraves 2008). This common phenomenon is referred to as Haldane's rule, and clearly applies to the *C.* sp. 9/*C. briggsae* hybrids: F1 males are either dead or sterile, depending upon cross direction, despite the presence of fertile F1 females in both directions. Many theories have been put forth to explain Haldane's rule, including the dominance theory, the "faster X" theory, and the "faster male" theory (Coyne and Orr 2004). Whichever applies, the leading candidate mechanism for incompatibility is the interaction of Dobzhansky–Muller incompatibility factors (Burke and Arnold 2001; Coyne and Orr 2004), and for the Caenorhabditis system several hypotheses can be framed with such factors in mind. The difference in the F1 sex ratio between reciprocal crosses (Figure 2, A and B) can be accounted for entirely by a difference in male-specific viability. This asymmetry in male viability could be explained by hemizygous X-linked *C. briggsae* factors that promote male inviability in the presence of *C.* sp. 9 autosomal factors. This explanation for this male-specific lethality is consistent with the dominance and faster-X theories of Haldane's rule.

In *C. briggsae/C. remanei* hybrid F1, an unusual Haldane's rule phenomenon is seen, in which XO male hybrids are transformed into females in a strain-dependent manner (Baird 2002). Although the possibility of male-to-female transformation was not specifically addressed here, the observation of hybrid males with bipolar gonads (Figure 3F) is suggestive that partial sexual transformation may occur in hybrids. Additionally, the segregation of both fertile and infertile males in the $B2_{b,9}$ generation suggests that this system can be utilized to determine the genetic basis of hybrid male infertility. Indeed, the number of fertile males in this hybrid generation (24%; Table 1) suggests that as few as two loci may be needed to restore fertility in this generation (but see discussion of genotyping results below). This hybrid system will likely prove useful in teasing apart the basis of Haldane's rule in Caenorhabditis.

In addition to Haldane's rule, another common pattern of asymmetry is the strong dependence of hybrid F1 viability and fertility upon the directionality of the parental cross. This phenomenon has recently been dubbed "Darwin's corollary to Haldane's rule" (Turelli and Moyle 2007), and it is in effect here. If *C.* sp. 9 is the mother in the parental cross, viable male progeny are produced (Figure 2A), a larger percentage of F1 females are fertile (Table 1), and more viable B2 progeny are produced (Figure 2, D *vs.* F). F1 progeny exhibit lower fitness with respect to all of these categories when *C. briggsae* is the P0 mother. The older age of the *C. briggsae* mothers used in these experiments (see materials and methods) may be partly responsible for the poor performance of their progeny. However, although this is a possible explanation for the lowered viability and fertility of the F1 females, it is unlikely that moderate aging of the *C. briggsae* P0 mother would sex-specifically reduce F1 male viability to zero. Indeed, crosses using L4 *C. briggsae* AF16 hermaphrodites and *C.* sp. 9 JU1325 males also failed to produce males (data not shown). It is also unlikely it could explain the lower viability of $B2_{9,9}$ than $B2_{b,9}$ embryos. This hybrid system then also has the potential to reveal potential causes of Darwin's corollary.

As an alternative to interactions between zygotic factors, there could be parental factors in the *C. briggsae* hermaphrodite or *C.* sp. 9 male gametes that adversely affect hybrid F1 fitness. The mutation rate of the *C. briggsae* mitochondrial genome has been reported to be much faster than that of other Caenorhabditis species (Howe and Denver 2008), and this difference could facilitate a nuclear–mitochondrial genome incompatibility between *C. briggsae* mitochondria and *C.* sp. 9 nuclear genes that is not reciprocal (Bolnick *et al.* 2008). This could provide a potential explanation for differences in F1 female fertility and backcross viability

normalized to 25% (50% for the X) to allow direct comparisons of the measurements of the hybrid markers. All DNA preparations used had at least 200 worms each. All tests had $N \geq 3$. The error bars represent one standard error of the mean. $^{*}P < 0.05$ for Mann–Whitney *U* test. [†]Genotyped using RFLP analyis. [‡]Indel of 18 bp. All other markers were SNPs genotyped through pyrosequencing.

in reciprocal crosses. Alternatively, *C. briggsae* paternal effect factors may explain why hybrid progeny are inviable when F1 and backcross females are crossed to *C. briggsae* males (Figure 2, C, E, and H). Since F1 and backcross females are capable of producing viable hybrid progeny with *C.* sp. 9 males (Figure 2, D, F, and I), and because backcross males can produce viable progeny with *C. briggsae* hermaphrodites (Figure 2G), there may be a *C. briggsae* paternal effect factor that is incompatible with a hybrid background. As *C. briggsae* males can produce viable F1 hybrids, however, the hybrid's zygotic genotype may also have to have substantial *C. briggsae* homozygosity for this particular incompatibility to arise. The ability of *C. briggsae* males to produce viable progeny with $B3_{b,9,9}$ females (Figure 2K and Table 2) is consistent with this.

The $B3_{b,9,bh}$ genotyping results also provide some insights into the genetic basis of hybrid male sterility in this system. That 24% of $B2_{b,9}$ males are fertile is consistent with the existence of two unlinked *C.* sp. 9 loci that must be homozygous to allow male fertility. However, all loci genotyped were detectably homozygous for the *C. briggsae* allele in the $B3_{b,9,bh}$ generation, implying that no region of the *C. briggsae* genome was absolutely excluded from fertile $B2_{b,9}$ males. Although clearly some genomic regions affect hybrid fitness more than others (particularly regions of chromosomes II, III, IV, and X; Figure 10), this suggests that there are multiple interactions between the *C. briggsae* and *C.* sp. 9 genomes that contribute to lowered hybrid fitness. Such polyfactorial interspecies incompatibility has been reported for both plants (*e.g.*, Rieseberg *et al.* 1999; Jiang *et al.* 2000; Taylor *et al.* 2009) and other animals (*e.g.*, Rogers and Bernatchez 2006; Good *et al.* 2008). In contrast, individual genes have been shown to play major roles in hybrid incompatibilities in Drosophila (Presgraves 2010), *C. elegans* (Seidel *et al.* 2008), and mice (Mihola *et al.* 2009).

Evolutionary implications for recessivity of the hermaphrodite germline: The discovery of productive hybridization between the gonochoristic *C.* sp. 9 and the androdioecious *C. briggsae* suggested that these species could help reveal the genetic architecture of hermaphrodite development. That selfing is almost completely recessive is itself an important and surprising insight. In particular, this implies that no *C. briggsae* gene (or set of genes) in a single copy is sufficient for robust hermaphroditism in a gonochoristic background. This observation suggests that the female germline state in *C.* sp. 9 is extremely canalized in its female fate and thus highly resistant to the action of factors that promote XX spermatogenesis. In *C. elegans* and *C. briggsae*, loss-of-function mutations in these factors are recessive (Hodgkin 1986; Schedl and Kimble 1988; Zhang *et al.* 1997; Li *et al.* 2000; Guo *et al.* 2009; A.V. Doty, unpublished results), indicating they are not individually dose sensitive in present-day hermaphrodites. Therefore, perhaps an important part of the evolution of selfing *C. briggsae* was the weakening of female germline sexual canalization, so that the "sexual oscillator" that has been proposed to effect limited spermatogenesis (Haag 2009) can function.

We also note that the defining attributes of hermaphrodite germline development are not always congruent in their expression in hybrid generations. In a few cases, the incidence of spermatogenesis is higher than the incidence of overt selfing within a given hybrid generation. This suggests that in the hybrids selfing can become defective at multiple stages in the process. It would also suggest that it is more difficult to complete the self-fertilization and laying of embryos than it is to simply produce sperm. This is consistent with recent studies in *C. remanei* that suggest that multiple steps are necessary for hermaphroditism to evolve (Baldi *et al.* 2009).

Opportunities and limitations for the genetic investigation of hermaphroditism: *C.* sp. 9 and *C. briggsae* open the possibility of mapping the historically crucial variants that led to *C. briggsae* hermaphroditism. However, the attempts to do this described here have so far been thwarted. One obvious problem is the extreme postzygotic isolation between these two species. Due to the inability to make self-fertile hybrid progeny, typical QTL designs based on recombinant inbred selfing lines are impossible (Doroszuk *et al.* 2009; Moyle and Payseur 2009). This necessitates the use of unconventional designs to tackle this problem in a genetic mapping context (Figure 9).

Another, larger problem is the low segregation of hermaphroditism in hybrid generations (Table 2), which falls to zero when inbred lines of *C.* sp. 9 are used. Further, in generations where hybrid hermaphrodites are observed, the incidence of hermaphroditism is no different from that seen in the F1 generation, indicating it is not due to rare combinations of homozygous *C. briggsae* alleles. The inability of *C.* sp. 9 inbred lines to yield any hybrid hermaphrodites suggests that either XX spermatogenesis in hybrids is especially sensitive to parental inbreeding depression or that there is standing genetic variation in *C.* sp. 9 for factors that specifically facilitate XX hybrid spermatogenesis. If the latter is true, then there should be heterogeneity among *C.* sp. 9 inbred lines with their ability to create hybrid hermaphrodites. This has not been observed, but only a small number of inbred lines have been investigated. However, even if such a polymorphic *C.* sp. 9 factor existed, its identity would not explain how *C. briggsae* became hermaphroditic. This would require the mapping of a *C. briggsae* hermaphroditizing factor that is not polymorphic.

Though it has proven difficult to use this system to map specific *C. briggsae* hermaphroditic factors, mechanisms patterning germline sex in this hybrid system can still be explored. One possibility is that this recessive trait has a complex (*i.e.*, polygenic) genetic architecture. However,

the presence of widespread segregation distortion biased against *C. briggsae* alleles in hybrids (Figure 10) prevents any such hypotheses about the nature of the genetic architecture of *C. briggsae* hermaphroditism from being rigorously evaluated. As an alternative approach, we screened 15,000 mutagenized haploid *C.* sp. 9 JU1422 genomes for a mutant that would facilitate the generation of hybrid F1 selfers. No such animal was observed. Given that loss-of-function alleles are generated for an average size gene about once in about every 2000 haploid mutagenized genomes (Anderson 1995), it is unlikely that there is any one *C.* sp. 9 feminizing factor that is responsible for the dominance of the female state in the hybrid germline. However, an increased dosage of *C. briggsae* factors to the hybrid background through the use of polyploids does elevate the incidence of hermaphroditism and in certain cases can almost triple it in the F1 (Table 2). This suggests that the haploinsufficiency of hermaphrodite-promoting *C. briggsae* alleles in the hybrid F1 is at least partly responsible for the recessivity of the hermaphrodite trait in this system.

Implications for the emergence of hermaphrodite lineages: Phylogenetic studies suggest that hermaphroditism evolved multiple times in Caenorhabditis (Cho *et al.* 2004; Kiontke *et al.* 2004). If the ability of *C.* sp. 9 and *C. briggsae* to produce fertile hybrids (whereas all other Caenorhabditis pairwise crosses cannot) can be used to infer that these two species are recently diverged sister taxa (an inference further supported by Cutter *et al.* 2010; Raboin *et al.* 2010), then the most parsimonious scenario is one in which the last common ancestor of these two species had a female germline, similar to *C.* sp. 9 today. If that ancestral female was as resistant to the effects of hermaphroditizing factors as *C.* sp. 9, then mating with gonochoristic relatives would have completely destroyed the nascent trait. How, then, was a hermaphrodite lineage successfully established?

One simple scenario posits a single dominant factor sufficient for hermaphroditisim arising in a gonochoristic population, which then rapidly fixes due to the reproductive fitness benefits of selfing (Smith 1978). Alternatively, multiple hermaphrodite-promoting factors (with one or more being recessive) arise and accumulate within a gonochoristic population and are fixed in the population by physical or ecological isolation and resulting inbreeding. That our results support the latter, more convoluted process is unexpected for two reasons. First, developmental genetic studies (Schedl and Kimble 1988; Baldi *et al.* 2009) indicate a small number of genetic changes can interconvert females and hermaphrodites in the laboratory. Second, the multiple independent gains of selfing within Caenorhabditis might suggest that the process that leads to the evolution of hermaphroditism would be relatively simple. However, it is important to note that this is predicated upon the notion that the germline state of *C.* sp. 9 is a good proxy for the common ancestor of *C.* sp. 9 and *C. briggsae*. Given the degree of reproductive isolation demonstrated to exist between these two species, it is entirely possible that such an assumption is unsound. The strongly canalized *C.* sp. 9 female germline state may have evolved since the origin of *C. briggsae* by a number of possible mechanisms, including nonadaptive developmental system drift (True and Haag 2001), selection for greater robustness, or to resist introgression of the selfing trait from *C. briggsae*. Indeed, *C. briggsae* is cosmopolitan and has been so for a long period of time (Dolgin *et al.* 2008), and the high standing load of deleterious recessive mutations in gonochoristic Caenorhabditis creates strong inbreeding depression (Dolgin *et al.* 2007; Barriére *et al.* 2009). Further, selfing lineages appear to be relatively short-lived (Kiontke *et al.* 2004; Cutter *et al.* 2008), and pure selfing is predicted to quickly lead to extinction due to the accumulation of new deleterious mutations (Loewe and Cutter 2008). Gonochoristic lineages which can resist conversion to selfing may therefore be favored when such conversion is associated with low rates of outcrossing.

Other differences between *C.* sp. 9 and *C. briggsae*: In addition to their reproductive barriers, other differences between *C. briggsae* and *C.* sp. 9 have been observed. One peculiar difference is that mated *C.* sp. 9 females stop laying embryos long before mated *C. briggsae* hermaphrodites do (Figure 4), even though the assays for the former initially placed multiple male mates with each female. The average *C.* sp. 9 total brood size of 259 is striking given that *C. elegans* hermaphrodites can lay >1000 embryos in a single mating assay (Hodgkin 1986). In the similarly gonochoristic *C. remanei*, outbred crosses of only 6-hr duration with single males (Dolgin *et al.* 2007) produce fecundities similar to those we report here for *C.* sp. 9, mated with multiple males for at least twice as long. This suggests that *C.* sp. 9 females cannot take full advantage of abundant sperm. This may be a general feature of the species, for example as a consequence of female spermatheca size, barriers to remating, male effects on female fecundity (Diaz *et al.* 2010), or as a consequence of unintended inbreeding in our laboratory stocks. Additionally, the reduction of *C. briggsae* hermaphrodite brood size by *C.* sp. 9 males may be in part due to a decrease in hermaphrodite survivorship after such matings (A. S. Chang, personal communication).

A more notable difference, however, is that *C.* sp. 9 animals have strikingly lower viabilities at low temperatures, whereas *C. briggsae* animals have relatively high viabilities at similar temperatures (Figure 5). Interestingly, this apparent pattern of *C.* sp. 9 as a high temperature specialist and *C. briggsae* as a temperature generalist correlates with these species' geographic distributions. *C. briggsae* has a relatively wide geographic range across at least five continents, and >100 isolates have been found in regions as disparate in temperature

as Iceland and India (Cutter *et al.* 2010). Thus far, *C.* sp. 9 has only been found in tropical nations such as India and Congo, but this may change with more sampling. Despite the small sample size, the locations of its isolation and its low fitness at low temperatures are consistent with this species having a geographic range restricted to high-temperature environments. If this is the case, then *C.* sp. 9 and *C. briggsae* provide a new system within which to potentially understand the evolution of generalist and specialist modes of ecological adaptation. In tandem with the reproductive isolation between these species, these differences will likely prove the comparative *C.* sp. 9/*C. briggsae* system to be fruitful for future studies.

Future prospects: We have described some attributes of the first hybrid genetic system in Caenorhabditis, formed between the gonochoristic species *C.* sp. 9 and the androdioecious species *C. briggsae*. Though mapping factors that distinguish hermaphrodites from true females has thus far been thwarted, a range of studies could be envisioned that would exploit this system's combination of speed and experimental resources to further studies of species formation, the evolution of mating systems, and any other phenotypic differences that may be discovered between the two species. Such experiments will be greatly aided by genomic tools for *C.* sp. 9. Indeed, a set of male- and female-specific transcriptome profiles (R. Jovelin and A. D. Cutter, personal communication) and a genome sequence (F. Piano, personal communication) of *C.* sp. 9 are currently being constructed. There is clearly great potential for this system that will begin to be realized in the near future.

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Insights Into Species Divergence and the Evolution of Hermaphroditism From Fertile Interspecies Hybrids of Caenorhabditis Nematodes

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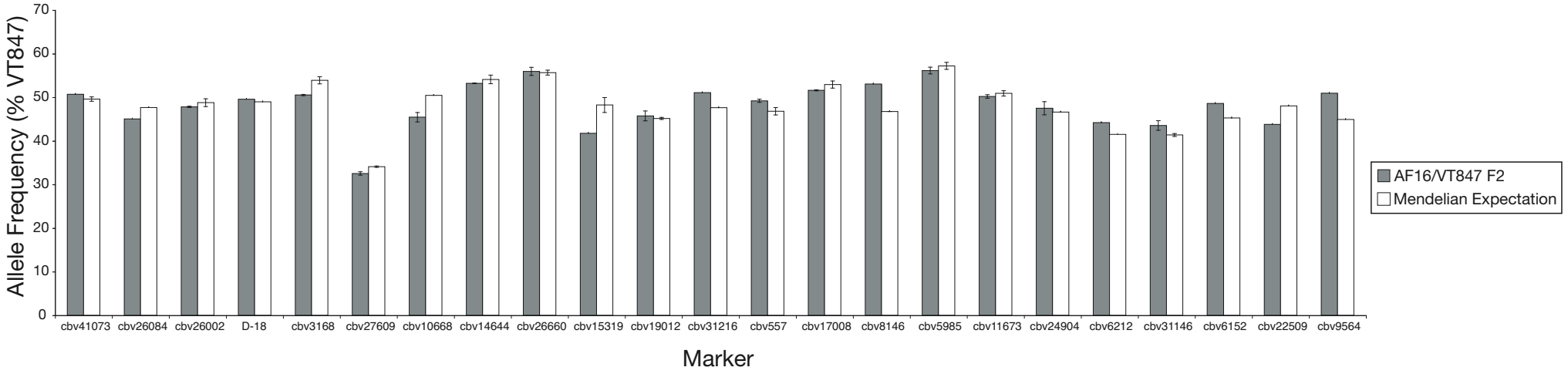

FIGURE S1.—Markers display no between-strain segregation distortion. The estimates of VT847 allele frequencies of an AF16/VT847 F2 generation. Gray bars represent the data for the AF16/VT847 F2 generation. The Mendelian expectation control (white bars) was a mixture of *C. briggsae* AF16 and *C. briggsae* VT847 worms in the proportions expected of these alleles under Mendelian segregation (50:50). Markers were genotyped using Pyrosequencing, RFLP, or indel analysis. All DNA preparations had at least 200 worms each. Error bars represent one standard error of the mean. Points without error bars represent one observation.

TABLE S1

Pyrosequencing Primers

Name	SNP ID	Sequence (5' to 3')
VTP-I-9F	cbv26002	AAATTCTGGGCCGTCTGGAT
VTP-I-9R		AGCGCTGCTCCGGTTCATAGATTGGTTTTAGCTGGTCCCGAGTTATT
VTP-I-9S		ACTGATCCTAATTGGTTAT
VTP-II-6F	cbv3168	AGCGCTGCTCCGGTTCATAGATTTCTCGGAAAATTTGAAATTGGA
VTP-II-6R		CCCTCACACTGCCAAAGTATTTA
VTP-II-6S		TACTTTTCACTTTTGAAAAT
VTP-II-9F	cbv27609	AAGTGGACAGTGTGGGGAACC
VTP-II-9R		AGCGCTGCTCCGGTTCATAGATTGACAAACAGATTGGGGCCACTAT
VTP-II-9S		CCATCCATTGGAAATT
VTP-III-2F	cbv14644	GGGCCATCCTCTTTTGTTAGCT
VTP-III-2R		AGCGCTGCTCCGGTTCATAGATTAGAGCCTACGATGCCTGGTATG
VTP-III-2S		TCTTTTGTTAGCTTCATTG
VTP-III-6F	cbv26660	AGCGCTGCTCCGGTTCATAGATTGGTTTTGAAAGAAGTTGCAGTGA
VTP-III-6R		GCTCGAAAAACATGACATTTTAA
VTP-III-6S		CTAAATTGCCTAAAATTGAT
VTP-III-7F	cbv19012	AGCGCTGCTCCGGTTCATAGATTGCTGCCGATGAGCAGAGAAA
VTP-III-7R		AGTAGCACCCCGGCCAAATT
VTP-III-7S		CCCTTACCTTATTGGTTG
VTP-IV-1F	cbv557	AGCGCTGCTCCGGTTCATAGATTAAATGGATGGGAATGCACTAATGA
VTP-IV-1R		TCTTTTTTGTTCCCATGAAGTCG
VTP-IV-1S		TTTACTTTGCTGGAAAAC
VTP-IV-2F	cbv17008	GGAGCCAAGATAATAAACCTCAAA
VTP-IV-2R		AGCGCTGCTCCGGTTCATAGATTCCCTTTAAAAAGAGATGCAGTGA
VTP-IV-2S		TTACGTTTTAAAAAGATGAG
VTP-IV-8F	cbv5985	TGCCCGAAAGTAGTCCTCCATA
VTP-IV-8R		AGCGCTGCTCCGGTTCATAGATTGGGAACGACTTGATTTTGTATCCA
VTP-IV-8S		TCTGACTGCGAACGA
VTP-V-2F	cbv11673	AGCGCTGCTCCGGTTCATAGATTTCCGATGTGTTCGTTTAGAAAGA
VTP-V-2R		CCATTATTCAAACTTCCGATGCTA
VTP-V-2S		TTTGTACCTGATTGAAA
VTP-V-6F	cbv24904	AGCGCTGCTCCGGTTCATAGATTGAATGTGGTCGGAAAAAAATTTA
VTP-V-6R		GCACTTTTGACCCCCATTTTA
VTP-V-6S		CCAAAACAAAAACCATG
VTP-V-8F	cbv31146	GTGAGCCGTTGATCTTCATATTCC
VTP-V-8R		AGCGCTGCTCCGGTTCATAGATTTCGGTCTTTGCACTGAAAAGTTT
VTP-V-8S		TCGATATTTTTTGTTCAATT
VTP-X-9F	cbv427	GAAAAATCAGTGTTCGAGGCTTAC
VTP-X-9R		AGCGCTGCTCCGGTTCATAGATTAAAGGTTTTCGGCTTCTGAGCT

VTP-X-9S	GCACTAGAATAAGTGAAAAG
VTP-UNIBIOT	Biotin-AGCGCTGCTCCGGTTCATAGATT

At the end of the primer name: "F" denotes it as a forward PCR primer, "R" denotes it as a reverse PCR primer, and "S" denotes it as the sequencing primer for the Pyrosequencing reaction. "VTP-UNIBIOT" is the biotinylated universal PCR primer (Aydin *et al.* 2005).