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Rapid genome shrinkage in a self-fertile nematode reveals sperm competition proteins

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Abstract

To reveal impacts of sexual mode on genome content, we compared chromosome-scale assemblies of the outcrossing nematode *Caenorhabditis nigoni* to its self-fertile sibling species, *C. briggsae*. *C. nigoni*'s genome resembles outcrossing relatives, but encodes 31% more protein-coding genes than *C. briggsae*. *C. nigoni* genes lacking *C. briggsae* orthologs were disproportionately small and male-biased in expression. These include the *male secreted short (mss)* gene family, which encodes sperm surface glycoproteins conserved only in outcrossing species. Sperm from *mss*-null males of outcrossing *C. remanei* failed to compete with wild-type sperm, despite normal fertility in non-competitive mating. Restoring *mss* to *C. briggsae* males was sufficient to enhance sperm competitiveness. Thus, sex has a pervasive influence on genome content that can be used to identify sperm competition factors.

Introduction

Sex between individuals is nearly ubiquitous in eukaryotic life (1). However, in multicellular organisms the costs of sex and scarcity of mates sometimes favor the evolution of

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Data availability and accession codes

C. nigoni genomic and transcriptomic data have been archived as NCBI BioProject accessions PRJNA384657 and PRJNA384658, and the *C. nigoni* genome assembly as DDBJ/ENA/GenBank accession PDUG00000000. Genome assembly, gene prediction, and gene expression data for *C. nigoni*, with supplementary datasets for other species, have been archived at the OSF (*C. nigoni*, https://osf.io/dkbwt and doi:10.17605/osf.io/dkbwt; other species, https://osf.io/b47r8 and doi:10.17605/osf.io/b47r8).

Supplementary Materials
Materials and Methods

uniparental reproduction through asexual parthenogenesis or self-fertilization (2). Such changes in sexual reproduction have consequences for both sexual traits and genome content. Comparative genomics using closely related species with different modes of sexual reproduction can reveal sex-related factors that might otherwise remain cryptic. In the nematode species *C. elegans, C. briggsae,* and *C. tropicalis*, animals with two X chromosomes that would normally be female have evolved into self-fertilizing hermaphrodites (Fig. 1a) (3). Nearly all progeny of these selfing XX hermaphrodites are themselves XX. Rare haplo-X (XO) male progeny experience weaker sexual selection than males from outcrossing species, exhibit atrophied traits required for efficient mating (4–7), and are hypersensitive to pheromone-induced mortality (8). Sexually antagonistic sperm-female interactions have also been relaxed in self-fertile *Caenorhabditis* (9).

Self-fertile *Caenorhabditis* have smaller genomes and transcriptomes than outcrossing *Caenorhabditis* (10, 11), as also observed in the selfing plant *Arabidopsis thaliana* (12). However, comparisons of self-fertilizing to outcrossing *Caenorhabditis* have involved species as divergent at the nucleotide level as humans are from mice (10, 13), making it unclear how quickly genomic shrinkage occurs. We hypothesized a direct link between the degradation of sexual traits and genome contraction in selfing species. Here we describe new genomic resources and functional experiments that confirm its existence.

Results

Of ~50 known *Caenorhabditis* species, the most closely related pair with different sexual modes are the outcrossing *C. nigoni* and the selfing *C. briggsae* (14–16). They remain partially interfertile, yet have numerous genetic and reproductive incompatibilities (9, 15, 17–19). To compare their genomes, we assembled the *C. nigoni* genome from 20-kb Pacific Biosciences (PacBio) and Illumina short-read libraries (table S1; (20)). The final *C. nigoni* chromosome-scale genome assembly totaled 129 Mb with an N50 contig length of 3.3 Mb; it was estimated as 99.6% complete (21). The genome was 19% larger than *C. briggsae*'s (108 Mb), but similar in size to genomes of the more distantly related outcrossing species *C. remanei, C. sinica, C. brenneri,* and *C. japonica,* which range from 131–135 Mb (Fig 1A) (10). Therefore, larger genome sizes were probably the ancestral condition, and genomic shrinkage occurred in the *C. briggsae* lineage after it diverged from *C. nigoni*. Over 90% (118 Mb) of the assembly can be aligned to the chromosomes of *C. briggsae* without large translocations or inversions, despite megabase-sized contigs (fig. S1). Thus, the two genomes are essentially colinear, but differ in many small species-specific segments. *C. nigoni*'s six chromosomes are 6.6–16.6% larger than their *C. briggsae* homologs (table S2).

We used whole-genome alignment to identify species-specific genomic segments (20). In *C. nigoni*, 47.7 Mb (36.9%) did not align with *C. briggsae*, and *C. briggsae* had 27.7 Mb (25.6% of 108.4 Mb) that did not align with *C. nigoni*. This 20.0 Mb difference accounted for 95% of the difference in genome sizes. Non-alignable genomic regions were concentrated on the distal arms of all six holocentric chromosomes, where small inversions and repetitive sequences were abundant and gene densities were low (Fig. 1b). These regions were mostly small (median ~500 bp; Fig. 2a), but larger (1–65 kb) insertions or deletions accounted for 17 Mb (81%) of the genome size difference (Fig. 2b). In both assemblies, non-

alignable sequences were most common in intergenic regions and introns (fig. S2). *C. nigoni* harbored 5.4 Mb more species-specific protein-coding sequences than *C. briggsae*, consistent with a net loss of genes in *C. briggsae* (see below). For orthologous genes in both species, exon lengths were highly correlated (Fig. 2c, table S3). In contrast, ortholog intron content was weakly correlated and was significantly larger in *C. briggsae.* Because both genomes had similar repetitive DNA fractions (*C. nigoni* 27% versus *C. briggsae* 25%), disproportionate loss of repetitive sequences (seen in plants) did not contribute to different genome sizes (table S1) (10, 12, 22).

We predicted 29,167 protein-coding genes for *C. nigoni* (table S4), with 88.9% (25,929) being expressed in adults (≥0.1 transcripts per million [TPM]). By equivalent methods, we predicted 22,313 genes in *C. briggsae* (20), 23.5% less than *C. nigoni*. The published gene annotations for *C. briggsae* (23) were even fewer (21,814 genes).

This 6,854-gene difference could have several causes, including gene family contraction and loss of sequence classes in *C. briggsae,* as well as *C. nigoni*-biased gain of novel sequences. We compared genes of *C. briggsae* and *C. nigoni* to genes of the outgroups *C. remanei*, *C. brenneri,* and *C. elegans* (20). In *C. nigoni*, 24,341 genes (83.5%) were orthologous to 21,124 *C. briggsae* genes, reflecting larger multigene families in *C. nigoni* versus *C. briggsae* (Fig. 3a; table S4) (24). Another 2,949 *C. nigoni* genes without *C. briggsae* orthologs (10.1%) represent losses in *C. briggsae* based on homologs in *Caenorhabditis* outgroups (fig. S3). Finally, 1,877 *C. nigoni* genes (6.4%) lacked homologs entirely and were classed as orphans. These genes could be exceptionally divergent, recently arisen in *C. nigoni*, or arisen shortly before the *C. nigoni-C. briggsae* split but then lost in *C. briggsae*. Overall, gene loss in *C. briggsae* appears to be the primary driver of the gene number difference.

To characterize genes lost in *C. briggsae*, we first compared Pfam protein domains encoded by *C. nigoni* versus *C. briggsae*. We found 26 Pfam domains that were overrepresented in *C. nigoni* (fig. S4; table S5); of these, seven were consistently overrepresented in outcrossing *C. nigoni*, *C. remanei*, and *C. brenneri* relative to the selfing species *C. briggsae* and *C. elegans*. Three of these domains (F-box, FBA_2/F-box associated, and BTB) are predicted to mediate protein-protein interactions. Male-female *Caenorhabditis* had 272–1,074 genes in these families, while hermaphroditic *Caenorhabditis* had only 101–258 genes per family. Two other domains (Peptidase_A17 and DNA_pol_B_2) are associated with repetitive DNA. The final two overrepresented domains were Asp_protease_2 (possibly associated with retroelements) and DUF3557 (a nematode-specific domain, currently of unknown function). One overrepresented domain specific to *C. nigoni* was zf.RING2_finger; the RING domain gene *spe-42* is important for sperm-egg interactions in *C. elegans* (25).

Because *C. nigoni*-specific genes might encode fast-evolving proteins that lacked known domains, we compared other gene properties. Genes encoding medium to large proteins (≥200 residues) were similar in frequency in both species, but *C. nigoni* encodes disproportionately more small proteins (<200 residues) than *C. briggsae* (Fig. 3b; table S6). As seen in other *Caenorhabditis* (11), genes with male-biased expression outnumbered female-biased genes (Fig. 3c; table S7). However, even against this background, *C. nigoni*

genes without *C. briggsae* homologs were disproportionately male-biased in expression. Preferential loss of small and fast-evolving proteins thus occurred in *C. briggsae* after the adoption of selfing.

We hypothesized that genes with highly male-biased expression that are present in outcrossing species, but lost in selfing species, might function in sexual selection. Among such genes we identified the *mss* (*male secreted short)* family. We found one to four *mss* genes in the outcrossing species *C. nigoni, C. sinica, C. remanei, C. brenneri*, *C.* sp. 34, *C. japonica*, and *C. afra,* but found none in the selfing *C. elegans, C. briggsae,* and *C. tropicalis*. The *mss* family encodes small proteins (median 111 residues) with N-terminal signal sequences, rapidly evolving central domains with several predicted O-glycosylation sites, and C-terminal glycosylphosphatidylinositol (GPI) anchor membrane attachment signals (Fig. 3d). Enzyme treatments confirmed that MSS proteins were heavily glycosylated (fig. S5).

Although we failed to detect *mss* genes in selfing species, we did discover a larger family of *mss*-related protein (*msrp)* genes*,* within which *mss* forms a monophyletic clade (fig. S6; (20)). Notably, *msrp* genes are found both in outcrossing *Caenorhabditis* and in the hermaphroditic *C. elegans*, *C. briggsae*, and *C. tropicalis* (fig. S6). Like MSS, MSRP proteins are small, and are predicted to be secreted, O-glycosylated, and (often) GPI-anchored. Both *mss* and *msrp* genes show male-biased expression in *C. nigoni* and other species (table S8). In cases where their chromosomal loci can be identified, *mss* and *msrp* genes are autosomal; this linkage fits a general pattern in heterogametic male species of male-biased genes being autosomal rather than X-chromosomal (ref. (26) and references therein).

Because we observed *mss* genes in two *C. elegans* outgroups (*C. japonica* and *C. afra*, fig. S6, table S8), their absence from hermaphrodites most likely reflects independent gene losses rather than phylogenetic restriction to close relatives of *C. nigoni*. Examination of the *C. briggsae* genomic region syntenic to the *C. nigoni mss* locus revealed fragments of *mss-1* and *mss-2* coding sequences and a nearly complete *mss-3* pseudogene (Fig. 3e, (20)). Mutations that ablate *Cbr-mss-3-ps* function in the AF16 reference strain also occur in 11 wild isolates that span the known diversity of *C. briggsae* (fig. S7 (20, 27)). Orthologs of all three *C. nigoni mss* genes were therefore present in the common ancestor of *C. nigoni* and *C. briggsae,* but were lost in *C. briggsae* before its global diversification.

In the outcrossing species *C. remanei, mss* transcripts were expressed only in adult males (Fig. 4a), with strongest expression in spermatocytes during mid-pachytene of meiosis I (Fig. 4b). To determine subcellular localization of MSS peptides, we used CRISPR/Cas9 editing to tag the *Cre-mss-1* gene of *C. remanei* with the hemagglutinin (HA) epitope. *Crem*-MSS-1::HA expression was first detected in large vesicles and on the plasma membrane of spermatocytes, with intensity increasing and localization restricted to secretory vesicles in mature spermatids (Fig. 4c, d, e). The secretory vesicles of nematode sperm, known as membranous organelles (MOs), fuse with the plasma membrane upon ejaculation and sperm activation (28).

MSS peptides might be processed by a signal peptidase to release a soluble fragment into the MO lumen, which could then be dumped into seminal fluid upon sperm activation. However, their transient plasma membrane localization in spermatocytes and predicted C-terminal GPI attachment signals (Fig. 3d; table S8) suggested that MSS peptides might instead be attached to membranes. Consistent with this latter hypothesis, *Crem*-MSS::HA remained associated with activated sperm dissected from inseminated females (Fig. 4f). We observed staining of the plasma membrane and of MO-derived punctae (Fig. 4g), which may be fused vesicles that remain as cup-like invaginations (29). Persistence of MSS on the surface of sperm after activation suggested that MSS acts cell-autonomously, rather than through the seminal fluid.

Because the four *C. remanei mss* paralogs form a 7-kb tandem array (fig. S8a), we deleted the entire *mss* cluster via CRISPR/Cas9 editing. To avoid inbreeding depression associated with homozygosity of entire chromosomes (30; fig. S8b), we generated the *mss* deletion in two different *C. remanei* strains and crossed them to create hybrid *mss*-null mutants. The resulting males showed no intrinsic fertility defects as judged by overall brood size (fig. S8c). However, when competing against heterozygous *mss*(null/+) males, *mss* mutants sired fewer progeny than non-mutants in both offense (mutant male second) and defense (mutant male first) scenarios (Fig. 5a, b). The *mss* family is therefore not required for fertility itself, but for male sperm competitiveness in multiple mating situations. Sperm lacking MSS compete poorly even when the female reproductive tract is conditioned by wild-type sperm. Thus, MSS proteins probably do not function as a secreted signal, but instead act cell-autonomously.

We then introduced *mss-1* and *mss-2* genes from *C. nigoni* into *C. briggsae* via a low-copy, germline-expressed MSS transgene; this transgene was strongly expressed in *C. briggsae* males, while also being detectable in hermaphrodites (fig. S9). Remarkably, sperm from transgenic *mss*(+) *C. briggsae* males outcompeted those of wild-type males (Fig. 5c, d). After *mss*(+) sperm were exhausted, however, wild-type *mss*(null) sperm were still fertilization-competent (Fig. 5c, d). In addition, *mss*(+) males were more consistently able to suppress use of a hermaphrodite mate's self-sperm (Fig. 5e).

Because 50% of outcross progeny are male, but selfed progeny are almost exclusively hermaphrodites, we examined the effect of transgenic *mss* on long-term sex ratios in *C. briggsae* populations. We started both wild-type and *mss*(+) *C. briggsae* populations with a 1:1 male-to-hermaphrodite sex ratio and examined them over time. Wild-type *C. briggsae* showed a rapid decline of males, as previously seen in *C. elegans* (7, 31). However, male frequency remained elevated in the *mss*(+) strain (Fig. 5f), only declining after 12 generations. The expression of MSS proteins was thus sufficient to shift population sex ratios towards parity.

Discussion

Comparison of the *C. nigoni* and *C. briggsae* genomes revealed that *C. briggsae* experienced rapid contraction of chromosomes and loss of protein-coding genes. However, loss of ancestral genomic content in *C. briggsae* does not fully explain their genomic divergence; the ongoing birth of novel sequences in both species, along with loss of ancestral DNA in *C.*

nigoni, is also important. Net shrinkage of the *C. briggsae* genome therefore resulted from a substantial increase in the ratio of losses to gains. These losses included many coding sequences, reducing the *C. briggsae* gene count by nearly one quarter.

Multiple observations implicate the evolution of selfing as the cause of genome shrinkage in *C. briggsae*. Reduced genome and transcriptome sizes are observed in all three selfing *Caenorhabditis* (10, 11). Continued interfertility of *C. briggsae* and *C. nigoni* (15) indicates that self-fertility and genome shrinkage evolved in quick succession. Genes with male-biased expression, such as the *mss* family, are disproportionately and consistently lost from selfing species (11). This suggests that genes with male reproductive functions that are either dispensable or maladaptive in the new sexual mode are purged from the genome. Finally, the net genome shrinkage we observed has been predicted to arise from a partially selfing mating system coupled with transmission distortion of autosomal deletion alleles (32, 33). Such distortion is driven by imbalanced chromatin during meiosis I of XO males, and causes preferential inheritance of shorter alleles by hermaphrodite progeny and their increased fixation in the population.

Larger autosomal deletions, influenced most by the deletion segregation distortion mechanism, are primarily responsible for the smaller genome of *C. briggsae* (Fig. 2). However, such deletions and net shrinkage were also found on the X chromosome (table S2), which should be unaffected. Moreover, orthologous genes have larger introns in *C. briggsae* than *C. nigoni* (Fig. 2), and introns comprised a greater fraction of the *C. briggsae* genome (fig. S2). X-chromosomal *C. briggsae* introns are also larger than those of the outgroup *C. remanei* (10; fig. S2c), suggesting that introns of many genes expanded in *C. briggsae*. Thus, additional processes must also contribute to shrinkage of the *C. briggsae* genome. Spontaneous short (1–5 nt) mutations in *C. elegans* are biased towards insertions rather than deletions (34), though biases in formation of larger indels remain uncharacterized. Regardless, the relative rates of insertion and deletion mutations likely evolve too slowly to explain *C. briggsae*'s reduced genome size, given its recent divergence from *C. nigoni* (27). Gene loss can sometimes be adaptive (35, 36), and has been proposed as a factor promoting genome shrinkage in selfing *Caenorhabditis* (10). Our results for the *C. nigoni-C. briggsae* pair support this hypothesis.

Genes encoding small proteins with male-biased expression are disproportionately lost in *C. briggsae*, with *mss* providing an instance affecting reproduction. Unlike *comp-1,* which encodes a kinase required for male versus hermaphrodite sperm competition in *C. elegans* (37), and which is conserved regardless of mating system, we found *mss* orthologs only in outcrossing species. In interspecies matings, sperm from males of outcrossing species rapidly invade the ovaries and body cavities of selfing hermaphrodites, sterilizing or killing them (9). This cryptic toxicity of outcrossing sperm is likely due to ongoing sexual selection in outcrossing species. Given their pronounced role in sperm competition, MSS proteins may contribute to sperm invasiveness.

How MSS improves sperm competitiveness remains unclear, but mature MSS proteins are substantially glycosylated (fig. S5). Such posttranslational modification may impose little constraint on MSS proteins, explaining how they can have weak sequence conservation yet

strong functional conservation. Another poorly conserved O-glycosylated protein, the mucin PLG-1, forms a copulatory plug found in all male-female *Caenorhabditis* species but lost in many wild isolates of *C. elegans* (4). Glycoproteins form the glycocalyx coat of mammalian sperm, and play important roles in fertility (38). *Caenorhabditis* provides a useful model for interactions between the glycocalyx and female tissues, and how they affect sperm competition.

Independent loss of *mss* in the three known hermaphroditic *Caenorhabditis* species could reflect either relaxed sexual selection coupled with mutation and drift, or adaptive convergence. Other changes in selfing species, such as loss of *plg-1* and of *plep-1,* which mediates reliable male discrimination between the vulva and excretory pore (4, 6), are likely due to relaxed selection. However, restoring *mss to C. briggsae* enhances male fitness (Fig. 5c,d), and mutations inactivating the *Cbr-mss-3-ps* pseudogene are not deletions that would be subject to loss via transmission ratio distortion (fig S7). These findings suggest that loss of *mss* may instead reflect adaptive convergence, permitting proto-hermaphrodites to adapt to a selfing lifestyle and resolve emergent sexual conflicts related to mating (39–41). Selfing *Caenorhabditis* lack inbreeding depression (42) and reproduce in spatially isolated habitats colonized by small numbers of founders (3). Reduced male mating success creates hermaphrodite-biased sex ratios (Fig. 5f), which may be adaptive under these conditions (41, 43–45). Thus, evolutionary transitions in reproductive mode may produce conditions for selection to rapidly eliminate formerly constrained reproductive genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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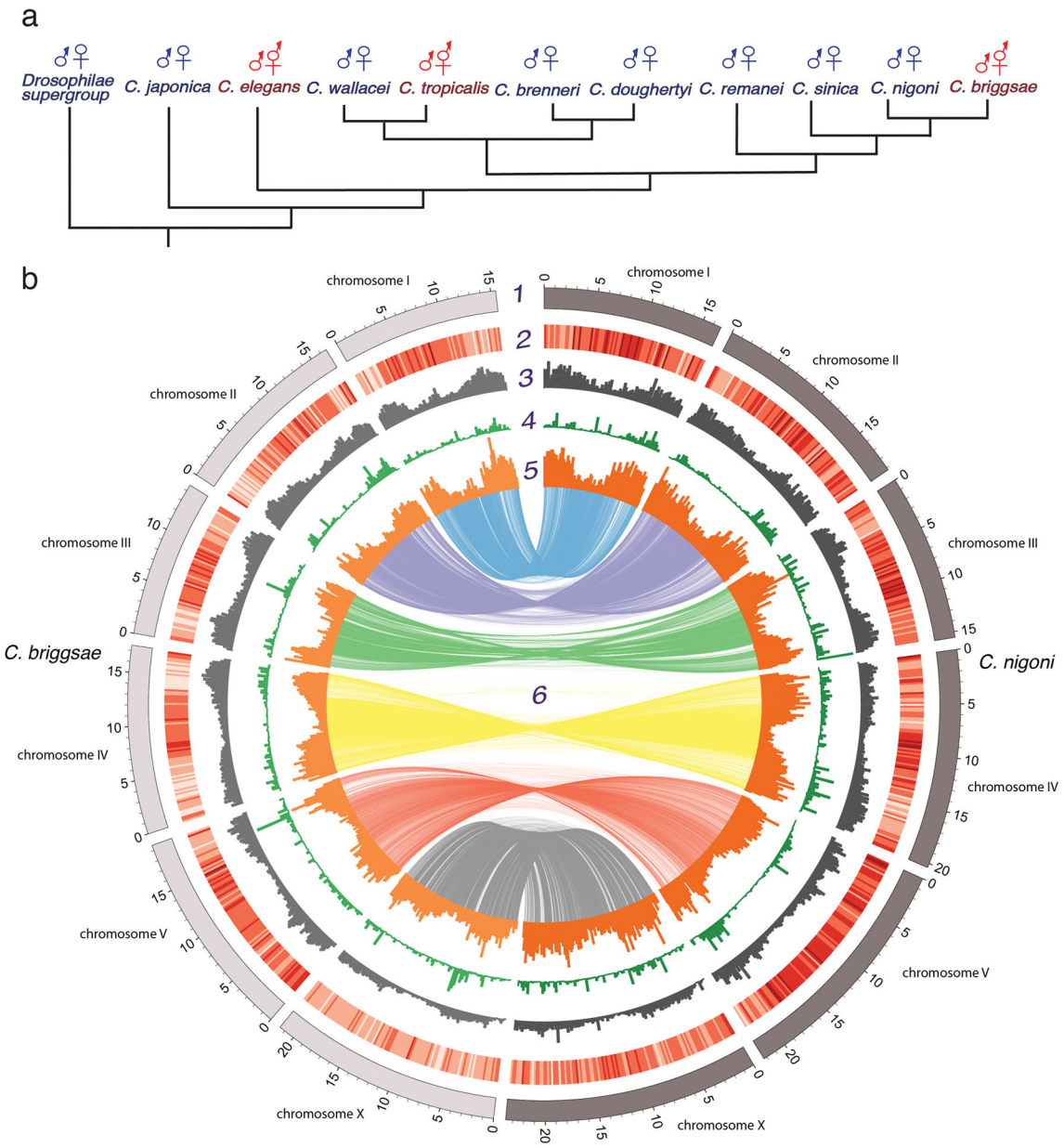

Figure 1. The phylogenetic relationship of *Caenorhabditis* and comparison of the *C. nigoni* and *C. briggsae* genome assemblies

a, Phylogeny of Elegans supergroup *Caenorhabditis* adapted from (3), with outcrossing species producing XX females indicated in blue, and self-fertile lineages with XX hermaphrodites indicated in red. **b,** Chromosomal alignments and genomic features over 200-kb chromosomal intervals. Tracks from outside to inside **1,** Positions (in Mb) of the six chromosomes of *C. nigoni* and *C. briggsae,* **2,** gene density heat map (darker shade means higher density), **3,** repeat frequency, **4,** inversion frequencies, **5,** percentage of sequence lacking homology in the other assembly (representing either deletions or species-specific gains), **6,** DNA sequence synteny.

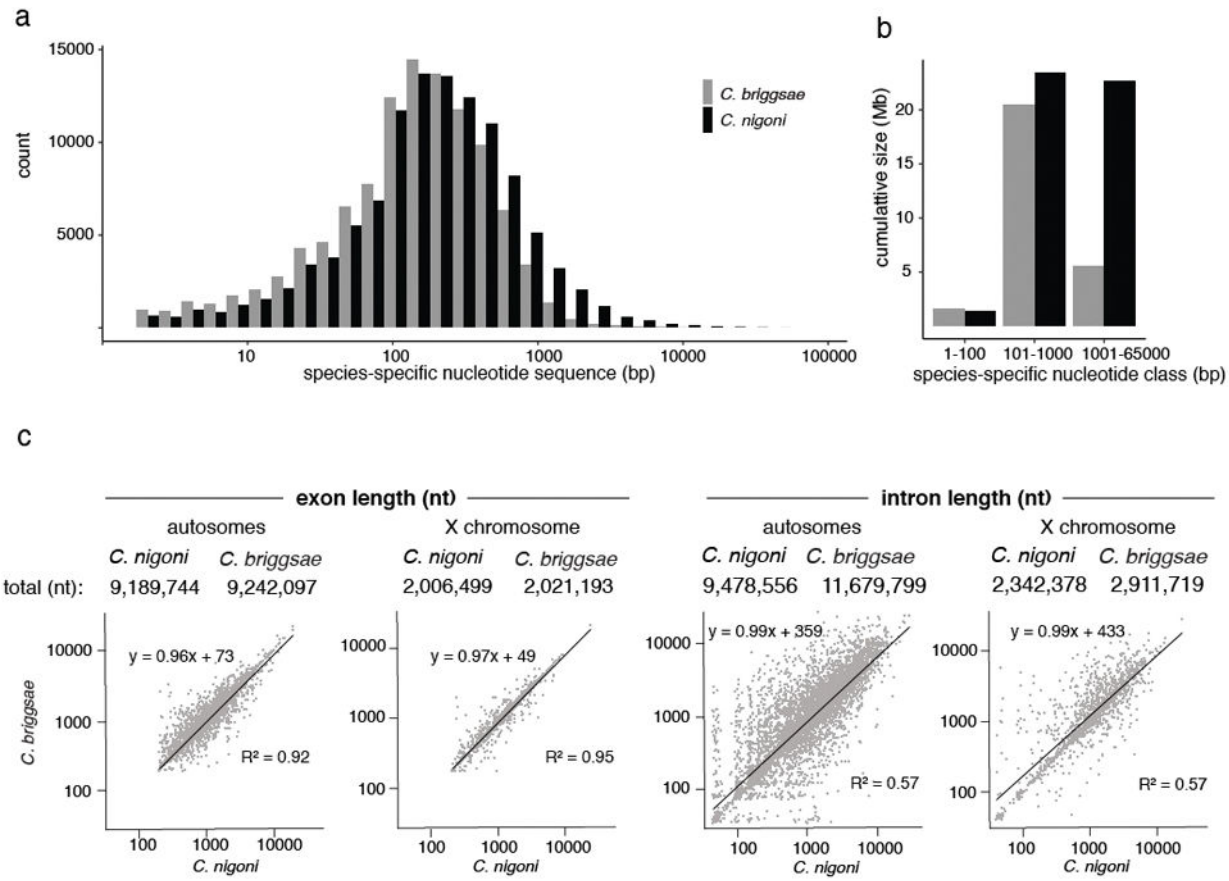

Figure 2. Size distributions of insertion-deletion variants

a, Size distribution of species-specific sequences in the *C. briggsae*-*C. nigoni* whole-genome alignment. Black, sequences present in *C. nigoni* alone; grey, sequences present in *C. briggsae* alone. **b,** Contribution of different species-specific sequence types to genome size. **c,** Regression analysis of total exon and intron lengths for 6,404 1:1 *C. briggsae*-*C. nigoni* orthologs on autosomes and 1,394 orthologs on the X chromosome. Interspecies differences were insignificant for either exon set (Wilcoxon rank-sum test with Bonferroni correction; $p = 0.378$ for autosomes; $p = 0.668$ for X), but introns on both autosomes ($p = 1.53 \cdot 10^{-10}$) and the X chromosome ($p = 1.2 \cdot 10^{-5}$) were significantly larger in *C. briggsae* (Wilcoxon rank-sum test with Bonferroni correction).

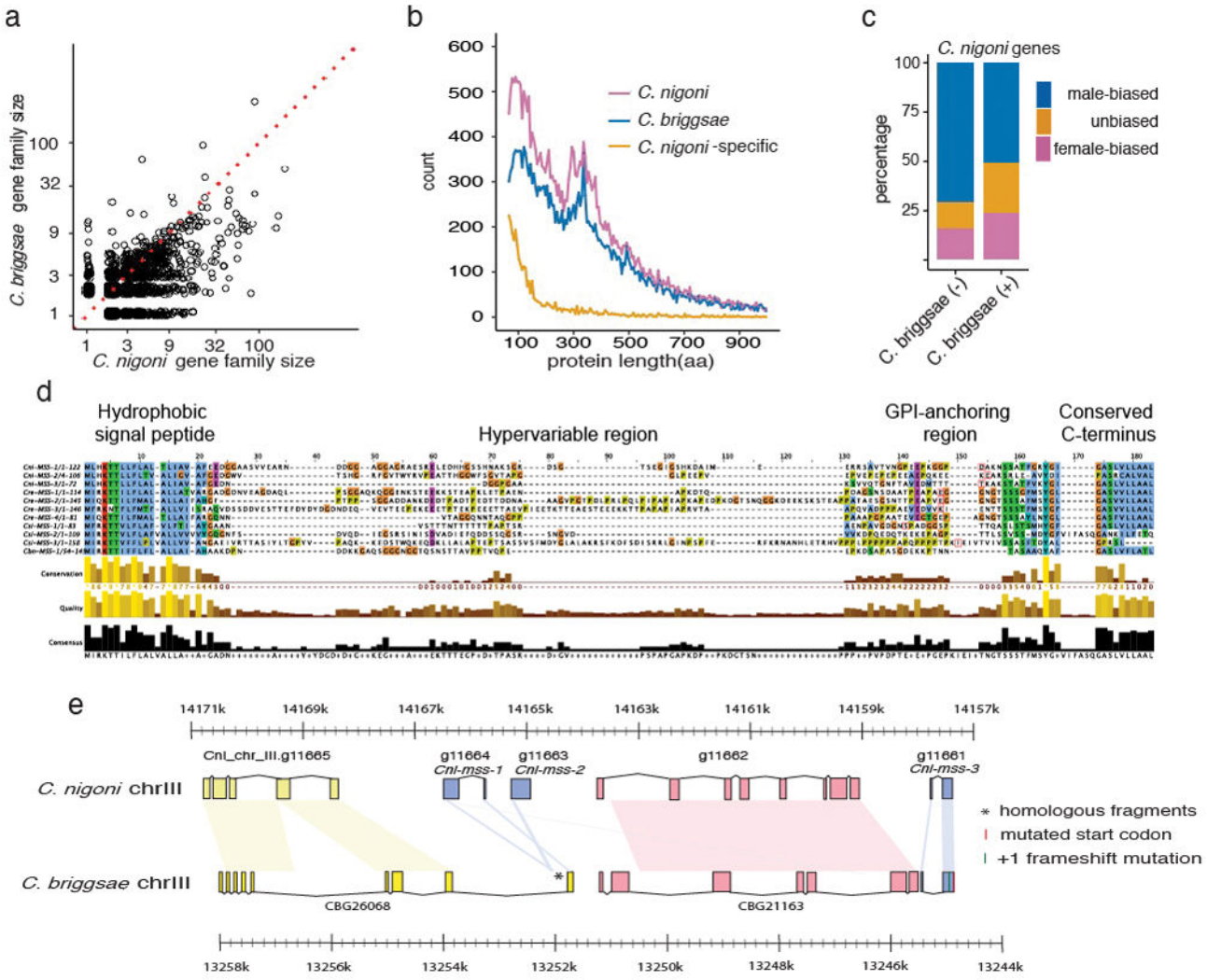

Figure 3. Comparison of the *C. nigoni* and *C. briggsae* proteomes

a, Scatter plot of sizes of OrthoFinder gene families, excluding one-to-one orthologs (table S4). Of 2,367 families with unequal numbers of *C. nigoni* and *C. briggsae* genes, the majority (1,624) were larger in *C. nigoni* than in *C. briggsae* (Wilcoxon signed rank test, $p < 2.2 \cdot 10^{-16}$). Dotted line indicates equal family sizes. **b,** Length distributions of *C. nigoni* and *C. briggsae* proteins and of *C. nigoni* proteins that lack *C. briggsae* homologs. **c,** For genes with sex-biased expression, male bias was seen for 50.9% of 6,804 genes with *C. briggsae* homologs ("*C. briggsae (+)*"), but significantly overrepresented (70.9%) among 605 genes lacking *C. briggsae* homologs ("*C. briggsae (-)*"; Fisher's exact test, $p < 0.0001$; table S9). **d,** Alignment of predicted MSS homologs from outcrossing *C. nigoni, C. sinica, C. remanei,* and *C. brenneri* (table S8; (20)), with protein domains indicated above. **e,** Comparison of *mss* gene regions in *C. nigoni* and *C. briggsae.* Pastel shapes connect homologous sequences. Except for *Cni-mss-3,* all genes are transcribed from left to right. Genes surrounding the three *C. nigoni mss* paralogs are conserved in *C. briggsae,* but only fragments and a pseudogene (*Cbr-mss-3-ps*) of the *mss* genes remain. The pseudogene has a

lost start codon and a +1 frameshift. CBG26068 has a novel 3′ exon derived from part of the *Cni-mss-1* second exon. See fig. S7 and (20) for details.

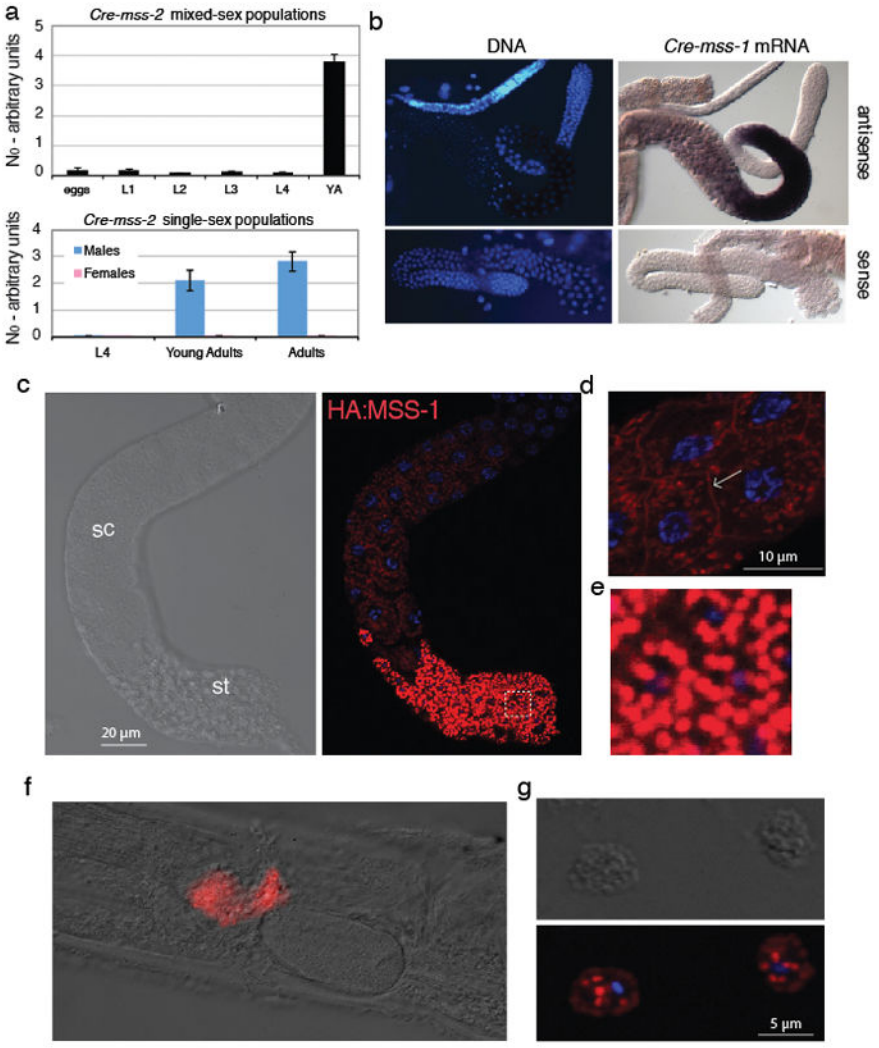

Figure 4. ***C. remanei*** **MSS is a male-specific protein localized to the surface of activated sperm**

a, qRT-PCR transcript quantification (for *Cre-mss-2*) of mixed-sex (top) versus larval and adult sex-specific populations (bottom), showing that *mss* expression is specific to adult males. Mean values are shown with standard error of the mean. Female data are 2–3 orders of magnitude below that for males. **b,** *Cre-mss-1* transcripts are detected in pachytene-stage primary spermatocytes. **c,** HA-tagged *Cre*-HA-MSS-1 is first detectable in spermatocytes (sc), and becomes enriched in spermatids (st). **d,** Some *Cre*-HA-MSS-1 is localized to the plasma membrane of spermatocytes, as indicated by the arrow. Blue fluorescence: Hoechst-stained DNA. **e,** Enlarged view of the boxed region in c, showing complete restriction to membranous organelles (MOs). **f,** *Cre*-HA-MSS-1 remains attached to sperm after activation and transfer to the female. **g,** Sperm cells dissected from a female show *Cre*-HA-MSS-1 in plasma membrane and fused MO remnants.

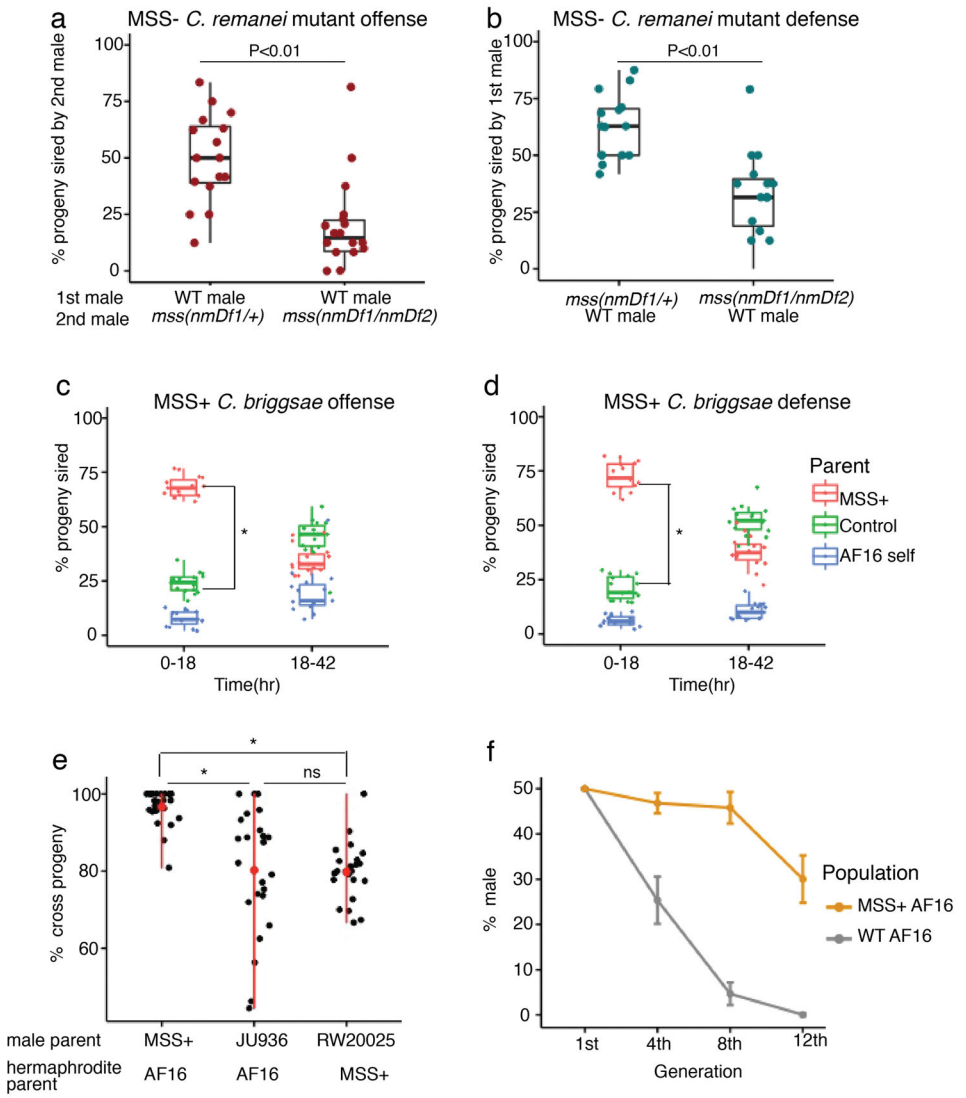

Figure 5. *mss* genes are necessary for sperm competitiveness in an outcrossing species, and sufficient to enhance it in a selfing species

a, When mated after a wild-type male ("offense"), *C. remanei mss(nmDf1/+)* males sire more than twice the progeny of *nmDf1/nmDf2 mss*-null mutants (N = 16 for both). **b,** When allowed to mate first ("defense"), heterozygous *C. remanei mss*(*nmDf1/+*) males have a slight advantage over wild-type males. *mss*-null mutants, in contrast, do not (N = 15 for both). Heterozygote success is assumed to be double the observed *nmDf1* frequency in their progeny. For both defense and offense, $p < 0.01$ (two-sample Kolmogorov–Smirnov test). **c, d,** Wild-type young *C. briggsae* hermaphrodites were mated sequentially (4 h each) with conspecific males carrying either an *C. nigoni mss(+)* transgene or control mCherry::histone reporter (RW0025). Progeny laid 0–18 h and 18–42 h after the second mating were scored for green (MSS+), red (RW0025), or no (self) fluorescent markers. In both offense (c) and defense (d), MSS+ males sire several-fold more progeny than control males in the first laying window. *, $p < 0.001$. **e,** MSS+ *C. briggsae* males suppress selfing more effectively than control AF16 (wild-type) males. Strain JU936 is a second control strain bearing two

transcriptional GFP reporters in the AF16 background. (*, $p < 0.001$; ns, not significant Kolmogorov–Smirnov test) **f,** Male frequency in MSS+ and wild-type AF16 *C. briggsae* populations in which male frequency was artificially elevated to 50% at the start of the experiment.