Sex-Biased Gene Expression and Evolution of the X Chromosome in Nematodes

Sarah Elizabeth Albritton,* Anna-Lena Kranz,* Prashant Rao,* Maxwell Kramer,* Christoph Dieterich,† and Sevinç Ercan*,1

*Department of Biology, Center for Genomics and Systems Biology, New York University, New York 10003 and †Max Planck Institute for Biology of Ageing, D-50931 Cologne, Germany

ABSTRACT Studies of X chromosome evolution in various organisms have indicated that sex-biased genes are nonrandomly distributed between the X and autosomes. Here, to extend these studies to nematodes, we annotated and analyzed X chromosome gene content in four *Caenorhabditis* species and in *Pristionchus pacificus*. Our gene expression analyses comparing young adult male and female mRNA-seq data indicate that, in general, nematode X chromosomes are enriched for genes with high female-biased expression and depleted of genes with high male-biased expression. Genes with low sex-biased expression do not show the same trend of X chromosome enrichment and depletion. Combined with the observation that highly sex-biased genes are primarily expressed in the gonad, differential distribution of sex-biased genes reflects differences in evolutionary pressures linked to tissue-specific regulation of X chromosome transcription. Our data also indicate that X dosage imbalance between males (XO) and females (XX) is influential in shaping both expression and gene content of the X chromosome. Predicted upregulation of the single male X to match autosomal transcription (Ohno's hypothesis) is supported by our observation that overall transcript levels from the X and autosomes are similar for highly expressed genes. However, comparison of differentially located one-to-one orthologs between *C. elegans* and *P. pacificus* indicates lower expression of X-linked orthologs, arguing against X upregulation. These contradicting observations may be reconciled if X upregulation is not a global mechanism but instead acts locally on a subset of tissues and X-linked genes that are dosage sensitive.

In an XY sex-determination system, male and female genomes are identical with the exception of the male-specific Y chromosome, which bears few genes (Charlesworth *et al.* 2005). This is particularly true when the Y chromosome is thought to be completely lost, as is the case for *C. elegans* and many other nematodes (Walton 1940). Because gene content is the same, phenotypic differences between males and females, termed "sexual dimorphisms," must be caused by differential gene expression between the two sexes (Connallon and Knowles 2005; Ellegren and Parsch 2007). Throughout the article, such differentially expressed genes are referred to as "sex biased."

As males and females have different fitness optima, a trait that is beneficial to one sex can be harmful to the other (termed sexual antagonism) (Rice and Chippindale 2001; Arnqvist 2004; Connallon and Knowles 2005; Ellegren and Parsch 2007; Mank *et al.* 2008a; Rice 1984). The evolution of sex-biased gene expression is thought to mediate the effects of sexual antagonism and allow for achievement of sex-specific fitness. Previous studies have indicated that anywhere between 30 and 60% of metazoan genes may be sex biased (Ranz *et al.* 2003; Parisi *et al.* 2004; Reinke *et al.* 2004; Yang *et al.* 2006; Reinius *et al.* 2008; Small *et al.* 2009; Innocenti and Morrow 2010; Assis *et al.* 2012; Reinius *et al.* 2012; Thomas *et al.* 2012). Genes with sex-biased expression contribute to both somatic and gonadal sexual dimorphisms (Ranz *et al.* 2003; Parisi *et al.* 2004; Yang *et al.* 2006; Mank *et al.* 2008a; Reinius *et al.* 2008).

As the number of X chromosomes differs between males and females, the X plays a large role in the evolution of sexual dimorphisms (Rice 1984; Mank *et al.* 2008a). Due to both sex-specific natural selection and the unique life cycle of the X chromosome, it is predicted that sex-biased genes should accumulate on the X (Rice 1984). Because males are monosomic for the X (they bear only one copy of the X chromosome

doi: 10.1534/genetics.114.163311
Manuscript received February 21, 2014; accepted for publication April 17, 2014; published Early Online May 2, 2014.
Sequence data from this article have been deposited with the GEO Data Libraries under accession no. GSE41367.
Supporting information is available online at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.163311/-/DC1.
[1]Corresponding author: Department of Biology, New York University, Center for Genomics and Systems Biolog, Brown Bldg., Room 852, New York, NY 10003. E-mail: se71@nyu.edu

to two copies of each autosome), recessive alleles that emerge on the X chromosome are immediately visible to male-specific selection. Recessive male-benefitting alleles are predicted to increase in frequency on the X, even if the fitness cost to females is much greater (Rice 1984). Additionally, because the X spends two-thirds of its evolutionary history in females, dominant female-benefitting alleles are predicted to accumulate on the X chromosome.

Indeed, in many organisms, sex-biased genes are nonrandomly distributed between the X chromosome and the autosomes (Saifi and Chandra 1999; Wang *et al.* 2001; Lercher *et al.* 2003; Reinke *et al.* 2004; Parisi *et al.* 2003; Ranz *et al.* 2003; Khil *et al.* 2004; Divina *et al.* 2005; Yang *et al.* 2006; Sturgill *et al.* 2007; Reinius *et al.* 2012; Meisel *et al.* 2012a; Allen *et al.* 2013). However, it is not always the case that both male and female-biased genes are enriched on the X. Enrichment of female-biased genes on the X chromosome ("feminization of the X") is observed in mammals, *Drosophila*, and *Caenorhabditis elegans* (Parisi *et al.* 2003; Ranz *et al.* 2003; Khil *et al.* 2004; Reinke *et al.* 2004 ; Reinius *et al.* 2012; Allen *et al.* 2013). The same studies also provide evidence for "demasculinization of the X chromosome," where male-biased genes are largely excluded from the X. The degree to which the *Drosophila* X chromosome is depleted of male-biased genes is still being studied (Vibranovski *et al.* 2009a,b; Meiklejohn and Presgraves 2012; Meisel *et al.* 2012a). Older male-biased genes are underrepresented on the *Drosophila* X chromosome, while younger male-biased genes [those emerged after the *melanogaster* split ~3–6 million years ago (Russo *et al.* 1995)] are enriched (Zhang *et al.* 2010). This suggests that X demasculinization is an evolutionary process (Gao *et al.* 2014). While enrichment of female-biased genes on the X is predicted, demasculinization of the X opposes the expectation that male-benefitting genes should accumulate on the X chromosome (Rice 1984).

One often-cited explanation for the depletion of male-biased genes from the X chromosome is meiotic sex chromosome inactivation (MSCI). In many species, the single unpaired X chromosome is transcriptionally silenced during male meiosis (Fong *et al.* 2002; Kelly *et al.* 2002; Turner 2007; Maine 2010). MSCI begins in early meiosis and persists throughout varying stages of spermatogenesis in different species (Turner 2007). Thus, genes necessary for spermatogenesis should move off of the X chromosome. Studies in mice and humans have found that male-biased genes expressed in the testes are enriched on the X chromosome (Wang *et al.* 2001; Lercher *et al.* 2003). It has been subsequently suggested that this enrichment may be true only for genes expressed during the earliest stages of spermatogenesis before the onset of MSCI (Khil *et al.* 2004). In *C. elegans*, sperm-enriched genes are depleted from the X chromosome (Reinke *et al.* 2000, 2004). Immunofluorescence analyses of various histone modifications in conjunction with microarray expression analyses provide strong evidence for the presence of MSCI in *C. elegans* (Kelly *et al.* 2002; Reuben and Lin 2002; Bean *et al.* 2004; Bessler *et al.* 2010). Throughout *C. elegans* spermatogenesis, histone modifications associated with active transcription are depleted from the X chromosome while those associated with transcriptional repression are enriched (Kelly *et al.* 2002; Reuben and Lin 2002; Bean *et al.* 2004; Bessler *et al.* 2010).

The *C. elegans* X chromosome is also repressed in the hermaphrodite germline (Reinke *et al.* 2000; Kelly *et al.* 2002; Reinke *et al.* 2004; Bender *et al.* 2006; Maine 2010). Germline X repression is restricted to early meiotic cells where the X is largely depleted of active chromatin marks, including H3K4me2 (Kelly *et al.* 2002). Early germline X silencing appears to be conserved in nematodes, since H3K4me2 is depleted from the X chromosome in each of the *Pristionchus pacificus*, *C. briggsae*, and *C. remanei* germlines (Kelly *et al.* 2002). Transcriptional repression of the X in both male and female germlines explains the observation that germline-expressed genes are underrepresented on the X chromosome in *C. elegans* (Reinke *et al.* 2000, 2004; Wang *et al.* 2009; Tabuchi *et al.* 2011; Gaydos *et al.* 2012).

Constraints on X chromosome dosage compensation have been cited as another potential explanation for the depletion of male-biased genes from the X chromosome (Bachtrog *et al.* 2010). Dosage compensation equalizes X chromosome transcript levels between XX females and single X males. Although strategies differ, dosage-compensation mechanisms have been found in mammals, *Drosophila*, and *C. elegans* (Meyer 2010; Conrad and Akhtar 2012; Disteche 2012; Ferrari *et al.* 2014). Mammalian females inactivate most of the genes on one of their two X chromosomes to match transcriptional output from the single male X (Pollex and Heard 2012; Dupont and Gribnau 2013). In *C. elegans*, the dosage-compensation complex (DCC) binds to both of the hermaphrodite X chromosomes and represses transcription of each by one-half (Csankovszki 2009; Ercan and Lieb 2009; Meyer 2010). The *Drosophila* dosage compensation machinery binds to the single X chromosome in males and increases transcription twofold (Gelbart and Kuroda 2009). In *Drosophila*, it has been hypothesized that functional or mechanistic constraints on the X could limit the upregulation of already transcriptionally upregulated genes in males, thus preventing them from becoming male biased (Bachtrog *et al.* 2010). However, the mechanism of such a constraint remains unclear.

Susumu Ohno hypothesized that, during evolution of sex chromosomes, transcription from the X was upregulated to compensate for the degeneration of the Y (Ohno 1967; Adler *et al.* 1997; Gupta *et al.* 2006; Nguyen and Disteche 2006; Lin *et al.* 2007; Deng *et al.* 2011; Gribnau and Grootegoed 2012). Ohno further hypothesized that X upregulation was not specific to males but also occurred in XX females, resulting in overexpression of the female X above autosomal levels. He predicted that dosage compensation in XX females (*i.e.*, the downregulation of X transcription in mammals) evolved to counter X upregulation. Supporting Ohno's hypothesis, microarray and mRNA-seq data from mouse, *Drosophila*, and *C. elegans* have indicated that overall transcript levels are similar between the X chromosome and the autosomes in both males (X:AA) and females (XX:AA). Further, hermaphrodite X chromosome

transcription increases to above autosomal levels when the DCC is mutated in *C. elegans* embryos (Kruesi *et al.* 2013).

To test Ohno's hypothesis more directly, attempts have been made to define those genes present on the autosomal progenitor of the X chromosome using species from major mammalian and bird lineages (Julien *et al.* 2012; Lin *et al.* 2012). If the X chromosome is upregulated, transcriptional output from the present-day X and its autosomal progenitor should be similar. These studies found transcription from the current eutherian (the mammalian clade that includes humans and other placental mammals) X chromosome to be significantly lower compared to its autosomal progenitor (Julien *et al.* 2012; Lin *et al.* 2012). However, X and autosomal progenitor expression levels were similar when analyses were limited to highly expressed genes and those encoding for proteins involved in large (more than seven subunits) complexes (Julien *et al.* 2012; Lin *et al.* 2012). This result suggests that X upregulation is restricted to dosage-sensitive genes.

Much of the comparative work regarding evolution of sex-biased gene expression and the X chromosome has been performed in *Drosophila* and mammals (Wang *et al.* 2001; Lercher *et al.* 2003; Parisi *et al.* 2003; Khil *et al.* 2004; Parisi *et al.* 2004; Yang *et al.* 2006; Sturgill *et al.* 2007; Vicoso and Charlesworth 2009; Ellegren 2011; Meisel *et al.* 2012a; Allen *et al.* 2013; Parsch and Ellegren 2013). Here, to expand our understanding of X evolution, we compared X-linked gene expression in five nematode species that include both gonochoristic (outcrossing) and hermaphroditic mating systems (Figure 1A). Hermaphroditism has arisen independently in several nematode species (Kiontke *et al.* 2011), a process that strongly influences evolution of the genome. The gonochoristic *Caenorhabditis* species that we analyzed have larger genomes and greater magnitude of sex-biased expression compared to the hermaphroditic species (Thomas *et al.* 2012).

For those species without a genetic map, we first assigned genes to the X chromosome using high-throughput sequencing of DNA isolated from XO males and XX females/hermaphrodites. Subsequently, we used mRNA-seq to analyze sex-biased gene expression in young adult males and females/hermaphrodites. Overall, we found that the genomic distribution of the sex-biased genes is conserved at varying degrees between *C. elegans* and the other four nematode species. Generally, those genes with high male-biased expression are underrepresented on the X chromosome. Genes with high female-biased expression are enriched on the X. To test Ohno's hypothesis, we compared expression of orthologous genes that are differentially located between *C. elegans* and *P. pacificus*. Transcription is higher from the autosomal-linked orthologs, suggesting that there is no upregulation of these genes on the X chromosome. However, in all nematode species analyzed here, the level of transcription from the single X chromosome is similar compared to the autosomes, supporting X upregulation. These contradicting observations can be reconciled if the mechanism of X upregulation does not act chromosome-wide but specifically regulates dosage-sensitive genes.

Materials and Methods

Worm strains and growth

C. elegans (*N2*), *C. brenneri* (*PB2801*), *C. briggsae* (*AF16*), *C. remanei* (*PB4641*), and *P. pacificus* (*PS312*) strains were maintained at 20° on NGM agar plates using standard *C. elegans* growth methods.

DNAseq

One female and two or more male replicates were collected per species (summarized in Supporting Information, Table S1). At least 50 young adult worms were hand picked per replicate. Worms were washed by settling three to five times with 1 ml of M9, starved overnight to eliminate gut bacterial contamination, and resuspended in 100 μl TE. A total of 400 μl of lysis buffer (0.1 M Tris–HCl; 0.1 M NaCl; 50 mM EDTA; 1.25% SDS) was added and worms were sonicated for 30 min using the Bioruptor at high setting, 30 sec on/off. Sonicated DNA was isolated and cleaned up using Qiagen MinElute kit. Illumina DNA sequencing libraries between 250 and 500 bp were prepared from the purified DNA using Illumina TruSeq DNA kit with the following modifications. Briefly, after end repair and A tailing, adapters were ligated and the resulting DNA was purified using AmpureXP beads. Ligated DNA was amplified by PCR and DNA library between 300 and 500 bp was gel purified. Fifty base pair paired-end or single-end sequencing (see Table S1) was performed using Illumina HiSeq-2000. The raw data can be found at Gene Expression Omnibus (GEO) under series number GSE53144. For paired-end data, quality scores of the reverse reads were much lower than those of the forward reads. As such, only forward reads were used for analysis.

Copy-number approach: X and autosomal gene assignments

For each species, forward reads were aligned to WS228 with Bowtie version 0.12.7 (Langmead *et al.* 2009). We supplied the parameter ($m = 4$) to suppress all alignments with more than four hits in the genome. The resulting alignment files (in BAM format, a binary file type containing sequence alignment data) were converted to SAM format (tab-delimited text version of a BAM file) using SAMtools v. 0.1.18 (Li *et al.* 2009). The SAM files were sorted and used to generate bedgraph files (BEDTools v. 2.15.0) (Quinlan and Hall 2010). For each species, the contigs were split into 5-kb windows and the bedgraph file was used to calculate the sequencing coverage for each window. The male-to-female coverage ratio was computed for each window by taking the $\log_2$ of male coverage divided by female coverage. Baseline was set as the mean male-to-female coverage ratio. Windows whose $\log_2$ ratio fell one standard deviation below the mean were initially assigned to the X chromosome. If the majority of 5-kb windows contained within a contig were assigned to the X, then the contig was assigned to the X. If there was no majority, or if the contig was <15 kb, we could not assign the contig. Assignments were given confidence scores ranging

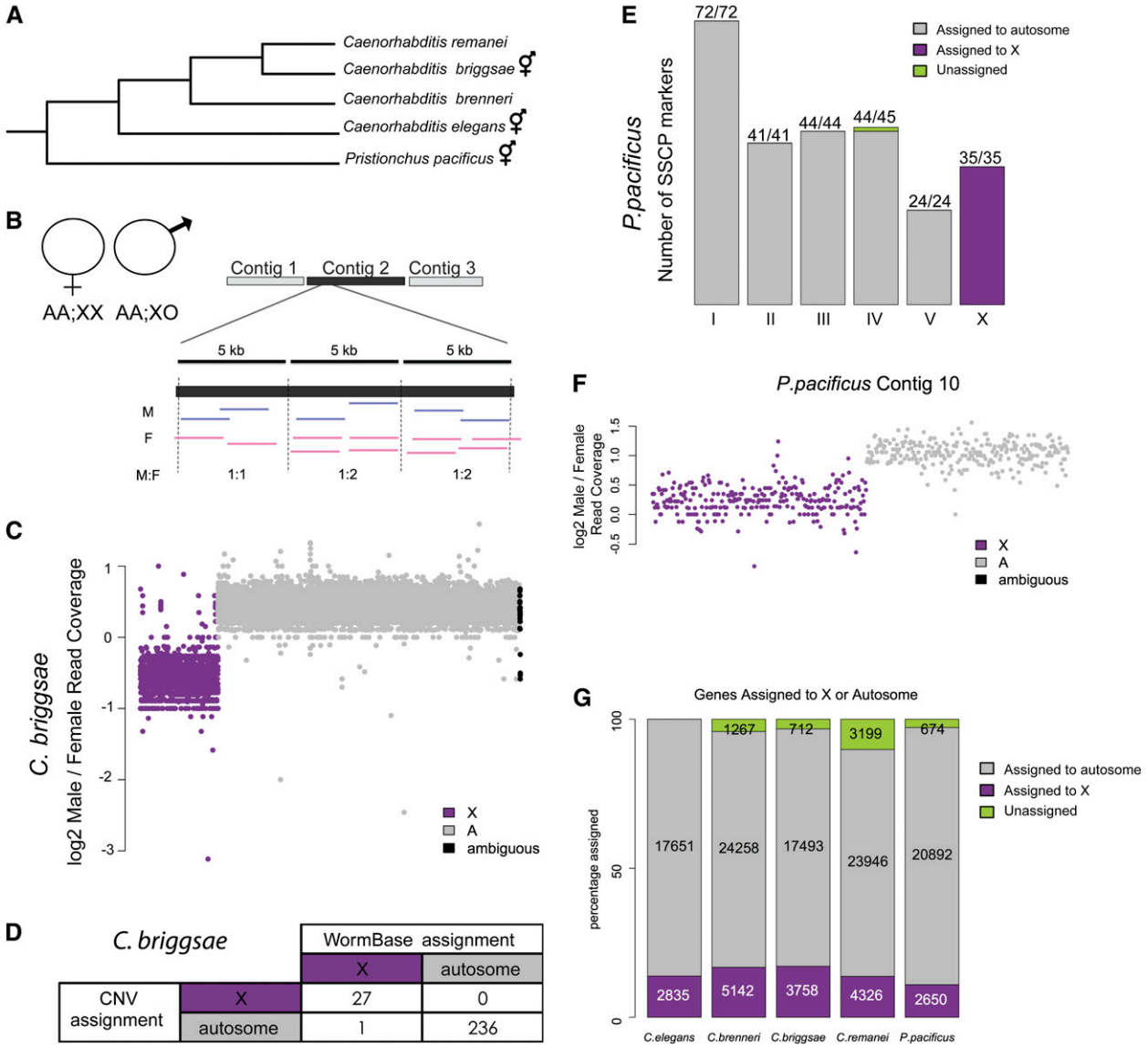

Figure 1 Copy-number analysis allows for assignment of sequencing contigs to either the X or autosomes. (A) Phylogeny showing the evolutionary relationship of the five nematode species (Kiontke *et al.* 2004, 2011). *Pristionchus pacificus* is estimated to have split from *Caenorhabditis* ~300 million years ago (Dieterich *et al.* 2006). *C. briggsae*, *C. elegans*, and *P. pacificus* are androgynous. *C. brenneri* and *C. remanei* are gonochoristic. Estimates of *C. elegans* and *C. briggsae* divergence times range between 40 and 80 million years. (B) Overview of the copy-number approach. DNA-seq data were generated from males (AA;XO) and females (AA;XX). Contigs were broken into 5-kb windows and male-to-female coverage ratio was evaluated for each window. (C) Plotting male-to-female coverage ratio shows clear difference between X and autosomal contigs. The $\log_2$ male-to-female coverage ratio is plotted for *C. briggsae*. Each point represents one 5-kb window. X chromosome (purple) and autosomal (gray) contigs are plotted along the *x*-axis. Contigs that could not be assigned unambiguously are plotted in black. (D) Overlap of *C. briggsae* copy-number assignment with WormBase assignment indicates high accuracy. (E) Overlap of *P. pacificus* copy-number assignment with previously reported SSCP markers (Srinivasan *et al.* 2002; Dieterich *et al.* 2006) indicates high accuracy. Chromosomal assignments by SSCP are indicated below each bar. Bars are colored to indicate copy-number assignment. Numbers above each bar indicate agreement. (F) Copy-number analysis allows for identification of a split contig in *P. pacificus*. The male-to-female coverage ratio of the left and right half of *P. pacificus* contig 10 are assigned to the X and autosome, respectively. (G) Ratio of X to autosomal gene content is roughly unchanged across species. The number of assigned genes in each category (X, purple; autosomal, gray; unassigned, green) is indicated. For each of the five species, ~10–14% of coding genes are assigned to the X chromosome.

from 0 to 2 based on both length of sequencing contig and agreement of assignment between replicates. One point was given to those contigs with lengths >50 kb. A second point was given if the final contig assignment obtained by combining all replicates matched the assignment obtained when the replicates were analyzed separately. The gene assignments and confidence scores are given in Table S2.

mRNA-seq

C. elegans (N2) and *P. pacificus (PS312)* worms were synchronized by bleaching gravid adults and allowing worms to hatch overnight. Larval worms were plated and grown at 20°. At least 750 young adult worms were hand picked for each of three biological replicates. Worms were washed three to five times in M9. Ten volumes of Trizol (Invitrogen)

was added. Samples were freeze-cracked five times and RNA purification was performed according to the manufacturer protocol. Isolated RNA was cleaned up using the Qiagen RNeasy kit. mRNA was purified using Sera-Mag oligo(dT) beads (ThermoScientific) from at least 1 μg of total RNA. Stranded mRNA-seq libraries were prepared based on incorporation of dUTPs during cDNA synthesis using a previously described protocol (Parkhomchuk *et al.* 2009). Single-end 50-bp sequencing was performed using the Illumina HiSeq-2000. Data for *C. elegans (fog-2)*, *C. brenneri*, *C. briggsae (she-1)*, and *C. remanei* were downloaded from GEO (accession no. GSE41367). Reads were aligned to genome version WS228 with tophat v. 2.0.0 (Trapnell *et al.* 2012). Default parameters allow up to 20 hits for each read. Read numbers and mapping percentages (which refer to the percentage of unique reads with at least one alignment) can be found in Table S1. Gene expression was estimated using Cufflinks v. 2.0.2 with default parameters and supplying gene annotations for WS228. Average male and female expression (FPKM, fragments per kilobase per million mapped reads) was determined (Table S6). The raw read data and the average Cufflinks FPKM data can be obtained from GEO accession no. GSE53144. Differential expression analysis between males and females was performed using the R package DESeq (Anders and Huber 2010; R Development Core Team 2012). These results are available in Table S6.

Defining sex-biased gene expression

To define sex bias, we first identified those genes that were differentially expressed between the two sexes (DESeq qvalue $<$ 0.05). Genes with FPKM >1 in at least one sex were considered "expressed" and used for subsequent analyses. We categorized "sex-biased" genes as those having FPKM >1 in one sex and FPKM >0 in the other. For each sex-biased gene, the magnitude of sex bias was calculated as the $\log_2$ ratio of FPKM values between the two sexes. Those genes that have FPKM >1 in one sex and FPKM $= 0$ in the other were categorized as "sex-specific" genes. We categorized "nonbiased" genes as those genes not called significant by DESeq (qvalue > 0.05) with FPKM >1 in both sexes and with less than twofold expression difference between the sexes. We categorized genes with high and low sex-biased expression based on a $\log_2$ sex-expression ratio cutoff of 3 (Table S3). The cutoff was selected based on a breakpoint in the distribution of sex-biased expression ratios driven largely by the gonadal expression of highly sex-biased genes (discussed further in *Results and Discussion*).

Results and Discussion

Copy-number approach for gene assignment to the X chromosome

To compare X chromosome gene content and sex-biased gene expression, we focused on five nematode species (Figure 1A). Four species are from the *Caenorhabditis* genus: *C. elegans*, *C. brenneri*, *C. briggsae*, and *C. remanei*. Determination of nematode divergence times is difficult owing to a lack of fossil records. Previous calculations based on the assumption of a universal molecular clock estimated that *C. elegans* and *C. briggsae* diverged 80–110 million years ago (Coghlan and Wolfe 2002; Stein *et al.* 2003). More recently, estimates of neutral mutation rates were used to approximate the divergence time between *C. elegans* and *C. briggsae* to ~40 million years (Cutter 2008). The fifth species, *P. pacificus*, is estimated to have diverged ~300 million years ago (Dieterich *et al.* 2006, 2008).

Three of the species, *C. elegans*, *C. briggsae*, and *P. pacificus*, are androdioecious and consist of self-fertile hermaphrodites and outcrossing males. The other two species, *C. remanei* and *C. brenneri*, are gonochoristic and have true outcrossing males and females. For simplicity, we refer to hermaphrodites as females except where the distinction is necessary. Although all five genomes have been previously sequenced, only the *C. elegans* and *C. briggsae* chromosomes are fully assembled. The other genomes remain on sequencing contigs.

To compare X and autosomal expression, we needed first to assign genes to either the X chromosome or the autosomes. To accomplish this task, we used a copy-number approach taking advantage of the fact that the X chromosome is present in one copy in males and two copies in females. The same principle was used recently for assigning genes to the Z chromosome of snakes and trematodes (Vicoso and Bachtrog 2011; Vicoso *et al.* 2013). We performed Illumina sequencing of DNA isolated from hand-picked male and female adults from *C. remanei*, *C. brenneri*, *C. briggsae*, and *P. pacificus*. Table S1 lists the number of reads and genome coverage for each replicate. We split each contig into 5-kb windows and calculated the $\log_2$ ratio of male-to-female read coverage for each window (Figure 1B). Figure 1C shows the $\log_2$ ratio of male-to-female read coverage for *C. briggsae*. A contig was assigned to the X chromosome if the majority of windows had a male-to-female coverage ratio one standard deviation below the mean.

When we compared our *C. briggsae* gene assignments to the current WormBase assignment [based on a high-density recombination map (Hillier *et al.* 2007; Koboldt *et al.* 2010; Ross *et al.* 2011)] we found that we correctly assigned 236 of 236 contigs to autosomes and 27 of 28 contigs to the X chromosome (Figure 1D). The inconsistent contig, cb25.fpc2310b, is assigned to an autosome by our method and to the X chromosome by WormBase. It contains no genes and was previously assigned to chromosome V as part of a larger sequencing contig, cb25.fpc2310. This contig was split into three parts for the current release, two of which are still assigned to chromosome V.

We also compared our *P. pacificus* contig assignments to a limited linkage map that was generated using a single-strand conformational polymorphism technique (SSCP; Orita *et al.* 1989). Recombinant inbred lines from two *P. pacificus* strains (wild-type *P. pacificus* var. California and the polymorphic Washington strain) were used to generate the linkage map (Srinivasan *et al.* 2002; Dieterich *et al.* 2006). Our assignment matched all of the X chromosome SSCP markers and all but one autosomal marker (Figure 1E). This last marker was unassigned

by our copy-number approach because its contig is less than the minimum 15 kb required by our method. Of note, our method also allowed for identification of contigs that may be incorrectly assembled. By our method, the left half of PPA contig10 (WS228) should be assigned to the X chromosome while the right half should be assigned to an autosome (Figure 1F).

If we assigned a contig to the X chromosome, then all of its annotated genes were also assigned to the X. We correctly assigned 3758 genes to the *C. briggsae* X chromosome and 17493 genes to the autosomes (Figure 1G). A total of 712 genes were left unassigned because their sequencing contigs were too short to be reliably called by our method. With this copy-number approach, we were able to assign between 90 and 97% of genes to either the X or the autosomes in *C. brenneri*, *C. briggsae*, *C. remanei*, and *P. pacificus*. A list of our gene assignments can be found in Table S2.

Gonochoristic nematode species (*C. brenneri* and *C. remanei*) have larger genomes than those of hermaphroditic species (*C. elegans*, *C. briggsae*, and *P. pacificus*) in terms of both sequence and gene content (Stein *et al.* 2003; Barriere *et al.* 2009; Thomas *et al.* 2012). Despite difference in size, all four *Caenorhabditis* genomes have roughly the same distribution of X and autosomal genes. *P. pacificus* stands out as having fewer X-linked genes than the other species (Figure 1G). This result is consistent with the observation that one arm of *P. pacificus* chromosome I contains genes orthologous to those located on the *C. elegans* X, indicative of a major translocation event that occurred after the *Pristionchus–Caenorhabditis* split (Srinivasan *et al.* 2002).

This type of translocation event is rare within Rhabditina (the phylogenetic clade that contains both *Pristionchus* and *Caenorhabditis*). Among the estimated 4000 rearrangement events that have occurred since *C. elegans* and *C. briggsae* split, few were interchromosomal (Coghlan and Wolfe 2002; Stein *et al.* 2003). Similarly, an 11-gene region on *P. pacificus* chromosome III has 10/11 orthologs on the *C. elegans* chromosome III (Lee *et al.* 2003). Further, members of Rhabditina have a stable chromosome number of six (Mitreva *et al.* 2005). These observations suggest that for the species analyzed here, the X chromosome descended from a common ancestor. Additionally, there is no apparent pseudoautosomal region on the nematode X chromosome. As the Y chromosome is absent in all five nematode species examined here (Walton 1940), all X chromosome genes are sex linked.

Determining genes with sex-biased gene expression

To investigate the relationship between sex-biased gene expression and the nematode X chromosome, we analyzed previously published mRNA-seq data from young adult males and females in each of the four *Caenorhabditis* species (Thomas *et al.* 2012; GEO accession no. GSE41367) and produced a similar data set for *P. pacificus*. The published data used *C. elegans (fog-2)* and *C. briggsae (she-1)* mutants, which lack XX spermatogenesis but produce otherwise normal developing female worms. Because a similar feminizing mutation was not available for *P. pacificus*, we used J4/young adult males and hermaphrodites of the wild-type California strain (PS312) and produced a comparable data set for wild-type *C. elegans* (*N2*) L4/young adult males and hermaphrodites. Expression values (FPKM) for each gene were calculated using Cufflinks (Trapnell *et al.* 2012) and differential expression was determined by DESeq (Anders and Huber 2010).

For differentially expressed genes, we calculated the magnitude of sex bias as the $\log_2$ ratio of expression between the two sexes (see *Materials and Methods*). In all five species, male-biased genes have a higher magnitude of bias compared to female-biased genes. Between 38 and 60% of male-biased genes are expressed at least eight times more in males than in females (Figure 2A). Conversely, the majority of female-biased genes have a less than twofold difference in expression between the sexes.

We categorized genes with high and low sex-biased expression using a $\log_2$ sex expression ratio cutoff of 3 (see *Materials and Methods*). For each species and sex, we plotted the magnitude of sex bias against the overall expression levels (Figure S1). Figure 2B shows the male/female expression ratio plotted against male expression (left) and female expression (right) for *C. elegans* (*fog-2*). As expected, genes that are highly sex biased have higher expression in the corresponding sex. Further, a large proportion of the high sex-biased genes have very low expression (FPKM <1) in the opposite sex (Figure 2B). However, regression analysis revealed low correlation between magnitude of sex-biased expression and overall expression levels (for all species, R-squared values ranged between 0.01 and 0.38). This relationship between sex bias and overall expression levels is similar for the X chromosome and autosomes (Figure S2A, Pearson correlation values between 0.6 and 0.9).

Overall tendency toward male-biased expression

Previous work on *Caenorhabditis* species noted a higher prevalence of genes with male-biased expression (Thomas *et al.* 2012) (Figure 3A). Our analyses replicated these findings and indicated the same trend in *Pristionchus* worms. In all five species, the number of male-specific and male-biased genes is greater than the number of female-specific and female-biased genes (Figure 3B). Overall, sex-biased expression is significantly more likely to be male-biased (P-value = 0.03 by paired t-test).

Compared to *Caenorhabditis* species, we identified fewer sex-biased genes in *P. pacificus*. (Figure 3B). We noted that *P. pacificus* mRNA-seq data were collected from male and hermaphrodite populations while *Caenorhabditis* data came from male and female populations. We reasoned that the use of hermaphrodite worms, which produce both sperm and eggs, might have reduced our ability to call sex bias in *P. pacificus*. To test this, we compared sex-biased gene expression in *C. elegans* using data from wild-type (N2) hermaphrodites or *fog-2* mutant females. More than 92% of the sex-biased genes identified in N2 were also identified as sex biased in the *fog-2* mutant analysis (Figure S3, A and B). Overall, we identified 8388 genes as differentially expressed between *C. elegans* males

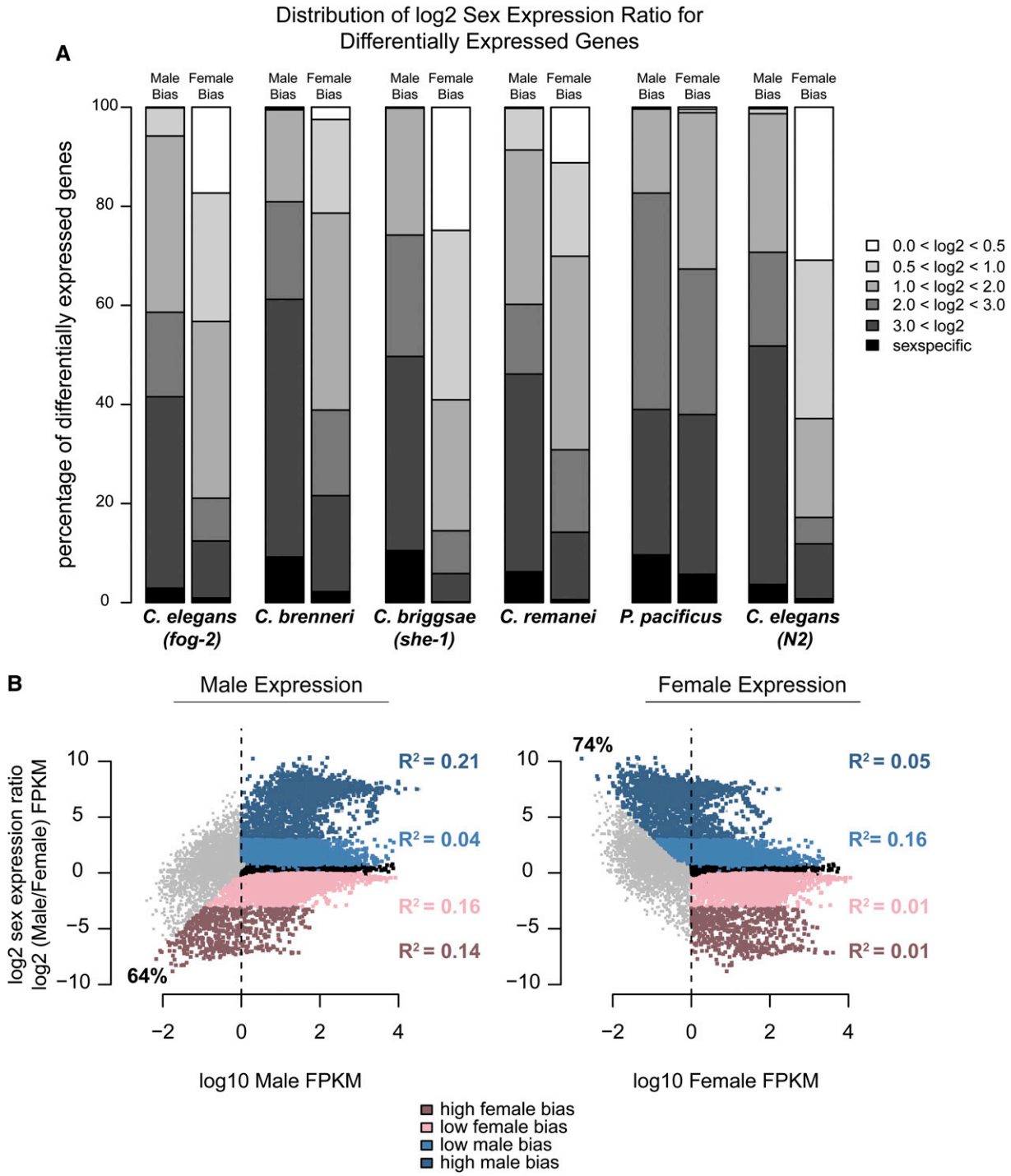

Figure 2 Distribution of the magnitude of sex bias ($\log_2$ sex expression ratio) is different for male- and female-biased genes. (A) Differential expression was determined using DESeq, *q*-value <0.05. Only genes with FPKM >1 in at least one sex were considered. Male and female expression was determined using cufflinks and reported as FPKM. Magnitude of sex bias was determined by taking the $\log_2$ of the sex expression ratio. For each category, male or female, genes were binned by magnitude of bias. Percentage of differentially expressed genes contained within each bin is plotted on the *y*-axis. (B) For *C. elegans* (*fog-2*) the magnitude of sex-biased expression is plotted against the level of expression in males (left) and females (right). Dashed line indicates FPKM = 1. Percentages of high-sex-biased genes with low expression (FPKM <1) in the opposite sex are indicated. Nonbiased genes are plotted in black. Male-biased genes are plotted in blue; female-biased genes are plotted in pink. Darker colors indicate high magnitude of bias. R^2 values between magnitude of bias and expression levels are indicated for each category.

and hermaphrodites. This was fewer than the number of sex-biased genes identified using *fog-2* females (11,753) but substantially more than the number of biased genes identified in our *P. pacificus* analysis (2241). We concluded that the reduced number of sex-biased genes identified in the *P. pacificus* genome was not entirely due to the use of hermaphrodite worms.

We also noted that our *P. pacificus* data sets contained fewer mRNA-seq reads compared to *C. elegans (fog-2)*. We randomly resampled the *C. elegans (fog-2)* reads to match *P. pacificus* and reanalyzed the distribution of biased genes. After resampling, we identified 11,484 sex-biased genes, slightly less than 11,753 genes identified before resampling (Figure S3C). The overlap of sex-biased genes was high: 5733 (97%) male-biased genes and 5555 (95%) female-biased genes were reidentified upon resampling (Figure S3, D and E). Thus, the reduced number of *P. pacificus* sex-biased genes was not a consequence of having fewer sequencing reads.

Gonad-specific genes show higher sex-biased expression

Previous work in *Drosophila* indicated that gonadal tissues account for most of the sex-biased expression, both in number of genes that are differentially expressed between the sexes and in magnitude of bias (Parisi *et al.* 2004). Using previously defined gene sets with somatic and gonadal expression in *C. elegans* (Spencer *et al.* 2011), we found that sex-biased genes expressed in the soma have a lower magnitude of bias compared to sex-biased genes expressed in the gonad (Figure 3C). Genes with low sex-biased expression are composed of both gonad and soma-expressed genes. Highly sex-biased genes are largely gonad specific (Figure S4A).

In contrast to *C. elegans*, we observed that the majority of *P. pacificus* sex-biased genes had a $\log_2$ sex expression ratio <3 (Figure 3D). We can think of two possible explanations for this. First, it could be that *Pristionchus* gonads are less sexually dimorphic than those of *Caenorhabditis*. Alternatively, a delay in the timing of gonad development in *Pristionchus* young adults (compared to the *Caenorhabditis* species) could mean that our *P. pacificus* collections contained young adults without full gonadal expression. In *C. elegans*, sperm production begins just prior to the L4/adult molt and hermaphrodites switch to oocyte production within the first 2 hr of young adulthood (Kimble 1981; Kimble and White 1981). In contrast, the first *P. pacificus* sperm are produced after the J4/adult molt. Hermaphrodite oogenesis does not switch on until 4 to 6 hr after this final molt (Rudel *et al.* 2005); thus young adults might not yet have full gonadal expression. Further experiments using older hermaphrodites are required to distinguish these two possibilities.

Demasculinization and feminization of the X chromosome

Genes with sex-biased expression tend to have nonrandom chromosomal distribution (Saifi and Chandra 1999; Wang *et al.* 2001; Khil *et al.* 2004; Lercher *et al.* 2003; Parisi *et al.* 2003; Ranz *et al.* 2003; Parisi *et al.* 2004; Reinke *et al.* 2004; Divina *et al.* 2005; Yang *et al.* 2006; Sturgill *et al.* 2007; Meisel *et al.* 2012a; Allen *et al.* 2013). Microarray analysis of gene expression in *C. elegans* has shown the X chromosome to be both feminized (enriched for genes with female-biased expression) and demasculinized (depleted of genes with male-biased expression) (Reinke *et al.* 2004). Consistent with this previous work, we found that only 4% (99/2317) of high male-biased genes are on the X chromosome in *C. elegans (fog-2)*. This is significantly less than expected by chance (14% of all *C. elegans* genes are on the X chromosome, *P*-value $<$ 1*e*–40 by Fisher test). Conversely, 23% (139/604) of the high female-biased genes are X linked, significantly more than expected (*P*-value $<$3.2 *e*–7 by Fisher test).

Figure 4A plots the percentage of genes on the X chromosome that show sex-biased expression for each of the five nematode species. In the *Caenorhabditis* species, highly sex-biased genes reveal a pattern of X chromosome demasculinization and feminization. Because those genes with high sex-biased expression are mainly expressed from the gonad, depletion of high male-biased genes from the X chromosome may be due to MSCI in the male germline.

Genes with low male-biased expression are enriched on the *Caenorhabditis* X chromosome. For *C. elegans (fog-2)*, 19% (681/3495) of genes with low male-biased expression are on the X (significant enrichment over the 14% expected, *P*-value $<$4.28 *e*–13 by Fisher test,), consistent with the predicted X accumulation of sex-biased genes (Rice 1984). However, only 8% (430/5199) of genes with low female-biased expression are on the X, indicating a surprising depletion (*P*-value $<$6.9 *e*–24). One explanation for this observation is that the female benefit for these low-biased genes is not large enough to drive X accumulation. Female-biased genes are predicted to accumulate on the X chromosome only if the fitness cost to males is minimal. It could be that the fitness cost of having a single copy of these genes in males is greater than the fitness benefit to females.

Unlike high-male-biased genes, male-specific genes are not particularly depleted from the X chromosome (Figure 4A), nor is their expressions specific to the gonad (Figure 4A). In addition, expression levels of sex-specific genes are, on average, lower than those of sex-biased genes (Figure 4B) This suggests that these genes may be expressed only in a small number of sex-specific somatic cells. For example, among the set of identified *C. elegans* male-specific genes is *ceh-7* (FPKM = 1.6), which encodes for a homeodomain transcription factor that is transcribed in cells around the rectum of the male tail (Kagoshima *et al.* 1999). The observation that male-specific genes with low somatic expression are not depleted from the X chromosome suggests that X demasculinization is largely due to highly expressed male-biased genes avoiding MSCI.

We observed the X chromosome of *P. pacificus* to be significantly enriched for both male and female highly biased genes. In *C. elegans*, gonad-specific genes tend to be autosomal whereas somatic genes tend to be X linked (Figure S4B) (Reinke *et al.* 2000; Spencer *et al.* 2011). The enrichment of both male and female-biased genes on the *Pristionchus* X might be due either to somatic bias in the data or to differences in regulation of X transcription between *Pristionchus* and *Caenorhabditis*.

Magnitude of sex bias differs between X and autosomes and between the sexes

We found that the magnitude of sex bias depends both on chromosomal location as well as on sex. For high-male-biased

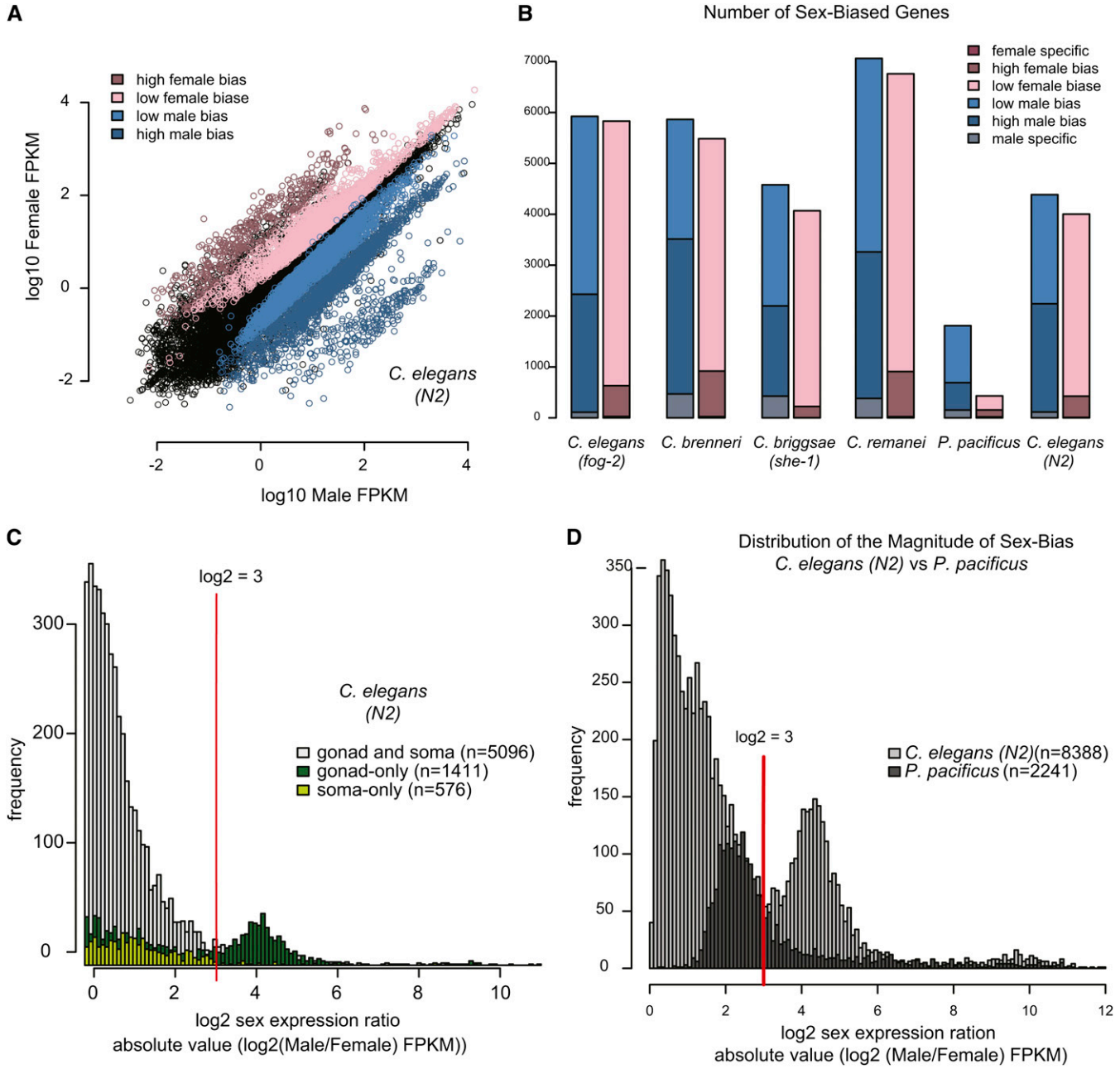

Figure 3 Majority of sex-biased genes are male biased. High magnitude of sex bias is linked to gonadal expression. (A) For *C. elegans (N2)*, male (*x*-axis) and female (*y*-axis) expression values ($\log_{10}$ FPKM) are plotted. (B) There is a greater tendency toward male bias in all five species. Number of genes with male (blue) and female (pink) biased expression is plotted for each species. (C) Genes expressed in the gonad show higher magnitude of sex-biased expression. Lists of genes with gonadal and somatic expression were taken from Spencer *et al.* (2011). Histogram shows the frequency of the magnitude of sex bias for gonad-only (dark green), soma-only (light green), and gonad and soma-expressed (gray) genes. Number of genes in each category is indicated. Red line indicates $\log_2$ expression ratio of 3. (D) Distribution of magnitude of sex bias for *P. pacificus* and *C. elegans* YA worms. Absolute value of the $\log_2$ sex expression ratio was calculated for all sex-biased genes. Histogram shows the frequency of the magnitude of sex bias for *C. elegans* (light gray) and *P. pacificus* (dark gray).

genes, the magnitude of sex bias is greater when the gene is autosomal (Figure 4C). Notably, in the two male/hermaphrodite data sets (*P. pacificus* and *C. elegans*, *N2*), the magnitude of male bias is similar for X and autosomal genes, suggesting that sperm production in hermaphrodites reduces the observable magnitude of autosomal male-biased expression. Overall, our analyses indicate that *Caenorhabditis* male-biased genes on the X chromosome tend to be more weakly biased than those located on the autosomes.

The opposite trend is true for high-female-biased genes (Figure 4D). Magnitude of female bias is greatest for those genes that are X linked. The overall trends were the same when we included all biased genes in the analysis (Figure S4, C and D). In summary, we found the nematode X chromosome to be

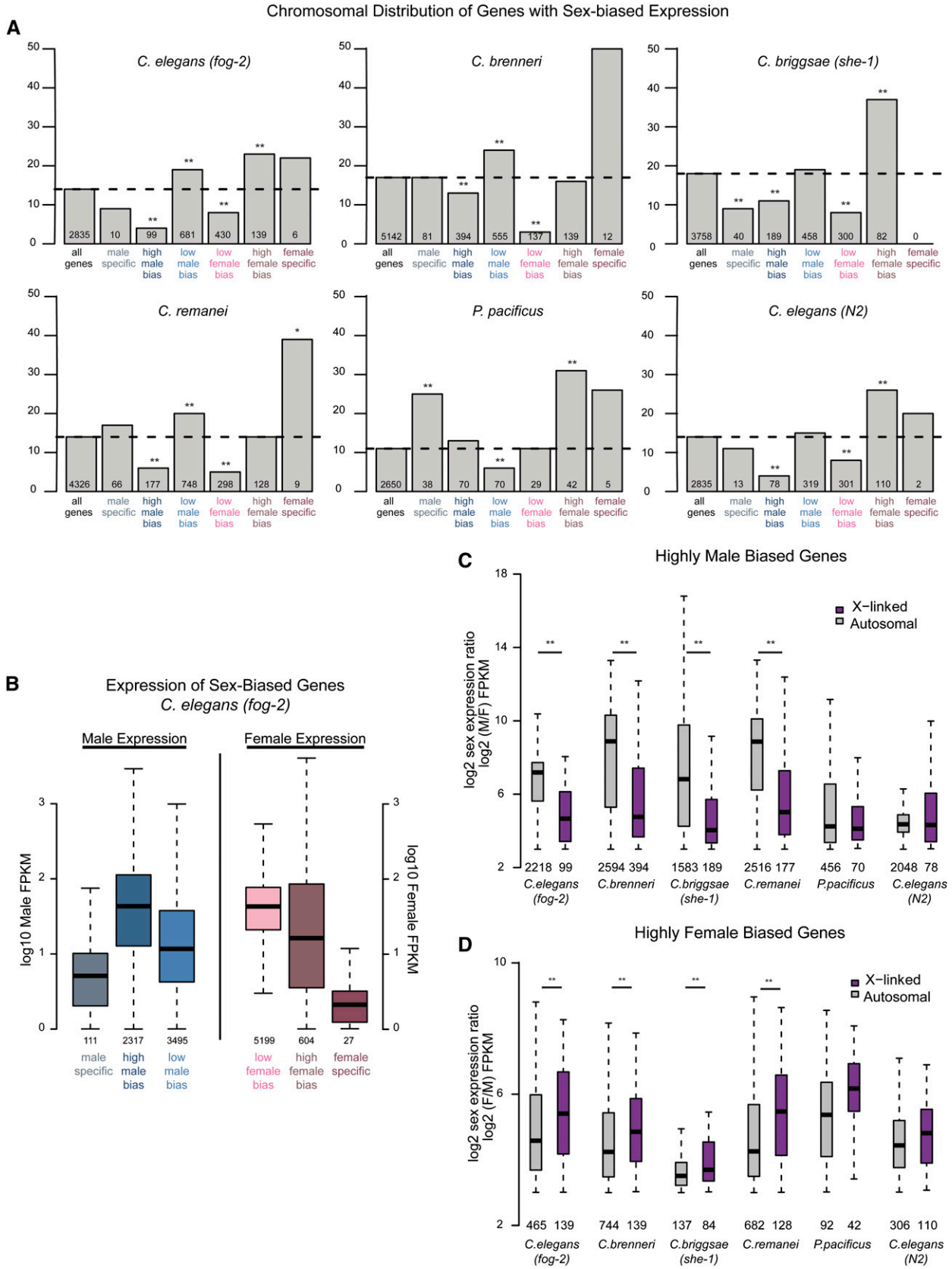

Figure 4 Sex-biased genes are nonrandomly distributed between the X and autosomes. (A) Genes for each species were placed into one of seven categories: all genes, male-specific, high male bias, low male bias, low female bias, high female bias, or female specific (see *Materials and Methods*). For each category, percentage of X-linked genes is plotted. Number of X-linked genes is indicated below each bar. Significance of enrichment or depletion

generally demasculinized and feminized with respect to both gene content and magnitude of bias.

Hermaphroditism and the preferential loss of autosomal male-biased genes

Gonochoristic species *C. remanei* and *C. brenneri* have substantially larger genomes than the hermaphroditic *C. elegans* and *C. briggsae* (Barriere *et al.* 2009). Hermaphrodite genome shrinking may be linked to a phenomenon whereby autosomes carrying large deletions preferentially segregate with the X chromosome during male meiosis (Wang *et al.* 2010). Previous work has also indicated that genes with high-sex-biased expression are preferentially lost in hermaphrodite lineages (Thomas *et al.* 2012). We wanted to see if there was a difference between X and autosomes with respect to this gene loss. To do so, we generated a list of gonochoristic-specific orthologs composed of genes that have a one-to-one ortholog in *C. brenneri*, *C. remanei*, and a third gonochorite, *C. japonica*, but lack an ortholog in both *C. elegans* and *C. briggsae* (Table S4). Similar to the previous report (Thomas *et al.* 2012), we found the set of gonochoristic-specific orthologs to be significantly enriched for genes with high male bias (Figure 5A). We also observed the set to be significantly depleted of X-linked genes, indicating that genes are preferentially lost from autosomes in the self-fertile species.

Because high-male-biased genes are significantly underrepresented on the X chromosome, we tested whether the tendency toward autosomal gene loss was simply a consequence of losing male-biased genes or if it reflected a true evolutionary difference between the X and autosomes. Among the high-male-biased genes, those that are gonochoristic specific are underrepresented on the X chromosome (Figure 5B). For example, in *C. remanei*, 6% of high-male-biased genes are on the X (177/2878), but only 2% (1 of 55) of male-biased gonochoristic-specific genes are on the X chromosome (Figure 5B). This result indicates that high-male-biased genes located on the X chromosome are better conserved among *Caenorhabditis* species, as further discussed below.

Conservation of chromosomal location and sex bias of one-to-one orthologs

Previous work comparing the *C. elegans* and *C. briggsae* genomes indicated substantial conservation of macrosynteny between the two organisms (Hillier *et al.* 2007). Overall, 95% of one-to-one orthologs are located on the same autosome and 97% on the X chromosome in both species. To understand how evolutionary pressures that maintain macrosynteny of sex-biased genes differ between the X and autosomes, we compared the conservation of chromosomal location for genes with male, female, and nonbiased expression. From orthologous gene data downloaded from WormBase, we extracted a set of 5383 1:1:1:1 orthologs between the four *Caenorhabditis* species (Table S5). We limited analysis to *Caenorhabditis* species because inclusion of evolutionarily distant *P. pacificus* to generate a 1:1:1:1:1 ortholog list greatly reduced the number of comparable orthologs. For male, female, and nonbiased genes, we determined the percentage of genes that had both a 1:1:1:1 ortholog and that also retained chromosomal location (X or autosome) across all four species (Figure 6A). For *C. elegans* and *C. briggsae*, we could compare each chromosome separately (Figure S4E). A high percentage indicates that most genes in the category are in single copy and remained on the X or autosome in all four *Caenorhabditis* species.

This analysis led to two main observations. First, genes on the X chromosome are better conserved compared to autosomal genes (Figure 6A, compare left to right). In *C. elegans* (*N2*), there are 2835 X-linked genes. Among these, 1423 (50.2%) have an X-linked 1:1:1:1 ortholog in all four *Caenorhabditis* species. Conversely, among the 17642 autosomal genes, only 3797 (21.5%) have an autosomal 1:1:1:1 ortholog in the other species. Because one-to-one orthologs usually have the same functional role in both species (Altenhoff and Dessimoz 2009; Verster *et al.* 2014), the observed high percentage of genes retaining X-linked single copy across the four *Caenorhabditis* species highlights the importance of conservation of X-linked genes. Second, genes with male-biased expression have higher incidence of conservation on the X chromosome than genes with high female bias. This observation agrees with a previous observation that male-related genes on the X have significantly lower rates of protein evolution compared to those on the autosomes (Cutter and Ward 2005). Lower conservation of female-biased genes on the X chromosome (with respect to both chromosomal location and copy number) may be due to greater functional pleiotropy of female-biased genes compared to male-biased genes (Zhang *et al.* 2007; Mank *et al.* 2008b; Mank and Ellegren 2009; Meiklejohn and Presgraves 2012). If an X-linked female-biased gene has a non-sex-biased role in many different tissues, there may be pressure to undergo gene duplication to allow one copy to carry out the sex-biased function and the other the pleiotropic functions (Maciejowski *et al.* 2005).

Better conservation of male-biased gene expression on the nematode X chromosomes

We next compared conservation of sex-biased gene expression between *C. elegans* (*fog-2*) and each of the other four nematode species. Among the 742 high male-biased genes in *C. elegans* (*fog*-2) that have a one-to-one ortholog in *C. briggsae*,

was calculated using Fisher test: (*)*P*-value <0.05; (**)*P*-value <0.001. (B) For *C. elegans (fog-2)*, expression levels of sex-biased genes in each category are plotted. Male expression is plotted for male-biased genes (left three boxes) and female expression is plotted for female-biased genes (right three boxes). (C) Magnitude of male-biased expression (log_2 male over female expression) was calculated for each high-male-biased gene. X-linked genes are plotted in purple; autosomal genes are plotted in gray. Number of genes analyzed is indicated below each box. High-male-biased genes located on autosomes showed significantly greater magnitude of sex bias compared to those located on the X chromosome as calculated by *t*-test: (*)*P*-value <0.01. (D) As in C, magnitude of female-biased expression (log_2 female over male expression) was calculated for each high female-biased gene.

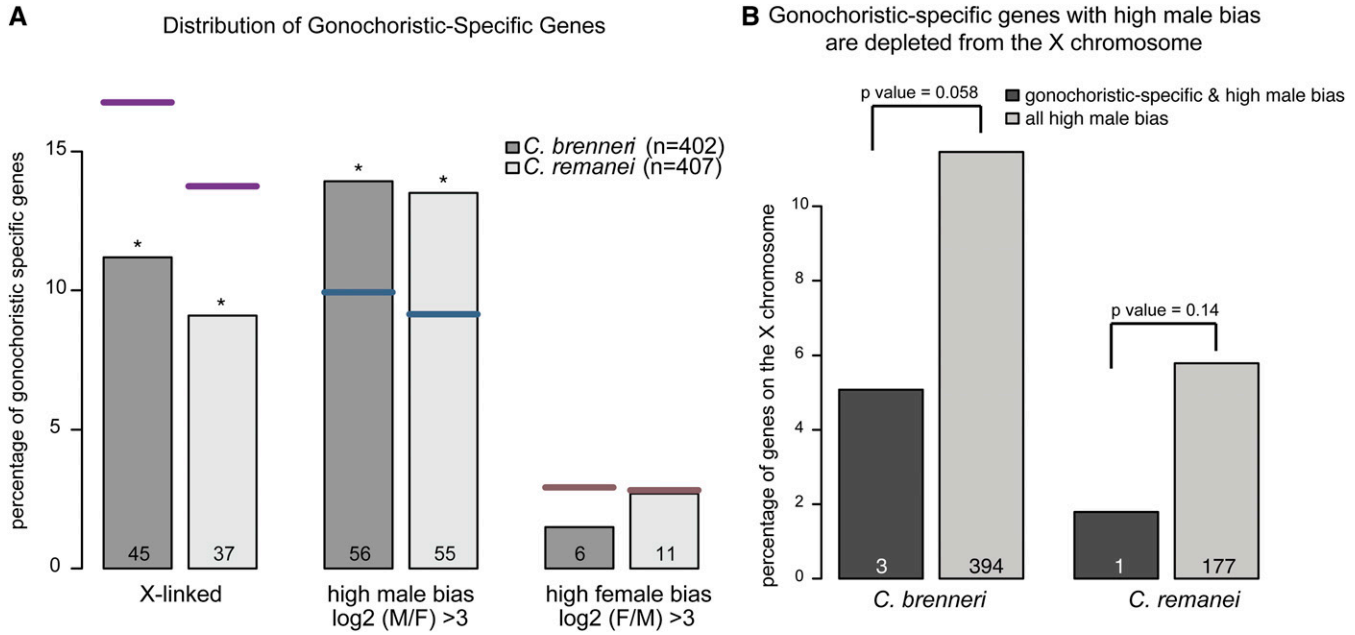

Figure 5 Evolution of hermaphroditism is linked to the preferential loss of autosomal-linked highly male-biased genes. (A) Gonochoristic-specific genes are enriched for genes with high male bias. Gonochoristic-specific genes were identified by taking the overlap of genes that had an ortholog in each of three gonochoristic species (*C. brenneri*, *C. remanei*, and *C. japonica*), but lacked an ortholog in two hermaphroditic species (*C. elegans* and *C. briggsae*). For *C. brenneri* (dark gray) and *C. remanei* (light gray), the percentage of gonochoristic-specific genes that are X linked, show high male bias, or show high female bias is plotted. The number of genes identified in each category is indicated in each bar. Significance of enrichment or depletion was calculated using Fisher test: (*)*P*-value <0.05; (**)*P*-value <0.001. From left to right: purple, blue, and pink lines demark the genome-wide percentage of X-linked, high-male-biased, and high-female-biased genes for *C. brenneri* and *C. remanei* respectively. (B) Gonochoristic-specific genes with high male bias are underrepresented on the X chromosome. Plotted is the percentage of high male-bias genes that are X linked. Gonochoristic-specific genes are plotted in dark gray. Numbers below each bar indicates the number of genes that are in each category. Significance of depletion was calculated using Fisher test.

474 (64%) also show high male bias in *C. briggsae* (Figure S5A). Conversely, only 32 of 164 (19.5%) *C. elegans (fog-2)* high female-biased orthologs also show high female-biased expression in *C. briggsae*. For orthologs with low-sex-biased expression, conservation is higher for female-biased genes.

Sex-biased genes often show greater amino acid sequence divergence than nonbiased genes (reviewed in Ellegren and Parsch 2007). This relationship between sex bias and the rate of molecular evolution has been observed in *Drosophila*, *C. elegans*, and mammals (Torgerson *et al.* 2002; Cutter and Ward 2005; Khaitovich *et al.* 2005; Richards *et al.* 2005). Although most of the previous studies focused on molecular evolution of sex-biased genes, recent studies in *Drosophila* have also indicated higher divergence of expression for male-biased genes, particularly for those located on the X chromosome (Meiklejohn *et al.* 2003; Llopart 2012).

To analyze expression divergence of sex-biased genes, we calculated the coefficient of variation (σ/μ) between species for each *Caenorhabditis* 1:1:1:1 ortholog. Unlike in the *Drosophila* studies, we did not observe higher divergence of male-biased gene expression (Figure S5B). Instead, we found that male-biased genes tend to have higher expression divergence in females, and female-biased genes have higher expression divergence in males. This suggests that sex-biased genes experience high selective pressure in the opposite sex (Jiang and Machado 2009).

Evaluation of the faster-X hypothesis based on gene expression

As it is present in only one copy in males, the X chromosome has a smaller effective population size compared to the autosomes, leaving it more susceptible to genetic drift (Avery 1984). This, in combination with the unique selective pressures acting on the X in males and females, led to the prediction that adaptive evolution may proceed faster on the X chromosome [the faster X hypothesis (Charlesworth *et al.* 1987; Wu and Davis 1993; Orr 1997; Turelli 1998)]. This hypothesis is supported by observations of higher rates of protein evolution for X-linked genes in both mammals and *Drosophila* (Thornton and Long 2002; Counterman *et al.* 2004; Lu and Wu 2005; Mikkelsen *et al.* 2005; Baines *et al.* 2008; Hvilsom *et al.* 2012; Kousathanas *et al.* 2013; Hu *et al.* 2013). Notably, no such faster X evolution was observed when comparing nonsynonymous substitution rates between *C. elegans* and *C. briggsae* (Artieri *et al.* 2008). However, adaptive changes in the genome frequently occur in *cis*-regulatory regions (Wittkopp *et al.* 2004; Schaefke *et al.* 2013) resulting in interspecies expression differences. Recent studies of gene expression evolution in *Drosophila* and mammals have found evidence of faster-X evolution in the form of greater divergence of X-linked expression across species (Brawand *et al.* 2011; Kayserili *et al.* 2012; Meisel *et al.* 2012b).

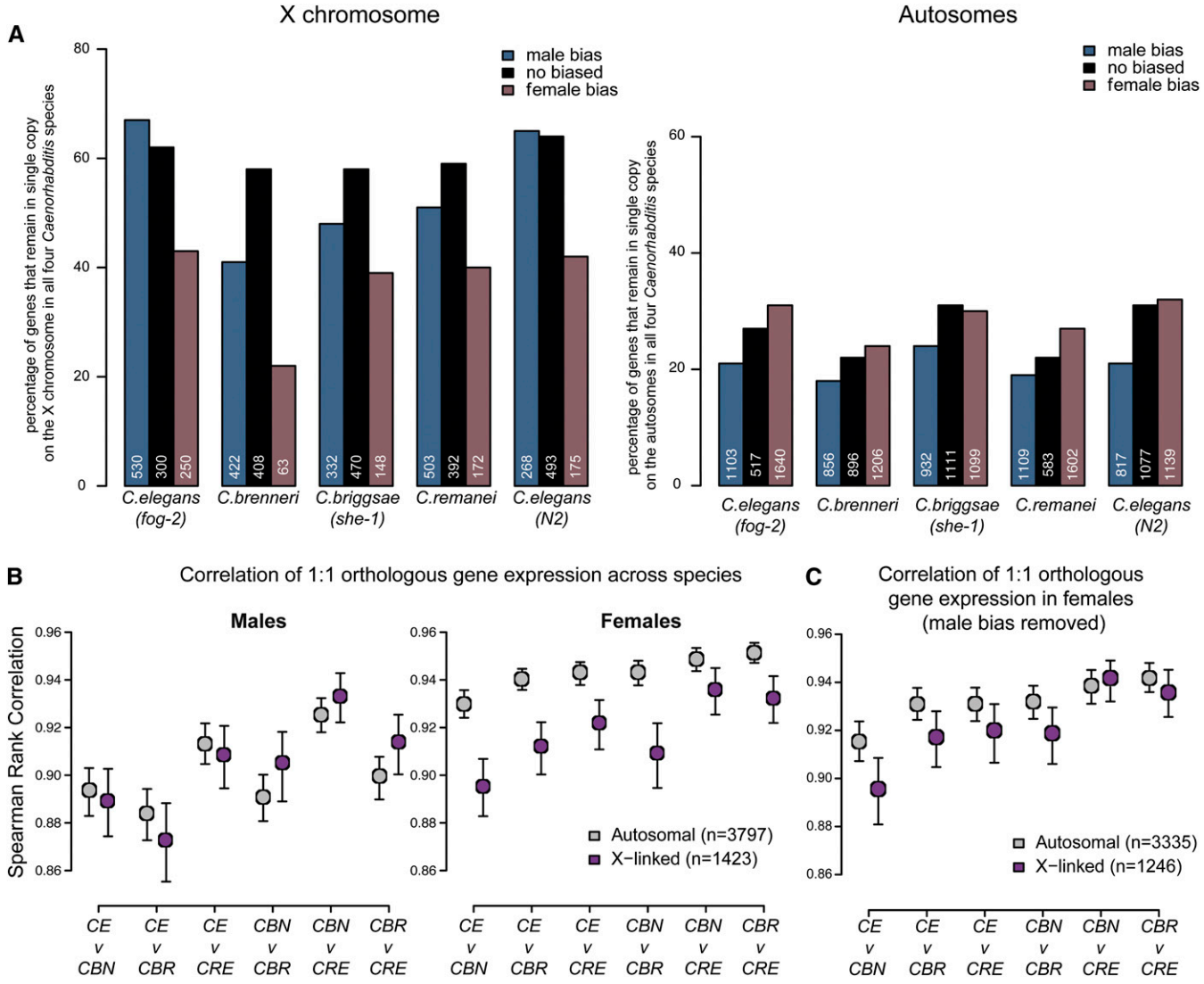

Figure 6 Conservation of orthologous gene expression across species. (A) On the X, male-biased genes are more likely to be conserved than female-biased genes. For each species, genes were divided into three categories: male-biased (blue), female-biased (purple), and nonbiased (black). Within each category, we identified 1:1:1:1 *Caenorhabditis* orthologs that are located on the same chromosome (X, left) or autosome (right) in all four species. X-linked genes have a higher tendency than autosomal genes to remain on the same chromosome and in single copy. Similarly, X-linked male-biased genes show greater tendency to remain in single copy compared to X-linked female-biased genes. (B) Correlation of 1:1 orthologous gene expression between *Caenorhabditis* indicates that X-linked expression is evolving faster in females. Between any two species, Spearman rank correlation of 1:1 orthologous gene expression was plotted for males (left) and females (right). As determined by bootstrapping, 95% confidence intervals are indicated. (CE, *C. elegans*; CBN, *C. brenneri*; CBR, *C. briggsae*; CRE, *C. remanei*). (C) High male-biased genes are removed from correlation analysis shown in B, right.

To evaluate faster-X evolution of gene expression in nematodes, we compared expression correlation of 1:1:1:1 orthologs between the four *Caenorhabditis* species. Between any two *Caenorhabditis* species, we calculated the Spearman rank correlation coefficient (as in Brawand *et al.* 2011 and Meisel *et al.* 2012b). In males, expression levels of orthologs on the X and autosomes are similarly correlated across species (Figure 6B, left). In females, X-linked ortholog expression is less correlated than autosomal ortholog expression (Figure 6B right, *P*-value = 0.00046, by paired *t*-test: Figure S5B). This result suggests that female gene expression diverges faster on the X compared to the autosomes.

Lower correlation of X expression in females may be due to differences in dosage compensation across species. Although orthologs of *C. elegans* dosage compensation complex subunits have been identified in the three other *Caenorhabditis* species, female X expression divergence may be due to differences in DCC binding or downstream effectors. It is worth noting that male-biased genes located on the X chromosome are largely responsible for the observed female expression divergence. When we removed high-male-biased genes from analysis, female X expression correlation between species resembled autosomal correlation (Figure 6C), consistent with the observation that male-biased genes

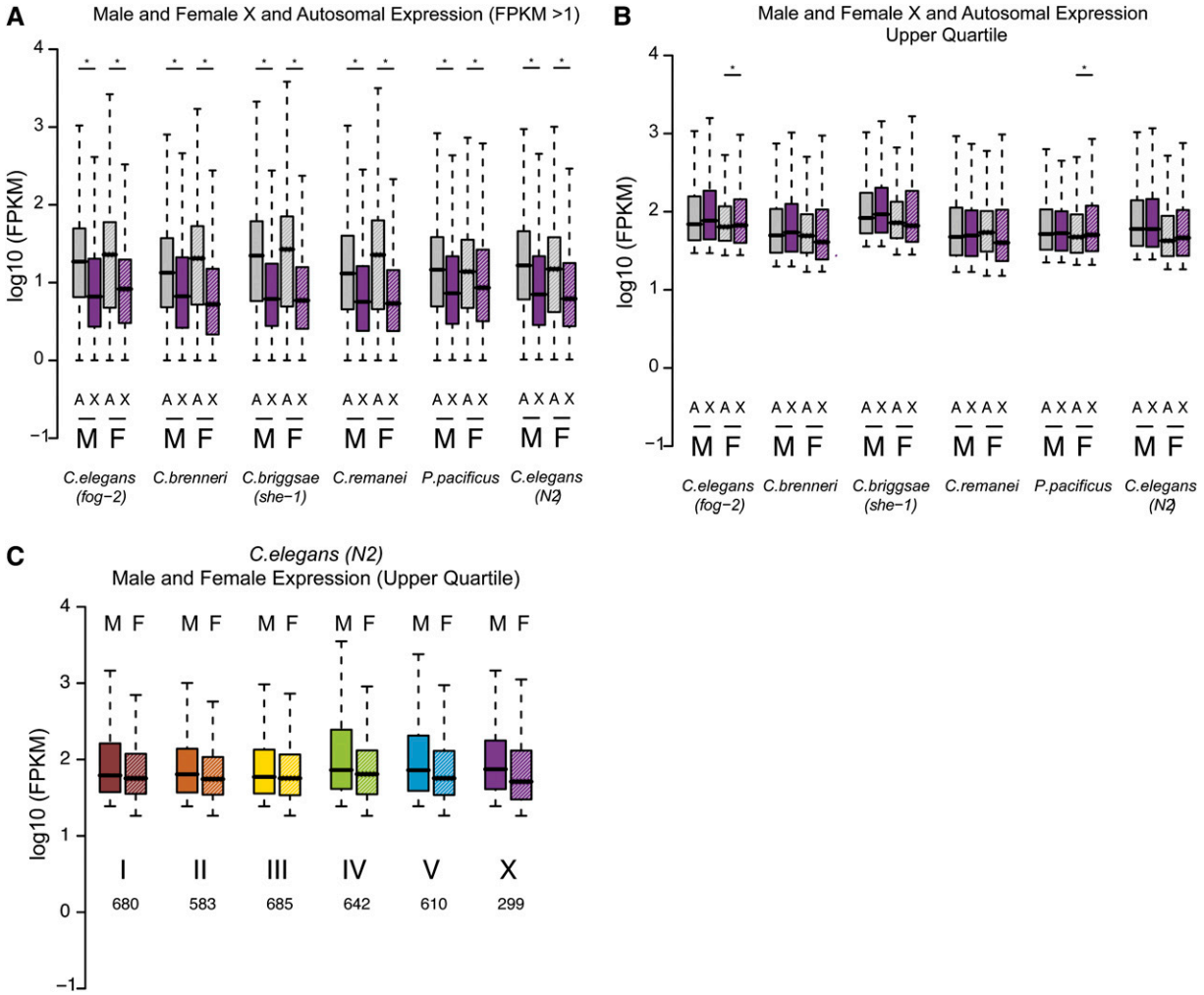

Figure 7 Analysis of overall expression levels from the X chromosome and autosomes. (A) Expression level of genes with FPKM >1 is plotted. X-linked genes are shown in purple. Autosomal genes are in gray. Male expression data are plotted in solid bars. Female data are in dashed bars. As measured in young adults, X expression is significantly lower than autosomal expression. (*)*P*-value <0.01 by *t*-test. (B) Genes whose expression is in the upper quartile in both males and females are plotted (at least 225 X-linked and 2890 autosomal-linked genes). X and autosomal expression is similar except in *C. brenneri* and *C. remanei*, where female X expression is significantly lower than autosomal expression. (*)*P*-value <0.01 by *t*-test. (C) *C. elegans (fog-2)* expression values plotted for each chromosome separately. Analysis is limited to the upper quartile genes. Number of genes analyzed is indicated below each bar.

show high levels of expression divergence in females (Figure S5B).

Effect of X localization on gene expression

Ohno's hypothesis and the extent of X upregulation are currently being debated in the field. The unique gene content of the X chromosome makes it difficult to compare X and autosomal transcription (Deng *et al.* 2011). In *C. elegans*, analysis is further complicated by germline repression of the X chromosome in young adult worms, which contain almost twice as many germline cells than somatic cells. As our data come from young adult worms, X chromosome repression in the germline explains the observation that overall X expression is lower than autosomal expression (Figure 7A). In support of this explanation, X and autosomal expression levels are similar in *C. elegans* worms lacking a germline (Deng *et al.* 2011). To remove those genes whose expression may be repressed in the germline, we limited analysis to only highly expressed genes (upper quartile of expression values). In agreement with previous observations (Deng *et al.* 2011), overall transcript levels of highly expressed genes are similar between X and autosomes in males and females (Figure 7B) and for each chromosome in *C. elegans* (Figure 7C).

To more directly test if the X chromosome is upregulated in males during the course of X evolution, we compared expression of one-to-one orthologs located on the autosome in one species and the X chromosome in another. Unfortunately, as reported above, macrosynteny among the *Caenorhabditis* species is high. Between *C. elegans* and each of the other three *Caenorhabditis* species, <50 genes have changed chromosomal location between X and autosomes (Figure S4F). However, owing to a large chromosomal translocation event that

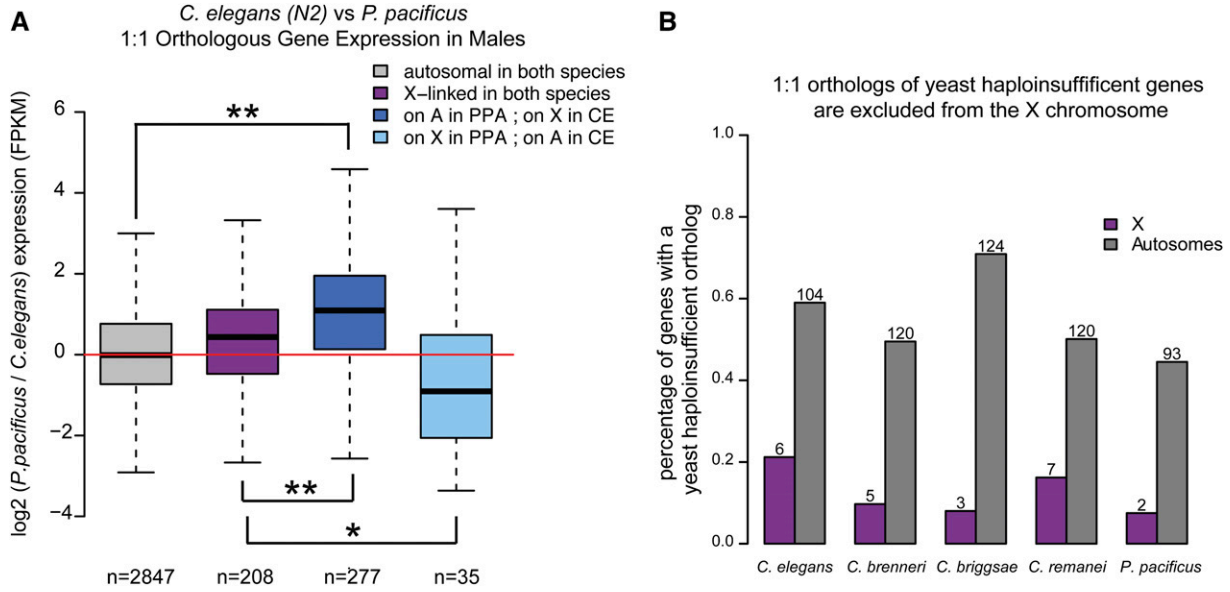

Figure 8 Monosomy of the male X results in lower expression of X-linked orthologs in males. (A) Comparison of gene expression between *C. elegans* and *P. pacificus* indicates higher expression of the autosomal ortholog. Log_2 male expression ratio (*P. pacificus*/*C. elegans*) is plotted for four groups of genes: autosomal in both species (gray), X-linked in both species (purple), autosomal in *P. pacificus* and X in *C. elegans* (dark blue), X in *P. pacificus* and autosomal in *C. elegans* (light blue). Number of genes in each category is indicated below each bar. (B) Haplo-insufficient genes are excluded from the X chromosome. Orthologs of yeast haplo-insifficient genes were identified in each of the five species (Table S7). The percentage of orthologs that are on X (purple) and autosomes (gray) is plotted. Numbers above each bar indicate the number of haplo-insufficient 1:1 orthologs identified.

occurred after their split, 466 genes have moved between X and autosomes in *C. elegans* and *P. pacificus*. Of these orthologs, 317 are expressed (FPKM >1) in the males of both species, which allowed us to analyze the effect of X and autosomal localization on gene expression. If the single male X chromosome is upregulated, there should be no difference in gene expression when the ortholog is differentially located on X and autosomes. To reduce any effects of sex bias on analysis of dosage compensation, we removed all genes with high sex-biased expression in either species. One-to-one orthologs that are similarly located (on either the X chromosome or on an autosome) are similarly expressed in both species (Figure 8A). For orthologs that are differentially located, expression is higher in the species where the ortholog is on an autosome. This result suggests that expression of the X-linked orthologs is not upregulated to match autosomal expression.

Orthologs of yeast haplo-insufficient genes are excluded from the X chromosome

If X upregulation does not compensate for the single X in males, then selective pressure should move dosage-sensitive genes off of the X chromosome. Haplo-insufficient genes are dosage sensitive. A reduction in their gene copy number from two to one results in a significant reduction of overall fitness. Haplo-insufficient genes have been well characterized in *Saccharomyces cerevisae* and can be used to predict haplo-insufficiency in other metazoan species (de Clare *et al.* 2011). Orthologs of yeast haplo-insufficient genes are excluded from the X chromosome in humans and *C. elegans* (de Clare *et al.* 2011). Here, we found that this trend holds for the four other nematode species (Figure 8B).

Previous studies have noted two additional differences in the organization of gene content between the X chromosome and the autosomes in *C. elegans*. First, genes essential for early embryonic development are underrepresented on the X chromosome (Piano *et al.* 2000). Second, ~15% of all *C. elegans* genes are expressed within operons, which are underrepresented on the X chromosome (Blumenthal *et al.* 2002). Underrepresentation of operons on the X is also observed in *C. briggsae* (Uyar *et al.* 2012).

Our analysis of X chromosome gene content with respect to sex-biased gene expression supports a general trend whereby the nematode X chromosome is enriched for female-biased genes and depleted of male-biased genes. This observation holds for highly sex-biased genes, which are mainly expressed in the gonad, but not for genes with low-sex-biased expression, which are expressed in both the soma and the gonad. In the male germline the X chromosome is silenced, potentially driving movement of high-male-biased genes from the X and resulting in the observed demasculinization. In the soma, X chromosome transcription is more subtly regulated by dosage compensation mechanisms that do not restrain the accumulation of male-biased genes on the X. Since the distribution of sex-biased genes is generally conserved between the five nematode species, regulation of X chromosome transcription in the germline and soma may also be conserved. However, we noted differences in sex-biased gene expression and X chromosome content between *C. elegans* and *P. pacificus*, as well as a lower correlation of X chromosome gene expression in females between the four *Caenorhabditis* species. These observations suggest the existence of species-specific differences in the mechanisms that regulate X chromosome

transcription. Our analyses indicate that monosomy of the X chromosome is an important player in shaping X chromosome gene content. While similarity of overall transcript levels from the X and autosomes support X chromosome upregulation, a shortage of haplo-insufficient genes and lower expression of differentially located X-linked orthologs suggests that potential mechanisms of X chromosome upregulation do not act on every X-linked gene.

Acknowledgments

We thank Karin Kiontke for an initial discussion on this project and Mark Siegal for comments on the manuscript. We thank New York University—Center for Genomics and Systems Biology High Throughput Sequencing Facility for sequencing and raw data processing. Some strains were provided by the Caenorhabditis Genetics Center (CGC), which is funded by National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). S.E. and S.E.A. conceived and designed the experiments. S.E.A., P.R., and M.K. performed the experiments. S.E.A., A.K., and C.D. analyzed the data. S.E. and S.E.A. wrote the manuscript.

Literature Cited

Adler, D. A., E. I. Rugarli, P. A. Lingenfelter, K. Tsuchiya, D. Poslinski *et al.*, 1997 Evidence of evolutionary up-regulation of the single active X chromosome in mammals based on Clc4 expression levels in *Mus spretus* and *Mus musculus*. Proc. Natl. Acad. Sci. USA 94: 9244–9248.

Allen, S. L., R. Bonduriansky, and S. F. Chenoweth, 2013 The genomic distribution of sex-biased genes in Drosophila serrata: X chromosome demasculinization, feminization, and hyperexpression in both sexes. Genome Biol. Evol. 5: 1986–1994.

Altenhoff, A. M., and C. Dessimoz, 2009 Phylogenetic and functional assessment of orthologs inference projects and methods. PLOS Comput. Biol. 5: e1000262.

Anders, S., and W. Huber, 2010 Differential expression analysis for sequence count data. Genome Biol. 11: R106.

Arnqvist, G., 2004 Sexual conflict and sexual selection: lost in the chase. Evolution 58: 1383–1393.

Artieri, C. G., W. Haerty, B. P. Gupta, and R. S. Singh, 2008 Sexual selection and maintenance of sex: evidence from comparisons of rates of genomic accumulation of mutations and divergence of sex-related genes in sexual and hermaphroditic species of Caenorhabditis. Mol. Biol. Evol. 25: 972–979.

Assis, R., Q. Zhou, and D. Bachtrog, 2012 Sex-biased transcriptome evolution in Drosophila. Genome Biol. Evol. 4: 1189–1200.

Avery, P. J., 1984 The population-genetics of haplo-diploids and X-linked genes. Genet. Res. 44: 321–341.

Bachtrog, D., N. R. Toda, and S. Lockton, 2010 Dosage compensation and demasculinization of X chromosomes in Drosophila. Curr. Biol. 20: 1476–1481.

Baines, J. F., S. A. Sawyer, D. L. Hartl, and J. Parsch, 2008 Effects of X-linkage and sex-biased gene expression on the rate of adaptive protein evolution in Drosophila. Mol. Biol. Evol. 25: 1639–1650.

Barriere, A., S. P. Yang, E. Pekarek, C. G. Thomas, E. S. Haag *et al.*, 2009 Detecting heterozygosity in shotgun genome assemblies: lessons from obligately outcrossing nematodes. Genome Res. 19: 470–480.

Bean, C. J., C. E. Schaner, and W. G. Kelly, 2004 Meiotic pairing and imprinted X chromatin assembly in *Caenorhabditis elegans*. Nat. Genet. 36: 100–105.

Bender, L. B., J. Suh, C. R. Carroll, Y. Fong, I. M. Fingerman *et al.*, 2006 MES-4: an autosome-associated histone methyltransferase that participates in silencing the X chromosomes in the *C. elegans* germ line. Development 133: 3907–3917.

Bessler, J. B., E. C. Andersen, and A. M. Villeneuve, 2010 Differential localization and independent acquisition of the H3K9me2 and H3K9me3 chromatin modifications in the Caenorhabditis elegans adult germ line. PLoS Genet. 6: e1000830.

Blumenthal, T., D. Evans, C. D. Link, A. Guffanti, D. Lawson *et al.*, 2002 A global analysis of *Caenorhabditis elegans* operons. Nature 417: 851–854.

Brawand, D., M. Soumillon, A. Necsulea, P. Julien, G. Csardi *et al.*, 2011 The evolution of gene expression levels in mammalian organs. Nature 478: 343–348.

Charlesworth, B., J. A. Coyne, and N. H. Barton, 1987 The relative rates of evolution of sex-chromosomes and autosomes. Am. Nat. 130: 113–146.

Charlesworth, D., B. Charlesworth, and G. Marais, 2005 Steps in the evolution of heteromorphic sex chromosomes. Heredity 95: 118–128.

Coghlan, A., and K. H. Wolfe, 2002 Fourfold faster rate of genome rearrangement in nematodes than in Drosophila. Genome Res. 12: 857–867.

Connallon, T., and L. L. Knowles, 2005 Intergenomic conflict revealed by patterns of sex-biased gene expression. Trends Genet. 21: 495–499.

Conrad, T., and A. Akhtar, 2012 Dosage compensation in *Drosophila melanogaster*: epigenetic fine-tuning of chromosome-wide transcription. Nat. Rev. Genet. 13: 123–134.

Counterman, B. A., D. Ortiz-Barrientos, and M. A. F. Noor, 2004 Using comparative genomic data to test for fast-x evolution. Evolution 58: 656–660.

Csankovszki, G., 2009 Condensin function in dosage compensation. Epigenetics 4: 212–215.

Cutter, A. D., 2008 Divergence times in Caenorhabditis and Drosophila inferred from direct estimates of the neutral mutation rate. Mol. Biol. Evol. 25: 778–786.

Cutter, A. D., and S. Ward, 2005 Sexual and temporal dynamics of molecular evolution in *C. elegans* development. Mol. Biol. Evol. 22: 178–188.

de Clare, M., P. Pir, and S. G. Oliver, 2011 Haploinsufficiency and the sex chromosomes from yeasts to humans. BMC Biol. 9: 15.

Deng, X. X., J. B. Hiatt, D. K. Nguyen, S. Ercan, D. Sturgill *et al.*, 2011 Evidence for compensatory upregulation of expressed X-linked genes in mammals, *Caenorhabditis elegans* and *Drosophila melanogaster*. Nat. Genet. 43: 1179–1185.

Dieterich, C., S. W. Clifton, L. N. Schuster, A. Chinwalla, K. Delehaunty *et al.*, 2008 The Pristionchus pacificus genome provides a unique perspective on nematode lifestyle and parasitism. Nat. Genet. 40: 1193–1198.

Dieterich, C., W. Roeseler, and J. Srinivasan, 2006 Pristionchus pacificus genomics: from genetics to genome sequence. *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.7.1, http://www.wormbook.org.

Disteche, C. M., 2012 Dosage compensation of the sex chromosomes. Annu. Rev. Genet. 46(46): 537–560.

Divina, P., C. Vlcek, P. Strnad, V. Paces, and J. Forejt, 2005 Global transcriptome analysis of the C57BL/6J mouse testis by SAGE: evidence for nonrandom gene order. BMC Genomics 6: 29.

Dupont, C., and J. Gribnau, 2013 Different flavors of X-chromosome inactivation in mammals. Curr. Opin. Cell Biol. 25: 314–321.

Ellegren, H., 2011 Sex-chromosome evolution: recent progress and the influence of male and female heterogamety. Nat. Rev. Genet. 12: 157–166.

Ellegren, H., and J. Parsch, 2007 The evolution of sex-biased genes and sex-biased gene expression. Nat. Rev. Genet. 8: 689–698.

Ercan, S., and J. D. Lieb, 2009 *C. elegans* dosage compensation: a window into mechanisms of domain-scale gene regulation. Chromosome Res. 17: 215–227.

Ferrari, F., A. A. Alekseyenko, P. J. Park, and M. I. Kuroda, 2014 Transcriptional control of a whole chromosome: emerging models for dosage compensation. Nat. Struct. Mol. Biol. 21: 118–125.

Fong, Y., L. Bender, W. Wang, and S. Strome, 2002 Regulation of the different chromatin states of autosomes and X chromosomes in the germ line of *C. elegans*. Science 296: 2235–2238.

Gao, G., M. D. Vibranovski, L. Zhang, Z. Li, M. Liu *et al.*, 2014 A long-term demasculinization of X-linked intergenic noncoding RNAs in *Drosophila melanogaster*. Genome Res. 4: 629–638.

Gaydos, L. J., A. Rechtsteiner, T. A. Egelhofer, C. R. Carroll, and S. Strome, 2012 Antagonism between MES-4 and Polycomb repressive complex 2 promotes appropriate gene expression in *C. elegans* germ cells. Cell Rep 2: 1169–1177.

Gelbart, M. E., and M. I. Kuroda, 2009 Drosophila dosage compensation: a complex voyage to the X chromosome. Development 136: 1399–1410.

Gribnau, J., and J. A. Grootegoed, 2012 Origin and evolution of X chromosome inactivation. Curr. Opin. Cell Biol. 24: 397–404.

Gupta, V., M. Parisi, D. Sturgill, R. Nuttall, M. Doctolero *et al.*, 2006 Global analysis of X-chromosome dosage compensation. J. Biol. 5: 3.

Hillier, L. W., R. D. Miller, S. E. Baird, A. Chinwalla, L. A. Fulton *et al.*, 2007 Comparison of *C. elegans* and *C. briggsae* genome sequences reveals extensive conservation of chromosome organization and synteny. PLoS Biol. 5: 1603–1616.

Hu, T. T., M. B. Eisen, K. R. Thornton, and P. Andolfatto, 2013 A second-generation assembly of the *Drosophila simulans* genome provides new insights into patterns of lineage-specific divergence. Genome Res. 23: 89–98.

Hvilsom, C., Y. Qian, T. Bataillon, Y. R. Li, T. Mailund *et al.*, 2012 Extensive X-linked adaptive evolution in central chimpanzees. Proc. Natl. Acad. Sci. USA 109: 2054–2059.

Innocenti, P., and E. H. Morrow, 2010 The sexually antagonistic genes of *Drosophila melanogaster*. PLoS Biol. 8: e1000335.

Jiang, Z. F., and C. A. Machado, 2009 Evolution of sex-dependent gene expression in three recently diverged species of Drosophila. Genetics 183: 1175–1185.

Julien, P., D. Brawand, M. Soumillon, A. Necsulea, A. Liechti *et al.*, 2012 Mechanisms and evolutionary patterns of mammalian and avian dosage compensation. PLoS Biol. 10: e1001328.

Kagoshima, H., G. Cassata, and T. R. Burglin, 1999 A *Caenorhabditis elegans* homeobox gene expressed in the male tail, a link between pattern formation and sexual dimorphism? Dev. Genes Evol. 209: 59–62.

Kayserili, M. A., D. T. Gerrard, P. Tomancak, and A. T. Kalinka, 2012 An excess of gene expression divergence on the X chromosome in Drosophila embryos: implications for the faster-x hypothesis. PLoS Genet. 8: e1003200.

Kelly, W. G., C. E. Schaner, A. F. Dernburg, M. H. Lee, S. K. Kim *et al.*, 2002 X-chromosome silencing in the germline of *C. elegans*. Development 129: 479–492.

Khaitovich, P., I. Hellmann, W. Enard, K. Nowick, M. Leinweber *et al.*, 2005 Parallel patterns of evolution in the genomes and transcriptomes of humans and chimpanzees. Science 309: 1850–1854.

Khil, P. P., N. A. Smirnova, P. J. Romanienko, and R. D. Camerini-Otero, 2004 The mouse X chromosome is enriched for sex-biased genes not subject to selection by meiotic sex chromosome inactivation. Nat. Genet. 36: 642–646.

Kimble, J., 1981 Alterations in cell lineage following laser ablation of cells in the somatic gonad of *Caenorhabditis elegans*. Dev. Biol. 87: 286–300.

Kimble, J. E., and J. G. White, 1981 On the control of germ cell development in *Caenorhabditis elegans*. Dev. Biol. 81: 208–219.

Kiontke, K., N. P. Gavin, Y. Raynes, C. Roehrig, F. Piano *et al.*, 2004 Caenorhabditis phylogeny predicts convergence of hermaphroditism and extensive intron loss. Proc. Natl. Acad. Sci. USA 101: 9003–9008.

Kiontke, K. C., M. A. Felix, M. Ailion, M. V. Rockman, C. Braendle *et al.*, 2011 A phylogeny and molecular barcodes for Caenorhabditis, with numerous new species from rotting fruits. BMC Evol. Biol. 11: 339.

Koboldt, D. C., J. Staisch, B. Thillainathan, K. Haines, S. E. Baird *et al.*, 2010 A toolkit for rapid gene mapping in the nematode *Caenorhabditis briggsae*. BMC Genomics 11: 236.

Kousathanas, A., D. L. Halligan, and P. D. Keightley, 2013 Faster-x adaptive protein evolution in house mice. Genetics 196: 1131–1143.

Kruesi, W. S., L. J. Core, C. T. Waters, J. T. Lis, and B. J. Meyer, 2013 Condensin controls recruitment of RNA polymerase II to achieve nematode X-chromosome dosage compensation. Elife 2: e00808.

Langmead, B., C. Trapnell, M. Pop, and S. L. Salzberg, 2009 Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10: R25.

Lee, K. Z., A. Eizinger, R. Nandakumar, S. C. Schuster, and R. J. Sommer, 2003 Limited microsynteny between the genomes of *Pristionchus pacificus* and *Caenorhabditis elegans*. Nucleic Acids Res. 31: 2553–2560.

Lercher, M. J., A. O. Urrutia, and L. D. Hurst, 2003 Evidence that the human X chromosome is enriched for male-specific but not female-specific genes. Mol. Biol. Evol. 20: 1113–1116.

Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan *et al.*, 2009 The sequence alignment/map format and SAMtools. Bioinformatics 25: 2078–2079.

Lin, F., K. Xing, J. Zhang, and X. He, 2012 Expression reduction in mammalian X chromosome evolution refutes Ohno's hypothesis of dosage compensation. Proc. Natl. Acad. Sci. USA 109: 11752–11757.

Lin, H., V. Gupta, M. D. VerMilyea, F. Falciani, J. T. Lee *et al.*, 2007 Dosage compensation in the mouse balances up-regulation and silencing of X-linked genes. PLoS Biol. 5: 2809–2820.

Llopart, A., 2012 The rapid evolution of X-linked male-biased gene expression and the large-X effect in *Drosophila yakuba*, *D. santomea*, and their hybrids. Mol. Biol. Evol. 29: 3873–3886.

Lu, J., and C. I. Wu, 2005 Weak selection revealed by the whole-genome comparison of the X chromosome and autosomes of human and chimpanzee. Proc. Natl. Acad. Sci. USA 102: 4063–4067.

Maciejowski, J., J. H. Ahn, P. G. Cipriani, D. J. Killian, A. L. Chaudhary *et al.*, 2005 Autosomal genes of autosomal/X-linked duplicated gene pairs and germ-line proliferation in *Caenorhabditis elegans*. Genetics 169: 1997–2011.

Maine, E. M., 2010 Meiotic silencing in *Caenorhabditis elegans*. Int. Rev. Cell Mol. Biol. 282(282): 91–134.

Mank, J. E., and H. Ellegren, 2009 Are sex-biased genes more dispensable? Biol. Lett. 5: 409–412.

Mank, J. E., L. Hultin-Rosenberg, M. T. Webster, and H. Ellegren, 2008a The unique genomic properties of sex-biased genes: insights from avian microarray data. BMC Genomics 9: 148.

Mank, J. E., L. Hultin-Rosenberg, M. Zwahlen, and H. Ellegren, 2008b Pleiotropic constraint hampers the resolution of sexual antagonism in vertebrate gene expression. Am. Nat. 171: 35–43.

Meiklejohn, C. D., and D. C. Presgraves, 2012 Little evidence for demasculinization of the drosophila x chromosome among genes expressed in the male germline. Genome Biol. Evol. 4: 1007–1016.

Meiklejohn, C. D., J. Parsch, J. M. Ranz, and D. L. Hartl, 2003 Rapid evolution of male-biased gene expression in Drosophila. Proc. Natl. Acad. Sci. USA 100: 9894–9899.

Meisel, R. P., J. H. Malone, and A. G. Clark, 2012a Disentangling the relationship between sex-biased gene expression and X-linkage. Genome Res. 22: 1255–1265.
Meisel, R. P., J. H. Malone, and A. G. Clark, 2012b Faster-X evolution of gene expression in Drosophila. PLoS Genet. 8: e1003013.
Meyer, B. J., 2010 Targeting X chromosomes for repression. Curr. Opin. Genet. Dev. 20: 179–189.
Mikkelsen, T. S., L. W. Hillier, E. E. Eichler, M. C. Zody, D. B. Jaffe *et al.*, 2005 Initial sequence of the chimpanzee genome and comparison with the human genome. Nature 437: 69–87.
Mitreva, M., M. L. Blaxter, D. M. Bird, and J. P. McCarter, 2005 Comparative genomics of nematodes. Trends Genet. 21: 573–581.
Nguyen, D. K., and C. M. Disteche, 2006 Dosage compensation of the active X chromosome in mammals. Nat. Genet. 38: 47–53.
Ohno, S., 1967 *Sex Chromosomes and Sex-Linked Genes*. Springer-Verlag, New York.
Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi, and T. Sekiya, 1989 Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc. Natl. Acad. Sci. USA 86: 2766–2770.
Orr, H. A., 1997 Haldane's rule. Annu. Rev. Ecol. Syst. 28: 195–218.
Parisi, M., R. Nuttall, P. Edwards, J. Minor, D. Naiman *et al.*, 2004 A survey of ovary-, testis-, and soma-biased gene expression in *Drosophila melanogaster* adults. Genome Biol. 5: R40.
Parisi, M., R. Nuttall, D. Naiman, G. Bouffard, J. Malley *et al.*, 2003 Paucity of genes on the Drosophila X chromosome showing male-biased expression. Science 299: 697–700.
Parkhomchuk, D., T. Borodina, V. Amstislavskiy, M. Banaru, L. Hallen *et al.*, 2009 Transcriptome analysis by strand-specific sequencing of complementary DNA. Nucleic Acids Res. 37: e123.
Parsch, J., and H. Ellegren, 2013 The evolutionary causes and consequences of sex-biased gene expression. Nat. Rev. Genet. 14: 83–87.
Piano, F., A. J. Schetter, M. Mangone, L. Stein, and K. J. Kemphues, 2000 RNAi analysis of genes expressed in the ovary of *Caenorhabditis elegans*. Curr. Biol. 10: 1619–1622.
Pollex, T., and E. Heard, 2012 Recent advances in X-chromosome inactivation research. Curr. Opin. Cell Biol. 24: 825–832.
Quinlan, A. R., and I. M. Hall, 2010 BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 26: 841–842.
R Development Core Team, 2012 *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
Ranz, J. M., C. I. Castillo-Davis, C. D. Meiklejohn, and D. L. Hartl, 2003 Sex-dependent gene expression and evolution of the Drosophila transcriptome. Science 300: 1742–1745.
Reinius, B., P. Saetre, J. A. Leonard, R. Blekhman, R. Merino-Martinez *et al.*, 2008 An evolutionarily conserved sexual signature in the primate brain. PLoS Genet. 4: e1000100.
Reinius, B., M. M. Johansson, K. J. Radomska, E. H. Morrow, G. K. Pandey *et al.*, 2012 Abundance of female-biased and paucity of male-biased somatically expressed genes on the mouse X-chromosome. BMC Genomics 13: 607.
Reinke, V., H. E. Smith, J. Nance, J. Wang, C. Van Doren *et al.*, 2000 A global profile of germline gene expression in *C. elegans*. Mol. Cell 6: 605–616.
Reinke, V., I. S. Gil, S. Ward, and K. Kazmer, 2004 Genome-wide germline-enriched and sex-biased expression profiles in *Caenorhabditis elegans*. Development 131: 311–323.
Reuben, M., and R. Lin, 2002 Germline X chromosomes exhibit contrasting patterns of histone H3 methylation in *Caenorhabditis elegans*. Dev. Biol. 245: 71–82.
Rice, W. R., 1984 Sex-chromosomes and the evolution of sexual dimorphism. Evolution 38: 735–742.
Rice, W. R., and A. K. Chippindale, 2001 Intersexual ontogenetic conflict. J. Evol. Biol. 14: 685–693.
Richards, S., Y. Liu, B. R. Bettencourt, P. Hradecky, S. Letovsky *et al.*, 2005 Comparative genome sequencing of *Drosophila pseudoobscura*: chromosomal, gene, and *cis*-element evolution. Genome Res. 15: 1–18.
Ross, J. A., D. C. Koboldt, J. E. Staisch, H. M. Chamberlin, B. P. Gupta *et al.*, 2011 *Caenorhabditis briggsae* recombinant inbred line genotypes reveal inter-strain incompatibility and the evolution of recombination. PLoS Genet. 7: e1002174.
Rudel, D., M. Riebesell, and R. J. Sommer, 2005 Gonadogenesis in *Pristionchus pacificus* and organ evolution: development, adult morphology and cell–cell interactions in the hermaphrodite gonad. Dev. Biol. 277: 200–221.
Russo, C. A., N. Takezaki, and M. Nei, 1995 Molecular phylogeny and divergence times of Drosophilid species. Mol. Biol. Evol. 12: 391–404.
Saifi, G. M., and H. S. Chandra, 1999 An apparent excess of sex- and reproduction-related genes on the human X chromosome. Proc. Biol. Sci. 266: 203–209.
Schaefke, B., J. J. Emerson, T. Y. Wang, M. Y. Lu, L. C. Hsieh *et al.*, 2013 Inheritance of gene expression level and selective constraints on *trans*- and *cis*-regulatory changes in yeast. Mol. Biol. Evol. 30: 2121–2133.
Small, C. M., G. E. Carney, Q. Mo, M. Vannucci, and A. G. Jones, 2009 A microarray analysis of sex- and gonad-biased gene expression in the zebrafish: evidence for masculinization of the transcriptome. BMC Genomics 10: 579.
Spencer, W. C., G. Zeller, J. D. Watson, S. R. Henz, K. L. Watkins *et al.*, 2011 A spatial and temporal map of *C. elegans* gene expression. Genome Res. 21: 325–341.
Srinivasan, J., W. Sinz, C. Lanz, A. Brand, R. Nandakumar *et al.*, 2002 A bacterial artificial chromosome-based genetic linkage map of the nematode *Pristionchus pacificus*. Genetics 162: 129–134.
Stein, L. D., Z. R. Bao, D. Blasiar, T. Blumenthal, M. R. Brent *et al.*, 2003 The genome sequence of *Caenorhabditis briggsae*: A platform for comparative genomics. PLoS Biol. 1: 166.
Sturgill, D., Y. Zhang, M. Parisi, and B. Oliver, 2007 Demasculinization of X chromosomes in the Drosophila genus. Nature 450: 238–241.
Tabuchi, T. M., B. Deplancke, N. Osato, L. J. Zhu, M. I. Barrasa *et al.*, 2011 Chromosome-biased binding and gene regulation by the *Caenorhabditis elegans* DRM complex. PLoS Genet. 7: e1002074.
Thomas, C. G., R. H. Li, H. E. Smith, G. C. Woodruff, B. Oliver *et al.*, 2012 Simplification and desexualization of gene expression in self-fertile nematodes. Curr. Biol. 22: 2167–2172.
Thornton, K., and M. Long, 2002 Rapid divergence of gene duplicates on the *Drosophila melanogaster* X chromosome. Mol. Biol. Evol. 19: 918–925.
Torgerson, D. G., R. J. Kulathinal, and R. S. Singh, 2002 Mammalian sperm proteins are rapidly evolving: evidence of positive selection in functionally diverse genes. Mol. Biol. Evol. 19: 1973–1980.
Trapnell, C., A. Roberts, L. Goff, G. Pertea, D. Kim *et al.*, 2012 Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7: 562–578.
Turelli, M., 1998 Evolutionary genetics: the causes of Haldane's rule. Science 282: 889–891.
Turner, J. M., 2007 Meiotic sex chromosome inactivation. Development 134: 1823–1831.
Uyar, B., J. S. Chu, I. A. Vergara, S. Y. Chua, M. R. Jones *et al.*, 2012 RNA-seq analysis of the *C. briggsae* transcriptome. Genome Res. 22: 1567–1580.
Verster, A. J., A. K. Ramani, S. J. McKay, and A. G. Fraser, 2014 Comparative RNAi screens in *C. elegans* and *C. briggsae* reveal the impact of developmental system drift on gene function. PLoS Genet. 10: e1004077.

Vibranovski, M. D., H. F. Lopes, T. L. Karr, and M. Long, 2009a Stage-specific expression profiling of Drosophila spermatogenesis suggests that meiotic sex chromosome inactivation drives genomic relocation of testis-expressed genes. PLoS Genet. 5: e1000731.

Vibranovski, M. D., Y. Zhang, and M. Long, 2009b General gene movement off the X chromosome in the Drosophila genus. Genome Res. 19: 897–903.

Vicoso, B., and D. Bachtrog, 2011 Lack of global dosage compensation in *Schistosoma mansoni*, a female-heterogametic parasite. Genome Biol. Evol. 3: 230–235.

Vicoso, B., and B. Charlesworth, 2009 The deficit of male-biased genes on the *D. melanogaster* X chromosome is expression-dependent: A consequence of dosage compensation? J. Mol. Evol. 68: 576–583.

Vicoso, B., J. J. Emerson, Y. Zektser, S. Mahajan, and D. Bachtrog, 2013 Comparative sex chromosome genomics in snakes: differentiation, evolutionary strata, and lack of global dosage compensation. PLoS Biol. 11: e1001643.

Walton, A. C., 1940 Gametogenesis, pp. 205–215 in *An Introduction to Nematology*, edited by G. B. Chitwood, and M. B. Chitwood. Babylon Press, New York.

Wang, J., P. J. Chen, G. J. Wang, and L. Keller, 2010 Chromosome size differences may affect meiosis and genome size. Science 329: 293.

Wang, P. J., J. R. McCarrey, F. Yang, and D. C. Page, 2001 An abundance of X-linked genes expressed in spermatogonia. Nat. Genet. 27: 422–426.

Wang, X., Y. Zhao, K. Wong, P. Ehlers, Y. Kohara *et al.*, 2009 Identification of genes expressed in the hermaphrodite germ line of *C. elegans* using SAGE. BMC Genomics 10: 213.

Wittkopp, P. J., B. K. Haerum, and A. G. Clark, 2004 Evolutionary changes in *cis* and *trans* gene regulation. Nature 430: 85–88.

Wu, C. I., and A. W. Davis, 1993 Evolution of postmating reproductive isolation: the composite nature of Haldane's rule and its genetic bases. Am. Nat. 142: 187–212.

Yang, X., E. E. Schadt, S. Wang, H. Wang, A. P. Arnold *et al.*, 2006 Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Res. 16: 995–1004.

Zhang, Y., D. Sturgill, M. Parisi, S. Kumar, and B. Oliver, 2007 Constraint and turnover in sex-biased gene expression in the genus Drosophila. Nature 450: 233–237.

Zhang, Y. E., M. D. Vibranovski, B. H. Krinsky, and M. Long, 2010 Age-dependent chromosomal distribution of male-biased genes in Drosophila. Genome Res. 20: 1526–1533.

Communicating editor: D. Charlesworth

GENETICS

Supporting Information
http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.163311/-/DC1

Sex-Biased Gene Expression and Evolution of the X Chromosome in Nematodes

Sarah Elizabeth Albritton, Anna-Lena Kranz, Prashant Rao, Maxwell Kramer, Christoph Dieterich, and Sevinç Ercan

DOI: 10.1534/genetics.114.163311

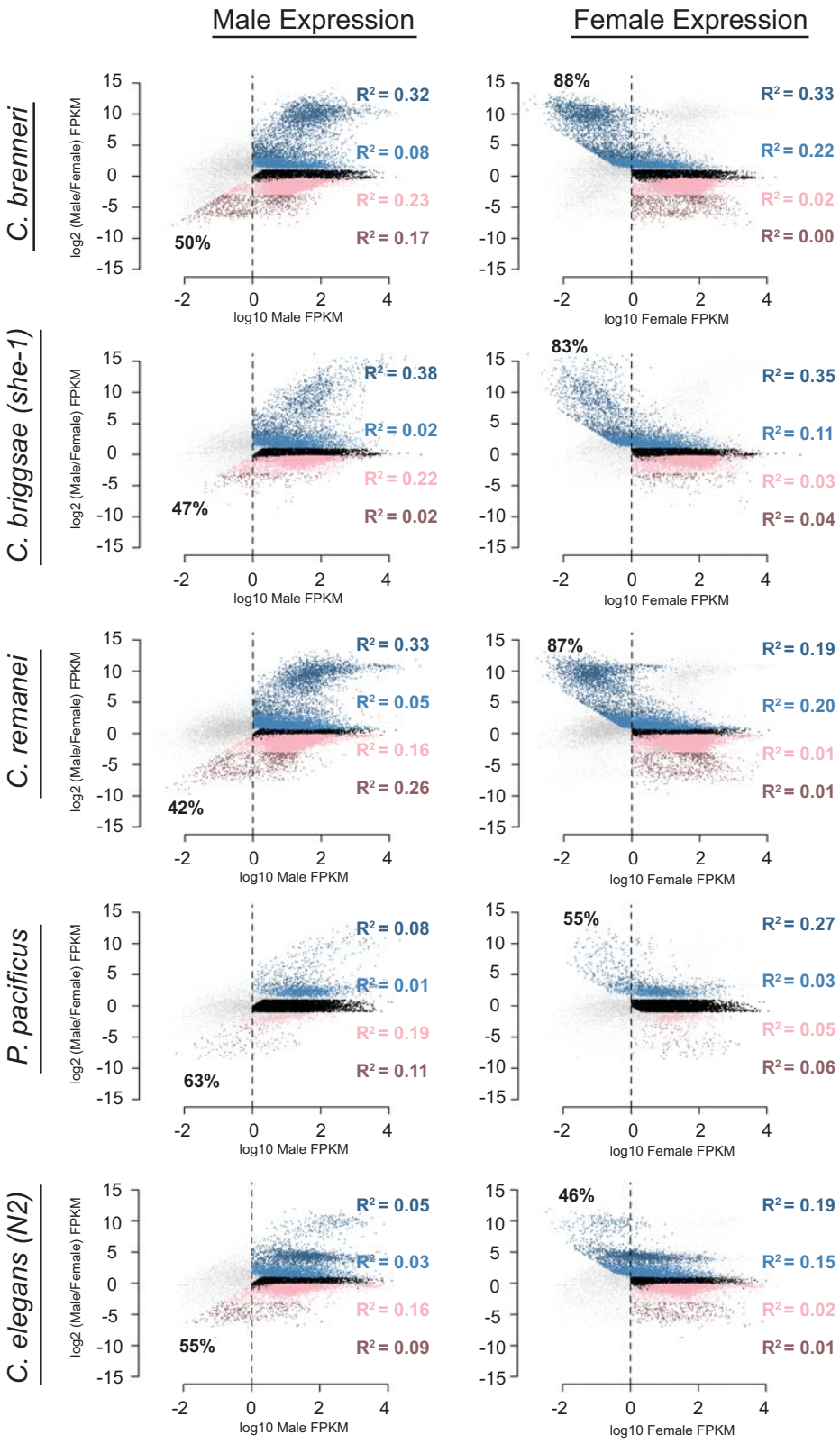

Figure S1 For each species, the magnitude of sex-biased expression (log2 sex expression ratio, (Male/Female) FPKM) is plotted against the level of expression (log10 FPKM) in males (left panels) and females (right panels). Dashed line indicates FPKM =1. Percentages of high sex-biased genes with low expression (FPKM < 1) in the opposite sex are indicated. Non-biased genes are plotted in black. Male-biased genes are plotted in blue; female-biased genes are plotted in pink. Darker colors indicate high magnitude of bias. R-squared values for each category are indicated.

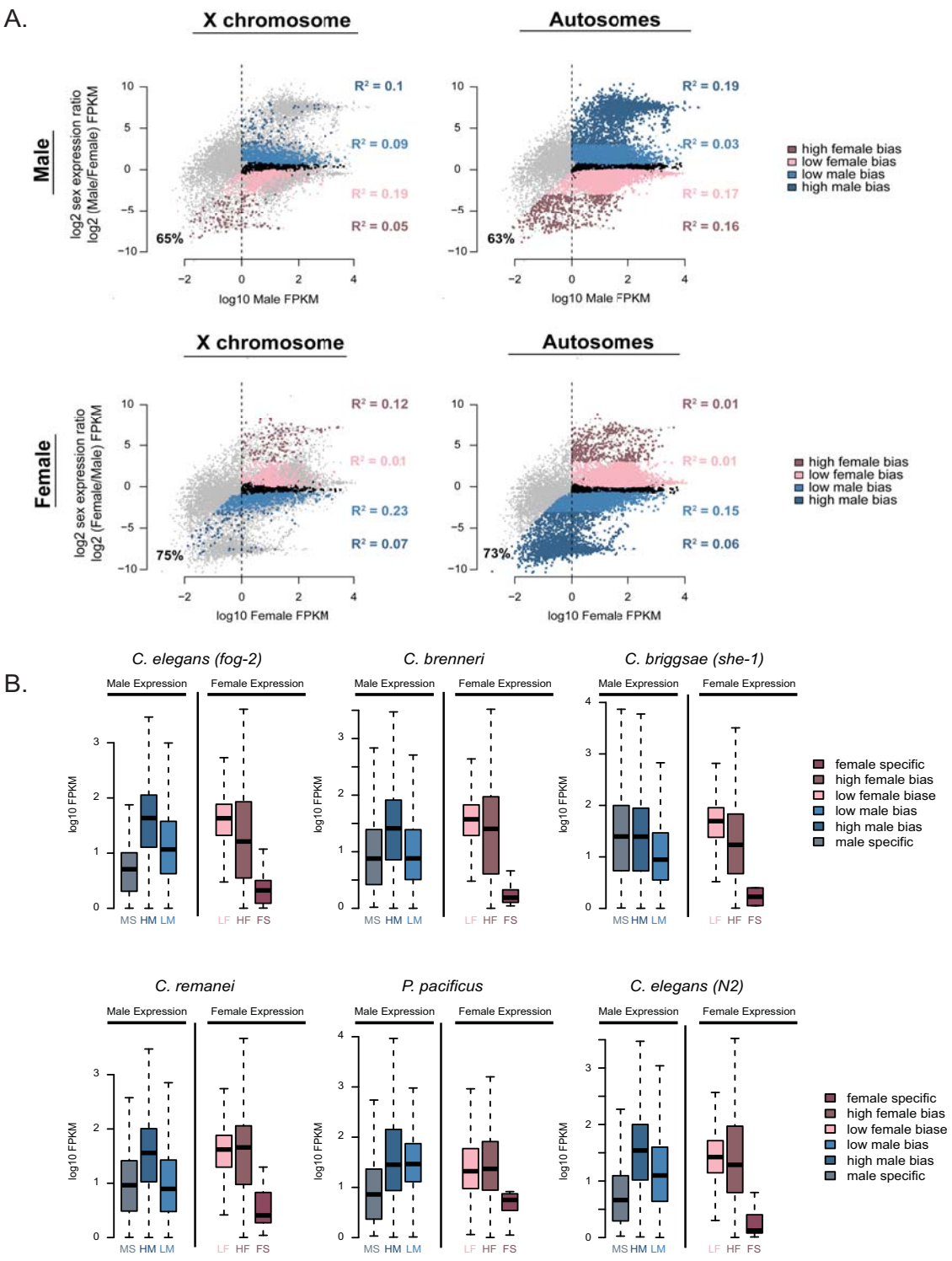

Figure S2 **A)** As in Supplemental Figure 1, for *C. elegans* (*fog-2*) data. Plots are separated by X (left panels) and autosomes (right panels). **B)** For all species, boxplots indicate the range of expression (calculated as the log10 FPKM) of each sex-bias category. Male expression is plotted for male-biased genes (left 3 boxes); female expression is plotted for female-biased genes (right 3 boxes). Overall, sex-specific genes are expressed at low levels in the corresponding sex. (MS – Male-Specific, HM – High Male, LM – Low Male, LF – Low Female, HF – High Female, FS – Female Specific).

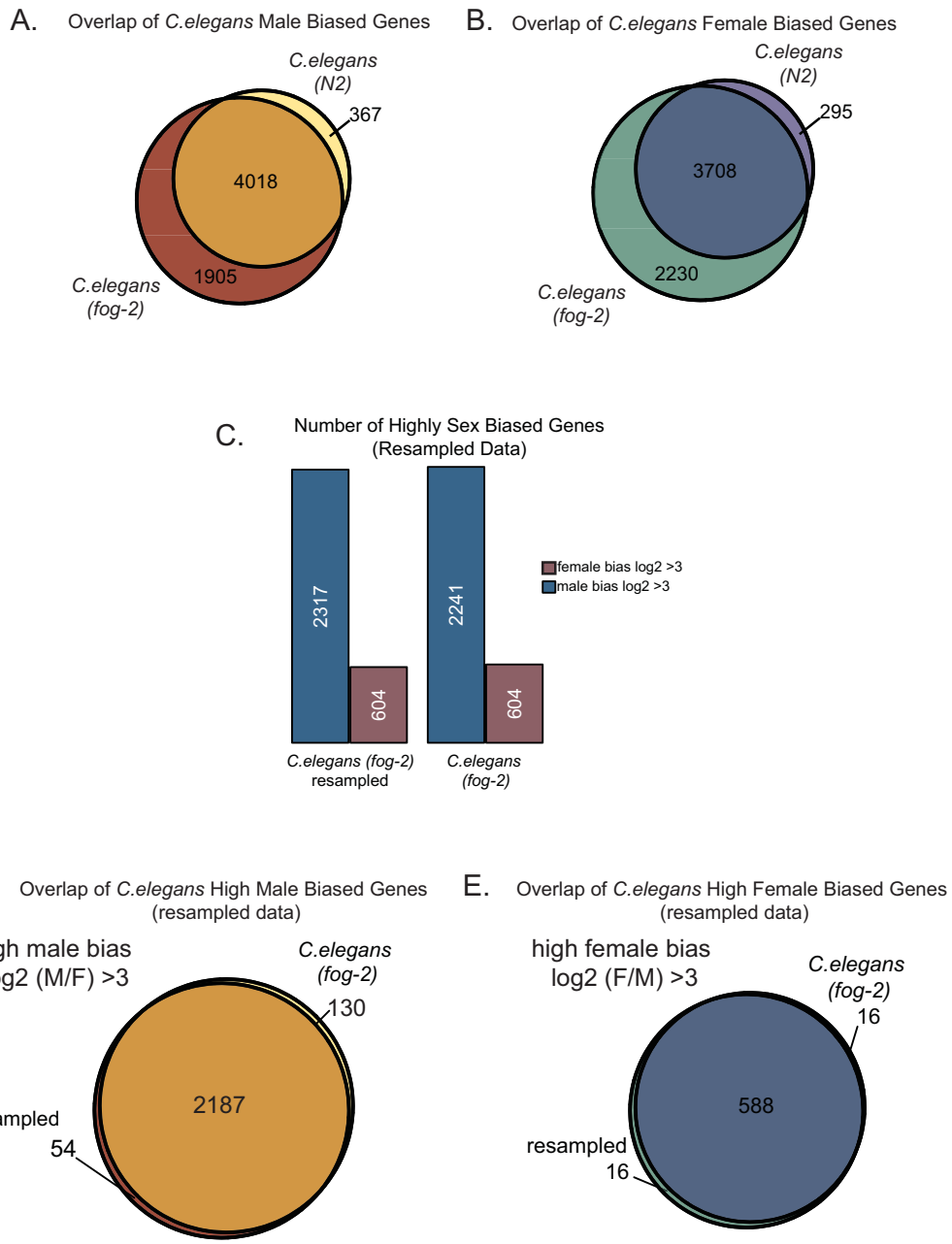

Figure S3 **A)** Sets of male and female-biased genes were determined individually using *C. elegans* (*N2)* (male-hermaphrodite) and (*fog-2)* (male-female) data. Sex-biased genes (including sex-specific genes) are those called differentially expressed by DESeq (q value < 0.05) and with FPKM > 1 in at least one sex. Overlap of male-biased genes is plotted. **B)** Same as in **A** but overlap of female-biased genes is plotted. **C)** *C. elegans (fog-2)* mapped reads were resampled to match the number of mapped reads in *P. pacificus*. Using the resampled data, we identified genes with sex-biased expression. Plot indicates the number of genes with high male and high female-bias (log2 sex expression ratio > 3) for the resampled data (left) and for the original *C. elegans (fog-2)* data. **D)** Overlap of high male-biased genes in the original and the resampled *C. elegans (fog-2)* data. **E)** Same as in **D** but for high female-biased genes**.**

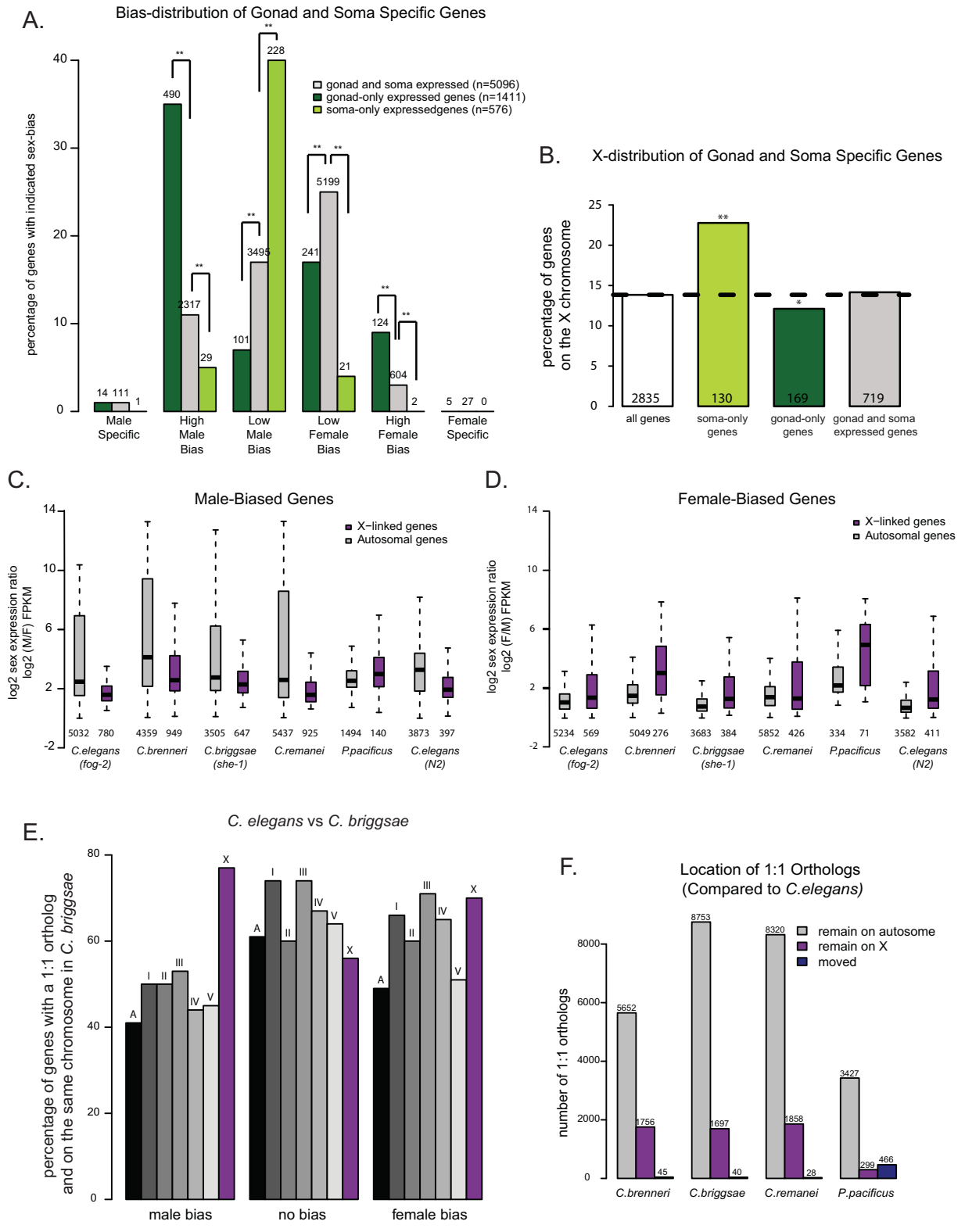

Figure S4 **A)** *C. elegans* genes were grouped into genes expressed in gonad and soma (gray), soma-only (light green), and gonad-only (dark green). For each category, the percentage of genes that fall into the indicated sex-bias category is plotted. Significance by Fisher test: (**) indicates p value less than 0.001. **B)** For each expression category the percentage of genes located on the X chromosome was plotted. Significance of enrichment or depletion was calculated using Fisher test: (*) indicates p value less than 0.05; (**) indicates p value less than 0.001. Number of X-linked genes is indicated at the bottom of each bar. **C)** As in Figure 4C, magnitude of male-biased expression (log2 male over female expression) was calculated for each

male-biased gene. Here, bias magnitude of male-biased genes is plotted. Number of genes analyzed is indicated below each box. **D)** Same as **C,** but for all female-biased genes. **E)** In Figure 6A we used a defined set of 1:1:1:1 orthologs to determine locational conservation of biased and unbiased genes. Here we define a set of 1:1 orthologs between *C. elegans* and *C. briggsae*. This less stringent definition of orthology gave a larger list of genes, and all chromosomes could be evaluated individually. The number of 1:1 orthologs located on the same chromosome was calculated. For each chromosome, the conservation is plotted as the percentage of *C. elegans* genes that have a 1:1 ortholog in *C. briggsae* located on the same chromosome. **F)** Macrosynteny is high amongst the *Caenorhabditis* species. Between *C. elegans* and each of the other four species, the number of 1:1 orthologs that remain autosomal (grey), remain X-linked (purple) or move between X and autosomes (dark blue) in the two species are plotted.

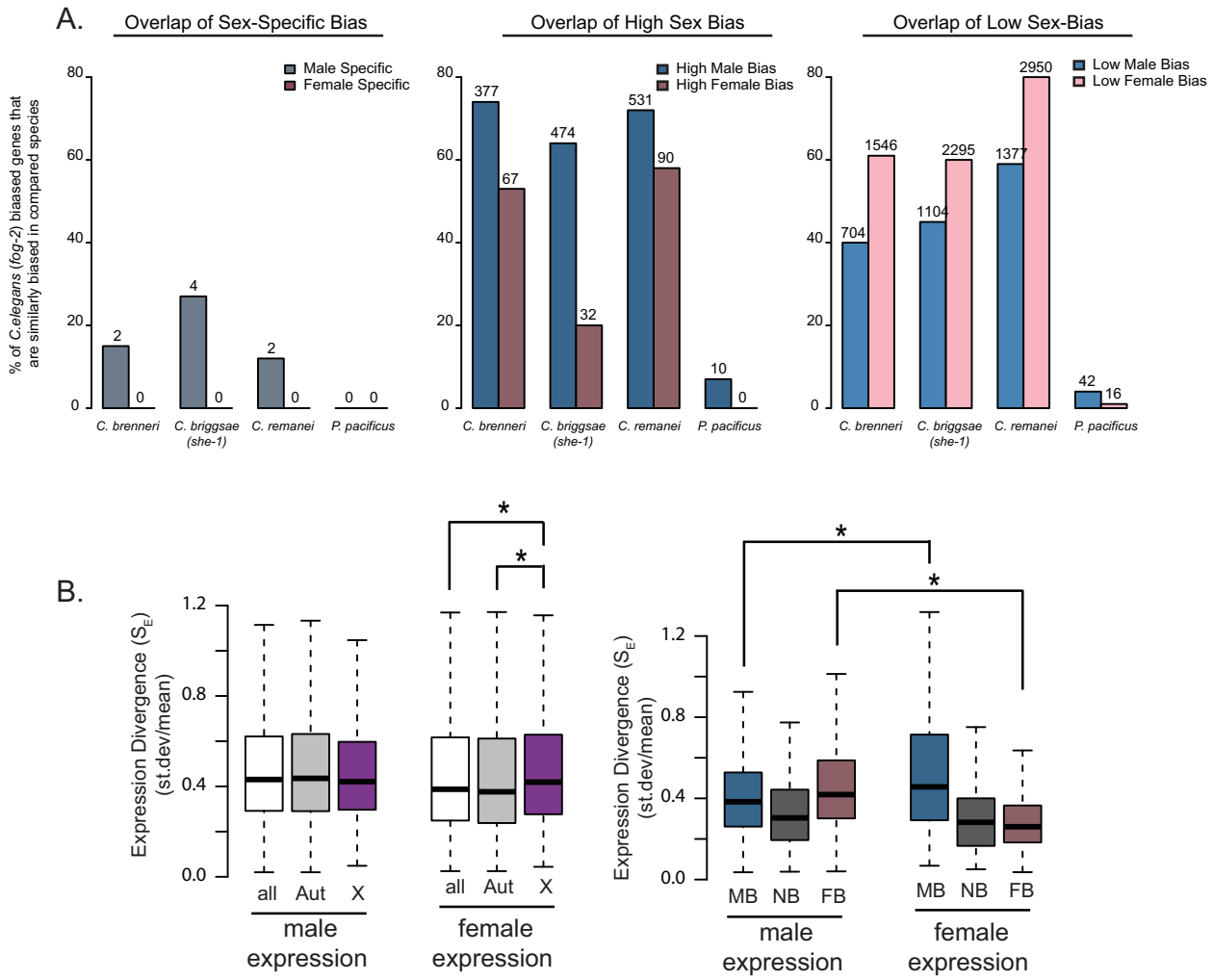

Figure S5 **A)** The percentage of genes in *C. elegans* with a similarly sex-biased 1:1 ortholog in the indicated species is plotted. Numbers above each bar indicated the number of 1:1 orthologs with similar bias. Percentage of overlap is plotted for *C. elegans (fog-2)* sex-specific genes **(Left)**, high sex-biased genes **(Middle)**, and low sex-biased genes **(Right)**. **B)** Using *Caenorhabditis* 1:1:1:1 orthologs, we calculated the interspecies expression divergence for each gene as the coefficient of variation (σ/μ). Left panel shows male and female expression divergence for all (white), autosomal (grey) and X-linked (purple) genes. Right panel shows male and female expression divergence for male-biased (MB, blue), female-biased (FB, pink), and non-biased (NB, dark grey) genes. (*) indicates significant difference in distribution (p-value < 0.01) as calculated by Mann-Whitney test.

Tables S1-S7
Available for download as Excel files at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.163311/-/DC1

Table S1 Overview of DNAseq Data, DNAseq Replicates, RNAseq data - read numbers, and GEO Numbers

Table S2 Contig Assignments

Table S3 Sex-Biased Genes

Table S4 Gonochorist-specific genes

Table S5 *Caenorhabditis* orthologs

Table S6 RNAseq proccesing - Cufflinks and DESeq

Table S7 Yeast 1:1 Orthologs