Submission intended as a **Letter** for the **Discoveries** section

The X chromosome of the German cockroach, *Blattella germanica*, is homologous to a fly X chromosome despite 400 million years divergence

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Abstract

The chromosomes that are sex-linked can differ between closely related species. Cases of long-term conservation could be informative of factors that prevent this sex chromosome turnover. We analyzed whole genome sequence data and found that many of the same genes are on the German cockroach, *Blattella germanica*, X chromosome and the ancestral X chromosome of higher flies. We also show that three regulators of transcription and chromatin on the fly X chromosome are conserved in the cockroach genome. We hypothesize that the common ancestor of cockroaches and flies had an X chromosome that resembled the extant cockroach/fly X. Cockroaches and flies diverged ∼400 million years ago, making this the longest documented conservation of a sex chromosome. Cockroaches and most flies have different mechanisms of sex determination, suggesting long-term conservation of the X chromosome despite evolution of the sex determination pathway.

Background

In species with separate sexes, genetic or environmental cues initiate sexually dimorphic developmental pathways (Bull, 1983; Beukeboom and Perrin, 2014). If the cue is genetic, a sex determining factor may reside on a sex chromosome (Bachtrog et al., 2014). For example, in most therian mammals, *SRY* on the Y chromosome initiates the development of male germline, gonad, and secondary sexual traits (Goodfellow and Lovell-Badge, 1993). In *Drosophila melanogaster*, the dosage of the X chromosome determines the initiation of male or female development (Baker and Belote, 1983; Cline, 1993; Erickson and Quintero, 2007). In both taxa, females have two X chromosomes (XX), and males have one X and one Y (XY). However, the sex chromosomes and genes that initiate the sex determination pathways are not homologous between mammals and *Drosophila* (Bachtrog et al., 2014).

Sex determining pathways and sex chromosomes can evolve rapidly, often differing between closely related species (Bachtrog et al., 2014; Beukeboom and Perrin, 2014). Evolutionary transitions in sex determination pathways are often accompanied by corresponding changes in the identity of the sex chromosomes (Bull, 1983; Beukeboom and Perrin, 2014; van Doorn, 2014). Transitions in sex determining pathways and turnover of sex chromosomes are well studied across insects, where there is a diversity of sex determination mechanisms (Gempe and Beye, 2011; Bopp et al., 2014; Vicoso and Bachtrog, 2015; Blackmon et al., 2017). For example, the genetic factors that initiate sex determination in *Drosophila* do not determine sex in other flies (Sievert et al., 1997; Meise et al., 1998; Saccone et al., 1998; Pane et al., 2002; Hall et al., 2015; Criscione et al., 2016; Krzywinska et al., 2016; Sharma et al., 2017), and the sex chromosomes of *Drosophila* are not homologous to the sex chromosomes of other flies (Foster et al., 1981; Vicoso and Bachtrog, 2013). It is most parsimonious to conclude that the ancestral sex determination system of higher dipterans (Brachycera, which includes flies but excludes mosquitoes, craneflies, midges, gnats, etc.) consists of a Y-linked male-determining factor that regulates the splicing of *transformer* (Pane et al., 2002, 2005; Bopp, 2010; Bopp et al., 2014; Scott et al., 2014; Sharma et al., 2017). The ancestral male-determiner of Brachycera is yet to be identified. The ancestral brachyceran X is a heterochromatin-enriched chromosome with <100 genes that is homologous to

D. melanogaster chromosome 4, i.e., the "dot" chromosome (Vicoso and Bachtrog, 2013). The evolution of a new sex determination mechanism in the lineage leading to *Drosophila* resulted in the transition of the ancestral X chromosome into an autosome and the creation of a new X chromosome from an ancestral autosome (Vicoso and Bachtrog, 2013).

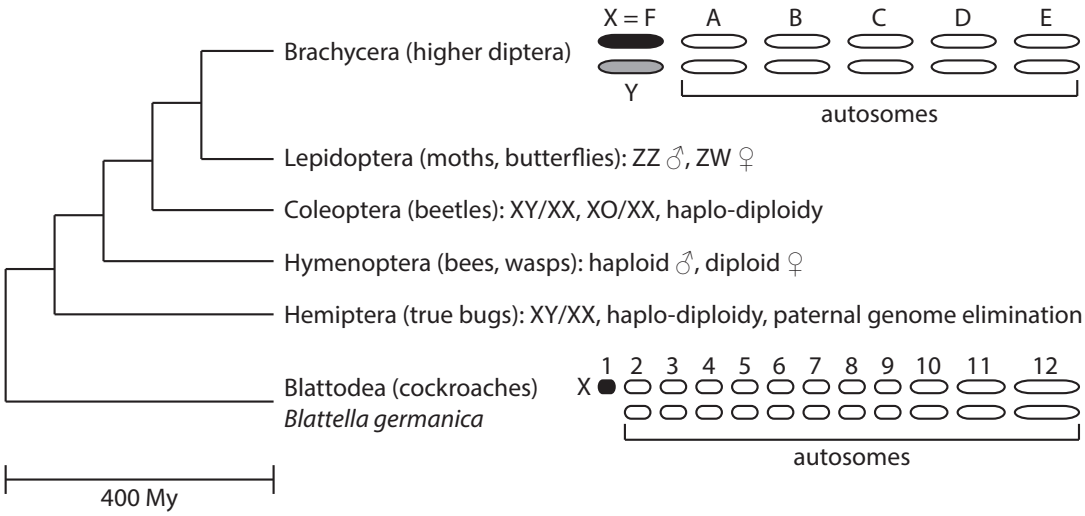

Figure 1: Evolutionary relationships and sex chromosome karyotypes of major insect groups. The phylogenetic topology and time to common ancestor are shown (Misof et al., 2014), but the relative branch lengths are not drawn to scale. Information on insect sex chromosomes and sex determination are reviewed elsewhere (Kaiser and Bachtrog, 2010; Vicoso and Bachtrog, 2013; Bachtrog et al., 2014; Blackmon et al., 2017).

The German cockroach, *Blattella germanica*, diverged from flies ∼400 million years ago (Mya) (Misof et al., 2014). Cockroach females are XX and males are XO, i.e., one X and no Y chromosome (Ross and Cochran, 1989; Kaiser and Bachtrog, 2010), which suggests that a dosage-sensitive X-linked factor determines sex, analogous to *D. melanogaster*. Many lineages more closely related to flies than cockroach have different sex determination mechanisms (**Figure 1**), suggesting *Drosophila* and cockroach dosage-dependent sex determiners arose independently. The cockroach X chromosome is heterochromatic along most of its length (Keil and Ross, 1984), reminiscent of the ancestral brachyceran X chromosome. We tested the hypothesis that the cockroach and brachyceran X chromosomes are homologous despite ∼400 My divergence.

Identifying X-linked cockroach genes

To test for X-linked genes in the cockroach genome assembly (JPZV00000000.1), we aligned paired-end reads from three male whole genome sequencing libraries (SRX693111, SRX693112, and SRX693113) and one female library (SRX693110) to the assembled scaffolds (Harrison et al., 2018) using BWA-MEM with default parameters (Li, 2013). We then assigned mapped read pairs to annotated genes if the first (forward) read aligned to any portion of a gene sequence. We summed across libraries to determine the total number of reads mapped to each gene for each sex. We next divided the number of male-derived (female-derived) reads aligned to each gene by the total number of male-derived (female-derived) reads aligned to all genes to determine a normalized mapping coverage of male-derived (female-derived) reads for each gene (**Supplemental Table S1**).

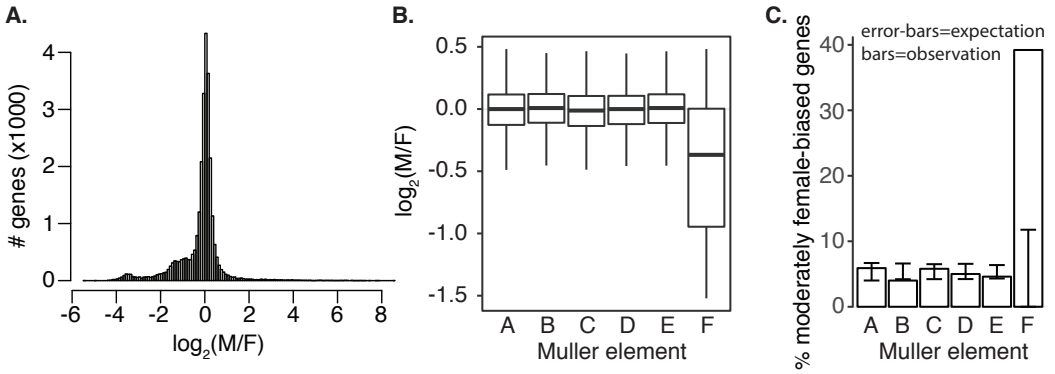

Figure 2: Muller element F is the cockroach X chromosome. The distributions of $\log_2 \frac{M}{F}$ for **(A)** annotated genes in the *B. germanica* genome and **(B)** genes with *D. melanogaster* homologs on each Muller element are shown. **(C)** The percent of *B. germanica* genes with moderately female-biased coverage ($\log_2 \frac{M}{F} < -0.5$) that have *D. melanogaster* homologs in each of the 6 Muller elements is plotted. The 95% confidence intervals (CIs) of the expected number of genes for each Muller element are shown by the error bars. Observed percentages that lie outside the CI indicate an excess or deficiency of genes on an element with moderately female-biased coverage.

X-linked genes are expected to have half as many male-derived reads mapped to them as female-derived reads (Vicoso and Bachtrog, 2013). Therefore, we calculated the $\log_2$ male:female read mapping coverage ($\log_2 \frac{M}{F}$) for each annotated cockroach gene (**Supplemental Table S1**), and then we normalized the data so that the median across all genes is zero. The

mode of the $\log_2 \frac{M}{F}$ distribution is at 0, consistent with a karyotype where most of the genome is autosomal (**Figure 2A**). However, there is a heavy shoulder $\log_2 \frac{M}{F} < 0$, suggesting that X-linked genes are also in the assembly (**Figure 2A**). In total, 3,499 of the 28,141 annotated genes have female-biased coverage ($\log_2 \frac{M}{F} \leq -1$), whereas only 1,363 genes have male-biased coverage ($\log_2 \frac{M}{F} \geq 1$), consistent with a heavy shoulder of X-linked genes. It is highly unlikely that all 3,499 female-biased genes are on the X chromosome. The cockroach X is the smallest of 12 chromosomes (Keil and Ross, 1984), which means a maximum of 2,345 genes ($\frac{1}{12} \times 28141$) should be X-linked.

Rather than identifying specific X-linked genes, we focus our remaining analysis on testing the hypothesis that the cockroach X chromosome is homologous to the ancestral brachyceran X. The ancestral brachyceran karyotype consists of six pairs of chromosomes, known as Muller elements (Muller, 1940). Five elements (A–E) are autosomal, and element F is the X chromosome (Vicoso and Bachtrog, 2013). We retrieved the *D. melanogaster* homologs of cockroach genes from the Baylor College of Medicine i5k Maker annotation (Harrison et al., 2018). We then assigned cockroach genes to fly Muller elements based on the Muller element of their *D. melanogaster* homolog (**Supplemental Table S1**).

We find two lines of evidence suggesting that the cockroach X chromosome is homologous to Muller element F. First, cockroach genes with *D. melanogaster* homologs on Muller elements A–E have distributions of $\log_2 \frac{M}{F}$ centered around 0, consistent with being autosomal (**Figure 2B**). In contrast, genes with homologs on element F have a $\log_2 \frac{M}{F}$ distribution centered <0 and significantly less than the other genes (P=10^{-10} using a Mann-Whitney U test). However, the distribution of $\log_2 \frac{M}{F}$ for element F genes is centered at ~0.5 (greater than the expectation of $\log_2 \frac{M}{F} = -1$), suggesting that some brachyceran X chromosome genes are autosomal in cockroach.

Second, we considered genes to be moderately female-biased if $\log_2 \frac{M}{F} < -0.5$ (the median of the distribution for genes with element F homologs). We then counted the number of moderately female-biased genes with homologs on each of the six Muller elements (**Supplemental Table S2**). To determine a null distribution of moderately female-biased genes on each chromosomes, we randomly assigned moderately-female biased genes to the six elements based on the number of genes with homologs on each element. A significant

excess of genes with moderately female-biased coverage have homologs on element F relative to our null expectation (**Figure 2C**), consistent with element F being the X chromosome in cockroach.

Conservation of element F transcriptional regulators in cockroach

There are three known proteins that interact to create a unique chromatin and transcriptional environment on element F in *D. melanogaster*. *Painting of fourth* (*Pof*) encodes an RNA binding protein that localizes over *D. melanogaster* element F gene bodies and binds nascent transcripts (Larsson et al., 2001; Johansson et al., 2007a,b, 2012). Because element F is the ancestral brachyceran X chromosome, it has been hypothesized that POF is part of an ancient X chromosome dosage compensation system that up-regulates expression in hemizygous males who only carry one copy of element F (Vicoso and Bachtrog, 2013). There is a *Pof* homolog in the cockroach genome (BGER016147) with a predicted RNA binding domain within the most conserved region of the protein relative to *D. melanogaster* (**Figure 3A–B**). Therefore, it is possible that cockroach and flies share both an ancient X chromosome and a conserved dosage compensation system.

The other two proteins are responsible for creating a chromatin environment around genes on *D. melanogaster* element F that resembles pericentromeric heterochromatin. The protein encoded by the SETDB1 homolog *eggless* (*egg*) is responsible for di-/tri-methylation of lysine 9 in histone H3 on the gene-dense region of *D. melanogaster* element F (Seum et al., 2007; Tzeng et al., 2007; Brower-Toland et al., 2009; Figueiredo et al., 2012; Lundberg et al., 2013). There are two predicted homologs of *egg* in the cockroach genome (BGER011023 and BGER011024). BGER011023 has a predicted SET lysine methyltransferase domain and a methyl-CpG binding domain commonly found in histone methyltransferases. BGER011024, on the other hand, has a tudor domain, which is found proximal to the SET domain in *D. melanogaster* Egg (Marchler-Bauer et al., 2017). These predicted functional domains overlap with the portions of the cockroach proteins that are most conserved relative to *D. melanogaster* Egg (**Figure 3C–D**). BGER011023 and BGER011024 are contiguous on a single *B. germanica* scaffold (Scaffold202; KN196692), suggesting that together they may constitute a single gene that encodes all Egg functional regions.

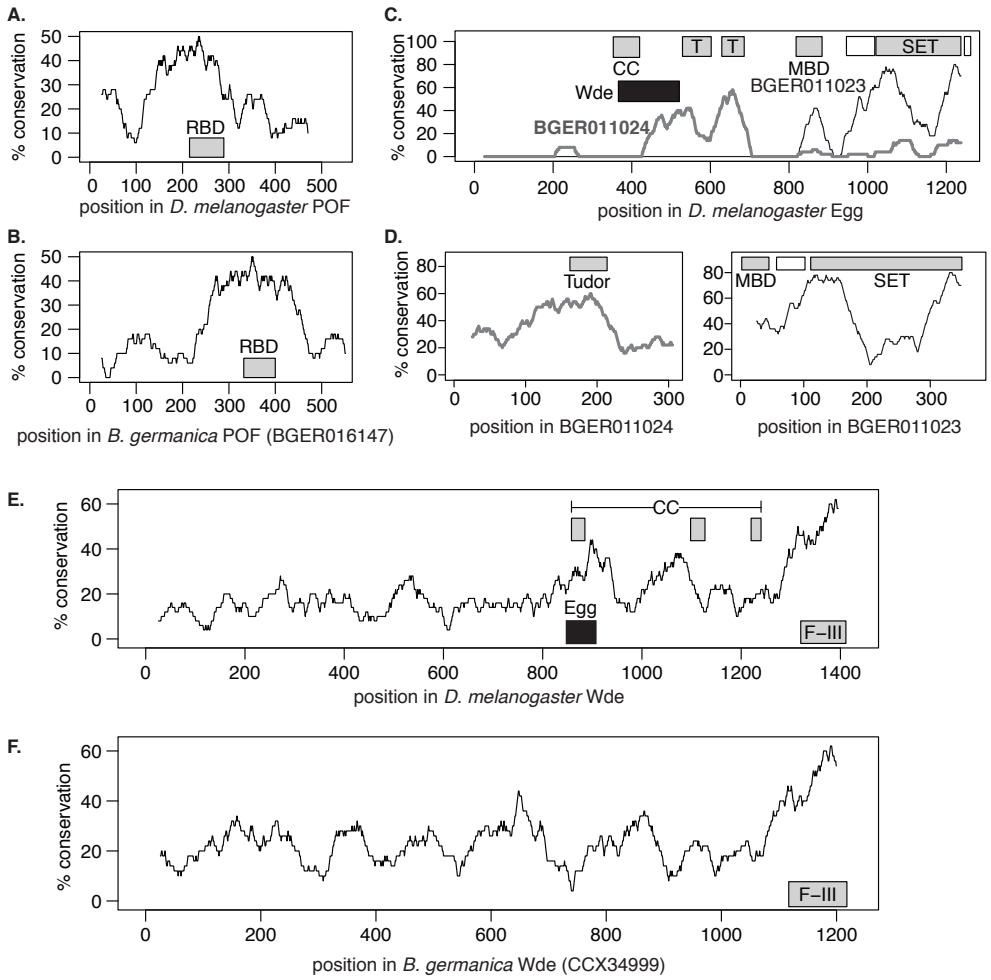

Figure 3: Three key regulators of element F transcription and chromatin are conserved in cockroach. Alignments between *D. melanogaster* and *B. germanica* proteins were performed using MUSCLE (Edgar, 2004). Lines show the percent amino acid (aa) sequence conservation at the midpoint of 50 aa sliding windows (1 aa increments) in the reference protein sequence listed along the bottom of each graph. Gaps in the non-reference aa sequence are counted as mismatches, and gaps in the reference sequence were ignored. The coordinates of the following predicted functional domains are shown as gray boxes in each graph: **(A-B)** RNA binding domain (RBD); **(C-D)** coiled-coil domain (CC), tudor domain (T), methyl-CpG-binding domain (MBD), and SET domain; **(E-F)** CC domain, and fibronectin type III repeats (F-III). **(C-D)** Predicted pre-SET domains are shown as white boxes next to SET domains. All functional domains were predicted by the NCBI Conserved Domain Database (Marchler-Bauer et al., 2017) or retrieved from UniProt (The UniProt Consortium, 2017). **(C)** The region of *D. melanogaster* Egg that interacts with Wde is shown by a black box, **(E)** as is the region of Wde that interacts with Egg.

The protein encoded by *windei* (*wde*) is an essential cofactor of Egg (Koch et al., 2009). There is one predicted homolog of *wde* in the cockroach genome (BGER025676), but an independently sequenced cockroach *wde* gene (CCX34999) is longer than the *wde* homolog predicted by the automated annotation (Herraiz et al., 2014). CCX34999 contains a predicted fibronectin type-III domain at the C-terminal end, similar to *D. melanogaster* Wde (Marchler-Bauer et al., 2017). The C-terminal end of CCX34999 is also the most conserved part of the protein relative to *D. melanogaster* Wde (**Figure 3E–F**). Finally, the coiled-coil region of *D. melanogaster* Wde that is required to interact with Egg and the region of Egg that interacts with Wde are among the most conserved regions of the proteins when compared to the cockroach homologs (**Figure 3C,E**).

Conclusions

We provide evidence that the X chromosome of the German cockroach, *B. germanica*, is homologous to the ancestral brachyceran X chromosome (**Figure 2**). There are two hypotheses that could explain this result. First, the most recent common ancestor (MRCA) of cockroaches and flies had an X chromosome that was conserved along the lineages leading to both cockroaches and flies. Second, the same genes could have independently become X-linked in both *B. germanica* and Brachycera. The heterochromatic composition of the *B. germanica* X chromosome (Keil and Ross, 1984), similar to the brachyceran X (Boyes and Van Brink, 1965), leads us to conclude that the former hypothesis is better supported.

Sex determination in *B. germanica* is likely regulated by X chromosome dosage, analogous to *Drosophila*, but different from the ancestral brachyceran sex determination system. It is unlikely that the same X-linked dosage sensitive factors determine sex in cockroach and *Drosophila* because the X chromosome is not homologous between the two taxa (element A is the ancstral X chromosome of *Drosophila*). In addition, the master regulators of *Drosophila* sex determination are derived in that function within flies. Therefore, we hypothesize that *B. germanica* has a homologous X chromosome with the MRCA of brachycera, but the sex determination system is not conserved between cockroach and flies. It is not clear which sex determination system is found in the MRCA of cockroach and flies because the diversity of insect sex determination prevents phylogenic inference (**Figure 1**). Regardless,

our results provide evidence that evolutionary transitions in sex determination pathways can be decoupled from X chromosome turnover (Meisel et al., 2017).

Finally, three genes that encode proteins responsible for creating a unique transcriptional and chromatin environment on *D. melanogaster* element F (which is homologous to the ancestral brachyceran X) are found in the cockroach genome, with their important functional domains conserved (**Figure 3**). One of these proteins (POF) may be part of an ancient X chromosome dosage compensation system in flies (Vicoso and Bachtrog, 2013). Cockroaches and flies diverged from their common ancestor 400 Mya (**Figure 1**). If element F was the X chromosome of the MRCA, this would represent the longest documented conservation of an X chromosome and corresponding dosage compensation system.

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Supplementary Material

Column name	Description
GeneID	i5k annotation ID for *B. germanica* gene
Scaffold	Scaffold identifier
beg	Beginning coordinate of gene in scaffold
end	End coordinate of gene in scaffold
Name	Name of *B. germanica* gene from i5k annotation
DmelHomolog	FlyBase protein ID of predicted *D. melanogaster* homolog from i5k annotation
FBgn	FlyBase gene ID of predicted *D. melanogaster* homolog
ME	Muller element of predicted *D. melanogaster* homolog
Alias	Gene name of predicted *D. melanogaster* homolog
GBid	GenBank ID of scaffold
female	Mapping coverage from female reads
male	Mapping coverage from male reads
log_mf	$\log_2 \frac{M}{F}$

Table S1: Description of column names for supplementary data reporting read mapping coverage for each cockroach gene.

	Muller element						
	A	B	C	D	E	F	total
# genes	898	999	1138	1159	1413	51	5658
female-biased	14	11	17	20	17	1	80
% female-biased	1.56	1.10	1.49	1.73	1.20	1.96	1.41
moderately female-biased	53	40	66	58	65	20	302
% moderately female-biased	5.90	4.00	5.80	5.00	4.60	39.22	5.34

Table S2: Cockroach genes with female-biased ($\log_2 \frac{M}{F} < -1$) and moderately female-biased ($\log_2 \frac{M}{F} < -0.5$) coverage with *D. melanogaster* homologs on each Muller element.

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