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Sex-specific and sex-chromosome regulatory evolution underlie widespread misregulation of inter-species hybrid transcriptomes

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Abstract

When gene regulatory networks diverge between species, their dysfunctional expression in inter-species hybrid individuals can create genetic incompatibilities that underlie the developmental defects responsible for intrinsic post-zygotic reproductive isolation. Divergence in *cis*- and *trans*-acting regulatory controls evolve despite stabilizing selection on gene expression, being hastened by directional selection with adaptation, sexual selection, and inter-sexual conflict. Dysfunctional sex-biased gene expression, in particular, may provide an important source of genetic incompatibilities, with more severe misregulation expected for the heterogametic sex. Here, we characterize and compare male and female transcriptome profiles for sibling species of *Caenorhabditis* nematodes, *C. briggsae* and *C. nigoni*, and allele-specific expression in their F_1 hybrids to deconvolve features of expression divergence and regulatory dysfunction. Despite evidence of widespread stabilizing selection on gene expression, we find broad misregulation of sex-biased genes in F_1 hybrids that is most pronounced for the X-chromosome, supporting a "large-X" effect, and that counters expectations by disproportionately affecting hybrid females. Hybrid male misexpression, however, is greater in magnitude, with spermatogenesis genes especially prone to high divergence in both expression and coding sequences that may explain elevated sterility of hybrid males, consistent with “faster male” and “fragile male” models for Haldane’s rule. Regulatory and coding divergence overall correlate only weakly, however, and downregulation of male-biased genes in females implicates *trans*-acting modifiers in the evolutionary resolution of inter-sexual conflicts. This work identifies important differences between the sexes in how regulatory networks diverge that contributes to sex-biases in how genetic incompatibilities manifest during the speciation process.

Keywords: *Caenorhabditis*, regulatory evolution, sex-biased expression, genome evolution, genetic incompatibilities, hybridization, *cis*-*trans* regulation, Haldane's rule

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Author's summary

Many mutations that affect traits as species diverge do so by altering gene expression. Such gene regulatory changes also accumulate in the control of static traits, due to compensatory effects of mutation on multiple regulatory elements. Theory predicts many of these changes to cause inter-species hybrids to experience dysfunctional gene expression that leads to reduced fitness, disproportionately affecting the sex chromosomes and sex-biased gene expression. Our analyses of genome-wide expression data from *Caenorhabditis* nematode roundworms support these predictions. We find widespread rewiring of gene regulation despite extensive morphological stasis, and conserved overall expression profiles, that is a hallmark of these animals. Misregulation of expression in both sexes is most severe for genes linked to the X-chromosome, sperm genes show distinctive signatures of divergence, and differences between the sexes in regulatory evolution implicate resolved historical sexual conflicts over gene expression. This work clarifies how distinct components of regulatory networks evolve and contribute to sex differences in the manifestation of genetic incompatibilities in the speciation process.

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Introduction

Many kinds of reproductive barriers can contribute to speciation (1,2), with genetically intrinsic post-zygotic barriers a kind that makes speciation irreversible. Such intrinsic barriers result from disrupted developmental programs due to divergence in the regulatory controls of and functional activity within genetic networks. Consequently, research for decades has aimed to decipher the identity and general features of genetic changes that accumulate by selection and genetic drift to lead to Dobzhansky-Muller (DM) incompatibilities in hybrids of diverging populations, due to non-additive, negatively-epistatic interactions among genes (1,3). It is therefore crucial to decipher how genes and gene expression evolve to understand how gene regulation influences post-zygotic reproductive isolation through misregulated gene interactions in hybrids (3–6).

Evolution of the regulatory controls over gene expression influences much phenotypic evolution (5,7), despite stabilizing selection as a prevailing force acting to preserve expression profiles (8–12). Expression differences between species accrue in predictable ways. Regulatory differences between species disproportionately involve the evolutionary accumulation of mutations to *cis*-regulatory elements, facilitated by such changes being predisposed to additivity and low pleiotropy of effects on traits and fitness (13,14). In contrast, larger, more pleiotropic effects can result from *trans*-regulatory changes that occur at distant genomic positions, such as to transcription factors, chromatin regulators, and small RNA genes. Consequently, theory predicts *trans*-regulatory mutations to fix less readily and to contribute fewer differences between species, despite their large mutational target size and disproportionate contribution to genetic variation within a species (4,13–15). Studies nevertheless commonly find both *cis*- and *trans*-regulatory differences between species (16–19). Indeed, the coevolution of changes to both *cis*- and *trans*-acting factors represents one plausible outcome of stabilizing selection on expression level. The compensatory effects of such coevolved *cis*- and *trans*-regulatory changes yield an overall conserved expression profile (4,11,20), but this multiplicity of changes are predisposed to generating misexpression in F_1 hybrids due to dysfunctional *cis*-by-*trans* regulatory interactions (5,21).

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Decomposing the changes of gene networks into their *cis*- and *trans*–regulatory components, however, presents a challenge to studying gene regulatory evolution. One way to address this problem is with hybrid cross experiments that assess differential expression between two closely related species and from allele-specific expression (ASE) in their F_1 hybrids (5,11,22). Differences in gene expression between species reveal the joint effects of *cis*- and *trans*-regulatory divergence, whereas differences in ASE within F_1 hybrids typically represent the effects of *cis*-regulatory divergence alone (22). Studies of this kind have unveiled broad empirical patterns of regulatory evolution, whether carried out in flies (16,23), mice (24), plants (17,18), or yeast (19,25). In particular, this work has shown substantial regulatory divergence in both *cis* and *trans*, extensive non-additivity, and disrupted regulation and misexpression in F_1 hybrids. Whether these patterns hold for the nematode phylum is, as yet, unknown, and the links between regulatory mechanisms and sex-biases in expression remain incompletely resolved.

Hybrid dysfunction of developmental programs, outward phenotypes, and fitness may often trace their origins to gene misregulation, from transcriptional to post-translational levels (5,6). Sex-biased misregulation, therefore, should underlie sex-biased developmental and fitness effects in hybrid individuals. Misexpression of male-biased genes and genes related to spermatogenesis links misregulation to male sterility in hybrids, with supporting empirical evidence in several kinds of animals (24,26–29). In organisms with chromosomal sex determination, more severe defects typically occur in hybrid individuals carrying heterogametic sex chromosomes (i.e., XO males in *Caenorhabditis* nematodes). This Haldane's rule pattern can arise from dominance effects (30), faster molecular evolution of genes with male-biased expression (31), greater sensitivity of male developmental programs to perturbation (31), and faster evolution of sex-linked loci (32), among other causes (33,34). Because of the prominent role that the X-chromosome plays in reproductive isolation (35–37), we might also expect to find greater expression divergence and misexpression for X-linked genes compared to autosomes (5,26,27,38,39), with the caveat that genes with male-specific expression might not

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114 necessarily be abundant on the X-chromosome (40–42). Thus, distinguishing between abnormal
115 expression in hybrids for X-linked genes overall and for sex-biased autosomal genes is
116 important to decipher the genetic mechanisms that underpin Haldane's rule in particular and
117 hybrid dysfunction in general.
118
119 *Caenorhabditis* nematode roundworms provide an especially tractable system to study
120 speciation genomics (34). The growing number of *Caenorhabditis* species known to science
121 conform to the biological species concept, with a few cases where sibling species can produce
122 some viable and fertile adult hybrid offspring (43,44). The *C. briggsae* × *C. nigoni* species pair is
123 one such case, where recent divergence (~3.5 Ma (45)) allows them to form hybrids of both
124 sexes. Haldane's rule is fulfilled: F_1 male hybrids exhibit both greater infertility and inviability in
125 crosses of *C. nigoni* females to *C. briggsae* males, with nearly total hybrid male inviability in the
126 reciprocal cross (46–48). The disproportionate loss of genes with male-biased expression in the
127 *C. briggsae* lineage has the potential to disrupt genetic networks in a sex-biased way to
128 disproportionately compromise hybrid male fitness (45,49–51). In an effort to identify and
129 locate incompatibility loci between these two species, Bi et al. (52) associated hybrid male
130 inviability and sterility with most of the X-chromosome, suggesting a large-X effect. Moreover,
131 analysis of X-chromosome introgression lines revealed that X-autosome incompatibility
132 involving misregulation of the 22G class of small RNAs led to down-regulation of
133 spermatogenesis genes as a contributor to hybrid male sterility, in addition to autosomal
134 factors responsible for hybrid inviability (29,53). Here, we analyze mRNA transcriptome
135 expression for each sex from each of *C. briggsae*, *C. nigoni*, and their F_1 hybrids. Using ASE
136 profiles, we then quantify *cis*- and *trans*-acting regulatory causes of expression divergence to
137 link genomic change to sex-biased expression, chromosomal features, and hybrid dysfunction.

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Results

Extensive expression divergence between species and between the sexes involve the X-chromosome

Each species and sex show distinctive overall transcriptome profiles that are further distinguishable from each sex of F_1 inter-species hybrids (**Fig. 1A**). In the contrast of pure *C. briggsae* and *C. nigoni* transcriptomes, we found more differentially expressed genes for females and hermaphrodites, hereafter jointly referred to as females for brevity, than for males. For the 12,115 one-to-one orthologs analyzed, females had a total of 66% (7,903) of genes differentially expressed between species, compared to 53% (6,391) for males (**Fig. 1B**). The X-chromosome shows the most extreme differences in expression between species, with both males and females showing significantly higher expression of X-linked genes in *C. briggsae* than in *C. nigoni* (**Fig. 1C**). Autosomes, by contrast, showed greater abundance of genes with higher expression in *C. nigoni*, albeit only significantly only for Chromosome I in females (**Fig. 1C**; Fisher's exact test; $P < 0.05$).

Within each species, approximately half of genes exhibited significant sex biases in expression, which, in turn, are roughly evenly divided between male- and female-biased expression: 28% male-biased genes in *C. briggsae* (3,353 genes), 26% male-biased genes in *C. nigoni* (3,188 genes), 26% female-biased genes in *C. briggsae* (3,205 genes), and 24% female-biased genes in *C. nigoni* (2,878 genes). Male-biased genes did not exhibit strong enrichment for any chromosome in either species, whereas female-biased genes in *C. briggsae* were 1.6-fold enriched on Chromosome I and 3.3-fold depleted on the X-chromosome (**Fig. 1E**), consistent with previous studies in *Caenorhabditis*. The X-chromosome is significantly enriched, however, for non-sexually differentiated genes in both *C. briggsae* and *C. nigoni* (Fisher's exact test, $P < 0.05$) (**Fig. 1E**). Overall, expression profiles for *C. briggsae* hermaphrodites were masculinized relative to *C. nigoni* females (**Fig. 1A**), consistent with hermaphrodite production of both sperm and oocytes in an otherwise morphologically female soma.

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Expression dominance in F_1 hybrids differs between males and females

We contrasted expression profiles of F_1 hybrids with their parent species to infer the expression inheritance of genes, i.e., to identify genes that exhibited additive, dominant (*C. briggsae*- and *C. nigoni*-like expression), or transgressive (overdominant and underdominant) expression patterns for each sex (**Fig. 2A**). Gene sets with distinct expression inheritance profiles revealed substantial differences between the sexes in terms of expression distance (**Fig. 2B**), number of genes (**Fig. 2C**), and enrichment across the genome (**Fig. 2D**).

The sexes differed most strikingly in their total number of transgressive genes (**Fig. 2C**). Transgressive genes are thought to be associated with hybrid dysfunction, as they represent expression phenotypes that are distinct from expression levels in either parent (28,54). Despite our expectation of an especially high number of misregulated genes in F_1 males due to their pronounced sterility, we found that just 21% (2,552) of genes show transgressive profiles in males (1,387 overdominant and 1,165 underdominant) compared to 55% (6,729) in females (3,284 overdominant and 3,445 underdominant) (**Fig. 2A,C**). However, we also found that Euclidean expression distances from the centroid of expression space are higher on average in males than in females for both overdominant (ordinary least-squared [OLS] regression, $t = 6.28$, $P < 0.001$) and underdominant genes (OLS, $t = 2.14$, $P < 0.05$), indicating especially deviant expression magnitude for those fewer transgressive genes in F_1 males (**Fig. 2A,B**). By contrast, genes with simple dominance or additive expression in F_1 males had consistently lower expression distance from the centroid than did F_1 females (**Fig. 2A,B**; OLS, $P < 0.05$). These results are consistent with our multidimensional scaling analysis (**Fig. 1A**) that showed shorter expression distances for F_1 males to parental males, in contrast to more dissimilar expression profiles of F1 females to parental females.

F_1 hybrids of both sexes had a relatively low percentage of genes with additive expression (6% or 698 genes in females; 7% or 872 genes in males) (**Fig. 2C**). By contrast, approximately 30% of genes expressed by each sex showed simple dominance, either matching *C. briggsae* or *C. nigoni* expression (27% or 3,878 genes in females; 31% or 3,297 genes in males). Hybrids of

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both sexes consistently showed a higher number of genes with expression dominance matching *C. briggsae* (20% or 2,408 genes in males and 16% or 1,904 genes in females), compared to expression dominance matching *C. nigoni* (**Fig. 2C**; 12% or 1470 genes in males and 11% or 1393 in females). In both sexes, the ratio of genes with simple dominance matching either *C. briggsae* or *C. nigoni* expression is close to 1:1 consistently across all five autosomes (mean ratio = 1.15, sd = 0.13). In F_1 females, the X-chromosome, however, was 4-fold biased towards expression dominance matching *C. briggsae* (**Fig. 2C,D**).

Genes and traits with dysfunctional expression are often associated with the X-chromosome, and differences between the sexes are expected due to Haldane's rule (24,26,27,38). Consistent with these expectations, we found expression heritability profiles in F_1 males and females to vary across the genome, and to differ most conspicuously for the X-chromosome. The X-chromosome was enriched for underdominant genes in both F_1 males and females (**Fig. 2C,D**; Fisher's exact test, $P < 0.05$). The X-chromosome was also enriched for overdominant genes in F_1 males, whereas females had significant depletions of such genes on the X (autosomal enrichments: V in males, I and III in females) (**Fig. 2C,D**). These data show clear differences in expression heritability across chromosomes between the sexes and reflect distinct hybrid expression dynamics between autosomal and X-linked genes.

Regulatory divergence is modulated by differences in *cis* and *trans* effects between sexes

Identifying the spectrum of changes in *cis*- and *trans*-acting regulators is important to understand how selection influences the evolution of gene expression and its effects on hybrid phenotypes. Correspondingly, we classified the types of regulatory changes and examined gene misregulation in F_1 hybrids for each sex. We found that most expression divergence between species results from *cis*-only, *trans*-only, and enhancing *cis-trans* gene regulatory divergence (**Fig. 2E,F**), consistent with other ASE studies in flies (16,23), mice (24), plants (17,18) and yeast (19,25). Comparing regulatory divergence between sexes, we found double the number of *trans*-only changes involving genes expressed in females (20% or 2,461 genes) compared to

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males (10% or 1,230 genes) (**Fig. 2G**). However, the sexes showed a reciprocal pattern for genes with *cis*-only divergence being more prevalent in males than in females (21% or 2,559 genes in males; 17% or 2,089 genes in females). Genes with *cis*-only and *trans*-only effects were not significantly enriched on any autosome for either sex, but genes expressed in males showed strong enrichment on the X-chromosome for *trans*-only regulatory changes as well as a depletion for *cis*-only changes (**Fig. 2H**; Fisher's exact test, $P < 0.05$). While genes with *C. briggsae* expression dominance were commonly associated with *cis*-only effects, especially in F_1 males, genes with *C. nigoni* dominant expression tended to show *trans*-only effects, especially for autosomes in F_1 females (**Fig. 3**). In contrast, X-linked genes in F_1 females rarely showed *C. nigoni* expression dominance displayed by either *cis*-only or *trans*-only effects (**Fig. 3**). In contrast, X-linked genes in both sexes have up to 3-fold higher proportion of genes showing *C. briggsae* expression dominance more often associated with *trans*-only effects (432:106 *C. briggsae : C. nigoni* in females; 329 : 274 in males) (**Fig. 3**). This skew is particularly notable for F_1 males given that they carry the *C. nigoni* X-chromosome, and therefore these genes would be both misexpressed and misregulated. These observations together illustrate how regulatory changes have evolved in sex-specific ways toward disproportionately *cis*-only changes in males and *trans*-only changes in females, in addition to divergent impacts on the X-chromosome and on overall expression profiles between the parent species.

Furthermore, we categorized genes into 13 groups based on distinct combinations of species differences and sex differences in expression, including their interactions and looked at the proportion of genes with different *cis*- and *trans*-effects (gene groups M0-M12; **Fig. 4A,E**). Consistent with the idea that *cis* and *trans* changes each play distinct roles in sex-biased expression and sexual dimorphism (55), we find, on one hand, that *trans*-only changes in females are more often associated with male-biased genes, and on the other hand, that female-biased genes show more *cis*-only regulatory changes in males (**Fig. 4E**). This pattern suggests that different sex-specific regulatory controls have evolved to repress expression of genes biased for the opposite sex.

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Hybrid misexpression is commonly due to overdominance involving genes with joint *cis-trans* regulatory changes

Genes exhibiting additive or transgressive expression profiles in F_1 hybrids are often associated with dysfunctional traits, due to radically different expression from those of parent species. Genes with additive expression heritability may commonly reflect *cis*-only regulatory divergence, such that allele-specific transcriptional differences generate intermediate expression profiles in F_1 hybrids (22,26,56). In line with this idea, we often found genes classified as additive to associate more often with significant *cis*-acting divergence in both sexes (i.e., *cis*-only and enhancing *cis-trans* effects; **Fig. 3**; **Supplementary Fig. S4**). However, expression additivity is not abundant in our analysis (**Fig. 2C**), suggesting that it is not a major source of phenotypic dysfunction in hybrids of this system.

Stabilizing selection on expression level is thought to be common in many species, at the molecular level enabled through the coevolutionary fine-tuning by changes to *cis*- and *trans*-acting factors (7,12). *cis* and *trans* changes with opposing effects can interact epistatically in hybrids to induce dysfunctional expression and allelic imbalance (21). Consistent with this idea, we found that transgressive genes with overdominant effects in hybrids often are associated with *cis-trans* regulatory changes in hybrids (43% or 539 genes in males and 42% or 1,312 genes in females; **Fig. 3**). In F_1 males, a higher fraction of changes was compensatory (26%) compared to enhancing (17%). F_1 females had the opposite pattern: a higher fraction of genes showed enhancing compared to compensatory changes (26% versus 16%). In contrast to genes with such overdominant expression profiles in F_1 hybrids, underdominant genes consistently exhibited more conserved regulatory controls in both sexes (53% or 577 genes in males and 50% or 1,541 genes in females identified as conserved or ambiguous), suggesting alternative mechanisms of regulatory dysfunction. These results highlight how stabilizing selection can act differently on sex-specific transcript abundance, leading to opposing *cis* and *trans* effects that are dysfunctional in F_1 hybrids.

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To further assess the role that different regulatory controls play in the origin and maintenance of divergent sex-biased expression, we contrasted expression heritability and patterns of *cis*- and *trans*-regulatory divergence for male-biased and female-biased genes (**Fig. 4D,E**). We found that male-biased genes expressed in F_1 females frequently show transgressive misexpression (underdominant) or have expression levels matching those of the species with lower expression (**Fig. 4A,D**). In contrast, male-biased genes expressed in F_1 males tend to show expression dominance matching *C. briggsae*, regardless of which pure species had higher gene expression (**Fig. 4A,D**).

Interestingly, genes expressed in F_1 males are more commonly underdominant when they correspond to male-biased genes than to genes that have a female-biased expression pattern (623 genes among male-biased genes vs 281 genes among female-biased genes), suggesting male-biased regulatory networks are more fragile. This idea is also supported by male-biased genes having a higher proportion of genes with enhancing or compensatory *cis-trans* changes (835 genes in male-biased genes vs 398 genes in female-biased genes). By contrast, female-biased genes in F_1 females are predominantly overdominant (**Fig. 4D**) and are more often associated with *cis* and enhancing *cis-trans* changes, which suggest that female gene regulatory networks can be more resilient to misexpression, which may translate to similar resilience for traits such as fertility (46).

Faster regulatory and molecular evolution of male-biased and spermatogenesis genes

Sexual selection and sexual conflict are predicted to drive faster rates of molecular evolution and expression divergence (36,57,58). Consistent with these predictions, we found that male-biased genes have higher average expression divergence (OLS, $P < 0.001$) and faster rates of molecular evolution (K_a/K_s, OLS, $P < 0.05$) than female-biased genes (**Fig. 4B,C**). Compared to sex-neutral genes, however, the signal for faster sequence evolution was weak (K_a/K_s OLS, $P = 0.3$), despite remaining strong for elevated expression divergence (OLS, $P < 0.001$).

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We observed the highest expression divergence as well as high average rates of molecular evolution in the distinctive set of genes with male-biased expression, higher expression in *C. briggsae* than *C. nigoni*, and with a species-by-sex interaction (M6) (**Fig. 4B,C**). The species-by sex interaction in M6 indicated a masculinized expression profile for *C. briggsae* hermaphrodites, implicating a role for them in sperm production. To test this idea, we looked at *C. elegans* genes previously identified as spermatogenesis genes (42) and found their orthologs in *C. briggsae* and *C. nigoni* to be 13-fold enriched in the M6 group (**Fig. 4F**; Fisher's exact test, $P < 0.05$) and depleted from the X-chromosome, consistent with previous observations for sperm genes in *Caenorhabditis* (34,40,41).

These orthologs to spermatogenesis genes, however, did not show strong misexpression (i.e., overdominant and underdominant genes) or enrichment of misexpressed genes in F_1 hybrid males among autosomes, except for chromosome V (**Fig. 4G,H** upper panel). More commonly, they showed expression dominance matching *C. nigoni* (24% or 225 genes vs. 12% or 109 *C. briggsae* dominant genes) or had no change in expression from either parent (38% or 360 genes for "no change" and "ambiguous" categories). For F1 males, X-linked genes showing expression divergence and dominance matching *C. briggsae* levels in hybrid males should be effectively misexpressed, as regulation is controlled mostly by *trans*-acting factors coming from the *C. briggsae* autosomal background that interact with the *C. nigoni*-derived X-chromosome, and therefore represent misexpression (**Supplementary Fig. S3**; also see (26)). We observed a 1.4-fold enrichment, although not significant (Fisher's exact test, $P = 0.31$, odds ratio = 1.42), of genes with these effects on the X-chromosome of hybrid males (**Fig. 4H**). Furthermore, transgressive expression on the X-chromosome in F_1 males should be driven by enhancing and/or compensatory *cis-trans* changes, for which we observed significant enrichments on the X (**Fig. 4H** lower panel; Fisher's exact test, $P < 0.05$). Together, these results show that, although there are fewer X-linked spermatogenesis genes compared to autosomes (**Fig. 4G**), they show high levels of X-linked expression dysfunction (61%, 37 genes of 61 X-linked genes). In addition, it suggests that modest number of key genes may dictate X-autosome incompatibilities and X-linked misregulation and misexpression with effects on hybrid male fertility.

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Genome architecture and molecular evolution moderately affect regulatory divergence

Given that protein-coding sequence evolution and gene composition vary non-randomly along chromosomes in many *Caenorhabditis* species in association with the chromosomal recombination landscape, we asked whether distinct chromosomal domains would also associate with the degree of *cis*-regulatory divergence. We find higher molecular divergence between the genomes of *C. briggsae* and *C. nigoni* in chromosomal arms compared to centers in noncoding sequences upstream of protein-coding genes (**Fig. 5A**), in addition to protein-coding sequence divergence (**Supplementary Fig. S5**; also see (45)). These observations are consistent with the idea of stronger purifying selection on mutations to genes and their *cis*-regulatory regions when linked to chromosome centers. Despite the elevated molecular divergence in arm regions (**Fig. 5A**), we only found modest elevation of ASE divergence for genes on arms, being strongest for chromosome V for both sexes (**Fig. 5B**). Moreover, we found no significant differences in the magnitude of regulatory divergence, in either *cis* or *trans*, between chromosome arms and centers (**Fig. 5C**; *cis*-only OLS, male P value = 0.51, female P value = 0.62; *trans*-only OLS, male P value = 0.18, female P value = 0.44). Similarly, we observed only a weak positive correlation between ASE divergence and rates of molecular evolution (**Fig. 5D-F**, linear regression for K_a/K_s': adjusted R = 0.019, m = 0.015, $P < 0.0001$; K_a: adjusted R = 0.023, m = 0.006, $P < 0.0001$; 1-P_{cons}: adjusted R = 0.003, m = 0.01, $P < 0.0001$). Overall, these patterns indicate that rates of divergence for gene expression and their *cis*-regulatory controls are largely decoupled from protein-coding sequence evolution.

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Discussion

Regulatory control over gene expression is an important component of phenotypic evolution (13). As species diverge and accumulate mutations, selection will permit regulatory changes that maintain transcript levels as well as changes that allow exploration of new phenotypic space when they confer a fitness advantage. Sexual selection and sexual conflict can further promote such genomic divergence, both in terms of molecular evolution (e.g., rapid coding or regulatory sequence evolution for male-biased genes) and in terms of gene expression (e.g., divergence in sex-biased gene expression levels) (36,57,58). In interspecies hybrids, sexually driven sources of genomic divergence can disrupt gene networks to create negative epistatic interactions that manifest as sex-biased hybrid sterility or inviability and generate reproductive isolation. Here we document extensive regulatory divergence in the face of both conserved and divergent gene expression, with prominent influences of sex-biases and genomic location on the potential to induce misexpression in interspecies hybrids.

Symmetry in species- and sex-specific ratios of differentially expressed genes implicates extensive developmental system drift

C. briggsae and *C. nigoni* acquired substantial divergence at the DNA level since they diverged from their common ancestor ~35 million generations ago (3.5 Mya assuming 10 generations per year), including ~20% sequence divergence for synonymous sites, changes to genome size, and disproportionate loss of short male-biased genes in *C. briggsae* since its transition in reproductive mode to androdioecy (45,51). Despite this genomic divergence, hybridization between these species yields viable and fertile F_1 females, while hybrid males suffer complete sterility and severe inviability depending on the cross direction (34,46). While in some systems, such as fruit files and plants, hybrids can exhibit an expression bias towards the parental species tending to show higher expression (16,18,23), we observe no such effect (**Fig. 1B**; Fisher's exact test, *P* value = 0.76). This symmetry in expression suggests that demographic effects do not bias regulatory changes toward either increased or decreased expression in this system, as could occur if regulatory changes fix more rapidly in species like *C. briggsae* with

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lower effective population sizes. However, autosomes tend to have more genes with slightly higher expression in *C. nigoni* whereas X-linked genes tend to have much higher expression in *C. briggsae*. This chromosomal pattern implicates a disproportionate role for divergence of the X-chromosome in mediating misexpression in *Caenorhabditis* hybrids.

Despite our evidence of substantial expression divergence, 34% of genes in females (4,212 genes) and 47% of genes in males (5,724 genes) show no differential expression between species. Stabilizing selection on transcript abundance is recognized as a common force acting to conserve expression levels between species (8–10,12). Mechanistically, conservation of the expression phenotype can occur, despite sequence evolution, when co-evolution changes both *cis*- and *trans*-regulatory elements: if a *trans*-acting mutation fixes due to a pleiotropic benefit on other loci, selection would favor fixation of any subsequent compensatory mutation in *cis* that returns expression to optimal levels at the focal locus (5,59). We find evidence of extensive compensatory *cis-trans* divergence in gene regulation between *C. briggsae* and *C. nigoni*. Such coevolution represents just one mechanism leading to "developmental system drift," in which the molecular controls over developmental pathways can diverge while resulting in little or no change to their phenotypic outputs (4,60,61). In *Caenorhabditis* nematodes, developmental system drift and stabilizing selection have been invoked as mechanisms leading to a high degree of phenotypic stasis and morphological constraint (62–65). Gene network conservation despite *cis*-regulatory divergence has been demonstrated by inter-species promoter swaps in *Caenorhabditis*, showing both robustness in regulatory networks and new functionalization in specific cell types (20,66,67). Our results overall support these views.

However, sequence divergence and developmental system drift in regulatory pathways occurring after speciation are expected to resolve deleterious transcriptomic changes differently and independently among diverging lineages. In hybrids, uncoupled regulatory mechanisms from the two parental genomes can lead hybrids to experience misregulation and therefore misexpression (6). Such a mechanism underlying misexpression could represent a Dobzhansky-Muller incompatibility because genetic interactions untested by natural selection

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will likely be detrimental (1). The clearest signal of misexpression in hybrids is the sharp contrast in the fraction of sex-biased genes: ~90% in hybrids vs ~50 in each parental species (**Fig. 1D**). Moreover, the usual depletion of female-biased gene expression from *Caenorhabditis* X-chromosomes is even more extreme in F_1s due in part to transgressive underdominance effects and, unusually, the X-chromosome is highly enriched for male-biased expression in F_1s (**Figs. 1E, 2C,D**) (40,41). The strong downward misexpression (underdominance) observed for the X in females, but not as strong for the X of males is likely to be responsible for this trend. In combination with our analyses showing extensive compensatory *cis-trans* regulatory divergence, these results implicate extensive developmental systems drift of genetic networks between *C. briggsae* and *C. nigoni*.

Sex-specific and chromosome-dependent cis- and trans-regulatory changes contribute to differences in F1 hybrid expression inheritance between sexes

Abundant transgressive expression is a signature of rampant misexpression in F_1 hybrids. In particular, studies in flies and mice have shown that misexpression of X-linked genes confers male sterility in F_1 interspecies hybrids (24,27,28,68), which contributes to more severe hybrid male dysfunction (Haldane's rule). *C. briggsae* × *C. nigoni* hybrids also obey Haldane's rule (46–48), so we expected more misexpression in hybrid males. In contrast, we find that it is hybrid females that experience more extensive transgressive misexpression of genes across the genome that exceed the expression extremes of either parental species (**Fig. 2A,C**). Most female-biased transgressive genes show overdominant misexpression whereas male-biased transgressive genes tend toward underdominant misexpression (M8-M12 vs M3-M7, **Fig. 4D**). Excluding spermatogenesis genes (most of which are male-biased, but also expressed in *C. briggsae* hermaphrodites; **Fig. 4F**), it is plausible that *cis*- and/or *trans*-regulatory changes acquired after speciation favoring female-biased expression experienced selection to sustain upregulation, behaving in an overdominant manner in hybrids. Indeed, overdominant genes with *cis-trans* divergence in females have disproportionately evolved "enhancing" regulatory changes (**Figs. 2G, 3, 4E**). The low magnitude of expression divergence among overdominant and female-biased genes (**Figs. 2B, 4B**), however, together with the fact that hybrid females are

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448 fertile, suggests that overdominant expression does not impact fitness as negatively as does
449 regulatory divergence that leads to underdominance in hybrids.
450
451 Interestingly, we find that regulatory controls suppressing or enhancing male-biased expression
452 in F_1 females is largely due to *trans*-only regulatory changes, particularly among genes that
453 show both male-biased expression and expression divergence between the parent species (**Fig.**
454 **4E**). Many of these *trans*-acting regulatory changes tend to be more strongly associated with *C.*
455 *nigoni* dominant expression in females among autosomes, contrasting with *cis*-regulatory
456 changes which are more strongly associated with *C. briggsae* dominant expression in males
457 (**Fig. 3**). These results align well with observations of downregulation of spermatogenesis genes,
458 such as *fog-1*, by specific transcription factors (i.e., *tra-1*), and sperm-specific expression
459 depending more on upstream promoter regions than 3-UTRs in *C. elegans* (69,70). One
460 potential explanation involves the fixation of regulatory changes that facilitate resolution of
461 genomic inter-sexual conflict through sex-biased expression (i.e., Rice's hypothesis (71)). For
462 example, more sexual conflict is expected in outcrossing than selfing species, such as *C. nigoni*,
463 due to stronger sexual selection of male traits (72). To avoid traits that are detrimental to
464 females but improve male performance, genomic conflict resolution by means of sex-biased
465 expression may be attained faster through *trans*-regulatory changes, which are more
466 pleiotropic, downregulating male-biased genes in females. This logic aligns with the hypothesis
467 that sex-biased expression is partly driven by selection acting to resolve sexual conflict by
468 means of modifier alleles or regulators (55,71). However, the fact that *trans*-only regulatory
469 changes do not predominate in the control of female-biased genes in males (**Fig. 4E**), suggests
470 that regulatory mechanism to resolve genomic sexual conflict act in different ways for the two
471 sexes.
472
473 Several studies associate regulatory divergence in *cis*-acting factors with genome-wide
474 expression profiles that enhance sex-specific traits and sex-biased expression (55,73,74). Our
475 analyses are consistent with these observations in terms of the higher proportion of genes
476 under *cis*-only compared to *trans*-only regulatory divergence among genes with significant sex-

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biased expression in their respective sex (i.e., male-biased in males; **Fig. 4E**). When also considering genes with *cis-trans* divergence, however, we see that a large portion of sex-biased genes show significant *trans*-divergence (**Fig. 4E**; **Supplementary Fig. S6**), highlighting the importance of both *cis* and *trans* effects for the development of sexually dimorphic traits in *Caenorhabditis*.

The abundance of underdominant genes in hybrid females, while perhaps counterintuitive, is not surprising in this system given that the egg-bearing sex in the *C. briggsae* parent is actually a hermaphrodite. Many of the genes in hybrid females that show underdominant effects would otherwise show male-biased expression (**Fig. 4B**), suggesting that they may compromise spermatogenesis to effectively convert F_1 hermaphrodites into females; a complementary view to the idea that hermaphroditism is 'recessive' to femaleness in a simple Mendelian manner (46).

The role of faster evolution in male-biased genes and autosome vs X-chromosome incompatibilities in hybrid male dysfunction

Because of sexual selection and sexual conflict, male-biased genes are expected to evolve faster, resulting in higher rates of protein-coding and gene expression divergence (36,57,58). Faster evolution of male-biased genes is the premise behind the "faster male" and “fragile male” hypotheses to explain the high incidence of hybrid male sterility in XY and X0 heterogametic systems (31). While we find that male-biased genes collectively do not show a strong signal of faster molecular evolution, the subset of male-biased genes that show exceptionally high expression divergence do have faster evolving coding sequences (M4 and M6; **Fig. 4A-C**). Additionally, these genes are implicated in spermatogenesis, based on *C. elegans* orthologs, and show upregulated expression in sperm-producing *C. briggsae* hermaphrodites as well as males of both species (M6; **Fig. 4F**). These findings accord with faster molecular evolution of spermatogenesis and male germline genes of *C. elegans* (75–77). Their rarity on the X-chromosome (**Fig. 4G**), however, suggests the "faster X" model does not provide a compelling explanation for Haldane's rule on hybrid sterility (32). Nevertheless, our

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506 transcriptome analyses support the idea that the X-chromosome plays an especially important
507 role in hybrid male sterility. This "large-X" effect arises despite just a few highly dysfunctional X-
508 autosome incompatibilities between *C. briggsae* x *C. nigoni* potentially explaining hybrid male
509 sterility (52,53), in contrast to the numerous X-linked hybrid male sterility factors reported for
510 *Drosophila* (35,37).
511
512 Gene expression in hybrid males predominantly shows either simple dominance or no change
513 (**Fig. 2C**). While it is tempting to speculate that regulatory changes affecting males tend to be
514 generally more conserved as males of different species share the same reproductive role,
515 reduced sexual selection in *C. briggsae* males (77), genomic divergence (50,51), and clear sex
516 differences in hybrid fertility (46,47), suggest otherwise. If most transgressive expression occurs
517 in the gonad, then the small and defective gonad development of F_1 males may have led to
518 their observed paucity of transgressive expression. Two non-mutually exclusive ways in which
519 hybrid male dysfunction (i.e., sterility) can arise are: 1) through misexpression and
520 misregulation of X-linked genes involved with male function, and 2) through negative epistatic
521 interactions (i.e., incompatibilities) between X-linked and autosome genes involved in male-
522 specific pathways. Our results suggest that both cases are plausible.
523
524 First, the paucity of X-linked sex-biased genes in parental genotypes of *Caenorhabditis* species
525 suggests that any misregulation and misexpression on the X might exert little downstream
526 impact (**Fig. 1E**; (40,41)). However, misexpression of X-linked genes in hybrids is relatively
527 common compared to autosomes in both sexes (**Fig. 2D**), with hybrid males having higher
528 relative incidence of effectively misregulated genes (*trans*-only, enhancing and compensatory
529 *cis*-*trans* changes) compared to female hybrids (**Fig. 2H**). We find that *trans*-acting factors often
530 contribute to misexpression in both sexes (**Figs. 2H, 3**). In hybrid females, *trans*-acting factors
531 largely drive the expression of X-linked genes with *C. briggsae* dominant and underdominant
532 expression, unlike autosomes (**Fig. 3**). In hybrid males, this effect is even more pronounced, in
533 part due to our inference that all X-linked genes with *C. briggsae* dominant expression arise
534 from *trans*-only effects. These findings are consistent with previous observations, particularly in

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Drosophila, of *trans*-acting changes sex-specific causing misregulation of X-linked genes (26,74,78).

Second, we find extensive expression dominance in F_1 males that disproportionately matches the *C. briggsae* expression level and that have strong *cis* effects (**Fig. 3**). Many of these genes also have biased expression of *C. briggsae* alleles (**Supplementary Fig. S7**) and therefore have the potential of disrupting gene networks as they may interact negatively with *C. nigoni* X-linked genes in hybrid males. Autosomal spermatogenesis genes, by contrast, tend to show *C. nigoni*-dominant expression in F_1 hybrid males (mean of 2.17-fold difference across autosomes relative to genes with *C. briggsae*-dominant expression) (**Fig. 4G**), consistent with previous work showing recessive effects of the *C. briggsae* autosomal portions of genetic incompatibilities (52). In addition, this prior work also showed that sterility in *C. nigoni* x *C. briggsae* hybrid males may not require many X-autosome incompatibilities (53). Despite their low abundance on the X-chromosome, X-linked spermatogenesis genes are often enriched for both misexpression and misregulation (**Fig. 5C**), potentially enhancing their role in hybrid dysfunction. Interestingly, we find that at least three X-linked spermatogenesis genes that are effectively misregulated (i.e. with *trans*-only, *cis-trans* enhancing and *cis-trans* compensatory regulatory changes) also have high rates of molecular evolution (**Supplementary Fig. S8**) and lie near to an X-chromosome segment implicated previously in hybrid male sterility (53). Our genome-wide transcriptome analysis of *cis*- and *trans*-regulatory divergence therefore reinforces some previous key inferences about hybrid dysfunction associated with males, spermatogenesis, and the X-chromosome.

Modest *cis*-regulatory divergence in spite of fast evolving chromosomal arms

Marked differences in recombination rates along chromosomes can modulate the rate and number of mutations fixed by direct selection as well as linked selection (79). *Caenorhabditis* nematodes have distinctive chromosomal arm regions with higher rates of molecular evolution and polymorphism compared to central regions (**Fig. 5A**; **Supplementary Fig. S5**; (45,80)), and therefore have a greater potential to facilitate fixation of weakly beneficial *cis*–regulatory

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564 mutations. We first predicted no effect of genomic region on differences in *trans* regulatory
565 divergence and confirmed this null expectation. *cis*-regulatory divergence, however, showed
566 only a weak elevation in chromosome arms compared to centers, with the signal being
567 somewhat stronger in females and for some autosomes (**Fig. 5B,C**). We further looked at
568 broader correlations for both coding and non-coding upstream sequence divergence with *cis*
569 regulatory divergence (**Fig. 5D**) and found only weak positive correlations. In line with previous
570 studies (81,82), these results indicate that rates of regulatory divergence due to *cis*-acting
571 elements are largely decoupled from rates of molecular evolution.

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Conclusion

We contrasted sex-specific transcriptomic profiles between *C. briggsae* and *C. nigoni* and their hybrids to understand how the evolution of *cis*- and *trans*-regulatory elements can drive F_1 hybrid dysfunction. Such evolution may arise from divergent expression changes as well as with stabilizing changes that lead overall expression to remain conserved between species. The sharp contrast of *Caenorhabditis* morphological stasis and extensive expression conservation between species with extensive misexpression in F_1 hybrids indicates substantial developmental system drift of regulatory networks that destabilize in hybrids to enforce reproductive isolation between species. Despite more extensive transgressive expression in hybrid females, they are fertile but unable to produce self-sperm compared to the entirely sterile hybrid males, suggesting that hybrid females may represent "demasculinized" hermaphrodites through the disruption of sperm-specific regulatory networks. Despite the rarity of sex-biased genes on the X-chromosome, the X is home to disproportionate misexpression in both sexes, with misregulation in hybrid males largely through *trans*-acting factors. X-autosome incompatibilities in hybrid males likely result from the propensity for *C. briggsae*-dominant autosomal expression via *cis*-acting factors yield allele-specific expression biases, and then interact negatively with *C. nigoni* X-linked genes. Moreover, *C. nigoni*-dominant *trans*-acting factors may act to downregulate male-biased genes in females, supporting the idea of genomic sexual conflict resolution through modifier alleles (71). Finally, we find only weak correlations of *cis*-regulatory divergence with chromosome architecture and protein-coding and non-coding sequence divergence, indicating that regulatory and protein evolution are largely decoupled. Consequently, regulatory and structural Dobzhansky-Muller incompatibilities may accumulate independently of one another, and in distinct ways in the regulatory networks of each sex, in the build-up of reproductive isolation in the speciation process.

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Material and Methods

Samples, RNA isolation, and sequencing

We cultured triplicate populations of isofemale *C. briggsae* (AF16) and *C. nigoni* (JU1421) on NGM-agar plates with *Escherichia coli* OP50 at 25°C, isolating total RNA via Trizol-chloroform-ethanol extraction from groups of approximately 500 individual age-synchronized young adult males or females (hermaphrodites) for each replicate sample. *C. briggsae* hermaphrodites are treated as the female sex for the purposes of this study, as their soma is phenotypically female despite the gonad producing a small number of sperm in addition to abundant oocytes. We also crossed in triplicate virgin *C. nigoni* females to male *C. briggsae* (isolated as L4 larvae) to produce F_1 hybrid progeny, with RNA isolated from male and female F_1 hybrids as for the parental pure species genotypes.

The triplicate mRNA samples for each sex and genetic group (*C. briggsae*, *C. nigoni*, F_1 hybrid) underwent 100bp read length, single-ended Illumina HiSeq sequencing at GenomeQuebec according to their standard TruSeq3 protocol. A total of ~250 million reads from these 18 barcoded samples spread across 4 lanes were cleaned for quality control using TRIMMOMATIC v0.38 (with arguments: ILLUMINACLIP:TruSeq3-SE:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36) (83).

Reference alignment and allele-specific read assignment

Following quality control trimming and filtering, we mapped sequence reads from each sample to the chromosome-level genome assembly and annotation of each species (*C. briggsae* WS271 https://osf.io/a4e8g/, *C. nigoni* 2018-01-WormBase https://osf.io/dkbwt/; (51)) using STAR v2.6 (https://github.com/alexdobin/STAR; (84)) with default parameters and adjusting for intron size (--alignIntronMin 40 --alignIntronMax 15600). Reads for all three genotype groups (*C. briggsae*, *C. nigoni*, F_1 hybrid) were mapped to both reference genomes.

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To obtain allele-specific read counts in F_1 hybrids, we applied a competitive read mapping approach using a custom Python script (https://github.com/santiagosnchez/CompMap) that uses the PYSAM library (https://github.com/pysam-developers/pysam). We then compared the alignment score (AS) and number of mismatches (nM) to both reference genomes, retaining the best single read alignments and excluding ambiguous reads (i.e., alignments with the same value in both parents). We have high power to detect ASE, given ~20% neutral sequence divergence between *C. briggsae* and *C. nigoni* (45) that confers an expected ~5 nucleotide differences for every 100 bp of coding sequence (0.2 divergence * 0.25 fraction of synonymous sites * 100 bp). To account for potential mapping bias (85) and unaccounted ambiguous reads, we subjected all samples to competitive read-mapping (hybrids and pure species) and retained only unambiguously mapped reads.

Ortholog identification and read abundance quantification

We quantified gene expression abundance for a set of 12,115 genes that we inferred to be one-to-one reciprocal orthologs between *C. briggsae* and *C. nigoni*. To identify orthologs, we applied a phylogenetic approach using ORTHOFINDER v2.2.6 (86,87), based on longest-isoform peptide sequence translations for gene annotations of 28 *Caenorhabditis* species (88) (http://caenorhabditis.org/). BLASTp all-by-all searches were done separately on SciNet's Niagara supercomputer cluster. ORTHOFINDER was run with default options, which included: -M dendroblast (gene tree reconstruction) and -I 1.5 (MCL inflation point). In further analysis of the final set of 12,115 orthologs, we excluded from a preliminary set of 15,461 orthologs those genes for *C. briggsae* and *C. nigoni* that could not be assigned to any of their six chromosomes (688 genes), that were associated with inter-chromosomal translocations (370 genes), that we could not estimate K_a/K_s reliably (275 genes), or that exhibited low mRNA-seq read abundance (2013 genes; see below).

We quantified gene expression with HTSEQ-count v0.11 (89) for each ortholog in each species, ignoring strand-specific, non-unique, secondary, and supplementary alignments (arguments: -s no --nonunique none --secondary-alignments ignore --supplementary-alignments ignore). Raw

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read counts were combined into a single table and imported into R (90) for normalization and statistical analyses.

Differential expression analyses: contrasts between species, hybrids, and sexes

We used the R Bioconductor packages LIMMA (91) and EDGER (92) to assess differential expression. Gene-level raw expression counts were first normalized with EDGER based on library size using the 'trimmed mean of *M*-values' (TMM) method with the *calcNormFactors* function. Genes were then filtered based on the amount of expression using EDGER's *filterByExpr* function (93). Expression counts were then $\log_2$-transformed using the *voom* mean-variance trend method to ensure consistent, normalized read counts across samples (94). Before statistically assessing differential expression, we summed the allele-specific counts from F_1 hybrids to yield a single count of transcripts per gene. We visualized the overall expression distance between samples using a non-metric multi-dimensional scaling plot, which showed all three biological replicates to cluster consistently within their corresponding treatment (Fig. 1A). We inferred sex-biased gene expression by comparing differential expression profiles between males and females (or hermaphrodites) in each genetic group (*C. briggsae*, *C. nigoni*, F_1 hybrids). We also quantified differential expression between the genetic groups in a pairwise manner (*C. briggsae* vs F_1, *C. briggsae* vs *C. nigoni*, *C. nigoni* vs F_1) for each sex separately. We then contrasted expression patterns between species (*C. briggsae* and *C. nigoni*) by looking at sex differences (sex-biased expression) and their interaction (expression ~ species * sex). Linear regression models were applied to make statistical inferences on differential expression with the *lmFit* and *eBayes* function in the LIMMA package with FDR = 0.05 for multiple test correction.

Mode of expression inheritance in F_1 hybrids

Based on patterns of expression in F_1 hybrids relative to parent species, we classified genes into those having **additive** (intermediate), **dominant** (matching either of the species), **overdominant** (higher that both parents), and **underdominant** (lower than both parents) profiles (16) (McManus et al. 2010). Genes with no significant differences in expression between F_1s and their parent species were deemed to have conserved regulatory controls resulting in **no change**

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in expression in F_1s. Genes with additive effects had intermediate expression in F_1s compared to both parental species, meaning that there were significant differences in expression between all groups in a manner where expression levels in F_1s fall in between both species. Genes with dominant allelic effects were those with expression levels in F_1s matching either one of the parent species (i.e. no significant differential expression), but with significant differential expression between species. Finally, genes with significant differential expression from both parents, but that were either significantly underexpressed (overdominant) or overexpressed (underdominant) compared to both species were regarded as transgressive. Genes falling outside any these specific categories were considered **ambiguous**.

We also measured absolute Euclidean distances in expression relative to the centroid or origin in expression space of F_1 hybrids relative to both parent species. For example, for every gene we took the expression difference between F_1s and *C. briggsae* and between F_1s and *C. nigoni* as an xy coordinate system. Then, we measured the Euclidean distance from that point in expression space to the origin (0,0), reflecting no change in expression:

$$d = \sqrt{(\Delta_{F1/Cbr})^2 + (\Delta_{F1/Cbr})^2}$$

Where $\Delta_{F1/Cbr}$ and $\Delta_{F1/Cni}$ are coefficients of differential expression between F_1 hybrids and each parent species. This metric allowed us to visualize the magnitude of expression distance from a "conserved" expression profile.

cis- and *trans*- regulatory divergence

We also used ASE in F_1s to quantify the extent and type of *cis*- and *trans*-regulatory differences between species. Expression divergence between parent species results from both *cis*- and *trans*-regulatory changes, whereas significant differential expression between alleles in F_1s results from *cis*-regulatory divergence only (16). To quantify the extent of *trans* effects, we applied a linear model to test for differences in gene expression between parent species (P) and between alleles in F_1 hybrids (ASE) using the following model: expression ~ species/group,

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where "group" represented categorical variables pointing to data from P and ASE. The division operator of the function "/" measures expression ratios independently for each category in "group". We then used a post-hoc Wald-type test (*linearHypothesis* from the CAR package) to test for significant differences between both coefficients (**P**[*C. nigoni*/*C. briggsae*] = **ASE**[*C. nigoni*/*C. briggsae*]). *P* values were considered significant after a 5% FDR analysis (95).

We inferred the influence of *cis*- and *trans*-regulatory divergence on genes linked to autosomes, as well as to the X-chromosome in females, following the criteria in McManus et al. (16). This procedure allowed us to designate genes having undergone significant regulatory divergence due to ***cis*-only**, ***trans*-only**, and **compensatory *cis-trans*** effects. Genes with either significant synergistic (*cis* + *trans*) or antagonistic (*cis* x *trans*) *cis-trans* effects were grouped together as representing changes with **enhancing *cis-trans*** effects. Genes expressed with no significant differences between parents, ASE, or *trans* effects were deemed as **conserved** and those that did not strictly fit into any of the previous groups were considered **ambiguous**.

Given the hemizygous condition of the X-chromosome in males, we cannot use F_1 ASE of X-linked genes in males to assess *cis* and *trans* regulatory divergence. However, we devised a scheme to assign different types of regulatory divergence to X-linked genes given differences in expression between male F_1 hybrids and parent species (Supplementary Fig. S3; Wayne et al. 2004). Given that F_1 males' X-chromosome derives solely from their maternal *C. nigoni*, X-linked genes that differ in expression between the parental species and showing *C. nigoni* dominant expression in hybrid males were considered as having significant *cis*-only effects, as differences in the *C. briggsae trans* autosomal environment did not lead to significant deviations from *C. nigoni* expression. Alternatively, X-linked genes in hybrid males found to be *C. briggsae* dominant reflect significant *trans*-only effects. This implies two things: 1) that X-linked *cis* elements in *C. nigoni* are not sufficiently different from their counterparts in *C. briggsae* to prevent *C. briggsae trans* regulators from acting on them; and 2) that *C. nigoni trans*-regulators on those pathways are potentially recessive. Supporting this assignment scheme, X-linked genes with *cis*-only effects on regulatory divergence have significantly higher rates of molecular

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739 evolution compared to genes with *trans*-only effects (Supplementary Fig. S2; ordinary linear
740 regression *P* value = 0.05 for K_a/K_s and *P* value < 0.05 for the proportion of conserved 5 bp
741 windows 500 bp upstream). Consequently, we inferred compensatory *cis-trans* effects for X-
742 linked genes in males where expression was not significantly different between parent species,
743 but significantly different from F_1 hybrid males. Lastly, we inferred significant enhancing *cis-*
744 *trans* effects for those X-linked genes with intermediate (additive) expression in F_1s, or with
745 significant differential expression between parent species coupled to significantly higher or
746 lower expression in F_1s than in both parent species (Supplementary Fig. S3; (26)).
747

748 ## Molecular evolution in coding sequences

749 Orthologs in the genomes of both *C. briggsae* and *C. nigoni* were first aligned as protein coding
750 sequences using MAFFT v7.407 (96). These alignments were then back-translated to coding
751 sequence (CDS) alignments using the python program CODONALIGN
752 (https://github.com/santiagosnchez/CodonAlign). We estimated rates of synonymous site (K_s)
753 and non-synonymous site divergence (K_a) between the two aligned sequences using a custom
754 Python script (https://github.com/santiagosnchez/DistKnKs) applying the Yang and Nielsen
755 (2000) model implemented in BioPython (Cock et al. 2009). We also corrected K_s values for
756 selection on codon usage using the effective number of codons (ENC; (97,98)) as a predictor in
757 a linear model. In short, we fitted a linear regression model (K_s ~ ENC), which we used to predict
758 K_s at the maximum value of ENC (=60). Then, we corrected the bias in K_s by adding the residuals
759 of the linear model to that idealized value of K_s at ENC = 60. We refer to these corrected set of
760 K_s estimates as K_s'.
761

762 ## Upstream non-coding sequence conservation

763 Chromosome-level FASTA sequences for *C. briggsae* and *C. nigoni* were aligned using LASTZ (99),
764 outputting alignment files for each chromosome in MAF format. We used BEDTOOLS's v2.27 (100)
765 *flank* function to generate 500 bp intervals of the 5' upstream flanking regions of each
766 orthologous gene. We then used *maf_parse* from PHAST (101) to extract overlapping alignment

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767 blocks of at least 500 bp long. We quantified sequence conservation as the average number of
768 identical 5 bp non-overlapping windows between aligned DNA in both sequences.
769

770 ## Spermatogenesis genes

771 To infer genes involved with spermatogenesis, we downloaded a list *C. elegans* genes
772 previously identified as spermatogenesis based on tissue-specific transcript abundance (42)
773 (Additional File 4). We then used the BioMart tool of the WormBase Parasite website (102) to
774 retrieve *C. briggsae* orthologs from the list of *C. elegans* genes. We cross-referenced *C. briggsae*
775 orthologs to our own set of orthologs between *C. briggsae* and *C. nigoni* and annotated the
776 1,089 gene matches with a spermatogenesis tag.
777

778 ## Data availability

779 Raw sequencing data is to be submitted to Short Read Archive upon acceptance under XXXXXX
780 accession number. Raw gene count data will be submitted to Gene Expression Omnibus. Scripts
781 detailing bioinformatic and analytical procedures will be hosted on GitHub
782 (https://github.com/santiagosnchez).
783

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Acknowledgements

We thank Katja Kasimatis and Karl Grieshop for comments on previous versions of the manuscript. Computations were performed on the Niagara supercomputer at the SciNet HPC Consortium. SciNet is funded by the Canada Foundation for Innovation, the Government of Ontario, the Ontario Research Fund - Research Excellence, and the University of Toronto. S.S.R. is supported by an EEB Departmental Postdoctoral Fellowship. A.D.C. is supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada.

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Figures

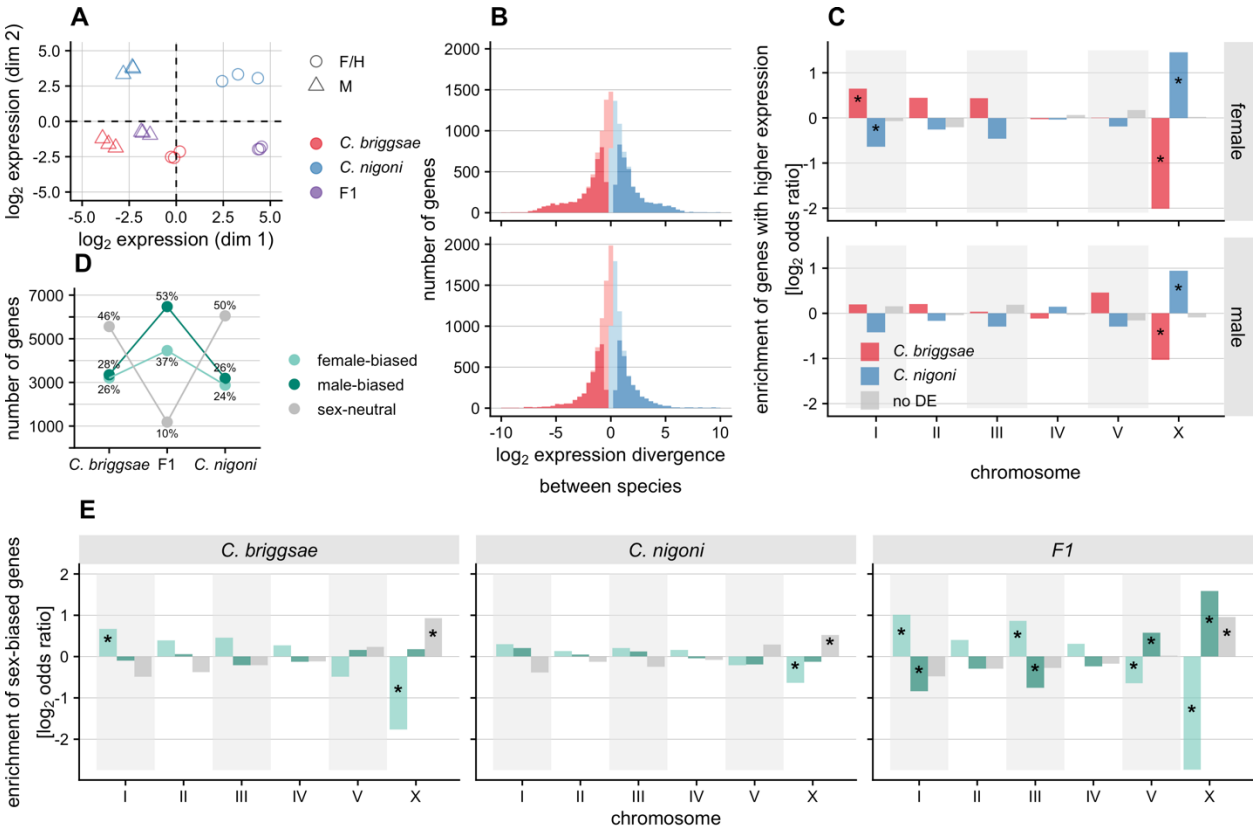

Figure 1. Incidences of differentially expressed genes for 12,115 orthologs between species and sexes. (**A**) Non-metric multidimensional scaling (MDS) plot showing $\log_2$ expression distances between samples. Sexes and species are well separated in expression space and hybrids present considerable distance from parental transcriptomic profiles (*C. nigoni* and hybrid female, F; *C. briggsae* hermaphrodite; male, M). (**B**) Histogram of $\log_2$ expression divergence (*C. nigoni*/*C. briggsae*) for female (top panel) and male transcriptomes (bottom panel). (**C**) Enrichment of differentially expressed genes between species for females and males ($\log_2$ odds ratio, i.e. observed/expected). Asterisks mark significant enrichments (positive values) or depletions (negative values) on chromosomes (*P* value < 0.05 and $|\log_2 \text{odds ratio}| > 0.5$). On the legend, "no DE" denotes genes that are not significantly differentially expressed. (**D**) Number and percentage of genes that show significant sex-bias is greater in F_1 hybrids than parent species. (**E**) Enrichment of genes with significant sex-bias and sex-neutrality in parent species and F_1 hybrids for each chromosome. Same legend as D and asterisks denote same statistics as C.

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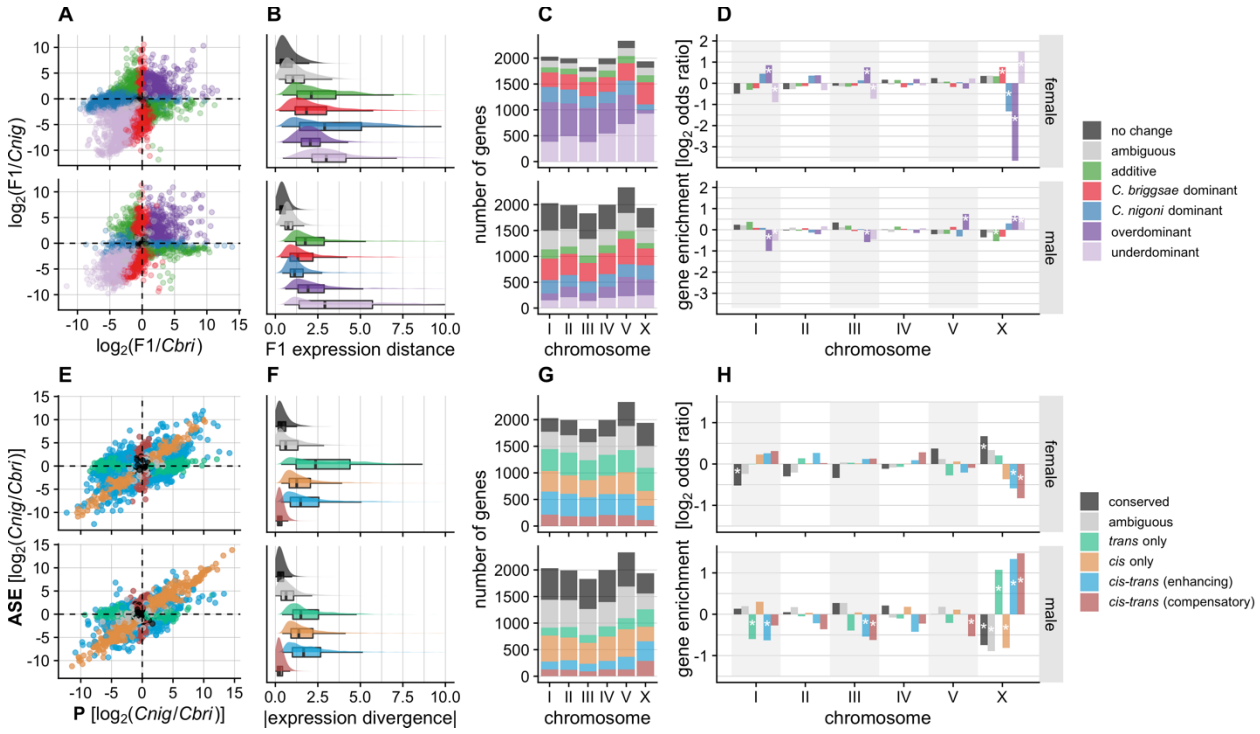

Figure 2. **Sex-specific differences in expression divergence and regulatory changes identify expression inheritance profiles between species**. (**A**) Per-gene biplot of $\log_2$ expression differences between F_1s and each parent species. (**B**) Box- and density plots of expression distance from the origin or centroid of F_1 hybrids for genes within a given expression inheritance category (see Materials and Methods). (**C**) Stacked barplot of gene counts in each expression inheritance group for each chromosome. (**D**) Per-chromosome enrichment ($\log_2$ odds ratio, i.e. observed/expected) of genes in a given expression inheritance group. Asterisks mark significant enrichments (positive values) or depletions (negative values) on chromosomes (*P* value < 0.05 and $|\log_2$ odds ratio$|$ > 0.5). (**E**) Biplot of expression divergence between species (x-axis) versus allele-specific expression (ASE) in hybrids that indicates the magnitude *cis*-acting expression difference between alleles (y-axis). (**F**) Box- and density plots of the magnitude of absolute expression divergence between species for each type of *cis* and *trans* regulatory changes. (**G**) and (**H**) as for C and D, but indicating different types of *cis* and *trans* regulatory-change profiles. In F, G, and H, X-linked genes in males followed the classification scheme described in the Methods section and in Supplementary Fig. S3. Colors indicate different groups of genes with different expression inheritance (see legend for A-D) and *cis* and *trans* regulatory changes (see legend for E-H). Top panels in each of A-H correspond to female transcriptomes, bottom panels to male transcriptomes.).

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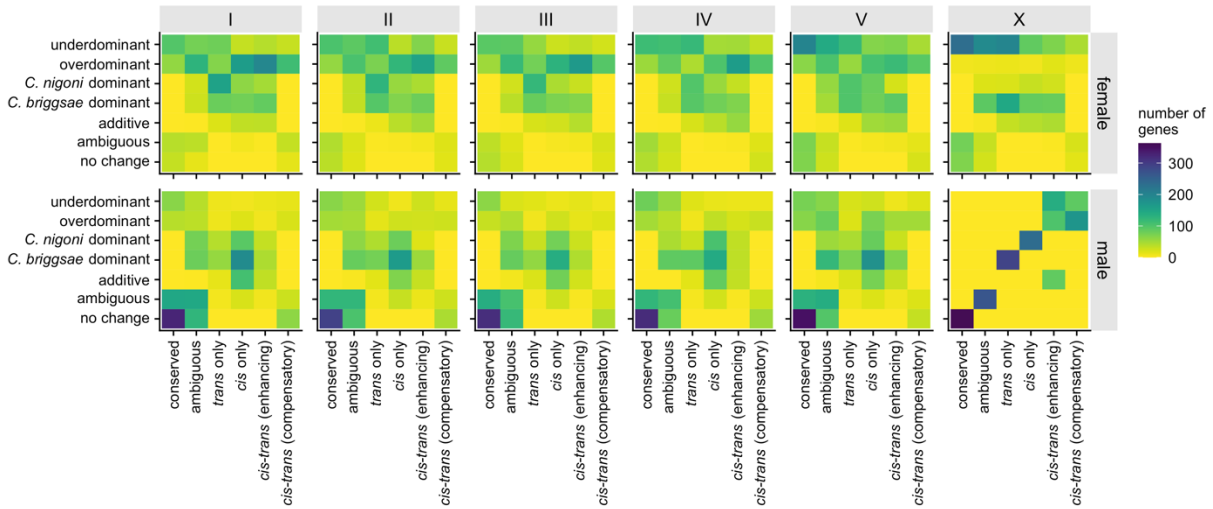

Figure 3. **X-autosome differences in regulatory controls between sexes underlie hybrid transcriptomic profiles relative to parent species**. Heatmap of the number of genes in each expression inheritance group (y-axis) for each type of *cis* and *trans* regulatory changes (x-axis) for each chromosome (I-V, X) and each sex. X-linked genes in males followed the classification scheme described in the Methods section and in Supplementary Fig. S3.

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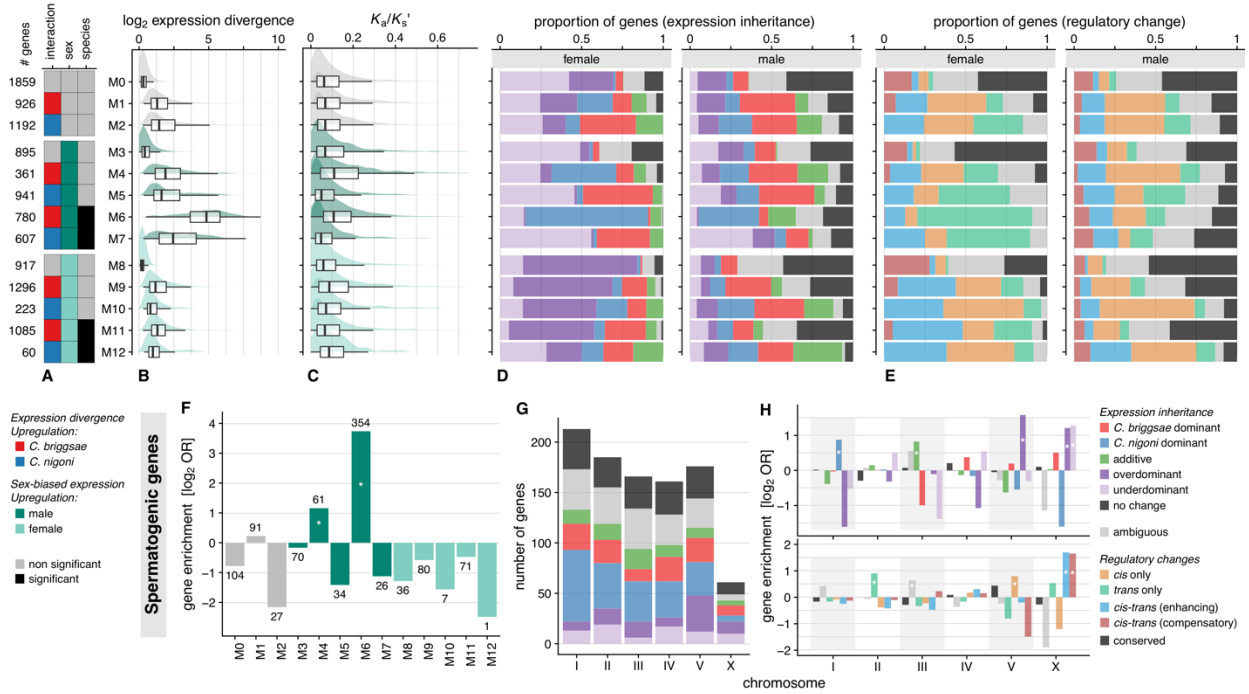

Figure 4. **Male-biased and spermatogenesis genes show higher expression divergence, molecular evolution, and sex-specific regulatory divergence**. (**A**) Thirteen distinct species-by-sex gene expression gene groups (M0-M12). Columns indicate significant expression divergence due to species (*C. briggsae* higher expression in red, *C. nigoni* in blue), sex (male dark green, female or hermaphrodite light green), or species-by-sex interaction (black); gray indicates no significant differential expression. (**B**) Absolute expression divergence and (**C**) protein sequence divergence (K_a/K_s') differs across the M0-M12 species-by-sex profiles. (**D**) Species Proportion of genes within each species-by-sex gene groups differ in the relative representation of expression inheritance categories and (**E**) types of *cis*- and *trans*-regulatory divergence, distinctly for females (left panels) and males (right panels). (**F**) Spermatogenesis genes are significantly enriched in male-biased gene groups M4 and M6 (orthologs of *C. elegans* genes in Ma et al. 2014; see Methods; *P* value < 0.05 and $|\log_2$ odds ratio$|$ > 0.5). (**G**) Spermatogenesis genes are rare on the X, with *C. nigoni* expression dominance in F_1s disproportionately common on autosomes. (**H**) Misexpressed and misregulated spermatogenesis genes are significantly enriched in the X chromosome.

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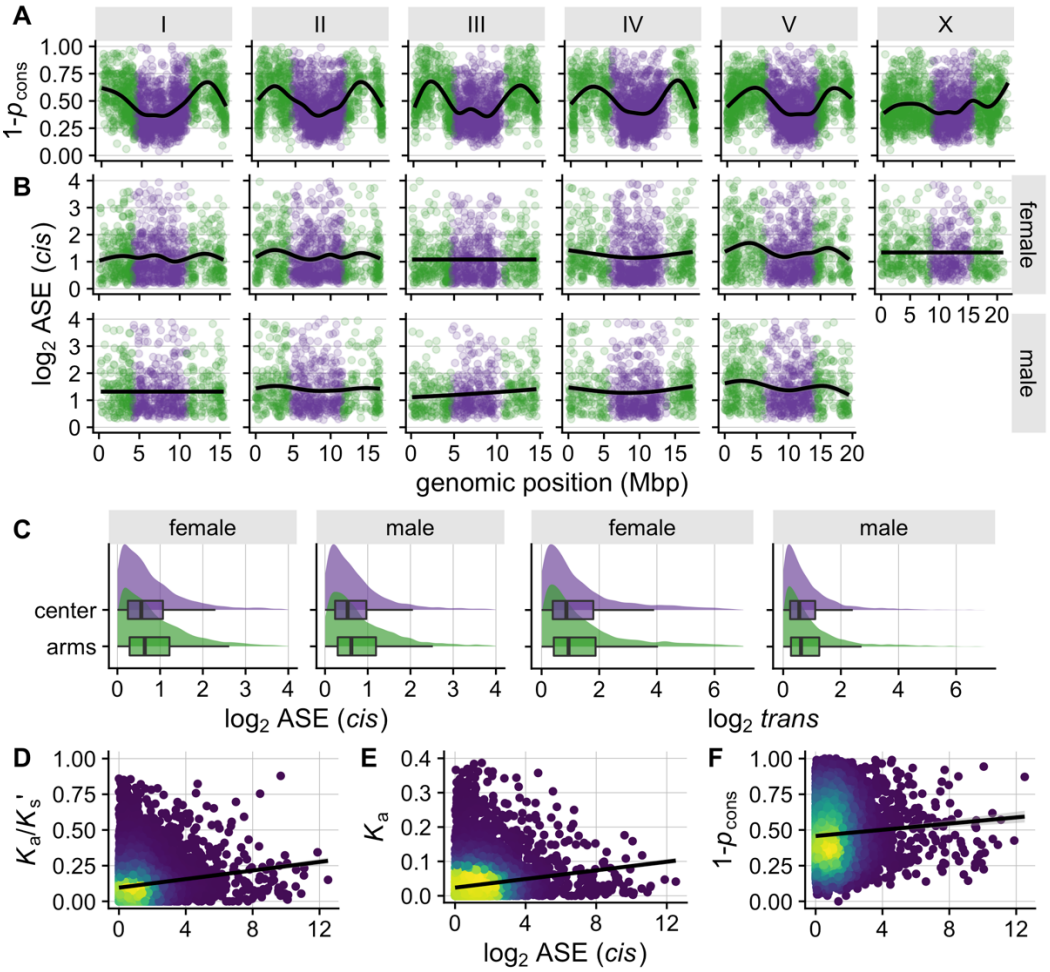

Figure 5. **Chromosomal arm (green) and center (purple) regions differ strongly in (A) upstream sequence divergence (1-P_{cons}; 5 bp windows within 500 bp upstream of each gene) but only moderately in (B) *cis*-regulatory divergence (log$_2$ allele-specific expression, ASE; females on top)**. (**A**) Proportion of non-conserved 5 bp windows within 500 bp upstream of each gene (1-P_{cons}) for each chromosome. (**B**) Absolute magnitude of log2 allele-specific expression or *cis*-regulatory divergence for each chromosome in females and for autosomes only in males. Arms and centers are colored with green and purple colors, respectively. Black lines indicate general additive regression (GAM) trendlines. (**C**) Arm and center regions do not differ greatly in magnitude of either box- and density plots of total (absolute) *cis*-regulatory divergence (left two panels) or *trans* regulatory divergence (right two panels) for either male or female gene expression. The magnitude of *cis*-regulatory divergence shows weak positive correlations with (**D**) rates of mutation-adjusted protein evolution (K_a/K_s', Spearman's $\rho = 0.068$, $P < 0.0001$; linear regression adjusted $R = 0.019$, $m = 0.015$, $P < 0.0001$), (**E**) overall protein evolution (K_a, Spearman's $\rho = 0.066$, $P < 0.0001$; linear regression adjusted $R = 0.023$, $m = 0.006$, $P < 0.0001$), and (**F**) non-coding upstream sequence evolution (1-P_{cons}, Spearman's $\rho = -0.0008$, $P > 0.05$; linear regression adjusted $R = 0.003$, $m = 0.01$, $P < 0.0001$). Color scale in D-F indicates density of points with brighter colors denoting higher density.

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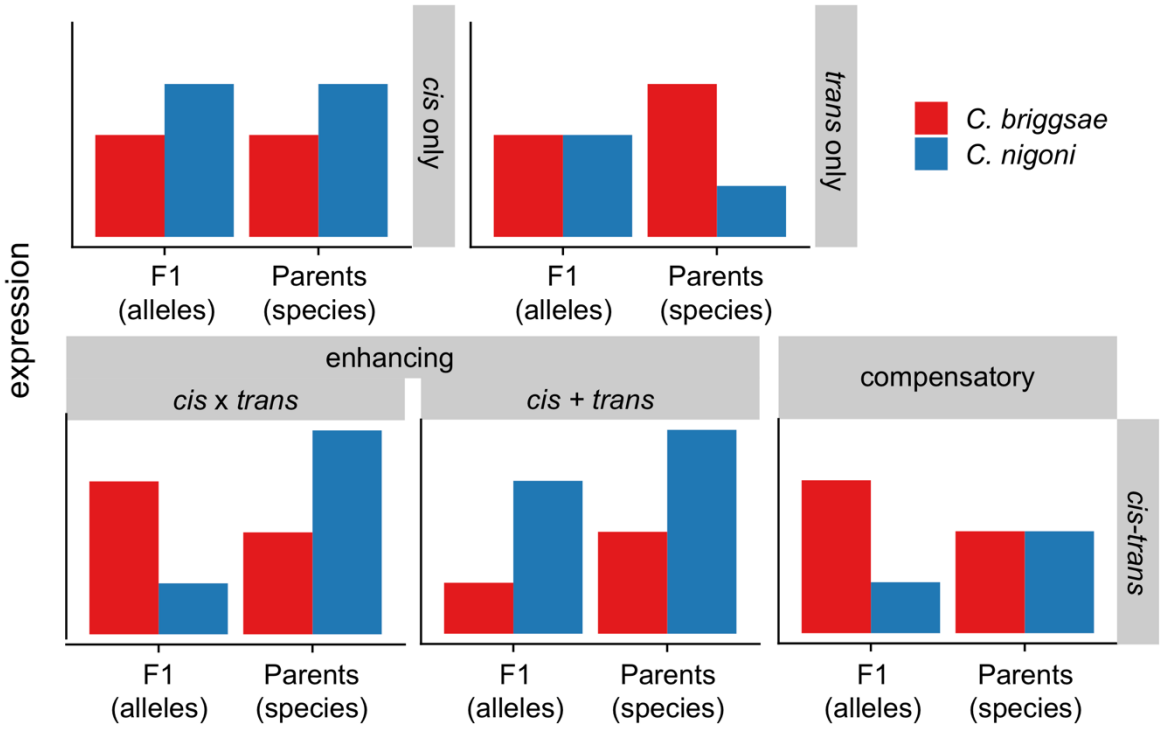
Supplementary figures



Supplementary Fig. S1. Qualitative examples of allele-specific expression with their classification of gene regulation type changes.

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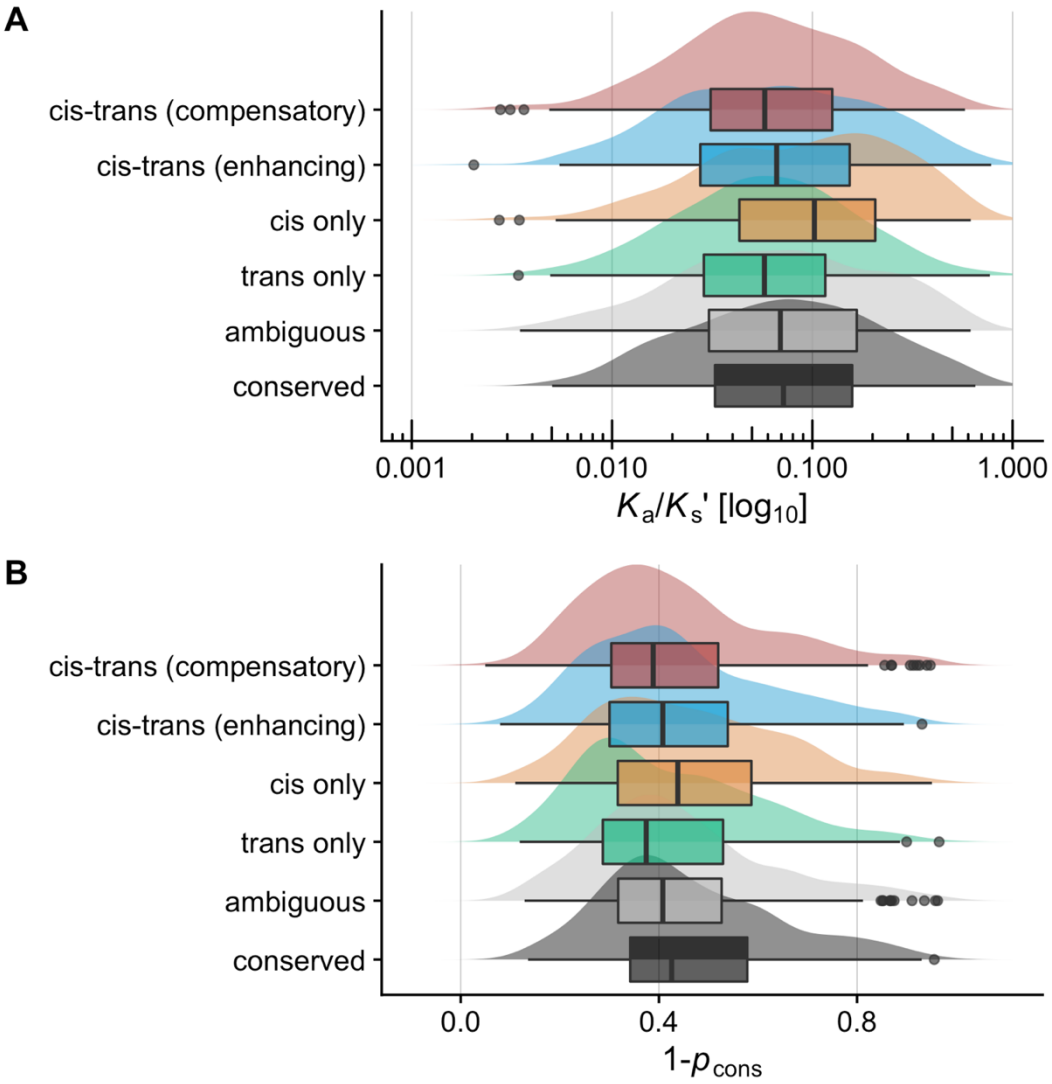

Supplementary Fig. S2. X-linked genes (n=1,939) with *cis*-acting effects have higher sequence divergence than other regulatory categories within (a) protein coding genes (K_a/K_s', ordinary least squares *cis*-only vs *trans*-only, T=2.02, $P < 0.05$) and (b) in upstream regions (1-p_{cons}, , ordinary least squares *cis*-only vs *trans*-only, T=2.48, $P < 0.05$).

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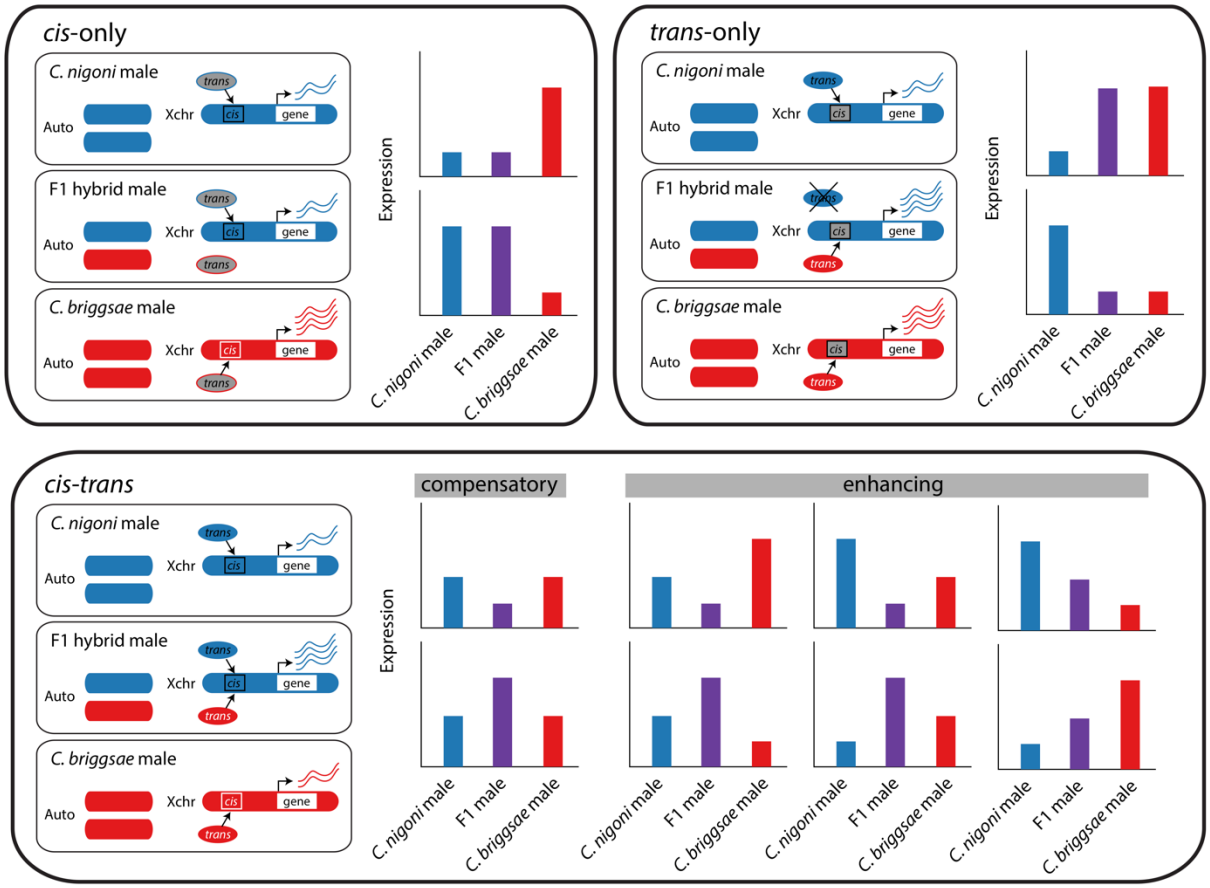

Supplementary Fig. S3. Qualitative examples to diagram regulatory divergence scoring for the X-chromosome in males that are hemizygous for the X.

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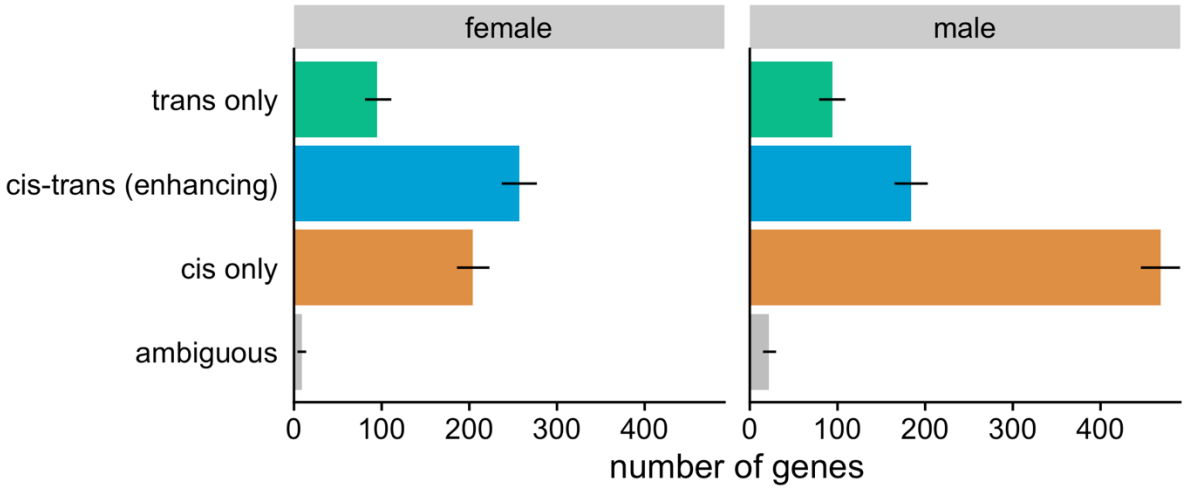

Supplementary Fig. S4. The number of genes with additive expression dominance showing *cis* and *trans* regulatory changes is distinct for females and males. See also Figure 3 in the main text. Horizontal bars show 95% interval confidence from non-parametric bootstrapping.

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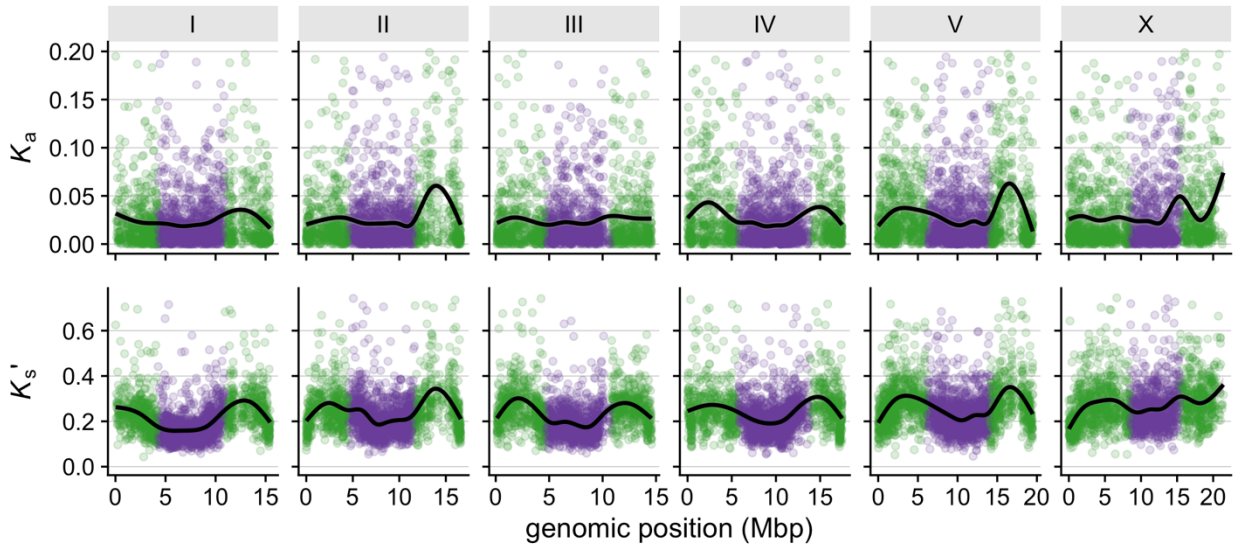

Supplementary Fig. S5. Coding sequence evolutionary rates for replacement sites (K_a) and synonymous sites (K_s', adjusted for selection on codon usage) for 12,115 orthologs between *C. briggsae* and *C. nigoni* along the chromosome positions of the *C. briggsae* genome. Colors mark chromosome arms (green) and center (purple); black lines indicate general additive regression (GAM) trends.

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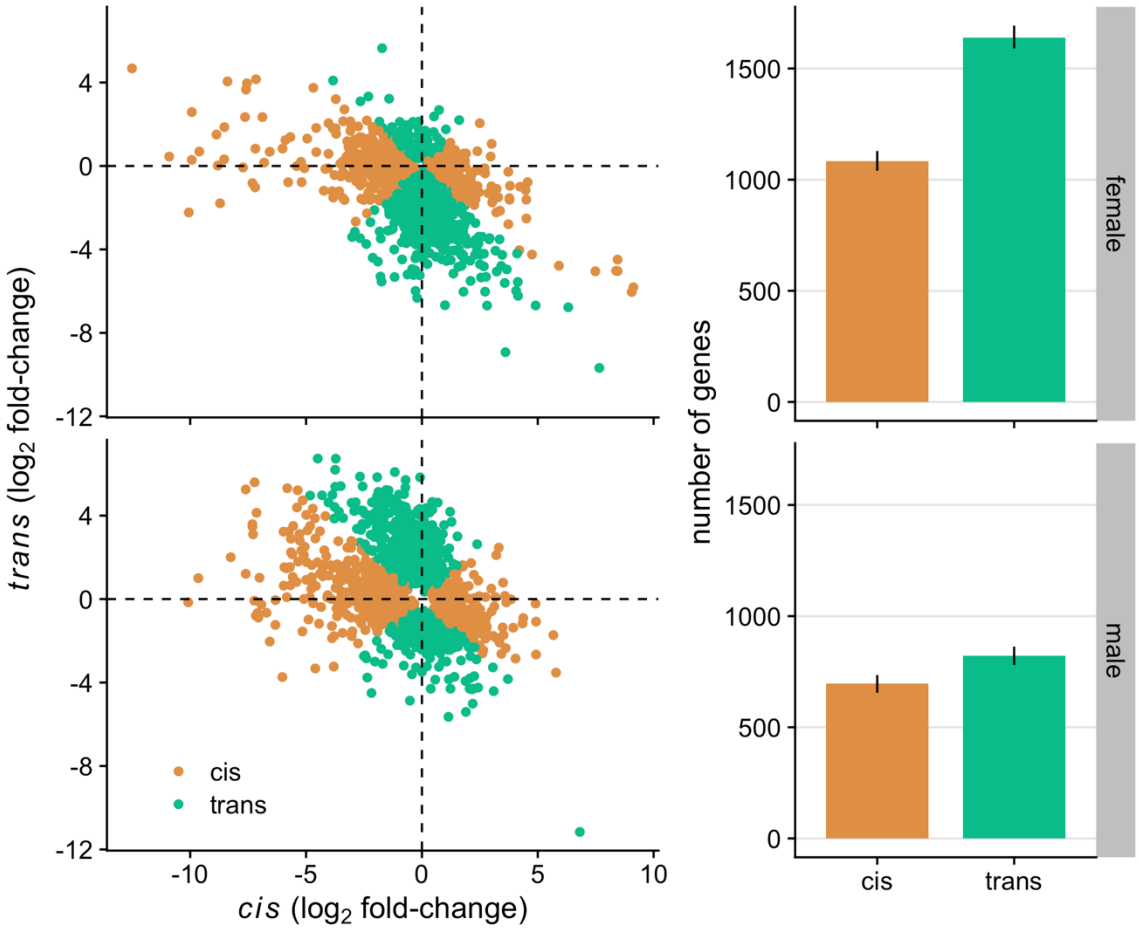

Supplementary Fig. S6. Biplot of *cis* vs *trans* effects for genes with significant sex-bias. *trans* divergence was measured as the $\log_2$ expression difference between parent species (*cis* and *trans*) - the $\log_2$ expression difference between alleles in F1 hybrids (*cis*). Genes with *cis*-only and *trans*-only effects were kept as *cis* and *trans* respectively, whereas genes with significant joint *cis-trans* effects in this analysis were deemed *cis* if abs(cis) > abs(*trans*) and vice versa to be deemed *trans*. Error bars were calculated using non-parametric bootstrapping (n=1000). Female transcriptomes in top panels; males in bottom panels.

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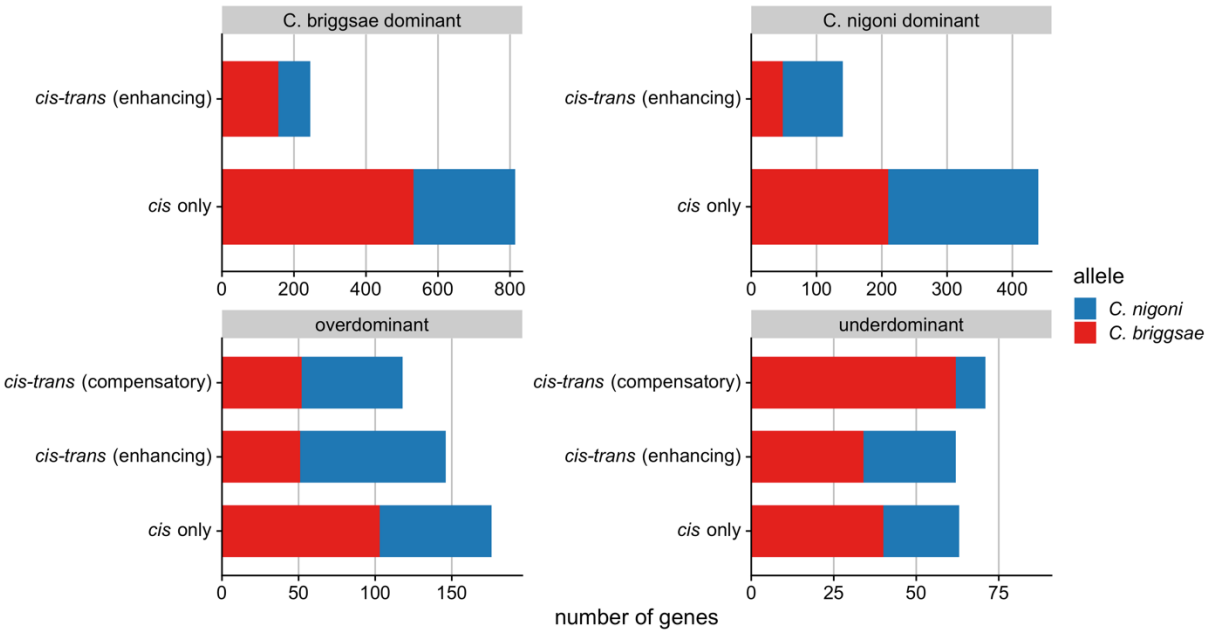

Supplementary Fig. S7. Number of autosomal genes with significant allele-specific expression showing simple expression dominance of one species (top panels) and transgressive over- or under-dominance. Genes with conserved regulation or *trans*-only effects were excluded for not having significant allele specific expression.

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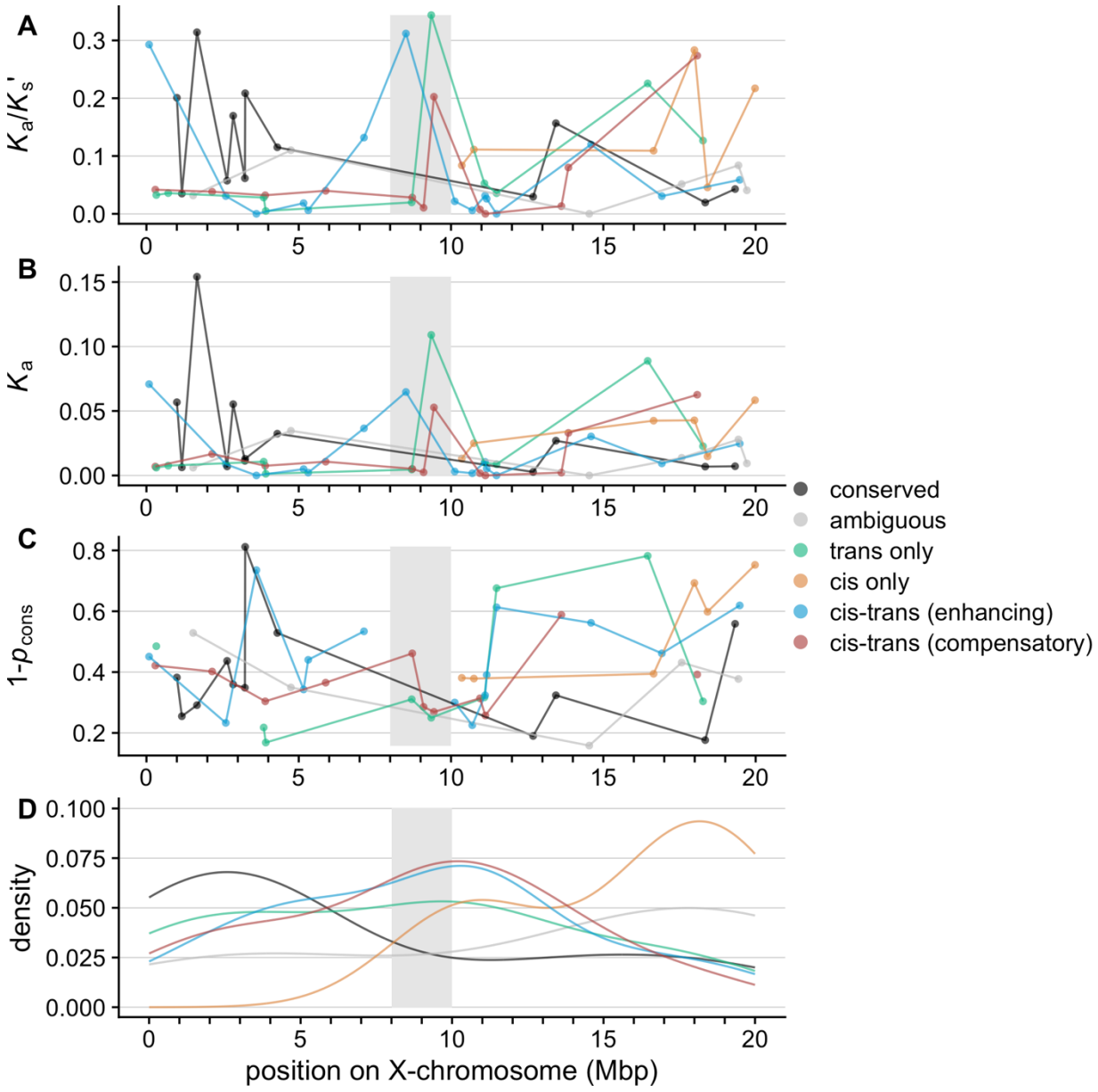

Supplementary Fig. S8. Protein-coding (A-B) and upstream non-coding (C) sequence divergence in X-linked spermatogenesis genes (n=61; also see Fig. 4F-H) with different types of regulatory divergence. (D) Shows gene density (n=1,939) for different *cis* and *trans* effects along Chromosome X. The shaded area near 10 Mbp marks genes with *trans*, enhancing *cis-trans*, and compensatory *cis-trans* effects with high protein-coding sequence divergence. This also overlaps with GFP-marked chromosome regions found to have sterility effects on hybrid males (Bi et al. 2019).