Widespread misregulation of inter-species hybrid transcriptomes due to sex-specific and sex-chromosome regulatory evolution

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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 generate the developmental defects responsible for intrinsic post-zygotic reproductive isolation. Both *cis*- and *trans*-acting regulatory divergence can be hastened by directional selection through adaptation, sexual selection, and inter-sexual conflict, in addition to inconspicuous evolution under stabilizing selection. Dysfunctional sex-biased gene expression, in particular, may provide an important source of sexually-dimorphic genetic incompatibilities. Here, we characterize and compare male and female/hermaphrodite transcriptome profiles for sibling nematode species *Caenorhabditis briggsae* and *C. nigoni*, along with 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, misexpression of sex-biased genes pervades F_1 hybrids of both sexes. This finding implicates greater fragility of male genetic networks to produce dysfunctional organismal phenotypes. Spermatogenesis genes are especially prone to high divergence in both expression and coding sequences, consistent with a "faster male" model for Haldane's rule and elevated sterility of hybrid males. Moreover, underdominant expression pervades male-biased genes compared to female-biased and sex-neutral genes and an excess of *cis*-*trans* compensatory regulatory divergence for X-linked genes underscores a "large-X effect" for hybrid male expression dysfunction. Extensive regulatory divergence in sex determination pathway genes likely contributes to feminization of XX hybrids. The evolution of genetic incompatibilities due to regulatory versus coding sequence divergence, however, are expected arise in an uncorrelated fashion. This study identifies important differences between the sexes in how regulatory networks diverge to contribute 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

Author's summary

As species diverge, many mutations that affect traits 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 genes biased toward expression in one sex and that localize to sex chromosomes. Our analyses of genome-wide gene expression from *Caenorhabditis* nematode roundworms support these predictions. We find widespread rewiring of gene regulation, despite the extensive morphological stasis and conserved expression profiles that are hallmarks of these animals. Misregulation of expression in inter-species hybrids of both sexes is most severe for genes linked to the X-chromosome, but male organismal phenotypes are most disrupted in hybrids. This fragility of male genetic networks and sex differences in regulatory evolution of local versus distant elements may underlie feminized and sterile phenotypes among hybrids. Our work clarifies how distinct components of regulatory networks evolve and contribute to sex differences in the manifestation of genetic incompatibilities in the speciation process.

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 (3–5).

Evolution of the regulatory controls over gene expression influences much phenotypic evolution (5,6), despite stabilizing selection as a prevailing force acting to preserve expression profiles (7–11). 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 (12,13). 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 (12–15). 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 (10,14,20,21), but this multiplicity of changes are predisposed to generating misexpression in F_1 hybrids due to dysfunctional *cis*-by-*trans* regulatory interactions (5,22).

88

89 Decomposing the changes of gene networks into their *cis*- and *trans*–regulatory components,
90 however, presents a challenge to studying gene regulatory evolution. One way to address this
91 problem is with hybrid cross experiments that assess differential expression between two
92 closely related species and from allele-specific expression (ASE) in their F_1 hybrids (5,10,23).
93 Differences in gene expression between species reveal the joint effects of *cis*- and *trans*-
94 regulatory divergence, whereas differences in ASE within F_1 hybrids typically represent the
95 effects of *cis*-regulatory divergence alone (23). Studies of this kind have unveiled broad
96 empirical patterns of regulatory evolution, whether carried out in flies (16,24), mice (25), plants
97 (17,18), or yeast (19,26). Overall, previous work has shown substantial regulatory divergence in
98 both *cis* and *trans*, extensive non-additivity, and disrupted regulation and misexpression in F_1
99 hybrids. Whether these patterns hold for the nematode phylum is, as yet, unknown, and the
100 links between regulatory mechanisms and sex-biases in expression remain incompletely
101 resolved.
102
103 Hybrid dysfunction of developmental programs, organismal traits, and fitness may often trace
104 their origins to gene misregulation, from transcriptional to post-translational levels (5,27). Sex-
105 biased misregulation, therefore, should underlie sex-biased developmental and fitness effects
106 in hybrid individuals. Misexpression of male-biased genes and genes related to
107 spermatogenesis links regulatory disruption to male sterility in hybrids, with supporting
108 empirical evidence in several kinds of animals (25,28–30). In organisms with chromosomal sex
109 determination, more severe defects typically occur in hybrid individuals carrying heterogametic
110 sex chromosomes (i.e., XO males in *Caenorhabditis* nematodes). This Haldane's rule pattern can
111 arise from dominance effects (31), faster molecular evolution of genes with male-biased
112 expression (32), greater sensitivity of male developmental programs to perturbation (32), and
113 faster evolution of sex-linked loci (33), among other causes (34,35). Because of the prominent
114 role that the X-chromosome plays in reproductive isolation (36–38), we might also expect to
115 find greater expression divergence and misexpression for X-linked genes compared to
116 autosomes (5,39–41), with the caveat that genes with male-specific expression might not

117 necessarily be abundant on the X-chromosome (42–44). Thus, distinguishing between abnormal
118 expression in hybrids for X-linked genes overall and for sex-biased autosomal genes is
119 important to decipher the genetic mechanisms that underpin Haldane's rule in particular and
120 hybrid dysfunction in general.
121
122 *Caenorhabditis* nematode roundworms provide an especially tractable system to study
123 speciation genomics (35). The growing number of *Caenorhabditis* species known to science
124 conform to the biological species concept, with a few cases where sibling species can produce
125 some viable and fertile adult hybrid offspring (45,46). The *C. briggsae* × *C. nigoni* species pair is
126 one such case, where recent divergence (~3.5 Ma (47)) allows them to form hybrids of both
127 sexes. In this system, *C. nigoni* has the ancestral gonochoristic reproductive mode (XX females
128 and X0 males) whereas XX hermaphrodites in the androdioecious *C. briggsae* (also with XO
129 males) are morphologically female and capable of self-fertilization due to each gonad arm
130 producing a set of sperm cells prior to an irreversible switch to oocyte production. In hybrids,
131 both Haldane's rule and Darwin's corollary are fulfilled: F_1 male hybrids are always sterile with
132 near complete inviability when arising from *C. briggsae* mothers (48–50). In contrast, F_1 females
133 from both reciprocal crosses are fertile, retaining the "female" condition (i.e., are not
134 hermaphrodites) (48). This transition in reproductive mode led to the evolution of a suite of
135 genomic and phenotypic traits in *C. briggsae* that define a "selfing syndrome" due to relaxed
136 selection on male function and adaptation to a selfing lifestyle (51,52), and has led to the
137 disproportionate loss of male-biased genes in the *C. briggsae* genome (47,51,53–56). Moreover,
138 extensive inclusion of X-linked loci in hybrid male sterility implicates a large-X effect (57).
139 Further analyses revealed that X-autosome incompatibilities involve misregulation of the 22G
140 class of small RNAs, leading to down-regulation of spermatogenesis genes and contributing to
141 hybrid male sterility (30,58). The full extent of hybrid misexpression throughout the genome of
142 each sex and its root causes in regulatory divergence, however, remain to be characterized.
143
144 Given the extensive phenotypic stasis within the genus (59) and previous work showing
145 prevalent action of *cis* and *trans* compensatory evolution, even between more distantly related

146 species (20), we expect to find substantial developmental systems drift within this system,
147 where sex-biased genetic networks will be disproportionately prone to misregulation and
148 misexpression. In particular, we expect greater dysfunctional expression in hybrid males, which
149 are sterile, compared to hybrid females, which are fertile. These effects also are expected to
150 associate most strongly with genetic networks that most depend on X-linked genes. To test
151 these ideas, we analyze mRNA transcriptome expression for each sex from each of *C. briggsae*,
152 *C. nigoni*, and their F_1 hybrids. Using ASE profiles, we then characterize and quantify *cis*- and
153 *trans*-acting regulatory causes of expression divergence linking genomic change to sex-biased
154 expression, chromosomal features, and hybrid dysfunction.

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 (**Supplementary Fig. S1A**). *C. briggsae* hermaphrodites resemble females phenotypically except that they are able to produce sperm in addition to eggs, and therefore show masculinized expression of some genes (**Supplementary Fig. S1A,2,3**). To more appropriately contrast “female” expression profiles between hermaphrodites and females, we identified 1,238 orthologous genes that are male-biased and up-regulated in hermaphrodites relative to sperm-less *C. briggsae* "pseudo-female" mutants (*she-1(v4)*; (53)) (9% of the 13,636 one-to-one orthologs analyzed). These 1,238 genes were then analyzed separately from most sex-based analyses that, for simplicity, we refer to hereafter as "female" expression contrasts.

The transcriptomes of pure *C. briggsae* and *C. nigoni* differed in expression for more than half of their genes, with slightly more genes differentially expressed for "females" than for males. Females had a total of 61% (7,598) of genes differentially expressed between species, compared to 54% (7,385) for males (**Fig. 1A**). The X-chromosome shows the most extreme differences in number of differentially expressed genes between species, with both males and "females" showing significantly higher ratios of upregulated X-linked genes in *C. nigoni* than in *C. briggsae* (**Fig. 1B**). Autosomes, by contrast, showed greater abundance of genes with higher expression in *C. briggsae*, albeit only significantly for Chromosome I in males (**Fig. 1B**; Fisher's exact test; $P < 0.05$). Similarly, we found more genes with significant ASE for autosomes among female hybrids (6,070 or 61%; n=9,932) than among hybrid males (5,402 or 53%; n=10,155). Which allele gets upregulated in hybrids was largely consistent between the autosomes of hybrid males and females (**Fig. 1B**), albeit slightly more consistent for males (higher expression of *C. briggsae* allele: 2,793 or 27%; *C. nigoni* allele: 2,609 or 26%) than for females (higher expression of *C. briggsae* allele: 3,215 or 32%; *C. nigoni* allele: 2,855 or 29%). The slightly

183 greater tendency in females for *C. briggsae* alleles to be upregulated could suggest more
184 regulatory changes fixed in the *C. briggsae* lineage that lead to higher expression, or some
185 degree of mapping bias in *C. briggsae*-derived reads compared to those from *C. nigoni*
186 (simulations suggest that mapping biases are an unlikely cause; see Methods).
187
188 Within each species, approximately 60% of genes showed significant sex biases in expression
189 (**Fig. 1C**). Male-biased and female-biased genes occurred with similar incidence in both species:
190 30% *C. nigoni* male-biased, 29% *C. briggsae* male-biased, 29% *C. nigoni* female-biased, 27% *C.*
191 *briggsae* "female"-biased. In stark contrast, over 80% of genes in F_1 hybrids were significantly
192 sex-biased, with a slightly higher incidence of male-biased genes (43% vs 40%). In *C. briggsae*
193 and F_1 hybrid transcriptomes, Chromosomes I and III were enriched for "female"-biased genes,
194 whereas Chromosomes V and X were enriched for male-biased and sex-neutral genes (**Fig. 1D;**
195 **Supplementary Fig. S2**). No *C. nigoni* chromosomes exhibited strong enrichment of sex-biased
196 genes and genes with "female"-biased expression in both species and in hybrids showed strong
197 depletions from the X-chromosome (**Fig. 1D; Supplementary Fig. S2**).
198

199 ## Expression dominance in F_1 hybrids differs between males and females

200 We contrasted expression profiles of F_1 hybrids with their parent species to infer the expression
201 inheritance of genes, i.e., to identify genes that exhibited additive, dominant (*C. briggsae*- and
202 *C. nigoni*-like expression), or transgressive (overdominant and underdominant) expression
203 patterns for each sex (**Fig. 2A**). Gene sets with distinct expression inheritance profiles revealed
204 substantial differences between the sexes in terms of expression distance (**Fig. 2B**), number of
205 genes (**Fig. 2C**), and enrichment across the genome (**Fig. 2D**).
206
207 The sexes differed most strikingly in their total number of transgressive genes: 26% (3,494) of
208 genes show transgressive profiles in males (1,788 overdominant and 1,706 underdominant)
209 versus 55% (6,881) in females (4,121 overdominant and 2,760 underdominant) (**Fig. 2A,C**).
210 Transgressive genes are thought to be associated with hybrid dysfunction, as such
211 underdominant and overdominant expression profiles represent misexpression phenotypes

212 that manifest values beyond the range of both parents (29,60). Given the pronounced sterility
213 of hybrid males, we were surprised that hybrid male transcriptomes showed only half the
214 incidence of misexpression as hybrid females. This finding suggests that the genetic networks
215 that control fitness are more robust to expression perturbation in female hybrids than in males.
216
217 In addition, genes classified as underdominant, overdominant, and additive had significantly
218 higher Euclidean expression distances from the centroid of expression space than genes with no
219 change in expression or with simple dominance (Gamma-distributed multiple generalized linear
220 least square regression [GLM], $P < 0.001$) (**Fig. 2A,B**), suggesting that genes with these
221 expression inheritance profiles are more prone to deviant expression phenotypes. However,
222 except for transgressively expressed genes, "females" had on average significantly larger
223 magnitude in expression distance from the centroid expression space than males (GLM, all $P <$
224 0.001 except transgressive $P > 0.1$) (**Fig. 2A,B**). These results are consistent with our
225 multidimensional scaling analysis that showed shorter expression distances for F_1 males to
226 parental males (**Supplementary Fig. S1A**), in contrast to more dissimilar expression profiles of
227 F1 females to parental females.
228
229 Genes with additive expression of alleles from both parental species were relatively rare in F_1
230 hybrids of both sexes (8% or 691 genes in females; 8% or 946 genes in males) (**Fig. 2C**). Genes
231 showing simple dominance were up to four-times more common, with approximately 20-30%
232 of genes expressed by each sex either matching *C. briggsae* or *C. nigoni* expression (23% or
233 2,869 genes in females; 30% or 3,698 genes in males). Expression dominance matching *C.*
234 *briggsae* was consistently more frequent in hybrids of both sexes (15% or 1,876 genes in
235 females and 18% or 2,228 genes in males), however, compared to expression dominance
236 matching *C. nigoni* (**Fig. 2C**; 8% or 993 genes in females and 12% or 1,470 in males), being more
237 extreme in female hybrids across autosomes (mean ratio *C. briggase* vs *C. nigoni* dominant
238 expression = 1.75 vs 1.14 in males) (**Fig. 2C,D**). The disproportionate dominance of the *C.*
239 *briggsae* copy in hybrid females was even more pronounced for the X-Chromosome (F_1 female
240 X ratio = 2.66 vs F_1 male X ratio = 1.13).

241

242 Expression dominance on the X-chromosome is distinct in hybrids of both sexes

243 Genes and traits with dysfunctional expression are often associated with the X-chromosome,
244 and we expect differences between the sexes due to Haldane's rule (25,28,39,40). Consistent
245 with these expectations, we found inheritance profiles associated with misexpression to differ
246 between F_1 males and females across the genome, and to differ most conspicuously for the X-
247 chromosome. In particular, the X-chromosome was enriched for underdominant genes in both
248 F_1 males and females (**Fig. 2C,D**), but only significantly so in females (Fisher's exact test, $P <$
249 0.05). The X-chromosome was also significantly enriched for overdominant genes in F_1 males,
250 whereas females had significant depletions of such genes on the X (autosomal enrichments: V
251 in males, and I and III in females) (**Fig. 2C,D**; Fisher's exact test, $P < 0.05$). These data show clear
252 differences in expression inheritance across chromosomes between the sexes and reflect
253 distinct hybrid expression dynamics between autosomal and X-linked genes.
254
255 Given the distinct misexpression of genes linked to the X-chromosome, we evaluated whether
256 disrupted dosage compensation in hybrids might have systematically perturbed X-linked
257 expression profiles. X-chromosome dosage compensation in *Caenorhabditis* acts to halve
258 expression of the two copies in females and hermaphrodites (61). The magnitude of expression
259 that we observed for the X-linked genes in hybrid females, however, does not differ on average
260 from either parental species (**Supplementary Fig. S1B**). Consistent with the implications of
261 conserved mechanistic features of the dosage compensation complex and its regulation across
262 multiple *Caenorhabditis* species, including *C. briggsae* and *C. nigoni* (62), this suggests that
263 dysfunctional dosage compensation is not a key driver of X-linked underdominant expression in
264 hybrid females.
265

266 *cis* and *trans* regulatory divergence modulates differences between sexes

267 Identifying the spectrum of changes to *cis*- and *trans*-acting regulators is important to
268 understand how selection influences the evolution of gene expression and its effects on hybrid
269 phenotypes. Correspondingly, we classified the types of regulatory changes and examined how

270 they perturbed gene expression in F_1 hybrids for each sex. Consistent with ASE studies in flies
271 (16,24), mice (25), plants (17,18) and yeast (19,26), we found substantial expression divergence
272 due to *cis-only* or *trans*-only regulatory changes in addition to joint effects of *cis* and *trans*
273 changes, with changes in *trans* and *cis* + *trans* conferring larger magnitudes of expression
274 divergence (**Fig. 2E,F**). Genes with *cis*-only and *trans*-only effects were not significantly enriched
275 on any autosome for either sex, although Chromosome V exhibited a trend toward enrichment
276 of genes with *trans*-only effects for males (**Fig. 2H**). Comparing regulatory divergence between
277 sexes, we found nearly 60% more genes involving *trans*-only changes in females (18% or 2,195
278 genes) compared to males (12% or 1,391 genes) (**Fig. 2G**). For genes with *cis*-only divergence,
279 however, the sexes showed a reciprocal pattern: *cis*-only divergence was more prevalent in
280 male than in female transcriptomes (17% or 1,944 genes in males; 14% or 1,647 genes in
281 females). Thus, the expectation that *cis*-regulatory divergence will be more prevalent than
282 *trans*-regulatory divergence holds true only when expression is measured in males, and points
283 to fundamental differences between the sexes in trends of regulatory evolution.
284
285 Furthermore, we categorized genes into 13 groups based on distinct combinations of species
286 differences and sex differences in expression, including their interactions, and looked at the
287 proportion of genes with different *cis*- and *trans*-effects (**Fig. 5A,B**). Our results are consistent
288 with the idea that *cis* and *trans* changes each play distinct roles in sex-biased expression and
289 sexual dimorphism (63). We find, on one hand, that *trans*-only changes in females are more
290 often associated with genes that have male-biased expression (37% *trans*-only in male-biased
291 genes vs. 15% in female-biased genes) and, on the other hand, that female-biased genes show
292 more conserved regulation (32% conserved in female-biased genes vs. 9% in male-biased
293 genes) when expressed in males (**Fig. 5D**; **Supplementary Fig. S2**). This pattern suggests that
294 distinct sex-specific regulatory controls may be asymmetric in this system and may have
295 evolved to repress expression of genes biased for the opposite sex. Together, these
296 observations highlight how the evolution sex-specific regulatory controls can underlie sexually
297 dimorphic expression phenotypes, with implications for sex biases in the speciation process.
298

299 Because of codominant effects of *trans*-acting factors controlling the expression of both alleles,
300 *trans*-only regulatory divergence is often associated with deviations from additivity (64).
301 Consistent with this idea, we found *trans*-regulatory changes more often associated with genes
302 having dominant expression patterns and that genes with additive expression more often
303 associated with genes having significant *cis*-effects (*cis*-only and *cis* + *trans*) (**Fig. 4**). However,
304 genes with *cis*-only effects often showed dominant patterns of expression. Genes that show *cis*-
305 only divergence on autosomes for male expression often have *C. briggsae* or *C. nigoni* dominant
306 expression in hybrids whereas, in females, they are more typically overdominantly expressed
307 (**Fig. 4**). We can think of three potential mechanisms that might contribute to this sex difference
308 and to the *cis*-dominant effect in general: (1) more extensive post-transcriptional regulation in
309 females (65) might limit transcript abundance through epistatic interactions; (2) greater
310 degradation or turnover of *cis*-acting binding sites for male-expressed genes (54,55) could
311 reduce affinity in transcription factor binding activity; and (3) the presence of species-specific
312 *trans* regulators that originate from lineage-specific gene duplication and/or loss resulting in
313 allele-specific *trans*-regulation.
314
315 Despite the limitations posed by the hemizygous condition of the X-chromosome in males, we
316 devised a strategy to confidently assign one of three categories of regulatory divergence to X-
317 linked genes in males based on their expression inheritance. In short, we inferred genes to have
318 i) *cis*-only* divergence (inferred from expression dominance with respect to *C. nigoni*, which
319 may include genes with X-linked *trans* regulators, with this distinction denoted by *), ii) *trans*-
320 only* divergence with recessive *C. nigoni trans*-acting factors (expression dominance with
321 respect to *C. briggsae*, which may exclude X-linked genes with codominant *trans* regulators,
322 denoted by *), which provides a lower-bound estimate of *trans*-acting regulatory divergence
323 affecting the X, and iii) *cis-trans* compensatory changes (no difference in expression between
324 parent species with hybrids showing over- or underdominance). We did not find enrichments of
325 *cis*-only* divergent genes on the X, as might be expected under a "large-X" effect model for
326 regulatory contributions to hybrid dysfunction. Similarly, we found only a non-significant trend
327 in both sexes toward enrichment of *trans*-only regulatory divergence affecting X-linked genes

328 (**Fig. 3**; Fisher exact test, $P > 0.05$). Instead, however, *cis-trans* compensatory changes were 1.9-
329 fold enriched on the X chromosome for males and 1.7-fold underrepresented on the X for
330 females (**Fig. 3**; Fisher exact test, $P < 0.05$). These results for regulatory divergence
331 "regroupings" are consistent with the full set of categories that could be defined for females
332 (**Fig. 2H**). This finding implicates stabilizing selection on X-linked male-specific networks as
333 being especially prone to disruption in F_1 hybrids, and contrasts with the 1.6-fold enrichment of
334 conserved regulatory controls for X-linked genes in females (**Fig. 3**; Fisher exact test, $P < 0.05$).
335

336 Hybrid misexpression commonly involves genes with joint *cis-trans* regulatory 337 changes

338 Genes exhibiting transgressive expression profiles in F_1 hybrids are often associated with
339 dysfunctional traits, due to radically different expression from those of parent species.
340 *cis* and *trans* changes with opposing effects can interact epistatically in hybrids to induce
341 transgressive expression and allelic imbalance (22). Such regulatory evolution can arise through
342 coevolutionary fine-tuning even when overall expression level is subject to stabilizing selection
343 (6,11). Consistent with this idea, we found that genes with transgressive effects in hybrids often
344 are associated with *cis-trans* regulatory changes (33% or 877 genes in males and 42% or 2,872
345 genes in females; **Fig. 4**). In both sexes, F_1 hybrids revealed a higher fraction of compensatory
346 changes (27% in males and 31% in females) compared to enhancing *cis-trans* changes (6% in
347 males and 11% in females). These results highlight how stabilizing selection can act differently
348 on sex-specific transcript abundance, leading to opposing *cis* and *trans* effects that are
349 dysfunctional in F_1 hybrids. In addition, we also found substantial *cis-trans* compensatory
350 changes among genes that show no change in expression between species (**Supplementary Fig.
351 S3**) and in those that do not show sex-biased expression (i.e., C-N-N group in **Fig. 5D**),
352 implicating extensive developmental systems drift in regulatory processes.
353

354 Genes with additive expression inheritance also may generate hybrid dysfunction by generating
355 intermediate expression profiles in F_1 hybrids, and are thought to commonly reflect *cis*-only
356 regulatory divergence (23,39,66). In line with this idea, we found that genes classified as

357 additive associated more often with significant *cis*-acting divergence in both sexes (*cis*-only:
358 43% in males, 26% in females; and enhancing *cis-trans* effects: 30% in males, 42% in females;
359 **Fig. 4**). However, expression additivity is not abundant in our analysis (**Fig. 2C**), suggesting that
360 it is not a major source of phenotypic dysfunction in hybrids of this system.
361
362 To further assess the role that different regulatory controls play in the origin and maintenance
363 of divergent sex-biased expression, we contrasted expression inheritance and patterns of *cis*-
364 and *trans*-regulatory divergence for male-biased and female-biased genes (**Fig. 5C,D**). We found
365 that male-biased genes expressed in F_1 hybrids of both sexes frequently show underdominant
366 transgressive misexpression compared to sex-neutral and female-biased genes (**Fig. 5A-C**).
367 Examining male-biased genes when expressed in females, we find that they often show
368 expression dominance matching the species with lower expression (**Fig. 5A-C**; N-M-I, B-M-I, N-
369 M-N, B-M-N; also M5, M8, and M14 in **Supplementary Fig. S2**). Additionally, genes that show
370 conserved expression between species, but with significant sex-bias (C-M-N for male-biased
371 and C-F-N for female-biased genes), also often are either misexpressed (over- or
372 underdominant) or have had no significant change in expression in hybrids (**Fig. 5C**). These
373 groups also have the highest proportion of genes with *cis-trans* compensatory changes (**Fig.
374 5D**), suggesting that many "conserved" sex-biased networks have undergone substantial
375 developmental systems drift.
376
377 Interestingly, genes expressed in F_1 males are more commonly underdominant when they
378 correspond to male-biased genes than to female-biased genes (957 genes among male-biased
379 genes vs 431 genes among female-biased genes), suggesting that male-biased regulatory
380 networks are more fragile. This idea is supported further by male-biased genes having a higher
381 proportion of genes with enhancing or compensatory *cis-trans* changes (1,086 or 23% genes in
382 male-biased genes vs 702 or 18% genes in female-biased genes). In addition, we find that male-
383 biased genes define more distinct expression profile modules than do female-biased genes (6
384 versus 3 co-expression modules), including one with male-specific underdominance
385 (**Supplementary Fig. S2**; M15). By contrast, female-biased genes expressed in F_1 females were

386 predominantly overdominant and are more often associated with *cis*-only and enhancing *cis-*
387 *trans* changes (**Fig. 5D; Fig. 5C, Supplementary Fig. S2**). These observations suggest that female
388 gene regulatory networks can be more resilient to regulatory divergence, potentially translating
389 into similar resilience for organismal traits such as fertility (48).
390

391 ## Faster regulatory and molecular evolution of male-biased and spermatogenesis
392 genes

393 Sexual selection and sexual conflict are predicted to drive faster rates of molecular evolution
394 and expression divergence (37,67,68). Consistent with these predictions, we found that male-
395 biased genes have higher average expression divergence (|$\log_2$-fold-change|, GLM, t=-23.639, P
396 < 0.001) and faster rates of molecular evolution in the two groups of male-biased genes that
397 are rare on the X-chromosome (K_a/K_s for B-M-I: GLM, t=-8.947, P < 0.001; B-M-N: GLM, t=-
398 8.764, P < 0.001) (**Fig. 5E,F**). Male-biased genes show elevated expression divergence compared
399 to sex-neutral genes and female-biased genes as a whole, though the signal for faster sequence
400 evolution was weak (K_a/K_s male-biased vs. sex-neutral GLM, t=-0.77, P = 0.44; male-biased vs.
401 female-biased GLM, t=-0.78, P = 0.43). Rapid sequence evolution for male-biased genes was not
402 associated with enrichment in chromosomal arms (**Fig. 5G**, Fisher's exact test, P < 0.05), regions
403 which are known to show higher divergence (47). The groups of male-biased genes with
404 enrichments on the X-chromosome, however, have either conserved expression between *C.*
405 *briggsae* and *C. nigoni* or have higher expression in *C. nigoni* males (**Fig. 5G**; C-M-N, N-M-I, N-M-
406 N; **Supplementary Fig. S2**; Fisher's exact test, P < 0.05), indicating that the X-chromosome is
407 home to a subset of genes that reflect ancestral male-biased gene networks.
408

409 We observed the highest expression divergence as well as high rates of molecular evolution in
410 the distinctive set of genes that combine male-biased expression, higher expression in *C.*
411 *briggsae* than *C. nigoni*, and a species-by-sex interaction (B-M-I; **Fig. 5E,F**). The species-by sex
412 interaction in this B-M-I group indicates a masculinized expression profile for *C. briggsae*
413 hermaphrodites, implicating a role for them in sperm production (**Supplementary Fig. S4**; and
414 M5 in **Supplementary Fig. S2**). To test this idea, we looked at *C. elegans* genes previously

415 identified as spermatogenesis genes (44) and found overlapping orthologs in *C. briggsae* and *C.*
416 *nigoni* to be 11-fold enriched in the B-M-I group (**Fig. 6B and 6C**; Fisher's exact test, $P < 0.05$)
417 and depleted from the X-chromosome (**Fig. 5G**), consistent with previous observations for
418 sperm genes in *Caenorhabditis* (35,42,43) and *Drosophila* (69). Further consistent with sperm-
419 related function, male-biased genes that show upregulated expression in *C. briggsae*
420 hermaphrodites relative to *C. briggsae* pseudo-females (**Fig. 6A-C**) were highly enriched (46-
421 fold) and overlapped extensively with the B-M-I group (**Fig. 6B and 6C**; Fisher's exact test, $P <$
422 0.05). Thus, the high divergence in both expression and sequence for these genes suggests
423 distinctive selection pressure on them, potentially reflecting the outcome of sexual selection
424 and sexual conflict on sperm.
425
426 The collection of male-biased genes that also show elevated expression in *C. briggsae*
427 hermaphrodites, putatively linked to spermatogenesis, showed expression phenotypes in
428 hybrids often resembling *C. nigoni*: extensive expression dominance for the *C. nigoni* copy and
429 underdominant expression in F_1 hybrids as well as high expression divergence (**Figs. 6D,E;**
430 **Supplementary Fig. S5)**. They also show an abundance of *trans*-only regulatory divergence (**Fig.**
431 **6F,G**).
432

433 Regulatory and expression divergence within the sex determination cascade

434 Sex determination of somatic and germline development involves a negative regulatory cascade
435 (70), including the secreted protein HER-1 that inhibits transcription factor TRA-2, and further
436 downstream, TRA-1 represses genes such as *fog-3* in controlling spermatogenesis (71,72).
437 Consistent with what is known about this pathway (70), our data shows male-biased expression
438 of *her-1* in both parental species and that it has evolved enhancing *cis* + *trans* regulatory
439 changes that implicate lineage-specific regulatory changes that promote its expression in males
440 of both species (**Supplementary Fig. S6**). Without HER-1, TRA-3 then cleaves the intracellular
441 domain of TRA-2 in XX individuals, which then interacts with FEM proteins preventing FEM–
442 TRA-1 interactions (72). We found the ortholog of *tra-3* to be similarly expressed and regulated
443 (*cis*-only) between sexes of pure species and hybrids. However, *fem* genes and *tra-2* seem to be

444 female-biased and overdominantly expressed (**Supplementary Fig. S6**), suggesting sex-specific
445 co-regulation; and at least two have potentially evolved *cis* and *trans* regulatory changes (*fem-*
446 *2*, *fem-3*). In addition, antagonistic *cis* x *trans* regulatory effects on *tra-1* seem to have evolved
447 in females, suggesting that that opposing regulatory changes have evolved on an important
448 transcription factor controlling germline and somatic sexual differentiation. However, the story
449 for *tra-1* regulatory divergence in males differs from females and is also less clear: our analysis
450 categorizes it as "ambiguous" (significant ASE, but non-significant regulatory divergence and
451 non-significant *trans* effects). In contrast, orthologs of *fog-3*, which is involved in
452 spermatogenesis, get upregulated in *C. briggsae* hermaphrodites and in males of both species
453 (B-M-I), as expected. In F_1 males, however, *fog-3* is misexpressed (underdominant) having a
454 non-significant *trans* effect (P = 0.28) and a marginally-significant *cis* ASE effect (P = 0.04). Its
455 expression in F_1 females shows *C. nigoni* dominant expression that is due to *trans*-only
456 regulatory divergence between the species (**Supplementary Fig. S6)**. The regulatory divergence
457 between *C. briggsae* and *C. nigoni* for genes involved with sex differentiation and development
458 point to plausible mechanisms for suppression of spermatogenesis in XX hybrids to produce a
459 "female" rather than a “hermaphrodite” phenotype, as well as yielding hybrid male sterility.
460

461 ## Genome architecture only modestly affects regulatory divergence

462 Given that protein-coding sequence evolution and gene composition vary non-randomly along
463 chromosomes in many *Caenorhabditis* species in association with the chromosomal
464 recombination landscape, we asked whether distinct chromosomal domains would also
465 associate with the degree of *cis*-regulatory divergence. We find higher molecular divergence
466 between the genomes of *C. briggsae* and *C. nigoni* in chromosomal arms compared to centers
467 in noncoding sequences upstream of protein-coding genes (**Supplementary Fig. S7A**), in
468 addition to protein-coding sequence divergence (**Supplementary Fig. S8**; also see (47)). These
469 observations are consistent with the idea of stronger purifying selection on mutations to genes
470 and their *cis*-regulatory regions when linked to chromosome centers or more effective positive
471 selection when linked to chromosome arms. Despite the elevated molecular divergence in arm
472 regions, we only found modest elevation of ASE divergence for genes on arms (**Supplementary**

473 **Fig. S7B**), as well as modest but significant differences in the magnitude of regulatory
474 divergence in *cis* and *trans* between chromosome arms and centers (**Supplementary Fig. S7C**;
475 *cis*-only GLM [females], coeff=0.06, t=1.99, P = 0.04; *trans*-only GLM [females], coeff=0.05,
476 t=4.45, P < 0.001). Notably, we observed only a weak positive correlation between ASE
477 divergence and rates of molecular evolution (**Fig. 7A,B**; linear regression for K_a/K_s': adjusted R =
478 0.035, m = 0.019, P < 0.0001; 1-P_{cons}: adjusted R = 0.01, m = 0.017, P < 0.0001). Overall, these
479 patterns indicate that rates of divergence for gene expression and their *cis*-regulatory controls
480 are largely decoupled from protein-coding sequence evolution.
481

482 # Discussion

483 Regulatory control over gene expression is an important component of phenotypic evolution
484 (12). As species diverge and accumulate mutations, selection will permit regulatory changes
485 that maintain transcript levels as well as changes that allow exploration of new phenotypic
486 space when they confer a fitness advantage. Sexual selection and sexual conflict can further
487 promote such genomic divergence, both in terms of molecular evolution (e.g., rapid coding or
488 regulatory sequence evolution for male-biased genes) and in terms of gene expression (e.g.,
489 divergence in sex-biased gene expression levels) (37,67,68). In interspecies hybrids, sexually
490 driven sources of genomic divergence can disrupt gene networks to create negative epistatic
491 interactions that manifest as sex-biased hybrid sterility or inviability and generate reproductive
492 isolation (5). Here we document extensive regulatory divergence in the face of both conserved
493 and divergent gene expression, with prominent influences of sex-biases and genomic location
494 on the potential to induce misexpression in interspecies hybrids.
495

496 ## Compensatory regulatory evolution implicates pervasive developmental system
497 drift

498 *C. briggsae* and *C. nigoni* acquired substantial divergence at the DNA level since they diverged
499 from their common ancestor ~35 million generations ago (3.5 Mya assuming 10 generations per
500 year), including ~20% sequence divergence for synonymous sites, changes to genome size, and
501 disproportionate loss of short male-biased genes in *C. briggsae* since its transition in
502 reproductive mode to androdioecy (47,55). Despite this genomic divergence, hybridization
503 between these species yields viable and fertile F_1 hybrid females, as well as viable hybrid males
504 that suffer complete sterility (35,48).
505

506 Despite observing substantial expression divergence, we find that 39% of genes expressed in
507 "females" (4,783 genes) and 46% in males (6,236 genes) show no differential expression
508 between species. Conserved expression between species may result from stabilizing selection,
509 recognized as a common force acting on transcript abundance (7–9,11). Mechanistically,

510 conservation of the expression phenotype can occur, despite sequence evolution, with co-
511 evolutionary changes to both *cis*- and *trans*-regulatory elements; for example, if a *trans*-acting
512 mutation fixes due to a pleiotropic benefit on other loci, selection would favor fixation of any
513 subsequent compensatory mutation in *cis* that returns expression to optimal levels at the focal
514 locus (5,20). We find evidence of widespread compensatory *cis-trans* divergence in gene
515 regulation between *C. briggsae* and *C. nigoni*. Such coevolution represents just one mechanism
516 leading to "developmental system drift," in which the molecular controls over developmental
517 pathways can diverge while resulting in little or no change to their phenotypic outputs
518 (14,21,73). In *Caenorhabditis* nematodes, developmental system drift and stabilizing selection
519 have been invoked to explain the high degree of phenotypic stasis and morphological constraint
520 among species (59,74–76). Gene network conservation despite *cis*-regulatory divergence has
521 been demonstrated by inter-species promoter swaps in *Caenorhabditis*, showing both
522 robustness in regulatory networks and neo-functionalization in specific cell types (20,77,78), as
523 well concerted action of *cis* and *trans* compensatory regulation between more distantly related
524 *Caenorhabditis* species (20). Our results reinforce this view of pervasive developmental system
525 drift: we show a high incidence of transgressively expressed genes (overdominant and
526 underdominant in hybrids) and *cis-trans* compensatory changes in the sexually-dimorphic
527 transcriptomes of each sex (**Fig. 4**), in addition to an abundance of transgressive hybrid
528 expression and *cis-trans* compensatory changes among genes with conserved sex-neutral
529 expression profiles (C-N-N) (**Fig. 5A-D**).
530
531 Sequence divergence and developmental system drift in regulatory pathways can lead hybrids
532 to experience misregulation due to the conflicting regulatory signals from the divergent
533 genomes, yielding misexpression in hybrid transcriptomes (27). This situation could present a
534 Dobzhansky-Muller incompatibility if the misexpression leads to reduced fitness; genetic
535 interactions like those experience by hybrids have been untested by natural selection and will
536 likely be detrimental (1). The most striking signal of misexpression in our hybrids is the sharp
537 contrast in the fraction of sex-biased genes: ~83% in hybrids vs ~60% in each parental species
538 (**Fig. 1C**). The degree of sex-biased gene expression is more extreme for the X chromosome in F_1

539 hybrids due in part to transgressive underdominance effects (**Figs. 1D, 2C,D**), though the
540 fraction of male-biased genes likely involved with spermatogenesis is, in fact, depleted on the X
541 (**Figs. 5G, 6C,E,G**), consistent with previous findings (42,43). The X-chromosome in hybrid males
542 is enriched, however, for genes showing overdominant expression, which can be linked directly
543 to *cis-trans* compensatory changes (**Figs. 2D, 3**; **Supplementary Fig. S2**). Overall, these results
544 implicate extensive sex-limited developmental systems drift that generates extensive
545 misexpression of genetic networks distinctly in each sex for hybrids of *C. briggsae* and *C. nigoni*.
546
547 While expression differences between species are often biased towards one species in systems
548 such as fruit files and plants (16,18,24), we do not observe much asymmetry toward one
549 species (**Fig. 1A**; binomial test: males, ratio = 0.5, $P = 0.26$; females, ratio = 0.47, $P < 0.001$). This
550 symmetry in expression suggests that demographic effects that exacerbate genetic drift are not
551 likely to bias regulatory changes toward either increased or decreased expression
552 disproportionately for one species, as could occur if regulatory changes fix more rapidly in
553 species like *C. briggsae* with lower effective population sizes due to selfing.
554

555 Sex differences in regulatory divergence expose sexual dimorphism of genetic
556 networks

557 Transgressive expression in F_1 hybrids beyond the bounds of parental expression levels is a
558 signature of misexpression, which we observe in abundance. Given that *C. briggsae* × *C. nigoni*
559 hybrids obey Haldane's rule (48–50), we expected more misexpression in hybrid males. Our
560 analyses revealed, however, that it is hybrid females that experience more extensive
561 transgressive gene misexpression (**Fig. 2A-C**). Studies in mice have shown that hybrid
562 misexpression of X-linked genes confers male sterility (5,28) and *Drosophila* demonstrates an
563 important influence of X-autosome incompatibilities for Haldane's rule and hybrid male sterility
564 (36,38), though with a more tenuous link to misexpression of X-linked genes (29,79). We found
565 enrichment of misexpressed loci on the X-chromosome for both sexes (**Fig. 2D**) and no
566 indication of compromised dosage compensation. Most female-biased transgressive genes
567 show overdominant misexpression, however, compared to more underdominant misexpression

568 in hybrid males (**Fig. 5A-C**). We hypothesize that *cis*- and/or *trans*-regulatory changes acquired
569 after speciation experienced selection to sustain upregulated expression of female-biased
570 genes, with those changes behaving in an overdominant manner in hybrids. Indeed,
571 overdominant genes with *cis*-*trans* divergence in females have disproportionately evolved
572 "enhancing" regulatory changes compared to males (**Figs. 2G, 3, 5D**). In spite of the extensive
573 overdominance in female-biased expression (**Figs. 2B,C, 5C**), the fact that hybrid females are
574 fertile suggests that overdominant expression does not impact fitness as negatively as does
575 regulatory divergence that leads to underdominance in hybrids. Consequently, this sexual
576 dimorphism in gene regulatory networks may contribute to the greater sensitivity of males to
577 manifesting hybrid dysfunction.
578
579 Interestingly, we find that regulatory controls over male-biased genes when they are expressed
580 in F_1 females largely reveals *trans*-only regulatory changes (**Fig. 5D,E, 6F,G**). These *trans*-acting
581 regulatory changes are more strongly associated with *C. nigoni* dominant expression in females
582 among autosomes, which contrasts with these same genes when expressed in males that reveal
583 extensive *cis*-regulatory changes and *C. briggsae* dominant expression (**Fig. 4**). Previous studies
584 have shown that *trans*-acting regulation often manifests expression dominantly in hybrids,
585 possibly as a result of masking effects between dominant and recessive *trans* alleles, whereas
586 *cis*-acting regulation often generates additive contributions in expression (64). However, *cis*-
587 dominant expression patterns are not uncommon in ASE studies (18) and may arise, potentially,
588 from cases where *cis* changes in one species decrease transcription factor binding affinity or
589 where significant post-transcriptional regulation in pure species is compromised in hybrids.
590 Merritt et al. (65) showed that *C. elegans* oogenic germline gene networks depend strongly on
591 3'UTR post-transcriptional regulation, but that genes showing spermatogenic expression rely
592 primarily on upstream transcriptional regulation. Curiously, disruption of endogenous 22G
593 smallRNA regulation of spermatogenesis in hybrids of *C. briggsae* x *C. nigoni* also is implicated
594 in male sterility (30). It may be that sex differences in the dominant mode of regulation
595 (transcriptional versus post-transcriptional) contributes to the sex differences in the *cis*-
596 dominance that we report. These results also align with observations of downregulation of

spermatogenesis genes, such as *fog-1* and *fog-3*, by specific transcription factors (i.e., *tra-1*), and sperm-specific expression depending more on upstream promoter regions than 3-UTRs in *C. elegans* (65,71,80).

We observed distinct signatures of regulatory divergence for genes with sex-biased expression when expressed in the opposite sex, raising the possibility that such divergence may reflect a history of sexual selection and sexual conflict (51,56). One possible explanation invokes Rice's hypothesis (81): the fixation of regulatory changes that create sex-biased expression serve as evolutionary solutions to inter-sexual genomic conflict. To avoid traits that are detrimental to females but improve male performance, genomic conflict resolution by means of sex-biased expression may be attained faster through *trans*-regulatory changes, which are more pleiotropic, downregulating male-biased genes in females. This logic aligns with the hypothesis that sex-biased expression is partly driven by selection acting to resolve sexual conflict by means of modifier alleles or regulators (63,81). However, the fact that *trans*-only regulatory changes do not predominate in the control of female-biased genes in males (**Fig. 4E**), suggests that regulatory mechanism to resolve genomic sexual conflict act in different ways for the two sexes. Direct manipulative experiments would prove valuable in testing these ideas further.

The fact that the egg-bearing sex in the *C. briggsae* parent is actually a hermaphrodite may help explain the presence of underdominant genes in hybrid females. Many of the genes in hybrid females that show underdominant effects would otherwise show male-biased expression (**Fig. 5C, 6E**), suggesting that they may compromise spermatogenesis to effectively convert F_1 hermaphrodites into females. This perspective provides a complementary view to the idea that hermaphroditism is 'recessive' to femaleness in a simple Mendelian manner (48). Our expression data characterizes *tra-1*, a master regulator of sexual development programs through its repression of genes such as *fog-3* that would promote spermatogenesis and male development (71), as a conserved sex-neutral gene that is overdominant in both male and female F_1 hybrids (C-N-N; **Supplementary Fig. S6**). Furthermore, *tra-2* and *fem* genes, which interact to facilitate TRA-1 activity as a repressor, are all female-biased and overdominantly

626 expressed in hybrids, which suggest an additional mechanism for hybrid XX "feminization".
627 Consequently, repression of male-biased genes involved with the *tra-1* pathway is likely to
628 cause both the "female" phenotype in hybrid females (i.e. absence of male function normally
629 seen in hermaphrodites) and reduced fertility in hybrid males.
630
631 Gene expression in hybrid males predominantly shows either simple dominance or no change
632 (**Fig. 2C**). While it is tempting to speculate that it might be a byproduct of males of different
633 species sharing the same reproductive role, reduced sexual selection in *C. briggsae* males
634 (52,56), genomic divergence (54,55), and clear sex differences in hybrid fertility (48,49), suggest
635 otherwise. If most transgressive expression occurs in the gonad, then the small and defective
636 gonad development of F_1 males may have led to their observed paucity of transgressive
637 expression. Distinct relative sizes of tissues between species, sexes, and hybrids could
638 potentially influence differences in expression and their interpretation (82). Several aspects of
639 these *Caenorhabditis* minimize the potential influence of allometric effects between species.
640 The morphology of *Caenorhabditis* species is highly conserved even between very distantly
641 related species, with body size being very similar for *C. briggsae* and *C. nigoni* in particular (83);
642 body size differences within and between species reflect differences in cell size not cell
643 numbers (59). Our data show symmetry in upregulated genes between species (**Fig. 1A**),
644 consistent with limited potential role of allometric bias between species. While hybrid females
645 experience delayed germline development (48), we analyzed adult animals with complete
646 development. However, the gonad of hybrid males is anatomically deformed, potentially
647 contributing to differences in expression due to tissue allometry. If disproportionately small
648 gonad size of hybrid males were to skew expression patterns, then we would expect a large
649 signal of genes showing low expression specifically in hybrid males. We observe two modules of
650 co-expression with such a pattern (M3 and M15 in **Supplementary Fig. S2**), but M15 comprises
651 just 437 (8.7%) of male-biased genes and M3 contains genes with female-biased expression
652 overall (672 genes, 15% of female-biased genes). Moreover, we observed a high number of
653 genes with conserved expression across males, including hybrids (**Fig. 2C**), counter to what
654 would be expected if tissue allometry introduced substantial bias.

655

656 ## Implications for Haldane's rule and the large-X effect

657 Male-biased genes are expected to evolve fast because of sexual selection and sexual conflict,
658 resulting in higher rates of protein-coding and gene expression divergence (37,67,68). Faster
659 evolution of male-biased genes is the premise behind the "faster male" hypothesis to explain
660 the high incidence of hybrid male sterility in Haldane's rule (32). We find faster evolving coding
661 sequences for the subset of male-biased genes that also show exceptionally high expression
662 divergence (B-M-I and B-M-N; **Fig. 5E,F**), many of which are implicated in spermatogenesis (**Fig.**
663 **6A-C**; **Supplementary Fig. S4,5**). This result is consistent with previous reports of faster
664 molecular evolution of spermatogenesis and male germline genes of *C. elegans* (56,84,85), and
665 suggests that "faster male" evolution may contribute to Haldane's rule in *Caenorhabditis*. To
666 the extent that rapid evolution predisposes genes to forming genetic incompatibilities,
667 however, the fact that we rarely observe such genes on the X-chromosome suggests that the
668 "faster X" model does not provide a compelling explanation for Haldane's rule for hybrid male
669 sterility (33) (**Fig. 5G**).
670

671 The "large-X" effect, where hybrid male sterility results from a disproportionate count of X-
672 linked loci involved in genetic incompatibilities (86), is evinced from deletion screen
673 experiments in *Drosophila* (36,38) and introgression experiments in *C. briggsae* x *C. nigoni*
674 (57,58). An analogous effect can result from X-linked regulatory divergence that causes
675 misexpression and hybrid dysfunction (25,28,87). Consistent with a large-X effect due to
676 regulatory evolution in *Caenorhabditis*, our analyses show that the X chromosome contains an
677 excess of genes that have undergone *cis-trans* compensatory changes causing misexpression in
678 hybrid males (**Fig. 3**). This pattern implicates a disproportionate role for regulatory divergence
679 of the X-chromosome in mediating misexpression in *Caenorhabditis* hybrids.
680

681 Two non-mutually exclusive ways in which hybrid male dysfunction (i.e., sterility) can arise are:
682 1) through misexpression and misregulation of X-linked genes involved with male function, and
683 2) through negative epistatic interactions (i.e., incompatibilities) between X-linked and

684 autosome genes involved in male-specific pathways. Our results suggest that both cases are
685 plausible.
686
687 First, the paucity of X-linked sex-biased genes in parental genotypes of *Caenorhabditis* species
688 suggests that any misregulation and misexpression on the X might exert little downstream
689 impact (**Fig. 1D**; (42,43)). However, misexpression of X-linked genes in hybrids is relatively
690 common compared to autosomes (with the exception of Chromosome V) (**Fig. 2D**), with hybrid
691 males having higher relative incidence of effectively misregulated genes (*trans*-only and
692 compensatory *cis*-*trans* changes) compared to female hybrids (**Fig. 3**). In fact, we find that
693 *trans*-acting factors often contribute to misexpression in both sexes (**Fig. 3, 4**). In hybrid
694 females, *trans*-acting factors largely drive the expression of X-linked genes with *C. briggsae*
695 dominant and underdominant expression, unlike autosomes (except Chromosome V) (**Fig. 4**). In
696 hybrid males, this effect is even more pronounced, in part due to our inference that all X-linked
697 genes with *C. briggsae* dominant expression arise from *trans*-only* effects that implicate
698 recessive *C. nigoni trans* regulators (**Supplementary Fig. S14**), which must be at minimum an
699 underestimation of all *trans*-acting regulatory divergence affecting the X-chromosome. These
700 findings are consistent with previous observations, particularly in *Drosophila*, of *trans*-acting
701 sex-specific changes causing misregulation of X-linked genes (39,88,89).
702
703 Second, the extensive expression dominance in F_1 males that disproportionately matches the *C.*
704 *briggsae* expression level due to *cis* effects have the potential to disrupt gene networks as they
705 may interact negatively with *C. nigoni* X-linked genes in hybrid males (**Fig. 4; Supplementary**
706 **Fig. S10**). Autosomal spermatogenesis genes, by contrast, tend to show *C. nigoni*-dominant
707 expression in F_1 hybrid males (**Supplementary Fig. S5B,C**), consistent with previous work
708 showing recessive effects of the *C. briggsae* autosomal portions of genetic incompatibilities
709 (57). In addition, this prior work also showed that sterility in *C. nigoni* x *C. briggsae* hybrid males
710 may not require many X-autosome incompatibilities (58). Despite their low abundance on the
711 X-chromosome, X-linked spermatogenesis genes are often enriched for both misexpression and
712 misregulation (**Fig. 3; Supplementary Fig. S5B,C**), potentially enhancing their role in hybrid

713 dysfunction. Our genome-wide transcriptome analysis of *cis*- and *trans*-regulatory divergence
714 therefore sheds new light on Haldane's rule, reinforcing some previous key inferences about
715 hybrid dysfunction associated with males, spermatogenesis, and the X-chromosome.
716

717 Decoupled coding vs regulatory divergence and the evolution of hybrid dysfunction

718 Genes that evolve fast may be predisposed to creating genetic incompatibilities, which could
719 result either from dysfunctional structural activities of the encoded proteins or controls over
720 the timing or location of gene expression. Studies to date indicate that rates of evolution of
721 coding sequences and regulatory regions do not correlate strongly (90,91). Supporting the idea
722 that regulatory divergence due to *cis*-acting elements is largely decoupled from rates of
723 molecular evolution, we found only weak positive correlations between rates of molecular for
724 protein structure and gene regulation (**Fig. 7**). *cis*-regulatory divergence also showed only a
725 weak elevation in chromosome arms compared to centers (**Supplementary Fig. S7B**), genomic
726 regions with marked differences in recombination rates, gene density, sequence conservation
727 within and between species (**Supplementary Fig. S7A,8**) (47,92) that influence the rate at which
728 mutations, especially weakly-selected regulatory mutations, can get fixed as a result of direct
729 selection and linked selection (93). The decoupled rates of evolution for protein structure and
730 gene regulation imply that genetic incompatibilities mediated through protein activity versus
731 gene regulation may follow different rules in the evolution of reproductive isolation.

732 # Conclusion

733 We contrasted sex-specific transcriptomic profiles between *C. briggsae* and *C. nigoni* and their
734 hybrids to understand how the evolution of *cis*- and *trans*-regulatory elements can contribute
735 to F_1 hybrid dysfunction. Regulatory evolution underlies divergent expression as well as
736 conserved expression subject to compensatory effects of changes to multiple elements. The
737 sharp contrast of extensive misexpression in F_1 hybrids with the morphological stasis and
738 expression conservation between *Caenorhabditis* species indicates substantial developmental
739 system drift of regulatory networks that destabilize in hybrids to enforce reproductive isolation
740 between species. Despite more extensive transgressive expression in hybrid females, they are
741 fertile but unable to produce self-sperm and hybrid males are entirely sterile, suggesting that 1)
742 genetic networks controlling “male” developmental pathways are more fragile in the face of
743 genetic perturbation and 2) hybrid females may represent "demasculinized" hermaphrodites
744 through the disruption of sperm-specific regulatory networks. Despite the rarity of sex-biased
745 genes on the X-chromosome, the X is home to disproportionate misexpression in both sexes,
746 with misregulation in hybrid males largely occurring through *trans*-acting factors and *cis*-*trans*
747 compensatory changes. X-autosome incompatibilities in hybrid males likely result from the
748 propensity for divergent *cis*-acting factors to drive *C. briggsae*-dominant autosomal expression
749 to yield allele-specific expression biases, which then interact negatively with *C. nigoni* X-linked
750 genes. Moreover, *C. nigoni*-dominant *trans*-acting factors may act to downregulate male-biased
751 genes in both males and females, through the misregulation of master controllers of sexual
752 development such as *tra-1*. Finally, we find only weak correlations of *cis*-regulatory divergence
753 with chromosome architecture and protein-coding and non-coding sequence divergence,
754 indicating that regulatory and protein evolution are largely decoupled. Consequently,
755 Dobzhansky-Muller incompatibilities involving regulatory and coding sequences may
756 accumulate independently of one another, and in distinct ways in the regulatory networks of
757 each sex, in the build-up of reproductive isolation in the speciation process.

758 # Material and Methods

759 ## Samples, RNA isolation, and sequencing

760 We cultured triplicate populations of isofemale *C. briggsae* (AF16) and *C. nigoni* (JU1421) on
761 NGM-agar plates with *Escherichia coli* OP50 at 25°C, isolating total RNA via Trizol-chloroform-
762 ethanol extraction from groups of approximately 500 individual age-synchronized young adult
763 males or females (hermaphrodites) for each replicate sample. *C. briggsae* hermaphrodites are
764 treated as the female sex for the purposes of this study (see below), as their soma is
765 phenotypically female despite the gonad producing a small number of sperm in addition to
766 abundant oocytes. We also crossed in triplicate virgin *C. nigoni* females to male *C. briggsae*
767 (isolated as L4 larvae) to produce F_1 hybrid progeny, with RNA isolated from male and female F_1
768 hybrids as for the parental pure species genotypes.
769
770 The triplicate mRNA samples for each sex and genetic group (*C. briggsae*, *C. nigoni*, F_1 hybrid)
771 underwent 100bp read length, single-ended Illumina HiSeq sequencing at GenomeQuebec
772 according to their standard TruSeq3 protocol. A total of ~250 million reads from these 18
773 barcoded samples spread across 4 lanes were cleaned for quality control using TRIMMOMATIC
774 v0.38 (with arguments: ILLUMINACLIP:TruSeq3-SE:2:30:10 LEADING:3 TRAILING:3
775 SLIDINGWINDOW:4:15 MINLEN:36) (94).
776

777 ## Reference alignment and allele-specific read assignment

778 Following quality control trimming and filtering, we mapped sequence reads from each sample
779 to the chromosome-level genome assembly and annotation of each species (*C. briggsae* WS271
780 https://osf.io/a4e8g/, *C. nigoni* 2018-01-WormBase https://osf.io/dkbwt/; (55)) using STAR v2.6
781 (https://github.com/alexdobin/STAR; (95)) with default parameters and adjusting for intron size
782 (--alignIntronMin 40 --alignIntronMax 15600). Reads from *C. briggsae* and *C. nigoni* were
783 mapped to their own reference, while reads from F_1 hybrids were mapped to both reference
784 genomes. Reference genomes and GFF files can also be found online
785 (https://github.com/santiagosnchez/competitive_mapping_workflow/tree/master/references).

786

787 To obtain allele-specific read counts in F_1 hybrids, we applied a competitive read mapping
788 approach by developing a Python implementation for competitive read-mapping we call
789 CompMap (https://github.com/santiagosnchez/CompMap). This approach uses the pysam library
790 (https://github.com/pysam-developers/pysam) internally for reading and indexing BAM
791 alignments by read name. Using BED files with transcript-level coordinates consistent between
792 references, read overlaps were then counted for each feature in both alignments. At each
793 feature, the alignment score (AS) and number of mismatches (nM) of each read to both
794 references were compared, assigning best aligned to reference-specific counts. Ambiguous
795 reads (i.e., those being equally good alignments) were also counted and redistributed
796 proportionally to the number of reads assigned to each reference. We validated our method
797 with simulated RNA-seq data using the R package polyester (96) finding high correlation
798 between true ASE and CompMap counts, as well as low type 1 and type 2 error rates among *cis*
799 and *trans* regulatory divergence categories (<5%; **Supplementary Fig. S11,S12**). We expected to
800 have high power to detect ASE, given ~20% neutral sequence divergence between *C. briggsae*
801 and *C. nigoni* (47) conferring on average ~5 nucleotide differences for every 100 bp of coding
802 sequence (0.2 divergence * 0.25 fraction of synonymous sites * 100 bp). Additionally, we did
803 not expect significant mapping bias (97,98) given that our data was strain-specific (i.e. our C.
804 *briggsae* RNA-seq data comes from strain AF16, which is the same as the reference genome and
805 our *C. nigoni* data comes from strain JU1421 which derives from the same wild isolate as the *C.*
806 *nigoni* reference genome; see supplementary data in (55)). Scripts, programs, and commands
807 used for bioinformatic analyses can be found online
808 (https://github.com/santiagosnchez/competitive_mapping_workflow).
809

810 Ortholog identification and read abundance quantification

811 The chromosomes in the *C. briggsae* and *C. nigoni* genomes are largely colinear, with only a few
812 small inversions and translocations reported (99). Therefore, we quantified gene expression
813 abundance for a set of 13,636 genes that we inferred to be one-to-one reciprocal orthologs
814 between *C. briggsae* and *C. nigoni*, for which upstream regulatory regions should also be

815 syntenic. To identify orthologs, we applied a phylogenetic approach using OrthoFinder v2.2.6
816 (100,101), based on longest-isoform peptide sequence translations for gene annotations of 28
817 *Caenorhabditis* species (102) (http://caenorhabditis.org/). BLASTp all-by-all searches were done
818 separately on SciNet's Niagara supercomputer cluster. OrthoFinder was run with default
819 options, which included: -M dendroblast (gene tree reconstruction) and -I 1.5 (MCL inflation
820 point). In further analysis of the final set of 13,636 orthologs, we excluded from a preliminary
821 set of 15,461 orthologs those genes for *C. briggsae* and *C. nigoni* that could not be assigned to
822 any of their six chromosomes (688 genes), that were associated with inter-chromosomal
823 translocations (370 genes), that we could not estimate K_a/K_s reliably (275 genes), or that
824 exhibited low mRNA-seq read abundance (492 genes; see below). A list of all the orthologs can
825 be found online
826 (https://github.com/santiagosnchez/competitive_mapping_workflow/blob/master/orthologs.tx
827 t).
828
829 We quantified gene expression in parent species for each ortholog with featureCounts v2.0.1
830 (103) as we found its read-counting method to be compatible with CompMap. Raw read counts
831 were combined into a single table and imported into R together with ASE counts (104) for
832 normalization and statistical analyses. All raw data counts can be found online
833 (https://github.com/santiagosnchez/competitive_mapping_workflow/tree/master/counts).
834

835 Identification of male-biased genes in *C. briggsae* hermaphrodites

836 *C. briggsae* hermaphrodites have the ability to produce low amounts of sperm in addition to
837 oocytes, while their soma phenotypically resembles that of a female. Therefore, to make more
838 biologically realistic contrasts between *C. briggsae* hermaphrodites, *C. nigoni* females, and
839 female hybrids, we used an *in silico* approach to identify male-biased genes, which are also
840 upregulated in hermaphrodites, through comparisons using previously published RNA-seq data.
841 We used RNA-seq read data derived from *C. briggsae* pseudo-females (AF16-derived *she-1(v47)*
842 mutant strain) (53), which are unable to produce self-sperm. We conducted differential
843 expression analyses using a similar pipeline as described below to identify genes with significant

844 sex-biased expression in our *C. briggsae* RNA-seq data and the one from pseudo-females in
845 Thomas et al. (53), in addition to being differentially expressed between datasets. These genes
846 where then excluded from analyses comparing hermaphrodites and females together and were
847 analyzed separately. Data for these analyses can be found online
848 (https://github.com/santiagosnchez/competitive_mapping_workflow/tree/master/analyses/ta
849 bles/Thomas_et_al_data).
850

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

852 We used the R Bioconductor package DESEQ2 (105) to assess differential expression. Before
853 statistically assessing differential expression, we summed the allele-specific counts from F_1
854 hybrids to yield a single count of transcripts per gene. After calculating library size factors, we
855 filtered out genes that did not meet the criterion of having at least 3 samples with more than
856 10 library-size scaled counts. We visualized the overall expression distance between samples
857 using a non-metric multi-dimensional scaling plot, which showed all three biological replicates
858 to cluster consistently within their corresponding treatment (**Supplementary Fig. S1A**). We
859 inferred sex-biased gene expression by comparing differential expression profiles between
860 males and females (or hermaphrodites) in each genetic group (*C. briggsae*, *C. nigoni*, F_1
861 hybrids). We also quantified differential expression between the genetic groups in a pairwise
862 manner (*C. briggsae* vs F_1, *C. briggsae* vs *C. nigoni*, *C. nigoni* vs F_1) for each sex separately. We
863 then contrasted expression patterns between species (*C. briggsae* and *C. nigoni*) by looking at
864 sex differences (sex-biased expression) and their interaction (expression ~ species * sex). Within
865 hybrid males and females, we compared allele-specific counts to measure ASE. For all of these
866 contrasts, we used the negative binomial generalized linear model fitting and Wald statistics to
867 determine differentially expressed genes, as implemented in DESEQ2. We used FDR-adjusted *P*-
868 values at the 5% level to assess significance. DESeq2 results can be found online
869 (https://github.com/santiagosnchez/competitive_mapping_workflow/tree/master/analyses/ta
870 bles/DESeq2).
871

872 ## Co-expression clustering

873 To identify groups of genes with shared co-expression trends, we averaged $\log_2$-transformed
874 normalized read counts with the *rlog* function from DESEQ2 for each sample. Then we
875 standardized gene-wise expression data by calculating Z-score values and used *K*-means to
876 cluster co-expression groups. We chose a sensible *k* value (*k*=15) approaching the asymptote by
877 plotting the within-group sum of squares for a range of *k* values (from 2 to 100). We then
878 calculated centroid expression levels by estimating the mean relative expression across samples
879 within each group. Our co-expression clustering results can be found online
880 (https://github.com/santiagosnchez/competitive_mapping_workflow/tree/master/analyses/ta
881 bles/clustering).
882

883 ## Mode of expression inheritance in F_1 hybrids

884 Based on patterns of expression in F_1 hybrids relative to parent species, we classified genes into
885 those having **additive** (intermediate), **dominant** (matching either of the species), **overdominant**
886 (higher that both parents), and **underdominant** (lower than both parents) profiles following the
887 logic in McManus et al. (16). Genes with no significant differences in expression between F_1s
888 and their parent species were deemed to have conserved regulatory controls resulting in **no**
889 **change** in expression in F_1s. Genes with additive effects had intermediate expression in F_1s
890 compared to both parental species, meaning that there were significant differences in
891 expression between all groups in a manner where expression levels in F_1s fall in between both
892 species. Genes with dominant allelic effects were those with expression levels in F_1s matching
893 either one of the parent species (i.e. no significant differential expression), but with significant
894 differential expression between species. Finally, genes with significant differential expression
895 from both parents, but that were either significantly underexpressed (underdominant) or
896 overexpressed (overdominant) compared to both species were regarded as transgressive.
897 Genes falling outside any these specific categories were considered **ambiguous**. A per-gene
898 summary table with all expression inheritance classification can be found online
899 (https://github.com/santiagosnchez/competitive_mapping_workflow/tree/master/analyses/ta
900 bles/expression_inheritance).

901

902 We also measured absolute Euclidean distances in expression relative to the centroid or origin
903 in expression space of F_1 hybrids relative to both parent species. For example, for every gene
904 we took the expression difference between F_1s and *C. briggsae* and between F_1s and *C. nigoni*
905 as an xy coordinate system. Then, we measured the Euclidean distance from that point in
906 expression space to the origin (0,0), reflecting no change in expression:
907

908 $$d = \sqrt{(\Delta_{F1/Cbr})^2 + (\Delta_{F1/Cbr})^2}$$

909 Where $\Delta_{F1/Cbr}$ and $\Delta_{F1/Cni}$ are coefficients of differential expression between F_1 hybrids and
910 each parent species. This metric allowed us to visualize the magnitude of expression distance
911 from a "conserved" expression profile.
912

913 ## *cis*- and *trans*- regulatory divergence

914 We used ASE in F_1s to quantify the extent and type of *cis*- and *trans*-regulatory differences
915 between species. Expression divergence between parent species results from both *cis*- and
916 *trans*-regulatory changes, whereas significant differential expression between alleles in F_1s
917 results from *cis*-regulatory divergence only (16). To quantify the extent of *trans* effects, we
918 applied a linear model to test for differences in gene expression between parent species (P) and
919 between alleles in F_1 hybrids (ASE) using the following model: expression ~ species/group,
920 where "group" represented categorical variables pointing to data from P and ASE. The division
921 operator of the function "/" measures expression ratios independently for each category in
922 "group". We then used a post-hoc Wald-type test (*linearHypothesis* from the CAR package) to
923 test for significant differences between both coefficients (**P**[*C. nigoni*/*C. briggsae*] = **ASE**[*C.*
924 *nigoni*/*C. briggsae*]). *P* values were considered significant after a 5% FDR analysis (106).
925

926 We inferred the influence of *cis*- and *trans*-regulatory divergence on genes linked to autosomes,
927 as well as to the X-chromosome in females, following the criteria in McManus et al. (16). This
928 procedure allowed us to designate genes having undergone significant regulatory divergence

929 due to ***cis*-only**, ***trans*-only**, and ***cis-trans*** effects. Genes with significant *cis* and *trans* effects
930 were split into those with synergistic (***cis* + *trans***), antagonistic (***cis* x *trans***), and **compensatory**
931 ***cis-trans***. Genes expressed with no significant differences in expression between parents, ASE,
932 or *trans* effects were deemed as **conserved** and those that did not strictly fit into any of the
933 previous groups were considered **ambiguous (Supplementary Fig. S13)**. A table with all gene-
934 wise classifications for expression inheritance in all chromosomes for females and for
935 autosomes in males can be found online
936 (https://github.com/santiagosnchez/competitive_mapping_workflow/tree/master/analyses/ta
937 bles/cis_trans).
938
939 Given the hemizygous condition of the X-chromosome in males, we cannot use F_1 ASE of X-
940 linked genes to assess *cis* and *trans* regulatory divergence. However, we devised a scheme to
941 assign different types of regulatory divergence to X-linked genes, with some limitations, based
942 on the differences in expression between male F_1 hybrids and parent species (**Supplementary**
943 **Fig. S14**; Wayne et al. 2004). F_1 males of our study carry their maternal *C. nigoni* X-
944 chromosome. Therefore, assuming that the bulk of the *trans* environment derives from
945 autosomes, we would expect that X-linked genes that differ in expression between parental
946 species but display *C. nigoni* dominant expression owe their regulatory divergence mostly to
947 *cis*-regulatory (***cis*-only***) changes, because any significant deviation from *C. nigoni* expression
948 levels would indicate *trans*-regulatory changes. An exception would involve the action of "local"
949 *trans* regulators found on the X, either way still considered "local". Genes with significant
950 regulatory divergence between species where hybrids display *C. briggsae* dominant expression
951 (***trans*-only***) would indicate no significant changes due to *cis* regulation, with the condition
952 that autosomal *C. nigoni trans* regulators affecting those genes are recessive, and therefore not
953 expressed. We consider these a subset of total *trans* regulation and is likely an underestimation
954 compared to autosomes and X-chromosomes expressed in females. The last category that we
955 can assign confidently is **compensatory *cis-trans* changes**. Genes with no differential expression
956 between species, but with significant up- or down-regulation in F1 males (i.e., overdominant or
957 underdominant) were considered as having *cis-trans* compensatory changes. For the purpose of

958 comparing regulatory divergence between autosomes and the X in males and females, we
959 lumped all other genes with significant expression divergence into "other", as they would be
960 difficult to disambiguate without additional data. These include genes with *trans*-only (i.e., with
961 codominant *trans* regulation), *cis* + *trans* (enhancing), *cis* x *trans* (compensatory), and
962 ambiguous. Genes with no expression change were kept as "conserved" (**Supplementary Fig.**
963 **S12**).
964

965 ## Molecular evolution in coding sequences

966 Orthologs in the genomes of both *C. briggsae* and *C. nigoni* were first aligned as protein coding
967 sequences using MAFFT v7.407 (107). These alignments were then back-translated to coding
968 sequence (CDS) alignments using the python program CODONALIGN
969 (https://github.com/santiagosnchez/CodonAlign). We estimated rates of synonymous site (K_s)
970 and non-synonymous site divergence (K_a) between the two aligned sequences using a custom
971 Python script (https://github.com/santiagosnchez/DistKnKs) applying the Yang and Nielsen
972 (2000) model implemented in BioPython (Cock et al. 2009). We also corrected K_s values for
973 selection on codon usage using the effective number of codons (ENC; (108,109)) as a predictor
974 in a linear model. In short, we fitted a linear regression model (K_s ~ ENC), which we used to
975 predict K_s at the maximum value of ENC (=60). Then, we corrected the bias in K_s by adding the
976 residuals of the linear model to that idealized value of K_s at ENC = 60. We refer to these
977 corrected set of K_s estimates as K_s'. A table with these estimates of molecular evolution for each
978 gene can be found online
979 (https://github.com/santiagosnchez/competitive_mapping_workflow/tree/master/analyses/ta
980 bles/molecular_evolution).
981

982 ## Upstream non-coding sequence conservation

983 Chromosome-level FASTA sequences for *C. briggsae* and *C. nigoni* were aligned using LASTZ
984 (110), outputting alignment files for each chromosome in MAF format. We used BEDTOOLS's
985 v2.27 (111) *flank* function to generate 500 bp intervals of the 5' upstream flanking regions of
986 each orthologous gene. We then used *maf_parse* from PHAST (112) to extract overlapping

987 alignment blocks of at least 500 bp long. We quantified sequence conservation as the average
988 number of identical 5 bp non-overlapping windows between aligned DNA in both sequences.
989

990 Spermatogenesis genes

991 To infer genes involved with spermatogenesis, we downloaded a list of *C. elegans* genes
992 previously identified as spermatogenesis-related based on tissue-specific transcript abundance
993 (44) (Additional File 4). We then used the BioMart tool of the WormBase Parasite website (113)
994 to retrieve *C. briggsae* orthologs from the list of *C. elegans* genes. We cross-referenced *C.*
995 *briggsae* orthologs to our own set of orthologs between *C. briggsae* and *C. nigoni* and
996 annotated the 1,089 gene matches with a spermatogenesis tag. The data obtained for *C.*
997 *elegans* spermatogenesis orthologs can be found online
998 (https://github.com/santiagosnchez/competitive_mapping_workflow/tree/master/analyses/ta
999 bles/spermatogenesis).
1000

1001 Data availability

1002 Raw sequencing data can be found on the Short Read Archive under accessions SRR12532505-
1003 SRR12532522 and BioProject PRJNA659254. Raw gene counts, workflow scripts, and main data
1004 tables and files can be found on GitHub under the following repository:
1005 https://github.com/santiagosnchez/competitive_mapping_workflow.

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1014 # References

1015 1. Coyne JA, Orr HA. Speciation. Sinauer Associates Incorporated: Sinauer Associates
1016 Incorporated; 2004.
1017 2. Rundle HD, Nosil P. Ecological speciation. Ecol Lett. 2005;8(3):336–52.
1018 3. Orr HA. The population genetics of speciation: the evolution of hybrid incompatibilities.
1019 Genetics. 1995;139(4):1805–13.
1020 4. Gordon KL, Ruvinsky I. Tempo and mode in evolution of transcriptional regulation. PLoS
1021 Genet. 2012;8(1):e1002432.
1022 5. Mack KL, Nachman MW. Gene regulation and speciation. Trends Genet. 2017;33(1):68–
1023 80.
1024 6. Lemos B, Meiklejohn CD, Cáceres M, Hartl DL. Rates of divergence in gene expression
1025 profiles of primates, mice, and flies: stabilizing selection and variability among functional
1026 categories. Evolution. 2005;59(1):126–37.
1027 7. Denver DR, Morris K, Streelman JT, Kim SK, Lynch M, Thomas WK. The transcriptional
1028 consequences of mutation and natural selection in *Caenorhabditis elegans*. Nat Genet.
1029 2005;37(5):544–8.
1030 8. Rifkin SA, Houle D, Kim J, White KP. A mutation accumulation assay reveals a broad
1031 capacity for rapid evolution of gene expression. Nature. 2005;438(7065):220–3.
1032 9. Gilad Y, Oshlack A, Rifkin SA. Natural selection on gene expression. Trends Genet.
1033 2006;22(8):456–61.
1034 10. Signor SA, Nuzhdin S V. The evolution of gene expression in *cis* and *trans*. Trends Genet.
1035 2018;34(7):532–44.
1036 11. Bedford T, Hartl DL. Optimization of gene expression by natural selection. Proc Natl Acad
1037 Sci U S A. 2009;106(4):1133–8.
1038 12. Wray GA. The evolutionary significance of *cis*-regulatory mutations. Nat Rev Genet.
1039 2007;8(3):206–16.
1040 13. Stern DL, Orgogozo V. The loci of evolution: how predictable is genetic evolution?
1041 Evolution. 2008;62(9):2155–77.
1042 14. Gordon KL, Ruvinsky I. Tempo and mode in evolution of transcriptional regulation. PLoS
1043 Genet. 2012;8(1):e1002432.
1044 15. Wittkopp PJ. Genomic sources of regulatory variation in *cis* and in *trans*. Cell Mol Life Sci.
1045 2005;62(16):1779–83.
1046 16. McManus CJ, Coolon JD, Duff MO, Eipper-Mains J, Graveley BR, Wittkopp PJ. Regulatory
1047 divergence in *Drosophila* revealed by mRNA-seq. Genome Res. 2010;20(6):816–25.
1048 17. Shi X, Ng DW-K, Zhang C, Comai L, Ye W, Chen ZJ. *cis*- and *trans*-regulatory divergence
1049 between progenitor species determines gene-expression novelty in *Arabidopsis*
1050 allopolyploids. Nat Commun. 2012;3(1):950–9.
1051 18. Bell GDM, Kane NC, Rieseberg LH, Adams KL. RNA-seq analysis of allele-specific
1052 expression, hybrid effects, and regulatory divergence in hybrids compared with their
1053 parents from natural populations. Genome Biol Evol. 2013;5(7):1309–23.
1054 19. Tirosh I, Reikhav S, Levy AA, Barkai N. A yeast hybrid provides insight into the evolution
1055 of gene expression regulation. Science. 2009;324(5927):659–62.

1056 20. Barrière A, Gordon KL, Ruvinsky I. Coevolution within and between regulatory loci can
1057 preserve promoter function despite evolutionary rate acceleration. PLoS Genet.
1058 2012;8(9):e1002961-13.
1059 21. True JR, Haag ES. Developmental system drift and flexibility in evolutionary trajectories.
1060 Evol Dev. 2001;3(2):109–19.
1061 22. Landry CR, Wittkopp PJ, Taubes CH, Ranz JM, Clark AG, Hartl DL. Compensatory *cis-trans*
1062 evolution and the dysregulation of gene expression in interspecific hybrids of *Drosophila*.
1063 Genetics. 2005;171(4):1813–22.
1064 23. Wittkopp PJ, Haerum BK, Clark AG. Evolutionary changes in *cis* and *trans* gene regulation.
1065 Nature. 2004;430(6995):85–8.
1066 24. Wittkopp PJ, Haerum BK, Clark AG. Regulatory changes underlying expression differences
1067 within and between *Drosophila* species. Nat Genet. 2008;40(3):346–50.
1068 25. Mack KL, Campbell P, Nachman MW. Gene regulation and speciation in house mice.
1069 Genome Res. 2016;26(4):451–61.
1070 26. Emerson JJ, Hsieh LC, Sung HM, Wang TY, Huang CJ, Lu HHS, et al. Natural selection on *cis*
1071 and *trans* regulation in yeasts. Genome Res. 2010;20(6):826–36.
1072 27. Ortiz-Barrientos D, Counterman BA, Noor MAF. Gene expression divergence and the
1073 origin of hybrid dysfunctions. Genetica. 2006;129(1):71–81.
1074 28. Turner LM, White MA, Tautz D, Payseur BA. Genomic networks of hybrid sterility. PLoS
1075 Genet. 2014;10(2):e1004162--23.
1076 29. Gomes S, Civetta A. Hybrid male sterility and genome- wide misexpression of male
1077 reproductive proteases. Sci Rep. 2015;5(1):1–11.
1078 30. Li R, Ren X, Bi Y, Ho VWS, Hsieh C-L, Young A, et al. Specific down-regulation of
1079 spermatogenesis genes targeted by 22G RNAs in hybrid sterile males associated with an
1080 X-Chromosome introgression. Genome Res. 2016;26(9):1219–32.
1081 31. Turelli M, Orr HA. The dominance theory of Haldane's rule. Genetics. 1995;140(1):389–
1082 402.
1083 32. Wu CI, Davis AW. Evolution of postmating reproductive isolation: the composite nature
1084 of Haldane's rule and its genetic bases. Am Nat. 1993;142(2):187–212.
1085 33. Charlesworth B, Coyne JA, Barton NH. The relative rates of evolution of sex
1086 chromosomes and autosomes. Am Nat. 1987;130(1):113–46.
1087 34. Delph LF, Demuth JP. Haldane's rule: genetic bases and their empirical support. J Hered.
1088 2016;107(5):383–91.
1089 35. Cutter AD. X exceptionalism in *Caenorhabditis* speciation. Mol Ecol. 2018;27(19):3925–
1090 34.
1091 36. Masly JP, Presgraves DC. High-resolution genome-wide dissection of the two rules of
1092 speciation in *Drosophila*. PLoS Biol. 2007;5(9):1890–8.
1093 37. Koevoets T, Beukeboom LW. Genetics of postzygotic isolation and Haldane's rule in
1094 haplodiploids. Heredity. 2008;102(1):16–23.
1095 38. Presgraves DC. Evaluating genomic signatures of "the large X-effect" during complex
1096 speciation. Mol Ecol. 2018;27(19):3822–30.
1097 39. Wayne ML, Pan YJ, Nuzhdin S V, McIntyre LM. Additivity and *trans*-acting effects on gene
1098 expression in male *Drosophila simulans*. Genetics. 2004;168(3):1413–20.
1099 40. Llopart A. The rapid evolution of X-linked male-biased gene expression and the large-X

1100 effect in *Drosophila yakuba*, *D. santomea*, and their hybrids. Mol Biol Evol.
1101 2012;29(12):3873–86.
1102 41. Meisel RP, Malone JH, Clark AG. Faster-X evolution of gene expression in *Drosophila*.
1103 PLoS Genet. 2012;8(10):e1003013.
1104 42. Reinke V, Gil IS, Ward S, Kazmer K. Genome-wide germline-enriched and sex-biased
1105 expression profiles in *Caenorhabditis elegans*. Development. 2004;131(2):311–23.
1106 43. Ortiz MA, Noble D, Sorokin EP, Kimble J. A new dataset of spermatogenic vs. oogenic
1107 transcriptomes in the nematode *Caenorhabditis elegans*. G3. 2014;4(9):1765–72.
1108 44. Ma X, Zhu Y, Li C, Xue P, Zhao Y, Chen S, et al. Characterisation of *Caenorhabditis elegans*
1109 sperm transcriptome and proteome. BMC Genomics. 2014;15(1):1–13.
1110 45. Baird SE, Seibert SR. Reproductive isolation in the Elegans-Group of *Caenorhabditis*. Nat
1111 Sci. 2013;05(04):18–25.
1112 46. Félix M-A, Braendle C, Cutter AD. A streamlined system for species diagnosis in
1113 *Caenorhabditis* (Nematoda: Rhabditidae) with name designations for 15 distinct
1114 biological species. PLoS One. 2014;9(4):e94723-9.
1115 47. Thomas CG, Wang W, Jovelin R, Ghosh R, Lomasko T, Trinh Q, et al. Full-genome
1116 evolutionary histories of selfing, splitting, and selection in *Caenorhabditis*. Genome Res.
1117 2015;25(5):667–78.
1118 48. Woodruff GC, Eke O, Baird SE, Félix M-A, Haag ES. Insights into species divergence and
1119 the evolution of hermaphroditism from fertile interspecies hybrids of *Caenorhabditis*
1120 nematodes. Genetics. 2010;186(3):997–1012.
1121 49. Kozlowska JL, Ahmad AR, Jahesh E, Cutter AD. Genetic variation for postzygotic
1122 reproductive isolation between *Caenorhabditis briggsae* and *Caenorhabditis* sp. 9.
1123 Evolution. 2011;66(4):1180–95.
1124 50. Bundus JD, Alaei R, Cutter AD. Gametic selection, developmental trajectories, and
1125 extrinsic heterogeneity in Haldane's rule. Evolution. 2015;69(8):2005–17.
1126 51. Cutter AD. Reproductive transitions in plants and animals: selfing syndrome, sexual
1127 selection and speciation. New Phytol. 2019;224(3):1080–94.
1128 52. Palopoli MF, Peden C, Woo C, Akiha K, Ary M, Cruze L, et al. Natural and experimental
1129 evolution of sexual conflict within Caenorhabditis nematodes. Int J Evol Biol. 2015;1–13.
1130 53. Thomas CG, Li R, Smith HE, Woodruff GC, Oliver B, Haag ES. Simplification and
1131 desexualization of gene expression in self-fertile nematodes. Curr Biol.
1132 2012;22(22):2167–72.
1133 54. Fierst JL, Willis JH, Thomas CG, Wang W, Reynolds RM, Ahearne TE, et al. Reproductive
1134 mode and the evolution of genome size and structure in *Caenorhabditis* nematodes.
1135 PLoS Genet. 2015;11(6):e1005323-25.
1136 55. Yin D, Schwarz EM, Thomas CG, Felde RL, Korf IF, Cutter AD, et al. Rapid genome
1137 shrinkage in a self-fertile nematode reveals sperm competition proteins. Science.
1138 2018;359(6371):55–61.
1139 56. Cutter AD, Morran LT, Phillips PC. Males, outcrossing, and sexual selection in
1140 *Caenorhabditis* nematodes. Genetics. 2019;213(1):27–57.
1141 57. Bi Y, Ren X, Yan C, Shao J, Xie D, Zhao Z. A genome-wide hybrid incompatibility landscape
1142 between Caenorhabditis briggsae and C. nigoni. PLoS Genet. 2015;11(2):e1004993-26.
1143 58. Bi Y, Ren X, Li R, Ding Q, Xie D, Zhao Z. Specific interactions between autosome and X-

1144 chromosomes cause hybrid male sterility in *Caenorhabditis* species. Genetics.
1145 2019;212(3):801–13.
1146 59. Woodruff GC, Willis JH, Phillips PC. Dramatic evolution of body length due to
1147 postembryonic changes in cell size in a newly discovered close relative of *Caenorhabditis*
1148 *elegans*. Evol Lett. 2018;2(4):427–41.
1149 60. Renaut S, Nolte AW, Bernatchez L. Gene expression divergence and hybrid misexpression
1150 between lake whitefish species pairs *Coregonus* spp. Salmonidae). Mol Biol Evol.
1151 2009;26(4):925–36.
1152 61. Meyer BJ. X-Chromosome dosage compensation. WormBook. 2005;1–14.
1153 62. Schartner CM. Evolutionary comparison of X-chromosome dosage compensation across
1154 *Caenorhabditis* species. [University of California, Berkeley]: University of California,
1155 Berkeley; 2016.
1156 63. Meiklejohn CD, Coolon JD, Hartl DL, Wittkopp PJ. The roles of *cis*- and *trans*-regulation in
1157 the evolution of regulatory incompatibilities and sexually dimorphic gene expression.
1158 Genome Res. 2014;24(1):84–95.
1159 64. Lemos B, Araripe LO, Fontanillas P, Hartl DL. Dominance and the evolutionary
1160 accumulation of cis- and trans-effects on gene expression. Proc Natl Acad Sci.
1161 2008;105(38):14471–6.
1162 65. Merritt C, Rasoloson D, Ko D, Seydoux G. 3'UTRs are the primary regulators of gene
1163 expression in the *C. elegans* germline. Curr Biol. 2008;18(19):1476–82.
1164 66. Gibson G, Weir B. The quantitative genetics of transcription. Trends Genet.
1165 2005;21(11):616–23.
1166 67. Kasimatis KR, Nelson TC, Phillips PC. Genomic signatures of sexual conflict. J Hered.
1167 2017;108(7):780–90.
1168 68. Rowe L, Chenoweth SF, Agrawal AF. The genomics of sexual conflict. Am Nat.
1169 2018;192(2):274–86.
1170 69. Sturgill D, Zhang Y, Parisi M, Oliver B. Demasculinization of X chromosomes in the
1171 Drosophila genus. Nature. 2007;450(7167):238–41.
1172 70. Kelleher DF, de Carvalho CE, Doty A V, Layton M, Cheng AT, Mathies LD, et al.
1173 Comparative genetics of sex determination: masculinizing mutations in *Caenorhabditis*
1174 *briggsae*. Genetics. 2008;178(3):1415–29.
1175 71. Ellis RE. Sex determination: TRA-1 Is a non-binary regulator of sexual identity. Curr Biol.
1176 2020;30(18):R1036--R1038.
1177 72. Goodwin EB, Biology REEC, 2002. Turning clustering loops: sex determination in
1178 *Caenorhabditis elegans*. Curr Biol. 2002;12(3):R111--R120.
1179 73. Verster AJ, Ramani AK, McKay SJ, Fraser AG. Comparative RNAi screens in *C. elegans* and
1180 *C. briggsae* reveal the impact of developmental system drift on gene function. PLoS
1181 Genet. 2014;10(2):e1004077-15.
1182 74. Kiontke K, Barrière A, Kolotuev I, Podbilewicz B, Sommer R, Fitch DHA, et al. Trends,
1183 stasis, and drift in the evolution of nematode vulva development. Curr Biol.
1184 2007;17(22):1925–37.
1185 75. Cutter AD. Divergence times in *Caenorhabditis* and *Drosophila* inferred from direct
1186 estimates of the neutral mutation rate. Mol Biol Evol. 2008;25(4):778–86.
1187 76. Mallard F, Noble L, Guzella T, Afonso B, Baer CF, Teotónio H. Selection and drift

1188 determine phenotypic stasis despite genetic divergence. 2019;
1189 77. Barrière A, Ruvinsky I. Pervasive divergence of transcriptional gene regulation in
1190 *Caenorhabditis* nematodes. PLoS Genet. 2014;10(6):e1004435-15.
1191 78. Barkoulas M, Vargas Velazquez AM, Peluffo AE, Félix M-A. Evolution of new cis-
1192 regulatory motifs required for cell-specific gene expression in *Caenorhabditis*. PLoS
1193 Genet. 2016;12(9):e1006278-23.
1194 79. Civetta A. Misregulation of gene expression and sterility in interspecies hybrids: causal
1195 links and alternative hypotheses. J Mol Evol. 2016;82(4):176–82.
1196 80. Lamont LB, Kimble J. Developmental expression of FOG-1/CPEB protein and its control in
1197 the *Caenorhabditis elegans* hermaphrodite germ line. Dev Dyn. 2007;236(3):871–9.
1198 81. Rice WR. Sex chromosomes and the evolution of sexual dimorphism. Evolution.
1199 1984;38(4):735–42.
1200 82. Ranz JM, Namgyal K, Gibson G, Hartl DL. Anomalies in the expression profile of
1201 interspecific hybrids of *Drosophila melanogaster* and *Drosophila simulans*. Genome Res.
1202 2004;14(3):373–9.
1203 83. Vielle A, Callemeyn-Torre N, Gimond C, Poullet N, Gray JC, Cutter AD, et al. Convergent
1204 evolution of sperm gigantism and the developmental origins of sperm size variability in
1205 *Caenorhabditis* nematodes. Evolution. 2016;70(11):2485–503.
1206 84. Cutter AD, Ward S. Sexual and temporal dynamics of molecular evolution in *C. elegans*
1207 development. Mol Biol Evol. 2005;22(1):178–88.
1208 85. Artieri CG, Haerty W, Gupta BP, Singh RS. Sexual selection and maintenance of sex:
1209 evidence from comparisons of rates of genomic accumulation of mutations and
1210 divergence of sex-related genes in sexual and hermaphroditic species of *Caenorhabditis*.
1211 Mol Biol Evol. 2008;25(5):972–9.
1212 86. Turelli M, Moyle LC. Asymmetric Postmating Isolation: Darwin's Corollary to Haldane's
1213 Rule. Genetics. 2007;176(2):1059–88.
1214 87. Lu X, Shapiro JA, Ting CT, LI Y, Li C, Xu J, et al. Genome-wide misexpression of X-linked
1215 versus autosomal genes associated with hybrid male sterility. Genome Res.
1216 2010;20(8):1097–102.
1217 88. Graze RM, McIntyre LM, Morse AM, Boyd BM, Nuzhdin S V, Wayne ML. What the X has
1218 to do with it: differences in regulatory variability between the sexes in *Drosophila*
1219 *simulans*. Genome Biol Evol. 2014;6(4):818–29.
1220 89. Stocks M, Dean R, Rogell B, Friberg U. Sex-specific *trans*-regulatory variation on the
1221 *Drosophila melanogaster* X chromosome. PLoS Genet. 2015;11(2):e1005015-19.
1222 90. Castillo-Davis CI, Hartl DL, Achaz G. *cis*-regulatory and protein evolution in orthologous
1223 and duplicate genes. Genome Res. 2004;14(8):1530–6.
1224 91. Tirosh I, Barkai N. Evolution of gene sequence and gene expression are not correlated in
1225 yeast. Trends Genet. 2008;24(3):109–13.
1226 92. Cutter AD, Choi JY. Natural selection shapes nucleotide polymorphism across the genome
1227 of the nematode *Caenorhabditis briggsae*. Genome Res. 2010;20(8):1103–11.
1228 93. Barton NH. The reduction in fixation probability caused by substitutions at linked loci.
1229 Genet Res. 1994;64(3):199–208.
1230 94. Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina sequence
1231 data. Bioinformatics. 2014;30(15):2114–20.

1232 95. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast
1233 universal RNA-seq aligner. Bioinformatics. 2012;29(1):15–21.
1234 96. Frazee AC, Jaffe AE, Langmead B, Leek JT. Polyester: Simulating RNA-seq datasets with
1235 differential transcript expression. Bioinformatics. 2015;31(17):2778–84.
1236 97. Stevenson KR, Coolon JD, Wittkopp PJ. Sources of bias in measures of allele-specific
1237 expression derived from RNA-seq data aligned to a single reference genome. BMC
1238 Genomics. 2013;14(1):513–36.
1239 98. Degner JF, Marioni JC, Pai AA, Pickrell JK, Nkadori E, Gilad Y, et al. Effect of read-mapping
1240 biases on detecting allele-specific expression from RNA-sequencing data. Bioinformatics.
1241 2009;25(24):3207–12.
1242 99. Ren X, Li R, Wei X, Bi Y, Ho VWS, Ding Q, et al. Genomic basis of recombination
1243 suppression in the hybrid between *Caenorhabditis briggsae* and *C. nigoni*. Nucleic Acids
1244 Res. 2018;46(3):1295–307.
1245 100. Fang G, Bhardwaj N, Robilotto R, Gerstein MB. Getting started in gene orthology and
1246 functional analysis. PLOS Comput Biol. 2010;6(3):e1000703-8.
1247 101. Emms DM, Kelly S. OrthoFinder: solving fundamental biases in whole genome
1248 comparisons dramatically improves orthogroup inference accuracy. Genome Biol.
1249 2015;16:157.
1250 102. Stevens L, Félix M-A, Beltran T, Braendle C, Caurcel C, Fausett S, et al. Comparative
1251 genomics of 10 new *Caenorhabditis* species. Evol Lett. 2019;3(2):217–36.
1252 103. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for
1253 assigning sequence reads to genomic features. Bioinformatics. 2014;30(7):923–30.
1254 104. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for
1255 Statistical Computing: R Foundation for Statistical Computing; 2019.
1256 105. Anders S, Huber W. Differential expression analysis for sequence count data. Genome
1257 Biol. 2010;11(10):1–12.
1258 106. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful
1259 approach to multiple testing. J R Stat Soc Ser B. 1995;57(1):289–300.
1260 107. Katoh K. MAFFT version 5: improvement in accuracy of multiple sequence alignment.
1261 Nucleic Acids Res. 2005;33(2):511–8.
1262 108. Wright F. The “effective number of codons” used in a gene. Gene. 1990;87(1):23–9.
1263 109. Fuglsang A. The “effective number of codons” revisited. Biochem Biophys Res Commun.
1264 2004;317(3):957–64.
1265 110. Harris RS. Improved pairwise alignment of genomic DNA. [Ph.D. Thesis, Pennsylvania
1266 State University]: Ph.D. Thesis, Pennsylvania State University; 1997.
1267 111. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic
1268 features. Bioinformatics. 2010;26(6):841–2.
1269 112. Hubisz MJ, Pollard KS, Siepel A. PHAST and RPHAST: phylogenetic analysis with
1270 space/time models. Brief Bioinform. 2011;12(1):41–51.
1271 113. Howe KL, Bolt BJ, Shafie M, Kersey P, Berriman M. WormBase ParaSite – a
1272 comprehensive resource for helminth genomics. Mol Biochem Parasitol. 2017;215:2–10.
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Figures

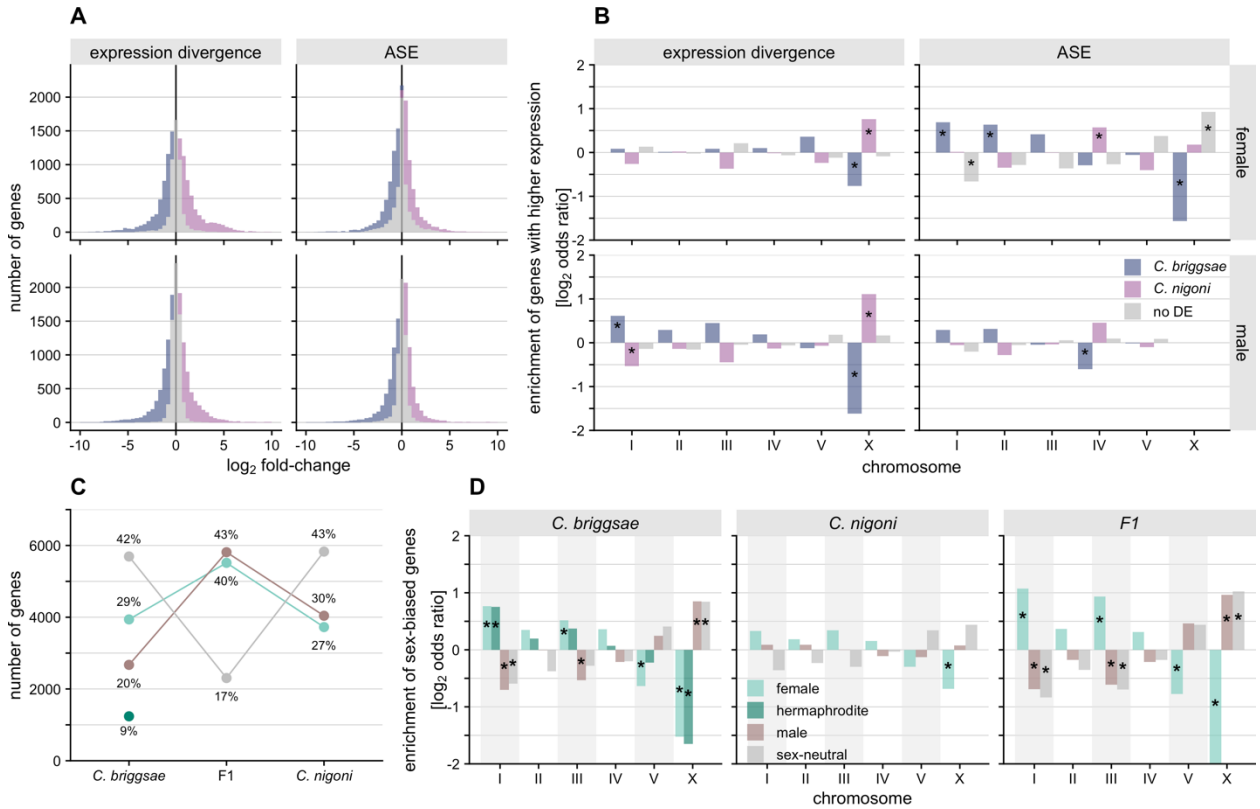


Figure 1. Incidences of differentially expressed genes for 13,636 orthologs between species and sexes. (**A**) Histogram of $\log_2$ expression divergence between species and allele-specific expression (ASE) in hybrids (*C. nigoni*/*C. briggsae*) for female (top row) and male transcriptomes (bottom row). Colored bars indicate significantly differentially expressed genes, while grey bars indicate counts of genes with non-significant expression differences. (**B**) Enrichment of differentially expressed genes between species and alleles 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. (**C**) Number and percentage of genes that show significant sex-bias is greater in F_1 hybrids than parent species. In *C. briggsae*, male-biased genes also expressed in hermaphrodites (dark green) are shown separately from male-biased only (brown) and from female-biased genes expressed in hermaphrodites (light green, "female") (see Figure 6). (**D**) 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.

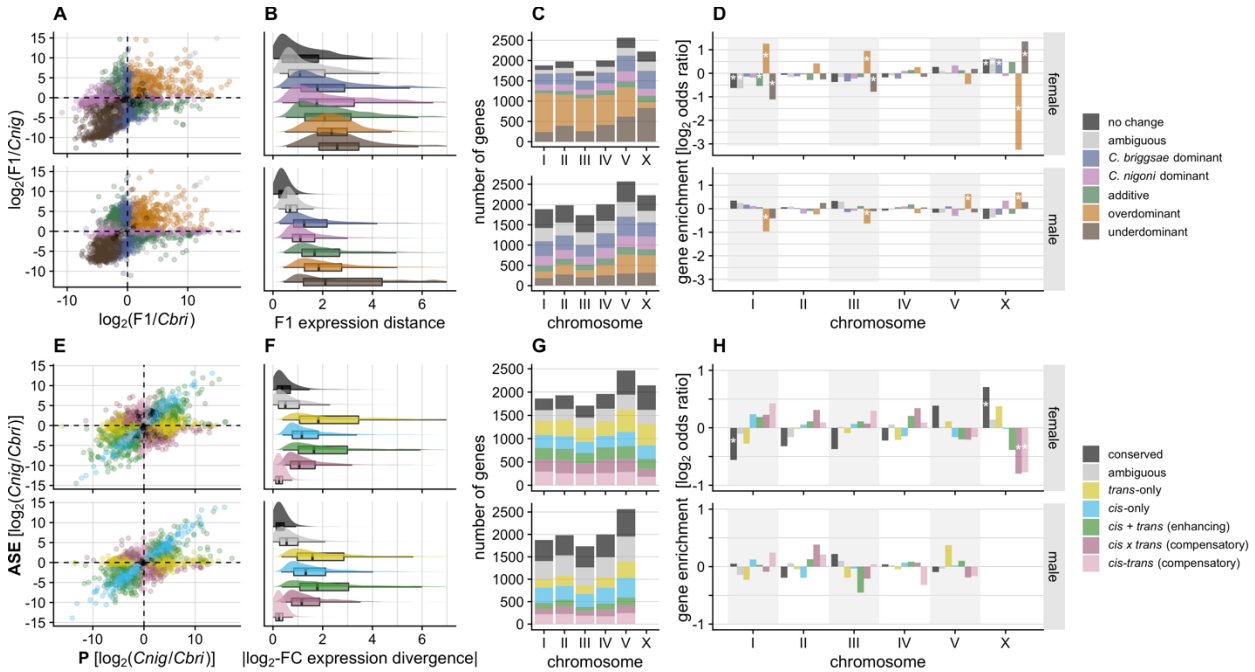
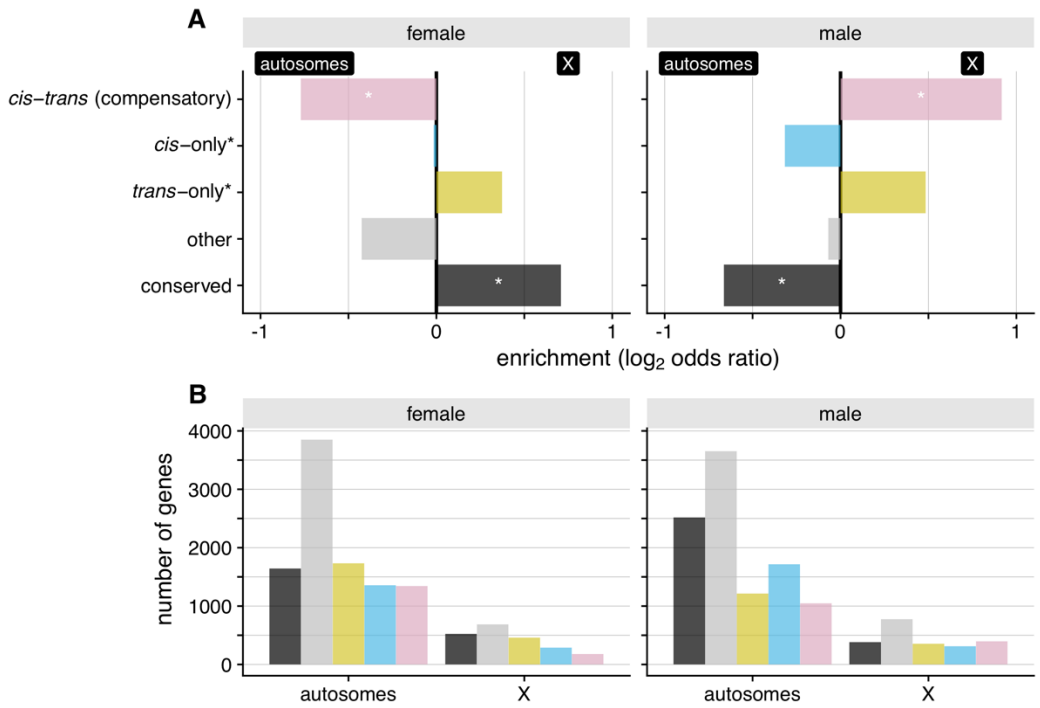


Figure 2. **Sex-specific differences in expression divergence and regulatory controls identify expression inheritance profiles between species**. (**A**) Per-gene biplot of $\log_2$ expression differences between F_1s and each parent species. (**B**) Box- and density plots of expression distance from 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. 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.



1312
1313 **Figure 3. The X-chromosome is enriched for compensatory *cis-trans* changes in males and**
1314 ***trans*-only* regulatory changes in both sexes.** (A) Positive values of the $\log_2$ odds ratios for
1315 females and males denote enrichment for the X-chromosome and negative values indicate
1316 enrichment for autosomes. (B) Gene counts for autosomes and the X-chromosome for
1317 regulatory divergence categories inferred from expression in males and females. In males, X-
1318 linked regulatory divergence was classified following Supplementary Fig. S12 (also see Material
1319 and Methods). Briefly, X-linked compensatory *cis-trans* changes in males represent genes that
1320 are not differentially expressed between species, but hybrids show over- or underdominance;
1321 *cis*-only* represent genes with expression divergence between species and hybrids matching *C.*
1322 *nigoni* expression; and *trans*-only* denote genes with expression divergence with hybrids
1323 matching *C. briggsae* expression. The * differentiates these categories from the *cis*-only and
1324 *trans*-only categories inferred from ASE in autosomes and XX individuals.
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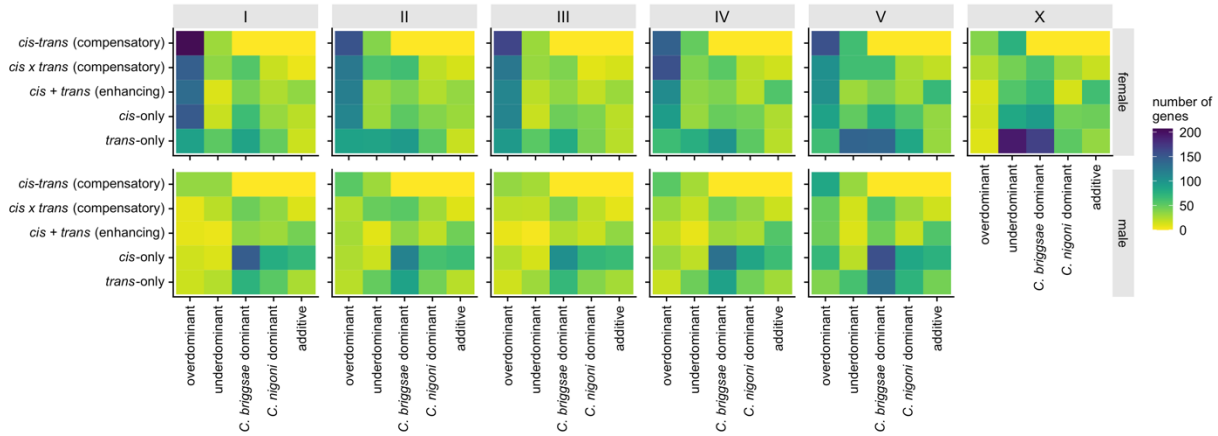

Figure 4. **Differences in regulatory controls between chromosomes and between sexes**. Heatmap of the number of genes in each expression inheritance group (x-axis) for each type of *cis* and *trans* regulatory changes (y-axis) for each chromosome (I-V, X) and each sex. Excludes conserved or ambiguous expression and regulation categories (Supplementary Figure S3).

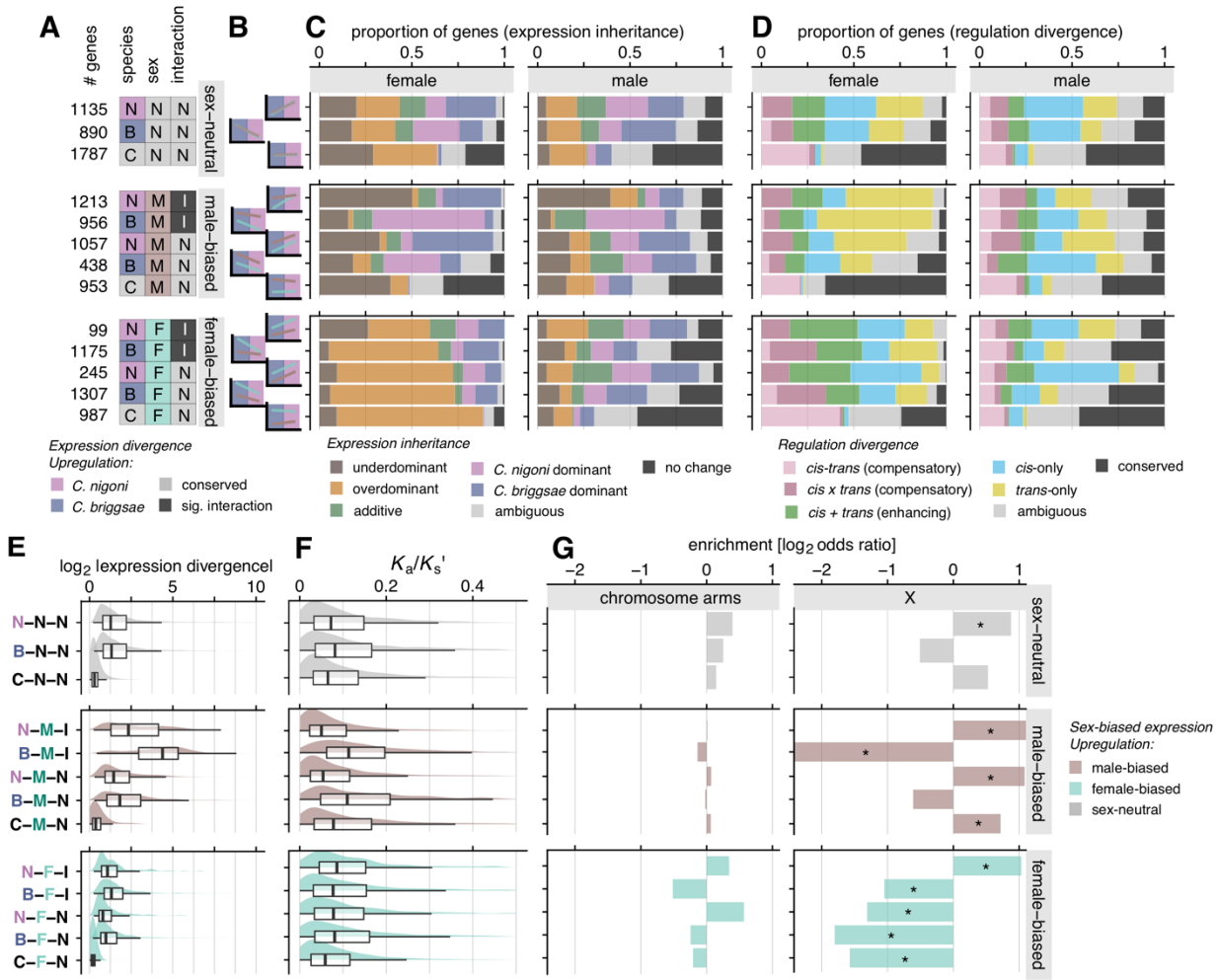

Figure 5. **Male-biased genes show higher expression divergence, molecular evolution, sex-specific regulatory divergence, and X enrichments**. (**A**) Thirteen distinct species-by-sex gene expression gene groups with respective gene counts. Matrix color indicates state with higher relative expression (N=*C. nigoni*, B=*C. briggsae*; M=male, F=female; I=significant interaction; gray cell indicates no difference between treatment groups: C=conserved, N=non-significant interaction). (**B**) Centroid relative expression reaction norm plots for each group indicating expression differences between species and sexes (Supplementary Figure S4). (**C**) Proportion of genes within each species-by-sex gene group differ in the relative representation of expression inheritance categories and (**D**) types of *cis*- and *trans*-regulatory divergence, distinctly for females (left panels) and males (right panels). (**E**) Absolute expression divergence and (**F**) protein sequence divergence (K_a/K_s') differs across species-by-sex expression profiles most strongly for male-biased genes. (**G**) Male-biased genes are not enriched in chromosome arms but are more abundant on the X-chromosome relative to autosomes (except for the BMI group that is rare on X, see Figure 6). * Fisher's exact test, $P < 0.05$ and $|\log_2 \text{odds ratio} > 0.5|$.

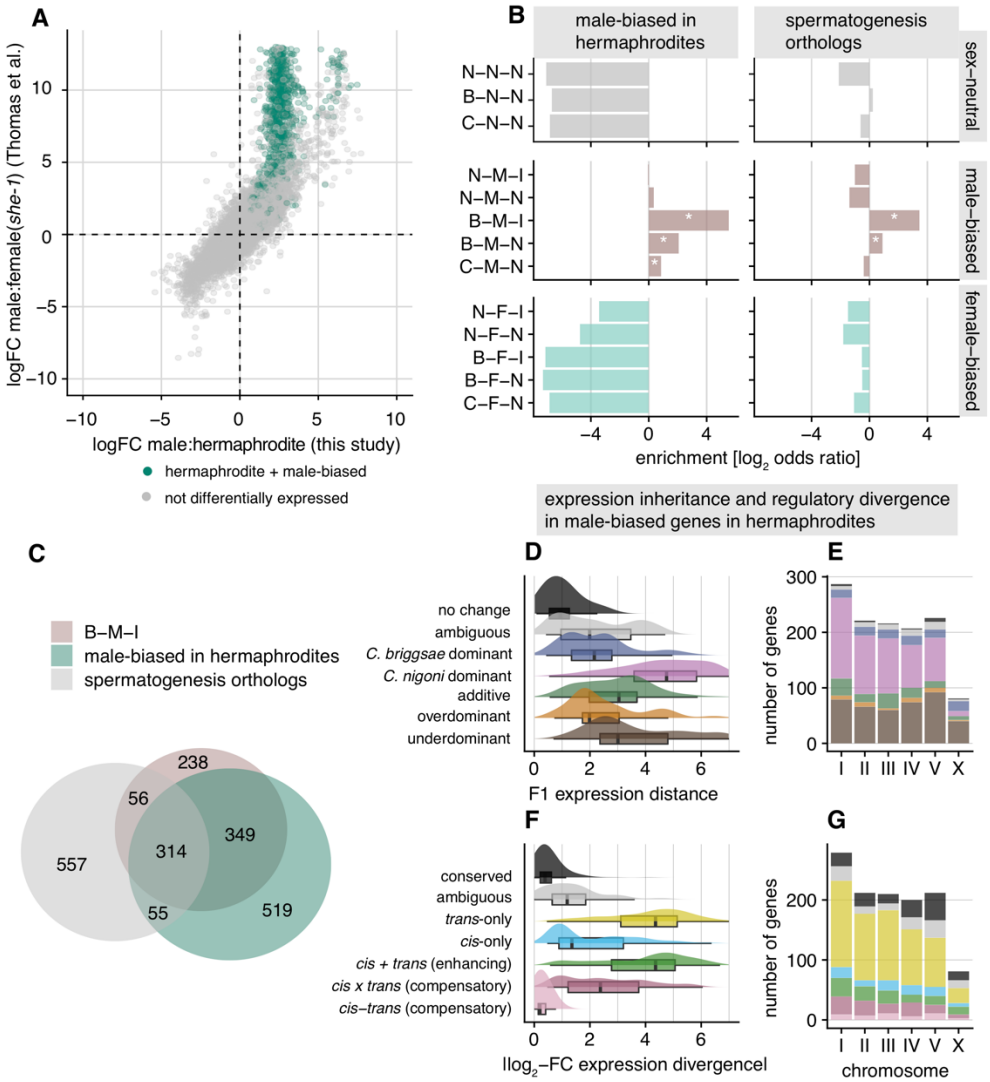

Figure 6. Male-biased genes that are upregulated in hermaphrodites are enriched for spermatogenesis genes and have distinct expression and regulatory patterns in hybrids. (**A**) Biplot of log-fold-change values in male:female contrasts (sex-biased expression) between our *C. briggsae* data and data from (53), which includes *C. briggsae* "pseudo-females". Green dots denote genes that were differentially expressed between hermaphrodites and "pseudo-females". (**B and C**) Both male-biased genes upregulated in hermaphrodites and orthologs to *C. elegans* spermatogenesis genes (44) are enriched for the B-M-I group that shows a species-by-sex interaction (Fig. 5; Supplementary Fig. S4). Divergence in expression and regulation (**D and E**) is driven largely by *trans*-only regulatory changes (**F**) resulting in abundant *C. nigoni* dominant expression and underdominant misexpression (**G**).

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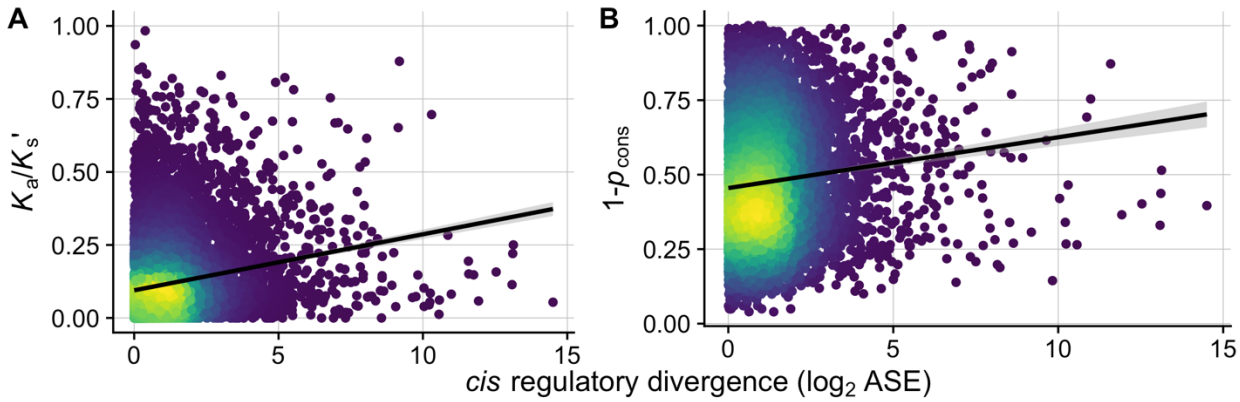
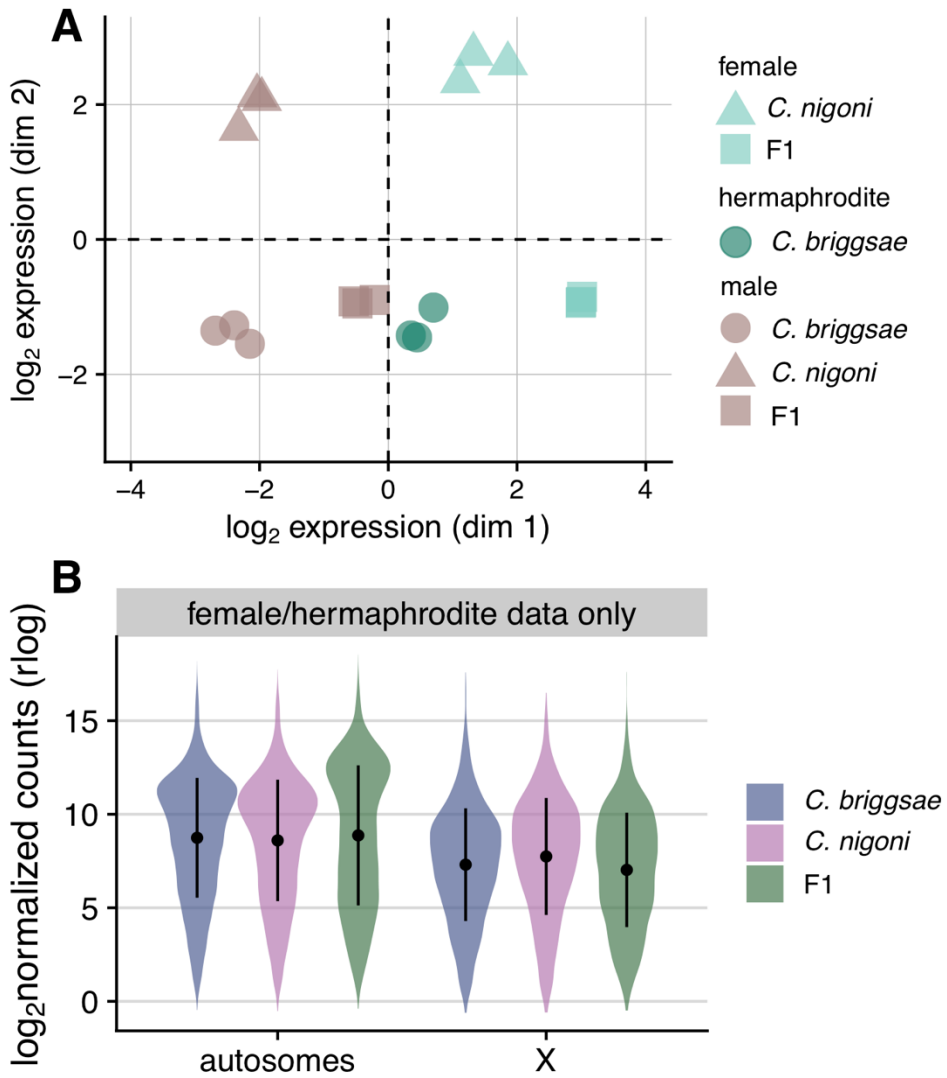


1363

1364 **Figure 7**. **The magnitude of *cis*-regulatory divergence shows positive but weak correlations**
1365 **with rates of molecular evolution**. (**A**) Scatter plot and linear regression of mutation-adjusted
1366 protein evolution rates (K_a/K_s', Spearman's $\rho = 0.17$, $P < 0.0001$; adjusted $R = 0.035$, $m = 0.019$,
1367 $P < 0.0001$) and (**B**) the proportion of non-conserved 5 bp windows within 500 bp upstream of
1368 each gene (1-P_{cons}, Spearman's $\rho = 0.12$, $P > 0.05$; 1-P_{cons}: adjusted $R = 0.01$, $m = 0.017$, $P <$
1369 0.0001) as a function of *cis*-regulatory divergence (allele-specific expression in hybrids, ASE).
1370 Color scale indicates density of points with brighter colors denoting higher density.

1371 # Supplementary figures



1372
1373 Supplementary Figure S1. Distinct expression profiles encompass species, sexes, F1 hybrids. (A)
1374 Multi-dimensional scaling plot showing overall expression distance between samples. (B)
1375 magnitude of expression (rlog-transformed counts) for autosomes and the X-chromosome
1376 across *C. briggsae* hermaphrodites, *C. nigoni* females, and F1 females. The X-chromosome has,
1377 on average, lower magnitude of gene expresion than autosomes, which is expected with
1378 dosage compensation. However, F1 hybrids do not show a strong pattern of over-active dosage
1379 compensation leading to generalized lower levels of expression.
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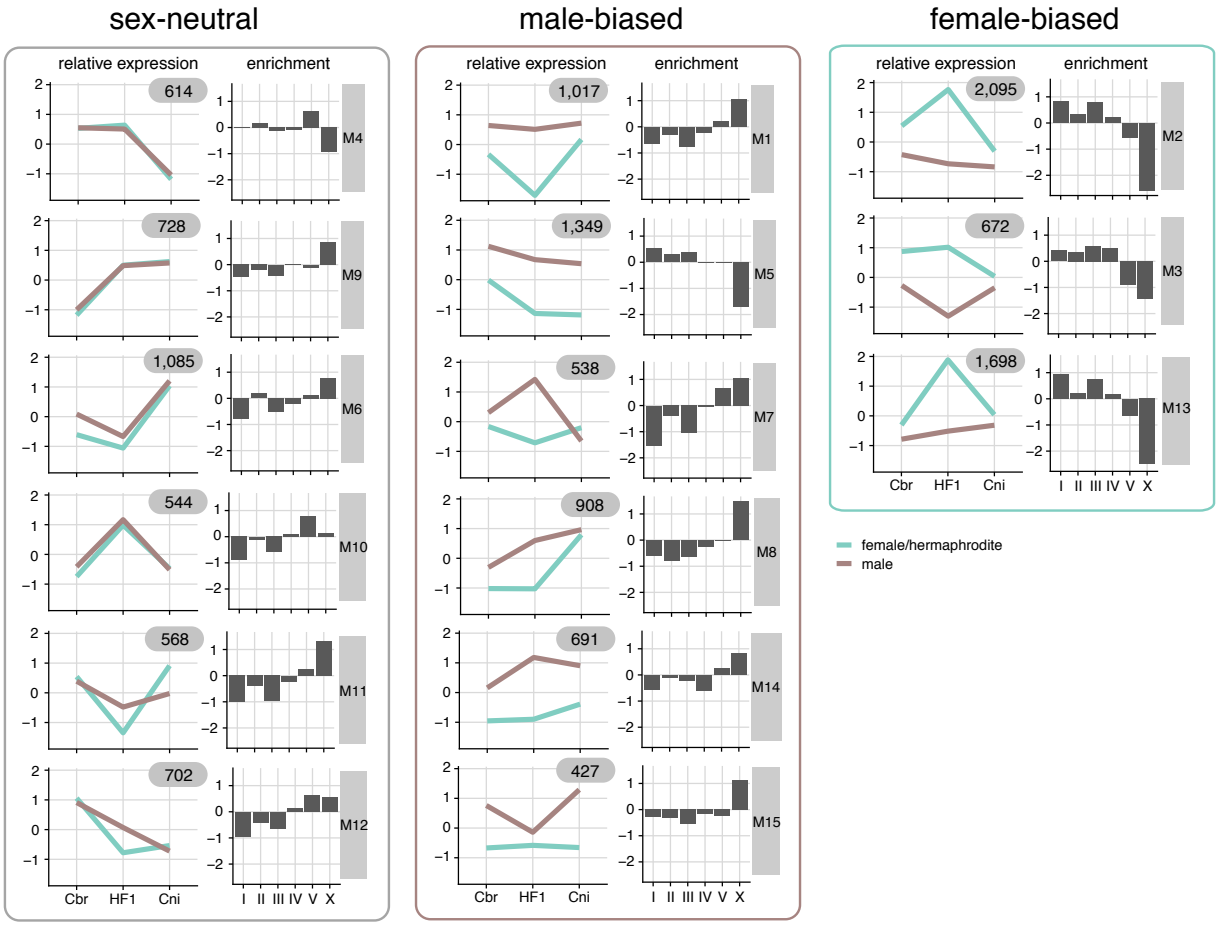
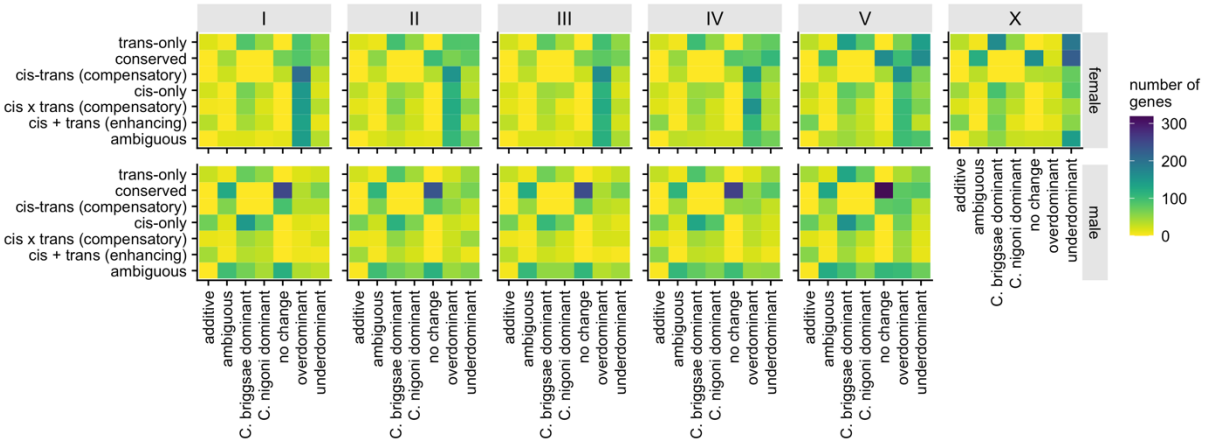
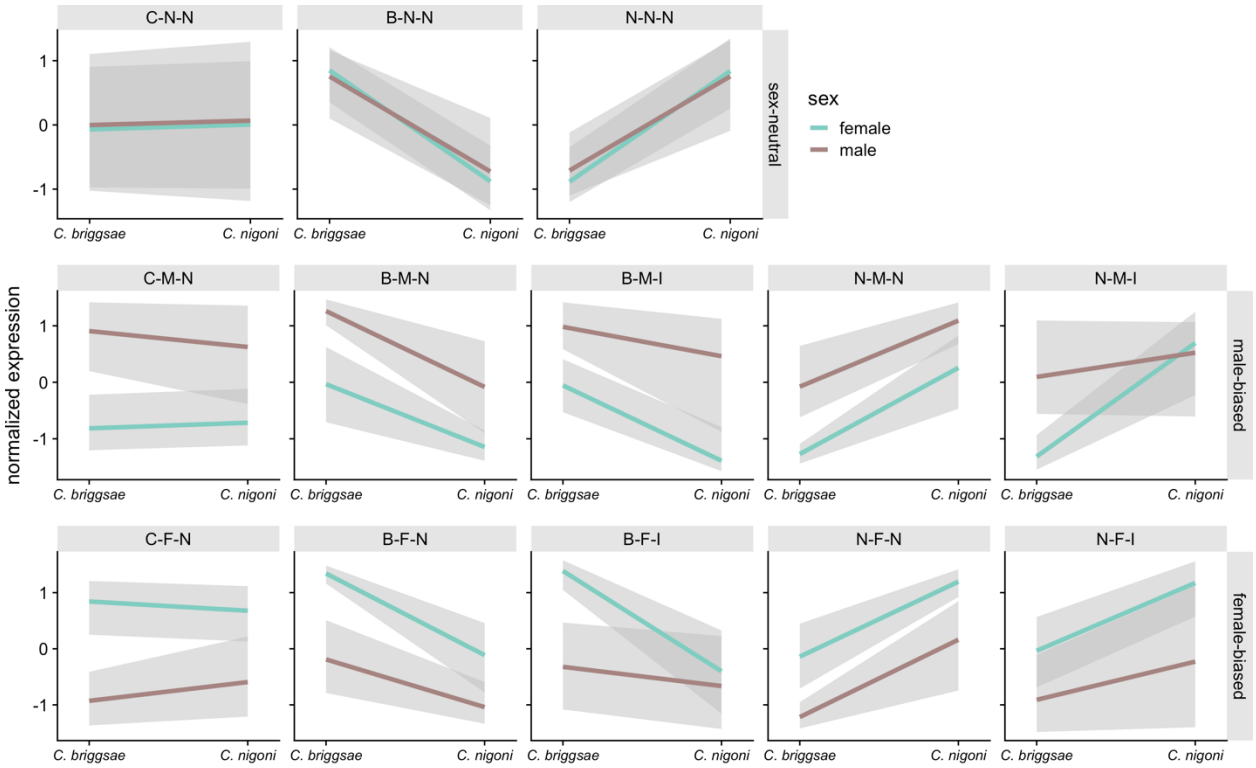


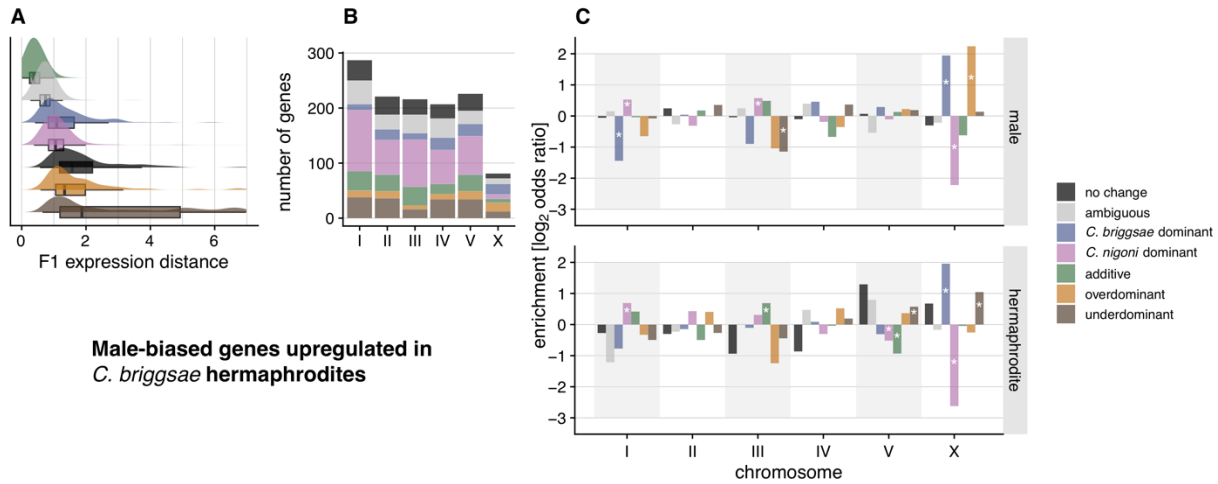
Supplementary Figure S2. Centroid relative expression of co-expression clusters and chromosomal enrichments grouped by sex-biased expression. The naming scheme for each of the clusters is arbitrary. Numbers inside circles indicate the number of genes in each cluster. Enrichment is represented by the $\log_2$ odds ratio (i.e., observed/expected), with positive values indicating enrichment and negative values depletion.



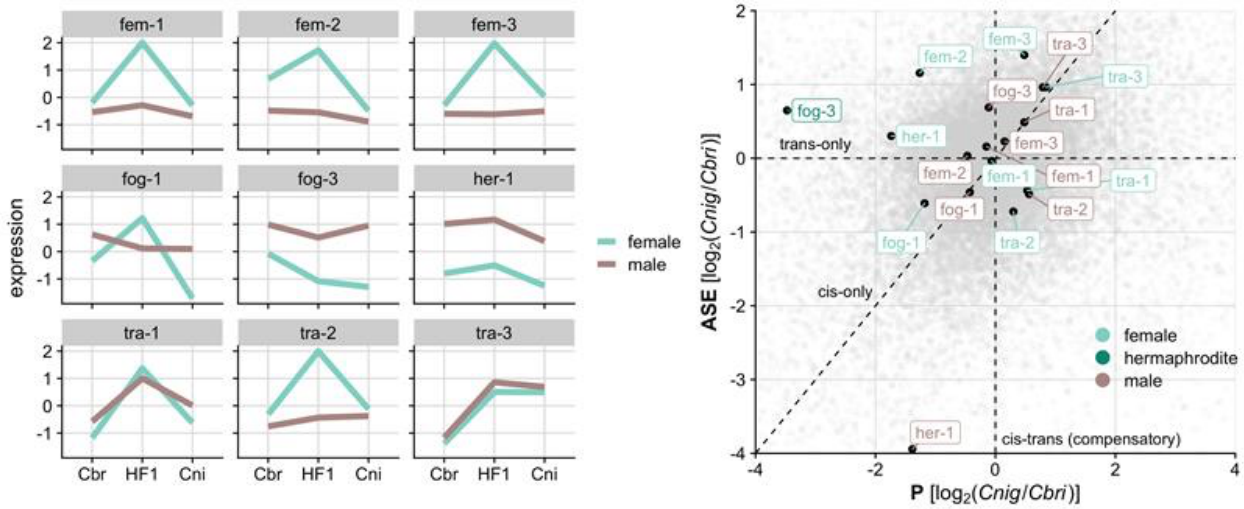
Supplementary Figure S3. X-autosome differences in regulatory controls in females and between sexes for autosomes underlie hybrid transcriptomic profiles relative to parent species. Heatmap of the number of genes in each expression inheritance group (x-axis) for each type of *cis* and *trans* regulatory changes (y-axis) for each chromosome (I-V, X) and each sex. Includes all categorizations.



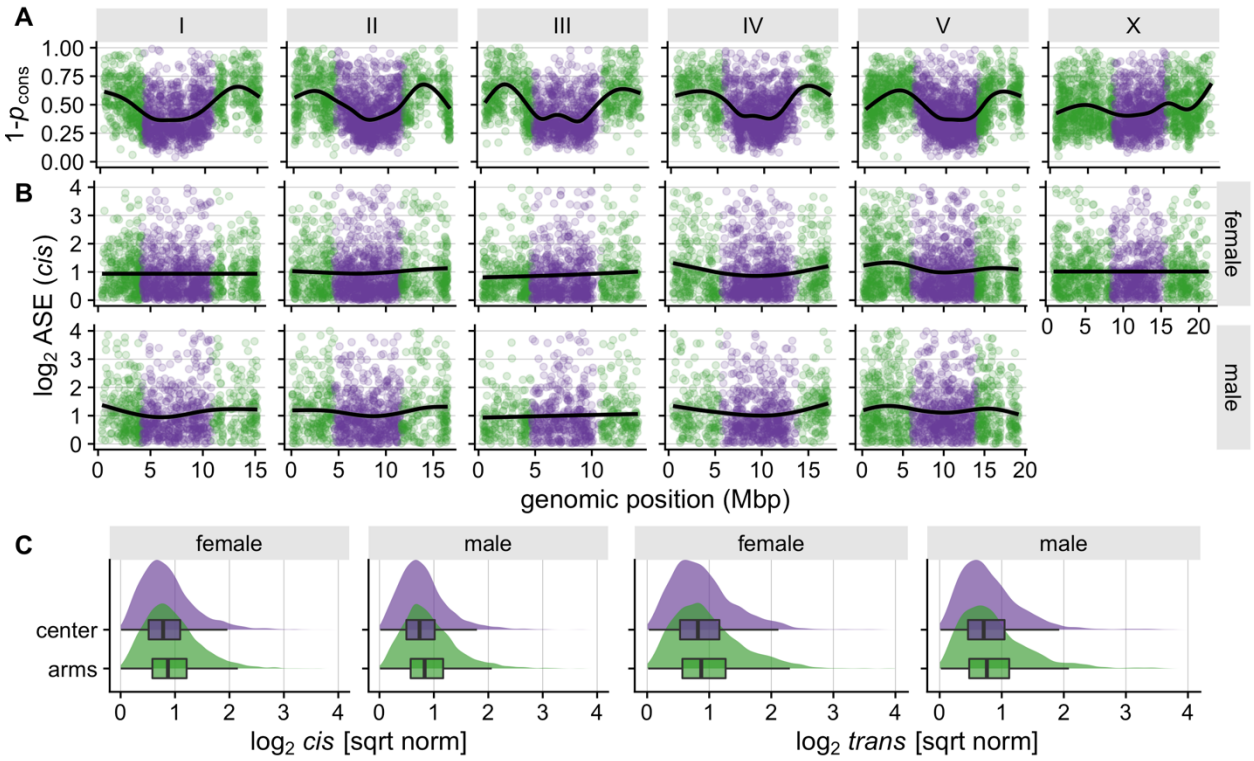
Supplementary Figure S4. Centroid of normalized expression and 95% CI of species-by-sex groups (see Fig. 5) plotted as reaction norms. Rows show relative expression of sex-neutral, male-biased, and female-biased genes. Groups that include an "I" at the end in the name code have significant species-by-sex interactions.



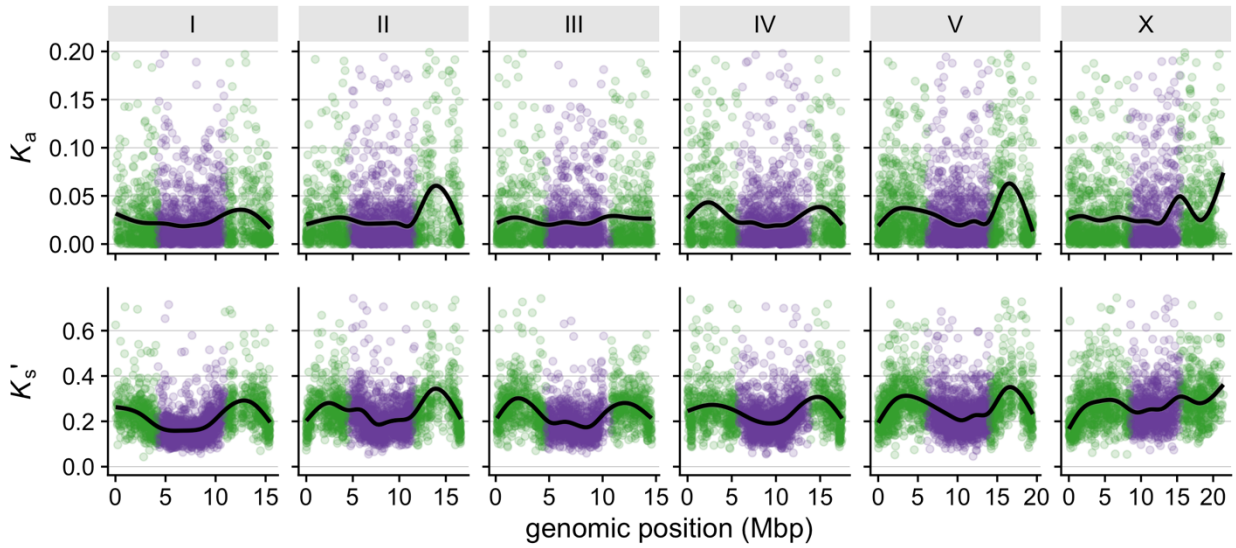
Supplementary Figure S5. Male-biased, hemaphrodite upregulated genes (see Fig. 6) in males have similar expression inheritance, (B) but not expression distance (A), to females (Fig. 6). The X-chromosome is depleted compared to autosomes (B) but has distinct relative enrichments of genes with *C. briggsae* expression dominance in both males and "females" in contrast to autosomes, and presents different misexpression categories between males (overdominant) and females (underdominant).



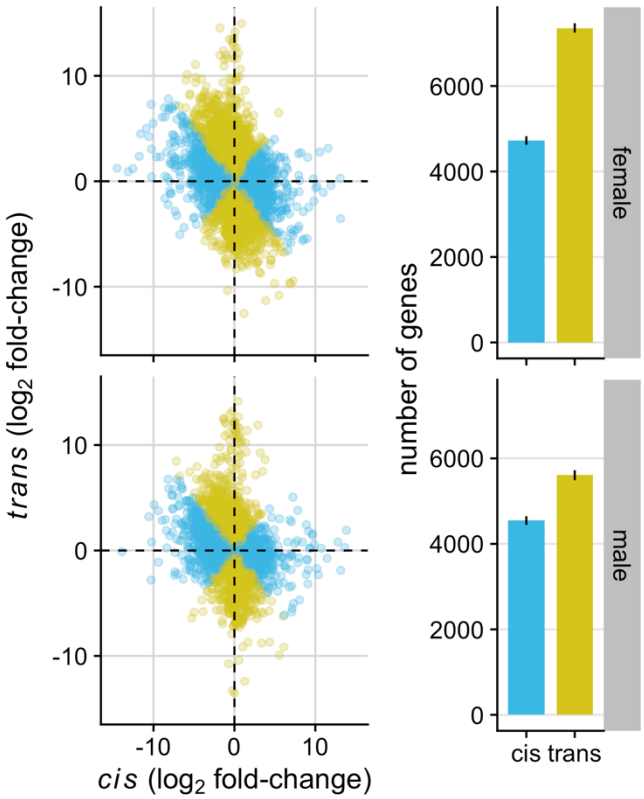
Supplementary Figure S6. Genes involved with sex determination and germline development have significant divergence in regulatory networks. The left panel shows relative (log-transformed and normalized) expression for males (brown) and females (light green) in *C. briggsae* (Cbr), F_1 hybrids (HF1), and *C. nigoni* (Cni). The right panel shows a biplot between the log-fold change in allele-specific expression (y-axis) and the log-fold change in expression divergence between species (x-axis). Dotted lines mark expected trajectories for *trans*-only (horizontal), *cis*-only (diagonal), and *cis-trans* compensatory (vertical) changes. Grey dots represent the regulatory space of other orthologs in our dataset.



Supplementary Figure S7. Chromosomal arm (green) and center (purple) regions differ strongly in (A) upstream sequence divergence (1-P_{cons}; proportion of non-conserved 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. Black lines in A and B indicate general additive regression (GAM) trendlines. (**C**) Arm and center regions do not differ greatly in magnitude of regulatory divergence. Box- and density plots of total (absolute) *cis*-regulatory divergence (left two panels) and *trans* regulatory divergence (right two panels) for either male or female gene expression. Allele-specific expression values are square-root-normalized in C to facilitate visual comparison.



Supplementary Figure S8. Coding sequence evolutionary rates for replacement sites (K_a) and synonymous sites (K_s', adjusted for selection on codon usage) for 13,636 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.



Supplementary Figure S9. 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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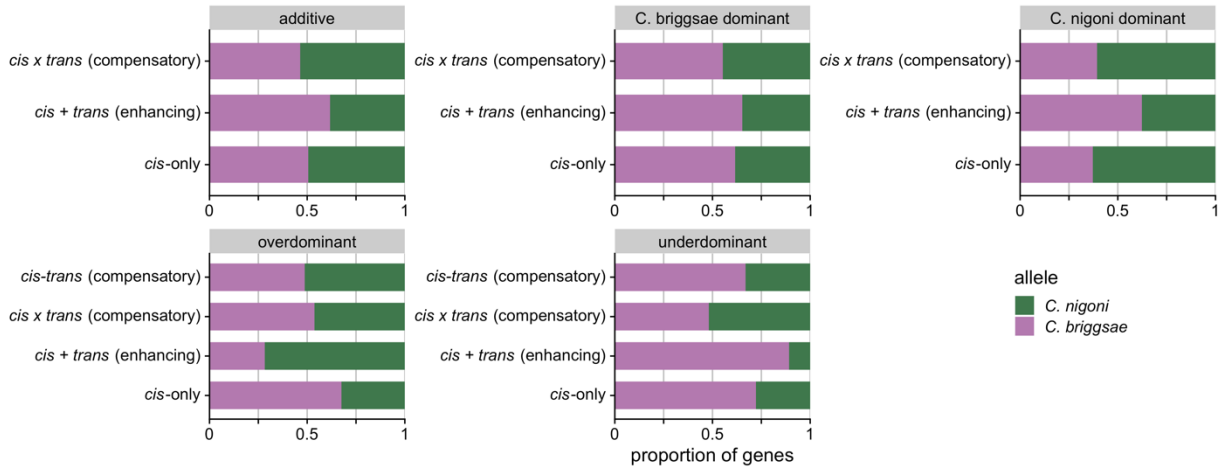

1460
1461 Supplementary Figure S10. Proportion of autosomal genes with significant allele-specific
1462 expression (y-axis) showing either additive expression (first panel, top row), simple expression
1463 dominance of one species (second and third panels, top row) and transgressive over- or under-
1464 dominant expression (bottom panels) in hybrid males. Genes with conserved regulation or
1465 *trans*-only effects were excluded for not having significant allele specific expression.
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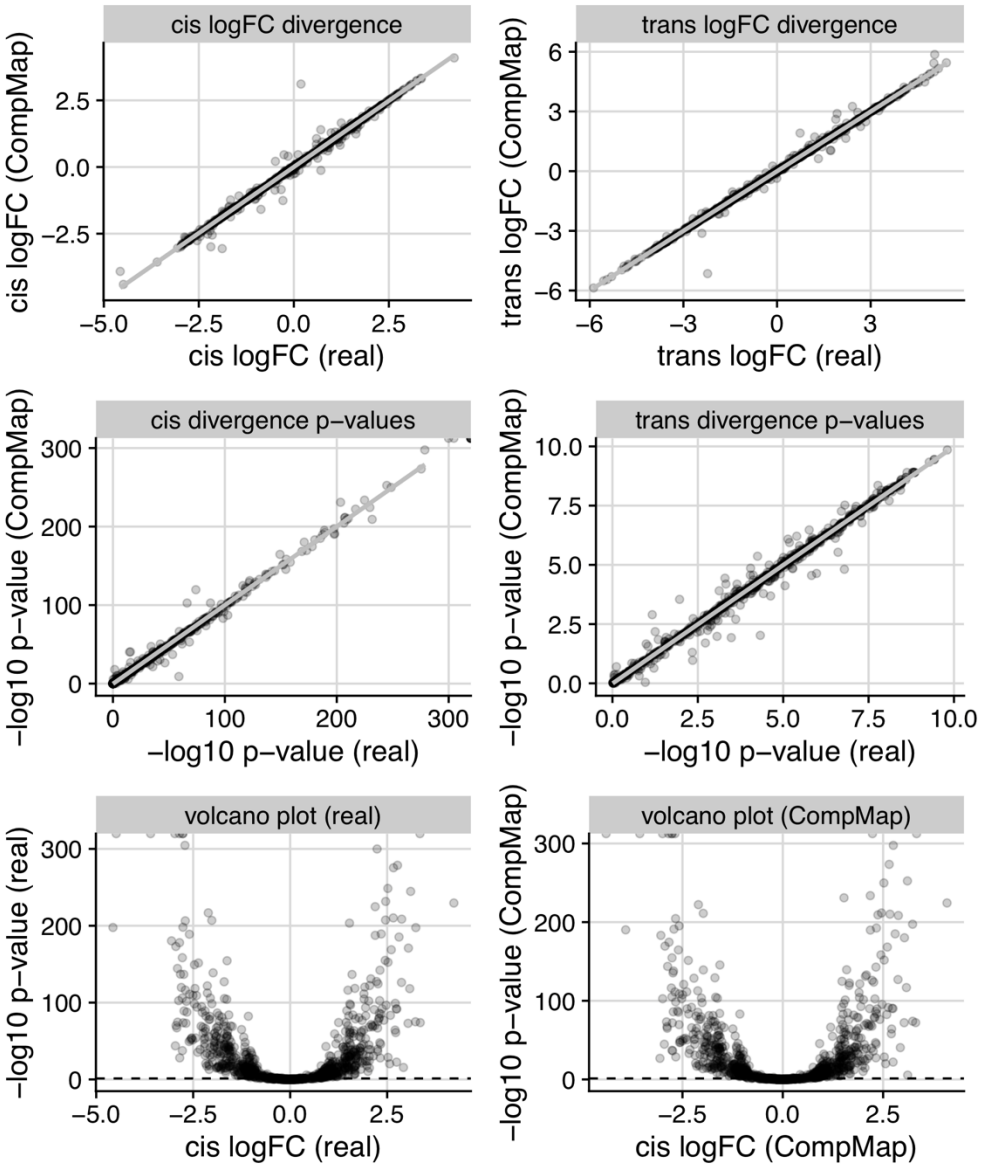

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1469 Supplementary Figure S11. Scatter plot and linear regressions (top 4 panels) between
1470 regulatory divergence (log-fold-change) due to allele-specific expression or *cis* changes and
1471 their FDR corrected *P*-values, regulatory divergence due to *trans* effects and their FDR
1472 corrected *P*-values, and volcano plots (lower panels) comparing results from simulated RNA-seq
1473 allele-specific expression data generated by *polyester* and allele-specific read counts by
1474 CompMap.

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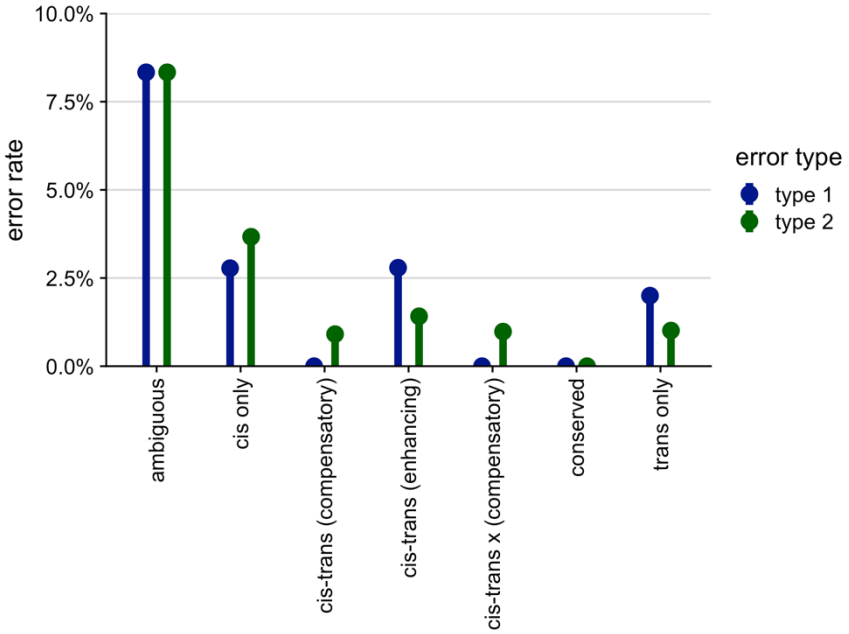

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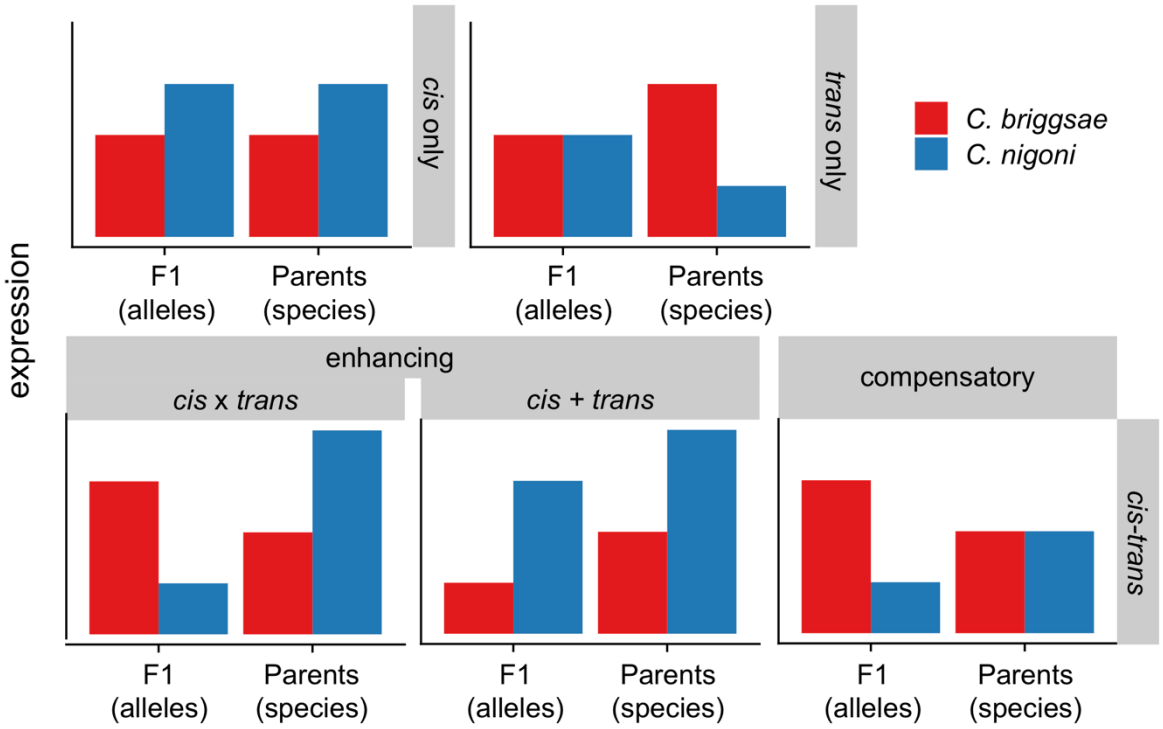
1477 Supplementary Figure S12. Type 1 (false positive) and type 2 (false negative) error rates for
1478 each category of regulatory divergence. Expected values were drawn from RNA-seq allele-
1479 specific expression data simulated with *polyester*. Observed values were drawn based on
1480 inferences drawn from CompMap allele-specific count data.



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1482 Supplementary Fig. S13. Qualitative examples of allele-specific expression with their
1483 classification of gene regulation type changes.
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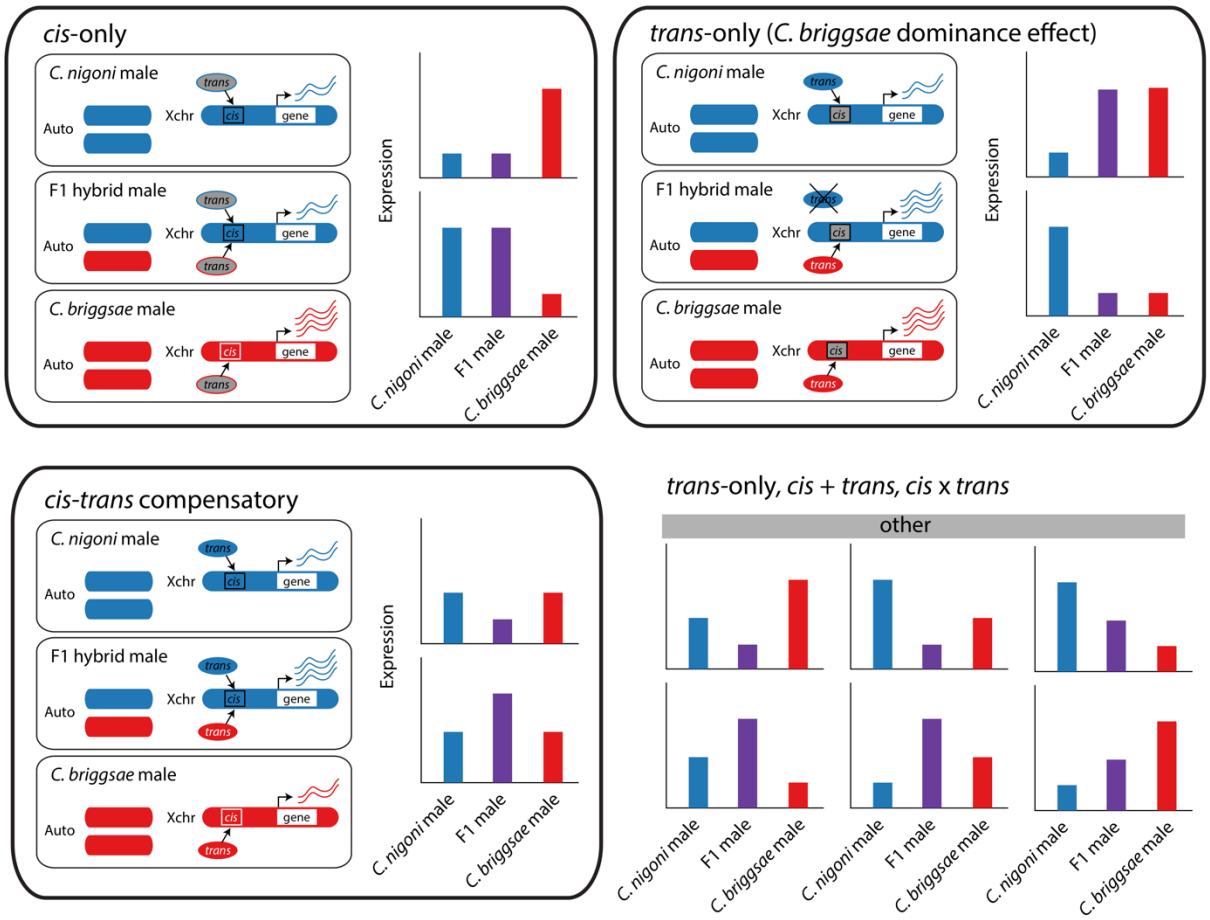

Supplementary Fig. S14. Qualitative examples to diagram regulatory divergence scoring for the X-chromosome in males that are hemizygous for the X (i.e., X0). Categories not fitting into either *cis*-only, *trans*-only, or compensatory *cis*-*trans* were lumped into "other".