Sex chromosomes control vertical transmission of feminizing *Wolbachia* symbionts in an isopod

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Abstract

Microbial endosymbiosis is widespread in animals, with major ecological and evolutionary implications. Successful symbiosis relies on efficient vertical transmission through host generations. However, when symbionts negatively affect host fitness, hosts are expected to evolve suppression of symbiont effects or transmission. Here we show that sex chromosomes control vertical transmission of feminizing *Wolbachia* endosymbionts in the isopod *Armadillidium nasatum*. Theory predicts that the invasion of an XY/XX species by cytoplasmic sex ratio distorters is unlikely because it leads to fixation of the unusual (and often lethal or infertile) YY genotype. We demonstrate that *A. nasatum* X and Y sex chromosomes are genetically highly similar and YY individuals are viable and fertile, thereby enabling *Wolbachia* spread in this XY-XX species. Nevertheless, we show that *Wolbachia* cannot drive fixation of YY individuals because infected YY females do not transmit *Wolbachia* to their offspring, unlike XX and XY females. The genetic basis fits the model of a Y-linked recessive allele (associated with an X-linked dominant allele), in which the homozygous state suppresses *Wolbachia* transmission. Moreover, production of all-male progenies by infected YY females restores a balanced sex ratio at the host population level. This suggests that blocking of *Wolbachia* transmission by YY females may have evolved to suppress feminization, thereby offering a whole new perspective on the evolutionary interplay between microbial symbionts and host sex chromosomes.

Keywords

Male heterogamety, sex chromosomes, endosymbiont, sex ratio distorter, *Wolbachia*, genetic conflicts, feminization, genome sequencing

Introduction

Microbial endosymbiosis is widespread in animals, with major effects on host ecology and evolution [1,2]. Successful symbiosis relies on efficient symbiont transmission through host generations. Vertical transmission occurs when symbionts are transferred from parent to offspring, usually through the maternal germ line [3,4]. From a symbiont perspective, faithful maternal inheritance can be achieved by conferring benefits to hosts, such as nutritional provisioning [5], defense against natural enemies [6] and pathogen resistance [7,8]. This transmission strategy leads to convergence of symbiont and host fitness. Conversely, symbionts may follow a selfish strategy consisting of favouring their transmission at the expense of host fitness. This is achieved through manipulation of host reproduction, which can result in highly distorted sex ratios [9,10]. As balanced sex ratios are optimal for most nuclear genes owing to biparental inheritance, hosts are predicted to evolve suppression mechanisms to control symbiont effects or transmission [10,11]. Genetic conflicts between sex ratio distorters, such as feminizing symbionts, and the rest of the genome are increasingly recognized as drivers of the evolution of host sex determination systems [10–16]. Here we take the reverse perspective and investigate whether host sex chromosome systems can influence symbiont transmission and dynamics.

In many animals, sex is determined by a locus located on sex chromosomes that is heterozygous in one sex (the heterogametic sex) and homozygous in the other sex (the homogametic sex). Two major types of sex chromosomes exist: male heterogamety (XY males and XX females) and female heterogamety (ZZ males and ZW females) [13,15]. Sex chromosomes evolve from autosomes that acquired a sex-determining locus characterized by sex-specific inheritance. Subsequently, genomic regions around sex-determining loci often stop recombining, leading to gradual accumulation of nucleotide and structural variation and repetitive DNA [13,15,17–19]. This so-called degeneration process also causes the formation of pseudogenes and gene loss, resulting in increasing differentiation of sex chromosomes over time. This is well illustrated by the human X and Y sex chromosomes, which dramatically differ in size and gene content [19].

Terrestrial isopods (crustaceans) are an ideal model to examine sex chromosome influence on symbiont transmission. This is because both female and male heterogametic systems of sex chromosomes are found in this group [20] and many species are naturally infected with *Wolbachia* bacteria [21,22]. *Wolbachia* are intracellular, maternally inherited endosymbionts of arthropods, often acting as reproductive parasites that manipulate host reproduction to favour infected females in host populations [23]. In terrestrial isopods, *Wolbachia* is best known as a sex ratio distorter due

to its ability to feminize genetic males into phenotypic females [10,24–26]. For example, the presence of feminizing *Wolbachia* in *Armadillidium vulgare* leads to highly female-biased progenies [27,28] because symbionts override the ZZ/ZW system of sex chromosomes [29,30]. As a result, genetic ZZ male embryos develop as phenotypic ZZ females when infected by *Wolbachia*. Theoretical models predict the extinction of the W sex chromosome in *A. vulgare* lines infected by *Wolbachia* [31,32] and empirical evidence verified this prediction [33,34]. Thus, all individuals of *Wolbachia*-infected lineages end up with ZZ sex chromosomes at equilibrium, those inheriting *Wolbachia* develop as females and those lacking *Wolbachia* develop as males. This phenomenon is known as cytoplasmic sex determination [10,24–26]. In sum, invasion of a ZZ/ZW species by feminizing *Wolbachia* is not problematic because it leads to the fixation of the ZZ genotype, which is the natural male genotype.

In sharp contrast with ZZ/ZW heterogamety, the conditions allowing feminizing *Wolbachia* to spread in an XY/XX heterogametic system are much more stringent. Indeed, theoretical work predicts that infection of an XY/XX system by a cytoplasmic sex ratio distorter should drive the loss of the X chromosome and concomitantly lead to fixation of the YY genotype at equilibrium [31]. In this case, YY individuals inheriting *Wolbachia* should develop as females and those lacking *Wolbachia* should develop as males. However, YY is not a standard genotype and it may often be lethal or infertile, owing to Y chromosome degeneration causing the loss of essential genes [13,15,17–19]. Therefore, XY/XX heterogamety should prevent the invasion of feminizing *Wolbachia* symbionts, unless the X and Y chromosomes are not substantially divergent, i.e. they both carry all vital loci and are genetically very similar. To the best of our knowledge, this prediction has never been explored empirically.

Here, we investigated the interplay between sex chromosomes and *Wolbachia* symbionts in *Armadillidium nasatum*, a terrestrial isopod species related to *A. vulgare* [20]. Analogous to *A. vulgare*, a feminizing *Wolbachia* strain (*w*Nas) is naturally present in *A. nasatum* [21,35,36]. However, unlike *A. vulgare*, chromosomal sex determination follows XY/XX heterogamety in *A. nasatum* [20,35]. This result was established using an original strategy consisting of experimentally reversing young genetic females into phenotypic males, crossing them with their sisters and analyzing sex ratios of the resulting progenies [20,35]. Here, based on whole-genome sequencing, pedigree analyses and simulations, we show that *Wolbachia* endosymbionts can spread in *A. nasatum* populations because *A. nasatum* sex chromosomes are genetically highly similar and YY males and females are both viable and fertile. Nevertheless, *Wolbachia* cannot drive the loss of the X chromosome because infected YY females do not transmit *Wolbachia* to their offspring, unlike XX and XY females. As infected YY females produce all-male progenies, a balanced sex ratio is

maintained at the host population level despite the presence of feminizing *Wolbachia*, suggesting that blocking of *Wolbachia* transmission by YY females may have evolved to suppress feminization.

Results

De novo *assembly and annotation of male (XY)* A. nasatum *genome*

We sequenced the male (XY) genome of an *A. nasatum* line derived from wild animals sampled in Thuré (France) in 2008. Males and females from this line have been consistently producing progenies with balanced sex ratios in our laboratory. In addition, sex reversal and crossing experiments demonstrated that sex determination in this line follows male heterogamety [20]. Absence of *Wolbachia* endosymbionts in the individuals selected for sequencing was confirmed by PCR.

We assembled the *A. nasatum* genome using a hybrid approach combining short paired-end Illumina reads and long PacBio reads (S1 Table). The initial assembly was processed through polishing, decontamination and scaffolding by a transcriptome assembly. The final assembly had a total length of 1,223,175,971 bp. It was composed of 25,196 contigs and scaffolds (hereafter collectively referred to as "contigs") with an N_{50} of 86,284 bp, and containing barely no (0.01%) undetermined nucleotides (Table 1). Genome completeness assessment using Benchmarking Universal Single Copy Orthologs (BUSCO 3.0.1) [37] revealed that 1,001 of 1,066 (94%) conserved specific arthropod genes were present in the assembly (Table 1). Furthermore, transcriptome assembly alignment on the genome assembly yielded 99.0% of transcripts longer than 1 kb aligned. Thus, we have obtained a reliable assembly of the XY male genome from *A. nasatum*.

131 **Table 1.** Summary information of *Armadillidium nasatum* genome assembly.

Genome assembly features	Assembly figures
Assembly statistics	
Number of contigs and scaffolds	25,196
Total size	1,223,175,971 bp
Longest contig/scaffold	877,941 bp
Number of contigs and scaffolds >1 kb	25,166
N_{50} contig size	75,116 bp
N_{50} scaffold size	86,284 bp
Undetermined nucleotides	0.01%
G+C content	25.77%
Analysis of BUSCO genes	
Complete genes	974/1066 (91.4%)
Complete and single-copy genes	932/1066 (87.4%)
Complete and duplicated genes	42/1066 (3.9%)
Fragmented genes	27/1066 (2.5%)
Missing genes	65/1066 (6.1%)

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133

134 The annotation of the *A. nasatum* assembly included 14,636 predicted gene models with an
135 average length of 9,422 bp and representing 11.3% of the total assembly length (S2 Table). Among
136 these predicted genes, 9,281 (63.4%) had Blastp hits to the UniProt-SwissProt database (release
137 September 2016) and 9,459 (64.6%) had InterProScan hits to Pfam domains (version 30.0) and 6,137
138 (41.9%) had Gene Ontology terms. Repeats accounted for 64.6% (or 790 Mb) of the *A. nasatum*
139 assembly (S3 Table). Specifically, transposable elements and simple tandem repeats accounted for
140 40.6% (or 496 Mb) and 23.1% (or 283 Mb) of the genome. The annotated genome sequence of *A.*
141 *nasatum* is available in DDBJ/ENA/GenBank under accession number SEYY00000000. The version
142 described in this article is version SEYY01000000.

143

144 *High genetic similarity of the X and Y chromosomes*

145 To investigate the extent of genomic differentiation between sex chromosomes, we searched
146 the *A. nasatum* assembly for contigs containing Y-specific sequences. We mapped short paired-end
147 Illumina reads generated for XY males and XX females (S1 Table) onto the *A. nasatum* assembly and
148 performed a Chromosome Quotient analysis [38]. This analysis consists of comparing the ratios of

149 female-to-male sequencing depths (CQ) for each contig, with the expectations that: (i) Y-specific
150 contigs should be mapped by male reads only (CQ ~0), (ii) X-specific contigs should be mapped by
151 female reads at twice the sequencing depth of male reads (CQ ~2), and (iii) autosomal contigs should
152 be mapped at similar sequencing depths by female and male reads (CQ ~1). The resulting frequency
153 distribution of CQ scores was unimodal and centered at CQ ~1 (mean: 1.03, median: 1.04), with no
154 peak at CQ scores of ~0 and ~2 (Fig 1a). In addition, we used the mapping-free Y chromosome
155 Genome Scan (YGS) method [39], which computes the proportion of single copy k-mers for each
156 contig of the genome assembly of the heterogametic (male) sex that are unmatched to sequencing
157 reads of the homogametic (female) sex. Y-specific contigs are expected to be unmatched by female
158 reads (YGS ~100%) while autosomal and X-linked contigs are expected to be matched entirely (YGS
159 ~0%). The resulting frequency distribution of YGS scores indicated that most contigs have very low
160 YGS scores (mean: 14.5%, median: 11.2%) and very few contigs had high YGS scores (i.e. only 43
161 contigs with YGS≥80% and just 2 with YGS≥90%) (Fig 1b). Thus, the CQ and YGS analyses consistently
162 indicated that the *A. nasatum* assembly mostly contains autosomal contigs and very few contigs
163 containing X- and Y-specific sequences. Intersecting the results of the CQ and YGS analyses identified
164 only 78 out of the 25,196 contigs of the assembly as containing Y-specific sequences, despite the use
165 of permissive thresholds, i.e. CQ≤0.35 and YGS≥35% (Fig 1c). The 78 contigs comprised a total length
166 of 1,327 kb, corresponding to just 0.1% of the *A. nasatum* genome assembly (S4 Table). While most
167 contigs lacked any gene (65/78, or 83%), a total of 20 genes (out of 14,636 genes in the assembly)
168 were annotated from 13 contigs (S4 Table). They constitute candidate master genes for sex
169 determination in *A. nasatum*. Thus, the Y-specific region of the *A. nasatum* genome is extremely
170 small (around one megabase) and contains very few genes, leading to the conclusion that the X and Y
171 chromosomes are poorly differentiated at the genomic and genic levels.

172

173 [Insert Fig 1 here]

174 **Fig 1.** Identification of sex-specific contigs in the *Armadillidium nasatum* genome assembly.
175 Frequency distribution of CQ (a) and YGS (b) scores calculated for each contig and scaffold of the
176 assembly. (c) Comparison of CQ and YGS scores. Each dot corresponds to one contig or scaffold
177 (those with CQ>2 are not represented). The red box contains 78 contigs with CQ≤0.35 and YGS≥35%.

178

179 To investigate sex chromosome differentiation at the molecular level, we analyzed the
180 patterns of sequence divergence and repeat content in the 78 contigs containing Y-specific

sequences relative to the other contigs of the *A. nasatum* genome. First, we analyzed the density of single nucleotide polymorphisms (SNP) in the 78 contigs (after removal of hemizygous regions, to focus on regions with orthologs on the X chromosome) versus the other contigs of the assembly. We calculated an average SNP density of 5.54 SNP/kb between allelic X/Y regions of the 78 contigs, compared to 3.40 SNP/kb across all other contigs of the assembly. Thus, we observed a slight (1.6-fold) but non-significant (Mann-Whitney bilateral U test, U=976,370, p-value=0.45) excess of SNP density in allelic X/Y regions relative to other regions of the genome. Furthermore, the repeat content in the 78 contigs was slightly higher than (66.1% on average, S4 Table), but not significantly different (Mann-Whitney bilateral U test, U= 1,048,600 p-value=0.28) from the repeat content of all other contigs (64.6%). In sum, our results indicated that *A. nasatum* sex chromosomes present patterns of molecular evolution that are quite similar to those of other genomic regions, with a slight elevation of SNP density and repeat content in contigs containing Y-specific sequences that is consistent with expectations for sex-specific sequences of the genome [40]. We conclude that the X and Y chromosomes of *A. nasatum* are highly similar not only at the genomic and genic levels, but also at the molecular level.

YY individuals are viable and fertile

To test for the existence and, if so, viability and fertility of YY individuals, we tracked Y chromosome inheritance in a *Wolbachia*-infected *A. nasatum* pedigree. First, we established robust Y-specific molecular markers using the Y-specific contigs identified previously. Reliable PCR assays were successfully designed for 42 of the 78 contigs containing Y-specific sequences, most of which (25/42) exhibited male-specific amplification with DNA samples from males and females closely related to those used for genome sequencing (S4 Table). However, only 9 of the 25 confirmed markers displayed male-specific amplification when tested in more distantly related individuals from the same population and in individuals from two other populations. Lack of sex specificity of a subset of markers in some populations indicated that recombination has occurred between some of the tested contigs and the sex-determining locus. This result provided further evidence that the Y-specific region of the genome is extremely small in *A. nasatum*. Concomitantly, the universal male specificity of 9 markers across all tested populations demonstrated that these *A. nasatum* populations possess a homologous Y chromosome. Hence, we used a subset of these robust markers to track the Y chromosome in an *A. nasatum* pedigree spanning three generations.

The pedigree was initiated by crossing a male with a female naturally infected by the feminizing *Wolbachia* strain *w*Nas, which produced an F1 progeny comprising 17 sisters (Fig 2). Two

F1 sisters were then crossed with genetic males (XY), to produce two F2 progenies: I-F2-1 comprising 24 females and I-F2-2 comprising 32 males. *Wolbachia* infection was tested in the 62 individuals of the pedigree (except the F0 father), indicating that all females carried *Wolbachia* while all males lacked *Wolbachia* infection, as expected. Next, we used previously designed Y-specific markers to assess the presence of the Y chromosome in the 62 individuals (except the F0 father). In the I-F2-1 progeny, half of the females showed amplification of the Y markers (indicating they are XY or YY) and half did not (indicating they are XX). The F2-1 mother carried XX chromosomes, as she did not amplify the Y markers. As all I-F2-1 offspring necessarily carry one X chromosome from their mother, the 11 I-F2-1 females amplifying the Y markers must be XY. In the I-F2-2 progeny, all individuals amplified the Y markers (indicating they are XY or YY), as did their mother. Given that the F2-2 father is XY, if the F2-2 mother was XY, we would have expected 25% XX individuals in the I-F2-2 progeny, but there was none. Thus, the I-F2-2 mother must be YY, which is the only genotype that can explain that the entire I-F2-2 progeny carries at least one Y chromosome. Given the parental genotypes, we predicted the I-F2-2 progeny is composed of half XY males and half YY males. Finally, the two F1 sisters being XX and YY, it followed that the F0 father and mother are XY. To independently assess these predictions, we devised a quantitative PCR assay measuring Y chromosome dose relative to autosomes (S1 Fig). This assay confirmed the predicted genotypes of the F0 and F1 individuals we tested and the composition of the I-F2-2 progeny (15 XY and 17 YY individuals) (Fig 2, S5 Table). In sum, the resolution of sex chromosome genotypes in the pedigree demonstrated that the YY genotype is viable both as male and as female in *A. nasatum*, as is the XY genotype. Moreover, the YY and XY genotypes are both fertile as females. Finally, we show below that the YY genotype is also fertile as male.

[Insert Fig 2 here]

Fig 2. *Armadillidium nasatum* pedigree used to track inheritance of the Y chromosome and *Wolbachia*. The pedigree spans three generations (F0-F2) and is comprised of 62 individuals (35 males and 27 females) for which sex chromosome genotype (XX, XY or YY) was identified and 15 F1 females not included in the molecular analyses (dotted circle). Males are shown as squares and females as circles. Individuals carrying *Wolbachia* are shown in purple. Progeny IDs are shown in grey. Sex chromosome genotype of individuals marked with an orange star (34 males and 2 females) was also assessed with a quantitative PCR assay.

Blocking of Wolbachia *transmission by YY females prevents X chromosome loss*

Surprisingly, the *Wolbachia*-infected YY mother in the previous pedigree analysis produced an all-male progeny (I-F2-2) entirely lacking *Wolbachia* endosymbionts (Fig 2). This is unexpected because infection by feminizing *Wolbachia* endosymbionts is usually associated with highly female-biased progenies, due to the maternal transmission of *Wolbachia* to usually >80% of the offspring [10,24–26]. To test whether lack of *Wolbachia* transmission from mother to I-F2-2 progeny was a random event or was due to the unusual YY maternal genotype, we extended our previous pedigree analysis to span five generations (S2 Fig) and analysed a second, independent pedigree spanning four generations (S3 Fig). In total, 20 families (defined as father, mother and progeny) were included in the two pedigrees, representing 799 individuals (252 males and 547 females). We tested 464 individuals for the presence of *Wolbachia* (Table 2). As expected, most tested females were infected by *Wolbachia* (180/214) while no tested male was (0/250). Based on the patterns of Y chromosome amplification and following a reasoning similar to that described in the previous section, we were able to infer sex chromosome genotypes for most analysed individuals. In addition, we independently verified sex chromosome genotypes of the parents of the 20 families using the aforementioned quantitative PCR assay (S5 Table). The sex chromosome genotypes of the 20 mothers included 11 XX, 5 XY and 4 YY females (S2 and S3 Fig, Table 2). All tested fathers were XY males, except the father of the II-F2-1 progeny which was a YY male. Interestingly, this individual demonstrated that the YY genotype is viable and fertile as male.

Table 2. Composition and frequency of *Wolbachia* infection in 20 families of *Armadillidium nasatum*. *Wolbachia* frequency in progenies was calculated as the *Wolbachia* frequency observed among tested females weighted by female proportion in the progenies (as all males lack *Wolbachia*).

Progeny ID	Parental genotypes		Progeny size	Female number	Female proportion	*Wolbachia* presence in males		*Wolbachia* presence in females		*Wolbachia* frequency
	Mother	Father				Nb. tested	Nb. infected	Nb. tested	Nb. infected	
I-F1-1	XY	XY	17	17	100%	0	0	3	3	100%
I-F2-1	XX	XY	24	24	100%	0	0	24	24	100%
I-F2-2	YY	XY	32	0	0%	32	0	0	0	0%
I-F2-3	XX	XY	52	52	100%	0	0	2	2	100%
I-F3-1	XY	XY	68	68	100%	0	0	12	12	100%
I-F3-2	XX	XY	33	33	100%	0	0	10	8	80%
I-F3-3	XX	XY	59	59	100%	0	0	12	12	100%
I-F3-4	XX	XY	40	40	100%	0	0	10	9	90%
I-F4-1	XX	XY	50	39	78%	11	0	15	12	62%
I-F4-2	XX	XY	41	24	59%	17	0	13	0	0%
I-F4-3	XX	XY	32	10	31%	22	0	10	2	6%
I-F4-4	XX	XY	16	16	100%	0	0	10	9	90%
I-F4-5	XX	XY	51	51	100%	0	0	15	15	100%
I-F4-6	XY	XY	42	19	45%	23	0	13	7	24%
II-F1-1	XX	Y?	13	13	100%	0	0	2	2	100%
II-F2-1	XY	YY	60	60	100%	0	0	60	60	100%
II-F2-2	XY	XY	20	20	100%	0	0	1	1	100%
II-F3-1	YY	XY	28	0	0%	28	0	0	0	0%
II-F3-2	YY	XY	42	0	0%	42	0	0	0	0%
II-F3-3	YY	XY	57	0	0%	57	0	0	0	0%

Wolbachia transmission rate from mother to offspring differed significantly between XX, XY and YY mothers, considering all 20 families (Kruskal-Wallis test, χ^2=8.84, p=0.01) or only the 16 families in which *Wolbachia* presence was tested in ≥10 individuals (Kruskal-Wallis test, χ^2=7.91, p=0.02). *Wolbachia* transmission rate was high (generally ≥80%) and did not differ significantly between XX and XY mothers (Dunn's post-hoc tests, p=0.73 for 20 families and p=0.56 for 16 families) (Fig 3). By contrast, *Wolbachia* infected none of the 159 offspring of the 4 YY mothers. This result cannot be ascribed to *Wolbachia* transmission to offspring and subsequent selective death of embryos carrying *Wolbachia* infection because progeny size did not differ significantly between XX, XY and YY mothers (Kruskal-Wallis tests, χ^2=0.18, p=0.91 for 20 families and χ^2= 3.32, p=0.19 for 16 families) (Table 2). Instead, the YY genotype is associated with a lack of *Wolbachia* transmission from mother to offspring. As a result, *Wolbachia* transmission rate differed significantly between YY mothers and both XX mothers (Dunn's post-hoc tests: p=0.006 for 20 families and p=0.01 for 16 families) and XY mothers (Dunn's post-hoc tests: p=0.008 for 20 families and p=0.01 for 16 families) (Fig 3). Consequently, as all offspring inherited a Y chromosome from their YY mother but no *Wolbachia* endosymbiont, they all developed as males (Table 2). Importantly, YY mothers originated from the two independent pedigrees we analysed (S2 and S3 Fig), indicating that the observed pattern is robust to *A. nasatum* genetic background.

[Insert Fig 3 here]

Fig 3. Boxplot of *Wolbachia* transmission rate from mother to offspring (measured as the frequency of *Wolbachia*-carrying individuals in each progeny) according to mother's sex chromosome genotype. The analysis is based on 16 progenies (n) for which *Wolbachia* presence was tested in ≥10 individuals. Thick lines and boxes depict median and interquartile range, respectively. Whiskers are bounded to the most extreme data point within 1.5 the interquartile range. Plots marked with the same letter (a, b) are not statistically different from each other (Kruskal-Wallis test, followed by pairwise comparison Dunn test).

As YY females exclusively produce males, only XX and XY females can produce female offspring in *Wolbachia*-infected lines of *A. nasatum*. Thus, the X chromosome is necessarily transmitted to at least a subset of each progeny of these *Wolbachia*-infected females. To evaluate consequences on sex chromosome frequencies in the long term, we extended Taylor's theoretical work [31] to enable the transmission rate of a cytoplasmic sex ratio distorter to vary depending on

female sex chromosome genotype. Specifically, we simulated the equilibrium frequencies of X and Y chromosomes in a population of a diploid genetic model containing a distorter with transmission rates of α, α and 0 in XX, XY and YY females (with α varying from 0 to 1), respectively, to reflect our empirical results. Under these conditions, equilibrium frequencies are 25% and 75% for the X and Y chromosomes for $\alpha<0.55$ (Fig 4a). In such cases, the distorter is lost from the population at equilibrium and the population is comprised of XY males and XX females in equal proportions. By contrast, for higher α values, equilibrium frequencies of the X and Y chromosomes vary in opposite directions, to the extent that the Y chromosome becomes more frequent than the X chromosome, but X equilibrium frequency is always ≥12.5%, whatever α (Fig 4a). Thus, a consequence of the lack of transmission of the distorter by YY females is that the X chromosome cannot be lost from the population. Another consequence evidenced by our simulations is that the equilibrium frequency of the distorter never exceeds 50%, whatever α (Fig 4a). Remarkably, a balanced sex ratio is maintained in the population at equilibrium, despite infected females individually producing progenies highly biased towards either females (XX and XY mothers) or males (YY mothers) (Fig 4b). This is in sharp contrast with the highly female-biased sex ratios at equilibrium expected for $\alpha>0.5$ when the distorter is transmitted by all three types of females[31] (Fig 4b).

[Insert Fig 4 here]

Fig 4. Evolutionary consequences of a cytoplasmic sex ratio distorter at the population level. (a) Equilibrium frequencies of X chromosome (pink line), Y chromosome (blue line) and distorter (dashed line), according to distorter transmission rate in XX and XY females (YY females do not transmit the distorter). (b) Evolution of the frequencies of females (solid lines) and individuals carrying the distorter (dashed lines) through time, with a distorter transmission rate of 0.9. Green: YY females do not transmit the distorter (only XX and XY females do); orange: all females transmit the distorter.

Discussion

We sequenced the genome of the terrestrial isopod *A. nasatum*. With an N_{50} of 86 kb, high completeness (BUSCO=94%) and near absence of unidentified nucleotides (0.01%), our ~1.2 Gb assembly is among the best assembled of all large-genome crustaceans sequenced to date [30]. Our results establish that *A. nasatum* X and Y chromosomes are genetically highly similar. The Y-specific

region of the genome may be as small as one megabase and it contains very few genes. Even at nucleotide resolution, sequence divergence and repeat content indicate very limited sex chromosome differentiation. This result explains why the unusual YY genotype is viable in this species. The underlying causes of the very high similarity of *A. nasatum* sex chromosomes include young evolutionary age, ongoing recombination or both. In any event, this high similarity was likely instrumental to the establishment of feminizing *Wolbachia* infection in the male heterogametic isopod *A. nasatum*.

An important outcome of the infection of *A. nasatum* by feminizing *Wolbachia* is the unusual production of YY individuals and XY females. This situation challenges the classical model of sex chromosome evolution by affecting chromosome effective population size (N_e) and recombination patterns. Classically, recombination arrest occurs between the X and Y chromosomes, leading to a drastic reduction of Y chromosome N_e to one third of X chromosome N_e and one fourth of autosome N_e (because there is one Y chromosome dose for three X chromosome doses and four autosome doses per mating pair). Lowered N_e results in enhanced genetic drift and concomitant reduction of selection efficiency, leading the Y chromosome to accumulate deleterious mutations and degenerate [13,15,17–19]. However, the existence of YY individuals and XY females implies that the standard expectation of sex chromosome N_e does not apply to *A. nasatum* in the presence of *Wolbachia*. Indeed, our simulations indicate that X chromosome N_e should stabilize at about one fourth of Y chromosome N_e and about one fifth of autosome N_e at observed *Wolbachia* transmission rates (Fig 4a). Thus, X chromosome N_e, not Y chromosome N_e, is predicted to be dramatically reduced in *A. nasatum* genetic backgrounds infected by feminizing *Wolbachia*. Furthermore, the occurrence of YY individuals opens the possibility of Y-Y recombination and more efficient selection, which may prevent the accumulation of deleterious mutations on the Y chromosome. By contrast, XX individuals are rare according to our simulations (<2%), thereby reducing the opportunity of X-X recombination. This suggests that the X chromosome, rather than the Y chromosome, may accumulate deleterious mutations and degenerate in the long term. Another possibility to consider is that recombination patterns may differ between sexes (heterochiasmy) in some species, so that the X and Y chromosomes may not recombine simply because males do not recombine (whereas females do) [15]. If so, the existence of XY females in *A. nasatum* as a consequence of *Wolbachia* infection would open the possibility of X-Y recombination in phenotypic females, as previously reported in frogs [41]. Unfortunately, male and female patterns of recombination are unknown in terrestrial isopods. Their investigation therefore represents a perspective for future research. In any event, there appears to be ample scope for microbial symbionts to drive the molecular evolution of their host sex chromosomes.

Remarkably, infected YY females did not transmit *Wolbachia* to any offspring, as confirmed in two independent pedigrees. An evolutionary consequence is that the YY genotype cannot become fixed (nor the X chromosome lost) in infected *A. nasatum* lines. Lack of *Wolbachia* transmission by YY females also raises the question of the underlying mechanism controlling vertical transmission of the symbionts. In sharp contrast with the YY genotype, the alternative XX and XY genotypes were associated with similarly high rates of *Wolbachia* transmission in both pedigrees. Thus, the genetic basis of *Wolbachia* transmission control fits the model of a recessive allele linked to the Y chromosome (associated with an X-linked dominant allele), in which the homozygous state blocks *Wolbachia* transmission. It has been shown previously that within-host microbial interactions can enhance or reduce symbiont vertical transmission [42]. This applies to *Wolbachia*, which vertical transmission is prevented by *Asaia* bacteria in *Anopheles* mosquitoes [43]. Host nuclear genotype is also an important factor that can affect symbiont vertical inheritance. For example, *Wolbachia* maternal transmission is strongly reduced by actin in *Drosophila* [44] and the *wds* gene in *Nasonia* [45]. Our results show that sex chromosome genotype represents yet another case of host nuclear control over symbiont transmission. *Wolbachia* symbionts were previously found to prevent sex chromosome transmission in *Eurema mandarina* butterflies [46]. Here we show that sex chromosomes can prevent *Wolbachia* transmission in *A. nasatum*, thereby offering a whole new perspective on the molecular interplay between feminizing symbionts and host sex chromosomes.

The mutation causing the suppression of *Wolbachia* transmission in *A. nasatum* may have existed in the standing pool of sex chromosome variants prior to *Wolbachia* infection, having evolved either neutrally or under natural selection. Alternatively, the mutation may have evolved as an adaptation subsequent to *Wolbachia* infection. While infection by feminizing symbionts normally leads to highly female-biased sex ratios, the ability of infected YY females to impede *Wolbachia* transmission in *A. nasatum* results in the production of all-male progenies, due to the systematic inheritance of a maternal Y chromosome. Interestingly, this situation restores balanced sex ratios at the population level, as indicated by our simulations (Fig 4) and consistent with empirical evidence [35,47]. This opens the possibility that blocking of *Wolbachia* transmission by YY females may have evolved to suppress feminization and ensuing sex ratio biases, as predicted by sex ratio selection [10–12,15]. Indeed, strong sex ratio biases towards females imposed by feminizing symbionts induce nucleo-cytoplasmic conflicts with most nuclear genes, which are biparentally inherited and optimally benefit from balanced sex ratios. In principle, conflict resolution may be achieved by restoring balanced sex ratios at the parental level, through selection of females producing balanced sex ratios [48]. Alternatively, resolution may occur at the population level, through selection of females producing male-biased sex ratios to compensate for the female-biased progenies of infected females

400 [48]. YY females in *A. nasatum* may represent an original example of conflict resolution at the
401 population level. Several cases of nuclear suppression preventing the action or transmission of sex
402 ratio distorting symbionts have been reported [11], including in the isopod *A. vulgare* [49,50]. A
403 distinguishing feature of symbiont suppression in *A. nasatum* is its connection with sex
404 chromosomes.

Materials & Methods

Genome sequencing and assembly

All *A. nasatum* individuals used for sequencing were from our inbred laboratory line ANa, which is originally derived from wild animals caught in Thuré, France, in 2008. Specifically, we used XY genetic males and XX genetic females descended from a single pair of grandparents of the family ANa2 (according to the crossing scheme shown in Fig S4) to minimize heterozygosity. Total genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit, according to the protocol for animal tissues (3 h of incubation in proteinase K at 56°C and 30 min of RNase treatment at 37°C). Absence of *Wolbachia* endosymbionts in all samples was confirmed by PCR using the *ftsZ* and *wsp* markers [21,36]. Short paired-end libraries with ~200 bp insert sizes were sequenced with the Illumina HiSeq2000 technology (S1 Table). In addition, PacBio RS II sequencing (P6C4 chemistry) was performed to obtain long sequencing reads (S1 Table). Accession numbers for Illumina and PacBio sequence datasets are provided in S1 Table.

Sequencing reads were subjected to quality control and filtering as described previously [30]. Male Illumina and PacBio sequencing reads were used in a hybrid strategy to assemble the male (XY) genome of *A. nasatum*, as described previously [30]. A summary of the workflow we used (including assembly, polishing, contaminant removal and scaffolding) is shown in Fig S5. Genome assembly completeness was evaluated using benchmarking for universal single copy orthologs (BUSCO, version 3.0.1) [37], with the arthropod profile library (-l arthropoda) composed of 1,066 arthropod core genes. Repeat identification and gene annotation were performed as described previously [30].

Identification and analyses of contigs containing Y-specific sequences

Contigs containing Y-specific sequences in the *A. nasatum* assembly were identified using the Chromosome Quotient (CQ) [38] and Y chromosome Genome Scan (YGS) [39], as described previously [30]. The maximum CQ score was set to 0.35 to retain contigs as Y-specific candidates, as recommended by CQ authors [38]. The minimum YGS score was set to 35% to retain contigs as Y-specific candidates. This threshold was selected to account for the high repetitive nature of the *A. nasatum* genome.To identify heterozygous SNPs in the contigs of the male genome assembly, we applied the Genome Analysis ToolKit (GATK) pipeline (version 3.8-0-ge9d806836) [51], as described previously [30].

436 PCR assays were designed on the candidate contigs as follows (S4 Table). Primers were
437 designed with Primer-BLAST [52] in unique regions of the contigs and primer specificity was checked
438 using Blastn (version 2.2.30+) [53] by aligning primers to the unmasked *A. nasatum* assembly. PCR
439 reactions were carried out in 25 µL with 5 µL of buffer 5×, 0.5 µL of dNTPs (2.15 mM), 0.7 µL of each
440 primer (10 µM), 0.25 µL of Taq polymerase 5 u/µL, and 1 µL of DNA. PCRs were conducted using the
441 following temperature cycle: 3 min at 94°C for initial denaturation, followed by 35 cycles of 30 s at
442 94°C, 30 s at 48/50/52°C (depending on primer annealing temperature) and 1 min at 72°C. The final
443 elongation step was 10 min at 72°C. PCR tests were then conducted in three successive steps: (i) test
444 on one male and three pools of two females of the ANa2 family (used for genome sequencing); (ii)
445 test on six males and twelve females from other families of our ANa laboratory line, and (iii) test on
446 two males and a pool of three females from Beauvoir-sur-Niort population (France) and on two
447 males and a pool of three females from Piriápolis population (Uruguay). After each step, loci
448 amplifying in all males and no female were retained for the next step. PCR tests targeting the
449 autosomal *18S rRNA* [30] and mitochondrial *COI* [54] genes were used as positive controls in all
450 samples. Absence of *Wolbachia* endosymbionts was also confirmed in all samples by PCR using the
451 *ftsZ* and *wsp* markers [21,36].

452

453 *Pedigree construction and analyses*

454 We generated two independent *A. nasatum* pedigrees spanning five (pedigree I, S2 Fig) and
455 four (pedigree II, S3 Fig) generations (*A. nasatum* has a generation time of one year). We started in
456 2013 with two F0 female founders isolated from a laboratory cage population (NASw) initiated in
457 2008 from wild animals (caught in Thuré, France) infected by the feminizing *Wolbachia* strain *w*Nas
458 [36]. Each F0 female produced an F1 progeny (resulting from mating with unknown F0 males from
459 the cage population). Each year, two to six females were selected from the previous generation and
460 crossed with XY genetic males from our ANa laboratory line (except the father of the II-F2-1 progeny,
461 which was selected from the NASw cage population and could carry XY or YY sex chromosomes).
462 Pedigrees I and II were composed of 14 and 6 families, respectively.

463 At each generation, total genomic DNA was extracted from the progenitors (except the F0
464 male founders and individuals which died prematurely) and from males and females of their
465 progenies. Presence or absence of *Wolbachia* was tested by PCR using the *ftsZ* and *wsp* markers
466 [21,36]. We also used two of the previously designed Y-specific markers (contig12740 and
467 contig18908) to assess the presence of the Y chromosome in the individuals, interpreting PCR results
468 as follows: amplification indicating the individual is XY or YY and lack of amplification indicating that

the individual is XX. PCR tests targeting the autosomal *18S rRNA* [30] and mitochondrial *COI* [54] genes were used as positive controls in all samples. Based on PCR amplification patterns and pedigree structures, we were able to infer sex chromosome genotypes (XX, XY or YY) for most analyzed individuals.

To independently assess sex chromosome genotypes (XX, XY or YY), we developed a quantitative PCR assay measuring Y chromosome abundance relative to autosomes. Y-specific primers were designed within the Y-specific PCR amplicon previously validated in contig10349 (primer sequences: 5'-CCCTACACAGCATACTTGACAG and 5'-CAGGTGCTCCTTCAGAGAAAC, product size: 129 bp). Contig10349 abundance was normalized against *EF2* gene abundance [55]. *EF2* is a single-copy gene in the *A. nasatum* assembly located in autosomal contig8976 (YGS=8.8% and CQ=1.09). PCR reactions were run in duplicates in a 480 Light-Cycler (Roche) using SYBR Green I assays, under the following conditions: 10 min at 95°C and 45 cycles of [10 sec at 95°C, 10 sec at 60°C, 20 sec at 72°C]. A melting curve (65°C to 97°C) was recorded at the end of each reaction to check PCR product uniqueness. Reaction mixture consisted of 0.5 µL of each primer (10 µM), 5 µL of Fast SYBR-Green Master Mix (Roche) 3 µL of bi-distilled water and 1 µL of extracted DNA. Fluorescence crossing points (C_t) were calculated with the second derivative maximum method using the LightCycler 1.5 software. PCR amplification efficiency was determined with a calibration curve for each primer pair. Only PCR reactions producing a single product and with $C_t \leq 35$ cycles were considered. Relative abundance of the Y-specific marker relative to autosomal marker was calculated as $2^{-\Delta Ct}$, where $\Delta C_t = C_{t(\text{Y chromosome})} - C_{t(\text{autosome})}$. XX, XY and YY genotypes corresponded to $2^{-\Delta Ct}$ values of ~0, ~0.5 and ~1, respectively.

Simulation of sex chromosome frequencies

To evaluate the evolutionary impact of cytoplasmic sex ratio distorters on sex chromosome frequencies, we extended Taylor's theoretical work[31] to enable the transmission rate of the distorter to vary according to female sex chromosome genotypes. Specifically, we simulated the frequencies of the X chromosome, the Y chromosome, the distorter and females in a population of a diploid genetic model with a distorter whose transmission rate can vary according to the sex chromosome genotype of females (i.e. XX, XY and YY). We used the following equations:

Genotypes

- Males: G_1=XY ; G_3=YY ; F_M=Males frequency
- Females: G_2=XX ; G_4=XX.Wo+ ; G_6=XY.Wo+ ; G_8=YY.Wo+ ; F_F=Females frequency

Gametes

- Males: P_1=Y; P_3=X
- Females: P_2=X; P_4=Y; P_6=X.Wo+; P_8=Y.Wo+

Wolbachia transmission rates by females

- t_X by XX.Wo+; t_{XY} by XY.Wo+; t_Y by YY.Wo+

Gametes production formula

- Male gametes:

$$P_1 = \frac{G_1}{2F_M} + \frac{G_3}{F_M} = \frac{(G_1 + 2G_3)}{2F_M}$$

$$P_3 = \frac{G_1}{F_M}$$

- Female gametes:

$$P_2 = \frac{G_2}{F_F} + \frac{G_4(1 - t_X)}{F_F} + \frac{G_6(1 - t_{XY})}{2F_F}$$

$$P_4 = \frac{G_6(1 - t_{XY})}{2F_F} + \frac{G_8(1 - t_Y)}{F_F}$$

$$P_6 = \frac{G_4 t_X}{F_F} + \frac{G_6 t_{XY}}{2F_F}$$

$$P_8 = \frac{G_8 t_Y}{F_F} + \frac{G_6 t_{XY}}{2F_F}$$

Next generation genotypes production formula

- Males:

$$G_1' = (P_1 P_2) + (P_3 P_4)$$

$$G_3' = (P_1 P_4)$$

- Females:

$$G_2' = (P_3 P_2)$$

$$G_4' = (P_3 P_6)$$

$$G_6' = (P_1 P_6) + (P_3 P_8)$$

$$G_8' = (P_1 P_8)$$

with $\sum_{i=1}^{8} G_i' = 1$

and $G_1' = G_1$; $G_2' = G_2$ … $G_8' = G_8$ at equilibrium.

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678 **Legends to supporting information**

679

680 **Fig. S1.** Characterization of sex chromosome genotypes (XX, XY or YY) of *A. nasatum*
681 individuals based on a quantitative PCR assay. Y chromosome to autosome ratios were
682 calculated for 60 individuals and compared to expected ratios: 1 for YY individuals
683 (corresponding to 18 males and 3 females), 0.5 for XX individuals (28 males and 5 females)
684 and 0 for XX individuals (6 females). Thick lines and boxes depict median and interquartile
685 range, respectively. Whiskers are bounded to the most extreme data point within 1.5 the
686 interquartile range.

687 **Fig. S2.** *Armadillidium nasatum* pedigree I used to track inheritance of the Y chromosome
688 and *Wolbachia*. The pedigree spans five generations (F0-F4) and is comprised of 572
689 individuals (119 males and 453 females), 269 of which were included in molecular analyses
690 (individuals not included in the molecular analyses are shown in dotted circles). Males are
691 shown as squares and females as circles. Individuals carrying *Wolbachia* are shown in purple.
692 Progeny IDs are shown in grey. Sex chromosome genotype of individuals marked with an
693 orange star was also assessed with a quantitative PCR assay.

694 **Fig. S3.** *Armadillidium nasatum* pedigree II used to track inheritance of the Y chromosome
695 and *Wolbachia*. The pedigree spans four generations (F0-F3) and is comprised of 226
696 individuals (132 males and 94 females), 196 of which were included in molecular analyses
697 (individuals not included in the molecular analyses are shown in dotted circles). Males are
698 shown as squares and females as circles. Individuals carrying *Wolbachia* are shown in purple.
699 Progeny IDs are shown in grey. Sex chromosome genotype of individuals marked with an
700 orange star was also assessed with a quantitative PCR assay.

701 **Fig. S4.** Workflow of the hybrid strategy used for assembling the *Armadillidium nasatum*
702 genome.

703 **Table S1**. Characteristics of *Armadillidium nasatum* sequencing datasets generated in this
704 study.

705 **Table S2**. Annotation statistics of the *Armadillidium nasatum* genome assembly.

706 **Table S3**. Repeat content of the *Armadillidium nasatum* genome.

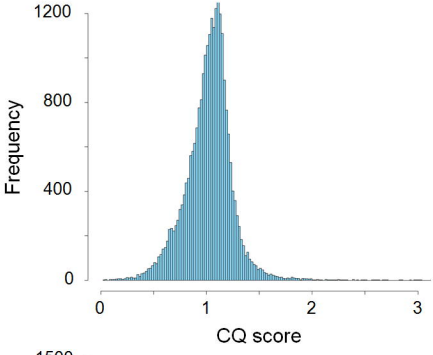
707 **Table S4**. Characteristics of the 78 contigs of the *Armadillidium nasatum* assembly
708 considered as Y-specific candidates.

709 **Table S5**. PCR results for Y chromosome and *Wolbachia* analyses of 72 *Armadillidium*
710 *nasatum* individuals. nt: not tested because male individual originating from *Wolbachia*-free

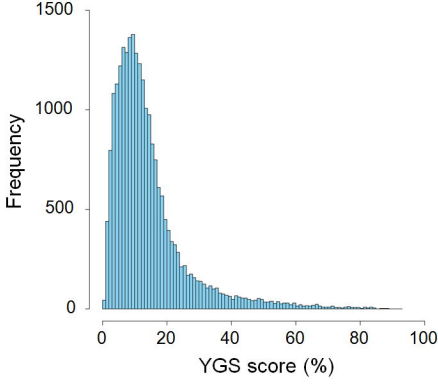
711 line, hence necessarily XY (except II-F1-1 father from *Wolbachia*-infected line, hence XY or

712 YY). Y chromosome to autosome ratio was calculated as 2-ΔCt.

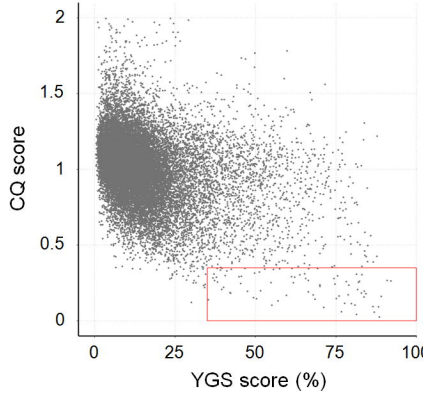
(a)

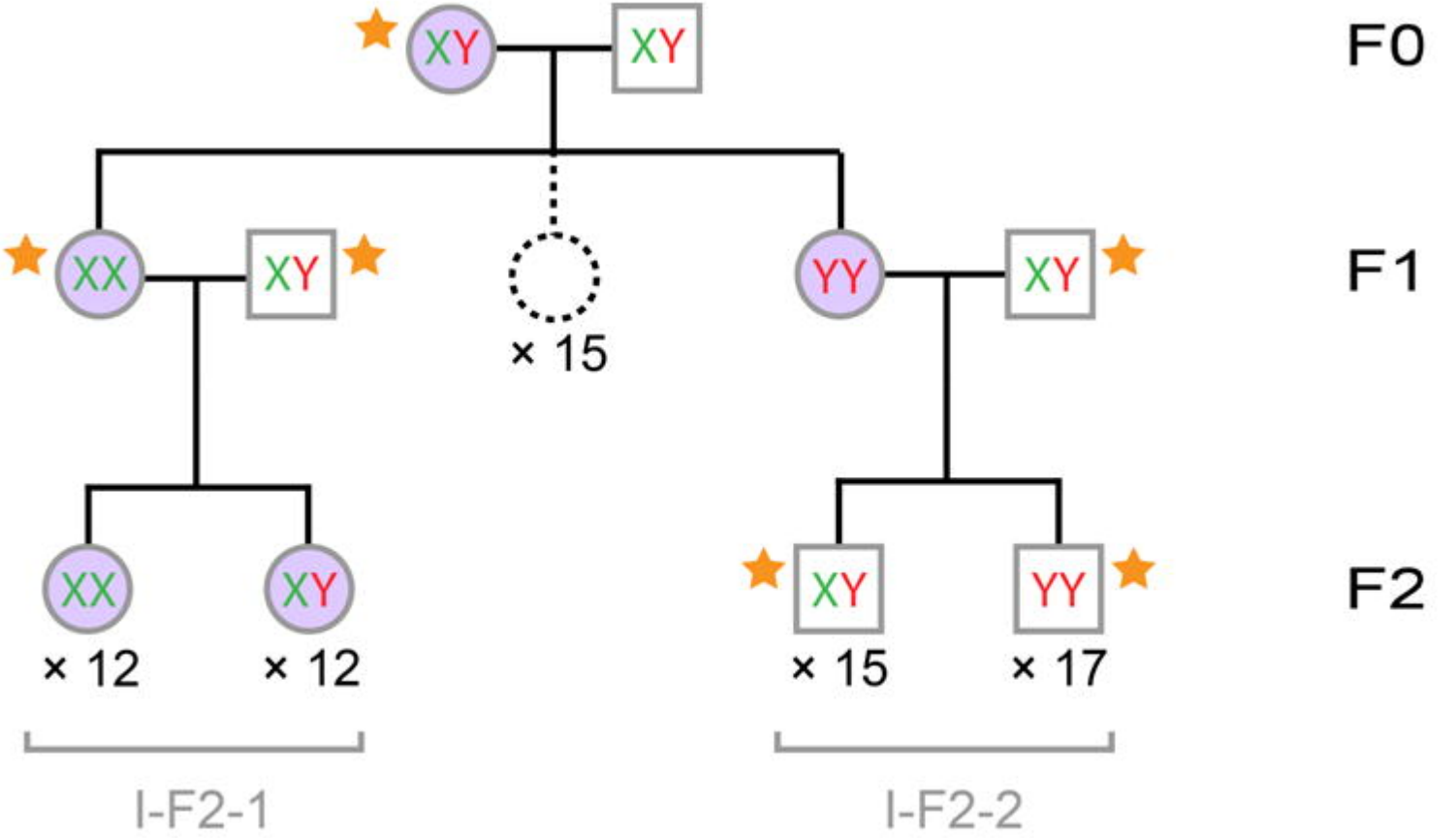

1200
800
400
0
Frequency
0
1
2
3
CQ score

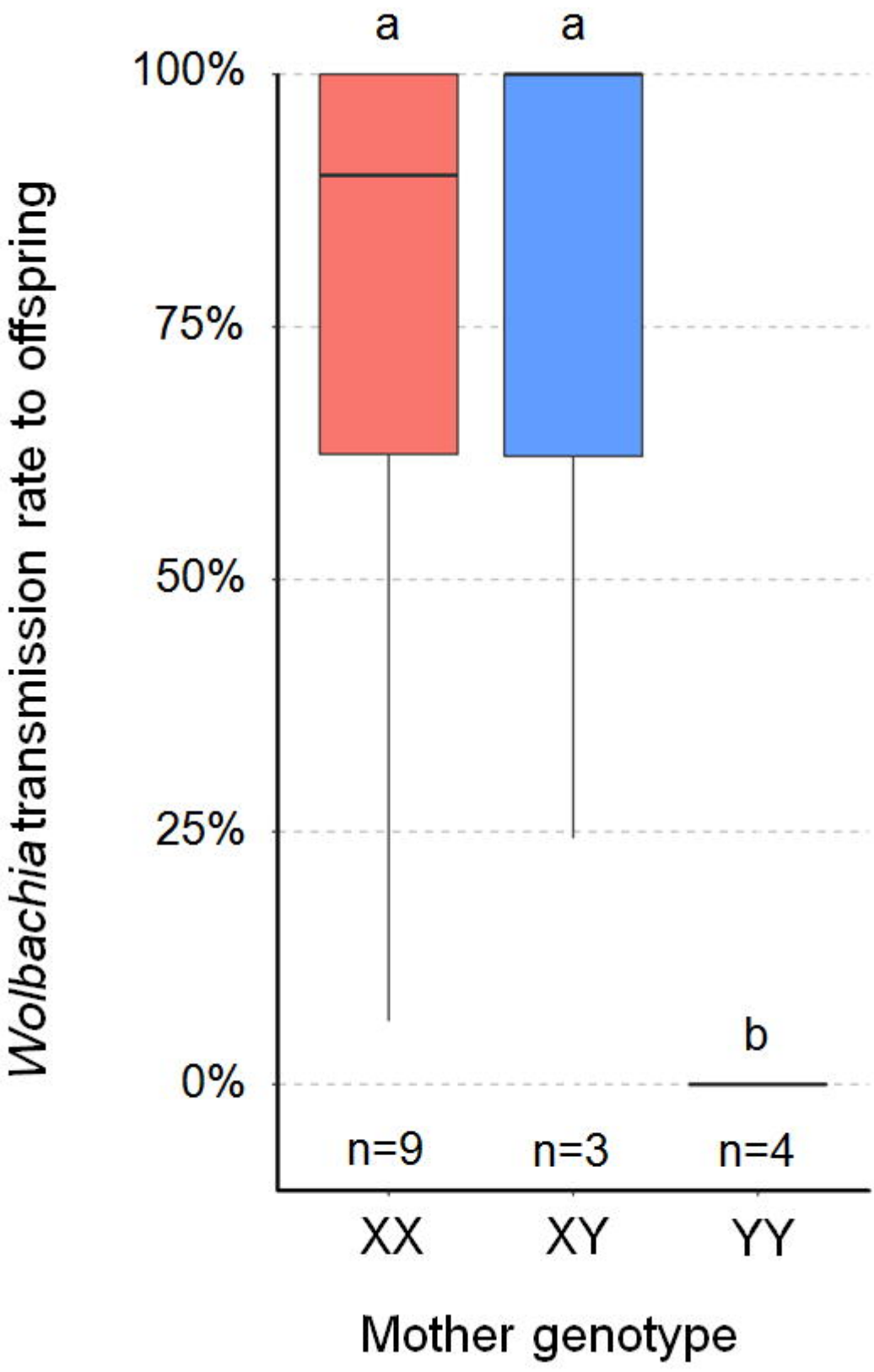
(b)

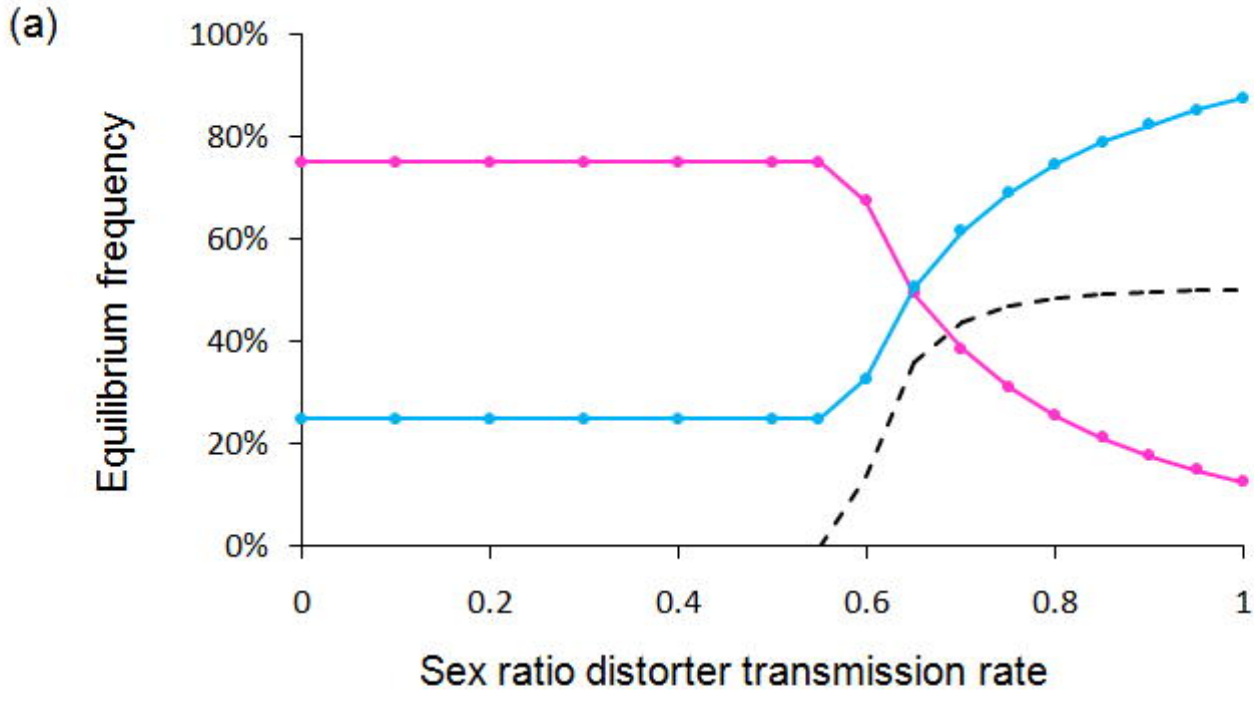

1500
1000
500
0
Frequency
0
20
40
60
80
100
YGS score (%)

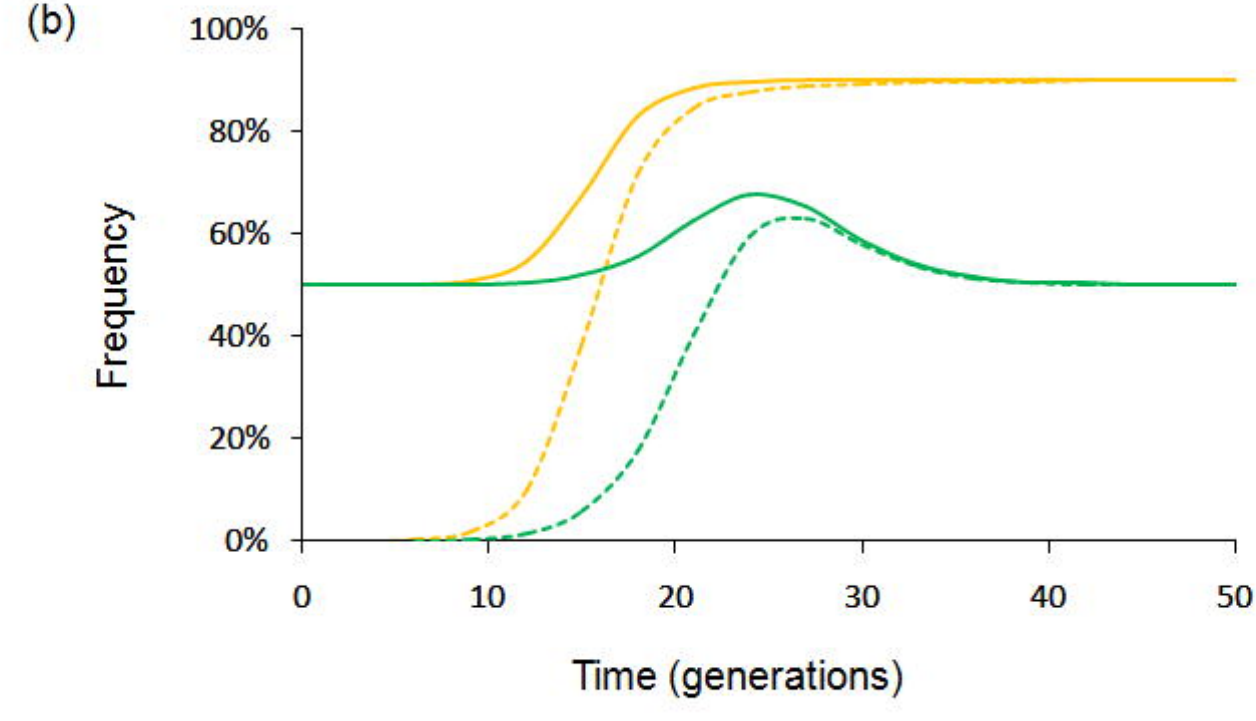
(c)


2
1.5
1
0.5
0
CQ score
0
25
50
75
100
YGS score (%)


XY
XY
F0
XX
XY
× 15
YY
XY
F1
XX
XY
XY
YY
× 12
× 12
× 15
× 17
F2
I-F2-1
I-F2-2


a
a
100%
75%
50%
25%
0%
b
n=9
n=3
n=4
XX
XY
YY
Mother genotype
Wolbachia transmission rate to offspring


(a)
100%
80%
60%
40%
20%
0%
Equilibrium frequency
0
0.2
0.4
0.6
0.8
1
Sex ratio distorter transmission rate


(b)
100%
80%
60%
40%
20%
0%
Frequency
0
10
20
30
40
50
Time (generations)