Heterogeneous distribution of sex ratio distorters in natural populations of the isopod *Armadillidium vulgare*

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Abstract

In the isopod *Armadillidium vulgare*, many females produce progenies with female-biased sex ratios, due to two feminizing sex ratio distorters (SRD): *Wolbachia* endosymbionts and the *f* element. We investigated the distribution and population dynamics of these SRD and mitochondrial DNA variation in 16 populations from Europe and Japan. Confirming and extending results from the 1990's, we found that the SRD are present at variable frequencies in populations, and that the *f* element is overall more frequent than *Wolbachia*. The two SRD never co-occur at high frequency in any population, suggesting an apparent mutual exclusion. We also detected *Wolbachia* or the *f* element in some males, which likely reflects insufficient titer to induce feminization or presence of masculinizing alleles. Our results are consistent with a single integration event of a *Wolbachia* genome in the *A. vulgare* genome at the origin of the *f* element, which contradicts an earlier hypothesis of frequent losses and gains. We identified strong linkage between *Wolbachia* strains and mitochondrial haplotypes, but no association between the *f* element and mitochondrial background. Our results open new perspectives on SRD evolutionary dynamics in *A. vulgare*, the evolution of genetic conflicts and their impact on the variability of sex determination systems.

Keywords

Sex ratio distorter, endosymbiont, *Wolbachia*, *f* element, sex determination

1. Introduction

Sex ratio distorters (SRD) are selfish genetic elements located on sex chromosomes or transmitted by a single sex, which skew the proportion of males and females in progenies towards the sex that enhances their own vertical transmission [1]. Major SRD types include sex chromosome meiotic drivers [2,3], some B chromosomes [4] and selfish cytoplasmic genetic entities [5,6,7]. Collectively, they are found in a wide range of animal and plant species and they have had a tremendous impact on the ecology and evolution of their host species [8,9]. One of the most emblematic SRD is the bacterial endosymbiont *Wolbachia* [10,11]. *Wolbachia* is a cytoplasmic, maternally inherited alpha-proteobacterium found in a wide range of arthropods and nematodes. In arthropods, *Wolbachia* often manipulates host reproduction in favor of infected females, thereby conferring itself a transmission advantage. This is achieved through various strategies, three of which causing sex ratio distortions towards females: male killing, thelytokous parthenogenesis and feminization of genetic males [6,7,10,11].

In the terrestrial isopod *Armadillidium vulgare*, chromosomal sex determination follows female heterogamety (ZZ males and ZW females) [12–14]. However, many females produce progenies with female-biased sex ratios, due to the presence of two feminizing SRD: *Wolbachia* endosymbionts and a locus called the *f* element [6,15,16]. *Wolbachia* symbionts cause ZZ genetic males to develop as phenotypic females [17]. Three *Wolbachia* strains have been described in *A. vulgare*, for which feminization induction has been demonstrated (*w*VulC and *w*VulM strains [18,19]) or is strongly suspected (*w*VulP strain [20]). The *f* element is a nuclear insert of a large portion of a feminizing *Wolbachia* genome in the *A. vulgare* genome [21]. The *f* element induces female development, as a W chromosome does, and it shows non-Mendelian inheritance, making it an SRD [21,22]. These SRD may cause turnovers in sex determination mechanisms [6,15,23] and they could explain why sex chromosome systems are so variable in terrestrial isopods [24–27].

Testing this hypothesis requires characterizing the evolutionary dynamics of SRD such as *Wolbachia* and the *f* element in natural populations. In *A. vulgare*, this characterization is quite limited because prior studies were mostly restricted to a narrow geographic area (western France), sometimes focusing solely on *Wolbachia* [20,28–31]. The only exception is a 1993 study [32], which collated and extended results from the early 1980's [33,34]. The main observations were that *Wolbachia* and the *f* element are present at variable frequencies in field populations, and the *f* element is more frequent than *Wolbachia*. However, earlier studies were limited by the lack of molecular tests for *Wolbachia* and/or the *f* element, preventing any direct assessment of SRD presence. Instead, the authors used a

complex, indirect procedure combining a physiological test and crossings [32]. In addition to being tedious and time-consuming (generation time is one year in this species), this procedure did not allow direct and undisputable assessment of SRD presence. Moreover, it could only be run on females and therefore provided no information on SRD presence in males. Finally, it could not reveal individuals potentially carrying both SRD.

Here, we took advantage of the availability of molecular markers to directly assess SRD presence in males and females from *A. vulgare* field populations from Europe and Japan. This approach allowed us to circumvent the limitations of previous studies, and to revisit the population dynamics of *Wolbachia* and the *f* element in this species and their association mitochondrial lineages.

2. Materials and Methods

647 *A. vulgare* individuals from 16 natural populations across Europe and Japan were collected by hand. Individuals were sexed and stored in alcohol or at -20°C prior to DNA extraction. Total genomic DNA was extracted from the head and legs of each individual, as described previously [21]. We used four molecular markers to assess the presence of *Wolbachia* and the *f* element in DNA extracts: *JtelI* [21], *wsp* [35], *recR* [36] and *ftsZ* [37] (Table S1). While *JtelI* is specific to the *f* element, *wsp* and *recR* are specific to *Wolbachia*, and *ftsZ* is present in both the *f* element and *Wolbachia* [21]. We assessed the presence or absence of these markers by PCR, as described previously [21]. Different amplification patterns were expected for individuals with *Wolbachia* only (*JtelI-, wsp+*, *recR+*, *ftsZ+*), the *f* element only (*JtelI+, wsp-, recR-*, *ftsZ+*), both *Wolbachia* and the *f* element present (*JtelI+, wsp+, recR+*, *ftsZ+*) or both *Wolbachia* and the *f* element lacking (*JtelI-, wsp-, recR-*, *ftsZ-*). The few individuals exhibiting other amplification patterns were classified as “undetermined status”. A quantitative-PCR assay was used to measure *Wolbachia* titer in some individuals (see supplementary Methods). To characterize *Wolbachia* strain diversity, *wsp* PCR products were purified and Sanger sequenced using both forward and reverse primers by GenoScreen (Lille, France). Forward and reverse reads were assembled using Geneious® v.7.1.9 to obtain one consensus sequence per individual. To evaluate mitochondrial diversity, we amplified by PCR a ~700 bp-long portion of the Cytochrome Oxidase I (*COI*) gene in all individuals [38]. PCR products were purified and Sanger sequenced as described above. Haplotype network analysis was performed using the *pegas* package [39]. All statistical analyses were performed with R v.3.6.0 [40]. Figures were realized with *ggplot2* [41].

102

103 **3. Results**

104

105 We tested the presence of *Wolbachia* and the *f* element in 423 females and 224 males from 16
106 populations across Europe and Japan (Tables 1, S2). While most males lacked both SRD, 48% of
107 females carried at least one of them. The remaining females presumably carry W chromosomes,
108 although the existence of other feminizing elements cannot be formally excluded. As expected for
109 feminizing elements, the SRD were mostly found in females, the *f* element being more frequent than
110 *Wolbachia* overall. Both SRD were found in the same individuals in only 3 females from a single
111 population (Chizé). *Wolbachia*-infected individuals carried one of the three previously known
112 *Wolbachia* strains of *A. vulgare*: *w*VulC (n=62), *w*VulM (n=23) or *w*VulP (n=4).

113 *Wolbachia* and *f* element distribution in females was highly heterogenous among populations (Figure
114 1a). These SRD were found in 10 and 11 out of 16 populations, but they reached frequencies >10% in
115 only 6 and 7 populations, respectively. The two SRD coexisted in 8 populations. A generalized linear
116 model predicting the frequency of the *f* element as a binomial response by the proportion of
117 individuals carrying *Wolbachia* (each statistical unit being a population) showed that the prevalence
118 of the two SRD was significantly negatively correlated (9.8% of deviance explained, Chi-squared test,
119 $p < 7.9 \times 10^{-8}$, 14 df) (Figure 1b). Hence, in Floirac, Poitiers, Saint Julien l'Ars and Pisa populations,
120 *Wolbachia* was frequent (23-94% frequency in females) and the *f* element was rare (0-8%). By
121 contrast, the *f* element was frequent (35-96%) and *Wolbachia* was rare (0-11%) in Prague, Beauvoir,
122 Chizé, Coulombiers and La Crèche populations. In the other populations, both SRD were found at low
123 to moderate frequency (0-19%), including 3 populations devoid of both SRD (Lastovo, Hyogo and
124 Bucharest).

125 Males carrying *Wolbachia* or the *f* element were found in 2 and 4 out of 16 populations, respectively.
126 In all cases, these males occurred in populations in which the corresponding SRD were the most
127 prevalent ones in females: Beauvoir, Chizé, Coulombiers and La Crèche for the *f* element, and Floirac
128 and Saint Julien l'Ars for *Wolbachia*. Overall, these males had much lower *Wolbachia* titer than
129 females from their respective populations (Figure S1, Table S3).

130 The 642 individuals sequenced at the *COI* gene presented a total of 92 segregating sites defining 23
131 haplotypes (named I to XXIII; GenBank accession numbers in Table S4), with 1 to 7 haplotypes per
132 population (Table S2, Figure 2). The most frequent and widespread haplotype (I) was found in 188
133 individuals from 10 populations. The second most frequent and widespread haplotype (V) was found

134 in 106 individuals from 7 populations. We found 21 out of the 23 haplotypes among individuals
135 lacking both *Wolbachia* and the *f* element (Table 2, Figure 2). Among individuals carrying the *f*
136 element, 6 haplotypes were found, all but one (I, II, III, V and VI) being shared with individuals lacking
137 both *Wolbachia* and the *f* element, and one (IV) being carried by a single individual in the entire
138 dataset. Among *Wolbachia*-infected individuals, all those carrying *w*VulC were associated with either
139 haplotype V or its close relatives (XI and XII). All individuals carrying *w*VulM were associated with
140 haplotype II and those carrying *w*VulP with haplotype VII. Of the 5 haplotypes found in *Wolbachia*-
141 infected individuals, 4 were shared with individuals lacking both *Wolbachia* and the *f* element (II, V,
142 VII and XII), 2 of which were also shared with individuals carrying the *f* element (II and V), and one
143 (XI) was present in a single individual in the entire dataset.

144

145 **4. Discussion**

146

147 Our results provide direct evidence that the *f* element is overall more frequent than *Wolbachia* in the
148 sampled *A. vulgare* populations. We detected the *f* element in 11 *A. vulgare* natural populations from
149 4 European countries (Czech Republic, France, Germany and The Netherlands) and Japan. Together
150 with its previous detection in Denmark [21], our results indicate that the *f* element has spread to a
151 wide geographical range. The relative frequencies of the *f* element and *Wolbachia* are highly variable
152 among populations and, in general, when one SRD is frequent, the other SRD is rare. Overall, these
153 results are consistent with earlier results from the 1990's [32], although no molecular assay allowing
154 direct testing was available at that time and SRD presence or absence was inferred indirectly.

155 As the *Jtel* marker is located across the site of integration of the *f* element in the *A. vulgare*
156 chromosome [21], *f* element presence in various populations can be explained by a single event of
157 integration of a *Wolbachia* genome in the *A. vulgare* genome. A less parsimonious scenario would
158 require independent insertions at the same chromosomal site, which is highly unlikely. The former
159 scenario contradicts an earlier hypothesis on the evolutionary dynamics of the *f* element, which
160 suggested that the *f* element was unstably integrated in the *A. vulgare* genome, experiencing
161 frequent loss from oocytes and recurrent gain from *Wolbachia* endosymbionts [22,23,42–44]. Under
162 this scenario, multiple independent *f*-like elements would be expected to segregate at low
163 frequencies in populations, they should be integrated in different genomic locations and they should
164 all be able to induce feminization [16]. While our results do not formally invalidate the possibility of
165 additional *f*-like integrations in *A. vulgare* populations, which the *Jtel* marker would not detect, it

166 does not appear to be the most parsimonious hypothesis. Examination of sex ratios from progenies
167 of wild-caught females lacking both SRD may offer further insight into this issue.

168 Using molecular assays allowed us to circumvent two limitations of the previously used physiological
169 test: the impossibility to detect *Wolbachia* and the *f* element in males, and the impossibility to detect
170 individuals carrying both SRD. Regarding *Wolbachia* presence in males, the historic protocol was only
171 applicable to females per design [29,30,32] and subsequent PCR screens for *Wolbachia* infection
172 have mostly focused on testing females [20,30,31]. In fact, males have seldom been tested and found
173 to carry *Wolbachia* [45]. Here, we detected *Wolbachia* in 7 males from 2 populations (Floirac and
174 Saint Julien l'Ars), carrying either *w*VulC or *w*VulM strains. The failure of feminization by *Wolbachia*
175 most certainly reflects insufficient bacterial titer to induce feminization (Figure S1). These field
176 observations hence support the view that titer is an important factor for successful feminization, as
177 low titer is linked to incomplete feminization and intersexual phenotypes [42,46].

178 We also detected the presence of the *f* element in 11 males from 4 populations. Historically, the
179 presence of the *f* element in males has been indirectly inferred from crossings and the resulting sex-
180 ratios biases of progenies [22,43,47]. Our results constitute the first direct evidence for the presence
181 of the *f* element in *A. vulgare* males. In all 4 populations in which *f*-carrying males were found, the *f*
182 element was also frequent in females. Altogether, these observations suggest that the 11 males
183 carrying the *f* element also carry the masculinizing dominant *M* allele [16,43,47]. Indeed, the *M* allele
184 is able to restore a male phenotype in individuals carrying the *f* element [16,43,47]. Because of sex
185 ratio selection, the *M* allele is thought to have been selected to restore males in response to female-
186 biased sex ratios caused by the *f* element [47]. Thus, the *M* allele is expected to rise in frequency
187 when the *f* element is frequent in a population [47], which is consistent with our observations.
188 Unfortunately, no molecular marker of the *M* allele is currently available, which prevents any direct
189 assessment of its actual presence in these populations. Thus, we cannot exclude that males carrying
190 the *f* element simply carry non-feminizing variants of this SRD.

191 Our results show that *Wolbachia* and the *f* element never co-occur at high frequency in any
192 population. This apparent mutual exclusion can be explained considering that co-occurrence of
193 multiple feminizing factors in a population should favor the most transmitted one [16,48]. Hence,
194 *Wolbachia* is expected to lead to the loss of nuclear feminizing elements in *A. vulgare* populations.
195 This situation does not result from an interference between chromosomes and *Wolbachia* within
196 individuals, but from counter selection of nuclear feminizing alleles in a population that becomes
197 increasingly biased towards females. Hence, the rise of *Wolbachia* would associate with the decline

198 of the *f* element in a population. Why, under these circumstances, *Wolbachia* has not invaded all *A.*
199 *vulgare* populations is still unclear and may reflect fitness effects or possible resistance genes.

200 As a result, only very few individuals were found to carry both *Wolbachia* and the *f* element. They
201 represent only 3 females, all from the Chizé population (Figure 1a). These were likely born from
202 mothers carrying *Wolbachia* and fathers carrying the *f* element, which are frequent at Chizé. The
203 apparent absence of carriers of both SRD in other populations where these SRD are present could
204 simply be explained by the paucity of males carrying the *f* element.

205 Mapping SRD distribution onto mitochondrial genealogy showed excellent congruence between
206 *Wolbachia* strains and mitochondrial haplotypes (*w*VulC-V, *w*VulM-II and *w*VulP-VII). Such strong
207 association has previously been noted in *A. vulgare*-*Wolbachia* interactions at a smaller geographic
208 scale [30,31] and, more generally, in many arthropod-*Wolbachia* interactions [49]. This result
209 corroborates the rarity of non-maternal transmission of *Wolbachia* in *A. vulgare*. By contrast, the *f*
210 element was found in 6 different mitochondrial backgrounds (I-VI) scattered across the
211 mitochondrial phylogeny, indicating no particular association between the *f* element and
212 mitochondria. This result confirms and extends earlier data focused on western France and in which *f*
213 element presence in females was indirectly inferred based on sex ratios of their progenies [30]. This
214 observation can be explained by the occasional paternal transmission of the *f* element, which breaks
215 its association with mitochondrial background [16,22,30].

216

Data accessibility

All data are provided in the electronic supplementary material.

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234 **Figure legends**

235

236 **Figure 1.** (**A**) Prevalence of *Wolbachia* and the *f* element in males (m) and females (f) from 16
237 *Armadillidium vulgare* populations. (**B**) Relative proportions of *Wolbachia* and the *f* element in 16 *A.*
238 *vulgare* populations (represented by open circles).

239

240 **Figure 2.** Haplotype network of 23 mitochondrial variants (I-XXIII) from 16 *Armadillidium vulgare*
241 populations. Each circle represents one haplotype and circle diameter is proportional to the number
242 of individuals carrying the haplotype. Branch lengths connecting circles are proportional to
243 divergence between haplotypes. Sex ratio distorter frequencies are color-coded for each haplotype.

244

245 **Table 1. Prevalence of *Wolbachia* and *f* element sex ratio distorters in 16 populations of *Armadillidium vulgare*.**

246

Population (Country)	Sampling year	Sample size	Sex	Number of individuals	No *f* element, no *Wolbachia*	Only *f* element	Only *Wolbachia*				Both *w*VulM and *f* element	Undetermined status
							*w*VulC	*w*VulM	*w*VulP	Undetermined		
Lastovo	2017	54	Males	30	30							
(Croatia)			Females	24	24							
Prague	2018	36	Males	9	9							
(Czech Republic)			Females	27	1	26						
Beauvoir	2017	31	Males	6	5	1						
(France)			Females	25	9	14		1				1
Chizé	2017	52	Males	8	2	6						
(France)			Females	44	3	36		2			3	
Coulombiers	2017	24	Males	4	2	2						
(France)			Females	20	6	13	1					
Floirac	2016	114	Males	38	34		2					2
(France)			Females	76	21	6	40	9				
Gript	2017	45	Males	15	15							
(France)			Females	30	26	2	2					
La Crèche	2017	58	Males	21	19	2						
(France)			Females	37	23	13	1					
Poitiers	2015	23	Males	4	4							
(France)			Females	19	10	1			4	4		
Saint Julien l'Ars	2016	31	Males	14	9		1	3		1		
(France)			Females	17	1		12	3				1
Göttingen	2017	24	Males	7	3							4
(Germany)			Females	17	11	3		2				1
Pisa	2017	28	Males	15	15							
(Italy)			Females	13	10		3					
Hyogo	2018	50	Males	21	18							3

(Japan)			Females	29	26							3
Tottori	2018	49	Males	21	21							
(Japan)			Females	28	26	2						
Bucharest	2017	17	Males	9	9							
(Romania)			Females	8	8							
Wageningen	2018	11	Males	2	2							
(The Netherlands)			Females	9	7	1						1
Total males				224	197	11	3	3		1		9
Total females				423	212	117	59	17	4	4	3	7
Total				647	409	128	62	20	4	5	3	16

249 **Table 2. Distribution of mitochondrial haplotypes in 642 *Armadillidium vulgare* individuals from 16 populations.**

250

Sex ratio distorter status	Number of individuals	Haplotype number	Haplotype list
No *f* element, no *Wolbachia*	404	21	I, II, III, V, VI, VII, VIII, IX, X, XII, XIII, XIV, XV, XVI, XVII, XVIII, XIX, XX, XXI, XXII, XXIII
f element only	128	6	I, II, III, IV, V, VI
Wolbachia (*w*VulC strain) only	62	3	V, XI, XII
Wolbachia (*w*VulM strain) only	20	1	II
Wolbachia (*w*VulP strain) only	4	1	VII
Wolbachia (undetermined strain) only	5	2	II, VII
Both *w*VulM and *f* element	3	1	II
Undetermined status	16	4	I, V, VI, XIX

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254

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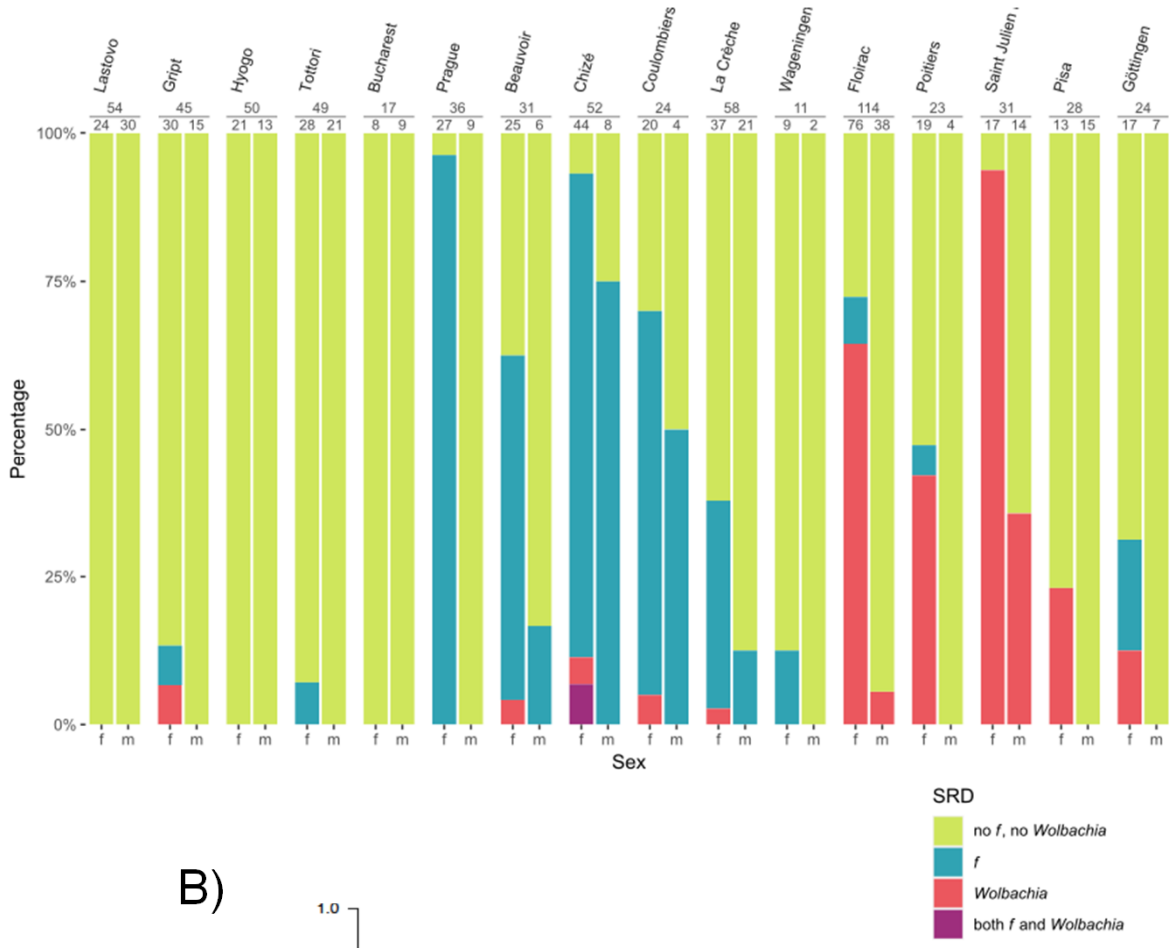
389

Figure 1

A)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28 13 15
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100%
75%
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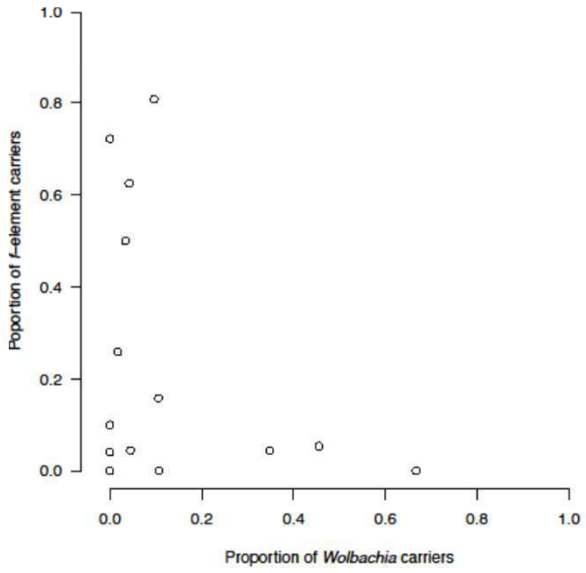
0%
Percentage
f m
Sex
SRD
no f, no Wolbachia
f
Wolbachia
both f and Wolbachia

B)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Poportion of f–element carriers
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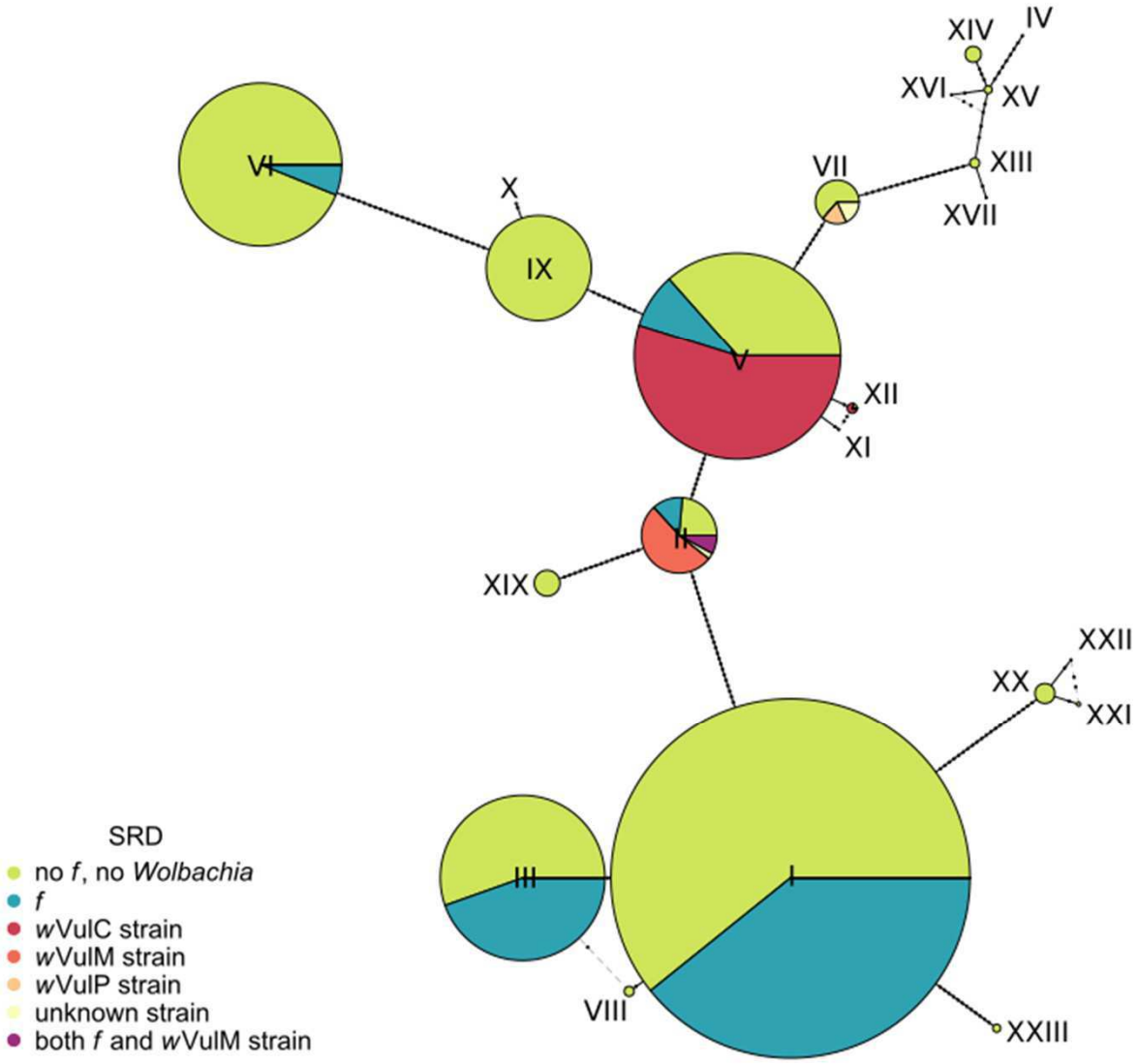
Proportion of Wolbachia carriers

Figure 2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VIII
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SRD
no f, no Wolbachia
f
wVulC strain
wVulM strain
wVulP strain
unknown strain
both f and wVulM strain