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1	Heterogeneous distribution of sex ratio distorters in natural populations of
2	the isopod Armadillidium vulgare
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5 6	Sylvine Durand ¹ , Baptiste Lheraud ¹ , Isabelle Giraud ¹ , Nicolas Bech ¹ , Frédéric Grandjean ¹ , Thierry Rigaud ² , Jean Peccoud ¹ and Richard Cordaux ¹
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9	¹ Laboratoire Ecologie et Biologie des Interactions, Equipe Ecologie Evolution Symbiose, Université de
10	Poitiers, UMR CNRS 7267, Bât. B31, 3 rue Jacques Fort, TSA 51106, 86073 Poitiers Cedex 9, France
11	² Laboratoire Biogéosciences, Université Bourgogne Franche-Comté, UMR CNRS 6282, 6 boulevard
12	Gabriel, 21000 Dijon, France
13	
14	
15	* Corresponding author: richard.cordaux@univ-poitiers.fr

17 Abstract

18

- 19 In the isopod Armadillidium vulgare, many females produce progenies with female-biased sex ratios, 20 due to two feminizing sex ratio distorters (SRD): Wolbachia endosymbionts and the f element. We 21 investigated the distribution and population dynamics of these SRD and mitochondrial DNA variation 22 in 16 populations from Europe and Japan. Confirming and extending results from the 1990's, we 23 found that the SRD are present at variable frequencies in populations, and that the f element is 24 overall more frequent than Wolbachia. The two SRD never co-occur at high frequency in any 25 population, suggesting an apparent mutual exclusion. We also detected Wolbachia or the f element 26 in some males, which likely reflects insufficient titer to induce feminization or presence of 27 masculinizing alleles. Our results are consistent with a single integration event of a Wolbachia 28 genome in the A. vulgare genome at the origin of the f element, which contradicts an earlier 29 hypothesis of frequent losses and gains. We identified strong linkage between Wolbachia strains and 30 mitochondrial haplotypes, but no association between the *f* element and mitochondrial background. 31 Our results open new perspectives on SRD evolutionary dynamics in A. vulgare, the evolution of 32 genetic conflicts and their impact on the variability of sex determination systems.
- 33

34 Keywords

- 35
- 36 Sex ratio distorter, endosymbiont, *Wolbachia*, *f* element, sex determination

37 1. Introduction

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39 Sex ratio distorters (SRD) are selfish genetic elements located on sex chromosomes or transmitted by 40 a single sex, which skew the proportion of males and females in progenies towards the sex that 41 enhances their own vertical transmission [1]. Major SRD types include sex chromosome meiotic 42 drivers [2,3], some B chromosomes [4] and selfish cytoplasmic genetic entities [5,6,7]. Collectively, 43 they are found in a wide range of animal and plant species and they have had a tremendous impact 44 on the ecology and evolution of their host species [8,9]. One of the most emblematic SRD is the 45 bacterial endosymbiont Wolbachia [10,11]. Wolbachia is a cytoplasmic, maternally inherited alpha-46 proteobacterium found in a wide range of arthropods and nematodes. In arthropods, Wolbachia 47 often manipulates host reproduction in favor of infected females, thereby conferring itself a 48 transmission advantage. This is achieved through various strategies, three of which causing sex ratio 49 distortions towards females: male killing, thelytokous parthenogenesis and feminization of genetic 50 males [6,7,10,11]. 51 In the terrestrial isopod Armadillidium vulgare, chromosomal sex determination follows female

52 heterogamety (ZZ males and ZW females) [12–14]. However, many females produce progenies with 53 female-biased sex ratios, due to the presence of two feminizing SRD: Wolbachia endosymbionts and 54 a locus called the f element [6,15,16]. Wolbachia symbionts cause ZZ genetic males to develop as 55 phenotypic females [17]. Three Wolbachia strains have been described in A. vulgare, for which 56 feminization induction has been demonstrated (wVulC and wVulM strains [18,19]) or is strongly 57 suspected (wVuIP strain [20]). The f element is a nuclear insert of a large portion of a feminizing 58 Wolbachia genome in the A. vulgare genome [21]. The f element induces female development, as a 59 W chromosome does, and it shows non-Mendelian inheritance, making it an SRD [21,22]. These SRD 60 may cause turnovers in sex determination mechanisms [6,15,23] and they could explain why sex 61 chromosome systems are so variable in terrestrial isopods [24–27].

62 Testing this hypothesis requires characterizing the evolutionary dynamics of SRD such as Wolbachia 63 and the f element in natural populations. In A. vulgare, this characterization is quite limited because 64 prior studies were mostly restricted to a narrow geographic area (western France), sometimes 65 focusing solely on Wolbachia [20,28–31]. The only exception is a 1993 study [32], which collated and 66 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 67 68 than Wolbachia. However, earlier studies were limited by the lack of molecular tests for Wolbachia 69 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.

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80 2. Materials and Methods

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82 647 A. vulgare individuals from 16 natural populations across Europe and Japan were collected by 83 hand. Individuals were sexed and stored in alcohol or at -20°C prior to DNA extraction. Total genomic 84 DNA was extracted from the head and legs of each individual, as described previously [21]. We used 85 four molecular markers to assess the presence of Wolbachia and the f element in DNA extracts: Jtel [21], wsp [35], recR [36] and ftsZ [37] (Table S1). While Jtel is specific to the f element, wsp and recR 86 87 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 88 89 amplification patterns were expected for individuals with Wolbachia only (Jtel-, wsp+, recR+, ftsZ+), 90 the f element only (Jtel+, wsp-, recR-, ftsZ+), both Wolbachia and the f element present (Jtel+, wsp+, 91 recR+, ftsZ+) or both Wolbachia and the f element lacking (Jtel-, wsp-, recR-, ftsZ-). The few 92 individuals exhibiting other amplification patterns were classified as "undetermined status". A 93 quantitative-PCR assay was used to measure Wolbachia titer in some individuals (see supplementary 94 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 95 96 reverse reads were assembled using Geneious[®] v.7.1.9 to obtain one consensus sequence per 97 individual. To evaluate mitochondrial diversity, we amplified by PCR a ~700 bp-long portion of the 98 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 99 100 [39]. All statistical analyses were performed with R v.3.6.0 [40]. Figures were realized with ggplot2 101 [41].

103 **3. Results**

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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: wVulC (n=62), wVulM (n=23) or wVulP (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).

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

The 642 individuals sequenced at the *COI* gene presented a total of 92 segregating sites defining 23
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 wVulC were associated with either 139 haplotype V or its close relatives (XI and XII). All individuals carrying wVulM were associated with 140 haplotype II and those carrying wVuIP 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**

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Our results provide direct evidence that the *f* element is overall more frequent than *Wolbachia* in the 147 148 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* chromosome [21], f element presence in various populations can be explained by a single event of 156 157 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 frequencies in populations, they should be integrated in different genomic locations and they should 163 164 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

does not appear to be the most parsimonious hypothesis. Examination of sex ratios from progeniesof 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 wVulC or wVulM 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 carrying the f element also carry the masculinizing dominant M allele [16,43,47]. Indeed, the M allele 183 184 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.

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

- of the *f* element in a population. Why, under these circumstances, *Wolbachia* has not invaded all *A*.
 vulgare populations is still unclear and may reflect fitness effects or possible resistance genes.
- As a result, only very few individuals were found to carry both *Wolbachia* and the *f* element. They represent only 3 females, all from the Chizé population (Figure 1a). These were likely born from mothers carrying *Wolbachia* and fathers carrying the *f* element, which are frequent at Chizé. The apparent absence of carriers of both SRD in other populations where these SRD are present could 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 (wVulC-V, wVulM-II and wVulP-VII). Such strong
- 207 association has previously been noted in *A. vulgare-Wolbachia* interactions at a smaller geographic
- 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
- element presence in females was indirectly inferred based on sex ratios of their progenies [30]. This
- observation can be explained by the occasional paternal transmission of the *f* element, which breaks
- its association with mitochondrial background [16,22,30].

217	Data accessibility
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219	All data are provided in the electronic supplementary material.
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234 Figure legends

235

- 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
- 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.

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

Population	Sampling	Sample	C	Number of	No f element,	Only f	Only Wa	olbachia			Both <i>w</i> VulM	Undetermined
(Country)	year	size	Sex	individuals	no Wolbachia	element	<i>w</i> VulC	<i>w</i> VulM	<i>w</i> VulP	Undetermined	and f element	status
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

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

Sex ratio distorter status	Number of individuals	Haplotype number	Haplotype list
No <i>f</i> element, no <i>Wolbachia</i>	404	21	I, II, III, V, VI, VII, VIII, IX, X, XII, XII
<i>f</i> element only	128	6	I, II, III, IV, V, VI
<i>Wolbachia</i> (wVulC strain) only	62	3	V, XI, XII
Wolbachia (wVulM strain) only	20	1	II
Wolbachia (wVulP strain) only	4	1	VII
Wolbachia (undetermined strain) only	5	2	II, VII
Both <i>w</i> VulM and <i>f</i> element	3	1	II
Undetermined status	16	4	I, V, VI, XIX

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Figure 1



