Evidence for a new feminizing *Wolbachia* strain in the isopod *Armadillidium vulgare*: evolutionary implications

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Wolbachia are intracellular maternally inherited alpha-Proteobacteria infecting a wide range of arthropods. In the common pill bug *Armadillidium vulgare*, the known *Wolbachia* strain is responsible for feminization of genetic males. We have investigated *Wolbachia* diversity in 20 populations of *A. vulgare* from west and east Europe, north Africa and north America. A new *Wolbachia* strain (wVulM) was identified through the variability of the *wsp* gene, distantly related to that previously known (wVulC) in this host species. No individual with multiple infections was detected. Inoculation experiments indicated that the new wVulM bacterial strain also induces feminization in

A. vulgare. However, the wVulC strain showed a higher transmission rate than the wVulM strain and was the most geographically widespread *Wolbachia* in *A. vulgare* populations. Mitochondrial 16SrDNA gene sequencing was conducted in *Wolbachia*-infected individuals, revealing the occurrence of four host lineages. The comparison of bacterial strains and their respective host mitochondrial phylogenies failed to show concordance, indicating horizontal transmission of the *Wolbachia* strains within populations of *A. vulgare*.

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Introduction

Wolbachia are maternally inherited endosymbiotic alpha-Proteobacteria (O'Neill *et al*, 1992) responsible for many effects on their hosts reproduction such as cytoplasmic incompatibility, thelytokous parthenogenesis, feminization of genetic males and male killing (reviewed in Stouthamer *et al*, 1999). Their abilities to interact with host biology have allowed *Wolbachia* to invade a wide spectrum of nematodes and arthropods (reviewed in Stouthamer *et al*, 1999). In isopod crustaceans, *Wolbachia* are responsible for cytoplasmic incompatibility in two species (Legrand and Juchault, 1986; Moret *et al*, 2001) and feminization in many species (Bouchon *et al*, 1998), including *Armadillidium vulgare* (Rigaud *et al*, 1991). In case of feminization, genetic males are converted into functional females, which typically produce, in turn, female-biased progenies (Rigaud *et al*, 1997).

PCR-based methods have allowed extensive phylogenetic studies of *Wolbachia* among host species during the last decade (eg O'Neill *et al*, 1992; Rousset *et al*, 1992a;

Werren *et al*, 1995b; Bouchon *et al*, 1998; Zhou *et al*, 1998; Cordaux *et al*, 2001). On the other hand, less information is available about *Wolbachia* at a within-host species scale. No *Wolbachia* variability was detected in symbionts of 11 populations of *Tribolium confusum* (Fialho and Stevens, 1997) or in symbionts of *Trichogramma kaykai* from California (Schiltuizen *et al*, 1998) using *ftsZ* gene sequences, suggesting single *Wolbachia* infection in these species. However, the presence of several *Wolbachia* strains has been reported in *Drosophila simulans* using bacterial 16SrDNA gene sequences (Rousset *et al*, 1992b). But, using the same marker, Bouchon *et al* (1998) could not detect any *Wolbachia* variability at the within-species level in isopods. The cloning of the *wsp* gene (Braig *et al*, 1998) opened a new era in the phylogenetic study of *Wolbachia* symbionts. This major outer surface protein-encoding gene has been shown to be about 10 times more variable than the bacterial 16SrDNA gene (Zhou *et al*, 1998), providing a better phylogenetic assay to investigate recent horizontal transfers. Using this marker, two male-killing *Wolbachia* strains were identified in the same host: the ladybird *Adalia bipunctata* (Hurst *et al*, 1999) and the butterfly *Acraea encedon* (Jiggins *et al*, 2001).

The evolutionary dynamics of the host-symbiont associations can be analysed by comparing the host mitochondrial DNA (mtDNA) with the symbiont genealogy. mtDNA is in linkage disequilibrium with the intracellular bacteria, because both are maternally inherited. The association between mitochondria and intracellular symbionts that manipulate host reproduction has already been documented in insects. In

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D. simulans, in which *Wolbachia* cause cytoplasmic incompatibility, mtDNA variants were found to hitchhike with *Wolbachia* strains, leading to congruence between mtDNA and *Wolbachia* phylogenies (Turelli et al, 1992; James and Ballard, 2000). A similar relationship was observed in the mosquito *Culex pipiens* (Guillemaud et al, 1997), the Asian rice gall midge *Orseolia oryzae* (Bethura et al, 2001) and fire ants of the genus *Solenopsis* (Shoemaker et al, 2001). In the two-spot ladybird beetle, in which *Wolbachia* induce male-killing, Schulenburg et al (2002) showed that mitochondrial haplotypes are correlated with the infection status, confirming linkage disequilibrium between mitochondria and bacteria.

In Crustacea, the association between feminizing *Wolbachia* and mtDNA has been investigated in two isopod species. Michel-Salzat et al (2001) detected three different *Wolbachia* strains within the species complex of the isopod *Porcellionides pruinosus*, but no congruence between *Wolbachia* gene (*wsp* gene) and mtDNA (mitochondrial 16SrDNA gene) gene genealogies. In *A. vulgare*, Rigaud et al (1999) investigated mtDNA variation through RFLP according to the presence of two sex ratio distorters, a *Wolbachia* strain and a non-mendelian feminizing factor (*f* element). The symbiont was associated with two closely related mitochondrial types, whereas no link was found between the *f* element and any mitochondrial lineage. However, the history of infection in this host species needs further investigations, since Rigaud et al (1999) sampled populations of *A. vulgare* on a small geographical scale (limited to France) and identified only one *Wolbachia* strain. The aims of the present study were: (i) to evaluate the diversity of *Wolbachia* strains infecting populations of the isopod *A. vulgare* on a large geographical scale, and (ii) to compare

it to host cytoplasmic genealogy, in order to reconstruct the evolution of *Wolbachia*/*A. vulgare* relationships. The *wsp* gene was used to detect *Wolbachia* strains and the mitochondrial 16SrDNA gene was chosen as host genetic marker. The latter appears to be well suited for the present investigation because it has previously been shown to be variable enough for population genetics analyses (Michel-Salzat and Bouchon, 2000; Michel-Salzat et al, 2001).

Materials and methods

Sampling

In all, 20 populations of the isopod *A. vulgare* were sampled (Table 1): 15 from Europe (France, Germany, Greece, Hungary, Spain and The Netherlands), one from north Africa (Tunisia) and four from north America (USA). Two different populations were sampled in Pittsford (USA) and Barcelona (Spain). The infection status of each population was assessed by testing several individuals for the presence of *Wolbachia*, using a PCR assay (see below).

Molecular data and phylogenetic analyses

Infected individuals randomly chosen from all *Wolbachia*-infected populations were sequenced for both the *Wolbachia* *wsp* gene and the mitochondrial 16SrDNA gene. In addition, uninfected individuals randomly chosen from uninfected populations were analysed for their mitochondrial sequences in order to compare infected vs uninfected lineages. DNA extraction, PCR amplification and sequencing of the *wsp* region of the *Wolbachia* DNA and of the 3' end of the 16SrDNA region of the host mtDNA were performed as described in Michel-Salzat et al (2001). *Wsp*

Table 1 Origin and characteristics of 20 populations of *A. vulgare*

Populations	Geographic coordinates	Wolbachia infection			Mitochondrial lineages	
		n	Strain	AC	Group	AC
<i>America</i>						
Pittsford-1 (U)	43°09'N, 77°36'W	5 (3)	wVulC	AJ419987	AvI	AJ419995
Pittsford-2 (U)	43°09'N, 77°36'W	5 (1)	wVulC	AJ419987	AvI	AJ419994
Rochester (U)	43°09'N, 77°36'W	3 (1)	wVulC	AJ419987	AvI	AJ419994
Slater-Creek (U)	43°15'N, 77°36'W	5 (3)	wVulC	AJ419987	AvIV	AJ419988
<i>Europe</i>						
Grebbeberg (N)	51°58'N, 5°40'E	6 (5)	wVulC	AJ419987	AvIV	AJ419989
Seewiesen (Ge)	48°08'N, 11°35'E	2 (0)	—	—	AvIV	AJ419989
Budapest (H)	47°30'N, 19°03'E	9 (7)	wVulM	AJ419984	AvIII	AJ419992
Mery-sur-Cher (F)	47°13'N, 1°58'E	30 (30)	wVulM	AJ419984	AvI	AJ419993
Saint-Cyr (F)	46°34'N, 0°21'E	6 (3)	wVulC	AJ419986	AvI	AJ419997
Celles-sur-Belle (F)	46°15'N, 0°12'W	1 (1)	wVulC	AJ419987	AvI	AJ419994
Angoulême (F)	45°40'N, 0°10'E	4 (4)	wVulC	AJ419987	AvI	AJ419994
Amou (F)	43°35'N, 0°45'W	4 (1)	wVulC	AJ419987	AvI	AJ419994
Rivesaltes (F)	42°46'N, 2°52'E	5 (2)	wVulC	AJ419987	AvII	AJ419990
Corte (F)	42°18'N, 9°08'E	5 (0)	—	—	AvI	AJ419994
Ribadeo (S)	43°32'N, 7°04'W	3 (3)	wVulC	AJ419987	AvIII	AJ419991
Barcelona-1 (S)	41°25'N, 2°10'E	5 (0)	—	—	AvI	AJ419996
Barcelona-2 (S)	41°25'N, 2°10'E	21 (10)	wVulM	AJ419985	AvIII	AJ419991
Sanlucar-de-B. (S)	36°46'N, 6°21'W	7 (0)	—	—	AvI	AJ419994
Athens (Gr)	37°58'N, 23°43'E	10 (3)	wVulM	AJ419984	AvI	AJ419993
<i>Africa</i>						
Tunis (T)	36°50'N, 10°13'E	3 (1)	wVulC	AJ419987	AvII	AJ419990

The presence of *Wolbachia* in females (number tested *n* and number infected in brackets), *Wolbachia* strain (if any), host mitochondrial lineage and accession numbers (AC) in EMBL, GenBank and DDBJ databases for *Wolbachia* and host mitochondrial genes. Origin of samples: USA (U), the Netherlands (N), Germany (Ge), Hungary (H), France (F), Spain (S), Greece (Gr) and Tunisia (T).

and mtDNA sequences were deposited in EMBL, Genbank and DDBJ databases under accession numbers AJ419984-AJ419987 and AJ419988-AJ419997, respectively.

Wsp sequences were manually aligned taking into account the coding structure of the gene with 11 previously determined *wsp* sequences from isopod *Wolbachia* (Cordaux *et al*, 2001), using the *Culex pipiens* symbionts (Zhou *et al*, 1998) as an outgroup. mtDNA sequences from *A. vulgare* were manually aligned with the sequence of *A. nasatum*, previously shown to be an adequate outgroup (Michel-Salzat and Bouchon, 2000). Regions including gaps and hypervariable portions that could not be aligned with confidence were removed from the analyses. Neighbour-joining analyses were conducted using MEGA 2.1 (Kumar *et al*, 2001) based on Kimura two-parameter distances. Bootstrap analyses were carried out with 1000 replicates. For *wsp* sequences, ratios of nonsynonymous to synonymous substitutions and tests for neutrality were computed using the Nei-Gojobori method implemented in MEGA 2.1 (Kumar *et al*, 2001). Standard errors (SE) were estimated via a bootstrap method (1000 replicates). Deviations from the expected standard variation under the neutral hypothesis were tested by a Z-test. Pairwise uncorrected distances between infected populations were computed for *Wolbachia wsp* sequences and host mtDNA sequences. These distances were then used to perform a Mantel test as implemented in GENETIX 4.01 (Belkhir, 2000), in order to test the correlation between host and *Wolbachia* genetic distances (10 000 permutations).

Effects of *Wolbachia* on host phenotypes

To investigate the effects of *Wolbachia* from the infected population of Mery-sur-Cher, bacteria were inoculated into 36 undifferentiated juveniles (20 days of post-embryonic development after releasing from the marsupium; Suzuki, 1999) from an asymbiotic lab strain of *A. vulgare*. Experiments were conducted as described in Bouchon *et al* (1998). Recipient specimens were reared and their sexual phenotype was checked every 2 weeks until their secondary sexual characters could be identified (120 days on average; Suzuki, 1999). Sexual phenotypes were defined as in Bouchon *et al* (1998). At the end of the experiment, the presence of *Wolbachia* in the surviving individuals was tested using a PCR assay.

In addition, infected females originating from four populations (Grebbeberg, Angoulême, Mery-sur-Cher and Barcelona-2) were reared in the lab at 20°C under a natural photoperiod. These females were tested positive for *Wolbachia* infection after they laid their clutches. The proportion of males and females of their progenies were recorded. Biases in the masculinity rate were tested according to the rate of 0.5, expected from the genetic sex determination known in *A. vulgare* (Juchault and Legrand, 1972). The statistical significance of differences in sex ratios was assessed using Likelihood-ratio (L-R) χ^2 tests as implemented in JMP version 3.2.2 (SAS Institute Inc., Cary, NC, USA).

Results

Wolbachia diversity

The *wsp* sequences were obtained from the *Wolbachia* of infected individuals from each of the 16 populations

found to harbour symbionts (Table 1). Each infected individual only harboured a single *Wolbachia* strain, as shown by the direct sequencing of the purified PCR products, which revealed no ambiguity or discrepancy within and between the analysed strands of each *wsp* sequence. To test for recombination events that may occur in the *wsp* gene (Werren and Bartos, 2001), dot-plot analysis of the *wsp* sequences was performed. No recombination signal was detected. In total, four different sequences were identified, which can be grouped into two *Wolbachia* strains.

The first strain was found in 12 populations from west Europe, north America and north Africa (Table 1), which corresponds to 75% of *Wolbachia*-infected populations investigated. Within this strain, *Wolbachia* from 11 populations (ie Celles-sur-Belle, Amou, Angoulême, Grebbeberg, Pittsford-1, Pittsford-2, Ribadeo, Rivesaltes, Rochester, Slater-Creek, Tunis) exhibited identical *wsp* sequences. This strain was named *wVulC* (for *Wolbachia A. vulgare* type Celles-sur-Belle) according to the nomenclature of Zhou *et al* (1998). The *wsp* sequence from Saint-Cyr differed from the *wsp* sequence from Celles-sur-Belle by only one substitution (based on the comparison of 493 positions of the *wsp* gene), and was therefore assigned to *wVulC*.

The second *Wolbachia* strain was found in four populations from Europe (Table 1), which corresponds to only 25% of *Wolbachia*-infected populations investigated. Within this strain, *Wolbachia* from three populations (ie Mery-sur-Cher, Athens, Budapest) exhibited identical *wsp* sequences. This strain was named *wVulM* (for *Wolbachia A. vulgare* type Mery-sur-Cher). The *wsp* sequence from Barcelona-2 differed from the *wsp* sequence from Mery-sur-Cher by only one substitution at a silent site and was therefore assigned to *wVulM*.

The pairwise distance between the two *Wolbachia* strains in *A. vulgare* was 5.3%. The estimated number of nonsynonymous substitutions (d_N = mean number of differences $\pm$ SE) between the two sequences was 9.5 ± 2.9 , whereas the estimated number of synonymous substitutions (d_S = mean number of differences $\pm$ SE) was 12.5 ± 3.1 . The phylogenetic relationships of *wVulC* and *wVulM* with other isopod *Wolbachia* strains were investigated (Figure 1). This analysis revealed that *wVulC* and *wVulM* belong to two different subclades (*Vul* and *Ase*, respectively) within the isopod-specific *Wolbachia* cluster of the B-supergroup of *Wolbachia* (Cordaux *et al*, 2001). These two subclades are separated by a high bootstrap score of 95% (Figure 1). Overall pairwise comparisons of the *wsp* sequences showed a significant deviation from the neutrality hypothesis ($Z = 3.69$, $P < 0.01$). The same analysis of the sequences clustering in the *Vul* subclade provided indication of evolution under selection ($Z = 2.19$, $P = 0.03$), whereas there was a neutral evolution in the *Ase* subclade ($Z = 0.67$, $P = 0.51$). Within the four, *Armadillidium Wolbachia* tests indicated a strong selection ($Z = 2.80$, $P < 0.01$) as well as between the *wVulM* and *wVulC* sequences ($Z = 2.82$, $P < 0.01$). Therefore, *wVulC* and *wVulM* are distantly related within the *Wolbachia* clade-infecting isopods.

A logistic regression testing the effects of both the geographical origin of populations and the symbiont strains on the prevalence of *Wolbachia* in females (Table 1) revealed only a significant effect of their interaction

Figure 1 Phylogenetic tree of isopod *Wolbachia* strains. The tree is based on the comparison of 493 bases of the *wsp* gene, using the neighbor-joining method and rooted with *C. pipiens* symbionts (AF020061). Numbers at nodes are bootstrap scores above 50%. Strains are identified by the host from which they were isolated. The two *Wolbachia* strains from *A. vulgare* (*wVulC* and *wVulM*) are shown in bold. Accession numbers of *wsp* sequences from other isopod symbionts are AJ276599–AJ276601, AJ276605–AJ276612.

(whole model: $\chi^2 = 53.5$, d.f. = 15, $P < 0.001$; effect of the *Wolbachia* strain: L-R $\chi^2 = 0$, d.f. = 1, $P = 1$; effect of the geographical origin: L-R $\chi^2 = 5.9$, d.f. = 11, $P = 0.88$; interaction: L-R $\chi^2 = 17.4$, d.f. = 3, $P < 0.001$). The proportion of infected females was similar (56% in average), whatever the geographical origin when infected by the *wVulC* *Wolbachia* strain (L-R $\chi^2 = 17.5$, d.f. = 11, $P = 0.09$), whereas there were differences in the infection pattern in females infected by the *wVulM* *Wolbachia* strain (L-R $\chi^2 = 32.9$, d.f. = 3, $P < 0.001$). However, the sample size for *wVulC* is relatively modest compared to *wVulM*, making it hard to resolve the issue of this heterogeneity in this infection.

Wolbachia-induced phenotype on hosts

The feminizing effect of the *wVulC* *Wolbachia* strain in *A. vulgare* has already been demonstrated (Rigaud et al, 1991). To investigate whether *wVulM* also induces feminization in *A. vulgare* or not, it was artificially inoculated into 36 asymbiotic undifferentiated juveniles as described in Bouchon et al (1998). Secondary sexual characters could be seen after 128 days of rearing: three males, 14 females and five intersexes were identified among the 22 surviving individuals. Time for secondary sexual differentiation and survival rate were similar to those recorded by Suzuki (1999). The intersex phenotype was defined as sterile males with genital female apertures and hypertrophied androgenic glands (Bouchon et al, 1998). The results were identical after 230 days and the PCR assay showed that all intersexes carried *Wolbachia*, whereas no male did. The intersex phenotype is therefore interpreted as resulting from a partial feminization of genetic males. The masculinity rate was 0.14 in the surviving individuals, which is significantly lower than the expected masculinity rate of 0.5 without inoculation ($\chi^2 = 11.6$, d.f. = 1, $P < 0.001$). Altogether,

these results demonstrate the feminizing effect of *wVulM* in *A. vulgare*. PCR assay in females showed that 10 of them harboured *Wolbachia*, whereas four lacked symbionts. According to the demonstration of the feminizing effect of *wVulM* in males, females lacking bacteria are genetic females (ie heterogametic females WZ) whereas females harbouring *Wolbachia* could be either feminized genetic males (ie homogametic females ZZ) or genetic females (Juchault and Legrand, 1972).

We also investigated possible differences in the feminization effects induced by *wVulM* and *wVulC* in *A. vulgare*. Females harbouring either *wVulC* or *wVulM* produced the same proportion of lines with biased sex ratios ($\chi^2 = 0.06$, d.f. = 1, $P = 0.81$). As expected, female-biased progenies were the most common (66.7% for females harbouring *wVulC* and 67.5% for those harbouring *wVulM*, Table 2). However, a small proportion of females produced male-biased progenies (11.1% for females harbouring *wVulC* and 15.0% for those harbouring *wVulM*). Male-biased progenies are most likely due to a lack of transmission of feminizing *Wolbachia* in homogametic females (ZZ), perhaps following the selection of host genes limiting *Wolbachia* transmission (Rigaud and Juchault, 1992). Lineages with tables 1:1 sex ratio can be explained by the presence of: (i) heterogametic females (WZ) lacking *Wolbachia* (Juchault and Legrand, 1972) and/or (ii) homogametic females (ZZ) with a *Wolbachia* transmission rate of 50%. Given that heterogametic females (WZ) rapidly disappear from *Wolbachia*-infected progenies (Taylor, 1990), explanation (ii) is most likely. Thus, the presence of males in the progenies of infected females suggests that both *wVulC* and *wVulM* are not successfully transmitted to all offspring.

In addition, we examined the efficiency of feminization of *wVulC* and *wVulM* within progenies. This efficiency was defined by two components: the transmission

Table 2 Progenies of *Wolbachia*-infected females in four European populations of *Armadillidium vulgare*

Population	Wolbachia strain	Male-biased progenies				Unbiased progenies				Female-biased progenies			
		n	Males	Females	MR	n	Males	Females	MR	n	Males	Females	MR
Grebbeberg	<i>wVulC</i>	1	33	6	0.85	1	17	16	0.52	3	10	192	0.05
Angoulême	<i>wVulC</i>	—	—	—	—	1	66	85	0.44	3	4	286	0.01
Overall	<i>wVulC</i>	1	33	6	0.85	2	83	101	0.45	6	14	478	0.03
Mery-sur-Cher	<i>wVulM</i>	6	204	55	0.79	6	80	92	0.47	18	88	781	0.10
Barcelona-2	<i>wVulM</i>	—	—	—	—	1	24	31	0.44	9	95	382	0.20
Overall	<i>wVulM</i>	6	204	55	0.79	7	104	123	0.46	27	183	1163	0.14

Number of progenies (*n*) and number of males and females in the progenies; MR = mean masculinity rate.

rate and the penetrance. The transmission rate corresponds to the proportion of individuals in a progeny which inherit *Wolbachia* from an infected mother. The penetrance refers to the levels of feminization expressed by the *Wolbachia* present in infected individuals. For example, all descendants of an infected mother might inherit *Wolbachia* (high transmission rate), but these might not express feminization in the descendants (low penetrance) because of a too small number of bacteria transmitted by the mother. However, all *Wolbachia*-infected individuals of *A. vulgare* analysed so far showed female (or at least intersexed) phenotype whatever the infecting strain (Juchault *et al*, 1980; Bouchon *et al*, 1998; this study), suggesting consistently high penetrance of both *wVulC* and *wVulM*. The comparison of the transmission rates of *wVulC* and *wVulM* was performed by measuring the proportion of males within the progenies of infected females, since individuals carrying *Wolbachia* are females whereas those lacking *Wolbachia* are males. Comparisons of progenies harbouring either *wVulC* or *wVulM* (Table 2) showed that the mean masculinity rate in *wVulC*-infected progenies (0.18) was significantly lower ($\chi^2 = 20.7$, d.f. = 1, $P < 0.001$) than the mean masculinity rate in *wVulM*-infected progenies (0.27). This indicated that *wVulC* has a higher vertical transmission rate than *wVulM*, or *wVulC* exhibits a more efficient feminizing effect than *wVulM*. Moreover, significant differences in the primary masculinity rates were recorded according to both *Wolbachia* strain and host populations (Table 2; whole model test: $\chi^2 = 33.3$, d.f. = 3, $P < 0.001$; effect of the *Wolbachia* strain: L-R $\chi^2 = 11.9$, d.f. = 1, $P < 0.001$; effect of the host population: L-R $\chi^2 = 9.9$, d.f. = 1, $P < 0.01$; interaction: L-R $\chi^2 = 0.08$, d.f. = 1, $P = 0.77$).

Host cytoplasmic types associated to *Wolbachia* in *A. vulgare*

The mitochondrial 16SrDNA sequences were obtained from the same individuals whose *Wolbachia wsp* gene was sequenced, as well as in additional individuals from uninfected populations for comparison. The aim was not to check the whole mtDNA diversity in these populations, but rather to track the history of *Wolbachia*-host co-evolution. Four mitochondrial lineages named AvI, AvII, AvIII and AvIV were identified (Figure 2). These monophyletic clusters were strongly supported by high bootstrap scores ranging from 97 to 100%. Lineage AvIV was the representative of the most basal lineage of the *A. vulgare* clade. Lineage AvI was the most common, while

Figure 2 Cytoplasmic genealogy of *A. vulgare*. The tree is based on the comparison of 399 bases of the mitochondrial 16SrDNA gene, using the neighbor-joining method and rooted with *A. nasatum* (AJ388098). Numbers at nodes are bootstrap scores above 50%. Populations harbouring the *Wolbachia* strain *wVulC* are identified by the symbol ○ and those harbouring *wVulM* are identified by the symbol ●. Other populations of *A. vulgare* are uninfected.

lineage AvII was the least common among the studied populations. The inferred tree also indicated a monophyletic origin of Athens and Mery-sur-Cher lineages, which could be considered as a subgroup of AvI lineage. Pairwise distances ranged from 0.0 to 1.0% within lineages, and from 3.0 to 6.3% between lineages (based on the comparison of 399 positions of the mitochondrial 16SrDNA gene). These distance ranges are similar to those observed for the same marker within other species of terrestrial isopods (Michel-Salzat and Bouchon, 2000; Michel-Salzat *et al*, 2001). The *wVulC* *Wolbachia* strain was found in association with the four host lineages. In contrast, the *wVulM* *Wolbachia* strain was only associated with AvIII and a subgroup of AvI. A Mantel test showed that the genetic distances of hosts and symbionts from the 16 infected populations were not correlated ($r^2 = 0.017$; $P = 0.39$). mtDNA sequences from uninfected individuals were found in the two most

distantly related lineages AvI and AvIV. Therefore, genetic differentiation of host mtDNA could not be related to the presence of different *Wolbachia* symbionts in *A. vulgare* assemblage, suggesting that the association of the two partners does not follow a strict co-evolutionary process.

Discussion

Based on the *wsp* sequences, two distantly related *Wolbachia* strains (*wVulC* and *wVulM*) were detected among *A. vulgare* symbionts sampled from three different continents. Similar to the first described *Wolbachia* strain (*wVulC*) in *A. vulgare* (Rigaud et al, 1991), the newly identified *wVulM* strain has a feminizing effect. The *wVulC* strain is widely spread, being associated to all host mitochondrial lineages (Figure 2). On the contrary, *wVulM* is restricted to two mitochondrial lineages (AvI and AvIII). Despite the large geographical distances between collection sites, mtDNA diversity was not found correlated with geographical origin, suggesting long-distance genetic exchanges between *A. vulgare* populations. No correlation between hosts and symbionts genetic distances was detected. Moreover, phylogenetic analysis showed that the divergence between the two *Wolbachia* strains does not result from strict co-evolution with their host, in which case we would have expected the two *Wolbachia* strains to be sister groups and/or at least some degrees of congruence between symbiont and host phylogenetic trees. These results suggest that *wVulC* and *wVulM* were acquired independently by *A. vulgare*, and strongly support the occurrence of horizontal transmission of *Wolbachia* within this host.

Inferring the evolutionary steps leading to such *Wolbachia* distribution in *A. vulgare* therefore requires gains and losses of symbionts. Gains of symbionts are the result of successful horizontal transfers to a new host. These successes crucially depend upon the propagation potential of the *Wolbachia* strain involved in the transfer. Looking to results of artificial transfers between isopod hosts (Rigaud and Juchault, 1995; Rigaud et al, 2001), *Wolbachia* seem to have a weak propagation potential, probably because strains are specialized to their natural hosts and are therefore maladapted to new ones. However, at the within-host species level, such maladaptation is not so strongly expected. Consequently, horizontal transfers within host species are more likely to be successful (Rigaud and Juchault, 1995; Rigaud et al, 2001) and our data in *A. vulgare* fully support this expectation.

The occurrence of *Wolbachia* transfers between populations supposes that a strain can occasionally be transferred to a population already harbouring another *Wolbachia* strain. This implies that, at a certain point, bi-infected individuals may exist. *Wolbachia* bi-infected and even tri-infected individuals have already been reported in numerous insects (Werren et al, 1995a; Vavre et al, 1999; Werren and Windsor, 2000). By contrast, bi-infected individuals have never been reported so far in isopod host species, including the present study, suggesting bi-infection should be unstable. Furthermore, minimum models of feminization suggest that coexistence of several feminizers in the same population could be unstable at equilibrium (Taylor, 1990). Such instability could result from the rapid loss of the *Wolbachia* strain

with the lower transmission efficiency whenever two strains are in competition within the same host. Our results in *A. vulgare* support this hypothesis since 75% of the infected samples harbour *wVulC*, which precisely happens to be associated with a higher transmission rate than *wVulM*.

Considered in combination with two studies by Rigaud et al (1999, 2001), our results may provide further insight into the infection dynamics of the two *Wolbachia* strains infecting *A. vulgare*. Rigaud et al (1999) analysed populations of *A. vulgare* from western France. Without regard to their geographical origin, all individuals harbouring *Wolbachia* exhibited closely related mtDNA haplotypes, suggesting an ancestral infection by a single *Wolbachia* strain. Moreover, Rigaud et al (1999) suggested the possible occurrence in these populations of an unknown *Wolbachia* strain prior to the arrival of the present-day strain. Our data reveal that the *Wolbachia*-infected populations in Rigaud et al (1999) study (ie Angoulême, Celles-sur-Belle and Saint-Cyr) harbour the *wVulC* strain and belong to the AvI mitochondrial lineage. In addition, experimental transfers of *Wolbachia* in different isopod hosts (Rigaud et al, 2001) revealed that the *Wolbachia* strain from Celles-sur-Belle (*wVulC* in the present study) has a wide potential for successfully infecting new hosts, compared to other *Wolbachia* strains present in isopods. This view is consistent with the results of the present study showing that *wVulC* has better transmission efficiency than *wVulM*. Taking into account these data, a possible scenario for the evolution of *Wolbachia* infection within mitochondrial lineages AvI and AvIII of *A. vulgare* can be proposed, in which the ancestor of AvI and AvIII harboured *wVulM* (the 'resident'). Later, *wVulC* (the 'intruder') invaded independently lineages AvI and AvIII, replacing the 'resident' *wVulM* in most populations, thanks to a better spread potential.

In the alternative scenario, where *wVulC* is the 'resident' and *wVulM* the 'intruder', *wVulM* would have replaced *wVulC* in some populations only. This would have required a better propagation potential for *wVulM* in order to replace *wVulC*, which is in contradiction with our data indicating a current weaker transmission rate and feminization ability of *wVulM* compared to *wVulC*. It is possible that the spread efficiency of a *Wolbachia* strain can vary over time and one might speculate that the propagation potential of *wVulM* strongly decreased since its successful competition with *wVulC* when initially invading *A. vulgare* populations. However, finding potential reasons for such decrease is not straightforward, especially when an already efficiently transmitted strain is facing competition. Therefore, given the present evidence, the scenario in which *wVulM* is the 'resident' seems more likely.

In conclusion, the discovery of two distantly related feminizing *Wolbachia* strains infecting the isopod *A. vulgare* opens new perspectives in the study of both hosts/symbionts and symbionts/symbionts interactions with regard to feminizing effects. Further experimental studies are in progress to examine the survival and spread of *Wolbachia* strains artificially injected into new host strains, according to the *Wolbachia* strain, the host mitotype and the previous infection status of the new host. They will provide a better understanding of the evolution of feminizing *Wolbachia* in isopods.

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