Mitochondrial DNA Polymorphism, Sex Ratio Distorters and Population Genetics in the Isopod Armadillidium vulgare

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ABSTRACT

Two maternally inherited sex ratio distorters (SRD) impose female-biased sex ratios on the wood louse Armadillidium vulgare by feminizing putative males. These SRD are (i) an intracytoplasmic bacterium of the genus Wolbachia, and (ii) another non-Mendelian element of unknown nature: the f element. Mitochondrial DNA variation was investigated in A. vulgare field populations to trace the evolution of host-SRD relationships and to investigate the effect of SRD on host cytoplasmic polymorphism. The Wolbachia endosymbionts showed no polymorphism in their ITS2 sequence and were associated with two closely related mitochondrial types. This situation probably reflects a single infection event followed by a slight differentiation of mitochondria. There was no association between the f element and a given mitochondrial type, which may confirm the fact that this element can be partially paternally transmitted. The spreading of a maternally inherited SRD in a population should reduce the mitochondrial diversity by a hitchhiking process. In A. vulgare, however, a within-population mtDNA polymorphism was often found, because of the deficient spread of Wolbachia and the partial paternal inheritance of the f element. The analysis of molecular variance indicated that A. vulgare populations are genetically structured, but without isolation by distance.

The last decade led to an ever-increasing amount of evidence of genetic elements altering the reproductive systems in arthropods (Werren et al. 1988; Hurst 1993; Werren and O'Neill 1997). These self-promoting elements, also called selfish genetic elements, are vertically transmitted and manipulate their host reproduction to promote their own spread. These “reproductive parasites” create a context for the occurrence of intragenomic conflicts, as reviewed by Hurst et al. (1996). Among these, elements disturbing the sex determination of the host have been discovered in several crustacean species (see review in Rigaud 1997). These elements, often intracytoplasmic microorganisms, reverse genotypic male hosts into functional phenotypic females (Martin et al. 1973; Ginsburger-Vogel and Desportes 1979; Dunn et al. 1993). As these females in turn transmit the feminizing elements transovarially, this leads to a high female bias in infected lines, promoting the sex by which the parasite can be transmitted to the next host generation.

Mitochondrial DNA is a useful tool for investigating the evolution of associations between cytoplasmic microorganisms and their hosts because both mitochondria and symbionts have the same inheritance pattern (i.e., maternal transmission). The screening and comparison of infected vs. uninfected lineages or individuals can provide data on the history and evolution of the infection. This has been successfully used in the Wolbachia/Drosophila simulans association (e.g., Rousset and Solignac 1995). Furthermore, empirical and theoretical studies revealed that reproductive parasites (Wolbachia-inducing cytoplasmic incompatibility [CI], male-killing microorganisms) dramatically decrease the within-population mtDNA diversity. This is due to a hitchhiking phenomenon: the cytoplasm infected by the inherited microbe is selected and the associated mtDNA reaches high prevalence in these populations (Turelli et al. 1992; Johnstone and Hurst 1996). This parasite-induced selective sweep could mimic the effect of population bottlenecks and therefore confuse the interpretation of population genetic data based on mtDNA measurements (Ballard and Kreitman 1995; Johnstone and Hurst 1996). Because of their strong selective advantage in host populations, the feminizing elements in crustaceans would potentially have the same effect on mtDNA diversity as male-killing or CI-inducing microbes (Taylor 1990; Grandjean et al. 1993).

Two feminizing sex ratio distorters are known in the wood louse Armadillidium vulgare: the intracytoplasmic Wolbachia bacterium (Rousset et al. 1992) and an unidentified non-Mendelian genetic element labeled f (Legrand and Juchault 1984). They are present at variable frequencies in natural populations, often leading to female-biased sex ratios in the wild (Juchault et al. 1993). These two feminizing elements do not have identical patterns of transmission. The Wolbachia is...
Mitochondrial DNA analysis: selected with the aim of investigating the impact of sex females (data not shown). Grandjean and association between aizing effect of f purely maternally transmitted and is therefore expected the female was assigned as an 1670 T. Rigaud et al. i.e. presence of Wolbachia endosymbionts by a physiological test of restriction sites, a maximum-likelihood analysis with boot-

e; Ar, Ars en Re (Charente-Maritime); Ce, Celles sur Belle (Deux-Sèvres); Ij, Île d’Oleron (Vienne); Lg, La Grière (Vendée); Lu, Luzac (Charente-Maritime); No, Noisiel (Essonne); Sc, St Cyr (Vienne); Sm, St. Martin du Fouilloux (Deux-Sèvres); Vi, Viré (Deux-Sèvres).

purely maternally transmitted and is therefore expected to be strictly associated with a given mitotype for a single infection event. The F element undergoes a partial amount of paternal transmission, mainly due to the selection of host autosomal genes repressing the feminizing effect of f (Rigaud and Juchault 1993). The association between f and a given mtDNA haplotype could therefore be broken by the possibility of paternal transmission, and thus the reduction of mtDNA diversity may be less severe (Grandjean et al. 1993).

In this study, 11 populations of A. vulgare were collected with the aim of investigating the impact of sex ratio distorters on the population genetics of the host at a limited geographic scale. We undertook a survey on (i) the prevalence of sex ratio distorters within each population, (ii) the association between mtDNA variation and feminizing elements, and (iii) the genetic population structure inferred from mtDNA variation.

MATERIALS AND METHODS

Animal collection and sex ratio distorter analysis: Wood lice were collected during the spring of 1993, mainly in the western central region of France, with the exception of the No sample, located near Paris (Figure 1). Each site consisted of enclosed gardens, except the Vi site (an open field), the Sc site (an open field near a golf course), and the Ar site (a beach). At each site, all gravid females (i.e., carrying embryos in their incubating pouch) present on the ground surface or under stones or vegetation were collected. They were allowed to produce offspring in the lab, at 20°C and under natural photoperiod conditions. Most of these females produced two successive broods without remating (females store sperm in the genital tracts). Three months after their birth, the sex ratio of the young (males/total offspring) was determined. After the release of their last brood, mothers were tested for the presence of Wolbachia endosymbionts by a physiological test (described in Juchault et al. 1993) and/or by specific PCR amplification of the 16S rDNA bacterial gene (method described in Bouchon et al. 1998). For each PCR run, a Wolbachia-infected female from the population of Niort (Rousset et al. 1992) was added as positive control. Unamplified individuals were tested with mitochondrial primers to test the quality and accessibility of the template DNA, as described in Bouchon et al. (1998). When Wolbachia were lacking in the mother, the sex ratio variation in the broods was the only means to discriminate between chromosomic females (heterogamic, WZ) and f-harboring females (no phenotypic or molecular marker is available to discriminate these female types). As described in Juchault et al. (1993), WZ females produce lineages with a stable 1:1 sex ratio, while f-harboring females produce lines with biased sex ratios. This bias (often toward females) can be constant for a single mother or may vary during the aging of the mother. In the latter case, young mothers often produce female-biased sex ratios, while they produce 1:1 and then male-biased sex ratios when they grow old (without remating). Then a single mother harboring f can produce a progeny with an overall 1:1 sex ratio, but consisting of a series of clutches with biased sex ratio. This increasing proportion of males is also often found in the following generations. In this study, when the overall brood sex ratio of a wild-caught female uninfected by Wolbachia significantly differed from 1:1 (probability for the observed ratio to fall within the confidence interval of a 1:1 binomial distribution), the female was assigned as an f-harboring female. Females producing <10 offspring were excluded from the analysis. On average, females produced 55.8 ± 2.4 offspring per brood (N = 257 females and 642 broods). The wild-caught females uninfected by Wolbachia that produced global a 1:1 brood sex ratio were more ambiguous. One daughter of these females was crossed with males from a reference strain lacking sex ratio distorters (Nice strain) to test the sex ratio in a second generation. The wild-caught females were assigned as WZ if all their single broods did not differ from the 1:1 sex ratio and if there were no significant sex ratio variation between the broods in the first and second generation. If sex ratios did not fit these conditions, females were assigned as f-harboring females (data not shown).

In the Vi site, collections were made in 1993 and 1994. Ten gravid females were collected each year, but, as the results were similar between years, the two samples were pooled for the analysis.

Mitochondrial DNA analysis: All offspring of each mother were pooled, and mtDNA was extracted from their gonads, fat tissue, and nervous system. This pool significantly increased the amount of DNA available for digestion by restriction enzymes. The total mtDNA was extracted as previously described (Souty-Grosset et al. 1992), and then digested with 11 enzymes recognizing unambiguous six-base sequences: BamHI, BglII, EcoRI, EcoRV, HinclI, PstI, PvuII, Smal, SstI, Stul, and XhoI. The digested DNA was run on 1.2% agarose gels in Trisphosphate EDTA (TPE) buffer for 15 hr at 30 V, stained with ethidium bromide, and visualized with UV light. The restriction patterns from each enzyme were labeled by a letter, each isofemale line (therefore each wild-caught female) being characterized by a haplotype (a series of letters).

The relationships between mitochondrial haplotypes were estimated using different methods. The divergence between pairs of haplotypes was estimated by computing the number of nucleotide substitutions per site (d) by the method of Nei and Tajima (Nei 1987). Cluster analysis based on d was performed by unweighted pair-group method of arithmetic average (UPGMA) and neighbor joining using the PHYLIP package (Felsenstein 1993). After coding the presence/absence of restriction sites, a maximum-likelihood analysis with boot-
strap resampling was also performed (REST ml and SEQBOOT programs in PHYLIP). The genetic structure of A. vulgare populations was estimated using analysis of molecular variance (AMOVA; Excoffier et al. 1992) with the software Arlequin 1.0 (written by L. Excoffier, S. Schneider, J. M. Kueffer and D. Roesli, University of Geneva). The $F_{ST}$, an analogue of Wright’s $F_{ST}$, was computed on the whole data set (only two hierarchical levels were recognized: within populations, i.e., within each sampling location, and among populations). The pairwise genetic distances between all pairs of populations were also estimated using $F_{ST}$. The correlation between genetic distance and geographic distance was tested by a Mantel test (Mantel 1967), using a program written by Manly (1991) and adapted by J. Goudet (personal communication).

Wolbachia diversity analysis: Three Wolbachia-positive samples were used for symbiont diversity analysis from An, Ce, and Sc locations (see Figure 1). Wolbachia symbionts of woodlice are closely related, especially in the Armadillidiae family. For example, the Wolbachia from A. vulgare, A. nasatum, and A. album had identical sequences for both the ribosomal RNA and ftsZ genes (Bouchon et al. 1998). A sequence reputedly poorly conserved was analyzed here: the internal transcribed spacer 2 (ITS2) between the 23S and 5S rRNA genes. The ITS2 region was amplified and sequenced using the following primers: Wol-23S, 5’-CCAGTTGATAGGCTA-3’ and Wol-5S1, 5’-CTTGCAACGACCTAC-3’ (Van Meer et al. 1999). Approximately 250 bp were amplified with this primer set, including the 23S 3’ end, the whole ITS2 spacer, and the whole 5S gene. Direct double-strand sequencing was performed as described by Rousset et al. (1992). Sequences are available in GenBank/EMBL/DDBJ databases under accession nos. AJ131642-AJ131644.

RESULTS

The prevalence of sex ratio distorters and the polymorphism of sex-determining systems: The sex-determining mechanisms (SDM) were investigated for each female collected from the wild (Figure 2). The sex ratio distorters were present in all locations. High prevalences of females infected by the f factor were found in all populations (ranging from 0.36 to 1), while females infected with the feminizing Wolbachia bacteria were
Mitochondrial DNA profiles produced by restriction enzymes in all the populations of *A. vulgare* (band size in kilobases)

<table>
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<th>(A)</th>
<th>(B)</th>
<th>(C)</th>
<th>(D)</th>
<th>(E)</th>
<th>(F)</th>
<th>(G)</th>
<th>(H)</th>
<th>(I)</th>
<th>(J)</th>
<th>(K)</th>
<th>(L)</th>
<th>(M)</th>
<th>(N)</th>
<th>(O)</th>
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<td>18.8</td>
<td>17.7</td>
<td>23.6</td>
<td>17.7</td>
<td>17.7</td>
<td>21.0</td>
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*(A) bands with high stoichiometry must be counted as three fragments to obtain the real mtDNA size (Table 1; Raimond et al. 1999). These fragments are superimposed on the agarose gels, leading to a single band with an unusually high stoichiometry. In profiles with more than two restriction sites, the bands with high stoichiometry have to be counted as three fragments to obtain a correct estimation of the mtDNA size (Table 1; Raimond et al. 1999). This atypical structure of the molecule induces difficulties in mapping restriction sites, but the presence/absence of sites for a given enzyme can be deduced from restriction profiles (Figure 3b). XhoI always produced the same restriction profile whatever the *A. vulgare* line tested while SmaI never cleaved the mtDNA. These enzymes were therefore excluded from the analysis. The combination of RFLPs of the nine remaining enzymes produced 11 haplotypes (Figure 4). The genetic relatedness between these haplotypes revealed that three main clusters of mtDNA are present in *A. vulgare* French populations, whatever the analysis method used (e.g., Figure 4). These clusters are (i) (Av9, Av10 (Av3, Av4)), (ii) Av11, and (iii) ((Av5, Av6), Av7 (Av1, Av8, Av2)). The bootstrap analysis gave contrasting levels of resolution, the relationships between haplotypes of the group (iii) being less clear than the general branching pattern and the relationships between haplotypes within the (i) group. Within each cluster, all haplotypes are nevertheless closely related (d < 0.01).

Relationships between sex determination and mtDNA polymorphism: The mtDNA haplotypes were distrib-
Sex Ratio Distorters and mtDNA in A. vulgare

Figure 4.—Mitochondrial haplotypes generated by the combination of restriction enzymes (a), and maximum likelihood unrooted network showing relationship between haplotypes (b). The letters of haplotypes are the profiles generated by the restriction enzymes in the following order: BamHI, EcoRI, BglII, EcoRV, StuI, HindII, PstI, PvuII, and SstI. The numbers next to the nodes refer to the bootstrap scores (percentage) in 1000 replicates (only values >50% are given).

Figure 3.—Structure of the mtDNA of A. vulgare (a) and restriction sites for each enzyme used in this study (b). The total molecule size is ~42 kb, but the molecule consists of three identical copies (see text). The restriction sites are given for the single linear copy. For a given enzyme, the letters labeling each restriction site refer to the letter used to define each restriction profile (e.g., for the EcoRI enzyme, the two restriction sites generate the A profile, while only the second site generates the B profile).
TABLE 2

Distribution of the mitochondrial haplotypes according to the location and the sex-determining mechanism of the lineages

<table>
<thead>
<tr>
<th>Location</th>
<th>SDM</th>
<th>Av1</th>
<th>Av2</th>
<th>Av7</th>
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<th>Av6</th>
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SDM, sex-determining mechanism found in the female; C, chromosomal sex determination (WZ female); Wo, female infected by Wolbachia; f, female infected by the f factor; Av1 . . . 11, mitochondrial haplotypes, as defined in Figure 4; the locations are as described in Figure 1.

the Av1 host haplotype, whereas the Ce Wolbachia strain (AJ131644) was associated with the Av2 host haplotype (see Table 2). No nucleotide variation was detected between these three Wolbachia sequences.

No link was found between a given mitochondrial lineage and the f factor. In fact, the f factor was detected in all the haplotypes found in this study, although it was found primarily associated with the common Av3 haplotype. In other words, the f-infected females had the greatest mtDNA polymorphism.

Two categories of WZ females were distinguished, according to the mitochondrial lines they harbored. The first category can be found in the Ar, Lu, and No locations. In these populations, WZ females were in a minority, were always associated with numerous f-infected females, and shared with these f-females the most common mtDNA found in these populations. In contrast, WZ females from the Sc population were more abundant and harbored only the Av11 haplotype, which is more distantly related to the others (Table 2, Figure 4).

Genetic differentiation in A. vulgare: The AMOVA analysis revealed that most of the haplotype diversity was found within populations. The Φ-statistics showed a high and significant level of genetic structure in our set of data (Table 3). The genetic distances (ΦST) between pairs of populations are given in Table 4. A Mantel test showed that there was no correlation between the genetic and the geographic distances (Table 4; r² = −0.146, P = 0.463). One could argue that the population No could have induced a bias in the results because...
of its greater geographic distance from all other populations. After removing this population from the analysis (i.e., by analyzing the structure only in the populations from the western central region of France), the results were similar: there was a high level of genetic structure (Table 3), and there was no correlation between geographic and genetic distances ($r^2 = 0.026$, $P = 0.874$). The links between the two other SDMs and mitochondrial haplotypes were much more complex. Females harboring the $f$ factor were found to possess all possible mitotypes. On the other hand, two types of associations were found between lineages harboring the $W$ chromosomal and $f$ factor were found to possess all possible mitotypes. On the other hand, two types of associations were found between lineages harboring the $W$ chromosomal and mitotypes: in the Sc population, chromosomal females were frequent and they were associated with a single mitotype of a peculiar type compared to all others. In other populations, the rare chromosomal females harbored various mitotypes, which were always the most abundant types in the population. These two categories of chromosomal females could therefore be of different origin.

These results may be explained by two independent phenomena. The absence of linkage between the $f$ factor and a given mtDNA lineage may confirm that this element does not possess pure maternal inheritance. If this was the case, a pattern of linkage similar to that observed with Wolbachia would have been observed. Paternal inheritance of $f$, even at low level, would break the link between the feminizing element and mtDNA (maternal) lineages because of the possibility of introgression of $f$ into foreign maternal lines by the paternal route (Grandjean et al. 1993). On the other hand, hypotheses about the evolution of SDM in A. vulgare (Juchault et al. 1993; Rigaud et al. 1997) may also explain some of our results. These hypotheses can be summarized as follows. Crossing experiments have suggested that the $f$ factor may be a part of the Wolbachia genome transferred into the host nuclear genome (a transposon-like factor; Legrand and Juchault 1984). As showed by Juchault et al. (1992), the $f$ factor increases in frequency in populations. After this stage,

### TABLE 4

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<th></th>
<th>No</th>
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<th>Lu</th>
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<td>Ij</td>
<td>0.04</td>
<td>0.70</td>
<td>0.01</td>
<td>0.16</td>
<td>0</td>
<td>0.94</td>
<td>0.40</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

The sample sites are as referred to in Figure 1.
the f factor might become integrated into the nuclear genome, generating a W-like chromosome (Juchault and Mocquard 1993), and consequently generating WZ-like females. But these “new” WZ females would be counterselected in populations as long as they produced fewer females than f-infected ones (Juchault et al. 1993). What does mtDNA variation tell us about this evolutionary scenario? The fact that Av1 and Av2 mitotypes are shared by Wolbachia and the f factor in some populations, with the latter always in a minority, could suggest that the transfer of the f factor from the bacteria to the host genome is a recurrent phenomenon. The f factor could secondarily be selected in the populations, a hypothesis strengthened by the evidence of high prevalence of the Av1 mitotype associated with the f factor in the Ar population. The recurrent stable insertion of the f factor, generating WZ-like females, may also be an accurate explanation for the occurrence of rare WZ females sharing the same mitotype as f-harboring females in populations Ar, Lu, and No. These WZ-like females could then be distinguished from “true” WZ females (as those observed in the Sc population) by their mitotypes. The two explanations for the mtDNA variability in f and WZ lines (i.e., paternal inheritance of f vs. evolution of SDM) are not mutually exclusive. Both might act in synergy to produce the observed variability, and one can argue that it is impossible to discriminate which phenomenon has been the most prevalent.

Another problem is that the most common mitochondrial type (Av3) found in all our populations was clearly not linked with Wolbachia. If Wolbachia were at the origin of the evolution of SDM at the geographic scale considered here, the most common mitotype would have been linked with these bacteria. We must therefore consider that the f factor was present in Western Central France before the Wolbachia. To clearly establish if another Wolbachia strain might have been at the origin of the f factor in these local populations, a wider sampling area is needed to find a Wolbachia associated with a mitotype closely related to Av3.

**Genetic differentiation of A. vulgare populations:** Owing to the presence of cytoplasmic sex ratio distorters, the interpretation of mtDNA variability in terms of genetic structure of natural populations should be made with care. This is because sex ratio distorters tend to spread in populations and the associated cytoplasmic elements are also spread by hitchhiking (Turelli et al. 1992; Johnstone and Hurst 1996). We must therefore wonder if the strong genetic structure found in our populations is due to the presence of sex ratio distorters that might mimic population bottleneck events or reflect intrinsic characteristics of the host species. However, the result of a study on worldwide genetic variability of A. vulgare based on nine enzyme loci (Garthwaite et al. 1995) revealed that population (deme) differentiation was variable in this species, but quite high in Europe. Because no genetic hitchhiking is expected between nuclear markers and reproductive parasites, this indicates that the high levels of population structure we found with the mtDNA marker were probably not an artefact due to feminizing sex ratio distorters. Furthermore, high degrees of genetic differentiation are often found between populations in terrestrial isopods (Cobolli-Sbordoni et al. 1997; Gentile and Sbordoni 1998). The reasons for these high levels of structure, including that found for A. vulgare in this study, might be due to the fact that woodlice are poor dispersers (Paris 1965). Also, A. vulgare populations could experience strong bottlenecks (Hassal and Dangerfeld 1997), which may reduce intrademe mtDNA diversity and increase interdeme diversity. The absence of genetic isolation by distance in A. vulgare may be due to its strong anthropophilic tendency, because soil or vegetable transportation may favor inactive migration of woodlice over long distances. The best example is the introduction of A. vulgare into the United States by European migrants around 1800 (Garthwaite et al. 1995). Such long migrations for woodlice, if not too frequent, could explain why the probability of finding two genetically closely related populations is the same between locations closely or distantly related geographically.

The results of mtDNA variation in A. vulgare contrast with other species harboring reproductive parasites. In D. simulans, Wolbachia inducing cytoplasmic incompatibility often invade populations and carry the mitotype with which it is associated (Turelli et al. 1992). In A. vulgare, Juchault et al. (1992) observed that the competition between Wolbachia and the f factor within a given population will often be in favor of the latter feminizing element. So a Wolbachia arriving by migration at low rate in a population harboring f (the more common population type) would have less chance to spread than in a population without sex ratio distorter. Therefore, in A. vulgare, the relatively low success of the Wolbachia spreading and the paternal transmission of the f factor (even incomplete) reduce the probability for a single mitochondrial variant to invade a polymorphic population.

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**LITERATURE CITED**


Bouchon, D., T. Rigaud and P. Juchault, 1998 Evidence for wide-


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