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Wolbachia diversity in the *Porcellionides pruinosus* complex of species (Crustacea: Oniscidea): evidence for host-dependent patterns of infection

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Porcellionides pruinosus is a cosmopolitan woodlouse. It is known to exhibit patterns of geographical variation between populations, and has been suspected to consist of several very closely related species. This species was found to carry *Wolbachia* endosymbionts, alpha-proteobacteria which are known to modify the reproduction of their crustacean hosts by inducing cytoplasmic incompatibility or feminization. In the *P. pruinosus* complex, *Wolbachia* induced feminization, but two different patterns of infection were reported: high prevalence and presence of infected males, or low prevalence and absence of infected males. In this study we investigated nine populations described as *Porcellionides pruinosus* carrying different *Wolbachia* strains, with the aim of investigating the possibility of coevolution between symbionts and hosts. Molecular analyses were carried out on both *Wolbachia* DNA (*wsp* gene) and host mitochondrial DNA (mt LSU rDNA). We show that (1) the nine host populations exhibited a high genetic differentiation so that they have to be split into two sibling species; (2) three different *Wolbachia* strains were found in the host complex but were not species-specific; and (3) the pattern of infection by *Wolbachia* was more host-dependent than symbiont-dependent.

Keywords: coevolution, genetic hitchhiking, molecular phylogenies, *Porcellionides pruinosus*, terrestrial isopod, *Wolbachia*, sibling species.

Introduction

The cosmopolitan terrestrial isopod *Porcellionides* (= *Metoponorthus*) *pruinosus* has a confused taxonomic history (Vandel, 1962). This species is highly synanthropic and is considered to be the most widely distributed species of terrestrial isopods (Vandel, 1962; Garthwaite & Sassaman, 1985). Originally native to Asia Minor (Vandel, 1962) and carried extensively elsewhere by humans, *P. pruinosus* has colonized the entire world except the polar regions. It presents important geographical variation and is considered to be a polytypic species. Based on morphological criteria about 20 subspecies were recognized throughout the world (Vandel, 1962) although the validity of these subspecies remains questionable (Böhme, 1978). More recently several observations supported the presence of distinct species (Garthwaite & Sassaman, 1985; Juchault *et al*., 1985; Marcadé *et al*., 1999).

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As far as is known, all the studied populations of this species complex harbour *Wolbachia* bacteria. *Wolbachia* are intracytoplasmic alpha-proteobacteria which are maternally inherited and can alter the reproduction of their hosts. In the case of *P. pruinosus*, they are believed to induce the feminization of genetic males (Rigaud *et al*., 1997; Marcadé *et al*., 1999). Two different patterns of infection seem to be present in *P. pruinosus* populations, named type 1 and type 2 in the following text. Type 1 was found in all the French host populations examined: *Wolbachia* were present both in males and females, with a 90% prevalence (Rigaud *et al*., 1997; Marcadé *et al*., 1999). Type 2 was found in Greece, Tunisia and Réunion Island host populations: *Wolbachia* were detected in females only, and the prevalence was only 60% (Marcadé *et al*., 1999). These two groups of host populations and their associated patterns of infection could be well separated by mitochondrial RFLP analysis. On the other hand, two *Wolbachia* strains belonging to the B super group of Wolbachiae were found in *P. pruinosus* (Marcadé *et al*.,

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1999; Cordaux *et al*., 2001). These results have led the authors to hypothesize a possible correlation between *Wolbachia* lineages inducing different phenotypes, and differentiated populations in the *P. pruinosus* group (Marcadé *et al*., 1999). However, the high genetic divergence recorded between the two *Wolbachia* strains involved suggested possible horizontal transmission rather than common origin.

The aims of the present study were (1) to check the genetic differentiation of both hosts and parasites on a larger scale; (2) to determine the infection patterns in the host populations; and (3) to test the hypothesis of coevolution of *P. pruinosus* and *Wolbachia* as previously suggested by Marcadé *et al*. (1999). This was accomplished by analysing the sequences of mitochondrial LSU rDNA host genes, shown to be an adequate marker for this taxonomic level (Michel-Salzat & Bouchon, 2000) and to be more reliable than RFLP analysis. At the same time, a molecular analysis of the *Wolbachia* strains found in the host populations was carried out by sequencing the *wsp* gene, a rapidly evolving gene encoding a *Wolbachia* surface protein (Braig *et al*., 1998). On the basis of these two analyses, the correlation between the infection pattern and the *Wolbachia* strains is then examined and discussed.

Materials and methods

Sampling and Wolbachia *detection*

Nine populations from Tunisia (Tunis), Spain (Manzanares), Réunion Island (Saint Paul), Israel (Haifa), Greece (Athens) and France (Nevers, Montamisé, Celles-sur-Belle, Camarade) were studied (see Table 1). Camarade, Manzanares and Haifa were new collecting sites, whereas additional sampling was studied in populations previously investigated (i.e. Nevers and Celles-sur-Belle, Rigaud *et al*., 1997; Athens, Tunis and Saint-Paul, Marcadé *et al*., 1999). Total DNA was obtained by standard phenol extraction and ethanol precipitation (Kocher *et al*., 1989) from muscles, nerve tissue and gonads of each individual. PCR assays were performed to test for the presence of *Wolbachia*, using two specific primer sets for the 16S rDNA and *wsp* genes (O'Neill *et al*., 1992; Zhou *et al*., 1998). Positive and negative controls using known infected and uninfected isopods (*Armadillidium vulgare*, laboratory strains) were performed with each set of PCR reactions.

Sequencing

Individuals testing positive for *Wolbachia* were used for both host mitochondrial and *Wolbachia* gene sequencing. The 3′-half end of the LSU rDNA region of the host's mitochondrial DNA was amplified using the specific primers 16Sar and 16Sbr (Xiong & Kocher, 1991). PCR was conducted as previously described (Michel-Salzat & Bouchon, 2000). The *wsp* region of the *Wolbachia* DNA was amplified using the specific primers *wsp81F* and *wsp691R* (Braig *et al*., 1998).

After purification, the double-stranded PCR products were sequenced on both strands with the amplification primer set, using the ABI Prism BigDye terminator cycle sequencing kit (PE Applied Biosystems, Foster City). Sequences were analysed on a ABI 310 automated sequencer. For each specimen, sequences were obtained independently at least twice from different PCR products.

Mitochondrial sequences were deposited in EMBL, GenBank and DDBJ databases under accession numbers AJ275207 to AJ275212 (Table 1). Two sequences used in analyses were previously obtained from the Celles-sur-Belle and Saint Paul populations (AJ388107 and AJ388108, Michel-Salzat & Bouchon, 2000). *Wsp* sequences were deposited in EMBL, GenBank and DDBJ databases under accession numbers AJ300579 to AJ300585 (Table 1). Two sequences were previously

Table 1 Origin and number of *Porcellionides pruinosus* sampled, presence of *Wolbachia* (the number testing positive is in parentheses) and accession numbers in EMBL, GenBank and DDBJ databases for host and *Wolbachia* genes

Locality	Geographical position	Presence of *Wolbachia*		Accession number	
		Males	Females	Mt LSU rDNA	*wsp* gene
Nevers (F)	46°59′N, 3°10′E	3 (2)	14 (10)	AJ300578	AJ300581
Montamisé (F)	46°37′N, 0°25′E	—	6 (4)	AJ275208	AJ276604
Celles-sur-Belle (F)	46°15′N, 0°14′W	2 (2)	9 (9)	AJ388107	AJ300585
Camarade (F)	43°04′N, 1°16′E	6 (1)	4 (1)	AJ275207	AJ300580
Manzanares (E)	40°00′N, 4°00′W	6 (4)	7 (7)	AJ275209	AJ300579
Athens (GR)	37°56′N, 23°36′E	10 (0)	10 (6)	AJ275211	AJ300583
Tunis (TN)	36°50′N, 10°15′E	5 (0)	5 (3)	AJ275210	AJ300582
Haifa (ISR)	32°50′N, 35°00′E	—	1 (1)	AJ275212	AJ300584
St Paul, Réunion (F)	21°00′N, 55°17′E	11 (0)	11 (8)	AJ388108	AJ276605

obtained from the Montamisé and Saint Paul populations (AJ276604 and AJ276605, Cordaux *et al*., 2001).

Alignment and phylogenetic inferences

LSU rDNA sequences from *P. pruinosus* were manually aligned with the sequence of *Porcellionides cingendus* (no. AJ388106), previously shown to be an appropriate outgroup (Michel-Salzat & Bouchon, 2000). *Wsp* sequences were manually aligned, taking into account the coding structure of the gene. The sequence of *Dysdera erythrina* symbionts (no. AJ276615) belonging to the A super group of Wolbachiae was added as outgroup (Cordaux *et al*., 2001). The corresponding data matrices (10 host sequences of 430 sites including gaps, 120 variable sites; 10 parasite sequences of 588 sites including gaps, 135 variable sites) were separately tested using the MODELTEST 3.0 software (Posada & Crandall, 1998) for a model of DNA substitution.

Phylogenetic trees were inferred using PAUP* 4.02B software (Swofford, 1998). Tree reconstructions were performed using maximum parsimony, maximum likelihood and distance methods using the General Time Reversible GTR model (Rodriguez *et al*., 1990). Bootstrap analyses were performed with 1000 replicates (Felsenstein, 1985). A Mantel test (Mantel, 1967) was performed with GENETIX 4.01 software (Belkhir, 2000) in order to estimate the correlation between the two distance matrices from parasite and host phylogenies (10 000 permutations).

Results

Infection status

All the populations were carrying *Wolbachia*. Infected males were only found in French and Spanish populations (i.e. Nevers, Celles-sur-Belle, Camarade and Manzanares) whereas no male was detected positive for *Wolbachia* in southern populations (i.e. Athens, Tunis and Saint-Paul; Table 1). The difference between these two infection patterns in males was significant ($\chi^2 = 17.4$, d.f. $= 1$, $P < 0.001$). The presence of infected males in French and Spanish populations indicated that they corresponded to the type 1 infection. There is no difference in infection rates in males in these four populations ($\chi^2 = 5.62$, d.f. $= 3$, $P > 0.05$). Southern populations with uninfected males corresponded to the type 2 infection. Assuming a 95% confidence limit, the maximum likelihood frequency of a positive male being present in these samples is therefore 10.8% (Post & Millest, 1991). Comparing the type of infection (type 1 vs. type 2) there was no difference in infection rate in females ($\chi^2 = 1.48$, d.f. $= 1$, $P > 0.05$). In the same way, when males and females were pooled, there was no significant difference in prevalence among populations ($\chi^2 = 11.99$, d.f. $= 6$, $P > 0.05$).

Host diversity

The alignment of LSU rDNA sequences from *P. pruinosus* revealed a first group of identical sequences that included French (Nevers, Montamisé, Celles-sur-Belle, Camarade) and Spanish (Manzanares) populations (Table 2). The second group of sequences corresponded to populations from Réunion Island (Saint Paul), Greece (Athens), and Tunisia (Tunis), with only one substitution between the Tunisian sequence and the others. The sequence obtained from the Israeli population (Haifa) exhibited a divergence of 22.5% with those of the second group and a divergence of 16.5% with those of the first group. The first and second groups differed by a mean divergence of 20.8% (Table 2).

All the procedures used for phylogenetic reconstruction resulted in the same inferred tree (Fig. 1b). Two groups of populations were clearly separated, the first one comprising the Montamisé, Celles-sur-Belle, Manzanares, Camarade, Nevers and Haifa populations

Table 2 Matrix of total nucleotide differences of mitochondrial sequences (below diagonal) and *Wolbachia* sequences (above diagonal) for populations of *Porcellionides pruinosus*

	N (F)	Mo (F)	Ce (F)	Ca (F)	Ma (E)	A (GR)	T (TN)	H (ISR)	S (F)
Nevers (F)	—	121	121	27	27	2	0	2	2
Montamisé (F)	0	—	0	121	121	121	121	121	121
Celles-sur-Belle (F)	0	0	—	121	121	121	121	121	121
Camarade (F)	0	0	0	—	0	29	27	29	29
Manzanares (E)	0	0	0	0	—	29	27	29	29
Athens (GR)	88	88	88	88	88	—	2	0	0
Tunis (TN)	89	89	89	89	89	1	—	2	2
Haifa (ISR)	71	71	71	71	71	96	97	—	0
St Paul, Réunion (F)	88	88	88	88	88	0	1	96	—

N, Nevers; Mo, Montamisé; Ce, Celles-sur-Belle; Ca, Camarade; Ma, Manzanares; A, Athens; T, Tunis; H, Haifa; S, St Paul.

(group *Pp*A, for *P. pruinosus* haplotype A), the second one comprising the Tunis, Athens and Saint Paul populations (group *Pp*B, for *P. pruinosus* haplotype B). Nodes were supported by high bootstrap values (>95%). The nucleotide divergence observed between the two mitochondrial lineages (20.8%) was similar to the distance separating all populations of *P. pruinosus* from the outgroup *P. cingendus* (from 22 to 24%). Assuming a molecular clock of about 2% of divergence per Myr (Simon *et al.*, 1994; Ballard & Kreitman, 1995), the two haplotypes could have diverged 10 Myr ago. However, the variation in the molecular clock across taxa suggests taking this result with caution. A calibration of the molecular clock in Oniscidea, with biogeographical and palaeontological data, would be necessary to obtain a fully reliable estimation.

Wolbachia *diversity*

The alignment of the *wsp* DNA sequences revealed four different sequences corresponding to three groups (Table 2). The first group corresponded to *Wolbachia* isolated from Athens, Tunis, Haifa, St-Paul and Nevers *P. pruinosus* populations. *Wolbachia* from Tunis and Nevers exhibited the same *wsp* sequence and showed a divergence of 0.3% with the other strains of this group. *Wolbachia* from Camarade and Manzanares exhibited the same *wsp* sequence and showed a divergence of 4.8% with the first group (Table 2). The third group included *Wolbachia* from the Celles-sur-Belle and Montamisé populations which exhibited the same *wsp* sequence. This last group was highly divergent from the others (pairwise distance = 20.6%).

All the procedures used for phylogenetic reconstruction gave the same inferred tree (Fig. 1a). Three strains were clearly identified and supported by high bootstrap values. The first strain included the symbionts found in southern populations (Athens, Tunis and Saint-Paul) as well as the symbionts from Haifa and Nevers. This strain was called *w*PruI (for *Wolbachia P. pruinosus* I) according to the nomenclature of Zhou *et al.* (1998). The second strain, called *w*PruII, grouped the bacteria found in the Camarade and Manzanares populations. The last strain comprising the Montamisé and Celles-sur-Belle symbionts was called *w*PruIII.

Comparison between host mitochondrial lineages and bacterial strains

Comparison of *P. pruinosus* and *Wolbachia* relationships (Fig. 1) revealed that the *Pp*B group of populations (St-Paul, Athens, and Tunis) was infected only by the *w*PruI strain of *Wolbachia*. The *Pp*A group of populations was found to carry the three different strains of *Wolbachia*. The Mantel test confirmed that genetic distances of hosts and symbionts were not correlated ($r^2 = 0.125$, $P = 0.761$), thus that the observed patterns of distribution can not be explained by co-speciation.

Discussion

We clearly identified three *Wolbachia* strains in the *P. pruinosus* complex. The comparison of the relationships of hosts and bacteria showed that the *Wolbachia* strains could not be strictly attributed to one or the other host lineages. The *w*PruI strain observed in the

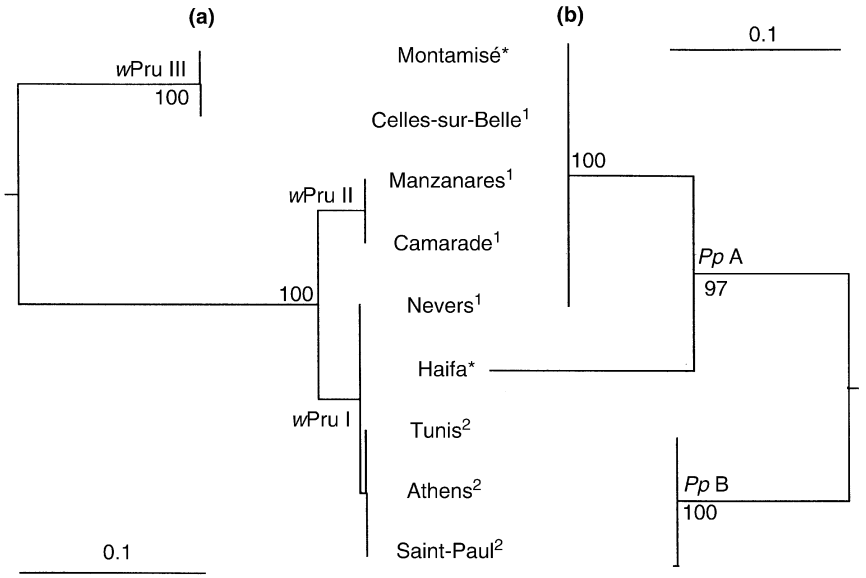

Fig. 1 Phylogenetic relationships among *Wolbachia* strains and *Porcellionides pruinosus* haplotypes, showing distribution of infection patterns. Infection patterns: [1], type 1, *Wolbachia* in males; [2], type 2, no *Wolbachia* in males; *, type of infection unknown.
Fig. 1(a) Maximum likelihood tree (−log likelihood = 1674.84) obtained from the *wsp* sequences. *w*PruI, *w*PruII, *w*PruIII are *Wolbachia* strains of *P. pruinosus* (see text). Bootstrap values (1000 replicates) are shown above nodes.
(b) Maximum likelihood tree (−log likelihood = 1382.77) obtained from partial sequences of mt LSU rDNA. *Pp* A and *Pp* B are mitochondrial lineages of *P. pruinosus* (see text). Bootstrap values (1000 replicates) are shown above nodes.

*Pp*B group of host populations was also found in two samples of the *Pp*A group (Haifa, Nevers) and host populations of the *Pp*A group harboured the three different strains of *Wolbachia*. Furthermore, the distribution of *Wolbachia* strains can not be explained only by coevolution and probably involved several infestation events. The most parsimonious hypothesis requires two infestation events: an ancestral infection by the *w*PruI strain might have occurred, followed by a cospeciation leading to the *w*PruII strain. More recently, a second infection might have occurred by host switching of the distantly related *w*PruIII strain. This scenario is strengthened by previous analyses of *Wolbachia* phylogenetic relationships, which revealed that the *w*PruIII strain is closely related to insect *Wolbachia* strains and could result from recent interspecific transfers (Bouchon *et al*., 1998; Cordaux *et al*., 2001). On the other hand, the *w*PruI and *w*PruII strains were closely related, belonging to a woodlice-specialized *Wolbachia* clade (Cordaux *et al*., 2001; Bouchon *et al*., 1998).

Two different infection patterns were recorded which can not be related to variation in prevalence, but instead only in presence or absence in males. Infection patterns were not correlated with *Wolbachia* distribution (Fig. 1), since the same pattern was associated with the two very distantly related strains *w*PruII and *w*PruIII, whereas the two most related strains *w*PruI and *w*PruII were associated with two different infection patterns. This suggests that the pattern of infection is mainly mediated by the host and not by the *Wolbachia* strain. The best evidence appears in the *w*PruI clade even if we can not totally preclude a slight *Wolbachia* effect because of the low differentiation of *wsp* sequences between Nevers strain (pattern 1) and the southern ones (pattern 2). As the *w*PruII strain belongs to the feminizer clade of *Wolbachia* (Bouchon *et al*., 1998; Cordaux *et al*., 2001), host mediated pattern is also probable in the Manzanares and Camarade populations. The presence of the *w*PruIII strain in both sexes in the Celles-sur-Belle population could either be because of a host effect or because this *Wolbachia* strain has never been a feminizer. A host mediated pattern of infection has already been suggested in woodlice (Rigaud *et al*., 1999) and in insects (Fialho & Stevens, 2000). Because the feminizing process involves competition between a bacterial product and the male hormone (Juchault & Legrand, 1985), the two infection patterns could be due to differences in hormonal target. A high variability of the androgenic hormone was recently reported in the Oniscidea (Martin & Juchault, 1999). The host mediated patterns of infection shown in this paper could therefore be the result of a differentiation of the androgenic hormone in the two *P. pruinosus* sibling species. Moreover, host resistance against feminizing *Wolbachia* has been hypothesized in some cases and might have occurred in the *P. pruinosus* complex (Rigaud *et al*., 1997).

The molecular analysis of a mitochondrial gene clearly revealed the existence of two distinct groups of *P. pruinosus* populations (*Pp*A vs. *Pp*B) separated by the high genetic distance of 20.8%. When comparing the same portion of the mitochondrial LSU rDNA in crustaceans, a divergence of 0.5% is observed between two branchiopod species (Palmero *et al*., 1988; Perez *et al*., 1994), whereas a divergence of 11% is observed between two decapod species (Machado *et al*., 1993). Within the Oniscidea, the pairwise distances were 3% between two populations of the same species (*Armadillidium vulgare*) and 7.4% between two subspecies (*Porcellio dilatatus dilatatus* and *P. d. petiti*) (Michel-Salzat & Bouchon, 2000). The higher divergence value found here in *P. pruinosus* corresponds to those observed between species within a given genus (e.g. from 22 to 24% between *P. cingendus* and *P. pruinosus* populations, this study; 22% between two *Armadillidium* species, Michel-Salzat & Bouchon, 2000). This result supports the existence of two distinct species in the studied populations of *P. pruinosus*, which is congruent with Marcadé *et al*. (1999). As no clear morphological features have been found to separate the two species, they correspond to the definition of sibling species (Dobzhansky *et al*., 1968). Our molecular data suggest that the divergence between the two species may have occurred during the Miocene (10 Myr ago). As the genus *Porcellionides* originates from Asia Minor (Vandel, 1962) the interfertility of populations from Tunis and Réunion Island (Marcadé *et al*., 1999) suggests a recent introduction of specimens from the Mediterranean zone to Réunion Island. This may have occurred by means of natural expansion due to the facility of woodlice for rafting on floating vegetation (Taiti *et al*., 1992), or more probably by way of human migration (Schmalfuss & Ferrara, 1978). According to our data, the sibling species seems to be present in Israel and Western Europe. However, mainly due to the synanthropy of these animals, phylogeographic considerations on their actual distribution are difficult to clarify.

There is some empirical evidence for the role of *Wolbachia* in speciation in the *Drosophila simulans* complex (Hoffmann & Turelli, 1988), between *Nasonia* species (Breeuwer & Werren, 1990) and in the terrestrial isopod *Porcellio dilatatus* (Legrand & Juchault, 1986). Host speciation by *Wolbachia*-induced incompatibility has been recently demonstrated (Bordenstein *et al*., 2001) between two closely related species of *Nasonia*. As all the studied samples were carrying *Wolbachia*, the reproductive isolation in *P. pruinosus* populations (Marcadé *et al*., 1999) could therefore be related to

Wolbachia effects on their hosts. However, by hitchhiking the mitochondria because they are associated cytoplasmic genomes, feminizing *Wolbachia* could promote the fixation of mitochondrial mutations (Werren, 1997). The only phenotype described in *P. pruinosus* up to date is feminization (Rigaud *et al*., 1997; Marcadé *et al*., 1999). Considering the distribution of *Wolbachia* strains in the two host species as well as the incongruent phylogenetic relationships of *Wolbachia* and their hosts (maybe due to recent *Wolbachia* horizontal transfer), we conclude that it is very unlikely that feminizing *Wolbachia* were involved in the differentiation of the two sibling species. Alternatively, we can suggest an ancestral CI-inducing *Wolbachia* acting in *P. pruinosus* speciation which could secondarily evolve to a feminizing phenotype. However this latter scenario seems highly hypothetical.

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