The Royal Society

Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization

Didier Bouchon*, Thierry Rigaud and Pierre Juchault

Laboratoire 'Génétique et Biologie des Populations de Crustacés', Université de Poitiers, CNRS UMR 6556, 40 avenue du Recteur Pineau, F-86022 Poitiers Cedex, France

Wolbachia are maternally inherited, intracellular, alpha proteobacteria that infect a wide range of arthropods. They cause three kinds of reproductive alterations in their hosts: cytoplasmic incompatibility, parthenogenesis and feminization. There have been many studies of the distribution of *Wolbachia* in arthropods, but very few crustacean species are known to be infected. We investigated the prevalence of *Wolbachia* in 85 species from five crustacean orders. Twenty-two isopod species were found to carry these bacteria. The bacteria were found mainly in terrestrial species, suggesting that *Wolbachia* came from a continental environment. The evolutionary relationships between these *Wolbachia* strains were determined by sequencing bacterial genes and by interspecific transfers. All the bacteria associated with isopods belonged to the *Wolbachiae* B group, based on 16S rDNA sequence data. All the terrestrial isopod symbionts in this group except one formed an independent clade. The results of interspecific transfers show evidence of specialization of *Wolbachia* symbionts to their isopod hosts. They also suggest that host species plays a more important role than bacterial phylogeny in determining the phenotype induced by *Wolbachia* infection.

Keywords: Crustacea; *Wolbachia*; feminization; phylogeny; 16S rRNA; *ftsZ*

1. INTRODUCTION

Many arthropods harbour intracellular prokaryotes with specialized adaptations to increase their spread. *Wolbachia* infect the reproductive tissues of arthropods and are transmitted to offspring via the egg cytoplasm. These bacteria cause three kinds of changes in arthropod reproduction (reviewed in Rigaud & Rousset (1996) and Werren & O'Neill (1997)). *Wolbachia* strains are associated with cytoplasmic incompatibility (CI), which occurs in many insects and in one isopod crustacean. *Wolbachia* infection also causes thelytokous parthenogenesis in haplodiploid wasps (reviewed in Stouthamer 1997). Feminization in terrestrial isopods (woodlice) is also induced by *Wolbachia* symbionts. Males infected with *Wolbachia* become functionally female in *Armadillidium vulgare* (Martin *et al*. 1973; Juchault *et al*. 1992) and *A. nasatum* (Juchault & Legrand 1989).

The phylogenetic position of these *Wolbachia* bacteria was first determined by sequencing bacterial ribosomal DNA genes (Breeuwer *et al*. 1992; O'Neill *et al*. 1992; Rousset *et al*. 1992; Stouthamer *et al*. 1993). A finer-scale phylogenetic analysis of *Wolbachia* has been done using the *ftsZ* gene (Werren *et al*. 1995). *Wolbachia* symbionts belong to the alpha proteobacteria and are closely related to the genus *Rickettsia* and other vectored arthropod microorganisms. They form a monophyletic group divided into two subgroups (designated A and B), which diverged 58–67 million years (Ma) ago based on *ftsZ* synonymous substitution rates. Phylogenies based on *ftsZ* and 16S rRNA are concordant, suggesting that they represent true bacterial strain phylogenies. It has been suggested that *Wolbachia* strains could be transmitted horizontally between insect taxa, because closely related bacteria have been found in distantly related hosts (O'Neill *et al*. 1992; Rousset *et al*. 1992).

The phylogenetic position of *Wolbachia* strains in isopods has been determined in only two species: *A. vulgare* (feminizing symbiont) and *Porcellio dilatatus* (CI symbiont). These two symbionts belong to the B subdivision of the *Wolbachia* assemblage and form a single cluster (Rousset *et al*. 1992; Werren *et al*. 1995), but the number of bacterial strains identified in isopods was too small and their effects were too different (feminization versus CI) for an accurate interpretation of their phylogenetic relationships. There is further evidence for symbiotic bacteria in isopods, the phylogenetic positions of which are unknown. Two woodlice, *Chaetophiloscia elongata* and *Porcellionides pruinosus*, and one estuarine isopod, *Sphaeroma rugicauda*, have been shown to harbour *Wolbachia* (Juchault *et al*. 1994; Martin *et al*. 1994). Feminizing microorganisms are also thought to be present in *A. album* (Juchault *et al*. 1974) and *Ligia oceanica* (Martin *et al*. 1974).

The aim of this study was to improve our understanding of the *Wolbachia*–crustacean association by (i) investigating the distribution of *Wolbachia* in a large sample of crustaceans; (ii) determining their phylogenetic relationships;

*Author for correspondence (didier.bouchon@campus.univ-poitiers.fr).

Received 6 February 1998 *Accepted* 9 March 1998

and (iii) testing their level of specialization by investigating the effects of these symbionts after interspecific transfers.

2. MATERIALS AND METHODS

(a) *Crustacean collection and handling*

Crustaceans were collected from several locations (see tables 1 and 2), primarily in Europe, from 1992 to 1997. Live terrestrial specimens were kept in laboratory culture until they were dissected. Marine specimens were fixed in 95% ethanol and kept refrigerated until DNA extraction. To investigate possible intertaxon transmission, other woodlice-associated species were also checked for *Wolbachia*: the woodlouse-parasitoid fly, *Phyto melanocephala*, two woodlouse-eating spiders, *Dysdera crochata* and *D. erythrina*, the woodlouse-phoretic mites *Gamasus* sp., and the commensal pill millipede, *Glomeris castanea*.

(b) *Template preparation*

Total DNA was extracted from the gonads, fat body, and nerve tissues of each individual as described by Kocher *et al*. (1989). The tissues were dissected and serially rinsed in sterile double-distilled water to avoid contamination. All solutions were filter-sterilized (pore size 0.22 µm). Control DNA samples were prepared for each extraction using ovaries from infected and uninfected strains of *A. vulgare*.

(c) *Polymerase chain reaction (PCR) assays*

PCR was performed to test for the presence of *Wolbachia*, using specific primer sets for the 16S rRNA (99f–994r, O'Neill *et al*. 1992) and *fts*Z genes (ftsZf1–ftsZr1, Werren *et al*. 1995). PCR conditions were as previously described. PCR reaction mixture was prepared as a single batch and then added to each sample. PCR reaction mixture alone was used as a negative control. rRNA primers amplified a 900 base pair (bp) fragment and *fts*Z primers, a 1040–1050 bp fragment. Crustaceans for which both products were detected were tentatively scored as positive for *Wolbachia*. The mitochondrial primer set (12Sai–12Sbi, Simon *et al*. 1991) was used in a separate reaction to ensure that the host's DNA was present and accessible in samples that tested negative for *Wolbachia*.

(d) *Sequencing*

Double-strand PCR products were sequenced as described in Rousset *et al*. (1992). The 16S rRNA gene sequencing primers were 27f, 685r, 530f and 973r (Rousset *et al*. 1992). Most of both strands of the central part of the rRNA gene were sequenced using these primers. The *fts*Z sequencing primers were based on the sequence of the *Wolbachia fts*Z gene (Holden *et al*. 1993) as follows: 5′-TCA ATT GAT GAG ATT ATG GAG CA-3′ (positions 263–285), 5′-TTT GCC CAT CTC GCT CAT-3′ (positions 712–696) and 5′-GCA TCA ACT TCA AAC AGA GTC AT-3′ (positions 877–855). Sequences are available in the GenBank, Embl and DDJ databases under accession numbers AJ001601–AJ001611 and AJ223238–AJ223246.

(e) *Phylogenetic analysis*

16S rRNA gene sequences were manually aligned with groups of prealigned sequences from the Ribosomal Database Project (Maidak *et al*. 1994), on the basis of conserved regions and secondary structure. A 783 bp region between positions 83 and 928 (*E. coli* numbering, gaps removed) was used in 42 species for phylogenetic analysis. This data matrix was used to calculate genetic distance as described by Kimura (1980) with nucleotide transversions to transitions weighted 2 : 1. 16S rRNA gene sequences contain sites with unequal substitution rates (Van de Peer *et al*. 1993), so we also used the Jin & Nei (1990) equations. Computation of distance matrices, construction of evolutionary trees, bootstrap analysis and the computation of consensus trees were done using the PHYLIP package (Felsenstein 1993). Phylogenetic relationships were also determined by the maximum likelihood method (DNAml program, PHYLIP package).

(f) *Inoculation experiments*

Wolbachia from five isopod species were used to inoculate males of several other species to investigate their effects on phenotype in an alien genotypic environment (table 3). Some of these *Wolbachia* strains induced unambiguous feminization in their own host (*A. vulgare* and *A. nasatum*), others are strongly suspected to induce feminization (*C. elongata* and *P. pruinosus*), while the phenotype induced by the symbionts from *Cylisticu convexus* was unknown. Tissues (ovaries, fat tissues and nerve chord) from ten females of each donor species were collected and crushed in 1 ml of Ringer solution. The resulting suspension was filtered through a 1.2 µm pore membrane, and 1 µl of filtrate was injected into recipient adult males (one-year-old) using a thin, glass needle. Recipient males were reared for at least 400 d, and their sexual phenotype checked monthly. Feminization was defined as (i) differentiation of oviducts and appearance of female genital apertures; and (ii) hypertrophy of the androgenic gland responsible for the synthesis of male hormone. At the end of the experiment (usually 400 d), a number of males were tested for the presence of *Wolbachia*, by PCR assays and/or transmission electron microscopy, as described by Martin *et al*. (1973).

3. RESULTS

(a) *Distribution of* Wolbachia

A total of 689 individuals (231 males and 458 females) from 85 arthropod species (80 crustacean, one insect, two arachnid, one acarid and one myriapod species) were screened for *Wolbachia* (table 1). Of the five crustacean orders tested (Amphipoda, Isopoda, Tanaidacea, Cumacea and Decapoda), *Wolbachia* were found only in the Isopoda group. Twenty-two (34.9%) of the 63 different isopods were found to be infected. The two Sphaeromatidae, *S. rugicauda* and *S. hookeri*, are estuarine isopods, and the Asellota, *Asellus aquaticus*, is a freshwater species, but all the remaining infected species (19) were land isopods (Oniscidea). The *Wolbachia* infection rate in this last group is therefore 46.3%. Infected individuals were found in eight of the major oniscidean families. The frequency of infection did not differ significantly between families ($\chi^2 = 5.18$, d.f. $= 7$, $p > 0.10$). However, this may be due to the sampling bias. For example, we tested the two known genera (*Tylos* and *Helleria*) of the Tylidae family, whereas the Armadillidiidae and the Porcellionidae are the most diverse families in Europe, with about 20 genera each. Several populations were checked for most of the infected species (table 2). The distribution of infected animals differed between locations, except for *P. pruinosus* and *L. oceanica* where the prevalence of *Wolbachia* was higher: all individuals of *P. pruinosus* and almost all females of *L. oceanica* were infected. The prevalence of *Wolbachia* was similar in the remaining host species ($\chi^2 = 5.83$, d.f. $= 6$, $p > 0.10$), and the mean infection rate per species was 20.3%. This result tends to overestimate

Table 1. *Distribution of* Wolbachia *by species*

(Presence of *Wolbachia* (W) is indicated by ⊕. Taxonomic position of tested samples refer to class, superorder, order, suborder, family and species. Some samples are listed under generic names only. The number of males (*M*) and females (*F*) tested is indicated for each species. For each category the number positive for *Wolbachia* is indicated in parentheses. The geographical origin of samples is shown as Antilles (An), France (F), Hungary (H), Israel (IS), Spain (SP), Sweden (S), Tunisia (T), United Kingdom (UK), USA (US). An asterisk (*) indicates that multiple locations were tested for a species (see table 2).)

taxon	W	*M*	*F*	loc
Crustacea				
Peracarida				
Amphipoda				
Corophiidae				
Corophium arenarium		1	1	F
Corophium volutator		1	1	F
Corophium curvispinum		3	2	F
Gammaridae				
Echinogammarus sp.		1	1	F
Gammarus duebeni		9	5	UK
Gammarus locusta		1	1	F
Gammarus pulex		5	5	F
Gammarus zaddachi		4	4	UK
Haustoriidae				
Haustorius arenarius		1	1	F
Urothoe brevicornis		1	1	F
Pontoporeiidae				
Bathyporeia pilosa		1	1	F
Talitridae				
Orchestia gammarellus		2	2	F
Isopoda				
Gnathiidea				
Gnathiidae				
Paragnathia formica		3	3	F
Anthuridea				
Anthuridae				
Cyathura carinata		2	4	F
Flabellifera				
Cirolanidae				
Eurydice affinis		5	2	F
Cymothoidae				
Anilocra frontalis		—	1	F
Sphaeromatidae				
Campecopea hirsuta		—	1	F
Dynamene bidentata		1	3	F
Sphaeroma hookeri	⊕	—	1 (1)	F
Sphaeroma rugicauda	⊕	*	*	*
Sphaeroma serratum		4	3	F
Sphaeroma tessieri		1	1	F
Asellota				
Asellidae				
Asellus aquaticus	⊕	5	6(1)	F
Proasellus meridianus		1	1	F
Janiridae				
Jaera albifrons		1	2	F
Jarina sp.		1	1	F
Valvifera				
Idoteidae				
Idotea baltica baltica		4	4	S
Idotea baltica tricuspidata		1	1	F
Idotea schmittii		2	2	US
Idotea woesnesienskii		2	2	US
Oniscidea				
Tylidae				
Tylos latreillei		1	1	F
Helleria brevicornis	⊕	*	*	*

(*Continued*)

Table 1 *(Continued)*

taxon	W	M	*F*	loc
Oniscidea (*continued*)				
Ligiidae				
Ligia oceanica	⊕	*	*	*
Ligidium hypnorum		—	4	F
Trichoniscidae				
Androniscus dentiger		6	6	F
Oritoniscus flavus		1	1	F
Trichoniscus pusillus		5	5	F
Haplophthalmus danicus	⊕	2	3(1)	F
Haplophthalmus mengei		—	1	F
Oniscidae				
Oniscus ancarensis		—	2	F
Oniscus asellus	⊕	*	*	*
Oniscus lusitanus	⊕	5	7(1)	SP
Philosciidae				
Chaetophiloscia elongata	⊕	3(1)	6(2)	F
Philoscia muscorum	⊕	*	*	*
Platyarthridae				
Platyarthrus hoffmannseggi		4	7	F
Armadillidae				
Armadillo officinalis		—	5	T
Cubaris murina		5	7	An
Armadillidiidae				
Armadillidium album	⊕	1	7(6)	F
Armadillidium depressum		1	4	F
Armadillidium maculatum		3	3	F
Armadillidium nasatum	⊕	*	*	*
Armadillidium vulgare	⊕	*	*	*
Eluma purpurascens		—	1	F
Schizidium tiberanium	⊕	—	1(1)	IS
Cylisticidae				
Cylisticus convexus	⊕	5(4)	8(8)	F
Porcellionidae				
Porcellio dilatatus dilatatus		—	3	F
Porcellio dilatatus petiti	⊕	—	3(3)	F
Porcellio dispar	⊕	3	4(2)	SP
Porcellio gallicus		1	2	F
Porcellio laevis		1	4	T
Porcellio monticola		1	1	F
Porcellio orarum		1	1	F
Porcellio scaber	⊕	*	*	*
Porcellio spinicornis	⊕	—	3(1)	F
Porcellio variabilis	⊕	—	3(2)	T
Porcellionides cingendus		4	7	F
Porcellionides pruinosus	⊕	*	*	*
Porcellionides sexfasciatus		1	2	T
Proporcellio quadriseriatus	⊕	—	1(1)	IS
Trachelipidae				
Hemilepistus reaumuri		4	9	T
Trachelipus rathkei		1	3	H
Tanaidacea				
Apseudidae				
Apseudes latreillii		2	4	F
Cumacea				
Bodotriidae				
Cumopsis fagei		1	—	F
Eocuma dolfusi		2	2	F
Iphinoe trispinosa		1	1	F

Table 1 (*Continued*)

taxon	W	*M*	*F*	loc
Eucarida				
Decapoda				
Caridea				
Alpheidae				
Athanas nithescens		1	1	F
Anomura				
Paguridae				
Pagurus sp.		1	1	F
Porcellanidae				
Porcellana platycheles		1	2	F
Brachyura				
Portunidae				
Carcinus maenas		1	1	F
Grapsidae				
Pachygrapsus marmoratus		2	2	F
Hexapoda				
Diptera				
Rhinophoridae				
Phyto melanocephala		1	1	F
Arachnida				
Aranae				
Dysderidae				
Dysdera crocata		1	—	F
Dysdera erythrina		1	3	F
Acari				
Gamasidae				
Gamasus sp.		ind.	ind.	F
Myriapoda				
Diplopoda				
Glomeridae				
Glomeris castanea		1	3	F

the prevalence of *Wolbachia* in *A. vulgare*, because Juchault *et al*. (1992) showed, with a more complete data set, that many populations lack the bacterium.

Wolbachia were mostly found in females (109 positive females versus 13 positive males). Positive males were found in *Oniscus asellus*, *C. elongata*, *Cylisticus convexus*, *Porcellio scaber* and *P. pruinosus*. Some intersex individuals (females with small penies) had *Wolbachia*, in the oniscidean *A. album* and *L. oceanica* and in the flabelliferan *S. rugicauda*. As these intersex individuals were functionally female, they were counted as females in tables 1 and 2.

In *Philoscia muscorum* and *O. asellus* from the Quincay population, some gravid females produced offspring in the laboratory, before they were tested for *Wolbachia*. In *P. muscorum*, the three *Wolbachia*-infected females produced no males (total number of offspring, 66 females), whereas the six uninfected females produced a mean of 53.3% males (total number of offspring, 105). In *O. asellus*, the six infected females produced a mean of 36.6% males (297 offspring) and the seven uninfected females produced a mean of 45.8% males (627 offspring) (ANOVA after arcsin transformation of the data: $F_{1,11}=1.34$, n.s.).

None of the woodlice-associate species was infected, although they were all collected from locations where *A. vulgare* populations harboured the bacterium (the parasitic dipteran *P. melanocephala* was taken from St Cyr, the spiders and the myriapod from Chizé, and the acarid came from Celles sur Belle; see table 2).

(b) *Molecular phylogeny*

The DNA from 16 of the 22 isopods infected by *Wolbachia* was amplified for sequencing. Thirteen 16S rRNA partial gene sequences were obtained. Symbionts of the Armadillidiidae, *A. vulgare*, *A. album* and *A. nasatum*, and of the Philosciidae, *C. elongata*, had identical partial sequences (AJ223238–AJ223241), which were identical to the previous *A. vulgare* sequence (X65669, Rousset *et al*. 1992). Sequences for the 940 bp *ftsZ* region were identical (AJ223243–AJ223246) in all these symbionts and also identical to the previous *A. vulgare* sequence (U28208, Werren *et al*. 1995). Similarly, the partial 16S rRNA gene sequence was identical in the symbionts of *P. pruinosus* and *S. hookeri* (AJ223242 and AJ001610).

The phylogenetic analysis involved the comparison of the *Wolbachia* sequences from woodlice with the 16S rRNA

Table 2. *Prevalence of* Wolbachia *in isopod populations*

(*M*, number of males; *F*, number of females; for each category the number testing positive for *Wolbachia* is indicated in parentheses. The geographical origins of samples are Canada (Can), France (F), Hungary (H), Spain (SP), Tunisia (T), United Kingdom (UK), Uruguay (UR).)

species	location	*M*	*F*	% infection	total number per species	% infection per species
Sphaeroma rugicauda	Graye-sur-mer (F)	0	4(1)	25.0	14(5)	35.7
	Arlesford Creek (UK)	2	3(3)	60.0	—	—
	Saint Osyth (UK)	1	2	0	—	—
	Woodbridge (UK)	0	2(1)	50.0	—	—
Helleria brevicornis	Eze (F)	3	4	0	18(2)	11.1
	Bastia (F)	2	5(1)	14.3	—	—
	Pietrocorbara (F)	3	1(1)	25.0	—	—
Ligia oceanica	Angoulins (F)	2	4(3)	50.0	14(7)	50.0
	Sables d'Olonne (F)	3	3(3)	50.0	—	—
	Santec (F)	0	2(1)	50.0	—	—
Oniscus asellus	Bussac (F)	1	5	0	64(13)	20.3
	Edinburgh (UK)	4	4(2)	25.0	—	—
	Quincay (F)	8	13(6)	28.6	—	—
	Romansville (F)	0	5	0	—	—
	Sainte Croix (F)	10(3)	10(2)	25.0	—	—
	Sepvret (F)	1	3	0	—	—
Philoscia muscorum	Boyardville (F)	1	1	0	32(4)	12.5
	Bussac (F)	3	3	0	—	—
	Edinburgh (UK)	1	1	0	—	—
	Noisiel (F)	0	1(1)	100	—	—
	Quincay (F)	7	9(3)	18.8	—	—
	Sepvret (F)	0	1	0	—	—
	Santa Maria del Sar (SP)	0	3	0	—	—
	Viré (F)	0	1	0	—	—
Armadillidium nasatum	Ahun (F)	1	1	0	29(5)	17.2
	Amou (F)	3	3(1)	16.7	—	—
	Bussac (F)	1	1	0	—	—
	Mignaloux (F)	0	19(4)	21.0	—	—
Armadillidium vulgare	Angoulême (F)	0	12(8)	66.7	97(24)	24.7
	Amou (F)	1	3(1)	25.0	—	—
	Belvédère (T)	0	3	0	—	—
	Bussac (F)	2	2(2)	50.0	—	—
	Camarade (F)	2	5(2)	28.6	—	—
	Celles sur Belle (F)	0	9(2)	22.2	—	—
	Chizé (F)	0	10(6)	60.0	—	—
	Nice (F)	0	3	0	—	—
	Niort (F)	0	3(1)	33.3	—	—
	Rocha (UR)	5	5	0	—	—
	Saint Cyr (F)	0	32(2)	6.3	—	—
Porcellio scaber	Ahun (F)	0	3(1)	33.3	57(10)	17.5
	Amou (F)	3(2)	3(1)	50.0	—	—
	Budapest (H)	4	4(2)	25.0	—	—
	Bussac (F)	3	3	0	—	—
	Camarade (F)	2	6(2)	25.0	—	—
	Celles sur Belle (F)	1	1	0	—	—
	Edinburgh (UK)	1(1)	1	50.0	—	—
	Kouchibouac (Can)	5	9	0	—	—
	Salamanque (SP)	3	3(1)	16.7	—	—
	Surrey (UK)	2	0	0	—	—
Porcellionides pruinosus	Celles sur Belle (F)	2(2)	1(1)	100	17(17)	100.0
	Nevers (F)	0	11(11)	100	—	—
	La Réunion (F)	0	3(3)	100	—	—

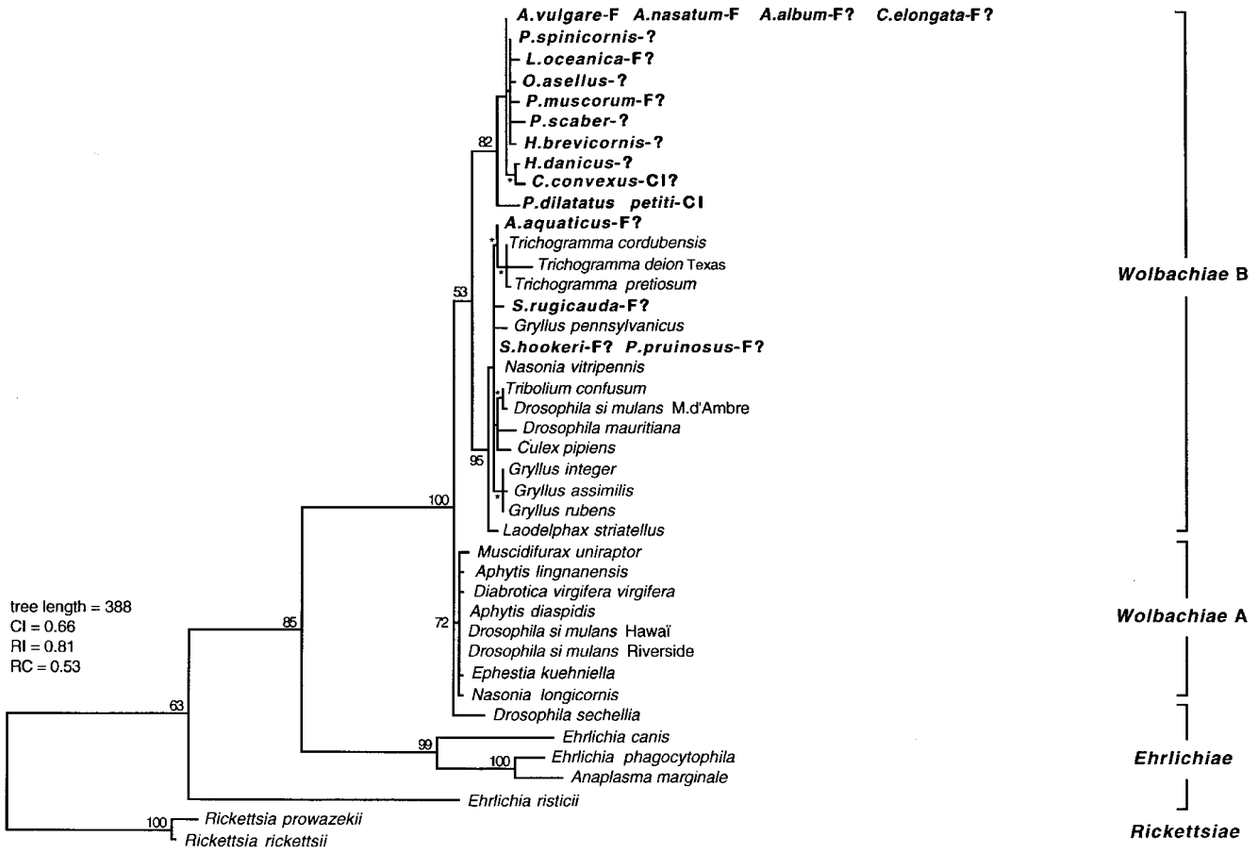

Figure 1. Inferred phylogenetic tree of *Wolbachia* based on 16S rDNA. The tree was generated by neighbour-joining using Jin & Nei (1990) distance excluding insertions and deletions. Sequences are identified by the host species from which they were isolated. Symbionts of isopods are shown in bold and come from the following locations: *A. vulgare* from Celles sur Belle (F), *A. album* from Yves (F), *A. nasatum* from Mignaloux (F), *C. elongata* from Celles sur Belle (F), *P. spinicornis* from Quincay (F), *L. oceanica* from Angoulins (F), *O. asellus* from Quincay (F), *P. muscorum* from Quincay (F), *H. brevicornis* from Bastia (F) and Pietrocorbara (F), *P. scaber* from Ahun (F), *P. dilatatus petiti* from St Honorat (F), *H. danicus* from Quincay (F), *C. convexus* from Avanton (F), *A. aquaticus* from Saint Cyr (F), *S. rugicauda* from Graye sur Mer (F) and Woodbridge (UK), *S. hookeri* from Graye sur Mer (F) and *P. pruinosus* from Celles sur Belle (F). The effect of *Wolbachia* on host phenotype is indicated in front of each host: CI = incompatibility, CI? = likely incompatibility, F = feminization, F? = likely feminization, ? = unknown. Numbers next to internal nodes refer to the bootstrap scores (%) in 1000 replicates (* = replicate numbers more than 55% in external nodes). Tree length, consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated excluding uninformative characters.

gene sequences of 22 *Wolbachia* strains from insects, six alpha proteobacteria (Rickettsiae and Ehrlichiae), and the gamma bacterium *Escherichia coli* as the outgroup. All the procedures used for phylogenetic reconstruction (distances matrix and maximum likelihood methods) place all the symbionts examined in a monophyletic group with other bacteria of the *Wolbachiae* B group (Werren *et al*. 1995) (e.g. figure 1). All the oniscidean endosymbionts, except bacteria from *P. pruinosus*, formed an independent clade. The symbionts in this group were closely related, the maximum pairwise distance being 1.4% and the minimum 0.13%. Symbionts of the Flabellifera, the Asellota and *P. pruinosus* were more distantly related, the pairwise distance being 2.29%.

(c) *Effects of* Wolbachia *on different hosts*

In most cases, the *Wolbachia* infected their new hosts after inoculation (table 3). There were some exceptions for *Armadillo officinalis*, *Helleria brevicornis* and in some cases in which the recipient host was taxonomically distant from the donor. The effect of bacteria on phenotype differed in the recipient species—qualitatively (feminization or not) and quantitatively (the number of days necessary to observe the phenotype)—depending on the recipient host and the origin of the bacteria.

The *Wolbachia* from *A. vulgare*, *A. nasatum* and *C. elongata* had identical sequences for the rRNA and *ftsZ* genes, but their effects after transinfection were quite different. *Wolbachia* from *C. elongata* did not cause feminization of *A. vulgare* males. Similarly, the feminizing *Wolbachia* from *A. vulgare* did not feminize the males of *O. asellus*, but caused feminization in the closely related species *A. nasatum*. However, feminizing *Wolbachia* from *A. nasatum* feminized *O. asellus* males. The symbionts from *P. pruinosus* had a feminizing effect in most other Oniscidea species, regardless of taxonomic position. The only exception to this was *H. brevicornis*, which is apparently not sensitive to feminization whatever the origin of the foreign bacterium.

Table 3. *Effect of interspecific transfers of* Wolbachia *bacteria on the sexual physiology of the recipient host*

(*n* = number of recipient males; S60 = number and phenotype of surviving individuals 60 days after *Wolbachia* injection (m, male phenotype); S400 = number and phenotype of surviving individuals 400 days after *Wolbachia* injection (m, male phenotype; i, intersex phenotype, i.e. sterile male with female genital apertures and hypertrophied androgenic glands); delay = time between *Wolbachia* injection and the first detection of an intersex in the trial; PCR and TEM assays = the number of surviving individuals tested is given with the result of the assay (+, presence of *Wolbachia*; −, absence of *Wolbachia*; nd, assay not done; *, presence of *Wolbachia*, but these individuals were originally infected).)

donor species	recipient species	*n*	S60	S400	delay	PCR	TEM
Armadillidium nasatum	*Armadillidium vulgare*	20	20 m	16 i	90	2+	nd
	Armadillo officinalis	10	10 m	10 m	—	2−	1−
	Porcellio scaber	20	18 m	13 i	150	2+	nd
	Porcellio dilatatus petiti	15	0	0	—	*	nd
	Oniscus asellus	13	12 m	11 i	150	2+	nd
	Helleria brevicornis	10	10 m	9 m	—	3−; 1+	1−
Armadillidium vulgare	*Armadillidium nasatum*[a]	15	15 m	13 i	85	nd	5+
	Armadillo officinalis[a]	20	19 m	18 m	—	nd	6−
	Porcellio scaber	14	14 m	14 i	260	3+	nd
	Porcellio gallicus	18	14 m	14 i	300	nd	5+
	Porcellio dilatatus dilatatus[a]	30	0	0	—	nd	2+
	Porcellio dilatatus petiti	15	0	0	—	*	nd
	Porcellionides pruinosus	20	15 m	13 m	—	*	nd
	Cylisticus convexus	13	10 m	9 i	300	*	nd
	Oniscus asellus[a]	15	14 m	12 m	—	nd	5+
	Helleria brevicornis[a]	10	10 m	8 m	—	nd	5+
Cylisticus convexus	*Armadillidium vulgare*	20	19 m	18 m	—	4+; 1−	1+
	Porcellio scaber	20	20 m	18 m	—	2+	nd
	Porcellio dilatatus dilatatus	22	18 m	13 m	—	2+	1+
	Cylisticus convexus	22	18 m	18 m	—	*	*
	Oniscus asellus	22	19 m	18 m	—	2−	1+
Chaetophiloscia elongata	*Armadillidium vulgare*	13	12 m	11 m	—	2+	1+
	Porcellio scaber	10	10 m	8 i	100	1+	1+
	Oniscus asellus	10	9 m	6 i	100	1+	1+
Porcellionides pruinosus	*Armadillidium vulgare*	13	12 m	10 i	250	2+	1+
	Armadillo officinalis	20	18 m	15 i	290	nd	3+
	Porcellio scaber	10	10 m	10 i	150	2+	nd
	Porcellio dilatatus dilatatus	20	13 m	5 i	220	2+	nd
	Porcellio dilatatus petiti	15	14 m	8 i	220	*	*
	Porcellio gallicus	21	18 m	15 i	180	3+	1+
	Cylisticus convexus	7	5 m	5 i	225	*	nd
	Cubaris murina	12	12 m	12 i	250	nd	1+
	Oniscus asellus	10	9 m	7 i	150	nd	2+
	Helleria brevicornis	13	12 m	12 m	—	2+	1+

[a] Results from Juchault *et al.* (1974).

Wolbachia from *Cylisticus convexus* did not feminize any recipient males even in the same species. The feminizing *Wolbachia* from *Armadillidium* species killed the recipient *P. dilatatus* males after about 30–60 days, whereas *Wolbachia* from *C. convexus* and *P. pruinosus* had no such effect.

4. DISCUSSION

In this study, we found that 16 species not previously known to harbour *Wolbachia* carry these bacteria. Five crustacean orders were tested, but all the infected species belonged to the Isopoda Order. The bacteria were found mainly in terrestrial species, suggesting that *Wolbachia* came from a continental environment. Symbionts found in aquatic species do not refute this hypothesis because *A. aquaticus* is a freshwater (therefore continental) isopod and the *Sphaeroma* are estuarine species living in the upper shore, only submerged at high tide.

Wolbachia are therefore widespread and common in land isopods. The rate of species infection (46.3%) in woodlice was the highest so far recorded in arthropods (Werren & O'Neill 1997). Infected species were found in all the main families of the Oniscidea, suggesting that *Wolbachia* infection affects the entire group. The frequency of infected individuals was low in populations (table 2) and varied with location. Negative results for *Wolbachia* detection in a given species may therefore be due to sampling insufficiency.

Most positive results in infected species (17 out of 22) were obtained in females only, suggesting that most isopod *Wolbachia* strains cause feminization. This is likely in *P. muscorum*, where all-female broods were associated with *Wolbachia* infection. Feminization also probably occurs in species where intersex individuals have been detected: *A. vulgare* (Martin *et al*. 1973), *A. album* (this study), *L. oceanica* (Martin *et al*. 1974; this study) and *S. rugicauda* (Martin *et al*. 1994; this study). Furthermore, Vitagliano *et al*. (1996) showed that sex determination and the female-biased sex ratio in *Asellus aquaticus* are influenced by a cytoplasmic factor, but the two studied populations were not tested for *Wolbachia*. The bacteria we detected in this species thus seem probable sex-ratio distorters. Infected males were detected in five species (*O. asellus*, *C. elongata*, *C. convexus*, *P. scaber* and *P. pruinosus*). The presence of *Wolbachia* in males may be due to host resistance to feminization. This is probable in *P. pruinosus* and *C. elongata* because their symbionts feminize males of other species and are linked to female-biased sex ratios (Juchault *et al*. 1994; Rigaud *et al*. 1997). The phenotypes caused by the symbionts of *O. asellus*, *C. convexus* and *P. scaber* are unknown. As *C. convexus* symbionts did not cause feminization in all the recipient males (table 3), even in intraspecific transmission, CI should be investigated in this species. The same hypothesis should be investigated in *O. asellus*, where infected females produced broods with a 1 : 1 sex ratio.

The transinfection experiments showed that the effect of the symbionts on isopod phenotype differed more according to host species than to bacterial phylogeny. Feminization is caused by relatively distantly related *Wolbachia* strains (*Armadillidium* sp. and *P. pruinosus* symbionts), whereas bacteria from *C. convexus*, closely related to those from *Armadillidium* sp., did not cause feminization. This may mean that mechanisms causing feminization were acquired at least twice, or that feminization was lost in the speciation/infection processes in terrestrial isopods. Trans-specific transfers also show that very closely related *Wolbachia* strains do not have the same potential to cause feminization and are, therefore, specialized. The feminizing process involves competition between a bacterial product and the male hormone (Juchault & Legrand 1985). Therefore, the extent of the feminizing specificity of a given *Wolbachia* strain may be determined by its ability to inhibit more than one type of male hormone. The systematic 'resistance' to feminization in the Tylidae, *H. brevicornis*, may be due to this species having a male hormone too different to be identified as a target by *Wolbachia*. This is consistent with the taxonomic position of the Tylidae, which form a distinct clade (infraorder Tylomorpha) in Oniscidea (Vandel 1962). Finally, the peculiar killing effect of *Armadillidium* symbionts in *P. dilatatus* is restricted to this particular host–*Wolbachia* association, because (i) *Wolbachiae* from other isopods do not kill *Porcellio*, and (ii) *Wolbachia* from *Armadillidium* do not kill other woodlice (table 3). The factor responsible for lethality in this interaction is unknown, but Juchault *et al*. (1974) observed a massive symbiont proliferation, followed by necrosis of the nervous tissues. In the present study we observed that death occurred after a sort of paralysis. Thus, interaction between the bacterium and the nervous system is probably involved in this mortality. This phenomenon appears to be similar to what was recently observed in *Drosophila melanogaster* infected by the *'popcorn' Wolbachia* strain (Min & Benzer 1997). The main difference is that the delay before death is long enough to allow *Drosophila* reproduction (and therefore *Wolbachia* vertical transmission), whereas death occurs in *P. dilatatus* too abruptly to allow reproduction (woodlice have a two-year lifespan with an annual reproductive cycle). Furthermore, we observed virulence after an interspecific transfer whereas the lethal *popcorn* symbiont was found naturally in its host. Comparing these data, we might suggest that the *popcorn* effect could be the result of a recent interspecies symbiont acquisition in *D. melanogaster*.

All the symbionts of isopods appear to be closely related in the B group of the *Wolbachia* assemblage. The DNA sequences of microorganisms are identical in different populations of a given species (e.g. symbionts of *S. rugicauda* from Graye sur Mer, France, and Woodbridge, UK). However, we found different *Wolbachia* strains in different woodlice hosts at a given location (e.g. *H. danicus*, *O. asellus*, *P. muscorum* and *P. spinicornis* from Quincay). The inferred phylogenetic tree clearly separates microorganisms of Flabellifera and Asellota from those of Oniscidea. The bacteria from Oniscidea form a monophyletic group, except the *P. dilatatus* symbiont, which is separated from others in an internal branch with a bootstrap value of 82%. Interestingly, this *Wolbachia* strain is the only unambiguous CI-inducing strain in woodlice.

Some horizontal transmission has nevertheless occurred between land isopods: *Wolbachia* 16S rRNA and *ftsZ* gene sequences are identical in infected species of the *Armadillidium* genus and in *C. elongata*, which belongs to a different family. Similarly, the 16S rRNA gene sequence of *Wolbachia* from *P. pruinosus*, a land isopod, is identical to that of *S. hookeri*, an estuarine isopod. The *P. pruinosus* symbiont therefore branches with those of Flabellifera and Asellota. This is the only exception to the independent clade of oniscidean symbionts. The closest relatives of the Flabellifera and Asellota symbionts are those of the parasitoid wasps, suggesting that *Wolbachia* lineages may sometimes be transmitted between insects and isopods. Sequence similarity is high, suggesting that this intertaxon transmission may have occurred very recently. Based on the molecular clock of the bacterial ribosomal operon (Rousset *et al*. 1992), the original event of divergence of the oniscidean *Wolbachia* occurred about 40 Ma ago. This time has been long enough for symbiont specialization on their hosts, as shown by the results of interspecific inoculations.

We thank Dr S. Gruppe, Dr M. Laulier, Dr S. Grenier and Dr C. Hollyday for assistance in the collection of material. We thank Dr F. Ferrara and Dr S. Taiti for assistance in identification of woodlice species. We thank F. Rousset and M. Solignac for sequencing facilities and helpful comments. We thank also S. O'Neill for comments on earlier versions of the paper. This research was supported in part by a grant from the Centre National de la Recherche Scientifique (ACC-SV3, no. 961098).

REFERENCES

Breeuwer, J. A. J., Stouthamer, R., Barns, S. M., Pelletier, D. A., Weisburg, W. G. & Werren, J. H. 1992 Phylogeny of cytoplasmic incompatibility microorganisms in the parasitoid

wasp genus *Nasonia* (Hymenoptera, Pteromalidae) based on 16S ribosomal DNA sequences. *Insect Molec. Biol*. **1**, 25–36.

Felsenstein, J. 1993 PHYLIP (Phylogeny Inference Package), version 3.5c. Department of Genetics, University of Washington, Seattle.

Holden, P. R., Brookfield, J. F. Y. & Jones, P. 1993 Cloning and characterization of an *ftsZ* cognate from a bacterial symbiont of *Drosophila melanogaster*. *Molec. Gen. Genet*. **240**, 213–220.

Jin, L. & Nei, M. 1990 Limitations of the evolutionary parsimony method of phylogenetic analysis. *Molec. Biol. Evol*. **7**, 82–102.

Juchault, P. & Legrand, J. J. 1985 Contribution à l'étude du mécanisme de l'état réfractaire à l'hormone androgène chez *Armadillidium vulgare* Latr. (Crustacé Isopode Oniscoïde) hébergeant une bactérie féminisante. *Gén. Comp. Endocrino*. **60**, 463–467.

Juchault, P. & Legrand, J. J. 1989 Sex determination and monogeny in terrestrial Isopods *Armadillidium vulgare* (Latreille, 1804) and *Armadillidium nasatum* (Budde-Lund, 1885). *Monitore Zool. Ital*. (*N.S.*) *Monogr*. **4**, 359–375.

Juchault, P., Legrand, J. J. & Martin, G. 1974 Action interspécifique du facteur féminisant responsable de la thélygénie et de l'intersexualité du Crustacé *Armadillidium vulgare* (Isopode Oniscoïde). *Ann. Embryol. Morph*. **7**, 265–276.

Juchault, P., Rigaud, T. & Mocquard, J. P. 1992 Evolution of sex-determining mechanisms in a wild population of *Armadillidium vulgare* Latr. (Crustacea, Isopoda): competition between two feminizing parasitic factors. *Heredity* **69**, 382–390.

Juchault, P., Frelon, M., Bouchon, D. & Rigaud, T. 1994 New evidence for feminizing bacteria in terrestrial isopods: evolutionary implications. *C. R. Acad. Sci. Paris III Life Sci*. **317**, 225–230.

Kimura, M. 1980 A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Molec. Evol*. **16**, 111–120.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X. & Wilson, A. C. 1989 Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natn. Acad. Sci. USA* **86**, 6196–6200.

Maidak, B. L., Larsen, N., McCaughey, M. J., Overbeek, R., Olsen, R., Fogel, G. J., Blandy, J. & Woese, C. R. 1994 The ribosomal data base project. *Nucl. Acids Res*. **22**, 3485–3487.

Martin, G., Juchault, P. & Legrand, J. J. 1973 Mise en évidence d'un micro-organisme intracytoplasmique symbiote de l'Oniscoïde *Armadillidium vulgare* L., dont la présence accompagne l'intersexualité ou la féminisation totale des mâles génétiques de la lignée thélygène. *C. R. Acad. Sci. Paris III* **276**, 2313–2316.

Martin, G., Maissiat, R., Juchault, P. & Legrand, J. J. 1974 Mise en évidence d'un micro-organisme intracytoplasmique symbiotique chez les intersexués (mâles à oostégites) du Crustacé *Ligia oceanica L.* (Isopode, Oniscoïde). *C. R. Acad. Sci. Paris III* **278**, 3375–3378.

Martin, G., Gruppe, S. G., Laulier, M., Bouchon, D., Rigaud, T. & Juchault, P. 1994 Evidence for *Wolbachia* spp. in the estuarine isopod *Sphaeroma rugicauda* (Crustacea): a likely cytoplasmic sex ratio distorter. *Endocytobiosis Cell Res*. **10**, 215–225.

Min, K. T. & Benzer, S. 1997 *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proc. Natn. Acad. Sci. USA* **94**, 10 792–10 796.

O'Neill, S. L., Gordiano, R., Colbert, A. M. E., Karr, T. L. & Robertson, H. M. 1992 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natn. Acad. Sci. USA* **89**, 2699–2702.

Rigaud, T. & Rouset, F. 1996 What generates the diversity of *Wolbachia*–arthropod interaction? *Biodiv. Conserv*. **5**, 999–1013.

Rigaud, T., Antoine, D., Marcadé, I. & Juchault, P. 1997 The effect of temperature on sex ratio in the isopod *Porcellionides pruinosus*: environmental sex determination or a by-product of cytoplasmic sex determination? *Evol. Ecol*. **11**, 205–215.

Rousset, F., Bouchon, D., Pintureau, B., Juchault, P. & Solignac, M. 1992 *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc. R. Soc. Lond*. B **250**, 91–98.

Simon, C., Franke, A. & Martin, A. 1991 The polymerase chain reaction: DNA extraction and amplification. In *Molecular techniques in taxonomy* (ed. G. M. Hewitt, A. W. B. Johnson & J. P. W. Young), pp. 329–355. Springer.

Stouthamer, R. 1997 *Wolbachia*-induced parthenogenesis. In *Influential passengers: inherited microorganisms and invertebrate reproduction* (ed. S. L. O'Neill, A. A. Hoffmann & J. H. Werren), pp. 102–124. Oxford University Press.

Stouthamer, R., Breeuwer, J. A. J., Luck, R. F. & Werren, J. H. 1993 Molecular indentification of microorganisms associated with parthenogenesis. *Nature* **353**, 440–442.

Van de Peer, Y., Neffs, J. M., De Rijk, P. & De Wachter, R. 1993 Reconstructing evolution from eukaryotic small ribosomal subunit RNA sequences: calibration of the molecular clock. *J. Molec. Evol*. **37**, 221–232.

Vandel, A. 1962 *Faune de France. 64*. Isopodes terrestres. Paris: P. Chevalier.

Vitagliano, E., Marchetti, E. & Vitagliano, G. 1996 Skewed sex-ratio, monogeny and maternal sex determination in two geographical populations of *Asellus aquaticus* (L., 1758) (Isopoda). *Crustaceana* **69**, 455–475.

Werren, J. H. & O'Neill, S. L. 1997 The evolution of heritable symbionts. In *Influential passengers: inherited microorganisms and invertebrate reproduction* (ed. S. L. O'Neill, A. A. Hoffmann & J. H. Werren), pp. 1–41. Oxford University Press.

Werren, J. H., Zhang, W. & Guo, L. 1995 Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proc. R. Soc. Lond*. B **261**, 55–71.

As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.