

Screening Mollusks for *Wolbachia* Infection

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We screened 38 species of mollusks for infection by *Wolbachia*, a bacterium that is a common endosymbiont in arthropods, where it induces alterations in reproduction. Using a PCR assay, we could not detect the symbiont in any of the samples, indicating that, in mollusks, it might be absent. © 1998 Academic Press

Key Words: *Wolbachia*; Mollusca; reproductive alteration; PCR; *ftsZ*.

INTRODUCTION

The α -proteobacterium *Wolbachia* is a well-known endosymbiont in various arthropods, including insects, isopods, and mites. It has figured prominently in the evolutionary literature over the past decade for its ability to interfere with host reproduction (see, for a review, Werren, 1997). Its most common manifestation is the induction of “cytoplasmic incompatibility” (CI), which is exhibited in many insects (Hoffmann and Turelli, 1997), 11 species of mites (Johanowicz and Hoy, 1996; Breeuwer and Jacobs 1996; Tsagkarakou *et al.*, 1996), and an isopod (Legrand *et al.*, 1986). In CI, the bacterium manipulates its host’s sperm in such a way that it can only fertilize egg cells that have already been infected with *Wolbachia*. If the sperm enter an uninfected egg, the zygote dies (Barr, 1980; Breeuwer and Werren, 1990). In many Hymenoptera, the symbiont has a different effect: it changes normally sexual individuals into parthenogens, which only produce female offspring. This feat is achieved by diploidisation of egg cells (Stouthamer *et al.*, 1990, 1993). In isopods, *Wolbachia* can turn males into functional “neofemales” (Rousset *et al.*, 1992; Juchault *et al.*, 1994; Martin *et al.*, 1994). These three effects (CI, parthenogenesis, and feminization) all result in an increased transmission of the symbiont.

Surveys have shown these bacteria to be quite common. In insects, circa 15% of all species appear to be infected (Werren *et al.*, 1995a), and in isopods even higher infection rates have been found (Bouchon, personal communication). Phylogenetic studies have also shown that, over evolutionary time, frequent horizon-

tal transmissions have taken place from one arthropod lineage to another (Stouthamer *et al.*, 1993; Werren *et al.*, 1995b; Schilthuizen and Stouthamer, 1997). However, although many authors have hinted at the possibility, *Wolbachia* has not been reported from non-arthropod hosts, with possibly one exception: a *Wolbachia*-like organism has recently been found in a nematode (Sironi *et al.*, 1995).

In this study, we report an initial survey for *Wolbachia* infection in a nonarthropod group, the Mollusca. Molluscan life histories offer good opportunities for endosymbiotic manipulation. Most are sexual, which would allow the action of CI. In the pulmonates, outcrossing hermaphrodites are the rule, which would even double the rate at which CI-inducing bacteria can spread. Also, like crustaceans, many gastropods show sex reversal from male to female (protandry) at some stage in their lives. This apparent flexibility in their sex determination might offer opportunities to feminizing *Wolbachia*. Parthenogenesis is also known, for example, in prosobranchs (Tompa *et al.*, 1984). Prosobranchs appear to have homogametic females (Tompa *et al.*, 1984), and diploidisation of unfertilized eggs would render the individual parthenogenetic. So, in theory, parthenogenesis in snails might be induced by a symbiont also.

MATERIAL AND METHODS

We obtained live material of 38 species of land and freshwater mollusks from The Netherlands, Greece, and Italy (see Table 1), including both bivalves and gastropods. We focused on terrestrial and limnic species since, in isopods at least, *Wolbachia* appears to be absent from marine habitats (Bouchon, personal communication). The material was stored at -80°C until further use.

We extracted DNA from three complete individuals for the smallest species (largest dimension less than 2 mm) and one complete individual for species of intermediate size (3–5 mm), while for the largest species, we used only fragments of the reproductive tissue (i.e., the albumen gland). In each extraction series, we included five specimens of the *Wolbachia*-infected parasitic wasp

TABLE 1
A List of the Species Studied in This Survey
and Their Provenances

Species	Locality
<i>Theodoxus fluviatilis</i>	Leiden, The Netherlands
<i>Viviparus viviparus</i>	Leiden, The Netherlands
<i>Pomatias elegans</i>	Ipiros, Greece
<i>Cochlostoma tessellatum</i>	Ipiros, Greece
<i>Potamopyrgus antipodarum</i> (= <i>jenkinsi</i>) ^a	Yerseke, The Netherlands
<i>Bithynia leachii</i>	Leiden, The Netherlands
<i>Bithynia tentaculata</i>	Leiden, The Netherlands
<i>Radix ovata</i>	Leiden, The Netherlands
<i>Lymnaea palustris</i>	Leiden, The Netherlands
<i>Lymnaea stagnalis</i>	Leiden, The Netherlands
<i>Planorbarius corneus</i>	Leiden, The Netherlands
<i>Anisus vorticulus</i>	Leiden, The Netherlands
<i>Segmentina nitida</i>	Leiden, The Netherlands
<i>Arion ater</i>	Leiden, The Netherlands
<i>Oxychilus cellarius</i>	Leiden, The Netherlands
<i>Deroceras reticulatum</i>	Leiden, The Netherlands
<i>Deroceras cf. laeve</i>	Leiden, The Netherlands
<i>Medora italiana</i>	Gargano, Italy
<i>Siciliaria gibbula</i>	Gargano, Italy
<i>Albinaria senilis</i>	Ipiros, Greece
<i>Albinaria hippolyti</i>	Crete, Greece
<i>Albinaria praeclara</i>	Crete, Greece
<i>Albinaria cretensis</i>	Crete, Greece
<i>Albinaria corrugata</i>	Crete, Greece
<i>Albinaria teres</i>	Crete, Greece
<i>Isabellaria praecipua</i>	Makedhonia, Greece
<i>Lindholmiola corcyrensis</i>	Ipiros, Greece
<i>Trichia hispida</i>	Leiden, The Netherlands
<i>Monacha cartusiana</i>	Ipiros, Greece
<i>Monacha parumcincta</i>	Ipiros, Greece
<i>Arianta arbustorum</i>	Leiden, The Netherlands
<i>Helicigona subzonata</i>	Ipiros, Greece
<i>Cepaea nemoralis</i>	Leiden, The Netherlands
<i>Theba pisana</i>	Gargano, Italy
<i>Helix</i> sp.	Ipiros, Greece
<i>Musculium lacustre</i>	Leiden, The Netherlands
<i>Dreissena polymorpha</i>	Leiden, The Netherlands
<i>Pisidium</i> sp.	Leiden, The Netherlands

^a Parthenogenetic population.

Leptopilina heterotoma as a positive control. The extraction method used was a phenol/chloroform protocol described previously (Schilthuizen and Stouthamer, 1997). The DNA was dissolved in 100 μ l of Tris-EDTA buffer (pH 7.8).

We carried out PCR reactions using the *Wolbachia*-specific primers for the *ftsZ* gene developed by Holden *et al.* (1993): 5'GGACCGGATCCGTATGCCGATTGCA-CAGCTTG3' and 5'GGACCGAATTCGCCATGAG-TATTCATTGGCT3'. To check the amplifiability of the DNA, we also performed reactions with the general primers for the first ribosomal internal transcribed spacer (ITS-1) as described in Schilthuizen *et al.* (1995). All PCR reactions were carried out in 25- μ l reaction volumes, using, apart from standard conditions, 0.5 U Super *Tth* DNA polymerase, 2.5 pmol of each primer, and a final [Mg²⁺] of 2.8 mM. As template, we took 2.5

μ l of undiluted (or, in some cases, 25 \times diluted) DNA sample. As negative controls, PCR reactions were also performed on blanks.

RESULTS AND DISCUSSION

All samples gave positive results with the ITS-1 primers, yielding products of between 400 and 800 bp, indicating that the extraction of amplifiable DNA had been successful. However, the *Wolbachia*-specific primers failed to give positive results with any of the mollusks, although the *Leptopilina* samples were always positive.

The negative results of this survey suggest that *Wolbachia* as it is known in arthropods is not present in mollusks. Alternatively, in view of our small sample sizes of one to three individuals, it might be present at such a low incidence that it could not be detected in this limited study. This might mean that either *Wolbachia* cannot easily adapt to the molluscan physiology, or there have not been sufficient opportunities for horizontal transfer from arthropods to mollusks. We suggest that the same sort of survey be undertaken for other major groups of Metazoa, in order to define the extent to which the symbiont has invaded hosts.

Finally, we want to emphasize that our survey used a *Wolbachia*-specific PCR. These negative results should not be taken to indicate that reproduction-altering microorganisms are altogether absent from mollusks, as our PCR primers would only have picked up strains closely related to the *Wolbachia* in arthropods. Other more remotely related symbionts might still be found in mollusks. In fact, the protozoan *Cryptobia* has been found associated with spermatozoa in pulmonates (Lind, 1973; Current, 1980), while vesicomid clams and possibly also other bivalves harbor obligate bacterial endosymbionts which are transovarially inherited (Cary and Giovannoni, 1993). Given the life histories of some mollusks, which may be particularly vulnerable to manipulation by endosymbionts (see above), we feel that additional studies should be undertaken.

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