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# Successful horizontal transfer of *Wolbachia* symbionts between *Trichogramma* wasps

**Simon Grenier[1]*, Bernard Pintureau[1], Abdelaziz Heddi[1], François Lassablière[1], Coenraad Jager[1], Claude Louis[2] and Chaqué Khatchadourian[1]**

[1]*Laboratoire de Biologie Appliquée, UA INRA 203, INSA, bât. 406, 20 Av. Albert Einstein, 69621 Villeurbanne Cedex, France*
[2]*Station de Recherches de Pathologie Comparée, INRA-CNRS, 30380 Saint Christol lez Alès, France*

Rickettsial symbionts of the genus *Wolbachia*, harboured by many arthropod species, are implicated in feminization, cytoplasmic incompatibility and parthenogenesis phenomena. These symbionts induce thelytokous parthenogenesis in some egg parasitoids of the *Trichogramma* genus. In our study of these minute wasps, purified *Wolbachia* from an infected species, *T. pretiosum*, were transferred by microinjection into *in vitro* developed pupae of an uninfected species, *T. dendrolimi*. We believe this to be the first successful transfer of *Wolbachia* in parasitoids. The presence or absence of *Wolbachia* was determined using DAPI staining, PCR and *ftsZ* gene sequencing. An *ftsZ* gene fragment from microinjected *T. dendrolimi* was shown to be identical to that of *T. pretiosum*, confirming that transfer was successful. *Wolbachia* were still present in the recipient species 26 generations after the transfer, although only partial induction of thelytoky was observed. Therefore, in *Trichogramma*, density of symbionts or symbiont–host interactions may be involved in the expression of parthenogenesis. The successful transfer of the symbiont within the Trichogrammatidae, a group of insects used in biological control strategies, could increase their agronomic importance by manipulating their reproductive system.

**Keywords:** symbiont; *Wolbachia*; *Trichogramma*; egg parasitoid; horizontal transfer; microinjection

## 1. INTRODUCTION

Rickettsial symbionts of the genus *Wolbachia* are harboured by many insects, mites and woodlice (Werren 1997; Rousset *et al*. 1992; Breeuwer & Jacobs 1996). These endosymbionts, belonging to the alpha subdivision of the Proteobacteria, infect different tissues of arthropod species and mainly alter reproductive processes, namely feminization of genetic males into functional females in isopods (Juchault *et al*. 1992), cytoplasmic incompatibility (CI) in isopods, insects and mites (Breeuwer & Jacobs 1996), and parthenogenesis in parasitoid insects (Stouthamer *et al*. 1990). Based on sequences of either the 16S rRNA gene (Rousset *et al*. 1992; Stouthamer *et al*. 1993) or the *ftsZ* gene (Werren *et al*. 1995), phylogenetic trees divide *Wolbachia* into two subgroups. In the genus *Trichogramma* Westwood (Hymenoptera, Trichogrammatidae), *Wolbachia* of the B subgroup change the host's bisexual mode of reproduction into the thelytokous mode (Werren *et al*. 1995), whereas *Wolbachia* of the A subgroup seem to increase female fecundity (Vavre & Girin 1997).

Comparative molecular phylogenies of 20 parthenogenetic *Trichogramma* species/strains and their symbiotic *Wolbachia* strongly suggest occasional horizontal transmission (Schilthuizen & Stouthamer 1997), but we believe that no experimental transfers have been previously performed. In an attempt to clarify the role of symbionts in the expression of parthenogenesis, we investigated microinjection techniques for transferring *Wolbachia* from infected to uninfected species. Elsewhere, transfer of *Wolbachia* between several species of isopods by ooplasm or ovary extract injections has been reported (Rigaud & Juchault 1995), as well as between *Drosophila* species and between *Drosophila* and mosquitoes by means of embryonic microinjections (Boyle *et al*. 1993; Braig *et al*. 1994; Rousset & de Stordeur 1994). A microinjection protocol was also developed to transfer *Wolbachia* from egg to egg between *Tribolium confusum* strains (Chang & Wade 1996). Attempts to transfer *Wolbachia* to parasitoid species, such as *Muscidifurax uniraptor* and *Nasonia vitripennis*, were unsuccessful (Van Meer *et al*. 1996). Because of the tiny size of *Trichogramma* adults, and therefore of their eggs, another technique had to be developed.

In the present paper we report, to our knowledge, the first successful transfer of *Wolbachia* in parasitoids, between two *Trichogramma* species, producing two newly infected isofemale lines of *T. dendrolimi*.

## 2. MATERIALS AND METHODS

### (a) *Source of* Wolbachia

Stock culture of *Trichogramma* spp. were maintained on *Ephestia kuehniella* Zeller eggs killed by UV irradiation ($23 \pm 0.5\,^{\circ}C$; $70 \pm 5\%$ relative humidity; 16:8 h L:D).

A regularly infected strain of *T. pretiosum* Riley from Uruguay was used as the donor species. Crudely purified *Wolbachia* fractions were prepared, from *E. kuehniella* eggs superparasitized

*Author for correspondence (sgrenier@jouy.inra.fr).

by *T. pretiosum*, from *T. pretiosum* pre-pupae grown on artificial media, or from *T. pretiosum* adults cultured on *E. kuehniella* eggs. The extraction protocol was modified from that of Jager *et al.* (1997). *Trichogramma pretiosum* were homogenized in a Dounce grinder in phosphate-buffered saline with 30% sucrose. The homogenate was filtered on tergal material (50–80 µm pore size) to retain large debris, then centrifuged twice for 5 min at 660 *g* to remove cellular debris. The supernatant was centrifuged once for 5 min at 4600 *g* for *Wolbachia* pelleting and the pellet was then resuspended in the buffer to be used as the injection suspension.

### (b) *Recipient species*

A naturally uninfected strain of *T. dendrolimi* Matsumura from China was used as the recipient species. This strain was cultured in artificial conditions, instead of breeding on *E. kuehniella* eggs, in order to facilitate the handling of the *Trichogramma* and to monitor the chosen stage to be injected: prepupae (PP), pupae with white eyes (PWE) or pupae with red eyes (PRE).

For *in vitro* rearing of *Trichogramma*, artificial host eggs consisting of plastic film in the form of hemispherical cupules were filled with medium comprising pupal insect haemolymph (40%), hen's egg yolk (20%), semi-skimmed cow milk (20%), Neisenheimer salt solution (10%) and distilled water (10%). Rearing was conducted in aseptical conditions (Grenier & Liu 1990; Grenier 1994).

### (c) *Injection method*

*Wolbachia* suspension was microinjected under an inverted microscope by a 20°-angled glass micropipette of about 7 µm in diameter (J.C. Diffusion) using a microinjector (Narishige IM6) connected to a micromanipulator. The prepupae or pupae, grown in artificial conditions, were extracted from the rearing cupules and disposed along a hooked glass rod glued onto the bottom of the injection box to prevent rolling; they were injected laterally into the abdomen (figure 1). A total of about 30 pl was injected in five *Trichogramma* laying in one box. After injections, the box was covered with a lid and placed in a larger, tight plastic box where relative humidity was maintained at *ca.* 75% by NaCl-saturated solution. The larger box, containing one set of injection boxes, was kept in a climatic chamber (23 ± 0.5 °C; 14:10 h L:D).

The surviving females were mated with stock-cultured males to establish isofemale lines. Beginning with the third generation, isofemale lines were regularly monitored for the presence of *Wolbachia*. Negative lines were discarded after the tenth generation.

### (d) *Test of presence/absence of* Wolbachia

The presence or absence of *Wolbachia* in injected suspension, in donor and in recipient species, was tested by four different methods.

1. Electron microscopy analyses, conducted as described previously (Louis & Nigro 1989; Louis *et al.* 1993).
2. DAPI staining, i.e. visual determination by fluorescent microscopy using the non-specific DNA-binding fluorochrome DAPI (4′,6-diamidino-2-phenylindole) (O'Neill & Karr 1990).
3. Diagnostic polymerase chain reaction (PCR) assays using specific primers for the *Wolbachia fts*Z gene (Holden *et al.* 1993). Total DNA from 20–30 frozen individual *Trichogramma* was extracted in a mixture of 40 µl of 5% Chelex solution and 2 µl of proteinase K (14.4 mg ml$^{-1}$; Boehringer), at 55 °C for 2 h. This extraction method is especially convenient with a small quantity of material. Reaction cocktails for PCR amplification consisted of 1.5 U of Taq polymerase (Appligene), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 0.3 µM primers (102 For, 969 Rev, amplifying an 867 base pair (bp) fragment, purchased from Eurogentec) and between 10 ng and 1 µg of DNA template in a final volume of 50 µl. The PCR parameters were 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 51 °C for 45 s, and 72 °C for 45 s, and finally 72 °C for 5 min.
4. DNA sequencing. For sequencing, DNA purification was assessed by centrifugation on a column using the QIA quick kit. Sequencing was performed on 500 ng of PCR products in an 'Alf express Pharmacia Biotech' autosequencer. The primers 'For 2' (5′cy5-GAG TTG TAA CTA ARC CGT TCG GTT 3′) and 'Rev 2' (5′cy5-AAC CGA ACG GYT TAG TTA CAA CTA 3′), located in positions 434 and 458 of the *fts*Z gene, respectively, were used for sequencing PCR. The parameters were 95 °C for 2 min followed by 35 cycles at 95 °C for 30 s, and 50 °C for 40 s, and finally 50 °C for 5 min. Samples were loaded onto a 5.7% denaturing polyacrylamide gel and run at 25 W for 12 h.

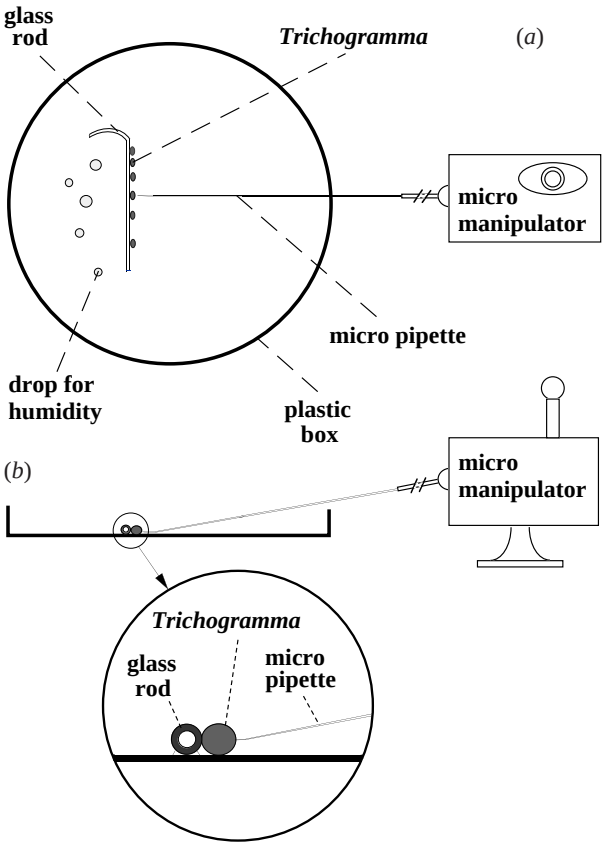


Figure 1. Microinjection material and device: (*a*) top view and (*b*) schematic transverse section. Plastic box 5 cm in diameter, with cover removed during the injection time.

## 3. RESULTS AND DISCUSSION

The presence of *Wolbachia* symbionts in injected solutions was confirmed by PCR and electron microscopy. The latter technique enabled us to monitor the

Table 1. *Survival of different stages of* Trichogramma dendrolimi *after injection of* Wolbachia *suspension purified from* Trichogramma pretiosum*, and infection success*

(Number of individuals surviving to adult = 80, number of normal females used as founders = 35, number of isofemale lines established = 18, and number of *Wolbachia* infected lines = 2. The results include all sets of experiments, even those from initial assays; in the last experiments, the percentage survival was higher for all three stages, reaching up to 28% for PRE.)

| injected stage[a] | number of injected insects | percentage of survival to adults[b] | infected isofemale lines |
|---|---|---|---|
| PP | 566 | 2.65 | — |
| PWE | 237 | 8.86 | 1 (line b) |
| PRE | 234 | 18.80 | 1 (line a) |

[a] PP, prepupal stage; PWE, pupal stage with white eyes; PRE, pupal stage with red eyes.
[b] Percentage survival differed significantly for each stage by Fisher exact test for $p < 0.002$.

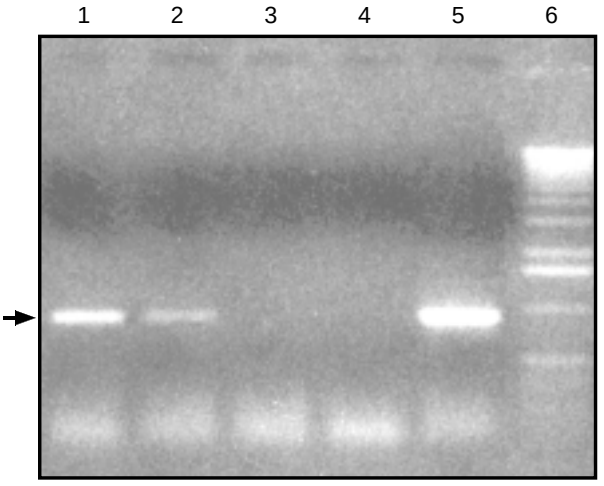


Figure 2. PCR amplification of an *fts*Z gene fragment was used to detect the presence of *Wolbachia* in transinfected and control *Trichogramma* lines. Characteristic band at 867 bp (arrow). Lanes: 1 and 2, transinfected injected *Trichogramma dendrolimi* line 'a'; 3, non-infected injected *T. dendrolimi*; 4, *T. dendrolimi* negative control; 5, *T. pretiosum* (naturally infected) positive control; 6, 1 kb ladder (Gibco).

presence of undamaged *Wolbachia* in the injected fraction. Microphotographs also allowed us to estimate the size of the *Wolbachia*: $0.69 \pm 0.04$ μm by $0.31 \pm 0.01$ μm (mean ± s.e., $n = 17$).

*Trichogramma* pupae microinjected at the oldest stage (PRE) showed a significantly higher survival rate to adulthood (table 1). However, a higher probability of infection could be expected in earlier stages because of the longer time available for *Wolbachia* to migrate from the injection site to the ovaries.

*Trichogramma dendrolimi*, the recipient species, was confirmed as being free of symbionts several times before injection using three independent methods for detection of *Wolbachia* infection: DAPI staining, electron microscopy and PCR (figure 2).

PCR amplification of the *fts*Z gene fragment was used to detect the presence of *Wolbachia* in transinfected and control *Trichogramma* lines. Three generations after injection, two isofemale lines tested positive for the presence of *Wolbachia*. Successful injections were performed with a five-month interval (figure 2). The isofemale line 'a' was injected as the PRE stage with *Wolbachia* purified from *T. pretiosum* eggs originating from superparasitized *E. kuehniella* eggs. The line 'b' was injected as PWE with *Wolbachia* purified from PP grown in artificial conditions. Subsequent positive PCR controls were performed with different amounts of total DNA extracted from both lines, at generations 6, 8, 10, 12, 15 and 26 for line 'a', and at generations 4, 5, 8 and 14 for line 'b'.

Line 'a' injected with *Wolbachia* from *T. pretiosum* eggs that possibly retained host material (*E. kuehniella* eggs) needed confirmation of *Wolbachia* origin. *Wolbachia* symbionts harboured by *E. kuehniella* and *T. pretiosum*, belonging to the A and B subgroups, respectively, were characterized from the 16S gene (Rousset *et al*. 1992; Stouthamer *et al*. 1993) or the *fts*Z gene (B. Pintureau and C. R. Jager, unpublished results; the *fts*Z gene sequence of *T. pretiosum* has been deposited at GenBank, accession number U 95753), and was thus easily discernible. PCR fragments of the *fts*Z gene were sequenced to confirm the identity of *Wolbachia* detected in line 'a'. Out of 633 bp, 64

Table 2. *Sex ratio in offspring of paired or virgin females and thelytoky in* Trichogramma dendrolimi *microinjected lines* (*a and b*) *compared with controls* (*C*)

(Results from 19, 18 and 23 paired females, and from 20, 22 and 20 virgin females, for line b, line a and control, respectively.)

| | | paired females | | | virgin females | | |
|---|---|---|---|---|---|---|---|
| lines | generations | total no. offspring | sex ratio[a] | sr < 0.15[b] | total no. offspring | sex ratio | % thely[c] |
| b | G13 | 921 | 0.182 | 0.474§ | 1073 | 0.995 | 5.0 |
| a | G25 | 854 | 0.212 | 0.278†§ | 1417 | 0.998 | 4.5 |
| C | — | 1212 | 0.225 | 0.130† | — | 1 | 0 |

[a] Sex ratio values (rate of males) do not differ significantly by ANOVA, $p = 0.21$ (after arcsin square-root transformation).
[b] Proportion of females having an offspring sex ratio of less than 0.15. Values followed by different symbols significantly differ by Fisher exact test for $p < 0.05$.
[c] Percentage of partly thelytokous females having between one and five females in their offspring.

differences appeared between *Wolbachia* of the injected line and *Wolbachia* of *E. kuehniella*, whereas no difference was shown between *Wolbachia* of the injected line and *Wolbachia* of *T. pretiosum*, confirming that the injected *Wolbachia* originated from *T. pretiosum*.

Hence, pupae (PWE or PRE) appeared to be a proper stage for successful transfer, even if the time-period seems shorter than in PP for migration of *Wolbachia* from the injection site to the ovaries.

To determine whether transinfected lines showed modification of the sex ratio and the appearance of parthenogenesis, offspring of paired and virgin females were analysed at different generations (table 2). Micro-injected line 'b' showed a percentage of mated females with low male offspring (less than 15%) higher than the control. Offspring of virgin females revealed a partial induction of thelytoky in only two females in both transinfected lines.

The limited expression of thelytoky is probably the result of a too-low density of symbionts in the micro-injected lines, possibly related to host influence. Indeed, a threshold effect on CI expression is well documented in *Drosophila* spp. and *Nasonia vitripennis*, i.e. a minimum density of *Wolbachia* is needed to obtain physiological perturbations (Breeuwer & Werren 1993; Karr 1994; Bourtzis *et al*. 1996). A second hypothesis would be that the interaction between the *Wolbachia* symbiont from *T. pretiosum* and the new host genome of *T. dendrolimi* is unfavourable for the expression of thelytoky. In other words, the expression of thelytokous parthenogenesis could be influenced by several factors, including bacterial strain and density, host genotype or interaction between each other. Further interspecific transfers will be necessary to clarify the role of each factor, especially by transferring a parthenogenetic *Wolbachia* type into diplodiploid insects and, conversely, CI *Wolbachia* type into haplodiploid insects (Van Meer *et al*. 1996).

Successful experimental transfer between the two *Trichogramma* species tested in the present work could lead to the transformation of the (bisexual) species used in biological control programmes by improving their efficiency, i.e. by increasing the number of females in progenies (Pintureau *et al*. 1993; Stouthamer 1993), thus reducing the production costs (Van Meer *et al*. 1996). Nevertheless, thelytoky as a beneficial characteristic in newly thelytokous infested strains has to be proved, as the actual female fecundity and daughter progenies were lower in parthenogenetic lines of *T. pretiosum* and *T. deion* Pinto & Oatman, compared with their bisexual counterparts (Stouthamer & Luck 1993). On the other hand, in *Encarsia formosa* Gahan, cured wasps (experimentally deprived of symbionts by antibiotic treatment) produced fewer offspring than the normally symbiotic wasps, whereas in *Muscidifurax uniraptor* no difference was found (Stouthamer *et al*. 1994).

The study of the possibility of transferring *Wolbachia* between more-or-less phylogenetically distant species appears to be a powerful tool for investigating the mode of action of this symbiont in relation to the host's genome. Transfer of *Wolbachia* could also be used as a vector for spreading beneficial genes in insect populations (Beard *et al*. 1993).

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