Entomologia Experimentalis et Applicata **96:** 177–184, 2000.
© 2000 *Kluwer Academic Publishers. Printed in the Netherlands.*

Wolbachia-induced parthenogenesis in the egg parasitoid *Telenomus nawai*

Norio Arakaki[1], Hiroaki Noda[2] & Kenzou Yamagishi[3]
[1]*Okinawa Prefectural Agricultural Experiment Station, Sakiyama, Naha, Okinawa 903-0814, Japan;* [2]*National Institute of Sericultural and Entomological Science, Tsukuba, Ibaraki 305-8634, Japan;* [3]*Meijo University, Shiogamaguchi, Tempaku-ku, Nagoya 468-0073, Japan*

Accepted: April 27, 2000

Key words: egg parasitoid, parthenogenesis, *Wolbachia*, *Telenomus*, Hymenoptera, Scelionidae

Abstract

Two egg parasitoid species, *Telenomus nawai* and *Telenomus* spec. are similar morphologically but they have been treated as different species because of their different reproductive forms, arrhenotoky and thelytoky. Male progeny were produced from the thelytokous colony of *Telenomus* spec. (TT) by heat and antibiotic treatments. These males mated successfully with females of arrhenotokous colony of *T. nawai* (AT) and the females produced on average 19.1% males. This percentage did not differ from that obtained from AT females mated with AT males (19.4%). Diagnostic PCR indicated that TT is infected with *Wolbachia*; antibiotic treatments eliminated *Wolbachia* from TT. These facts suggest that *Wolbachia* causes thelytoky and *Telenomus* spec. (TT) is conspecific with *T. nawai* (AT).

Introduction

The most common reproductive mode in Hymenoptera is arrhenotoky, a form of parthenogenesis in which males arise from unfertilized eggs and are haploid and females arise from fertilized eggs and are diploid. Another mode of reproduction, thelytoky, is also found in many hymenopterous lineages (Luck et al., 1992). Thelytokous females produce diploid female offspring from unfertilized eggs. Consequently, thelytokous lines can persist for multiple generations in the absence of males.

Two forms of thelytoky are recognized in Hymenoptera, revertible or microbe-associated thelytoky and non-revertible thelytoky (Stouthamer & Kazmer, 1994). In microbe-associated thelytoky, bacteria of the genus *Wolbachia* cause parthenogenesis, and removal of these microbes by antibiotic or high-temperature treatments induces the production of males (Stouthamer et al., 1993). Microbes are absent in non-revertible parthenogenesis. Neither temperature nor antibiotic treatments causes them to revert to arrhenotoky (Stouthamer et al., 1990; Stouthamer & Werren, 1993). The sexual functioning of antibiotic-induced males ranges from possessing sperm although lacking the ability to transmit it in *Encarsia formosa* (Zchori-Fein et al., 1992) to the successful production of female offspring in *Trichogramma* (Stouthamer et al., 1990; Stouthamer & Kazmer, 1994).

The egg parasitoid, *Telenomus nawai* Ashmead (Hymenoptera: Scelionidae), is an important natural enemy of cutworms, belonging to the genus *Spodoptera* (Lepidoptera: Noctuidae) in Japan. We collected two egg parasitoid species, arrhenotokous *Telenomus nawai* Ashmead (Hymenoptera: Scelionidae) (AT) from Ibaraki and thelytokous *Telenomus* spec. (TT) from Okinawa. These two species were indistinguishable by morphological examination but they differ in reproductive forms, arrhenotoky and thelytoky.

This paper reports that thelytoky in *Telenomus* spec. (TT) is caused by the infection with *Wolbachia* and AT female and TT male are conspecific.

Materials and methods

Insects. We collected thelytokous *Telenomus* spec. (TT) from an egg mass of *S. litura* in Ginowan, Okinawa Pref., May 1997. Arrhenotokous *T. nawai*

(AT) was collected, from egg masses of *S. depravata* in Tsukuba, Ibaraki Pref., September 1997. These two strains were subsequently maintained on eggs of *S. litura* in the laboratory in Okinawa.

High-temperature treatment. Wasps were reared at a high temperature because the heat is often deleterious for microbes associated with arthropods. TT females were placed in a glass test tube (1.7 cm diameter × 10.5 cm long) containing an *S. litura* egg-mass as hosts and plugged with a silicon sponge (=standard vial). Females were allowed to oviposit for 24 h. The immature offspring from these thelytokous females were incubated at 25 °C for ten days and then exposed to 35 °C during the last three days of their pupal development. A similar group of thelytokous females served as a control. This latter group was incubated at 25 °C until they emerged. Twenty females in both groups were individually isolated in a standard vial, provided with honey and an *S. litura* egg-mass having excess hosts, and allowed to oviposit for 24 h. These parasitized eggs were then incubated at 25 °C and L16:D8 until the next generation emerged. The number of emerged wasps and their sexes were recorded daily. We tested whether the sex ratios differed among the offspring of wasps subject to the two temperature treatments using a *t*-test after arcsine transformation (arcsine $\sqrt{M/F + M \times 100}$, where F is the number of female offspring and M is number of male offspring).

Antibiotic treatment and sex ratio. These experiments sought to determine whether parthenogenesis in TT is associated with a microbe such as *Wolbachia*. An experiment involving four treatments was therefore conducted to observe the effects of antibiotic treatment and mating on the sex ratio of TT. Each treatment consisted of 20 newly emerged females. Males of TT used for mating were obtained by previous tetracycline treatment on mother wasps as mentioned-below. The treatment were: (i) Fifty mg/ml of the antibiotic tetracycline hydrochloride was dissolved in honey solution and a cotton ball containing this antibiotic solution was provided to the newly emerged females for 24 h inside the vial. (ii) Newly emerged females were provided with the antibiotic in honey solution for 24 h and each female was then allowed to mate with one male of TT. The male was then removed and the females were placed individually into the vials. (iii) Newly emerged females were directly allowed to mate with males of TT without antibiotic treatment. (iv) Twenty untreated virgin females were provided as a control. The wasps of the four treatments were individually provided with honey and an egg mass (an excess number of eggs) of *S. litura* and allowed to oviposit for 24 h in the vial. These parasitized eggs were incubated at 25 °C under a L16-D8 cycle until the emergence of wasps. Number of the next generation and their sexes were recorded daily. When a wasp laid on unfertilized eggs of *S. litura* (unfertilized eggs turned brown, became deflated), their offspring failed to emerge; therefore, the data from unfertilized host egg masses were discarded when calculating the mean number of offspring and mean percentage of males.

Crossing experiments. Four crosses and a control were conducted to determine the taxonomic relationship between AT and TT. One virgin female and one virgin male from AT or TT were put together in a vial with diluted honey. The following crosses were made: (i) Twenty untreated AT females that were unmated were placed individually in the vial (=control). (ii) Twenty AT females were mated with AT males. (iii) Twenty AT females were mated with TT males (antibiotic-induced males). (iv) F_1 females from the cross involving AT female × TT male were randomly mated with the F_1 males, and 20 F_1 females randomly selected. (v) Twenty TT females were mated with AT males. The males were removed after mating and the females were placed individually into the vials. Female wasps were provided with honey and an egg mass of *S. litura* and allowed to oviposit for 24 h. These parasitized eggs were incubated at 25 °C under a L16-D8 cycle until the emergence of wasps. The number of offspring and their sexes were recorded daily.

Test for Wolbachia *infection.* We detected *Wolbachia* by amplifying 16S rDNA (99F/994R primers, O'Neill et al., 1992), *ftsZ* gene (513F/1242R primers, Holden et al., 1993; f1/r1 primers, Werren et al., 1995), and *wsp* gene (81F/ 691R primers, Zhou et al., 1998). Two pairs of PCR primers, Adf/Adr and Bf/Br (Werren et al., 1995), were also used to distinguish between the A and B groups of *Wolbachia*. Wasps were individually homogenized in STE buffer (100 mM NaCl, 1 mM EDTA [pH 8.0], 10 mM Tris-HCl [pH 8.0]), and incubated with proteinase K (0.5 mg ml^{-1}) at 37 °C. The homogenates were boiled for three min to inactivate the proteinase K and used for templates of PCR. The PCR cycle consisted of an initial 30 s exposure at 95 °C, followed by 30 cycles each at 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 90 s with a final extension at 72 °C for 5 min. Rickettsial infection was

also examined by a primer pair for citrate synthase of rickettsia (Regnery et al., 1991).

Samples lacking a PCR product of *Wolbachia* genes by agarose gel electrophoresis were further tested using mitochondrial CO1 primers (772 and 773, Navajas et al., 1994) to examine the quality of template solution. The samples showing a DNA products of CO1 were regarded as *Wolbachia* negative and those lacking a DNA product were excluded from the analysis.

Nucleotide sequencing and phylogenetic analysis. The nucleotide sequences of *ftsZ* and *wsp* were determined to examine the phylogenetic affiliation of *Wolbachia*. A *ftsZ* gene was amplified by f1 primer (Werren et al., 1995) and Holden's reverse primer (1242R) (Holden et al., 1993), which amplified a 1110 bp fragment. The *wsp* gene was amplified by *wsp* 81F and *wsp* 691R primers (Zhou et al., 1998). The PCR products were cloned into a manually-made T-vector plasmid of pBluescript II (Stratagene) or pGEM-T Vector (Promega). Sequencing templates were amplified by PCR using M13-20 and reverse primers. The sequences were determined by the Cy5 AutoCycle Sequencing method with DNA sequencer ALF Express (Pharmacia Biotech) or the BigDye Terminator Cycle Sequencing method with a DNA Sequence System (model 377, Perkin Elmer). The sequences were determined for at least three clones.

Nucleotide sequences of *ftsZ* were aligned with CLUSTAL X (Thompson et al., 1994) for phylogenetic analysis and preliminarily analyzed by a neighbor-joining procedure in CLUSTAL X. Since this microbe belonged to the A-group, the *wsp* gene was used for further analysis to observe the precise affiliation among *Wolbachia* belonging to the A-group. The gene sequences were aligned with the clustal algorithm followed by manual modification. Third hypervariable region of the *wsp* gene could not be aligned with certainty, and therefore the region was deleted from the analysis as suggested by Zhou et al. (1998). The resulting alignment contained 570 bases including two outgroups from the B-group of *Wolbachia*. A maximum parsimony analysis, as implemented in PAUP v.4.0b2a (Swofford, 1999), was based on a heuristic search. A bootstrap analysis was performed with 500 replications. The same data set was analyzed by maximum likelihood using PUZZLE v.4.02 (Strimmer & von Haessler, 1996).

Results

High-temperature treatment. After exposing the pupal stage of TT to 35 °C, 15 of 20 females produced offspring, whereas all non-treated females ($N = 20$) produced offspring (Table 1). The mean number of offspring produced by high temperature treated females (4.6±4.4, mean±SD) was significantly smaller than that of the control females (54.8±17.4) ($P<0.0001$, *t*-test). The percentage males in the offspring produced after heat-treatment was 16.4%, whereas the offspring produced by the control females were only 0.1% male. There was a significant difference in the mean percentage of male offspring produced by these two groups ($P<0.01$, *t*-test).

Antibiotic treatment and sex ratio. Antibiotic treatment had a remarkable effect on male production in TT. Male progeny were preliminarily obtained from TT by giving tetracycline with honey to newly emerged females. Table 2 shows the effect of antibiotic treatment and mating on the sex ratio of the progeny. Untreated control females produced exclusively female progeny, with only 0.1% of the offspring male did. The untreated and mated females, however, produced only 0.2% male. Tetracycline-treated females, however, produced many male progeny with and without mating (27.1% and 29.7%). No significant difference was observed in the mean percentage of males between the mating and non-mating females ($P>0.05$, Scheffe's multiple comparisons). There was no significant difference in the mean numbers of offspring produced among the four treatments ($P>0.05$, one-way ANOVA).

Crossing experiments between AT and TT. Untreated virgin females of AT produced only male progeny (100%) (Table 3). Females of AT produced 19.4% and 19.1% males when mated with AT and TT males, respectively. AT females showed no significant difference in the mean percentage of male offspring production regardless of whether the mating partner was AT or TT ($P>0.05$, Scheffe's multiple comparisons). F_1 adults from the crosses between AT females and TT males produced 18.7% males. No biased sex ratio was observed, not even in the F_2 generation. In contrast, TT females mated with AT males produced only 0.3% males and the male percentage was significantly different from that in the other four treatments ($P<0.05$, Scheffe's multiple comparisons).

Table 1. Influence of high-temperature treatment (35 °C) on the sex ratio of offspring produced by *Telenomus* spec.

Treatment	No. of female replicate	No. of females producing offspring	Mean no. of offspring[a]	Mean % of males[b]
High temperature (35 °C)	20	15	4.6 ± 4.4 a	16.4 ± 22.7 a
Control (25 °C)	20	20	54.8 ± 17.4 b	0.1 ± 0.6 b

[a]Mean(±SD) numbers of offspring are significantly different ($P<0.0001$, *t*-test).
[b]Mean(±SD) percentage of males was analyzed after arcsine transformed (see text). Means followed by different letters differ significantly ($P<0.01$, *t*-test).

Table 2. Effects of antibiotic treatment and mating on the sex ratio of *Telenomus* spec.

Treatment	No. of female replicate	No. of host egg masses fertilized	Mean no. of offspring[a,b]	Mean % of males[a,c]
Antibiotic	20	19	49.6 ± 14.9 a	27.1 ± 4.7 a
Antibiotic and mated with male	20	20	44.7 ± 17.0 a	29.7 ± 8.7 a
Untreated and mated with male	20	20	51.4 ± 13.5 a	0.2 ± 0.6 b
Control	20	20	54.8 ± 17.4 a	0.1 ± 0.6 b

[a]The data from the unfertilized host egg mass were excluded in calculating the mean number of offspring and mean percentage of males.
[b]Mean(±SD) numbers of offspring are not significantly different by comparisons at the 5% level by one-way ANOVA.
[c]The mean(±SD) percentage of males was analyzed after arcsine transformation. Means followed by the same letter did not differ at the 5% level by Scheffe's multiple comparisons.

Table 3. Crossing experiment between *Telenomus nawai* (AT) and *Telenomus* spec. (TT)

Crossing combination ♀ × ♂	No. of pairs tested	No. of host egg masses fertilized	Mean no. of offspring[a,b]	Mean% of males[a,c]
AT♀	20	18	47.4 ± 18.1 a	100 a
AT♀ × AT♂	20	20	50.5 ± 16.0 a	19.4 ± 7.3 b
AT♀ × TT♂	20	20	50.9 ± 20.1 a	19.1 ± 11.5 b
F1(AT♀ × TT♂)	20	19	55.1 ± 20.0 a	18.7 ± 5.2 b
TT♀ × AT♂	20	20	51.5 ± 16.4 a	0.3 ± 0.9 c

[a]The data from the unfertilized host egg mass were excluded in calculating the mean number of offspring and mean percentage of males.
[b]Mean (±SD) numbers of offspring did not significantly differ at 5% level by one-way ANOVA.
[c]The mean (±SD) percentage of males was analyzed after arcsine transformation. Means followed by the same letter did not differ at the 5% level by Scheffe's multiple comparisons.

PCR amplification of Wolbachia *genes.* Three genes of *Wolbachia*–16S rDNA, the *ftsZ* gene and the *wsp* gene–were amplified by PCR from TT, whereas AT was negative for these *Wolbachia* genes (data not shown). PCR product was also observed from TT by a primer pair, Adf and Adr, but not by Bf and Br, indicating that *Wolbachia* infecting TT belongs to the A-group (Werren et al., 1995). Primers for citrate synthase of rickettsia did not amplify specific products from TT, suggesting that TT does not harbor microorganisms belonging to the genus *Rickettsia*.

Males and females of the next generation produced after tetracycline-treated females were examined by PCR using 99F/994R primers for 16S rDNA for *Wolbachia* infection. It appeared that 91.7% (33/36) and 41.7% (15/36) of the female and male progeny were infected with *Wolbachia*.

Phylogenetic relationship of Wolbachia *from* Telenomus *spec.* The nucleotide sequence of the *ftsZ* gene of *Wolbachia* from TT was determined (accession number AB037892). A neighbor-joining tree was constructed using 9 and 11 *ftsZ* gene sequences from the *Wolbachia* of the A- and B-groups and the resulting tree was midpoint-rooted. The *Wolbachia* in TT was monophyletic with members of the A-group (data not shown), as already shown by PCR using an A-group-specific *ftsZ* gene primer set. Since the *ftsZ* gene does not show high resolution among the members, the *wsp* gene (accession number AB037893) was used to determine its affiliation in A-group. Twenty-five sequences, including the *Telenomus Wolbachia* gene and two outgroups which belong to the B-group, were aligned and analyzed by maximum parsimony and maximum likelihood algorithms. The branching positions of the *Wolbachia* from *Muscidefurax uniraptor* differed among the analyses, but the overall topology was similar. *Telenomus Wolbachia* was monophyletic with *Glossina austeni Wolbachia* in the maximum likelihood analysis (Figure 1), but the maximum parsimony analysis showed that *G. austeni Wolbachia* branched earlier and *Telenomus Wolbachia* formed a monophyletic group with the *Wolbachia* of *Asobora tabida* 1 and *Trichopria drosophilae*. The quartet puzzling support value of this internal branch of four members, *Wolbachia* from *Telenomus* spec., *G. austeni*, *A. tabida* and *T. drosophilae*, was 62 and the bootstrap value by maximum parsimony analysis in this branch was 98. These four members are phylogenetically closely related.

Discussion

Stouthamer et al. (1990) discovered that antibiotic treatments cause some female parthenogenetic (thelytokous) strains of *Trichogramma* wasps to revert to the production of male progeny. Subsequent work revealed that cytoplasmically inherited bacteria of the genus *Wolbachia* were associated with this form of thelytoky (Stouthamer et al., 1993, Stouthamer & Werren, 1993). *Wolbachia*-associated parthenogenesis has since then been reported in a wide range of hymenopterous families, including *Trichogramma* spp. (Trichogrammatidae) (Stouthamer et al., 1990, 1993; Stouthamer & Werren, 1993), *Muscidifurax uniraptor* (Pteromalidae) (Stouthamer et al., 1993), *Encarsia formosa* (Aphelinidae) (Zchori-Fein et al., 1992; Stouthamer et al., 1993) and *Aphitis linignanensis* (Aphelinidae) (Kajita, 1993; Zchori-Fein et al., 1995). However, it has not yet been detected in the family Scelionidae (Stouthamer, 1997; Cook & Butcher, 1999).

The present results suggest that *Wolbachia* is the causative agent of thelytoky in TT. Males appeared in significant numbers in the offspring following an exposure of pupal stage of TT to a high temperature (35 °C) (Table 1). The mean number of offspring produced by high-temperature-treated females (4.6±4.4, mean±SD) was smaller than that of the control females (54.8±17.4) ($P<0.0001$, *t*-test). This may be due to the high temperature having a harmful effect on the development of reproductive organs in the pupal stage. Antibiotic treatment also produced males in the next generation of the treated TT female (Table 2). Untreated females produced a few males in the offspring (0.1%), but tetracycline-treated females produced many male progeny (27.1%). In contrast to heat treatments, there were no significant differences in the mean number of offspring produced between the treatments. This result and that from the heat treatment strongly suggest that microorganisms infecting TT are the causative agents of parthenogenesis. Some offspring from the treated TT female (27.1%) were unable to develop thelytokously without the aid of the microorganisms, and were reverted to arrhenotoky, resulting in becoming males. The other offspring (72.9%) appear to retain the microorganisms even after the antibiotic treatement and show thelytokous reproduction. Diagnostic PCR indicated that TT was infected with *Wolbachia*, which is known to cause parthenogenesis in some wasp species. However, AT was not infected with *Wolbachia*, and some

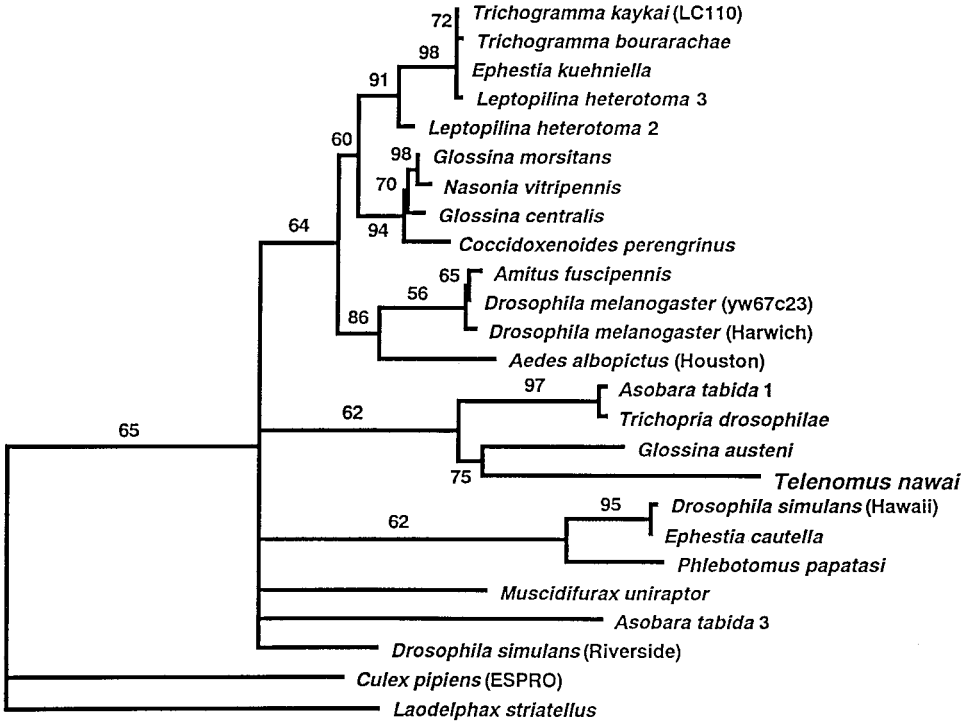

Figure 1. Phylogenetic tree of *Wolbachia* based on sequences of the *wsp* gene. The name of the host species is followed by the strain designation. Maximum likelihood distances were estimated in PUZZLE 4.02 using the '1 invariable + 1 variable 1' model of rate heterogeneity. The Numbers indicate the percentage quartet puzzling. The database accession numbers are as follows; (shown in host species): A-group, AF020058 *Aedes albopictus* (Houston strain), AF020066 *Drosophila melanogaster* (Harwich), AF020068 *Drosophila simulans* (Hawaii), AF020070 *Drosophila simulans* (Riverside), AF020071 *Muscidifurax uniraptor*, AF020072 *Drosophila melanogaster* (yw67c23), AF020075 *Ephestia cautella*, AF020077 *Glossina austeni*, AF020078 *Glossina centralis*, AF020079 *Glossina morsitans*, AF020081 *Nasonia vitripennis*, AF020082 *Phlebotomus papatasi*, AF071909 *Amitus fuscipennis*, AF071910 *Trichopria drosophilae*, AF071911 *Ephestia kuehniella*, AF071912 *Trichogramma kaykai* (LC110), AF071913 *Trichogramma bourarachae*, AF071914 *Coccidoxenoides perengrinus*, AF124856 *Asobara tabida* 1, AF124858 *Leptopilina heterotoma* 2, AF124859 *Asobara tabida* 3, AF124860 *Leptopilina heterotoma* 3, AB037893*Telonomus nawai*; B group, AF020061 *Culex pipiens* (ESPRO), AF020080 *Laodelphax striatellus*.

males from the antibiotic-treated TT were also free of *Wolbachia*. In addition, rickettsia (genus *Rickettsia*), which is tetracycline-sensitive and often found in insects, was not observed in TT. These facts suggest that *Wolbachia* cause parthenogenesis in TT. The *Wolbachia* in TT was phylogenetically related to those in *Asobara tabida*, *Trichorpria drosophilae*, and *Glossina austeni*.

The parasitoid species, *E. formosa* and *M. uniraptor* are known to harbor *Wolbachia* and to reproduce by thelytoky (Zchori-Fein et al., 1992; Stouthamer et al., 1993). However, when antibiotics are fed to these females, the males obtained are not sexually functional and consequently a sexual line can not be established (Zchori-Fein et al., 1992; Van den Assem & Povel, 1973). Stouthamer (1990) reviewed the functionality of heat-induced males in thelytokous species. Such males were generally considered non-functional. Several *Trichogramma* species are, however, able to produce offspring when mated with their conspecific females (e.g., Orphanides & Gonzalez, 1970). Antibiotic-induced males of *Trichogramma* have the ability to produce female offspring (Stouthamer et al., 1990; Stouthamer & Kazmer, 1994).

Males from TT were functionally normal. Males could inseminate AT females and the sperm fertilized eggs. AT females produced 19.4% males in their offspring when mated with AT males (Table 3). This appears to be the result of successful mating and fertilization; arrhenotokous wasps usually show few male offspring after mating. AT females produced 19.1% males when mated with TT males (antibiotic induced). F_1 adults produced 18.7% males. No significant differences were observed in the mean percentage of males in the offspring of these crosses ($P>0.05$, Scheffe's multiple comparisons). This indicates that TT males

successfully inseminated AT females and produced diploid females. If TT males fail to inseminate the AT females or male sperm fails to fertilize the eggs of AT female, all progeny should be male, as shown in the control of Table 3. There is no reproductive barrier between AT females and TT males; therefore, they must be conspecific species. Similar results are reported in the parasitoid species, *Apoanagyrus diversicornis* where males could be generated following antibiotic treatment and females of the arrhenotokous line were successfully fertilized by the antibiotic-cured males (Pijls et al., 1996).

In contrast, TT females did not showed any sign of fertilization. The TT males, which appeared following tetracycline treatment, successfully mated with antibiotic-treated or -untreated females. However, *Wolbachia*-infected females exclusively produced female progeny after mating with the tetracycline-induced males (male ratio = 0.2%, Table 2), suggesting that the eggs developed parthenogenetically and sperm did not participate in egg development (Table 2). If the sperm was functional, male ratio would have been around 20%, as shown in Table 3. A similar result was also observed in the crosses between TT females and AT males (0.3%, Table 3). In the latter case, AT males should produce functional sperm; nevertheless, TT showed a very high female ratio, indicating parthenogenetic reproduction after successful mating. These results suggest that *Wolbachia*-infected females and/or eggs ignore the sperm and begin parthenogenetic reproduction. No participation of sperm in reproduction was also observed in antibiotic-treated TT. The mean percentage of males produced was not significantly different between mated and unmated females (29.7% and 27.1%, $P > 0.05$, Scheffe's multiple comparisons) (Table 2). If sperm participated in reproduction, the male ratio in the offspring would presumably be lower in the mated females than in the unmated ones. Therefore, a certain reproductive barrier still prevents the successful completion of fertilization in females of TT. Since there seems to be a certain barrier for fertilization in TT females, it may not be easy to establish an arrhenotokous colony from TT. More studies on fertilization of *Wolbachia*-infected wasps are required to elucidate the function of parthenogenesis-inducing *Wolbachia*.

Antibiotic treatment in the present study was transient and did not completely eliminate *Wolbachia* from TT females. Some male progeny (15 out of 36) from tetracycline-treated females possessed *Wolbachia*. *Wolbachia* in the mother should be suppressed by tetracycline but not completely eliminated, and a small amount of remaining *Wolbachia* seems to be inherited by the progeny. The amount of inherited *Wolbachia* in the eggs must be too low to express parthenogenesis. In contrast, a few female progeny (3 out of 36) produced from antibiotic-treated virgin females did not possess *Wolbachia*. The reason that females appeared without the *Wolbachia* infection is presently not clear, but there seem to be two explanations. One is that the small amount of *Wolbachia* that could cause gamete duplication failed to further propagate in the female progeny. The other is that the presence of *Wolbachia* in eggs is not a prerequisite to gamete duplication but product(s) of *Wolbachia* are necessary in the eggs for thelytokous production.

Acknowledgements

We thank Dr S. Wakamura, National Institute of Sericultural and Entomological Science (NISES), and Dr K. Yasuda and Mr. Sadoyama, Okinawa Prefectural Agricultural Experiment Station (OPAES), for their kind discussion regarding this work. Thanks also to Mr. H. Shiroma of OPAES and Dr Q. Zhang of NISES for their assistance in the experiments. This work was partly supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences to H.N.

References

Assem, J. van den & G. D. E. Povel, 1973. Courtship behavior of *Muscidifurax* species: A possible example of a recently evolved ethological isolating mechanism. Netherlands Journal of Zoology 23: 465–487.

Cook, J. M. & R. D. J. Butcher, 1999. The transmission and effects of *Wolbachia* bacteria in parasitoids. Researches on Population Ecology 41: 15–28.

Holden, P. R., J. F. Y. Brookfield & P. Jones, 1993. Cloning and characterization of an *ftsZ* homologue from a bacterial symbiont of *Drosophila melanogaster*. Molecular and General Genetics 240: 213–220.

Kajita, H., 1993. Induction of males in the thelytokous wasp *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae). Applied Entomology and Zoology 28: 115–117.

Luck, R. F., R. Stouthamer & L. Nunney, 1992. Sex determination and sex ratio patterns in parasitic hymenoptera. In: D.L.Wrench & M.A.Ebbert (eds), Evolution and Diversity of Sex Ratio in Haplodiploid Insects and Mites. Chapman & Hall, New York, pp. 442–476.

Navajas, M., J. Gutierrez, O. Bonato, H. R. Bolland & S. Mapangou-Divassa, 1994. Intraspecific diversity of the cassava green mite *Mononychellus progresivus* (Acari: Tetranychidae) using comparisons of mitochondrial and nuclear ribosomal DNA

sequences and cross-breeding. Experimental & Applied Acarology 18: 351–360.

O'Neill, S. L., R. Giordano, A. M. E. Colbert, T. L. Karr & H. M. Robertson, 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proceeding of the National Academy of Sciences, USA 89: 2699–2702.

Orphanides, G. M. & D. Gonzalez, 1970. Identity of a Uniparental race of *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae). Annals of the Entomological Society of America, 63: 1784–1785.

Pijls, J. W. A. M., H. J. van Steenbergen & J. J. M. van Alphen, 1996. Asexuality cured: the relations and differences between sexual and asexual *Apoanagyrus diversicornis*. Heredity, 76: 506–513.

Regnery, R. C., C. L. Spruill & B. D. Plikaytis, 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. Journal of Bacteriology 173: 1576–1589.

Stouthamer, R., 1997. *Wolbachia*-induced parthenogenesis. In: S. L. O'Neill, A. A. Hoffman & J. H. Werren (eds), Influential Passengers. Oxford University Press, New York, pp. 102–124.

Stouthamer, R. & D. J. Kazmer, 1994. Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. Heredity 73: 317–327.

Stouthamer, R. & J. H. Werren, 1993. Microbes associated with parthenogenesis in wasps of the genus *Trichogramma*. Journal of Invertebrate Pathology 61: 6–9.

Stouthamer, R., R. F. Luck & W. D. Hamilton, 1990. Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera: Trichogrammatidae) to revert to sex. Proceedings of the National Academy of Sciences, USA 87: 2424–2427.

Stouthamer, R., J. A. J. Breeuwer, R. F. Luck & J. H. Werren, 1993. Molecular identification of microorganisms associated with parthenogenesis. Nature 631: 66–68.

Strimmer, K. & A. von Haessler, 1996. Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. Molecular Biology and Evolution 13: 964–969.

Swofford, D. L., 1999. PAUP: Phylogenetic Analysis using Parsimony, Version 4.0b2, Sinauer Associated, Sunderland, Massachusetts.

Thompson, J. D., D. G. Higgins & T. J. Gibson, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

Werren, J. H., W. Zhang and L. R. Guo, 1995. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proceedings of Royal Entomological Society of London, Series B 261: 55–71.

Zchori-Fein, E., R. T. Rousch & M. S. Hunter, 1992. Male production induced by antibiotic treatment in *Encarsia formosa*, an asexual species. Experientia 48:102–105.

Zchori-Fein, E., O. Faktor, M. Zeidan, Y. Gottlieb, H. Czosnek & D. Rosen, 1995. Parthenogenesis-inducing microorganisms in *Aphitis* (Hymenoptera: Aphelinidae). Insect Molecular Biology 4: 173–178.

Zhou, W., F. Rousset & S. O'Neill, 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proceedings of Royal Entomological Society of London, Series B 265: 509–515.