Heredity (2003) 91, 208–216

www.nature.com/hdy

Wolbachia distribution and cytoplasmic incompatibility based on a survey of 42 spider mite species (Acari: Tetranychidae) in Japan

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Wolbachia are a group of maternally inherited bacteria that infect a wide range of arthropods. *Wolbachia* infections are known to result in the expression of various abnormal reproductive phenotypes, the best known being cytoplasmic incompatibility. The first systematic survey of 42 spider mite species in Japan revealed that seven species (16.7%) were infected with *Wolbachia*. *Wolbachia* in the spider mites were grouped into three subgroups in supergroup B by phylogenetic analyses of the *wsp* gene. Most spider mites did not show cytoplasmic incompatibility when infected males were crossed with uninfected females. However, all infected populations of *Panonychus mori* and *Oligonychus gotohi* (five and four populations, respectively) possessed modification-positive strains of *Wolbachia*, and the cytoplasmic incompatibility decreased egg hatchability and female ratio of the spider mites. Thus, some *Wolbachia* strains cause sex ratio distortion in their hosts.
Heredity (2003) **91,** 208–216. doi:10.1038/sj.hdy.6800329

Keywords: *Wolbachia*; distribution; spider mite; cytoplasmic incompatibility

Introduction

Wolbachia are alpha proteobacteria that infect a wide range of arthropods (Werren *et al*, 1995a; Jeyaprakash and Hoy, 2000) and filarial nematodes (Bandi *et al*, 1998). *Wolbachia* infect at least 16.9% of neotropic insects (Werren *et al*, 1995a), and 19.3% of temperate insect species sampled in North America (Werren and Windsor, 2000). *Wolbachia* infection is very common in some insect groups. For instance, 50% of a sample of 50 Indonesian ant species (Wenseleers *et al*, 1998) and 28.1% of 89 wild-caught mosquito species in Southeast Asia (Kittayapong *et al*, 2000) are infected with *Wolbachia*. Other invertebrates besides insects are commonly infected with *Wolbachia*. A total of 22 out of 85 (26%) species of isopod crustaceans (Bouchon *et al*, 1998) and nine of 10 species of filarial nematodes were found to be infected with *Wolbachia* (Bandi *et al*, 1998). However, none of the mollusk species tested were infected (Schilthuizen and Gittenberger, 1998). *Wolbachia* were subdivided into six supergroups from A to F (Lo *et al*, 2002) based on the *ftsZ* gene sequence (Werren *et al*, 1995b). Arthropods are mainly infected with *Wolbachia* belonging to supergroups A and B, nematodes are infected with supergroups C and D, springtail is infected with supergroup E (Vandekerchove *et al*, 1999) and termites are infected with supergroup F (Lo *et al*, 2002).

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Received 22 April 2003

In Acari (mites), *Wolbachia* have been detected in *Metaseiulus occidentalis* (Nesbitt) and *Tetranychus urticae* Koch (Johanowicz and Hoy, 1996), in a *T. urticae* strain from Athens (Tsagkarakou *et al*, 1996), and in six out of 16 species of spider mites and four out of 11 predatory mites (Breeuwer and Jacobs, 1996). These *Wolbachia* strains belong to the B-supergroup. However, not many species of Acari have been surveyed for *Wolbachia*.

Reproductive alterations by *Wolbachia* are known as cytoplasmic incompatibility, parthenogenesis, feminization and male-killing (O'Neill *et al*, 1997; Stouthamer *et al*, 1999). Among these phenomena, only cytoplasmic incompatibility is known in the spider mites. Breeuwer (1997) first reported cytoplasmic incompatibility in *T. urticae* and *T. turkestani*. The effect of *Wolbachia* on their host mites was then studied by several researchers (Breeuwer, 1997; Gomi *et al*, 1997; Perrot-Minnot and Norton, 1997; Johanowicz and Hoy, 1998, 1999; Gotoh *et al*, 1999a, b; Vala *et al*, 2000). The incompatibility cross in spider mites shows F_1 zygotic mortality among females. Therefore, cytoplasmic incompatibility causes a reduced egg hatchability and a reduced female ratio. In contrast, three *Wolbachia*-infected species of *Tetranychus* did not show cytoplasmic incompatibility (Gomi *et al*, 1997; Gotoh *et al*, 1999a, b).

The aims of this study were to investigate the distribution of *Wolbachia* of the spider mites of Japan, to test the effects of *Wolbachia* on the reproductive traits of Japanese spider mites, and to determine the phylogenetic relationships of the different strains of *Wolbachia*. This is the first systematic survey of *Wolbachia* in spider mites.

Materials and methods

Spider mites collection and rearing

A total of 42 of the 80 known species of spider mites in Japan were examined (Table 1; Ehara, 1999; Ehara and Yamaguchi, 2001; Ehara and Ohashi, 2002). Details of how these populations were collected are available at http://wwwa.agr.ibaraki.ac.jp/~shokubutu/gotoh/Cdata.pdf or from the author. The number of populations in each species used ranged from 1 to 75. Mites were reared on detached common bean leaves (*Phaseolus vulgaris*) or, when available, the original host leaves in a climate-controlled room (25°C, L:D = 16:8, RH 60%).

PCR

Two pairs of *Wolbachia*-specific primers were used to detect the presence of *Wolbachia*. One amplifies a part of the *fts*Z gene (Holden *et al*, 1993) and the other amplifies 16S rDNA (O'Neill *et al*, 1992). PCR templates were made by homogenizing a single female adult in a 25-µl mixture of STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with proteinase K (10 mg/ml, 2 µl). The mixture was incubated at 37°C for 30 min and proteinase K was inactivated at 95°C for 5 min. The sample was briefly centrifuged in a microfuge tube, and used immediately for the PCR reaction or stored at –20°C for later use. All PCR reactions were run in 26.25 µl of buffer: 16 µl H_2O, 2.5 µl 10 × buffer, 1.5 µl dNTP (2.5 mM each), 0.25 µl *Taq* polymerase (1.25 U, TaKaRa r*Taq*, Tokyo), 2 µl sample and 2 µl of primers (20 pmol each). Reactions were cycled 40 times for 30 s at 95°C, for 30 s at 52°C and for 2 min at 72°C. The techniques used here were the same as those used by Gomi *et al* (1997).

Table 1 *Wolbachia* infection in Japanese spider mites[a]

Genus *Species*	N[b]	*Wolbachia*[c] (%)	*Genus* *Species*	N[b]	*Wolbachia*[c] (%)
Aponychus			*suginamensis*	1	0
corpuzae	1	0	*tiliarium*	2	0
firmianae	1	0	*uchidai*	1	0
Panonychus			*uncatus*	2	0
bambusicola	1	0	*Oligonychus*		
citri	72	0	*biharensis*	1	0
mori	52	9.6	*coffeae*	1	0
osmanthi	44	0	*formosanus*	1	0
thelytokus	1	0	*gotohi* (on Pasania)	4	100.0
ulmi	2	0	*gotohi* (on chestnut)	2	50.0
Sasanychus			*ilicis*	1	0
akitanus	2	0	*Amphitetranychus*		
Schizotetranychus			*quercivorus*	2	0
bambusae	1	0	*viennensis*	2	0
cercidiphylli	2	100.0	*Tetranychus*		
leguminosus	2	0	*ezoensis*	1	0
longus	3	0	*kanzawai*	75	29.3[d]
recki	1	0	*ludeni*	4	0
schizopus	2	0	*neocaledonicus*	2	0
Yezonychus			*parakanzawai*	16	31.3[e]
sapporensis	1	0	*phaselus*	1	0
Eotetranychus			*piercei*	2	0
asiaticus	1	0	*pueraricola*	38	47.4
cornicola	1	0	*takafujii*	1	0
dissectus	1	0	*urticae* (green form)	3	66.7[f]
rubricans	1	0	*urticae* (red form)	6	0[f]
smithi	1	0			

[a]*Wolbachia* were detected by PCR using the primers for 16S rDNA (O'Neill *et al*, 1992) and the *ftsZ* gene (Holden *et al*, 1993). A total of 3–20 female adults were tested for each population. [b]Number of populations tested. [c]Percentage of the populations infected with *Wolbachia*. [d]Details of collection for 63 out of 75 populations are given in Gotoh *et al* (1999a; in this paper, *T. kanzawai* was referred to as the T strain of *T. kanzawai*). [e]Details of collection for 11 out of 16 populations are given in Gotoh *et al* (1999a; in this paper, *T. parakanzawai* was referred to as the K strain of *T. kanzawai*). [f]Details of collection for two out of three populations of the green form of *T. urticae* and one out of six populations of the red form of *T. urticae* are given in Gotoh *et al* (1999b).

Sequencing and phylogenetic analysis

The phylogenetic relationship of *Wolbachia* in spider mites was based on the nucleotide sequence of the *wsp* gene. The *wsp* gene was amplified by 81F and 691R primers (Zhou *et al*, 1998). The PCR product was cloned into a pGEM-T Vector (Promega). The template DNA was amplified by PCR using M13-20 and reverse primers. The sequence was determined by the Dye Terminator Sequencing method with a DNA Sequencer (model 377 and 3700, PE Applied Biosystems).

The *wsp* sequences were aligned with the CLUSTAL X program (Thompson *et al*, 1997). Two parts of the variable region were removed and a data set of 494 nucleotide sites was used for the phylogenetic study. A neighbor-joining analysis was performed with CLUSTAL X and a maximum-parsimony analysis was performed based on a heuristic search using PAUP v. 4.0b10 (Swofford, 1999). A bootstrap analysis was performed with 1000 replications.

Cross experiments

Two series of intraspecies crosses were carried out to determine the effects of *Wolbachia* on the reproduction of their hosts. One series of crosses was between a *Wolbachia*-infected population and a *Wolbachia*-uninfected population. The other series of crosses was between a *Wolbachia*-infected population and a *Wolbachia*-infected population that had been cured with antibiotic treatment. Tetracycline was administered to spider mites through the leaves by dissolving it in the water used to moisten the cotton bet under the leaves (Gotoh *et al*, 1995; Gomi *et al*, 1997). Rifampicin was also used for eliminating *Wolbachia* from *Panonychus mori*. The number of eggs laid by females during the first 5 days of oviposition was counted. We designated a crossing pair by the female/male population names. For example, Ami/Tsukuba indicates a cross between an Ami female and a Tsukuba male. In this paper, '$W^-/W^{+\prime}$' means a cross between a *Wolbachia* uninfected female and an infected male. Antibiotic-cured colonies are designated as '$W^=$.'

Females in the teleiochrysalis stage (the final immature stage) obtained from each stock culture were transferred onto a small leaf disc (ca 4 cm^2) with a male adult (1- to 5-day-old) either from the same or a different culture. Males were removed 2 days after adult emergence of the females. After oviposition started, each female was allowed to lay eggs for 5 days and then removed: that is, young mites were used. Eggs on leaf discs were checked daily to determine the hatchability, survival rate and sex ratio (% females). All experiments were carried out at 25°C and L:D = 16:8.

Results

Distribution of *Wolbachia* among mite species

We detected *Wolbachia* in seven (16.7%) of the 42 mite species surveyed (Table 1). The seven species were members of the genera *Panonychus*, *Shizotetranychus*, *Oligonychus* and *Tetranychus*. *Wolbachia* have not yet been found in the genera *Aponychus*, *Sasanychus*, *Yezonychus*, *Eotetranychus* or *Amphitetranychus*.

Five out of 52 populations of *P. mori* were infected with *Wolbachia*. However, *Wolbachia* were not found in 72 populations of *P. citri* or in 44 populations of *P. osmanthi*. Two populations in *Schizotetranychus cercidiphylli* possessed *Wolbachia*. Both the chestnut and *Pasania* populations of *Oligonychus gotohi* were infected with *Wolbachia*. The infection frequencies of two closely related species, *T. kanzawai* and *T. parakanzawai*, were similar (29.3 and 31.3%, respectively). Previously, 14 out of 63 populations of *T. kanzawai* and four out of 11 populations of *T. parakanzawai* were found to be infected with *Wolbachia* (Gotoh *et al*, 1999a; in this paper, *T. kanzawai* and *T. parakanzawai* were referred to as the T and K strains of *T. kanzawai*, respectively). In the present study, we included an additional eight infected populations of *T. kanzawai* and one additional infected population of *T. parakanzawai*. We detected *Wolbachia* in two of three populations of the green form of *T. urticae*, one of which was reported previously (Gotoh *et al*, 1999b). On the contrary, we failed to detect *Wolbachia* in six populations of the red form of *T. urticae*. Out of 38 populations of *T. pueraricola*, which morphologically resembles *T. urticae*, 18 were infected with *Wolbachia*. Thus, 59 (16.3%) of the 362 populations examined were infected with *Wolbachia*.

Wolbachia effects on spider mites

We carried out crossing experiments and observed the effects of *Wolbachia* infection on egg hatchability, survival rate and sex ratio in all but one *Wolbachia*-infected species. The exception was *S. cercidiphylli*, which did not complete development because leaf discs of its host plant, *Cercidiphyllum japonicum*, swiftly deteriorated as a result of the tetracycline treatment. Two mite species, *P. mori* and *O. gotohi* (on *Pasania*), showed cytoplasmic incompatibility and four *Tetranychus* species did not show any reproductive abnormalities.

Effects on *P. mori*: *Wolbachia*-infected Toyama populations of *P. mori* showed unidirectional cytoplasmic incompatibility when females cured with rifampicin were crossed with infected males ($To^{=}/To^{+}$) (Table 2). The hatchability was quite normal, but cytoplasmic incompatibility was revealed by the male-biased sex ratio of the offspring. The Toyama population was infected with a modification-positive strain (CI-*Wolbachia*). Incompatibility was also observed in intrapopulation crosses between infected males and antibiotic-treated females in three other populations, Hanayama, Tsuruoka and Haruno, but not in the Sendai population (data not shown). Some local populations were also crossed, but the results were not simple. The spider mites had, in addition to *Wolbachia*-mediated CI, some inherent reproductive incompatibilities that appear to affect the reproductive biology among the populations (unpublished data).

Table 2 Compatibility of crosses between *Wolbachia*-infected (To^{+}) and antibiotic-treated ($To^{=}$) colonies of the Toyama population of *Panonychus mori*

Cross			N[a]	*No. of eggs/ female*	*Hatchability (%)*	*Survival rate in immature stages (%)*	*% Female offspring*
Female	×	*Male*					
To^{+}	×	To^{+}	15	32.0±0.95	97.9±0.50	97.8±0.63	81.6±4.88 c
To^{+}	×	$To^{=}$	24	32.6±1.40	96.8±0.52	96.5±0.68	71.9±1.03 b
$To^{=}$	×	To^{+}	15	30.3±1.60	96.5±0.35	98.8±0.59	25.5±1.39 a
$To^{=}$	×	$To^{=}$	24	34.0±1.28	98.8±0.42	97.4±0.55	72.2±0.94 b
χ^2-value[b]				5.508 ns	6.431 ns	4.128 ns	50.842***

[a]Number of females tested. [b]Means (±SE) differ significantly at $P<0.001$ (***) (Kruskal–Wallis test); ns, not significant at $P>0.05$. The data of % female offspring followed by different letters are significantly different at $P<0.05$ (Scheffé's test). Arcsin-transformed values were used for analyzing the percentages of egg hatchability, survival rate and % female offspring.

Effects on *O. gotohi* from *Pasania*: The populations of *O. gotohi* collected from *Pasania*, at four different locations, Futtsu (Fu^{+}), Kasumigaura (Ka^{+}), Ami (Am^{+}) and Hasaki (Ha^{+}), were all infected with *Wolbachia*. Therefore, members from the infected original populations were crossed with antibiotic-cured colonies (Table 3). We observed unidirectional cytoplasmic incompatibility between tetracycline-treated females and infected males. A male-biased sex ratio was observed in all incompatible crosses, $Fu^{=}/Fu^{+}$, $Fu^{=}/Ka^{+}$, $Fu^{=}/Am^{+}$ and $Fu^{=}/Ha^{+}$, and significantly reduced hatchability in the former three crosses. On the other hand, crosses among four *Wolbachia*-infected populations (Ka^{+}/Fu^{+}, Fu^{+}/Ka^{+}, Fu^{+}/Am^{+} and Fu^{+}/Ha^{+}) did not cause any cytoplasmic incompatibility, indicating that the *Wolbachia* infecting these mites were the same strain.

Effects on *O. gotohi* from chestnut: In contrast, *O. gotohi* collected from chestnuts did not show cytoplasmic incompatibility. Two populations, one infected (Ami, Am^{+}) and the other uninfected (Tsukuba, Ts^{-}), were crossed, but no difference was found in the number of eggs, hatchability, survival rate in immature stages or female ratio among four crosses: Am^{+}/Am^{+}, Ami^{+}/Ts^{-}, Ts^{-}/Am^{+}, and Ts^{-}/Ts^{-}(Table 4). As *O. gotohi* on chestnuts was reproductively isolated from the same species on *Pasania* (Gotoh, unpublished data), populations from different host plants were not crossed.

Effects on *T. kanzawai*: For *T. kanzawai*, we previously reported that *Wolbachia* strains from 14 populations in Japan were all modification-negative or did not cause CI (Gomi *et al*, 1997; Gotoh *et al*, 1999a; in these papers, *T. kanzawai* was referred to as the T strain of *T. kanzawai*). This time, we tested another eight *Wolbachia*-infected strains: Tsukuba, Kimitsu, Haibara, Miza, Takeyano, Shidoshi, Sumiyoshi and Miyanoura. The males of the eight *Wolbachia*-infected populations of *T. kanzawai* were crossed with females of either the *Wolbachia*-infected population (Ka^{+}) or the tetracycline-treated Kanaya population ($Ka^{=}$) to clarify whether the *Wolbachia* strains infecting these populations are modification-positive or -negative. The cross between $Ka^{=}$ females

Table 3 Compatibility in crosses among *Wolbachia*-infected populations (Fu^+, Ka^+, Am^+ or Ha^+) and tetracycline-treated Futtsu population ($Fu^=$) of *Oligonychus gotohi* on *Pasania*

Cross[a]			N[b]	No. of eggs/female	Hatchability (%)	Survival rate in immatures (%)	% Female offspring
Female	×	Male					
Fu^+	×	Fu^+	15	13.9±0.71 a	96.0±1.66 a	86.1±1.93	74.7±2.46 a
Fu^+	×	$Fu^=$	16	12.9±0.52 a	92.6±1.70 a	81.0±1.93	74.0±1.96 a
$Fu^=$	×	Fu^+	19	14.3±0.56 a	59.6±3.51 c	87.4±2.13	27.7±5.38 b
$Fu^=$	×	$Fu^=$	17	13.7±0.49 a	96.5±1.27 a	81.7±2.10	70.3±1.60 a
Ka^+	×	Ka^+	19	15.0±0.67 a	97.2±0.85 a	85.6±2.08	74.7±1.45 a
Ka^+	×	Fu^+	14	15.4±0.66 a	94.9±1.87 a	85.7±2.55	74.6±2.38 a
Fu^+	×	Ka^+	14	13.6±0.57 a	91.8±1.95 a	85.0 ±2.77	73.1±1.90 a
Ka^+	×	$Fu^=$	18	13.7±0.65 a	96.6±1.29 a	91.8±1.95	72.2±1.67 a
$Fu^=$	×	Ka^+	17	12.1±0.59 a	56.4±5.03 c	85.8±3.07	33.8±4.68 b
Fu^+	×	Am^+	13	12.9±0.58 a	93.0±2.19 a	82.1±2.48	72.3±1.51 a
$Fu^=$	×	Am^+	15	13.1±0.61 a	64.6±5.82 c	84.2±2.59	35.6±6.79 b
Fu^+	×	Ha^+	15	13.0±0.60 a	89.4±2.59 ab	87.5±2.17	72.6±1.74 a
$Fu^=$	×	Ha^+	15	11.9±0.47 a	71.8±3.81 bc	84.3±2.60	45.8±5.81 b
χ^2-valve[c]				26.231*	120.731***	11.229 ns	127.010***

[a]Fu: Futtsu, Chiba; Ka: Kasumigaura, Ibaraki; Am: Ami, Ibaraki; Ha: Hasaki, Ibaraki. [b]Number of females tested. [c]Means (±SE) differ significantly at $P<0.001$ (***) and $P<0.05$ (*) (Kruskal–Wallis test); ns: not significant at $P>0.05$. Values in a column followed by different letters are significantly different at $P<0.05$ (Scheffé's test).

Table 4 Compatibility of crosses between the *Wolbachia*-infected Ami population (Am^+) and the *Wolbachia*-uninfected Tsukuba population (Ts^-) in *Oligonychus gotohi* on chestnut

Cross[a]			N[b]	No. of eggs/females	Hatchability (%)	Survival rate in immatures (%)	% Female offspring
Female	×	Male					
Am^+	×	Am^+	15	26.4±0.86	96.8±0.84	96.0±1.06	76.9±1.75
Ts^-	×	Ts^-	17	26.4±0.56	96.4±0.67	96.4±0.99	79.3±1.66
Am^+	×	Ts^-	21	25.1±0.61	96.5±0.78	94.8±1.16	75.3±1.51
Ts^-	×	Am^+	15	26.0±0.91	96.7±0.70	94.0±1.21	75.5±1.83
χ^2-value[c]				3.142 ns	0.926 ns	2.199 ns	3.110 ns

[a]Am: Ami, Ibaraki; Ts: Tsukuba, Ibaraki. [b]Number of females tested. [c]Means (±SE) are not significantly different at $P>0.05$ (Kruskal–Wallis test); ns: not significant.

and the infected males of the test population did not result in an egg hatchability or sex ratio that was significantly different from those obtained from a cross between Ka^+ females and the infected males (Table 5).

Effects on *T. parakanzawai*: Four populations of *T. parakanzawai* were previously found to harbor a modification-negative strain of *Wolbachia* (Gotoh *et al*, 1999a; in this paper, *T. parakanzawai* was referred to as the K strain of *T. kanzawai*). The *Wolbachia*-infected Futtsu population of *T. parakanzawai* was newly examined here. Mites of the Futtsu population (Fu^+) were crossed with those of the *Wolbachia*-free Ami (Am^-) population, and hatchability and the sex ratio of the next generation were compared among the four crosses (Table 6). No significant differences were found in the crosses, indicating that *Wolbachia* in *T. parakanzawai* did not have cytoplasmic incompatibility.

Effects on *T. pueraricola*: A total of 18 *Wolbachia*-infected populations of *T. pueraricola* were crossed with the *Wolbachia*-free Ohta population. Reciprocal crosses between females of the test population and Ohta males and between Ohta females and males of the test population were similar with respect to hatchability, survival rate in immature stages and female ratios (Figure 1). Thus, the reciprocal crosses produced essentially the same results with some exceptions. When *Wolbachia*-free Ohta males were mated with Namerikawa (Nm), Ikaruga (Ik) or Ube (Ub) females, the female ratio was significantly lower and Ikaruga females did not produce any females by mating with Ohta males (Figure 1). Although a reduced sex ratio resulted from these crosses, it did not appear to be due to *Wolbachia*-induced CI. Similar results were obtained from crosses using antibiotic-treated females, suggesting that there is a nucleus-to-nucleus incompatibility in these mating combinations (unpublished observation). For example, the cross between tetracycline-treated Ik females and Ohta males produced no female offspring with normal values in hatchability (97.4±0.50 (SE), $n=13$) and survival rate (94.2±0.94).

Effects on *T. urticae*: One *Wolbachia*-infected population in the green form of *T. urticae* (Kitsuregawa population) was found to harbor a modification-negative *Wolbachia* (Gotoh *et al*, 1999b). In another population (Yasato, Ya^+), a *Wolbachia*-free colony was established by tetracycline treatment, and four combinations of crosses were observed between *Wolbachia*-infected and -free colonies. No differences were observed in hatchability, survival rate in immature stages or sex ratio among the four crosses (Table 7). As was observed to be the case in the Kitsuregawa population, *Wolbachia* in the Yasato population did not cause CI.

Phylogenetic relationships

Nucleotide sequences of *wsp* genes of *Wolbachia* were determined for 21 mite populations (five *P. mori* populations, two *S. cercidiphylli* populations, two *O. gotohi* populations, four *T. kanzawai* populations, two *T. para-*

Table 5 Compatibility of crosses between males of each *Wolbachia*-infected local population and females of either *Wolbachia*-infected Kanaya population (Ka^+) or tetracycline-treated Kanaya population ($Ka^=$) in *Tetranychus kanzawai*

Cross[a]			N[b]	*No. of eggs/females*[c]		*Hatchability (%)*[c]		*Survival rate in immatures (%)*[c]		*% Female offspring*[c]	
Female	×	*Male*									
Ka^+	×	Ts^+	12	25.5±2.37		96.0±0.93		96.2±0.90		82.3±1.07	
					**		ns		ns		*
$Ka^=$	×	Ts^+	9	36.7±2.72		96.2±1.04		99.1±1.17		86.8±1.10	
Ka^+	×	Ki^+	10	26.6±2.71		97.3±0.70		96.8±0.88		81.6±1.44	
					***		ns		*		ns
$Ka^=$	×	Ki^+	12	45.1±1.98		96.8±0.72		98.9±0.34		81.2±1.38	
Ka^+	×	Ha^+	18	38.3±1.20		98.1±0.67		94.8±1.16		77.9±0.90	
					ns		ns		ns		ns
$Ka^=$	×	Ha^+	16	35.2±1.12		97.2±0.52		92.8±1.32		75.6±1.11	
Ka^+	×	Mz^+	11	36.7±1.40		97.8±0.49		99.2±0.43		75.7±2.05	
					**		ns		ns		ns
$Ka^=$	×	Mz^+	11	44.5±1.83		97.2±0.61		99.2±0.35		78.8±2.46	
Ka^+	×	Ta^+	12	32.6±0.66		98.2±0.59		99.5±0.33		76.8±1.05	
					ns		ns		ns		ns
$Ka^=$	×	Ta^+	9	32.0±0.58		98.9±0.76		97.9±0.74		78.0±1.58	
Ka^+	×	Sh^+	11	40.2±1.08		97.4±0.77		97.5±0.32		72.1±2.34	
					***		ns		ns		**
$Ka^=$	×	Sh^+	11	30.1±0.96		98.5±0.51		97.8±0.68		82.1±1.65	
Ka^+	×	Su^+	13	34.8±2.01		96.6±0.92		97.8±0.61		82.8±0.95	
					ns		ns		ns		ns
$Ka^=$	×	Su^+	11	33.9±1.40		95.7±1.15		98.0±0.49		83.5±1.26	
Ka^+	×	Mi^+	10	34.4±1.98		96.5±1.11		92.6±0.82		84.4±1.11	
					**		ns		*		ns
$Ka^=$	×	Mi^+	13	44.4±1.96		98.7±0.39		98.9±0.45		83.9±1.03	

[a]Ka: Kanaya, Shizuoka; Ts: Tsukuba, Ibaraki; Ki: Kimitsu, Chiba; Ha: Haibara, Shizuoka; Mz: Miza, Kagoshima; Ta: Takeyano, Kagoshima; Sh: Shidoshi, Kagoshima; Su: Sumiyoshi, Kagoshima; Mi: Miyanoura, Kagoshima. [b]Number of females tested. [c]Means (±SE) differ significantly at $P<0.001$ (***), $P<0.01$ (**) and $P<0.05$ (*); ns: not significantly different at $P>0.05$ (Mann–Whitney *U*-test).

Table 6 Combatibility of crosses between *Wolbachia*-infected Futtsu population (Fu^+) and *Wolbachia*-uninfected Ami population (Am^-) in *Tetranychus parakanzawai*

Cross[a]			N[b]	*No. of eggs/ females*	*Hatchability (%)*	*Survival rate in immatures (%)*	*% Female offspring*
Female	×	*Male*					
Fu^+	×	Fu^+	15	37.5±1.44 ab	94.4±0.75 c	97.6±0.80	82.1±1.38
Fu^+	×	Am^-	12	34.8±2.25 b	96.6±0.93 bc	97.9±1.01	83.4±1.44
Am^-	×	Fu^+	15	37.7±1.87 ab	98.0±0.58 ab	97.6±1.46	81.9±1.22
Am^-	×	Am^-	34	43.9±1.24 a	99.2±0.37 a	97.8±0.66	82.7±0.47
χ^2-value[c]				15.058**	28.493***	2.846 ns	1.996 ns

[a]Fu^+: Futtsu, Chiba; Am: Ami, Ibaraki. [b]Number of females tested. [c]Means (±SE) differ significantly at $P<0.001$ (***) and $P<0.01$ (**) (Kruskal–Wallis test); ns: not significant at $P>0.05$. Values in a column followed by different letters are significantly different at $P<0.05$ (Scheffé's test).

kanzawai populations, four *T. pueraricola* populations and two *T. urticae* populations). The *wsp*, 81F and 691R primer pairs amplified 555-, 552- and 549-bp DNA fragments (not including the primer sequences). Phylogenetic analyses of *Wolbachia* based on the *wsp* gene sequences indicated that all *Wolbachia* strains in spider mites from Japan (21 populations) and from two other regions belonged to supergroup B (Figure 2) (Werren *et al*, 1995b). All nine *Wolbachia* strains that possess the 555-bp size gene belonged to the Con subgroup and 10 *Wolbachia* strains of the 552-bp size gene were members of the Ori subgroup (see Zhou *et al*, 1998; van Meer *et al*, 1999). Two *Wolbachia* strains, which were found in *O. gotohi* and had the 549-bp size gene, formed a new and distinct group in the phylogenetic tree. This group was named the Epo subgroup (after *Acraea eponina*). The *Wolbachia* strains in the spider mites formed six clades within the B supergroup: three in the Con subgroup, two in the Ori subgroup and one in the Epo subgroup (Figure 2). Parsimony analysis also supported the six clades of *Wolbachia* (data not shown).

The CI-*Wolbachia* strains in the Sendai, Toyama and Hanayama populations of *P. mori* had identical *wsp* gene sequences and were placed in the Ori subgroup. The *wsp* sequence was the same as the sequences in two populations of *T. kanzawai* and three populations of *T. pueraricola*. Thus, this *wsp* sequence was shared by *Wolbachia* from eight Japanese populations of *P. mori*, *T. kanzawai* and *T. pueraricola*. It is also identical to the

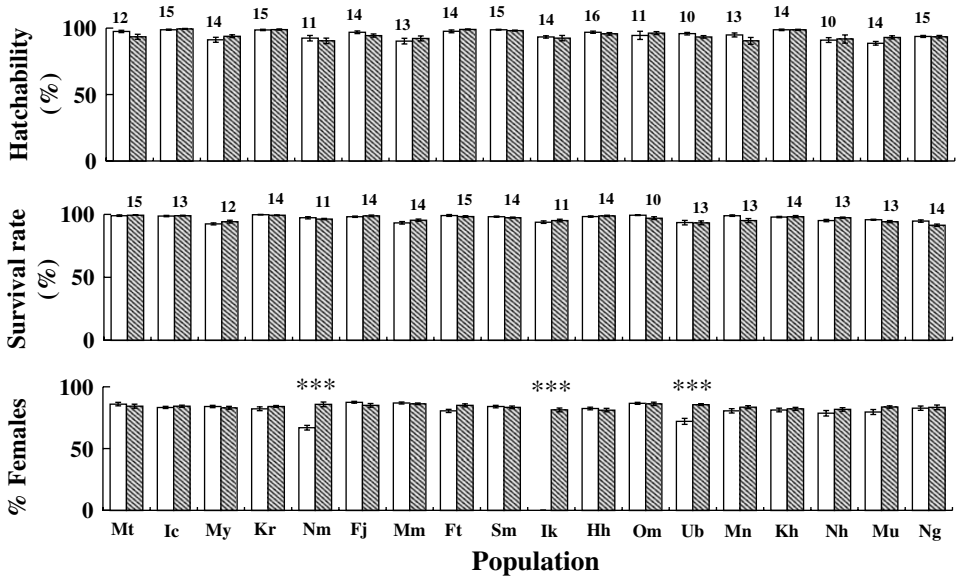

Figure 1 Hatchability, survival rate in immature stages and sex ratio (% females) in crosses between the *Wolbachia*-free Ohta population and the *Wolbachia*-infected local populations in *Tetranychus pueraricola*. Open bars indicate crosses between females of each test populations and males of the Ohta population, and hatched bars indicate the reciprocal crosses. Numbers on the bars indicate the number of pairs tested. Vertical lines indicate the standard error. Mt: Matsuo, Iwate; Ic; Ichinoseki, Iwate; My: Matsuyama, Yamagata; Kr: Kiryu, Gunma; Nm: Namerikawa, Toyama; Fj: Fujioka, Tochigi; Mm: Matsumoto, Nagano; Ft: Futtsu, Chiba; Sm: Shimada, Shizuoka; Ik: Ikaruga, Nara; Hh: Higashi-Hiroshima, Hiroshima; Om: Onomichi, Hiroshima; Ub: Ube, Yamaguchi; Mn: Mononobe, Kohchi; Kh: Kahoku, Kohchi; Nh: Nishihara, Kumamoto; Mu: Miyanoura, Kagoshima; Ng: Nagata, Kagoshima.

Table 7 Compatibility of crosses between *Wolbachia*-infected (Ya^+) and tetracycline-treated (Ya^-) colonies of Yasato population in *Tetranychus urticae*

Cross[a]			N[b]	*No. of eggs/ females*	*Hatchability (%)*	*Survival rate in immatures (%)*	*% Female offspring*
Female	×	*Male*					
Ya^+	×	Ya^+	13	53.3±1.33	96.6±1.06	97.8±0.81	67.9±1.58
Ya^+	×	Ya^-	15	50.0±1.77	95.8±0.81	96.7±1.34	70.3±1.42
Ya^-	×	Ya^+	13	49.8±2.23	97.4±0.36	98.1±0.84	72.6±1.84
Ya^-	×	Ya^-	11	51.5±2.09	96.8±0.83	98.0±1.26	72.6±1.28
χ^2-value[c]				2.468 ns	1.237 ns	0.961 ns	5.220 ns

[a]Ya: Yasato, Ibaraki. [b]Number of females tested. [c]Means (±SE) are not significantly different at $P>0.05$ (Kruskal–Wallis test).

sequence of *Wolbachia* in *T. urticae* in the United States (AF217719), which has a length of more than 536 bp, and differs by one nucleotide from the sequence in the European population of *T. urticae* (AJ437288) (AF217719 is not shown in Figure 2 because of its shorter length). The *wsp* sequence of *T. urticae* (Yasato and Kitsuregawa populations) was identical to that of *T. urticae* from Europe (AF404766). The *wsp* sequences of mite *Wolbachia* in the Ori subgroup are similar. At most, the sequences in the Japanese, European and American populations differ from each other by three nucleotides.

Discussion

In our survey of 42 species and 362 populations of spider mites from Japan, the frequency of infected species was 16.7% (seven of 42 species). For most species (36/42), we tested more than 10 individuals for *Wolbachia* infection. Similar frequencies of infection have been reported for other arthropods (eg Werren *et al*, 1995a (16.9%); West *et al*, 1998 (21.7%); Werren and Windsor, 2000 (19.3%); Jiggins *et al*, 2001 (16.7%)). However, Breeuwer and Jacobs (1996) detected *Wolbachia* in six out of 16 species (37.5%). Among mites of the genus *Tetranychus*, they detected *Wolbachia* in four out of 12 species (33.3%). We also detected *Wolbachia* at a high rate (40%; 4/10) in the genus *Tetranychus*. Our data revealed that the infection rate in genera other than *Tetranychus* was low (9.4%; 3/32). *Wolbachia* was unequally distributed among the genera of the spider mites. No *Wolbachia* infection was found in *Aponychus*, *Sasanychus*, *Yezonychus*, *Eotetranychus* or *Amphitetranychus*, although the number of the test species in these genera is not sufficient to say that these genera are *Wolbachia* free. Unequal distributions were also observed within a single genus. For example, we did not find *Wolbachia* in *P. citri* or *P. osmanthi*, even though 72 populations of the former and 44 populations of the latter were tested. Only one species of *Panonychus*, *P. mori*, was infected with *Wolbachia*.

The phylogenetic analysis of *wsp* genes revealed that *Wolbachia* in the spider mites in Japan belong to the subgroups Ori, Con and Epo in the supergroup B (Figure 2). The Ori and Con subgroups were first proposed by Zhou *et al* (1998) based on the *wsp* gene phylogeny. Zhou *et al* (1998) proposed that members of any subgroup should have a similarity in the *wsp* sequence greater than 97.5%. Therefore, we now propose a new subgroup, the Epo subgroup, named after the host species *Acraea eponina* (accession number AJ271194), which was the first member of this subgroup to be deposited in the database. The *Wolbachia* strains in

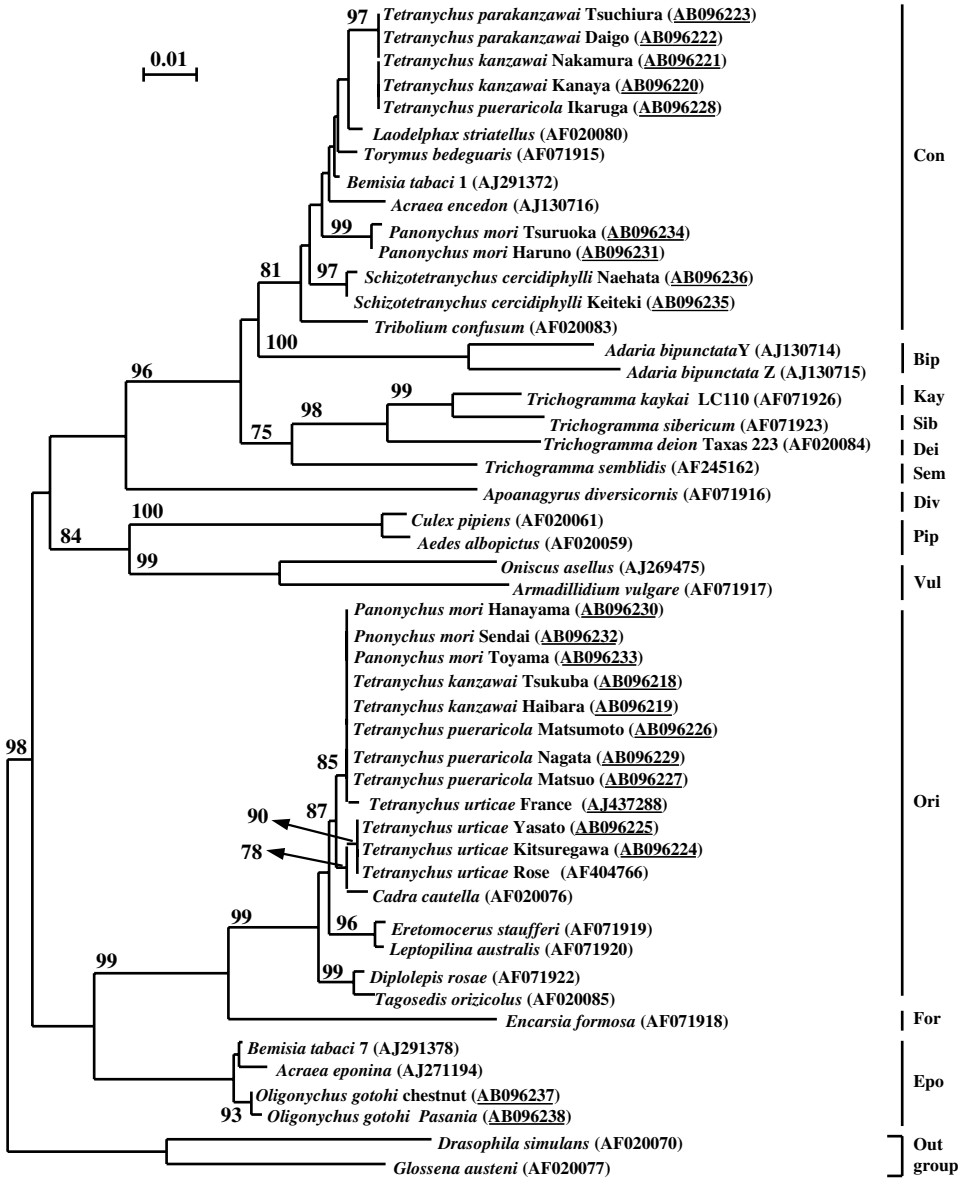

Figure 2 Phylogenetic tree based on *wsp* sequences of *Wolbachia*, constructed by a neighbor-joining procedure with Clustal X. Accession numbers with underlines indicate *Wolbachia* strains of the spider mites examined in this study. *Wolbachia* strains are indicated by the host name. DNA database accession numbers are shown after the host name in parentheses. Numbers on the nodes indicate bootstrap percent confidence values (more than 70). The tree, restricted to the B supergroup of *Wolbachia*, was rooted from two outgroups: *Wolbachia* from *Glossina austeni* (AF020077) and *Drosophila simulans* (Riverside) (AF020070).

O. gotohi belong to the subgroup Epo, and are distantly related to other *Wolbachia* strains in spider mites. *Wolbachia* in *S. cercidiphylli* belongs to the subgroup Con, and the strains in *T. urticae* including the European populations belong to the subgroup Ori. *Wolbachia* in the other four species among the populations were separated into the subgroups Con and Ori. These results suggest that *Wolbachia* in the spider mites originated from a few or several ancestral strains and have been inherited for many generations of the spider mites. As has been pointed out by many authors (eg Werren *et al*, 1995b; van Meer *et al*, 1999), acquisition of *Wolbachia* in the spider mites appears to be due to horizontal infection, because the phylogeny of *Wolbachia* is not concordant with that of the host species, and the same mite species had different *Wolbachia* strains. Identical nucleotide sequences of *wsp* genes were detected in eight populations from three species in the subgroup Ori, suggesting recent horizontal transmission of *Wolbachia* among the spider mites. As was pointed out by Jiggins *et al* (2002), *Wolbachia* are more likely to move horizontally within the host groups than between distantly related hosts. Horizontal transmission is considered to be caused by parasites or parasitoids (Heath *et al*, 1999; Huigens *et al*, 2000; Noda *et al*, 2001b), but it is unknown how the bacteria have spread among the spider mites.

An important result of the phylogenetic tree is that the same species possess the same strain or a very close strain of *Wolbachia* despite the location of the host mites. Closely related strains of *Wolbachia* were found in

T. urticae of the European and Japanese populations. Based on the *wsp* gene sequences, the same *Wolbachia* strain was detected in the Sendai, Toyama and Hanayama populations of *P. mori*. These observations indicate that different species of spider mites are associated with specific strains of *Wolbachia*. One possible explanation of this phenomenon is that the relationship between the mites and *Wolbachia* is old and *Wolbachia* have been inherited from many generations in the same species. This means *Wolbachia* have spread geographically through the parasite population. Another possible explanation is that the infection comes from parasites of spider mites, because *Wolbachia*-infected populations distribute patchily and do not show any continuous distribution even on cultivated crops, which is different from the continuous distribution caused by spreading of *Drosophila simulans* (Turelli and Hoffmann, 1991) or *Laodelphax striatellus* (Noda *et al*, 2001b). That is, a parasite that is specific to a given species of spider mite might carry a specific strain of *Wolbachia*. However, the latter explanation needs further evidence that similar parasites with the same *Wolbachia* strain attack the spider mites in Japan and Europe. *Wolbachia* is distributed among the spider mites by horizontal and vertical transmissions, although it is hard to fully explain how this transmission occurs.

Cytoplasmic incompatibility in spider mites was first reported in *T. urticae* (Breeuwer, 1997). However, Japanese *T. urticae* or *T. kanzawai* did not show cytoplasmic incompatibility (Gomi *et al*, 1997; Gotoh *et al*, 1999a, b). These two species shared an identical *wsp* sequence with *P. mori*, which showed cytoplasmic incompatibility. Although cytoplasmic incompatibility may require a certain density of *Wolbachia* rather than just the presence of *Wolbachia* (Noda *et al*, 2001a), our preliminary real-time PCR studies did not support the different density effect of *Wolbachia* between *P. mori* and *Tetranychus* species. There are two possible explanations for the phenomenon that *Wolbachia* of indistinguishable or the closely related gene sequences show different phenotypes. One is that the expression of cytoplasmic incompatibility depends on the mite strains that are hosts of *Wolbachia*. Another one is that *Wolbachia* have recently changed in phenotype, which is suggested by Jiggins *et al* (2002). This phenotypic change in *Wolbachia* may support the difference in cytoplasmic incompatibility phenotype of *T. urticae* between Europe and Japan.

Acknowledgements

We thank K Iwadate and S Kawai of Ibaraki University for their technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research (nos. 09660040 and 12460019) from the Ministry of Education, Science, Sports and Culture of Japan.

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