Bulletin of Entomological Research (2010) **100**, 231–239
doi:10.1017/S000748530999023X

First published online 24 August 2009

Superinfection of five *Wolbachia* in the alnus ambrosia beetle, *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae)

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Abstract

Wolbachia bacteria are among the most common endosymbionts in insects. In *Wolbachia* research, the *Wolbachia* surface protein (*wsp*) gene has been used as a phylogenetic tool, but relationships inferred by single-locus analysis can be unreliable because of the extensive genome recombination among *Wolbachia* strains. Therefore, a multilocus sequence typing (MLST) method for *Wolbachia*, which relies upon a set of five conserved genes, is recommended. In this study, we examined whether the alnus ambrosia beetle, *Xylosandrus germanus* (Blandford), is infected with *Wolbachia* using *wsp* and MLST genes. *Wolbachia* was detected from all tested specimens of *X. germanus* ($n = 120$) by *wsp* amplification. Five distinct sequences (i.e. five alleles) for *wsp* were found, and labeled as *w*Xge1–5. MLST analysis and molecular phylogeny of concatenated sequences of MLST genes identified *w*Xge3 and *w*Xge5 as closely-related strains. The detection rate of *w*Xge4 and *w*Xge1 was 100% and 63.3%, respectively; *w*Xge2, *w*Xge3 and *w*Xge5 were detected from less than 15% of specimens. We performed mitochondrial haplotype analyses that identified three genetic types of *X. germanus*, i.e. Clades A, B and C. *Wsp* alleles *w*Xge1, *w*Xge2 and *w*Xge4 were detected in all clade A beetles; *w*Xge2 allele was absent from Clades B and C. We concluded that (i) five *wsp* alleles were found from *X. germanus*, (ii) use of MLST genes, rather than the *wsp* gene, are more suited to construct *Wolbachia* phylogenies and (iii) *wsp* alleles *w*Xge2 and *w*Xge3/*w*Xge5 would infect clade A and clade B/C of *X. germanus*, respectively.

Keywords: endosymbiotic bacteria, Scolytinae, multilocus sequence typing method, multiple infections, infection polymorphism

(Accepted 21 May 2009)

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Introduction

Wolbachia is an intracellular alpha-proteobacterium that infects an estimated 66% of arthropod species (Hilgenboecker *et al*., 2008). *Wolbachia* are vertically transmitted from parents to offspring only through the egg cytoplasm. In order to enhance maternal transmission, *Wolbachia* manipulates host reproduction by inducing cytoplasmic incompatibility (CI), male-killing, parthenogenesis and feminization of genetic males (O'Neill *et al*., 1997).

Previous studies have reported different sequence types (i.e. alleles) for the *wsp* gene within a host species. Some host species are often detected with two or more alleles of a *Wolbachia* gene, referred to as multiple infections. Double or triple infection in a single host species are well documented (Breeuwer *et al*., 1992; Vavre *et al*., 1999; Kondo *et al*., 2002a). Detection of four or more alleles (especially defined as superinfections here) are reported in a few insects, such as ants, fruit flies and beetles (Malloch *et al*., 2000; Jamnongluk *et al*., 2002; Reuter & Keller, 2003; Dedeine *et al*., 2005). Superinfection is caused by frequent occurrence of horizontal transmission of *Wolbachia* from other host species (Jamnongluk *et al*., 2002), mutation of *Wolbachia* genome (Malloch *et al*., 2000) and/or recombination between different *Wolbachia* strains co-existing in a single individual (Reuter & Keller, 2003). Genetic recombination confounds phylogenetic analyses of *Wolbachia* based on single loci, e.g. 16S rDNA, *ftsZ* or *wsp* (Werren & Bartos, 2001; Baldo *et al*., 2005). Therefore, Baldo *et al*. (2006) has proposed a multilocus sequence typing (MLST) scheme for *Wolbachia*, which uses five housekeeping and ubiquitous genes to study *Wolbachia* strain relationships.

The subfamily Scolytinae (Curculionidae) is composed of subcortical-feeding insects (bark beetles) and fungus-feeding beetles (ambrosia beetles) (Rudinsky, 1962). Some of them seriously damage forest trees, and their ecology and evolutionary history have been extensively studied for the purpose of pest control (e.g. Kirkendall, 1983, 1993; Beaver, 1989; Normark *et al*., 1999; Farrell *et al*., 2001). The alnus ambrosia beetle, *Xylosandrus germanus*, is one such species. It is highly inbred with female-biased sex ratio (>0.9) (Kaneko, 1965) that is reduced with outbreeding (Peer & Taborsky, 2004). However, outbreeding reduces egg viability (Peer & Taborsky, 2005). This observation is suggestive of *Wolbachia* infections (Peer & Taborsky, 2005).

The objectives of this study were to reveal whether *Wolbachia* infects *X. germanus*; and, if so, to investigate the *Wolbachia* infection pattern and evolutionary history in *X. germanus*, which may provide information on outbreeding depression. We performed amplification and sequence of five MLST and *wsp Wolbachia* genes and conducted molecular phylogenetic analyses. The revealed pattern of *Wolbachia* infection was then compared to the *X. germanus* phylogeny based on mitochondrial COI.

Materials and methods

Insect collection

We collected flying adults of this species at nine sites in Japan in 2005 and 2006 (table S1). Ethanol-bait traps, 10-ml vials filled with 99.5% ethanol (Ito *et al*., 2008), were set up in a mixed stand of broad-leaved trees and shrubs and trapped insects were collected two weeks post set up. Trapped live insects were all females because males of *X. germanus* are not capable of flying in forests (Kaneko, 1965). Captured insects were placed in absolute ethanol and stored at −30°C until DNA was extracted. Numbers of samples used in this study are shown in table S1.

DNA extraction and PCR

DNA was extracted from the abdomens of individual specimens. After the abdomens were crushed, each homogenate was incubated with 200 µl of 5% (wt/wt) Chelex-100 sodium (sigma) and 4 µl of 20 mg µl^{-1} Proteinase K at 56°C overnight. After boiling, the supernatant was used directly as the PCR template.

Because, in this study, we used two distinguishable regions of *ftsZ* (Holden *et al*., 1993; Baldo *et al*., 2006), we described *ftsZ-a* and *ftsZ-b*, respectively (table S2). We amplified three *Wolbachia* genes (*wsp*, 16S rDNA and *ftsZ-a*) and a mitochondrial gene (COI) of *X. germanus* by PCR using specific primer pairs (table S2). Each 10-µl reaction volume consisted of 1 µl of DNA extract, 0.5 µl of dNTPs (2.5 mM each), 0.05 µl of Taq polymerase (5 U µl^{-1}), 5.45 µl of sterile water, 1 µl of 10 × buffer (TAKARA) and 1 µl of forward and reverse primers for the target gene (100 µM). We carried out the standard PCR following conditions: denaturation for 3 min at 94°C, 35 cycles of 94°C for 1 min, the optimal annealing temperature (55°C for *wsp*, 50°C for *ftsZ-a* and 16S rDNA, and 48°C for COI) for 1 min and 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were visualized in 1.5% agarose gel under natural light by staining with Mupid Blue (ADVANCE-BIO) or under UV illumination by staining with ethidium bromide.

Cloning and sequencing

PCR products of *wsp* (550–600 bp) in 3–6 individuals from Furano, Sapporo, Iwate and Aichi, respectively, were cloned with the p-GEMT Easy Vector (Promega) using ampicillin and X-gal blue-white selection system. About ten white colonies expected to contain the inserted plasmid from each product were directly subjected to PCR using the primers M13M4 (5′-GTT TTC CCA GTC ACG AC-3′) and M13RV (5′-CAG GAA ACA GCT ATG AC-3′), useful for determining the length of the inserted DNA fragment. The colonies containing the expected fragment were isolated and cultured in 2 ml of LB medium with ampicillin, and the purified plasmid DNA were directly sequenced using M13M4 and M13RV primers. From the medium, purified plasmids (50 µl) were eluted using a QIAprep-Spin Miniprep Kit (Qiagen). A dye terminator-labeled cycle sequencing reaction was conducted with BigDye DNA Sequencing Kit ver. 3.1 (PE Applied Biosystems). Reaction products were analyzed using an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). The temperature profile was 96°C for 10 s followed by 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. We assigned the *wsp* alleles in *X. germanus* the names '*w*Xge1-5', according to *wsp* sequences.

Amplicons of 16S rDNA, *ftsZ-a* and COI were purified using QIAprep-Spin Miniprep Kit (Qiagen) and bi-directionally sequenced using BigDye DNA Sequencing Kit ver. 3.1 (PE Applied Biosystems). Reaction products of these genes then were analyzed by the same method as those of *wsp*.

The *wsp*, *ftsZ-a*, 16S rDNA and COI sequences determined were deposited in the DDBJ/EMML/GenBank

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nucleotide sequence databases. Accession numbers are shown in table S3a, b.

Phylogenetic analysis

Multiple alignments of *wsp* and COI sequences were conducted using the program package CLUSTALW (Thompson *et al*., 1994). The final alignment was inspected and corrected manually using the sequence analysis software BioEdit 7.0.5.3 (Hall, 1999). Ambiguously-aligned regions were excluded from phylogenetic analysis. Nucleotide sites, including alignment gaps, were also omitted from the analysis. Phylogenetic trees were constructed with neighbor-joining (NJ), maximum parsimony and UPGMA methods, using the program package MEGA 3 (Kumar *et al*., 2004) or PHYLIP 3.65 (Felsenstein, 2004). Bootstrap tests were conducted with 1000 resamplings.

For the phylogenetic tree of the COI sequence, we selected only five *X. germanus* populations (Furano, Sapporo, Iwate, Aichi and Tottori). The other four populations (Yamagata, Saitama, Kochi and Miyazaki) were very similar in haplotypes to Aichi and Tottori (Ito *et al*., 2008). As an outgroup species, we used the closely-related species, *Xylosandrus crassiusculus* (Motshulsky), captured in the traps placed in Aichi.

Detection of Wolbachia *alleles infecting each individual*

Wsp alleles in each *X. germanus* sample were detected by diagnostic PCR. Allele-specific reverse primers (table S4) were designed according to *wsp* or 16S rDNA sequences. Multiplex-PCR for detection of *w*Xge1, *w*Xge2 and *w*Xge3/*w*Xge5 (lengths of expected PCR products were 312, 245 and 406 bp, respectively) was performed in 20-μl reaction volumes consisting of 1 μl of DNA extract, 1 μl of dNTPs (2.5 mM each), 0.2 μl of Taq polymerase (5 U μl^{-1}), 9.8 μl of sterile water, 2 μl of 10 × buffer (TAKARA), 3 μl of wsp81F and 1 μl of each reverse primer (wxge1wr, wxge2wr and wxge3/5wr) (100 μM). *w*Xge4 was detected by standard PCR using another reverse primer (wxge4wr) (255 bp). For wxge3/5wr-positive individuals, standard PCRs for detection of *w*Xge3 and *w*Xge5 were also conducted using different reverse primers, wxge3wr and wxge5_16sr, respectively. The conditions of multiplex PCR and standard PCR for *w*Xge4 detection were identical to those of *wsp* PCR. To discriminate between *w*Xge3 and *w*Xge5 (489 and 632 bp, respectively), we carried out touchdown PCR using the following conditions: denaturation for 3 min at 94°C; 94°C for 1 min, 70°C decreasing by 2°C for 1 min and 72°C for 1 min; 35 cycles of ten cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min and final extension at 72°C for 10 min. All PCR products were visualized in 1.5% or 2% agarose gel for standard PCR and multiplex PCR, respectively, under natural light by staining with Mupid Blue (ADVANCE-BIO) or under UV illumination by staining with ethidium bromide. To check whether accurate alleles were detected, a few randomly selected PCR products were sequenced directly.

Multilocus sequence typing method for Wolbachia

The MSLT method consists of (i) PCR, (ii) sequenced, (iii) assigned and (iv) reconstruction of the MLST tree. MLST genes (*gatB*, *hcpA*, *coxA*, *ftsZ-b*, *fbpA*) were amplified by touchdown PCR using specific primers (table S2). The PCR conditions were as follows: denaturation for 3 min at 94°C; 94°C for 30 s, 70–50°C decreasing by 2°C for 45 s and 72°C for 1 min; 35 cycles of 94°C for 30 s, 50°C for 45 s and 72°C for 1 min and final extension at 72°C for 10 min. PCR products of these genes were purified and bi-directionally sequenced by the same method as 16S rDNA, *ftsZ-a* and COI. Because MLST genes of *w*Xge4 could not be amplified and sequenced, we removed the strain from MLST analysis.

Wolbachia strains were assigned to their own sequence types (ST), defined as the combination of five alleles in MLST genes. Strain and host information was deposited in the MLST database at http://pubmlst.org/wolbachia (see table S5).

UPGMA and NJ trees were reconstructed from MLST allelic profiles (table S5) using START2 program (Jolley *et al*., 2001). Moreover, the concatenated alignment of MLST genes (2079 bp) for the phylogenetic tree was analyzed as described above.

Results

Wolbachia *infection in local populations of* X. germanus

The *wsp* gene was successfully amplified from all the specimens analyzed, confirming fixation of *Wolbachia* infections in all *X. germanus* populations.

Wolbachia *alleles in* X. germanus

Five alleles for *wsp* were detected. Lengths of the five *wsp* sequences, coded as *w*Xge1to *w*Xge5, ranged from 538 to 580 bp. Based on molecular phylogenetic analysis using *wsp* sequences, our five *wsp* sequences were apparently independent of each other (fig. 1).

*w*Xge3 differed from *w*Xge5 by only 2 bp. With respect to *ftsZ-a* and 16S rDNA, however, genetic differences between *w*Xge3 and *w*Xge5 were greater (*ftsZ-a*: 4 bp/728 bp; 16S rDNA: 5 bp/853 bp). Therefore, we regarded *w*Xge3 and *w*Xge5 as different alleles and designed primers for detecting infecting *Wolbachia* based on the polymorphisms existing between these sequences for either the *wsp* and 16S rDNA (table S4) (see Materials and methods).

Multilocus sequence typing for Wolbachia

We obtained a phylogeny of the five *Wolbachia* strains using an MLST method based on *Wolbachia*-housekeeping genes with strains from Baldo & Werren (2007) and the MLST database (fig. 2a). Sequence tagging (ST) profiles are shown in table S5. Because MLST genes of *w*Xge4 could not be amplified, we excluded this strain from MLST analysis. *w*Xge1 (ST-131) and *w*Xge2 (ST-138) were closely related to ST-82 and ST-119, respectively (fig. 2a), containing two common alleles (table S5). *w*Xge3 and *w*Xge5 belonged to the ST-139 and ST-140 complex, which shares three alleles with each other (table S5). The complex is phylogenetically similar to the ST-88 and ST-130 complex (fig. 2a).

A molecular phylogenetic tree using the concatenated alignment of MLST genes (2079 bp) is shown in fig. 2b. All the *Wolbachia* strains in this analysis belonged to the A supergroup as defined by Werren *et al*. (1995). *w*Xge1 was closely related to ST-12, ST-66 and ST-73, and *w*Xge2 to ST-65. *w*Xge3 and *w*Xge5 showed the greatest similarity to ST-2, not to ST-88 or ST-130.

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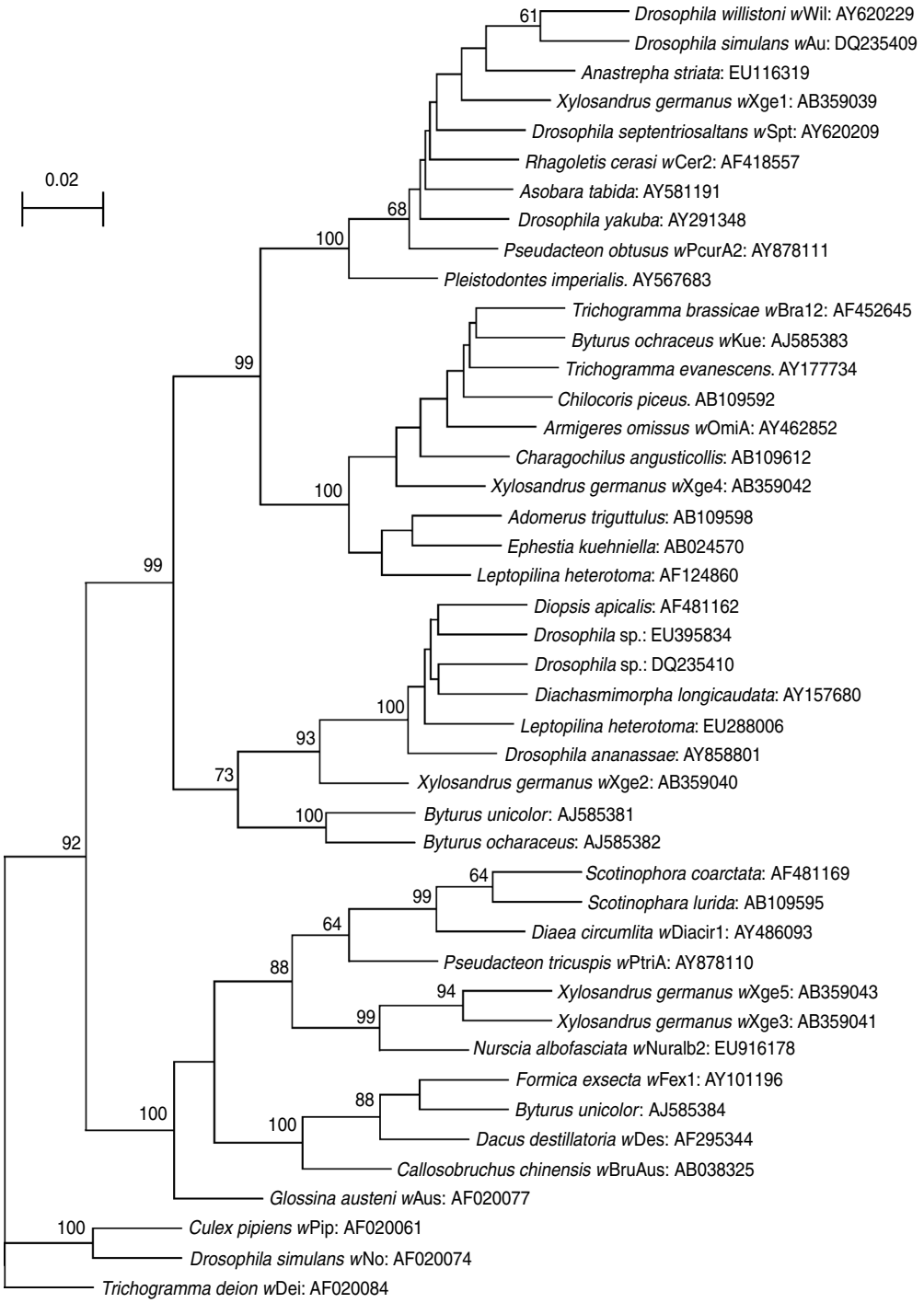

Fig. 1. Molecular phylogenetic tree of five *Wolbachia* alleles infecting *X. germanus* based on the *wsp* gene.

Host insect species, accession number of *wsp* sequence and name of each *Wolbachia* are described in the neighbor-joining tree. Bootstrap values of more than 60% which were obtained with 1000 resamplings are shown at the nodes. Both maximum-parsimony analysis and UPGMA analysis essentially produced the same result. No amplification was obtained with *ftsZ-a* and 16S rDNA primers in the *w*Xge4.

Detection rates and polymorphism of Wolbachia

Prevalence of each *Wolbachia* alleles found by diagnostic PCR is summarized in table 1. *w*Xge4 was detected in all the insects tested. *w*Xge1 also occurred in all the populations accounting for >55% of the infection rates except that from Sapporo. *w*Xge2 was detected in Furano, Sapporo and Iwate, *w*Xge3 in Furano and Iwate and *w*Xge5 only in Furano.

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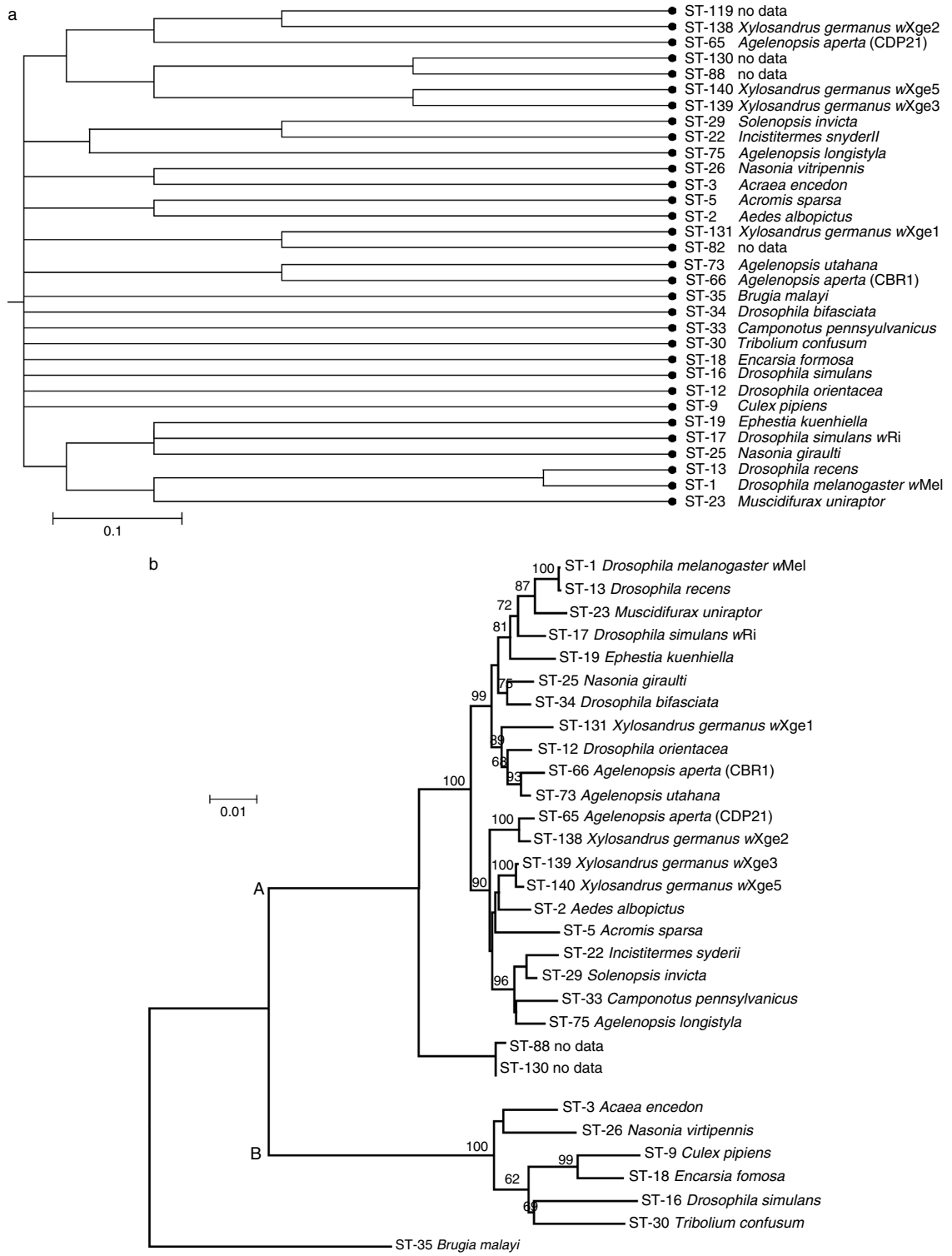

Fig. 2. (a) MLST tree based on sequence type (ST) profiles and (b) neighbor-joining (NJ) tree based on concatenated alignment of MLST genes of four *Wolbachia* strains infecting *X. germanus* without *w*Xge4, including other strains from Baldo *et al*. (2007) and the database http://www.pubmlst/wolbachia.

ST numbers, host species and names of *Wolbachia* strains are described in both trees. ST profiles are shown in table S5. Bootstrap values of more than 60% which were obtained with 1000 resamplings, and delineation of supergroup (A, B) are shown at the nodes of the NJ tree. No amplification is found in five MLST genes of *w*Xge4.

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Table 1. Detection rates (%) of each *Wolbachia* allele in Japanese *X. germanus* populations.

Locality	Detection rate (%)				
	*w*Xge1	*w*Xge2	*w*Xge3	*w*Xge4	*w*Xge5
Furano	66.7	4.8	76.2	100.0	23.8
Sapporo	10.0	70.0	0.0	100.0	0.0
Iwate	61.1	38.9	5.6	100.0	0.0
Yamagata	60.0	0.0	0.0	100.0	0.0
Saitama	60.0	0.0	0.0	100.0	0.0
Aichi	94.1	0.0	0.0	100.0	0.0
Tottori	55.6	0.0	0.0	100.0	0.0
Kochi	71.4	0.0	0.0	100.0	0.0
Miyazaki	75.0	0.0	0.0	100.0	0.0
Total	63.3	12.5	14.2	100.0	4.2

The detection rates for these alleles were lower than those for *w*Xge1, except for *w*Xge2 in Sapporo and *w*Xge3 in Furano.

The maximum number of *Wolbachia* combinations in *X. germanus* was 16 ($=2^4$) because *w*Xge4 was detected from all the individuals. We have developed an abbreviation system for the possible combinations (e.g. detection of three alleles, *w*Xge1, *w*Xge2 and *w*Xge3, is represented as X123). At least nine combinations out of 16 were detected from nine populations (table 2): one pattern of a allele (X4); four combinations of two alleles (X14, X24, X34 and X45); three combinations of three alleles (X124, X134 and X345); and one combination of four alleles (X1345). The Furano population showed the greatest combination of *Wolbachia* alleles (seven combinations). In Sapporo and Iwate, there were three and five *Wolbachia* combinations, respectively. In contrast, Yamagata, Saitama, Aichi, Tottori, Kochi and Miyazaki were less polymorphic (X4 and X14).

Relationship of Wolbachia *combinations with* X. germanus *haplotypes*

A molecular phylogenetic tree based on COI of mtDNA in *X. germanus* is shown in fig. 3 (cf. table S6). Description of haplotypes (Xg2–36) and clades (A–C) was determined according to Ito *et al*. (2008). Six new haplotypes (Xg31–36) were found in this study. Clade A (Xg02–04, 10, 31, 35 and 36; $N=40$) was consistently found in all five populations, whereas clade B (Xg22–24, 27, 32–34; $N=17$) belonged to individuals from Furano and Iwate. Clade C (only Xg30; $N=3$) was found only in Furano.

Figure 3 also illustrates relationship between *Wolbachia* combination and COI haplotypes. Individuals in clade A had *w*Xge1 and/or *w*Xge2 in addition to *w*Xge4 (X4, X14, X24 and X124). On the other hand, *w*Xge2 was absent from Clades B and C: X14, X34, X45, X134, X345 and X1345.

Discussion

Wolbachia *infection in an ambrosia beetle*

This is the first report of *Wolbachia* infection in ambrosia beetles. *Wolbachia* infections had already been found in four species of bark beetles: *Ips typographus* (Stauffer *et al*., 1997), *Hypothenemus hampei* (Vega *et al*., 2002), *Coccotrypes dactyliperda* (Zchori-Fein *et al*., 2006) and *Pityogenes chalcographus* (Arthofer *et al*., in press). However, there are no sequence data of *Wolbachia* infecting *I. typographus*, *C. dactyliperda* and *P. chalcographus*, and the data for *H. hampei* (accession number: AF389084) is too short to compare with our *wsp* sequences.

In *X. germanus*, outbreeding depression reduces egg viability (Peer & Taborsky, 2005), suggesting CI induced by *Wolbachia*. In our results (figs 1 and 2), *X. germanus* was determined to be infected with at least five *Wolbachia* strains and with a total of nine allele combinations (table 2). Although we did not investigate *Wolbachia* phenotypes in the present study, our results imply outbreeding depression is probably caused by *Wolbachia*-induced multi-directional CI, which occurs in crosses where both males and females are infected with different CI-inducing *Wolbachia* (e.g. Hoffmann & Turelli 1997). We recognize the possibility that female-biased sex ratios in *X. germanus* may be caused by infection of other sex-altering bacteria (e.g. *Cardinium*: Gotoh *et al*., 2007). Surveys of such bacteria are planned in the future.

Comparison of MLST analysis with wsp*-based phylogeny*

In the present study, we determined *Wolbachia* taxonomy by both *wsp*-based phylogeny (fig. 1) and MLST analysis (fig. 2). Although molecular phylogenetic analysis, based on *wsp*, has often been reported since Zhou *et al*. (1998), the surface protein *wsp* is highly recombinant and *wsp*-based inferences are not reliable (Baldo & Werren, 2007). After the MLST method was proposed as the better analysis for *Wolbachia* taxonomy (Baldo *et al*., 2006), some studies using MLST have been carried out (Baldo & Werren, 2007; Baldo *et al*., 2007, 2008; Zabalou *et al*., 2008; Narita *et al*., 2009; Ratchoudhury *et al*., 2009 (cf. MLST database)). However, the MLST tree (fig. 2a) has low resolution in the point of phylogeny because it focused on differentiation of each-gene sequences not sequences themselves (Maiden *et al*., 1998). Therefore, at the moment, molecular phylogenetic analysis using concatenated alignment of MLST genes may reveal *Wolbachia* evolution more clearly.

Historical dynamics of Wolbachia *strains infecting* X. germanus

We have detected five alleles of *wsp* gene from *X. germanus*. Detection of distinct *wsp* alleles in a single specimen is common (Breeuwer *et al*., 1992; Vavre *et al*., 1999; Kondo *et al*., 2002a). However, only four studies reported detection of more than five alleles in one insect species: ants *Formica exsecta* (five alleles: Reuter & Keller, 2003), *Solenopsis daguerrei* (nine alleles: Dedeine *et al*., 2005), the fruit fly *Bactrocera ascita* (five alleles: Jamnongluk *et al*., 2002) and the raspberry beetle *Byturus tomentosus* (seven alleles: Malloch *et al*., 2000).

*w*Xge4 is unique because only *wsp* gene was amplified in all the insects tested. Some recent papers have reported lateral gene transfer from *Wolbachia* to host insects (Kondo *et al*., 2002b; Hotopp *et al*., 2007). Although we do not have any direct evidence, *w*Xge4 might be the only gene fragment inserted in the host chromosome.

The phylogenetic analyses (figs 1 and 2) show that *Wolbachia* infecting *X. germanus* are divergent between *w*Xge3 and *w*Xge5; the difference in the *wsp* sequence between *w*Xge3 and *w*Xge5 was only 2 bp, and *w*Xge3 was identical in three out of five MLST genes with *w*Xge5

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Table 2. *Wolbachia* infection polymorphism in Japanese *X. germanus* populations.

Locality	No. of individuals									
	with an allele	with two alleles				with three alleles			with four alleles	Total
	X4	X14	X24	X34	X45	X124	X134	X345	X1345	
Furano	–	2	1	3	2	–	10	1	2	21
Sapporo	2	1	7	–	–	–	–	–	–	10
Iwate	6	4	1	–	–	6	1	–	–	18
Yamagata	6	9	–	–	–	–	–	–	–	15
Saitama	6	9	–	–	–	–	–	–	–	15
Aichi	1	16	–	–	–	–	–	–	–	17
Tottori	4	5	–	–	–	–	–	–	–	9
Kochi	2	5	–	–	–	–	–	–	–	7
Miyazaki	2	6	–	–	–	–	–	–	–	8
Total	29	57	9	3	2	6	11	1	2	120

Combinations of *Wolbachia* alleles are shown in abbreviations (e.g. triple detection of *w*Xge1, *w*Xge2 and *w*Xge3 as X123).

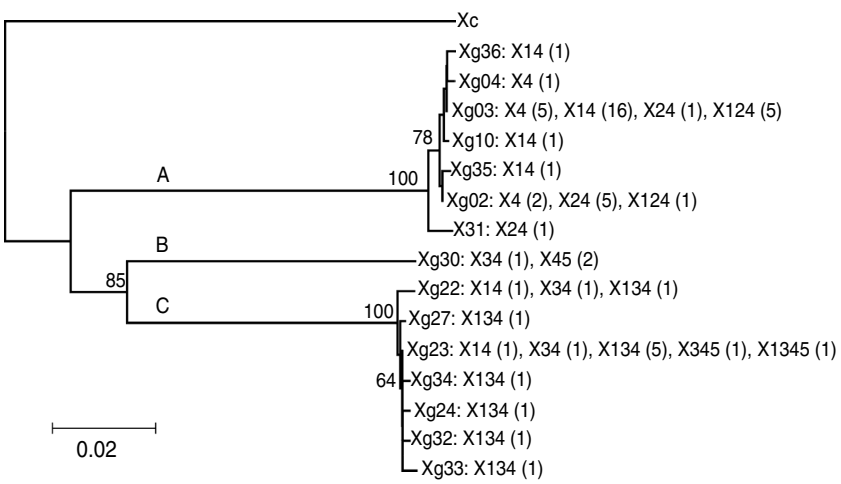

Fig. 3. Molecular phylogenetic tree of *X. germanus* haplotypes based on *CO1* of mtDNA with reference to *Wolbachia* combinations.

Bootstrap values of more than 60% which were obtained with 1000 resamplings and clade of *X. germanus* (A–C) are shown at the nodes. Localities of the haplotypes (in abbreviation as Xg02–36) are shown in table S6. *Wolbachia* combinations in *X. germanus*, belonging to each haplotype are described in the same manner as table 2, together with number of individuals in parentheses. *Xylosandrus crassiusculus* (Xc) was used as an outgroup.

(cf. table S5). These results suggest that a common strain differentiated to *w*Xge3 or *w*Xge5. Although when they were differentiated has not been clear, it may have occurred in the current host, *X. germanus*.

We have found nine different combinations with four *Wolbachia* alleles, without *w*Xge4, in *X. germanus* (table 2). To date, this species has the most variable combinations of *Wolbachia* among the reported hosts. This combination may have been formed by multiple horizontal transmissions to different host lineages. *w*Xge2 and *w*Xge3/*w*Xge5 did not infect the same individuals (table 2) and their combinations were related to the beetle phylogeny (fig. 3). Ito *et al*. (2008) implied that *X. germanus* has already developed into three lineages (clades A, B and C) before colonization of Japan. Thus, *w*Xge1 and *w*Xge4 may infect the common ancestor of three clades of *X. germanus* before its differentiation. After that, *w*Xge2 and the ancestor strain of *w*Xge3 and *w*Xge5 may infect its descendants, clades A and B/C. Finally, *w*Xge3 and *w*Xge5 would differentiate from the common *Wolbachia*.

Acknowledgements

We thank A. Davies, T. Hagimori-Adachi, T. Kikuchi and Y. Oba for providing technical advices and information on *Wolbachia*. We also thank L. Baldo, F.E. Vega and H. Anbutsu for their critical reading of the manuscript. We are grateful to the following people for collecting the beetle specimens: K. Iguchi, A. Ueda, S. Saito, K. Nakamura, K. Ishida, M. Inoue, S. Sato and K. Araya.

This study was supported by Grants-in-Aid for Scientific Research from JSPS (18380090, 18405012, 20405025), the Fujiwara Natural History Foundation (2004), the Inamori Foundation (2005), the IFO Foundation (Institute for Fermentation, Osaka) (2007), and the Shouwahoukoukai Foundation (Ito Chube'e) (2008).

Supplementary material

The following online table can be viewed at http://journals.cambridge.org/ber:

Table S1. Location of sampling sites and numbers of individuals tested in each year.
Table S2. Information on primers for amplifying *Wolbachia* and host insect *X. germanus* DNA.
Table S3a. Accession numbers of three genes (*wsp*, *ftsZ-a* and 16S rDNA) of each *Wolbachia* strain.
Table S3b. Accession numbers of COI of mtDNA in each haplotypes of *X. germanus* and *X. crassiusculus*.
Table S4. Information on reverse primers for differentiating between *Wolbachia* alleles.
Table S5. Sequence type profile of each *Wolbachia* strain used in MLST.
Table S6. Composition of *X. germanus* haplotype on COI of mtDNA in each locality.

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