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High Prevalence of *Wolbachia* in the Azuki Bean Beetle *Callosobruchus chinensis* (Coleoptera, Bruchidae)

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ABSTRACT—Members of the family Bruchidae (Insecta, Coleoptera) were examined for *Wolbachia* infection. Out of seven species investigated, which represented three subfamilies and five genera, amplified bands were detected only from *Callosobruchus chinensis* by diagnostic PCR with *Wolbachia*-specific primers. Bacterial 16S rDNA was amplified by PCR, cloned and sequenced from the total DNA of *C. chinensis*. Molecular phylogenetic analysis demonstrated that the sequence belongs to a monophyletic group of the genus *Wolbachia* in the α-*Proteobacteria*. The *Wolbachia* was detected from all tissues and body parts such as ovary, gut, fat body, muscle, wing, leg, head and antenna. In six geographic populations from central Japan, all the individuals examined, more than 400 in total, possessed the *Wolbachia*, indicating high prevalence of *Wolbachia* among natural populations of *C. chinensis*.

INTRODUCTION

Endosymbiotic associations with microorganisms are quite ubiquitous in many groups of insects and other arthropods (Buchner, 1965; Baumann and Moran, 1997). There is an enormous variety of endosymbiotic relationships in which the host and symbiont interact with various degrees of interdependency. Some endosymbionts are obligate and essential endosymbiotic companions for the host, whereas others are regarded as facultative guest microbes of a commensal or rather parasitic nature. Members of the genus *Wolbachia*, which are widely distributed among various groups of insects, crustaceans and nematodes (Werren *et al*., 1995a; Werren, 1997; Werren and O'Neill, 1997), are the most intensively studied endosymbiont of the latter type. It has been shown that *Wolbachia* infection causes various sex-related aberrations in their arthropod hosts such as cytoplasmic incompatibility (Hoffmann *et al*., 1990; Hoffmann and Turelli, 1997), parthenogenesis (Stouthamer *et al*., 1990; Stouthamer, 1997) and feminization of genetic males (Rigaud *et al*., 1991; Rigaud, 1997; Kageyama *et al*., 1998). Because *Wolbachia* is inherited solely through maternal lineage of the host by vertical transmission, these reproductive manipulations can be interpreted as the strategy by which *Wolbachia* increases the frequency of infected females in populations of the host, often at the expense of host's fitness. In this context, *Wolbachia* is regarded as a selfish genetic element (Werren *et al*., 1988), is expected to conflict with the host in an evolutionary sense, and, therefore, is an attractive subject in evolutionary biology.

The azuki bean beetle, *Callosobruchus chinensis* (Coleoptera, Bruchidae), is known as a pest of stored azuki bean, *Vigna angularis*. In addition to the importance as a pest insect, this beetle has been widely used as a model organism in population biology because various parameters related to its population dynamics can easily be measured, quantified and controlled under laboratory conditions (Utida, 1941; Hassell *et al*., 1989; Shimada and Tuda, 1996). Thus, if *C. chinensis* harbors an endosymbiotic microorganism, it will provide us with a good system to investigate the dynamics of host-symbiont interactions.

In this paper, we report the discovery of *Wolbachia* in *C. chinensis* based on molecular phylogenetic and PCR analyses. We also report high prevalence of *Wolbachia* among natural populations in Japan and widespread distribution in host's entire body.

MATERIALS AND METHODS

Materials

Laboratory strains of bruchid beetles used in this study are listed in Table 1(a). They were maintained on their host seeds at 30°C and 70% r.h. in a long-day regimen 16 hr light- 8 hr dark. Natural populations of *C. chinensis* examined in this study are shown in Table 1(b). We collected adult beetles in fields of the azuki bean (*Vigna angularis*) and/or the sasage bean (*V. sinensis*) at six localities in central Japan (see Table 1(b) and later Fig. 4 for a map).

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Table 1. Species, strain codes, original places of collection and their year in bruchids examined.

Subfamily	Species name	Strain code	Original place	Year	Host seeds
(a) Laboratory strains					
Amblycerinae					
	Zabrotes subfasciatus	C100	CIAT[1)], Colombia	1984	*Phaseolus vulgaris*
		US	Kampala,Uganda	1980	ditto
Bruchinae					
	Callosobruchus chinensis	jC	Kyoto, Japan	1936	*Vigna angularis*
		mrC97	Hatakenaka, Maruoka, Fukui, Japan	1997	ditto
	C. maculatus	hQ	Hongo, Bunkyo,Tokyo, Japan	1988	ditto
		IQ	Tirunelveli, India	1979	*V. radiata*
	C. rhodesianus		Zinbabwe[2)]	1993	*V. angularis*
	Acanthoselides obtectus		Xalapa, Veracruz, Mexico	1998	*P. vulgaris*
	Bruchidius dorsalis		Harataima, Kanagawa, Japan	1998	*Gleditsia japonica*
			Tatsuno, Nagano, Japan	1998	ditto
Kytorhininae					
	Kytorhinus sharpianus		Mitsuma, Ibaraki, Japan	1997	*Sophola flavescens*
			Yoneyama, Niigata, Japan	1997	ditto
(b) Natural populations in Japan					
	C. chinensis	skC98	Saiki, Tsukuba, Ibaraki	1998	*V. angularis*
		tsC98	Mt.Tsukuba,Tsukuba, Ibaraki	1998	ditto
		kzC98	Koizumi, Tsukuba, Ibaraki	1998	ditto
		mrC98	Hatakenaka, Maruoka, Fukui	1998	ditto
		mgC98	Gokurakuji, Mino, Gifu	1998	ditto
		kkC98	Uchimaki, Kasukabe, Saitama	1998	ditto

[1)] Centro Internacional de Agricultura Tropical
[2)] District is unknown

Sample preparation

For standard DNA analyses, adult insects were preserved in 100% acetone until DNA extraction. In order to examine the distribution of *Wolbachia* among tissues, live female adults of *C. chinensis*, jC strain were subjected to the following dissection procedure. The body of *C. chinensis* was divided into eleven parts: antennae, head, fore and hind wings, legs (fore-, mid-, and hind-), prothorax, muscle (from thorax), meso- and metathorax, gut, ovarioles with eggs, fat body, and abdomen. The dissection of body parts/ tissues was carefully conducted in 100% ethanol with clean forceps. To minimize the contamination from other tissues, after a body part was carefully isolated from an insect, it was transferred to and rinsed with fresh ethanol.

DNA extraction

For standard DNA analyses, a single whole adult beetle preserved in acetone was subjected to DNA extraction. To examine the distribution of *Wolbachia* in the insect body, DNA was extracted from the isolated body part/ tissue from a single insect. QIAamp Tissue Kit (QIAGEN) was used for DNA extraction essentially according to manufacturer's instruction. The material was put into a 1.5 ml plastic tube, and the preservative was removed well with a micropipette. Then, the material was homogenized with a plastic pestle in 200 µl of lysis buffer containing Proteinase K. After 3 hr or longer incubation at 55°C, DNA was purified from the lysate using QIAamp micro-spin columns.

PCR

PCR was conducted using Takara Taq polymerase (Takara) and its supplemented buffer system, under temperature profiles of 94°C for 2 min followed by 30 amplification cycles. Following primer sets and conditions were used. Around 1.6 kb of insect mitochondrial small subunit ribosomal RNA gene fragment (MtrDNA) was amplified with primers MtrA1 [5'-AAWAAACTAGGATTAGATACCCTA-3'] and MtrB1 [5'-TCTTAATYCAACATCGAGGTCGCAA-3'] (Fukatsu and Shimada, 1999) with cycles of 1 min at 94°C, 1 min at 48°C and 3 min at 68°C. About 1.5 kb of eubacterial 16S ribosomal RNA gene fragment (16S rDNA) was produced with primers 16SA1 [5'-AGAGTTTGATCMTGGCTCAG-3'] and 16SB1 [5'-TACGGYTACCTTGTTACGACTT-3'] (Fukatsu and Nikoh, 1998) with cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 70°C. A 0.75 kb fragment of *Wolbachia* homolog of *ftsZ*, a cell-division related gene of bacteria, was specifically amplified with primers fts1 [5'-GTATGCCGATTGCAGAGCTTG-3'] and fts2 [5' GCCATGAGTATTCACTTGGCT-3'], which were designed according to a previous report (Holden *et al.*, 1993), with cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 70°C. About 0.6 kb of *Wolbachia* surface protein gene fragment (*wsp*) was specifically produced with primers wsp1 [5'-GGATCCGGGTCCAATAAGTGATGAAGAAAC-3'] and wsp2 [5'-GGATCCTTAAAACGCTACTCCAGCTTCTGC-3'], which were provided by Dr. T. Sasaki, with cycles of 1min at 94°C, 1 min at 50°C and 1 min at 70°C. Although the wsp primers contained an additional BamHI site at the 5' end for cloning, it did not affect specificity of the PCR reaction. The PCR products were electrophoresed in TAE-agarose gels, stained with ethidium bromide, and observed under an UV transilluminator.

DNA cloning and sequencing

Amplified product of 16S rDNA from *C. chinensis*, jC strain was subjected to cloning and sequencing essentially as described previously (Fukatsu and Nikoh, 1998). The product was directly cloned with TA-cloning vector pT7Blue (Novagen) and *E. coli* JM109 competent cell (Takara) using ampicillin and X-gal blue-white selection system. White colonies that were expected to contain inserted plasmid were directly subjected to PCR using primers on the flanking region of the vector's cloning site, U-19 [5'-GTTTTCCCAGTCACGACGT-3'] and BT7 [5'-TAATACGACTCACTATAGGG-3'], in order to check the length of the inserted DNA fragment (1.5 kb). The colonies identified to contain the gene fragment were isolated and cultured in 1.5 ml LB medium with ampicillin overnight, and subjected to plasmid extraction using QIAprep-Spin Miniprep Kit (QIAGEN). The purified plasmids, eluted with 30 µl TE buffer, were used for sequencing template DNA. Dye terminator labelled cycle sequencing reaction was conducted with BigDye DNA Sequencing Kit (PE Applied Biosystems). Six primers, 16SA1, 16SA2 [5'-GTGCCAGCAGCCGCGGTAATAC-

3'], 16SA3 [5' TGCATGGYTGTCGTCAGCTCG-3'], 16SB1, 16SB2 [5'-CGAGCTGACGACARCCATGCA-3'] and 16SB3 [5'-GTATTA-CCGCGGCTGCTGGCAC-3'] were used for sequencing. The temperature profile was 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 70°C. Reaction products were analyzed by ABI PRISM 377 DNA sequencer (PE Applied Biosystems). Determined partial sequences were edited and connected using DNASIS V. 3.0 (Hitachi Software Engineering Co., Ltd.).

Nucleotide sequence accession number

The sequence of 16S rDNA of *Wolbachia* sp. from *C. chinensis*, jC strain has been deposited in the DDBJ, EMBL, and GenBank nucle-

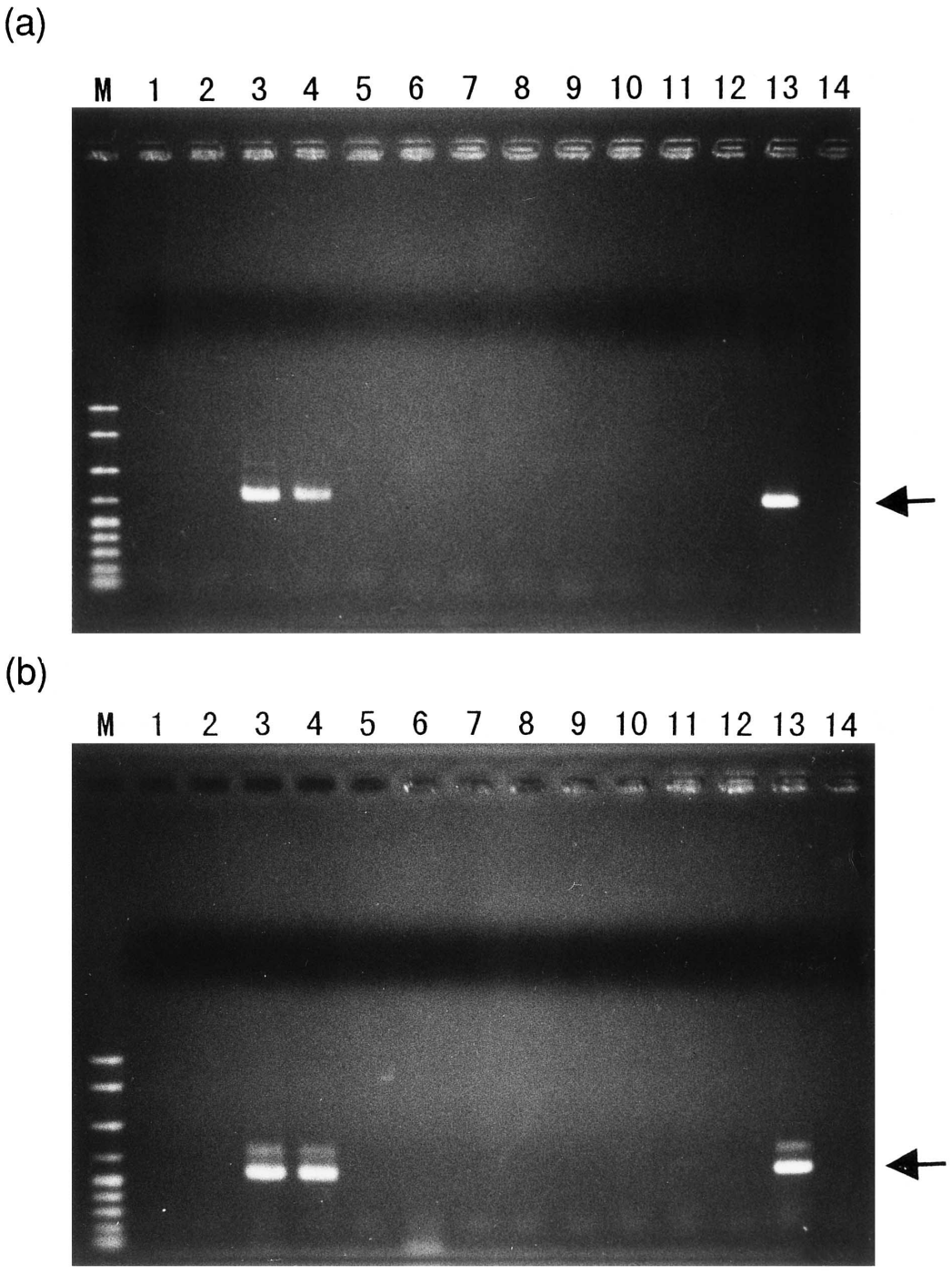

Fig. 1. Diagnostic PCR detection of *Wolbachia* with specific primer sets from laboratory strains of bruchid beetles. (a) *ftsZ* homolog specific to *Wolbachia*. (b) *wsp* gene specific to *Wolbachia*. Lane 1, *Z. subfasciatus*, C100 strain; lane 2, *Z. subfasciatus*, US strain; lane 3, *C. chinensis*, jC strain; lane 4, *C. chinensis*, mrC97 strain; lane 5, *C. maculatus*, hQ strain; lane 6, *C. maculatus*, iQ strain; lane 7, *C. rhodesianus*; lane 8, *A. obtectus*; lane 9, *B. dorsalis*, Harataima strain; lane 10, *B. dorsalis*, Tatsuno strain; lane 11, *K. sharpianus*, Mitsuma strain; lane 12, *K. sharpianus*, Yoneyama strain; lane 13, *Ephestia kuehniella* with *Wolbachia* sp.for a positive control; lane 14, no template control; lane M, DNA size markers (2000, 1500, 1000, 700, 525/500, 400, 300, 200 and 100 bps from the top to the bottom). Arrow indicates the specific PCR product. Other faint bands are due to non-specific amplification.

otide sequence databases under accession number AB025965.

Molecular phylogenetic analysis

The sequence data used for molecular phylogenetic analyses were searched for and retrieved from the DDBJ sequence database. Multiple alignment was performed by Clustal W (Thompson *et al.*, 1994). The final alignment was inspected and corrected manually. Ambiguously aligned regions were excluded from the phylogenetic analysis. Nucleotide sites that included alignment gap(s) were also omitted from the aligned data set. Neighbor-joining tree (Saitou and Nei, 1987) was constructed with Kimura's two parameter distance (Kimura, 1980) using the program package Clustal W (Thompson *et al.*, 1994), and bootstrap test (Felsenstein, 1981) was conducted with 1,000 resamplings.

RESULTS

Detection of *Wolbachia* in the Bruchidae

Twelve laboratory strains of bruchid beetles, which represented seven species, five genera and three subfamilies of Bruchidae, were subjected to DNA extraction to conduct diagnostic PCR experiments with *Wolbachia*-specific primers. DNA of a large molecular size was extracted from these insects (data not shown). Quality of the extracted DNA was confirmed by successful PCR amplification of 1.6 kb segment of insect MtrDNA (data not shown). When PCR was conducted with the specific primers to amplify *ftsZ* homolog of *Wolbachia*, an amplified product of expected size, approximately 0.75kb, was detected only from *C. chinensis*, strains jC and mrC97 (Fig. 1a). With another specific primer set to detect *wsp* gene fragment (0.6 kb) of *Wolbachia*, the same result was obtained (Fig. 1b). Therefore, it was strongly suggested that only *C. chinensis* was infected by *Wolbachia* among the bruchid species examined.

Molecular phylogeny of *Wolbachia* of *C. chinensis* based on 16S rDNA

To confirm the diagnostic PCR result, we determined the nucleotide sequence of bacterial 16S rDNA fragment amplified from *C. chinensis*, jC strain. The sequence showed high similarity to the 16S rDNA sequences of *Wolbachia* spp. deposited in the DNA databases. In the molecular phylogenetic tree (Fig. 2), the sequence constituted a compact monophyletic group in the α-*Proteobacteria*, supported by 100%

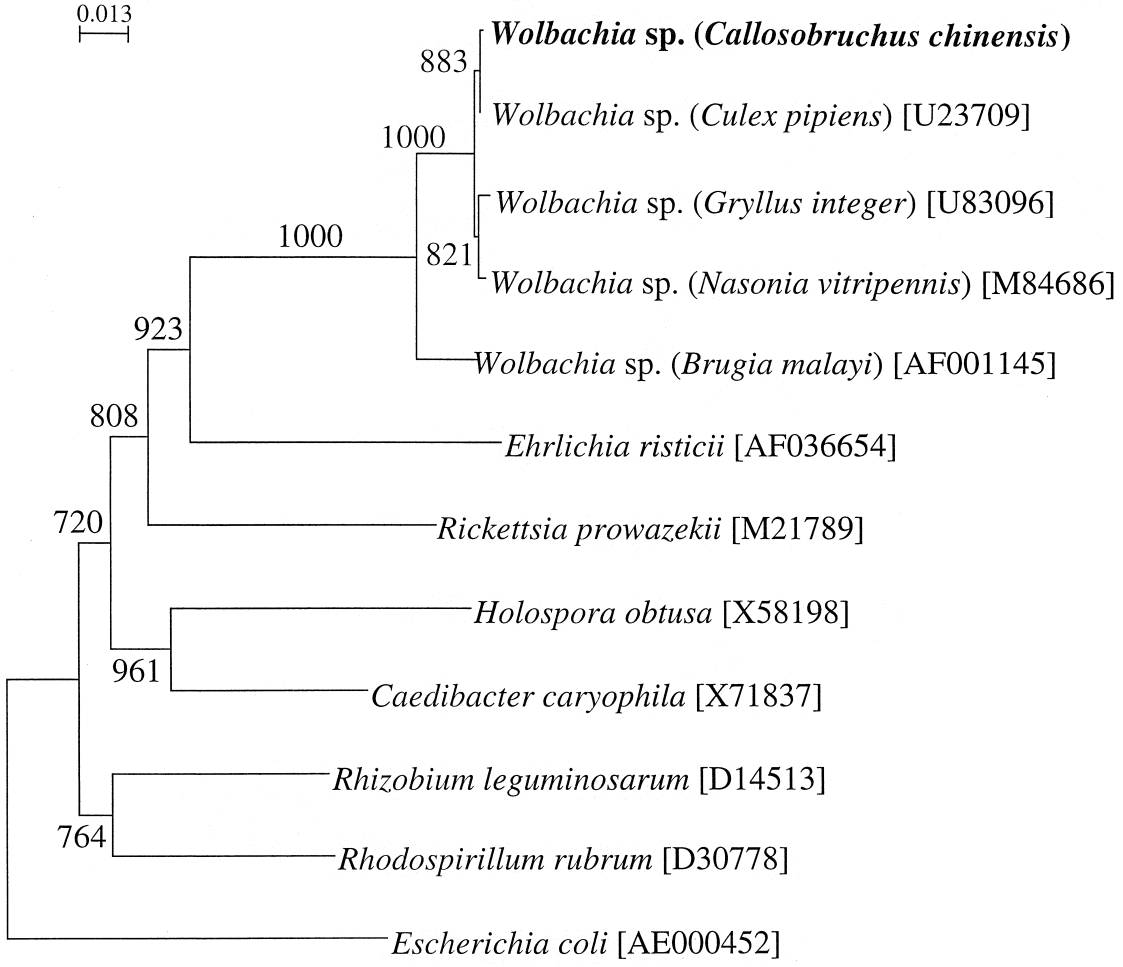

Fig. 2. Phylogenetic position of the bacterial 16S rDNA sequence (1,592 bp) amplified and cloned from *C. chinensis*, jC strain. The 16S rDNA sequence was analyzed by neighbor-joining method with sequences of representatives of the α-*Proteobacteria* and *E. coli* as an outgroup. A total of 1,271 unambiguously aligned nucleotide sites were subjected to the analysis. Bootstrap values obtained with 1,000 resamplings are shown at nodes. Numbers in brackets are the accession numbers of the sequence data.

bootstrap value, with *Wolbachia* sequences derived from other insects.

Wolbachia infection in the body of *C. chinensis*

The distribution of the *Wolbachia* in the body of *C. chinensis* was checked by PCR experiments with *Wolbachia*-specific primers. Using *ftsZ* primers (Fig. 3a), the amplified product was detected from all the body parts/ tissues examined; antennae, head, wings, legs, prothorax, muscle, meso- and metathorax, gut, ovarioles with eggs, fat body, and

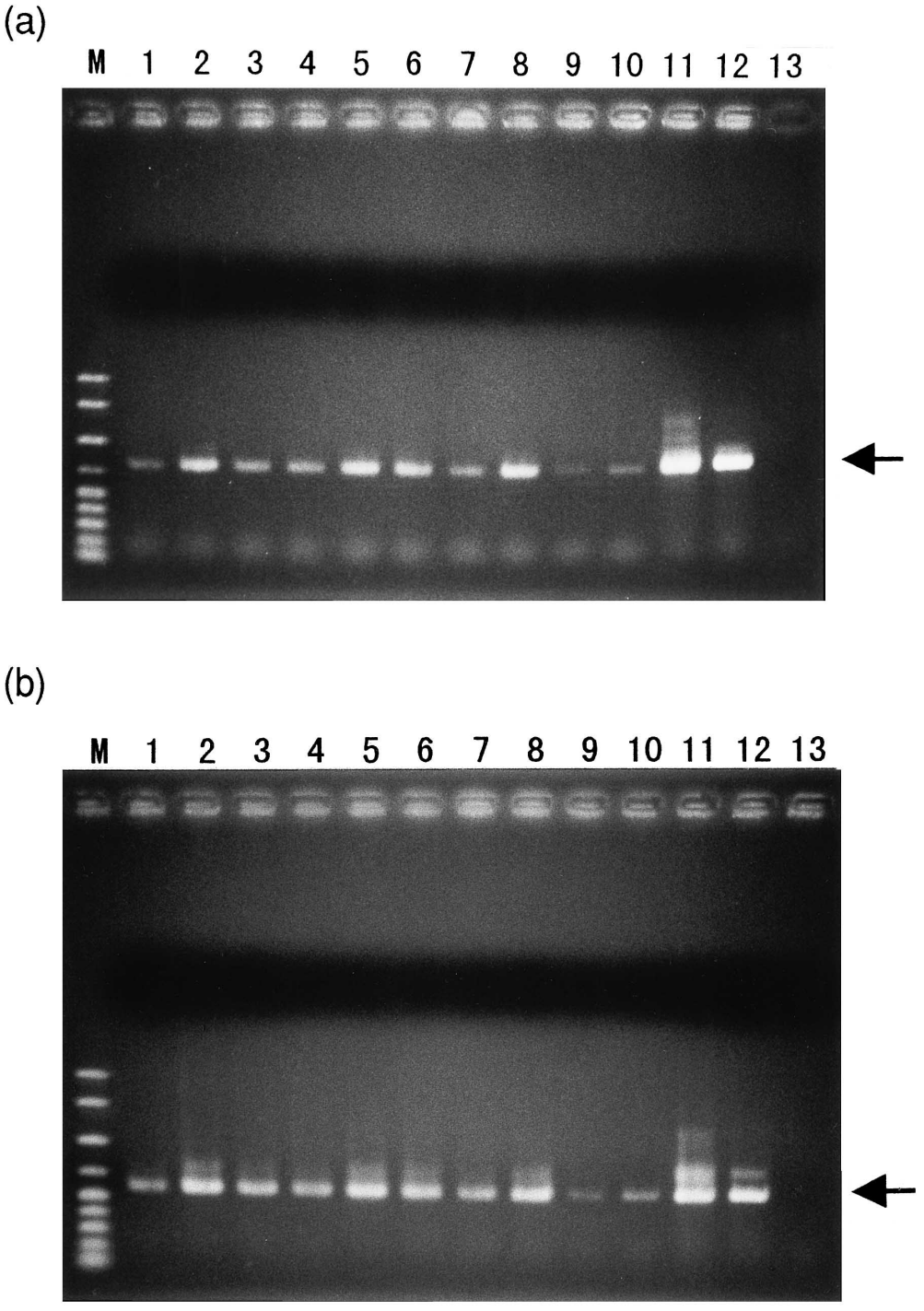

Fig. 3. Distribution of the *Wolbachia* in the body of *C. chinensis*, examined by PCR with *Wolbachia* specific primers. (a) *ftsZ*, and (b) *wsp*. Lane 1, antennae; lane 2, head; lane 3, fore and hind wings; lane 4, fore-, mid- and hindlegs; lane 5, prothorax; lane 6, muscle from thorax; lane 7, mesothorax and metathorax; lane 8, gut; lane 9, ovarioles with eggs; lane 10, fat body; lane 11, abdomen; lane 12, *Ephestia kuehniella* with *Wolbachia* sp. for a positive control; lane 13, no template control; lane M, DNA size markers (2000, 1500, 1000, 700, 525/500, 400, 300, 200 and 100 bps from the top to the bottom). Arrow indicates the specific PCR product. Other faint bands are due to non-specific amplification.

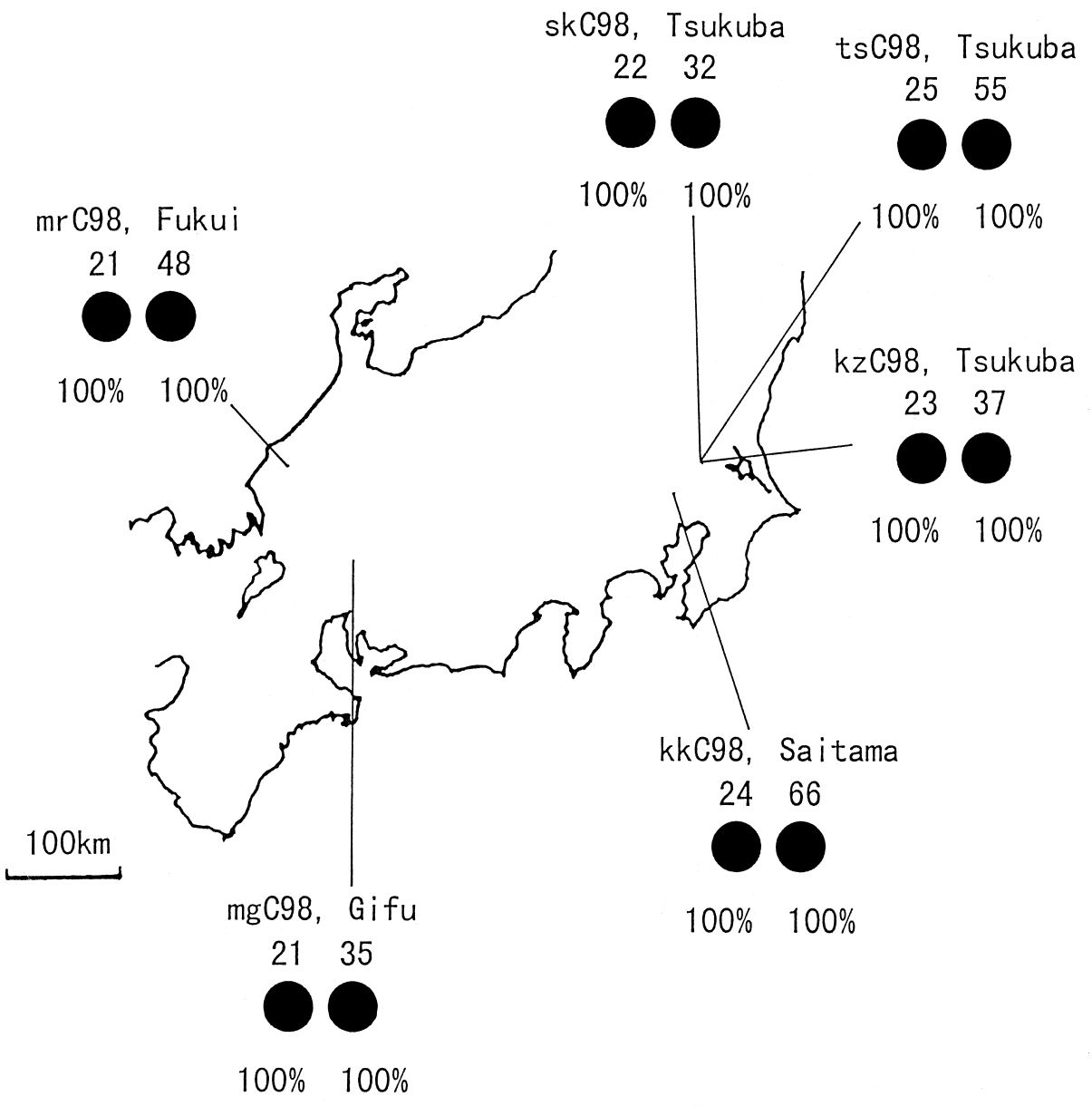

Fig. 4. Locations of six collection sites with the infection rates of *Wolbachia* in *C. chinensis* in central Japan. Solid regions in pie diagrams represent the infection rates. The number of samples and the infection rates (%) in males (left) and females (right) are shown above and beneath each pie diagram, respectively. See Table 1(b) for detailed information on localities of the populations.

abdomen. Using *wsp* primers (Fig. 3b), the same result was obtained.

Wolbachia infection among natural populations of *C. chinensis*

In our field survey, occurrence of *Wolbachia* in natural populations of *C. chinensis* in Japan was investigated by PCR experiments (Fig. 4). All the individuals, 136 males and 273 females in total, collected at the six localities were Wolbachia-positive with *ftsZ* primers.

DISCUSSION

Among seven species of Bruchidae examined in this study, only *C. chinensis* exhibited the amplified bands by diagnostic PCR with two *Wolbachia*-specific primer sets, *ftsZ* and *wsp* (Fig. 1). Molecular phylogenetic analysis confirmed that *C. chinensis* possesses a bacterium of the genus *Wolbachia* (Fig. 2). So far as we know, there has been no report on the endosymbiont, including *Wolbachia*, from the family Bruchidae except for *Rickettsia* sp. in *Kytorhinus sharpianus* (Fukatsu and Shimada, 1999), although various types of endosymbiotic microorganisms have been found from related coleopteran groups such as Chrysomelidae,

Curculionidae and Rhynchophoridae (Buchner, 1965; Campbell *et al*., 1992; Heddi *et al*., 1998).

Although our sampling of bruchid species was far from complete, the result that *Wolbachia* was detected only in *C. chinensis* suggests that the infection with *Wolbachia* may not be common in the family Bruchidae. Even in the genus *Callosobruchus*, *Wolbachia* was not detected in *C. maculatus* nor *C. rhodesianus* but found only in *C. chinensis*. On the other hand, in the species *C. chinensis*, the *Wolbachia* was universally distributed among natural populations in central Japan. From these results, it is conceivable that the *Wolbachia* was acquired by a common ancestor of *C. chinensis*, possibly from either an unexamined bruchid carrier or a phylogenetically distant arthropod. In fact, several studies have claimed that interspecific transmissions must have occurred throughout the evolutionary history of *Wolbachia* (O'Neill *et al*., 1992; Werren *et al*., 1995b).

Since *Wolbachia* is passed to the next host generation by vertical transmission and causes sex-related aberrations in the host, it has been widely assumed that *Wolbachia* specifically infect reproductive tissues. Recently, however, it was reported that *Wolbachia* are much more widely distributed among host tissues in insects than previously appreciated (Dobson *et al*. 1999), which is concordant with our finding that *Wolbachia* was detected by PCR not only from an ovary but from all the other tissues and body parts of *C. chinensis*.

So far, several works have been conducted on *Wolbachia* infection rates in natural populations, *Drosophila simulans* in California (Turelli and Hoffman 1995), *D. melanogaster* in Australia (Hoffmann *et al*. 1994), and *Laodelphax striatellus* in northeastern Japan (Noda 1984; Hoshizaki and Shimada 1995), in which most of local populations were not infected perfectly. Compared with these reports, perfect infection of *C. chinensis* by *Wolbachia* (Fig. 4) appears quite remarkable, because complete infection rate has not been reported except the population of *L. striatellus* in southwestern Japan (Noda 1984; Hoshizaki and Shimada 1995).

In theoretical models, factors determining an infection rate of *Wolbachia* in a host population are, first, fidelity of vertical transmission of *Wolbachia*, secondly, fitness cost of an infected host such as decrease in viability and fecundity, thirdly, the degree of cytoplasmic incompatibility, and finally, initial frequency of invasion (Caspari and Watson, 1959; Fine 1978; Turelli and Hoffmann, 1995). These factors in *C. chinensis* populations should be investigated in detail to identify the determinants of the remarkable complete infection. In addition, we plan to analyse the *wsp* sequence and phylogenetic differences of the *Wolbachia* in *C. chinensis* from different geographical populations.

The azuki bean beetle *C. chinensis* is a useful material for population biological studies because it is easy to establish and maintain experimental populations in order to examine its life history parameters. Therefore, this species will provide us with a model system to understand the interaction and dynamics between *Wolbachia* and its host insect.

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