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Dynamics of infection with *Wolbachia* in *Hypera postica* (Coleoptera: Curculionidae) during invasion and establishment

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Abstract The process of loss or gain of parasites during invasion of new lands is not well understood. The alfalfa weevil *Hypera postica* is an invasive pest of various leguminous crops and consists of three major mitochondrial haplotypes, 'Western', 'Egyptian' and 'Eastern'. The Western strain is infected with the endosymbiotic proteobacteria *Wolbachia*, that cause unidirectional complete reproductive incompatibility, in its native (Europe) and an introduced (the United States) ranges. However, our preliminary screening of a few introduced populations in Northern Kyushu, southwestern Japan, failed to detect *Wolbachia* from the Western strain. A larger-scale and historical assessment of *Wolbachia* infection may allow to estimate when and how the bacteria were lost, and current geographical distribution of infection among host haplotypes. In this study, we aim to assess the *Wolbachia*-infection status of *H. postica* populations throughout Northern Kyushu, where *H. postica* invasion to Japan was first found. A total of 228 individuals from seven regions in Northern Kyushu collected in different time periods from 1982–2015 and 14 individuals from Europe were subjected to PCR diagnostics for *Wolbachia*. *Wolbachia* from the Western strain was not detected, irrespective of the time periods and geographic areas in Northern Kyushu. We found 'Egyptian'-strain *H. postica* collected most recently from an island off Kyushu harboured a supergroup-B *Wolbachia* variant. This variant was genetically different from the European *Wolbachia* variant infecting Western-strain *H. postica*. The infection was new to the Egyptian haplotype and was estimated to have taken place independently of the loss in the Western strain.

Keywords: intracellular bacteria · phylogeny · infection history · enemy release hypothesis · ftsZ ·MLST

Introduction

Invading species tend to reach at high densities in new locations resulting from decreased number of their natural enemy parasites/predators, known as the enemy release hypothesis (Keane and Crawley 2002; Mitchell and Power 2003; Torchin et al. 2003). This is probably due to reduced probability of introduction of native parasites/predators with their hosts/prey and/or absence of new parasites/predators in invaded areas (Keane and Crawley 2002; Mitchell and Power 2003; Torchin et al. 2003). Process of loss or gain of parasites during invasion of new lands, however, is not well understood.

Wolbachia (Rickettsiales: Rickettsiaceae) are maternally inherited endosymbiotic proteobacteria that inhabit in arthropods (Werren et al. 1995; Cordaux et al. 2001; Gotoh et al. 2003) and filarial nematodes (Bandi et al. 1998). About 40–65% of insects harbour *Wolbachia* (Hilgenboecker et al. 2008; Kondo et al. 2011; Zug and Hammerstein 2012). Diversity of *Wolbachia*, both genetically and functionally, has been studied (Werren 1997; Werren et al. 2008). *Wolbachia* control host reproduction and thus called reproductive parasites (Werren 1997), though beneficial effects (Moreira et al. 2009; Darby et al. 2012) and no manipulative effect (Hamm et al. 2014) of *Wolbachia* on their hosts are found recently. One of the phenotypes of *Wolbachia* is cytoplasmic incompatibility that decreases reproductive fitness of uninfected females as they cross with infected males. Infection with *Wolbachia* may incur costs that eventually deter prevalence in host populations (e.g., Sarakatsanou et al. 2011; Suh and Dobson 2013; Dykstra et al. 2014; theoretical study, Crain et al. 2011).

The weevil *Hypera postica* (Gyllenhal) is an invasive pest of various leguminous crops (Skuhrovec 2005). *Hypera postica* is native to Palaearctic and accidentally introduced into the United States and Japan (Wood et al. 1978; Kimura et al. 1988). Three major mitochondrial

haplotypes, the ‘Western’, ‘Egyptian’ and ’Eastern’, have been reported (Hsiao 1996; Erney et al. 1996; Böttger et al. 2013). The Western-strain/haplotype is infected with *Wolbachia* in its native range (Europe) and an introduced range (the United States) (Hsiao and Hsiao 1985). The *Wolbachia* variant belongs to the supergroup B (sensu Werren et al. 1995) (according to the *16S rRNA* gene fragment, O’Neill et al. 1992).

In Japan, *H. postica* was first found in Fukuoka and Okinawa Prefectures in 1982 (Kimura et al. 1988) and eventually has become a serious pest, indirectly affecting the apiculture industry (Okumura 1991) reaching to higher densities on chinese milk vetch than on other host plants (Iwase et al. 2015). Our previous survey detected no infected *H. postica* irrespective of its haplotypes in its introduced local populations from Fukuoka Prefecture (Iwase et al. 2015). Since mating between uninfected females and infected males causes unidirectional complete reproductive incompatibility (Hsiao and Hsiao 1985), the absence of *Wolbachia* enables higher production of inter-strain hybrids in *H. postica* induced by compatible crossbreeding between haplotypes (Iwase and Tani, in press). Crossbreeding among pest strains may produce offspring with resistance against pesticides or parasitoids and may change control efficiency of the pest (Oliver et al. 2003; Augustin et al. 2004).

In this study, we aim to quantify geographic distribution and change over time of *Wolbachia* infection of *H. postica* haplotypes to estimate inter-strain crossbreeding throughout Northern Kyushu, where *H. postica* invasion of Japan was first found. We use PCR diagnostics with *ftsZ* and *wsp* gene fragments of *Wolbachia*. To estimate the origin of *Wolbachia* infection and transmission route, we compare the nucleotide sequences of *Wolbachia* obtained from Japanese and European *H. postica* and reconstruct a molecular phylogeny.

Materials and Methods

The insect

Either larvae or adults of *H. postica* were collected from five populations in Fukuoka Prefecture and two populations in Oita Prefecture, by beating their host plants [either toothed medic (*Medicago polymorpha* L.), narrow-leaved vetch (*Vicia angustifolia*; senior synonym *V. sativa* L. subsp. *nigra* (L.) Ehrh.), or chinese milk vetch (*Astragalus sinicus* L.)] in May, 2013, 2014 and 2015 (Table 1, Fig. 1a). Our previous and on-going identification of *H. postica* haplotypes based on the mitochondrial gene, cytochrome b (*CYB*), sequence indicated that about 60%, 39% and 1.5% of the individuals collected in Northern Kyushu in 2013/2014 were the Western-, Egyptian- and Eastern-type, respectively (Iwase et al. 2015; Iwase and Tani, in press; see the next section for the method).

In addition, to assess any change over time in the infection distribution, *H. postica* specimens collected in 1982, 1985, 2001 and 2002 were obtained (Table 1, Fig. 1a). The specimens from 2001 and 2002 were preserved in acetone and earlier specimens were either dried (for specimens from 1982) or preserved in ethanol (for those from 1985). We included these old specimens in our analysis only if PCR products of their nuclear *28S rRNA* were confirmed, with primers described in Kim et al. (2000).

We also obtained *H. postica* from its native range (Czech Republic, the Netherlands and France) in April/May, 2012–2014 to check for infection with *Wolbachia*, its genetic identity, and its host's haplotype (Table 1, Fig. 1b). The *H. postica* from Czech were collected from *Medicago falcata* L. Those from the Netherlands were collected by sweeping cultivated *Medicago*. The *H. postica* from Chaussy were collected by pitfall traps and those from Auradé were obtained by sweeping non-host, *Brassica rapa* subsp. *oleifera*. We stored the collected *H.*

118 *postica* in acetone until use.

119

120 *Wolbachia* infection

121 Genomic DNA was extracted from the whole larval body or adult abdomen of a total of 242 *H.*
122 *postica* individuals (Table 1), using a DNeasy Blood and Tissue kit (Qiagen, Tokyo, Japan). The
123 DNA samples were subjected to PCR diagnostics to detect *Wolbachia* infection, based on two
124 *Wolbachia*-specific regions, *ftsZ*, *wsp* and, one supergroup-A *Wolbachia*-specific region, ARM
125 that can detect the supergroup even at low concentrations. We used the following primers;
126 fts-Z-f and fts-Z-r to amplify the *ftsZ* gene fragment (Holden et al. 1993), wspF and wspR to
127 amplify the *wsp* gene fragment (Kondo et al. 2002), and ARM-F1 and ARM-R1 for ARM
128 (Schneider et al. 2014). The PCR amplifications for *ftsZ*, *wsp* and ARM consisted of initial
129 preheating at 95°C for 2 min, followed by 32 cycles of denaturation at 94°C for 30 s (45 s for
130 ARM), annealing at 55°C for 40 s (45 s for ARM), and extension at 70°C (72°C for ARM) for 1
131 min (slightly modified from Kondo et al. 2002, 2011, Iwase et al. 2015, and Schneider et al.
132 2014). The *H. postica* from Europe that were found to be the Western strain naturally infected
133 with *Wolbachia* was used as a positive control and autoclaved water was used as a negative
134 control. If *Wolbachia* was detected, we further sequenced the PCR products of *ftsZ* and *wsp*, and
135 other MLST loci, *coxA* and *hcpA* in addition to *ftsZ* (Baldo et al. 2006), following Iwase et al.
136 (2015). To eliminate possible false positive result because of parasitization by parasitoid
137 infected with *Wolbachia*, we performed additional PCR diagnostics on the DNA extracted from
138 a foreleg and a midleg of a *Wolbachia*-positive *H. postica*. PCR products were checked by
139 electrophoresis on 1.3% agarose gel containing Midori Green DNA stain (Nippon Genetics,
140 Tokyo) for 13.5 min, with positive and negative controls and a molecular marker, followed by
141 illumination by 480–510-nm LED light.

142 To estimate the origin of *Wolbachia* infection and transmission, we compared the *ftsZ*,
143 *coxA*, *hcpA* and *wsp* sequences obtained from Japanese and European *H. postica*. We also
144 searched for nucleotide sequence(s) similar to our *ftsZ* and *wsp* sequences using BLAST
145 (National Library of Medicine). For *wsp*, the following sequences were compared; *Culex*
146 *quinquefasciatus* Pel strain (Diptera: Culicidae) (*w*Pip strain, GenBank accession, AM999887),
147 *Nilaparvata muiri* isolate Jinhua (Hemiptera: Delphacidae) (HQ404755), *Callosobruchus*
148 *chinensis* (Coleoptera: Chrysomelidae: Bruchinae) (*w*BruCon, AB038326), *Conotrachelus*
149 *nenuphar* (Coleoptera: Curculionidae) (*w*Cne1 strain, GU013550), *Diplolepis rosae*
150 (Hymenoptera: Cynipidae) (AF071922), *Nasonia vitripennis* isolate 34 (Hymenoptera:
151 Pteromalidae) (DQ842480), *Tetranychus urticae* ST279 (Trombidiformes: Tetranychidae)
152 (AF404766), *Bactocera pyrifoliae* (Diptera: Tephritidae) (*w*Pyr, AF295350) *Tomosvaryella*
153 *subvirescens* (Diptera: Pipunculidae) (*w*Hyd, AF481166), *Eurema hecabe* (Lepidoptera:
154 Pieridae) (*w*HecFem from strain Okinawa 4, AB094396), *Epirrita autumnata* (Lepidoptera:
155 Geometridae) (JX310340) and *Nephopterix tomisawai* (Lepidoptera: Pyralidae) (FJ441058). For
156 *ftsZ*, see a later section.
157
158 Identification of haplotype of infected *H. postica*
159 Individuals diagnosed as infected with *Wolbachia* were further identified for their haplotypes,
160 based on the mitochondrial *CYB*, because the nucleotide sequences of this coding region are
161 different among the three strains (Hsiao 1996) (Western-strain from Utah, GenBank accession
162 number U61174.1; Egyptian-strain from Arizona, U61173.1; Eastern-strain from Maryland,
163 U61172.1). In addition, haplotypes of a few randomly chosen uninfected individuals were
164 identified likewise. We amplified and sequenced *CYB*, using a primer set CB-J-11545mod
165 (5′-ACATGAATTGGAGCTCGACCA-3′) and N1-N-11841modCB

(5′-GGTACATTACCTCGGTTTCG-3′) [modified from Hsiao (1996)]. PCRs were performed following Tuda et al. (2004). Cycling conditions for *CYB* amplification were as follows; preheating at 95°C for 2 min, followed by 38 cycles of denaturation at 94°C for 50 s, annealing at 54°C for 1 min and extension at 60°C for 1 min. Resultant PCR products were purified and sequenced.

Reconstruction of molecular phylogeny of *Wolbachia* variants

We reconstructed a molecular phylogeny based on *ftsZ*, *coxA* and *hcpA* with additional sequence data of supergroup-B *Wolbachia* infecting *Culex quinquefasciatus* Pel strain (*w*Pip strain, GenBank accession, AM999887 for *ftsZ* and *coxA*), *Nilaparvata muiri* isolate Jinhua (HQ404753 for *ftsZ*, HQ404751 for *coxA*, and HQ404752 for *hcpA*), *Callosobruchus chinensis* (Yunnan strain, Tuda unpublished for *ftsZ*, *coxA* and *hcpA*), *Conotrachelus nenuphar* (*w*Cne1 strain, GU013553 for *ftsZ*), *Tribolium confusum* isolate 20 (Coleoptera: Tenebrionidae) (DQ842337 for *ftsZ*, DQ842301 for *coxA*, and DQ842412 for *hcpA*), *Diplolepis rosae* (U83888 for *ftsZ*), *Torymus bedeguaris* (Hymenoptera: Torymidae) (U83893 for *ftsZ*), *Encarsia formosa* isolate 33 (Hymenoptera: Aphelinidae) (DQ842324 for *ftsZ*, DQ842288 for *coxA*, and DQ842399 for *hcpA*), *Nasonia vitripennis* isolate 34 (DQ842333 for *ftsZ*, DQ842297 for *coxA*, and DQ842408 for *hcpA*), *Nasonia longicornis* isolate 16 (DQ842331 for *ftsZ*, DQ842295 for *coxA*, and DQ842406 for *hcpA*), *Tetranychus urticae* ST279 (JX094400 for *ftsZ*, JX094413 for *coxA*, and JX094405 for *hcpA*), and *Eurema hecabe* (*w*HecFem from strain Okinawa 4, AB107225 for *ftsZ*) (*w*Fem from strain ISG1, AB592920 for *ftsZ*, AB592910 for *coxA*, and AB592915 for *hcpA*).

Sequence data from the *Wolbachia* supergroup A were also included; *Wolbachia* from *C. chinensis* (*w*BruAus, AB080665 for *ftsZ*), *Drosophila melanogaster* (Diptera: Drosophilidae)

(*w*Mel, AE017196 for *ftsZ*, *coxA* and *hcpA*) and*N. vitripennis* strain 12.1 (U28188 for *ftsZ*, and FJ390240 for *coxA*). Supergroup-D *Wolbachia* infecting *Brugia malayi* isolate 37 (DQ842341 for *ftsZ*, DQ842273 for *coxA*, and DQ842384 for *hcpA*) was used as outgroup.

An evolutionary model for reconstruction of the molecular phylogeny was selected for each gene from the models employed in MrBayes using MrAIC.pl 1.3.1 (Nylander 2004). The GTR (general time reversible) model was supported by AICc. The phylogenetic relationships among *Wolbachia* variants were estimated with Bayesian inference using MrBayes 3.2 (Ronquist and Huelsenbeck 2003). For all model parameters, we used the default priors. The MCMC (Markov chain Monte Carlo) simulation was performed, with a partition by genes, sampled every 1,000 generations for 2,000,000 generations, of which initial 25% generations were discarded as a burn-in. The convergence of parameters among runs was checked using Tracer 1.5.0 (Rambaut and Drummond 2009).

Results

The PCR diagnostics using *ftsZ* and *wsp* fragments of expected sizes showed that none of the *H. postica* individuals from Northern Kyushu, irrespective of the year of collection, were infected with *Wolbachia*, except the ones from Ainoshima Island off Fukuoka collected in 2014 and 2015 (Fig. 2). They were a male Egyptian strain from the 2014 collection and a female Egyptian strain from the 2015 collection. The DNA additionally extracted from legs of the male was also *Wolbachia*-positive (Fig. 2). In Northern Kyushu, *Wolbachia* prevalence in the Western and Eastern strains was thus 0% and prevalence in the Egyptian strain was 1.43% in 2013/2014. All 14 *H. postica* individuals from Europe were positive with *Wolbachia* (i.e., prevalence = 100.0%,

Fig. 2). All of the European *H. postica* were confirmed to be the Western strain. None of the *H. postica* from Northern Kyushu and Europe were positive with supergroup-A *Wolbachia* basing ARM.

The 699-bp *ftsZ*, 432-bp *coxA* and 463-bp *hcpA* sequences of *Wolbachia* infecting the Egyptian-strains on Ainoshima Island was 100% identical to each other and to the variant infecting Yunnan-strain *C. chinensis*. The *ftsZ* sequence was 99.4% identical to the *w*Pip strain. The *ftsZ*, *coxA* and *hcpA* sequences of *Wolbachia* infecting the Western strain from the four European populations were identical to each other and the *ftsZ* showed 99.1% identity to the sequence of *Wolbachia* infecting *E. autumnata* and *N. tomisawai*. The two *Wolbachia* variants found infecting different strains of *H. postica* in Japan and Europe differed by 2.00% for *ftsZ*, 8.10% for *coxA*, and 1.73% for *hcpA*. Based on the mean MLST loci sequence difference of 3.58%, the genetic distance between the two *Wolbachia* variants was estimated as 26.9 million *Drosophila melanogaster* generations of divergence (95% confidence interval, 14.3–63.5 million years), based on the estimate of 6.65×10^{-10} (95% C.I., 2.82×10^{-10}–1.25×10^{-9}) substitutions per site per *D. melanogaster* generation by Richardson et al. (2012) (mean of the substitution rates at the $1^{st}/2^{nd}$ codon positions and the 3^{rd} position of *Wolbachia* genome).

The 541-bp *wsp* fragment of *Wolbachia* infecting the Egyptian strain from Ainoshima Island was 100.0% identical to *w*Pyr [host: *B. pyrifoliae*], *w*BruCon [host: *C. chinensis*], and *w*HecFem [host: *E. hecabe* Okinawa 4 strain]. The sequences of *Wolbachia* from the four European *H. postica* populations were identical to each other and were 97.0% identical to *Wolbachia* infecting *E. autumnata*, *N. tomisawai* and *Liriomyza trifolii* (Diptera: Agromyzidae). The *Wolbachia* variants infecting *H. postica* in Europe and Japan differed by 8.69% in this gene fragment.

Reconstructed molecular phylogeny of *Wolbachia* basing MLST loci showed both of

238 the two variants infecting *H. postica* belong to the supergroup B (Fig. 3). The Ainoshima variant
239 that was identical to the variant infecting Yunnan-strain *C. chinensis* belonged to a
240 well-supported clade of *w*Pip and its relatives (i.e., the *Wolbachia* variants associated with *T.*
241 *urticae* and *N. vitripennis*). The European variant formed a clade with the variants infecting
242 Okinawa islands strain of *E. hecabe* (Fig. 3).
243
244
245 **Discussion**
246
247 Among the three haplotypes of the invasive weevil *H. postica*, we found two Egyptian-strain
248 individuals on an island in Kyushu, Japan were infected with a *Wolbachia* variant. This genetic
249 variant was different from a European variant infecting the Western-strain of *H. postica* in its
250 native (Europe) and the other introduced (the US) areas. The infection was new to the Egyptian
251 strain in its native and invaded ranges. Our finding may indicate horizontal transfer of the
252 bacterium from other hosts, most likely insects, on the island. It is also possible that the infected
253 *H. postica* was introduced via Kyushu or directly from East Asia by anthropogenic (e.g. ferry
254 boats) or natural (e.g. airborne flight or drift through sea current) means. Different geographic
255 strains of an insect species can possess different *Wolbachia* variants (e.g. Kondo et al. 2002;
256 Muller et al. 2013) likely through independent horizontal transfers of each variant or, less likely,
257 loss of each different variant as doubly infected ancestral host population differentiated into
258 geographic strains. In *H. postica*, the loss of *Wolbachia* in a host strain (i.e., the Western strain)
259 occurred at a very early stage of invasion to Japan, while new infection in another strain (i.e.,
260 the Egyptian strain) was most likely quite recent. Loss of microbacteria including *Wolbachia*
261 during the process of invasions of new lands may frequently take place in host organisms

262 [Torchin et al. 2003; Mitchell and Power 2003; recent examples include the Argentine ant
263 (Tsutsui et al. 2003) and the fire ant (Ahrens and Shoemaker 2005; Yang et al. 2010)].

264 Phenotypes (host reproductive incompatibility, feminization, parthenogenesis, male
265 killing, resistance against pathogens, or no manipulative effect) (Werren 1997; Moreira et al.
266 2009; Hamm et al. 2014) of the *Wolbachia* variant newly found in the Egyptian strain are not
267 yet known in this study and requires further studies. The extremely low prevalence of the
268 *Wolbachia* variant in the haplotype (1.4%) may indicate that the variant is not beneficial for the
269 host or that the *Wolbachia* introduction/infection event was quite recent in Egyptian-strain *H.*
270 *postica*.

271 The *Wolbachia* infection not only in the abdomen but also in the legs of the host *H.*
272 *postica* (Fig. 2) excludes the possibility of false detection of *Wolbachia* infecting parasites or
273 parasitoids of *H. postica* [e.g. the adult parasitoid *Microctonus aethiopoides* (Hymenoptera:
274 Braconidae), whose establishment in Japan failed after repeated release during 1990–1999 (Moji
275 Plant Protection Station 2007)]. Infection of somatic tissues including legs is found in many
276 organisms (Dobson et al. 1999; Narita et al. 2007) and this may increase the rate of horizontal
277 transfer of *Wolbachia*.

278 Our on-going investigation of mitochondrial haplotypes of *H. postica* in Northern
279 Kyushu indicates that about 60% of the individuals were the Western type and the proportion is
280 stable through time [Iwase et al. 2015; see also Kuwata et al. (2005)'s result for 2001]. This
281 study showed none of these were infected with *Wolbachia*, supporting the result found for local
282 populations in Fukuoka Prefecture (Iwase et al. 2015). The absence of *Wolbachia* in the Western
283 strain may allow the increase in inheritance of Egyptian-strain haplotypes when the Egyptian
284 strain is crossbred with the Western strain, compared to the situation in which the Western strain
285 is infected with *Wolbachia*. This absence of infection indicates either invasion by uninfected

286 founders or lost infection following invasion, possibly because of fitness costs incurred by
287 infection (Sarakatsanou et al. 2011; Suh and Dobson 2013; Dykstra et al. 2014), high
288 temperature (Clancy and Hoffmann 1998), imperfect maternal transmission and/or agricultural
289 application of bactericide (McManus et al. 2002). With our current knowledge, all explanations
290 remain possibilities. Stochasticity may also add to these deterministic factors and cause loss of
291 *Wolbachia* especially in small populations (Jansen et al. 2008).

292 No association between mitochondrial haplotypes and host plant species in *H. postica*
293 (Iwase et al. 2015) suggests no effects of the absence of *Wolbachia* in the Western-strain on their
294 host plant usage. None of the *H. postica* strains had resistance against currently-used parasitoid
295 introduced to Japan [*Bathyplectes anurus* (Thomson) (Hymenoptera: Ichneumonidae), Okumura
296 and Shiraishi 2002] (Maund and Hsiao 1991). Further studies investigating whether *H. postica*
297 has not achieved resistance against the parasitoid, and whether there is any difference in
298 resistance against currently-used pesticides (Mori et al. 1991; Yamaguchi et al. 1993;
299 Hayashikawa et al. 2010) among strains will contribute to better control of *H. postica*.

300 In conclusion, this study suggested that the loss of *Wolbachia* occurred at a very early
301 stage of host insect's invasion to a new land or that the founder was uninfected. The new
302 infection in the new area may have taken place after 20–30 years of the first invasion. Both of
303 these possibilities support the enemy release hypothesis of invading organisms but with
304 additional direct time estimates.

305

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Table 1 Collected populations of *Hypera postica*

Location	Date	Stage	*n*
1 Moji, Fukuoka Prefecture	May 6, 2014	Larva	30
2 Onga, Fukuoka Prefecture	May 5, 2014	Larva	30
	April 11, 2002	Larva	12
3 Fukuoka Airport, Fukuoka Prefecture	May 3 and 4, 2014	Larva	30
	April 28, 1985	Larva	8
4 Chikuzen, Fukuoka Prefecture	May 5 and 9, 2014	Larva	30
	July 15, 1982	Adult	4
5 Ainoshima Island, Fukuoka Prefecture	May 21, 2014	Adult	15
	May 9, 2015	Adult	15
6 Usa, Oita Prefecture	May 9, 2014	Larva	30
	September 10, 2002	Adult	8
7 Oita city, Oita Prefecture	May 8, 2013	Adult	8
	May, 2001	Adult	8
8 Prague, Czech Republic	May 11, 2012	Adult	5
9 Amsterdam, the Netherlands	May 30, 2014	Adult	2
10 Chaussy, France	May 6, 2013	Adult	4
11 Auradé, France	April 14, 2014	Adult	3

NA: Not available

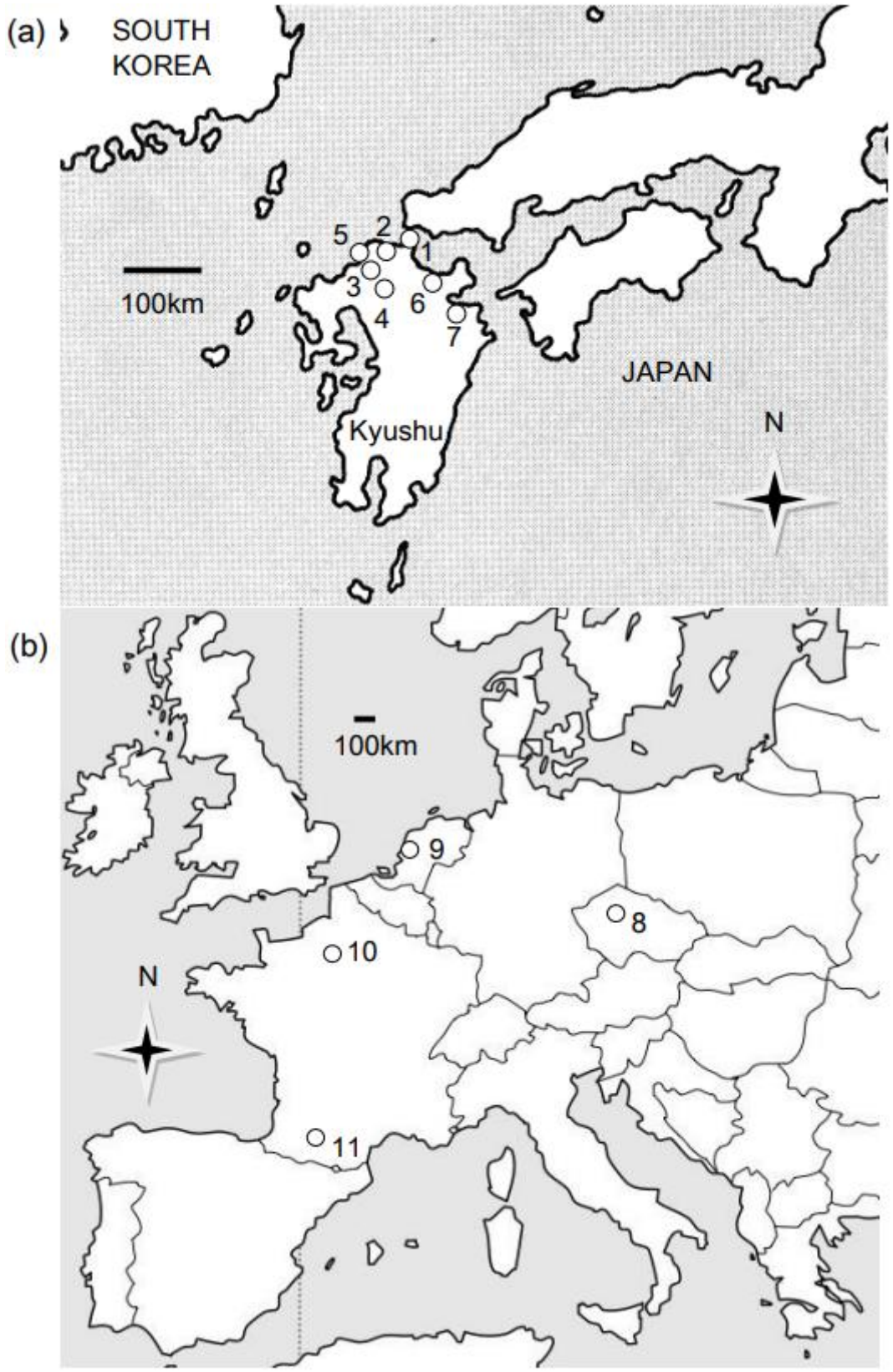
Figure legends

Fig. 1 Collection sites of *Hypera postica* in (a) Northern Kyushu and (b) Europe. 1, Moji; 2, Onga; 3, Fukuoka Airport; 4, Chikuzen; 5, Ainoshima Island; 6, Usa; 7, Oita City; 8, Prague, Czech Republic; 9, Amsterdam, the Netherlands; 10, Chaussy, France; 11, Auradé, France

Fig. 2 Gel electrophoresis of PCR products of *Wolbachia ftsZ* and *wsp* fragments. M: Molecular marker, T284 and T338: the Egyptian-strain *H. postica* from Ainoshima Island [(abd), abdomen; (leg), legs], T297: the Western-strain *H. postica* from Ainoshima Island, T210: the Egyptian-strain *H. postica* from Fukuoka Airport, CzW: the Western-strain *H. postica* from Czech, as a positive control, (-): a negative control

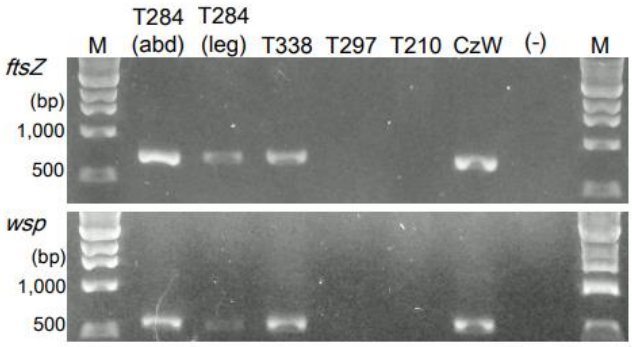
Fig. 3 Consensus molecular phylogeny of *Wolbachia* based on *ftsZ, coxA* and *hcpA.* Names of host insects are unitalicized, following the *Wolbachia* strain designation. *Wolbachia* variants found infecting *H. postica* are in bold. Numbers above the branches indicate Bayesian posterior probabilities (only $\geq$0.7 are shown)

Fig. 1



489

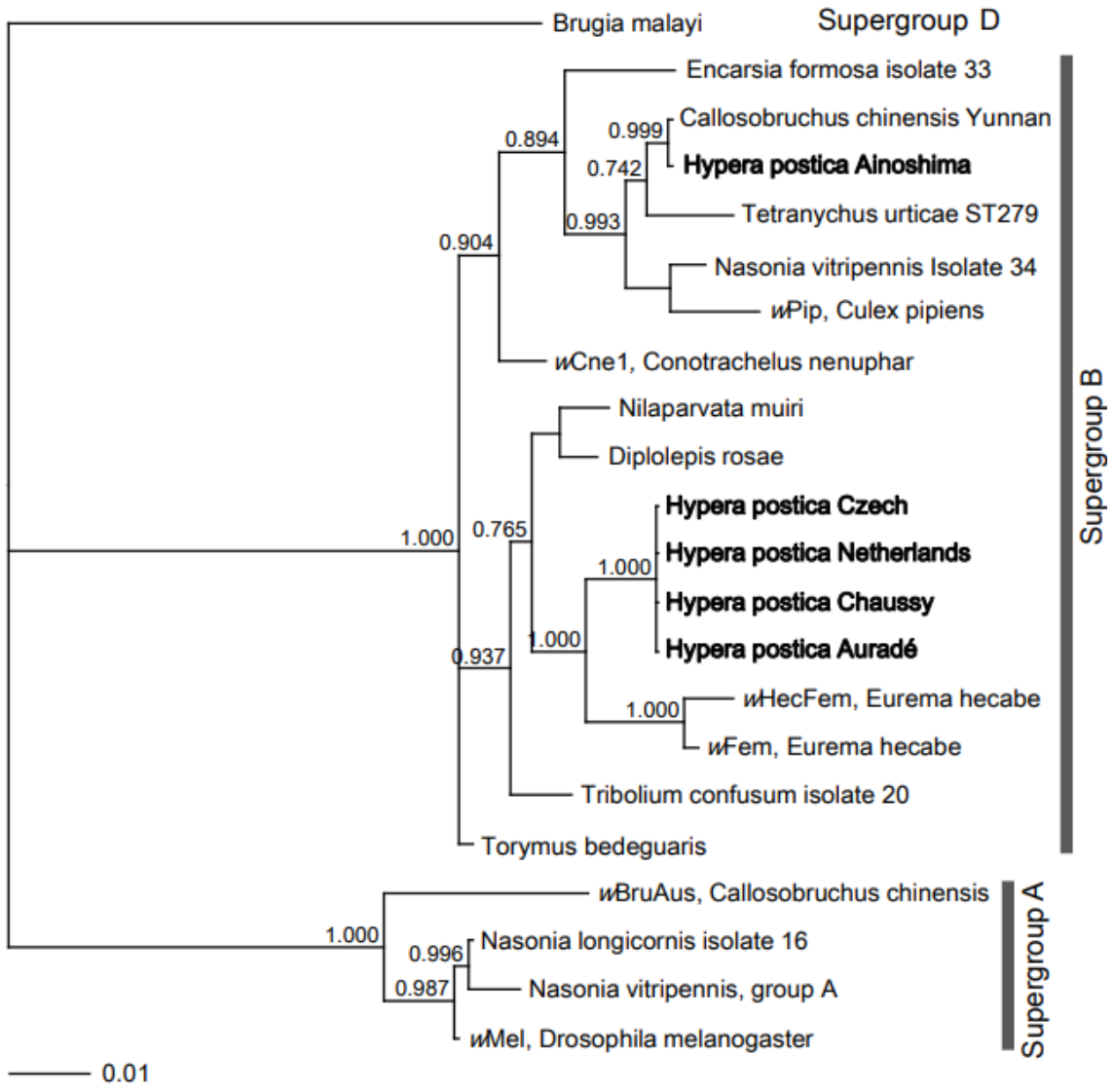
Fig. 2


M
T284 (abd)
T284 (leg)
T338
T297
T210
CzW
(-)
M
ftsZ
(bp)
1,000
500
wsp
(bp)
1,000
500

490

491

Fig. 3


Brugia malayi
Supergroup D
Encarsia formosa isolate 33
Callosobruchus chinensis Yunnan
Hypera postica Ainoshima
Tetranychus urticae ST279
Nasonia vitripennis Isolate 34
wPip, Culex pipiens
wCne1, Conotrachelus nenuphar
Nilaparvata muiri
Diplolepis rosae
Hypera postica Czech
Hypera postica Netherlands
Hypera postica Chaussy
Hypera postica Auradé
wHecFem, Eurema hecabe
wFem, Eurema hecabe
Tribolium confusum isolate 20
Torymus bedeguaris
wBruAus, Callosobruchus chinensis
Nasonia longicornis isolate 16
Nasonia vitripennis, group A
wMel, Drosophila melanogaster
Supergroup B
Supergroup A
0.894
0.999
0.742
0.993
0.904
1.000
0.765
1.000
1.000
0.937
1.000
1.000
0.996
0.987
0.01

492