

Obligate symbiont involved in pest status of host insect

Takahiro Hosokawa^{1,2}, Yoshitomo Kikuchi^{1,3}, Masakazu Shimada²
and Takema Fukatsu^{1,2,*}

¹*Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan*

²*Department of Systems Sciences, University of Tokyo, Tokyo 153-8902, Japan*

³*Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269-3125, USA*

The origin of specific insect genotypes that enable efficient use of agricultural plants is an important subject not only in applied fields like pest control and management but also in basic disciplines like evolutionary biology. Conventionally, it has been presupposed that such pest-related ecological traits are attributed to genes encoded in the insect genomes. Here, however, we report that pest status of an insect is principally determined by symbiont genotype rather than by insect genotype. A pest stinkbug species, *Megacopta punctatissima*, performed well on crop legumes, while a closely related non-pest species, *Megacopta cribraria*, suffered low egg hatch rate on the plants. When their obligate gut symbiotic bacteria were experimentally exchanged between the species, their performance on the crop legumes was, strikingly, completely reversed: the pest species suffered low egg hatch rate, whereas the non-pest species restored normal egg hatch rate and showed good performance. The low egg hatch rates were attributed to nymphal mortality before or upon hatching, which were associated with the symbiont from the non-pest stinkbug irrespective of the host insect species. Our finding sheds new light on the evolutionary origin of insect pests, potentially leading to novel approaches to pest control and management.

Keywords: *Megacopta punctatissima*; *Megacopta cribraria*; *Candidatus* Ishikawaella capsulata; symbiont capsule; plant adaptation; pest evolution

1. INTRODUCTION

In many herbivorous insects, different populations of the same species often use different food plants, which are referred to as host races, biotypes or ecotypes. Formation of such intraspecific plant specialization must have evolved through acquisition of a new food plant by a local population of the insect. In the case that the new food plant is an agricultural plant, the insect population will be recognized as an emergent pest. Hence, the origin of specific insect genotypes that enable efficient use of agricultural plants is an important subject not only in applied fields like pest control and management but also in basic disciplines like evolutionary biology (Via 1990; Berlocher & Feder 2002; Karban & Agrawal 2002; Coyne & Orr 2004; Shoonhoven *et al.* 2005). Conventionally, it has been presupposed that such ecological traits are attributed to genes encoded in the insect genomes (Feder *et al.* 1988; Hawthorne & Via 2001).

However, recent studies have revealed that facultative bacterial symbionts may substantially affect various ecological traits of herbivorous insects. For example, several species of facultative symbionts play important biological roles for the pea aphid *Acyrtosiphon pisum* in specific ecological

contexts, including tolerance to high temperature (Montllor *et al.* 2002; Russell & Moran 2006), resistance to parasitoid wasps (Oliver *et al.* 2003, 2005), resistance to pathogenic fungi (Scarborough *et al.* 2005), broadening of food plant range (Tsuchida *et al.* 2004), and others. Here, an idea arises that the symbiont could be involved in emergence of insect pest. However, although many pest insects of agricultural, economical and medical importance are known to be associated with bacterial symbionts (Bourtzis & Miller 2003, 2006), there have been no studies demonstrating that the symbiont is responsible for pest status of its host insect.

Many stinkbugs (Insecta: Heteroptera) are, by sucking plant sap and tissues, known as notorious agricultural pests (Schaefer & Panizzi 2000). These plant-feeding stinkbugs are generally in close association with gut symbiotic bacteria: they have a specialized midgut section bearing a number of caecal evaginations, in which a copious amount of symbiont is harboured (Buchner 1965). When experimentally deprived of the symbiont, host stinkbugs suffer retarded growth and high mortality (Buchner 1965; Abe *et al.* 1995; Fukatsu & Hosokawa 2002; Hosokawa *et al.* 2006). Probably the host stinkbugs are provided by the symbionts with essential nutrients that are lacking in their diet, as aphids are nutritionally dependent on the endocellular symbiont *Buchnera aphidicola* (Douglas 1989; Baumann *et al.* 2000).

Stinkbugs of the family Plataspidae harbour an obligate γ -proteobacterial symbiont '*Candidatus* Ishikawaella capsulata' in the cavity of crypt-bearing posterior midgut

* Author and address for correspondence: Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan (t-fukatsu@aist.go.jp).

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(Hosokawa *et al.* 2006). The plataspid stinkbugs have been known for their unique mechanism for vertical transmission called 'symbiont capsule'. When adult females lay eggs on their host plant, small brownish particles are always deposited under the egg mass. The particles encase a copious amount of the symbiont inside, and hatchlings from the eggs orally acquire the symbiont from the capsule (Schneider 1940; Müller 1956; Fukatsu & Hosokawa 2002; Hosokawa *et al.* 2005, 2006; figure 1a; electronic supplementary material, movie 1). When deprived of the symbiont capsule, the symbiont-free insects suffer abnormal coloration, retarded growth, smaller body size, higher mortality and sterility (Müller 1956; Fukatsu & Hosokawa 2002; Hosokawa *et al.* 2006). Hence, despite the extracellular location in the gut cavity, the symbionts are regarded as obligate mutualistic associates for the host stinkbugs. The symbiont phylogeny shows a perfect agreement with the host phylogeny, indicating strict host-symbiont cospeciation over evolutionary time (Hosokawa *et al.* 2006). A number of plataspid species have been reported as pests of various legumes and other agricultural plants (Tomokuni 1993; Schaefer & Panizzi 2000).

In this study, by making use of the unique transmission system mediated by symbiont capsules, we experimentally demonstrated that pest status of a plataspid stinkbug is determined by the symbiont genotype rather than by the insect genotype.

2. MATERIAL AND METHODS

(a) Insect sampling and rearing

Freshly laid egg masses of *Megacopta punctatissima* were collected from *Pueraria lobata* at Tsukuba, Ibaraki, Japan. Egg masses of *Megacopta cribraria* were obtained from sexually mature females that had been collected from *Pueraria montana* at Naha, Okinawa, Japan. Each of the field-collected females of *M. cribraria* was allowed to oviposit in a Petri dish containing several pea pods (*Pisum sativum*). To avoid pseudoreplication, all of the egg masses of *M. cribraria* used for experiments were derived from different females. These insects were maintained in climatic chambers at 25°C in a long-day regimen (16 h light–8 h dark).

(b) Experimental manipulation

Only newly laid egg masses containing 24 or more eggs and 7 or more capsules were used for experiments. All of these egg masses exhibited normal egg hatch rates ranging from 80 to 100%. To minimize confounding effects of the insect genetic backgrounds, two experimental groups were produced from each of the egg masses for control treatment and symbiont-replacing treatment, respectively. Each of the egg masses was separated into individual eggs and capsules by using fine forceps under a binocular microscope. To sterilize remnant symbiont cells possibly adhering to the egg surface, the eggs were treated with 70% ethanol for 5 min, 4% formalin for 30 min and 70% ethanol for 10 s, and were dried in air. For control treatment, randomly chosen 12 eggs and 7 capsules from an egg mass were realigned like a natural egg mass and were glued on a piece of filter paper. For symbiont-replacing treatment, the other 12 eggs from the egg mass were realigned with 7 heterospecific capsules in the same way. These experimental egg masses were individually kept in a Petri dish with a wet cotton ball.

(c) Fitness measurement

For each of the stinkbug species, nymphs from 18 sets (families) of a pair of experimental egg masses were subjected to fitness measurements. After a day of egg hatch, 10 nymphs were randomly chosen from each of the egg masses and reared on a potted soya bean plant (*Glycine max*). These insects were examined for growth rate (nymphal period), adult emergence rate (%) and adult body size (thorax width).

Adult insects that emerged from the 17 out of 18 sets for *M. punctatissima* and those from the 16 out of 18 sets for *M. cribraria* were examined for their reproductive performance. Each of several (1–5) pairs of a male and a female from the same egg masses was separately kept in a Petri dish and provided with pea pods. The dishes were inspected everyday and the pea pods were periodically replaced with fresh ones. All egg masses laid by each of the females were collected during 30 days after the first oviposition. Each of the egg masses was kept in a Petri dish and inspected, and the eggs were categorized into hatched eggs, fertilized unhatched eggs and unfertilized eggs. Nymphal body segments and/or red eyespots were seen in fertilized eggs but not in unfertilized eggs. Nymphs that died during hatching were counted as fertilized unhatched eggs. For each of the females, total number of eggs, egg fertilization rate (%) and egg hatch rate (%) were calculated. Females that died without producing eggs were excluded from the analyses. Proportions of such females were statistically not different between the control and symbiont-replaced treatments (5 out of 62 control females versus 5 out of 58 symbiont-replaced females in *M. punctatissima*, Fisher's exact probability test, $p=1$; 4 out of 45 control females versus 6 out of 43 symbiont-replaced females in *M. cribraria*, Fisher's exact probability test, $p=0.517$).

(d) Cloning and sequencing

DNA was extracted from isolated symbiont capsules by using a QIAamp tissue mini kit (QIAGEN), from which a 1.7 kbp segment of eubacterial *groE* gene was amplified by polymerase chain reaction (PCR) with primers Gro-F1 (5'-ATG GCA GCW AAA GAC GTA AAT TYG G-3') and Gro-R1 (5'-TTA CAT CAT KCC GCC CAT GC-3'). The PCR product was cloned and sequenced as described previously (Kikuchi & Fukatsu 2003). The *groE* sequences of the symbiont from *M. punctatissima* and *M. cribraria* were deposited in the DNA Data Bank of Japan database with accession numbers AB231904 and AB264337, respectively.

(e) Symbiont quantification

We constructed seven and six sets of control and symbiont-replaced egg masses for *M. punctatissima* and *M. cribraria*, respectively. After a day of egg hatch, 10 nymphs from each of the experimental egg masses were individually subjected to DNA extraction and symbiont quantification. The symbiont titres were measured in terms of bacterial *groE* gene copies by using a quantitative PCR technique as described previously (Koga *et al.* 2003). Since the symbiont *groE* gene sequences were completely identical between *M. punctatissima* and *M. cribraria* (accession nos. AB231904 and AB264337, respectively), the same primers MEGAgro-F1 (5'-GGT GCT GCC ACT GAA GTT GA-3') and MEGAgro-R1 (5'-CCG CTA CAC GCA CTA ACG C-3') and the same probe MEGAgro-P1 (5'-TGA AGA AGG TGT CGT TCC CGG AGG-3') were used.

(f) Statistics

To statistically evaluate the differences between the control and the symbiont-replacing treatment, the Wilcoxon signed-rank test was adopted for adult emergence rate, and a generalized linear model (GLM) framework was applied to the other fitness parameters (McCullagh & Nelder 1989). In the GLM analyses, for egg fertilization rates and egg hatch rates, we used binomial error or, if overdispersion was detected, quasi-binomial error (Crawley 1993, 2005), whereas for the other data, an appropriate error distribution was selected from normal, Poisson, gamma, inverse normal and negative binomial errors according to the Akaike information criterion. Two terms, namely symbiont types (control and replaced) and family, were included in the models, and the effects of the terms were evaluated by an analysis of deviance (Crawley 1993). All the statistical analyses were conducted by using a software R v. 2.3.1 (R Development Core Team 2006).

3. RESULTS AND DISCUSSION**(a) Closely related pest and non-pest plataspid stinkbugs**

The plataspid stinkbug *M. punctatissima* (figure 1b) is commonly found in the mainland Japan, while a closely related stinkbug *M. cribraria* (figure 1c) is distributed across the southwestern islands of Japan. They are classified into different species morphologically: e.g. *M. cribraria* is smaller in size and paler in colour than *M. punctatissima* (figure 1b,c). However, they are no doubt close genetically and biologically: their mitochondrial 16S *rRNA* genes showed 99.3% (1565/1576 nucleotide sites) sequence identity (Hosokawa *et al.* 2006); reciprocal crosses between the species resulted in F_1 offspring with intermediate morphological traits; and crosses between the F_1 insects could produce F_2 offspring (T. Hosokawa 2005, unpublished data). The main host plants of *M. punctatissima* and *M. cribraria* are wild leguminous vines *P. lobata* and *P. montana*, respectively, while these insects also use other leguminous plants (Tomokuni 1993). In particular, *M. punctatissima* has been known as pest of soya bean, pea and other legumes. The insects often gregariously infest the plants and damage the crops, and without spraying, lay eggs and proliferate in the legume fields (Kono 1990; Tomokuni 1993; Endo *et al.* 2002). On the other hand, *M. cribraria* scarcely causes such serious problems in Japan, although infestation on soya bean and other legumes has been occasionally reported (Tomokuni 1993). What is the basis of the difference between the pest and non-pest insects?

(b) Fitness parameters of pest and non-pest stinkbugs on crop legumes

In order to address the question, we evaluated the general performance of the pest species *M. punctatissima* and the non-pest species *M. cribraria* on potted soya bean plants and pea pods. Both species normally grew to adult and laid eggs (figure 2b–e). The eggs were certainly fertilized (figure 2f). However, egg hatch rates were strikingly different between the species; around 80% in *M. punctatissima* in contrast to only 50% in *M. cribraria* (figure 2g). The difference was statistically significant (median test, $p < 0.0001$). A characteristic mortality symptom was observed in the egg masses of *M. cribraria*, wherein many nymphs failed to escape from the eggshell

and died (figure 3b). These dead nymphs were generally frail in size and morphology, probably due to developmental abnormality (data not shown).

(c) Low egg hatch rate of non-pest stinkbug on crop legumes

These results indicated that the non-pest species *M. cribraria* suffers low egg hatch rate on the crop legumes while the pest species *M. punctatissima* does not, which is probably relevant to their different pest status. The crop legumes, although used under the laboratory condition, are unsuitable host plants for *M. cribraria*.

(d) Experimental symbiont exchange between pest and non-pest stinkbugs

In most of the obligate endosymbiotic systems in insects, such as those in aphids and tsetse flies, the host and the symbiont are structurally, functionally and developmentally integrated into an almost inseparable biological entity (Buchner 1965; Douglas 1989; Baumann *et al.* 2000; Braendle *et al.* 2003). Thus, it has been practically impossible to manipulate these obligate host–symbiont associations experimentally. However, the unique capsule-mediated transmission system in the plataspid stinkbugs enabled us to attempt such experiments despite the obligate nature of the symbiosis. The host eggs and the symbiont capsules were separated by using forceps under a binocular microscope, and the eggs of *M. punctatissima* were combined with the capsules of *M. cribraria*, and vice versa. The hatchlings readily accepted the heterospecific capsules and ingested the content. Quantitative PCR assays confirmed that the nymphs certainly acquired the heterospecific symbiont cells, and the acquired amount was equivalent to that of the conspecific symbiont cells (figure 2a).

(e) Fitness parameters of pest and non-pest stinkbugs on crop legumes after symbiont exchange

The symbiont-replaced insects normally grew to adult and laid eggs (figure 2b–e). Although symbiont-eliminated adults of these stinkbugs were reported to severely suffer abnormal coloration and reduced body size (Fukatsu & Hosokawa 2002; Hosokawa *et al.* 2006), the symbiont-replaced adults were almost indistinguishable from the control adults (figure 1b,c), except that growth rate and body size of *M. cribraria* were slightly but significantly reduced in association with the symbiont replacement (figure 2c,d). In both the species, the eggs laid by the symbiont-replaced adults were certainly fertilized (figure 2f), but egg hatch rates were strikingly different between the species; around 90% in *M. cribraria* in contrast to only 25% in *M. punctatissima* (figure 2g). The difference was statistically significant (median test, $p < 0.0001$). In the egg masses deposited by the symbiont-replaced females of *M. punctatissima*, many nymphs failed to escape from the eggshell and died (figure 3c), which was reminiscent of the symptom observed with the egg masses deposited by the normal females of *M. cribraria* (figure 3b).

(f) Pest status of *Megacopta punctatissima* determined by symbiont genotype

In summary, the fitness measurements on the crop legumes in combination with the symbiont-replacing experiments demonstrated that (i) *M. punctatissima* that

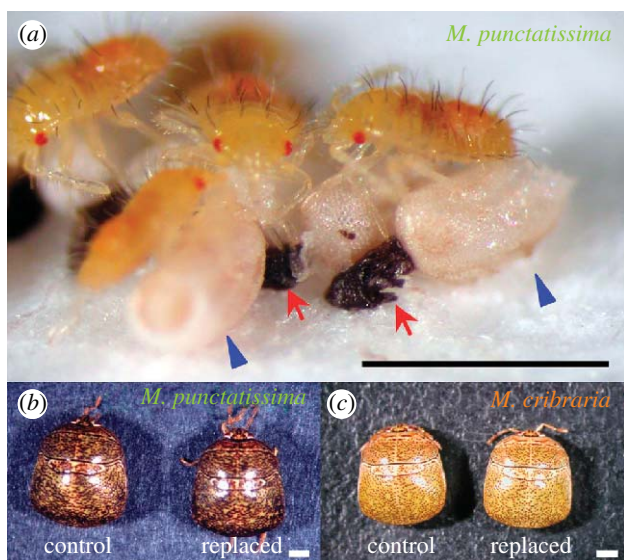


Figure 1. Pest and non-pest plataspid stinkbugs. (a) Newborn nymphs of *Megacopta punctatissima* probing capsules for symbiont acquisition. Arrows and arrowheads indicate symbiont capsules and eggshells, respectively. (b) Pest species *M. punctatissima*. (c) Non-pest species *Megacopta cribraria*. Normal adult females with their original symbiont (control) and manipulated adult females whose symbiont was experimentally replaced by the heterospecific one (replaced) are shown. Scale bars, 1 mm.

originally exhibited a normal egg hatch rate suffered a low egg hatch rate when infected with the symbiont from *M. cribraria*, (ii) *M. cribraria* that originally exhibited a low egg hatch rate restored a normal egg hatch rate when infected with the symbiont from *M. punctatissima*, (iii) the mortality symptom in hatchlings was similar irrespective of the stinkbug species and was associated with the symbiont from *M. cribraria*, and (iv) hence, the normal egg hatch rate and the good performance of the stinkbugs on the crop legumes were attributed to the symbiont from *M. punctatissima*. These results strongly suggest that the pest status of *M. punctatissima* is principally determined by the symbiont genotype rather than by the insect genotype.

(g) Evolutionary origin of pest-related symbiont genotype?

The mechanism whereby the symbiont from *M. punctatissima* can support the normal development of the host insects on the crop legumes is unknown. In plataspid stinkbugs, the host insect phylogeny perfectly agreed with the symbiont phylogeny, indicating stable host–symbiont association over evolutionary time (Hosokawa *et al.* 2006). Among the plataspid stinkbugs phylogenetically analysed, *M. punctatissima* and *M. cribraria* are the closest genetically (Hosokawa *et al.* 2006). The symbionts from *M. punctatissima* and *M. cribraria* are also very close genetically as their host insects are: their 16S *rRNA* genes showed 99.9% (1308/1309 nucleotide sites) sequence identity, and their genome size was estimated to be 0.82 Mbp in common (Hosokawa *et al.* 2006). It appears probable, although speculative, that mutations occurring in the symbiont genomes after the host speciation have modulated their capability of using different host plants, which predisposed the host insects to potentially become pests or not.

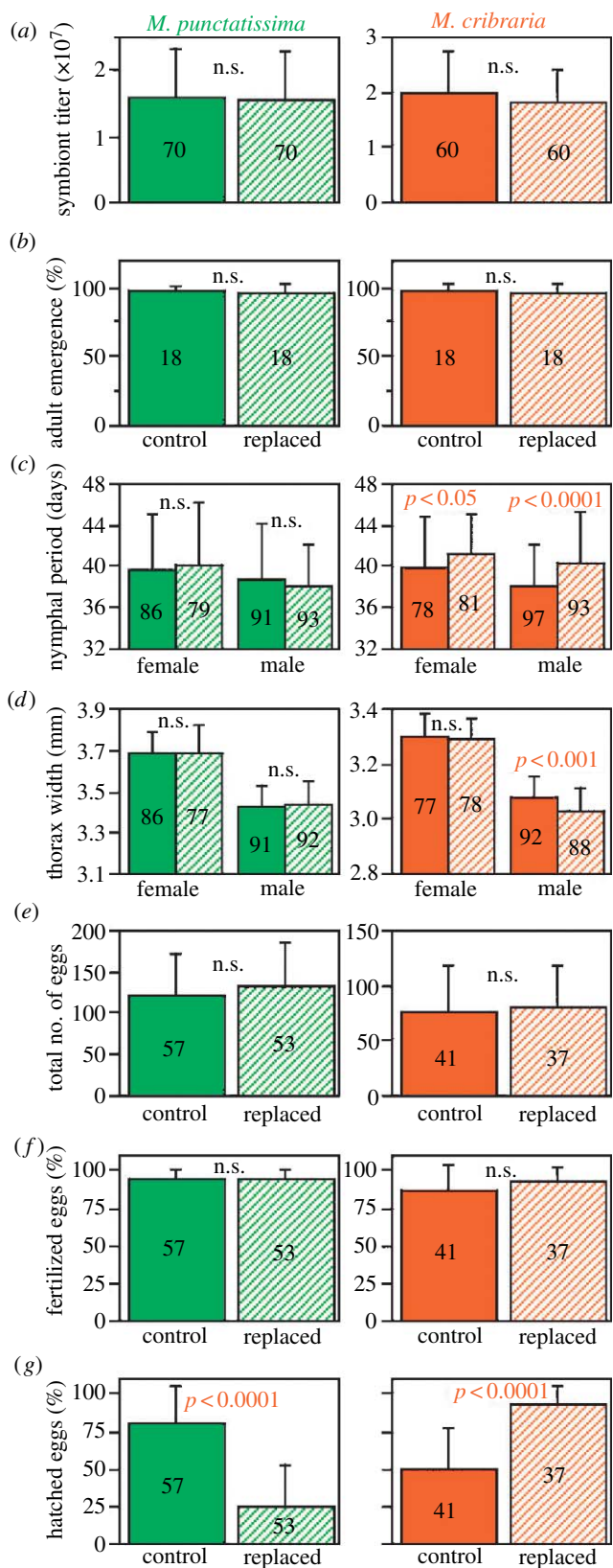


Figure 2. Fitness measurements of normal and symbiont-replaced plataspid stinkbugs. (a) Symbiont titre acquired by newborn nymphs, in terms of symbiont *groE* gene copies per insect. (b) Adult emergence rate (%). (c) Growth rate, in terms of nymphal period (days). (d) Adult body size, in terms of thorax width (mm). (e) Total number of eggs produced by an adult female. (f) Fertilization rate of eggs (%). (g) Hatch rate of eggs (%). Means and standard deviations are shown. Sample sizes are indicated on columns. Statistically significant differences between control treatment and symbiont-replacing treatment are shown in red.

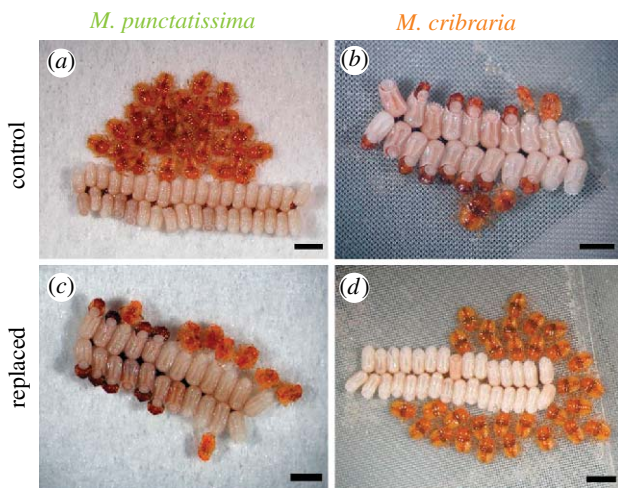


Figure 3. Mortality symptom observed with hatchlings of plataspid stinkbugs reared on the crop legumes. (a,c) *Megacopta punctatissima*, (b,d) *M. cribraria*, (a,b) egg masses laid by normal females and (c,d) egg masses laid by symbiont-replaced females. Scale bars, 1 mm.

(h) What underlies symbiont-mediated fitness effect on host insect: plant-specific difference or general difference in vigour?

For herbivorous insects, being pests basically requires the ability to use an alternative suboptimal host plant (Karban & Agrawal 2002; Shoonhoven *et al.* 2005). For *M. punctatissima* and *M. cribraria*, wild leguminous vines *Pueraria* spp. are the main optimal host plants. Both species perform well on their original host plant, attaining over 90% egg hatch rates under natural conditions (T. Hosokawa 2005, unpublished data). Meanwhile, soya bean and other crop legumes are regarded as auxiliary suboptimal host plants for these insects. We found that normal *M. cribraria* and symbiont-replaced *M. punctatissima* suffer low egg hatch rates on the crop legumes (figures 2g and 3), indicating that the symbiont from *M. cribraria* is responsible for the poor performance of the insects on suboptimal host plants. In other words, the symbiont from *M. punctatissima* was superior to the symbiont from *M. cribraria* in supporting normal development of the host insects on the suboptimal host plants. There are two mechanisms as to how these symbionts differ in their effects on the host fitness. One mechanism is a plant-specific difference, wherein the symbiont from *M. punctatissima* physiologically performs better for the host insects than the symbiont from *M. cribraria* specifically on the suboptimal host plants. The other mechanism is a general difference in vigour, wherein the symbiont from *M. punctatissima* generally shows better performance for the host insects than the symbiont from *M. cribraria*, and the difference becomes obvious on the suboptimal host plants but not on the optimal host plants. If the former scenario is true, we should consider the possibility that the biological trait of the symbiont from *M. punctatissima* might have been selected for in the mainland Japan where the crop legumes are widely cultivated. On the other hand, if the latter scenario is true, the possibility becomes less likely, and the difference might be attributed to more general aspects of the symbiont genomes. To verify which of these mechanisms better accounts for the phenomena, further experimental studies, in particular fitness measurements of the

symbiont-manipulated insects on the optimal host plants, are needed.

(i) Plant specialization of herbivorous insect mediated by obligate symbiont

Recent studies have revealed that some facultative microbial symbionts substantially affect various ecological traits of herbivorous insects including plant specialization (Montllor *et al.* 2002; Oliver *et al.* 2003, 2005; Tsuchida *et al.* 2004; Scarborough *et al.* 2005; Leonardo & Mondor 2006; Russell & Moran 2006). Our discovery indicates that even obligate symbionts that play essential biological roles for their host may also affect plant specialization, and suggests that such symbionts could potentially be causal agents of emergent insect pests. It is currently unknown how prevalent similar cases of symbiont-mediated plant specialization are in natural and agricultural ecosystems. In this context, it is of both evolutionary and practical importance to survey the correlation between symbiont genotypes and host races/biotypes/ecotypes in various insect–microbe symbiotic systems.

(j) Perspective for pest control and management

A number of agriculturally, economically and medically notorious insect pests harbour symbiotic micro-organisms (Bourtzis & Miller 2003, 2006). In some of these cases, the symbionts have been suggested as possible agents for controlling the pests by using paratransgenic approach (Durvasula *et al.* 1997; Ben Beard *et al.* 2002), symbiont-driven population replacement (Dobson 2003; Sinkins & Gould 2006) and incompatible insect technique (Zabalou *et al.* 2004). Strikingly, it was reported that the most widely applied biological insecticide, *Bacillus thuringiensis*, is effective to lepidopteran larvae only when the insects harbour a gut microbial community (Broderick *et al.* 2006), illuminating profound relevance of insect gut bacteria to pest control. The gut symbiotic bacteria of the plataspid stinkbugs provide a model system for understanding the mechanisms underlying the symbiont-mediated pest evolution, which would potentially lead to novel means of pest control and management. Functional and genomic analyses of the stinkbug symbiont would lead to further insights into how the symbionts affect such ecological traits of the host insects.

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