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Detection of ecological hybrid inviability in a pair of sympatric phytophagous ladybird beetles (*Henosepilachna* spp.)

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Abstract

Ecological speciation is a process by which reproductive isolation evolves as the result of divergent natural selection between populations inhabiting distinct environments or exploiting alternative resources. Ecological hybrid inviability provides direct evidence for ecological speciation. To detect ecological hybrid inviability, we examined survival rates to the second instar of F1 hybrids and backcross hybrids in a pair of sympatric phytophagous ladybird beetles, *Henosepilachna niponica* Lewis and *Henosepilachna yasutomii* Katakura (Coleoptera: Coccinellidae: Epilachninae), reared on their respective host plants, thistle [*Cirsium alpicola* Nakai (Asteraceae)] and blue cohosh [*Caulophyllum robustum* Maxim. (Berberidaceae)], and on a common food plant, Japanese nightshade [*Solanum japonense* Nakai (Solanaceae)]. Hybrid larvae reared on leaves of the Japanese nightshade always had high rates of survival, irrespective of the crossing type of their parents, suggesting a lack of intrinsic hybrid inviability between the two species. In contrast, survival rates on thistle and blue cohosh varied greatly. On blue cohosh, the survival rate of F1 hybrids was nearly as high as that of *H. yasutomii*, but on thistle, survival was significantly lower than of *H. niponica*. Survival rates of backcross hybrids on the two host plants were intermediate between those of the parents, showing a reversed rank order of different types of backcross hybrids on the two food plant species. These results suggest that ecological hybrid inviability exists between *H. niponica* and *H. yasutomii*, although the two species do not show intrinsic hybrid inviability. Thus, our study supports the hypothesis that *H. niponica* and *H. yasutomii* underwent ecological speciation by divergent selection.

Introduction

Ecological speciation is defined as the process by which reproductive isolation evolves as the result of divergent natural selection between populations inhabiting distinct environments or exploiting alternative resources (Mayr, 1942; Endler, 1977; Rice & Hostert, 1993; Schluter, 2000; Rundle & Nosil, 2005). This divergent natural selection may yield ecological hybrid inviability, in which the poor adaptation of hybrids to either of the parental habitats rather than intrinsic genetic incompatibility reduces hybrid fitness (Coyne & Orr, 2004; Kawecki, 2004). Ecological hybrid inviability is a unique expectation of, and provides direct evidence for, ecological speciation (Schluter, 2000; Rundle & Nosil, 2005). However, only a few studies have demonstrated ecological hybrid inviability, distinguishing it from genetic incompatibility that can result not only from ecological but also from non-ecological speciation processes (Schluter, 2000; Rundle & Nosil, 2005). In phytophagous insects, ecological hybrid inviability has been reported or suggested for the apple and hawthorn races of the apple maggot fly, *Rhagoletis pomonella* (Walsh) (Linn et al., 2004), two host races of the goldenrod gall fly, *Eurosta solidaginis* Fitch (Craig et al., 1997, 2007), and host races of the pea aphid, *Acyrthosiphon pisum* (Harris), on alfalfa and red clover (Via et al., 2000).

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In the present study, we examined ecological hybrid inviability in a pair of Japanese phytophagous ladybird beetles adapted to different host plants. *Henosepilachna niponica* (Lewis) (Coleoptera: Coccinellidae: Epilachninae), feeding on wild thistles [*Cirsium* spp. (Asteraceae)], and *Henosepilachna yasutomii* Katakura, feeding on blue cohosh [*Caulophyllum robustum* Maxim. (Berberidaceae)], are sympatric from southernmost Hokkaido (the northern main island of Japan) to the middle of Honshu (the central main island) (Katakura, 1981, 1997). Their reproductive isolation has been studied in detail, mainly in populations sampled at Nanae-cho, Hokkaido, Japan. Katakura et al. have argued that the two species are reproductively isolated solely by fidelity to their respective host plants (Katakura et al., 1981, 1989; Katakura & Hosogai, 1994, 1997; Katakura, 1997), and subsequent field cage experiments supported this view (Hirai et al., 2006). Existing evidence thus strongly suggests that *H. niponica* and *H. yasutomii* have undergone ecological speciation. However, no previous study has detected ecological hybrid inviability between these two ladybird species (Katakura & Hosogai, 1994, 1997).

One way to partition total hybrid inviability into ecological hybrid inviability and genetic incompatibility is to compare the fitness of hybrids in the wild to that under benign conditions, where ecological factors have been removed (Rundle & Nosil, 2005). Another way is to compare the fitness of the two types of backcrosses in the respective parental environments. With this latter approach, if there were ecological hybrid inviability, the fitness of backcross progeny relative to each parental species in its native habitat would be greater than that of backcross progeny relative to each parental species outside its native habitat (Rundle, 2002). In the present study, we estimated ecological hybrid inviability between *H. niponica* and *H. yasutomii* by combining these two approaches. We first compared survival rates between progeny of backcrosses with the thistle-adapted species (*H. niponica*) and progeny of backcrosses with the blue cohosh-adapted species (*H. yasutomii*), to test whether the reduction in hybrid fitness depends on differences between the food plants. We then compared the results with those obtained by rearing the beetles on an alternative food plant, Japanese nightshade [*Solanum japonense* Nakai (Solanaceae)]. Japanese nightshade is used for the laboratory rearing of larvae of both *H. niponica* and *H. yasutomii* (Fujiyama & Katakura, 2002b). It is eaten by adults of both species of ladybird beetle, even when presented along with native food plants in a field cage (Hirai et al., 2006), although no population of either ladybird species has been reported feeding on Japanese nightshade in the wild. We consider that the Japanese nightshade provides a ‘benign’ condition suitable for detecting ecological hybrid inviability and for estimating genetic incompatibility between *H. niponica* and *H. yasutomii*.

Katakura & Hosogai (1994) previously reported no reduction in survival of F1 hybrids between *H. niponica* and *H. yasutomii*, compared with their parents, when the hybrids were reared on the parental host plants. That study, however, used ladybird beetles from around Lake Ohnuma in Nanae-cho, Hokkaido, Japan, and *Cirsium kamtschaticum* Ledeb (the commonest, most widespread thistle species on Hokkaido) from Sapporo, where the experiment was conducted, because the major host plant of *H. niponica* in the Nanae area (*Cirsium alpicola* Nakai) was not available in the Sapporo area. More recent studies, however, have shown that the response of thistle-eating ladybird beetles can vary with the species of thistle or even among different individuals of the same thistle species (Koizumi et al., 1999; Fujiyama & Katakura, 2002a; Fujiyama et al., 2003; Koji et al., 2004). Some studies on other phytophagous insects have likewise reported that the fitness of hybrids between host races varies according to the species or genotype of the host plant (Craig et al., 2007; Ferrari et al., 2008). Therefore, in this study, we reexamined hybrid survival on the thistle species actually utilized by *H. niponica* populations in the wild.

Materials and methods

In practice, it was very difficult to prepare enough high-quality food plants to rear thousands of ladybird larvae to adulthood. Therefore, we used the survival rate to the second instar as a measure of fitness. Although this treatment may limit our interpretation of the results obtained, we consider the limitation to be minor, because essentially the same results were obtained previously between survival to second instar and survival to adulthood when the performance of larvae was compared among *H. niponica*, *H. yasutomii*, and their F1 hybrids (Katakura & Hosogai, 1994). Furthermore, in a pilot study conducted in 2003 with another set of sympatric populations of *H. niponica* and *H. yasutomii*, we found a very strong positive relationship between survival to the second instar and survival to adulthood (N Kobayashi, N Fujiyama & H Katakura, unpubl.) (Figure 1).

Food plants

Thistles (*Cirsium* spp.) are very diverse in Japan, with many locally distributed species (Kadota, 1995). Populations of *H. niponica* usually utilize particular local species of thistles. For this reason, we used the local thistle

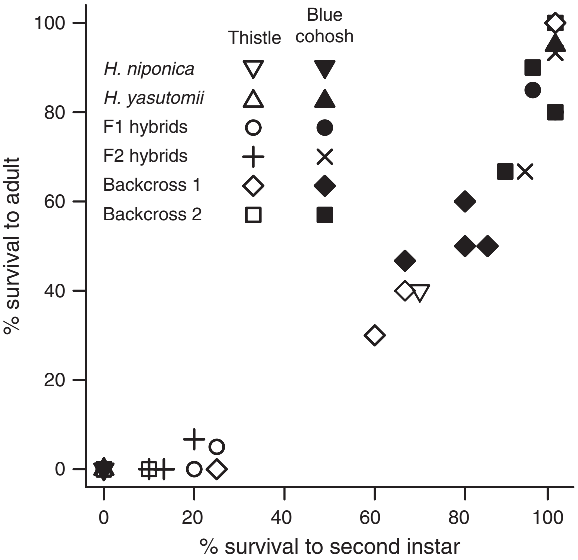

Figure 1 Relationship between survival to the second instar and survival to adulthood on local thistle (*Cirsium nipponicum*) and on blue cohosh for *Henosepilachna niponica*, *H. yasutomii*, F1 and F2 hybrids, and backcross progeny. The experimental populations were from Sakunami, Tohoku District, northern Honshu Island. Each symbol represents a particular cross. Offspring (5–25) from 1 to 4 mothers were reared on either thistle or blue cohosh for each cross.

species actually utilized by the population of *H. niponica* studied in the wild (*C. alpicola*). We transplanted thistles from around Lake Ohnuma in Nanae-cho (41°59′N, 140°37′E) onto the campus of Hokkaido University in Sapporo (43°04′N, 141°20′E). In contrast, blue cohosh (*C. robustum*) comprises a single species, subspecies, and variety throughout Japan; hence, we used plants transplanted from the suburbs of Sapporo onto the university campus. Plants of the Japanese nightshade (*S. japonense*), a 'common' food plant of the two ladybird species (Fujiyama & Katakura, 2002b), were derived from an individual that originally grew on the campus of Hokkaido University and has been maintained through cuttings for more than 10 years in a greenhouse at Hokkaido University.

Beetles

Both *H. niponica* and *H. yasutomii* are univoltine in the field. New adults emerge from mid summer to early fall and hibernate from late fall to the next spring. Usually only overwintered females oviposit, but both males and females mate repeatedly during their lives, even before entering hibernation (Katakura, 1981; Katakura et al., 1989). Two or more generations per year can be raised under laboratory conditions.

In the present study, we used the laboratory-reared offspring of overwintered beetles collected on their respective host plants (*H. niponica* on thistle and *H. yasutomii* on blue cohosh). Collected beetles and their offspring were maintained in the laboratory by using Japanese nightshade as the food plant. The experiments to examine survival on each food plant were all conducted in the laboratory at 23 °C under a regulated light regime of L16:D8 photoperiod indication.

All individuals were derived from 17 females and 22 males of *H. niponica* and 22 females and 18 males of *H. yasutomii*. These stocks were collected in 2003 from around Lake Ohnuma in Nanae-cho (41°59′N, 140°37′E for *H. niponica*; 41°57′N, 140°39′E for *H. yasutomii*). The collecting sites for the two species were approximately 5 km apart. We kept the beetles in transparent styrene cases (15.5 × 8.0 × 2.0 cm), the bottoms of which were covered with moist filter paper to maintain humidity.

Beetles of the two species were confined separately in mass in the transparent styrene cases and were allowed to copulate freely. From summer to autumn 2003, we collected eggs from each species, reared the newly hatched larvae, and obtained virgin adults. The virgin adults were kept outdoors so that they would hibernate. In 2004, unmated adults (five females and five males) for each of four crosses (*H. niponica* × *H. niponica*, *H. niponic* × *H. yasutomii*, *H. yasutomii* × *H. niponica*, *H. yasutomii* × *H. yasutomii*) were confined in mass in styrene cases and were allowed to copulate freely. From summer to autumn 2004, we obtained virgin adults for crossing (*H. niponica*, *H. yasutomii*, and F1 hybrids). We kept them outdoors so that they would hibernate and used them for experiments the following year.

Crossing

All crossing experiments were conducted in the laboratory from late spring to early summer, using *H. niponica*, *H. yasutomii*, and the two types of F1 hybrids (NY, hybrids between *H. niponica* female and *H. yasutomii* male; YN, hybrids between *H. yasutomii* female and *H. niponica* male), all of which had emerged the previous year in the laboratory and had hibernated outdoors. We prepared the following 12 combinations of parental types (Table 1): two conspecific combinations, two heterospecific combinations, and eight types of backcross (i.e., *H. niponica* female × NY male, *H. niponica* female × YN male, NY female × *H. niponica* male, *H. yasutomii* female × NY male, *H. yasutomii* female × YN male, NY female × *H. yasutomii* male, YN female × *H. yasutomii* male, and YN female × *H. niponica* male). Beetles were reared in pairs in transparent styrene cases and fed fresh Japanese nightshade leaves. Eggs were collected daily.

Table 1 Number of individuals (and number of pairs of parents) used in the rearing experiment with *Henosepilachna niponica* (N) and *H. yasutomii* (Y)

Beetle species (or type of hybrid)	No. individuals reared on Japanese nightshade	Thistle	Blue cohosh	Total (no. pairs of parents)
Henosepilachna niponica (NN)	124	125	125	374 (5)
Backcrosses to *H. niponica*				
NN–NY hybrids	87	89	89	265 (5)
NN–YN hybrids	180	181	181	542 (5)
NY–NN hybrids	253	248	248	749 (5)
YN–NN hybrids	200	197	197	594 (5)
F1 hybrids				
NY hybrids	189	189	189	567 (4)
YN hybrids	83	82	82	247 (4)
Backcrosses to *H. yasutomii*				
YY–NY hybrids	66	63	63	192 (4)
YY–YN hybrids	93	97	97	287 (5)
NY–YY hybrids	194	189	189	572 (4)
YN–YY hybrids	136	150	150	436 (5)
H. yasutomii (YY)	206	206	206	618 (5)
Total (no. pairs of parents)	1 811 (56)	1 816 (56)	1 816 (56)	5 443 (56)

Larvae from the same parents were divided into three groups, each of which was reared on one of the three food plants.

Rearing

We divided newly hatched first instars from each egg clutch (3–30 larvae per clutch) into three groups, and reared one group on thistle leaves, one on blue cohosh leaves, and one on Japanese nightshade leaves. Survival rates to the second instar on the respective plants were recorded for offspring from every pair of parents. Two to 10 egg clutches (3–66 larvae for each food plant) were examined for each pair of parents. Two to 10 larvae were reared in each case. In all, 5 443 larvae from 56 pairs of parents were examined (Table 1).

Statistical analysis

For statistical analyzes, we used the software package R 2.9.0 (R Development Core Team, 2009) and treated the 12 combinations of parental types as five crossing types: *H. niponica*, *H. yasutomii*, F1 hybrids, backcross hybrids with *H. niponica*, and backcross hybrids with *H. yasutomii*. To test the effect of each food plant on the crossing types, we analyzed survival to the second instar with a mixed logistic regression using the ‘lmer’ function (family = binomial, logit link) in the lme4 package (Bates et al., 2008), with the five crossing types as fixed effects and parental pair as a random effect nested within crossing type. We then compared the alternative model to the null model without a fixed effect by analyzing deviance with the ANOVA function (test = ‘Chisq’) to compute P- and χ^2-values. We performed a subsequent post-hoc test using the sequential Bonferroni method (Rice, 1989).

Results

When larvae were reared on leaves of the Japanese nightshade, survival rates to the second instar were consistently high (80% or higher) in *H. niponica*, *H. yasutomii*, and all of the hybrids tested. No significant difference was detected in the rate of larval survival on Japanese nightshade among five types of cross ($\chi^2 = 2.96$, d.f. = 4, $P = 0.56$) (Figure 2A). By contrast, survival rates on thistle and on blue cohosh were variable among combinations of parental types. Survival rates were significantly different among the five types of cross depending on whether the beetles were reared on thistle ($\chi^2 = 67.58$, d.f. = 4, $P<0.001$) (Figure 2B) or blue cohosh ($\chi^2 = 78.14$, d.f. = 4, $P<0.001$) (Figure 2C). On thistle, *H. niponica* showed a relatively high rate of survival (mean among pairs = 66.3%), whereas *H. yasutomii* and backcrosses to *H. yasutomii* showed extremely low rates (0–1.2%). The survival rate of F1 hybrids on thistle was significantly lower than for *H. niponica*, but was significantly higher than for *H. yasutomii* and backcross hybrids with *H. yasutomii* (8.2%). In backcross hybrids with *H. niponica*, the survival rate on thistle was intermediate between that of *H. niponica* and F1 hybrids (49.1%), but was not significantly different from that of *H. niponica*. The survival rate of backcross hybrids with *H. niponica* was significantly higher than that of F1 hybrids. When larvae were reared on blue cohosh, F1 hybrids and backcross hybrids with *H. yasutomii* showed quite high survival (84.1 and 90.9%, respec-

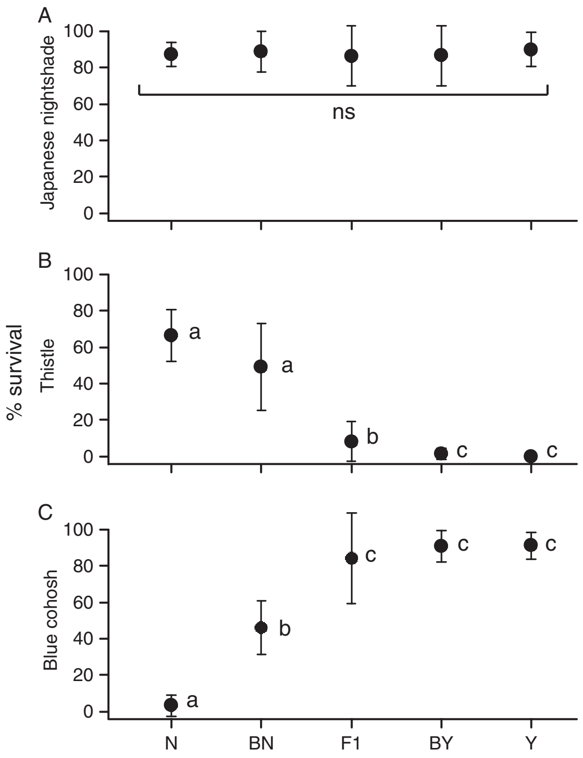

Figure 2 Mean (± SEM) survival of *Henosepilachna niponica*, *H. yasutomii*, and their hybrids on (A) Japanese nightshade, (B) thistle, and (C) blue cohosh. N, *H. niponica*; BN, backcross to *H. niponica*; BY, backcross to *H. yasutomii*; Y, *H. yasutomii*. In (A), no significant difference was detected (ns, non-significant). In B and C, no significant difference was detected between crosses with the same letter (post-hoc comparison using a mixed logistic regression, corrected with the sequential Bonferroni method; $\alpha = 0.05$).

tively), with no significant difference from *H. yasutomii* (91.2%), whereas *H. niponica* showed the lowest survival (3.2%). The survival rate on blue cohosh of backcross hybrids with *H. niponica* was significantly lower than that for *H. yasutomii*, but was significantly higher than that for *H. niponica* (45.9%). Survival rate of F1 hybrids on thistle was significantly lower than on blue cohosh ($\chi^2 = 393.98$, d.f. = 1, $P<0.001$).

Discussion

Previous studies have shown that *H. niponica* and *H. yasutomii* achieve reproductive isolation solely through fidelity to their respective host plants (Katakura et al., 1981, 1989; Katakura & Hosogai, 1994, 1997; Katakura, 1997; Hirai et al., 2006), suggesting that these species underwent ecological speciation. However, hybrid ecological inviability, a unique possible outcome of ecological speciation, had not been detected in this species pair. According to Katakura & Hosogai (1994), F1 hybrids of *H. niponica* and *H. yasutomii* performed as well as their parental species on both thistle and blue cohosh.

The present study showed lower survival of F1 hybrids only on thistle; F1 hybrids survived well on blue cohosh but were nearly unable to grow on thistle. On thistle, the survival rate of backcross hybrids with *H. niponica* was higher than that of backcross hybrids with *H. yasutomii*. The opposite result was seen on blue cohosh, where the survival rate of backcross hybrids with *H. yasutomii* was lower than that of backcross hybrids with *H. niponica*. The reversed rank order of different types of backcross hybrids on the two food plant species suggests that hybrid inviability depends on differences between these food plants. In addition, the consistently high performance on Japanese nightshade of all types of beetles tested indicates that the hybrids' lower survival rates on thistle and on blue cohosh are not because of intrinsic physiologic weaknesses deriving from the incompatibility between the different genetic systems, but instead are because of a kind of ecological hybrid inviability, specifically, a mismatch between hybrid larvae and particular types of food plants. These results thus provide solid evidence for ecological hybrid inviability between *H. niponica* and *H. yasutomii*. This host-dependent hybrid inviability supports the hypothesis that *H. niponica* and *H. yasutomii* underwent ecological speciation by divergent selection.

Our results for F1 hybrids between *H. niponica* and *H. yasutomii* were different from previous results (Katakura & Hosogai, 1994) in which F1 hybrids survived as well as their parental species on both parental host plants. Katakura & Hosogai (1994) used ladybird beetles from Nanae-cho, Hokkaido, but used *C. kamtschaticum*, a thistle species widespread on Hokkaido, instead of *C. alpicola*, the local host plant of *H. niponica* at Nanae. In contrast, we used transplanted *C. alpicola* plants. As the responses of thistle-eating ladybird beetles can vary according to the species of thistle or even among different individuals of the same thistle species (Koizumi et al., 1999; Fujiyama & Katakura, 2002a; Fujiyama et al., 2003), this difference in results might be attributable to the respective use of different thistle species in experiments, although this needs further confirmation. The same pattern of survival on blue cohosh (*H. yasutomii* = F1 hybrids > *H. niponica*) between this and the previous study, and the consistently high survival on Japanese nightshade in this study, refute the alternative possibility that differences in the design and the execution of the rearing experiment led to the result showing a reduction in hybrid survival.

The present study also showed that ecological hybrid inviability between *H. niponica* and *H. yasutomii* is asymmetric. Survival rates of F1 hybrids were lower on thistle than on blue cohosh. Furthermore, backcross hybrids with *H. niponica* showed survival rates on thistle equal to or lower than that of *H. niponica*, and survival rates on blue cohosh lower than that of *H. yasutomii*. On the other hand, survival rates for F1 hybrids and backcross hybrids with *H. yasutomii* on blue cohosh were as high as for *H. yasutomii*. Some plant chemicals are likely involved in this asymmetry, but no data exist on the relationships between host selection by the *Henosepilachna* beetles studied here and chemicals in their host plants. Chemical ecology is a yet unexplored but promising field of study for the system involving *Henosepilachna* beetles and their host plants.

It is also not clear how much this asymmetric ecological hybrid inviability contributes to reproductive isolation between *H. niponica* and *H. yasutomii* in the wild. As females of *H. niponica* and *H. yasutomii* lay eggs strictly on their natal host plants (Hirai et al., 2006), there would be a difference in mortality between the two types of F1 hybrids, if they occur, with higher survivorship for hybrids derived from crossing *H. yasutomii* females with *H. niponica* males than for hybrids from the reciprocal cross. This would result in much more gene flow from *H. niponica* on thistle to *H. yasutomii* on blue cohosh than vice versa. However, premating isolation between *H. niponica* and *H. yasutomii* because of host fidelity has proven to be very strong (Hirai et al., 2006), and natural hybridization is extremely limited. Moreover, no evidence in support of biased gene flow was obtained by analyzes of the mitochondrial COI gene (Kobayashi, 2000, 2004). Those analyses detected no current gene flow in four of five sympatric or neighboring population pairs of *H. niponica* and *H. yasutomii*, including the pair from Nanae studied here, but suggested gene flow from *H. yasutomii* to *H. niponica* in one pair.

Ecological hybrid inviability has been reported or suggested for several closely related phytophagous insect pairs (Matsubayashi et al., 2010). The apple maggot fly (*R. pomonella*) has host races associated with apple and hawthorn (Feder et al., 1994). Hybrids between these two host races exhibit lower ability to respond to the odors of the parental host fruits than their parents (Linn et al., 2004). The goldenrod gall fly (*E. solidaginis*) has host races adapted to two *Solidago* species, and multiple factors such as hybrid inviability, temporal isolation, and habitat isolation contribute to reproductive isolation between these races (Craig et al., 1993, 1997). Craig et al. (1997, 2007) have shown that hybrid inviability between *Eurosta* host races is ecological, and is variable depending on the particular species and genotypes of the host plants. The pea aphid (*A. pisum*) feeds on alfalfa and red clover in North America. F1 hybrids between two host-associated populations exhibit lower fitness than their specialized parents on either of the parental host plants (Via et al., 2000). The present study provides another example of ecological hybrid inviability, host-dependent hybrid inviability in *Henosepilachna* beetles.

Potential limitations of our study are that we investigated survival only until the second instar and used a low number of experimental replicates. In particular, tracking survival only to the second instar ignores possible mortality in later instars. However, we consider that this possibility was small, although not excluded (see Material and methods and Figure 1). In the present study, Japanese nightshade provided a 'benign' condition suitable for detecting ecological hybrid inviability. We note, however, that the ecological hybrid inviability we detected in this study might not represent the overall fitness of hybrids in natural populations, because not only the quality of host plants but also many other ecological factors may affect the fitness of phytophagous insects. We need further studies on the ecology and life history of sympatric populations of *H. niponica* and *H. yasutomii* to resolve these issues.

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References

Bates D, Maechler M, Dai B & Vasishth S (2008) lme4: Linear Mixed-Effects Models Using S4 Classes R Package Version 0.99375-28. Available at: http://lme4.r-forge.r-project.org/ (accessed 28 April 2009).

Coyne JA & Orr HA (2004) Speciation. Sinauer Associates, Sunderland, MA, USA.

Craig TP, Itami JK, Abrahamson WG & Horner JD (1993) Behavioral evidence for host–race formation in *Eurosta solidaginis*. Evolution 47: 1696–1710.

Craig TP, Horner JD & Itami JK (1997) Hybridization studies on the host races of *Eurosta solidaginis*: implications for sympatric speciation. Evolution 51: 1552–1560.

Craig TP, Itami JK & Craig JV (2007) Host plant genotype influences survival of hybrids between *Eurosta solidaginis* host races. Evolution 61: 2607–2613.

Endler JA (1977) Geographic Variation, Speciation, and Clines. Princeton University Press, Princeton, NJ, USA.

Feder JL, Opp SB, Wlazlo B, Reynolds K, Go W & Spisak S (1994) Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. Proceedings of the National Academy of Sciences of the USA 91: 7990–7994.

Ferrari J, Via S & Godfray HCJ (2008) Population differentiation and genetic variation in performance on eight hosts in the pea aphid complex. Evolution 62: 2508–2524.

Fujiyama N & Katakura H (2002a) Host plant suitability of a recently naturalized thistle *Cirsium vulgare* (Asteraceae) for a phytophagous ladybird beetle, *Epilachna pustulosa* (Coleoptera: Coccinellidae). Ecological Research 17: 275–282.

Fujiyama N & Katakura H (2002b) Host plant suitability of *Solanum japonense* (Solanaceae) as an alternative larval food for three closely related *Epilachna* ladybird beetles (Coleoptera: Coccinellidae). Applied Entomology and Zoology 37: 551–557.

Fujiyama N, Koizumi T & Katakura H (2003) Conspecific thistle plant selection by a herbivorous ladybird beetle, *Epilachna pustulosa*. Entomologia Experimentalis et Applicata 108: 33–42.

Hirai Y, Kobayashi H, Koizumi T & Katakura H (2006) Field-cage experiments on host fidelity in a pair of sympatric phytophagous ladybird beetles. Entomologia Experimentalis et Applicata 118: 129–135.

Kadota Y (1995) *Cirsium*: flora of Japan IIIb. Angiospermae, Dicotypedoneae, Sympetalae (ed. by Y Iwatsuki, Y Yamazaki, D Boufford & H Ohba), pp. 119–151. Kodansha, Tokyo, Japan.

Katakura H (1981) Classification and evolution of the phytophagous ladybirds belonging to *Henosepilachna vigintioctomaculata* complex (Coleoptera, Coccinellidae). Journal of the Faculty of Science, Hokkaido University, Series VI, Zoology 22: 301–378.

Katakura H (1997) Species of *Epilachna* ladybird beetles. Zoological Science 14: 869–881.

Katakura H & Hosogai T (1994) Performance of hybrid ladybird beetles (*Epilachna* spp.) on the host plants of parental species. Entomologia Experimentalis et Applicata 71: 81–85.

Katakura H & Hosogai T (1997) Host preference of F1 hybrids between two host specific phytophagous ladybird beetles (*Epilachna* spp., Epilachninae, Coccinellidae). Japanese Journal of Entomology 65: 75–79.

Katakura H, Hinomizu H & Yamamoto M (1981) Crossing experiments among three ladybird 'species' of *Henosepilachna vigintioctomaculata* complex (Coleoptera, Coccinellidae) feeding on thistles and/or blue cohosh. Japanese Journal of Entomology 49: 482–490.

Katakura H, Shioi M & Kira Y (1989) Reproductive isolation by host specificity in a pair of phytophagous ladybird beetles. Evolution 43: 1045–1053.

Kawecki TJ (2004) Genetic theories of sympatric speciation. Adaptive Speciation (ed. by U Dieckmann), pp. 36–53. Cambridge University Press, Cambridge, UK.

Kobayashi N (2000) Molecular Population Genetic Analyses of the Phytophagous Ladybird Beetles, the *Epilachna vigintioctomaculata* Species Complex (Coleoptera: Coccinellidae). PhD dissertation, Hokkaido University, Hokkaido, Japan.

Kobayashi N (2004) Speciation of insects derived by the difference of host plant preferences. Chemistry & Biology 42: 287–289 (In Japanese).

Koizumi T, Fujiyama N & Katakura H (1999) Host-plant specificity limits the geographic distribution of thistle feeding ladybird beetles. Entomologia Experimentalis et Applicata 93: 165–171.

Koji S, Nakamura K & Yamashita M (2004) Adaptive change and conservatism in host specificity in two local populations of the thistle-feeding ladybird beetle *Epilachna niponica*. Entomologia Experimentalis et Applicata 112: 145–153.

Linn CE Jr, Dambroski HR, Feder JL, Berlocher SH, Nojima S & Roelofs WL (2004) Postzygotic isolating factor in sympatric speciation in *Rhagoletis* flies: reduced response of hybrids to parental host-fruit odors. Proceedings of the National Academy of Sciences of the USA 101: 17753–17758.

Matsubayashi KW, Ohsihma I & Nosil P (2010) Ecological speciation in phytophagous insects. Entomologia Experimentalis et Applicata 134: 1–27.

Mayr E (1942) Systematics and the Origin of Species from the Viewpoint of a Zoologist. Columbia University Press, New York, NY, USA.

R Development Core Team (2009) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-project.org. ISBN 3-900051-07-0 (accessed on 28 April 2009).

Rice WR (1989) Analysing tables of statistical tests. Evolution 43: 223–225.

Rice WR & Hostert EE (1993) Laboratory experiments on speciation: what have we learned in 40 years? Evolution 47: 1637–1653.

Rundle HD (2002) A test of ecologically dependent postmating isolation between sympatric sticklebacks. Evolution 56: 322–329.

Rundle HD & Nosil P (2005) Ecological speciation. Ecology Letters 8: 336–352.

Schluter D (2000) The Ecology of Adaptive Radiation. Oxford University Press, Oxford, UK.

Via S, Bouck AC & Skillman S (2000) Reproductive isolation between divergent races of pea aphids on two hosts. II. Selection against migrants and hybrids in the parental environments. Evolution 54: 1626–1637.