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Divergent host plant specialization as the critical driving force in speciation between populations of a phytophagous ladybird beetle

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Abstract

Detecting the isolating barrier that arises earliest in speciation is critically important to understanding the mechanism of species formation. We tested isolating barriers between host races of a phytophagous ladybird beetle, *Henosepilachna diekei* (Coleoptera: Coccinellidae: Epilachnine), that occur sympatrically on distinct host plants. We conducted field surveys for the distribution of the beetles and host plants, rearing experiments to measure six potential isolating factors (adult host preference, adult and larval host performance, sexual isolation, egg hatchability, F_1 hybrid inviability, and sexual selection against F_1 hybrids), and molecular analyses of mitochondrial ND2 and the nuclear ITS2 sequences. We found significant genetic divergence between the host races, and extremely divergent host preference (i.e. habitat isolation) and host performance (i.e. immigrant inviability), but no other isolating barriers. The fidelity to particular host plants arises first and alone can prevent gene flow between differentiating populations of phytophagous specialists.

Introduction

The speciation process is generally driven by the evolution of several barriers to gene flow between interbreeding populations (Dobzhansky, 1937; Mayr, 1963). In this process, which isolating barrier actually triggers the population divergence is a fundamental question. We must elucidate the order in which isolating barriers evolve to understand the mechanisms driving speciation (e.g. Coyne & Orr, 1989, 2004; Gleason & Ritchie, 1998; Mendelson, 2003). However, the barrier triggering speciation is not always the barrier that contributes most intensely to present-day reproductive isolation (Nosil *et al.*, 2005), especially after populations have developed multiple other forms of isolating barriers, the action of which can obscure the contribution of the barrier that initiated the speciation process.

To detect barriers likely to be involved in the very early phase of speciation, the most frequently applied method is the comparative analysis of data gathered from multiple taxa. For example, analyses of fruit flies (Coyne & Orr, 1989, 1997) and darter fish (Mendelson, 2003) have shown faster evolution of premating isolation (e.g. sexual isolation) than post-mating isolation (e.g. hybrid inviability). In addition to the comparative approach, it is possible to investigate sympatric population pairs that are partially or completely isolated by only a few isolating barriers (Coyne & Orr, 2004: p. 64). However, this approach has rarely been applied because of the difficulty of finding suitable study systems, because closely related species pairs are usually isolated by multiple barriers to gene flow.

Many cases are known in both animals and plants in which ecological divergence is involved in incipient speciation (Schluter, 2000, 2001; Nosil *et al.*, 2005; Funk *et al.*, 2006; Nosil, 2007; Funk & Nosil, 2008; Lowry *et al.*, 2008; Funk, 2009; Matsubayashi & Katakura, 2009; Matsubayashi *et al.*, 2010). The recent conceptual synthesis of ecological speciation, in which ecologically based divergent natural selection drives the evolution of reproductive isolation (Schluter, 1998, 2000, 2001), highlights the contribution of ecological adaptation to speciation. Divergent selection often causes the

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incidental evolution of reproductive isolation among populations inhabiting different environments via both direct and indirect effects on reproductive characters. We thus consider ecological divergence ensuring ecological isolation (i.e. habitat isolation, immigrant inviability, seasonal isolation or pollinator isolation) as the most likely candidate for an isolating barrier that triggers population divergence and speciation.

Phytophagous specialist, especially phytophagous insects, in which the life history usually depends on a specific host plant species, can attain reproductive isolation mainly by the difference in host plant preference (i.e. host fidelity *sensu* Funk *et al.*, 2002), because habitat isolation caused by divergent host preference is often associated with preferences in mating and oviposition sites (Bush, 1969; Katakura *et al.*, 1989; Craig *et al.*, 1993; Feder *et al.*, 1994; Funk, 1998; Via, 1999; Pappers *et al.*, 2002; Malausa *et al.*, 2005; Ohshima, 2008). Several theoretical and empirical studies have also stressed the importance of a positive association between habitat preference and assortative mating for population divergence (Maynard Smith, 1966; Diehl & Bush, 1984, 1989; Rice & Salt, 1990; Rice & Hostert, 1993; Kirkpatrick & Ravigné, 2002; Dieckmann *et al.*, 2004; Gavrilets, 2004). These lines of evidence strongly support the idea that the factor initiating population divergence leading to speciation in phytophagous insect specialists involves adaptation to different host plants. Nonetheless, the actual evolutionary forces that split populations and prevent gene flow after divergence remain unclear.

The phytophagous ladybird beetle *Henosepilachna diekei* Jadwiszczak & Wegrzynowics (*Henosepilachna* sp. 3 in Katakura *et al.*, 2001; see Kobayashi *et al.*, 2009) is widely distributed in Indonesia, feeding on some species in the families Asteraceae and Lamiaceae (Katakura *et al.*, 2001). In West Java, Indonesia, the beetle occurs on *Leucas lavandulifolia* Sm. (Lamiaceae) and the climbing hemp weed, *Mikania micrantha* Kunth (Asteraceae), which grow in close proximity (Fujiyama *et al.*, 2001; Katakura *et al.*, 2001). *Leucas lavandulifolia* is native to Southeast Asia (Wardani, 2001), whereas *M. micrantha* was introduced to West Java (into the Bogor Botanic Garden) from Paraguay in 1949 (Kostermans *et al.*, 1987; Whitten *et al.*, 1996).

The two types of populations of the beetle demonstrate strong preferences for, and high larval survivorship on, their natal host plants (N. Fujiyama, submitted; S. Nakano, unpublished); beetles collected from the different host plants are crossable and produce viable offspring, without sex distortion. F_1 hybrid larvae show slightly reduced survivorship on *M. micrantha* but not on *L. lavandulifolia* (S. Nakano, unpublished). Based on these data, Nakano hypothesized that these two host-dependent beetle populations occurring on either *M. micrantha* or *L. lavandulifolia* are reproductively isolated merely by the divergent host use and that they possibly represent host races (S. Nakano, personal communication) or intraspecific populations partially reproductively isolated by adaptation to different hosts (Berlocher & Feder, 2002; Drés & Mallet, 2002).

In this study, we estimated various potential isolating factors between these putative host races of *H. diekei* in West Java, Indonesia, through field observations, laboratory experiments, and molecular analyses. Our goal was to elucidate the isolating barrier that developed in the earliest stage of speciation, with particular attention to the importance of divergent host plant use.

Materials and methods

Microspatial distribution of host plants and beetles

We surveyed the distributions of the two host plant species, *M. micrantha* and *L. lavandulifolia*, and the ladybird beetles, *H. diekei*, that feed on them, in an area approximately 20×20 km square in the vicinity of Bogor, West Java, from 2005 to 2007. In the study area, *M. micrantha*, *L. lavandulifolia* and the two host races of *H. diekei* occurred abundantly and in close proximity (Fig. 1). We considered beetles collected within a continuous patch of a single host species to belong to a single population. We term beetles collected on *M. micrantha* in the wild as the *Mikania* race, and those collected on *L. lavandulifolia* as the *Leucas* race.

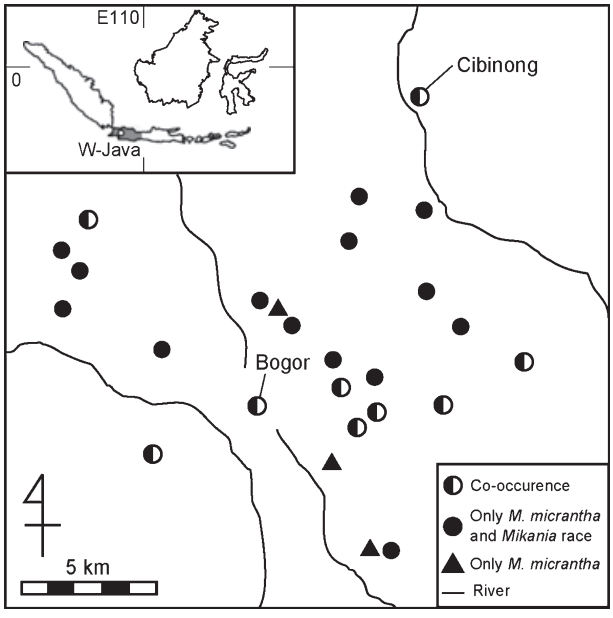

Fig. 1 Distribution of the two host races of *Henosepilachna diekei* and the host plants, *Mikania micrantha* and *Leucas lavandulifolia*, in the vicinity of Bogor, West Java, Indonesia. Sites of co-occurrence, where patches of both *M. micrantha* and *L. lavandulifolia* were within sight, are shown by half-solid circles. Single patches of *M. micrantha* with beetles are shown by solid circles.

Beetle collection and maintenance for feeding tests and laboratory stocks

We kept the collected beetles individually in styrene boxes (6 cm × 5.5 cm × 2 cm) with the bottom covered with a sheet of moist filter paper to maintain adequate moisture and housed them in a noncontrolled room under conditions roughly equivalent to ambient outdoor conditions (27.5–31.4 °C and approximately 12L : 12D) in the Research Center for Biology, Indonesian Institute of Science – LIPI, Cibinong ('Cibinong' in Fig. 1; 06°29′45″S, 106°51′10″E). We provided them daily with fresh leaves of the original host plants. We maintained these conditions throughout the experiments described later. For laboratory experiments, we collected adult beetles from both host plants in July 2005 at Cibinong. After confirmatory matings between females and males of the same host race in the laboratory, we collected egg batches daily from more than 40 females. We reared newly emerged larvae from these eggs to adulthood on the natal host plant and used these virgin adult beetles as the laboratory stock. Adult beetles were kept individually and were fed fresh leaves of the respective host plants daily. We note that our results might have been influenced both by a conditioning effect and genetic differences between the host races, because these races were fed only their original host plants.

We designate crosses with the maternal type first; for example, M × L means a cross between a female of the *Mikania* race and a male of the *Leucas* race. We use the abbreviations F_1ML and F_1LM for F_1 hybrids mothered by individuals of the *Mikania* and *Leucas* races, respectively.

Test of adult host preference in the wild beetle populations

We tested the preference of adult beetles for the two host plants by using two sympatric population pairs of the host races from Cibinong (45 females and 39 males of *Mikania* race, 41 females and 30 males of *Leucas* race) and Bogor (42 females and 27 males of *Mikania* race, 27 females and 26 males of *Leucas* race) in 2006. We placed adult beetles collected in the wild individually into boxes, placed a piece (approximately 6 cm^2) of fresh leaf from each of *M. micrantha* and *L. lavandulifolia* into each box and allowed the beetles to feed freely on the leaf pieces for 24 h. We recorded whether the individual ate only *M. micrantha*, only *L. lavandulifolia*, both host plants, or neither (null choice), and the amount of leaves consumed. We compared between the host races the numbers of individuals that accepted only *M. micrantha*, only *L. lavandulifolia* or both hosts. We tested the significance of differential host preference between the host races using a Fisher's exact probability tests with the 3 × 2 design.

Test of host survivorship in wild beetle populations (immigrant inviability)

To evaluate whether the host races used the alternative host plants under duress, we conducted a long-term nonchoice acceptance test in which beetles collected from the respective host plants were continuously served only *M. micrantha* or only *L. lavandulifolia*. We collected more than 20 individuals each of adults and larvae from the respective host plants in December 2006 at Cibinong. The adults possibly included both those raised on the host plant species on which they were collected and immigrants from the alternative host species (e.g. Via, 1999). To examine the magnitude of interhost immigration, we used two groups of adults, ones collected as adults and others collected as larvae and then reared to adulthood on the host plant from which they were collected. Discordant results between the two groups would indicate that immigration between the two host plants had occurred. We put a fresh leaf of *M. micrantha* or *L. lavandulifolia* cut to approximately the same area (12 cm^2) into a small box, released an individual beetle into the box and allowed the beetle to feed freely on the leaf piece for 24 h. We recorded the beetle's condition (dead or alive) and acceptance (accepted or not) on the respective host plants daily throughout the test period. When a beetle consumed even just a small portion of the leaf served, we treated this as acceptance. We counted the number of days of acceptance for each beetle during the test period (up to 10 days or, in cases where a beetle died within 10 days, the number of days from the onset of the experiment to the death of the beetle). Because all beetles offered only the alternative host plant died within 10 days, the test was conducted only for this period.

We compared the 10-day survivorship of adult beetles on the respective host plants by using survival analysis (the Kaplan–Meier method with the Wilcoxon test) conducted with JMP statistical software (ver. 6.0.3; SAS institute, Cary, NC, USA, 2005). We corrected statistically significant values in multiple comparisons by using Holm's method (Rice, 1989). We also constructed a generalized linear model (GLM) with R 2.10.0 statistical software (R Core Development Team, 2009) to determine the variable(s) responsible for acceptance on the respective host plants. The response variable was the number of beetles showing acceptance, and the main explanatory variables were the host plant species from which the beetles were collected ('host'), stage of the beetles (adult or larva) upon collection ('collection'), and whether the host plant served was the natal host or the alternative host for the individual tested ('treatment'). Days within the test period (fewer than 10 days) were regarded as replicates of the single acceptance test for individuals. The error structure and link function were the binomial and logit link, respectively.

Genetic differentiation between sympatric populations

To analyse genetic divergence between the host races, we used nucleotide sequences of the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) and internal transcribed spacer region 2 (ITS2) between nuclear ribosomal RNA genes from adult beetles from two sympatric host race populations (Cibinong and Bogor in Fig. 1). We also used these markers to estimate whether there is current gene flow between the host races, although the markers were not suitable for detecting the precise amount of gene flow. We extracted total DNA from muscle tissue by Boom's (1990) method. We used primers 5′-AAGCTACTGGGTTCATACC-3′ (forward) and 5′-TYATYCAYTTRGGGAARAATCCTAA-3′ (reverse) (Wang *et al.*, 2006; Kobayashi *et al.*, 2009) for PCR amplification of ND2, and 5′-GCATCGATGAAGAACG CAGC-3′ (forward) and 5′-TCCTCCGCTTATTGATATG C-3′ (reverse) (White *et al.*, 1990; N. Kobayashi, unpublished) for ITS2. We performed cycle-sequencing reactions with an ABI PRISM BigDye Terminator Cycle Sequencing Kit Ver. 3.1 and determined the sequences with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). We aligned sequences using Clustal W in MEGA 4 (Tamura *et al.*, 2007) and adjusted them visually. As ITS2 is a nuclear marker, it is necessary to determine its diploid genotype to analyse genetic differentiation. There was no polymorphism in sequence length (insertions or deletions), nor any samples with more than two heterosubstitution sites within this region, so we determined the genotypes visually. We estimated genetic divergence between host races for the two molecular markers by net between population genetic distances with MEGA 4, by analysis of molecular variance (locus-by-locus AMOVA; group = host, population = locality) (Excoffier *et al.*, 1992) with 1000 permutations, and by calculating pairwise F_{st} values, both with Arlequin ver. 3.5.1.2 software (Excoffier *et al.*, 2005). Median-joining haplotype network trees for ND2 and ITS2 haplotypes were calculated with NETWORK 4.6.0.0 (Bandelt *et al.*, 1999).

Nonchoice mating test (sexual isolation and mate discrimination against F_1 hybrids)

We crossed virgin adults from the stock population in August 2005 and obtained newly emerged adults of the *Mikania* and *Leucas* races, and their F_1 hybrids, in September 2005. We reared reciprocal F_1 hybrids on the host plant of the maternal race, because we expected females to oviposit on their natal host plant. We conducted mating tests with a nonchoice design using these stocks. We placed one female and one male from among four types of adults (*Mikania* race, *Leucas* race, F_1LM, and F_1ML) together in a small box. We did not attempt crosses between F_1 hybrids, because we assumed hybridization to be rare or nonexistent in the wild. We conducted the mating test on 50 pairs for each mating combination.

By direct observation for 60 min, we recorded whether each male attempted to mate (male attempt) and whether mating actually occurred. When no mating behaviour occurred during the observation period, we returned the test beetles to their respective stocks and used them in the experiment on another day. We regarded mating as successful when it lasted for more than 30 min, because sperm transfer in another species of *Henosepilachna* does not usually occur in matings lasting < 30 min (Katakura, 1985). We regarded matings that lasted < 30 min as having failed. Throughout the experiment, we maintained more than 200 individuals of each sex for both parental races and more than 100 individuals of each sex for the F_1 hybrids. We used adult beetles 2 weeks after emergence in all the tests in this experiment to assure sexual maturity. We conducted all tests between 22 September and 7 October 2005 to reduce bias because of age and/or seasonal effects.

We assessed the significance of sexual isolation between the two races from the results of the mating tests by using I_{PSI} coefficients (Rolán-Alvarez & Caballero, 2000) based on a bootstrapping procedure. The I_{PSI} coefficient is a sexual isolation index and can distinguish between real mate choice and differential mating propensity by comparing the mating frequency between combinations (the I_{PSI} coefficient ranges from −1 to +1, with −1 = complete disassortative mating, 0 = random mating, and +1 = complete assortative mating). We determined the significance of the I_{PSI} estimates by resampling 10 000 times with JMATING software (Carvajal-Rodriguez & Rolan-Alvarez, 2005).

We also examined sexual selection against F_1 hybrids, using data on female mating attractiveness and male mating success as measures. We defined the mating attractiveness of each type of F_1 female as the total number of males of the parental races that attempted to mate with the females divided by the total number of trials (100 replicates for each type of female in the test). For F_1 hybrid females, low mating attractiveness means reduced fitness, because males of the parental races are unwilling to mate with them. We defined the mating success of F_1 hybrid males as the number of mating pairs achieved for an F_1 male type divided by the number of mating attempts by that male type towards females of the parental races. In F_1 hybrid males, low mating success also means reduced fitness, because of unsuccessful attempts to mate with females of the parental races. We compared these two values among hybrids and the parental races using multiple comparisons of the *G*-test (Sokal & Rohlf, 1995).

Egg hatchability test (reduced egg hatchability)

We tested the effect of reduced egg hatchability as a postzygotic isolating barrier between races. We mated virgin

females and males of both races only once and kept the females individually in boxes for oviposition. In this experiment, we made four combinations of matings, including intraracial matings (M × M, L × L) and interracial matings (M × L, L × M). We collected new egg batches and recorded daily the numbers of eggs and newly hatched larvae for each female. We excluded from analyses data from females that laid fewer than three batches and egg batches damaged by cannibalism of the female.

We compared the number of eggs and the number of hatched larvae per batch among intra- and inter-racial matings. We tested the significance of differences in the number of eggs per batch among the four crossing combinations using the Tukey–Kramer test conducted with JMP statistical software, and the significance of differences in the number of hatched larvae by multiple comparisons with the *G*-test.

Larval performance test (F_1 hybrid inviability)

We tested the performance of larvae of the two host races and the reciprocal F_1 hybrids on *M. micrantha* and *L. lavandulifolia* to measure the relative fitness on the two host plants. We mated virgin females singly with intra- or inter-racial males and collected the egg batches daily. We reared ten newly hatched larvae from each mother on leaves of each of the host plants (split-brood design), placing the newly hatched larvae on leaves within a day of hatching and rearing them individually. We supplied fresh host plant leaves daily and checked the development of the larvae and their initial acceptance of the respective host plants daily.

To detect differences in performance among the host races and their reciprocal F_1 hybrids on the two host plants, we used Fisher's exact probability test followed by Holm's method to test for statistically significant differences in fitness components, including initial acceptance by the first instar larvae, survivorship to the second instar and survivorship to adulthood.

Results

Microspatial distribution of host plants and beetles

We observed the host plants *M. micrantha* and/or *L. lavandulifolia* at 26 sites (Fig. 1) and detected 23 populations of the *Mikania* race and nine populations of the *Leucas* race among these sites. The minimum distance between patches of different host plants was 3 m. In West Java, *M. micrantha* is a dominant weed covering other plants or buildings, and we detected it throughout the study area. Our sampling was thus biased towards rarely sighted patches of *L. lavandulifolia*. Because *M. micrantha* occurred abundantly, all patches of *L. lavandulifolia* we found were in close proximity (i.e. sympatric) to patches of *M. micrantha*. *Mikania micrantha* occurred mainly in relatively moist habitats with moderate sunshine, whereas *L. lavandulifolia* was found in relatively dry habitats exposed to the sun, particularly in open fields. Despite this difference in habitat, the two host plants often grew side by side in the study area.

Test of adult host preference for the wild beetle populations

Mikania race individuals chose only *M. micrantha* (100% of 84 individuals from the Cibinong population; 98.6% of 69 individuals from the Bogor population) (Table 1). Conversely, the *Leucas* race chose only *L. lavandulifolia* (95.8% of 71 individuals from the Cibinong population; 100% of 53 individuals from the Bogor population). A small proportion of both host races demonstrated the null choice (4.2% of the *Leucas* race from Cibinong; 1.4% of the *Mikania* race from Bogor). Population pairwise Fisher's exact probability tests with the 3 × 2 design (chose only *M. micrantha*; chose only *L. lavandulifolia*; null choice) showed no significant difference between same-host population comparisons ($P = 0.451$ for the *Leucas* race between Cibinong and Bogor; $P = 0.300$ for the *Mikania* race between Cibinong and Bogor), whereas

Table 1 Leaf areas consumed in the two-choice feeding test of the host races offered both host plants, *Mikania micrantha* and *Leucas lavandulifolia*. The number of individuals that accepted neither host plant is denoted as 'null choice.' No individuals accepted both host plants in this test.

				Fed only on *M. micrantha*			Fed only on *L. lavandulifolia*			
Beetle type	Site	Sex	*N*	*N*	Leaf area consumed (mm^2)	SE	*N*	Leaf area consumed (mm^2)	SE	No. of null choice
Mikania race	Cibinong	Female	45	45	232.86	19.32	0	0	–	0
Mikania race	Cibinong	Male	39	39	149.30	17.09	0	0	–	0
Leucas race	Cibinong	Female	41	0	0	–	41	376.86	42.24	0
Leucas race	Cibinong	Male	30	0	0	–	27	182.94	29.38	3
Mikania race	Bogor	Female	42	41	208.78	21.99	0	0	–	1
Mikania race	Bogor	Male	27	27	188.65	24.42	0	0	–	0
Leucas race	Bogor	Female	27	0	0	–	27	195.98	34.68	0
Leucas race	Bogor	Male	26	0	0	–	26	187.60	36.29	0

there was a significant difference between populations on the different hosts regardless of the collection site ($P < 0.0001$).

Test of host survivorship for the wild beetle populations

The long-term nonchoice acceptance test also detected extremely divergent host acceptability (Table 2). Adult beetles showed higher longevity on the host plant on which they were collected than on the alternative host plant ($P < 0.05$; Kaplan–Meier method with the Wilcoxon test followed by Holm's method). A GLM showed that the frequency of acceptance was significantly determined by the difference in host race ('host') and whether the test plant was the natal or the alternative host ('treatment'), but not by the developmental stage in which the beetles were collected ('collection') (Table S1). Adults of both host races starved to death on the alternative host plant, and the consumed leaf area was quite small on the alternative host, if any. We detected no presumed inter-host immigrants among the beetles tested.

Genetic differentiation between sympatric populations

We detected 17 unique haplotypes with 10 variable substitution sites among the 669-bp ND2 sequences and eight genotypes with three variable substitution sites among the 447-bp ITS2 sequences from the two host races (Tables S2a,b and S3; GenBank accession numbers ND2: AB620109–AB620125, ITS2: AB620051–AB620056). For these molecular markers, the two host races did not share haplotypes or genotypes even when they were sympatric (Table S2). Population pairwise F_{st} values between the two host races from two sites revealed restricted gene flow among populations except for the *Mikania* race between Bogor and Cibinong (Table 3). For both markers, F_{st} values were high between beetle populations on the different host plants regardless of the geographical distance. On the other hand, we detected moderate but significant F_{st} values for the *Leucas* races between Bogor and Cibinong. Net between population genetic distances based on ND2 sequences demonstrated large genetic divergence between *Leucas* race and *Mikania* race ($d_{XY} = 0.0059$). In addition, we detected larger genetic divergence between populations of *Leucas* race ($d_{XY} = 0.0011$) than that between *Mikania* race ($d_{XY} = 0$) from Bogor and Cibinong. For both markers, AMOVA detected significantly large genetic divergence between the two host races (68.5% of the total molecular variation, $P < 0.001$ for ND2, Table S4a; 83.61% of the total molecular variation, $P = 0.014$ for ITS2, Table S4b). Haplotype network trees based on ND2 and ITS2 haplotypes represented close relationship and clear differentiation in the neutral genetic regions of these host races (Fig. S1, S2).

Nonchoice mating test

The outcomes of nonchoice mating test (19 pairs of *Mikania* race × *Mikania* race, 27 pairs of *Leucas* race × *Leucas* race, 26 pairs of *Mikania* race × *Leucas* race and 22 pairs of *Leucas* race × *Mikania* race) among 50 replicate trials for each mating combination showed approximately random mate choice of the host races. Sexual isolation between the host races was not significant (*I*psi = −0.0286, SD = 0.104, $P = 0.776$).

Female mating attractiveness and male mating success demonstrated that there was no sexual selection against F_1 hybrids for either sex (Table 4). F_1 hybrid females were sufficiently attractive to be approached by males of both parental races ($P > 0.05$; multiple comparisons by *G*-test), and F_1 hybrid males were as successful in mating as males of either parental race ($P > 0.05$; multiple comparisons by *G*-test).

Table 2 Adult survivorship tests for the two host races of *Henosepilachna diekei*. '*N*' indicates the number of adult beetles tested. Adult beetles were collected from either *Mikania micrantha* or *Leucas lavandulifolia* as adults or larvae (collection) and were served either the natal host or the alternative host (treatment) for 10 days. Different superscript letters indicate a significant difference in survivorship between categories ($\alpha = 0.05$; Kaplan–Meier method with the Wilcoxon test, followed by Holm's method). The acceptance rate within the test period for each individual was estimated as the number of days on which the treatment plant was accepted divided by the total days in the test period (maximum 10 days).

Adult type	*N*	Collection	Treatment	Mean survivorship (days ± SD)	Acceptance rate (days) within the test period
Mikania race	24	Adult	*M. micrantha*	9.36 ± 0.27[a]	0.87
	20	Larvae	*M. micrantha*		0.91
	20	Adult	*L. lavandulifolia*	5.38 ± 0.29[b]	0
	20	Larvae	*L. lavandulifolia*		0.02
Leucas race	23	Adult	*M. micrantha*	5.26 ± 0.31[b]	0.04
	23	Larvae	*M. micrantha*		0.01
	20	Adult	*L. lavandulifolia*	8.43 ± 0.37[a]	0.93
	36	Larvae	*L. lavandulifolia*		0.94

Table 3 Population pairwise F_{st} table based on mitochondrial ND2 haplotypes (upper right) and nucleic ITS2 genotypes (lower left). Population name abbreviations are as follows: CL, *Leucas* race at Cibinong; CM, *Mikania* race at Cibinong; BL, *Leucas* race at Bogor, BM, *Mikania* race at Bogor.

ITS2\ND2	CL	CM	BL	BM
CL	–	0.807***	0.263**	0.873***
CM	0.849***	–	0.606***	0.041^{NS}
BL	0.165**	0.851***	–	0.671***
BM	0.866***	0.015^{NS}	0.877***	–

Statistically significant F_{st} values are denoted with asterisks (***$P < 0.001$; **$P \leq 0.05$; $^{NS}P > 0.05$).

Egg hatchability test

We detected no differences in the number of eggs produced (Table 5; $P > 0.05$, Tukey–Kramer test) or in egg hatchability among the four mating combinations ($P > 0.05$, multiple comparisons by *G*-test).

Larval performance test

We detected extremely divergent host plant performance between the two host races. Larvae of neither host race in the first instar stage accepted leaves of the alternative host plant (initial acceptance), nor survived even to the second instar on the alternative host plant (Table 6).

Reciprocal F_1 hybrids survived on both host plants (Table 6). On *M. micrantha*, larvae of the *Mikania* race showed significantly higher initial acceptance and survivorship to the second instar than the reciprocal F_1 hybrids; however, there was no significant difference in survivorship to adulthood. On *L. lavandulifolia*, larvae of the *Leucas* race and the reciprocal F_1 hybrids demonstrated similar levels of initial acceptance, survivorship to the second instar, and survivorship to adulthood. There was no F_1 hybrid inviability, as measured by larval survivorship to adulthood.

Discussion

Factors relating population divergence and speciation in *Henosepilachna diekei*

We detected only two types of positive isolating barriers, which could have arisen as a direct consequence of divergent host plant specialization between host races of *H. diekei* in West Java. These host races demonstrated extremely divergent host preference (habitat isolation) and reduced performance of individuals on the alternative host (immigrant inviability *sensu* Nosil *et al.*, 2005) in

Table 4 Female mating attractiveness of four types of females (a) and male mating success of four types of males (b). There were no significant differences in either index among the four types of females and males tested (multiple comparisons by *G*-test; $\alpha = 0.05$).

			Female mating attractiveness from		
(a) Female type	Total no. replicates	Total no. attempts from males	*Mikania* race male	*Leucas* race male	Total
Mikania race	100	65	0.60	0.70	0.65^{NS}
F_1ML	100	63	0.68	0.58	0.63^{NS}
Leucas race	100	63	0.58	0.68	0.63^{NS}
F_1LM	100	79	0.82	0.76	0.79^{NS}
			Male mating successfulness with		
(b) Male type	Total no. attempts	Total mating pairs	*Mikania* race female	*Leucas* race female	Total
Mikania race	59	41	0.63	0.76	0.69^{NS}
F_1ML	64	53	0.78	0.81	0.80^{NS}
Leucas race	64	51	0.74	0.79	0.77^{NS}
F_1LM	69	50	0.76	0.81	0.78^{NS}

Table 5 Hatchability of eggs resulting from intra- and inter-racial matings between the *Mikania* and *Leucas* races of *Henosepilachna diekei* at Cibinong, West Java. There were no significant differences in the number of eggs per batch (Tukey–Kramer test) or hatchability (multiple comparisons by *G*-test) among the four mating combinations.

Crossing combination					Mean no. of eggs per	
Female race	Male race	No. of females	No. of egg batches	No. of eggs	Egg batch ± SD	Hatching rate
Mikania race	*Mikania* race	11	66	1302	20.2 ± 2.7^{NS}	0.723^{NS}
Mikania race	*Leucas* race	10	60	1130	17.6 ± 3.9^{NS}	0.702^{NS}
Leucas race	*Leucas* race	13	73	1075	19.0 ± 2.0^{NS}	0.643^{NS}
Leucas race	*Mikania* race	10	56	978	17.9 ± 3.7^{NS}	0.678^{NS}

Table 6 Survivorship during the larval stage for the two host races and reciprocal F_1 hybrids on *Mikania micrantha* and *Leucas lavandulifolia*. Fitness components were measured as initial acceptance by first instar larvae, survivorship to the second instar, and survivorship to adulthood. Different superscript letters indicate significant differences among the four types of larvae (Fisher's exact probability test followed by Holm's method; $\alpha = 0.05$).

		Fitness components on *M. micrantha*				Fitness components on *L. lavandulifolia*			
				Survivorship to				Survivorship to	
Larvae type	No. of families	No. of larvae	Acceptance in first instar	Second instar	Adulthood	No. of larvae	Acceptance in first instar	Second instar	Adulthood
Mikania race	10	100	1.00^a	0.92^a	0.64^a	100	0^a	0^a	0^a
F_1ML	6	60	0.93^b	0.77^b	0.65^a	60	0.98^b	0.97^b	0.67^b
Leucas race	10	100	0^c	0^c	0^b	100	0.96^b	0.90^b	0.81^b
F_1LM	7	70	0.87^b	0.77^b	0.63^a	70	0.97^b	0.90^b	0.79^b

the adult and larval stages. The fact that the field-caught adults exhibited the strong preference for the original host plant and reduced survivorships on the alternative host (Tables 1 and 2) shows that these traits keeps the beetles to stay on respective hosts in the wild condition. Our results thus indicate that the first isolating barriers to develop were habitat isolation and immigrant inviability. We consider this pattern of population divergence of the host races to be a simple case of ecological speciation, because both these barriers were unlikely to have arisen solely by nonecological processes, and specifically, immigrant inviability definitely evolves because of divergent natural selection (Rundle & Nosil, 2005; Funk & Nosil, 2008). In this study, however, we did not detect hybrid inviability, which can potentially evolve in adaptive divergence (Nosil *et al.*, 2005; Funk, 2009) between host races.

Here, we postulate that the divergent host use is genetically determined, at least to some extent, because even newly hatched larvae demonstrated extremely divergent host acceptability. Although this can also be caused by the maternal effect, high acceptance and high performance of reciprocal F1 hybrid larvae discount this possibility. We further postulate that the divergent host acceptability shown by newly emerged adults is also genetically determined at least partly; however, the possibility of a conditioning effect because of feeding experience during larval stages could not be ruled out in this case. To elucidate the extent of genetic determination for the divergent host use of these host races is needed in the future study.

There are relatively few examples in which such extremely ecological divergence developed prior to the development of other isolating barriers (Bush, 1969; Coyne & Orr, 2004; Gavrilets & Vose, 2005), but the host races in *H. diekei* are the second case for phytophagous ladybird beetles. Host races of the congeneric Japanese phytophagous ladybird beetles *Henosepilachna niponica* and *Henosepilachna yasutomii* are reproductively isolated only by fidelity to different host plants (Katakura *et al.*, 1989; Katakura & Hosogai, 1994). Field cage experiments have shown that adult host preference functions as an extremely strong isolating barrier by limiting the beetles' mating sites to the respective natal host plants (Hirai *et al.*, 2006). Notable congruent features between the two cases of host race formation in *Henosepilachna* beetles are extreme specialization on the natal host plant and the absence of other isolating barriers. In these cases, an extreme, single-dimensional niche shift (i.e. occurring along one ecological axis, such as habitat; reviewed in Nosil & Harman, 2009) may drive population divergence and prevent gene flow even when the races are in close proximity. When ecological divergence is strong enough to prevent gene flow, the evolution of other isolating barriers might not be necessary to permit co-occurrence of two diverged populations or species.

Development of isolating barriers as a consequence of divergent host plant specialization

We will now consider how these two isolating barriers jointly accomplished reproductive isolation. Different habitat preference acts as habitat isolation by limiting the probability of interhost immigration and by limiting mating sites to on or near the respective natal host plants. This host fidelity strongly contributes to preventing current gene exchange between populations utilizing different host plants (Bush, 1969; Katakura *et al.*, 1989; Feder *et al.*, 1994; Funk *et al.*, 2002; Hirai *et al.*, 2006; Funk & Nosil, 2008). In addition, reduced fitness of interhost immigrants on the alternative host plant through immigrant inviability reduces opportunities for interpopulation mating, because poorly adapted immigrants are likely to die or emigrate from the habitat before encountering potential mates among the different host race individuals (Feder *et al.*, 1997; Filchak *et al.*, 2000; Via *et al.*, 2000; Nosil, 2004; Nosil *et al.*, 2005).

Current reproductive isolation between the host races is evidently achieved more by habitat isolation than by immigrant inviability, because the host races are extremely divergent in host preference, and the different host preference acts prior to the reduced fitness of interhost immigrants. Here, we considered only adult beetles as potential interhost immigrants, because the larvae are less

mobile over long distances (they can travel at most several metres, if at all). If individuals are mobile enough, interhost immigrants in cases of sympatry can return to their natal host from the inferior alternative host. The effects of immigrant inviability may thus generally be obscured by the effects of habitat isolation (i.e. host fidelity), and in any case, we regard the intensity of reproductive isolation because of immigrant inviability to be rather limited in these sympatric host races.

Although the effect of immigrant inviability on current reproductive isolation may be limited, immigrant inviability could have played a crucial role in the development of divergent host use in the host races. Reduced viability of immigrants in foreign habitats may favour individuals that prefer the natal (i.e. suitable) host plant to others, yielding habitat isolation (Ballkau & Feldman, 1973; Rice & Hostert, 1993; Nosil *et al.*, 2005). We consider this positive feedback loop of increasing habitat isolation and divergence between populations (Nosil *et al.*, 2005; Hendry *et al.*, 2007) to be what actually happened in the earliest stage of host race formation in these beetles. We thus consider immigrant inviability to be a potential generator of adaptive divergence rather than contributing to current reproductive isolation.

The two isolating barriers can arise most quickly (possibly simultaneously) with specialization to a particular host when there is physical linkage between alleles controlling the two traits (habitat preference and habitat performance), and more specifically, when a single gene region determines the two traits pleiotropically (Futuyma & Moreno, 1988; Joshi & Thompson, 1995; Fry, 1996). The former occurs in host race formation in *Acyrthosiphon* pea aphids (Hawthorne & Via, 2001), and the latter is consistent with the following statement by Coyne & Orr (2004: p. 163): ''... acceptance and performance are probably products of the *same* genes – those genes that determine whether a plant provides the correct feeding stimulus.'' Our results suggest that performance (longevity and survivorship) on the host plants depended on acceptance by both the adult and larval stages of the beetles. For example, adult beetles refused the alternative host even if they starved to death (Table 2). Similarly, the first instar larvae of neither host race accepted the alternative host or survived to the second instar on it (Table 6). These lines of evidence indicate that the reduced survivorship of each host race on the alternative host plant involved divergent host acceptance rather than divergent host performance, and in any case, we could not evaluate the actual ability of the beetles to use the alternative host when they would not accept it.

Strength of the extremely divergent host specialization as reproductive isolation

We conclude that the two isolating barriers are strong enough to prevent gene flow between the host races, for three reasons. First, F_{st} values based on mitochondrial ND2 haplotypes and nuclear ITS2 genotypes were quite large, indicating strong restriction of current gene flow between the host races, which occur in close proximity to one another. Second, sympatric occurrence of the host races was observed as early as 1990 in the vicinity of Bogor (Kahono *et al.*, 2002), and continues to the present (February 2011); the divergent host plant utilization has thus been maintained for more than 20 years. Finally, relatively few wild-caught individuals of the host races showed an intermediate preference for the two host plants (Table 1). Because reciprocal F_1 hybrid adults accepted both host plants in the laboratory (K.W. Matsubayashi, unpublished), we infer F_1 hybrids to have been quite rare in the wild. The test comparing feeding acceptance between wild-caught adults and reared adults that were collected as larvae on the respective host plants demonstrated that adult immigrants between the races are also quite rare (Table 2). These lines of evidences suggest that the isolating barriers nearly completely prevent gene flow between the two host races, even in sympatry.

Despite the extreme specialization of the beetles on the natal host plants, Fujiyama *et al.* (2001) reported a noteworthy exception in preliminary feeding tests on *H. diekei* at Cibinong. These authors observed that the *Mikania* race showed a strong preference for the original host plant, whereas the nearby *Leucas* race accepted both *M. micrantha* and *L. lavandulifolia*. Unfortunately, because of small sample sizes and a mass design for the host choice test, the percentage of individuals of the *Leucas* race that chose the alternative host was unclear. This observation needs particular attention, because it evidently suggests two possibilities, either occasional hybridization or an intermediate stage of development of host specialization in the two host races.

How these host races with extremely divergent host use formed remains unresolved. Because *M. micrantha* is first introduced in W-Java (in Bogor Botanical Garden) from Paraguay at 1949 (Kostermans *et al.*, 1987; Whitten *et al.*, 1996), the use of *M. micrantha* by *H. diekei* must have started within 50 years. On the other hand, *L. lavandulifolia* is native in Southeast Asia (Wardani, 2001). Lower genetic diversity and lack of genetic differences between populations of recently introduced *Mikania* race for mtDNA and nuclear ITS is consistent with a recent founder event and a rapid expansion of beetles associated to *M. micrantha*. For the reason, the secondary contact scenario is the most likely explanation for the coexistence of host races with extremely divergent host specialization in West Java. Even in this scenario, we suggested that the observed divergent host specialization strongly contributes to population divergence by preventing gene exchange after secondary contact of diverged host races.

Detailed analyses on population phylogeny using much more molecular markers and populations are needed to resolve this issue. Addressing their origin will

provide valuable insight into how species arise by divergent specialization.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Generalized linear model (GLM) tables for adult survivorship tests.

Table S2 (a) Mitochondrial ND2 haplotypes from the host races at Cibinong and Bogor. (b) Unique nuclear ITS2 genotypes from the host races at Cibinong and Bogor.

Table S3 Population molecular diversity based on mitochondrial ND2 haplotypes and nuclear ITS2 sequences from the host races at Cibinong and Bogor.

Table S4 Analysis of molecular variance (locus-by-locus AMOVA) based on (a) mitochondrial ND2 and (b) nuclear ITS2 haplotypes from the host races at Cibinong and Bogor.

Figure S1 Median-joining haplotype network tree based on ND2 sequences from host races of ladybird beetle, *Henosepilachna diekei* collected in Bogor and Cibinong.

Figure S2 Median-joining haplotype network tree based on nuclear ITS2 sequences from host races of *Henosepilachna diekei* collected in Bogor and Cibinong.

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