Unilateral Mate Choice Causes Bilateral Behavioral Isolation Between Two Closely Related Phytophagous Ladybird Beetles (Coleoptera: Coccinellidae: Epilachninae)

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Abstract

Behavioral isolation between two sympatric and closely related phytophagous ladybird beetles, *Henosepilachna vigintioctomaculata* and *Henosepilachana pustulosa*, was studied in laboratory. Mating behavior was investigated using the male-choice test. Males of *H. vigintioctomaculata* preferred conspecific females to heterospecific females as mates, whereas males of *H. pustulosa* did not show such preference. Females of *H. vigintioctomaculata* appeared more fastidious at mating than females of *H. pustulosa*, irrespective of the species of males. Males of neither species showed tenaciousness following rejection by females. The choice of conspecifics by *H. vigintioctomaculata* males and a difference in the intensity of rejection between *H. vigintioctomaculata* females (strong) and *H. pustulosa* females (weak) result in positive behavioral isolation in both directions of heterospecific matings. Our results thus indicated that positive unilateral mate choice yields bilateral behavioral isolation between these two species.

Introduction

Reproductive isolation between species results from barriers against gene flow (Coyne & Orr 2004). Of these barriers, behavioral isolation plays a critical role in various groups of animals (Mayr 1963; Wiernasz & Kingsolver 1992; Herring & Verrell 1996). Many studies have shown that animals use mating cues (acoustic, optic, olfactory, etc.) for identification of conspecific mates, and that differences in these cues cause a mismatch in mate recognition between species (Hoy & Paul 1973; Martínez Wells & Henry 1992; Wiernasz & Kingsolver 1992).

The importance of interaction between male and female behavioral factors in successful matings has been suggested by studies of Hawaiian *Drosophila* (Kaneshiro & Boake 1987; Boake 1989; Hoikkala & Kaneshiro 1993; Boake & Hoikkala 1995). These studies have shown that behavioral isolation results from failures in the interactions between the mating behaviors of both sexes. Hence, to understand how

behavioral isolation is accomplished, we need to know both the relevant factors in the behavior of each sex and the interactions of these factors.

In the present study, we analyzed the factors of behavioral isolation in a sympatric phytophagous ladybird beetle pair, *Henosepilachna vigintioctomaculata* (Motschulsky) and *H. pustulosa* (Kôno) belonging to the *H. vigintioctomaculata* species complex (Katakura 1997). We especially sought to determine which behavioral factor and which sex are responsible for their behavioral isolation.

H. vigintioctomaculata is widespread in cool-temperate eastern Asia, including all the main islands of Japan, and occurs on crops and weeds belonging to the family Solanaceae (Katakura 1981) and also on a cucurbit, *Schizopepon bryoniaefolius* (Katakura 1976). *H. pustulosa* is endemic to Hokkaido, northern Japan (Katakura 1981), and occurs on wild thistles (*Cirsium* spp.) and blue cohosh (*Caulophyllum robustum*, Berberidaceae). The two species are thus sympatric over the whole range of *H. pustulosa*

(Hokkaido except for the eastern and the southern parts), utilizing different host plants. The genetic distance (Nei's D) between the two species measured at 14 allozyme loci is 0.286 (S. Saitoh & H. Katakura unpubl. data), and the species are clearly differentiated in mitochondrial CO1 and nuclear ITS2-region gene sequences (Kobayashi et al. 1998; N. Kobayashi unpubl. data).

Under laboratory conditions, the two species interbreed and produce fully viable and fertile F_1 hybrids (Katakura & Nakano 1979; Nakano 1981; Katakura 1997). Furthermore, occasional heterospecific matings between the two species have been observed in the wild (S. Nakano pers. comm.), and putative hybrid individuals determined by morphological criteria have also been discovered at low frequency (Nakano 1981).

However, there exists certain degree of behavioral isolation between the two species. Given a choice between females of the two species under laboratory conditions (male-choice test), males of these species tended to choose conspecifics (Katakura & Nakano 1979). Mate choice by females (female choice) has not been investigated in these species. No study has determined what kind of behavioral mismatch is responsible for the reproductive isolation in these species.

Methods

Beetle Collection and Handling

The beetles used in the present study were the offspring of overwintered beetles collected from May to Jun. 2003 in the Toyoura area of southwestern Hokkaido. Individuals of *H. pustulosa* were collected at the margin of a thistle patch (*Cirsium kamtschaticum* Ledeb.), and individuals of *H. vigintioctomaculata* were collected from potato plants in a crop field. The two sampling locations were located approx. 8 km apart. The beetles and their offspring were maintained in the laboratory in plastic cases at 23°C and 16:8 h (light:dark) photoperiod, and were fed fresh leaves of the Japanese nightshade (*Solanum japonense* Nakai; Solanaceae), a plant preferentially eaten by both species under laboratory conditions (Fujiyama & Katakura 2002). Larvae were reared on potted Japanese nightshade in a greenhouse. We crossed virgin adults of these laboratory stocks in Apr. 2004 and obtained newly emerged adults of *H. vigintioctomaculata* and *H. pustulosa* in Jun. 2004. We kept these individuals on *S. japonense* and used them for our experiments. Females began to oviposit, males

showed mating attempts within two weeks of emergence. Hence, we used only adults more than 2 wk old after emergence, to insure the beetles' sexual maturity. We maintained more than 80 individuals each of females and males of both species throughout this study.

Mate-choice test

Mate-choice tests were conducted with a male-choice design. One female each of *H. vigintioctomaculata* and *H. pustulosa* and one male of either species were put together in a clear plastic case (6 cm × 5.5 cm × 2 cm), with the bottom covered by moist filter paper, and the male was allowed to choose freely between the females. All mate-choice tests were conducted between 12 Jul. and 10 Aug. 2004, in order to reduce any bias due to age and/or seasonal effects. Thus, we used adult beetles from 2–6 wk after emergence in all tests in this experiment.

Observation of Mating Behavior

The mating behavior of *H. vigintioctomaculata* and *H. pustulosa* is simple (Katakura & Nakano 1979). When a male finds a female, it approaches the female and tries to mount. The female sometimes shows rejection behavior against the male's approach, such as by shaking its body or by kicking the male.

Mating behavior was recorded by direct observation for a period of 120 min. When a pair was established, the remaining female was removed from the case and was returned to the respective stocks of beetles according to the species. If no mating behavior occurred during the observation period, the beetles were returned to the respective stocks of beetles according to the sex and species for the subsequent experiment conducted on another day. Mating was defined as having been completed when it lasted for more than 30 min, as sperm transfer does not usually occur in matings lasting <30 min (Katakura 1985). Matings that lasted <30 min were regarded as having failed (however, no failed matings were observed in this experiment). Females that made genital contact with males even once were not used again in subsequent experiments.

Scoring of Behavioral Factors

For each trio, we recorded 'male attempt' (which female the male approached first), 'female response' (response of the female to the approaching male: accept or reject), 'male tenaciousness' (response of

Table 1: Number of first mating attempts of males of *H. vigintioctomaculata* (Hv) and *H. pustulosa* (Hp) when they were individually given a choice between females of the two species: n indicates the number of males that attempted to mate

| | | No. attempts | | Difference between conspecifics and heterospecifics | |
| | | Female | | | |
Male	n	Conspecific	Heterospecific	Chi-squared test for each male type	Fisher's exact probability test for comparison between two types of males
Hv	40	32	8	p < 0.0001	p = 0.0024
Hp	40	18	22	p = 0.64	

the male to rejection by the female: give up or not), and the combination of the pair finally established.

Males are sometimes not reproductively active under experimental conditions. We used data obtained only for trios in which the male tried to copulate with a female at least once. We continued the experiment until data were obtained for 40 such trios for each male type.

Statistical Analysis and Measurement of Behavioral Isolation

We performed chi-squared test to examine if there is a preference of each male species for a particular species of females at mating. We compared the number of conspecific and heterospecific attempts (or finally established mating pairs) for each male species. The null hypothesis was no difference between the number of conspecific and heterospecific attempts (or mating pairs). Then, we compared the results of mate choices between males of *H. vigintioctomaculata* and *H. pustulosa* by using Fisher's exact probability test to detect the difference in preference between males of the two species. Both tests mentioned above were implemented in MS Excel 2003 software.

We performed a logistic regression analysis with JMP statistical software (ver. 5.1; SAS institute Inc, Cary, NC, USA, 2004) to determine the variable(s) responsible for female rejection to males. Response variable was the number of female rejection, and the explanatory variables were: (a) female species being attempted by males, (b) male species that attempted to females, and (c) interaction of these two variables (henceforth designated as 'female species × male species').

Results

Males of *H. vigintioctomaculata* chose conspecific females significantly more often than heterospecifics (chi-squared test, $\chi^2 = 14.40$, df = 1, p < 0.0001; Table 1), whereas *H. pustulosa* males showed no

Table 2: Responses of females to first mating attempts by males: n indicates the number of males that attempted to mate with females

| Female | Male | Female response | | | |
		n	Reject	Accept	Percent rejection
Hv	Hv	32	18	14	56.3
	Hp	22	18	4	81.8
Total (overall percent)		54	36	18	(66.7)
Hp	Hv	8	1	7	12.5
	Hp	18	5	13	27.8
Total (overall percent)		26	6	20	(23.1)

preference (chi-squared test, $\chi^2 = 0.40$, df = 1, p = 0.53; Table 1). Significant statistical difference was detected in the frequency of conspecific attempts between the two species males (Fisher's exact probability test, p = 0.0024; Table 1), indicating asymmetrical mate choice of males.

Females of *H. vigintioctomaculata* rejected males more often (66.7% rejection) than did *H. pustulosa* females (23.1% rejection; Table 2). The logistic regression analysis (Table 3) showed that 'female species' significantly contributed to the difference in female rejection behavior, whereas 'male species' did not. This means that females of *H. vigintioctomaculata* were more fastidious than *H. pustulosa* females, irrespective of the species of males that attempted to

Table 3: Results of logistic regression analysis to determine which variable is responsible for female rejection of male mating attempts. Explanatory variables were (a) female species; (b) male species; and (c) female species × male species

| Source | Likelihood | | | |
	df	χ^2	p-value	r^2
Frequency of female rejection of male attempts				
Full model	3	24.3411	<0.0001	0.2289
(a) Female species	1	23.9396	<0.0001	
(b) Male species	1	1.5025	0.2203	
(c) Female species × Male species	1	6.8422	0.0089	

Table 4: Number of finally established mating pairs when males of *H. vigintioctomaculata* (Hv) and *H. pustulosa* (Hp) were given a choice between females of the two species: n indicates the number of mated males

| Male | N | No. pairs | | Difference between conspecifics and heterospecifics | |
| | | Female | | | |
		Conspecific	Heterospecific	Chi-squared test for each male type	Fisher's exact probability test for comparison between two types of males
Hv	19	17	2	p < 0.0001	p = 0.23
Hp	18	13	5	p = 0.06	

mate. The regression analysis also showed a significant interaction between 'female species × male species,' probably suggesting a weak preference of *H. vigintioctomaculata* females for conspecific males, as they rejected heterospecific males more frequently (81.8%) than conspecific males (56.3%). However, when analyzed separately, this effect was marginally non-significant; i.e. *H. vigintioctomaculata* females did not reject heterospecific males significantly more often than conspecific ones (Fisher's exact probability test, p = 0.08).

Males of neither species were tenacious at mating. Seventeen of 19 (89.5%) *H. vigintioctomaculata* males and 22 of 23 (95.7%) *H. pustulosa* males gave up after the first rejection by females. Males of *H. vigintioctomaculata* mated significantly more often with conspecific than with heterospecific females (chi-squared test, $\chi^2 = 11.84$, df = 1, p < 0.0001; Table 4). Similarly, *H. pustulosa* males mated more often with conspecific females, but this effect was only marginally significant (chi-squared test, $\chi^2 = 3.56$, df = 1, p = 0.06; Table 4). Furthermore, no statistical difference was detected in the frequency of finally established conspecific pairs between the two species (Fisher's exact probability test p = 0.23; Table 4), indicating not asymmetrical behavioral isolation.

Discussion

In our experiments using male-choice tests, *H. vigintioctomaculata* and *H. pustulosa* showed 'bilateral' behavioral isolation. Katakura & Nakano (1979) also reported significant assortative mating in experiments of the same design using allopatric and sympatric population pairs of these species. Despite some degrees of difference in intensity of behavioral isolation, the three population pairings examined by Katakura & Nakano (1979) and in the present study all showed significant bilateral behavioral isolation between *H. vigintioctomaculata* and *H. pustulosa*. Furthermore, no population pairings showed complete behavioral isolation.

Our analyses of behavioral factors show that two major factors are responsible for this bilateral

isolation. The first factor is the strong preference only of *H. vigintioctomaculata* males for conspecific females. Males of *H. pustulosa* did not show such a preference. Females of *H. vigintioctomaculata* showed a slight but non-significant preference for conspecific males, whereas *H. pustulosa* females appeared not to discriminate between conspecifics and heterospecifics. The second crucial factor is the difference in female fastidiousness at mating. Females of *H. vigintioctomaculata* behaved more fastidiously than *H. pustulosa* females, irrespective of the species of male attempting to mate (Tables 2 and 3).

As a result of these factors, heterospecific matings are reduced in both directions. Mating between *H. pustulosa* females and *H. vigintioctomaculata* males is suppressed because *H. vigintioctomaculata* males are choosy; mating between *H. vigintioctomaculata* females and *H. pustulosa* males is reduced because *H. vigintioctomaculata* females are fastidious at mating. Female rejection is effective because male tenaciousness against female rejection is very weak.

Thus, the present study showed that the bilateral behavioral isolation between *H. vigintioctomaculata* and *H. pustulosa* results largely from the behavior of *H. vigintioctomaculata*, i.e. the choosiness of males and fastidiousness (and possibly choosiness) of females. No behavioral factors of *H. pustulosa* seem to be involved in the behavioral isolation. Despite the unilateral mate choice by only *H. vigintioctomaculata*, the mating pairs finally established showed bilateral and not asymmetric behavioral isolation.

Gray (2005) pointed out that behavior-based reproductive isolation between closely related species may be driven by females rather than males, since natural selection should favor females that avoid costly heterospecific matings. However, contrary to this expectation, our results suggest that behavioral factors of both sexes or of males of *H. vigintioctomaculata* contribute almost equally to the overall reproductive isolation between *H. vigintioctomaculata* and *H. pustulosa*.

One behavior observed during the present study suggests that *H. vigintioctomaculata* males use an olfactory cue (pheromone and/or contact pheromone) to

recognize conspecifics. When a male approached a female (possibly mediated by visual cues), it usually stroked the female with its antennae. If the female was a conspecific, the male touched surface of the female's elytra and tried to mate, extending its abdomen and penis. However, if the female was a heterospecific, the male often ceased its attempt and withdrew from the female, without touching the female with its forelegs. In contrast, females of neither species showed this behavior. Detailed investigation of behavioral elements and chemical cues will be necessary to fully understand the mechanism of behavioral isolation between these species.

Our results indicate that males of *H. vigintioctomaculata* recognize conspecific females as legitimate mating partners by some specific cues. On the other hand, neither sex of *H. pustulosa* appeared capable of distinguishing between *H. vigintioctomaculata* and conspecifics as appropriate partners. Hence, mating in *H. vigintioctomaculata* may involve specific cues not included in the repertory of mating cues of *H. pustulosa*.

Contribution of mismatch of mating cues to behavioral isolation is studied in detail using several species pairs of Hawaiian *Drosophila*. For example, *Drosophila heteroneura* and *D. silvestris* show an asymmetry in the utilization of mating cues similar to that we observed in the beetles. Price & Boake (1995) suggested that *D. heteroneura* males do not require sex-specific cues to initiate courtship. In that study, the probability of pairings of *D. silvestris* females and *D. heteroneura* males was much higher than that of the reciprocal combination; pairings of *D. heteroneura* females and *D. silvestris* males were very rare. A failure of one of the combinations of heterospecific matings is the factor most often responsible for an obviously asymmetrical behavioral isolation (Craddock 1974; Kaneshiro 1976, 1980, 1983; Val 1977; Boake et al. 2000).

Our results highlight a point rarely recognized in studies on behavioral isolation; that mate choice and behavioral isolation are not synonymous, but represent different levels of phenomena. The asymmetries in mate choice between our study species hold for both sexes, yet the establishment of mating pairs is symmetric. This is because behavioral interactions between males and females can conceal between-species asymmetries in mate choice. The results also suggested that the final number of established mating pairs does not always reflect the asymmetry of mate choice. To elucidate the mechanisms of behavioral isolation, we should analyze in detail the behavioral factors (including genetic and chemical factors) and behavioral interaction between males and females.

Acknowledgements

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