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Reproductive Isolation in Four Phytophagous Ladybeetles (*Epilachna*, Coccinellidae, Coleoptera) in West Sumatra

Susumu NAKANO Faculty of Commercial Sciences, Hiroshima Shudo University, Numata, Asaminami, Hiroshima 731-31, Japan

Idrus ABBAS Department of Biology, Faculty of Science, Andalas University, Padang, West Sumatra, Indonesia

ABSTRACT Reproductive isolation in four Sumatran *Epilachna* species was studied by testing 1) food preference, 2) mate choice, 3) sperm viability and 4) hybrid viability.

Food habits of two solanum feeders, *E. vigintioctopunctata* and *E. enneasticta* (abbreviated as Ev, Ee) overlapped, while two cucurbit feeders, *E. dodecastigma* and *E. septima* (Ed and Es) preferred different food plants. Complete or strong ethological isolation was observed in all but one combination consisting of Ev and Es females and Es males. In this combination, half of the Ev females and all Es females received sperm of Es male(s), but sperm of Es male(s) was inactivated in Ev females. The average percentage of egg hatch for four conspecific combinations was 65.9%, while in 12 interspecific ones none hatched. These results show that the four *Epilachna* species are completely isolated by the combination of 1) difference of food preference, 2) strong preferences for conspecific matings, 3) inactivation of sperm in heterospecific females, and 4) inviability of eggs from interspecific matings.

Food plants of Ev, Ee, and Es were introduced to Sumatra relatively recently and the presumed native food plants of these three species have not been found. Thus, the four *Epilachna* species had probably been isolated when the present relationship between *Epilachna* and their food plants was established in West Sumatra.

Key words: *Epilachna* / Coccinellidae / Coleoptera / reproductive isolation / West Sumatra

The phytophagous ladybeetles belonging to the subfamily Epilachninae are characterized by considerable variation in food habits both among and within species and the relatively small differences in external morphology among species (Katakura, 1981, 1988; Hoshikawa, 1983). Thus, they are suitable materials for the study of speciation by shifts in host plant usage (Katakura, 1981; Hoshikawa, 1983). Although many studies on reproductive isolation have been carried out using Japanese sympatric *Epilachna* species (Katakura, 1981; Nakano, 1987; Katakura *et al.*, 1989), reproductive isolation in *Epilachna* in other regions has not been studied. To elucidate the speciation process in Epilachninae, discerning reproductive isolation mechanisms between species with various levels of divergence is indispensable.

In West Sumatra four *Epilachna* species, that is, *E. vigintioctopunctata, E. enneasticta, E. septima*, and *E. dodecastigma* (abbreviated as Ev, Ee, Es, and Ed, respectively), occur abundantly and attack solanaceous and cucurbitaceous crops (Katakura *et al.*, 1988). These four species reproduce all the year round (Nakano, unpubl.). Further, their altitudinal distributions largely overlap between 400 and 1000 m (Katakura *et al.*, 1988) in regions where food plants are cultivated side by side in small gardens (Nakano, unpubl.). Under these conditions, therefore, Ev and Ee share common food plants (cf. Katakura *et al.*, 1988), seem to encounter frequently and may carry out interspecific matings. On the other hand, Ed and Es were collected from different food plants (Katakura *et al.*, 1988). However, the coexistence of Es and Ed or that of solanum feeders and cucurbit feeders may also occur if they can mature on food plants other than their own.

The aim of this paper is to clarify whether the four sympatric *Epilachna* species are isolated by host choice of larvae and adults, mate choice, sperm inviability in interspecific matings, or by hybrid inviability.

MATERIALS AND METHODS

Adult beetles of four *Epilachna* species were collected in and near Padang, West Sumatra from January to April, 1988. The origins of the beetles are as follows: Ev on *Solanum tuberosum* and Ee on *Solanum torvum* both collected at Kayu Jao (1250 m alt.), 46 km southeast of Padang; Es on *Momordica charantia* collected at Limau Manis (150 m) in Padang; Ed on *Cucurbita moschata* collected at Kayu Jao and Sukarami (950 m), 41 km east of Padang. A part of these field-collected adults were used to examine adult food preference (see 1-1 in Materials and Methods). Since *Epilachna* beetles mate many times during their lives (Katakura, 1982; Nakano, 1987), field-collected adults were also used for the observation of mate choice behavior (2-1). The remaining adults of each species were reared in mass and allowed to lay eggs. First instar larvae and virgin adults obtained from these eggs were used in experiments on larval food preference (1-2) and mate choice (2-2), observation of sperm viability (3) and test of hybrid viability (4). Rearing of Ee, Ed and Es was done on the host species they were collected, while Ev was reared with *Solanum torvum*, one of the known food plants in West Sumatra (Katakura *et al.*, 1988). Food was renewed every day. The rearing and experiments were carried out under a relatively constant temperature (24.5-27.0°C) and natural day length (12L12D) at the Sumatra Nature Study Laboratory, Andalas University, Padang.

1. Food preference experiment

Food preference experiments were aimed to examine the effectiveness of isolation by habitat or host plants. The following nine food plant species were offered to first instar larvae and adults of four *Epilachna* species: four solanaceous (*Solanum torvum, S. tuberosum, S. melongena,* and *Datura metel*) and five cucurbitaceous species (*Momordica charantia, Cucurbita moschata, Benincasa cerifera, Cucumis sativus,* and *Luffa acutangula*). Four solanaceous species are food plants of Ev and three *Solanum* species are those of Ee (Katakura *et al.*, 1988; see Table 1). Es utilizes *Momordica charantia* and Ed feeds on other four cucurbits in the field (Katakura *et al.*, 1988; see Table 1).

1-1. Adult food preference

Six adults (three females and three males) of each *Epilachna* species were individually put into transparent plastic cages (10.0 cm in diameter and 4.3 cm in depth). A piece of the leaf of a plant species (one of above nine plant species) was given for one day. When an adult fed on the leaf of that plant species on that day, the leaf of another species was offered the next day. If after 24 hr the adult did not eat the leaf of the first plant species, a leaf of second species was placed in the cage. By the same procedure, food preference for nine plant species were examined. Daily leaf consumption by each individual was scored as follows: 0 = not eaten, 1= nibbled, 2= eaten, 3= eaten avidly. Feeding intensity (I) in each species was shown by the average score of six adults.

1-2. Larval food preference

Twenty first instar larvae of an *Epilachna* species (one of four *Epilachna* species) were put on a leaf of a single plant species within one day of hatching. They were reared with the leaves of that plant species until they proceeded to the second instar. Suitability of nine food plants to the larvae of four *Epilachna* species was examined by the same procedure. Suitability (S) of food plant was indicated by the percentage of larvae reaching the second instar: (number of larvae that reached the second instar/20) × 100.

Table 1. Result of food preference experiments. Feeding Intensity (I) and Suitability (S) show the index of the food preference of adults and larvae, respectively. Food plant in the field (FP) is cited from Katakura *et al.* (1988).

Name of plant	Ev		Ee		Es		Ed	
(English name)	I	S	I	S	I	S	I	S
Solanaceae								
Solanum torvum	3.0 FP	100	3.0 FP	100	0	0	0	0
S. tuberosum (potato)	2.7 FP	100	2.6 FP	100	0	0	0	0
S. melongena (egg plant)	3.0 FP	100	3.0 FP	100	0	0	0	0
Datura metel (thorn apple)	1.8 FP	95	0	25	0	0	0	0
Cucurbitaceae								
Momordica charantia (bitter cucumber)	0	0	0	0	3.0 FP	100	0.7	0
Cucurbita moschata (squash)	0	0	0	0	0	40	2.8 FP	100
Benincasa cerifera (wax gourd)	0	0	0	0	0.3	0	2.5 FP	100
Cucumis sativus (cucumber)	0	15	0	15	2.3	90	3.0 FP	100
Luffa acutangula (loufah)	0	0	0	0	0.3	45	2.5 FP	100

2. Mating experiments

The degree of ethological isolation among four species was examined by two mate choice experiments.

2-1. Mate choice experiment 1

Adult beetles collected in the field were separated by the sex and reared for one week in the laboratory before the experiment. One male of a species was confined with one conspecific female and one heterospecific female in a transparent polystyrene cage (6.5 × 5.0 × 2.5 cm) and mating behavior was observed for 90 min. All combinations of *Epilachna* species were tested. The detailed method of observation was described in Katakura & Nakano (1979). This observation was repeated until the number of successful matings reached more than 20 per combination. Chi-square tests (d.f.=1) were adopted for statistical analysis; the expected values were calculated by assuming random mating.

2-2. Mate choice experiment 2

As soon as the progeny of the field-collected adults emerged, they were sexed and females and males were reared separately. Newly emerged adults begin to mate within one week after emergence (Nakano, unpubl.). Thus, three or four days after emergence, four virgin females from each of two species and four unmated males from one of these two species (a total of 12 individuals) were put together and reared with the food plants of both species in the transparent plastic cage (11.3 cm in diameter and 8.0 cm in depth). All combinations of *Epilachna* species were tested. After rearing for twenty days, all females were dissected and the presence of sperm was checked with the aid of a microscope (cf. Katakura, 1986).

3. Observation of sperm viability

When sperm of different species was preserved in mate choice experiment 2, activity of heterospecific sperm was recorded by comparing with that of conspecific sperm.

4. Hatchability test

By the same procedure as that in (2-2), virgin females and males were reared separately. Two weeks after emergence one unmated male and one virgin female were placed together in a transparent plastic cage (10.0 cm in diameter and 4.3 cm in depth), reared with fresh leaves and allowed to lay eggs. Oviposition was checked every day and egg masses were collected to determine percentage of egg hatch. Four conspecific and twelve interspecific combinations were examined and each combination included 5-10 pairs. Egg masses in interspecific combinations were collected until the death of females, while ten egg masses were collected at maximum in conspecific combinations.

RESULTS

Result of food preference experiments is shown in Table 1. Ev and Ee did not feed on cucurbits and Ed and Es did not eat solanaceous plants. The only exception was 15% of the Ev and Ee larvae that proceeded to the second instar on *Cucumis sativus*, although no adults

Table 2. Result of mate choice experiment 1.

Combination	No. of pairs examined	No. of mated pairs	
		conspecific mating	interspecific mating
(Ev♀+ Ee♀)×Ev♂	129	30	0
(Ev♀+ Ee♀)×Ee♂	187	28	1
(Ev♀+ Es ♀)×Ev♂	110	30	0
(Ev♀+ Es ♀)×Es♂	90	32	0
(Ev♀+ Ed♀)×Ev♂	133	33	0
(Ev♀+ Ed♀)×Ed♂	208	31	1
(Ee♀+ Es ♀)×Ee♂	147	22	1
(Ee ♀+ Es ♀)×Es♂	80	32	0
(Ee♀+ Ed♀)×Ee♂	236	20	0
(Ee♀+ Ed♀)×Ed♂	115	30	5
(Es♀+ Ed♀)×Es♂	90	35	0
(Es♀+ Ed♀)×Ed♂	172	31	0

fed on this food plant. Between the two solanum feeders, there was no difference in food preference except for the preference for *Datura metel*. Es and Ed preferred their own food plants. Besides its own food plant Es showed a strong preference for *Cucumis sativus*, one of the food plants of Ed, but did not accept other food plants of Ed.

Result of mate choice experiment 1 is shown in Table 2. Comparison between the expected and the observed numbers of matings showed conspecific matings were more frequent in all combinations including (Ee+Ed) × Ed (<0.001). Table 3 shows the result of mate choice experiment 2. Ethological isolation was complete in 11 out of 12 combinations. In the combination (Ev + Es) × Es , half of the Ev females (two individuals) and all Es females (four individuals) received sperm from Es male(s). However, sperm of Es male(s) in one Ev female was not motile and in another Ev female motility was lower than that in

Table 3. Result of mate choice experiment 2. Numerator and denominator in fractional number show the number of females receiving sperm and the number of females dissected, respectively.

Combination	Conspecific Combination	Interspecific Combination
(Ev♀+Ee♀)×Ev♂	4/4	0/4
(Ev♀+Ee♀)×Ee♂	4/4	0/4
(Ev♀+Es ♀)×Ev♂	4/4	0/4
(Ev♀+Es ♀)×Es♂	4/4	2/4
(Ev♀+Ed♀)×Ev♂	4/4	0/4
(Ev♀+Ed♀)×Ed♂	4/4	0/4
(Ee♀+Es ♀)×Ee♂	2/3*	0/4
(Ee♀+Es ♀)×Es♂	4/4	0/4
(Ee♀+Ed♀)×Ee♂	3/4	0/4
(Ee♀+Ed♀)×Ed♂	4/4	0/4
(Es♀+Ed♀)×Es♂	4/4	0/4
(Es♀+Ed♀)×Ed♂	4/4	0/4

Table 4. Result of hatchability test. Average and range of percentage egg hatch show average value and range for each pair.

Combination	No. of pairs examined	No. of eggs obtained	average	% hatch range
Conspecific				
Ev ♀ × Ev ♂	6	1208	70.8	84.2 - 25.0
Ee ♀ × Ee ♂	5	492	78.8	90.5 - 60.0
Es ♀ × Es ♂	10	1259	40.0	81.7 - 0
Ed ♀ × Ed ♂	7	2101	74.0	92.8 - 54.6
Interspecific				
Ev ♀ × Ee ♂	5	534	0	
Ev ♀ × Es ♂	5	546	0	
Ev ♀ × Ed ♂	5	780	0	
Ee ♀ × Ev ♂	5	357	0	
Ee ♀ × Es ♂	5	329	0	
Ee ♀ × Ed ♂	6	394	0	
Es ♀ × Ev ♂	5	446	0	
Es ♀ × Ee ♂	5	332	0	
Es ♀ × Ed ♂	7	460	0	
Ed ♀ × Ev ♂	5	826	0	
Ed ♀ × Ee ♂	9	579	0	
Ed ♀ × Es ♂	6	335	0	

conspecific females.

Table 4 shows the result of hatchability test. The percent egg hatch in four conspecific combinations was high (average 65.9%), while none hatched in twelve interspecific crosses.

DISCUSSION

Four *Epilachna* species used in the present study were collected in and near Padang situated close to the equator (0°53' S, 100°21' E). The climate in this area lacks distinct cycle of wet and dry seasons and annual rainfall reaches 4100 mm (Inoue & Nakamura, 1990). Since food plants of four *Epilachna* species grow and the four *Epilachna* species also reproduce all the year round (Nakano, unpubl.), seasonal isolation is not an effective potential isolating mechanism.

Katakura *et al.* (1988) clarified the altitudinal distribution and food plants of four *Epilachna* species. Their results and ours (Table 1) provide information on habitat isolation through food plant preference. According to Katakura *et al.* (1988), the altitudinal distributions of four species largely overlapped between 400 and 1000 m: Ev 0-1,400 m, Ee 400-1,400 m, Es and Ed 0-1000 m. Further, food plants of four species are frequently cultivated side by side in small gardens (Nakano, unpubl.) In this condition, habitat isolation is ineffective in the following combinations: Ev and Ee sharing common *Solanum* species and Es and Ed having the possibility of coexistence on *Cucumis sativus* (Table 1). On the other hand, the difference in food plant preference acts as an effective isolating factor between cucurbit feeders and solanum feeders and between Es and Ed on all cucurbits other than *Cucumis sativus* (Table 1), although they may have a slight chance to encounter each other on their food plants growing nearby.

In contrast to a lack of seasonal isolation and partial habitat isolation, ethological isolation is complete or strong. Complete or strong isolation in all combinations in mate choice experiment 1 (Table 2) and one exception in experiment 2 (Table 3) suggest that hybridization is unlikely. Even when Es and Ed have a chance to encounter on *Cucumis sativus* or Ev and Ee on three *Solanum* species, successful interspecific matings appear not to occur (Tables 2 and 3).

Then, how is the fate of rare interspecific matings? In combination (Ev + Es) × Es in mate choice experiment 2, two Ev females received the sperm of Es male(s). However, sperm of Es male(s) was inactivated in sperm storage organs of Ev females. Further, the percent egg hatch in twelve interspecific combinations was 0% (Table 4). Although it is not clear whether inviability of eggs in twelve interspecific combinations was caused by unsuccessful matings, inactivation of heterospecific sperm in reproductive organs of females, the death of hybrid eggs after fertilization or combined effects of them, the present study suggests that hybrid production is prevented completely among four *Epilachna* species.

To summarize, four *Epilachna* species are completely isolated by the combination of 1) difference in food preference, 2) strong preferences for conspecific matings, 3) inactivation of sperm in heterospecific females, and 4) inviability of eggs from interspecific crosses.

Finally we will mention the relationship between the origin of food plants and reproductive isolation in four *Epilachna* species. Among nine plants used in the present study, all food plants of Ev, Ee, and Es and a part of food plants of Ed are not native to Sumatra but introduced: *S. torvum*, *Datura metel* , and *Cucurbita moschata* from Central America, *S. tuberosum* from South America , *Momordica charantia* and *Cucumis sativus* from India, *S. melongena* from India or N.E. Africa (Symon, pers. comm. ; Hotta *et al.*, 1989). Further, presumed native food plants of Ev, Ee, and Es have not been found among 17 solanaceous and 11 cucurbitaceous plants examined by us in West Sumatra (Nakano *et al.*, unpubl.). Thus, it is suggested that sympatric relationships among four species were formed relatively recently and four *Epilachna* species probably had already been isolated when the present relationship between *Epilachna* and their food plants was established.

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中野　進，Idrus ABBAS　西スマトラの4種の食植性テントウムシ(マダラテントウ属、テントウムシ科，甲虫目)の生殖的隔離

西スマトラに同所的に分布する4種の食植性テントウムシの生殖的隔離を明らかにするため 1) 食草選択，2) 交尾選択，3) 雌体内における異種精子の活性，4) 異種間ペアーから得られた卵の孵化率を調べた。*Epilachna vigintioctopunctata* と *E. enneasticta* (以下 Ev, Ee と略) は同じナス科の作物を好んだが *E. septima* と *E. dodecastigma* (Es, Ed と略) は異なるウリ科植物を好んだ。交尾選択実験では 12 の組み合せのうち 11 で完全あるいは強い行動的隔離がみられた。行動的隔離が不完全な Ev, Es の雌と Es の雄の組み合せでは，種間交尾と種内交尾の割合が1対2であった。しかし種間交尾をした雌の体内の精子は種内交尾のものより活性が低かった。同種間ペアーの卵の平均孵化率は 65.9% だったが異種間ペアーでは0%だった。以上の結果，食性の違い，極めてまれな種間交尾，異種精子の雌体内での活性低下，異種間ペアーの卵が孵化しないことの組み合せにより4種は完全に隔離されていると考えられる。

ところで今回の実験で使用した Ev, Ee, Es の食草のすべて，および Ed の食草の一部は比較的最近スマトラに導入されたものである。また我々が西スマトラで採集した上記以外のウリ科とナス科植物 28 種の中には，スマトラ固有で Ev, Ee, Es の食草になっているのものが見つかっていない。したがって現在の食草と4種の *Epilachna* の関係ができたときにはすでに4種間の生殖的隔離が存在していたと考えられる。