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Authors: Kobayashi, Norio, Shirai, Yoichi, Tsurusaki, Nobuo, Tamura, Koichiro, Aotsuka, Tadashi, et al.

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Two Cryptic Species of the Phytophagous Ladybird Beetle *Epilachna vigintioctopunctata* (Coleoptera: Coccinellidae) Detected by Analyses of Mitochondrial DNA and Karyotypes, and Crossing Experiments

Norio Kobayashi[1], Yoichi Shirai[2], Nobuo Tsurusaki[3], Koichiro Tamura[4], Tadashi Aotsuka[4] and Haruo Katakura[1]*

[1]*Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan,*
[2]*National Institute of Agro-Environmental Sciences, Tsukuba, Ibaraki 305-0856, Japan,*
[3]*Laboratory of Biology, Faculty of Education and Regional Sciences, Tottori University, Tottori 680-8551, Japan and*
[4]*Department of Biology, Graduate School of Science, Tokyo Metropolitan University, Hachioji, Tokyo 192-0397, Japan*

ABSTRACT—Analyses of a part of mitochondrial cytochrome c oxidase I gene sequences (645 bp) for seventeen individuals of *Epilachna vigintioctopunctata* (Fabricius) from eight localities in east and southeast Asia revealed that the populations are divided into two genetically distinct groups (Chiba, Tokyo, Naha, Iriomote, Bangkok vs. Kuala Lumpur, Padang, Bogor). The number of nucleotide substitutions between sequences of different groups was 57–60, while that between sequences within each group was 1–8. Karyotypes of the two groups were also distinctly different. Crossing experiments showed that there exist strong postmating barriers between the two groups: eggs obtained from between-group crossings usually did not hatch, whereas more than 90% of eggs from within-group crossings hatched. It is concluded that *E. vigintioctopunctata*, a notorious pest of solanaceous crops in Asia and Australia, is composed of at least two reproductively isolated biological species that probably occupy different geographic ranges.

INTRODUCTION

Species are often viewed as reproductively isolated entities (Dobzhansky, 1937; Mayr, 1942). Such "biological species" are not always morphologically distinct. There are many cases of cryptic species that can only be recognized by crossing experiments or molecular characterization (Mayr, 1963; Futuyma, 1997). In phytophagous insects, there has been intense debate over the importance of sympatric speciation (Mayr, 1963; Otto and Endler, 1989; Futuyma, 1997; Howard and Berlocher, 1998). For this reason, great effort has been expended characterizing cryptic phytophagous species occurring in the same geographic area (sympatric species), whereas less effort has been concentrated on allopatric or parapatric cryptic species. Hence, the literature may exhibit a bias towards sympatric examples. Here we will present a possible example of allopatric/parapatric cryptic species.

* Corresponding author: Tel. +81-11-706-2751;
FAX. +81-11-746-0862.
E-mail: katakura@sci.hokudai.ac.jp

The phytophagous ladybird beetle *Epilachna vigintioctopunctata* (Fabricius) is widespread in Asia and Australia, where it is notorious for causing severe damage to solanaceous crops such as eggplants and tomatoes (Dieke, 1947; Li and Cook, 1961; Pang and Mao, 1979; Hoang, 1983; Richards, 1983; Katakura *et al.*, 1988; Richards and Filewood, 1990; Park and Yoon, 1991; Li, 1993; Shirai and Katakura, 1999). Furthermore, occurrence of this species on a leguminous weed *Centrosema pubescens* in Java and Sumatra was recently reported (Nishida *et al.*, 1997; Shirai and Katakura, 1999). This species is known to exhibit a considerable degree of geographic variation in some external features, and thus, it was previously treated as many different species or subspecies (cf. Dieke, 1947). However, most of these variations were in external appearance, i.e., elytral spot patterns and degree of melanism, which are now known to vary even within a single population (Katakura *et al.*, 1988, 1994). Subsequent studies uncovered very close resemblances in important structural characters, including genitalia of both sexes, among the "species" and "subspecies". *Epilachna vigintioctopunctata* is thus currently treated as a widely distributed highly polymorphic

species (Li, 1993).

In the course of analyzing the phylogenetic relationships of certain species of Asian epilachnines using mitochondrial DNA sequences, we found that Japanese and Javanese samples of *E. vigintioctopunctata* showed considerable genetic difference comparable to that found between some distinct congeneric species. Preliminary crossing experiments also suggested the presence of a strong post-mating barrier between the two populations. We hence undertook an intensive comparative study using several population samples obtained from Japan and southeastern Asia. Results have demonstrated that *E. vigintioctopunctata* is composed of two biological species that may occupy mutually exclusive distribution.

MATERIALS AND METHODS

Insects

The locations of samples used in the present study are given in Table 1 and Fig. 1. The samples included some field collected specimens and six laboratory-reared strains, the latter of which were derived from approximately eleven to twenty-five pairs of beetles collected from 1994 -1997 and maintained for 3 to 20 generations in the laboratory of the National Institute of Agro-Environmental Sciences under the following rearing procedures and conditions.

Thirty females and 30 males of newly emerged adults were introduced into a cage (30 cm×35 cm×50 cm high). They were fed potted black nightshade (*Solanum nigrum* L.), irrespective of their original host plants. For two days between 20 and 25 days after emergence of the adults, when the oviposition activity peaked, a total of 30 egg masses were collected and transferred to another cage. Hatched larvae were then reared on black nightshade foliage. After the emergence of new adults, 30 females and 30 males were randomly chosen and used as parental stock of the next generation. The beetles from temperate and subtropical regions (Chiba and Naha) were kept under 24°C and 16L8D, and other populations, all from Asian tropics, were kept under 26°C and 14L10D.

Voucher specimens will be deposited in the Zoological Institute, Graduate School of Science, Hokkaido University, Sapporo.

DNA analysis

One individual each from five localities, i.e., Chiba, Naha, Bangkok, Kuala Lumpur, and Bogor, three individuals from Padang, and ten individuals collected at Funaura on Iriomote Island, the Ryukyus, were investigated (Table 1). Beetles used were those collected on solanaceous plants or their laboratory-reared progenies, except those from Padang, which included two individuals derived from beetles collected on a legume *Centrosema pubescens* (Padang A) and one from *Solanum torvum* (Padang B).

Mitochondrial DNA was extracted from living adult specimens following the method of Tamura and Aotsuka (1988). Using the nucleotide sequence of a congener, *E. vigintioctomaculata* Motschulsky as a reference, we designed a set of PCR primers to amplify a region containing the whole cytochrome c oxidase subunit I (COI) gene as described in Kobayashi *et al.* (1998). Then we determined the nucleotide sequences of a part of COI gene by the direct sequencing method using an ABI PRISM 377 autosequencer.

The number of nucleotide sites determined and used for analyses was 645 bp comprizing a 16th of the gene. Jukes and Cantor's (1969) method was used for estimating the number of substitutions per site for all possible pairs of the sequences. On the basis of the distance matrix, we constructed a phylogenetic tree by the neighbor-joining (NJ) method (Saitou and Nei, 1987) using the software MEGA (Kumar *et al.*, 1993). We also constructed a phylogenetic tree by the maximum parsimony method with branch and bound search algorithm (Hendy and Penny, 1982) using the same software. The nucleotide sequence of another individual of *E. vigintioctopunctata* from Tokyo (Tables 1 and 2) published in Kobayashi *et al.* (1998) (accession number: AB002180) was also incorporated into the analysis. In addition, two congeners, *E.* sp. 3 (AB002174) and *E. pusillanima* Mulsant (AB002177) (Kobayashi *et al.*, 1998) were used as outgroups. To test the confidence probability for each interior branch, the bootstrap method (Felsenstein, 1985) was performed with 1,000 replications.

Table 1. Sources of materials examined for *Epilachna vigintioctopunctata*.

Locality	Date collected[1]	Collected on	Number of specimens examined or strains used for		
			mtDNA	Karyotype[2]	Crossing
Honshu, Japan					
1) Higashi-Matsuyama	September - October 1982	Eggplant (*Solanum melongena*)	–	11L 4m	–
2) Tokyo*	September 1994	Eggplant	1	–	–
3) Chiba	(July 1997)	Eggplant	1	–	+
Ryukyu Islands, Japan					
4) Naha, Okinawa Is.	(May 1997)	*Solanun photeinocarpum*	1	1f 8m	+
5) Iriomote Is.	March 1997	*Solanum* sp.	10	–	–
Thailand					
6) Bangkok	(November 1996)	*Solanum torvum*	1	5f 10m	+
Malaysia					
7) Kuala Lumpur	(November 1996)	*S. torvum*	1	9m	+
Sumatra, Indonesia					
8a) Padang (A)	(March 1995)	*Centrosema pubescens*	1	6f 9m	+
8b) Padang (B)	March 1995	*S. torvum*	2	–	–
9) Kayu Jao, near Padang	August 1988	*Solanum* sp.	–	3m	–
Java, Indonesia					
10) Bogor	(February 1994)	*S. torvum*	1	3f 10m	+

[1] Dates of collection for the founders of the laboratory strains are given in parentheses.
[2] L, 4th instar larvae; f, female; m, male.
* Cited from Kobayashi *et al.* (1998).

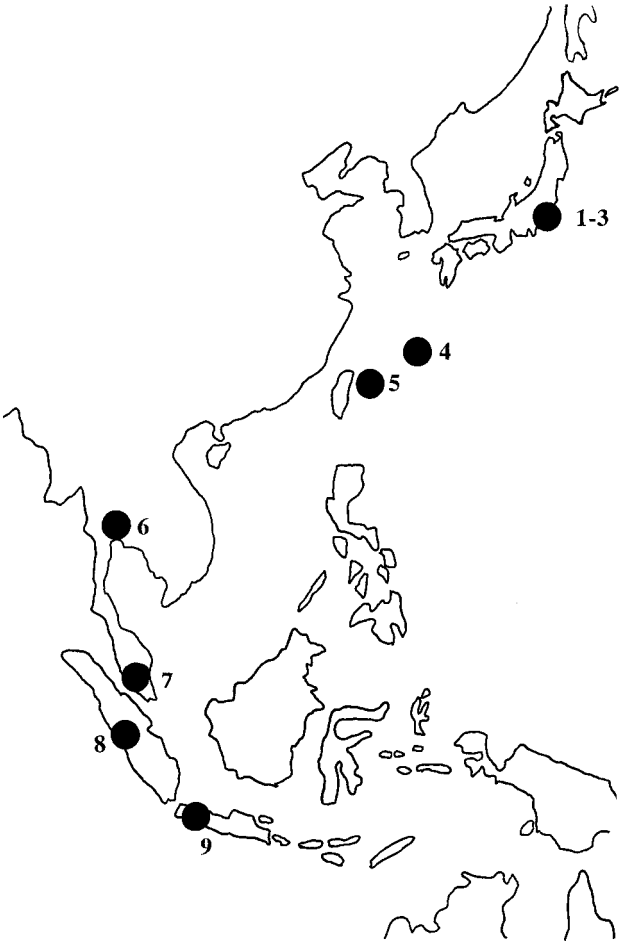

Fig. 1. Collection sites of *Epilachna vigintioctopunctata* examined in the present study. 1. Higashi-Matsuyama, 2. Tokyo, 3. Chiba, 4. Naha, 5. Iriomote, 6. Bangkok, 7. Kuala Lumpur, 8. Padang, 9. Bogor.

Karyotype analyses

Chromosomes of *E. vigintioctopunctata* have been investigated several times by different authors (Japanese specimens: Yosida, 1948; Tanaka and Sasaji, 1992; Indian specimens: Bose, 1948; Agarwal, 1961; Yadav and Pillai, 1974), though none of these studies used modern air-drying techniques.

We restudied chromosomes of the species for seven samples (Table 1). The cytological data were obtained from air-dried preparations of testes or ovaries of adults just after eclosion, or supraoesophageal ganglia of the fourth instar larvae. We dissected materials from living individuals in a hypotonic solution containing colchichine (mixture of 0.5 volume of 0.1% colchichine solution and 9.5 volume of 1% sodium citrate) on a hollow slide. Then we macerated the materials for ca. 10 min. in the same solution before fixation with Carnoy's solution (3:1 volumes of absolute methanol and glacial acetic acid). We prepared chromosome slides using mainly an air-drying technique with 30% acetic acid treatment for dissociation of cells (Dietrich and Mulder, 1981). For the materials from Higashi-Matsuyama (Honshu, Japan) and Kayu Jao (Sumatra), we employed another cell dissociation treatment using lactic acid, which is the same as that used for harvestman and epilachnine chromosomes (Tsurusaki, 1985; Tsurusaki and Cokendolpher, 1990; Tsurusaki *et al.*, 1993). There was no detectable difference in the quality of chromosome strands obtained between the two treatments for dissociation of cells, though the method using 30% acetic acid was easier to perform when a centrifuge was available. In both treatments, chromosomes were stained by 5% Giemsa solution (Merck) in Sörensen's buffer (p.H. 6.8).

We serially arranged mitotic metaphase chromosomes according to the descending order of length (Figs. 3, 4). Haploid idiograms (Fig. 4) were drawn by calculating the ratio of the length of each chromosome to the TCL, the total length of a haploid set of autosomes plus an X chromosome. We followed Levan *et al.* (1964) for chromosome classification.

Crossing experiments

Crossing experiments were made using six laboratory-reared strains (Table 1). Beetles were sexed within three days after emergence, and then, 25 virgin females of one strain and 25 males of another strain were placed together in a cage and were given black nightshade as food. Adults did not show any mating behavior for more than three days after emergence under the present experimental condition. During three successive days between 20 and 25 days after emergence, 40 egg masses were collected and their hatching rates were checked. Also, two to five additional egg masses were collected and fixed with Carnoy's solution at the age of three days. After removing the chorion, the eggs were stained with corbol-thionin, dehydrated with alcohol, cleared with benzene, and mounted with ceder oil (Katakura and Sobu, 1986). Then the condition of the embryonic development was observed. Furthermore, we dissected all the females surviving at the end of the experiments to confirm whether they retained sperm or not. Control experiments (with males and females of the same strain) were also performed following the procedures described above.

Crossing experiments were made under 24°C and 16L8D when one of the parents was either Chiba or Naha, and under 26°C and 14L10D for other combinations.

Table 2. Distribution of the eight COI gene haplotypes across samples studied for mitochondrial DNA analyses.

	Locality*								
Haplotype	Chiba (1)	Tokyo** (1)	Naha (1)	Iriomote (10)	Bangkok (1)	Kuala Lumpur (1)	Padang A (1)	Padang B (2)	Bogor (1)
hap 1	1								
hap 2		1							
hap 3			1	8					
hap 4				2					
hap 5					1				
hap 6						1		1	1
hap 7							1		
hap 8								1	

* The number of specimens examined is noted in parentheses.
** Cited from Kabayashi *et al.* (1998).

RESULTS

DNA analysis

Eight haplotypes were discriminated (Table 2). A total of 65 nucleotide and 4 amino acid sites were variable. Variations such as insertion and deletion were not found. Haplotype 3 was shared by individuals from Naha and Iriomote, and haplotype 6 was shared by individuals from Kuala Lumpur, Padang (B), and Bogor. Other haplotypes were unique. Two haplotypes were found in Iriomote individuals, and three in Padang (A + B).

Figure 2 is a phylogenetic tree for the eight haplotypes constructed by the NJ method with *E. pusillanima* and *E.* sp. 3 as outgroups. The bootstrap probabilities are given in the upper or lower side of each branch. The eight haplotypes of *E. vigintioctopunctata* are clearly divided into two groups with a very high bootstrap value (100%): haplotypes 1–5 comprising Chiba, Tokyo, Naha, Iriomote, and Bangkok samples, and haplotypes 6–8 comprising Kuala Lumpur, Padang (A + B), and Bogor samples. This dichotomy was also supported by a phylogenetic tree constructed using the maximum parsimony method (data not shown). The number of nucleotide substitutions between haplotypes of the same groups was 1–8, whereas that between haplotypes of different groups was 57–60.

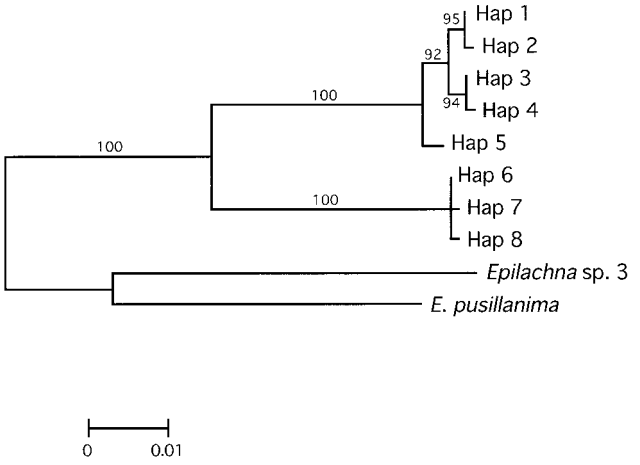

Fig. 2. A phylogenetic tree constructed using the NJ method for the eight haplotypes (Table 2) of the sequence of a part of cytochrome oxidase I gene (645bp) detected in *Epilachna vigintioctopunctata* populations. Numerals at branching points show bootstrap values (1000 replications).

Karyotype analyses

Results of the present study are summarized in Table 3 together with those of previous studies. Representative karyotypes are given in Fig. 3. The previous studies revealed that the chromosome composition of *E. vigintioctopunctata* was 2n=18 with male heterogametic XY_p-XX in sex determination. The present study confirmed the basic chromosome number of *E. vigintioctopunctata* to be 2n=18, although some individuals from Padang and Bogor possessed supernumerary chromosomes (2n=19, 20, 21, 22). Detailed accounts of variations relating to supernumerary chromosomes will be published elsewhere.

The two groups of samples recognized on the basis of the mtDNA analysis also differed from each other karyologically (Table 3, Figs 3, 4). The specimens from Japanese localities and Bangkok (designated as “northern populations” in Fig. 4) possessed an obviously larger Y chromosome compared with those from Kuala Lumpur and Indonesian localities (“southern populations”). On the other hand, there was no detectable difference in the size of the X chromosome between the two groups. Owing to these facts, two distinctly different types

Table 3. Chromosome numbers of *Epilachna vigintioctopunctata* collected from diverse localities of Asia.

Locality	Chromosome number			Reference (PS=present study)
	Male		Female	
	2n	MI[1)]	2n	
Higashi-Matsuyama	18	8+XY_p	18	PS
Fukui, Honshu, Japan	18	8+XY_p	–	Tanaka and Sasaji, 1992
Aichi Pref., Honshu, Japan	18	8+XY_p	–	Yosida, 1948
Naha	18	8+XY_p	18	PS
Culcutta, India	18	8+XY_p	–	Bose, 1948
Allahabad, India	18	8+XY_p	–	Agarwal, 1961
Kurukshetra, India	18	?[2)]	–	Yadav and Pillai, 1974
Bangkok	18	8+XY_p	18	PS
Kuala Lumpur	18	8+Xy_p	–	PS
Kayu Jao, Sumatra	18/21	8+Xy_p	–	PS
Padang, Sumatra	18–21	8+Xy_p	18	PS
Bogor, Java	18–22	8+Xy_p	18/20	PS

[1)] First meiotic metaphase. Y in lower and upper cases stands for a minute or a large Y chromosome, respectively, and p in subscript for a “parachute” which represents the parachute-like configuration formed by a “canopy” X chromosome and a “parachutist” Y.

[2)] To which type this population belongs is not clear. No illustration of MI (as well as somatic chromosomes) was presented in this report, though the authors described it to be “8+XY_p”.

of the X-Y sex bivalents were observed in the first meiotic metaphases: XY_p, exclusively found in the "northern populations" and Xy_p, in the "southern populations" (Table 3). The subscript p stands for a "parachute" that denotes the parachute-like configuration formed by a "canopy" X chromosome and a "load or parachutist" Y.

The karyotypes of the two groups were strikingly different from one another in the arm ratio of each chromosome of the complement as well. Namely, smaller six autosomes were acro- or subtelocentric in the "northern populations" and meta-

Fig. 3. Representative karyotypes of *Epilachna vigintioctopunctata*. B, E, H and I from females (sex chromosome composition: XX); others from males (XY). A–B: Higashi-Matsuyama. C: Naha. D–E: Bangkok. F: Kuala Lumpur. G - H: Padang. I: Bogor. Scale=0.01mm. Note that the difference in relative size of the two sex chromosomes between the northern three populations (A–E) and southern three populations (F–I). Also note laterally united sister chromatids in the paracentric portion of No. 1 pair of chromosomes in karyotypes of the southern three populations. Other chromosomes of the karyotypes of the southern populations also exhibit similar configurations in their short arms.

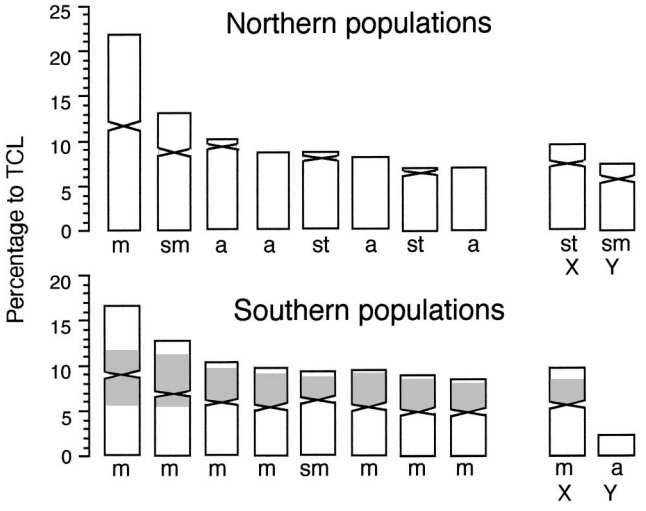

Fig. 4. Schematic representation of karyotypes of *E. vigintioctomaculata*. TCL = total chromosome length; m, sm, st, a = metacentric, submetacentric, subtelocentric, and acrocentric chromosomes, respectively. Distribution of possible heterochromatic (shaded) and euchromatic (unshaded) blocks was inferred from the chromosomal configurations shown in the karyotypes of the southern populations.

or submetacentric in the "southern populations". Moreover, the karyotypes of the latter group possessed a large block, where two sister chromatids were tightly associated side by side, on each of all the chromosomes, except for a minute Y chromosome. The block was pericentric in the first and second autosomes, whereas it was confined to short arms in the remaining autosomes and an X chromosome (Fig. 4). This type of chromosome configuration is typical for the diphasic chromosomes often encountered in various groups of Coleoptera including *Epilachna* (Drets *et al.*, 1983; Tsurusaki *et al.*, 1993), and the block can be inferred to be heterochromatic, although no C-banding technique was employed in the present study.

The mode of karyotypic differentiation between the two forms is highly reminiscent of that found between two closely related forms of the *Epilachna vigintioctomaculata* species complex (Tsurusaki *et al.*, 1993).

Crossing experiments

Results were very straightforward as summarized in Table 4. The six populations were divided into the following two distinct groups with respect to the crossability: Group I: Chiba, Naha, and Bangkok; group II: Kuala Lumpur, Padang, and Bogor. The mean hatching rates of eggs were consistently high (89.1–100%) in the crossings within each group. On the other hand, no eggs hatched in the crossings between the two groups except the crossing between Chiba females and Bogor males, in which only 0.2% of eggs hatched (two individuals from an egg mass). These two larvae died in the second instar.

We could not detect any trace of embryonic development in eggs from between-group crossings (Table 5). On the other hand, dissection of females demonstrated that sperm transfer was largely successful between the two groups, although there were several females that possessed no sperm (Table 6).

Table 4. Percentage hatching of eggs produced by various combinations of crossings between six strains of *E. vigintioctopunctata*. The number of egg masses examined was 40 for every combination of crossings.

	Female					
Male	Chiba	Naha	Bangkok	Kuala Lumpur	Padang	Bogor
Chiba	97.6±6.3	92.5±26.7	90.4±27.0	0	0	0
Naha	97.7±6.8	93.7±22.4	99.0± 4.8	0	0	0
Bangkok	97.5±7.6	89.1±30.4	94.6±10.8	0	0	0
Kuala Lumpur	0	0	0	92.9±22.6	98.4± 4.9	93.9±9.7
Padang	0	0	0	95.4±10.2	94.9±16.7	96.5±7.5
Bogor	0.2±1.1	0	0	98.0± 4.9	98.7± 4.2	100.0±0.0

Table 5. Percentage of eggs containing developing embryos, which were produced by various combinations of crossings between six strains of *E. vigintioctopunctata*. The numbers of eggs and egg masses (in *Italic*) examined are shown in parentheses.

	Female					
Male	Chiba	Naha	Bangkok	Kuala Lumpur	Padang	Bogor
Chiba	98.3 (58, *6*)	–	–	0 (39, *2*)	0 (29, *2*)	0 (105, *5*)
Naha	–	–	–	0 (37, *2*)	0 (43, *2*)	0 (38, *2*)
Bangkok	–	–	–	0 (53, *2*)	0 (26, *2*)	0 (25, *2*)
Kuala Lumpur	0 (32, *2*)	0 (21, *2*)	0 (48, *2*)	–	–	–
Padang	0 (43, *2*)	0 (31, *2*)	0 (47, *2*)	–	–	–
Bogor	0 (120, *5*)	0 (39, *2*)	0 (38, *2*)	–	–	–

Table 6. Percentage of females that possessed spermatozoa after various combinations of crossings between six strains of *E. vigintioctopunctata*. The number of females examined is shown in parentheses.

	Female					
Male	Chiba	Naha	Bangkok	Kuala Lumpur	Padang	Bogor
Chiba	–	–	–	66.7 (15)	38.9 (18)	100 (13)
Naha	–	–	–	100 (9)	60.0 (25)	100 (16)
Bangkok	–	–	–	50.0 (14)	100 (7)	100 (12)
Kuala Lumpur	100 (18)	81.0 (21)	100 (16)	–	–	–
Padang	95.5 (22)	100 (14)	100 (5)	–	–	–
Bogor	100 (24)	85.0 (20)	100 (11)	–	–	–

DISCUSSION

Epilachna vigintioctopunctata is currently treated as a widely distributed highly polymorphic species (Li, 1993). In the present materials, too, there exist large interpopulational variations in spot patterns and some other external features (Abbas *et al*., 1988; Katakura *et al*., 1988, 1994), but all of them share identically shaped genitalia, a characteristic of *E. vigintioctopunctata* (cf. Katakura *et al*., 1988). However, the present results consistently indicated that the samples of *E. vigintioctopunctata* studied here are composed of two biological species. One of them, here tentatively called "the northern form", is consisted of the samples from Japan through Thailand (Bangkok), and the other one, "the southern form", is comprised of samples from the peninsular part of Malaysia to the two large islands (Sumatra, Java) of Indonesia. The two forms are genetically (Fig. 2) and karyologically (Fig. 4) well differentiated. Furthermore, they are potentially reproductively isolated from each other by a very strong postmating barrier (failure of fertilization or death of hybrid embryos in a very early stage; Tables 4, 5). The results also suggested that there might be a certain degree of sexual isolation, since some females did not retain sperm when they were kept for nearly four weeks with males of the other form (Table 6).

Available evidence suggests that the two forms are allopatric or parapatric, and the two forms seem to replace to each other in the Malay Peninsula, somewhere between Bangkok and Kuala Lumpur (Fig. 1). If this interpretation is correct, *E. vigintioctopunctata*, a common pest species widespread in East and Southeast Asia, may provide an extremely suitable situation for the studies of various controversial issues of evolutionary biology, in particular those concerning the mode of speciation and reinforcement of reproductive isolation (*sensu* Howard, 1993). However, entire picture of the distributional pattern of the two forms remains unresolved. Further intensive studies that aim to clarify the detail of their geographic distribution are indispensable. Judging from the previous data on karyotypes in which meiotic metaphases were illustrated (Bose, 1948; Agarwal, 1961), populations in India seemed to belong to the northern form (Table 3) by having an XY_p association in meiosis. But this must be ascertained through careful analyses of DNA and crossing experiments. Moreover, the distribution range of *E. vigintioctopunctata* is enormous (Dieke, 1947; Li and Cook, 1961; Pang and Mao, 1979; Hoang, 1983; Richards, 1983). Hence it is possible that *E. vigintioctopunctata* comprises other "biological species" that occupy unsurveyed areas.

There are a number of available names that have been synonymized with *E. vigintioctopunctata* (Richards, 1983; Li, 1993), and it is likely that the two forms were already described under different names. However, no obvious correspondence between the two forms and previously recognized taxa of *E. vigintioctopunctata* has been ascertained. We will refrain from adopting any scientific names for our two forms until a thorough revision of this species complex based on sufficient amount of information is possible.

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REFERENCE

Abbas I, Nakamura K, Katakura H, Sasaji H (1988) Geographical variation of elytral spot patterns in the phytophagous ladybird, *Epilachna vigintioctopunctata* (Coleoptera: Coccinellidae) in the Province of Sumatera Barat, Indonesia. Res Popul Ecol 30: 43–56

Agarwal U (1961) Studies on the chromosomes of five species of Coccinellidae (Coleoptera). Cytologia 26: 285–293

Bose I (1948) The association of non-homologue chromosomes in the spermatogenesis of the ladybird beetle, *Epilachna vigintioctopunctata* (Fab.). Proc Zool Soc Bengal 1: 131–140

Dieke GH (1947) Ladybeetles of the genus *Epilachna* (sens. lat.) in Asia, Europe, and Australia. Smiths Misc Collect 106: 1–183

Dietrich AJJ, Mulder RJP (1981) A light microscopic study of the development and behaviour of the synaptonemal complex in sper-

matocytes of the mouse. Chromosoma (Berlin) 83: 409–418
Dobzhansky T (1937) Genetics and the Origin of Species. Columbia University Press, New York
Drets ME, Corbella E, Panzera F, Folle GA (1983) C-banding and non-homologous associations. II. The "parachute" Xy_p sex bivalent and the behavior of heterochromatic segments in *Epilachna paenulata*. Chromosoma (Berlin) 88: 249–255
Felsenstein J (1985) Confidence limits on phylogenies. Evolution 39: 783–791
Futuyma DJ (1997) Evolutionary Biology 3rd ed. Sunderland, Mass: Sinauer
Hendy MD, Penny D (1982) Branch and bound algorithms to determine minimal evolutionary trees. Math Biosci 59: 277–290
Howard DJ (1993) Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis. In "Hybrid Zones and the Evolutionary Process" Ed by RG Harrison, Oxford University Press, Oxford, pp 46–69
Howard DJ, Berlocher SH (1998) Endless Forms. Oxford University Press, Oxford
Hoang DN (1983) Ladybeetles (Coleoptera: Coccinellidae) of Viet Nam, Tome II. Hanoi (in Vietnamese)
Jukes TH, Cantor CR (1969) Evolution of protein molecules. In "Mammalian Protein Metabolism" Ed by NH Munro, Academic Press, New York, pp 21–123
Katakura H, Abbas I, Nakamura K, Sasaji H (1988) Records of epilachnine crop pests (Coleoptera, Coccinellidae) in Sumatera Barat, Sumatra, Indonesia. Kontyû (Tokyo) 56: 281–297
Katakura H, Saitoh S, Nakamura K, Abbas I (1994) Multivariate analyses of elytral spot patterns in the phytophagous ladybird beetle *Epilachna vigintioctopunctata* (Coleoptera, Coccinellidae) in the Province of Sumatera Barat, Indonesia. Zool Sci 11: 889–894
Katakura H, Sobu Y (1986) Cause of low hatchability by the interspecific mating in a pair of sympatric ladybirds (Insecta, Coleoptera, Coccinellidae): incapacitation of alien sperm and death of hybrid embryos. Zool Sci 3: 315–322
Kobayashi N, Tamura K, Aotsuka T, Katakura H (1998) Molecular phylogeny of twelve Asian species of epilachnine ladybird beetles (Coleoptera, Coccinellidae) with notes on the direction of host shifts. Zool Sci 15: 147–151
Kumar S, Tamura K, Nei M (1993) MEGA: Molecular Evolutionary Genetics Analysis. Institute of molecular evolutionary genetics, University Park, Pennsylvania
Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centric position on chromosomes. Hereditas (Lund), 52: 201–220
Li CS (1993) Review of the Australian Epilachninae (Coleoptera: Coccinellidae). J Australian Entomol Soc 32: 209–224
Li CS, Cook EF (1961) The Epilachninae of Taiwan (Col.: Coccinellidae). Pacific Insects 3: 31–91
Mayr E (1942) Systematics and the Origin of Species. Columbia University Press, New York
Mayr E (1963) Animal Species and Evolution. Harvard University Press, Cambridge, MA
Nishida T, Pudjiastuti LE, Nakano S, Abbas I, Kahono S, Nakamura K, Katakura H (1997) The eggplant beetle on a leguminous weed: host race formation in progress? Tropics 7: 115–121
Otto D, Endler JA (1989) Speciation and Its Consequences. Sinauer, Sunderland, MA
Pang XF, Mao JL (1979) Economic Insects of China 14, Coleoptera-Coccinellidae, II. Beijing (in Chinese)
Park HC, Yoon IB (1991) A taxonomic revision of subfamily Epilachninae in Korea (Coleoptera: Coccinellidae). Entomol Res Bull (Korea) 17: 81–92
Richards AM (1983) The *Epilachna vigintioctopunctata* complex (Coleoptera: Coccinellidae). Intern J Entomol 25: 11–41
Richards AM, Filewood LW (1990) Feeding behavior and food preferences of the pest species comprising the *Epilachna vigintioctopunctata* (F.) complex (Col., Coccinellidae). J Appl Entomol 110: 501–515
Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425
Shirai Y, Katakura H (1999) Host plants of the phytophagous ladybird beetle, *Epilachna vigintioctopunctata* (Coleoptera: Coccinellidae), in southeast Asia and Japan. Appl Entomol Zool 34: 75–83
Tamura K, Aotsuka T (1988) Rapid isolation method of animal mitochondrial DNA by alkaline lysis procedure. Biochemical Genetics 26: 11–12
Tanaka H, Sasaji H (1992) Karyotypes of twenty-seven species of the Coccinellidae (Coleoptera) of Fukui Prefecture, Japan. Entomol J Fukui 11: 25–38 (In Japanese with English synopsis)
Tsurusaki N (1985) Taxonomic revision of the *Leiobunum curvipalpe*-group (Arachnida, Opiliones, Phalangiidae). I. *hikocola-, hiasai-, kohyai-*, and *platypenis*-subgroups. J Fac Sci Hokkaido Univ VI Zool 24: 1–42
Tsurusaki N, Cokendolpher JC (1990) Chromosomes of sixteen species of harvestmen (Arachnida, Opiliones, Caddidae and Phalangiidae). J Arachnol 18: 151–166
Tsurusaki N, Nakano S, Katakura H (1993) Karyotypic differentiation in the phytophagous ladybird beetles *Epilachna vigintioctomaculata* complex and its possible relevance to the reproductive isolation, with a note on supernumerary Y chromosomes found in *E. pustulosa*. Zool Sci 10: 997–1015
Yadav, JS., Pillai, RK (1974) Chromosome numbers and sex-determining mechanism in 28 species of Indian Coleoptera. Chromosomal Information Services 16: 20–22
Yosida T (1948) Distinction of three species of *Epilachna* based on the cytological evidence. Matsumushi 2: 167–169 (In Japanese)

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