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Molecular Phylogeny of Twelve Asian Species of Epilachnine Ladybird Beetles (Coleoptera, Coccinellidae) with Notes on the Direction of Host Shifts

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ABSTRACT—We determined the nucleotide sequences of a part of the mitochondrial cytochrome c oxidase I gene (1,000 bp) for twelve species of Asian phytophagous ladybird beetles belonging to the genus *Epilachna*, and constructed molecular phylogenetic trees for ten "*Henosepilachna*" species, using two "*Epilachna*" species as outgroups. Based on the suggested phylogenetic trees, we discussed taxonomic issues and the direction of host shift in these epilachnines.

INTRODUCTION

Ladybird beetles of the subfamily Epilachninae are phytophagous, and most species are distributed in tropical and subtropical zones of the world (Gordon, 1975). Most species are host specific, and their host plants cover diverse taxonomic groups of angiosperms (Schaefer, 1983; Pang and Mao, 1979; Katakura *et al.*, 1992). Some species are notorious for causing serious damage to important crops such as legumes, solanums and cucurbits (Dieke, 1947; Schaefer, 1983).

The phylogenetic position of Epilachninae in the family Coccinellidae seems well established. The subfamily is considered to represent a lineage of advanced coccinellids that has a close relationship with the higher predaceous coccinellid group, the subfamily Coccinellinae (Sasaji, 1968). However, the supraspecific classification of Epilachninae, especially of Old World species, is still controversial (Iablokoff-Khnzorian, 1980; Richards, 1983; Fürsch, 1991). There are two morphologically distinct groups in Asian and African species: one is characterized by toothless tarsal claws and the lack of a spilt in the sixth visible abdominal sternite of females; whereas the other is characterized by toothed tarsal claws and a divided sixth sternite. The two groups are further divided into species groups (Dieke, 1947). Some authors treat the two groups as two distinct genera, *Epilachna* Dejean and *Henosepilachna* Li (Li and Cook, 1961; Fürsch, 1990, 1991), respectively. On the other hand, others assign them to the single genus *Epilachna* (Iablokoff-Khnzorian, 1980; Richards, 1983), considering that the separation by these characters was unreliable. Although the present study is mainly concerned with *Henosepilachna*, all the species treated here are tentatively placed in *Epilachna* to avoid nomenclatural problems by yielding new combinations. When necessary, however, the species belonging to *Henosepilachna* and *Epilachna* (*sensu* Li and Cook, 1961) are indicated as "*Henosepilachna*" and "*Epilachna*", respectively (see Katakura *et al.*, 1994).

In order to establish a satisfactory classification system, it is necessary to clarify phylogenetic relationships between various species and species groups (Katakura *et al.*, 1994). Reconstruction of phylogenetic relationships among extant taxa of epilachnines is also indispensable to understand the evolutionary changes in relationships between ladybird beetles and their host plants.

Katakura *et al.* (1994) analyzed the phylogenetic relationships of several species groups of Asian epilachnines based on the female internal reproductive organs and modes of sperm transfer. They suggested that "*Henosepilachna*" and "*Epilachna*" are sister groups and that "*Epilachna*" is further divided into two sister groups. Unfortunately, however, their analysis provided little information on the phylogenetic relationships between the members of "*Henosepilachna*".

In the present paper, we analyzed the phylogenetic relationships of ten species of Asian epilachnines belonging to "*Henosepilachna*" using mitochondorial DNA sequences, assuming two species of "*Epilachna*" (*sensu* Li and Cook, 1961) as outgroups. Since the rate of nucleotide substitution in animal mitochondrial DNA is generally higher than that in nuclear DNA (Brown *et al.*, 1979), it is suitable for the analysis of phylogenetic relationships among closely related taxa such as

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Table 1. *Epilachna* beetles used in the present study, and their sampling locations and host plants

Species group	Species	Collection sites	Host plants
"*Epilachna*" *admirabilis* group	*Epilachna admilabilis* Crotch	Hachiôji, Tokyo, Japan	Cucurbitaceae
	E. sp. H	Padan, West Sumatra, Indonesia	Cucurbitaceae
"*Henosepilachna*" *vigintioctopunctata* group	*E. vigintioctomaculata* Motschulsky*	Sapporo, Hokkaido, Japan	Solanaceae & Cucurbitaceae
	E. pustulosa Kôno*	Sapporo, Hokkaido, Japan	Compositae & Berberidaceae
	E. niponica Lewis*	Ohnuma, Hokkaido, Japan	Compositae
	E. yasutomii (Katakura)*	Ohnuma, Hokkaido, Japan	Berberidaceae
	E. vigintioctopunctata (Fabricius)	Hachiôji, Tokyo, Japan	Solanaceae
	E. sp. 3	Bogor, West Java, Indonesia	Compositae
	E. pusillanima Mulsant	Sukarami, West Sumatra, Indonesia	Cucurbitaceae
	E. boisduvali Mulsant	Okinawa, Japan	Cucurbitaceae
	E. septima Dieke	Padang, West Sumatra, Indonesia	Cucurbitaceae
"*Henosepilachna*" *enneasticta* group	*E. enneasticta* Mulsant	Sukarami, West Sumatra, Indonesia	Solanaceae

Members of the *Epilachna vigintioctomaculata* complex are asterisked.

those used in this study. Based on the suggested phylogeny, we discuss taxonomic issues and infer the host plants of the ancestral species of these epilachnines.

MATERIALS AND METHODS

Beetle samples

We used ten species of "*Henosepilachna*" and two species of "*Epilachna*". The species, species groups, provenance and host plants of these specimens are given in Table 1. The name *E. pusillanima* is used for the species referred to as *E. dodecastigma* in Katakura *et al.* (1988, 1994), because the identity of *E. dodecastigma* (*sensu* Wiedemann, 1823) is still ambiguous (Booth and Pope, 1989). Two Indonesian species, whose taxonomic status are not yet determined, are referred to using the species-specific code number or code letter (*E.* sp. 3 and *E.* sp. H) (cf. Katakura *et al.*, 1994). All the ladybird beetles used for the phylogenetic study were collected from 1994–95. Of the ten "*Henosepilachna*" species, nine belong to the *vigintioctopunctata* group and one to the *enneasticta* group according to Dieke (1947). Four species, i.e., *E. vigintioctomaculata*, *E. niponica*, *E. pustulosa* and *E. yasutomii* are very closely related and are assigned to the so-called *Epilachna vigintioctomaculata* species complex, which is further classified into two groups (group A: *E. vigintioctomaculata*; group B: other three species) based on their morphological characters (Katakura, 1981). Both of the "*Epilachna*" species belong to the *admirabilis* group (Dieke, 1947).

Laboratory procedures

For *E. vigintioctomaculata*, *E. niponica*, *E. pustulosa*, *E. yasutomii*, *E. vigintioctopunctata* and *E. admirabilis*, mitochondrial DNA (mtDNA) was extracted from a living adult specimen following the method of Tamura and Aotsuka (1988). For other species, total DNA was extracted from a specimen stored in alcohol by using Steller's (1990) method. The mtDNA samples from *E. vigintioctomaculata* were digested by appropriate restriction enzymes to fractionate cytochrome c oxdase subunit I (COI) gene and were then cloned into plasmid vector pUC118 with *E. coli* K12.MV1184 as a host. The mtDNA recombinants were subcloned by using exonuclease III and mungbean nuclease (Henikoff, 1984) and/or appropriate restriction enzymes. Single strand templates for sequencing were obtained from these subclones by taking advantage of the pUC118/119-M13KO7 system (Vieria and Messing, 1987). The nucleotide sequences were determined by using an ABI autosequencer according to the protcol supplied by the manufacturer.

Using the *E. vigintioctomaculata* sequence obtained as a reference, we designed a set of PCR primers to amplify a region containing the whole COI gene. The primer sequences were: 5'-TTTACCGCCTAATTCAGCCA-3' and 5'-AGAATTCATGGGGTTTAAATCCAGTGC-3', in the latter of which the sequence of seven bases from the 5'terminal was an adapter to facilitate subsequent cloning procedures. Depending on the species mentioned above, either mtDNA or a total DNA sample was used for PCR templates. The reaction mixture (100 µl) for PCR contained: Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 200 µM each dNTP, 200 nM each primer, approximately 50 ng template DNA, 2.5 units *Taq* polymerase. Since an addition of *Pfu* polymerase improves the fidelity of PCR (Barnes, 1994), we used a mixture of 2.5 units *Taq* and 0.0125 units *Pfu* polymerases. Amplifications were performed for 25 cycles in a DNA thermalcycler using the following parameters: 94°C for 30 sec; 60°C for 1 min; 72°C for 1 min, except for the last cycle where 72°C for 8 min. The DNA fragments amplified were then cloned and sequenced by essentially the same method as that for the sequence of *E. vigintioctomaculata*.

Since we determined nucleotide sequences from only a single cloned DNA, they are subject to PCR error. Nevertheless, as shown later, PCR error is negligible in the phylogenetic analyses, because the differences in sequence among the species used are much greater than those expected by PCR error.

Phylogenetic analysis for sequence data

We determined the nucleotide sequences of a part of mitochondrial cytochrome c oxidase subunit I gene (COI) for 12 individuals

(Table 1). The number of nucleotide sites determined and used for phylogenetic analyses was 1,000 bp from the initial codon on the sense strand. Jukes and Cantor's (1969) method was used for estimating the number of substitutions per site for all possible pair of sequences. Using the distance matrix, we constructed a phylogenetic tree by the minimum-evolution (ME) method (Rzhetsky and Nei, 1992). To estimate the confidence probability for each interior branch, the bootstrap method (Felsenstein, 1985) was performed with 1,000 replications.

RESULTS

The nucleotide sequence of COI region (1,000 bp) was determined for each "*Henosepilachna*" and "*Epilachna*" individual. The sequences have been deposited in databases (DDBJ, EMBL and GenBank) under the following accesion numbers: *Epilachna enneasticta*, AB002173; *E.* sp. 3, AB002174; *E. boisduvali*, AB002175; *E. septima*, AB002176; *E. pusillanima*, AB002177; *E. admirabilis*, AB002178; *E.* sp. H, AB002179; *E. vigintioctopunctata*, AB002180; *E. pustulosa*, AB002183; *E. yasutomii*, AB002184; *E. niponica*, AB002185.

A total of 343 nucleotide substitution sites and 38 amino acid replacement sites were detected in the 12 sequences. Variations such as insertion and deletion were not found. Within "*Henosepilachna*," the number of nucleotide substitutions was fewest between the members of the group B of the *E. vigintioctomaculata* complex (one site: *E. yasutomii* vs. *E. niponica*), and most numerous between *E. boisduvali* and *E. septima* (180 sites).

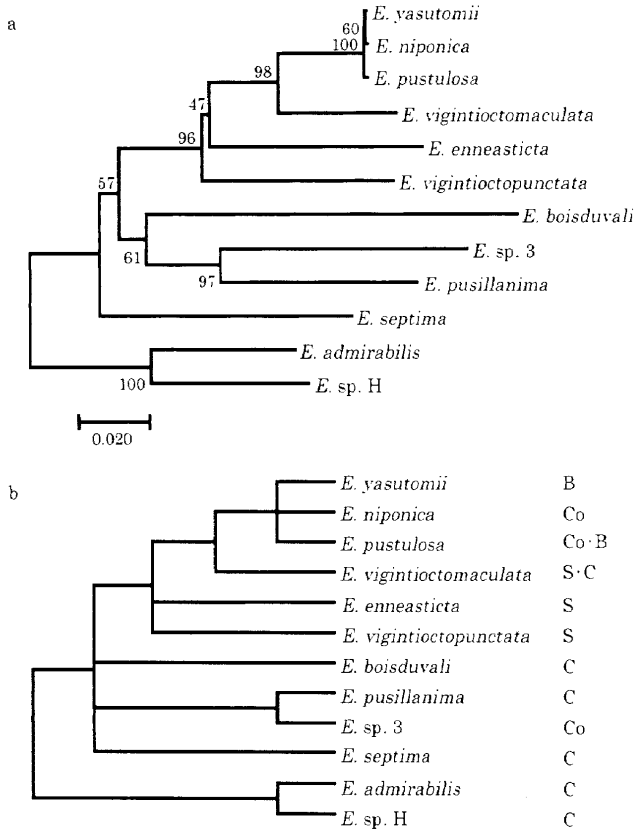

Fig. 1. Phylogenetic trees reconstructed from the sequence of a part of cytochrome oxdase gene I (1,000 bp) of twelve *Epilachna* species. (**a**) minimum-evolution (ME) tree; numerals at branching points show bootstrap values (1,000 replications). (**b**) condensed tree; host plants abbreviated: B, Berberidaceae; Co, Compositae; S, Solanaceae; C, Cucurbitaceae.

Figure 1a is a phylogenetic tree for the ten "*Henosepilachna*" sequences constructed using the ME method. Two "*Epilachna*" sequences were used as outgroups. The bootstrap probabilities were given in the upper or lower side of each branch. To reconstruct the condensed tree (Nei, 1996) showing only the reliable topology of the specimen, branches whose bootstrap values were lower than 95% in Fig. 1a were multifurcated (Fig. 1b). These trees convey the following information:

1) "*Henosepilachna*" diverged into four groups: (a) *E. pusillanima* and *E.* sp. 3, (b) *E. septima*, (c) *E. boisduvali* and (d) other six species (the *E. vigintioctomaculata* complex, *E. vigintioctopunctata* and *E. enneasticta*) (Fig. 1b).

2) In group (d), the *E. vigintioctomaculata* complex (which includes four species), *E. vigintioctopunctata* and *E. enneasticta* were trifurcated and their phylogenetic relationships were not resolved.

3) The *E. vigintioctomaculata* complex diverged into two subclusters corresponding to two morphologically defined groups. One subcluster consists of the *E. vigintioctomaculata* sequence, which is classified in group A, and the other subcluster consists of the members of group B, namely *E. pustulosa*, *E. niponica* and *E. yasutomii*. In the latter subcluster, only one or two nucleotide substitutions were detected by pairwise comparisons between the three sequences.

DISCUSSION

Because the number of species analyzed in the study was very small and limited to paticular groups, we could not refer to the phylogenetic relationship between "*Henosepilachna*" and "*Epilachna*". However, the present study clarified some noteworthy aspects of the "*Henosepilachna*".

One was the phylogenetic position of *E. enneasticta*. Among "*Henosepilachna*", *E. enneasticta* and related species (the *E. enneasticta* group, *sensu* Dieke, 1947) are distinct in genitalia morphology in both sexes and the associated abdominal segments of females (Dieke, 1947). The last visible sternite of the female is of particular taxonomic interest. In *E. enneasticta*, the last visible sternite of the female is split but fused on the suture. This characteristic might be an intermediate condition between "*Epilachna*", which has a non-split last sternite, and typical "*Henosepilachna*", which has split sternite (Kapur, 1967). The occurrence of such an equivocal condition is one of the reasons why some authors reject "*Henosepilachna*" as a valid genus (Richards, 1983). Furthermore, *E. enneasticta* showed a somewhat different condition in the morphology of the female internal reproductive organ (Katakura *et al.*, 1994).

The present study showed that *Epilachna enneasticta* is not an intermediate form that links members of "*Epilachna*" with those of "*Henosepilachna*". It is rather an advanced form

of "*Henosepilachna*" derived from a solanum-feeding lineage (Fig. 1b). Morphological features characteristic to *E. enneasticta* (modified genitalia in both sexes and the abdominal segments in females) can thus be regarded as autoapomorphies.

Another point clarified by the present study is the phylogenetic relationships among the members of the *E. vigintioctomaculata* complex. The *E. vigintioctomaculata* complex is composed of a number of closely related but morphologically and/or biologically different forms (Katakura, 1981). It has been paid much attention from students of evolutionary biology, in particular those who have specific interest in speciation (Katakura, 1997). Based on morphological evidence and information on the host plants, the *E. vigintioctomaculata* complex has been classified into two groups, A and B. In group B, three species have been recognized by differences in host plants and the geographic allopatric-sympatric relationships (Katakura, 1981). The present study supports the dichotomy of the species complex (Fig. 1b). As the genetic divergence between the three species of the group B was very small, our data could not provide positive evidence for a trichotomy. This means that the differences in morphology and/or the host plants (Katakura, 1981) must have developed during a short geological time. Detailed analyses of the phylogenetic relationships between various members of the *E. vigintioctomaculata* complex will be reported elsewhere (Kobayashi *et al.*, in preparation).

Finally, we discuss the direction of host shifts in the studied groups of epilachnines based on the suggested phylogeny (Fig. 1a, b). Such an attempt is indispensable to understand the evolutionary relationships between ladybird beetles and their host plants. Indeed, reconstruction of phylogenetic relationships among species of insects has often succeeded in detecting the direction of their adaptation to particular host species in the course of phyletic evolution (Futuyma and McCafferty, 1990; Futuyma *et al.*, 1995).

It has been known that the majority of "*Henosepilachna*" species feed on either cucubitaceous or solanaceous plants (Shaefer, 1983; Katakura *et al.*, 1992). The most parsimonious interpretation of Fig. 1a and b with respect to the direction of host shifts is that the host plant of the ancestral species of "*Henosepilachna*" was cucurbits. Later, two types of host shifts occurred in the two lineages. One was a shift toward the species of Compositae in the *E.* sp. 3 lineage, and the other was a shift toward solanaceous plants in the common ancestor of the cluster comprizing *E. enneasticta*, *E. vigintioctopunctata* and the *E. vigintioctomaculata* complex. In the latter group, further shifts to other plants followed. The ability of *E. vigintictomaculata* in the *E. vigintioctomaculata* complex to feed on cucurbits (Katakura, 1981) can be interpreted in two different ways. It may be a plesiomorphic condition inherited from a cucurbits-feeding ancestor. Alternatively, it may have been acquired in a recurrent evolution after once shifting to solanaceous plants occurred.

The validity of the scenario of the host shift presented above, however, must be examined by further extensive phylogenetic analyses of various "*Henosepilachna*" species.

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