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Molecular phylogenetic analysis of three groups of Asian epilachnine ladybird beetles recognized by the female internal reproductive organs and modes of sperm transfer

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We determined partial sequences of the nuclear 28S ribosomal RNA gene (717 base pairs) and mitochondrial DNA NADH-dehydrogenase subunit 2 gene (535 base pairs) of 25 species of phytophagous ladybird beetles from Asia that comprise 16 species of the genus *Henosepilachna* and nine species of the genus *Epilachna*, and reconstructed the phylogenetic trees for each gene by the maximum parsimony and maximum likelihood methods. The estimated phylogenetic relationships were consistent with those obtained by the mode of sperm transfer and female internal reproductive system, and supported an earlier assumption that very similar elytral spot patterns of some sympatric members of epilachnine beetles evolved independently.

Keywords: gene tree; epilachnine beetles; female internal reproductive organ; modes of sperm transfer; elytral convergent evolution

Introduction

Phytophagous ladybird beetles of the subfamily Epilachinae are varied and abundant in tropical and subtropical regions worldwide (Gordon 1975). More than one thousand species of epilachnines have so far been described, and they are classified into four tribes and 23 genera (Jadwiszczak and Węgrzynowcz 2003). *Epilachna* (including about 600 species) and *Henosepilachna* (including about 250 species) are two large genera. The former genus is distributed in Africa, Asia, Oceania and America, and the latter is found in Africa, Europe, Oceania and Asia. In these two genera, several infrageneric groups have been further recognized (Dieke 1947; Gordon 1975; Fürsch 1991; Katakura et al. 2001). However, the supraspecific classification and phylogenetic relationships of Epilachninae are still not firmly settled. For the Asian species, only two studies dealt with the phylogeny of some species of the genera *Epilachna* and *Henosepilachna* (Katakura et al. 1994; Kobayashi et al. 1998). Katakura et al. (1994) recognized three groups (I–III) based on the female internal reproductive systems and mode of sperm transfer in 12 species of *Henosepilachna* and nine species covering four species groups

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of *Epilachna* beetles recognized by Dieke (1947), and they estimated the phylogenetic relationship of these three groups (Figure 1 and Table 1). Katakura et al. (2001) later recognized four species groups (groups 1–4) of *Epilachna* for Indonesian and Japanese species, based on the genital morphology of both sexes and on reproductive traits. Kobayashi et al. (1998) presented a phylogenetic relationship of 10 species of Asian *Henosepilachna* beetles based on mitochondrial DNA (mtDNA).

In the present study, we aimed to clarify the phylogenetic relationships among and within the three groups (I–III) of Asian epilachnines discussed by Katakura et al. (1994, 2001) based on mitochondrial and nuclear DNA sequences. We determined the nucleotide sequences of part of the mtDNA ND2 gene and 28S ribosomal RNA (rRNA) gene of 25 species sampled from Japan and Indonesia covering the genus *Henosepilachna* and four Asian groups of the genus *Epilachna* treated by Katakura et al. (1994), and constructed a phylogenetic gene tree of these epilachnines.

Reconstruction of phylogenetic relationships among extant taxa is also indispensable when addressing various evolutionary issues concerning epilachnine beetles. In this paper, we focus on the phylogenetic relationships of the three types of reproductive traits defined by the combination of female internal reproductive systems and modes of sperm transfer (Figure 1, Table 1; Katakura et al. 1994). Another topic we address in the present paper is the presumably convergent evolution of elytral spot patterns in some sympatric epilachnines. Katakura et al. (2001) reported that some Indonesian beetles that are distributed in sympatry have very similar elytral spot patterns; nevertheless these species are classified into different groups. They argued that these similar spot patterns were caused by the convergence evolution representing possible cases of Müllerian mimicry. Then, we test the hypothesis based on molecular phylogenetic evidence.

Materials and methods

Beetle samples

The species, species groups, localities and host plant families of the beetle specimens examined are listed in Table 1. Four species, whose taxonomic statuses are not yet settled, are referred to using the species-specific code numbers or code letters consistently

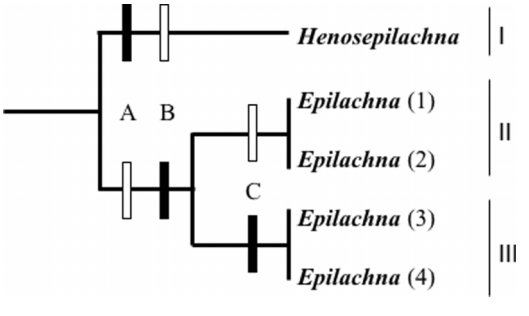

Figure 1. A possible phylogenetic relationship between the three types of east Asian epilachnines differentiated by the difference in female internal reproductive organs and modes of sperm transfer (see Table 1). Letters represent: A, position of spermatheca; B, spermatophore formation; C, bursa copulatrix. Open symbol indicates ancestral condition; solid symbol indicates derived condition (after Katakura et al. 1994.). Roman and Arabic figures indicate group means defined by Katakura et al. (1994) and Katakura et al. (2001), respectively.

Table 1. List of *Henosepilachna* and *Epilachna* beetles used in this study, and their sampling points.

Genus / group* / group**	Species	Female internal reproductive systems and modes of sperm transfer			Sampling location	Host plant family
		Bursa copulatrix (A)	Position of spermatheca (B)	Spermatophore formation (C)		
Henosepilachna / I / -	*H. vigintioctomaculata* (Motschulsky)	Functional†	Lateral‡	Present†	Gifu, Japan	Solanaceae
	H. pustulosa (Kôno)				Hokkaido, Japan	Asteraceae
	H. niponica (Lewis)				Miyagi, Japan	Asteraceae
	H. yasutomii Katakura				Miyagi, Japan	Berberidaceae
	H. coalescens (Mader)				Viet Nam	Solanaceae
	H. boisduvali (Mulsant)				Okinawa, Japan	Cucurbitaceae
	H. vigintioctopunctata (Fabricius) N form				Okinawa, Japan	Solanaceae
	H. vigontioctopunctata (F.) S form				West Java, Indonesia	Solanaceae
	H. enneasticta (Mulsant)				West Java, Indonesia	Solanaceae
	H. sp. 10				Sulawesi, Indonesia	Solanaceae
	H. diekei				West Java, Indonesia	Asteraceae
	H. septima (Dieke)				West Java, Indonesia	Cucurbitaceae
	H.pusillanima (Mulsant)				Central Java, Indonesia	Cucurbitaceae
	H. bifasciata (Fabricius)				Central Java, Indonesia	Solanaceae
	H. pytho (Mulsant)				West Java, Indonesia	Cucurbitaceae
	H. sp. 5				West Java, Indonesia	Acanthaceae
Epilachna / II / (1)	*E. admirabilis* Crotch	Functional†	Terminal†	Absent‡	Hokkaido, Japan	Cucurbitaceae
	E. alternans Mulsant				West Java, Indonesia	Cucurbitaceae
Epilachna / II / (2)	*E. decipiens* Crotch				West Java, Indonesia	Ranunculaceae
	E. sp. G				West Java, Indonesia	Ranunculaceae
Epilachna / III / (3)	*E. orthofasciata* (Dieke)	Reduced‡			West Java, Indonesia	Vitaceae
	E. sp. K				West Java, Indonesia	Vitaceae
	E. chinensis (Weise)				Tsushima, Nagasaki, Japan	Rubiaceae
Epilachna / III / (4)	*E. incauta* (Mulsant)				Central Java, Indonesia	Urticaceae
	E. gedeensis (Dieke)				West Java, Indonesia	Urticaceae
Coccinella	*C. septempunctata* (Mulsant)	Functional†	Terminal†	Present†	Hokkaido, Japan	–

The * and ** indicate group names defined by Katakura et al. (1994) and Katakura et al. (2001), respectively. Supposed ancestral (†) and derived (‡) conditions by Katakura et al. (1994).

used in our previous studies (*E.* sp. G, *E.* sp. K, *H.* sp. 5, *H.* sp. 10) (cf. Katakura et al. 1994, 2001). The beetles used for the phylogenetic study were collected from 1996 to 2006, and they had been stored in absolute ethyl alcohol until DNA purification. Phylogenetic relationships among higher taxa of Coccinellidae are still not clear. Based on the morphological features, Sasaji (1968) suggested that Coccinellinae was considered a sister group of Epilachninae. Recently, on the basis of molecular phylogenetic analysis, Hunt et al. (2007) and Robertson et al. (2008) indicated that Coccinellinae, Scymninae and Chilicorinae were closely related to Epilachninae, but the relationships between them were not firmly settled. We used an aphidophagous species, *Coccinella septempunctata* (Coccinellinae), as an out-group because the three studies above consistently suggested close relatedness of Coccinellinae and Epilachninae.

Some additional comments are necessary.

(1) *Henosepilachna coalescens* was once treated as a well-defined subspecies of *H. vigintioctomaculata* distributed in Szechwan and Tibet (Dieke 1947; incorrectly referred to as *Epilachna niponica*; for the taxonomy of *H. vigintioctomaculata* and *H. niponica*, see Katakura 1981) and later treated as a mere individual variation of *H. vigintioctomaculata* not worthy to be ranked subspecifically (Li and Cook 1961; Pang and Mao 1979). In the present study, however, we treat it as an independent species because our results, mentioned later, have demonstrated that it is not only different in elytral pattern, but also is genetically well-differentiated from *H. vigintioctomaculata*.

(2) We recognize two species in a nominal species *H. vigintioctopunctata*, which will be referred to as form N and form S, respectively. They are morphologically indistinguishable, but DNA analysis and crossing experiments showed that they undoubtedly represent two reproductively isolated biological species (Kobayashi et al. 2000).

(3) We referred to a 12-spotted species as *Henosepilachna* sp. 3 in our previous studies (cf. Katakura et al. 1994, 2001), which is distributed in various parts of Indonesia. This time we identified this species with the taxon originally described from the Philippines as *Epilachna emarginata* Dieke, 1947, or more strictly its 12-spotted subspecies *Epilachna emarginata emarginata* described from Samar Island. Since the name *Epilachna emarginata* was already in use for another species, i.e. *Epilachna emarginata* Montrouzier in Perroud and Montrouzier, 1864 (now in the genus *Scymnus* of the subfamily Scymninae), Jadwiszczak and Węgrzynowcz (2003) proposed the use of the name *Epilachna altera* Dieke, originally given to a multipunctate (26-spotted) subspecies of *Epilachna emarginata* Dieke occurring on Luzon and Mindanao. Since *E. emarginata emarginata* Dieke needs a name by this treatment, Jadwiszczak and Węgrzynowcz (2003) further proposed a replacement name *Henosepilachna emarginata diekei* (sic) for this subspecies. However, it should be *H. altera diekei* so the situation is somewhat confusing. Moreover, in the light of the current knowledge of the infraspecific variation of spot patterns in Asian epilachnines, it is not clear whether Dieke's two subspecies really represent two locally differentiated infraspecific variations or not. They might represent a mere individual variation, or represent two distinct species (cf. notes on *H. coalescens* above). We here adopt the name *Henosepilachna diekei* for our 12-spotted taxon, tentatively regarding it as specifically different from

Henosepilachna altera altera (Dieke, 1947) (*sensu* Jadwiszczak and Węgrzynowcz 2003).

(4) Our material of *Epilachna chinensis* came from Tsushima Island, southern Japan, and is treated as a distinct subspecies *E. chinensis tsushimana* (Nakane et Araki) endemic to this island.

Laboratory procedures

Total DNAs were extracted by the method of Boom et al. (1990) with the following modifications: We used guanidium thiocyanate buffer with pH 7.0 for DNA purification (originally pH 6.4) and TE buffer with 0.1 mM ethylenediamine tetraacetic acid for purified DNA (originally 1 mM). We first tried to amplify the mtDNA cyclooxygenase 1 (COI) region for phylogenetic analysis by the primers described by Kobayashi et al. (1998), by primers (UEA 1 for sense strand, UEA 8 or UEA 10 for anti-sense strand) described by Lunt et al. (1996), or by their combinations, but we failed to obtain polymerase chain reaction products for species of groups 2, 3, and 4 in the genus *Epilachna*. Instead of COI gene, then, we targeted the nuclear 28S rRNA (mainly domain V) gene and mtDNA ND2 gene for use in the present phylogenetic analysis. We used the following primers (Palumbi 1996) to amplify the nuclear 28S rRNA gene: 28S-H, 5′-AAGGTAGCCAAATGCCTCATC-3′; 28S-T, 5′-AGTAGGGTAAAACTAACCT-3′.

For amplifying mtDNA ND2 gene of *Henosepilachna* ladybird beetles, we used the following primers (Wang et al. 2006): ND2-H, 5′-AAGCTACTGGGTTCATACC-3′; ND2-T, 5′-ATATTKAYARCTTTGAAGG-3′. We failed to amplify nucleotide sequences of ND2 of some *Epilachna* beetles. Then, for the ND2 gene of *Epilachna* beetles, on the basis of sequence data of *Henosepilachna* beetles, we designed the following two primers to amplify some *Epilachna* beetles: ND2-sub1f, 5′-TWATGGGAACCCTYATTWCCAT-3′; ND2-sub9.5r, 5′-TYATYCAYTTRGGGAARAATCCTAA-3′.

Amplifications were performed by using a DNA thermal cycler with the following parameters: first step, 94°C 7 min.; next step, 35 cycles with 94°C 45 seconds, 42°C 90 seconds, 72°C 2 min and last step, 7 min. All nucleotide sequences were determined by direct sequencing methods by using Big Dye Terminator Kit ver. 3.1 with ABI 3100 Avant autosequencer (Applied Biosystems, Foster City, CA). The above primers were used for direct sequencing.

Phylogenetic analysis for sequence data

Length polymorphisms among sequences were observed and were aligned using the software CLUSTAL W (Thompson et al. 1994) with the default setting: gap opening cost = 15, gap extension cost = 6.66 and transition weight = 0.5. The chi-squared tests were performed for the sequences of ND2, 28S and the combined data sets to detect nucleotide composition bias among taxa. The congruence/incongruence between molecular regions was evaluated by the Incongruence Length Difference (ILD) test (Farris et al. 1994) with software PAUP* (Swofford 2002). The ILD test by maximum parsimony (MP) heuristic search was performed with 1000 replications and 100 initial maximum trees. Phylogenetic trees were constructed for ND2 and 28S rRNA genes and their combined data set was analysed by MP and maximum likelihood (ML) methods using the software PAUP version 4.0b (Swofford 2002). In the

MP method, a heuristic search was used to reconstruct gene trees for each data partition and for combined data sets, and 100 replicate random addition searches with tree bisection–reconstruction branch swapping. In the ML method for constructing trees for each gene and combined data, we used a neighbour joining tree (Saitou and Nei 1987) for the starting tree to search the optimized substitution model with AIC criteria (Akaike 1974), which was performed by using software MODELTEST (Posada and Crandall 1998). To estimate the confidence probability for each interior branch, the bootstrap method (Felsenstein 1985) was performed with 1000 replications for two different methods.

Results

Data characteristics

The partial sequences of the two genes determined by the present study have been deposited in databases (DDBJ, EMBL and GenBank), and their accession numbers were given in AB353860–AB353885 and AB359199–AB359224. Properties of the examined sequences of these genes are summarized in Table 2. Length variations were not observed in the ND2 gene. On the other hand, nine indels were recognized in 28S rRNA. Information of indels in the 28S rRNA gene was eliminated following phylogenetic analyses. There is no evidence for nucleotide composition bias among taxa (Table 2).

Phylogenetic trees

The molecular phylogenetic trees of 28S rRNA and ND2 genes were reconstructed by the MP and ML methods (data not shown). Heuristic searches under the MP criterion resulted in 368 and two MP trees for 28S rRNA and ND2, respectively. For the ML tree, a GTR-I model and a GTR-G+I model were used for 28S rRNA and ND2 gene, respectively, using software MODELTEST (Posada and Crandall 1998). Because the ILD test (Farris et al. 1994) yielded no significant difference between the 28S and ND2 gene regions at a 0.05 significant level (28S versus ND2, $P = 0.932$) using the criteria of Darlu and Lecointre (2002), the MP and ML trees were reconstructed using combined data sets (Figures 2 and 3). The MP analysis of the combined data yielded 10 MP trees, and the strict consensus of these trees is illustrated (Figure 2). To reconstruct the ML tree, a GTR-G+I model was selected and the base frequencies for combined data sets were as follows: A–C, 2.5702; A–G, 8.0510; A–T, 5.1134; C–G, 1.2939; C–T, 20.0512; G–T: 1.000. The proportion of invariable sites was 0.5094, and their gamma-distribution shape parameter was 0.6910.

The MP and ML trees for the combined data set (Figures 2, 3) showed: (1) 16 species of *Henosepilachna* and nine species of *Epilachna* were respectively monophyletic with high bootstrap value supports, (2) *H. boisduvali* was divergent from the other 15 species in the genus *Henosepilachna*, although *H. septima* had been the first to diverge in Kobayashi et al. (1998), and the phylogenetic positions of *H. septima* drawn by MP and ML were different, and (3) members of each group (1–4) in the genus *Epilachna* formed a distinct cluster although the phylogenetic relationships of groups 1 and 2 were ambiguous by MP and ML methods. Based on the suggested ML phylogenetic tree, character traces for some morphological features are shown in Figure 4.

Table 2. Number of length variation sites and polymorphic sites in each gene.

Gene	Nucleotide sites used	Length polymorphism	Polymorphic sites	Parsimoniously informative sites	Base frequencies (%)			Compositional heterogeneity		
					A	T	G	C	χ^2 (df,75)	*P*-value
28S rRNA	718	0-9	82	47	36.6	41.9	8.3	13.3	8.0298	1.000
ND2	533	0	348	281	22.5	22.6	29.4	25.5	0.7210	1.000
Combined	1251	0-9	430	328	28.8	31.2	20.0	20.1	1.4654	1.000

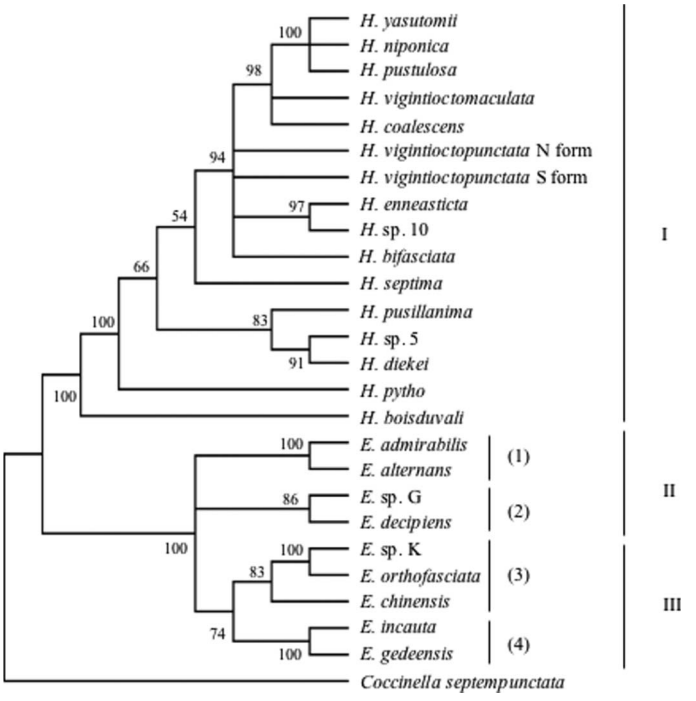

Figure 2. A strict consensus tree based on the combined data sets for 25 species of Asian epilachnines reconstructed by maximum parsimony method of heuristic search.

Discussion

Phylogenetic relationship of Asian Epilachninae

Female internal reproductive organs are important for the classification of coccinellids including epilachnines (Dobzhansky 1924, 1926; Katakura et al. 1994). Katakura et al. (1994) estimated the phylogenetic relationships of Asian species of the genus *Henosepilachna* and four species groups in the genus *Epilachna* on the basis of the presence or absence of bursa copulatrix, position of spermatheca and spermatophore formation (Figure 1). According to this system, *Henosepilachna* (group I) and *Epilachna* diverged first, and then two clades diverged within the genus *Epilachna* (group II = groups 1+2, group III = groups 3+4) (Figure 1).

The results of the present study supported the dichotomy to *Henosepilachna* and *Epilachna*. The two groups were monophyletic, respectively, with high bootstrap values (Figures 2 and 3). Furthermore, it was indicated that the four groups of *Epilachna* were monophyletic, respectively, with high bootstrap values (Figures 2 and 3). The monophyly of group III (= groups 3+4) was also supported. However, the present analyses did not resolve the relationship of groups 1 and 2, which were included in group II, and their relation to group III. In the MP and ML trees, the relationship of group 1, group 2 and the common ancestor of group 3 and 4 was not valid. In the strict consensus MP tree, group 1, group 2 and the common ancestor of groups 3 and 4 were unresolved (Figure 2). Also, in the ML tree, group 2 split from the other groups first, followed by the splitting of group 1 (Figure 3). Consequently, the relationships

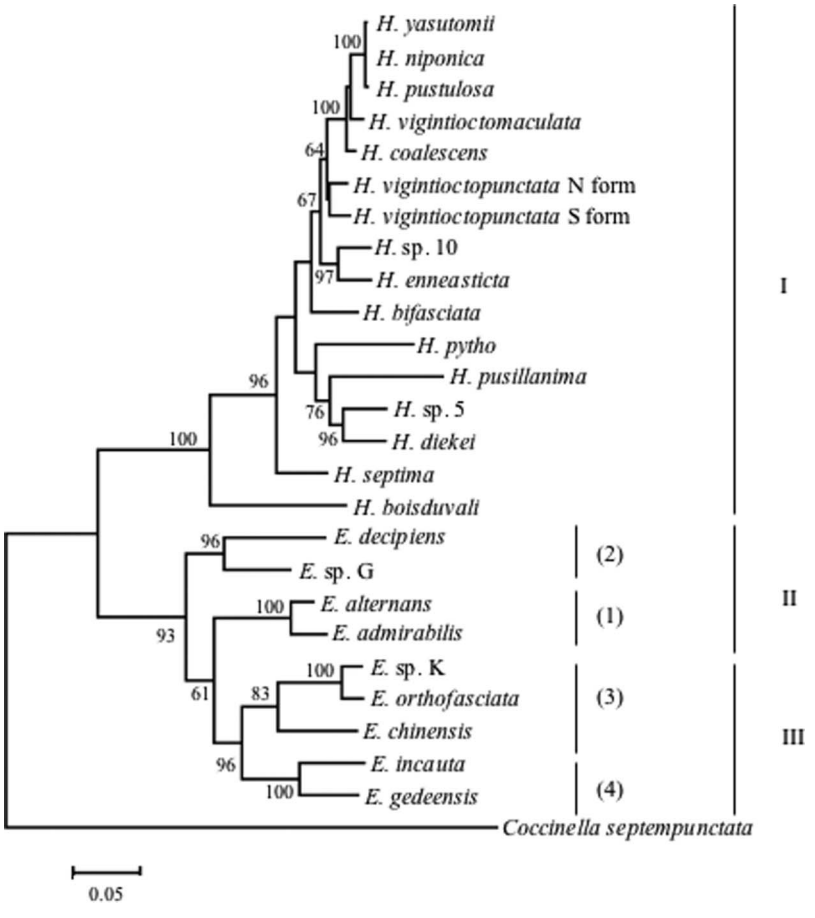

Figure 3. A phylogenetic gene tree based on the combined data sets for 25 species of Asian epilachnines reconstructed by maximum likelihood method with a GTR-G+I model. Bootstrap values were shown when more than 50% support was obtained.

of group 1, group 2 and the common ancestor of groups 3 and 4 were not clarified. Group II, which was defined by the possession of presumably plesiomorphic characters, i.e., functional bursa copulatrix (Table 1), may or may not be monophyletic.

Within *Henosepilachna*, the present study generally supported our previous interpretation of the phylogenetic relationships among 10 Asian species (Kobayashi et al. 1998), except for the positions of *H. boisduvali* and *H. septima*. On the basis of a minimum evolution tree (Rzhetsky and Nei 1992) obtained for the mtDNA COI gene (1000 base pairs long), Kobayashi et al. (1998) suggested that *H. septima* would have first diverged in the 10 species of *Henosepilachna* they studied, and *H. boisduvali* formed a clade with the common ancestor of *H. pusillanima* and *H. diekei*. However, the present study did not support these relationships. The combined gene trees (a total of 1251 base pairs long; see Table 2) constructed by two different methods (Figures 2 and 3) indicated that *H. boisduvali* had first diverged from the rest with extremely high bootstrap support. Because the phylogenetic position of *H. boisduvali* was supported by results using two independent genes (ND2 and 28S rRNA), the

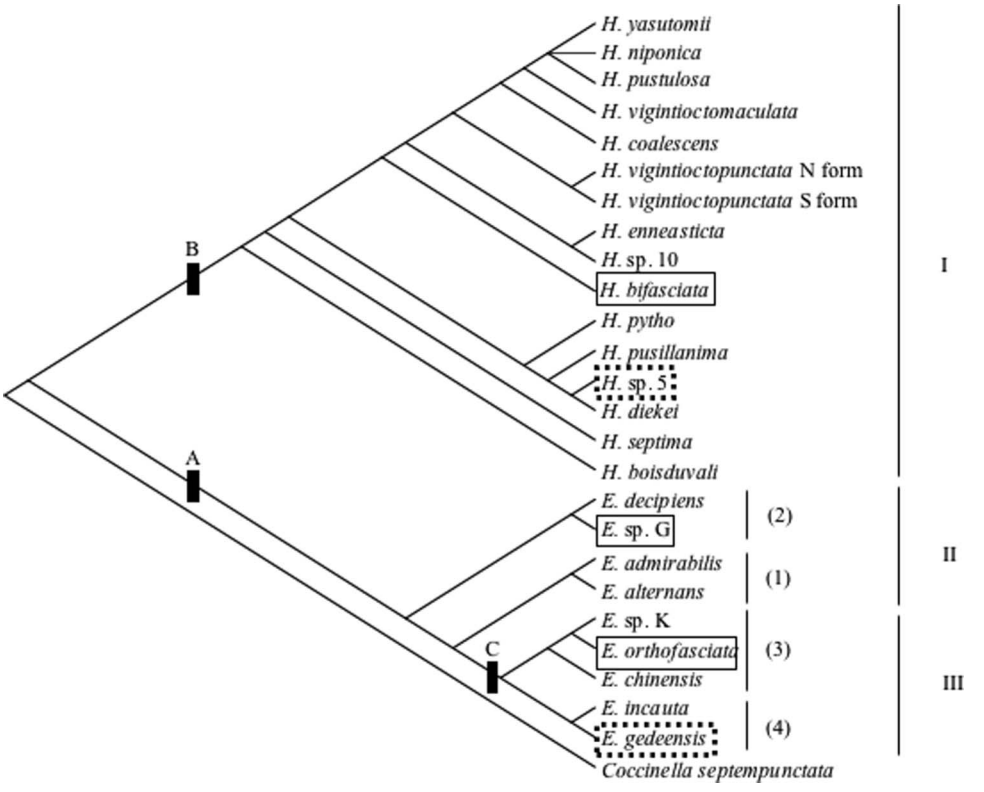

Figure 4. A topology illustrated by maximum likelihood method. Solid symbol indicates derived condition of female internal reproductive organs and modes of sperm transfer (see Table 1 and Figure 1). Two sets of sympatric species that have presumably convergent elytral patterns are respectively enclosed by a solid line and a dotted line.

present finding would be more plausible than that of Kobayashi et al. (1998). On the other hand, the position of *H. septima* was not stable in the present study. In the ML tree, *H. septima* diverged following *H. boisduvali* and, in the MP tree, this species formed a cluster with the most recent common ancestor of four species of the *H. vigintioctomaculata* species complex, two forms of *H. vigintioctopunctata*, *H. coalescens*, *H.* sp. 10, *H. enneasticta* and *H. bifasciata*. Because their bootstrap values concerned with the position of *H. septima* are not so high, its phylogenetic position has not yet been clarified.

Character evolution in Epilachninae

Katakura et al. (1994) assumed the polarity of reproductive traits as shown in Table 1. The present analysis supported their assumption very well and indicated that these traits are phylogenetically informative (Figure 4). The degeneration of the spermatophore would occur on the lineage of the common ancestor of groups II and III, and the reduction of the bursa copulatrix would occur on the most recent common ancestor of group III. The position of spermatheca would change from a terminal position to a lateral one in *Henosepilachna*.

Convergent evolution has occurred of spot patterns in some epilachnines. Many Asian species of epilachnines have a similar external appearance with 10 or 12 black spots on the brownish background on the elytra. Some species of *Henosepilachna* have many more – up to 28 spots. In most cases, this similar appearance of epilachnine beetles could be explained by the sharing of ancestral characters, namely the common ancestor possessed such multi-spotted elytra. However, Katakura et al. (2001) pointed out that sympatric distantly related species of Indonesian epilachnines often showed a very similar but apparently derived elytral pattern, and interpreted this phenomenon as convergence. They further postulated this convergence to be Müllerian mimicry, in which two or more unpalatable species reduce the risk of predation by their shared aposematic appearance. Indeed, coccinellid beetles very often possess defensive chemicals (Hodek and Honek 1996), and there are many putative cases of Batesian mimicry in the Asian tropics, in which various groups of insects, such as hemipterans and chrysomelids, resemble sympatric epilachnine beetles (H. Katakura et al., personal observations). The present study strongly supported the independent origin of similar elytral patterns for at least two sets of sympatric species previously examined by Katakura et al. (2001) (Figure 4): one composed of *H. bifasciata*, *E.* sp. G (*Epilachna* group 2) and *E. orthofasciata* (*Epilachna* group 3), and the other composed of *H.* sp. 5 and *E. gedeensis* (*Epilachna* group 4), all occurring sympatrically in the forest habitats of Mount Gede in West Java on different types of host plants (Katakura et al. 2001). The three species in the first set are of approximately equal sizes and are characterized by elytra with two transverse fasciae and a pair of apical spots, a pattern that is rare in epilachnines in other parts of Indonesia (Katakura et al. 2001). Of the three species, *H. bifasciata* and *E.* sp. G were thought endemic to West Java and are so far known to be sympatric only on the slope of Mount Gede. *Epilachna orthofasciata*, or its close relatives, were collected in Sumatra and Java, but except for those from Mount Gede, their elytral spots 3 and 4 were always separate, spot 1 was usually separate, and none of them formed fasciae. In the latter set, the two species are smaller than those in the former set and have elytra with characteristic medial spots arranged in "V", again a pattern uncommon for Indonesian epilachnines.

The distribution of these species on the obtained phylogeny clearly showed that the members within each of these two sets of epilachnines are indeed distantly related, suggesting convergent evolution of their similar elytral spot patterns. Whether these putatively convergent elytral patterns are really attributable to Müllerian mimicry as postulated by Katakura et al. (2001) or not will be clarified through further studies dealing with broad ranges of information concerning the biology of these ladybird beetles, including the identification of defensive chemicals of ladybirds and detection of predators.

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