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Citation	Journal of Natural History, 43(27-28), 1637-1649 https://doi.org/10.1080/00222930902968817
Issue Date	2009-07
Doc URL	http://hdl.handle.net/2115/43178
Rights	This is an electronic version of an article published in Journal of Natural History, 43(27-28) July 2009, 1637-1649. Journal of Natural History is available online at: http://www.informaworld.com/openurl?genre=article&issn=0022-2933&volume=43&issue=27&spage=1637
Type	article (author version)
File Information	JNH43-27-28_1637-1649.pdf

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Title: 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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Abstract

We determined partial sequences of nuclear 28S ribosomal RNA gene (717 bp) and mitochondrial DNA NADH-dehydrogenase subunit 2 gene (535 bp) of 25 species of phytophagous ladybird beetles from Asia that comprise sixteen 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.

Key words: gene tree - epilachnine beetles - female internal reproductive organ – modes of sperm transfer - elytral convergent evolution

Introduction

Phytophagous ladybird beetles of the subfamily Epilachinae are rich and abundant in tropical and subtropical regions worldwide (Gordon, 1975). More than one thousand species of epilachnines have so far been described, and 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 in Africa, Europe, Oceania,

and Asia. In these two genera, several infrageneric groups have been further recognized (Dieke, 1947; Fürsch, 1991; Gordon, 1975, Katakura *et al*., 2001). However, 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 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 genitalic morphology of both sexes and reproductive traits. Kobayashi *et al*. (1998) presented a phylogenetic relationship of ten species of Asian *Henosepilachna* beetles based on mtDNA.

In the present study, we aimed to clarify 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 a part of mtDNA ND2 gene and 28S 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 to address various evolutionary issues concerning epilachnine beetles. In this paper, we focused 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 presumable 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 erytral 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 given 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 used by 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 purifications. 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 Coccinellinae, Scymninae, and Chilicorinae were closely related to Epilachninae, but the relationships of them were not firmly settled yet. We used an aphidophagous species *Coccinella septempunctata* (Coccinellinae) as an outgroup, because above three studies consistently suggested a close relatedness of Coccinellinae and Epilachninae.

Some additional comments are necessary to this list.

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 since our results mentioned later have demonstrated that it is not only different in elytral pattern, but also 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 twelve-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 twelve-spotted subspecies *Epilachna emarginata emarginata* described from Samar Island. Since the name *Epilachna*

emarginata was preoccupied by another species, i.e. *Epilachna Emarginata* Montrouzier in Perroud et 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. But it should be *H. altera diekei*. The situation is thus 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 twelve-spotted taxon, tentatively regarding it as specifically different from *Henosepilachna altera altera* (Dieke, 1947) (*sens.* Jadwiszczak and Węgrzynowcz, 2003).

4) Our material of *Epilachna chinensis* came from Tsushima Island, southern Japan, which 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 0.1mM EDTA for purified DNA (originally 1mM). We first tried to amplify mtDNA 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 PCR 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 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 7min.; next step, 35 cycles with 94° C 45 sec., 42° C 90 sec., 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 by 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 χ^2 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) tests (Farris *et al*., 1994) with software PAUP* (Swofford, 2002). The ILD test by MP heuristic search was performed with 1000 replications and 100 initial max trees. Phylogenetic trees were constructed for ND2 and 28S rRNA genes and their combined data set by Maximum parsimonious (MP) and Maximum likelihood (ML) methods using the software PAUP ver. 4.0b (Swofford, 2002). In 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 (TBR) branch swapping. In ML method for constructing trees for each gene and combined data, we used NJ tree (Saitou and Nei, 1987) for starting tree to search 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 ND2 gene. On the other hand, nine indels were recognized in 28S rRNA. Information of indels in 28S rRNA gene was eliminated for 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 most parsimonious trees for 28S rRNA and ND2 respectively. For ML tree, GTR-I model and GTR-G+I model were used for 28S rRNA and ND2 gene respectively, by 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 with 0.05 significant level (28S vs. ND2, $P = 0.932$) by Darlu & Lecointre criteria (2002), the MP and ML trees were reconstructed by combined data sets (figures 2 and 3). MP analysis of the combined data yielded ten most parsimonious trees, and the strict consensus of these trees is

illustrated (figure 2). For the reconstructing the ML tree, GTR-G+I model was selected. And their 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 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 diverged from other 15 species in the genus *Henosepilachna*, although *H. septima* had been firstly diverged in Kobayashi *et al*. (1998), and the phylogenetic position of *H. septima* drawn by MP and ML was different from each other, and (3) members of each group (1-4) in the genus *Epilachna* formed a distinct cluster although the phylogenetic relationships of group 1 and 2 were ambiguous by MP and ML methods. Based on the suggested ML phylogenetic tree, character traces for some morphological features were shown in figure 4.

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 was included in group II, and the 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 were 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). And, in the ML tree,

group 2 spilt from other groups first, followed by the splitting of group 1 (figure 3). Thus, the relationships of group 1, 2 and the common ancestor of groups 3 and 4 were not clarified yet. 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 ten 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 mtDNA COI gene (1000 bp long), Kobayashi *et al*. (1998) suggested that *H. septima* would have firstly diverged in the ten 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 bp long; see table 2) constructed by two different methods (figures 2 and 3) indicated that *H. boisduvali* had firstly diverged from the rest with extremely high bootstrap support. Because the phylogenetic position of *H. boisduvali* was supported by two independent genes (ND2 and 28S rRNA), the present result would be more plausible than that by Kobayashi *et al*. (1998). On the

other hand, the position of *H. septima* was not stable in the present study. In ML tree, *H. septima* diverged following *H. boisduvali*. And, in 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 was not clarified yet.

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 spermatophore would occur on the lineage of the common ancestor of groups II and III, and the reduction of bursa copulatrix on the most recent common ancestor of group III. The position of spermatheca would change from terminal position to lateral one in *Henosepilachna*.

Here we move to another topic, convergent evolution 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 much more number of up to 28 spots. In most cases, such similar appearance of epilachnines 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 due to 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 Asian tropics, in which various groups of insects, such as hemipterans and chrysomelids, resemble sympatric epilachine beetles (H. Katakura *et al*., pers. obs.). 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 Mt. Gede in West Java on different kind of host

plants (Katakura *et al*., 2001). The three species in the former set are approximately of equal sizes and 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 seemed endemic to West Java and so far known to be sympatric only on the slope of Mt. Gede. *Epilachna orthofasciata* or its close relatives were collected in Sumatra and Java, but except for those from Mt. Gede, their elytral spots 3 and 4 were always, and spots 1 and were usually separate and did not form fasciae. In the latter set, the two species are smaller than the former set and have elytra with characteristic medial spots arranged in "V", again the 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.

Acknowledgements

We wish to express our thanks to Dr. Susumu Nakano, and Dr. Naoyuki Fujiyama for collecting samples. This study was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 14000007 to NK, Nos. 14204081, 18207005 to HK), and 21st Century Center of Excellence (COE) Program of Hokkaido University, "The Neo-Science of Natural History – Origin and Evolution of Natural Diversity."

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Figures Legends

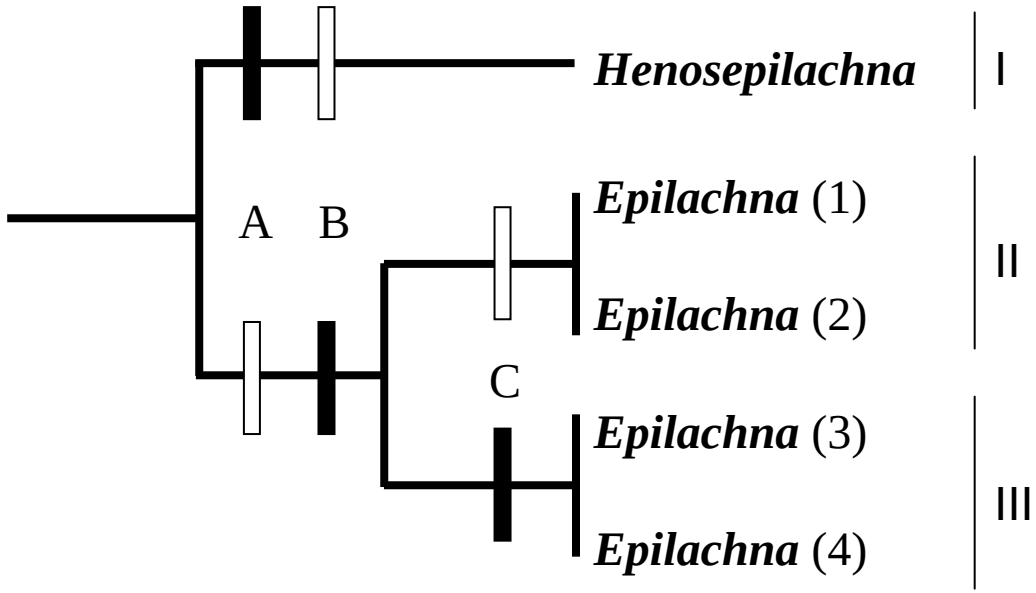
Fig. 1. A possible phylogenetic relationship between the three types of east Asian epilachnines discriminated by the difference in female internal reproductive organs and modes of sperm transfer (See table 1). A, position of spermatheca; B, spermatophore formation; C, bursa copulatrix. Open symbol, ancestral condition; solid symbol, derived condition (After Katakura *et al*. 1994.). Roman and Arabic figures indicate group manes defined by Katakura *et al*. (1994) and Katakura *et al*. (2001), respectively.

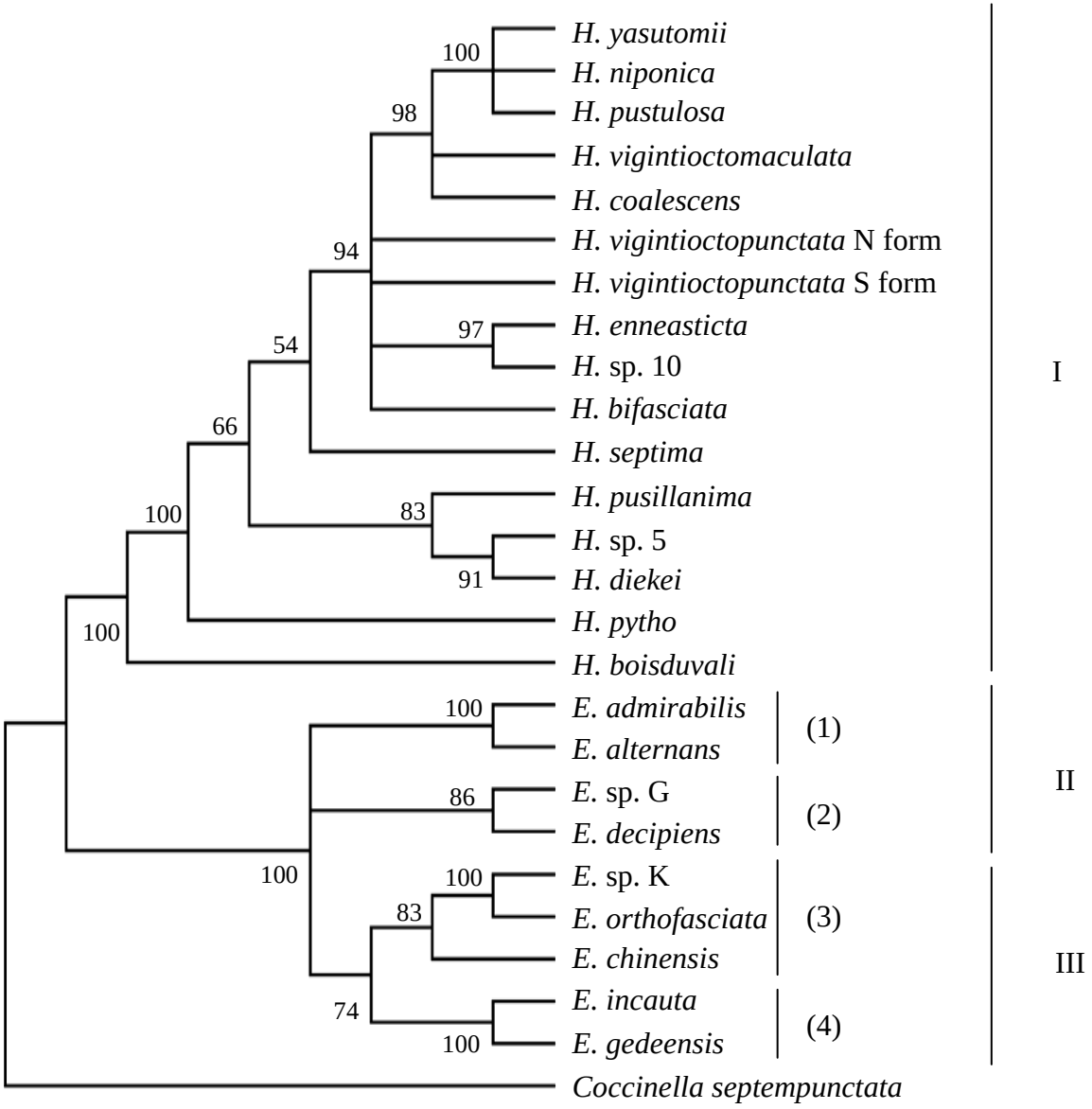
Fig. 2. A strict consensus tree based on the combined data sets for 25 species of Asian epilachnines reconstructed by MP method of heuristic search.

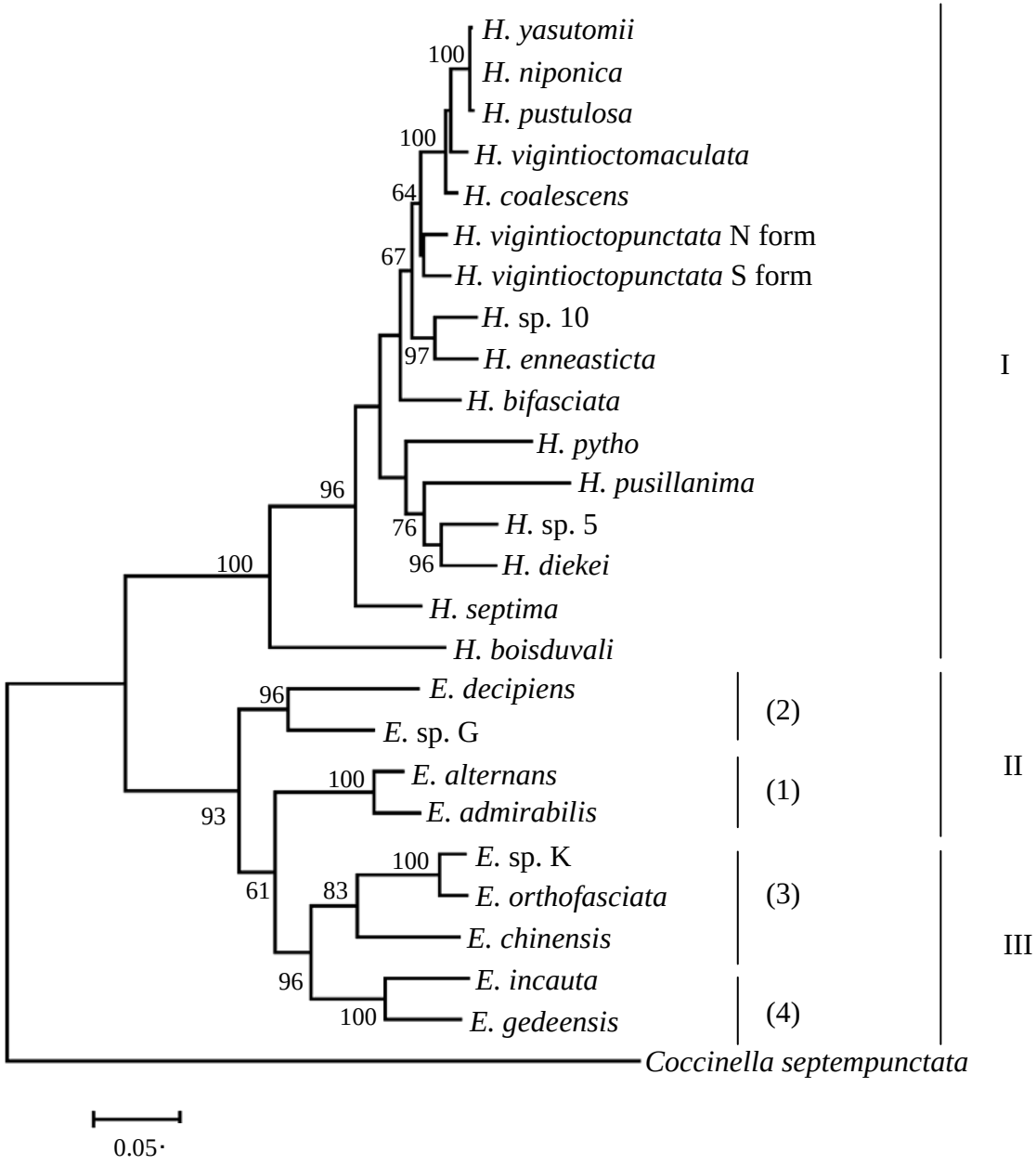
Fig. 3. A phylogenetic gene tree based on the combined data sets for 25 species of Asian epilachnines reconstructed by ML method

with GTR-G+I model. Bootstrap values were shown when more than 50% support was obtained.

Fig. 4. A topology illustrated by ML 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 solid line and dotted line.


Henosepilachna
I
A
B
Epilachna (1)
Epilachna (2)
II
C
Epilachna (3)
Epilachna (4)
III


H. yasutomii
H. niponica
H. pustulosa
H. vigintioctomaculata
H. coalescens
H. vigintioctopunctata N form
H. vigintioctopunctata S form
H. enneasticta
H. sp. 10
H. bifasciata
H. septima
H. pusillanima
H. sp. 5
H. diekei
H. pytho
H. boisduvali
E. admirabilis
E. alternans
E. sp. G
E. decipiens
E. sp. K
E. orthofasciata
E. chinensis
E. incauta
E. gedeensis
Coccinella septempunctata
100
98
94
97
54
66
100
83
91
100
100
86
100
100
83
74
100
I
II
III
(1)
(2)
(3)
(4)


H. yasutomii
H. niponica
H. pustulosa
H. vigintioctomaculata
H. coalescens
H. vigintioctopunctata N form
H. vigintioctopunctata S form
H. sp. 10
H. enneasticta
H. bifasciata
H. pytho
H. pusillanima
H. sp. 5
H. diekei
H. septima
H. boisduvali
E. decipiens
E. sp. G
E. alternans
E. admirabilis
E. sp. K
E. orthofasciata
E. chinensis
E. incauta
E. gedeensis
Coccinella septempunctata
100
100
64
67
97
96
76
96
100
96
100
93
100
61
83
96
100
I
II
III
(2)
(1)
(3)
(4)
0.05

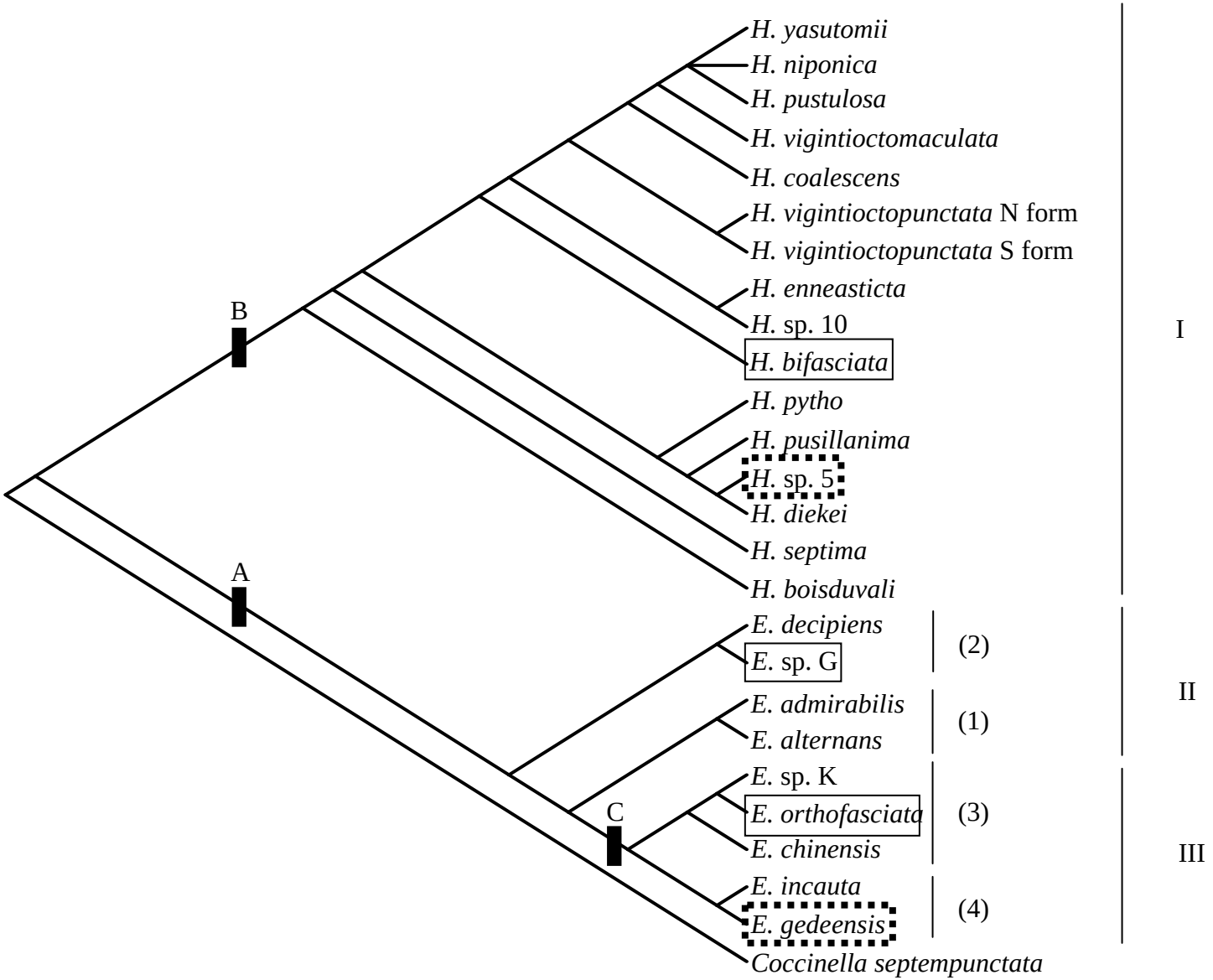

H. yasutomii
H. niponica
H. pustulosa
H. vigintioctomaculata
H. coalescens
H. vigintioctopunctata N form
H. vigintioctopunctata S form
H. enneasticta
H. sp. 10
H. bifasciata
H. pytho
H. pusillanima
H. sp. 5
H. diekei
H. septima
H. boisduvali
E. decipiens
E. sp. G
E. admirabilis
E. alternans
E. sp. K
E. orthofasciata
E. chinensis
E. incauta
E. gedeensis
Coccinella septempunctata
A
B
C
(1)
(2)
(3)
(4)
I
II
III

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

Genus / group* / group**	Species	Female internal reproductive systems and modes of sperm trensfer			Sampling location	Host plant family
		Bursa copulatrix (A)	Position of spermatheca	Spermatophore formation (C)		
Henosepilachna / I / -	*H. vigintioctomaculata* (Motschulsky)	Functional[#]	Lateral[$]	Present[#]	Gifu, Japan	Solanaceae
	H. pustulosa (Kôno)				Hokkaido, Japan	Asteraceae
	H. niponica (Lewis)				Miyagi, Japan	Asteraceae
	H. yasutomii Katakura				Miyagi, Japan	Berberidaceae
	H. coalescens (Mader)				Viet Nam	Solanaceae
	H. boisduvali (Mulsant)				Okinawa, Japan	Cucurbitaceae
	H. vigintioctopunctata (Fabricius) N forr				Okinawa, Japan	Solanaceae
	H. vigontioctopunctata (F.) S form				West Java, Indonesia	Solanaceae
	H. enneasticta (Mulsant)				West Java, Indonesia	Solanaceae
	H. sp. 10				Sulawesi, Indonesia	Solanaceae
	H. diekei				West Java, Indonesia	Asteraceae
	H. septima (Dieke)				West Java, Indonesia	Cucurbitaceae
	H.pusillanima (Mulsant)				Central Java, Indonesia	Cucurbitaceae
	H. bifasciata (Fabricius)				Central Java, Indonesia	Solanaceae
	H. pytho (Mulsant)				West Java, Indonesia	Cucurbitaceae
	H. sp. 5				West Java, Indonesia	Acanthaceae
Epilachna / II / (1)	*E. admirabilis* Crotch	Functional[#]	Terminal[#]	Absent[$]	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	–

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

Gene	Nucleotide sites used	Length Polymorphism	Polymorphic sites	Parsimoniously informative	Base frequencies (%)				Compositional heter	
					A	T	G	C	X^2 (df, 75)	
28S rRNA	718	0-9	82	47	36.6	41.9	8.3	13.3	8.0298	1.
ND2	533	0	348	281	22.5	22.6	29.4	25.5	0.7210	1.
combined	1251	0-9	430	328	28.8	31.2	20.0	20.1	1.4654	1.