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Phylogeny of *Epilachna*, *Henosepilachna*, and Some Minor Genera of Phytophagous Ladybird Beetles (Coleoptera: Coccinellidae: Coccinellinae: Epilachnini), with an Analysis of Ancestral Biogeography and Host-Plant Utilization

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Ladybird beetles in the tribe Epilachnini include notorious crop pests and model species studied intensively in various fields of evolutionary biology. From a combined dataset of mitochondrial (*ND2*) and nuclear (*28S*) DNA sequences, we reconstructed the phylogeny of 46 species of Epilachnini from Asia, Africa, America, and the Australian region: 16 species in *Epilachna*, 24 species in *Henosepilachna*, and one species each in *Adira*, *Afidenta*, *Afidentula*, *Afissula*, *Chnootriba*, and *Epiverta*. In our phylogenetic trees, both *Epilachna* and *Henosepilachna* were reciprocally polyphyletic. Asian *Epilachna* species were monophyletic, except for the inclusion of *Afissula* sp. Asian and Australian *Henosepilachna* species likewise formed a monophyletic group, excluding *H. boisduvali*. African *Epilachna* and *Henosepilachna* species did not group with their respective Asian and American congeners, but were paraphyletic to other clades (*Epilachna* species) or formed a separate monophyletic group (*Henosepilachna* species) together with *Chnootriba similis*. The American *Epilachna* species were monophyletic and formed a clade with American *Adira clarkii* and Asian *Afidentula manderstjernae bielawskii*; this clade was the sister group to Asian and Australian *Henosepilachna*, but was distant from Asian *Epilachna*. *Chnootriba* was embedded in the African *Henosepilachna* clade, and *Afissula* in the Asian *Epilachna* clade. *Epiverta*, which is morphologically unique, was the sister group to Asian *Epilachna*, although with weak support. From reconstructions of biogeographical distribution and host-plant utilization at ancestral nodes, we inferred an African origin for the common ancestor of the species studied, and found the frequency of host shifts to differ greatly between the two major lineages of Epilachnini examined.

Key words: Epilachnini, *Epilachna*, *Henosepilachna*, *Epiverta*, historical biogeography, host shift

INTRODUCTION

Insects are an exceptionally abundant and diverse group of metazoans (Grimaldi and Engel, 2005; Resh and Cardé, 2009), with the number of recognized species estimated to be slightly more than one million (Adler and Foottit, 2009) and still increasing (Grimaldi and Engel, 2005; Resh and Cardé, 2009). Nearly half of all insect species are phytophagous, and they exhibit great ecological dominance on Earth (Strong et al., 1984; Grimaldi and Engel, 2005; Schoonhoven et al., 2005). The majority of phytophagous insects are specialists, feeding on only one or a few host-

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plant species (Strong et al., 1984; Schoonhoven et al., 2005). The study of evolutionary processes in phytophagous insects, particularly those leading to specialization on specific host plants, is thus important for understanding the diversity of life.

Most species of phytophagous ladybird beetles are considered to be host-specific (cf. Pang and Mao, 1979; Katakura et al., 2001). There are over 1000 described species, previously classified in 23 genera and four tribes in the subfamily Epilachninae of the family Coccinellidae (Jadwiszczak and Węgrzynowicz, 2003; two additional genera were recently described by Szawaryn and Tomaszewska, 2013). Nearly all the species inhabit tropical and subtropical regions, with only a few occurring in temperate regions (Gordon, 1975). Previous morphological studies have presented alternative views concerning the phylogenetic position of Epilachninae. Sasaji (1968, 1971) and Kovář (1996) suggested that Epilachninae is a sister taxon to the subfamily Coccinellinae, while Yu (1994) proposed that it is the most basal group in Coccinellidae. Likewise, analyses of molecular data have not unambiguously resolved the phylogenetic position of Epilachninae (Giorgi et al., 2009; Aruggoda et al., 2010; Magro et al., 2010; Seago et al., 2011; Nedvěd and Kovář, 2012). From an analysis of a combined molecular and morphological dataset, Seago et al. (2011) relegated Epilachninae to the rank of a tribe, Epilachnini, in a broadly defined subfamily Coccinellinae within Coccinellidae. The newly defined Epilachnini includes all four previously recognized tribes, i.e., Epilachnini with 13 genera, Cynegetini with 10 genera, and Epivertini and Eremochilini with one genus each (Jadwiszczak and Węgrzynowicz, 2003; Szawaryn and Tomaszewska, 2013). In this article, we generally follow this new classification system, except for the use of Epilachnini or Epilachnini (*s.l.*) instead of Epilachninae. When necessary, we refer to Epilachnini in the previous sense as Epilachnini (*s.s.*), and refer to other, previously recognized tribes as before.

Both larvae and adults of all species in Epilachnini (*s.l.*) are phytophagous, utilizing host plants in diverse taxonomic groups of angiosperms, including Cucurbitaceae, Solanaceae, Fabaceae, Asteraceae, Urticaceae, and Vitaceae (Pang and Mao, 1979; Schaefer, 1983; Katakura et al., 2001). Some species are serious pests of important crops, including eggplant, potato, squash, and beans (Dieke, 1947; Li and Cook, 1961; Schaefer, 1983). Some closely related species of *Henosepilachna* are of particular interest as candidates for sympatric speciation via host races, and have been the subject of intensive studies on speciation and/or reproductive isolation (e.g., Katakura et al., 1989; Katakura and Hosogai, 1994, 1997; Katakura, 1997; Hirai et al., 2006; Matsubayashi and Katakura, 2007, 2009; Kuwajima et al., 2010; Kobayashi et al., 2011; Matsubayashi et al., 2011).

Despite the ecological, agricultural, and evolutionary importance of epilachnine beetles as a model system, their taxonomy and phylogenetic relationships have remained largely unclear. Although Seago et al. (2011) grouped all four previously recognized tribes into a single tribe, Epilachnini (*s.l.*), that study included only one representative species from each of three genera in Epilachnini (*s.s.*), two genera in Cynegetini, one genus in Epivertini, and one genus in Eremochilini. Moreover, they used only morphological data to infer relationships for Epivertini and Eremochilini. The relationships among, and taxonomic status of, the four tribes are thus still not firmly settled. The taxonomy and phylogeny of various genera are also unclear. Studies besides Seago et al. (2011) have examined phylogenetic relationships only in some species in Epilachnini (*s.s.*): three European species in genera *Epilachna*, *Henosepilachna*, and *Subcoccinella* (Magro et al., 2010) and some Asian species in *Epilachna*, *Henosepilachna*, and *Afissula* (Katakura et al., 1994; Kobayashi et al., 1998, 2009; Aruggoda et al., 2010).

To resolve the taxonomic positions of the various species, species groups, and genera in Epilachnini (*s.l.*), it is first necessary to resolve the phylogeny (Katakura et al., 1994; Pang et al., 2012), which will in turn be indispensable for tracing evolutionary changes in the relationships between the beetles and their host plants. The primary goal of the present study was to reconstruct phylogenetic relationships within Epilachnini (*s.s.*), the largest of the four previously recognized tribes, using DNA sequences from the mitochondrial NADH dehydrogenase subunit 2 (*ND2*) gene and the nuclear 28S rRNA gene (*28S*). We paid special attention to resolving phylogenetic relationships in *Epilachna* (the largest genus, comprising some 580 species) and *Henosepilachna* (the second largest, with ca. 250 species). Based on the phylogeny, we also reconstructed ancestral states for geographical distribution and host-plant-family utilization. Although the former tribal classification of Epilachnini (*s.l.*) was likely not valid (Seago et al., 2011), Epilachnini (*s.s.*) comprises the largest number of genera and species, and so we surmised that an analysis of this group might provide a firm foundation for broadly understanding taxonomy and evolutionary history across Epilachnini (*s.l.*). We also incorporated into this study *Epiverta chelonia*, the only species in Epivertini, which Seago et al. (2011) transferred to Epilachnini (*s.l.*) on the basis of morphological data.

MATERIALS AND METHODS

Specimens

Table 1 lists the species included in this study, their global distributional ranges, and the host-plant families they utilize. We collected all the specimens ourselves except for *Epiverta chelonia*, for which only one dried and pinned specimen was available. Supplementary Table S1 online provides additional information on species groups, collection localities, and host plants. Genus names follow the classification of Jadwiszczak and Węgrzynowicz (2003), although we realize that the current generic classification of Epilachnini (*s.l.*) needs a thorough revision. We refer to three species whose taxonomic status has not been resolved by the code number or letter used in previous studies (*Epilachna* sp. G, *Epilachna* sp. K, *Henosepilachna* sp. 5) (Katakura et al., 1994, 2001; Kobayashi et al., 2009), and leave *Afissula* sp. undetermined, as only a female specimen was available. Our study included 46 species in Epilachnini (*s.l.*) collected from Asia, Africa, and the New World, with one species from New Guinea (Australian region). Taxonomic coverage was 16 species in *Epilachna*, 24 species in *Henosepilachna*, and one species each in *Adira*, *Afidenta*, *Afidentula*, *Afissula*, *Chnootriba,* and *Epiverta*—in all, representing seven of the thirteen genera in Epilachnini (*s.s.*) (Jadwiszczak and Węgrzynowicz, 2003; Szawaryn and Tomaszewska, 2013) and the only genus and species in Epivertini. While the majority of samples were from Asia, African species of Epilachnini (*s.s.*) were also well represented (six of the seven species groups of African *Epilachna* and *Henosepilachna*, along with *Chnootriba*; see Supplementary Table S1 online). By

Table 1. Taxa included in this study and GenBank accession numbers for *ND2* and *28S* sequences. Sequences obtained in this study are underlined.

Species	Global distribution ranges used for biogeographical analyses	Host-plant family	Accession No.	
			ND2	*28S*
Adira clarkii (Crotch)	America	Aristolochiaceae	AB872224	AB872014
Afidenta misera (Weise)	Asia	Fabaceae	AB872225	AB872015
Afidentula manderstjernae bielawskii Tomaszewska et Szawaryn	Asia	Poaceae	AB872226	AB872016
Afissula sp.	Asia	Cucurbitaceae	AB872227	AB872017
Chnootriba similis (Thunberg)	Africa	Poaceae	AB872228	AB872018
Epilachna admirabilis Crotch	Asia	Cucurbitaceae	AB359221	AB353862
E. alternans Mulsant	Asia	Cucurbitaceae	AB359199	AB353861
E. cacica (Guérin-Méneville)	America	Cucurbitaceae	AB872229	AB872019
E. chinensis tsushimana (Nakane et Araki)	Asia	Rubiaceae	AB359216	AB353882
E. clandestina Mulsant	America	Cucurbitaceae	AB872230	AB872020
E. gedeensis (Dieke)	Asia	Urticaceae	AB359206	AB353871
E. incauta Mulsant	Asia	Urticaceae	AB359203	AB353868
E. kaestneri kaestneri Fürsch	Africa	Asteraceae	AB872231	AB872021
E. lupina Mulsant	Africa	Amaranthaceae	AB872232	AB872022
E. ocellataemaculata (Mader)	Asia	Asteraceae	AB872233	AB872023
E. orthofasciata (Dieke)	Asia	Vitaceae	AB359208	AB353873
E. paykulli Mulsant[a]	Africa	Solanaceae	AB872234	AB872024
E. sp. G	Asia	Ranunculaceae	AB359207	AB353872
E. sp. K	Asia	Vitaceae	AB359202	AB353867
E. tredecimnotata (Latreille)	America	Cucurbitaceae	AB872235	AB872025
E. varivestis (Mulsant)	America	Fabaceae	AB872236	AB872026
Henosepilachna bacthaiensis Hoang	Asia	Cucurbitaceae	AB872237	AB872027
H. bifasciata (Fabricius)	Asia	Solanaceae	AB359205	AB353870
H. boisuduvali (Mulsant)	Asia, Australia	Cucurbitaceae	AB359215	AB353881
H. callipepla (Gerstaecker)	Africa	Cucurbitaceae	AB872238	AB872028
H. chenoni mombonensis (Weise)	Africa	Solanaceae	AB872239	AB872029
H. diekei Jadwiszczak et Węgrzynowicz	Asia	Asteraceae, Lamiaceae	AB359200	AB353863
H. elaterii (P. Rossi)	Africa	Cucurbitaceae	AB872240	AB872030
H. enneasticta (Mulsant)	Asia	Solanaceae	AB359222	AB353864
H. indica (Mulsant)	Asia	Solanaceae	AB872241	AB872031
H. kaszabi (Bielawski et Fürsch)	Asia	Cucurbitaceae,	AB872242	AB872032
H. maunsonica Jadwiszczak et Węgrzynowicz	Asia	Solanaceae	AB872243	AB872033
H. pusillanima (Mulsant)	Asia	Cucurbitaceae	AB359204	AB353869
H. pustulosa (Kôno)	Asia	Asteraceae, Berberidaceae	AB359212	AB353877
H. pytho (Mulsant)	Asia	Cucurbitaceae	AB359209	AB353874
H. quatuordecimsignata (Reiche)	Africa	Cucurbitaceae	AB872244	AB872034
H. reticulata limbicollis (Sicard)	Africa	Cucurbitaceae	AB872245	AB872035
H. septima (Dieke)	Asia	Cucurbitaceae	AB359201	AB353866
H. signatipennis (Boisduval)	Australia[b]	Fabaceae	AB872246	AB872036
H. sp. 5	Asia	Acanthaceae	AB359210	AB353875
H. vigintioctomaculata (Motschulsky)	Asia	Solanaceae, Cucurbitaceae	AB359211	AB353876
H. vigintioctopunctata (Fabricius) N form	Asia	Solanaceae	AB359214	AB353880
H. vigintioctopunctata (Fabricius) S form	Asia, Australia[c]	Solanaceae, Fabaceae	AB359223	AB353865
H. wissmanni (Mulsant)	Asia	Cucurbitaceae	AB872247	AB872037
H. yasutomii Katakura	Asia	Berberidaceae, Solanaceae	AB359213	AB353879
Epiverta chelonia (Mader)	Asia	Asteraceae, Ranunculaceae	AB971827	AB971828
Chilocorus kuwanae (Silvestri)	Asia	–	AB872248	AB872038
Coccinella septempunctata L.	Asia, Africa	–	AB359217	AB353860
Harmonia axyridis (Pallas)	Asia	–	AB872249	AB872039
Amida tricolor (Harold)	Asia	–	AB872250	AB872040
Rodolia cardinalis (Mulsant)	Australia	–	AB872251	AB872041

[a] Originally spelled as *Epilachna paykullii* by Mulsant (1850: 833), but *Epilachna paykulli* Mulsant is the prevailing usage (cf. Fürsch, 1990, 1991a, b) (cf. Articles 33.3 and 33.4 of the Code (ICZN, 1990)).

[b] The actual distributional range of *H. signatipennis* is New Guinea, New Britain, New Ireland, and the Solomon Is. (Jadwiszczak and Węgrzynowicz, 2003).

[c] "*H. vigintioctopunctata*" has also been reported from Australia and South Pacific islands. These records are tentatively treated here as the S form of *H. vigintioctopunctata*.

contrast, New World species of Epilachnini (*s.s.*) were poorly represented. Although the Australian region, particularly New Guinea, harbors a number of endemic species (cf., Bielawski, 1963; Szawaryn and Tomaszewska, 2013), we included only *Henosepilachna signatipennis* from this region.

We determined *ND2* and *28S* sequences for 25 species in Epilachnini (*s.l.*). Our phylogenetic analyses included nucleotide sequences for another 21 species from a previous study (Kobayashi et al., 2009), and sequences from five carnivorous species (*Amida tricolor*, *Chilocorus kuwanae*, *Coccinella septempunctata*, *Harmonia axyridis*, and *Rodolia cardinalis*) in Coccinellinae as outgroup taxa.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted by the method of Boom et al. (1990), with some modifications. PCR amplifications of the *ND2* and *28S* sequences were performed by using the same primers as Kobayashi et al. (2009). Amplifications were carried out in 10-μl reaction volumes, each containing 1× Ex *Taq* buffer (Takara Bio), 200 μM each dNTP, 0.5 μM each primer, 0.25 U Ex *Taq* polymerase (Takara Bio), and approximately 10 ng of genomic DNA, under the following cycling conditions: 95°C for 3 min; 35 cycles of 95°C for 30 s, 45–50°C for 90 s, and 72°C for 90 s; and 72°C for 7 min. PCR products were directly sequenced in both directions with a BigDye Terminator Sequencing Kit and an ABI 3100-Avant, 3130, or 3730 Genetic Analyzer (Life Technologies), according to the manufacturer's protocols.

Phylogenetic analysis

Nucleotide sequences were aligned by using the MUSCLE algorithm (Edgar, 2004) implemented in MEGA 5.10 (Tamura et al., 2011) with default settings. The resulting alignments were checked by eye and manually adjusted in some regions that contained gaps. Indel sites that remained in both the *ND2* and *28S* alignments were ignored for phylogenetic analyses. To determine whether the nucleotide composition was biased among taxa, χ^2 goodness-of-fit tests were performed on the sequence data by using PAUP* 4.0b10 (Swofford, 2002). The *ND2* and *28S* data were first analyzed separately and then concatenated for analysis. Before the datasets were concatenated, an incongruence length difference (ILD) test (Farris et al., 1995) (referred to as the partition homogeneity test [PHT] in PAUP*) was performed to detect possible incongruence between the two datasets. To generate the null distribution, parsimony analyses of 1000 heuristic search replicates for each of 100 starting trees generated by random stepwise addition were performed with TBR branch swapping.

Maximum likelihood (ML) analyses were performed with PAUP*. The best-fit model for each dataset was determined with the corrected Akaike information criterion (AICc) implemented in jModeltest 2.1.1 (Darriba et al., 2012); Table 2 lists the optimal models used for the ML analyses with PAUP*. The optimal ML trees were found by tree bisection recombination (TBR) searches, with starting trees determined by the neighbor-joining (NJ) method (Saitou and Nei, 1987). Bootstrap values for ML trees were calculated from nearest neighbor interchange (NNI) searches of 1000 pseudoreplicates (Felsenstein, 1985), with the starting topology in each case given by an NJ tree.

To assess the effect of data partitions on the phylogenetic analyses, a ML analysis was conducted by using raxmlGUI 1.3 (Stamatakis, 2006; Silvestro and Michalak, 2012), in which the GTRGAMMAI (GTR+I+G) model was applied respectively for the 1st, 2nd, and 3rd-codon positions of *ND2* and *28S* data partitions. A bootstrap analysis of 1000 pseudoreplicates was performed by using the "ML + thorough bootstrap" search.

Bayesian analyses were conducted by using MrBayes 3.2.1 (Ronquist et al., 2012), in which the TIM+I+G (for *ND2*) and SYM+I+G (for *28S*) models (Table 2) were respectively applied to the 1st, 2nd, and 3rd-codon positions of *ND2* and *28S* data partitions. A Markov-Chain Monte-Carlo (MCMC) search was performed with four chains, each of which was run for 1,000,000 to 2,000,000 generations. Trees were sampled every 100 generations, with those from the first 100,000 generations discarded as burn-in to ensure that a stable likelihood had been reached. The trace file generated by the Bayesian MCMC runs was inspected in TRACER 1.5.0 (Rambaut and Drummond, 2009) to check that the number of sampling generations and effective sample sizes were large enough for reliable parameter estimates. A consensus of sampled trees was computed, and the posterior probability for each interior node was obtained to assess the robustness of the inferred relationships.

Table 2. Optimal substitution models used for the ML analyses with PAUP*, selected by AICc in jModeltest 2.1.1 (Darriba et al., 2012).

Partition	Model[a]	Base frequencies	Rate matrix	I	G
All data	GTR+I+G	A = 0.3128	A-C = 1.7154	0.4960	0.6350
		C = 0.1596	A-G = 5.1985		
		G = 0.1743	A-T = 3.0574		
		T = 0.3534	C-G = 1.2194		
			C-T = 12.2172		
			G-T = 1.0000		
28S	SYM+I+G	Equal frequencies	A-C = 0.6374	0.6840	0.6180
			A-G = 1.7615		
			A-T = 1.6022		
			C-G = 0.2834		
			C-T = 4.6376		
			G-T = 1.0000		
ND2	TIM2+I+G	A = 0.4036	A-C = 0.3475	0.1770	0.6380
		C = 0.1266	A-G = 5.4775		
		G = 0.0586	A-T = 0.3475		
		T = 0.4112	C-G = 1.0000		
			C-T = 3.4297		
			G-T = 1.0000		

[a] GTR, general time reversible model (Tavaré, 1986); SYM, symmetrical model (Zharkikh, 1994); TIM, transitional model (Posada, 2003); I, proportion of invariant sites; G, gamma distribution of the shape parameter.

Biogeographical analyses

The distributional ranges of species were divided into four areas for the analyses: Africa, Asia, America, and Australia (Table 1). Although the specimens of *E. varivestis* and *R. cardinalis* were collected in Japan, their distribution areas were assigned as America and Australia, respectively, because these species were introduced to Japan from their original distributional areas (Sasaji, 1971; Fujiyama et al., 1998). *Henosepilachna signatipennis*, endemic to New Guinea and nearby islands, was treated as an Australian species. The range of *H. boisduvali* was treated as Asia and Australia. Likewise, the range of the *H. vigintioctopunctata* S form was tentatively regarded as Asia and Australia, although it is yet uncertain whether "*H. vigintioctopunctata*" in the Australian region is really the S form; at present, precise identification of the S and N forms of *H. vigintioctopunctata* is possible only by examining mitochondrial DNA sequences (see Kobayashi et al., 2000).

The possible ancestral ranges of species were reconstructed by using statistical dispersal-vicariance (S-DIVA) and Bayesian binary MCMC (BBM) analyses implemented in RASP 2.1 beta (Yu et al., 2010, 2013). These methods average across all trees the frequency of an ancestral range at each node. To take into account phylogenetic uncertainty, 15,000 trees generated from the MCMC search in MrBayes from the concatenated dataset were used as input trees. S-DIVA and BBM analyses were then run on a ML tree topology. The maximum number of areas was set to four (not restricted) for both analyses. For the BBM analysis, ten MCMC

chains were run simultaneously for 500,000 generations, and the state was sampled every 100 generations. The fixed Jukes-Cantor model with equal among-site rate variation was used for the BBM analysis, with the root distribution set to "null".

Reconstruction of host-plant utilization

The history of host association was also reconstructed by using BBM analysis implemented in RASP. The families of host plants of the respective species in Tables 1 and S1 were used as character states; we directly confirmed host-plant utilization in the field in the course of this study or in previous studies (Katakura, 1997; Fujiyama et al., 1998; Kobayashi et al., 2000; Katakura et al., 2001; Nakano et al., 2001), except for *Epiverta chelonia*, for which host-plant information was obtained from Pang and Mao (1979). The following beetle species are known to occur on more than one plant family: *H. pustulosa*, *H. yasutomii*, *H. vigintioctomaculata*, *H. vigintioctopunctata* (S form), and *H. diekei*; Katakura (1997) and Katakura et al. (2001) provided detailed host-plant information for these species. *Epiverta chelonia* also reportedly feeds on plants in two families (Pang and Mao, 1979). As with the biogeographical analyses, 15,000 trees generated from an MCMC search in MrBayes based on the concatenated dataset were used as the input, and the BBM analysis was run on a ML tree topology under the same conditions as the biogeographical analyses, except that the maximum number of areas was set at six (the default value in RASP).

RESULTS

Data characteristics

The nucleotide sequences we determined have been deposited in DDBJ; see Table 1 for accession numbers. The concatenated sequence data excluding gap sites comprised 453 bp from *ND2* and 698 bp from *28S*. The G+C content for the *ND2* and *28S* regions across all taxa was 23.8 ± 2.7% and 54.4 ± 0.7%, respectively. A χ^2 test of base frequencies across taxa revealed no heterogeneity among the samples (for *ND2*, χ^2 = 112.5, df = 150, P = 0.95; for *28S*, χ^2 = 10.3, df = 150, P = 1.00). The ILD test yielded no significant incongruence between the *ND2* and *28S* datasets (sum of tree length, 2961; P = 0.54).

Phylogenetic analysis

Figure 1 shows the ML tree obtained from the concatenated *ND2* and *28S* dataset. RaxmlGUI and MrBayes generated similar tree topologies, with only minor differences (Supplementary Figures S1, S2 online) that did not affect our conclusions. The ML trees obtained from separate analyses of *ND2* and *28S* (Supplementary Figures S3, S4 online) generated somewhat different topologies, but most nodes received low bootstrap support. Hereafter we focus on the ML tree generated from the concatenated dataset. In Fig. 1, the African species *Epilachna kaestneri kaestneri* is the sister taxon to a clade containing all remaining species in the ingroup, with 70% bootstrap support (BS) and 1.0 posterior probability (PP). After *E. kaestneri kaestneri* branches off, two basal clades emerge. Clade A, with low nodal support (52% BS; < 0.50 PP), contains *Epiverta chelonia*, nine *Epilachna* species, and *Afissula* sp., all from Asia. Within clade A, *Epiverta chelonia* is the sister group to clade C (91% BS; 1.0 PP), which contains all the other species. Clade B (79% BS; 1.0 PP) contains the representatives of *Adira*, *Afidenta*, *Afidentula*, and *Chnootriba*; six species in *Epilachna*; and all species in *Henosepilachna*. Within clade B, clade D (*E. lupina* + *E. paykulli*) forms the sister group to clade E. Within clade E, clade F comprises six African species (*Chnootriba similis* and five species in *Henosepilachna*), although the nodal support is low (< 50% BS; 0.98 PP), and clade G comprises the representatives of *Adira*, *Afidenta*, and *Afidentula*; four American species in *Epilachna*; and all Asian and Australian species in *Henosepilachna*. Within clade G, *Afidenta misera* and *Henosepilachna boisduvali* are placed at the basal position; the remaining species comprise two clades, H and I. Clade H comprises one Asian (*Afidentula manderstjernae bielawskii*) and five American species (clade J; *Adira clarkii*, and four *Epilachna* species comprising clade K), although nodal support is low for clades H (57% BS; 0.73 PP) and J (57% BS; 0.99 PP). Clade I (99% BS; 1.0 PP) contains all the Asian and Australian *Henosepilachna* species, except for *H. boisduvali*.

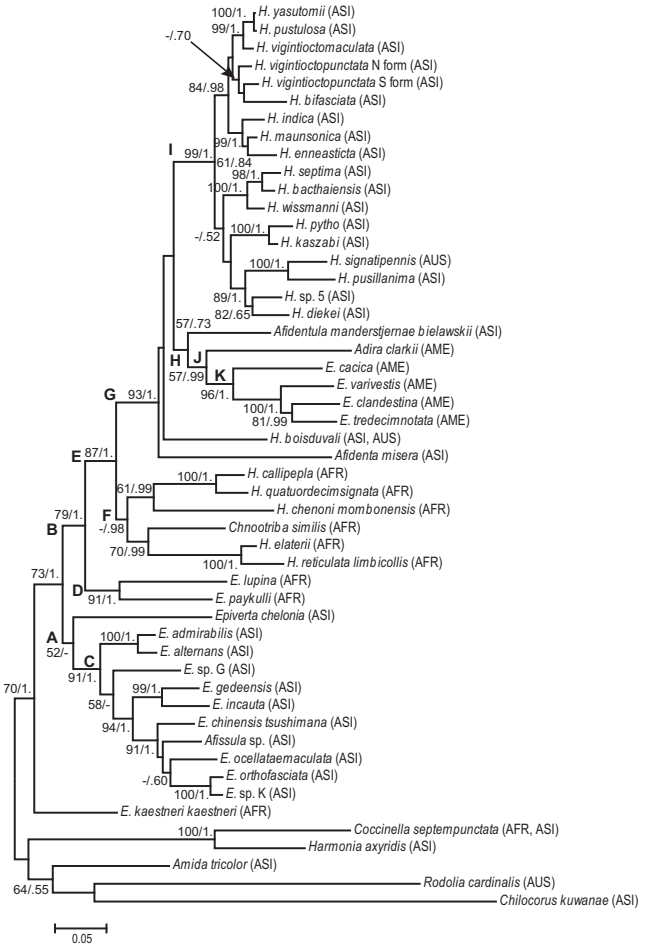

Fig. 1. Maximum-likelihood (ML) tree for the combined *ND2* and *28S* dataset, based on the GTR+I+G substitution model, conducted with PAUP*. Nodal support values are presented as the bootstrap value (≥ 50%, above diagonal) followed by the Bayesian posterior probability (≥ 0.5, below diagonal). Nodal support values < 50% (bootstrap) or < 0.5 (posterior probability) are indicated by hyphens. The scale bar indicates branch length in substitutions per site. To facilitate description in the text, selected nodes are labeled with capital letters. Distributional ranges are indicated in parentheses following the species names (AFR, Africa; ASI, Asia; AME, America; AUS, Australia).

Biogeographical analysis

Ancestral distributions were inferred from S-DIVA and BBM analyses (Figs. 2 and 3, respectively). In both reconstructions, the most recent common ancestor of the epilachnine beetles included in the study was distributed in Africa (node 45), with relative probabilities of 100% (S-DIVA) and 82.0% (BBM analysis). For the subsequent history, the two analyses showed somewhat different results. In the S-DIVA reconstruction (Fig. 2), the ancestors of *Epilachna*, *Epiverta*, and *Henosepilacha* in Asia were once distributed in both Africa and Asia (nodes 44 and 31), followed by vicariance between the two regions, whereas in the BBM reconstruction (Fig. 3), the ancestors of Asian *Epilachna* (plus *Epiverta*) and *Henosepilacha* respectively migrated from Africa (nodes 44 and 31). In the S-DIVA reconstruction, the ancestor of American species was once distributed in both Asia and America (node 22), with subsequent vicariance between the two regions, whereas in the BBM reconstruction the ancestors of American species migrated from Asia (node 22).

Reconstruction of host-plant utilization

We reconstructed the ancestral states of host-plant utilization, optimized on the ML tree based on the combined dataset (Fig. 4). The ancestral states for the most basal node (45) and the next-most-basal node (44) were inferred to be Asteraceae (88.4% and 61.7% probabilities, respectively).

The ancestral state at node 43 leading to *Epiverta chelonia* and Asian species of *Epilachna* was ambiguous: Ranunculaceae (45.5%), Asteraceae (34.5%), Ranunculaceae and Asteraceae (11.5%), and other host-plant families (8.5%). Host shifts were frequent in this clade, and Cucurbitaceae, Ranunculaceae, Ulticaceae, Rubiaceae, Asteraceae, and Vitaceae were finally adopted as host plants, although the direction of host shifts was not clear.

By contrast, the most likely ancestral state was inferred to be Cucurbitaceae at node 33 and most subsequent inter-

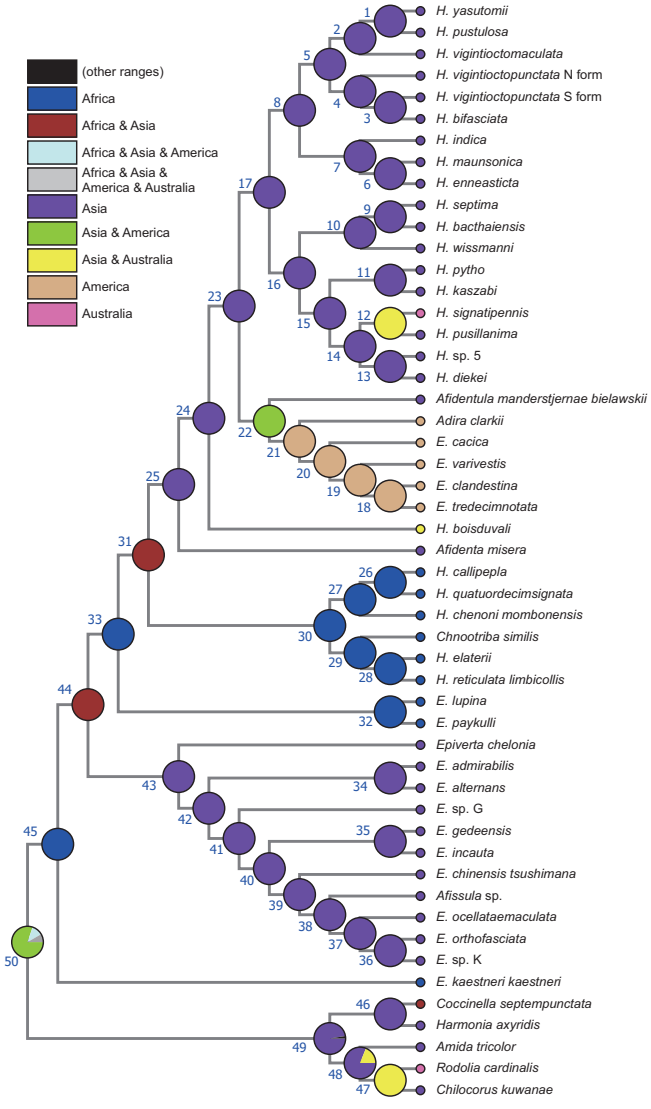

Fig. 2. Geographical areas mapped onto the ML tree, with ancestral states reconstructed by an S-DIVA analysis. Nodes are numbered.

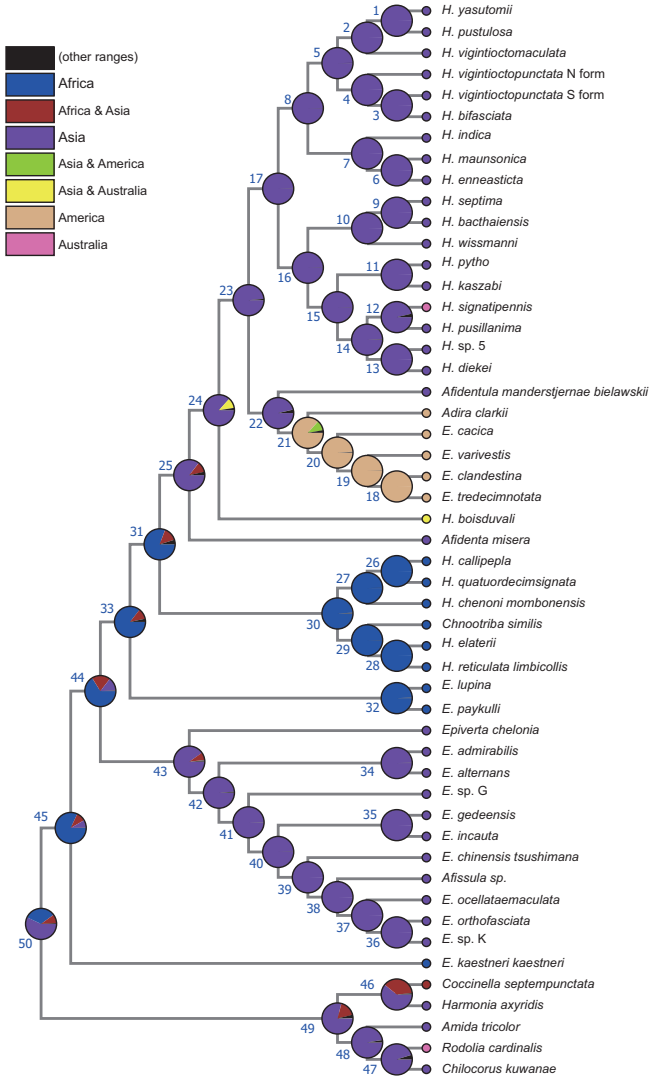

Fig. 3. Geographical areas mapped onto the ML tree, with ancestral states reconstructed by a BBM analysis. Nodes are numbered.

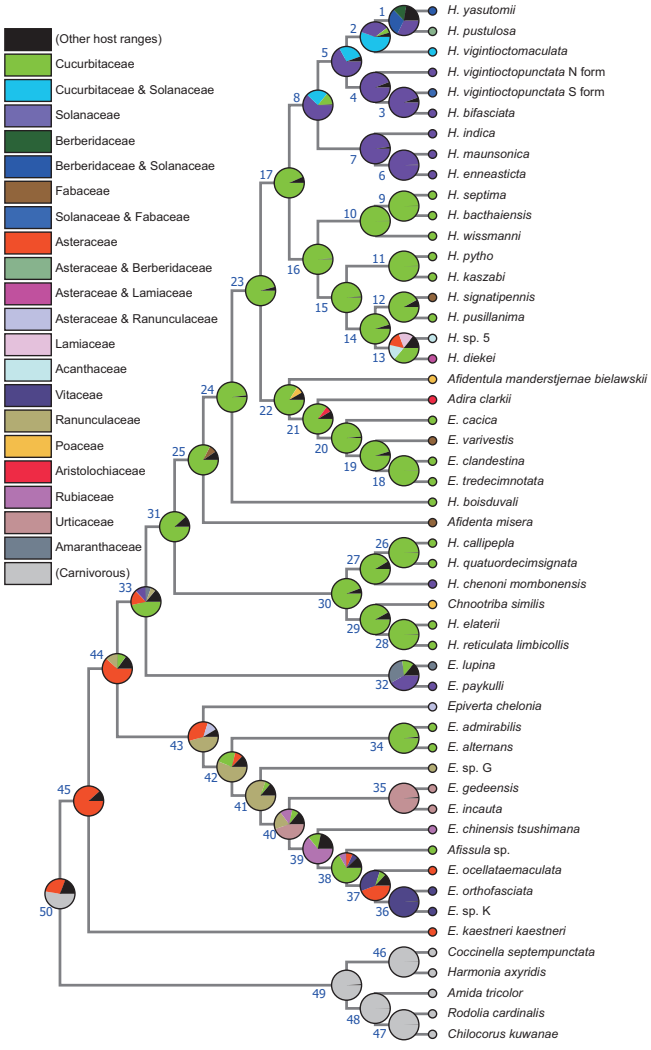

Fig. 4. Utilization of host-plant families mapped onto the ML tree, with ancestral states reconstructed by a BBM analysis. Nodes are numbered.

nal nodes, and shifts from Cucurbitaceae to other plant families followed. The reconstruction shows a host shift from Cucurbitaceae (node 17) to Solanaceae (node 8, the common ancestor of nine Asian species of *Henosepilachna*). Within this clade, further shifts from Solanaceae to Asteraceae and/or Berberidaceae were found in the *H. pustulosa* – *H. yasutomii* clade (node 1). A host shift from Cucurbitaceae to Solanaceae was also found for *H. chenoni mombonensis*. Independent host shifts from Cucurbitaceae to Fabaceae were indicated for *H. signatipennis*, *Afidenta misera*, and *E. varivestis*. Host shifts from Cucurbitaceae to Acanthaceae and/or Asteraceae + Lamiaceae were found for the *H.* sp. 5 – *H. diekei* clade (node 13). Independent host shifts from Cucurbitaceae to Poaceae were indicated for *Afidentula manderstjernae bielawskii* and *Chnootriba similis*. A host shift from Cucurbitaceae to Aristolochiaceae was found for *Adira clarkii*. Probable host shifts from Cucurbitaceae to Amaranthaceae and/or Solanaceae were found for the *E. lupina* – *E. paykulli* clade (node 32).

DISCUSSION

Epiverta and genera in Epilachnini (*s.s.*), and the relationship between *Epilachna* and *Henosepilachna*

Although the phytophagous ladybird beetles in the tribe Epilachnini (*s.s.*) constitute 13 genera (Jadwiszczak and Węgrzynowicz, 2003; Szawaryn and Tomaszewska, 2013), most species belong to either *Epilachna* or *Henosepilachna* (Li and Cock, 1961; Fürsch, 1990, 1991a, b; Jadwiszczak and Węgrzynowicz, 2003). However, the taxonomic status of these two large genera has long been controversial. Some authors have treated them as valid genera (e.g., Li and Cook, 1961; Bielawski, 1965; Sasaji, 1971; Fürsch, 1990, 1991a, b; Li, 1993; Jadwiszczak and Węgrzynowicz, 2003; Poorani, 2004; Szawaryn, 2011; Nedvěd and Kovář, 2012), whereas others have treated *Henosepilachna* as a synonym of *Epilachna* (e.g., Kapur, 1967; Iablokoff-Khnzorian, 1980; Richards, 1983; Ślipiński, 2007). These two genera are distinguished by the morphology of the tarsal claws and the abdominal sternite in females (Li and Cook, 1961). However, whether the evolution of these characters correlates with the species phylogeny has been quite controversial, and the phylogenetic relationships among species remain largely unresolved. To address this issue, Katakura et al. (1994) reconstructed the phylogenetic relationships of Asian *Epilachna* and *Henosepilachna* based on the mode of sperm transfer and the internal morphology of the female reproductive system. That report suggested that *Epilachna* and *Henosepilachna* are reciprocally monophyletic, a result that is largely compatible with a recent molecular phylogenetic study by Kobayashi et al. (2009). Previous studies, however, have dealt with only the Asian species in these two genera. In fact, Aruggoda et al. (2010) obtained a result using mitochondrial 16S rRNA gene sequences that showed five Asian species of *Epilachna* to be paraphyletic with respect to both three Asian *Henosepilachna* and one *Afissula* species. Our study had much broader geographical coverage, as it included additional species in these two genera and representatives of some minor genera from Asia, Australia, Africa, and America. Our study also included *Epiverta chelonia*, the only representative of Epivertini. Some conclusions are as follows.

Polyphyly of *Epilachna* and *Henosepilachna*. Our phylogenetic tree (Fig. 1; see Fig. 2 for distributional areas) shows clearly that the current generic classification of Epilachnini (*s.s.*) does not reflect phylogenetic relationships, but it also explains why a previous study (Kobayashi et al., 2009) that included only a limited number of Asian species suggested monophyly for both *Epilachna* and *Henosepilachna*. If non-Asian species and representatives of other nominal genera are removed from our tree, Asian *Epilachna* (clade C) and Asian–Australian *Henosepilachna* (clade I) emerge as reciprocally monophyletic, with *H. boisduvali* collapsing into clade I. This is congruent with the results of previous phylogenetic studies that included fewer species (Katakura et al., 1994; Kobayashi et al., 2009). When the species from other nominal genera, and from Africa and America, are included (Fig. 1), however, both *Epilachna* and *Henosepilachna* emerge as polyphyletic groups. Species of *Henosepilachna* are separated into clade I, *H. boisduvali*, and the five African species in clade F (Fig. 1), and *Epilachna* comprises three

separate, relatively well-supported clades (Fig. 1: C, D, and K). The four American species of *Epilachna* form a clade (K) with another American species (*Adira clarkii*) as the sister group. These five American species are more closely related to Asian *Afidentula manderstjernae bielawskii* and Asian *Henosepilachna* than to *Epilachna* species from regions other than America. Many of the backbone nodes in Fig. 1 received low or no nodal support, but some nodes indicating polyphyly for *Henosepilachna* and *Epilachna* are well supported: for example, node G, which groups American *Epilachna* species in the same clade with Asian *Henosepilachna* species, and node C, which groups Asian *Epilachna* species to the exclusion of African and American *Epilachna* species.

Minor genera. We included in our study one species each in the genera *Adira, Afidenta*, *Afidentula*, *Afissula*, and *Chnootriba*. *Adira* is restricted to the New World; *Afidentula* and *Afissula* are restricted to Southeast Asia; and *Chnootriba* is African (Jadwiszczak and Węgrzynowicz, 2003). *Afidenta* is mostly restricted to Africa, with only a few species in Asia (Jadwiszczak and Węgrzynowicz, 2003). Among these genera, *Adira* and *Afidentula* grouped with the American *Epilachna* species as clade H (Fig. 1), although with low nodal support. *Afidenta* emerged as a basal branch in clade G, which comprised *Adira*, *Afidentula*, all the Asian and Australian *Henosepilachna* species (including *H. boisduvali*), and the American *Epilachna* species. *Afissula* was embedded in clade C, which otherwise contained Asian *Epilachna*; *Afissula* was the sister group to species in the *E. flavicollis* group of Dieke (1947), although there was no nodal support for this relationship. *Chnootriba* (from Africa) fell into clade F together with African *Henosepilachna* and emerged as the sister group to representatives of the *H. elaterii* species group (Fürsch, 1964, 1990).

***Epiverta chelonia*.** In our tree, *Epiverta chelonia* was embedded in a basal position in the clade that contained all the epilachnine species except for *E. kaestneri kaestneri*; it formed a clade with Asian species of *Epilachna*, although with low nodal support (52% BP, < 0.5 PP). This result is consistent with Seago et al. (2011), who transferred *E. chelonia* to Epilachnini by lumping the monotypic group Epivertini with Epilachnini (*s.l.*) on the basis of morphology.

Taxonomy. Our results indicate that neither *Epilachna* nor *Henosepilachna* is monophyletic, and the phylogenetic positions of the minor genera (especially of *Afissula* and *Chnootriba*) calls into question their validity as distinct genera. Our study did detect some well-supported clades, such as Asian *Epilachna* (Fig. 1: clade C), American *Epilachna* (clade K), and Asian and Australian *Henosepilachna* (clade I), although this last result is inconclusive because our samples contained only one representative from the Australian region. While we make no specific recommendations for taxonomic revision here, our study indicates that the genera in Epilachnini (*s.l.*) need thorough revision based both on a careful evaluation of morphological characters and molecular phylogenetic analyses with broad taxonomic representation, especially including members of Cynegetini and Eremochilini.

Biogeographical origin of Epilachnini (*s.s.*)

Representatives of Epilachnini (*s.l.*) are distributed worldwide in tropical and subtropical regions (Gordon, 1975), but the historical biogeography of these species has remained unclear. Although this study covered only Epilachnini (*s.s.*) and Epivertini, our phylogenetic trees consistently showed African representatives to have relatively long branches, and to be paraphyletic to a clade containing both Asian and American lineages. The most parsimonious explanation for this pattern is that both Asian and American species derived from a common ancestor in Africa; other explanations require the assumption that more migratory events occurred between continents. Our S-DVIA and BBM reconstructions of ancestral distributional areas consistently indicated that the most recent common ancestor of Epilachnini (*s.s.*) plus *Epiverta* likely resided in Africa. This must be regarded as a tentative hypothesis, however, because the number of American and Australian representatives examined was small, and further studies using additional loci and taxa will be required to test it. Furthermore, the geographic origin of Epilachnini (*s.l*) is still open to question, since no species in Cynegetini or Eremochilini were included in our phylogenetic tree.

Evolutionary shifts in host use

Kobayashi et al. (1998) inferred ancestral host-plant utilization for several lineages in Epilachnini. From a phylogeny including four Indonesian and six Japanese *Henosepilachna* species, they concluded that the most recent common ancestor of these species fed on Cucurbitaceae. However, their study dealt with only a small number of species in a single genus. In the present study, we examined the direction of host shifts in Epilachnini (*s.s.*) plus Epivertini by including more species and genera from a broader geographical range (Africa, Asia, Australia, and America), and analyzing host-plant use at the level of plant family rather than individual species (Fig. 4).

Our reconstruction indicated that the common ancestor of the species included (node 45) utilized host plants in Asteraceae, and also that the common ancestor excluding African *E. kaestneri kaestneri* (node 44) probably utilized Asteraceae. However, these inferences were apparently influenced by the sporadic occurrence of Asteraceae as host plants in various lineages of epilachnine beetles, including the most basal one (*E. kaestneri kaestneri*). They remain inconclusive because the species of Asteraceae actually utilized by the beetles were different (*Henosepilachna pustulosa*, *Cirsium* spp.; *H. diekei*, *Mikania* spp.; *Epilachna ocellataemaculata*, *Artemisia* sp.; *E. kaestneri kaestneri*, Asteraceae sp.; *Epiverta chelonia*, *Artemisia* sp.). Therefore, independent adoption of various species in Asteraceae by phylogenetically remote beetles cannot be ruled out.

Epilachnines frequently use members of Cucurbitaceae and Solanaceae as host-plants (e.g., Schaefer, 1983), but as shown in Fig. 4, the origins of usage differ between the two families. The frequent utilization of Cucurbitaceae is due in most cases to this trait being ancestral, especially in the species in clade B. In contrast, the utilization of solanaceous plants is a derived trait; the Asian species of *Henosepilachna* that feed on solanaceous plants are descendants of a single ancestor (node 8) that shifted in host-plant use from Cucurbitaceae to Solanaceae.

Determining the factors affecting the direction of host

shifts by phytophagous insects has become a central issue in the field of insect-plant associations. To date, three main scenarios have been proposed to explain host shifts (Becerra and Vebable, 1999). First, host shifts might be mediated by chemical similarity between the new and old host plants (Ehlich and Raven, 1964; Futuyma and McCafferty, 1990; Becerra, 1997; Ohshima and Yoshizawa, 2006). Second, host shifts could occur through parallel cladogenesis, in which the isolation of hosts together with their associated insects eventually results in allopatric co-speciation (Farrell and Mitter, 1990; Mitter et al., 1991). Third, a particular insect species might shift among host plants depending on the geographical availability of the latter (Bernays and Chapman, 1994; Dobler et al., 1996).

Molecular phylogenies of angiosperms (Soltis et al., 1999, 2000; Hilu et al., 2003) have indicated a number of cases in which several plant families commonly represented among host plants are relatively closely related to one another, but distantly related to other host-plant clades; examples include (Cucurbitaceae, Urticaceae, and Fabaceae), (Solanaceae, Acanthaceae, and Rubiaceae), and (Berberidaceae and Ranunculaceae). Asteraceae and Vitaceae are isolated families distant from the other host-plant clades. Host shifts in Epilachnini have occurred irrespective of a close phylogenetic relationship between the host plants (e.g., the shift from Curcurbitaceae to Solanaceae). It thus seems more likely that similar host chemistry and/or host-range coincidence determined the direction of host shifts in this group of beetles, although the possibility of parallel cladogenesis cannot be ruled out when we focus on host shifts occurring among plants closely related phylogenetically.

Another important question is whether host shifts play a major role in beetle speciation. Host shifts have occurred in many taxa of phytophagous insects, although the frequency or importance of host shifts relative to other modes of speciation remains unclear (Futuyma, 2008; Winkler and Mitter, 2008; Nosil, 2012). Nyman et al. (2010) recently addressed this question in a phylogenetic study of sawflies (Nematinae), in which the number of host shifts was estimated relative to the number of speciation events (= sum of internal nodes for ingroup taxa) on a tree with reconstructed ancestral states. A similar analysis applied to our phylogenetic tree (Fig. 4) shows 46 ingroup taxa requiring 45 past speciation events, whereas our reconstruction of ancestral host-plant use indicates 21 shifts, which means that 46.7% of lineage splits were correlated with host shifts. However, our reconstruction indicated a notable difference in the frequency of host shifts between major clades A (node 43 and descendants) and B (node 33 and descendants) (Figs. 1, 4). Host shifts were evidently more frequent in clade A comprising Asian *Epilachna*, *Afissula* sp. and *Epiverta chelonia* than in clade B, which contains the remaining species except for *E. kaestneri kaestneri*. In other words, clade B is more conservative than clade A with respect to host-plant utilization. When we excluded clade A, speciation events correlated with host shifts were not so frequent (39.4%). This result seems congruent with the study by Nyman et al. (2010), which suggested that the importance of niche shifts in the diversification of phytophagous insects has been implicitly and explicitly overestimated. However, our study demonstrated that the importance of host shift in speciation events may differ greatly between even closely related lineages. Relevant phylogenetic information from other insects is insufficient, and additional studies across many insect taxa will be required to assess the importance of host shifts in speciation.

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