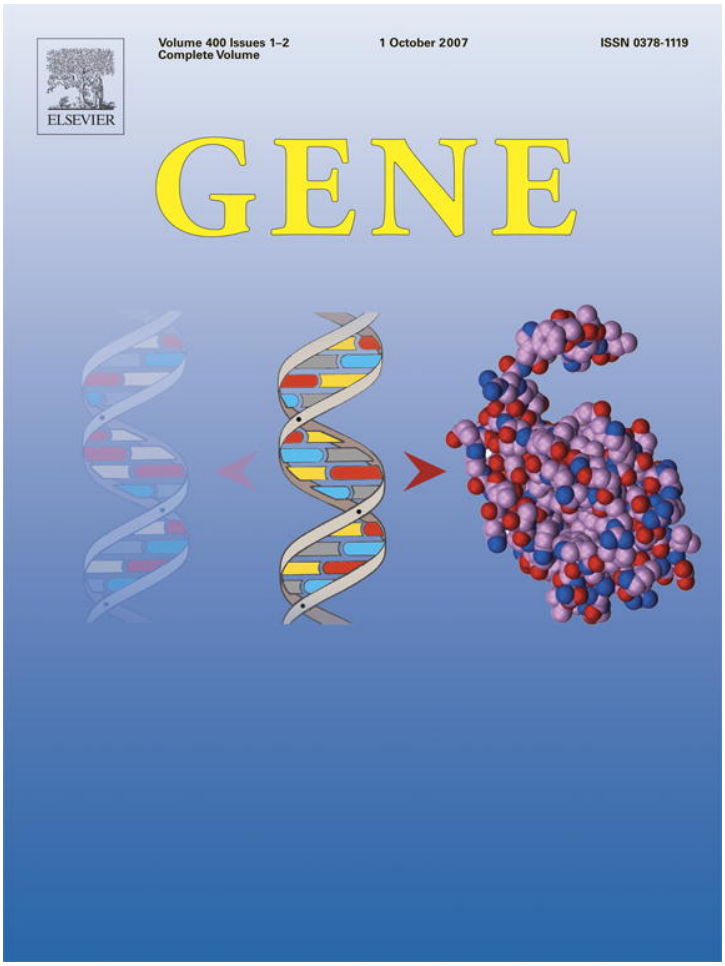

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The evolutionary process of bioluminescence and aposematism in cantharoid beetles (Coleoptera: Elateroidea) inferred by the analysis of 18S ribosomal DNA

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Abstract

Cantharoid beetles are distinctive for their leathery soft elytra and conspicuous color or bioluminescence, and many of the members are equipped with chemical defenses. Thus, the vivid coloration of Cantharidae and Lycidae and the bioluminescence in Lampyridae and Phengodidae appear to be aposematic signals. However, the evolutionary aspect of their aposematism is not well understood, because the classification of the families remains controversial. In this study, we performed molecular phylogenetic analyses of species from cantharoid families, based on nucleotide sequence comparisons of nuclear 18S ribosomal DNA. The results shows that the luminous species *Rhagophthalmus ohbai*, which had sometimes been classified in Lampyridae, is excluded from a lampyrid clade and associates with the taxa of Phengodidae. The molecular data also suggests that four major subfamilies of Cantharidae (Cantharinae, Chauliognathinae, Malthininae, and Silinae) form a clade. The six subfamilies of Lampyridae are grouped and classified into two sublineages: Amydetinae+Lampyrinae+Photurinae and Cyphonocerinae+Luciolinae+Ototretinae. Genera *Drilaster* and *Stenocladius* are the members of Ototretinae in Lampyridae. These results conform to traditional taxonomy but disagree with more recent cladistic analyses. Based on these findings, we propose an evolutionary process of bioluminescence and aposematism in cantharoids: the clades of Cantharidae, Lampyridae, Lycidae, and Phengodidae have evolved aposematic coloration; subsequently Lampyridae and Phengodidae acquired bioluminescence; and these four major cantharoid families achieved their current adaptive diversities.

Keywords: Cantharidae; Cantharoidea; Lampyridae; Lycidae; Phengodidae; Phylogeny

1. Introduction

The cantharoid beetles, which formerly composed the superfamily Cantharoidea (Crowson, 1972), include the families Cantharidae (soldier beetle), Lampyridae (firefly), Lycidae (net-winged beetle), Phengodidae (glowworm beetle), and six other small families (Lawrence, 1982). All members are currently classified in a part of Elateroidea under series Elateriformia (Lawrence and Newton, 1995).

The members of these four major cantharoid families (Cantharidae, Lampyridae, Lycidae, and Phengodidae) show several phenotypic attributes associated with chemical defense (Grimaldi and Engel, 2005). Cantharidae are often brightly colored and are known to be distasteful to vertebrate and invertebrate predators. They possess repugnatorial secretion glands at the prothorax and abdominal segments (Crowson, 1972; Brancucci, 1980; Lawrence, 1982; Brown et al., 1988). Luminosity has not been reported at any stage in any species of this family (Crowson, 1972). The chemical component of the defensive secretion in the genus *Chauliognathus* was identified

Abbreviations: LBA, long-branch attraction; ML, maximum likelihood; MP, maximum parsimony; rDNA, ribosomal DNA; TBR, tree bisection-reconnection.

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as 8-*cis*-dihydromatricaria acid (Brown et al., 1988, and references therein). Odorous 2-methoxy-3-*sec*-butylpyrazine was detected in the aposematic cantharid *Rhagonycha fulva* (Moore et al., 1990). Lycidae possess a characteristic bitter taste and are generally marked in contrasting patterns of black and red, orange, or yellow (Moore and Brown, 1981). Because of these warning colorations (Crowson, 1972), lycids are apparently mimiced by other beetles and also by certain flies and moths (Moore and Brown, 1981). No luminous species have been described in this family (Crowson, 1972; Lawrence, 1982). The major chemical components of the odor in the Australian lycid species *Metriorrhynchus rhipidius* were identified as 2-metyoxy-3-isopropylpyrazine and the bitter principles were 3-phenylpropanamide and 1-methyl-2-quinolone (Moore and Brown, 1981). Lampyridae are well known as bioluminescence, and also known to be distasteful to a number of predators (Lloyd, 1973). All known species are luminous, at least in the larval stages (Branham and Wenzel, 2003). Adults of various firefly species exhibit reflex bleeding from elytral and pronotal margins and around the antennal sockets. The viscid blood has a very bitter taste and characteristically very pungent (Ohba and Hidaka, 2002, and references therein). The eggs and larvae of lampyrids also produce light as an aposematic display to convey to predators that they are chemically defended (Underwood et al., 1997; González et al., 1999a). Thus, it seems that bioluminescence in Lampyridae had primarily evolved through a function of aposematism and was subsequently applied to courtship behavior (De Cock and Matthysen, 1999). Fireflies of the genera *Photinus* and *Photuris* contain mixtures of steroidal pyrones, lucibufagins, and *N*-methylquinolinium-2-carboxylate, which at least partially explain their distastefulness and toxicity (González et al., 1999b). The color patterns with combination of black, red, and yellow in some species of adult lampyrids appear to act as aposematic warnings (De Cock and Matthysen, 1999; Ohba and Hidaka, 2002). The bioluminescence in phengodid species is probably aposematic rather than courtship function (Grimaldi and Engel, 2005). Colored fluid secretion was observed in the genera *Phengodes*, *Zarhipis*, and *Phrixothrix*. A brownish substance in *Phrixothrix* is inflammatory (Sivinski, 1981), and a caustic odor is characteristic of *Rhagophthalmus* (Raj, 1957).

Based on morphological characteristics, evolutionary relationships within or around the cantharoid families have been proposed (Crowson, 1972; Pototskaja, 1983; Beutel, 1995; Branham and Wenzel, 2001, 2003), but the results have thus far been inconsistent. In the present study, we examined the molecular phylogeny of cantharoid species, especially of Cantharidae and Lampyridae, the most diversified families in cantharoids, based on nucleotide sequence comparisons of nuclear 18S ribosomal DNA (rDNA). We then discuss the origin of their aposematic coloration and bioluminescence.

Cantharidae are the largest and most widely distributed family in cantharoids (Crowson, 1972), comprised of approximately 135 genera and 5000 species (Lawrence, 1982). Brancucci (1980) divided Cantharidae into five subfamilies: Cantharinae, Chauliognathinae, Dysmorphocerinae, Malthininae, and Silinae. This revision is currently accepted worldwide (Lawrence and Newton, 1995). Based on the analysis of synapomorphic characteristics of adults, Brancucci (1980) also inferred the phylogenetic relationships among five subfamilies of Cantharidae. Recently, Imasaka (2004) attempted to reconstruct the phylogenetic relationships among the four major subfamilies (excluding Dysmorphocerinae) based on the morphological features of adults. His phylogram is incongruent with that of Brancucci (1980). Thus, the higher classification of the Cantharidae has not been sufficiently elucidated. In an attempt to resolve the various problems associated with the systematics of Cantharidae, we also conducted a phylogenetic analysis of the ingroup taxa based on 18S rDNA sequences.

Lampyridae occur throughout the world, with the richest faunas being in South America and Asia, comprised of approximately 100 genera and 2000 species (Lawrence, 1982). The taxonomic history of Lampyridae is complicated as reviewed in McDermott (1964) and Branham and Wenzel (2001, 2003), but eight subfamilies are currently recognized: Amydetinae, Cyphonocerinae, Lampyrinae, Luciolinae, Ototretadrilinae, Ototretinae, Photurinae, and Pterotinae (Crowson, 1972; Lawrence and Newton, 1995). Phylogenetic analyses of lampyrid species have been conducted based on the morphological characteristics of adults (Branham and Wenzel, 2001, 2003) and mitochondrial 16S rDNA sequences (Suzuki, 1997; Li et al., 2006). However, the resultant trees are inconsistent with the established taxonomy of Lampyridae. To attempt to reconcile these conflicts, we performed a phylogenetic analysis of the ingroup taxa using 18S rDNA data.

2. Materials and methods

2.1. Taxa and DNA isolation

The taxa used in this study are listed in Supplemental Table 1. To prevent the degradation of genomic DNA, collected specimens were immediately killed by immersion in 99.5% ethanol (Wako, Osaka, Japan) and stored at 4 °C until use. Total DNA was extracted from the legs of a single specimen using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2. PCR amplification and DNA sequencing

Almost complete sequences of 18S rDNA (approximately 1900 bp) were amplified using the following primers: 18S5′, 5′-GAC AAC CTG GTT GAT CCT GCC AGT-3′; 18S3′I, 5′-CAC CTA CGG AAA CCT TGT TAC GAC-3′ (Shull et al., 2001); 18S ai, 5′-CCT GAG AAA CGG CTA CCA CAT C-3′; and 18S bi, 5′-GAG TCT CGT TCG TTA TCG GA-3′ (Whiting et al., 1997). PCR was performed using a thermal cycler GeneAmp 9700 (Applied Biosystems, Foster City, CA) or a Dice Gradient TP600 (Takara, Shiga, Japan). The reaction was carried out in a volume of 10.5 μl, containing 0.38 μM of each primer, 0.19 mM of each dNTP, and 0.25 units of Ex Taq polymerase (Takara) in Ex Taq buffer (Takara). PCR cycles were as follows: 94 °C for 1 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. The amplicons

were directly sequenced using a BigDye Terminator kit (Applied Biosystems) and an ABI PRISM 3130 genetic analyzer (Applied Biosystems). All sequences were deposited in GenBank under accession nos. AB298808–AB298874.

2.3. Phylogenetic analysis

The sequences were aligned using the L-INS-i strategy of MAFFT ver. 5.734 (Katoh et al., 2002) and manually inspected. The regions of gap, ambiguity code, and uncertain alignment were eliminated from the data matrix. PAUP*4.0beta 10 (Swofford, 2002) and MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) were used to conduct phylogenetic analyses. A χ^2 test in PAUP* for homogeneity of the base frequencies across the taxa was applied to the aligned datasets.

For distance (neighbor-joining) method, we chose both standard Kimura's two-parameter and LogDet/Paralinear models, as LogDet is effective at solving the nonstationarity problem (Lockhart et al., 1994). The robustness of each branch was determined by a nonparametric bootstrap test with 1000 replicates and a tree bisection-reconnection (TBR) branch-swapping algorithm.

For maximum parsimony (MP) analysis, all sites were equally weighted. Optimal MP trees were searched by a heuristic strategy with 1000 random sequence additions and TBR branch swapping. Bootstrap values were calculated using 1000 replicates, 10 random additions per replicate, and TBR branch swapping.

For Bayesian inference, the program MrModeltest 2.2 (Posada and Crandall, 2001) was used to determine the available substitution model with the best fit to each partitioned dataset (by Akaike information criterion).

For maximum likelihood (ML) analysis, the best-fit model of nucleotide substitution by Akaike information criterion was selected using Modeltest 3.5 (Posada and Crandall, 1998). The optimal ML tree was searched using a heuristic strategy with 10 random sequence additions and TBR branch swapping.

2.4. Analysis of cantharoid beetles

Of the ten families of cantharoid beetles (Lawrence, 1982), we were able to sample specimens from five: Cantharidae, Lampyridae, Lycidae, Omethidae, and Phengodidae. In addition, the sequences of 18S rDNA of the taxa in Drilidae and Omalisidae are available in GenBank; in total, seven cantharoid families were included in the present analysis (Supplemental Table 1). As outgroups, data from other families in Elateriformia were also included (only one data of the Eucnemidae *Melasis* sp., AF451949 in GenBank, was excluded from the dataset because of its unusual long branch). Within Elateriformia, a clade comprising Scirtoidea (=Eucinetoidea; including Clambidae, Decliniidae, Eucinetidae, and Scirtidae) was assumed to be a sister to all other families (Vogler and Caterino, 2003). We therefore chose this group for a basal clade in Fig. 1. Uncorrected pairwise nucleotide differences among all taxa ranged from 0 to 11.6%. As the branch lengths of Cantharidae and Lampyridae were relatively long, the analyses were also performed after removing these taxa from the matrix (Table 1; Telford and Copley, 2005). For Bayesian analysis, we conducted two simultaneous chains for 5,000,000 generations, sampling trees every 100 cycles, and the first 12,500 trees were discarded. Majority rule consensus of the remaining trees was used to determine clade posterior probabilities.

2.5. Analysis of the Cantharidae taxa

Of the five subfamilies in Cantharidae (Brancucci, 1980), we were able to sample specimens from four: Cantharinae, Chauliognathinae, Malthininae, and Silinae (Supplemental Table 1). In this analysis, we reconstructed an unrooted tree (Fig. 2) as the rooting position was affected by the outgroup taxa chosen. The aligned matrix comprised a total of 1731 positions, of which 198 were parsimony-informative. The χ^2 test of base homogeneity indicated that the base composition is not significantly different across ingroup taxa (*P* value of the all sites is 1.000, *P* value of the informative sites is 1.000). Uncorrected pairwise nucleotide differences ranged from 0 to 6.9%. For Bayesian analysis, we conducted two simultaneous chains for 1,000,000 generations, sampling trees every 100 cycles, and discarded the first 2500 trees, and majority rule consensus of the remaining trees was used to determine clade posterior probabilities. The sequences of cantharid 28S rDNA were analyzed but not combined in this study because of the high nonstationarity across the ingroup taxa (the χ^2 test indicated that the *P* value of the informative sites was 0.000).

2.6. Analysis of the Lampyridae taxa

Of the eight subfamilies in Lampyridae (Lawrence and Newton, 1995), we were able to sample specimens from the four—Cyphonocerinae, Lampyrinae, Luciolinae, and Ototretinae—that occur in Japan (Supplemental Table 1). The partial sequences of 18S rDNA in *Photuris pennsylvanica* (Photurinae) and *Vesta* sp. (Amydetinae) available in GenBank were included in this analysis (Fig. 3). Addition of these partial data (*P. pennsylvanica*, 960 bp; *Vesta* sp., 1370 bp) decreases the position numbers in the matrix, but not affects the basal topology of the tree (see dataset L in Table 1). As outgroups, *Malthinus nakanei* and *Liponia quadricollis* are chosen. The aligned matrix comprised a total of 911 positions, of which 64 were parsimony-informative. The χ^2 test of base homogeneity indicated that the base composition was not significantly different (*P* value of the all sites is 1.000, *P* value of the informative sites is 0.989). Uncorrected pairwise nucleotide differences among ingroup taxa ranged from 0 to 4.5%. For Bayesian analysis, we conducted two simultaneous chains for 1,000,000 generations, sampling trees every 100 cycles, and discarded the first 2500 first trees, and majority rule consensus of the remaining trees was used to determine clade posterior probabilities. The sequences of lampyrid 28S rDNA were preliminary analyzed but not combined in the analysis because of the high nonstationarity across the ingroup taxa (the χ^2 test indicated that the *P* value of the informative sites was 0.003).

3. Results

3.1. Cantharoids

18S rDNA has been utilized for phylogenetic analyses of various taxonomic levels in eukaryotes, including Coleoptera (Korte et al., 2004, and references therein). In this study, we conducted molecular phylogenetic analyses of cantharoid beetles using 18S rDNA sequences from Elateriformia. We also analyzed the other molecular markers preliminarily, such as EF-1α and COI, but these data did not resolve the relationships between the taxa (data not shown). Although 28S rDNA partially resolve the

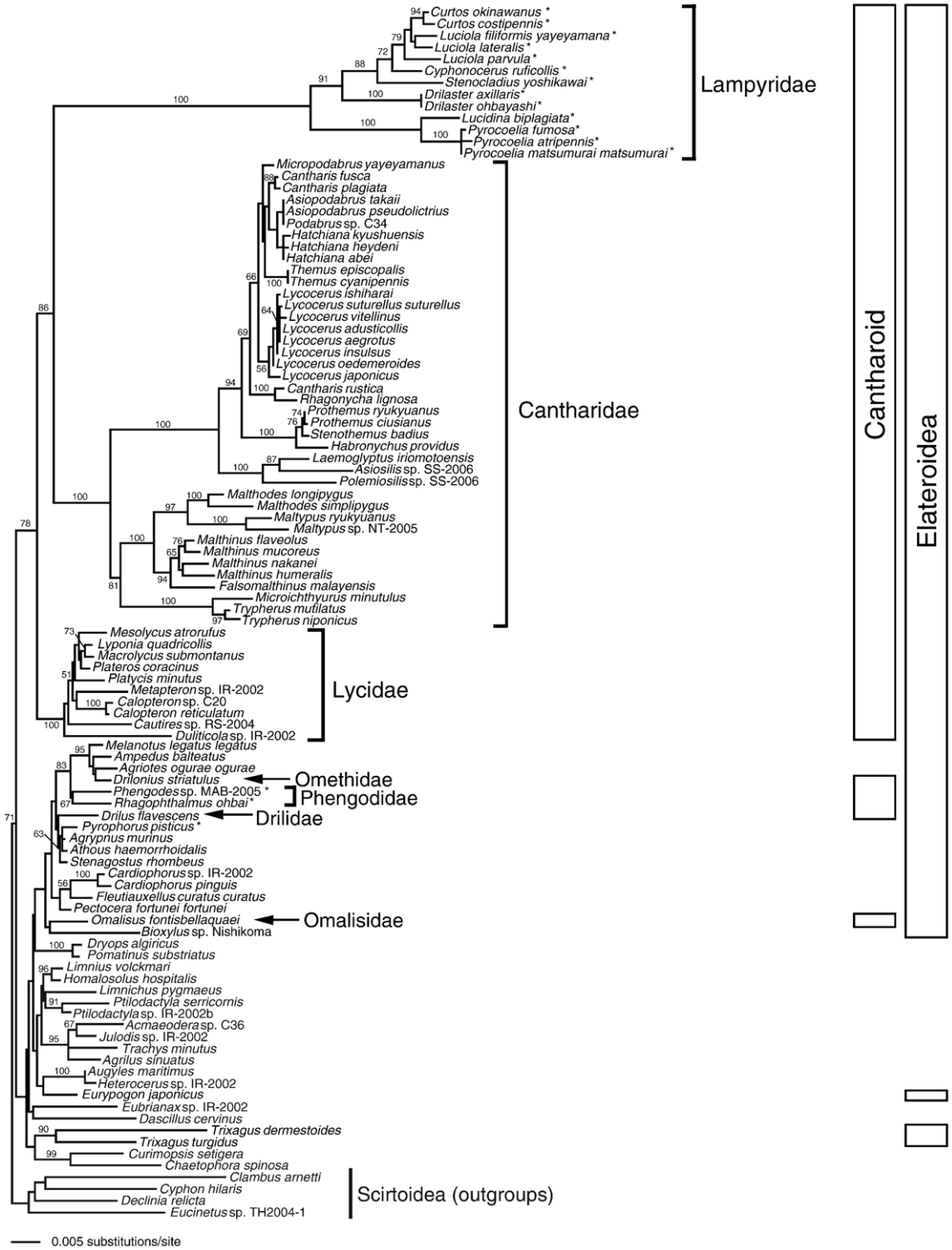

Fig. 1. Phylogenetic tree of cantharoid beetles and related taxa resulting from the distance method (LogDet) based on analysis of the 18S rDNA (dataset E in Table 1). Bootstrap values above 50% are shown on the nodes, and the taxa not belonging to Elateroidea are shown in gray. The luminous taxa are indicated by asterisks.

Table 1
Statistical supports for the nodes

Dataset	Taxon number	Position no. (informative)	*P* value of χ^2 test (informative)	Node	Bootstrap % (distance)		Bootstrap % (MP)	Posterior probability
					Kimura	LogDet		
E (Fig. 1)	104	1624 (338)	1.000 (0.000)	L	**100**	**100**	**100**	**1.00**
				La	**100**	**100**	**100**	**1.00**
				Lu+Cy+Ot	80	**91**	**93**	**1.00**
				C	**100**	**100**	**100**	**1.00**
				Ch+Ma	76	81	–	–
				Ca+Si	**100**	**100**	**100**	**1.00**
				Ca+Si+Ch	–	–	–	0.91
				Ma	**99**	**100**	**92**	**1.00**
				L+C	60	86	–	–
				Y	**99**	**100**	**93**	**0.99**
				P	70	67	–	0.69
E–L	91	1704 (297)	1.000 (0.000)	C	**100**	**100**	**100**	**1.00**
				Ch+Ma	66	70	–	–
				Ca+Si	**100**	**100**	**100**	**1.00**
				Ca+Si+Ch	–	–	59	**0.95**
				Ma	**100**	**100**	**96**	**1.00**
				Y	**100**	**100**	**100**	**1.00**
				P	70	69	–	**0.97**
E–C	64	1631 (262)	1.000 (0.203)	L	**100**	**100**	**100**	**1.00**
				La	**100**	**100**	**100**	**1.00**
				Lu+Cy+Ot	**92**	**97**	**94**	**1.00**
				Y	**100**	**99**	**95**	**1.00**
				P	89	87	–	0.90
E–L–C	51	1718 (201)	1.000 (0.819)	Y	**100**	**100**	**100**	**1.00**
				P	81	81	63	**1.00**
L	13	1775 (167)	1.000 (0.553)	La	**100**	**100**	**100**	**1.00**
				Lu	**99**	**99**	**94**	**1.00**
				Ot	84	83	63	**1.00**
				Lu+Cy	**92**	**91**	75	0.78

E = Elateriformia (excl. *Photuris pennsylvanica* and *Vesta* sp.); L = Lampyridae (excl. *P. pennsylvanica* and *Vesta* sp.), C = Cantharidae, Y = Lycidae, P = Phengodidae, La = Lampyrinae, Lu = Luciolinae, Cy = Cyphonocerinae, Ot = Ototretinae, Ch = Chauliognathinae, Ma = Malthininae, Ca = Cantharinae, Si = Silinae.
Bootstrap values of >90% and Bayesian posterior probabilities of >0.95 are shown in bold letters.
All Bayesian inferences were performed under a model of GTR+I+Γ.

tree, the combination analysis with 18S rDNA didn't improve the statistical support values for the nodes.

As a result, all the phylogenetic analyses reject the monophyly of both Elateroidea and cantharoids. The affinity between Cantharidae and Omethidae inferred by adult morphological characteristics (Crowson, 1972; Branham and Wenzel, 2001, 2003; Imasaka, 2004) is not supported by our analyses (Fig. 1). Sister grouping of Cantharidae and Lampyridae is supported by the distance analyses of 18S rDNA (Fig. 1 and Table 1) and also by 28S rDNA analysis (Sagegami-Oba et al., 2007). However, this grouping may result from long-branch attraction (LBA; Telford and Copley, 2005). The χ^2 test indicated that nucleotide frequencies of informative sites are nonstationary across the taxa in Elateriformia (Table 1); when the taxa of Cantharidae and Lampyridae are eliminated from the matrix, the frequencies of the remaining sequences become stationary (Table 1). To detect the LBA artifact, comparing molecular results with morphological evidence might be useful (Bergsten, 2005). In this regard, no morphological study had implied their close relationship (Crowson, 1972; Pototskaja, 1983; Beutel, 1995; Branham and Wenzel, 2001, 2003). Therefore, we conclude that the affinity of Cantharidae and Lampyridae inferred by the present molecular data will be an artifact.

3.2. Cantharidae

The taxonomic status of Cantharidae has been occasionally disputed; Miskimen (1961) raised Chauliognathinae to a family, and Branham and Wenzel (2001, 2003) recovered a polyphyletic Cantharidae by cladistic analysis. On the other hand, our phylogenetic analysis suggested that the taxa in four major subfamilies of Cantharidae—Cantharinae, Chauliognathinae, Malthininae, and Silinae—form a clade (Fig. 1 and Table 1). Of the four subfamilies, Cantharinae and Silinae are more closely related (Fig. 2). The same result was obtained by the analysis of the 28S rDNA dataset (data not shown). The root position of Cantharidae was estimated from the results shown in Table 1. Two possible roots are proposed as shown in Fig. 2. The same root positions were also estimated by 28S rDNA analysis (data not shown). The phylogeny of Cantharidae proposed here is incongruent with the previous inference by Brancucci (1980; Fig. 4A), while is in agreement with the phylogram proposed by Imasaka (2004; Fig. 4B). Based on synapomorphic characteristics of adults, Brancucci (1980) divided Malthininae into three tribes: Malthinini, Malthodini, and Malchinini. In this analysis, the taxa of *Falsomalthinus* and *Malthinus* (both belonging to Malthinini) form a clade, and the taxa of *Malthodes* and

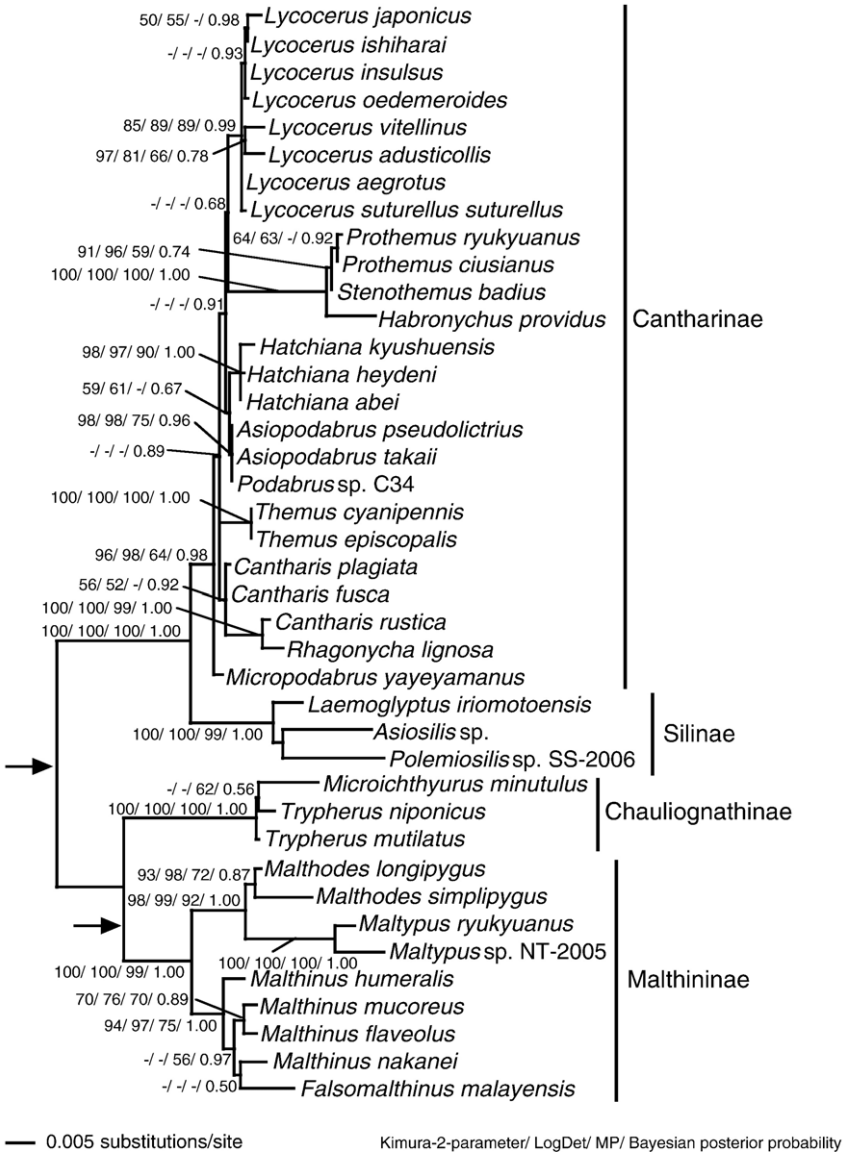

Fig. 2. Unrooted tree of the taxa in Cantharidae resulting from the ML analysis based on the 18S rDNA under the GTR+I+Γ model of substitution. Bayesian inference was also performed under a model of GTR+I+Γ. Values of distance (Kimura's two-parameter) bootstrap/distance (LogDet) bootstrap/MP bootstrap/Bayesian posterior probabilities (above 50%) are indicated on the nodes. The arrows indicate two possible root positions.

Maltypus (both belonging to Malthodini) form another clade (Fig. 2). Malchinini (the single genus *Malchinus*=*Macrocerus*) was not collected. Recently, the genera *Lycocerus*, *Athemus*, *Athemellus*, *Mikadocantharis*, *Andrathemus*, and *Isathemus* were combined into single genus *Lycocerus* (Okushima, 2005). This revision fits our present results in that all *Lycocerus* analyzed in this study (consisting of former *Athemus*, *Athemellus*, *Mikadocantharis*, and *Andrathemus*) are closely associated (Fig. 2).

3.3. Lampyridae

The taxonomic status of Lampyridae has been argued by several authors (McDermott, 1964; Crowson, 1972; Suzuki, 1997; Branham and Wenzel, 2001, 2003; Li et al., 2006). In our molecular analysis of cantharoids, the taxa in the four subfamilies of Lampyridae (Cyphonocerinae, Lampyrinae, Luciolinae, and Ototretinae) form a clade with strong statistical support (Fig. 1). When the partial sequences of 18S rDNA in *P. pennsylvanic*a (Photurinae) and *Vesta* sp. (Amydetinae) were included in the analysis, these taxa placed in a clade of Lampyridae (data not shown). Next, using 18S rDNA sequences of ingroup taxa, phylogenetic relationship was reconstructed between the four subfamilies in Lampyridae (dataset L in Table 1), and the six subfamilies (+Amydetinae and Photurinae, Fig. 3). The result indicates the association of the genera *Stenocladius* (Ototretinae) and *Drilaster* (Ototretinae), which was not supported by 16S rDNA data (Suzuki, 1997), with good statistical values (Fig. 3 and Table 1). Based on male morphological characteristics of 85 taxa, Branham and Wenzel (2001, 2003) inferred the phylogeny of cantharoid beetles. In their analysis, the taxa of Lampyridae were indicated

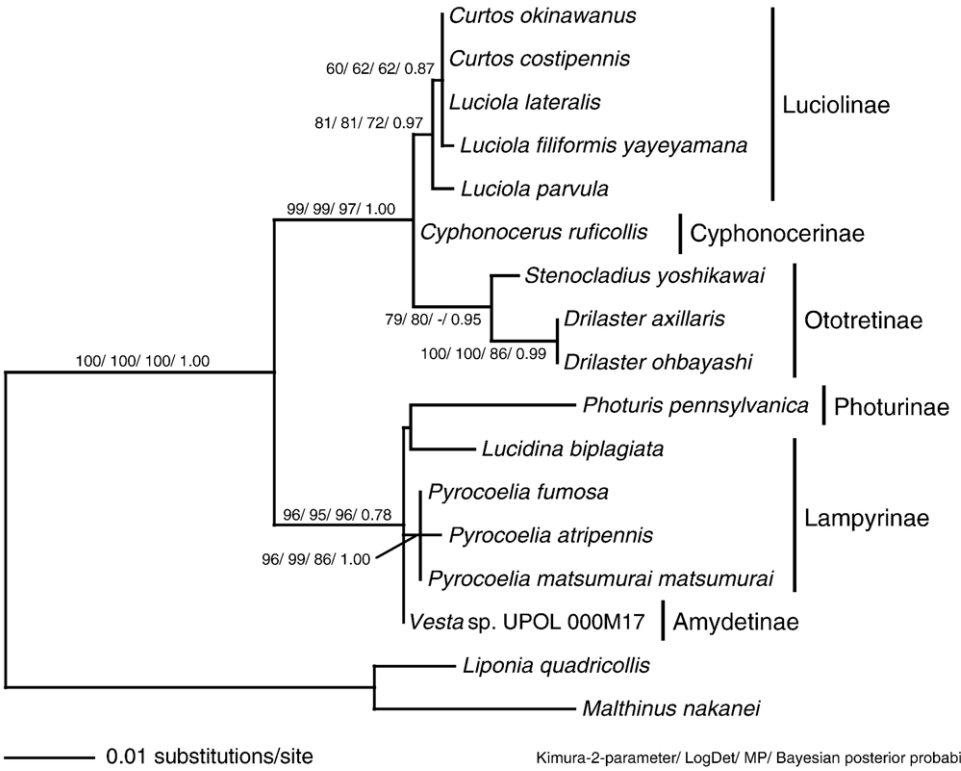

Fig. 3. Phylogenetic tree of the taxa in Lampyridae resulting from the ML analysis based on the 18S rDNA under the TrN+I+Γ model of substitution. Bayesian inference was performed under a model of GTR+I+Γ . Values of distance (Kimura's two-parameter) bootstrap/distance (LogDet) bootstrap/MP bootstrap/Bayesian posterior probabilities (above 50%) are indicated on the nodes.

to be polyphyletic; *Stenocladius* formed a clade with Phengodidae but not with Lampyridae, while *Drilaster* was not placed within any existing family group (Branham and Wenzel, 2003; Table 2). In order to assess these inconsistencies, it may be necessary in the future to compare morphologies between the specimens used in our analysis and used by Branham and Wenzel (2001, 2003). In our analysis, Lampyridae are resolved into two major lineages: Amydetinae+Lampyrinae+Photurinae and Luciolinae+Cyphonocerinae+Ototretinae (Fig. 3). The rooting position was estimated between these two lineages. This rooting was quite stable even when any outgroup taxa were chosen (data not shown), and was also supported by preliminary analysis of 28S rDNA (Sagegami-Oba et al., 2007). As the number of taxa analyzed in this study is unsatisfactory, we will not discuss the evolution of the various photic behavior in Lampyridae, as was done by Suzuki (1997) and Branham and Wenzel (2003).

3.4. Phengodidae

The family Phengodidae consists of two subfamilies (Crowson, 1972; Lawrence, 1982; Lawrence and Newton, 1995): Phengodinae, which are geographically restricted to the New World, and Rhagophthalminae, which are restricted to the Old World (Lawrence, 1982). The taxonomic positions of these two subfamilies have been discussed at length; McDermott (1964) placed Rhagophthalminae under Lampyridae, while Wittmer and Ohba (1994) raised it to a separate family, Rhagophthalmidae. Molecular analysis of 16S rDNA indicated that Rhagophthalminae is a member of Lampyridae (Suzuki, 1997; Li et al., 2006, 2007), although the bootstrapping value was low. Branham and Wenzel (2001, 2003) considered Rhagophthalminae a sister group of Lampyridae and separate from Phengodinae. On the other hand, our 18S rDNA data indicate that *R. ohbai* (Rhagophthalminae) is a sister group of

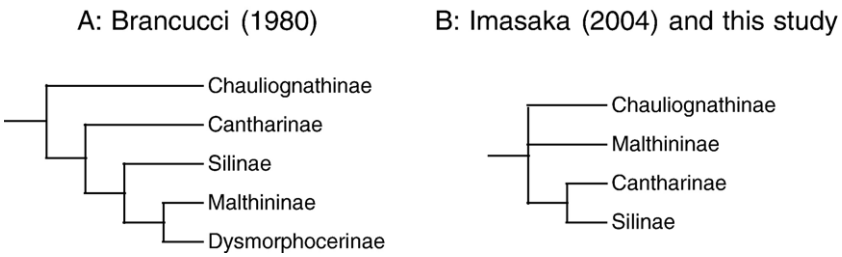

Fig. 4. Hypotheses of basal cantharid relationships by Brancucci (1980, A), and by Imasaka (2004) and the present molecular study (B).

Table 2
The cladistic and taxonomic placement of *Drilaster*, *Stenocladius*, Rhagophthalminae, and Phengodinae

	Crowson (1972), Lawrence and Newton (1995)	Suzuki (1997)	Branham and Wenzel (2001)	This study
Drilaster	Lampyridae (Ototretinae)	Grouped under Lampyridae	*Incertae sedis*	Grouped under Lampyridae
Stenocladius		Grouped under Lampyridae	Phengodidae	
Rhagophthalminae	Phengodidae		Rhagophthalmidae	Single group
Phengodinae		–	Phengodidae	

Phengodes sp. (Phengodinae) (Fig. 1, Tables 1 and 2). When the taxa of Cantharidae and Lampyridae, which have long branches, were removed from dataset, the supporting values increased (Table 1). The rhagophthalmid–phengodid association is also indicated by the analysis of 28S rDNA (data not shown). Moreover, comparisons among beetle luciferase genes indicated that the amino acid sequence of *R. ohbai* is more similar to those of *Phrixothrix* (Phengodinae) than those of Lampyridae (Viviani, 2002). These molecular data indicates that Rhagophthalminae and Phengodinae are members of the family Phengodidae sensu Crowson (1972).

3.5. Lycidae

Lycidae, along with Cantharidae and Lampyridae, is one of the three largest families in cantharoids. This family is a sharply defined and easily recognizable group in both adult and larval stages (Crowson, 1972), so the monophyly of Lycidae has not been questioned. Lycid species are currently divided into seven subfamilies (Bocak and Matsuda, 2003). In our molecular analysis, the taxa of five subfamilies and the single taxon *incertae sedis*, *Duliticola* sp., form a clade with high bootstrap support (Fig. 1 and Table 1), but their intrarelationships are not clearly resolved.

4. Discussion

In this study, we performed molecular phylogenetic analyses of species from cantharoid families based on nucleotide sequence comparisons of nuclear 18S rDNA to elucidate the evolutionary process of their bioluminescence and aposematism. The results showed that: four major subfamilies of Cantharidae form a clade; *R. ohbai* (Rhagophthalminae) is a member of Phengodidae; genera *Drilaster* and *Stenocladius* are in the clade of Lampyridae; the taxa in Lycidae form a clade. Our present findings conform well to traditional systematics, thus reconciling some recent systematic inconsistencies (Table 2), suggesting that reconsideration of the evolution of bioluminescence and aposematism in cantharoids is warranted.

4.1. Evolution of bioluminescence

One of the most remarkable characteristics found in Coleoptera is bioluminescence, and its origin is an interesting and significant problem (Crowson, 1972). Six families of luminous beetles have been described worldwide: Lampyridae, Omalisidae, Phengodidae, Elateridae (genus *Pyrophorus* and others), Throscidae (the single species *Balgus schnusei*, which is sometimes included in Elateridae; Chassain, 2003), and Staphylinidae (Costa et al., 1986), of which the first five belong in Elateroidea, and the first three are cantharoids. Among these, the luminosity of Omalisidae is questionable (Burakowski, 1988). Crowson (1972) wrote, "it is unlikely that the luminosity of *Pyrophorus* and of various Cantharoidea derived from a common ancestor." Recently, based on molecular phylogenetic analysis of 28S rDNA, we proposed that the ancestral state of Elateridae was non-luminous, despite the common mechanisms of bioluminescence among Lampyridae, Phengodidae, and Elateridae (Sagegami-Oba et al., 2007). Our present molecular data indicate that luminous taxa of cantharoids are arranged into two groups: Lampyridae and Phengodidae. Hence, we consider herein that luminous cantharoids are categorized into two distinct families that originated from a non-luminous ancestor.

Based on analyses of morphological characteristics, Crowson (1972) suggested that Lampyridae and Phengodidae were directly related and speculated that their luminosity would be attributed to inheritance from a common ancestor. These close relationships were also supported by Beutel (1995) primarily because both families are bioluminescent. On the other hand, our present results do not support the Lampyridae–Phengodidae affinity. Therefore, it is expected that the origin of bioluminescence in cantharoids is either once, as Crowson (1972) speculated, or twice—that is, Lampyridae and Phengodidae acquired luminosity independently. We speculate that the former (single origin of the luminosity in Lampyridae and Phengodidae) is more likely, because luciferases in these taxa are apparently similar compared to that of Elateridae (Viviani, 2002). Previously, we found that the homologous genes of beetle luciferase in *Drosophila melanogaster* and *Tenebrio molitor* (Tenebrionidae, Coleoptera) encode fatty acyl-CoA synthetase, suggesting that beetle luciferase evolved from fatty acyl-CoA synthetase (Oba et al., 2006). Thus, the parallel evolution of enzyme, from fatty acyl-CoA synthetase to luciferase, might occur in two independent lineages (Lampyridae + Phengodidae and Pyrophorinae in Elateridae).

4.2. Evolution of aposematism

Several warning defenses are found in insects, such as stings, disagreeable secretions, and odors. These insects are explicable to evolve the aposematic display. The evolution of distastefulness, on the other hand, presents a problem, since any individual tasted would almost certainly perish. In this regard, Fisher (1930) interpreted that distasteful aposematism has evolved by kin selection. The conspicuous visual signal is realized not only by vivid color pigmentation but also by iridescence (Vulinec,

1997) and bioluminescence (De Cock and Matthysen, 1999). The aposematic display was observed in four major cantharoid families: Cantharidae and Lycidae (in which coloration occurs with distastefulness and odor), and Lampyridae and Phengodidae (in which coloration and bioluminescence occur with distastefulness and odor). The aposematism has not been described in other small families of cantharoids. The evolutionary aspects of aposematism in cantharoids are still not fully understood because the systematics of cantharoids has remained controversial.

Branham and Wenzel (2001, 2003) hypothesized based on their cladistic analysis that bioluminescence arose once in the early evolutionary history of the cantharoid clade (which contains Omalisidae, Rhagophthalminae, and the major part of Lampyridae) and was subsequently lost in the glade of taxa (which contains Lycidae, Cantharidae, Omethidae, and Telegeusidae), then later regained in the clade of Phengodidae and *Stenocladius*. Thus, their hypothesis indicates that chemical defenses and warning colors in Cantharidae and Lycidae have taken over the original role of bioluminescent aposematism seen in other cantharoids, including Rhagophthalminae and Lampyridae (Grimaldi and Engel, 2005). On the other hand, our present results demonstrate that genera *Drilaster* and *Stenocladius* are included in Lampyridae, and that Rhagophthalminae is grouped with Phengodinae (Table 2). It is therefore more conceivable that Cantharidae, Lampyridae, Lycidae, and Phengodidae have evolved aposematic displays (such as bright coloration and/or bioluminescence) independently or carried over the common ancestral aposematic coloration (and Lampyridae and Phengodidae subsequently acquired bioluminescence). In fact, species in Cantharidae, Lampyridae, and Lycidae employ unrelated components for their distastefulness (the component in Phengodidae is unknown). We presume that four major families of cantharoids—Cantharidae, Lampyridae, Lycidae, and Phengodidae—had developed distasteful, odorous or toxic components (and their own detoxification mechanism) independently, although they might originally possess a common defensive component, and achieved their current adaptive diversities. In this regard, it will be interesting to analyze the components of the chemical defense in Phengodidae.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2007.06.004.

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