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Molecular phylogeny of the leaf beetle subfamily Criocerinae (Coleoptera: Chrysomelidae) and the correlated evolution of reproductive organs

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Abstract

Phylogenetic relationships among major groups of Criocerinae were reconstructed using molecular data (mitochondrial cytochrome oxidase I and 12S rDNA, and nuclear histone 3). The monophyly of Criocerinae was consistently and robustly supported. The *Lema* group including *Lema*, *Oulema* and *Neolema* was recovered as a clade, with the latter two genera imbedded within *Lema*. The *Lilioceris* group was placed as the sister taxon of the *Lema* group, and the genus *Crioceris* was identified as the sister taxon of the *Lilioceris* + *Lema* groups. The monophyly and/or validity of *Mecoprosopus* Chûjô, 1951 and the subgenera *Lema*, *Petauristes* Latreille, 1829, *Quasilema* Monrós, 1960, *Microlema* Pic, 1932, and *Bradyceris* Chûjô, 1951 were not confirmed. The monophyly of the subgenus *Lema* except for the type species *L. cyanea* was supported by molecular and morphological data, and we termed it the *cyanella* clade. The present molecular phylogeny was compared with previous concepts with respect to the validity of each genus/subgenus. A revision of several genera is necessary. Based on the phylogenic result, the character evolution of the reproductive organs was analyzed. The ancestral states of this character system were parsimoniously reconstructed. Various shapes of the spermatheca were observed in the subfamily. A convoluted spermatheca evolved once, and reversals to the ancestral state took place several times independently. An elongation of a part of the intromittent organ also occurred several times independently. The length of the male and female reproductive ducts, which are in physical contact during copulation, showed a tight positive correlation even after removing phylogenetic effects. This strongly suggests coevolution between the male and female genital length.

Key words

Lema, *Lilioceris*, *Crioceris*, *Neolema*, *Mecoprosopus*, *Oulema*, flagellum, spermatheca.

1. Introduction

The subfamily Criocerinae Latreille, 1804 (Coleoptera: Polyphaga: Chrysomelidae) is one of the possible basal branches of the mega-diverse herbivorous family Chrysomelidae (Farrell & Sequeira 2004; Gómez-Zurita et al. 2007, 2008 but see Lee 1993; Farrell 1998; Reid 1995, 2000, see also Schmitt 1996 and Suzuki 1996 for a historical review of inter-subfamily relationships). The subfamily comprises ca. 1,200–1,500 species (Monrós 1960; Schmitt 1988, 1996; Vencl & Leschen 2014) and is divided into three tribes and ca. 20 genera (Seeno & Wilcox 1982). Most species belong to five species-rich genera, *Crioceris* Muller, 1764, *Lilioceris* Reitter, 1912, *Lema* Fabricius, 1798, *Oulema* Gozis, 1886, and *Neolema* Monrós, 1951 (Vencl & Leschen 2014). These taxa

have a worldwide distribution except for *Neolema*, which is known only from the New World. The smaller genera include only few species (less than 20) and show a more restricted distribution (Monrós 1960).

The subfamily was established in the early 19th century, but the species belonging to the group have already been described in the 18th century, and most of the tribes, genera, and subgenera were established in this early era of entomology (Monrós 1960; Seeno & Wilcox 1982). In the field many species are difficult to obtain in large series. As a result, the intraspecific variability has not been fully assessed, and several synonyms exist for many species (see Schmitt 2010; Warchałowski 2010, 2011). Additionally, older descriptions are not always sufficient to identify species unambiguously. The body shape is relatively uniform within the subfamily, and differences in color have been used as the most important diagnostic feature at the species level, without considering intraspecific variation (Warchałowski 2011). These factors have impeded the establishment of a reliable classification. In addition, the demarcation of genera and subgenera is insufficient, especially in *Lema* and potentially related genera. *Oulema* and *Neolema*, for instance, were treated as independent genera in some studies (Gressitt & Kimoto 1961; Kimoto & Gressitt 1979; Schmitt 1985a,b, 1990, 2010; Kimoto & Takizawa 1994; White 1993; Warchałowski 2010, 2011), while other authors classified them under the genus *Lema* (Monrós 1951, 1960; Mohr 1966, 1985; Warchałowski 1985). Another problematic case is the subgenus *Microlema*, which was treated as a synonym of the subgenus *Lema* by Seeno & Wilcox (1982), then as an independent subgenus by Kimoto & Takizawa (1994) and Warchałowski (2010, 2011), and again as a synonym of the subgenus *Lema* by Schmitt (2010). These taxonomic concepts and changes were proposed without giving explicit reasons, and the validity of each genus is still insufficiently established. The major genera are mainly defined based on inconspicuous features, such as for instance fused versus separate bases of claws, arrangements of punctures on the elytra, the angle of the X-shaped groove on the vertex, and the length/width ratio of the head capsule. The number of taxonomic revisions focused on Criocerinae or broader higher taxa including Criocerinae is increasing (Kimoto & Gressitt 1979; White 1981, 1993; Schmitt 1990, 2010; Vencl et al. 2004; Warchałowski 2010, 2011; Matsumura et al. 2011; Tishechkin et al. 2011; Lee & Matsumura 2013), but a solid phylogenetic background based on formal analysis with a broad sampling of taxa and characters is urgently required.

In contrast to the unsatisfying taxonomic and systematic situation, the morphology and ecology of members of the subfamily are well documented (stridulatory organs: Schmitt & Traue 1990; reproductive organs: Düngelhoef & Schmitt 2005; Matsumura & Suzuki 2008; Matsumura & Yoshizawa 2012; host plants: Schmitt 1988; Jolivet & Hawkeswood 1995; Vencl et al. 2004; plant-insect interactions: Schmitt 1988; Aiello & Vencl 2006; Vencl & Nishida 2008; chemical defense: Morton & Vencl 1998; Vencl & Morton 1998, 1999). This wealth of data is an excellent basis for evolutionary studies on the group, but a solid phylogenetic hypothesis for the subfamily is a necessary pre-requisite for well-founded interpretations. Evolutionary hypotheses presented by Schmitt (1985a,b), Teo (1999), Vencl & Morton (1998) and Vencl et al. (2004) (Fig. 1) are valuable contributions, but not fully convincing due to insufficient sampling of taxa and/or data, and the lack of a well-supported phylogeny of Criocerinae.

In the present study we address the phylogenetic relationships in the subfamily with molecular data covering all major genera. Based on the obtained tree we discuss the validity of the systematic concepts proposed in previous studies. In an evolutionary context, the extreme elongation of genitalic structures observed in this subfamily is one of the most conspicuous phenomena. Some species in Criocerinae have an extremely elongated spermathecal duct and flagellum, i.e. a prolonged sclerotized tube at the end of the ejaculatory duct (Lindroth 1957), and the male flagellum is accommodated in a specialized pocket of the internal sac (Matsumura & Suzuki 2008; Matsumura & Yoshizawa 2012). The flagellum is inserted into the spermathecal duct during copulation (Matsumura & Akimoto 2009). Even though correlated evolution between male and female genital traits in this group has already been suggested by Matsumura & Suzuki (2008), phylogenetic effects were not taken into consideration in that study. The structural diversity of the spermatheca is also known as a conspicuous feature in this group (Matsumura & Suzuki 2008). Based on the phylogenetic hypothesis presented here, we formally evaluate the evolutionary transitions of both the male and female reproductive organs.

2. Materials and methods

2.1. Specimens examined and molecular data acquisition

We used specimens collected recently (2006–2011) and preserved in 99.5% ethanol. Only in one case (*Lema* (*Lema*) *saigonensis* Pic, 1923 collected in Malaysia) we used a dried specimen killed with ethyl acetate. The ingroup included 42 individuals from 38 species. Six species of other chrysomelid subfamilies and one species of Cerambycidae were chosen as outgroup taxa (Tab. 1). Except for two ingroup species belonging to a New World subgenus, all species were collected in the Palearctic region. The voucher repository is shown in Table 1.

DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan) or the modified Chelex method (Walsh et al. 1991). Primer sets CI-J-2183 (Si-

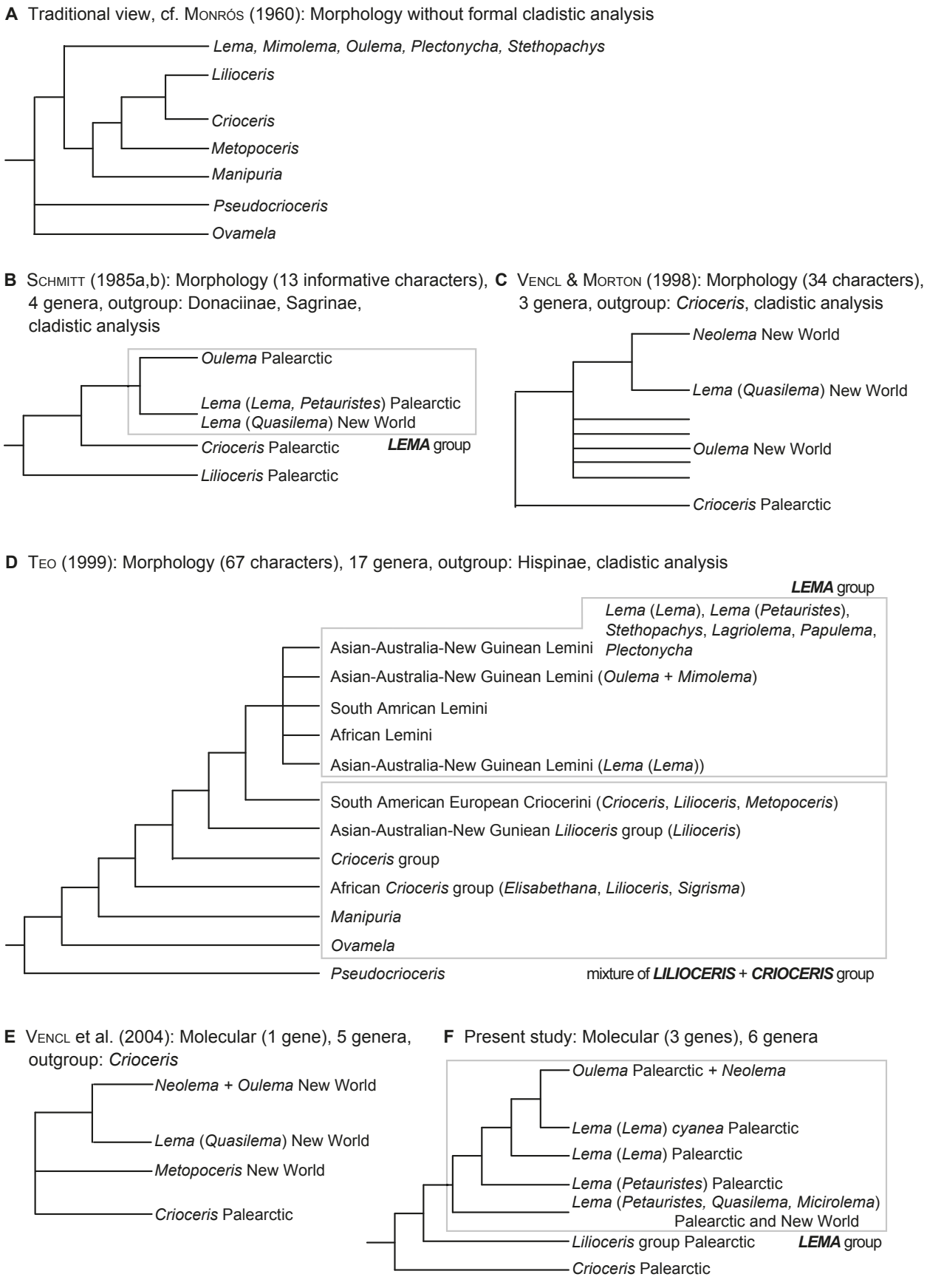


Fig. 1. Comparison of preceding and present phylogenetic hypotheses.

Table 1. Taxa studied. SEHU: Systematic Entomology of Hokkaido University, KSP: Kunio Suzuki Private Collection.

Taxa	Collection locality	Voucher (YK-)	Voucher repository
Tribe Criocerini			
Crioceris orientalis Jacoby, 1885	Japan, 2008	56	SEHU
Crioceris quatuordecimpunctata (Scopoli, 1763)	Japan, 2006	51	KSP
Lilioceris (*Bradyceris*) *lewisi* (Jacoby, 1885)	Japan, 2006	50	KSP
Lilioceris (*Lilioceris*) *impressa* (Fabricius, 1787)	Thailand, 2011	30	SEHU
Lilioceris (*Lilioceris*) cf. *impressa*	Malaysia, 2007	35	SEHU
Lilioceris (*Lilioceris*) *rugata* (Baly, 1865)	Japan, 2006	49	KSP
Lilioceris (*Lilioceris*) *schneideri* (Weise, 1990)	Hungary, 2010	37	SEHU
Lilioceris (*Lilioceris*) *subpolita* (Motschulsky, 1861)	Japan, 2006	47	KSP
Mecoprosopus sp.1	Malaysia, 2007	36	SEHU
Mecoprosopus sp.2	Thailand, 2011	34	SEHU
Tribe Lemiini			
Lema (*Lema*) *cambodiae* Kimoto & Gressitt, 1979	Malaysia, 2009	42	SEHU
Lema (*Lema*) *cirsicola* Chûjô, 1959	Japan, 2008	10	SEHU
Lema (*Lema*) *concinnipennis* Baly, 1865	Japan, 2008	5	SEHU
Lema (*Lema*) *coronata* Baly, 1873	Japan, 2008	7	SEHU
Lema (*Lema*) *cyanea* Fabricius, 1798	Malaysia, 2009	44	SEHU
Lema (*Lema*) *cyanea* Fabricius, 1798	Thailand, 2011	31	SEHU
Lema (*Lema*) *cyanella* (Linnaeus, 1758)	Japan, 2009	4	SEHU
Lema (*Lema*) *delauneyi* Baly, 1889	Malaysia, 2009	43	SEHU
Lema (*Lema*) *delicatula* Baly, 1873	Japan, 2008	1	SEHU
Lema (*Lema*) *dilecta* Baly, 1873	Japan, 2008	8	SEHU
Lema (*Lema*) *diversa* Baly, 1873	Japan, 2008	9	SEHU
Lema (*Lema*) *lacertosa* Lacordire, 1845	Malaysia, 2009	40	SEHU
Lema (*Lema*) *praeus*ta (Fabricius, 1792)	Vietnam, 2009	11	SEHU
Lema (*Lema*) *rondoniana* Kimoto & Gressitt, 1979	Vietnam, 2009	12	SEHU
Lema (*Lema*) *saigonensis* Pic, 1923	Malaysia, 2009	14	SEHU
Lema (*Lema*) *saigonensis* Pic, 1923	Thailand, 2011	29	SEHU
Lema (*Lema*) *scutellaris* (Kraatz, 1879)	Japan, 2008	6	SEHU
Lema (*Lema*) sp. 1	Malaysia, 2009	17	SEHU
Lema (*Lema*) sp. 2	Malaysia, 2009	41	SEHU
Lema (*Microlema*) *decempunctata* (Gebler, 1830)	Japan, 2006	46	KSP
Lema (*Microlema*) *decempunctata* (Gebler, 1830)	Japan, 2009	2	SEHU
Lema (*Petauristes*) *honorata* Baly, 1873	Japan, 2006	45	KSP
Lema (*Petauristes*) *indica* Jacoby, 1895	Thailand, 2011	32	SEHU
Lema (*Petauristes*) *palpalis* Lacordaire, 1845	Thailand, 2011	33	SEHU
Lema (*Petauristes*) *quadripunctata* (Olivier, 1808)	Malaysia	15	SEHU
Lema (*Quasilema*) *trilinea* White, 1981	USA	13	SEHU
Neolema eremita (Jacoby, 1888)	USA	71	SEHU
Neolema eremita (Jacoby, 1888)	USA	72	SEHU
Neolema eremita (Jacoby, 1888)	USA	73	SEHU
Oulema atrosuturalis (Pic, 1923)	Japan, 2008	3	SEHU
Oulema erichsonii Suffrian, 1841	Romania, 2010	39	SEHU
Oulema rufocyanea (Suffrian, 1847)	Hungary, 2010	38	SEHU
Outgroup taxa			
Plateumaris sericea (Linnaeus, 1760) (Donaciinae)	Japan, 2007	53	SEHU
Plateumaris weisei (Duvivier, 1885) (Donaciinae)	Japan, 2011	55	SEHU
Sagra femorata (Drury, 1773) (Sagrinae)	Japan, 2009	52	SEHU
Chlamisus laticollis (Chûjô, 1942) (Chlamisinae)	Japan, 2011	54	SEHU
Syneta adamsi Baly, 1877 (Synetinae)	Japan, 2010	57	SEHU
Plagosterma aenea (Linnaeus, 1758) (Chrysomelinae)	Japan, 2007	58	SEHU
Lemula rufithorax (Pic, 1901) (Lepturinae, Cerambycidae)	Japan, 2006	48	KSP

mon et al. 1994) and R2760 (CGA CGA GGC ATA CCT CTA AGT CCT: K. Odagiri, personal communication) or COI-internal-R (CCA TGT ARD GTT CCY ATT CA), 12Sai and 12Sbi (Simon et al. 1994), HexAF + HexAR (Colgan et al. 1998) were used for amplification of partial regions of COI, 12S, and Histone 3 (= H3), respectively. PCR condition was as follow: 94°C for 1 minute followed by 40 cycles of 94°C for 30 s, 45–50°C for 30–45 s, and 72°C for 60 s. PCR products were purified and sequenced following the methods described in Yoshizawa & Johnson (2003).

Alignment of the partial sequences of COI (266 bp), 12S (277–340 bp), and H3 (330 bp) was performed with ClustalW (Larkin et al. 2007). Alignments of 12S were manually adjusted according to the similarity criterion (Simmons 2004). Although the utility of structural alignment for rDNA was discussed in Marvaldi et al. (2009), here it was not adopted because the region analyzed was quite conservative. Ambiguously aligned regions in 12S sequences were selected manually and excluded from the analyses. Then all sequence data were combined and analyzed using maximum likelihood (ML) and Bayesian inference. For ML analyses we used PAUP* (Swofford 2002) with TBR branch swapping and a NJ starting tree. The best fit substitution model was estimated using Akaike Information Criterion (AIC) as implemented in jModeltest 2.1.1 (Darriba et al. 2012) and the TMP1uf+I+G model was selected. We also performed 1000 ML bootstrap pseudoreplicates in PhyML using the same substitution model. For Bayesian analysis, we separated characters by gene and then by codon position for COI and H3, resulting in seven partitions (12S and the three codon positions of the COI and H3 genes). The best fit model was estimated independently for each partition using AIC as implemented in MrModeltest (Nylander 2004), resulting in 12S and H3 third position (GTR+G), H3 first position (SYM), and COI and H3 second position (JC). Detailed parameters and commands for ML and Bayesian analyses are all described in the online supplementary data matrix. Bayesian analysis was conducted in MrBayes 3.2 (Ronquist & Huelsenbeck 2012) with two runs of four chains each for 2,000,000 generations and trees sampled every 1000 generations. Stationarity and convergence of runs were judged based on Average Standard Deviation of Split Frequencies, which was below 0.01 after 2 million MCMC generations. The first 50% of trees were discarded as a burnin, and a 50% majority consensus tree of the remaining trees was used to calculate posterior probabilities. In addition to the bootstrapping and posterior probability, the robustness of the tree was tested using an approximately unbiased test (AU test) using CONSEL (Shimodaira 2002), by contrasting the best ML tree with those estimated by constraining some alternative relationships (see below). Nexus files of the aligned sequences are available online (El. Suppl. 1). Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number(s): AB862319–AB862360 for 12S, AB862361–AB862399 for COI, and AB862400–AB862437 for H3.

Although homologous sequences of partial COI used in the present study were found for several species in GenBank, the added matrix of the data only increased instability of tree estimation. Therefore we did not include those sequences for the analyses.

2.2. Morphological data acquisition and character transformation

We analyzed evolutionary transformations of characters of the male and female copulatory contact area: length of flagellum and spermathecal duct and shape of the spermatheca. The data used in the study were mainly acquired from Berti & Rapilly (1976), Hayashi (2004, 2005), Matsumura & Suzuki (2008), Matsumura et al. (2011), Matsumura & Yoshizawa (2012), and Lee & Matsumura (2013). Additionally we studied relevant anatomical features by manual dissection under a stereomicroscope (Leica MZ 125; Wetzlar, Germany) and a scanning electronic microscope (Philips XL 30 ESEM, Royal Philips, Amsterdam, The Netherlands). The measurements of the male and female elongated parts of the reproductive organs were performed using the method described in Matsumura & Yoshizawa (2010). Extracted genitalic parts were put on a glass slide using euparal or glycerin as an embedding medium. Then we took pictures of the mounted specimens and printed them. The length was measured using the printed images and a curvimeter (Koizumi COMCURVE-9 Junior, Japan). The elytral lengths were measured using micrometers as indicators of a body size.

We reconstructed ancestral states of the morphological characters by parsimony criterion using Mesquite 2.75 (Maddison & Maddison 2011). For continuous traits the ancestral states were reconstructed using linear-change parsimony. Felsenstein's (1985) method of comparing phylogentically independent contrasts was used for testing correlated evolution among characters using PDAP package (Midford et al. 2010). As the phylogenetic trees reconstructed with Bayesian and ML analyses differ only slightly and the ML tree is better resolved, we used the latter for reconstructing evolutionary transitions.

3. Results

3.1. Molecular phylogeny among major clades

The data matrix contained a total of 460 variable sites. Bayesian and maximum likelihood analyses of the data matrix yielded almost identical topologies except for

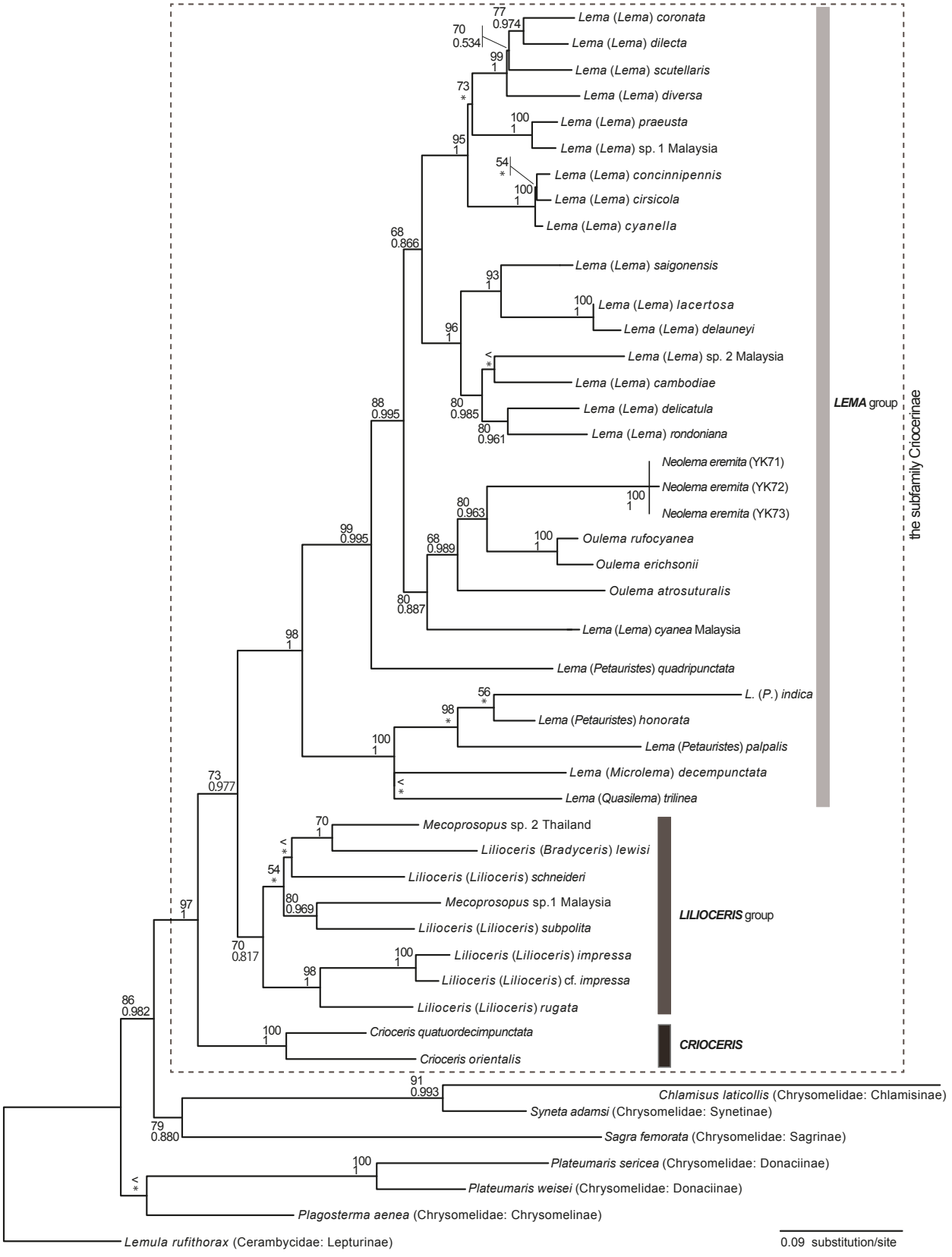


Fig. 2. A tree estimated by the most likelihood analysis. Branch lengths are proportional to reconstructed changes. The numbers on the branches indicate the bootstrap supports (upper) and posterior probability values (lower) of the nodes. An asterisk indicates that the node is not supported by Bayesian analysis and '<' indicates bootstrap support lower than 50%.

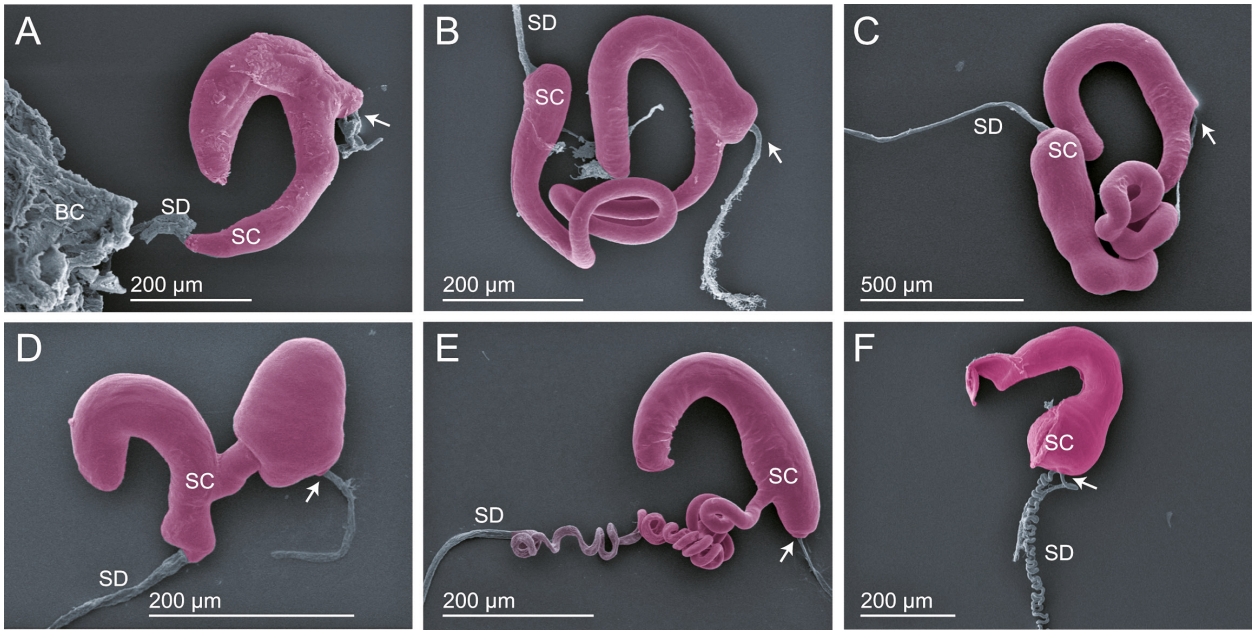


Fig. 3. Variations of spermatheca. **A**: *Crioceris quatuordecimpunctata*; **B**: *Lema* (*Microlema*) *decempunctata*; **C**: *Lilioceris* (*Lilioceris*) cf. *impressa*; **D**: *Lema* (*Lema*) *delauneyi*; **E**: *Neolema eremita*; **F**: *Cassida denticollis*. BC – bursa copulatrix, SC – spermathecal capsule, SD – spermathecal duct. Arrows indicate opening of gland to spermatheca. The pink colored area indicates the spermathecal capsule.

the arrangements of few weakly supported clades. Fig. 2 shows the tree obtained with the ML analysis, and the Bayesian tree is available online: El. Suppl. 2 and 3. The monophyly of the subfamily is well supported (bootstrap value > 90%, posterior probability = 1) (Fig. 2).

The genus *Crioceris* was recovered as the sister group of the remaining Criocerinae (*Lema* group + *Lilioceris* group) (Fig. 2). This relationship was only moderately supported (73 BS, 0.98 PP). An alternative pattern (*Crioceris* + *Lema* group) could not be rejected statistically but the p-value is marginal ($P = 0.064$ by AU test). The monophyly of both the *Lilioceris* group and the *Lema* group was supported. *Neolema* and *Oulema* were imbedded within the genus *Lema*, and the monophyly of *Oulema* was not supported. The monophyly of the genus *Lema* (excluding *Neolema* and *Oulema*) was rejected statistically by AU test ($P = 0.003$). The subgenus *Lema* was supported excluding *Lema* (*Lema*) *cyanea* Fabricius, 1798, although the bootstrap value and posterior probability for this branch were low (ML = 68; Bayesian = 0.86; Fig. 2). The subgenus *Petauristes* of *Lema* was not supported as *Lema* (*Petauristes*) *quadripunctata* (Olivier, 1808) was placed as sister group of *Lema* (*Lema*) + *Oulema* + *Neolema* with high support values (99 BS, 0.995 PP). The other three of the four species of *Petauristes* examined here formed a clade with *Lema* (*Microlema*) *decempunctata* (Gebler, 1830) and *Lema* (*Quasilema*) *trilinea* White, 1981. The monophyly of the subgenus *Petauristes* was rejected statistically by AU test ($P < 0.001$). *Lilioceris* and *Mecoprosopus* were not supported by ML and Bayesian analyses (Fig. 2). The monophyly of *Lilioceris* was also rejected by AU test ($P = 0.028$) but not the monophyly of *Mecoprosopus* ($P = 0.102$ by AU test).

3.2. Spermathecal shape

The character states and measurements of the intromittent organs, spermathecae, and male and female body size are summarized in Table 2, El. Suppl. 4, and Fig. 3. In particular the shape and degree of complexity of the spermathecae varies conspicuously between the species (Fig. 3). In some of them a simple spermatheca shaped like a question-mark is present (Fig. 3A), whereas some others have the proximal part of the spermatheca elongated and convoluted (Fig. 3B,C). The presence of a peculiar, large bulb (Fig. 3D) was observed in two species of the genus *Lema*. The spermatheca of *Neolema eremita* (Jacoby, 1888) (Fig. 3E) shows a quite different condition as the demarcation between the spermathecal capsule and duct is indistinct (i.e., a coiled part can be interpreted either as a part of the spermatheca or the spermathecal duct). The coiled area of *N. eremita* is relatively strongly sclerotized with a brown coloration which is the typical feature for the spermathecal capsule of Criocerinae. Therefore we tentatively address the coiled part of *Neolema eremita* (Fig. 3E) as a part of the spermathecal capsule.

The shape of the spermatheca in the examined outgroup taxa is simple except for *Chlamisus laticollis* Chûjô, 1942 (Chrysomelidae: Chlamisinae), which is characterized by an irregularly folded spermathecal duct (not shown here, Fig. 3F is an image of a different but closely related species; in *C. laticollis* the spermathecal duct has a more irregular shape).

Table 2. The list of morphological characters and their states. Data references 1) Berti & Rapilly (1976); 2) Hayashi (2004, 2005); 3) Matsumura & Suzuki (2008); 4) Matsumura et al. (2011); 5) Matsumura & Yoshizawa (2012); 6) Lee & Matsumura (2013). * A simple spermatheca shaped like a question-mark; ** the proximal part of the spermatheca elongated and convoluted; *** the data came from *Mecoprosopus* sp. 3 (West Sumatra). **** A tube was found in outgroup taxa, but it was not sure whether the tube was homologous to the flagellum in Criocerinae or not.

	Flagellum accommodated in a pocket?	**Shape of spermatheca**	**Flagellum length** [mm]	**Sperma-thecal duct length** [mm]	**Male elytral length** [mm]	**Female elytral length** [mm]
Tribe Criocerini						
Crioceris orientalis	[5] no	–	–	–	–	–
Crioceris quatuordecimpunctata	[5] no	[3] simple *	–	–	–	–
Lilioceris (*Bradyceris*) *lewisi*	[5] no	[3] simple *	–	–	–	–
Lilioceris (*Lilioceris*) *impressa*	[5] no	convoluted **	–	–	–	–
Lilioceris (*Lilioceris*) near *impressa*	–	convoluted **	–	–	–	–
Lilioceris (*Lilioceris*) *rugata*	no	[3] convoluted **	–	–	–	–
Lilioceris (*Lilioceris*) *schneideri*	no	[1] simple *	–	–	–	–
Lilioceris (*Lilioceris*) *subpolita*	[5] no	[3] convoluted **	–	–	–	–
Mecoprosopus sp.1	–	–	–	–	–	–
Mecoprosopus sp.2	no	simple ***	–	–	–	–
Tribe Lemiini						
Lema (*Lema*) *cambodiae*	[5] yes	simple *	[5] 10.35	10.35	3.52	4.55
Lema (*Lema*) *cirsicola*	[4] yes	[3] simple *	[3] 3.24	[3] 3.48	[4] 4.34	[4] 4.68
Lema (*Lema*) *concinnipennis*	[4] yes	[3] simple *	[3] 3.68	[3] 4.12	[4] 3.81	[4] 4.14
Lema (*Lema*) *coronata*	[5] yes	[3] simple *	[3] 11.15	[3] 13.90	3.59	3.56
Lema (*Lema*) *cyanea*	[5] no	convoluted **	–	–	–	–
Lema (*Lema*) *cyanella*	[4] yes	[4] simple *	[4] 2.05	[4] 1.90	[4] 3.54	[4] 3.30
Lema (*Lema*) *delauneyi*	[5] yes	simple with large bulb near gland opening	[5] 1.52	0.33	3.25	3.59
Lema (*Lema*) *delicatura*	[5] yes	[3] simple *	[3] 0.60	[3] 0.41	3.18	2.55
Lema (*Lema*) *dilecta*	[5] yes	[3] simple *	[3] 4.20	[3] 4.96	2.33	2.65
Lema (*Lema*) *diversa*	[5] yes	[3] simple *	[3] 0.93	[3] 1.62	3.72	3.65
Lema (*Lema*) *lacertosa*	[5,6] yes	[6] simple with large bulb near gland opening	[6] 1.58	[6] 0.43	[6] 3.15	[6] 3.57
Lema (*Lema*) *praeusta*	[5] yes	simple *	[5] 4.65	–	–	–
Lema (*Lema*) *rondoniana*	–	–	–	–	–	–
Lema (*Lema*) *saigonensis*	yes	simple *	[5] 2.08	0.33	3.98	4.11
Lema (*Lema*) *scutellaris*	[5] yes	[3] simple *	[3] 2.12	[3] 2.50	3.54	3.65
Lema (*Lema*) sp. 1	–	–	–	–	–	–
Lema (*Lema*) sp. 2	yes	–	[5] 1.95	–	3.18	–
Lema (*Microlema*) *decempunctata*	[5] no	[3] convoluted **	–	–	–	–
Lema (*Petauristes*) *honorata*	[5] no	[3] convoluted **	–	–	–	–
Lema (*Petauristes*) *indica*	no	convoluted **	–	–	–	–
Lema (*Petauristes*) *palpalis*	[5] no	convoluted **	–	–	–	–
Lema (*Petauristes*) *quadripunctata*	[5] no	convoluted **	–	–	–	–
Lema (*Quasilema*) *trilinea*	[5] no	–	–	–	–	–
Neolema eremita	yes	convoluted **	3.25	2.70	3.00	3.31
Oulema atrosuturalis	no	–	–	–	–	–
Oulema erichsoni	[5] short flagellum	convoluted **	–	–	–	–
Oulema rufocyanea	short flagellum	convoluted **	–	–	–	–
Outgroup taxa						
Plateumaris sericea (Donaciinae)	short tube ****	[2] simple *	–	–	–	–
Plateumaris weisei (Donaciinae)	short tube ****	[2] simple *	–	–	–	–
Sagra femorata (Sagrinae)	short tube ****	simple *	–	–	–	–
Chlamisus laticollis (Chlamisinae)	tube in lumen of ejaculatory duct ****	simple *	–	–	–	–
Syneta adamsi (Synetinae)	short tube ****	simple *	–	–	–	–
Plagosterma aenea (Chrysomelinae)	thick short tube ****	–	–	–	–	–
Lemula rufithorax (Cerambycidae: Lepturinae)	–	–	–	–	–	–

3.3. Evolution of male and female reproductive traits

Parsimony reconstructions were performed for (1) the elongated flagellum accommodated in a specialized pocket of the internal sac (Fig. 4), (2) the shape of the female spermathecal capsule (Fig. 4), and (3) the length of the flagellum (Fig. 5). The ancestral state was unambiguously reconstructed for all characters.

The elongated flagellum evolved independently in the clades formed by *Neolema* and the subgenus *Lema* (Fig. 4). The shape of the spermatheca is more variable, and a simple spermathecal capsule was identified as the plesiomorphic state in the Criocerinae (Fig. 4). The convoluted spermathecal capsule was acquired in the common ancestor of the *Lema* + *Lilioceris* group (Fig. 4). In the common ancestor of the subgenus *Lema* and a part of the *Lilioceris* group, this character state was reversed to the ancestral condition. The large bulb on the spermathecal capsule was acquired by the common ancestor of *Lema lacertosa* Lacordaire, 1845 + *L. delauneyi* Baly, 1889 (Figs. 3D, 4). The analyses show no distinct correlated pattern between acquisitions of the flagellum + pocket and the spermathecal shape (Fig. 4).

In spite of a relative uniformity in body size, the length of the flagellum and spermathecal duct was highly variable (Table 2 and El. Suppl. 4). There were no significant correlations between the genital size and body size in both sexes (the flagellum vs the male body size: $r^2 = 0.06$, $F_{1,11} = 0.75$, $P = 0.40$; the spermathecal duct vs the female body size: $r^2 = 0.19$, $F_{1,11} = 2.587$, $P = 0.14$, Fig. 5A,B). The flagellum length and the spermathecal length were highly correlated ($r^2 = 0.98$, $F_{1,11} = 596$, $P < 0.0001$, Fig. 5C), and a least squared regression revealed the flagellum to be positively correlated to the spermathecal duct (slope: 0.83).

The ancestral state reconstruction of the male flagellum length is shown in Fig. 6. An overall trend is the increasing elongation of the flagellum in the clade of the subgenus *Lema*. Especially in *Lema coronata* Baly, 1873 and *L. cambodiae* Kimoto & Gressitt, 1979, the flagellum was extremely elongated independently.

4. Discussion

4.1. Phylogeny and systematics of Criocerinae

Because of the limited taxon sampling, the discussion will be focused on estimated relationships among major clades. Especially, some potentially basal groups of the subfamily (*Ovamela* and *Pseudocrioceris*) (Monrós 1960; Teo 1999) and endemic groups were not included in our analyses. A more complete taxon sampling is necessary for recovering the deepest branching events as discussed below. However we consider a comparison of our phylogeny based on molecular data with the previous contributions (Monrós 1960; Schmitt 1985a,b; Vencl & Morton 1998; Teo 1999; Vencl et al. 2004; summarized in Fig. 1) is a start point for establishing reliable criocerine relationships.

The monophyly of the subfamily Criocerinae was consistently and strongly supported by the molecular data (Figs. 1F, 2). Morphologically, the subfamily has been characterized by the following apomorphies: stridulatory organs on pygidium present, dorsally opening larval anus (Monrós 1960; Schmitt 1985a,b, 1988; Vencl et al. 2004), three setae on larval labral disc, and larval segments I–VIII with ambulatory warts (cf. Schmitt 1985b, 1988).

Three major clades, the genus *Crioceris*, the *Lilioceris* group and the *Lema* group (sensu Schmitt 1985a,b), were identified in the present analyses (Figs. 1F, 2). The placement of the *Lilioceris* group as sister of the *Lema* group is in contrast to Schmitt (1985a,b), who proposed a clade *Crioceris* + *Lema* group and suggested the following synapomorphies for it: (1) stridulatory file on pygidium undivided, (2) two or three pairs of setae inserted on external angle of larval labrum, (3) postcubital vein of hind wings reduced, (4) reduced number of setae on the mesoscutum. However, as already discussed in Schmitt (1985a,b), the latter two character states (3, 4) are reductions and may have evolved independently (Schmitt 1985b). As the complexity of the second character is low and information is unavailable for many species it is quite unreliable (Schmitt 1985a). After extensive study of the stridulatory organ on the pygidium Schmitt (1990) suggested that the first character may also have evolved convergently, and Teo (1999) also found this feature in *Crioceris* species. Apparently there is little morphological evidence supporting a clade *Crioceris* + *Lema* group (Fig. 1A). In contrast to this, the sister group relationship between *Lilioceris* and the *Lema* group received moderate to strong support values (73 BS, 0.977 PP). This suggests that the widely accepted tribe Criocerini (Seeno & Wilcox 1982) including *Crioceris* and *Lilioceris* is probably paraphyletic. Our morphological survey suggests that an elongated and convoluted spermathecal capsule is a synapomorphy of *Lilioceris* and the *Lema* group (Fig. 4), and Suzuki (1969) suggested the relationship based on hind wing venations. An alternative relationship (*Crioceris* + *Lema* group) could not be rejected statistically but the *P*-value was marginal ($P = 0.064$ by AU test). Teo (1999) which included broader taxa pointed out that *Crioceris* may be imbedded in the *Lilioceris* group and either the *Lilioceris* group and *Crioceris* may be paraphyletic (Fig. 1D), and the grouping of three major clades is still problematic.

The monophyly of the genus *Crioceris* was well supported (99.8 BS, 1.0 PP) and morphological and ecological data tentatively support this (Schmitt 1985a,b). Potential autapomorphies are (1) asparagus-feeding and (2)

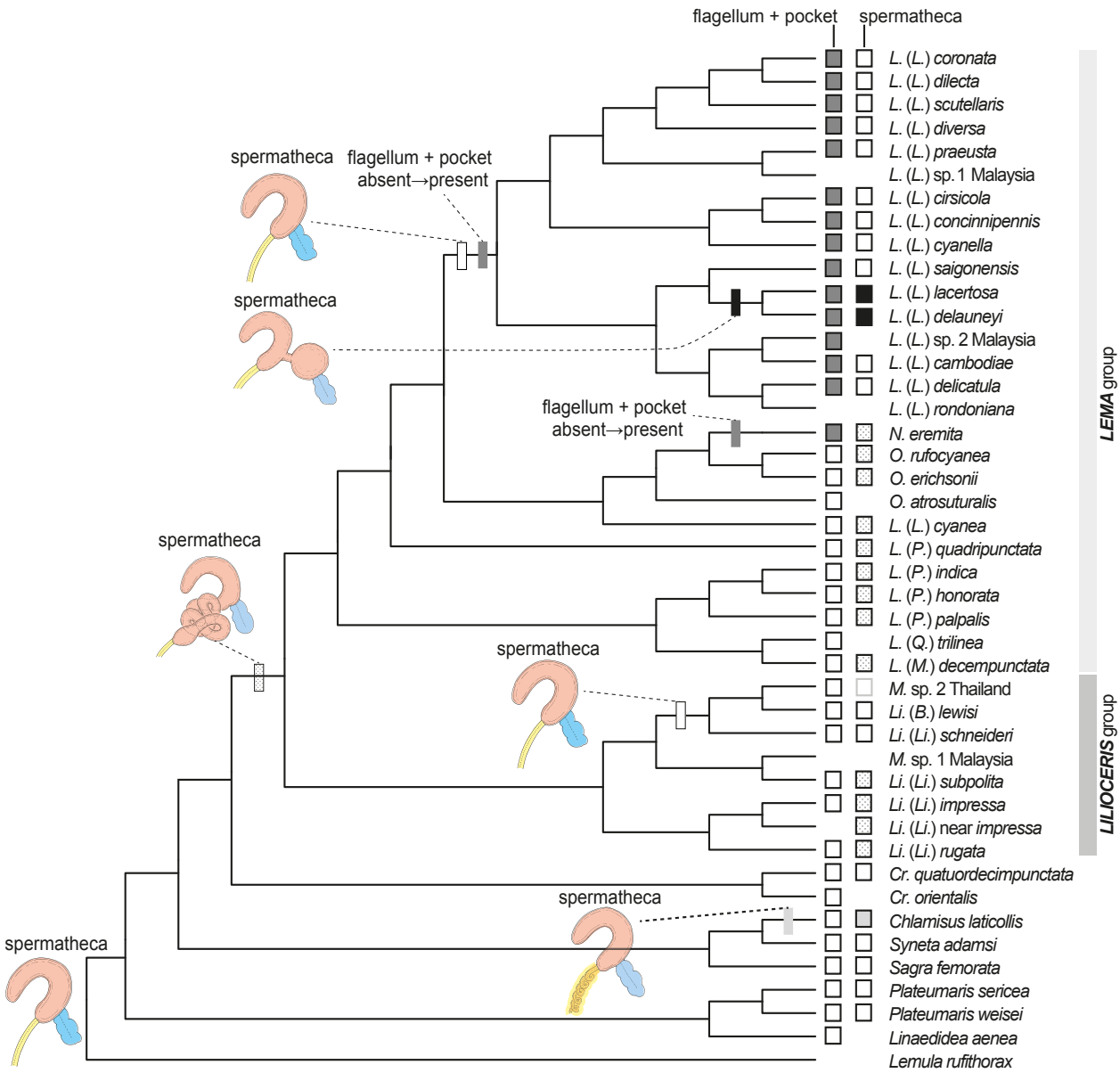


Fig. 4. Most parsimonious optimization of male and female reproductive organs on the ML tree. Colors in the squares indicate character states of the internal sac (left column) and spermatheca (right column). In the left column, gray squares mean the flagellum and specialized pocket is present, and white means absence of these features. In the right column, white indicates simple (Fig. 3A), black indicates simple plus a large bulb (Fig. 3D), stippling indicates elongated and convoluted (Fig. 3B,C,E), and gray indicates that the spermathecal duct is convoluted (Fig. 3F); gray lines in *Mecoprosopus* sp. 2 tentatively assigns simple type as this character state was observed in morphologically similar species (YM pers. obs.). Pink in the spermatheca indicates the spermathecal duct, yellow the spermathecal duct, and blue the spermathecal gland.

aedeagus with a notched apex (a similar state was also observed in Sagrinae; SCHMITT 1985a,b). A single spur on the tibial apex (two spurs in others) is an additional potential autoapomorphy (SCHMITT 1985a,b). This result is compatible with the results of the analyses of TEO (1999) who suggested additional potential autapomorphies: (3) the epipleuron neither reaching the apex nor the base, (4) hind tibiae with one spur apically, and (5) the AA_{1+2} vein connected to CuA_{3+4}. Although so far relatively few species were covered in phylogenetic analyses, the type species *Crioceris asparagi* (Linnaeus, 1758) was included in SCHMITT (1985a,b) and TEO (1999). Therefore we should provisionally classify species possessing the above mentioned character states as a part of a monophyletic group *Crioceris*, as long as phylogenetic analyses with a dense taxon sampling are not available.

Lilioceris + *Mecoprosopus* (= *Lilioceris* group: Figs. 1D, 2) received only low to moderate support (70 BS, 0.82 PP). Based on *Lilioceris* (*Lilioceris*) and *Lilioceris* (*Chujoita*) Monrós, 1960, SCHMITT (1985a,b) proposed a deeply divided vertex as an autapomorphy of the *Lilioceris* group. The blunt dorsal plate of the aedeagus was also mentioned as a derived character state, but this feature is poorly investigated and presently not sufficiently

established as an autapomorphy (Schmitt 1985a). Teo (1999) suggested paraphyletic *Crioceris* + the *Lilioceris* group containing several monophyletic subunits and emphasized the necessity of a revision of these groups. The ambiguity of our results supports her point of view.

The subgenus *Bradyceris* and the genus *Mecoprosopus*, both included in the *Lilioceris* group, were established by Chûjô (1951). However, some authors did not accept their independent status (Monrós 1960; Schmitt 2010). Diagnostic characters of the subgenus *Bradyceris* (only *Lilioceris* (*Bradyceris*) *lewisi* (Jacoby, 1885)) are the lack of a scutellar row of punctures on the elytra and some other less significant features (Monrós 1960). In our phylogeny this subgenus is placed inside the subgenus *Lilioceris*, which supports Schmitt's (2010) taxonomic treatment. *Mecoprosopus* comprises two described species (Schmitt 2010). Although they were not included in the present analyses, *Mecoprosopus* sp. 1 and *M.* sp. 2 analyzed here can apparently be assigned to this genus based on unusual morphological features such as an elongated head and protruding compound eyes, which are diagnostic for the taxon (Chûjô 1951). As both features are quantitative and evolutionary transformations of proportions of the head shape can be explained easily, Monrós (1960) treated this genus as a subgenus of the genus *Lilioceris*. The present analyses did not support the monophyly of *Mecoprosopus*, although this could not be rejected statistically (P = 0.102 by AU test). The conspicuous elongation of the head probably evolved independently in the genus *Lilioceris* (Fig. 2).

As previously suggested by Schmitt (1985a,b), Vencl & Morton (1998), Teo (1999) and Vencl et al. (2004), the monophyly of the *Lema* group including the genera *Lema*, *Oulema*, and *Neolema* was strongly supported by our data (Figs. 1, 2; 98 BS, 1.0 PP). A possible synapomorphy is the fused base of the claws (Schmitt 1985a,b; Vencl & Morton 1998). The clade *Neolema* + *Oulema* (Teo 1999; Vencl et al. 2004) is imbedded within the genus *Lema*, implying its paraphyly (Figs. 1F, 2). The monophyly of *Lema* was also rejected statistically by AU test. The genus *Lema* is highly diverse and difficult to define based on consistent diagnostic features (Monrós 1960). In contrast, *Neolema* and *Oulema* are relatively easily distinguished from *Lema* by conspicuous differences in the shape of the pronotum and the angle of the X-shaped groove on the vertex (Monrós 1960; Schmitt 1990). Our results suggest that a pronotum shaped as in *Lema* is plesiomorphic whereas the *Oulema/Neolema*-type is specialized and derived within the *Lema* clade.

The present analyses suggest that the subgenera, *Microlema-Petauristes-Quasilema* are closely related with each other, with very strong bootstrap and Bayesian supports (Fig. 2). Monrós (1951) established the subgenus *Quasilema* within the genus *Lema* based exclusively on vicariance with *Petauristes* (New World vs Old World). There are no distinct morphological features to distinguish the two taxa, but *Petauristes* species mainly feed on Monocotyledoneae whereas *Quasilema* species mainly prefer Solanaceae (Schmitt 1988; but certain overlap is known: Schmitt 1988; Vencl & Leschen 2014). The subgenus *Microlema* consisting only of *Lema* (*Microlema*) *decempunctata* was established by Pic (1932), characterized by the following inconspicuous features: 1) punctures of scutellar row much smaller than those of the other rows and 2) pronotum without any transverse impression. Schmitt (2010) treated the species as a member of the subgenus *Lema* but our molecular data did not support this concept. Given the absence of clear diagnostic characters, it is evident that a revision of these subgenera is necessary.

The type species of the genus *Lema*, *L.* (*L.*) *cyanea* (Selman & Smith 1967; ICZN 1970) was placed as sister group of the *Neolema* + *Oulema* clade, and the remaining species of the subgenus *Lema* formed a separate clade (Figs. 1F, 2). The subgenus *Lema* including *L. cyanea* was defined by obscure morphological features, such as the number of elytral rows of punctures or their arrangement. In contrast the subgenus *Lema* excluding *L. cyanea* supported by our molecular data can be defined by a peculiar feature of the internal sac, the flagellum and the specialized pocket (Table 2 and El. Suppl. 4) (see Matsumura & Yoshizawa 2012 for detailed anatomical data). This is a useful and distinct diagnostic character to distinguish members of this large clade. It should be treated as a separate subgenus. There exist several synonyms of the genus *Lema*, and it was impossible from a literature survey to trace whether previously proposed type species (of the synonymized genera) possessed this specialized character defining the clade. Therefore, we do not propose an official nomenclatural act at this point. For convenience, the clade is termed here as *cyanella* clade based on the oldest species name within the clade.

4.2. Character evolution

The shape of the spermathecae in groups more or less closely related with Criocerinae was well investigated (i.e. Donaciinae: Chrysomelidae, e.g. Gómez-Zurita et al. 2007, 2008; Marvaldi et al. 2009). All examined species of Donaciinae possess a simple spermatheca shaped like a question mark as shown in Fig. 3A (Hayashi 2004, 2005), whereas the shape of the spermathecal capsule is highly variable in Criocerinae (Fig. 3). Recent studies have revealed a cryptic diversity of the female reproductive organs in contrast to previous predictions (e.g. Arnqvist & Rowe 2002; Puniamoorthy et al. 2010; Yassin & Orgogozo 2013; Simmons 2014). It was shown that the female reproductive structure affects the shape of the male reproductive system (e.g. Miller & Pitnick 2002; Córdoba-Aguilar 2005; Higginson et al. 2012). Manier et al. (2010) recently established a technique of visualizing and discriminating spermatozoa from different males in a spermatheca of *Drosophila*. Using this approach it will be possible to evaluate how shape differences affect sperm or spermatozoa dynamics. This will likely uncover

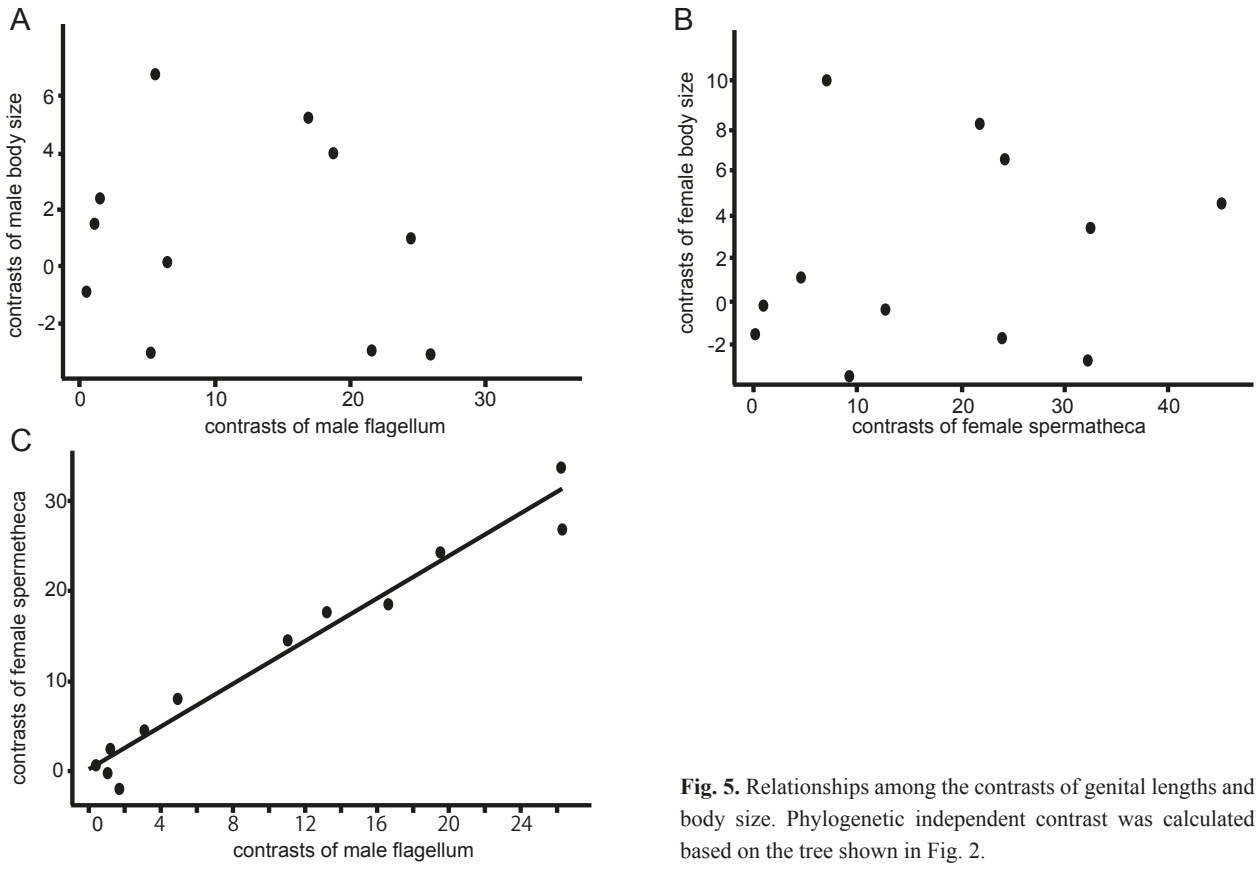
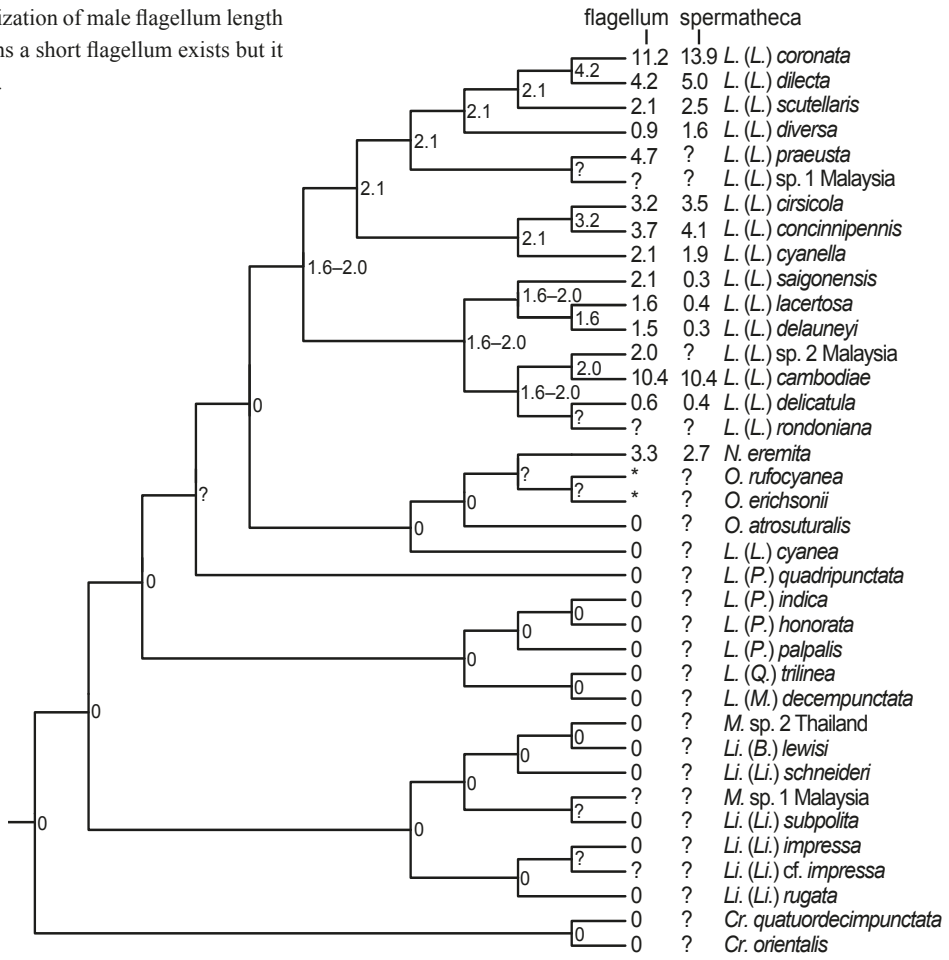


Fig. 5. Relationships among the contrasts of genital lengths and body size. Phylogenetic independent contrast was calculated based on the tree shown in Fig. 2.

Fig. 6. Most parsimonious optimization of male flagellum length in Criocerinae. Unit: mm. * means a short flagellum exists but it is not accommodated in a pocket.



functional aspects of the diverse spermatheca in Criocerinae, evolutionary mechanisms behind this system, and more generally the evolutionary significance of the diversity of female genitalia.

The present study showed a correlation of the genital length between sexes even after correction of phylogenetic effects, whereas the genital length does not correlate with the body length of either males or females. This indicates coevolution of the flagellum and spermathecal duct length between the sexes. Such a phenomenon is widely known in insects (e.g. intromittent organs: Ilango & Lane 2000; Rodriguez et al. 2004; spermatozoa: Dybas & Dybas 1981; Morrow & Gage 2000; Pitnick et al. 2003; Rugman-Jones & Eady 2008). In the case of coevolution between the male intromittent organ and the female spermathecal duct, it is known that females choose actively males with a longer intromittent organ (e.g. Rodriguez 1994, 1995; Rodriguez et al. 2004), or males can replace rival sperm using the elongated organ (e.g. Kamimura 2000). However, it is still unclear why such a conspicuous variation of the length evolved. The genus *Lema* could be one of the potential groups providing an answer to this question. The length of the flagellum and spermathecal duct is highly variable, although the body size of the species is relatively uniform (Table 2 and El. Suppl. 4). A reconstruction of the evolutionary transition of the flagellum length showed that extreme elongation happened at least twice, in *Lema* (*Lema*) *coronata* and *L.* (*L.*) *cambodiae* (Fig. 6). Especially *Lema* (*Lema*) *coronata*, *L. dilecta* Baly, 1873, *L. scutellaris* (Kraatz, 1879) and *L. diversa* Baly, 1873 forming a clade live in similar habitats at the same locality and are very similar in their life style (Kimoto & Takizawa 1994). The variance is likely related to differences in sexual selection mechanisms or different intensities of it among species. Genetic markers suitable for paternity determination and widely used in studies on sexual selection are available for these species (Matsumura & Yao 2011). This group is apparently also a good model for behavioral ecological experiments and detailed ecological investigations to reveal the evolutionary mechanisms triggering the astonishing variability among closely related species.

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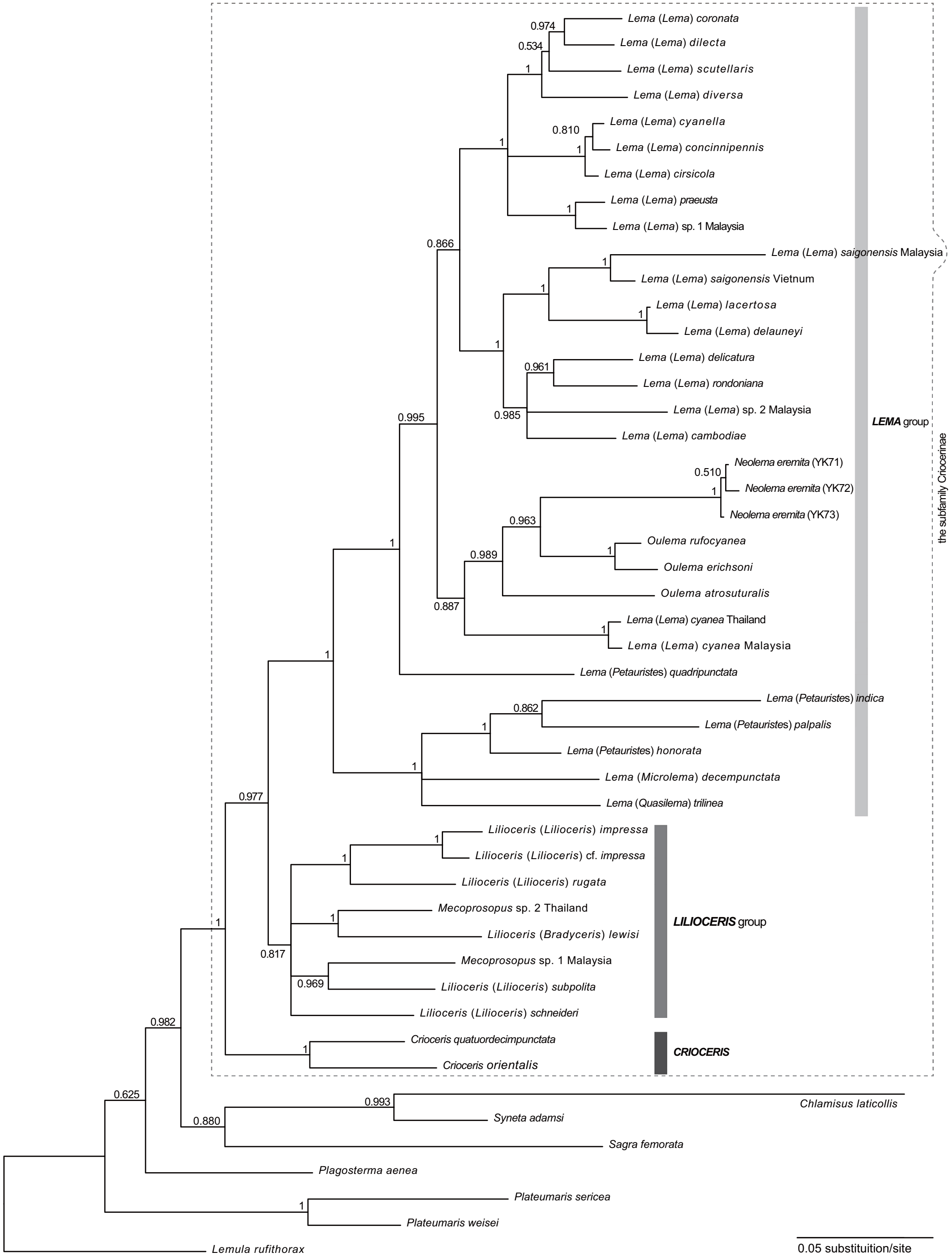
Electronic Supplement Files

at http://www.senckenberg.de/arthropod-systematics ("Contents")

File 1: matsumura&al-criocerinaephylogeny-asp2014-electronicsupplement-1.nex. – Nexus file with the combined aligned sequences. The partial sequences of COI (266 bp), 12S (277–340 bp), and Histone 3 (330 bp) are included.
File 2: matsumura&al-criocerinaephylogeny-asp2014-electronicsupplement-2.pdf. – A tree estimated by Bayesian analysis. Branch lengths are proportional to reconstructed changes, and the numbers on each branch indicate posterior probabilities.
File 3: matsumura&al-criocerinaephylogeny-asp2014-electronicsupplement-3.tre. – A tree file estimated by Bayesian analysis.
File 4: matsumura&al-criocerinaephylogeny-asp2014-electronicsupplement-4.nex. – Character state matrix.


Lema (Lema) coronata
Lema (Lema) dilecta
Lema (Lema) scutellaris
Lema (Lema) diversa
Lema (Lema) cyanella
Lema (Lema) concinnipennis
Lema (Lema) cirsicola
Lema (Lema) praeusta
Lema (Lema) sp. 1 Malaysia
Lema (Lema) saigonensis Malaysia
Lema (Lema) saigonensis Vietnum
Lema (Lema) lacertosa
Lema (Lema) delauneyi
Lema (Lema) delicatura
Lema (Lema) rondoniana
Lema (Lema) sp. 2 Malaysia
Lema (Lema) cambodiae
Neolema eremita (YK71)
Neolema eremita (YK72)
Neolema eremita (YK73)
Oulema rufocyanea
Oulema erichsoni
Oulema atrosuturalis
Lema (Lema) cyanea Thailand
Lema (Lema) cyanea Malaysia
Lema (Petauristes) quadripunctata
Lema (Petauristes) indica
Lema (Petauristes) palpalis
Lema (Petauristes) honorata
Lema (Microlema) decempunctata
Lema (Quasilema) trilinea
Lilioceris (Lilioceris) impressa
Lilioceris (Lilioceris) cf. impressa
Lilioceris (Lilioceris) rugata
Mecoprosopus sp. 2 Thailand
Lilioceris (Bradyceris) lewisi
Mecoprosopus sp. 1 Malaysia
Lilioceris (Lilioceris) subpolita
Lilioceris (Lilioceris) schneideri
Crioceris quatuordecimpunctata
Crioceris orientalis
Chlamisus laticollis
Syneta adamsi
Sagra femorata
Plagosterma aenea
Plateumaris sericea
Plateumaris weisei
Lemula rufithorax
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LEMA group
LILIOCERIS group
CRIOCERIS
the subfamily Criocerinae
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