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1 A title: Homology of the internal sac components in the leaf beetle subfamily Criocerinae
2 and evolutionary novelties related to the extremely elongated flagellum.

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8 A short title: Homology of the internal sac components

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ABSTRACT

Extremely elongated intromittent organs are found in a wide range of taxa, especially among insects. This curious phenomenon is generally thought to result from sexual selection, but it is predicted that limited storage space in the body cavity and the difficulty of using these elongated organs should have constrained the evolution of this extreme elongation, neutralizing any selective advantage. Therefore, in groups with long intromittent organs, evolutionary novelties to overcome these constraints should have occurred pre-adaptively or in co-evolution with extreme elongation. Using a comparative morphological approach and outgroup comparisons, we identified potential constraints and key novelties that would have neutralized such constraints in the leaf beetle subfamily Criocerinae. Observations of the internal sac structure throughout Criocerinae were performed. Comparing the results with preceding studies from outgroups, a ground plan of the criocerine internal sac was constructed. Our analysis also identified specific features that are obligatorily correlated with the extreme elongation: the rotation of whole internal-sac sclerites and the possession of a pocket in which to store the elongated flagellum. The pocket is thought to be formed by the rotation of the sclerites, markedly altering internal sac shape from the criocerine ground plan. Only the clades that have acquired this derived state have species with an elongated flagellum that distinctly exceeds the median lobe length. It is presumed that these character correlations evolved independently three times. The detected character correlations corroborate the hypothesis that there are latent adaptive

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constraints for the evolution of extremely elongated intromittent organs. The constraints may have been neutralized by the dramatic alteration from the criocerine ground plan, resulting in the formation of a storage pocket. In conclusion, deviation from the criocerine ground plan is considered to be the evolutionary innovation that neutralized the latent adaptive constraints of flagellum elongation in the subfamily Criocerinae.

Key words: adaptive constraints, deviation, genitalia, ground plan, *Lema*

INTRODUCTION

Animal genitalia, especially intromittent organs, often show fantastically ornate variations, and detecting the selection pressures that may have promoted such structural diversity has attracted many biologists (Eberhard, 1985; Arnqvist, 1998; Hosken and Stockley, 2004; Eberhard, 2010a, b; Leonard, 2010). Extremely elongated intromittent organs, the length of which can surpass the length of the body, are observed throughout the animal kingdom in species from ducks to snails, barnacles, ostracods, spiders, and insects (Neufeld and Palmer, 2008). Especially within the extremely diverse insects, the phenomenon occurs in many orders (Table 1). Available phylogenetic hypotheses for insects (e.g., Ishiwata *et al.*, 2011) indicate that extreme elongation of intromittent organs has independently evolved many times.

It is generally recognized that the evolution of genital structures, particularly copulatory organs, is promoted by sexual selection and/or sexual conflict (Eberhard, 1985; Arnqvist,

1998; Hosken and Stockley, 2004; Eberhard, 2010a, b; Leonard, 2010). Empirical studies suggest that sexual selection by cryptic female choice and/or sperm competition has promoted the elongation of intromittent organs (Tadler, 1999; Gschwentner and Tadler, 2000; Rodriguez *et al.*, 2004; Kamimura, 2005). Although these findings explain the selective advantage of longer intromittent organs, they do not account for the origin of extreme elongation.

In many animals with internal fertilization, the male intromittent organ is stored in the body cavity, where available space is usually limited. Additionally, use of these organs requires dramatic movements during copulation (e.g., insertion into and withdrawal from the female genital cavity). Thus, even if males with longer intromittent organs are favored by sexual selection, limited storage space and the difficulty of handling elongated intromittent organs should constrain the evolution of extreme elongation (e.g., Gack and Peschke, 2005; Neufeld and Palmer, 2008) by neutralizing positive directional selection. Nevertheless, extremely long intromittent organs occur in many animals, and evolutionary innovations to overcome these adaptive constraints should have emerged either pre-adaptively or in co-evolution with extreme elongation. However, to date, such evolutionary novelties have rarely been addressed.

A tube-like element of the intromittent organ, termed a flagellum, is present in species of *Lema* (*Lema*) of the leaf beetle subfamily Criocerinae (Fig. 1A), and this organ varies greatly in length (Matsumura and Suzuki, 2008). Remarkably, in *L. (L.) coronata* Baly, the

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flagellum is nearly twice as long as the entire body and is inserted into the elongated female genital tube (i.e., the spermathecal duct) during copulation (Matsumura and Akimoto, 2009). Matsumura and Yoshizawa (2010) found that the male internal sac (the intromittent organ) in this species has an unusual structure (Fig. 2) that facilitates insertion and withdrawal of the greatly elongated flagellum. In contrast, no specialized copulatory behavior has been observed (Matsumura and Akimoto, 2009; Matsumura and Yoshizawa, 2010). Therefore, it can be predicted that certain anatomical modifications have been acquired as preconditions for the evolution of the greatly elongated flagellum in this species. Some other criocerines also reportedly possess an elongated flagellum (e.g., Mann and Crowson, 1996; Dngelhoef and Schmitt, 2006), whereas it is absent in others (Fig. 1; Mann and Crowson, 1996). Therefore, the flagellum was probably acquired independently in this subfamily, which provides an opportunity to study correlated character evolution in relation to extreme elongation of the flagellum.

In the present study, we compared the internal sac structures of over 130 criocerine species from geographically and generically diverse groups and homologized the internal sac components with a comparative morphological approach. Based on the results of comparing closely related taxa, we discuss the evolutionary history of structural transformations and their implications for the evolution of extremely elongated flagellum.

MATERIAL AND METHODS

Species examined

The subfamily Criocerinae includes approximately 1100 species (Monrós, 1959) and is divided into three tribes and approximately 20 genera (Table 1; Monrós, 1959; Seeno and Wilcox, 1982). One hundred thirty-three species representing most genera were examined using dried and alcohol-preserved specimens (see Table 1; the species used are listed in the appendix). For most species, one or two specimens were observed, but when it was possible, we observed more than ten specimens per species to detect intraspecific variation. To examine the soft tissue in detail (i.e., the ejaculatory duct and muscles), we used representative fresh specimens for five Japanese species that included species with a flagellum [*Lema (Lema) diversa*, *L. (L.) scutellaris*, *L. (L.) coronata*] and species without [*Lema (Microlema) decempunctata*, *Oulema oryzae*]. Flagellum length varies greatly among the species (i.e., the flagellum is ca. 0.15 and 0.4 times the length of the body in *diversa* and *scutellaris*, respectively, and nearly twice the length of the body in *coronata*), whereas body length differs only slightly (ca. 5 - 5.5 mm) (Matsumura and Suzuki 2008).

Phylogenetic hypotheses for inter-generic relationships in the subfamily Criocerinae have been proposed by Schmitt (1985a, b), Vencl and Morton (1996) and Vencl *et al.*, (2004). Although these studies have been based on a limited number of species from only *Lema*, *Neolema*, *Oulema*, *Crioceris*, and *Lilioceris*, these hypotheses have been accepted here for the purpose of discussion and are visually represented in Fig. 3

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120 **Dissection, illustration and measurements.**

121 We performed structural observations based solely on manual dissection under binocular

122 microscopes (Olympus SZ60 and SZX12, Japan). Dried specimens were softened by

123 soaking in distilled water at 50°C for one night, after which the abdomen was removed and

124 soaked in 5–10% KOH solution. We incubated the abdomen at 50°C for two days. Next,

125 we removed the aedeagus and carefully pulled the internal sac from the orifice of the

126 median lobe using fine forceps. The aedeagus was preserved in glycerine, and observation

127 and illustration of specimens was conducted using glycerine and/ or massage oil (Soft

128 demand, Japan) under a binocular microscope. When we investigated thin or fine structures,

129 specimens were mounted on a slide and observed using a light microscope (Zeiss Axiophot,

130 Germany).

131 To clearly observe the soft tissue (i.e., the ejaculatory duct and muscles), we also used

132 freshly collected specimens. Live insects were frozen, which facilitated the observation of

133 soft tissue structures. When possible, we observed individuals anaesthetized with ether and

134 mating pairs immobilized with a cooling spray.

135 In same cases, aedeagus were cleared in BABB (Benzyl Alcohol + Benzyl Benzoate) (e.g.

136 McGurk *et al.*, 2007; Kamimura and Mitsumoto, 2011) for a week. This procedure makes

137 the darkly colored median lobe transparent and enables us to observe the inner structure of

138 the median lobe (as in Figs. 1A, B).

139 To examine character correlations related to extreme elongation of the flagellum (defined
140 as flagellum length exceeding the length of the storage organ, i.e., the median lobe, Fig.
141 2A), the ratio of flagellum length to median lobe length was calculated. The flagellum
142 length was measured using photographs from a slide-mounted specimen. We measured the
143 lengths with a curvimeter (Koizumi COMCURVE-9 Junior, Japan) according to the
144 methods of Matsumura and Yoshizawa (2010). We measured at least one or two
145 individuals for each species.

147 **Terminology**

148 In the closely related group Donaciinae (e.g., Gomez-Zurita *et al.* 2008, see also
149 Discussion), anatomical studies have been extensively conducted, and so terminology has
150 been strongly established (Askevold, 1988, 1990, 1991; Hayashi, 2004, 2005). However, as
151 mentioned in Matsumura and Yoshizawa (2010), some of the criocerine and donacine
152 species examined to this point show structural differences in their internal sac components.
153 Therefore, we termed each sclerite of criocerine internal sac based on topographical
154 correspondence, and sclerites which share a similar relative internal sac position were
155 drawn in the same color. With one exception, we adopted the common term ‘flagellum’ for
156 the elongated organ following Lindroth (1957). An elastic connection of sclerites was
157 observed in the criocerine internal sac. Then we termed it an elastic bridge.

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159 **Homologization**

160 Homologous relationships among the internal sac components were evaluated with a
161 comparative morphological approach. Although it is widely accepted that the concept of
162 homology is a foundation in evolutionary biology, its definition remains widely debated
163 (e.g., Tautz, 1998; Brigandt and Griffiths, 2007). In the present study, we used the term
164 'homology' to refer to characters that have a common origin and are detectable using a
165 comparative morphological approach. We adopted the following four criteria for evaluating
166 homology based on the criteria established by Remane (1952) and utilized more recently
167 (e.g., Rieppel and Kearney 2002; Richter 2005): similarity (e.g., Patterson, 1988; Wägele,
168 2005), compatibility (e.g., Wägele, 2005), conjunction (e.g., Patterson, 1988), and
169 complexity (e.g., Wägele, 2005).

170 The position of the opening of the ejaculatory duct was used as an initial landmark to
171 homologize subsequent components. The ejaculatory duct is formed by an ectodermal
172 invagination (Sánchez and Guerrero, 2001), which implies that the position of its opening is
173 determined early in genital morphogenesis (Heming, 2003; YM *et al.*, unpublished).
174 Therefore, we reasoned that this duct should be homologous throughout the subfamily
175 Criocerinae.

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177 **RESULTS**

178 **Internal-sac sclerites and a membranous sheet**

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7 179 The internal sac consists of a membranous sheet, three sclerites connected by an elastic
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10 180 bridge, an ejaculatory duct, and three bundles of muscles. The external appearance of the
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12 181 internal sac is not detectably different among the species. In contrast, the shapes of the
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14 182 internal-sac sclerites markedly differ, especially between species that do or do not have
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17 183 elongated flagellum (Fig. 3). The shape of the internal sac sclerites is also variable among
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19 184 the genera, including species with the flagellum (e.g., Figs. 3C, H, I, and N). In contrast,
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21 185 the shape of the internal sac is relatively uniform among the species without a flagellum
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23 186 (e.g., Fig. 3), even if the species are distantly related. The character states of each species
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25 187 are tabled in the appendix.
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30 188 To compare the structures more easily, we made schematic drawings for representative
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32 189 species in which the membranous sheet and internal-sac sclerites were aligned on a straight
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34 190 line without altering their relative positions (Figs. 5F-J). Homologous sclerites (see
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36 191 Discussion) were highlighted in the same color. The most ventrally positioned sclerite
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38 192 (ventral sclerite) was drawn in blue. The yellow-colored sclerite (medial sclerite) is
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40 193 connected to the ventral sclerite by the elastic bridge, and the dorsal sclerite (pink-colored)
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42 194 is positioned below the other sclerites (Figs. 5F-J). The positional relationships are stable in
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50 196 In all species with the flagellum, the flagellum is formed by the middle or middle and
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52 197 dorsal sclerites; the middle sclerite has a concave shape in species without the flagellum
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54 198 (Figs. 6A, B). Some species with the flagellum have character states similar to those in
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species that do not have the flagellum, except in the shape of the middle sclerite (e.g. Fig. 3H). On the other hand, many species with the flagellum do have character states differing from the species without the flagellum, i.e. the dorsal sclerite tightly encloses the middle sclerite (e.g. Figs. 3 C, I, and N). In addition, in species with the elongated flagellum, whole sclerites rotate on a large scale along the longitudinal axis (compare Figs. 4A and D), which accompanies with an invaginated membranous sheet (i.e. a pocket). The invaginated membranous sheet corresponds to the area indicated by the red-colored membrane in Figs. 5F - J. In terms of these differences, some species with the flagellum superficially show a dorso-ventrally opposite arrangement of their ventral and dorsal sclerites (Fig. 3). The condition does not change during copulation in the species with the flagellum, as far as we observed for *Lema coronata*.

The above-mentioned character states were observed in three lineages belonging to different genera i.e. most species of the subgenus *Lema* (e.g. Fig. 3I), *Neolema* sp. near *elemita* 1 (Fig. 3N), and *Lilioceris (Chujoita) quadripustulata* (Fig. 3C). The subgenus *Lema* includes many species with the flagellum, and its length is variable (Appendix). Some of the species have only a moderately elongated flagellum, but they also have internal sac characters that are identical with those observed in species with the extremely elongated flagellum (Fig. 3I). The character states between the subgenus *Lema* and *Lilioceris quadripustulata* are apparently similar (Figs. 3C, I), but the inflection point of the flagellum differs dramatically; the inflection occurs in the area in which the middle sclerite

219 is fused with the dorsal sclerite in *Lema* and in a more basal area of the flagellum in which
220 the sclerites are not fused with the dorsal sclerite in *Lilioceris quadripustulata* (see Figs. 3
221 C, I). *Neolema* sp. near *elemita* 1 (Fig. 3N) shows rotation twice over although the
222 flagellum length is much shorter than in some species of the subgenus *Lema* (see below,
223 Fig. 7). In addition, the direction of flagellum inflection in *Neolema* sp. near *elemita* 1 is
224 different from other species (see Figs. 3 C, I, and N).

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226 **Ejaculatory duct and muscles**

227 The ejaculatory duct opens onto the elastic bridge joined to the ventral sclerites (Fig. 6). In
228 the frontal area of the ejaculatory duct, the duct passes through a groove or hole on the
229 dorsal side of the ventral sclerite (Figs. 6B, D). In species with the flagellum, the opening is
230 entirely surrounded by the elastic bridge and continues to the tube-shaped middle sclerite
231 (i.e., the flagellum) (Figs. 6C, D).

232 Figs. 2B, C shows the insertion points of muscles in the normal condition, and Figs. 5F-J
233 shows those schematically in which the components are aligned on a straightened line.
234 Three pairs of muscle bundles are inserted on the internal sac. In the species without the
235 flagellum, all the muscles are inserted onto the upper area to the sclerites (Figs. 5F, G),
236 including a pair directly attached to the upper tip of the ventral sclerite (Figs. 5F, G). The
237 condition is mostly preserved in the species with the flagellum, except that an inserted
238 position of a pair of the muscles was found on the lower area of the sclerites (Figs. 5H-J):

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i.e., the pocket membrane (Figs. 2B, C).

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Character correlations

We coded the following qualitative differences in character states (see Fig. 4):

- 1. A tube-like element formed by the middle sclerite: (1) present; (0) absent.
- 2. Fusion of the dorsal and middle sclerites: (1) present; (0) absent.
- 3. Inflection of the middle sclerite or the fused middle and dorsal sclerite: (1) present in the basal-most part of the middle sclerite; (2) present in the fused middle and dorsal sclerites; (0) absent.
- 4. Inward rotation of whole sclerites along the longitudinal axis: (1) 180°; (2) more than 720°; (0) absent (see Figs. 4A and D, stars indicate the same site).
- 5. A pocket formed by an invagination of the membranous sheet: (1) present; (0) absent.

The distribution of character states for each genus is listed in Table 3. All species without the flagellum (Char. 1-0) show “0” for all characters, whereas species with the flagellum (Char. 1-1) are variable. Additionally, characters 4 and 5 in particular show similar distribution patterns in the character state matrix.

The ratio of flagellum length to median lobe length ranged from 0.06 to 32.39 (Fig. 7, Appendix). In groups with rotation of whole sclerites (Char. 4-1, 2) and a pocket for storing the flagellum (Char. 5-1), the ratio ranged from 1.54 to 32.39, whereas in groups without rotation (Char. 4-0) or pockets (Char. 5-0) the ratio was much smaller (0.06 to 1.35).

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260 DISCUSSION

261 Homology hypothesis in Criocerinae

262 The internal sac consists of components that are common throughout the subfamily. These
263 components include the ejaculatory duct, three bundles of muscles, the membranous sheet,
264 three sclerites, and the elastic bridge all within a small space approximately 0.5 mm^3 in area.
265 In addition, three bundles of muscles were commonly observed in the fresh specimens;
266 therefore, the internal sac itself is regarded as homologous (frame homologies; Wägele,
267 2005) based on the criteria of complexity, similarity and conjunction.

268 The components of the internal sac retain identical positions relative to each other
269 across the subfamily. In addition, the ventral sclerites have a groove or channel for the
270 ejaculatory duct, and the ejaculatory duct opens onto the area between the sclerite and
271 elastic bridge (Fig. 5). Therefore, these sclerites are considered to be homologous.
272 In contrast, shape of the middle sclerites is significantly different between the species with
273 and without forming the flagellum. From a morphological viewpoint, the tube-shaped
274 middle sclerite (i.e., the flagellum) is the vehicle for sperm transfer during copulation. In
275 species without the flagellum, the middle sclerite assumes a concave shape positioned just
276 below the opening of the ejaculatory duct, where it acts as a basin for ejaculate. Therefore,
277 based on their positional and functional congruence, the middle sclerites are also
278 considered to be homologous. Finally, the ventral and middle sclerites are weakly

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279 connected to the dorsal sclerite by the elastic bridge, and the dorsal sclerite occupies a
280 similar position (Fig. 5). Thus, the dorsal sclerites were also regarded to be homologous
281 throughout Criocerinae.

282 Based on these observations, the homology hypothesis for sclerites and ducts can be
283 reasonably supported. However, the homology of one muscle attachment requires further
284 clarification. The species with the flagellum exhibit differences in the location of one
285 muscle attachment compared with species that do not have the flagellum (Figs. 5F-J). This
286 incongruence is strongly associated with the modification in the positioning of membranous
287 sheets (i.e., the invaginated membranous sheets in species with the flagellum; Figs. 2; 4 red
288 areas). Therefore, it is reasonable to conclude that the different musculature between the
289 species with and without the flagellum does not reject our homology hypothesis.

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291 **The flagellum and its storage pocket in the family Chrysomelidae**

292 The subfamily Criocerinae is a member of the clade also comprising Donaciinae, Sagrinae
293 and Bruchinae. Its monophyly is relatively well-supported by molecular data, and the clade
294 is considered to have arisen in the basal splitting event within the Chrysomelidae (Farrell
295 1998; Duckett *et al.* 2004; Farrell and Sequeira 2004; Gómez-Zurita *et al.* 2007; Marvaldi
296 *et al.* 2009, partly by Reid 1995, 2000; but see Lee 1993, Reid 1995, 2000; Gómez-Zurita
297 *et al.* 2008). Internal sac structures have been relatively well-investigated for Donaciinae
298 (Sharp and Muir, 1912; Harusawa, 1985; Mann and Crowson, 1983, 1996; Askevold, 1988,

1990, 1991; Hayashi, 2004, 2005), Sagrinae (Sharp and Muir, 1912; Mann and Crowson, 1991, 1996), and Bruchinae (Sharp and Muir, 1912; Kingsolver, 1970).

Flagellum-like structures have not been reported in Bruchinae. In contrast, a projection from the internal sac (termed a 'median ejaculatory guide' in Askevold, 1988 and a 'flagellum' in Mann and Crowson, 1991, 1996) has been reported in almost all sagrine and donacine species examined, and states of the internal-sac sclerites are relatively uniform within each subfamily. In the donacine species *Plateumaris constricticollis constricticollis*, the ejaculatory duct opens onto the base of a tube-shaped element (a 'flagellum' of Lindroth, 1957), which is enveloped by the median ejaculatory guide (YM pers. obs.). *Sagra* sp. of Sagrinae has similar elements, although the opening of the ejaculatory duct could not be detected (YM pers. obs.). Therefore, as in some species of Criocerinae, most species of the subfamilies Sagrinae and Donaciinae probably have the sclerotized terminal prolongations of the ejaculatory duct (i.e., flagellum), although these observations are limited and tentative.

However, there have been no reports of the fusion of the dorsal and middle sclerites (Char. 2-1), inflection of the middle sclerite (Char. 3-1) or the fused middle and dorsal sclerite (Char. 3-2), rotation of whole sclerites (Char. 4-1, 2), or possession of a pocket in which to store the flagellum (Char. 5-1) in Sagrinae and Donaciinae. The present study shows that these characteristics are exclusively found in some species of Criocerinae. In addition, extreme elongation of the flagellum, in which its length exceeds its median lobe length, is

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also uniquely found in Criocerinae. Furthermore, in the other subfamilies of Chrysomelidae and the related families (Gómez-Zurita et al. 2008; Marvaldi et al. 2009) Megalopodidae, Orsodacnidae, and Cerambycidae, there are no species that show rotation of the sclerites and/ or possession of a pocket in the internal sac (e.g., Sharp and Muir, 1912; Ehara, 1954; Mann and Crowson, 1996; Kasatkin 2006; Yamasako and Ohbayashi, 2011). Therefore, although the phylogenetic relationships among the subfamilies of the clade encompassing Criocerinae, Donaciinae, Sagrinae, and Bruchinae have not been resolved, the extreme elongation in Criocerinae is apparently a novel state in which states of the characters 3–5 have changed from ‘0’ to ‘1, 2’.

The derived states of characters 4 and 5 (the rotation of whole sclerites and the possession of a pocket) were observed in only three lineages: the subgenus *Lema*, *Neolema* sp. near *elemita* 1 and *Lilioceris* (*Chujoita*) *quadripustulata*. The degree of rotation for the whole sclerite in *Neolema* sp. (Char. 4–2) is quite different from that of the subgenus *Lema* and *Lilioceris quadripustulata* (Char. 4–1). Additionally, the subgenus *Lema* and the genus *Lilioceris* are distantly related (Schmitt, 1985a, b), and they show differences in the inflection points of their flagella (Char. 3–1, 2). These results indicate independent origins of these character states for each genus. Therefore, the derived condition is considered to have evolved at least three times in Criocerinae. Because we could not decide the polarity for the character 1, the ground plan of Criocerinae is considered to be Figs. 4A or B, and the character states in Figs. 4 C and D are considered to be derived from the plesiomorphic

339 state.

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341 **Character correlations and their implications for evolution.**

342 Our analysis identified two specific character states that are associated with the extreme
343 elongation of the flagellum (Table 3, Fig. 7; the rotation of whole sclerites and the
344 possession of a pocket; Char, 4-1, 2 and 5-1). The ratio of flagellum length to median lobe
345 length is 1.54 to 32.39 in species with a pocket and rotation of the sclerites, whereas this
346 ratio in species without these characters is 0.06 to 1.35. Thus the length of the flagellum is
347 sometimes dramatically greater than the median lobe length in species with a pocket and
348 rotation of the sclerites, but it is less than or approximately equal to the median lobe length
349 in species without these modifications. The flagellum is most frequently observed in the
350 subgenus *Lema*, and its length greatly varies among species (Fig. 7-VI, appendix). Even the
351 species with shortly elongated flagellum have the same character states as in the species
352 with extremely elongated flagellum. This suggests that evolution of the rotation of whole
353 sclerites and a storage pocket preceded the origin of extreme flagellum elongation.

354 Morphologically, the pocket is thought to be formed by the rotation of the sclerites and
355 the resulting invaginated membranous sheet. The pocket greatly expands the storage space
356 in the taxa and, at least in the subgenus *Lema*, the pocket makes it possible to control the
357 extremely elongated flagellum during the copulation (Matsumura and Yoshizawa, 2010).

358 As discussed above, the rotation of whole sclerites and formation of the pocket are

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359 considered to be acquired independently at least three times in the different taxa (the
360 subgenus *Lema*, the genera *Neolema*, and *Lilioceris*). The independent origins of character
361 correlations between the formations of the pocket and extremely elongated flagellum
362 corroborate the hypothesis that limited storage space and an inability to handle the
363 flagellum are latent adaptive constraints for extreme flagellum elongation in Criocerinae.
364 The presence of the numerous pleats (Fig. 8) that were observed in the subgenus *Lema*
365 (e.g. Fig. 3I) is crucial from a functional viewpoint because the pleats greatly increase
366 storage space for the flagellum in the median lobe. In fact, all species with a flagellum that
367 exceeds median lobe length show this condition (e.g. Fig. 3I). In contrast, in species
368 retaining the plesiomorphic condition in the characters 2-5 (Fig. 4B), the length of the
369 flagellum does not exceed, or is only slightly longer, than the maximum length of the
370 median lobe (Fig. 8). Matsumura and Yoshizawa (2010) verified that the highly modified
371 condition imparts the ability to insert and withdraw the flagellum efficiently during
372 copulation in species with the extremely elongated flagellum. From a morphological
373 standpoint, the marked deviation from the criocerine ground plan, namely, the rotation of
374 all sclerites resulting in a invaginated membranous sheet (Char. 4-1, 2 and 5-1), is
375 considered to be the evolutionary event that neutralized the latent adaptive constraints on
376 extreme elongation of the flagellum in this subfamily.

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378 **Conclusions**

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7 379 In the beetle subfamily Criocerinae, we identified specific features that are obligatorily
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10 380 correlated with extreme elongation; these features are the rotation of whole sclerites and the
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12 381 possession of a pocket in which to store the elongated flagellum. Importantly, only lineages
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14 382 that have acquired these derived states show extremely elongated flagellum that distinctly
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17 383 exceeds the median lobe in length. Additionally, the character correlation has evolved
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20 384 independently three times. Therefore, the detected character correlation corroborates the
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22 385 hypothesis that there are latent adaptive constraints on the evolution of extreme elongation
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24 386 of the flagellum, and the potential constraints were neutralized by the dramatic alteration
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27 387 from the criocerine ground plan resulting in the formation of a storage pocket.
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576

577 Figure 1. Aedeagus of *Lema* (*Lema*) *coronata* (A, C, E) and *Lema* (*Microlema*)

578 *decempunctata* (B, D, F). (A, B) Whole aedeagus in lateral view. (C, D) Everted internal

579 sac in lateral view. (E, F) *id.*, in dorsal view. Scale bars indicate 0.50 mm in A and B, and

580 0.25 mm in C-F.

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582 Figure 2. Comparison of the male intromittent organ between the species with and without

583 the flagellum in lateral view. (A) Schematic drawings of a movement of the male

584 intromittent organ. The bold line on the median lobe shows the length measured as a

585 storage size. (B-D) The internal sac structure during copulation and drawn in sagittal plane.

586 (B) *Lema* (*Microlema*) *decempunctata*, just after the initiation of copulation. (C) *Lema*

587 (*Lema*) *coronata*, corresponding to stage (B). (D) *id.*, the elongated flagellum is fully

588 inserted into the female; the arrow indicates a track of the membranous sheet everted. Red

589 broken lines indicate the ejaculatory duct, and green lines show the insertion areas of

590 muscles. Green broken line in (C) shows that the insertion of muscles is on the surface of

591 the pocket.

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593 Figure 3 (A). The comparative morphology of the internal sac of the presumed clades
594 including *Lilioceris* and *Crioceris* (see Schmitt, 1985a, b). Areas presumed to be
595 homologous are highlighted with the same color, and gray colored areas show the elastic
596 bridge. The red broken line indicates the ejaculatory duct.

597 Figure 3 (B). Continued. The presumed clade including the genus *Lema* and its related taxa
598 (see Schmitt, 1985a, b).

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600 Figure 4. Schematic drawings of the character coding and observed sets of character states.
601 (A) The most widely observed pattern. (B) With forming shortly elongated flagellum. (C)
602 With shortly elongated flagellum, inflection of the middle sclerite is present. (D) The
603 pattern observed in the majority of species in the subgenus *Lema*. The numbers in the
604 drawings correspond to the characters and character state codes given in the main text.
605 Stars in (A) and (D) indicate the corresponding sites of the ventral sclerites.

606

607 Figure 5A. Comparative morphology of the internal sac. (A–E) Photos and drawings of the
608 internal-sac sclerites. (F–J) The membranous sheet and sclerites were aligned on a
609 straightened line without changing their relative positions. The same colored components
610 indicate homologous parts. Red broken lines indicate the ejaculatory duct. Green lines and
611 areas indicate the insertion points of muscles. Red area corresponds to the pocket for the
612 flagellum in normal condition (compare with Fig. 2). Scale bars indicate 0.10 mm.

613 Figure 5B. Continued.

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615 Figure 6. Detailed morphology of the internal-sac sclerites. (A, B) *Lema* (*Microlema*)

616 *decempunctata*, (C, D) and *Lema* (*Lema*) *diversa*. (A, C) dorso-lateral view, (B) dorsal

617 view in which the dorsal sclerites was removed, (D) and an enlarged drawing of the

618 opening of the ejaculatory duct. Red broken lines indicate the ejaculatory duct. Scale bars

619 indicate 0.10 mm.

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621 Figure 7. Comparison of the ratio of flagellum length to median lobe length among the

622 following genera, with sample size for each genus given in parentheses: I: *Stethopachys* (1),

623 II: *Lilioceris* (3), III: *Lema* (10), IV: *Oulema* (2), V: *Neolema* (6), VI: *Lema* (27), VII:

624 *Lilioceris* (1), VIII: *Neolema* (1). The lines r-1 and r-2 indicate the ratio is 1 and 2,

625 respectively.

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627 Figure 8. Schematics of the internal sac, which is located in the storage organ. (A)

628 Plesiomorphic structure of the whole intromittent organ in Criocerinae. (B) In the case that

629 elongation occurs in taxa retaining the plesiomorphic condition. (C) In the case that

630 elongation occurs in taxa with derived states in the internal sac.

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632 Appendix. A list of species studied and characters states of their internal sac components.

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For Peer Review

209x297mm (300 x 300 DPI)

281x408mm (300 x 300 DPI)

Lilioceris* lineage**A. *Lilioceris infraticornis* C. *Lilioceris (Chujoita) quadripustulata* B. *Metopocerus gemmans* D. *Mecoprosopus* sp. E. *Ovamela ornatipennis* Crioceris* lineage**F. *Elizabethana inornata* G. *Crioceris multicaculata*

163x133mm (300 x 300 DPI)

The genus *Lema* and its related groups

297x420mm (300 x 300 DPI)

105x84mm (300 x 300 DPI)

176x287mm (300 x 300 DPI)

Lema (Lema) diversa *Lema (Lema) scuteraliis* *Lema (Lema) coronata*

185x190mm (300 x 300 DPI)

129x81mm (300 x 300 DPI)

70x45mm (300 x 300 DPI)

155x230mm (300 x 300 DPI)

Table 1. A taxonomic list of groups of insects with long tube-like organs.

orders	references
Dermaptera	e.g. Jamet & Caussanel 1995; Ramamurthi 1958
Zoraptera	e.g. New 2000
Hemiptera	e.g. Carayon 1989; Deckert 1990
Neuroptera	e.g. Sziráki 2002
Diptera	e.g. Spencer 1976
Siphonaptera	from Neufeld & Palmer 2008
Coleoptera	e.g. Peschke 1978; Klimaszewski, 1984, Holloway 1960

Table 2. Taxonomy and number of species examined in the subfamily Criocerinae.

Tribe	Genus	Subgenus	species	distribution
Pseudocriocerini				
	<i>Pseudocrioceris</i>		- (6)	Java, Madagascar
Criocerini				
	<i>Ovamela</i>		1 (1)	Madagascar
	<i>Metopoceris</i>		1 (19)	C. America
	<i>Lilioceris</i>	<i>Lilioceris</i>	23 (133)	World Wide
		<i>Bradyceris</i>	1 (1)	Japan
		<i>Chujoita</i>	1 (8)	Australia, Asia
	<i>Mecoprosopus</i>		2 (2)	China
	<i>Crioceris</i>		10 (46)	World Wide
	<i>Elisabethana</i>		1 (13)	Africa
	<i>Sigrisma</i>		- (4)	Africa
	<i>Manipuria</i>		- (1)	India
Lemiini				
	<i>Lema</i>	<i>Lema</i>	42 (516)	World Wide
		<i>Petauristes</i>	11 (103)	Europe, Asia, Africa
		<i>Microlema</i>	1 (1)	Japan
		<i>Quasilema</i>	9 (269)	N., C. and S. America
		<i>Pachylema</i>	- (35)	C. and S. America
	<i>Neolema</i>		11 (148)	N., C. and S. America
	<i>Mimolema</i>		- (1)	S. and E. Africa
	<i>Oulema</i>	<i>Oulema</i>	11 (72)	Holarctic, Oriental
		<i>Gracilema</i>		India, S.eE. Asia, S. China
		<i>Parhapsidolema</i>		N. and S. America
		<i>Hapsidolemoides</i>		Asia
	<i>Ortholema</i>		3 (4)	Asia
	<i>Incisolema</i>		- (2)	Africa
	<i>Plectonycha</i>		1 (6)	S. America
	<i>Stethopachys</i>		4 (4)	Asia, Australia
	<i>Lagriolema</i>		-	New Guinea
	<i>Papulema</i>		-	New Guinea

The number in parentheses indicates the number of described species belonging to each genus
-: the species of the genus was not available for the present study.

Table 3. Character matrix. Some genera show a noticeable bias in their distribution of polymorphic states. The predominant states for each genus are shown in bold.

			Characters				
			1	2	3	4	5
Criocerini	<i>Ovamela</i>		0	0	0	0	0
	<i>Metopocerus</i>		0	0	0	0	0
	<i>Liliocerus</i> (<i>Liliocerus</i>)	A	0	0	0	0	0
		B	1	0	1	0	0
		C	1	1	2	0	0
	<i>Liliocerus</i> (<i>Bradycerus</i>)		0	0	0	0	0
	<i>Liliocerus</i> (<i>Chujoita</i>)		1	1	1	1	1
	<i>Mecoprosopus</i>		0	0	0	0	0
	<i>Criocerus</i>		0	0	0	0	0
	<i>Elisabethana</i>		0	0	0	0	0
Lemiini		A	1	1	2	1	1
	<i>Lema</i> (<i>Lema</i>)	B	1	0	0	0	0
		C	0	0	0	0	0
	<i>Lema</i> (<i>Petauristes</i>)	A	0	0	0	0	0
		B	1	0	0	0	0
	<i>Lema</i> (<i>Microlema</i>)		0	0	0	0	0
	<i>Lema</i> (<i>Quasilema</i>)	A	0	0	0	0	0
		B	1	0	0	0	0
		A	0	0	0	0	0
	<i>Neolema</i>	B	1	0	0	0	0
		C	1	1	2	2	1
	<i>Oulema</i>	A	0	0	0	0	0
		B	1	0	0	0	0
		C	1	0	1	0	0
	<i>Ortholema</i>		0	0	0	0	0
	<i>Plectonycha</i>		0	0	0	0	0
	<i>Stethopachys</i>	A	0	0	0	0	0
		B	1	1	2	0	0