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Title	Exploration of the homology among the muscles associated with the female genitalia of the three suborders of Psocodea (Insecta)
Author(s)	Cheng, Zixin; Yoshizawa, Kazunori
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Instructions for use

1   **Exploration of the homology among the muscles associated with the female**

2   **genitalia of the three suborders of Psocodea (Insecta)**

3   **Zixin Cheng & Kazunori Yoshizawa**

4   Systematic Entomology, Graduate School of Agriculture, Hokkaido University

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16   **ABSTRCT**

17   By using µCT technology, we reconstructed 3D models of the female genital structures

18   and associated muscles of seven species from three suborders of Psocodea (free-living

19   species only, formerly known as independent insect order "Psocoptera"). The homology

20   of the female genital structures and associated muscles of different species is discussed.

21   A total of 21 muscle groups were observed, and except for one muscle, all were

22   homologized throughout the order. Moreover, some of the homologous muscles could

23   be identified confidently in holometabolan insects. Using the muscles as landmarks, we

24   discuss the homology of the ovipositor valves between Psocodea and other neopteran

25   insects. Most importantly, the ovipositor of the suborder Trogiomorpha was identified

26   to consist of the well-developed external valve (V3) plus a remnant of the dorsal valve

27   (V2). We also examined the phylogenetic information included in the female genital

28   muscles and found that certain muscles provide useful information and support deeper

29   nodes (e.g., monophyly of the suborder Psocomorpha). The present study of female

30   genital muscles not only helps us to better understand the phylogeny of Psocodea but

31   also provides a solid foundation for research on muscle evolution.

32   **KEYWORDS**

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1. INTRODUCTION

36

37   Insect genitalia are extremely diversified structures, and their morphology and

38   evolution have received great interest from variable perspectives (Boudinot, 2019),

39   such as taxonomic species recognition (e.g., Tuxen, 1970), lower- to higher-level

40   phylogenetic estimation (e.g., Yoshizawa & Johnson, 2006), selection forces acting on

41   these structures (e.g., Eberhard, 1985), or biomechanics (e.g., Matsumura *et al*., 2017).

42   In most cases, male genital structures are the subject of these studies because male

43   genitalia are more strongly sclerotized than female genitalia, and their shapes and

44   variations are more easily observable and detectable. In contrast, insect female genitalia,

45   especially the structures having direct contact with male intromittent organ, are largely

46   membranous, and their importance has long been overlooked. However, recent studies

47   have shown that an accurate understanding of female genitalia, including the

48   membranous parts, is very important for elucidating genital evolution (Kamimura, 2016;

49   Muto, 2018; Simmons, 2019; Sloan, 2019; Genevcius, 2020; Onuma *et al*., 2021) and

50   coupling mechanisms (Matsumura *et al*., 2021).

51   Psocodea is one of the hemimetabolous insect orders composed of nonparasitic

52   barklice and booklice (formerly known as Psocoptera, hereafter called psocopterans)

53   and parasitic lice (Phthiraptera) (de Moya *et al*., 2021). The order is subdivided into

54   three monophyletic suborders, Trogiomorpha, Troctomorpha (in which parasitic lice

55   are classified), and Psocomorpha. Over 6500 species in 41 families of psocopterans are

56   known to date (Lienhard & Smithers, 2002; Lienhard, 2016, 2021). As in the other

57   insect groups, male genitalia are the most important characters for diagnosing species

58   and recognizing some higher taxa (e.g., Smithers, 1972). Female genital characters are

59   also important taxonomically and phylogenetically. In particular, this character system

60   is known to be less homoplasious than male genital characters in estimating the

61   phylogenetic relationships of the suborder Psocomorpha (Yoshizawa & Johnson, 2014).

62   Moreover, two female genital apomorphies are the only morphological characters that

63   support the monophyly of the suborder Trogiomorpha (Yoshizawa & Lienhard, 2020).

64   These results suggest the potential importance of this character system for phylogenetic

65   estimations. However, ambiguities remain in the interpretation of homology among

66   certain key female genital structures; for example, simplified ovipositors of

67   Trogiomorpha have usually been interpreted as composed of external valves (Lienhard,

68   1998) but alternatively of ventral valves (Azar *et al*., 2017).

69   In addition, female genitalia of Psocodea are of special interest. Recently, a penis-

70   like genital structure (termed gynosome) was discovered in females of the Brazilian

71   cave-dwelling psocopteran genus *Neotrogla* (Lienhard *et al*., 2010). Subsequent studies

72   showed that the gynosome is inserted into the male genital chamber during copulation,

73   i.e., copulatory functions are completely reversed in *Neotrogla* (Yoshizawa *et al*., 2014;

74   2019). This is a very prominent novel structure that has only been identified thus far in

75   this group of insects in the animal kingdom. However, the morphological origin and the

76   functional background of the coupling role reversals in *Neotrogla* are completely

77   unknown.

78   To elucidate the origin of the novel coupling structures and improve the utility of

79   the female genital character system for taxonomic, phylogenetic, evolutionary and

80   biomechanical studies, providing a detailed morphology and establishing a stable

81   homology interpretation of female genitalia, including both external and internal

82   structures, are essential. However, the detailed musculature related to female genital

83   structures has only been examined for a very limited number of species (Badonnel,

84   1934).

85   In this study, we examined the skeletal morphology and musculature of a wide

86   variety of psocopterans using the synchrotron micro-computed tomography (μCT)

87   technique. Seven species from all three suborders were selected for examination. Based

88   on the examinations, we established a homology scheme of female genitalia throughout

89   psocopterans.

90

91   **2. MATERIALS AND METHODS**

92   **2. 1. Micro-CT imaging**

93   Seven species of psocopterans, *Prionoglaris stygia* Enderlein, 1909,

94   *Psyllipsocus clunjunctus* Lienhard, 2013 (Lienhard & Ferreira, 2013), *Lepinotus*

95   *reticulatus* Enderlein, 1904, Amphientomidae Gen. sp., *Archipsocus* sp.*, Valenzuela*

96   *badiostigma* Okamoto, 1910 and *Trichadenotecnum pseudomedium* Yoshizawa, 2001

97   were examined (Table 1). All voucher specimens are stored in the Hokkaido University

98   Insect Collection. An uncopulated female of each species was used for μCT

99   examination. Samples were fixed with FAA solution (formalin:alcohol:acetic acid =

100   6:16:1) or 80% ethanol and then preserved in 80% ethanol. Dehydration was conducted

101   in ascending order with 80–100% ethanol before drying them at the critical point (EM

102   CPD300, Leica, Wetzlar, Germany) to remove water without serious organ shrinkage.

103   Samples were then scanned using the synchrotron μCT at the BL47XU (Uesugi *et al*.,

104   2012) beamline of the Super Photon ring-8 GeV (SPring-8; Hyogo, Japan) using a

105   stable beam energy of 8 keV in absorption-contrast mode. The tomography system

106   consists of a full-field X-ray microscope with Fresnel zone plate optics (Uesugi *et al*.,

107   2017). We used semiautomatic segmentation algorithms based on grey-value

108   differences in the software ITK-SNAP (Yushkevich *et al*., 2006) to obtain 3D

109   representations of the terminalia of all six species.

110

111   **2. 2. Examination of phylogenetic signals**

112   We examined the phylogenetic significance of the female genital musculature by

113   reconstructing the character information on the phylogenetic tree that was previously

114   estimated using molecular and phylogenomic data (Yoshizawa *et al*., 2006; Yoshizawa

115   & Johnson, 2014; Johnson *et al*., 2018; de Moya *et al*., 2021). The presence (coded as

116   1) or absence (0) of each muscle was used as the character state (Table 2), and this

117   dataset was parsimoniously reconstructed on the tree using MacClade version 4

118   (Maddison & Maddison, 2001). All characters were treated as unordered. Because the

119   homology of many muscles between psocopterans and outgroups was ambiguous (see

120 Discussion), no outgroup taxa were included in the analysis. Therefore, the character

121 changes at the deepest node (between Trogiomorpha and Troctomorpha + Psocomorpha)

122 could not be reconstructed.

123

124 **3. RESULTS**

125 **Skeletal and muscle structures of female terminalia.**

126 We describe the basic structure of the female terminalia and muscles related to them. We

127 grouped those muscles according to their origin as follows: muscles of the epiproct [ep];

128 paraproct [pa]; subgenital plate [sg]; ventral valve [ve]; dorsal valve [do]; external valve

129 [ex]; spermapore plate or the membrane surrounding it [sp]. The distribution of the

130 terminal muscles is summarized in Table 2.

131 Abbreviations: O – origin; I – insertion; and F – assumed function (based on

132 morphological conditions).

133 3. 1. Clunium

134 The clunium is composed of the fused tergites of segments IX and X and houses many

135 attachments of muscles originating from the epiproct, paraproct, gonapophyses,

136 subgenital plate, and spermapore plate (e.g. Fig. 7). Three valves of gonapophyses arise

137 from the ventral end of the clunium and articulate posteriorly with the epiproct and

138 paraproct.

139   3. 2. Epiproct and paraprcot

140   The epiproct is a structure located dorsal to the anus. The paraproct is a paired structure

141   located ventrolateral to the epiproct and involves a reduced cercus (trichobothrial field).

142   These two structures are thought to represent segment XI and together surround the

143   anus. Males also have these two structures, usually with some characteristic protrusions

144   on them, which play an important role in the copulation process. The paraproct and

145   epiproct of females is usually simpler and less variable, with sparse bristles and no

146   protrusions (Fig. 7C, D).

147   Muscle originating from the epiproct

148   **01** epX01 (Fig. 2B); O: posterior end of the epiproct; I: mid-dorsal site of clunium

149   (segment IX); and F: closure and/or flipping of the epiproct.

150   This is the only muscle originating from the epiproct observed throughout Psocopterans.

151   However, this muscle is absent in *Prionoglaris* (Fig. 1B). In *Lepinotus*, additional

152   paired muscles (paX02: see below; Fig. 3B) are also inserted into the epiproct.

153   Muscles originating from the paraproct

154   **02** paX01 (Fig. 8); O: anterodorsal end of the paraproct, very close to the posterolateral

155   margin of epiproct; I: anterolateral region of clunium (segment X); and F: involved in

156   opening the paraproct.

157   **03** paX02 (Fig. 8); O: anterodorsal end of the paraproct, very close to the anterolateral

158   corner of epiproct; I: mediodorsal region of clunium (segment X); and F: involved in

159   opening the paraproct.

160   **04** paX03 (Fig. 9); O: internal margin of the paraproct near the anal opening; I:

161   mediolateral region of clunium (segment X); and F: involved in opening the paraproct.

162   **05** paX04 (Fig. 9); O: anterolateral margin of the paraproct; I: mediolateral region of

163   clunium (segment IX); and F: involved in restoring the paraproct.

164   **06** paX05 (Fig. 10); O: anteroventral end of the paraproct; I: anteroventral margin of

165   clunium (segment IX), near the base of the dorsal valve; and F: involved in opening the

166   paraproct.

167   The paX01–05 muscles are widely observed throughout psocopterans, although

168   paX03 is absent in *Prionoglaris*, *Lepinotus*, *Amphientomidae*, *Archipsocus* and

169   *Valenzuela* (Fig. 9), while paX05 is absent in *Psyllipsocus* (Fig. 2B).

170   **07** papa01 (Fig. 9); O: internal margin of the paraproct near the anal opening; I:

171   anterolateral margin of the paraproct; and F: involved in opening the anus.

172   **08** papa02 (Fig. 9); O: internal margin of the paraproct near the junction of the two

173   paraprocts; I: internal margin of the anteroventral region of the paraproct; and F:

174   involved in opening the anus.

175   The papa01 muscle is widely observed throughout psocopterans but is absent in

176   *Trichadenotecnum*. The papa02 muscle is only observed in *Prionoglaris* and coexists

177   with papa01 (Fig. 9A).

178

179   3. 3. Gonapophyses

180   The female gonapophyses arise from sternum VIII (ventral valve) or the IX segment

181   (dorsal and external valves), and the following three pairs of valves are tightly

182   associated to each other: the most external valve on the outside, the dorsal valve at the

183   middle and the most internal ventral valve on the inside. The shape and degree of

184   development vary greatly among different taxa. A reduction of the gonapophyses is

185   especially distinct in Trogiomorpha: in most members, the ventral and dorsal valves are

186   highly reduced or completely absent. In contrast, in *Valenzuela* (Psocomorpha), the

187   external valve is greatly reduced, although the ventral and dorsal valves are well

188   developed (Fig. 6A). In *Archipsocus* (Psocomorpha), all gonapophyses are greatly

189   reduced (Fig. 5A).

190   Muscle originating from the ventral valve

191   **09** veVII01 (Fig. 11A, B); O: base of the ventral valve; I: ventral region of segment

192   VII; and F: involved in opening the ventral valve.

193   This is the only muscle in psocopterans that originates from the ventral valve. The

194   muscle is observed in *Amphientomidae* and *Valenzuella* but is absent in all other taxa

195   examined here, which is partly due to the reduction of the ventral valve

196   (Trogiomorpha and *Archipsocus*). However, in *Trichadenotecnum*, this muscle is

197   absent, despite the species possessing a well-developed ventral valve.

198

199   Muscles originating from the dorsal valves

200   **10** dosp01 (Fig. 11C, D); O: base of the dorsal valve; I: on the membrane connected to

201 the spermapore plate, near the posterior tip of the spermapore plate; and F: involved in

202 restoring the position of the spermapore plate.

203 **11** doIX01 (Fig. 11C, D); O: anterior end of the dorsal valve; I: posterolateral margin

204 of segment IX; and F: involved in opening the dorsal valve.

205 These two muscles originate from the dorsal valve and are inserted into segment

206 IX at either the spermapore plate (dosp01) or the clunium (doIX01). They are only

207 observed in *Valenzuela* and *Trichadenotecnum* (Psocomorpha) but are absent in all

208 other taxa examined.

209

210 Muscles originating from the external valves

211 **12** exsp01 (Fig. 12); O: base of the external valve; I: on the membrane connected to the

212 spermapore plate, near the posterior tip of the spermapore plate, and partially

213 overlapping dosp01; and F: involved in restoring the position of the spermapore plate.

214 **13** exIX01 (Fig. 12); O: lateral margin of the external valve; I: posterolateral margin of

215 segment IX, and partially overlapping doIX01; and F: involved in opening the external

216 valve.

217 **14** exIX02 (Fig. 12); O: base of the external valve, near the middle of the junction

218 with the clunium; I: anterolateral margin of the clunium; and F: involved in opening

219 the external valve.

220 These three muscles originate from the external valve and are inserted on segment

221 IX at either the spermapore plate (exsp01) or the clunium (exIX01–02). These

222 muscles are widely observed throughout psocopterans but completely absent in

223 *Archipsocus* and *Valenzuela*. The exIX01 muscle is absent in *Prionoglaris,*

224 *Archipsocus* and *Valenzuela*. In addition, the exIX02 muscle is absent in Psocomorpha

225 (Fig. 12).

226

227 **15** doex01 (Fig. 11E, F); O: base of the dorsal valve; I: base of the external valve; and

228 F: involved in opening the dorsal valve.

229   This is the only muscle connecting two gonapophyses (dorsal and external)

230 observed in psocopterans. The muscle is only observed in *Lepinotus* (Trogiomorpha)

231 and Amphientomidae (Troctomorpha).

232

233 3. 4. Subgenital plate

234 The subgenital plate is formed by sternum VIII and covers the gonapophyses

235 ventrally. It is variable in shape between taxa, and it frequently bears a posterior

236 extension (egg guide). In contrast, the subgenital plate is greatly reduced in

237 Trogiomorpha and only covers the anterior part of the gonapophyses.

238 **16** sgVIII01 (Fig. 13); O: middle of the dorsal surface of the subgenital plate; I: middle

239 of the ventral surface of the egg guide; and F: involved in closing the subgenital plate

240 **17** sgVIII02 (Fig. 13); O: middle of the dorsal surface of the subgenital plate; I: anterior

241 margin of sternum VIII, and partially overlapping sgVIII01; and F: involved in opening

242 the subgenital plate.

243   These two groups of muscles originate from the subgenital plate and are only

244   observed in Psocomorpha. No muscles are attached on the subgenital plate in the

245   suborders Trogiomorpha and Troctomorpha. The subgenital plate of Trogiomorpha is

246   greatly reduced and the absence of the subgenital muscles may be partly linked to this

247   condition. The sgVIII02 muscle was present in all Psocomorpha taxa examined,

248   whereas sgVIII01 was absent in *Archipsocus* (Fig. 13).

249   3. 5. Spermapore plate

250   The spermapore plate is a sclerite bearing the spermapore (e.g., Figs. 1B and 12).

251   According to Bitsch (1979) the spermapore plate is situated on the posterior part of

252   sternum VIII (see Lienhard *et al.*, 2010). However, by observing the 3D modeling, in

253   most cases, the spermapore plate is located behind the subgenital plate, but is not

254   connected to it. The skeletal part of the spermapore plate is not connected to the

255   surrounding structures directly but is connected to the genital structures of the segment

256   IX through the spermapore membrane, so we judged it to be a part of the sternum IX.

257   Its degree of sclerotization is highly variable, and it is almost completely membranous

258   in some taxa (*Archipsocus* and *Valenzuela*; Figs. 5B and 6B). Its lateral margins loosely

259   articulate with surrounding structures (paraproct, gonapophyses, and subgenital plate)

260   via the spermapore membrane.

261   In addition to the muscles described below, the muscles dosp01 and exsp01, both

262   originating on the gonapophyses, are also associated with the membranous part of the

263   spermapore plate in many taxa examined (see above).

264 **18** spVIII01 (Fig. 14C, E); O: on the spermapore membrane; I: anteroventral margin

265 of segment VIII, near the junction of the clunium; and F: involved in restoring the

266 position of the spermapore plate.

267   This is the only muscle that originates from the spermapore plate; it is inserted

268 onto sternum VIII the examined if present. It is present in *Lepinotus* (Trogiomorpha)

269 and *Valenzuela* (Psocomorpha).

270 **19** spIX01 (Fig. 14); O: on the spermapore membrane; I: mediolateral region of the

271 clunium; and F: involved in restoring the position of the spermapore plate.

272 **20** spIX02 (Fig. 14); O: on the spermapore membrane; I: mediolateral region of the

273 clunium, and partially overlapping with exIX01; and F: involved in restoring the

274 position of the spermapore plate.

275 **21** spIX03 (Fig. 14); O: the membranous part of the spermapore plate, near the

276 internal part of the dorsal valve; I: anterolateral margin of segment IX, and partially

277 overlapping with doVIII02 and exIX02; and F: involve in stretching the spermapore

278 plate.

279   The spIX03 muscle is often present but missing in *Prionoglaris* and

280 Amphientomidae. In contrast, muscle spIX02 is restricted to Trogiomorpha and spIX01

281 was only found in *Psyllipsocus* and *Lepinotus*.

282

283 **4. DISCUSSION**

284 **4. 1. Homology of the muscles**

285   Apart from the ovipositor valves (discussed below), the homology of the female

286   terminal sclerites (i.e., clunium, epiproct, paraproct, subgenital plate and spermapore

287   plate) is almost unambiguously identifiable throughout psocopterans (Figs. 1–7).

288   Therefore, based on the origin and insertion points of each muscle, the homology of the

289   female genital muscles of psocopterans can be established confidently throughout the

290   order (Table 2). Badonnel (1934) also examined the female genital musculature of

291   *Stenopsocus stigmaticus*, a close relative of *Valenzuela* examined here, and the muscles

292   that he observed can be homologized with the muscles identified here (see also the

293   ovipositor section below). However, the homology of several muscles is debatable.

294   For example, paX02 (Fig. 8) was consistently observed throughout psocopterans

295   and almost always originates from the dorsal margin of the paraproct, very close to the

296   lateral margin of the epiproct. However, in *Lepinotus*, the site of origin of the

297   corresponding muscle is changed to the epiproct (Fig. 3B). Therefore, judging only

298   from origin, this muscle may be regarded as an epiproctal muscle. However, in

299   *Lepinotus*, no other muscle corresponding to paX02 could be detected. In addition, its

300   site of origin is very close to that of paX02 observed in other psocopterans, and its

301   insertion site is consistent with that of paX02. Therefore, although its origin and

302   probably also its function are different from that of paX02 in other psocopterans, the

303   muscle originating from the lateral margin of the epiproct of *Lepinotus* was identified

304   here as paX02 (Table 2).

305   A similar situation was observed for spIX02 (Fig. 14A–C), which only occurs in

306   Trogiomorpha. The insertion site of the muscle in *Psyllipsocus* and *Lepinotus* is the

307   mediolateral region of the clunium. The spIX02 muscle of *Lepinotus* consists of a pair

308   of bundles, whereas spIX02 of *Prionoglaris* and *Psyllipsocus* consists of a single bundle.

309   Moreover, the insertion site of spIX02 of *Prionoglaris* is the middle region of the

310   external valve. Except for spIX02, no other muscles of *Prionoglaris* are connected to

311   the spermapore membrane, and no muscles similar to those on the external valve are

312   found in other psocopterans. In *Prionoglaris,* the external valve is significantly enlarged

313   and thus expands dorsally in comparison to other trogiomorphan genera. The

314   orientation of the muscles (horizontally directed towards its origin on the spermapore)

315   is similar in *Prionoglaris* and *Psyllipsocus/Lepinotus*. Therefore, the muscle originating

316   from the spermapore plate and inserted on the middle region of the external valve of

317   *Prionoglaris* was identified as spIX02 (Table 2).

318   In addition, some muscles are confirmed only in few distantly related taxa. For

319   example, veVII01 (Fig. 11A, B) was only observed in Amphientomidae (Troctomorpha)

320   and *Valenzuela* (Psocomorpha), and doex01 (Fig. 11E, F) was only found in *Lepinotus*

321   (Trogiomorpha) and Amphientomidae (Table 2). As discussed below (see the

322   phylogenetic significance section), independent gains of these muscles are the most

323   parsimonious interpretation (i.e., Fig. 15), but corresponding muscles are also observed

324   in some holometabolans (Table 2: Hünefeld *et al.*, 2012). It is generally regarded that

325   the loss of a character occurs much more frequently than the independent gain of a

326   novel feature (e.g., Trueman *et al.*, 2004). Therefore, although this remains ambiguous,

327   we tentatively recognized the muscles only observed in distantly related psocopteran

328   taxa and holometabolans as homologous (Table 2), suggesting multiple loss rather than

329   more parsimonious independent gain.

330   Homology identification of the female genital muscles between psocopterans and

331   other insects is complicated. Psocopterans are traditionally included in the superorder

332   Paraneoptera (this view is still most strongly supported morphologically and also could

333   not be rejected also by phylogenomics: Hennig, 1969; Yoshizawa & Lienhard 2016;

334   Johnson *et al.*, 2018), but the order Psocodea was interpreted as the sister taxon of the

335   Holometabola by phylogenomics (Misof *et al.*, 2014; Johnson *et al*., 2018). The ground

336   plan condition of the female genital musculature in Holometabola has been estimated

337   by Hünefeld *et al*. (2012). Therefore, we tried to homologize the psocopteran female

338   genital muscles according to the system of Hünefeld *et al*. (2012). As shown in Table

339   2, homology of only a few muscles (e.g., epiproctal epX01 muscle) can be identified

340   confidently   between   psocopterans   and   Holometabola.   In   contrast,   although

341   homologous muscles are probably present in Holometabola (e.g., paraproctal muscles

342   paX01–05), the homology of many psocopteran muscles cannot be specified with

343   reasonable certainty, for which more than one candidate homologous muscle is

344   indicated in Table 2. Examination of the other paraneopteran and holometabolan taxa

345   may improve these ambiguities.

346   **4. 2. Homology of the psocopteran ovipositor valves**

347   In the neopteran ground plan condition, the ovipositor is composed of three sets of

348   elongated valves derived from postabdominal appendages, namely, V1 (segment VIII),

349 V2 and V3 (both segment IX). Frequently, a short valvifer (or gonocoxite) is also

350 present basal to the valves of each segment (VIII and IX) (Beutel *et al*., 2014).

351 The psocopteran ovipositor valves are also usually composed of three valves,

352 namely, ventral (segment VIII), dorsal and external valves (both segment IX), which

353 are generally considered to be homologous to V1–V3 of the neopterans groundplan.

354 When all valves are well developed, they can be addressed very clearly. Even though

355 the dorsal and external valves are fused with each other, they can be clearly identified

356 as elongated dorsal valve and basally bulged external valve (e.g., Yoshizawa, 2005). In

357 contrast, the valvifer is never developed as a separated sclerite in psocopterans.

358 Hünefeld *et al*. (2012) examined the musculature of the female postabdomen of a

359 wide variety of taxa and estimated the ground plan of the abdominal musculature of the

360 Holometabola. According to the interpretation presented in Hünefeld *et al*. (2012), all

361 intersegmental and dorsoventral muscles associated with the ovipositor valves are

362 inserted on the valvifer (i.e., the coxal segment of the appendage).

363 In psocopterans, one intersegmental muscle (veVII01: homologous to the muscle

364 "c" of Badonnel, 1934: fig. 69) is inserted to the base of the ventral valve (segment VIII:

365 Fig. 11A, B). This muscle is apparently homologous to either muscle 08 or 09

366 (intersegment VII) of Hünefeld *et al*. (2012) (Table 2), suggesting that the psocopteran

367 ventral valve is composed of fused valvifer VIII and V1. In psocopteran segment IX,

368 three dorsoventral muscles are observed (Figs 11C, D and 12): one is inserted on the

369 dorsal valve (doIX01: muscle "e" of Badonnel, 1934) and the other two on the base of

370   the external valve (exIX01 and 02: muscle "f" of Badonnel, 1934). They very likely

371   correspond to either the muscles 50, 51, 52 or 53 (dorsoventral IX) of Hünefeld *et al.*

372   (2012), which are usually inserted on valvifer IX in neopterans. Therefore, it is also

373   likely that the psocopteran dorsal and external valves are possibly a composite structure

374   formed by fusion of V2, V3, and valvifer IX.

375   In addition, a muscle named doex01 was observed in *Lepinotus* and

376   Amphientomidae, which connect the 65s (Fig. 11E, F). In the holometabolan ground

377   plan condition, two muscles connecting valvifers IX and V2 (57 and 58) are present

378   (Hünefeld *et al.*, 2012), and doex01 is very likely homologous to either 57 or 58 of

379   Hünefeld *et al.* (2012) (Table 2). In Amphientomidae (with three well-developed

380   valves), this muscle originates from the base of the dorsal valve and is inserted to the

381   external margin of the external valve (Fig. 11F). It also provides additional support that

382   valvifer IX is fused to V3 in psocopterans, together forming the external valve.

383   The presence of doex01 also provides interesting insights for the morphological

384   interpretation of ovipositor valves in psocopterans. In the suborder Trogiomorpha, they

385   are greatly reduced and are usually represented by a single pair of valves (Figs 1–3). It

386   is generally assumed that these single pair of valves are homologous to the external

387   ones. However, the presence of the doex01 muscle (= either 57 or 58 of Hünefeld *et al.*,

388   2012) strongly suggests that the well-developed valve of *Lepinotus* involves at least a

389   vestigial element of the dorsal valve as the insertion site of the muscle doex01 (Fig.

390   11E).

391   Different morphological interpretations for the female ovipositor valves were also

392   proposed for the mid-Cretaceous trogiomorphan genus *Palaeosiamoglaris* by Azar *et*

393   *al.* (2017). *Palaeosiamoglaris* is thought to be most closely related to the extant genus

394   *Siamoglaris* from Thailand, and they together form a monophyletic subfamily

395   *Prionoglaridinae* with *Prionoglaris*. The basic female genital structures of *Siamoglaris*

396   and *Prionoglaris* (examined here) are very similar. Azar *et al.* (2017) identified the most

397   well-developed ovipositor valve of *Palaeosiamoglaris* as the ventral valve, and opaque

398   structures presented next and dorsal to these structures were identified as external and

399   dorsal valves, respectively (Azar *et al.*, 2017: figs 5B, 15B). However, as discussed

400   above, the most distinct ovipositor valve of *Prionoglaris* and other trogiomorphan taxa

401   should be homologized with the external valve (with some vestigial element of the

402   dorsal valve), and reduction of the ventral and dorsal valves should be considered as an

403   autapomorphy of Trogiomorpha (Yoshizawa & Lienhard, 2020). As mentioned above,

404   *Palaeosiamoglaris* is placed "within" a subfamily of a trogiomorphan family and is

405   very closely related to *Prionoglaris*. Under such conditions, it is less likely that a well-

406   developed ventral valve is retained in *Palaeosiamoglaris*; thus, the structure is

407   interpreted here as the external valve (plus some vestigial element of a dorsal valve), as

408   also seen in *Prionoglaris*.

409   **4. 3. Phylogenetic significance of the musculature**

410

411   The data presented in Table 2 were treated as a character state matrix and

412   reconstructed on the phylogenetic tree of psocopterans estimated based on multiple

413   genes (Yoshizawa *et al.*, 2006; Yoshizawa & Johnson, 2014) or transcriptomes (Johnson

414   *et al.*, 2018; de Moya *et al*., 2021) to visualize the phylogenetic signal contained in the

415   female genital musculature (Fig. 15). The consistency index (CI) was calculated as 0.69,

416   and the retention index (RI) was 0.53. This CI value is relatively high compared to other

417   morphological character system used for the phylogenetic reconstruction of

418   Psocomorpha, e.g., 0.41 for the total morphology, 0.36 for male external genitalia, and

419   0.45 for female external genitalia (Yoshizawa & Johnson, 2014), suggesting that the

420   characteristic system includes useful phylogenetic signals with relatively low levels of

421   homoplasies.

422   The most parsimonious reconstruction of the character matrix on the tree yielded

423   some useful characters supporting deep psocopteran phylogenetic splits (Fig. 15): the

424   absence of exIX01 (14) and the presence of sgVIII01 (16) support the monophyly of

425   the suborder Psocomorpha, and the presence of dosp01 (10), doIX01 (11) and sgVIII02

426   (17) (all non-homoplasious) support the monophyly of Psocomorpha excluding

427   Archipsocetae. In addition, the condition of spIX02 (20) was consistently different

428   between Trogiomorpha (present) and Troctomorpha + Psocomorpha (absent) (Table 2).

429   Therefore, this muscle likely contains a signal to support either of these clades

430   (depending on the condition of the outgroup, but if the reduction of muscles is a general

431   tendency, this may rather support Troctomorpha + Psocomorpha: Table 2). Some

432   homoplasious and nonhomoplasious character states are only identified at the terminal

433   branches (i.e., phylogenetically noninformative) (Fig. 15). However, with denser

434   taxonomic sampling, these characters may be phylogenetically informative on a lower

435   taxonomic level (i.e., infraorder, family, or genus).

436   It has to be noted that the female genital musculature may contain more

437   homoplasies than discussed above. As already mentioned, independent losses of a

438   character are generally considered to be much more frequent than independent gains of

439   a novel feature. The present results suggested several independent gains of new muscles

440   (Fig. 15), for which the alternative interpretation (multiple loss of the muscles) should

441   also be considered. For example, exIX01 is absent in *Valenzuela* but was observed in

442   *Stenopsocus* (a close relative of *Valenzuela*) by Badonnel (1934: fig. 69). Although the

443   ancestral condition of this muscle is ambiguous for *Valenzuela* (Fig. 15), the presence

444   of exIX01 in *Stenopsocus* strongly suggests that the absence of this muscle is an

445   apomorphic condition independently derived in *Prionoglaris*, *Archipsocus*, and

446   *Valenzuela*. Much denser sampling of psocopterans is needed to accurately identify the

447   evolutionary trend of the female genital muscles. The present study provides the

448   foundation for such a future project.

449

450

451   **ACKNOWLEDGEMENTS**

459

460

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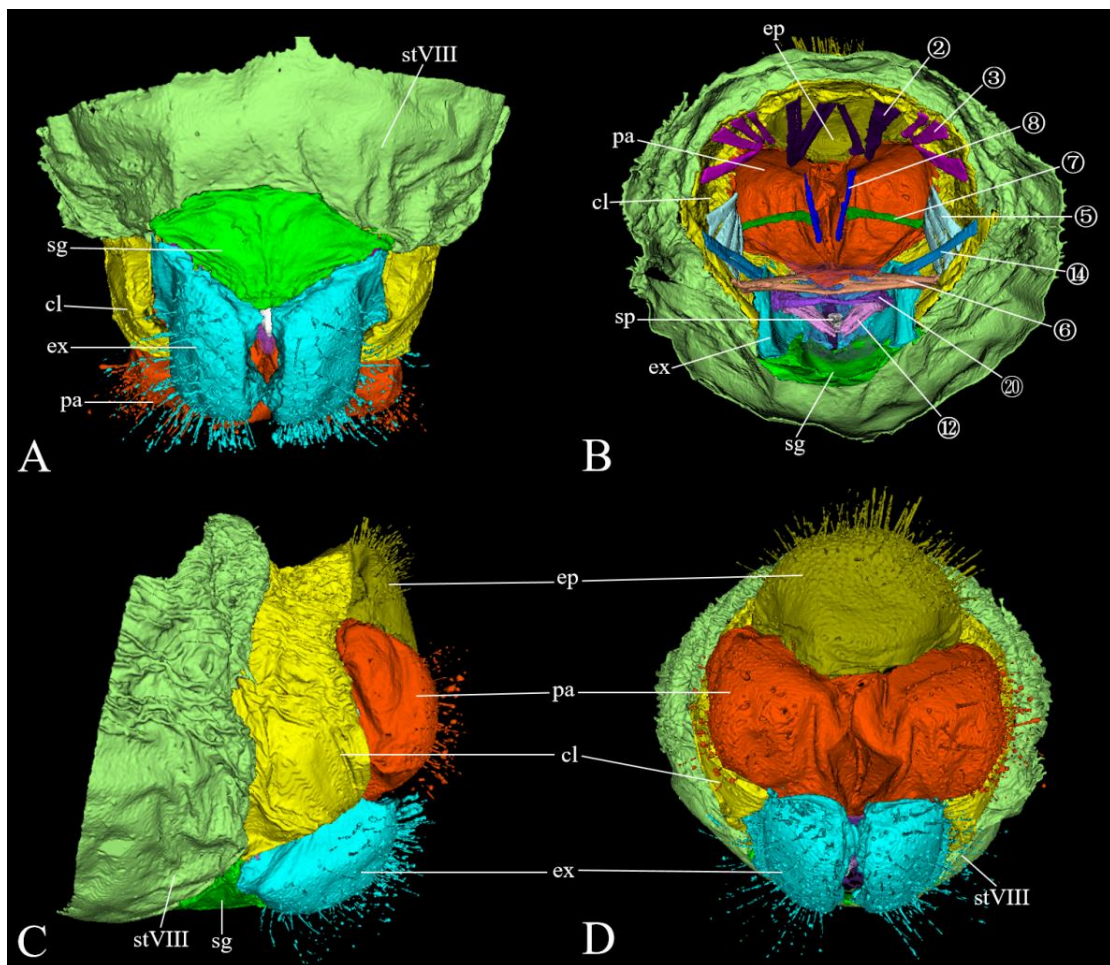
Fig. 1.

3D reconstruction of the female terminalia of *Prionoglaris stygia*: (A) ventral view; (B)

578   internal view: (2) paX01; (3) paX02; (5) paX04; (6) paX05; (7) papa01; (8) papa02;

579   (12) exsp01; (14) exIX02; (20) spIX02; (C) lateral view; and (D) posterior view.

580   Abbreviations: cl = clunium; ex = external valve; sg = subgenital plate; ep = epiproct;

581   pa = paraproct; sp = spermapore plate; mb = membrane; st = sternum.
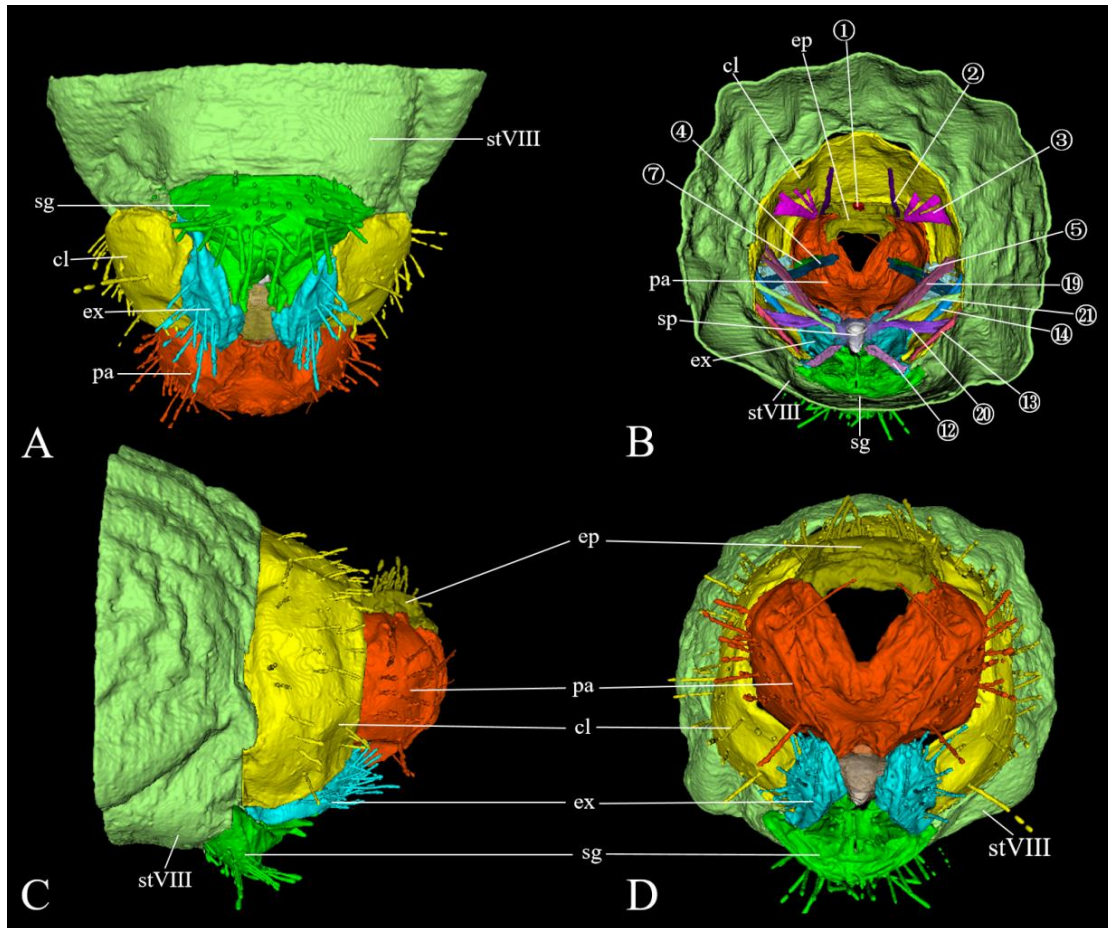
583   **Fig. 2.**

584   3D reconstruction of the female terminalia of *Psyllipsocus clunjunctus*: (A) ventral

585   view; (B) internal view: (1) epX01; (2) paX01; (3) paX02; (4) paX03; (5) paX04; (7)

586   papa01; (12) exsp01; (13) exIX01; (14) exIX02; (19) spIX01; (20) spIX02; (21) spIX03;

587   (C) lateral view; and (D) posterior view. See Fig. 1 for abbreviations.
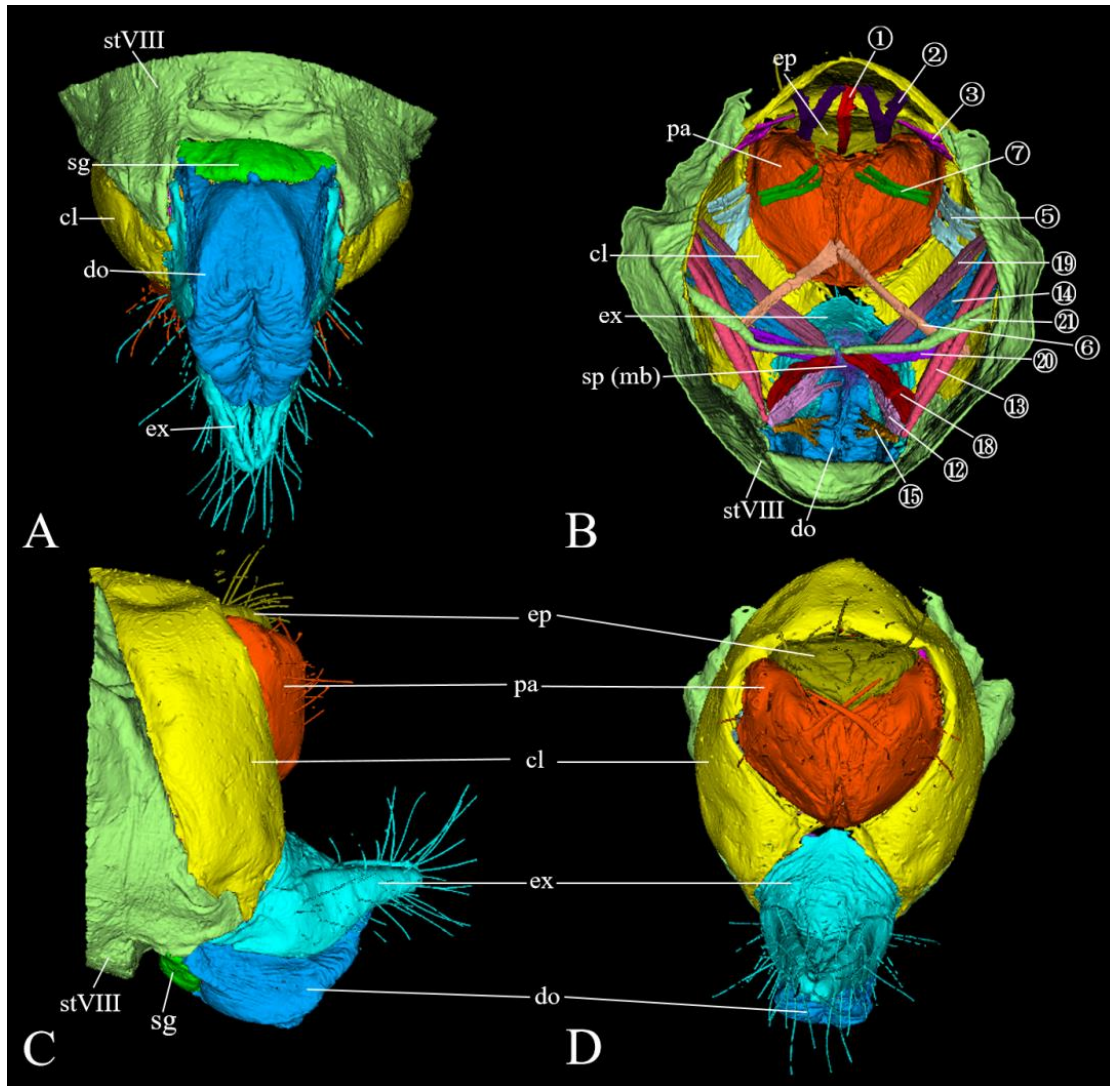
Fig. 3.

3D reconstruction of the female terminalia of *Lepinotus reticulatus*: (A) ventral view;
(B) internal view: (1) epX01; (2) paX01; (3) paX02; (5) paX04; (6) paX05; (7) papa01;
(12) exsp01; (13) exIX01; (14) exIX02; (15) doex01; (18) spVIII01; (19) spIX01; (20)
spIX02; (21) spIX03; (C) lateral view; and (D) posterior view. See Fig. 1 for
abbreviations.
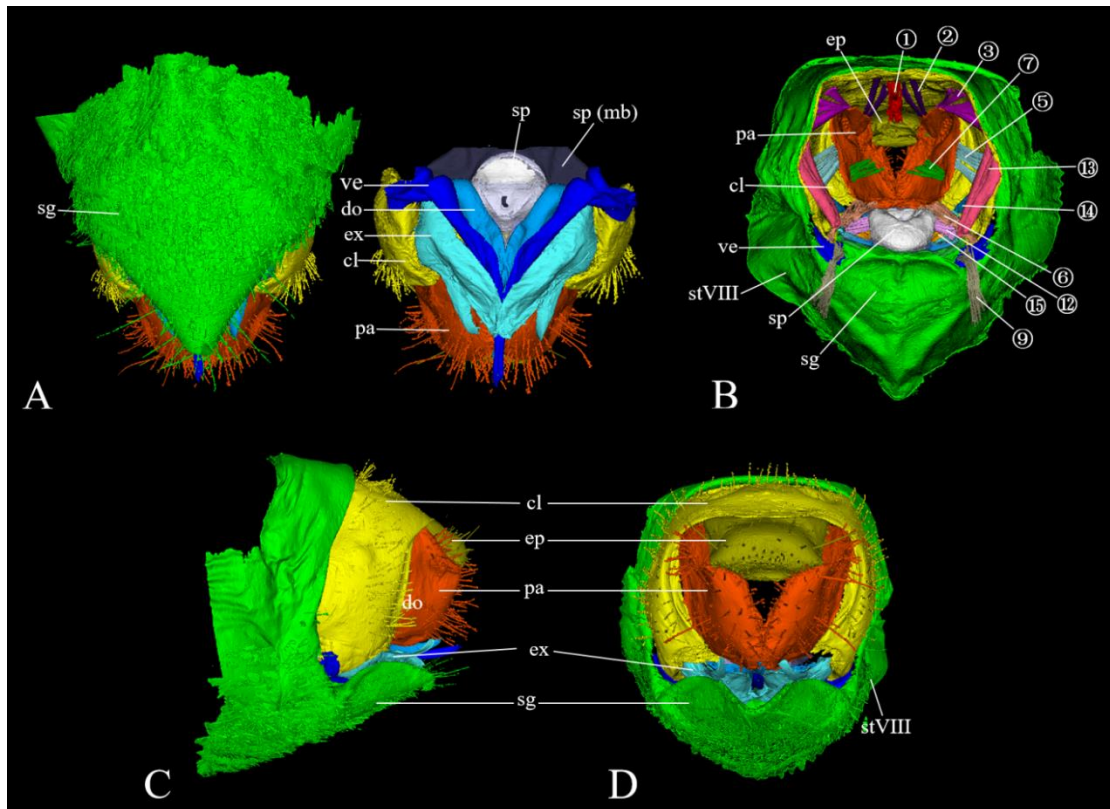
Fig. 4.

3D reconstruction of the female terminalia of Amphientomidae Gen. sp.: (A) ventral view (right: the subgenital plate is invisible); (B) internal view: (1) epX01; (2) paX01; (3) paX02; (5) paX04; (6) paX05; (7) papa01; (9) veVII01; (12) exsp01; (13) exIX01; (14) exIX02; (15) doex01; (C) lateral view; and (D) posterior view. See Fig. 1 for abbreviations.
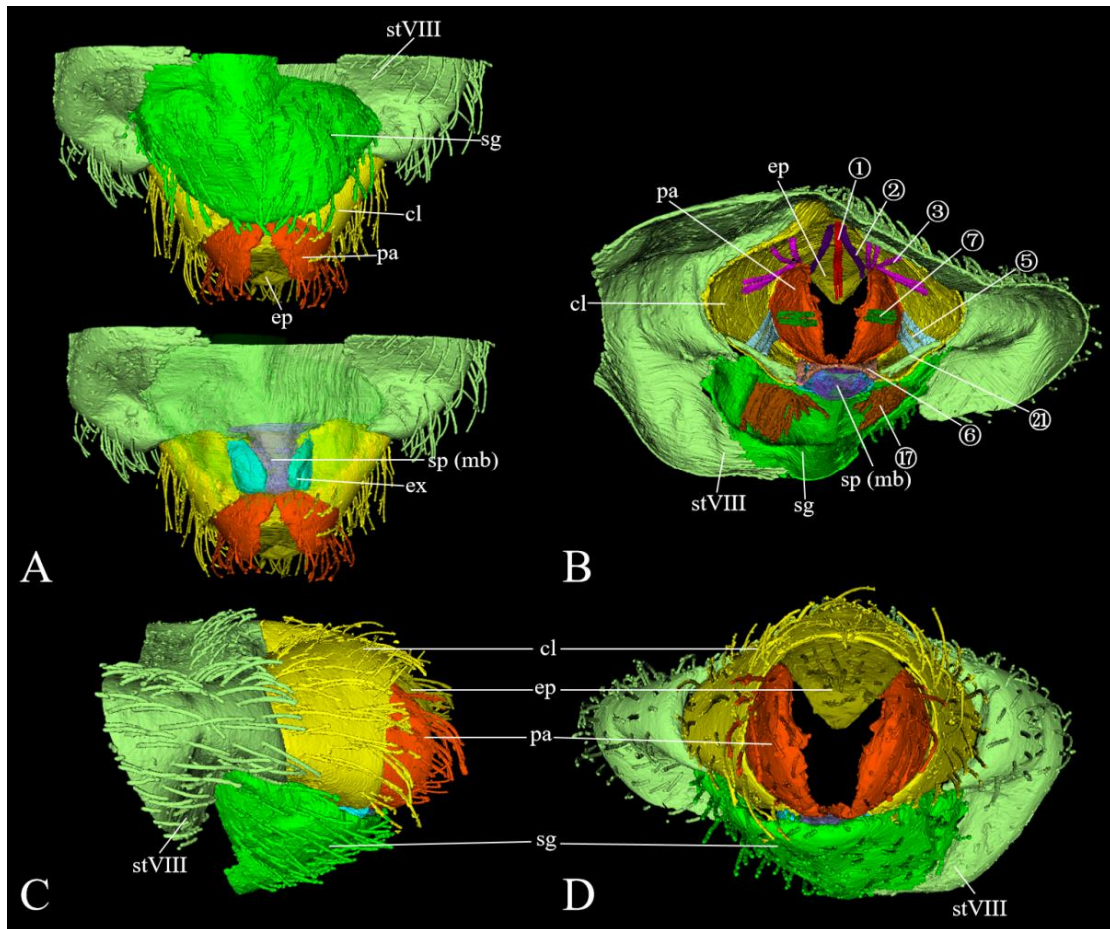
Fig. 5.

3D reconstruction of the female terminalia of *Archipsocus* sp.: (A) ventral view (below: the subgenital plate is invisible); (B) internal view: (1) epX01; (2) paX01; (3) paX02; (5) paX04; (6) paX05; (10) dosp01; (11) doIX01; (12) exsp01; (13) exIX01; (17) sgVIII02; (21) spIX03; (C) lateral view; and (D) posterior view. See Fig. 1 for abbreviations.

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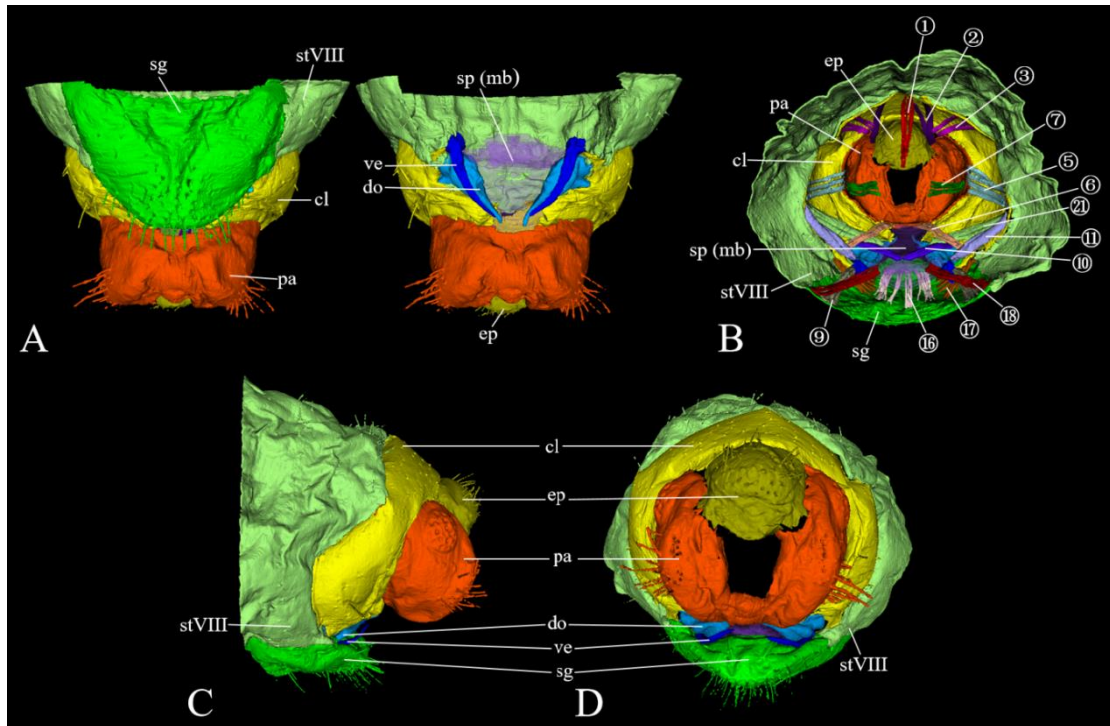
Fig. 6.

3D reconstruction of the female terminalia of *Valenzuela badiostigma*: (A) ventral view
(right: the subgenital plate is invisible); (B) internal view: (1) epX01; (2) paX01; (3)
paX02; (5) paX04; (6) paX05; (7) papa01; (9) veVII01; (10) dosp01; (11) doIX01; (16)
sgVIII01; (17) sgVIII02; (18) spVIII01; (21) spIX03; (C) lateral view; and (D) posterior
view. See Fig. 1 for abbreviations.

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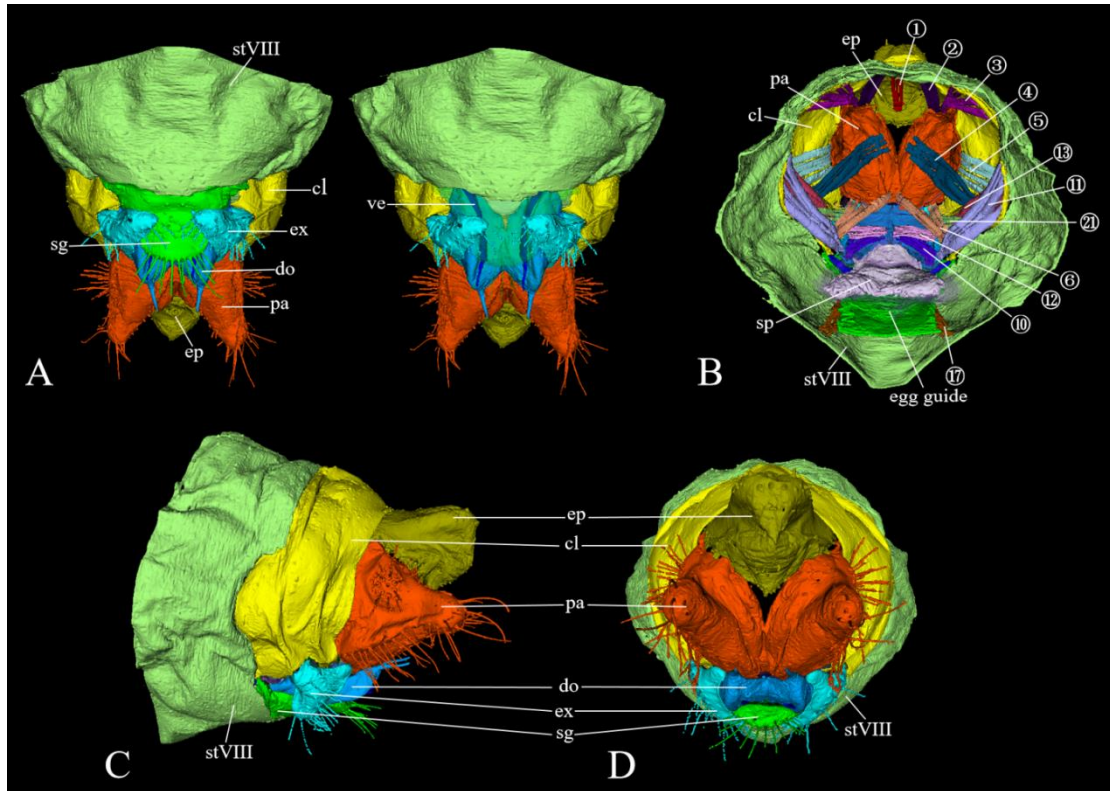
Fig. 7.

3D reconstruction of the female terminalia of *Trichadenotecnum pseudomedium*: (A)

ventral view (right: subgenital plate is invisible); (B) internal view: (1) epX01; (2)

paX01; (3) paX02; (4) paX03; (5) paX04; (6) paX05; (10) dosp01; (11) doIX01; (12)

exsp01; (13) exIX01; (17) sgVIII02; (21) spIX03; (C) lateral view; and (D) posterior

view. See Fig. 1 for abbreviations.
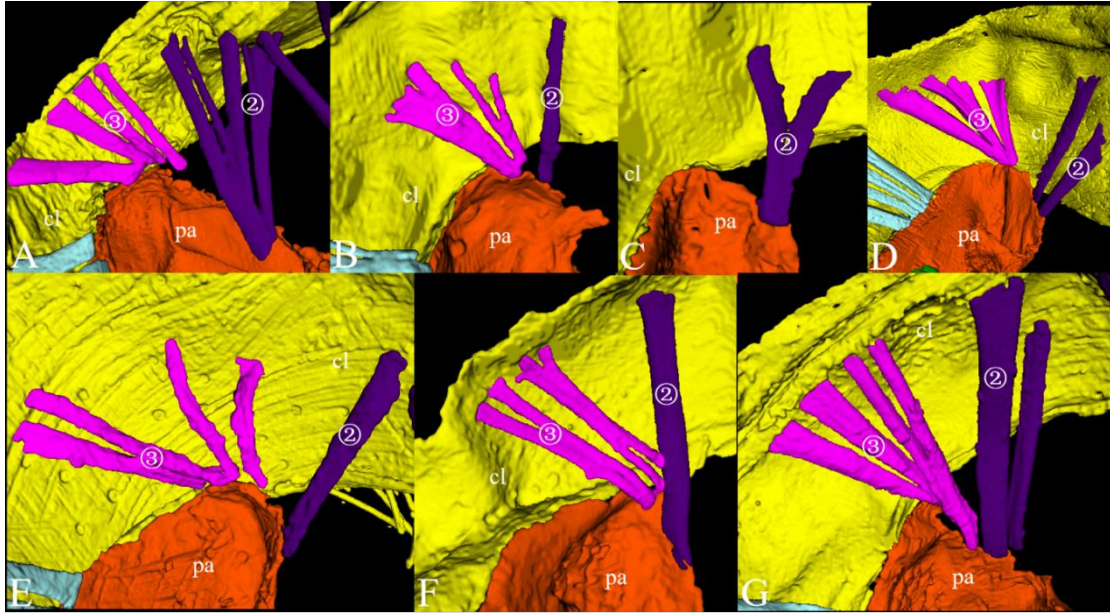
Fig. 8.
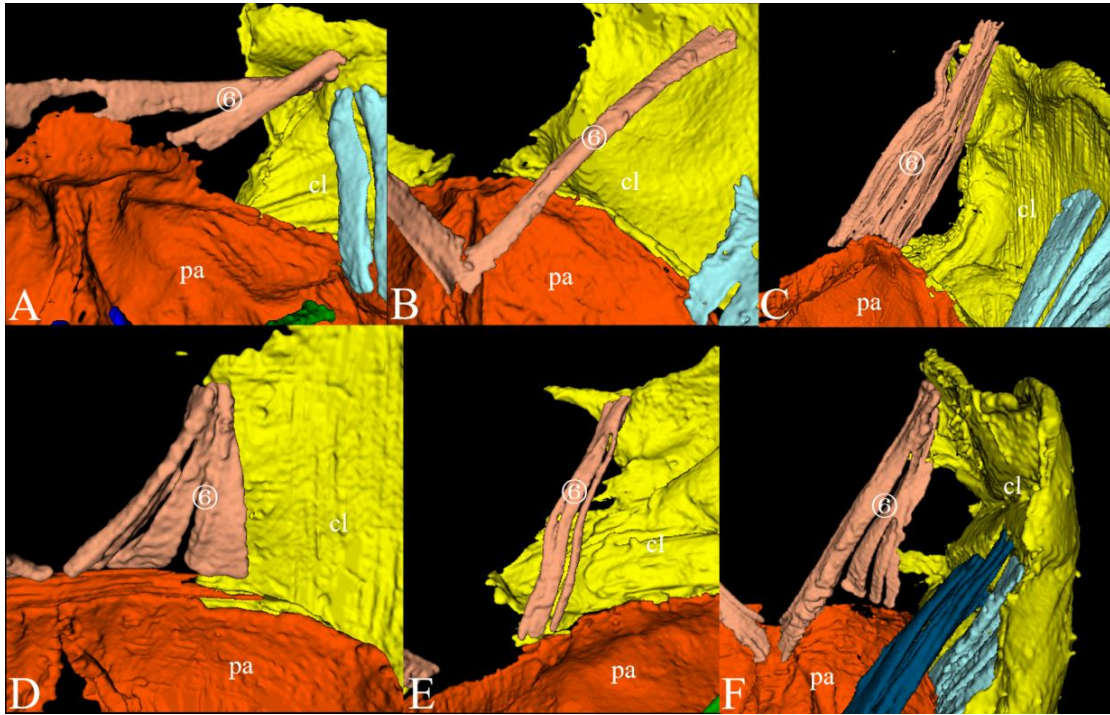
Close up of the paraproct (dorsal part) and clunium, internal view: (A) *Prionoglaris stygia*; (B) *Psyllipsocus clunjunctus*; (C) *Lepinotus reticulatus*; (D) Amphientomidae Gen. sp.*;* (E) *Archipsocus* sp.*;* (F) *Valenzuela badiostigma*; and (G) *Trichadenotecnum pseudomedium*. (2) paX01; (3) paX02. See Fig. 1 for abbreviations.

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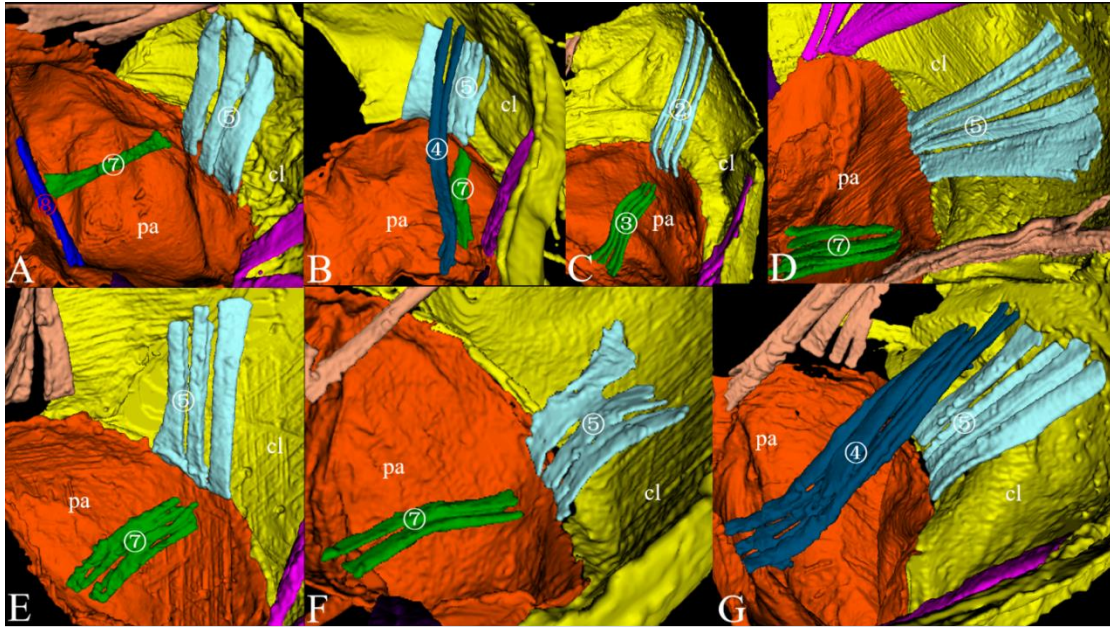
Fig. 9.

Close up of the paraproct (lateral part) and clunium, internal view: (A) *Prionoglaris stygia*; (B) *Psyllipsocus clunjunctus*; (C) *Lepinotus reticulatus*; (D) Amphientomidae Gen. sp.*;* (E) *Archipsocus* sp.*;* (F) *Valenzuela badiostigma*; and (G) *Trichadenotecnum pseudomedium*. (4) paX03; (5) paX04; (7) papa01; (8) papa02. See Fig. 1 for abbreviations.
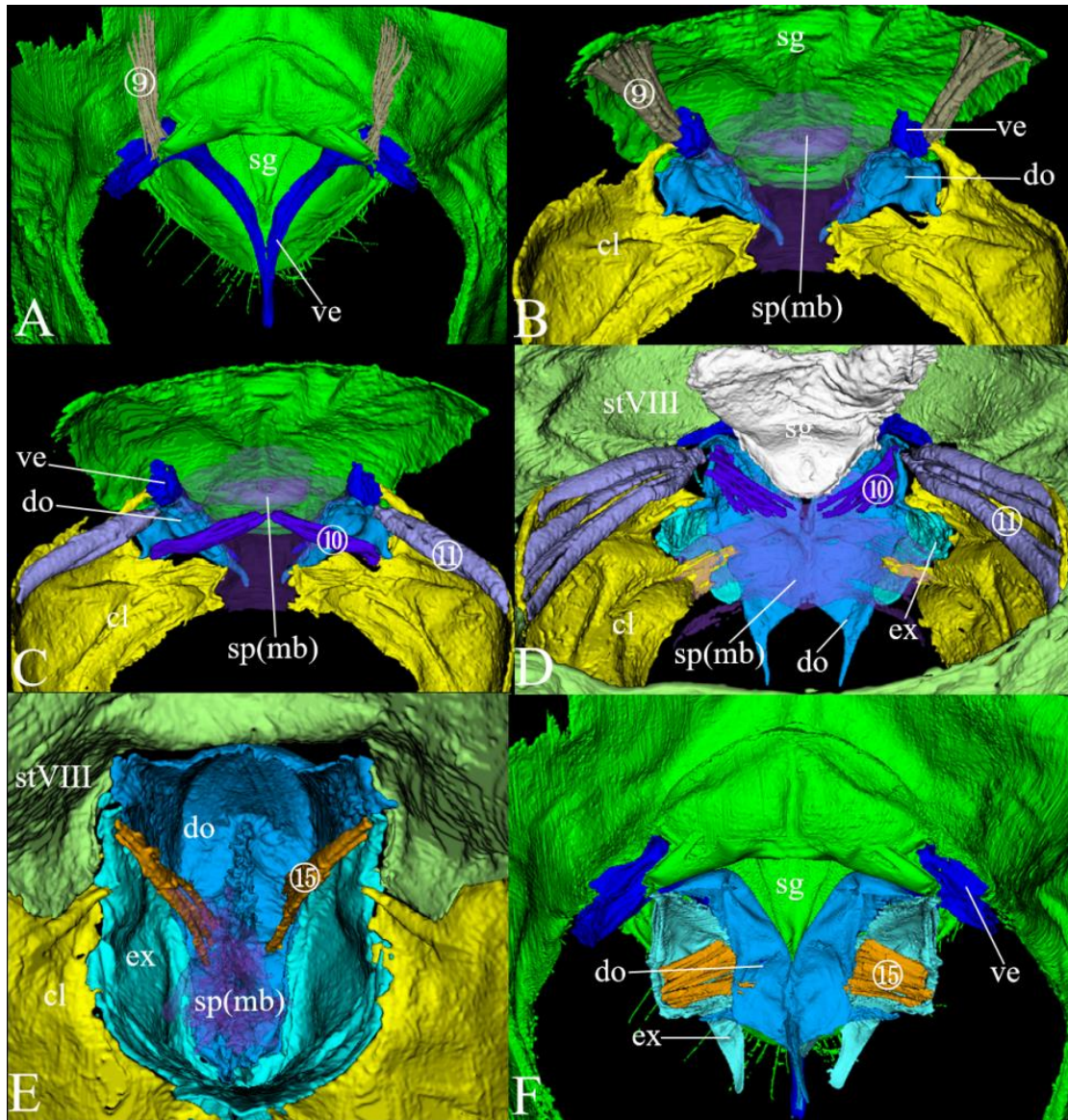
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Fig. 10.

Close up of the paraproct (ventral part) and clunium, internal view: (A) *Prionoglaris*

stygia; (B) *Lepinotus reticulatus*; (C) Amphientomidae Gen. sp.*;* (D) *Archipsocus* sp.*;*

(E) *Valenzuela badiostigma*; and (F) *Trichadenotecnum pseudomedium*. (6) paX05.

Abbreviations: cl = clunium; pa = paraproct.

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Fig. 11.

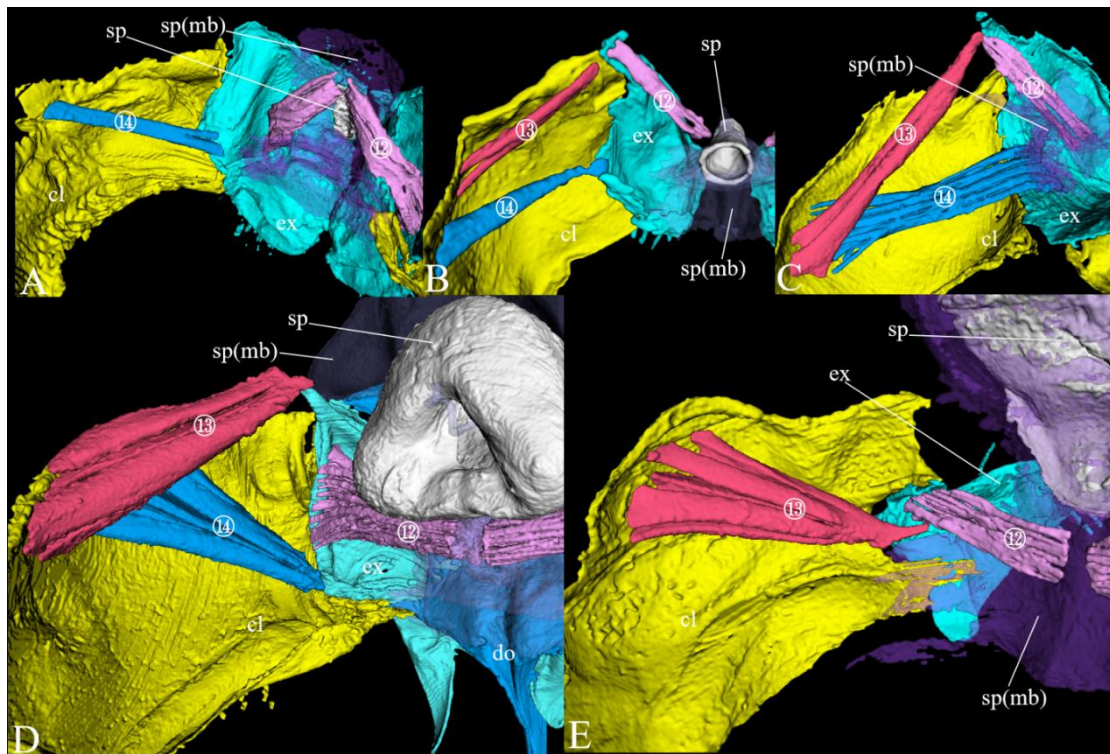Close-up of the gonapophyses and related structures, internal view: (A) Amphientomidae Gen. sp.*;* (B) *Valenzuela badiostigma*; (C) *Valenzuela badiostigma*; (D) *Trichadenotecnum pseudomedium*; (E) *Lepinotus reticulatus*; and (F) Amphientomidae Gen. sp. (9) veVII01; (10) dosp01; (11) doIX01; (15) doex01. See Fig. 1 for abbreviations.

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Fig. 12.
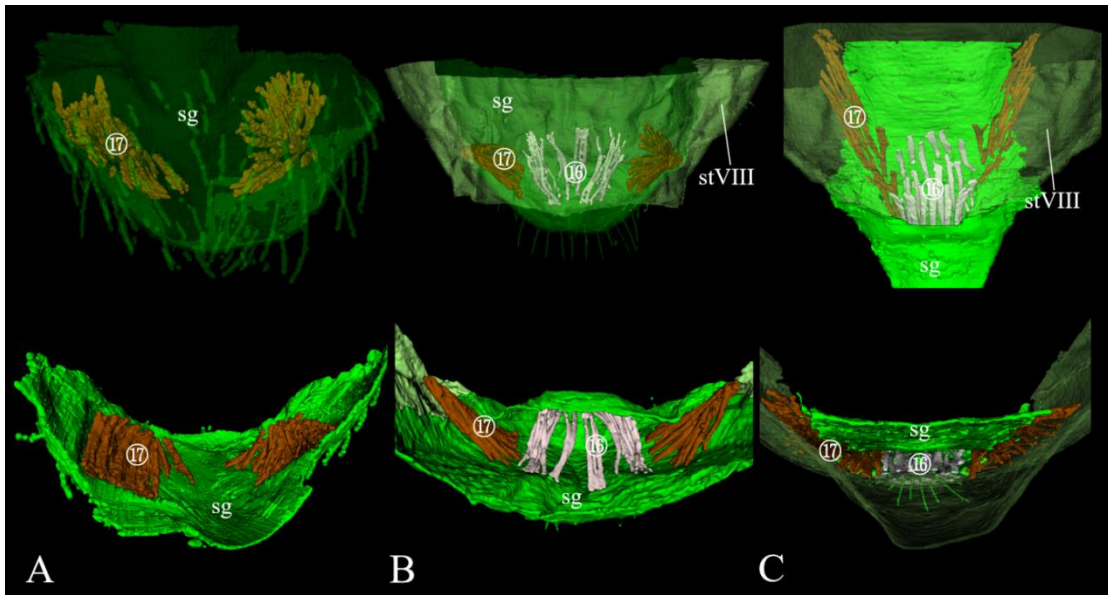
Close-up of the gonapophyses and related structures, internal view: (A) *Prionoglaris stygia*; (B) *Psyllipsocus clunjunctus*; (C) *Lepinotus reticulatus*; (D) Amphientomidae Gen. sp; and (E) *Trichadenotecnum pseudomedium*. (12) exsp01; (13) exIX01; (14) exIx02. See Fig. 1 for abbreviations.
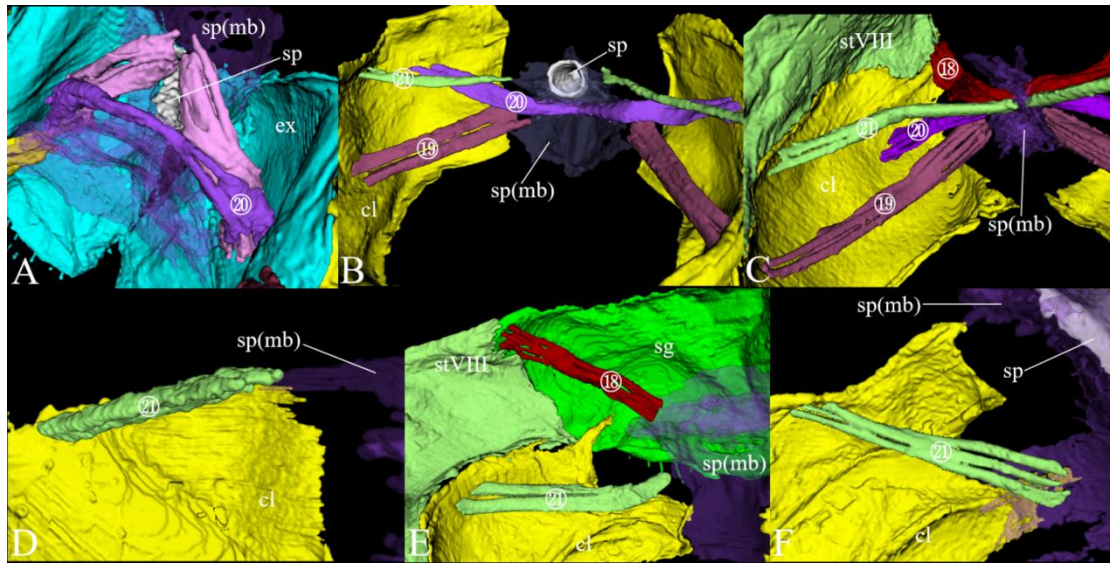
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Fig. 13.

Subgenital plate, internal view: (A) *Archipsocus* sp. (top: ventral view; below: internal view); (B) *Valenzuela badiostigma* (top: ventral view; below: internal view); and (C) *Trichadenotecnum pseudomedium* (top: ventral view; below: internal view). (16) sgVIII01; (17) sgVIII02. See Fig. 1 for abbreviations.
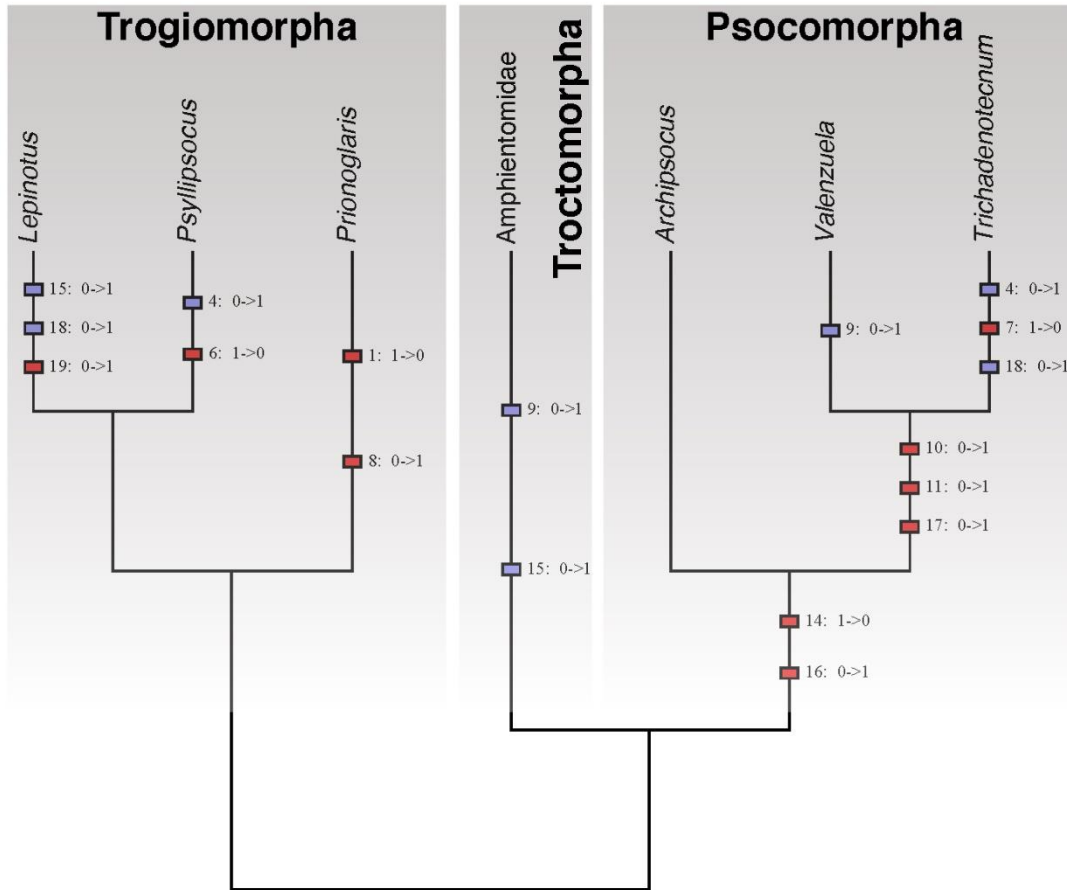
Fig. 14.

Muscles related to the spermapore membrane, internal view: (A) *Prionoglaris stygia*; (B) *Psyllipsocus clunjunctus*; (C) *Lepinotus reticulatus*; (D) *Archipsocus* sp*.;* (E) *Valenzuela badiostigma*; and (F) *Trichadenotecnum pseudomedium*. (18) spVIII01; (19) spIX01; (20) spIX02; (21) spIX03. See Fig. 1 for abbreviations.

Fig. 15.

Most parsimonious reconstruction of the presence (1) or absence (0) of the female genital muscles. Tree topology is from the phylogenomic tree estimated by de Moya *et al*. (2021). See Table 2 for the character table. Red bars indicate nonhomoplasious characters, and blue bars indicate characters that include homoplasy.

Suborder	Infraorder	Family	Species	Locality	Specimen ID	Collector
Trogiomorpha	Prionoglaridetae	Prionoglarididae	*Prionoglaris stygia*	France	S8KY37	B. Horser
	Psyllipsocetae	Psyllipsocidae	*Psyllipsocus clunjinctus*	Brazil	S8KY10	R. Ferreira
	Atropetae	Trogiidae	*Lepinotus reticulatus*	Japan	S8KY32	K. Yoshizawa
Troctomorpha	Amphientometae	Amphientomidae	Genus sp.	Malaysia	S8KY14	K. Yoshizawa
Psocomorpha	Archipsocetae	Archipsocidae	*Archipsocus* sp.	Mexico	S8KY75	K. Yoshizawa
	Caeciliusetae	Caeciliusidae	*Valenzuela badiostigma*	Japan	S8KY18	K. Yoshizawa
	Psocetae	Psocidae	*Trichadenotecnum pseudomedium*	Japan	S8KY28	K. Yoshizawa

Table 1

Taxa examined in this study.

Muscle #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
	epIX01	paIX01	paIX02	paIX03	paIX04	paIX05	papa01	papa02	veVIII01	dosp01	doIX01	exsp01	exIX01	exIX02	doex01	sgVIII01	sgVIII02	spVIII01	spIX01	spIX02	spIX03
Prionoglaris	0	1	1	0	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0
Psyllipsocus	1	1	1	1	1	0	1	0	0	0	0	1	1	1	0	0	0	0	0	1	1
Lepinotus	1	1	1	0	1	1	1	0	0	0	0	1	1	1	1	0	0	1	1	1	1
Amphientomidae	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	0	0	0	0	0	0
Archipsocus	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Valenzuela	1	1	1	0	1	1	1	0	1	1	1	0	0	0	0	1	1	0	0	0	1
Trichadenotecnum	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	1	1	1	0	0	1
# in Hünefeld et al.	59		65, 67–69				66	64	08, 09		53		50–52		57,58	27, 33	30, 31	70		75–77	
Badonnel (1934)									c	d	e		f								

Table 2

List of the muscles identified in the psocopteran female terminalia and their distribution throughout the taxa examined (present 1/absent 0). The muscle numbers correspond to the character numbers shown in Figure 15. Homology with the holometabolan female genital muscles (as proposed in Hünefeld *et al*. 2012) and homology of the gonapophyses muscles observed by Badonnel (1934) are also shown at the bottom of the table (for holometabolan muscles, more than one candidate homologous muscle is listed for many cases).