1 **Divergent sperm traits in Carabidae ground beetles (Insecta: Coleoptera)**

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6 **Running head:** Divergent sperm traits in Carabid beetles

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13 **Abstract**

14 Sperm exhibit marked morphological diversity, and investigations into sperm diversity can further

15 the understanding of many areas of evolutionary biology. In this study, using light microscopy,

16 sperm morphology was examined in 39 species of Carabidae from eight subfamilies, including five

17 subfamilies in which sperm morphology has not previously been examined. In all but one of the

18 subfamilies, the subfamily members shared the same type of sperm: single sperm were observed in

19 Cicindelinae, Nebriinae, and Trechinae; sperm conjugates, in which numerous sperm adhere together,

20 were observed in Elaphrinae, Patrobinae, and Brachinae; and both single sperm and sperm

21 conjugates were observed in Broscinae. In the remaining subfamily, Harpalinae, most species

22 formed sperm conjugates, but some species formed single sperm. Some noteworthy sperm were also

23 observed: the shortest single sperm in the order Coleoptera was found; multiflagellated sperm were

24 observed, which had previously been reported from only one species in the class Insecta; and size

25 variation of sperm conjugates, which may represent size dimorphism, was observed. Based on the

26 results of this and previous studies, the evolutionary pattern of sperm traits and the phylogenetic

27 utility of sperm morphologies in Carabidae are discussed.

28

INTRODUCTION

Insects possess divergent reproductive traits, which are of interest in many areas of evolutionary biology. In these reproductive traits, male sperm show marked morphological diversity, and this diversity is associated with two research topics. The first addresses the evolution of reproductive traits. Reproductive traits often exhibit correlated evolution, and sperm also demonstrates morphological association with other reproductive traits (Simmons, 2001; Pitnick *et al.*, 2009). Thus, the study of sperm morphology is necessary to develop an understanding of the mechanisms by which diversity in reproductive traits occurred. The second research topic involves phylogenetic analyses. For example, among higher taxa, it is often difficult to determine the homology of morphological characters, which hampers phylogenetic reconstruction based on morphological data. However, even in such cases, the homology of sperm morphology can often be determined, and comparative morphology can provide insights into the reconstruction of phylogenies (i.e., spermiocladistics) (Jamieson *et al.*, 1999). Nevertheless, despite the utility of these studies, sperm morphology remains unexamined in many insect groups.

The beetle family Carabidae is one such group. It comprises more than 40,000 species that are classified into about 20 subfamilies according to current taxonomic systems; members of the group inhabit a variety of habitats and exhibit a diverse range of morphologies and life histories (Lövei & Sunderland, 1993; Löbl & Löbl, 2017). Despite this diversity, sperm morphology has been examined in only four subfamilies (Cicindelinae, Scaritinae, Carabinae, and Harpalinae) (Sasakawa & Toki, 2008; Sasakawa, 2009a). In addition, sufficient numbers of species for within-group comparative studies have only been examined for two groups, the Carabinae tribe Carabini and the Harpalinae tribe Pterostichini; only one or a few species have been examined in other taxa. However, the results of previous studies indicate the importance of studying sperm morphology in Carabidae. For example, in the Carabini species studied (genus *Carabus* subgenus *Ohomopterus*), the size of a male genital organ, the "copulatory piece," is associated with the size and dimorphism of the sperm bundle, which is a type of conjugated sperm (Takami & Sota, 2007). In the Pterostichini, the size of the female spermatheca is positively correlated with the size of the sperm bundle (Sasakawa, 2007). These reports suggest correlated evolution between the sperm and genitalia, and indicate that examination of sperm morphology is necessary in studies investigating the evolution of reproductive traits in Carabidae. Studies of sperm morphology are also important for elucidation of the within-family phylogenies of Carabidae. Sasakawa & Toki (2008) compared sperm morphology among species from five tribes of three subfamilies, whose phylogenetic positions within the family were elucidated by molecular phylogenetic analysis; their results revealed that conjugated sperm with an elongated central rod may be an autapomorphy of Harpalinae, the largest and most derivative subfamily of Carabidae (Löbl & Löbl, 2017). This assumption was supported by subsequent studies of a species from an additional tribe within Harpalinae (Sasakawa, 2009b), and a

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67 species from an additional subfamily from basal lineages (Sasakawa, 2009a). These reports imply

68 the utility of sperm morphology for reconstructing phylogenies.

69 In this study, using light microscopy sperm morphology was examined in 39 carabid species of

70 eight subfamilies, including five subfamilies for which sperm morphology has not been examined.

71 Although the numbers of species examined in each group were limited, the results of this and

72 previous studies encompass many major lineages of Carabidae. Thus, the results provide insight into

73 the evolutionary patterns and phylogenetic utility of sperm morphology, as well as enabling further

74 detailed studies of each group.

75

76 **MATERIALS AND METHODS**

77 Three species of Nebriinae, one species of Cicindelinae, two species of Elaphriinae, one species of

78 Broscinae, four species of Trechinae, two species of Patrobinae, one species of Brachinae, and

79 twenty-five species of Harpalinae were examined (Table 1). Live reproductive adult males were

80 dissected within 2 days after field collection; the seminal vesicles were removed from the

81 reproductive organs, and sperm obtained from the seminal vesicles were examined. Separation of the

82 seminal vesicles from the body was performed in a Petri dish, and dissection of the seminal vesicles

83 and subsequent observation of live sperm were performed on a glass slide, both in Ringer's solution

84 using sharp tweezers. Live sperm were observed and photographed with a light microscope,

85 followed by Giemsa staining for a detailed morphological investigation. The sperm obtained from

86 the seminal vesicles were considered to reflect the characteristics of the sperm within the ejaculate,

87 such as the spermatophore, considering the results of previous studies in other species of Carabidae,

88 in which no morphological differences were found between sperm obtained surgically from the

89 seminal vesicles and that found in the ejaculate (Takami, 2002; Sasakawa, 2009a).

90 To describe the morphology, two terms are defined herein. First, all conjugated sperm are

91 referred to as "sperm conjugate". Higginson & Pitnick (2011) noted that many different, often

92 taxon-specific terms have been used to refer to conjugated sperm and, for the avoidance of confusion,

93 proposed the use of two categories, primary conjugate and secondary conjugate, both of which

94 include several subcategories, based on their developmental mechanisms. Because it is not yet clear

95 whether the species studied here fall into the primary or secondary conjugate categories, this report

96 uses the broad category that includes both types of conjugate, which is also used in Higginson &

97 Pitnick (2011). Additionally, the structure to which individual sperm adhere in the sperm conjugate

98 is referred to in this report as "spermatostyle". Although various terms have been used to describe

99 this structure, mainly depending on the shape (e.g., rod or cap), a single term is used here,

100 irrespective of the shape. In some species, the structure that appears to correspond to the

101 spermatostyle was observed, but adhesion of individual sperm on the surface was not. Here, those

102 structures are also referred to as spermatostyle for convenience.

103   Length measurements were performed on Giemsa-stained specimens for both single sperm and

104   sperm conjugate. Typically, even species that form sperm conjugate have a small number of single

105   sperm in the seminal vesicle, as reported in Dytiscidae beetles (Higginson *et al.*, 2012). In addition,

106   on Giemsa-stained specimens of sperm conjugate, single sperm that dissociated from the

107   spermatostyle during the staining procedure were often observed. These sperm were used for the

108   measurement of single sperm, in species with sperm conjugate. For multiflagellated sperm that were

109   found in a species of Platynini, the measurement was taken as the anterior end of the sperm head to

110   the posterior end of the longest flagellum. In all but two species, the sperm conjugate size was

111   measured from the anterior end to the posterior end of the spermatostyle; in the exceptions (*Elaphrus*

112   *punctatus* and *Craspedonotus tibialis*), due to the indistinct border between the spermatostyle

113   posterior end and the attached sperm, the posterior end used for measurement was newly established

114   on the rearmost attached sperm (the posterior end of the sperm head for *E. punctatus*, and the tail end

115   for *C. tibialis*). Spermatostyle length was also measured for species in which sperm were not

116   attached to the spermatostyle. Because the sample size from single individuals was small in most

117   cases, data from multiple individuals were pooled by species, assuming homogeneity of data among

118   individuals. For single sperm and sperm conjugate with a sample size ≥ 15, the size distribution was

119   assessed by testing goodness-of-fit to a normal distribution using the Kolmogorov-Smirnov test,

120   with subsequent fitting of smooth curves to the histograms. ImageJ software (version 1.50i)

121   (Rasband, 2016) was used to obtain the measurements, and R software (version 3.2.1) (R

122   Development Core Team, 2015) was used for analysis thereof. Body length information was

123   obtained from published literatures.

124

125   **RESULTS**

126   **DESCRIPTIONS OF SPERM MORPHOLOGY**

127   **SUBFAMILY NEBRIINAE**

128   (Fig. 1A, B)

129   In all three species, only motile single sperm were observed (Fig. 1A, B). The heads of the sperm of

130   the two *Nebria* species were slightly broader than the other parts of the sperm (Fig. 1B), but in the

131   *Leistus* species, the head and other parts did not differ in width.

132

133   **SUBFAMILY CICINDINAE**

134   (Fig. 1C, D)

135   Only single sperm were observed (Fig. 1C). The sperm were motile and had heads that were only

136   slightly broader than the tails (Fig. 1D).

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138 **SUBFAMILY ELAPHRINAE**

139 (Fig. 1E–G)

140 In both species, sperm conjugate formed, in which the heads of numerous sperm were attached to a
141 spermatostyle, while the tail of each sperm was free-moving (Figs. 1E, F), and single sperm that
142 dissociated from the conjugate had an obviously broad head (Fig. 1G).

143

144 **SUBFAMILY BROSCINAE**

145 (Fig. 1H–J)

146 Both sperm conjugate and single sperm were observed (Fig. 1H). In sperm conjugate, the heads of
147 numerous sperm were glued together, while the tail of each sperm was free-moving (Fig. 1I). Unlike
148 the free single sperm described below, conjugate-forming single sperm did not have a differentiated
149 head. Single sperm that co-occurred with the sperm conjugate had an elongated drop-shaped head,
150 and a motile tail was attached to the head at a position in front of the posterior end of the head (Fig.
151 1J). On Giemsa-stained specimens, single sperm detached from the conjugate and free single sperm
152 that lost their heads during specimen preparation were virtually indistinguishable.

153

154 **SUBFAMILY TRECHINAE**

155 (Fig. 1K–N)

156 Only motile single sperm were observed in both Bembidiini (Fig. 1K, L) and Trechini (Fig. 1M, N).
157 The heads of the Bembidiini sperm were slightly broader than the other parts (Fig. 1L), whereas the
158 heads of the Trechini sperm did not broaden (Fig. 1N).

159

160 **SUBFAMILY PATROBINAE**

161 (Fig. 1O–Q)

162 Sperm conjugate was observed, in which the heads of numerous sperm were attached to an elongated
163 spermatostyle, while the tail of each sperm was free-moving (Fig. 1O, P). The spermatostyle was
164 twisted in a right-handed direction at regular intervals, but was flexible and showed no conspicuous
165 spiral structure. The head of the spermatostyle was spoon-shaped. The head of the detached single
166 sperm was slightly broader than the other parts (Fig. 1Q).

167

168 **SUBFAMILY BRACHINAE**

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169   (Fig. 1R, S)

170   Sperm conjugate was observed, in which the heads of numerous sperm were attached to a

171   spermatostyle, while the tail of each sperm was free-moving (Fig. 1R). The spermatostyle was

172   markedly elongated, without a conspicuous spiral structure. The head of the spermatostyle had a

173   match-head shape. The head of the single sperm that dissociated from the conjugate was slightly

174   broader than the other parts (Fig. 1S).

175

176   **SUBFAMILY HARPALINAE**

177   **TRIBE CHLAENIINI**

178   (Fig. 1T–W)

179   In *Callistoides pericallus*, sperm conjugate was observed, in which the heads of numerous sperm

180   were attached to the spermatostyle, while the tail of each sperm was free-moving (Fig. 1T). The

181   spermatostyle was a straight, rigid rod, and its head had a match-head shape. In the other three

182   species, although spermatostyles were observed, only single sperm were present, which were

183   unattached to the spermatostyles. These three species shared a feature not found in the other species

184   examined: single sperm were longer than the spermatostyle. However, the shape of the spermatostyle

185   differed between the species, with a wave form in *Chlaenius micans* (Fig. 1U), a short rod in

186   *Chlaenius inops*, and a long rod in *Chlaenius pallipes* (Fig. 1V). The head of single sperm did not

187   broaden (Fig. 1W).

188

189   **TRIBE MASOREINI**

190   (Fig. 2A–C)

191   Sperm conjugate with a right-handed helical spermatostyle was observed (Fig. 2A). The sperm

192   formed a sheet-like structure with a wavy edge, and adhered to both lateral sides of the

193   spermatostyle (Fig. 2B). The head of the spermatostyle narrowed at the anterior end. The head of the

194   detached single sperm was slightly broader than the other parts (Fig. 2C).

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196 **TRIBE DRYPTINI**

197 (Fig. 2D–G)

198 Although some sperm were attached to the spermatostyle by their head (Fig. 2D), while their tail was

199 free-moving, most sperm did not attach to the spermatostyle (Fig. 2E, F). The spermatostyle was

200 elongated, without a conspicuous spiral structure. The spermatostyle head was slightly broader than

201 the other parts. The heads of the single sperm did not broaden, and thus it was unclear which end

202 was the head (Fig. 2G).

203

204 **TRIBE GALERITINI**

205 (Fig. 2H–J)

206 Although a spermatostyle was observed, the single sperm that were present were not attached to the

207 spermatostyle surface. The spermatostyle was elongated, without a conspicuous spiral structure, and

208 its head was slightly broader than the other parts (Fig. 2H, I). The head of single sperm was slightly

209 broader than the other parts (Fig. 2J).

210

211 **TRIBE HARPALINI**

212 (Fig. 2K–P)

213 Sperm conjugate was observed in all species. The spermatostyle had a left-handed helical shape (Fig.

214 2K–M) or a rod shape (Fig. 2N, O). In both types, the head of the spermatostyle did not broaden, and

215 numerous sperm were attached by their heads to the spermatostyle surface, while the tails were

216 free-moving. The heads of single sperm that dissociated from the spermatostyle did not broaden, and

217 thus it was unclear which end was the head (Fig. 2P).

218

219 **TRIBE LEBIINI**

220 (Fig. 2Q–T)

221 In both species, sperm conjugate with an elongated spermatostyle was observed, and the head of the

222 spermatostyle narrowed at the anterior end. In the Dromiusina, the spermatostyle had a right-handed

223 helical shape, and the sperm formed a sheet-like structure with a wavy edge and adhered to both

224 lateral sides of the spermatostyle (Fig. 2Q, R). The sheet-like structures had firm adhesion, and

225 single sperm that dissociated from the spermatostyle were not observed in the specimens examined.

226 In the Metallicina, the spermatostyle was flexible, without a conspicuous spiral structure, and

227 numerous sperm were attached by their heads to the spermatostyle surface, while the tails were

228 free-moving (Fig. 2S). The head of the single sperm detached from the spermatostyle was slightly

229 broader than the other parts (Fig. 2T).

230

231 **TRIBE LICININI**

232   (Fig. 2U–X)

233   The formation of long spermatostyles was observed (Fig. 2U–W). Adhesion of the sperm was

234   observed in a small part of the spermatostyle; the head of each sperm was attached to the

235   spermatostyle, while the tail was free-moving (Fig. 2U). Other sperm were present that were not

236   attached to the spermatostyle. The head of the spermatostyle was simple and did not broaden. The

237   head of the single sperm did not broaden, and thus it was unclear which end was the head (Fig. 2X).

238

239   **TRIBE OODINI**

240   (Fig. 3A–C)

241   Wave-shaped spermatostyles were observed; however, adhesion of the sperm to the spermatostyle

242   was not observed (Fig. 3A, B). The head of the single sperm did not broaden, and thus it was unclear

243   which end was the head (Fig. 3C).

244

245   **TRIBE PLATYNINI**

246   (Fig. 3D–J)

247   Two types of sperm were observed. The first type was observed in *Agonum suavissimum*, and only a

248   single sperm was produced (Fig. 3D–G). The sperm had a long, drop-shaped head and two tails

249   attached slightly behind the anterior end of the head. The tail was bifurcated in the rear half, and one

250   of the bifurcated tails was broader than the rest of the tail and shorter than the other bifurcated tail

251   (Fig. 3E–G). Motility was observed in Ringer's solution (Fig. 3D). The other type of sperm was

252   observed in the other two species, and sperm conjugate was found. The spermatostyles had a long,

253   left-handed helical shape and a spoon-shaped head (Fig. 3H, I). Numerous sperm attached their

254   heads to the spermatostyle surface, while the tails were free-moving. The head of the single sperm

255   detached from the spermatostyle was slightly broader than the other parts (Fig. 3J).

256

257   **TRIBE SPHODRINI**

258   (Fig. 3K–P)

259   Sperm conjugate, in which sperm were attached to spermatostyles, was observed, but the shape of

260   the spermatostyles and the condition of the attached sperm differed between the species. In *Synuchus*

261   *arcuaticollis* (Fig. 3K, L), the anterior and posterior halves of the spermatostyle had a left- and

262   right-handed helical shape, respectively. Sperm were attached by their heads to the spermatostyle,

263   while their tails were free-moving. The head of single sperm detached from the spermatostyle was

264   distinctly broader than the other parts. In *Synuchus dulcigradus* (Fig. 3M, N), the posterior half of

265   the spermatostyle had a left-handed helical shape, and the anterior half was oriented backward

266   (compared to the helical direction of the posterior half). The sperm attached to the lateral sides of the

267   spermatostyle along the midline and formed a sheet-like structure, except near the posterior end,

268 where the tails of the sperm unraveled. The head of the detached single sperm did not broaden.

269 *Synuchus orbicollis* formed a straight spermatostyle, and the sperm attached to the lateral sides of the

270 spermatostyle, forming a sheet-like structure (Fig. 3O). The sheet-like structures undulated actively

271 in Ringer's solution. The heads of the single sperm detached from the spermatostyle were broadened

272 (Fig. 3P).

273

274 **TRIBE ZABRINI**

275 (Fig. 3Q–T)

276 Sperm conjugate, in which sperm were attached to the spermatostyles, was observed; however, the

277 shape of the spermatostyles and condition of the attached sperm differed between the species. In

278 *Amara chalcites* (Fig. 3Q) and *Amara sinuaticollis* (Fig. 3R), the spermatostyles had a left-handed

279 helical shape, and numerous sperm were attached by their heads to the spermatostyle surface, while

280 the tails were free-moving. In *Amara macronota*, the anterior half of the spermatostyle formed a

281 rigid and circular arc, while the posterior half was markedly slender and flexible (Fig. 3S). The

282 sperm adhered to both of the lateral sides of the posterior half of the arc portion of the spermatostyle,

283 while their tails were free-moving (Fig. 3T). The heads of the sperm that dissociated from the

284 spermatostyle did not broaden in any of the three species.

285

286 **SPERM SIZE VARIATION**

287 Single sperm from 18 species, sperm conjugate from 15 species, and spermatostyles without

288 attached sperm from 3 species were analyzed (Table 1). The size distribution of the sperm conjugate

289 in *E. punctatus* deviated from the normal distribution ($D = 0.106$, $P = 0.034$), whereas the size

290 distributions of all others did not deviate from normality ($P > 0.05$; Appendix S1). Comparison of

291 the histograms and fitted smooth curves of the various sperm conjugate sizes between *E. punctatus*

292 and the related species, *Elaphrus sugai*, revealed that the *E. punctatus* sperm conjugate had a

293 positively skewed size distribution (Fig. 4).

294

295 **DISCUSSION**

296 Several notable sperm traits were uncovered in this study. First, the average length of the single

297 sperm of *E. punctatus* was 55.10 μm. Prior to this study, the shortest single sperm reported in the

298 order Coleoptera was that of the carabid beetle *Carabus maiyasanus*, which has an average length of

299 67.33 μm (Takami & Sota, 2007; Pitnick *et al.*, 2009). Thus, the *E. punctatus* single sperm is now

300 the smallest single sperm reported in Coleoptera. Furthermore, *E. punctatus* single sperm is also the

301 second shortest in the class Insecta (Pitnick *et al.*, 2009). The size variation in *E. punctatus* sperm

302 conjugate is also notable. To date, size variation in sperm conjugate has been reported in various

303 animal species, but only the sperm conjugate of carabid beetles of the genus *Carabus*, subgenus

9

304 *Ohomopterus* has been quantitatively and statistically evaluated (Takami, 2002; Takami & Sota,

305 2007, both reported as "sperm bundles"). In *Ohomopterus*, the sperm conjugate exhibited

306 species-specific variation in size dimorphism, ranging from a distinct bimodality to a positively

307 skewed distribution (a lesser degree of bimodality); regardless of the degree of dimorphism, the

308 sperm conjugate could be divided into two types: large and small (Takami & Sota, 2007). The

309 positively skewed size distribution of the *E. punctatus* sperm conjugate may reflect a low degree of

310 dimorphism; if so, this is the first report of dimorphism in the size of sperm conjugate, other than in

311 *Ohomopterus*.

312 In the Harpalinae tribes Chlaeniini (except for one species), Dryptini, Galeritini, Licinini, and

313 Oodini, few to none of the sperm were attached to the spermatostyles. One possible explanation for

314 this observation is that, while loss of conjugation occurred in the species, the process of

315 spermatostyle formation was maintained. If so, this sperm condition may represent a new type of

316 sperm that evolved from the normal type of sperm conjugate. Another possible interpretation is that

317 dissociation of the sperm from the spermatostyle is an artifact of the experimental procedures that

318 were used. In this study, the sperm was surgically removed from the male seminal vesicles and

319 observed in Ringer's solution, which is the usual procedure for observing insect sperm (e.g., Hayashi

320 & Kamimura, 2002; Higginson *et al.*, 2012; Hodgson *et al.*, 2013; Schubert *et al.*, 2017). However,

321 when sperm are transferred to the female, they are combined with other ejaculates, such as

322 spermatophores. Physiological and/or chemical effects resulting from this difference may have

323 caused the dissociation of sperm from the spermatostyles. Future studies should be conducted to

324 examine the sperm in ejaculate collected from females immediately after mating in these species, as

325 has been performed in other species (Takami, 2002; Takami & Sota, 2007; Sasakawa, 2007).

326 In the Harpalinae tribe Platynini, multiflagellated sperm were found, which have previously

327 only been reported in one species in the class Insecta (Pitnick *et al.*, 2009). The multiflagellated

328 sperm previously reported were found in the Australian termite *Mastotermes darwiniensis* and had a

329 conical head and about 100 simple (i.e., not branched) flagella (Baccetti & Dallai, 1978). Thus, the

330 biflagellate sperm of *A. suavissimum* represent a second example of multiflagellated sperm in insects.

331 The Platynini, which includes *A. suavissimum*, could serve as a model system for elucidating the

332 evolutionary relationship between multiflagellated sperm and sperm conjugate, which may be

333 mutually exclusive in terms of increased sperm motility; this is because aside from *A. suavissimum*,

334 all Platynini species examined to date produced sperm conjugate consisting of uniflagellate sperm

335 (Sasakawa & Toki, 2008; Schubert et al., 2017; Sasakawa, this study). Studies of sperm traits from

336 additional Platynini species may provide further insights into this phenomenon.

337 In the coleopteran family Dytiscidae, which is the only example (to my knowledge) in which

338 the evolution of sperm conjugation has been examined from a phylogenetic perspective, the same

339 type of sperm conjugate as that observed in Carabidae was found to be the ancestral sperm trait, and

340 other types of sperm conjugate evolved from it, followed by the loss of sperm conjugation in several
341 lineages (Higginson *et al.*, 2012). Compared to the findings for Dytiscidae, the current information
342 regarding Carabidae sperm is insufficient, in that some phylogenetic relationships within the family
343 are uncertain and the number of species examined for sperm traits is smaller. However, the available
344 information revealed some of the evolutionary patterns of sperm traits in Carabidae. For the
345 phylogenetic relationships, the latest subfamily-level phylogenetic trees based on mitochondrial
346 genomes and nuclear 18S rRNA genes, which attempt to minimize the effects of various
347 methodological problems (López-López & Vogler, 2017), and some robust sister group relationships
348 that were confirmed in other molecular phylogenetic studies (Trechinae + Patrobinae: Maddison *et*
349 *al.*, 1999; Ribera *et al.*, 2005; Brachinae + Harpalinae: Ribera *et al.*, 2005; Ober & Maddison, 2008)
350 are now available. These show that the relationship of taxa with known sperm traits is (Cicindelinae,
351 (Carabidae, (Nebriinae, (Elaphrinae, ((Trechinae, Patrobinae), (Broscinae, (Scaritinae, (Brachinae,
352 Harpalinae)))))))). Importantly, taxa with single sperm (i.e., Cicindelinae, Nebriinae, and Trechinae),
353 and those with sperm conjugate (i.e., Carabidae, Elaphrinae, and Patrobinae), are placed alternately
354 in basal lineages of the phylogeny. This implies that whether the ancestor of Carabidae formed single
355 sperm or sperm conjugate is, at present, indeterminable; however, in either case, the transition
356 between single sperm and sperm conjugate occurred several times in basal lineages. On the other
357 hand, in Harpalinae, the largest subfamily that includes about half of the species of Carabidae, sperm
358 conjugate with a long spermatostyle was considered most likely to be the ancestral trait, because
359 most examined species of the subfamily and a species of Brachinae, the subfamily sister to
360 Harpalinae, form sperm conjugate with a long spermatostyle.

361 The observed putative evolutionary pattern provides a definitive result for the phylogenetic
362 utility of sperm morphology. Because two clades composed of species producing sperm conjugate
363 with a long spermatostyle are separated in the phylogeny (i.e., Patrobinae in basal lineages, and
364 Bachinae + Harpalinae at the most derived position), and an unambiguous transition from
365 conjugated to single sperm has occurred in one of the two clades (*A. suavissimum* in the Harpalinae
366 tribe Platynini), the current findings do not support Sasakawa & Toki's (2008) assumption that a
367 long spermatostyle contains phylogenetic information as an apomorphy. Also, sperm conjugate and
368 single sperm are placed alternately on the basal lineages; thus, the macromorphology of sperm does
369 not appear to contain useful phylogenetic information in Carabidae. To uncover phylogenetic
370 information from sperm morphology, it will be necessary to examine micromorphology, such as the
371 internal structure of sperm.

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Table 1. Species data

Taxa[†]	Body length (mm)[‡]	No. males examined	Length of sperm conjugate (µm)[§¶]	Length of single sperm (µm)[§¶]
Subfamily Nebriinae				
Leistus (*Pogonophorus*) *kozakai* Perrault, 1984[1)]	10.9	1	–	1684.33 ± 198.02 (3)
Nebria (*Sadonebria*) *asahina* Sasakawa, 2009[2)]	13.2/14.2	2	–	718.20 ± 62.66 (5)
Nebria (*Reductonebria*) *ochotica* Sahlberg, 1844[3)]	9.5−12.0	1	–	906.75 ± 132.13 (4)
Subfamily Cicindelinae				
Apterodela ovipennis (Bates, 1883)[4)]	14−15	1	–	126.18 ± 8.66 (27)[ns]
Subfamily Elaphrinae				
Elaphrus (*Elaphroterus*) *punctatus* Motschulsky, 1844[5)]	6	1	70.61 ± 26.92 (182)*	55.10 ± 2.73 (56)[ns]
Elaphrus (*Neoelaphrus*) *sugai* Nakane, 1987[5)]	8	1	59.51 ± 6.30 (170)[ns]	163.92 ± 4.88 (34)[ns]
Subfamily Broscinae				
Craspedonotus tibialis Schaum, 1863[6)] [sperm conjugate]	20−24	1	393.22 ± 23.24 (4)	Undeterminable[#]
Ditto [single sperm]	Ditto	2	–	460.81 ± 19.55 (30)[ns]
Subfamily Trechinae				
Tribe Bembidiini				
Bembidion (*Ocydromus*) *echigonum* Habu & Baba, 1957[7)]	5.7	1	–	605.67 ± 54.20 (9)
Bembidion pseudolucillum Netolitzky, 1938[7)]	3.7	1	–	464.79 ± 9.17 (5)
Tribe Trechini				
Blemus discus (Fabricius, 1792)[5)]	4.2−5.5	1	–	136.97 ± 5.54 (21)[ns]
Trechus (*Epaphius*) *ephippiatus* Bates, 1873[5)]	3.5−4.8	1	–	91.49 ± 1.83 (24)[ns]
Subfamily Patrobinae				

Apatrobus (*Apatrobus*) *osuzuyamanus* Sasakawa & Toki, 2007[8]	10.31/10.97	1	749.86 $\pm$ 121.38 (80) [ns]	105.81 $\pm$ 4.30 (35) [ns]
Archipatrobus flavipes (Motschulsky, 1864)[5]	15	1	463.89 $\pm$ 49.74 (89) [ns]	78.77 $\pm$ 5.67 (32) [ns]
Subfamily Brachinae				
Pheropsophus (*Stenaptinus*) *jessoensis* Morawitz, 1862[9]	11−18	1	18251.20 $\pm$ 7971.70 (2)	151.46 $\pm$ 8.65 (7)
Subfamily Harpalinae				
Tribe Chlaeniini				
Callistoides pericallus (Redtenbacher, 1867)[10]	11.3−12	1	2057.96 $\pm$ 220.02 (4)	508.15 $\pm$ 58.03 (5)
Chlaenius (*Chlaeniellus*) *inops* Chaudoir, 1856[5]	10.3−11	1	137.08 $\pm$ 30.15 (30) [ns]	710.35 $\pm$ 110.32 (7)
Chlaenius (*Achlaenius*) *micans* (Fabricius, 1792)[5]	15−17.5	1	309.72 $\pm$ 32.91 (6)	639.01 $\pm$ 32.01 (2)
Chlaenius (*Chlaenius*) *pallipes* (Gebler, 1823)[5]	13.5−14.5	3	803.03 $\pm$ 265.55 (23) [ns]	1107.92 $\pm$ 201.76 (5)
Tribe Masoreini				
Aephnidius (*Aephnidius*) *adelioides* (MacLeay, 1825)[11]	6−7	2	973.47 $\pm$ 107.30 (8)	211.56 $\pm$ 11.92 (8)
Tribe Dryptini				
Drypta (*Drypta*) *fulveola* Bates, 1883[12]	8−9.5	2	2120.88 $\pm$ 256.84 (23) [ns]	215.54 $\pm$ 8.78 (43) [ns]
Tribe Galeritini				
Planetes (*Planetes*) *puncticeps* Andrewes, 1919[5]	12−13	1	7559.29 $\pm$ 325.80 (2)	848.66 $\pm$ 48.13 (26) [ns]
Tribe Harpalini				
Subtribe Harpalina				
Harpalus (*Pseudoophonus*) *eous* Tschitschérine, 1901[5]	12.5−15	1	1456.87 $\pm$ 89.68 (23) [ns]	730.60 $\pm$ 86.78 (5)
Harpalus (*Zangoharpalus*) *tinctulus* Bates, 1873[10]	6.5−8	1	944.28 $\pm$ 181.61 (53) [ns]	479.97 $\pm$ 6.31 (11)
Nipponoharpalus discrepans (Morawitz, 1862)[13]	10.5−12	1	3805.65 $\pm$ 302.76 (10) [ns]	905.63 $\pm$ 220.21 (10)
Subtribe Stenolophina				
Loxoncus (*Loxoncus*) *circumcinctus* (Motschulsky, 1858)[5]	8.3−9.5	1	224.28 $\pm$ 103.04 (8) [ns]	184.91 $\pm$ 11.78 (8)

Tribe Lebiini				
Subtribe Dromiusina				
Dromius (*Klepterus*) *prolixus* Bates, 1883[14]	6−6.5	1	$1099.57 \pm 63.07 (33)^{ns}$	Undeterminable[#]
Subtribe Metallicina				
Parena amamiooshimaensis Habu, 1964[15]	8−9.5	2	$1413.45 \pm 184.48 (44)^{ns}$	$217.37 \pm 7.93 (34)^{ns}$
Tribe Licinini				
Diplocheila (*Isorembus*) *macromandibularis* (Habu & Tanaka, 1956)[16]	16.2−17	1	$12543.66 \pm 1534.73 (7)$	$510.41 \pm 15.45 (9)$
Diplocheila (*Isorembus*) *zeelandica* (Redtenbacher, 1867)[10]	20.5−26	1	$18197.70 (1)$	$288.59 \pm 8.02 (23)^{ns}$
Tribe Oodini				
Lachnocrepis prolixa (Bates, 1873)[5]	10.5−11.5	1	$910.13 \pm 122.62 (6)$	$523.96 \pm 2.56 (2)$
Tribe Platynini				
Agonum (*Olisares*) *suavissimum* (Bates, 1883)[5]	7.5−10	1	−	$138.72 \pm 5.45 (24)^{ns}$
Metacolpodes buchanani (Hope, 1831)[17]	10.5−13	1	$3002.49 \pm 314.65 (9)$	$410.23 \pm 6.26 (11)$
Xestagonum xestum (Bates, 1883)[18]	8−11	1	$3202.05 \pm 184.17 (3)$	$638.86 \pm 22.48 (9)$
Tribe Sphodrini				
Synuchus (*Synuchus*) *arcuaticollis* (Motschulsky, 1860)[5]	8−10.5	1	$607.63 \pm 52.02 (29)^{ns}$	$344.70 \pm 16.26 (22)^{ns}$
Synuchus (*Synuchus*) *dulcigradus* (Bates, 1873)[11]	8−10	3	$1077.71 \pm 62.14 (24)^{ns}$	$1123.31 \pm 101.30 (15)^{ns}$
Synuchus (*Synuchus*) *orbicollis* (Morawitz, 1862)[5]	8.5−11.5	1	$293.04 \pm 6.24 (31)^{ns}$	$277.53 \pm 11.76 (19)^{ns}$
Tribe Zabrini				
Amara (*Amara*) *chalcites* Dejean, 1828[5]	8.0−10.2	2	$760.58 \pm 88.60 (24)^{ns}$	$1282.65 \pm 105.38 (6)$
Amara (*Curtonotus*) *macronota* Solsky, 1875[11]	10.5−13.5	6	$2318.11 \pm 365.79 (30)^{ns}$	$696.18 \pm 14.82 (27)^{ns}$
Amara (*Bradytus*) *sinuaticolis* Morawitz, 1862[11]	8−10	2	$5358.26 \pm 381.20 (6)$	$667.53 \pm 19.14 (30)^{ns}$

[†]Collection site: [1]Mt. Tsurugi, Tokushima; [2]Mts. Asahi, Yamagata; [3]My. Zaô, Yamagata; [4]Mt. Gassan, Yamagata; [5]Watarase wetland, Tochigi; [6]Asahikawa, Hokkaido; [7]Mt. Chôkai, Yamagata; [8]Mt. Osuzu, Miyazaki; [9]Tsuno, Miyazaki; [10]Noda, Chiba; [11]Narashino, Chiba; [12]Riv. Obitsu, Chiba; [13]Hokuto, Yamanashi; [14]Mt. Tairoku, Hokkaido; [15]Is. Takara, Kagoshima; [16]Is. Tobi, Yamagata; [17]Chichibu, Saitama; [18]Tsuchiyu, Fukushima.

[†]Source: Perrault (1984) for *L. kozakai*; Sasakawa (2009c) for *N. asahina*; Ohkawa & Kurihara (2018) for *E. punctatus* and *E. sugai*; Sasakawa & Toki (2007) for *A. osuzuyamanus*; Sasakawa *et al.* (2010) for *A. chalcites*; Uéno *et al.* (1985) for the other species.

[§]Mean ± SD (number of sperm measured)

[¶]Superscript letters indicate a statistically significant difference in size distribution from normality (*$P < 0.05$; [ns] nonsignificant at $\alpha = 0.05$).

[#]Single sperm that dissociated from the conjugate could not be determined unambiguously for *C. tibialis* and were not observed for *D. prolixus*.

[Figure legends]

Figure 1. Sperm of various species of Carabidae observed in Ringer's solution (RS) or with Giemsa stain (GS): *Leistus kozakai* single sperm in RS (A); *Nebria asahina* single sperm with GS (B); *Apterodela ovipennis* single sperm in RS (C) and with GS (D); an *Elaphrus sugai* sperm conjugate in RS (E); *Elaphrus punctatus* sperm conjugate (F) and single sperm (G) with GS; *Craspedonotus tibialis* sperm conjugate and single sperm in RS (H), and sperm conjugate (I) and single sperm (J) with GS; *Bembidion echigonum* single sperm in RS (K); a *Bembidion pseudolucillum* single sperm with GS (L); *Blemus discus* single sperm in RS (M); *Trechus ephippiatus* single sperm with GS (N); an *Archipatrobus flavipes* sperm conjugate in RS (O); *Apatrobus osuzuyamanu*s sperm conjugate (P) and single sperm (Q) with GS; *Pheropsophus jessoensis* sperm conjugate (R) and single sperm (S) with GS; a *Callistoides pericallus* sperm conjugate in RS (T); *Chlaenius micans* single sperm and spermatostyles in RS (U); *Chlaenius pallipes* spermatostyle (V) and single sperm (W) with GS. The heads of the single sperm and sperm conjugate, and the anterior ends of the spermatostyles without attached sperm, are indicated by an asterisk. The "ss" in the photos including sperm conjugate indicates single sperm that co-occur (for *C. tibialis*) or single sperm that dissociated from sperm conjugate (for the other species).

Figure 2. Sperm of various species of Carabidae: *Aephnidius adelioides* sperm conjugate in RS (A) and with GS (B), and single sperm with GS (C); *Drypta fulveola* spermatostyle with (D) and without (E, F) sperm on the surface, and single sperm (G) observed in RS (D, E) and with GS (F, G); *Planetes puncticeps* spermatostyles and single sperm in RS (H) and with GS (I), and single sperm with GS (J); *Harpalus eous* sperm conjugate in RS (K) and with GS (L); a *Nipponoharpalus discrepans* sperm conjugate in RS (M); *Harpalus tinctulus* sperm conjugate in RS (N); *Loxoncus circumcinctus* sperm conjugate in RS (O) and single sperm with GS (P); *Dromius prolixus* sperm conjugate in RS (Q) and with GS (R); *Parena amamiooshimaensis* sperm conjugate (S) and single sperm (T) with GS; *Diplocheila macromandibularis* spermatostyle with (U) and without (V) sperm on the surface in RS, and spermatostyles and single sperm with GS (W); *Diplocheila zeelandica* single sperm with GS (X). Explanations of the markings are the same as in Figure 1.

Figure 3. Sperm of various species of Carabidae: *Lachnocrepis prolixa* spermatostyles and single sperm in RS (A) and with GS (B), and single sperm with GS (C); *Agonum suavissimum* single sperm in RS (D) and with GS (E–G); *Xestagonum xestum* sperm conjugate with GS (H); *Metacolpodes buchanani* sperm conjugate (I) and single sperm (J) with GS; *Synuchus arcuaticollis* sperm conjugate in RS (K) and sperm conjugate and detached single sperm with GS (L); *Synuchus dulcigradus* sperm conjugate in RS (M) and sperm conjugate and detached single sperm with GS (N); *Synuchus orbicollis* sperm conjugate in RS (O) and sperm conjugate and single sperm with GS

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(P); an *Amara chalcites* sperm conjugate in RS (Q); an *Amara sinuaticollis* sperm conjugate with GS (R); *Amara macronota* sperm conjugate in RS (S) and sperm conjugate and detached single sperm with GS (T). Explanations of the markings are the same as in Figure 1.

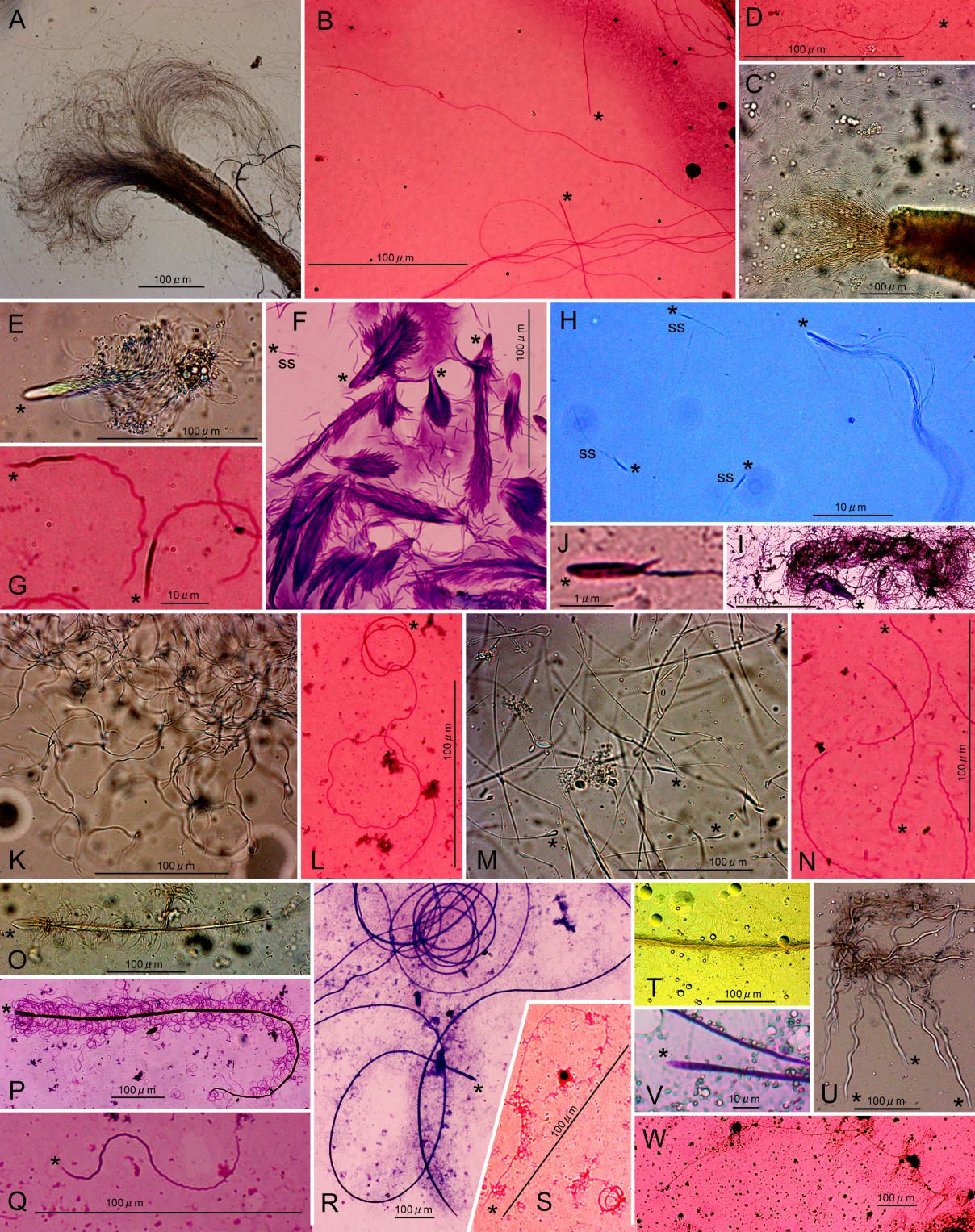**Figure 4.** Variation in sperm conjugate length in *E. punctatus* (A) and *E. sugai* (B).
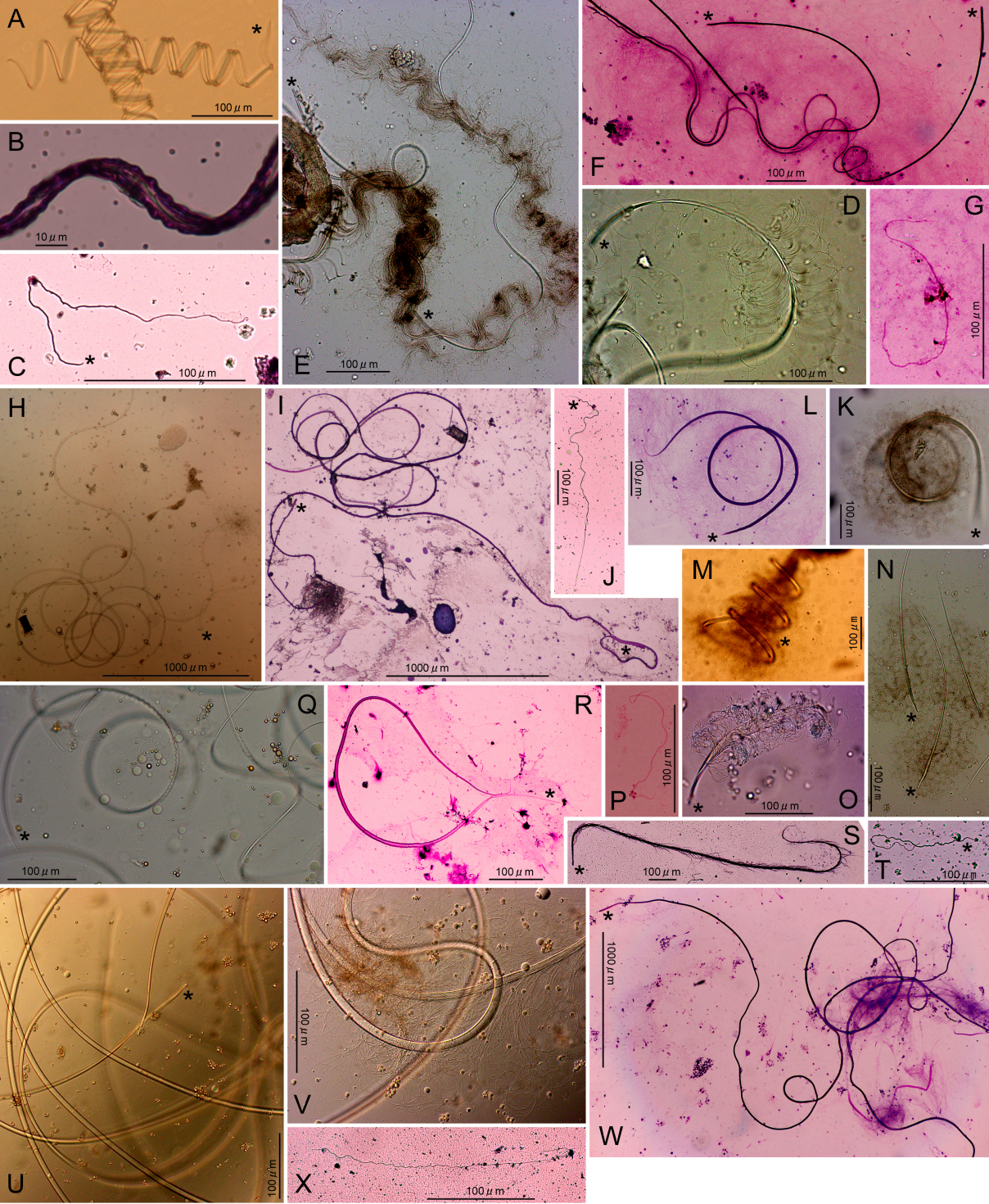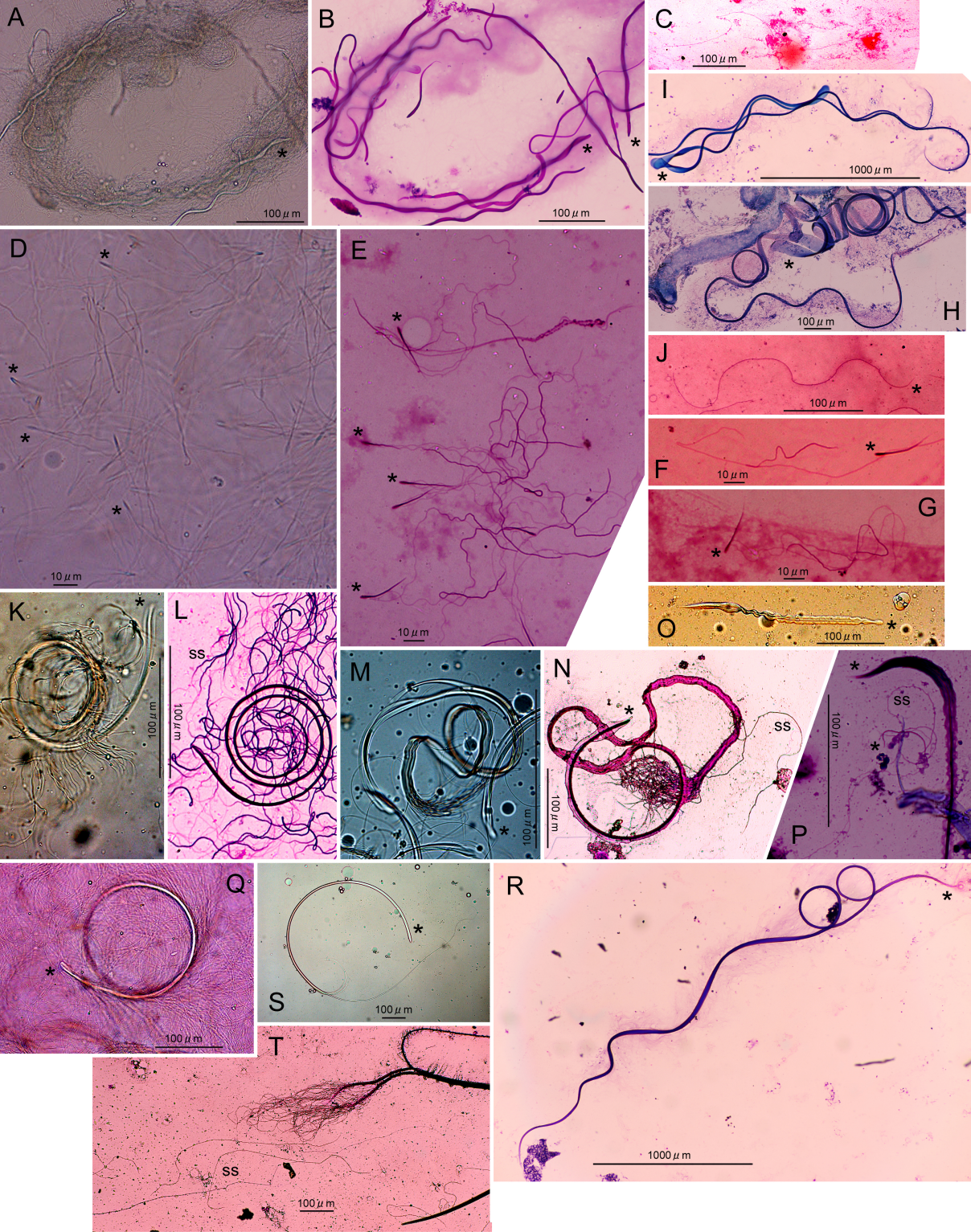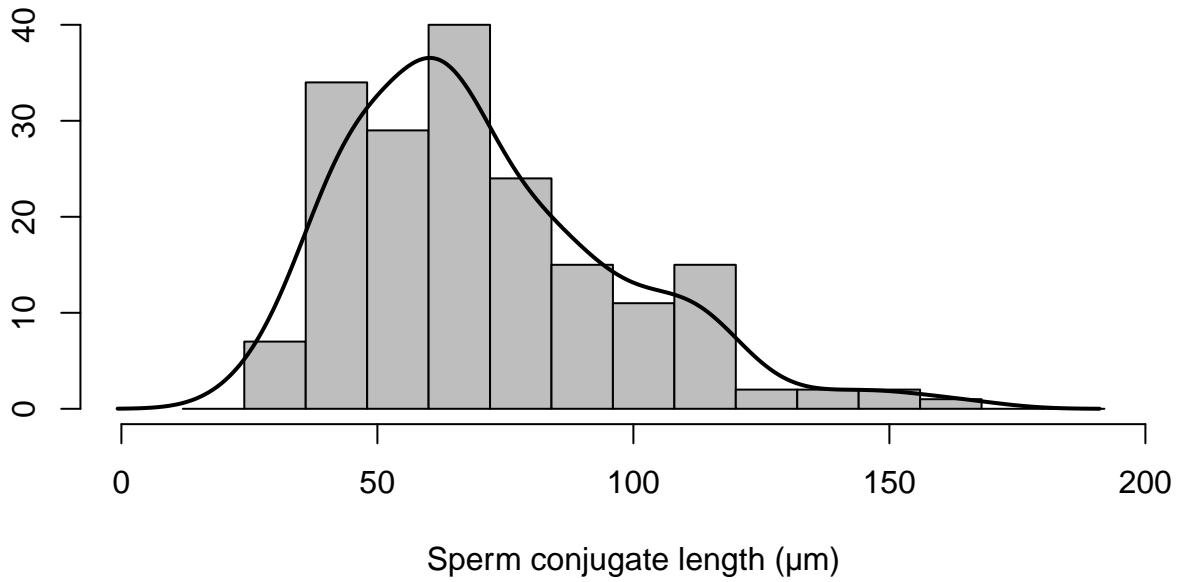
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Figure 1

Figure 2

Figure 3

A

Sperm conjugate length (μm)

B

Sperm conjugate length (μm)