

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

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3 **Kôji Sasakawa**

4 Department of Science Education, Faculty of Education, Chiba University, Chiba, Japan

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

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8 **Correspondence:** Kôji Sasakawa, Laboratory of Zoology, Department of Science
9 Education, Faculty of Education, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba
10 263-8522, Japan

11 E-mail: ksasa@chiba-u.jp

12
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
29 **Keywords:** Dimorphism; multiflagellated sperm; reproductive traits; single sperm; size variation;
30 spermatostyle; sperm conjugate; spermiocladistics; sperm morphological evolution

31 **INTRODUCTION**

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

45 The beetle family Carabidae is one such group. It comprises more than 40,000 species that are
46 classified into about 20 subfamilies according to current taxonomic systems; members of the group
47 inhabit a variety of habitats and exhibit a diverse range of morphologies and life histories (Lövei &
48 Sunderland, 1993; Löbl & Löbl, 2017). Despite this diversity, sperm morphology has been examined
49 in only four subfamilies (Cicindelinae, Scaritinae, Carabinae, and Harpalinae) (Sasakawa & Toki,
50 2008; Sasakawa, 2009a). In addition, sufficient numbers of species for within-group comparative
51 studies have only been examined for two groups, the Carabinae tribe Carabini and the Harpalinae
52 tribe Pterostichini; only one or a few species have been examined in other taxa. However, the results
53 of previous studies indicate the importance of studying sperm morphology in Carabidae. For
54 example, in the Carabini species studied (genus *Carabus* subgenus *Ohomopterus*), the size of a male
55 genital organ, the “copulatory piece,” is associated with the size and dimorphism of the sperm
56 bundle, which is a type of conjugated sperm (Takami & Sota, 2007). In the Pterostichini, the size of
57 the female spermatheca is positively correlated with the size of the sperm bundle (Sasakawa, 2007).
58 These reports suggest correlated evolution between the sperm and genitalia, and indicate that
59 examination of sperm morphology is necessary in studies investigating the evolution of reproductive
60 traits in Carabidae. Studies of sperm morphology are also important for elucidation of the
61 within-family phylogenies of Carabidae. Sasakawa & Toki (2008) compared sperm morphology
62 among species from five tribes of three subfamilies, whose phylogenetic positions within the family
63 were elucidated by molecular phylogenetic analysis; their results revealed that conjugated sperm
64 with an elongated central rod may be an autapomorphy of Harpalinae, the largest and most
65 derivative subfamily of Carabidae (Löbl & Löbl, 2017). This assumption was supported by
66 subsequent studies of a species from an additional tribe within Harpalinae (Sasakawa, 2009b), and a

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).

137

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**

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).

195

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

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 unflagellate 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, (Nebrinae, (Elaphrinae, ((Trechinae, Patrobinae), (Broscinae, (Scaritinae, (Brachinae,
352 Harpalinae)))))). Importantly, taxa with single sperm (i.e., Cicindelinae, Nebrinae, 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 Brachinae + 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.

372

373

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378

379

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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 Nebrinae				
<i>Leistus (Pogonophorus) kozakai</i> Perrault, 1984 ¹⁾	10.9	1	–	1684.33 \pm 198.02 (3)
<i>Nebria (Sadonebria) asahina</i> Sasakawa, 2009 ²⁾	13.2/14.2	2	–	718.20 \pm 62.66 (5)
<i>Nebria (Reductonebria) ochotica</i> Sahlberg, 1844 ³⁾	9.5–12.0	1	–	906.75 \pm 132.13 (4)
Subfamily Cicindelinae				
<i>Apterodela ovipennis</i> (Bates, 1883) ⁴⁾	14–15	1	–	126.18 \pm 8.66 (27) ^{ns}
Subfamily Elaphrinae				
<i>Elaphrus (Elaphroterus) punctatus</i> Motschulsky, 1844 ⁵⁾	6	1	70.61 \pm 26.92 (182)*	55.10 \pm 2.73 (56) ^{ns}
<i>Elaphrus (Neoelaphrus) sugai</i> Nakane, 1987 ⁵⁾	8	1	59.51 \pm 6.30 (170) ^{ns}	163.92 \pm 4.88 (34) ^{ns}
Subfamily Broscinae				
<i>Craspedonotus tibialis</i> Schaum, 1863 ⁶⁾ [sperm conjugate]	20–24	1	393.22 \pm 23.24 (4)	Undeterminable [#]
Ditto [single sperm]	Ditto	2	–	460.81 \pm 19.55 (30) ^{ns}
Subfamily Trechinae				
Tribe Bembidiini				
<i>Bembidion (Ocydromus) echigonum</i> Habu & Baba, 1957 ⁷⁾	5.7	1	–	605.67 \pm 54.20 (9)
<i>Bembidion pseudolucillum</i> Netolitzky, 1938 ⁷⁾	3.7	1	–	464.79 \pm 9.17 (5)
Tribe Trechini				
<i>Blemus discus</i> (Fabricius, 1792) ⁵⁾	4.2–5.5	1	–	136.97 \pm 5.54 (21) ^{ns}
<i>Trechus (Epaphius) ephippiatus</i> Bates, 1873 ⁵⁾	3.5–4.8	1	–	91.49 \pm 1.83 (24) ^{ns}
Subfamily Patrobinae				

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

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

(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).

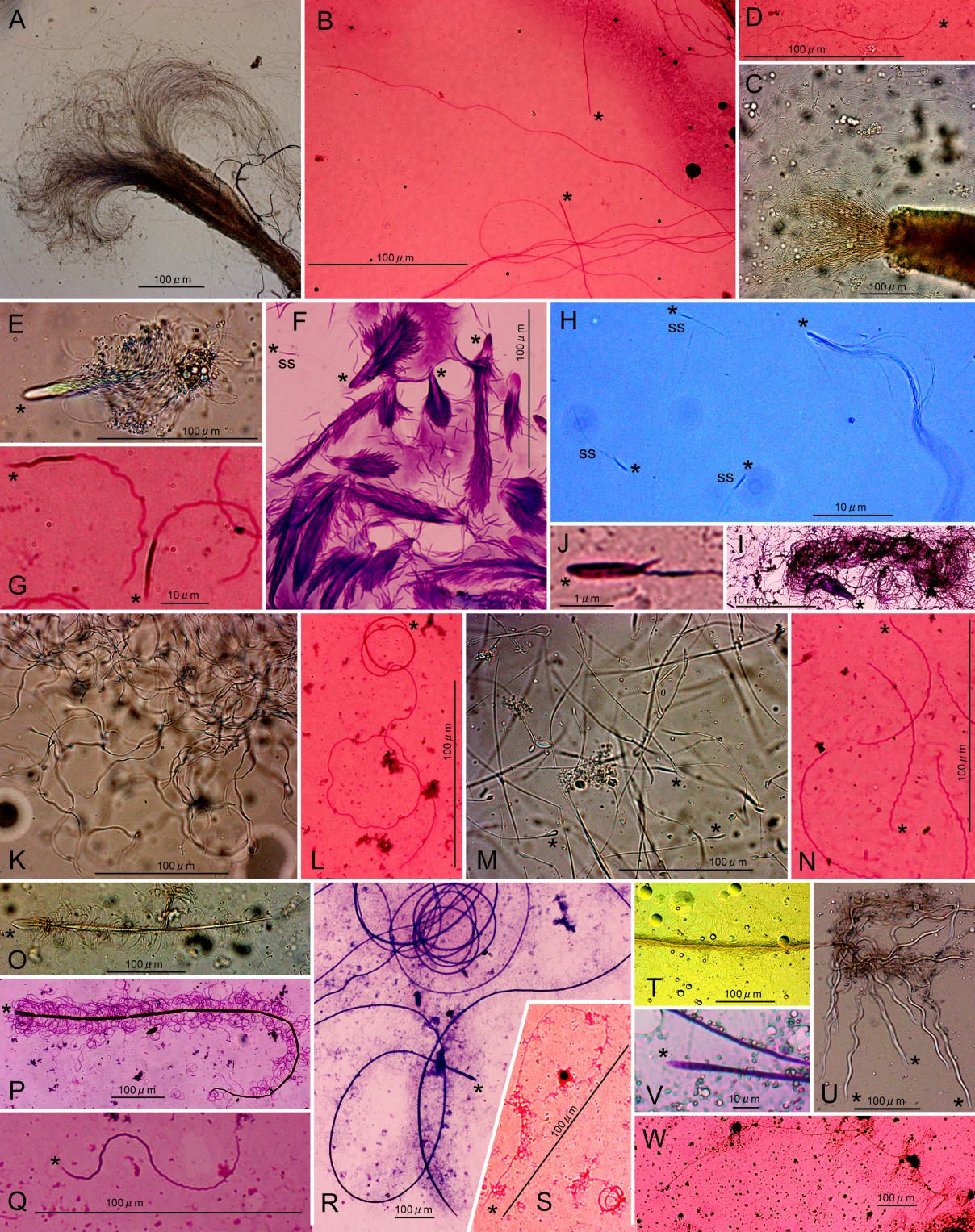


Figure 1

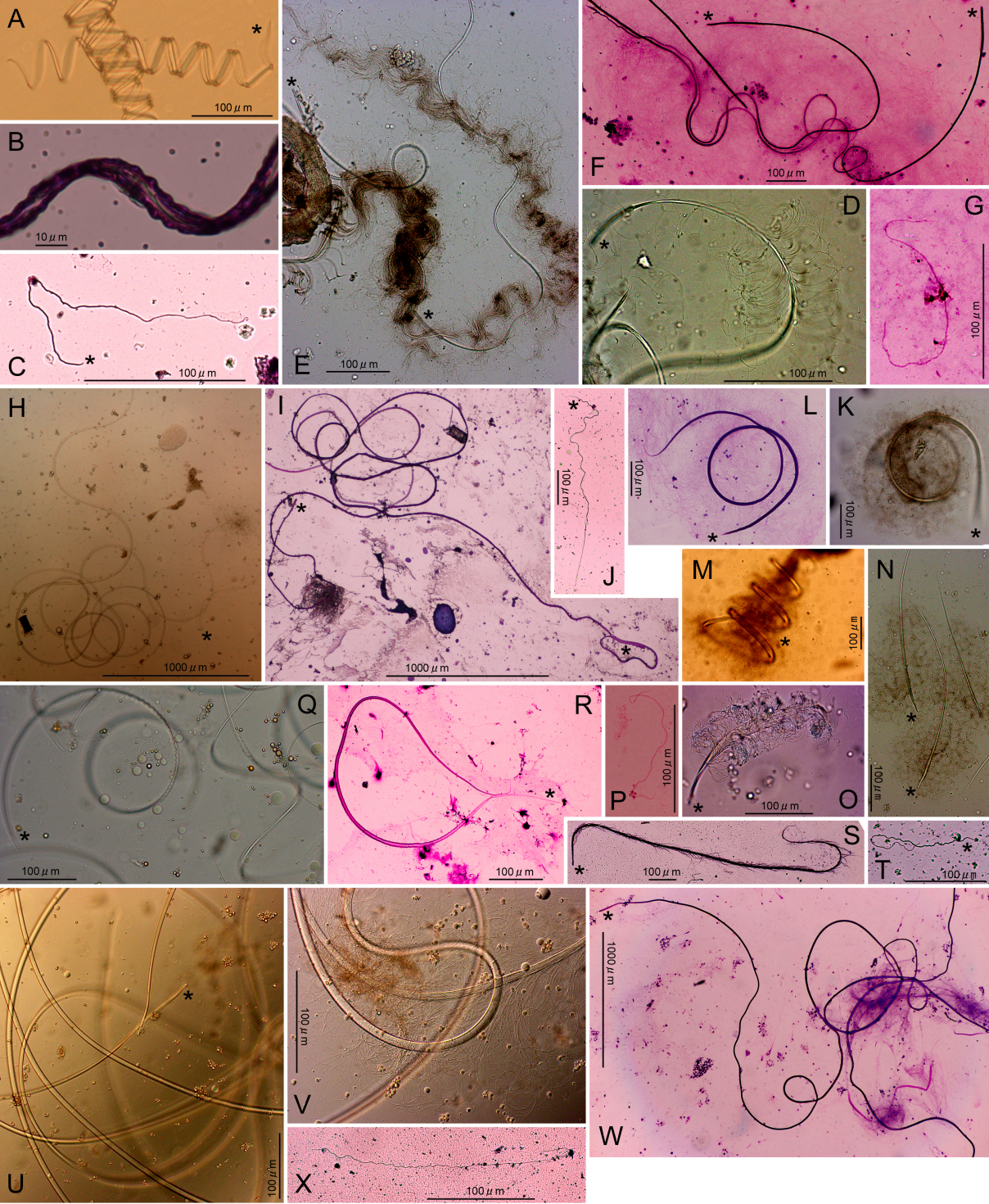


Figure 2

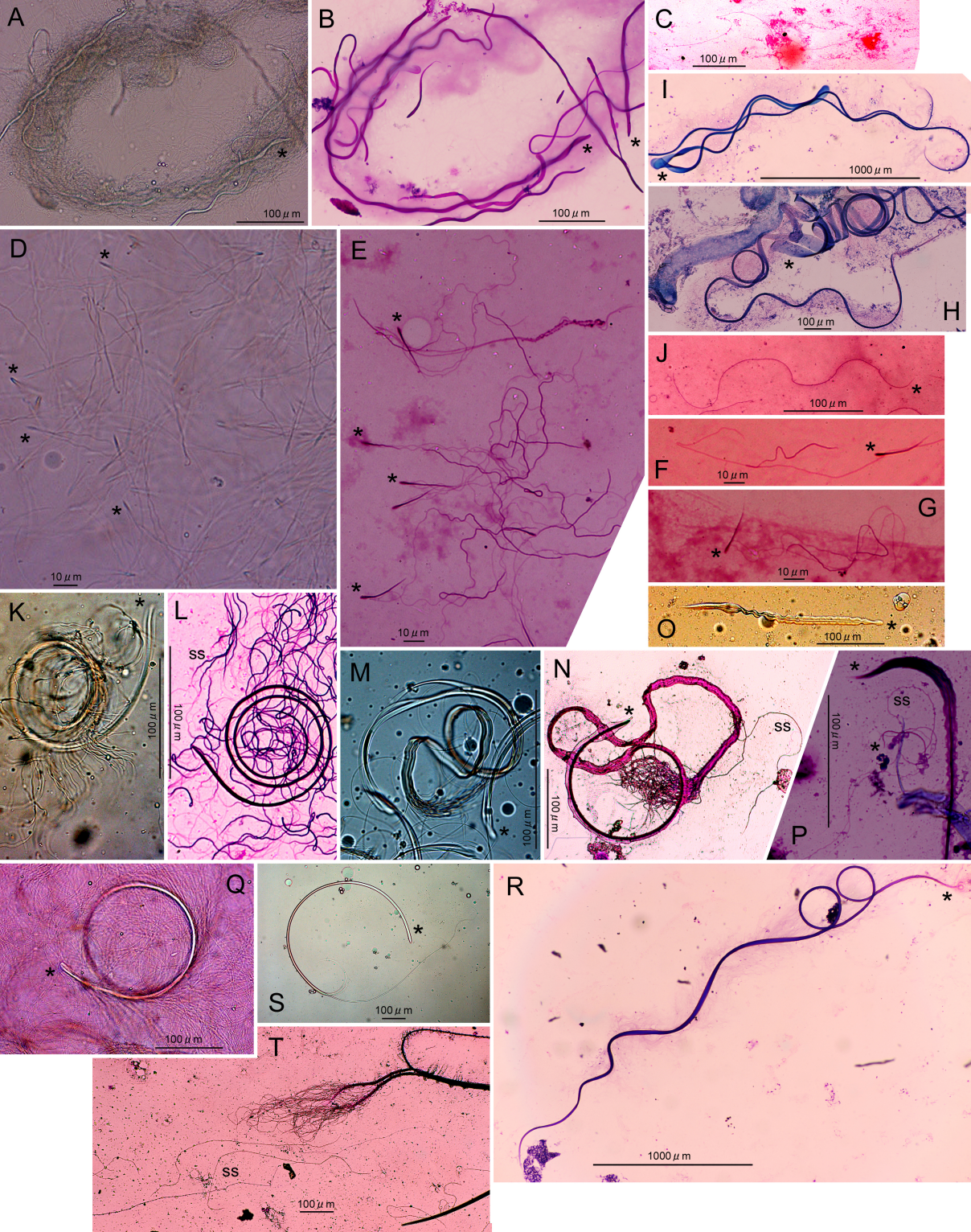
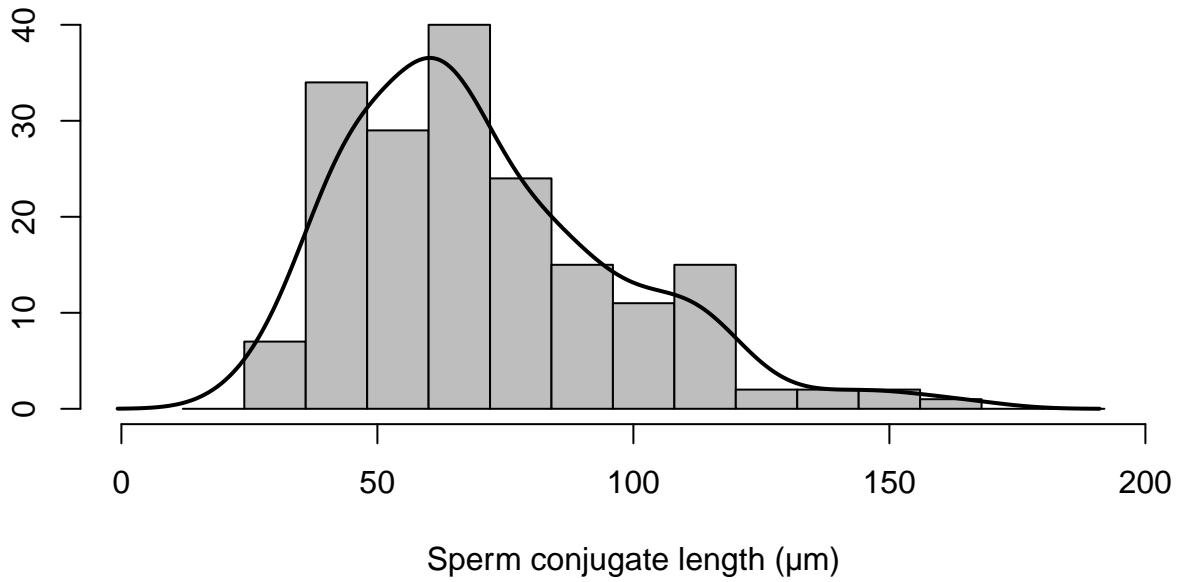


Figure 3

A**B**