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1 **Repeated evolution of asymmetric genitalia and right-sided**  
2 **mating behavior in the *Drosophila nannoptera* species group**

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14 Research article

## 15 **Abstract**

16 **Background:** Male genitals have repeatedly evolved left-right asymmetries, and the  
17 causes of such evolution remain unclear. The *Drosophila nannoptera* group contains four  
18 species, among which three exhibit left-right asymmetries of distinct genital organs. In the  
19 most studied species, *Drosophila pachea*, males display asymmetric genital lobes and  
20 they mate right-sided on top of the female. Copulation position of the other species is  
21 unknown.

22 **Results:** To assess whether the evolution of genital asymmetry could be linked to the  
23 evolution of one-sided mating, we examined phallus morphology and copulation position in  
24 *D. pachea* and closely related species. The phallus was found to be symmetric in all  
25 investigated species except *D. pachea*, which displays an asymmetric phallus with a right-  
26 sided gonopore, and *D. acanthoptera*, which harbors an asymmetrically bent phallus. In all  
27 examined species, males were found to position themselves symmetrically on top of the  
28 female, except in *D. pachea* and *D. nannoptera*, where males mated right-sided, in  
29 distinctive, species-specific positions. In addition, the copulation duration was found to be  
30 increased in the *nannoptera* group species compared to closely related outgroup species.

31 **Conclusion:** Our study shows that gains, and possibly losses, of asymmetry in genital  
32 morphology and mating position have evolved repeatedly in the *nannoptera* group. Current  
33 data does not allow us to conclude whether genital asymmetry has evolved in response to  
34 changes in mating position, or vice versa.

35

36 **Keywords:** phallus asymmetry, *Drosophila nannoptera* species group, one-sided mating  
37 position, copulation behavior

## 38 **Background**

39           Changes in behavior are thought to play important roles in animal evolution [1]–[3].  
40 How new behaviors evolve and how they are encoded in the genome is little understood.  
41 New behaviors can initiate secondary evolutionary shifts in morphology, physiology or  
42 ecology (“behavioral drive”) [1]–[9], for example when they bring an organism into contact  
43 with new environmental factors. Behavior can also prevent evolutionary changes because  
44 plasticity in behavior might enable individuals to adjust for changed environmental  
45 conditions [10]–[12]. Other investigations suggest that behavior and morphology are both  
46 subject to selection and that their responses to changes in the environment are perhaps  
47 independent [13], [14], or that behavior could simultaneously impede and drive  
48 evolutionary diversification of different characters [12], [15], [16]. So far, it appears that the  
49 effects of behavioral changes on the evolution of morphological traits cannot be  
50 generalized and that they require case-specific assessments.

51           The evolution of left-right asymmetric genitalia in insects is a case where  
52 morphology was proposed to have evolved in response to changes in mating behavior  
53 [17]. Asymmetric genitalia are observed in many species and phylogenetic studies indicate  
54 that they have evolved multiple times independently from symmetric ancestors [18], [19].  
55 While most extant insect species copulate with the male being on top of the female  
56 abdomen, the ancestral mating position in insects is inferred to be a configuration with the  
57 female on top of the male [18], [20], [21]. The extant male-on-top configuration has likely  
58 evolved multiple times in insects [20]. Such changes in mating position probably altered  
59 the efficiency of male and female genital coupling, and may have led to the evolution of  
60 genital asymmetries to optimize the coupling of genitalia [17]. Furthermore, these changes  
61 in position may have been driven by male-male competition, or by sexual conflict between

62 males and females.

63 The *nannoptera* species group belongs to the genus *Drosophila* and consists of four  
64 described species that feed and breed on rotten pouches of columnar cacti of the genus  
65 *Stenocereus* and *Pachycereus* in Northern and Central America [22]–[24]. These species  
66 are particularly interesting to study the evolution of genital asymmetry because distinct  
67 genital structures were identified to be asymmetric in three out of the four described  
68 species of this group. *Drosophila acanthoptera* males have an asymmetric phallus, *D.*  
69 *pachea* males have a pair of asymmetric external lobes with the left lobe being  
70 approximately 1.5 times longer than the right lobe [25], [26], and in the sister species *D.*  
71 *wassermani* males have a pair of asymmetric anal plates (cerci) [25]. In contrast, no  
72 asymmetries are known in the fourth described species *D. nannoptera* [27]. The four  
73 species separated about 3-6 Ma and lineage-specific changes likely led to the distinct and  
74 elaborated asymmetries in each species [28]. Interestingly, *D. pachea* mates in a right-  
75 sided copulation position where the male rests on top of the female abdomen with its  
76 antero-posterior midline shifted about 6-8 degrees to the right side of the female midline  
77 [26], [29]. This one-sided mating posture is associated with asymmetric coupling of female  
78 and male genitalia during copulation, with the male genital arch being rotated about 6  
79 degrees towards the female's right side. Apart from our previous investigations of the *D.*  
80 *pachea* copulation position [26], [29], little is known about mating positions in other  
81 *Drosophila* species. In Diptera, several mating positions are known and all involve a  
82 symmetric alignment of male and female genitalia. Male and female genitalia are usually  
83 inversely positioned relative to each other with the dorsal surface of the aedeagus  
84 (phallus) contacting the ventral side of the female reproductive tract [30]. *Drosophila*  
85 *melanogaster*, *D. simulans* and *D. sechellia* were reported to adopt such a symmetric  
86 copulation posture, with the male aligned along the female midline [31]–[33]. A one-sided

87 mating position was generated artificially in *D. melanogaster* by unilateral ablation of a  
88 long bristle located on the genital claspers [31]. In any case, no data is currently available  
89 regarding mating positions of the closely related species of *D. pachea*.

90 The observation of a right-sided mating posture and asymmetric male genitalia in  
91 *D. pachea* led us to wonder whether morphological asymmetry in the *nannoptera* group  
92 species might have evolved in response to the evolution of one-sided mating [17]. We  
93 therefore decided to investigate copulation position and aedeagus asymmetry in species  
94 closely-related to *D. pachea*, and to reconstruct their most likely evolutionary history.

95

## 96 **Results**

### 97 **The phallus of *D. pachea* is asymmetric**

98 The shape of the aedeagus/phallus of *D. pachea* has not been described  
99 previously. We examined the aedeagus of two dissected *D. pachea* males using scanning  
100 electron microscopy (SEM) and found that both were strikingly asymmetric (Fig. 1).  
101 Aedeagi were strongly bent, dorsally flattened and pointed at the dorsal tip. Their ventral  
102 region bore two ventrally pointing asymmetric spurs, one positioned apically, the other sub-  
103 apically. The gonopore was positioned dorso-apically on the right side of the aedeagus.  
104 The aedeagal parameres broke off during dissection and were not visualized. In order to  
105 corroborate the SEM observations, we dissected and examined 10 aedeagi of *D. pachea*  
106 males using light microscopy. Apical and subapical spurs, as well as a right-sided  
107 gonopore, were consistently observed in all preparations (n=10, Additional file 1: Fig. S1).  
108 Our results indicate that the *D. pachea* phallus is directionally asymmetric (Fig. 2b).

109

110

111 **Aedagus asymmetry is observed in *D. acanthoptera* but not in *D. nannoptera*, *D.*  
112 *machalilla* and *D. bromeliae***

113 We compared aedeagus shapes in several species that are closely related to *D.*  
114 *pachea* (Fig. 2). As previously described [27], the aedeagus of *D. acanthoptera* was found  
115 to be asymmetrically bent (n=10). Two asymmetric spurs were found at the ventral apical  
116 tip of the aedeagus, with the right spur being consistently longer than the left spur (Fig. 2e,  
117 Additional file 1: Fig. S2, Additional file 2). However, in contrast to *D. pachea*, no dorso-  
118 apical gonopore was observed on the right side of the apex. Aedeagi of *D. nannoptera*  
119 males (Fig. 2k, Additional file 1: Fig. S3, Additional file 2) were found to be symmetric  
120 (n=15). The ventral side of the apex revealed two apical elongations with slightly variable  
121 lengths at the left and right side (n=15, Additional file 1: Fig. S3, Additional file 2). The  
122 variation in length was not directional and thus considered to reflect random fluctuating  
123 asymmetry. The ventral tip of the aedeagus of *D. machalilla* (*atalaia* species group) (n=10)  
124 displayed two lateral hooks (Fig. 2n, Additional file 1: Fig. S4, Additional file 2), of the same  
125 length on both sides. The aedeagus of *D. bromeliae* showed two lateral symmetric ridges  
126 (n=10) (Fig. 2q, Additional file 1: Fig. S5, Additional file 2). In summary, aedeagus  
127 asymmetry was only observed in *D. pachea* and *D. acanthoptera*, and distinct phallus  
128 structures were found to be asymmetric in these species.

129 ***D. pachea* and *D. nannoptera* males mate right-sided**

130 The position of the male during copulation has not been described for any of the  
131 closely related species of *D. pachea*. In this study, we assessed copulation postures in *D.*  
132 *pachea* and nine related species: *D. acanthoptera* and *D. nannoptera* (sister species of *D.*  
133 *pachea*), *D. machalilla* and *D. bromeliae* (representatives of close outgroup lineages), *D.*  
134 *buzzatii* and *D. mojavenis* (members of the *repleta* species group), as well as

135 representatives of other *Drosophila* species groups (*D. tripunctata*, *D. willistoni* and *D.*  
136 *melanogaster*). Phylogenetic relationships between the ten studied species were  
137 estimated with a Bayesian phylogeny (Additional file 1: Fig. S6) based on a previously  
138 published sequence dataset [28], supplemented with publicly available sequence data (this  
139 study) for *D. tripunctata* and *D. willistoni* (Additional file 3). The obtained phylogeny is  
140 congruent with previous findings [28] that *D. nannoptera*, *D. acanthoptera* and *D. pachea*  
141 form a monophyletic group with a short internode branch length between the split of the *D.*  
142 *nannoptera* lineage and the separation of *D. acanthoptera* and *D. pachea*. Also, *D.*  
143 *machalilla* and *D. bromeliae* form two close outgroup lineages of the *nannoptera* clade  
144 [28], [34], [35], followed by the *repleta* group species *D. buzzatii* and *D. mojavensis* [28].

145 For each species, we introduced a single virgin female and a single virgin male into  
146 a circular mating chamber and recorded the couple until copulation ended or for 45 min  
147 when no copulation was detectable. We obtained 315 movies, of which 111 were used for  
148 assessing courtship duration, 146 for copulation duration and 124 for copulation posture  
149 analysis (Additional file 4). Most movies were discarded because no copulation occurred  
150 or individuals had damaged wings or legs (all reasons listed in Additional file 4). As  
151 previously described [36]–[38], copulation duration varied significantly among species  
152 (ANOVA,  $df_1 = 9$ ,  $df_2 = 136$ ,  $F = 73.38$ ,  $p < 2e-16$ ) (Table 1). We could reproduce a  
153 previously reported trend that copulation duration in *nannoptera* group species was  
154 remarkably long compared to *D. mojavensis* and *D. buzzatii* of the *repleta* group, with  
155 copulation duration of  $88.49 \text{ min} \pm 35.18 \text{ min}$  for *D. acanthoptera*,  $29.58 \pm 7.86 \text{ min}$  for *D.*  
156 *pachea* and  $11.9 \pm 4.2 \text{ min}$  for *D. nannoptera* (mean  $\pm$  standard deviation (SD)). In  
157 comparison, copulation duration of *D. buzzatii*  $1.79 \pm 0.65 \text{ min}$  and *D. mojavensis*  $2.3 \pm$   
158  $0.35 \text{ min}$  (mean  $\pm$  SD) of the *repleta* species group was shorter and similar to *D.*  
159 *machalilla*  $2.28 \pm 0.53 \text{ min}$  and *D. bromeliae*  $0.92 \pm 0.28 \text{ min}$  (mean  $\pm$  SD) (Table 1).

160 To assess mating posture, we calculated the angle between a line drawn through  
161 the female head midline and the female scutellum tip and a second line drawn through the  
162 female head midline and the female scutellum tip (Additional file 1: Fig. S7a). The angle  
163 was set positive when male head lies on the right side of the female and negative when on  
164 the left. The camera view relative to the fly couple position within the mating cell may affect  
165 the measured angle in each experiment but the sign of the average mating angle taken  
166 from different recordings for each species should accurately reflect the one-sidedness of  
167 the male mating position. As a consequence, we expected a one-sided copulation position  
168 to produce a consistent positive or negative distribution of angle values, while symmetric  
169 mating positions should result in an angle distribution around zero.

170 To compare mating angles between species, it is necessary to examine copulation  
171 postures at the same corresponding time point during copulation. At copulation start, the  
172 male position on top of the female was found to be greatly variable between couples, even  
173 within a single species, so this time point was not considered appropriate for our  
174 comparative analysis. Since copulation duration varies greatly between species, finding  
175 another comparable time point across species was not trivial. We subdivided copulation  
176 into two phases, an initial phase where the male is on top of the female abdomen but  
177 consistently moving legs and abdomen, and a second phase when the male maintains an  
178 invariant position relative to the female, which can sometimes walk or move its legs  
179 (Additional file 1: Fig. S6). The “settling time point” is defined as the time point between the  
180 first and second phase, when the male adopts an invariant position relative to the female.  
181 For our cross-species analysis we chose to assess copulation angle at two time points: (1)  
182 right after the male had settled into an initial invariant copulation position (the settling time  
183 point) and (2) at 10% of elapsed time between the settling time point and the end of  
184 copulation (10% stable copulation time point). These two time points were assumed to

185 represent moments during copulation that are comparable between species. For species  
186 with a mean copulation duration > 2.5 min, > 15 min or > 60 min, we also measured the  
187 angles every 2.5 min, 5 min or 10 min, respectively. This allowed us to follow mating  
188 postures of each species over the course of copulation.

189 Significant one-sided mating positions were observed in *D. pachea* and *D.*  
190 *nannoptera*, both at the settling time point and at the 10% stable copulation time point  
191 (Fig.3a,b, Table 2). No significant one-sided copulation postures were detected in *D.*  
192 *acanthoptera* and the other seven tested species including *D. melanogaster* (Fig. 3a,b).

193 Over the course of copulation, mating angles continued to range over zero for *D.*  
194 *melanogaster* and *D. acanthoptera* (Fig. 3c,d), indicating a relatively steady symmetric  
195 copulation position without any left- or right-sidedness. Similar to previous investigations  
196 [26], [29], *D. pachea* revealed right-sided angles that were highest at the beginning of  
197 copulation at 0-10 min after settling (Fig. 3e). At later time points, the angles tended to  
198 range over zero. In *D. nannoptera*, mating angles tended to be right-sided throughout  
199 copulation (Fig. 3f). In summary, *D. pachea* and *D. nannoptera* revealed a right-sided  
200 copulation posture whereas all the other tested species displayed a symmetric mating  
201 posture.

## 202 **Male *D. nannoptera* tilt to the right side of the female abdomen during copulation**

203 To further investigate the right-sided copulation posture in *D. nannoptera* and better  
204 observe the male position relative to the female dorso-ventral midline, we filmed the  
205 couples from a frontal perspective (Additional file 1: Fig. S8). In particular, we assessed  
206 the inclination of the male body relative to the female dorso-ventral axis by measuring the  
207 angle P4-P5-P6, with P4 as the medial most dorsal edge of the female head (often visible  
208 by the ocelli), P5 being the most ventral medial position of the female head (the female

209 proboscis) and P6 as the medial most dorsal edge of the male head (often visible by the  
210 ocelli) (Additional file 1: Fig. S8).

211 *D. nannoptera* mating positions were on average strikingly right-sided (Additional  
212 file 1: Fig. S8), with a considerable variation of observed angles, ranging from slightly left-  
213 to strongly inclined right-sided ( $-8.42^{\circ} - 57.7^{\circ}$ ) over the course of copulation. Left-sided  
214 angles were only observed during the first two minutes of copulation. On average, the  
215 male tended to initially adopt a right-sided copulation posture with an angle of  $10.36^{\circ} \pm$   
216  $6.88^{\circ}$  (mean  $\pm$  SD) (n=25) between 0-1 min after copulation start (Table 3). Over the  
217 course of copulation, the angle then increased to  $27.16^{\circ} \pm 10.81^{\circ}$  (n=29) between 3-4 min  
218 after copulation start (Table 3), which was visible by an inclination of the male head  
219 towards the female's right side. This tilt-movement was not observed in *D. pachea*, where  
220 all males remained on top of the female abdomen [29]. We therefore conclude that *D.*  
221 *pachea* and *D. nannoptera* adopt distinct copulation postures, even though both of them  
222 are right-sided.

223

## 224 Discussion

### 225 Phallus asymmetries differ between *D. pachea* and *D. acanthoptera*

226 The currently published data suggest that genital asymmetries are rare among  
227 *Drosophila* species. The genus *Drosophila* encompasses over 1500 described species  
228 [39] and only 8 species have been shown without doubt to display an asymmetric phallus:  
229 *D. marieaehelenae* and *D. hollisae* of the flavopilosa group [40], [41], *D. asymmetrica* and  
230 *D. quinarensis* of the guarani group [42], [43], *D. endobanchia* of the *canalinea* group [44],  
231 *D. acuminanus* and *D. freilejoni* of the onychophora group [27], [45], [46] and the  
232 *nannoptera* group species *D. acanthoptera* [27]. Genital asymmetry might be more

233 widespread than what is reported in the literature across *Drosophila*, as certain species  
234 are only described based on a few specimens, and subtle asymmetric characters might  
235 have been overlooked and interpreted as fluctuating variation between left and right sides.  
236 Here, we compared aedeagus morphology of at least 10 specimens of five different  
237 species that belong to the *nannoptera* species group and closely related species. We did  
238 not detect aedeagus asymmetry in the tested species outside of the *nannoptera* species  
239 group and found that within the *nannoptera* group only *D. acanthoptera* and *D. pachea* but  
240 not *D. nannoptera* reveal striking left-right asymmetries (Fig. 4). We did not evaluate  
241 aedeagus asymmetry of *D. wassermani*, as this species is not available for examination  
242 and our attempts to catch specimens in the wild were not successful (see materials and  
243 methods). Asymmetries differed between *D. pachea* and *D. acanthoptera*. Whereas ventral  
244 spurs on the *D. pachea* aedeagus were apart from each other, with one being apical and  
245 the other subapical, the aedaegus of *D. acanthoptera* had a pair of apical spurs that  
246 differed in length. In addition, the gonopore was visible and right-sided in *D. pachea* while  
247 it was not visible in *D. acanthoptera*. Our results thus highlight that the asymmetric phallus  
248 structures of *D. pachea* and *D. acanthoptera* are derived morphologies that have little in  
249 common and diversified independently after the split of the two species about 3-6 Ma ago  
250 [28]. It is impossible to infer whether the asymmetries observed in both species derived  
251 from a pre-existing asymmetric phallus in their ancestor or if asymmetry evolved *de novo*  
252 in both lineages.

253         The outer genitalia (epandrium) has been reported to be asymmetrical in *D. pachea*  
254 (where the left lobe is longer than the right lobe [25], [26] and in *D. wassermani* (where the  
255 right anal plate is larger than the left one [25]). Our inspection of a few dissected epandria  
256 of *D. nannoptera*, *D. acanthoptera* and *D. machalilla* revealed no obvious asymmetry (Fig.  
257 2). However, a quantitative comparison remains to be done to confirm the absence of

258 asymmetry in the epandrium of these species.

259

### 260 **Long copulation duration is specific to the *nannoptera* group species**

261 We observed that *nannoptera* species copulated considerably longer than any  
262 representative species of the close outgroup lineages (Fig. 4). This trend was previously  
263 reported by Pitnick and Markow (1991) [36], [37], where the authors compared copulation  
264 duration of *nannoptera* group species with *repleta* group and other species. Here we  
265 included two additional closely related species, *D. machalilla* and *D. bromeliae*, and  
266 observed that their copulation durations were relatively short. Our observations therefore  
267 indicate that a long copulation duration is specific to the *nannoptera* group.

268

### 269 **Right-sided mating positions differ between *D. pachea* and *D. nannoptera***

270 We assessed copulation postures of *D. pachea* and a range of related species to  
271 track the extent of right-sided mating position in the *nannoptera* group. Two aspects of  
272 copulation behavior made cross-species comparisons difficult. First, copulation duration  
273 was extremely diverse and ranged from less than a minute in *D. bromeliae* to more than  
274 two hours in *D. acanthoptera*. Second, the movements of the male and female during  
275 copulation varied between species. In *D. melanogaster* and *D. willistoni*, we observed  
276 vigorous movements of the male during copulation accompanied by female hindleg  
277 kicking. These phases were interrupted by periods without movements. In contrast, males  
278 of most other species initially moved upon mounting the female and then settled into an  
279 invariant copulation posture relative to the female.

280 At the two measured time points during the initial stable copulation period (settling  
281 time point, 10% stable copulation time point), the angle between the male midline and the

282 female midline was distributed symmetrically around zero, indicating a symmetric mating  
283 position in all tested species except *D. pachea* and *D. nannoptera*. Our previous data from  
284 *D. pachea* [26], [29] was re-analyzed in this study with a different measurement approach  
285 and led to the same conclusion as our earlier reports. In addition, we found that *D.*  
286 *nannoptera* adopts a right-sided mating position with angle values that were slightly higher  
287 than in *D. pachea* (Fig. 3). Assessment of homology of behaviors is difficult compared to  
288 morphological characters, because Owen's position criterion for homology [47] does not  
289 exist for behavioral traits. Observation of similar behaviors does not necessarily mean  
290 common descent [6]. Our precise examination of the mating position of *D. nannoptera*  
291 from a frontal perspective revealed that *D. nannoptera* males strongly tilt to the female's  
292 right side during copulation, a behavior that is not observed in *D. pachea* [26], [29].  
293 Therefore, mating postures can be regarded as distinct between the two species.  
294 Interestingly, a comparable tilting behavior during copulation was observed in experiments  
295 with *D. pachea* males that had surgically modified external genital lobes [29]. Male lobes  
296 are considered to be important in grasping the female abdomen beneath the oviscapt  
297 valves and to keep *D. pachea* upright on the female abdomen. A hypothetical scenario is  
298 thus that the ancestral mating position in shared ancestor of the two species may have  
299 been right-tilted but the evolution of asymmetric external lobes in *D. pachea* led to a  
300 derived right-sided copulation posture, which is upright. Alternatively, right-sided mating  
301 position may have evolved independently in the two lineages leading to *D. pachea* and *D.*  
302 *nannoptera*. In all scenarios, at least two evolutionary changes in mating position must be  
303 considered to account for the distinct, species-specific right-sided mating positions in the  
304 *nannoptera* group.

305

306 **Asymmetry in mating position and in phallus have evolved in different branches of**  
307 **the *nannoptera* group phylogeny**

308         Across the *nannoptera* group, we find no striking correspondence between right-  
309 sided mating posture and asymmetric male genitalia. For example, *D. acanthoptera* has an  
310 asymmetric aedeagus but mates in a symmetric overall posture. Conversely, no directional  
311 asymmetry is detected in the male (external and internal) genitalia of *D. nannoptera*, but  
312 males adopt a right-sided copulation posture (Fig. 4). Based on our phylogeny, *D.*  
313 *nannoptera* presents the earliest branching lineage within the *nannoptera* group. In this  
314 sense, right-sided mating postures could have originated earlier during evolution than  
315 asymmetric morphologies and may have been lost in *D. acanthoptera*. However, the  
316 internode branch length between the split of the *D. nannoptera* lineage and the separation  
317 of *D. acanthoptera* and *D. pachea* is short and statistical support is weak [28]. Thus,  
318 phylogenetic relationships within the *nannoptera* group remain to be resolved and it is  
319 more appropriate to regard all *nannoptera* species as sister species.

320         So far, we conclude that both right-sided copulation behavior and asymmetric male  
321 genitalia evolved within the *nannoptera* species group and that diversification of both traits  
322 have involved lineage-specific evolutionary changes. They may have evolved by  
323 modifications of pre-existing right-sided mating behavior and/or asymmetric genital  
324 morphologies already present in the ancestor. Alternatively, they can have appeared *de*  
325 *novo* in each extant lineage.

326

327 **One-sided mating and asymmetric phallus are correlated with giant sperm and**  
328 **female sperm storage, respectively**

329         Asymmetric genital morphology and right-sided mating behavior may also be  
330 associated with other characters that are special to the *nannoptera* species group. *D.*

331 *pachea* and *D. nannoptera* are among the *Drosophila* species that produce the longest  
332 (giant) sperm [48], [49] (Fig. 4). The association of right-sided mating with giant sperm  
333 production actually holds better than with asymmetric male genital morphology because *D.*  
334 *acanthoptera* has an asymmetric aedeagus but has relatively small sperm [48] and mates  
335 in a symmetric overall posture (Fig. 4). Again, a specific one-sided mating posture might  
336 be necessary for optimal transfer of giant sperm. Examining mating postures in *Drosophila*  
337 species which harbor even longer sperm (*D. bifurca* 58 mm, *D. kanekoi* 24 mm, *D. hydei*  
338 23 mm, *D. eohydei* 18 mm) [50], [51] would be interesting to test further the possible  
339 association between sperm length and one-sided mating.

340         The species *D. pachea*, *D. acanthoptera* and *D. wassermani* are also special in the  
341 way the female stores sperm after copulation (Fig. 4). They are the only *Drosophila*  
342 species that store sperm exclusively inside the spermathecae but not in the seminal  
343 receptacle as most other species [49]. In contrast, *D. nannoptera* stores sperm exclusively  
344 inside the seminal receptacle [49]. Morphological phallus asymmetry is thus observed in  
345 those species that reveal exclusive sperm storage in the spermathecae. It would be  
346 interesting to collect male specimens of *D. wassermani* to analyze their phallus shapes to  
347 test this trend. In any case, it is hard to generalize from our observations as only three  
348 species are concerned.

349

### 350 **Evolution of left-right asymmetric genitalia and one-sided mating in the *nannoptera*** 351 **group**

352         During *D. pachea* copulation, the male is on top on the female and oriented towards  
353 the female right side [29]. Since the male gonopore is located on the right side of the  
354 aedeagus (this study), the opening of the male reproductive tract during copulation is  
355 expected not to be aligned with the female opening. In *D. nannoptera*, male structures are

356 symmetric but the male and female openings are probably also unaligned because of the  
357 tilted mating position. One-sided copulatory behavior and asymmetric genital morphology  
358 can thus both contribute to asymmetric complexing of male and female structures in an  
359 additive or compensatory way. The evolution of mating posture and genital asymmetry  
360 might therefore both be driven by selection on the sterical characteristics of the complex of  
361 male and female genitalia. Why would asymmetric male-female genitalia complexing  
362 evolve? As mentioned above, asymmetric complexing might facilitate the transfer of giant  
363 sperm into the female tract. Another possibility would be because of sexual conflict  
364 between males and females [52]–[54], as asymmetric alignments of the male and female  
365 reproductive structures might overcome internal barriers in the female uterus that  
366 otherwise prevent sperm entry. Asymmetric genital complexing could also be a stimulus for  
367 the female to preferentially store or use sperm, thereby driving the evolution of mating  
368 position and genital asymmetry by cryptic female choice [53], [55], [56]. For example,  
369 males of the fly *Dryomyza anilis* tap the female external genitalia shortly after insemination  
370 with a pair of genital claspers, which can be symmetric or asymmetric [57], [58]. Males with  
371 asymmetric small claspers have higher fertilization success compared to males with large  
372 symmetric ones, suggesting that asymmetric male tapping can bias sperm distribution in  
373 the spermathecae [59]. Females of the *nannoptera* group store sperm exclusively in the  
374 spermathecae, except for *D. nannoptera* females that only use the receptacle [49]. Such  
375 rapid evolution of post-copulatory sperm processing is indicative of sexual selection and  
376 cryptic female choice. The exact underlying mechanisms are unknown but it is possible  
377 that they also contributed to the evolution of asymmetric structures and one-sided  
378 copulation behaviors. Future studies of the positioning of the phallus gonopore inside of  
379 the female uterus and of sperm transfer during copulation in the various *nannoptera* group  
380 species would be helpful to test the various scenarios.

381

## 382 **Conclusion**

383 Phallus asymmetries were identified in *D. pachea* and *D. acanthoptera* of the *nannoptera*  
384 species group and distinct structures were observed to be asymmetric in both species. An  
385 increased copulation duration was found to be specific to *nannoptera* group species and  
386 was not observed in the closely related outgroup species *D. machalilla* and *D. bromeliae*.  
387 Right-sided mating positions were detected in *D. pachea* and *D. nannoptera* and were  
388 found to be distinct between them. Our data does not allow us to conclude whether the  
389 evolution of the right-sided copulation position may have promoted the evolution of genital  
390 asymmetry, or vice versa. Our results nevertheless indicate that asymmetry in genital  
391 morphology and in copulation behavior have evolved through multiple evolutionary steps in  
392 the *nannoptera* group, revealing a complex history of sexual trait changes, maybe in  
393 relationship with the evolution of giant sperm and unique sperm storage in the *nannoptera*  
394 group.

395

## 396 **Methods**

### 397 **Fly sampling and maintenance**

398 An isofemale stock of *Drosophila machalilla* was established from a collection of A.  
399 A. in December 2015 at San Jose Beach (GPS coordinates: 01°13'46.4"S, 80°49'14.6"W)  
400 Ecuador, using a modified version of the fly traps described in [60]. Our baits contained  
401 rotten pieces of the columnar cactus *Armatocereus carwrightianus* and yeast solution. The  
402 *D. machallila* stock was raised on standard *Drosophila* medium (60 g/L brewer's yeast,  
403 66.6 g/L cornmeal, 8.6 g/L agar, 5 g/L methyl-4-hydroxybenzoate and 2.5% v/v ethanol) and

404 a piece of fresh *Opuntia ficus-indica* (prickly pear opuntia) or *Hylocereus undatus* (dragon  
405 fruit) in the medium. The isofemale stock was raised for two generations before  
406 experiments started and it was maintained for a total of 36 generations.

407 We also intended to collect *D. wassermani* in August 2016 in Oaxaca, Mexico. Six  
408 localities were sampled based on previous records: Reserva de la Biosfera Tehuacan-  
409 Cuicatlan (GPS coordinates 18°11'21.30" N, 97°14' 51.7" W), Huajuapán de León  
410 (17°48'25.6" N, 97°14' 56.7" W), San Luis del Río (16°46'30" N, 96°10' 49.9" W), and four  
411 sites along the Carretera Internacional 190: kilometer stones Km 73 (16°42'57.2" N,  
412 96°19'41.9" W), km 89 (16°40'41.3" N, 96°14'41.7" W), km 102 (16°42'11.3" N, 96°11'32.4"  
413 W) and km 111 (16°39'48.4" N, 96°07'31.8" W). We used banana traps, cactus baits that  
414 contained rotten organ pipe cactus *Stenocereus prinosus* and mixed food traps that  
415 additionally contained banana and yeast. Besides the invasive species *Zaprionus indianus*  
416 and cosmopolitan species *D. melanogaster* and *D. simulans*, we identified several species  
417 of the *repleta* group, about 100 individuals of *D. nanoptera*, three males of *D.*  
418 *wassermani* and one female of *D. acanthoptera*. Unfortunately, we were not successful in  
419 establishing iso-female strains from *D. nanoptera* and *D. acanthoptera*.

420 All other stocks were retrieved from the San Diego Drosophila Species Stock  
421 Center or were provided by Jean David (Additional file 1: Table S1). Flies were maintained  
422 at 25 °C, except for *D. melanogaster*, *D. tripunctata* and *D. willistoni*, which were either  
423 maintained at 22 °C or 25 °C (details in Additional file 4). Flies were kept in vials with 10 mL  
424 of standard *Drosophila* food medium (see above) inside incubators with a 12 h light: 12h  
425 dark photo-periodic cycle combined with a 30-min linear illumination change between light  
426 (1080 lumen) and dark (0 lumen). For maintenance of *D. pachea*, we mixed standard  
427 *Drosophila* food medium in the food vial with 40 µL of 5 mg/mL 7-dehydrocholesterol  
428 (dissolved in ethanol) [61].

429

430 **SEM analysis of the *D. pachea* aedeagus**

431 Virgin males of at least 14 days after hatching from the pupa were transferred into a  
432 2 mL reaction tube, snap frozen in liquid nitrogen and stored in ethanol at -20 °C. For  
433 dissection, frozen and fixed males were placed in 80% ethanol at room temperature and  
434 the aedeagus was dissected out with fine needles. Tissues were dried using an EM  
435 CPD300 automated critical point dryer (Leica) and mounted on aluminium stubs with the  
436 distal end facing upwards and coated with platinum/palladium (20 nm). Each aedeagus  
437 was SEM-imaged with a JSM-7500F field emission scanning electron microscope (Jeol) at  
438 270x magnification.

439

440 **Analysis of aedeagus asymmetry by light microscopy**

441 The terminal segments of the male abdomen were picked out with fine forceps and  
442 boiled for 10 min in two drops of 30% KOH. Genital parts were further dissected on a  
443 microscope slide (Thermo Scientific Menzel) in a drop of water using 0.1 mm Minutien  
444 Pins (Fine Science Tools) under the stereo-microscope K-500 (VWR). Dissected structures  
445 were mounted in pure glycerol on 1.5 mm concave microscope slides (Marienfeld). Images  
446 were acquired with a light microscope VHX2000 (Keyence) equipped with a zoom lens  
447 VH-Z100UR/W at 350-550 fold magnification. For storage, male genitalia were mounted in  
448 DMHF medium on microscope slides (Entomopraxis).

449

450 **Phylogenetic analysis**

451 We used data of eight species from a multi-locus dataset of Lang et al. (2014) [28],

452 and added corresponding sequences for *D. willistoni* and *D. tripunctata* (Additional file 1:  
453 Table S2, Additional file 3, BEAST input file in DRYAD). For *D. tripunctata*, only  
454 mitochondrial data was available at GenBank (<https://www.ncbi.nlm.nih.gov/nucleotide/>)  
455 and missing data was annotated by '?'. Phylogenetic analysis was performed in BEAST  
456 [62] according to the settings described in Lang et al (2014) [28]. Markov-Chain Monte-  
457 Carlo (MCMC) runs were performed with a chain length of  $10^7$  generations and recorded  
458 every 1000 generations. MCMC output analysis was carried out using TreeAnnotator  
459 [62] and the tree was visualized and edited with FigTree [63]. We chose a strict molecular  
460 clock and set priors for most recent common ancestors according to the divergence  
461 estimates of Lang et al. (2014) [28] for the splits of *D. nannoptera* - *D. pachea*  $3.7 \pm 1.5$   
462 Ma, *D. bromeliae* - *D. pachea*  $8 \pm 3$  Ma. The divergence estimate for all analyzed species  
463 was set to  $40 \pm 5$  Ma [64].

464

### 465 **Copulation recording**

466 Emerged flies (0-14 h) were anesthetized with CO<sub>2</sub>, separated according to sex and  
467 transferred into food vials in groups of either 5 females or 5 males using a Stemi 2000  
468 (Zeiss) stereo microscope and a CO<sub>2</sub>-pad (Inject+Matic sleeper). Flies were maintained at  
469 22°C or 25°C until they reached sexual maturity (Additional file 1: Table S1). Males of *D.*  
470 *bromeliae*, *D. melanogaster*, *D. pachea* and *D. nannoptera* were isolated into single vials  
471 for at least two days before the experiment was performed. For video recording, one male  
472 and one female were introduced with a self-made fly aspirator by mouth suction into a  
473 circular plastic mating cell with a diameter of 10-12 mm, a depth of 4mm and a transparent  
474 1-mm Plexiglas cover [26]. For copulation recording of *D. acanthoptera*, flies were let to  
475 initiate copulation in a food vial and were then rapidly transferred to the mating cell.

476 Movies were recorded in a climate controlled chamber [26] at 22 or  $25 \pm 0.1^\circ\text{C}$  and

477 60% or 85%  $\pm$  5% humidity (Additional file 4). Flies were filmed from above using digital  
478 microscope cameras 191251-62 (Conrad), DigiMicro Profi (DNT) or MIRAZOOM MZ902  
479 (OWL). Movies were recorded with the program GUVVIEW (version 0.9.9) GTK UVC or  
480 Cheese (version 3.18.1) (<https://wiki.gnome.org/Apps/Cheese>) at a resolution of 800 X 600  
481 pixels on a Linux Ubuntu operating system. Movies were recorded until copulation ended  
482 or for at least 45 min when no copulation was detectable. After movie recording, flies were  
483 dissected or stored in ethanol at -20°C.

484

### 485 **Multiple species mating position analysis**

486 Each movie name consisted of a three-letter abbreviation for the species filmed, an  
487 additional two-digit number that also indicated the species and a two-digit number for each  
488 respective experiment. Movies were analyzed with the video editor OpenShot 1.4.3 (Open  
489 Shot Studios, Texas, USA). Courtship start, copulation start, the settling time point and the  
490 end of copulation were annotated manually by two different persons, except for movies of  
491 *D. pachea* and *D. melanogaster*, which were annotated only by one person (Additional file  
492 4). Courtship was defined to start when the male displayed at least three consecutive  
493 typical courtship behaviors, such as tapping the female, following the female's abdomen,  
494 licking the female oviscapt or the ground beneath the female abdomen, wing rowing (*D.*  
495 *melanogaster*) or other wing vibrations [65]. Courtship was defined to end with the start of  
496 copulation, when a male started to mount the female abdomen. Only cases where the  
497 male remained mounted on the female for at least 15 sec were counted as copulation  
498 starts. Copulation was defined to end when the male had completely descended from the  
499 female abdomen with the forelegs detached from the female dorsum and female and male  
500 genitalia being separated. As mentioned above, the male moved its legs and abdomen for  
501 a certain time period (considered as the settling phase) until adopting an invariant

502 abdomen posture at the settling time point (Additional file 1: Fig. S6, Additional file 4). The  
503 remaining copulation period was defined as the stable copulation period (Additional file 1:  
504 Fig. S6). In fact, this period was often interrupted by periods of vigorous movements in *D.*  
505 *melanogaster*, *D. tripunctata* and *D. willistoni*. In the other species, males remained rather  
506 invariant on the female abdomen after the settling time point.

507 We video-recorded 315 movies, of which 111 were used for assessing courtship  
508 duration (Additional file 4). Reasons for discarding 204 movies for courtship duration  
509 measurements were: wrong handling of the camera or the software, damaged files: 4;  
510 incomplete recording of courtship: 43; fly leg or wing damaged: 27; no copulation after 45  
511 min of experiment start: 129; wing damaged and incomplete recording of courtship: 1. A  
512 total of 146 movies were used for the analysis of copulation duration. Reasons for  
513 discarding 169 movies for copulation duration measurements were: wrong handling of the  
514 camera or the software, damaged files: 4, incomplete recording of copulation: 7, fly leg or  
515 wing damaged: 27, no copulation after 45 min of experiment start: 129; multiple reasons: 2  
516 (Additional file 4). From these 146 movies, we had to exclude 22 movies for the  
517 assessment of the copulation posture because landmark positions could not be observed.  
518 This was mainly due to couples being recorded from the ventral view. As a result, 124  
519 experiments were used for assessment of the copulation posture (Additional file 4). One  
520 additional movie was discarded for posture assessment at the 10% stable copulation time  
521 point because the female head was not in the camera field of view.

522 Movie names were replaced by a seven-digit random number (Additional file 5) so  
523 that mating postures were quantified in a blind fashion with respect to the species name.  
524 Time points for position analysis (Additional file 1: Fig. S6, Additional file 4) were calculated  
525 with a custom R script and exported values were used as an input for a bash script to  
526 extract images from each movie at particular time points with avconv (libav tools,

527 <https://www.libav.org>).

528         The angle was measured using three landmarks on the female and male body: the  
529 anterior tip of the female head along its mid-line (P1), the distal tip of the female scutellum  
530 (P2) and the most posterior medial point of the male head (P3) (Additional file 1: Fig. S7a).  
531 In cases where images were too dark, positions of P1 and P3 were approximated as the  
532 anterior and posterior mid distances between the eyes and the position of the scutellum tip  
533 (P2) was approximated by the medial dorsal point at the body constriction observed  
534 between the third thoracic and first abdominal segment. Position landmarks were placed  
535 manually on each image using imageJ and data analysis was done using R. Briefly,  
536 coordinates (Additional file 6) were rotated and scaled, so that all P1 points were  
537 superimposed and all P2 points as well (Additional file 1: Fig. S7b-k). The angle P1-P2-P3  
538 (Additional file 1: Fig. S7a) was used to measure one-sidedness of mating positions (Fig.  
539 3). Repeatability of landmark positioning was assessed by two independent rounds of  
540 coordinate acquisition for all species at one specific time point during copulation, the 10%  
541 stable copulation time point (see text) (2x 124 images). Variation in angle estimates was  
542 found to be attributable mostly to individual images and not to replicate measurement  
543 (ANOVA, linear model: angle ~ image + replicate, image: df1 = 122, df2 = 123, F = 87.174,  
544  $p < 2e-16$ , replicate: df1 = 1, df2 = 244, F = 0.077,  $p = 0.782$ ).

545         Hypothesis testing was performed in R to compare mating postures across species  
546 (Fig. 3) with the null hypothesis: angle = 0, using the functions glm for generalized linear  
547 model fits, and glht to derive estimated contrasts.

548

### 549 **Analysis of the *D. nanoptera* copulation posture**

550         Flies were reared and isolated before copulation as described above. One female  
551 and one male were CO<sub>2</sub> anesthetized and transferred onto a white plastic support (mating

552 cap) and were caged with a transparent plastic cylindrical 25 mm x 7 mm cap. Once  
553 courtship was observed, mating caps were put on a motorized horizontally turning stage  
554 (0-30 rpm) (grinding stone 8215, Dremel) in front of a camera MIRAZOOM MZ902 (OWL)  
555 and copulation was recorded with the camera being put into an optimized frontal view  
556 towards the female head by rotating or turning the mating cap. The transparent cap was  
557 optionally removed once copulation had started. The yield of informative experiments with  
558 these settings was poor as we performed 167 mating experiments but only 29 experiments  
559 were informative for our data analysis (Additional file 7, reasons for discarding the  
560 experiments are listed). Images were extracted with avconv (see above) every 15-30 sec  
561 or when the flies were visible in a frontal view. We measured the inclination of the male  
562 body relative to the female dorso-ventral axis by using three landmarks: P4 as the medial  
563 most dorsal edge of the female head (often visible by the ocelli), P5 being the most ventral  
564 medial position of the female head (the female proboscis) and P6 as the medial most  
565 dorsal edge of the male head (often visible by the ocelli) and measuring the angle between  
566 the lines drawn through P4-P5 and P5-P6 (Additional file 1: Fig. S8, Additional file 8).

## 567 **List of abbreviations**

568	SEM	scanning electron microscopy
569	ANOVA	Analysis of Variance, statistics
570	df	degrees of freedom
571	df1	degrees of freedom, numerator
572	df2	degrees of freedom, denominator
573	t	t-value
574	F	F-value
575	p	probability

576	SD	standard deviation
577	GPS	global positioning system
578	Ma	million years
579	MCMC	Markov-Chain Monte-Carlo

## 580 **Declarations**

### 581 **Ethics approval and consent to participate**

582 “Not applicable” --- This research focuses on invertebrate insects. There are no ethical  
583 considerations mentioned for these species according to EU Directive 86/609-STE123.

### 584 **Consent for publication**

585 “Not applicable”

### 586 **Availability of data and material**

587 The data sets supporting the results of this article are available in the DRYAD repository,  
588 <https://datadryad.org/review?doi=doi:10.5061/dryad.5h1q6mc>

### 589 **Competing interests**

590 The authors declare no competing financial interests

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597 analysis, interpretation of the data and writing the manuscript.

598

## 599 **Authors' contributions**

600 ML and VCO designed the experiments, AA collected fly specimen, AA, SP and ML  
601 recorded fly copulation, AA performed light microscopy analysis of *Drosophila* male  
602 genitalia, FTR performed SEM analysis of *D. pachea* male genitalia. ML, AA and SP  
603 analyzed the movie datasets, ML and VCO wrote the manuscript with AA. All authors have  
604 read and approved the manuscript.

605

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## 790 **Supporting Information**

791 **Additional file 1:** Supplementary Figures and Tables. **Figure S1:** The aedeagus of *D.*  
792 *pachea* is asymmetric. **Figure S2:** The aedeagus of *D. acanthoptera* is asymmetric.  
793 **Figure S3:** No asymmetry is detected in the aedeagus of *D. nannoptera*. **Figure S4:** No  
794 asymmetry is detected in the aedeagus of *D. machalilla*. **Figure S5:** No asymmetry is  
795 detected in the aedeagus of *D. bromeliae*. **Figure S6:** Courtship and copulation duration in  
796 *D. pachea* and related species. **Figure S7:** Multi-species mating position measurements.

797 **Figure S8:** *D. nannoptera* tilts to the right side of the female abdomen. **Table S1:** Species  
798 Resources, **Table S2:** GenBank Accession Numbers of the phylogeny dataset.

799

800 **Additional file 2:** Length measurements at the left and right sides of the ventral aedeagus  
801 tip of *D. acanthoptera*, *D. nannoptera*, *D. machalilla* and *D. bromeliae*

802

803 **Additional file 3:** Multilocus DNA sequence dataset for the molecular phylogeny shown in  
804 Additional file 1: Fig. S6.

805

806 **Additional file 4:** Multi-species analysis of courtship and copulation periods, shown in  
807 Additional file 1: Fig. S6.

808

809 **Additional file 5:** Randomization of experiment names for the multi-species mating  
810 position analysis. Original names and random number substitutes are listed for each  
811 movie.

812

813 **Additional file 6:** Landmark position measurements, used to calculate angle values for  
814 the multi-species mating position analysis, shown in Fig. 3.

815

816 **Additional file 7:** Copulation times of couples filmed for the position analysis of *D.*  
817 *nannoptera* from a frontal perspective, shown in Additional file 1: Fig. S8.

818

819 **Additional file 8:** Angle measurements for the position analysis of *D. nannoptera* from a  
820 frontal perspective, shown in Fig. 3.

## 821 **Figures and Tables**

822

823 **Figure 1: The aedeagus of male *Drosophila pachea* is asymmetric.** SEM images of a  
824 single phallus in lateral-dorsal and dorsal-apical view. Note the asymmetric position of two  
825 subapical spurs, located on the ventral side of the aedeagus, and the asymmetric position  
826 of the gonopore. The white arrows point to the gonopore. The scale bar is equivalent to  
827 100  $\mu\text{m}$ .

828

829 **Figure 2: Genital and aedeagus shapes in *D. pachea* and closely related species.**  
830 External genitalia and aedeagus shapes are compared across closely related species of  
831 *D. pachea*. Aedeagus asymmetries are only found in *D. acanthoptera* and *D. pachea* (a)  
832 ventral view of a *D. acanthoptera* male. The black frame indicates the position of male  
833 genitalia and the box with a dashed frame shows a magnification with an erected penis.  
834 (b-d, e-g, h-j, k-m, n-p) Lateral views of male specimens and male genitalia of *D.*  
835 *acanthoptera*, *D. pachea*, *D. nannoptera*, *D. machalilla*, and *D. bromeliae*, respectively. (c,  
836 f, i, l, o) Male terminalia in lateral and posterior view. (d, g, j, m, p) Aedeagus in lateral and  
837 ventral view. The scale bar is 100  $\mu\text{m}$ .

838 **Figure 3: The copulation position of *D. nannoptera* and *D. pachea* is asymmetric.**  
839 Copulation angles of *D. pachea* couples and of nine related *Drosophila* species; aca: *D.*  
840 *acanthoptera*, bro: *D. bromeliae*, buz: *D. buzzatii*, mac: *D. machalilla*, mel: *D.*  
841 *melanogaster*, moj: *D. mojavensis*, nan: *D. nannoptera*, pac: *D. pachea*, tri: *D. tripunctata*,  
842 wil: *D. willistoni*. (a,b) Copulation angle at the settling time point (settling, see material and  
843 methods) and at the 10% stable copulation time point (10pct), respectively. Stars indicate  
844 significant rejection of the null hypothesis: angle = 0 (Table 2, GLM fit angle~species \*\*:)

845  $p < 0.001$ , \*\*\*:  $p < 0.0001$ ). Numbers below each boxplot indicate the number of  
846 observations. The dashed lines indicate an angle of zero degrees. **(c-e)** Copulation angles  
847 over the course of copulation of *D. melanogaster* (mel), *D. acanthoptera* (aca), *D. pachea*  
848 (pac) and *D. nanoptera* (nan). n indicates the number of observations. Grey lines connect  
849 points obtained from the same copulation couple over time. The dashed lines indicate an  
850 angle of zero degrees.

851 **Figure 4: Evolution of sexual characters in the *nanoptera* species group.** The  
852 cladogram was established based on the phylogeny of this study (Additional file1: Fig. S6),  
853 combined with data from Lang et al (2014) [28]. AS, asymmetric states; S, symmetric  
854 states; nd, not determined, sp, spermathecae; rec, female seminal receptacle.

**Table 1: Courtship and copulation duration**

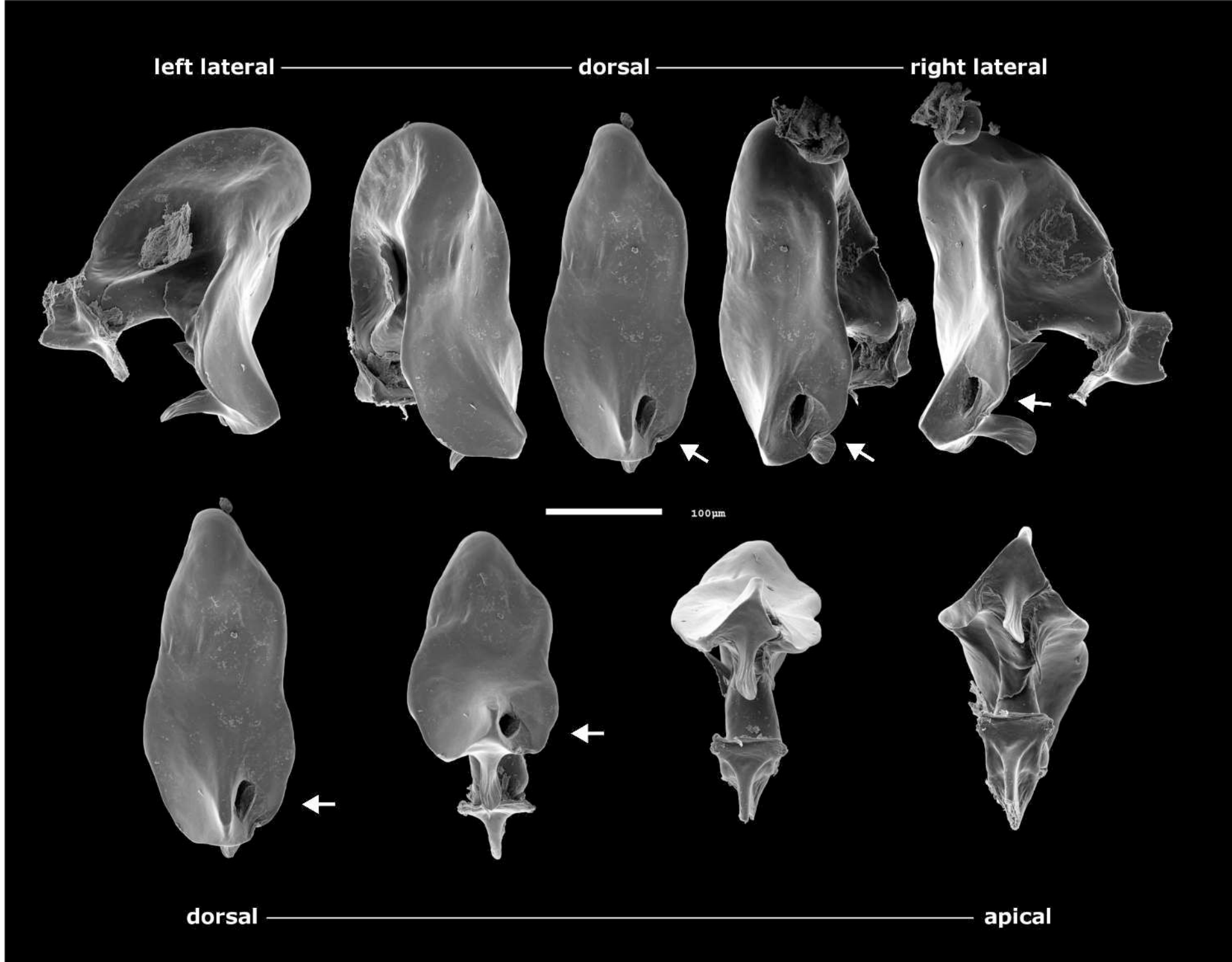
<i>species</i>	courtship duration [min] (mean $\pm$ SD)	range [min]	n	copulation duration [min] (mean $\pm$ SD)	range [min]	n
<i>D. acanthoptera</i>	---	---	---	88.49 $\pm$ 35.18	38.85 - 144.32	12
<i>D. pachea</i>	4.67 $\pm$ 3.91	0.17 - 12.37	18	29.58 $\pm$ 7.86	7.33 - 42.63	21
<i>D. nannoptera</i>	1.89 $\pm$ 3.25	0.05 - 12.67	15	11.9 $\pm$ 4.2	4.03 - 20.1	21
<i>D. machalilla</i>	1.97 $\pm$ 3.44	0.08 - 11.85	13	2.28 $\pm$ 0.53	1.07 - 3.55	18
<i>D. bromeliae</i>	2.07 $\pm$ 2.4	0.23 - 8.37	10	0.92 $\pm$ 0.28	0.65 - 1.73	12
<i>D. mojavenensis</i>	1.56 $\pm$ 2.71	0.13 - 5.63	4	2.3 $\pm$ 0.35	1.83 - 2.57	4
<i>D. buzzatii</i>	2.87 $\pm$ 5.26	0.08 - 18.87	15	1.79 $\pm$ 0.65	1.13 - 3.42	17
<i>D. tripunctata</i>	5.17 $\pm$ 5.98	0.68 - 13.82	4	33.34 $\pm$ 9.54	20.47 - 42.15	4
<i>D. willistoni</i>	5.72 $\pm$ 5.39	0.53 - 14.65	5	16.88 $\pm$ 2.58	13.9 - 21.55	6
<i>D. melanogaster</i>	13.48 $\pm$ 8.9	2.55 - 40.23	27	13.83 $\pm$ 4.33	7.57 - 24.55	31

**Table 2: Test for one-sided mating positions.** Fit: GLM (angle ~ species), family = "gaussian", hypothesis: angle = 0, Bonferroni corrected p-values

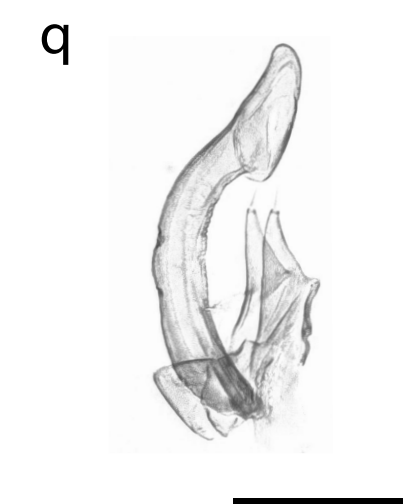
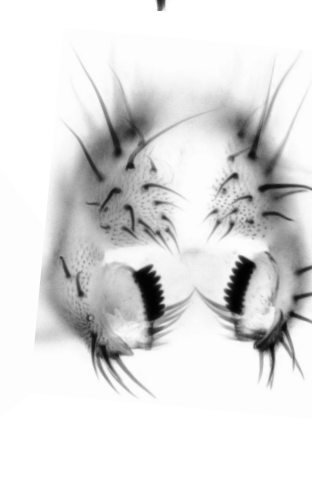
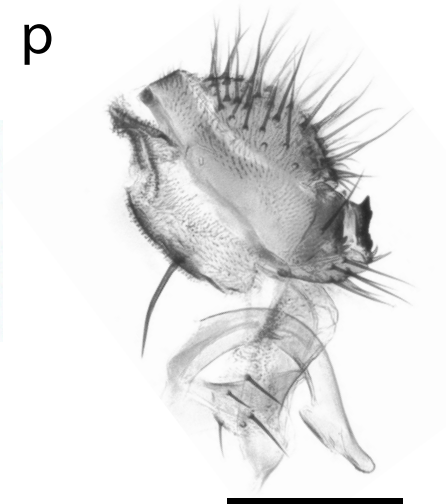
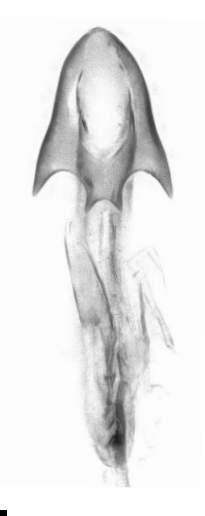
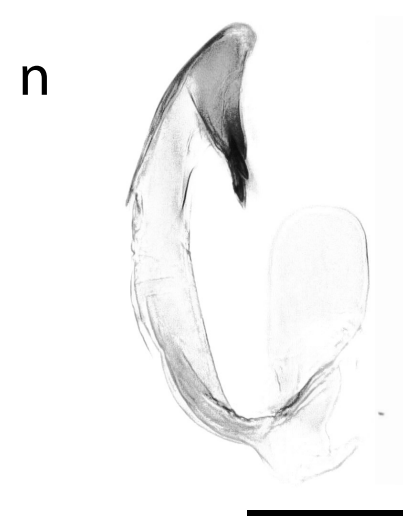
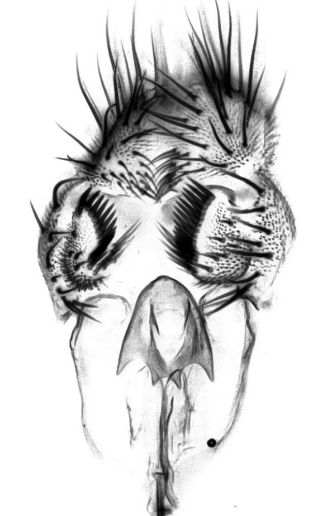
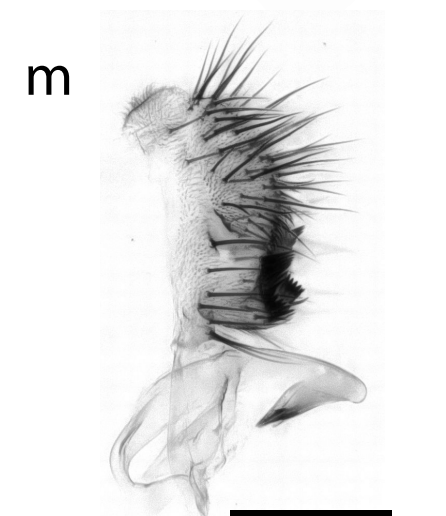
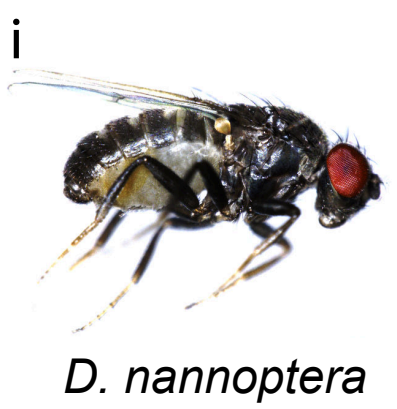
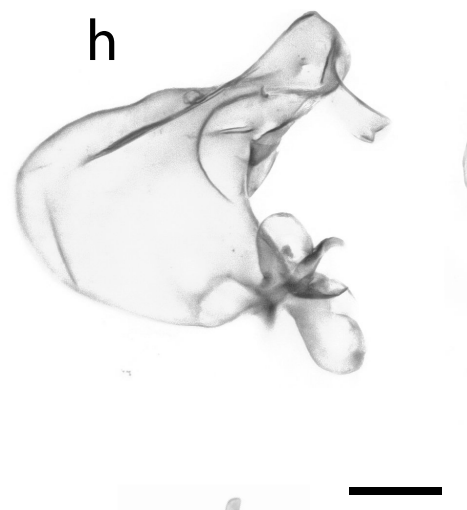
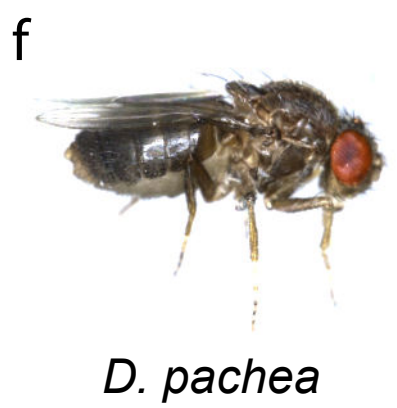
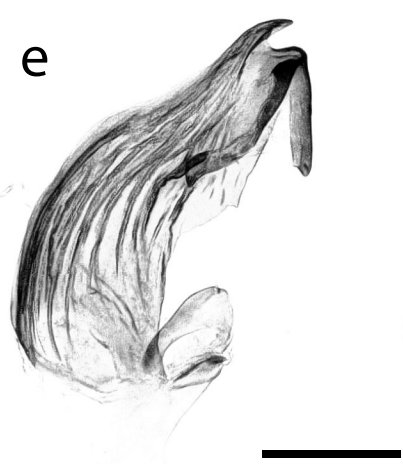
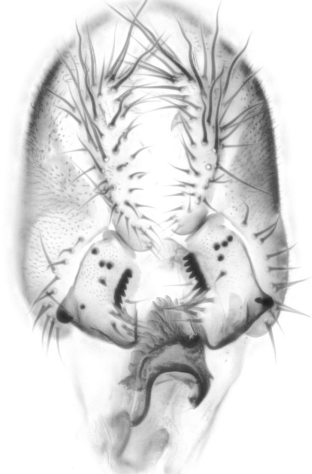
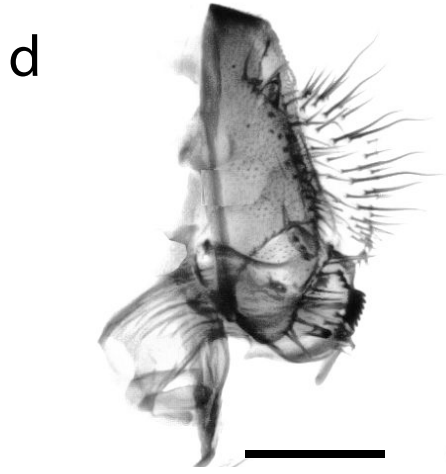
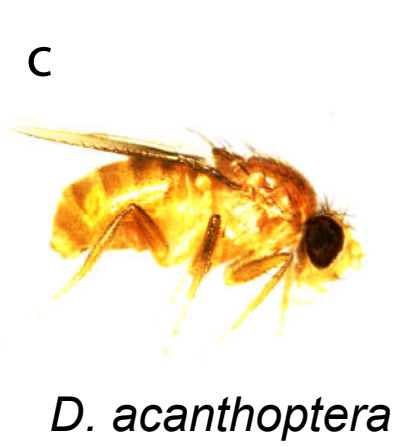
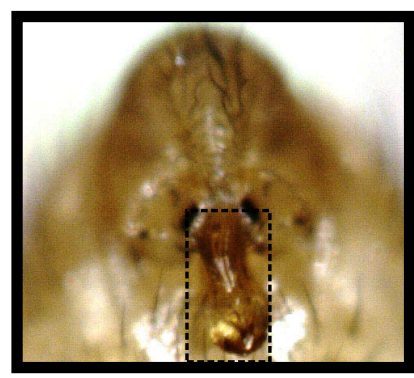
<i>species</i>	settling			10% stable copulation time point replicate measurement 1			10% stable copulation time point replicate measurement 2		
	est. contrast	z value	p	est. contrast	z value	p	est. contrast	z value	p
<i>D. acanthoptera</i>	-1.9105	-0.282	1	0.5919	0.091	1	-0.4980	-0.075	1
<b><i>D. pachea</i></b>	<b>21.4011</b>	<b>4.048</b>	<b>0.000517</b>	<b>18.8120</b>	<b>3.704</b>	<b>0.00212</b>	<b>17.8498</b>	<b>3.449</b>	<b>0.00564</b>
<b><i>D. nanoptera</i></b>	<b>32.5346</b>	<b>6.646</b>	<b>3.00e-10</b>	<b>34.4231</b>	<b>7.321</b>	<b>2.46e-12</b>	<b>35.0479</b>	<b>7.314</b>	<b>2.60e-12</b>
<i>D. machalilla</i>	5.7585	0.851	1	6.5023	1.001	1	8.2219	1.242	1
<i>D. bromeliae</i>	-3.9911	-0.590	1	-2.9702	-0.457	1	-0.7399	-0.112	1
<i>D. mojavensis</i>	3.0034	0.268	1	4.2370	0.393	1	6.3650	0.580	1
<i>D. buzzatii</i>	2.4878	0.430	1	3.0853	0.555	1	1.2320	0.217	1
<i>D. tripunctata</i>	3.6189	0.323	1	-2.9770	-0.239	1	-5.4349	-0.429	1
<i>D. willistoni</i>	0.7288	0.080	1	0.4118	0.047	1	0.6928	0.077	1
<i>D. melanogaster</i>	6.6156	1.414	1	1.6010	0.356	1	0.7997	0.175	1

**Table 3: *D. nanoptera* frontal mating angles.** The mean estimates for each time interval were calculated with average values when multiple measurement points were available for a given experiment

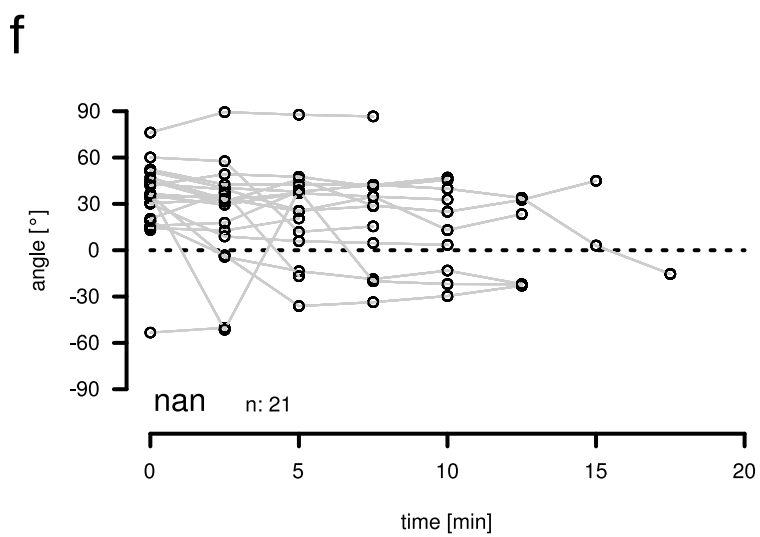
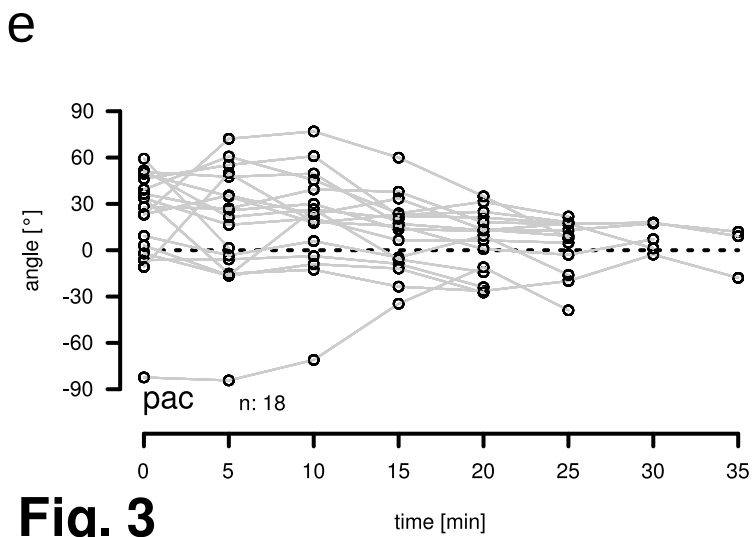
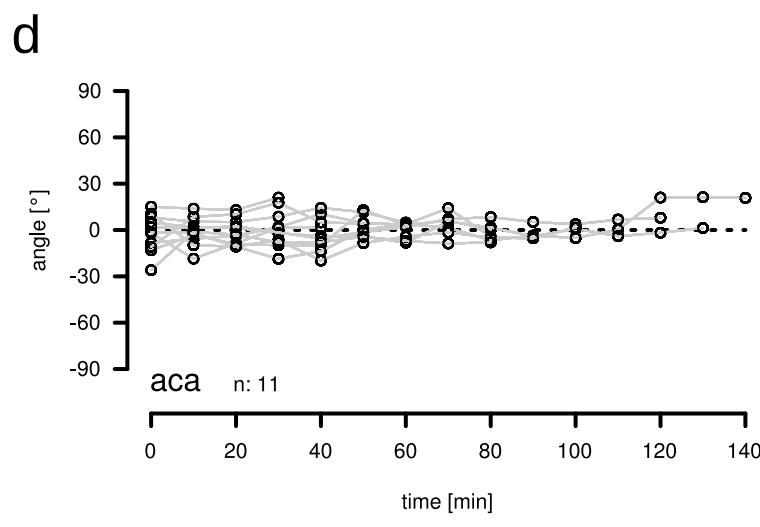
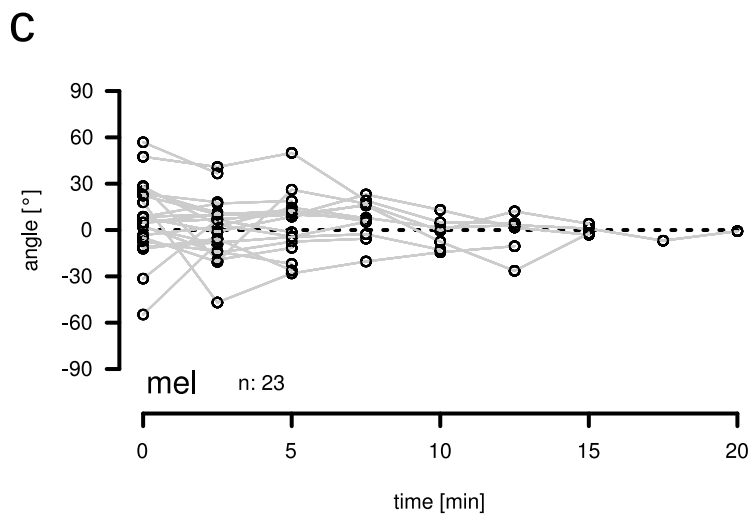
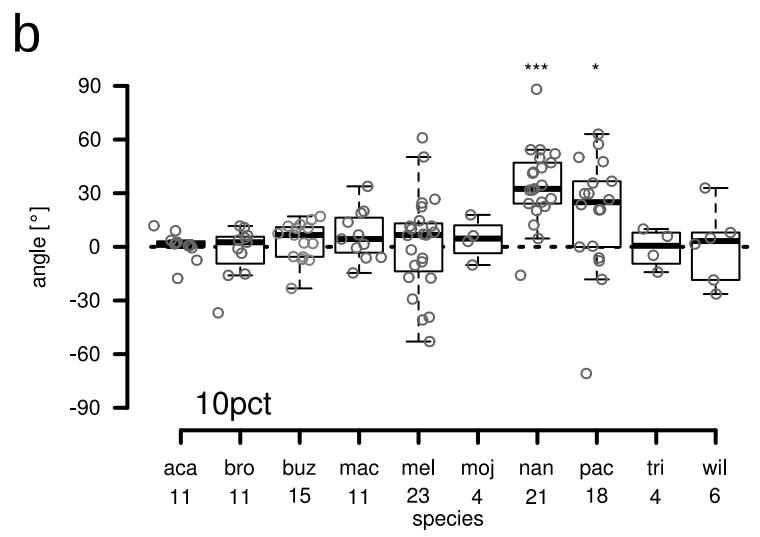
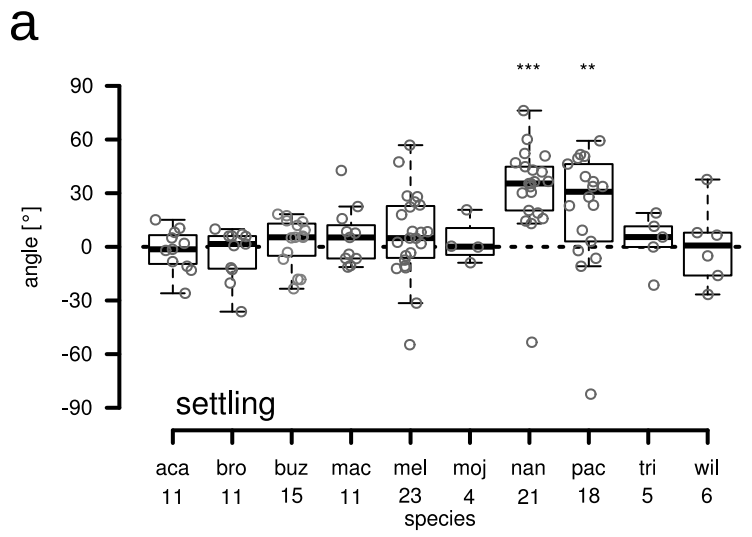
<b>time interval after cop. start [ min]</b>	<b>angle (mean <math>\pm</math> SD)</b>	<b>n</b>
0 - 1	10.36 $\pm$ 6.88	25
1 - 2	15.46 $\pm$ 8.82	29
2 - 3	23.44 $\pm$ 12.15	29
3 - 4	27.16 $\pm$ 10.81	27
4 - 5	29.1 $\pm$ 11.62	25
5 - 6	28.97 $\pm$ 11.92	21
6 - 7	32.48 $\pm$ 8.29	13
7 - 8	26.44 $\pm$ 10.24	5
8 - 9	23.21 $\pm$ 11.88	4
9 - 10	24.7 $\pm$ 13.66	3
10 - 11	18.65 $\pm$ 14.03	2
11 - 12	8.07 $\pm$ NA	1



**Fig. 1**



**Fig. 2**



**Fig. 3**



	<b>Aedagus</b>	<b>External Genitalia</b>	<b>Mating Position</b>	<b>Mating Duration</b>	<b>Sperm Length</b>	<b>Sperm Storage</b>
<i>D. pachea</i>	AS	AS (lobes)	right-sided	30 min	16.6	sp
<i>D. wassermani</i>	nd	AS (anal plates)	nd	nd	4.5	sp
<i>D. acanthoptera</i>	AS	S	S	88 min	5.8	sp
<i>D. nanoptera</i>	S	S	right-tilted	12 min	15.7	rec
<i>D. machalilla</i>	S	S	S	< 3 min	nd	nd
<i>D. bromeliae</i>	S	S	S	< 3 min	nd	sp + rec
<i>D. mojavensis</i>	S	S	S	< 3 min	1.9	rec
<i>D. melanogaster</i>	S	S	S	14 min	1.9	sp + rec

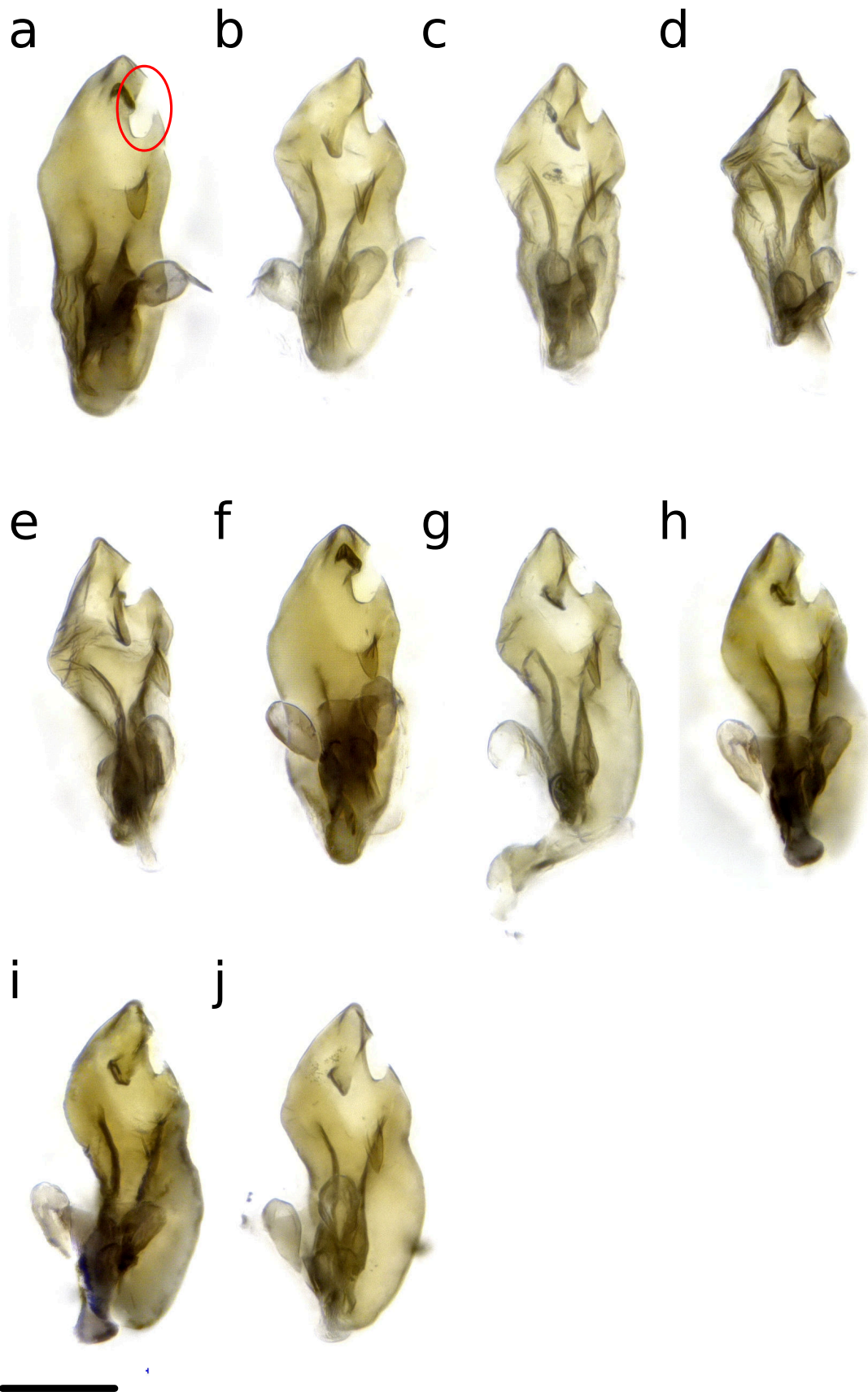
**Fig. 4**

**Table S1: Species Resources**

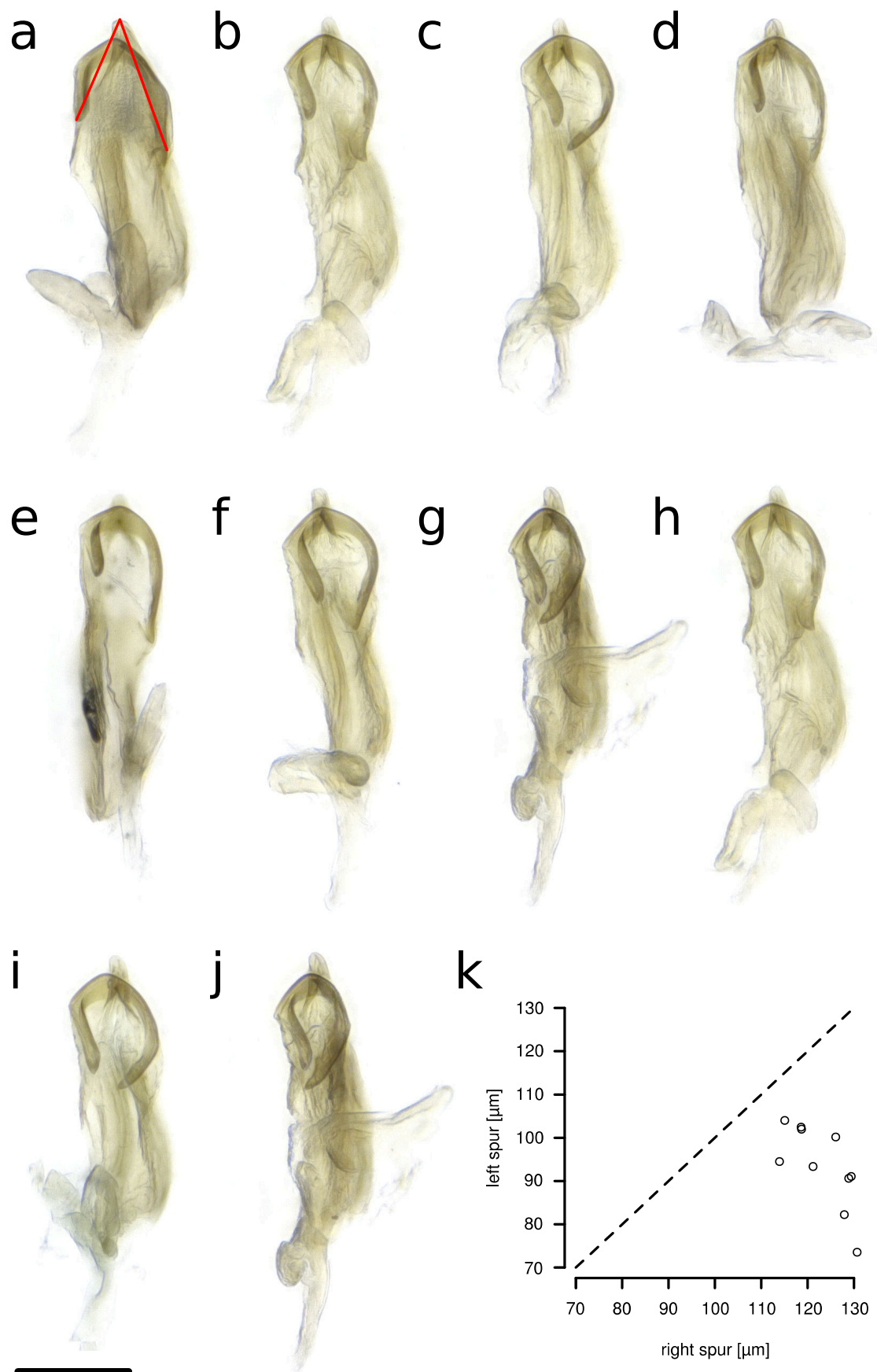
Species	Source	Stock number	collection locality	collection year
<i>D. acanthoptera</i>	Drosophila Species Stock Center	15090-1693.00	Oaxaca, Mexico	1976
<i>D. pachea</i>	Drosophila Species Stock Center	15090-1698.02	Sonora, Mexico	1996
<i>D. nanoptera</i>	Drosophila Species Stock Center	15090-1692.10 15090-1698.12	Oaxaca/Puebla, Mexico	1992
<i>D. machalilla</i>	Andrea Acurio		San Jose, Ecuador	2015
<i>D. bromeliae</i>	Drosophila Species Stock Center	15085-1682.00	Grand Cayman Island, UK	1985
<i>D. buzzatii</i>	Jean David		Bahia, Brazil	2010
<i>D. mojavensis</i>	Drosophila Species Stock Center	15081-1352.22	Catalina Island, USA	2002
<i>D. tripunctata</i>	Drosophila Species Stock Center	15020-2401,02	New Orleans, USA	1950
<i>D. willistoni</i>	Jean David		Rio de Janeiro, Brazil	2010
<i>D. melanogaster</i>	Drosophila Species Stock Center	14021-0231.07	Taiwan	1968

**Table S2: GenBank Accession Numbers of the phylogeny dataset**

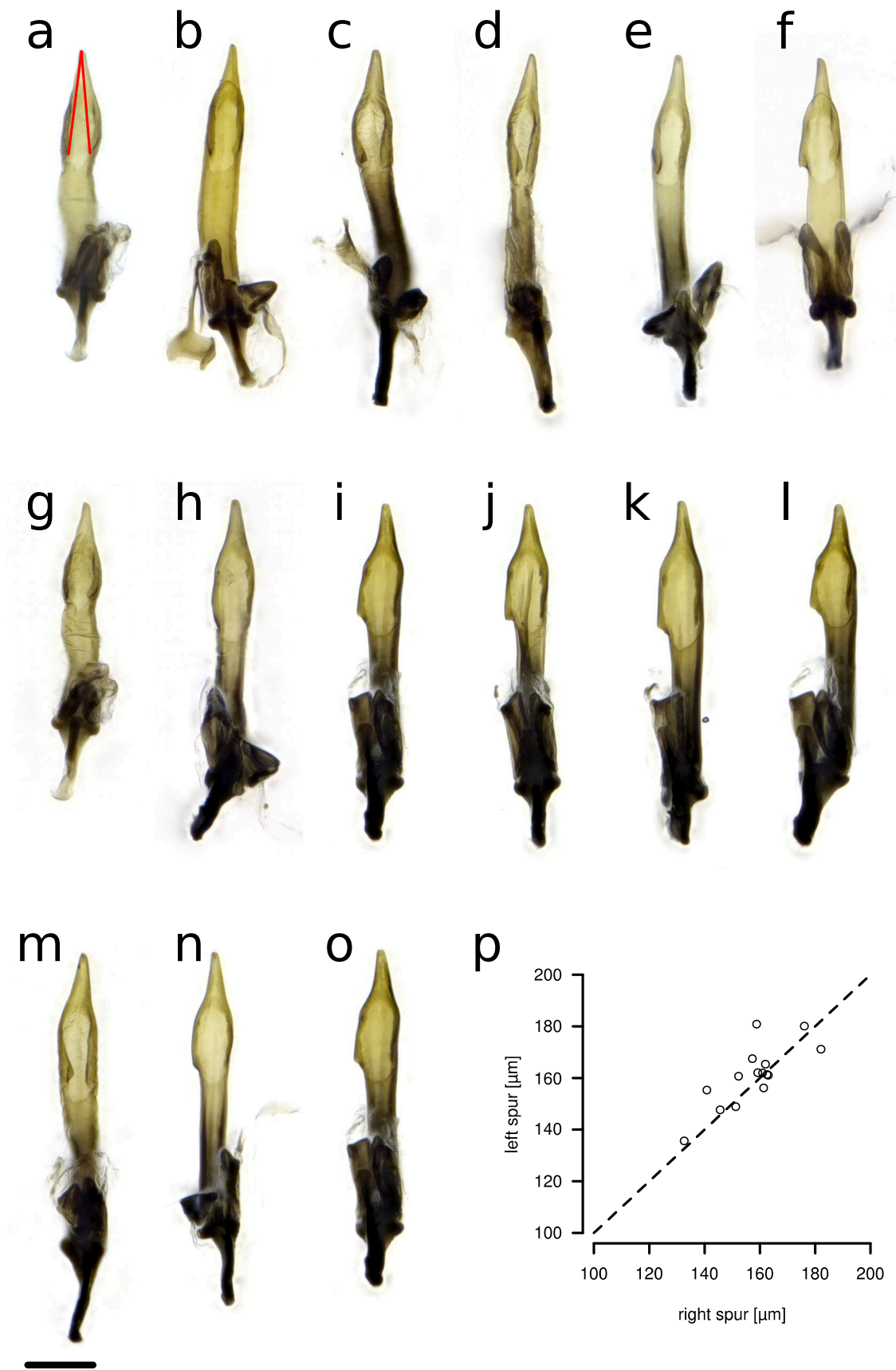
Species	Locus, Accession Number										
	<i>amy</i>	<i>amyrel</i>	<i>boss</i>	<i>fkh</i>	<i>marf</i>	<i>sinA</i>	<i>snf</i>	<i>wee</i>	ND2	COI	COII
<i>D. acanthoptera</i>	KF632687	KF632675	JF736442	KF632652	KF632638	EU341611	JF736382	KF632612	KF632701	KF632601	AF183968
<i>D. pachea</i>	KF632697	KF632683	KF632672	KF632662	KF632648	KF632595	KF632634	KF632622	KF632709	KF632609	KF632600
<i>D. nanoptera</i>	KF632696	KF632682	JF736456	KF632661	KF632647	JF736334	KF632633	KF632621	KF632708	DQ471531	AF183971
<i>D. machalilla</i>	KF632694	KF632680	KF632671	KF632659	KF632645	KF632594	KF632631	KF632619	KF632706	KF632607	KF632599
<i>D. bromeliae</i>	KF632689	AY733049	KF632666	KF632654	KF632640	KF632591	KF632627	KF632614	KF632702	KF632602	AF478418
<i>D. buzzatii</i>	KF632690	KF632677	KF632667	KF632655	KF632641	EU341621	JF736384	KF632615	KF632703	KF632603	DQ202011
<i>D. mojavensis</i>	XM002004425	XM002006561	XM001999692	XM001999791	XM002009489	XM002007289	XM002011475	XM002003093	BK006339	BK006339	BK006339
<i>D. tripunctata</i>	---	---	---	---	---	---	---	---	EU493508	EF570023	EU493748
<i>D. willistoni</i>	CH963849	CH963719	CH964272	CH964232	CH963925	CH963876	CH964239	CH963920	consensus of: NW002031144 NW002033407 NW002033850 NW002036038 NW002038401	JQ679116	EU532097
<i>D. melanogaster</i>	NM079044	NM057914	NM080709	NM079818	AF355475	NM057377	NM078490	NM057687	NC024511	NC024511	NC024511



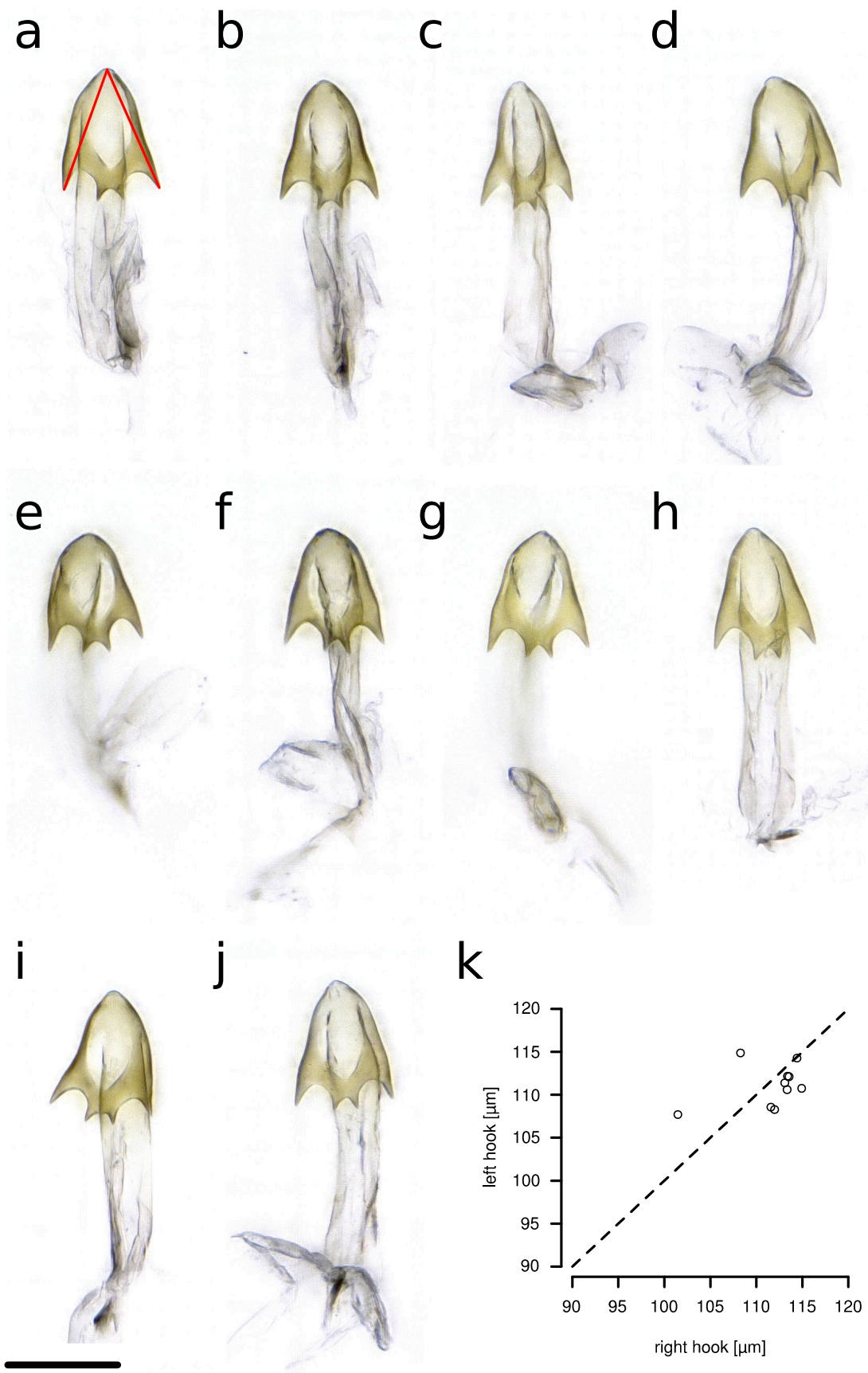
**Figure S1: The aedeagus of *D. pachea* is asymmetric.** Preparations in ventral view (a) The red circle indicates the right-sided position of the gonopore. (a-j) Ten preparations. The scale bar is 100  $\mu$ m.



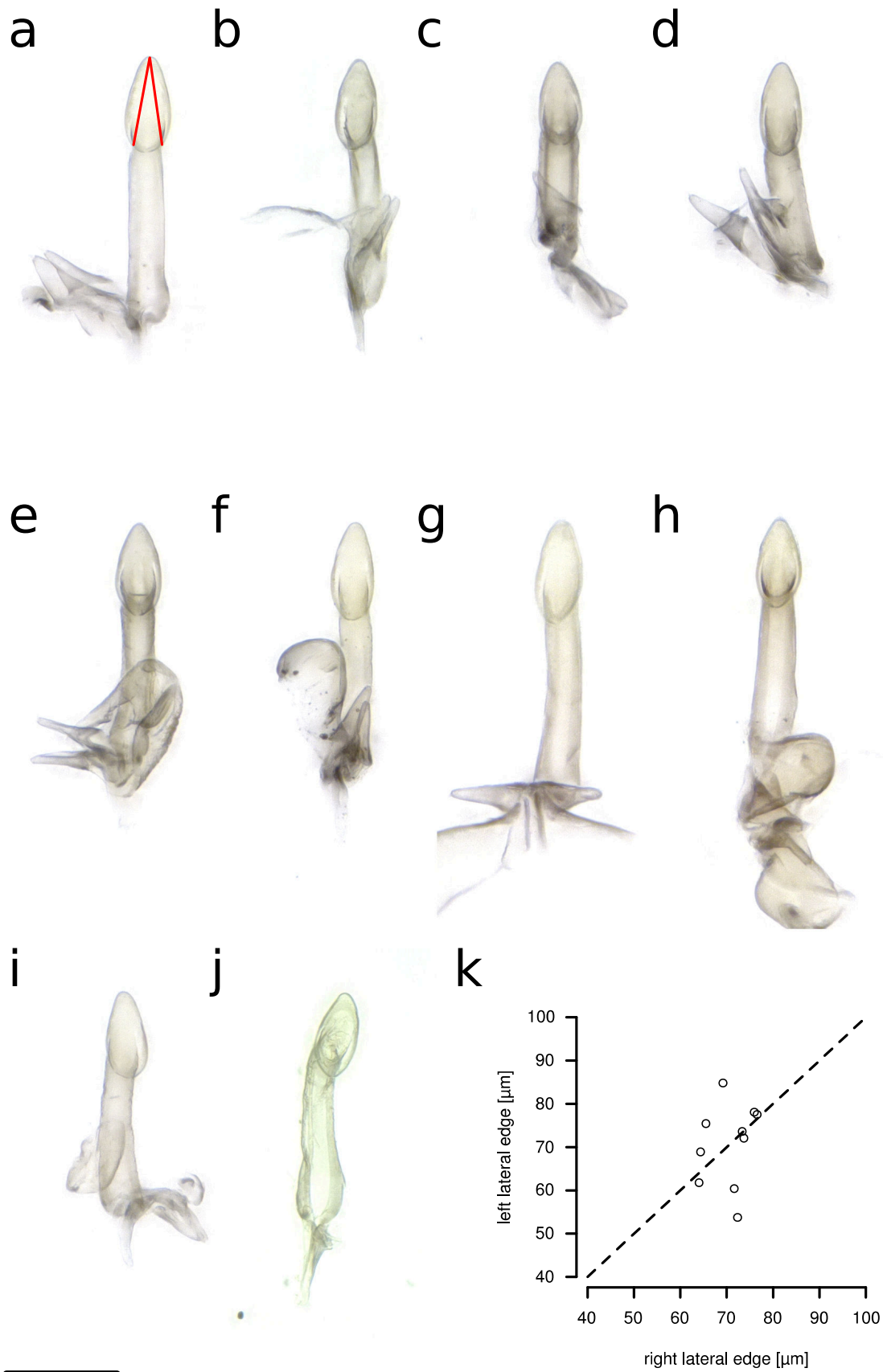
**Figure S2: The aedeagus of *D. acanthoptera* is asymmetric.** Preparations in ventral view. (a) The red lines indicate the length measurements of ventral apex spurs (see materials and methods). (a-j) preparations. (k) Length measurements of apical spurs. The dashed line corresponds to the 1:1 length ratio of left and right spurs. The scale bar is 100  $\mu\text{m}$ .



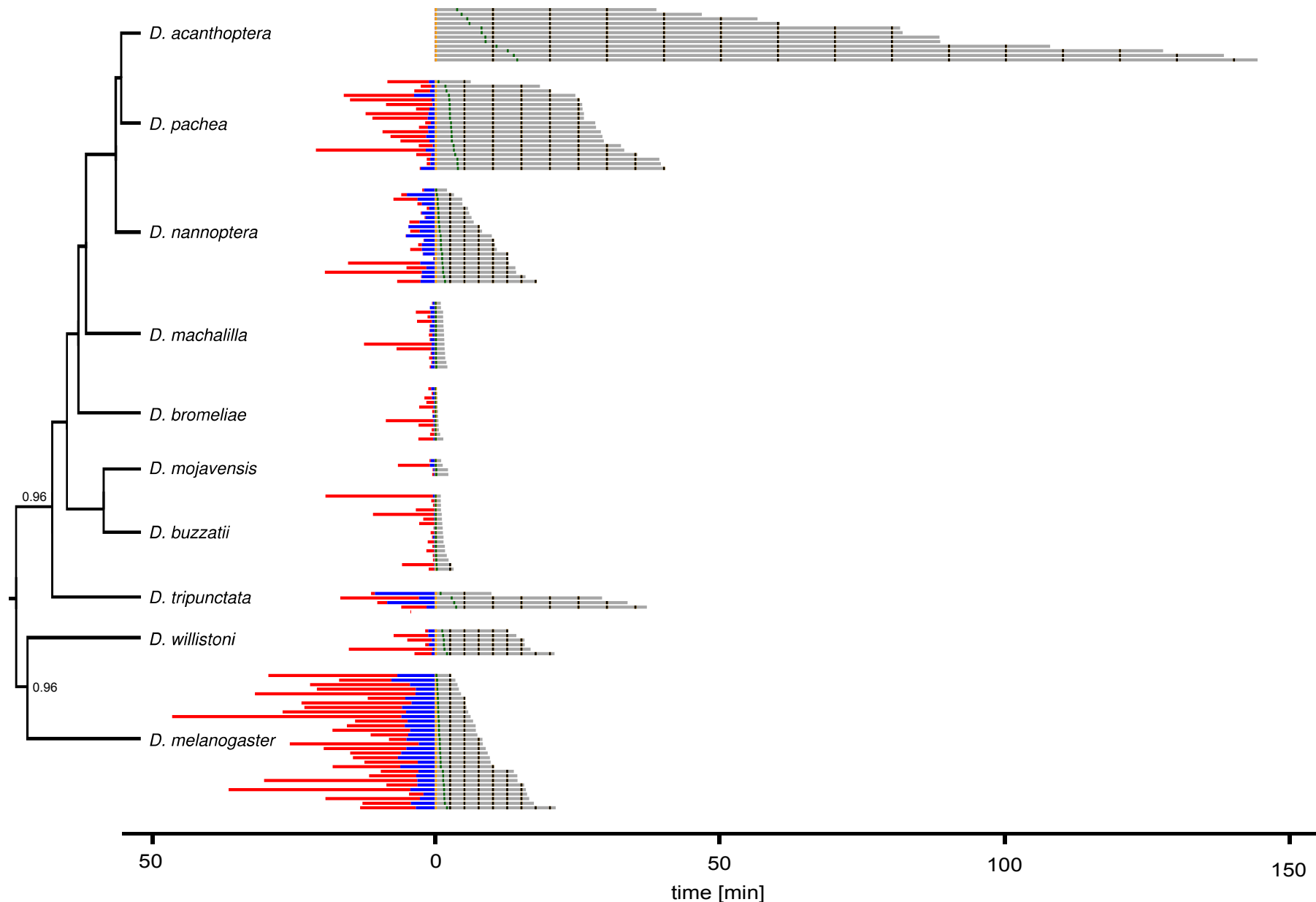
**Figure S3: No asymmetry is detected in the aedeagus of *D. nanoptera*.** Preparations in ventral view (a) The red lines indicate the length measurements of ventral apex spurs (see materials and methods). (a-o) Fifteen preparations. (p) Length measurements of apical spurs. The dashed line corresponds to the 1:1 length ratio of left and right spurs. The scale bar is 100  $\mu\text{m}$ .



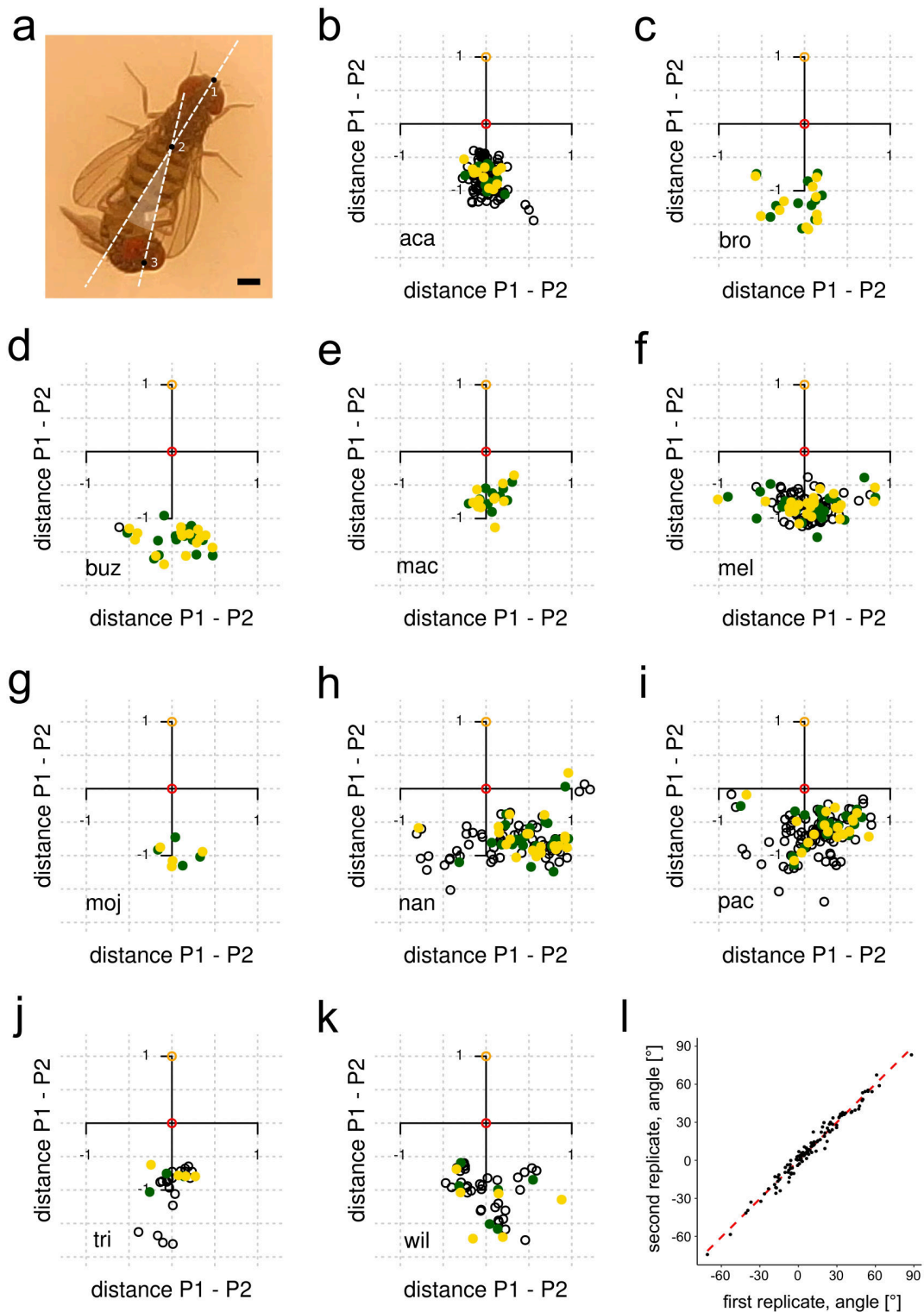
**Figure S4: No asymmetry is detected in the aedeagus of *D. machalilla*.** Preparations in ventral view (a) The red lines indicate the length measurements of ventral apical hooks (see materials and methods). (a-j) Ten preparations. (k) Length measurements of apical hooks. The dashed line corresponds to the 1:1 length ratio of left and right hooks. The scale bar is 100 μm.



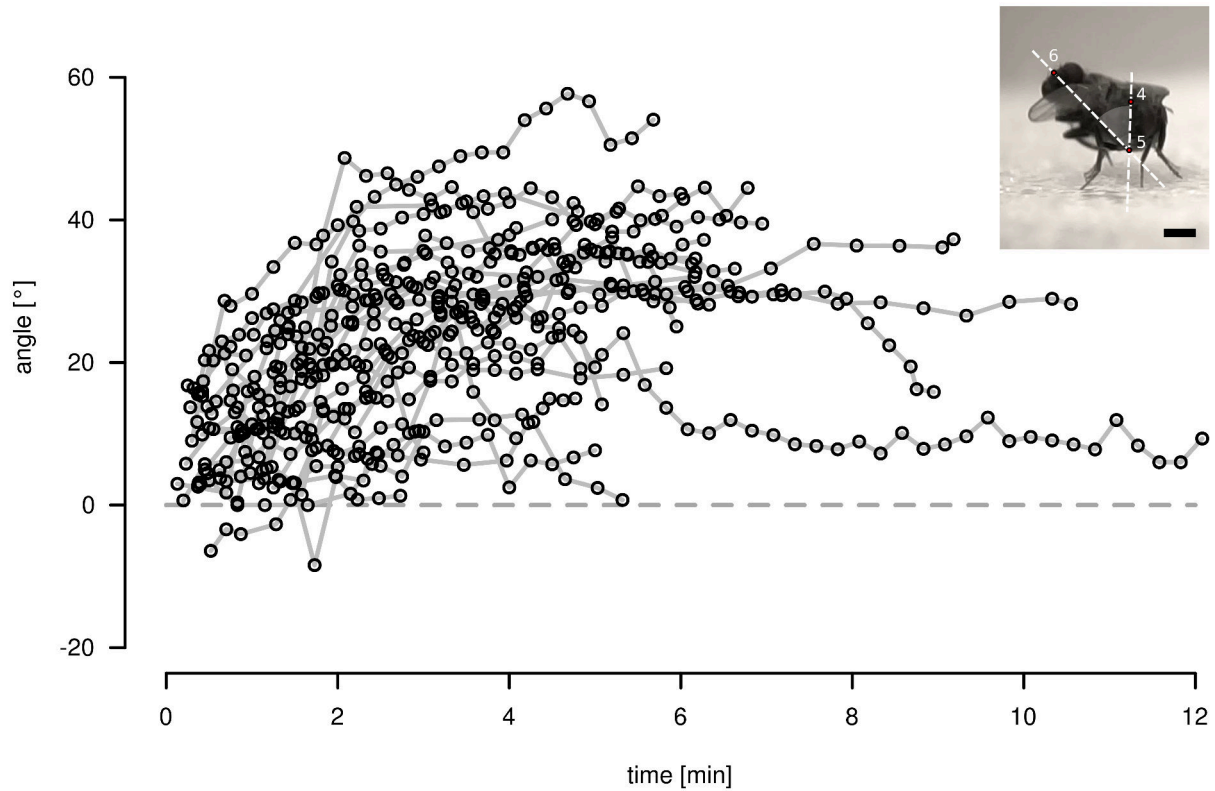
**Figure S5: No asymmetry is detected in the aedeagus of *D. bromeliae*.** Preparations in ventral view (a) The red lines indicate the length measurements of ventral apex ridges (see materials and methods). (a-j) Replicate preparations. (k) Length measurements of apex ridges. The dashed line corresponds to the 1:1 length ratio of left and right ridges. The scale bar is 100 μm.



**Figure S6: Courtship and copulation duration in *D. pachea* and related species.** The phylogenetic relationships of analyzed species are indicated on the left with a bayesian phylogeny based on a multilocus dataset of Lang et al. 2014 [28], and additional data for *D. willistoni* and *D. tripunctata* (see material and methods, Additional file 1: Table S1). Numbers indicate posterior probabilities for node supports < 1. Each line represents an experiment. Courtship is indicated in red, initial copulation with variable positions in blue and copulation after the settling time point in grey. Experiments are aligned by the settling time point. Time points at which the mating angle was calculated are indicated as tick marks: the settling time point in yellow; 10% stable copulation time point in green and measurements at later regular time intervals in black.



**Figure S7: Multi-species mating position measurements.** (a) Mating couple of *D. buzzatii* the scale bar 500  $\mu\text{m}$ . Landmarks P1 (1), P2 (2), and P3 (3) are indicated. The dashed white lines (P1,P2) and (P2,P3) form an acute angle (semi-transparent circle sectors) which was measured to assess copulation posture. (b-k) Position coordinates for angle measurements of *D. pachea* and nine related *Drosophila* species; aca: *D. acanthoptera*, bro: *D. bromeliae*, buz: *D. buzzatii*, mac: *D. machalilla*, mel: *D. melanogaster*, moj: *D. mojavensis*, nan: *D. nanoptera*, pac: *D. pachea*, tri: *D. tripunctata*, wil: *D. willistoni*. Points P1 (orange circle) are placed at coordinates (0,1) and P2 (red circle) at coordinates (0,0). P3 points are shown for the settling time-point in yellow dots, for the 10% stable copulation time point in green dots and for later time points in black circles. (l) Correlation of angle values calculated from two replicate measurements ( $n=124$ ) at the 10% copulation time point (Pearson correlation coefficient = 0.988,  $df = 121$ ,  $t = 69.231$ ,  $p < 10e^{-16}$ ). The red dashed line indicates the linear regression line.



**Figure S8: *D. nanoptera* tilts to the right side of the female abdomen.** Frontal copulation angles (black circles) are plotted over the course of copulation. Positive and negative values indicate left-sided and right-sided angles, respectively. Grey lines connect points obtained from the same copulation couple over time. The dashed line indicates an angle of zero degrees. The position analysis from a frontal perspective is indicated on the image of the copulating couple on the right. Points and numbers indicate position landmarks P4 (4), P5 (5), and P6 (6). The dashed white lines (P1,P2) and (P2,P3) form an acute angle (semi-transparent circle sector) which corresponds to the frontal copulation angle. The scale bar is 500  $\mu\text{m}$ .