Convergent evolution of sex-specific leg ornaments in Drosophilidae – from cells to structures.

K. Tanaka [1*], O. Barmina [1], J. H. Massey [2,3], and A. Kopp [1]

[1] Department of Evolution and Ecology, University of California – Davis, Davis CA USA
[2] Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor MI USA
[3] Janelia Research Campus, Howard Hughes Medical Institute, Ashburn VA USA
* Corresponding author: kohtanaka@ucdavis.edu

Abstract

Sexually dimorphic morphological traits are among the fastest evolving animal features. Similar sex-specific structures have sometimes evolved independently in multiple lineages, presumably as targets of parallel sexual selection. In such cases, comparing the cellular mechanisms that generate these structures in different species can elucidate the interplay between selection and developmental constraint in evolution. In Drosophilidae, male-specific tarsal brushes on the front legs are found in at least four separate lineages. In this study, we combine phylogenetic reconstruction with developmental analyses and behavioral observations to investigate the evolutionary origin of these structures. We show that the sex brush has evolved independently at least three times from sexually monomorphic ancestral morphology. However, all sex brushes have very similar fine structure and develop through indistinguishable cellular processes, providing a striking example of developmental convergence. In all examined species, males use their sex brushes to grasp the female abdomen prior to copulation. We discuss potential reasons why convergent evolution of novel structures is rare even in the face of similar functional demands.

Keywords: *Drosophila* phylogeny; sexual dimorphism; convergent evolution; mating behavior; bristle development

Introduction

Most animals are sexually dimorphic. Perhaps the most fascinating feature of sexual dimorphism is the rapid evolutionary turnover of sex-specific traits. Even among close relatives, the characters that distinguish males from females vary greatly from species to species. This simple observation implies that new sexual characters are gained, and ancestral ones are often lost, during the evolution of many if not most animal lineages. Understanding the genetic and developmental basis of this turnover is necessary to shed light on one of the most important drivers of biological diversity. Examples where similar traits have evolved multiple times are of particular value, as they often provide insights into the general patterns and mechanisms of evolution (Kopp 2009).

Most higher Diptera mate with the male on top of the female, and the male front (T1) legs are often involved in grasping or stimulating the female (Huber et al., 2007; McAlpine, 1981). Perhaps for this reason, male-specific ornaments or grasping structures are found on the T1 legs of many dipteran species (Daugeron et al., 2011; Eberhard, 2001; Ingram et al., 2008; Sivinski, 1997). In Drosophilidae, the most obvious male-specific leg modifications include the sex combs found in the *Drosophila melanogaster* and *obscura* species groups and in the genus *Lordiphosa* (Katoh et al., 2018; Kopp, 2011); branched or spoon-shaped tarsi of some Hawaiian *Drosophila* species (Hardy, 1965; Stark and O'Grady, 2009); and tarsal brushes that are the focus of this study (Figure 1). Sex brushes are found in at least four separate groups within the Drosophilidae: the *Drosophila immigrans* species group, the *loiciana* species complex, *D. repletoides*, and the genus *Zaprionus.* Due to uncertain relationships among these lineages, the evolutionary origin(s) of this structure remain unclear.

In the *immigrans* group, male sex brushes are found in some but not all of the species; the most likely scenario is that the brush was present in the last common ancestor of this clade, but was secondarily lost in the *nasuta* subgroup and greatly reduced in several other species (Rice et al., 2018). In *Zaprionus*, male brushes are present in most African species, with the exception of *Z. neglectus*, *Z. spineus*, and *Z. spinosus* (Tsacas and Chassagnard, 1990; Yassin et al., 2008; Yassin and David, 2010). The *Zaprionus* phylogeny is not fully resolved, but the distant relationship between the first species and the last two

suggests that their lack of brushes is likely to reflect independent secondary losses. The situation is more complicated among species assigned to the Oriental *Anaprionus* subgenus of *Zaprionus*. Many of its members, including *Z. lineosus, Z. spinilineosus, Z. orissaensis, Z. multistriatus, Z. grandis*, and *Z. aungsani*, lack leg brushes (Gupta, 1972; Kikkawa and Peng, 1938; Okada and Carson, 1983; Wynn and Toda, 1988). However, *Anaprionus* is now thought to be polyphyletic (Yassin, 2007; Yassin et al., 2010), and these species appear to be more closely related to the genus *Xenophorticella* than to *Zaprionus* sensu stricto (M. Toda, pers. comm.). Other *Anaprionus* species such as *Z. bogoriensis, Z. obscuricornis,* and *Z. pyinoolwinensis* have leg brushes (Mainx, 1958; Okada, 1964; Wynn and Toda, 1988) and likely form a clade with the Afrotropical *Zaprionus* (M. Toda, pers. comm.). Thus, the leg brush has evolved either at or near the base of *Zaprionus*.

D. pruinosa belongs to the *loiciana* species complex, which also includes *D. loiciana*, *D. allochroa*, *D. pachneissa*, *D. semipruinosa*, and *D. xanthochroa*. All of these species have male leg brushes of different sizes (Tsacas, 2002; Tsacas and Chassagnard, 2000). The fourth lineage where a male leg brush is found consists of a single species, *D. repletoides,* which does not have any known close relatives; a species described originally as *D. tumiditarsus* (Tan et al., 1949) was later synonymized with *D. repletoides* (Hsu, 1943; Wheeler, 1981). Yassin (Yassin, 2007) suggested that some species currently classified as *Zaprionus* (*Z. multistriatus*, *Z. flavofasciatus*, and *Z. cercociliaris*) could in fact be more closely related to *D. repletoides* than to *Zaprionus*; unfortunately, these species have not been included in any molecular phylogenies.

The four clades of interest – *Zaprionus*, the *immigrans* species group, *D. repletoides*, and the *loiciana* complex – have never been included together in the same molecular phylogeny. Different combinations of these taxa have been examined in several phylogenetic studies, which were based on a small number of loci and produced different results. Da Lage et al (Da Lage et al., 2007) and Yassin et al (Yassin et al., 2010) provided some evidence for a distant relationship among *Zaprionus*, *D. repletoides*, and the *immigrans* species group. Russo et al (Russo et al., 2013) placed *D. pruinosa* as sister to *D. sternopleuralis* (a member of the *histrio* species group that lacks a sex brush), and the resulting clade as sister to the *immigrans* species group. The phylogenies of Da Lage et al (Da Lage et al., 2007) and Izumitani et al (Izumitani et al., 2016) did not include *D. pruinosa*,

106 but did not support a sister-group relationship between *D. sternopleuralis* and the
107 *immigrans* species group.
108 In this study, we used a larger multilocus dataset to test whether the male leg brush
109 evolved independently in each of these four clades, or whether its distribution could be
110 better explained by shared origin in some of these lineages. To facilitate this analysis, we
111 included one or more representatives of each clade, as well as several brush-less species
112 that have been suggested by previous studies to be closely related to the brush-bearing
113 clades. In parallel, we compared the cellular mechanisms that produce the male leg
114 brushes in different species, as well as the role of these ornaments in mating behavior. Our
115 results strongly suggest that the sex brushes evolved convergently in several distantly
116 related lineages, but develop through virtually identical mechanisms.
117
118
119 **Materials and Methods**
120
121 *Leg imaging*
122
123 For brightfield imaging, male front legs were dissected, mounted in Hoyers media between
124 two coverslips, and photographed on a Leica DM500B microscope with a Leica DC500
125 camera. For electron microscopy, adult legs were dehydrated in 100% ethanol, critical
126 point dried, and coated with gold. Scanning electron micrographs were taken on Thermo
127 Fisher Quattro S and Philips XL30 TMP.
128
129 *Sequence data collection*
130
131 The sources of live *Drosophila* strains, fixed specimens, and unpublished genome
132 assemblies used in this study are listed in Table S1. DNA was extracted from live or
133 alcohol-fixed flies using an affinity resin based protocol (Hi Yield® Genomic DNA Mini Kit,
134 Süd-Laborbedarf Gauting, Germany). PCR was carried out using DreamTaq polymerase
135 (Thermo Fisher) and the following cycling conditions: 95° 5' => (95° 30" => 55° 30" => 72°
136 80")x35 => 72° 5' =>12°; the loci and primer sequences are listed in Table S2. In some

137 cases, two rounds of PCR with nested primers were needed to obtain amplicons from fixed
138 specimens. Amplified fragments were gel-purified and sequenced from both ends using
139 amplification primers. Sequence chromatograms were trimmed in SnapGene Viewer, and
140 the two end reads were aligned and edited in Geneious. Heterozygous nucleotide positions,
141 if present, were represented by IUPAC ambiguity codes. All new sequences were deposited
142 in Genbank under accession number listed in Table S1. Additional sequences were
143 obtained from Genbank or extracted from whole-genome assemblies using Blast v2.2.23
144 (Table S1).
145
146 *Sequence analysis*
147
148 The sequences of each locus were aligned using the MUSCLE algorithm (Edgar, 2004)
149 implemented in Geneious (Kearse et al., 2012). The alignments were trimmed at the ends,
150 and poorly aligning intronic regions were removed. The alignments of all eight loci were
151 then concatenated for combined analysis. Combined Bayesian analysis was carried out in
152 MrBayes v3.2.6 (Ronquist et al., 2012). Two sets of analyses were conducted. In the first,
153 the dataset was partitioned by gene, and each locus was allowed to follow a different
154 nucleotide substitution model with empirically estimated parameters. In the second, we
155 partitioned the dataset by gene and by codon position, and used PartitionFinder with the
156 PhyML algorithm (Guindon et al., 2010; Lanfear et al., 2012; Lanfear et al., 2017) to identify
157 the appropriate partitioning scheme; this resulted in a total of 21 character subsets, each of
158 which was subsequently allowed to follow its own substitution model with empirically
159 estimated parameters. For each analysis, two parallel runs of 1,500,000 generations, each
160 starting from a different random tree, were carried out, and convergence was confirmed by
161 comparing tree likelihoods and model parameters between the two runs. *D. melanogaster*
162 was used as outgroup. Trees were sampled every 1000 generations and summarized after
163 a 20% relative burn-in. Each analysis was also repeated after excluding *D. quadrilineata*
164 (see Results). Samples of probable trees were extracted from the .tprobs file, and a strict
165 consensus of most probable trees with combined posterior probabilities of 95% or 99%
166 was constructed from these sets of trees in Geneious. Consensus trees were then formatted
167 using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

168

169 *Immunocytochemistry and microscopy*

170

171 Fly cultures were raised on standard *Drosophila* media at room temperature. Since each
172 species develops at a different rate, the timing of pupal stages was determined empirically
173 based on the morphology of transverse bristle rows (TBRs). Each species was imaged at an
174 early stage when TBR bristles of the tibia and the first tarsal segment are separated by one
175 or more intervening epithelial cells; this stage corresponds to 16 - 21 hours after
176 pupariation (AP) in *D. melanogaster*. Each species was also imaged at a late stage, after the
177 packing of TBRs is completed and bristle shaft differentiation is underway (corresponding
178 to 36+ hrs AP in *D. melanogaster*). Pupal legs were dissected, processed and
179 immunostained as in Tanaka et al (2009). The primary antibodies used were rat anti-E-
180 cadherin (DCAD2, from the Developmental Studies Hybridoma Bank, at 1:20) for *D.*
181 *pruinosa* and *Z. tuberculatus,* and mouse anti-Armadillo (N2 7A1, DSHB; 1:30) for *D.*
182 *immigrans* and *D. repletoides*. AlexaFluor 488 secondary antibodies (Invitrogen) were used
183 at 1:400. Fluorescent images were taken on an Olympus 1000 confocal microscope and
184 processed using Image J and Adobe Photoshop.

185

186 *Analysis of mating behavior*

187

188 High-speed videos of mating behavior were recorded using a Fascam Photron SA4 mounted
189 with a 105 mm AF Micro Nikkor lens. In brief, individual virgin males were isolated upon
190 eclosion in food vials and aged for up to two weeks. Virgin females were isolated upon
191 eclosion and housed in groups of 20-30. Pairs of males and females were then gently
192 aspirated into single wells of a 96 well culture plate (Corning 05-539-200) filled halfway
193 with a hardened 2% agarose solution and sealed using a glass microscope slide and tape.
194 Video clips were captured at 1000 frames per second (fps) using Photron Fastcam Viewer
195 software.

196

197

198

Results

Sex brush morphology is highly similar in distantly related species

In all examined species of *Zaprionus*, the *immigrans* species group, *loiciana* species complex, and *D. repletoides*, the male-specific leg brush is found on the anterior-ventral surface of the first (ta1) and sometimes also the second (ta2) tarsal segment of the prothoracic leg. In females and in other species, this area is occupied by transverse bristle rows (TBRs), which the flies use to clean their head and eyes (Tokunaga 1962; Kopp 2011). The male brush replaces the distal TBRs, with a few TBRs remaining at the proximal end of ta1 (Figure 1). In the *immigrans* group (Rice et al. 2018) and in the other species (Figure 1), the brush shows several major differences from the TBRs. The bristles of each TBR are aligned into a straight, tightly packed row that is nearly perpendicular to the proximo-distal (PD) leg axis, while the consecutive TBRs along the PD axis are separated by many cell diameters. In contrast, the modified bristles ("hairs") that make up the brush are packed closely together in all directions and do not show any regular arrangement. The shafts of the TBR bristles are robust and straight, with ridges and grooves running their length and a triangular bract at the base; the brush hairs are thin and wavy with a smooth surface, and lack bracts (Figure 1).

Although the morphology of the male leg brush is similar across species, detailed scanning electron microscopy (SEM) analysis reveals a small but consistent difference. In *D. immigrans* and *D. pruinosa,* the tips of brush hairs are thin and flat, taper to a point, and form hooks that curve toward the base of the leg (Figure 1B, D). In *D. repletoides* and in *Zaprionus*, the hair tips are noticeably thicker and curve away from the leg base (Figure 1 F, H, J, L); in *Zaprionus*, they are also flattened into paddle-like shapes (Figure 1 H, J, L). Thus, while the spatial arrangement of bristles appears to be similar in all species, some differences exist in the morphology of bristle shafts. These differences may reflect the phylogenetic relationships among these taxa, especially the close relationship between *D. pruinosa* and the *immigrans* species group (see next section).

230 *Male leg brushes evolved independently at least three times*

231

232 We sequenced partial coding sequences of eight nuclear, protein-coding loci: *acon,*
233 *eno, glyp, Amyrel, Ddc, Gpdh, Pepck*, and *Pgm*. Separate analyses of each locus produced
234 very poorly resolved trees. We therefore combined the data from all loci (up to 9060
235 nucleotides per species) for a partitioned Bayesian analysis where each locus was allowed
236 to follow its own, empirically estimated substitution model but all loci were constrained to
237 the same tree topology. The resulting tree (Figure 2A; brush-bearing clades labeled in
238 blue) suggests a close relationship of *D. pruinosa* to the *immigrans* species group, with the
239 (*D. sternopleuralis* + *D. trisetosa*) clade, which belongs to the *histrio* species group, as the
240 next outgroup. In contrast, *D. repletoides* is placed as sister group to the (*D. busckii* + *D.*
241 *brachytarsa*) clade, well away from the *immigrans-pruinosa* lineage. Finally, the *Zaprionus*
242 genus is distantly related to both *D. repletoides* and the *immigrans-pruinosa* lineage, and is
243 placed near the base of the tree, separately from the *Drosophila* and *Sophophora* subgenera.
244 We also note that *D. curviceps* and *D. annulipes* appear as sister groups with 100% support,
245 while there is no support for clustering the *immigrans* species group either with the (*D.*
246 *curviceps* + *D. annulipes*) clade or with *D. quadrilineata* (see Supplementary text). A strict
247 consensus of 11 trees with the cumulative posterior probability of 95% is not resolved near
248 the base, but does not support a close relationship among the brush-bearing lineages: *D.*
249 *repletoides*, the *immigrans-pruinosa* clade, and *Zaprionus* (Figure S1).

250 We then examined 27 most probable trees, with the cumulative posterior
251 probability of 99%, to determine the probability of each bipartition of interest to our study.
252 The *Zaprionus* genus (occasionally together with *D. quadrilineata*; see below) was placed at
253 the base of the tree, well separated from *D. repletoides* and the *immigrans-pruinosa* lineage
254 (Figure 2A, Table S3). *D. pruinosa* clustered with the *immigrans* species group, to the
255 exclusion of the (*D. sternopleuralis* + *D. trisetosa*) clade, with ~87% probability; the
256 alternative grouping, of *D. pruinosa* with the (*D. sternopleuralis* + *D. trisetosa*) clade to the
257 exclusion of the *immigrans* species group, was observed with ~13% probability. *D.*
258 *repletoides* was grouped with the (*D. busckii* + *D. brachytarsa*) clade with 95% probability,
259 and this group was separated from the *immigrans-pruinosa* lineage by multiple internal
260 branches. In contrast, potential groupings of brush-bearing lineages – for example, of

261 *Zaprionus* with either *D. repletoides* or the *immigrans-pruinosa* clade, or of the *repletoides-*
262 *busckii-brachytarsa* clade with the *immigrans-pruinosa* clade – were never observed among
263 this set of probable trees.

264 We noticed that among these 27 probable trees, the position of *D. quadrilineata* was
265 by far the most unstable. We therefore repeated the analysis after excluding this species.
266 The resulting tree (Figure 2B, Table S3) shows the same relationships as the full analysis
267 (Figure 2A), but with stronger support for most basal nodes. This tree, as well as the strict
268 consensus of the 11 most probable trees with the cumulative posterior probability of 99%
269 (Figure S2) places *Zaprionus* near the base of the tree, separately from the *Drosophila* and
270 *Sophophora* subgenera; *D. pruinosa* together with the *immigrans* species group and the (*D.*
271 *sternopleuralis* + *D. trisetosa*) clade; and *D. repletoides* in a clade that is clearly separated
272 both from *Zaprionus* and from the *immigrans-pruinosa* lineage. The probability of sister-
273 group relationship between *D. pruinosa* and the *immigrans* species groups is estimated at
274 93.8% ± 1.32% across these trees.

275 Finally, we carried out an analysis with a more complicated partitioning scheme,
276 where each locus and each codon position was allowed to follow its own substitution
277 model; this analysis was also performed with and without *D. quadrilineata*. In both cases, it
278 produced a tree with the same topology as in the simpler partitioning scheme, but with
279 slightly different levels of node support (Figure S3, S4 and Table S3). In summary, we find
280 substantial though not overwhelming support for a close relationship between *D. pruinosa,*
281 and by implication the *loiciana* species complex, and the *immigrans* species group.
282 However, based on our data, the probability of a close relationship among the different
283 brush-bearing clades – *D. repletoides*, *Zaprionus*, and the *immigrans-pruinosa* lineage – is
284 very low for each of the possible pairwise relationships.

285

286 *The same cellular processes underlie sex brush development in all species*

287

288 In *D. melanogaster,* TBR bristle precursors are specified between 6 and 12 hours
289 after pupariation (AP) (Joshi and Orenic 2006, Schroff 2007). Initially, these cells are
290 specified in sparse, loosely organized rows, and separated from one another by several
291 epithelial cells. By 20-21 hrs AP, the bristle cells that are destined to make each TBR

292 migrate toward each other to form a straight, contiguous row, while the intervening
293 epithelial cells are expelled distally and proximally from the TBRs (Atallah et al. 2009;
294 Tanaka et al. 2009). This mechanism suggests two potential explanations for the tight
295 packing of brush hairs. One possibility is that the hair progenitor cells are specified with
296 minimal spacing; in this case, the initial spacing between hair cells is expected to be much
297 denser than the spacing of TBR progenitors. Alternatively, hair progenitors may first be
298 specified similarly to TBR bristles, with wide separation by epithelial cells, but expel the
299 epithelial cells (either laterally or basally) at later stages to form a densely packed brush.
300 In principle, independently evolved brushes in different species could utilize different
301 cellular mechanisms to produce adult structures that are essentially indistinguishable.
302 In order to characterize and compare brush development in different species, we
303 used antibodies against membrane-localized proteins to visualize cell arrangement in
304 pupal legs. When labeled with antibodies against the beta-catenin Armadillo (Arm) or the
305 E-cadherin Shotgun (DE-cad), bristle cells can be distinguished from the surrounding
306 epithelial cells by their unique membrane shape (Figure 3). We examined brush
307 development at two timepoints: an early stage roughly corresponding to ~16-21 hr AP in *D.*
308 *melanogaster*, when the bristle cells of the future TBRs begin to migrate toward each other
309 and expel the intervening epithelial cells, and a later stage when cell migration is
310 completed. We found that at the early stage, most bristle cells in the developing brush are
311 each surrounded by four to six epithelial cells in all species. In effect, the bristle cells are
312 separated from one another by one to two epithelial cells (Figure 3). At the late stage, this
313 spacing remains virtually invariant, although the cells appear more organized compared to
314 the early stage (Figure 3). We did not see evidence of cell migration in any of the examined
315 species. These observations indicate that despite the packed appearance similar to TBRs,
316 the brush cells are not directly adjacent to each other, are specified at high density, and
317 undergo minimal if any migration during development. Importantly, the cellular
318 mechanism of brush development is very similar in all species.
319
320
321
322

323 *Leg brushes are used in male mating behavior*

324

325 High-speed video recordings show that the proximal tarsal segments of the male T1 legs,
326 including the sex brushes, are used to grab the female abdomen and resist the female's
327 efforts to dislodge the male during copulation attempts in *Z. tuberculatus*, *D. immigrans*,
328 and *D. repletoides* (Supplementary movies 1-3); unfortunately, no mating attempts were
329 observed in *D. pruinosa*. In the *Drosophila melanogaster* species group, males use their sex
330 combs, which are also located on the T1 tarsus, to grab the female genitalia as in *D.*
331 *melanogaster,* or the middle abdominal segments as in *D. kikkawai, D. ananassae*, and *D.*
332 *bipectinata* (Massey et al., 2019). In these species, male T2 and T3 legs remain on the
333 substrate and are not involved in mating. Typically, these males proceed very quickly from
334 mounting to attempted copulation; females may resist by walking away or using their hind
335 legs to kick the male off. Interestingly, similar behavior is observed in *D. willistoni*, one of
336 the closest relatives of the *melanogaster* species group that lacks sex combs (Massey et al.,
337 2019), suggesting that the origin of the sex comb did not radically change this aspect of
338 mating. In contrast, *D. immigrans, Z. tuberculatus*, and *D. repletoides* males use their sex
339 brushes to grab females more anteriorly, near the constriction between the thorax and
340 abdomen (Supplementary movies 1-3). In the former two species, males also use their T2
341 legs to grab the female mid-abdomen, while in *D. repletoides* T1, T2 and T3 legs are all used
342 to grab the female so that the male "rides" on the female and is not in contact with the
343 substrate. In all these species, females appear to resist mating attempts more vigorously
344 than in the *melanogaster* group, using side-to-side bucking and wing vibrations in apparent
345 efforts to dislodge the male, while the males use their legs to resist these efforts. The delay
346 between mounting and attempted copulation is longer in the brush-bearing species,
347 especially in *Z. tuberculatus*, than in the comb-bearing species; most mountings result in
348 the male being eventually dislodged and do not lead to copulation attempts.

349 A more systematic analysis, including many lineages that lack male-specific leg
350 modifications, will be needed to test whether morphological evolution correlates with the
351 evolution of behavior. At this point, we can only speculate that male leg brushes, which
352 consist of hundreds of thin hairs that are hooked at the tips and have a very large combined

surface area, may have evolved to provide a more secure grip of the female abdomen, especially if stronger grip is needed to counteract the female attempts to dislodge the male.

Discussion

Repeated evolution of leg brushes

Our results suggest that the male-specific brush on the tarsal segments of front legs has evolved independently at least three times in Drosophilidae: in *Zaprionus*, in *D. repletoides*, and in the common ancestor of the *immigrans* species group and the *loiciana* complex, which includes *D. pruinosa*. Although our study does not provide unequivocal support for a sister-group relationship between *D. pruinosa* and the *immigrans* group (a closer relationship of *D. pruinosa* to the *sternopleuralis-trisetosa* clade cannot be completely ruled out, and of course there may exist other closely related species that we have not sampled), independent evolution of brushes in the *immigrans* species group and the *loiciana* species complex appears less likely than a single origin in a common ancestor of these clades. The proximally curving tips of brush hairs in *D. pruinosa* and *D. immigrans*, distinct from the distally curving tips in *Zaprionus* and *D. repletoides* (Fig. 1), are consistent with a close relationship between the *immigrans* group and the *loiciana* species complex.

Convergent origin of leg brushes in *Zaprionus*, *D. repletoides*, and the *immigrans-loiciana* clade is remarkable given the strong structural similarities of these brushes, and especially the fact that the cellular mechanisms that produce them in different species are essentially identical. In all species examined, the bristles that make up the sex brush are specified with only one or two intervening epithelial cells between them. Bristle specification in *Drosophila* and other insects is governed by a lateral inhibition mechanism, which is based on contact signaling between adjacent cells, and prevents two adjacent cells from both assuming the fate of bristle precursors (Simpson, 1990). Later in development, a tighter packing of bristles can be achieved through cell migration, as observed for example in the transverse bristle rows of the front legs (Atallah et al., 2009; Tanaka et al., 2009). However, no cell migration is observed during sex brush development; instead, the future

384 brush hairs are always specified at the maximum density allowed by lateral inhibition.
385 Independent evolution of this highly derived spatial pattern in multiple lineages suggests
386 that similar selective pressures may elicit not only similar structures, but also similar
387 changes in the underlying developmental mechanisms.
388
389 *A hierarchy of convergence, from structures to genes*
390
391 Convergence is a pervasive phenomenon in evolution. Pictures comparing bats and
392 birds, fish and dolphins, or the vertebrate and cephalopod eyes are textbook clichés. Less
393 obvious but equally striking examples of convergent forms shaped by a common function
394 include the asymmetrical ears of owls (Nishikawa, 2002), Mullerian mimicry in butterflies
395 (Brower, 1994; Naisbit et al., 2003), trophic morphology in cichlid fishes (Ruber and Adams,
396 2001), and many others (Moore and Willmer, 1997). A fundamental question raised by the
397 widespread occurrence of convergent traits is to what extent phenotypic convergence
398 reflects an underlying similarity of the molecular and cellular mechanisms that control
399 individual development.
400 The mechanisms of convergence can be viewed in either genetic or developmental
401 terms. From the genetic standpoint, convergent phenotypes are controlled by the same
402 mechanism if they are the result of changes in the same loci ("genetic convergence")
403 (Gompel and Prud'homme, 2009; Martin and Orgogozo, 2013). Alternatively, convergent
404 changes may be considered to share a similar mechanism if they are produced by the same
405 changes in development, regardless of the ultimate genetic causes ("developmental
406 convergence") (Wake, 1991; Wake et al., 2011; Wray, 2002). We can describe convergence
407 in hierarchical terms by applying three increasingly stringent criteria: phenotypic,
408 developmental, and genetic. Phenotypic convergence can occur without developmental
409 convergence: for example, the disproportionally elongated bodies of burrowing
410 salamanders can be caused either by an increase in the number of vertebrae, or by
411 increased length of the individual vertebrae – a superficial phenotypic resemblance
412 produced by entirely different developmental mechanisms (Parra-Olea and Wake, 2001).
413 Similarly, larval abdominal prolegs of butterflies and sawflies, while superficially similar
414 and playing similar roles in locomotion, develop from non-homologous leg segments

415 (Suzuki and Palopoli, 2001). In *D. melanogaster*, latitudinal clines in wing size have evolved
416 on several continents, but the differences in wing size are due largely to differences in cell
417 number in some populations, while in other populations differences in the size of
418 individual cells make a larger contribution (James et al., 1997; Zwaan et al., 2000).

419 Developmental convergence, in turn, does not necessarily reflect genetic
420 convergence. In *Drosophila*, similar color patterns can evolve in different species through
421 changes at different loci (Signor et al., 2016; Wittkopp et al., 2003b; Yassin et al., 2016), but
422 in the end all of these genetic changes are translated into adult phenotypes through the
423 same set of enzymatic reactions in the catecholamine metabolism pathway (Kronforst et al.,
424 2012; Wittkopp and Beldade, 2009; Wittkopp et al., 2003a), presenting an example of
425 developmental convergence in the absence of genetic convergence. In other cases, the
426 evolution of similar phenotypes in distantly related species is clearly caused by
427 independent changes in the same genes – as, for example, in the loss of larval cuticular
428 hairs through regulatory mutations in the *shavenbaby* gene in two *Drosophila* species
429 separated by >40 million years (Sucena et al., 2003), or in the evolution of mimetic wing
430 color patterns in different species of *Heliconius* butterflies through parallel changes in the
431 *optix* and *WntA* genes (Gallant et al., 2014; Hines et al., 2011; Martin et al., 2012; Reed et al.,
432 2011; Supple et al., 2013).

433 Going even deeper from the genetic to the molecular level, we can ask whether
434 recurrent involvement of the same gene in multiple instances of convergent evolution
435 reflects repeated appearance of the same mutations, or whether different mutations can
436 alter the function of the causative gene in similar ways, leading to the evolution of similar
437 phenotypes. Again, we can find examples in support of either scenario. A variety of
438 proteins have evolved similar amino acid residues and active sites repeatedly in different
439 taxa (Gasparini et al., 1998; Hill et al., 2019; Kriener et al., 2000; Lopreato et al., 2001;
440 Yokoyama, 2002). Although non-coding DNA sequences are clearly less constrained in
441 their function than proteins, similar regulatory mutations can nevertheless appear and be
442 fixed independently in different populations or species in response to similar selective
443 pressures (Chan et al., 2010; Loehlin et al., 2019; Xie et al., 2019). Conversely, mutations
444 that affect different amino acids, or different *cis*-regulatory elements, can produce similar

445 phenotypic outcomes in different populations or species (Gross et al., 2009; Kingsley et al.,
446 2009; Manceau et al., 2010; Protas et al., 2006; Rosenblum et al., 2009; Yassin et al., 2016).
447 The sex brush represents a clear case of developmental convergence, but the genetic
448 control of its development remains to be determined. Specification and positioning of the
449 sex brush are likely to involve the HOX gene *Scr* and the sex determination gene *doublesex*
450 (Rice et al., 2018; Tanaka et al., 2011). However, the identity of the downstream genes that
451 translate the regulatory information provided by *dsx* and *Scr* into the final 3-dimensional
452 structure is unknown. In principle, these genes need not be the same in different lineages.
453 Future work may show whether the convergence we observe at the level of cell behavior
454 extends to the genetic and molecular level.
455
456 *Convergent innovation and the failure to innovate*
457
458 The evolution of similar structures in response to similar functional demands is perhaps
459 not surprising; should we instead be surprised when organisms faced with similar
460 demands *fail to evolve* convergent phenotypes (Blount et al., 2018)? Males grasp female
461 abdomen with their front legs in many *Drosophila* species, including those that primitively
462 lack any male-specific leg ornaments (Massey et al., 2019; Spieth, 1952). Why don't all
463 *Drosophila* species evolve sex combs, brushes, or other grasping structures? Development
464 does not provide any clues. At the level of cell behavior, both the ancestral/female
465 condition (cell migration that produces tightly packed bristle rows from sparsely spaced
466 precursors) and the derived/male condition (specification of bristle precursors at the
467 maximum density permitted by lateral inhibition) are the same in all lineages where the
468 sex brush is present. There is no a priori reason to think that the transition between these
469 modes of development is easier in some species than others.
470 The answer may lie instead in either behavior or population genetics. Although
471 males of different species use their sex brushes in at least superficially similar ways, we
472 don't know the female side of the story. If females of different species vary in their
473 responses to male grasping, the evolution of specialized leg structures in males may not be
474 universally favored. This may also explain why both the sex brushes and the sex combs
475 (Kopp, 2011) have been secondarily lost multiple times. Moreover, it is difficult to know

476 whether the female preferences observed today are the same as they were in the distant
477 past when the male-specific structures evolved (Watts et al., 2019).
478 Alternatively, the origin of a new trait such as the leg brush may require such an
479 unlikely series of genetic changes that it may often fail to occur even in response to strong
480 selective pressure. For example, it is possible that while a single mutation is sufficient to
481 modify or eliminate an existing morphological structure, the origin of a *new* structure may
482 require simultaneous changes in multiple genes. From the population-genetic perspective,
483 this would mean that functionally novel and positively interacting alleles at multiple loci
484 must segregate in the same population at the same time in order for selection in favor of a
485 new structure to be effective. Naturally, this would greatly reduce the probability of
486 evolutionary innovations compared to other types of phenotypic change. This is of course
487 pure speculation; we do not know why convergent innovations evolve in some lineages but
488 fail to evolve in others. We hope that research models where both the functional roles and
489 the genetic basis of novel traits can be studied in parallel will shed some light on this
490 intriguing question in the future.
491
492 **Acknowledgements**
493
494 We thank the US *Drosophila* species stock center, Ehime University *Drosophila* stock center,
495 J.-R. David, S. Prigent, and M. Watada for *Drosophila* strains and specimens; Developmental
496 Studies Hybridoma Bank for antibodies; A. Comeault, A. Gloss, M. Lang, D. Matute, D. Miller,
497 V. Orgogozo, and N. Whiteman for genome assemblies; M. Toda for advice on *Zaprionus*
498 phylogeny; G. Rice for *D. immigrans* images; and A. Yassin for comments on the manuscript.
499 We also thank the MCB Light Microscopy Imaging Facility, Department of Cell Biology and
500 Human Anatomy EM Lab, and Materials Science and Engineering AMCaT Lab at UC Davis
501 for equipment and imaging assistance, and Gwyneth Card and W. Ryan Williamson for use
502 of the Photron camera. The Olympus FV1000 confocal used in this study was purchased
503 using NIH Shared Instrumentation Grant 1S10RR019266-01. AK and OB are grateful to N.
504 Gompel for allowing some of this work to be conducted in his lab while on a sabbatical visit,
505 and to members of the Gompel lab for treating them as their labmates. This work was

supported by NIH grant 5R35GM122592 to AK and by funds from Ludwig-Maximilians-Universität München to Nicolas Gompel.

508 Supplemental text - The *immigrans* species group

509

510 The *immigrans* species group and the wider *immigrans-tripunctata* radiation have long
511 played a pivotal role in *Drosophila* systematics (Throckmorton, 1975; Yassin, 2013).
512 However, the composition of the *immigrans* group has not been entirely clear. Historically,
513 this group was proposed to include five subgroups: *immigrans, hypocausta, nasuta,*
514 *quadrilineata*, and *curviceps* (Huang et al., 2002; Zhang and Toda, 1988, 1992; Zhang et al.,
515 1995). Recent studies have shown, however, that both the *hypocausta* and the *immigrans*
516 subgroups are likely polyphyletic, as some species assigned to these subgroups are actually
517 closer to the *nasuta* subgroup (Da Lage et al., 2007; Katoh et al., 2007; Rice et al., 2018).
518 The *quadrilineata* and *curviceps* subgroups have been the source of even greater
519 complications. Previous phylogenetic studies have provided evidence against a sister-
520 group relationship between *D. quadrilineata* and the rest of the *immigrans* species group
521 (Katoh et al., 2007; Morales-Hojas and Vieira, 2012; Yassin, 2013). Moreover, *D. annulipes*,
522 traditionally assigned to the *quadrilineata* subgroup (Lin and Tseng, 1973), was found to be
523 distantly related to *D. quadrilineata*, and closer to the *virilis-repleta* radiation (Katoh et al.,
524 2007). The phylogeny of Huang et al (Huang et al., 2002) showed a close relationship
525 between *D. annulipes* and the clade composed of *D. curviceps* and *D. oritisa*, another species
526 assigned to the *curviceps* subgroup. Morales-Hojas and Vieira (Morales-Hojas and Vieira,
527 2012) confirmed the distant relationship between *D. annulipes* and *D. quadrilineata*, as well
528 as between *D. annulipes* and the *immigrans* species group *sensu stricto*. Yassin (Yassin,
529 2013) pointed out important differences between *D. quadrilineata* and the *curviceps*
530 subgroup and the *immigrans* group *s.s*. Finally, Pradhan et al (Pradhan et al., 2015)
531 removed the *curviceps* subgroup from the *immigrans* species group, elevating it to the
532 status of a separate species group.

533 Our analysis, based on a larger amount of sequence data than previous studies,
534 confirms these observations, namely that (1) *D. quadrilineata* is not closely related either to
535 the *immigrans* species group *s.s.* or to *D. annulipes*, (2) *D. curviceps* is not closely related to
536 the *immigrans* species group *s.s.*, and (3) *D. annulipes* is related to *D. curviceps*. Based on
537 these results, we support the conclusions on other authors (Katoh et al., 2007; Pradhan et
538 al., 2015; Yassin, 2013) that the definition of the *immigrans* species group should be

539 restricted to the *immigrans*, *hypocausta*, and *nasuta* subgroups. Further work, with a more
540 extensive sampling of the *immigrans* group *s.s.*, is needed to revise its internal taxonomy
541 and re-establish monophyletic subgroups. Similarly, better taxon sampling will be needed
542 to determine whether *D. annulipes* could be reassigned to the *curviceps* species group
543 (Pradhan et al., 2015), or whether it is possible to establish a monophyletic *quadrilineata*
544 species group.
545

546 **References**
547
548 Atallah, J., Liu, N.H., Dennis, P., Hon, A., Godt, D., and Larsen, E.W. (2009). Cell dynamics and
549 developmental bias in the ontogeny of a complex sexually dimorphic trait in Drosophila
550 melanogaster. Evol Dev *11*, 191-204.
551 Blount, Z.D., Lenski, R.E., and Losos, J.B. (2018). Contingency and determinism in evolution:
552 Replaying life's tape. Science *362*.
553 Brower, A.V. (1994). Rapid morphological radiation and convergence among races of the
554 butterfly Heliconius erato inferred from patterns of mitochondrial DNA evolution. Proc Natl
555 Acad Sci U S A *91*, 6491-6495.
556 Chan, Y.F., Marks, M.E., Jones, F.C., Villarreal, G., Jr., Shapiro, M.D., Brady, S.D., Southwick,
557 A.M., Absher, D.M., Grimwood, J., Schmutz, J.*, et al.* (2010). Adaptive evolution of pelvic
558 reduction in sticklebacks by recurrent deletion of a Pitx1 enhancer. Science *327*, 302-305.
559 Da Lage, J.-L., Kergoat, G.J., Maczkowiak, F., Silvain, J.-F., Cariou, M.-L., and Lachaise, D.
560 (2007). A phylogeny of Drosophilidae using the *Amyrel* gene: questioning the *Drosophila*
561 *melanogaster* species group boundaries. J Zool Syst Evol Res *45*, 47-63.
562 Daugeron, C., Plant, A., Winkler, I., Stark, A., and Baylac, M. (2011). Extreme male leg
563 polymorphic asymmetry in a new empidine dance fly (Diptera: Empididae). Biology letters
564 *7*, 11-14.
565 Eberhard, W.G. (2001). The functional morphology of species-specific clasping structures
566 on the front legs of male Archisepsis and Palaeosepsis flies (Diptera, Sepsidae). Zoological
567 Journal of the Linnean Society *133*, 335-368.
568 Edgar, R.C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and
569 space complexity. BMC Bioinformatics *5*, 113.
570 Gallant, J.R., Imhoff, V.E., Martin, A., Savage, W.K., Chamberlain, N.L., Pote, B.L., Peterson, C.,
571 Smith, G.E., Evans, B., Reed, R.D.*, et al.* (2014). Ancient homology underlies adaptive
572 mimetic diversity across butterflies. Nature communications *5*, 4817.
573 Gasparini, S., Danse, J.M., Lecoq, A., Pinkasfeld, S., Zinn-Justin, S., Young, L.C., de Medeiros,
574 C.C., Rowan, E.G., Harvey, A.L., and Menez, A. (1998). Delineation of the functional site of
575 alpha-dendrotoxin. The functional topographies of dendrotoxins are different but share a
576 conserved core with those of other Kv1 potassium channel-blocking toxins. J Biol Chem *273*,
577 25393-25403.
578 Gompel, N., and Prud'homme, B. (2009). The causes of repeated genetic evolution. Dev Biol
579 *332*, 36-47.
580 Gross, J.B., Borowsky, R., and Tabin, C.J. (2009). A novel role for Mc1r in the parallel
581 evolution of depigmentation in independent populations of the cavefish Astyanax
582 mexicanus. PLoS Genet *5*, e1000326.
583 Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010).
584 New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the
585 performance of PhyML 3.0. Syst Biol *59*, 307-321.
586 Gupta, J.P. (1972). D. orissaensis, a new species of Drosophila from Orissa, India. Oriental
587 Insects *6*, 561-563.
588 Hardy, D.E. (1965). Diptera: Cyclorrhapha II, series Schizophora, section Acalypterae I,
589 family Drosophilidae. . In Insects of Hawaii, E.C. Zimmerman, ed. (Honolulu: University of
590 Hawaii Press).

591 Hill, J., Enbody, E.D., Pettersson, M.E., Sprehn, C.G., Bekkevold, D., Folkvord, A., Laikre, L.,
592 Kleinau, G., Scheerer, P., and Andersson, L. (2019). Recurrent convergent evolution at
593 amino acid residue 261 in fish rhodopsin. Proc Natl Acad Sci U S A *116*, 18473-18478.
594 Hines, H.M., Counterman, B.A., Papa, R., Albuquerque de Moura, P., Cardoso, M.Z., Linares,
595 M., Mallet, J., Reed, R.D., Jiggins, C.D., Kronforst, M.R.*, et al.* (2011). Wing patterning gene
596 redefines the mimetic history of Heliconius butterflies. Proc Natl Acad Sci U S A *108*, 19666-
597 19671.
598 Hsu, T.C. (1943). Chromosomes of Drosophila repletoides. Kwangsi Agric *4*, 155-160.
599 Huang, J., Hao, L., Liu, S., Li, L., Zhang, W.-X., and Dai, Z.-H. (2002). Phylogenetic position of
600 Chinese endemic Drosophila curviceps species subgroup in the Drosophila immigrans
601 group. Acta Genetica Sinica *29*, 417-423.
602 Huber, B.A., Sinclair, B.J., and Schmitt, M. (2007). The evolution of asymmetric genitalia in
603 spiders and insects. Biol Rev *82*, 647-698.
604 Ingram, K.K., Laamanen, T., Puniamoorthy, N., and Meier, R. (2008). Lack of morphological
605 coevolution between male forelegs and female wings in Themira (Sepsidae : Diptera :
606 Insecta). Biological Journal of the Linnean Society *93*, 227-238.
607 Izumitani, H.F., Kusaka, Y., Koshikawa, S., Toda, M.J., and Katoh, T. (2016). Phylogeography
608 of the Subgenus Drosophila (Diptera: Drosophilidae): Evolutionary History of Faunal
609 Divergence between the Old and the New Worlds. PLoS ONE *11*, e0160051.
610 James, A.C., Azevedo, R.B., and Partridge, L. (1997). Genetic and environmental responses to
611 temperature of Drosophila melanogaster from a latitudinal cline. Genetics *146*, 881-890.
612 Katoh, T., Nakaya, D., Tamura, K., and Aotsuka, T. (2007). Phylogeny of the Drosophila
613 immigrans species group (Diptera: Drosophilidae) based on Adh and Gpdh sequences.
614 Zoolog Sci *24*, 913-921.
615 Katoh, T.K., Zhang, G., Toda, M.J., Zhang, W.X., and Gao, J.J. (2018). The Lordiphosa denticeps
616 species group (Diptera: Drosophilidae) in China, with redescriptions of four known species
617 and descriptions of nine new species. Zootaxa *4471*, 37-75.
618 Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper,
619 A., Markowitz, S., Duran, C.*, et al.* (2012). Geneious Basic: an integrated and extendable
620 desktop software platform for the organization and analysis of sequence data.
621 Bioinformatics *28*, 1647-1649.
622 Kikkawa, H., and Peng, F.T. (1938). Drosophila species of Japan and adjacent localities.
623 Japanese Journal of Zoology *7*, 507-552.
624 Kingsley, E.P., Manceau, M., Wiley, C.D., and Hoekstra, H.E. (2009). Melanism in peromyscus
625 is caused by independent mutations in agouti. PLoS ONE *4*, e6435.
626 Kopp, A. (2011). Drosophila Sex Combs as a Model of Evolutionary Innovations. Evol Dev *13*,
627 504-522.
628 Kriener, K., O'HUigin, C., Tichy, H., and Klein, J. (2000). Convergent evolution of major
629 histocompatibility complex molecules in humans and New World monkeys.
630 Immunogenetics *51*, 169-178.
631 Kronforst, M.R., Barsh, G.S., Kopp, A., Mallet, J., Monteiro, A., Mullen, S.P., Protas, M.,
632 Rosenblum, E.B., Schneider, C.J., and Hoekstra, H.E. (2012). Unraveling the thread of
633 nature's tapestry: the genetics of diversity and convergence in animal pigmentation.
634 Pigment cell & melanoma research *25*, 411-433.

Lanfear, R., Calcott, B., Ho, S.Y., and Guindon, S. (2012). Partitionfinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol Biol Evol *29*, 1695-1701.

Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T., and Calcott, B. (2017). PartitionFinder 2: New Methods for Selecting Partitioned Models of Evolution for Molecular and Morphological Phylogenetic Analyses. Mol Biol Evol *34*, 772-773.

Lin, F.-J., and Tseng, H.-C. (1973). The Drosophila immigrans species group in Taiwan with descriptions of five new species. Bull Inst Zool, Academia Sinica *12*, 13-26.

Loehlin, D.W., Ames, J.R., Vaccaro, K., and Carroll, S.B. (2019). A major role for noncoding regulatory mutations in the evolution of enzyme activity. Proc Natl Acad Sci U S A *116*, 12383-12389.

Lopreato, G.F., Lu, Y., Southwell, A., Atkinson, N.S., Hillis, D.M., Wilcox, T.P., and Zakon, H.H. (2001). Evolution and divergence of sodium channel genes in vertebrates. Proc Natl Acad Sci U S A *98*, 7588-7592.

Mainx, F. (1958). Zaprionus bogoriensis spec. nov.,eine neue Drosophilide aus Java. Zoologischer Anzeiger *161*, 126-130.

Manceau, M., Domingues, V.S., Linnen, C.R., Rosenblum, E.B., and Hoekstra, H.E. (2010). Convergence in pigmentation at multiple levels: mutations, genes and function. Philos T R Soc B *365*, 2439-2450.

Martin, A., and Orgogozo, V. (2013). The Loci of repeated evolution: a catalog of genetic hotspots of phenotypic variation. Evolution *67*, 1235-1250.

Martin, A., Papa, R., Nadeau, N.J., Hill, R.I., Counterman, B.A., Halder, G., Jiggins, C.D., Kronforst, M.R., Long, A.D., McMillan, W.O.*, et al.* (2012). Diversification of complex butterfly wing patterns by repeated regulatory evolution of a Wnt ligand. Proc Natl Acad Sci U S A *109*, 12632-12637.

Massey, J.H., Chung, D., Siwanowicz, I., Stern, D.L., and Wittkopp, P. (2019). The yellow gene influences Drosophila male mating 1 success through sex comb melanizatio. eLife *in press*.

McAlpine, J.F. (1981). Morphology and terminology - adults. In Manual of Nearctic Diptera, J.F. McAlpine, B.V. Peterson, G.E. Shewell, H.J. Teskey, J.R. Vockeroth, and D.M. Wood, eds. (Ottawa: Research Branch, Agriculture Canada), pp. 9--63.

Moore, J., and Willmer, P. (1997). Convergent evolution in invertebrates. Biol Rev Camb Philos Soc *72*, 1-60.

Morales-Hojas, R., and Vieira, J. (2012). Phylogenetic patterns of geographical and ecological diversification in the subgenus Drosophila. PLoS ONE *7*, e49552.

Naisbit, R.E., Jiggins, C.D., and Mallet, J. (2003). Mimicry: developmental genes that contribute to speciation. Evol Dev *5*, 269-280.

Nishikawa, K.C. (2002). Evolutionary convergence in nervous systems: insights from comparative phylogenetic studies. Brain Behav Evol *59*, 240-249.

Okada, T. (1964). Drosophilidae (Diptera) of Southeast Asia collected by the Thai-Japanese Biological Expedition 1961-62. In Nature and Life in Southeast Asia, T.a.U. Kira, T., ed. (Kyoto: Fauna and Flora Research Society), pp. 439-466.

Okada, T., and Carson, H.L. (1983). The genera Phorticella Duda and Zaprionus Coquillett (Diptera, Drosophilidae) of the Oriental region and New Guinea. Kontyu *51*, 539-553.

Parra-Olea, G., and Wake, D.B. (2001). Extreme morphological and ecological homoplasy in tropical salamanders. Proc Natl Acad Sci U S A *98*, 7888-7891.

680 Pradhan, S., Sati, P.C., Fartyal., R.S., Chatterjee, R.N., Sarswat, M., Kandpal, M.C., and Singh,
681 B.K. (2015). Drosophila curviceps Species-Group (Diptera: Drosophilidae) from India, with
682 Description of a New Species and Redescription of a Known Species. Proceedings of the
683 Zoological Society *68*, 178-183.
684 Protas, M.E., Hersey, C., Kochanek, D., Zhou, Y., Wilkens, H., Jeffery, W.R., Zon, L.I., Borowsky,
685 R., and Tabin, C.J. (2006). Genetic analysis of cavefish reveals molecular convergence in the
686 evolution of albinism. Nat Genet *38*, 107-111.
687 Reed, R.D., Papa, R., Martin, A., Hines, H.M., Counterman, B.A., Pardo-Diaz, C., Jiggins, C.D.,
688 Chamberlain, N.L., Kronforst, M.R., Chen, R.*, et al.* (2011). optix drives the repeated
689 convergent evolution of butterfly wing pattern mimicry. Science *333*, 1137-1141.
690 Rice, G., Barmina, O., Hu, K., and Kopp, A. (2018). Evolving doublesex expression correlates
691 with the origin and diversification of male sexual ornaments in the Drosophila immigrans
692 species group. Evol Dev *20*, 78-88.
693 Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Hohna, S., Larget, B., Liu,
694 L., Suchard, M.A., and Huelsenbeck, J.P. (2012). MrBayes 3.2: efficient Bayesian
695 phylogenetic inference and model choice across a large model space. Syst Biol *61*, 539-542.
696 Rosenblum, E.B., Rompler, H., Schoneberg, T., and Hoekstra, H.E. (2009). Molecular and
697 functional basis of phenotypic convergence in white lizards at White Sands. Proc Natl Acad
698 Sci U S A.
699 Ruber, L., and Adams, D.C. (2001). Evolutionary convergence of body shape and trophic
700 morphology in cichlids from Lake Tanganyika. J Evol Biol *13*, 325.
701 Russo, C.A., Mello, B., Frazao, A., and Voloch, C.M. (2013). Phylogenetic analysis and a time
702 tree for a large drosophilid data set (Diptera: Drosophilidae). Zoological Journal of the
703 Linnean Society *169*, 765-775.
704 Signor, S.A., Liu, Y., Rebeiz, M., and Kopp, A. (2016). Genetic Convergence in the Evolution of
705 Male-Specific Color Patterns in Drosophila. Curr Biol *26*, 2423-2433.
706 Simpson, P. (1990). Lateral inhibition and the development of the sensory bristles of the
707 adult peripheral nervous system of Drosophila. Development *109*, 509-519.
708 Sivinski, J. (1997). Ornaments in the Diptera. Florida Entomologist *80*, 142-164.
709 Spieth, H.T. (1952). Mating behavior within the genus Drosophila (Diptera). Bulletin of the
710 American Museum of Natural History *99*, 395-474.
711 Stark, J.B., and O'Grady, P.M. (2009). Morphological variation in the forelegs of the
712 Hawaiian Drosophilidae. I. The AMC clade. J Morphol.
713 Sucena, E., Delon, I., Jones, I., Payre, F., and Stern, D.L. (2003). Regulatory evolution of
714 shavenbaby/ovo underlies multiple cases of morphological parallelism. Nature *424*, 935-
715 938.
716 Supple, M.A., Hines, H.M., Dasmahapatra, K.K., Lewis, J.J., Nielsen, D.M., Lavoie, C., Ray, D.A.,
717 Salazar, C., McMillan, W.O., and Counterman, B.A. (2013). Genomic architecture of adaptive
718 color pattern divergence and convergence in Heliconius butterflies. Genome Res *23*, 1248-
719 1257.
720 Suzuki, Y., and Palopoli, M.F. (2001). Evolution of insect abdominal appendages: are prolegs
721 homologous or convergent traits? Development Genes and Evolution *211*, 486-492.
722 Tan, C.C., Hsu, T.C., and Sheng, T.C. (1949). Known drosophila species in China with
723 descriptions of twelve new species. In Studies in the genetics of Drosophila VI Articles on
724 genetics, cytology, and taxonomy, J.T. Patterson, ed. (Austin, TX: University of Texas
725 Publications), pp. 196-206.

726 Tanaka, K., Barmina, O., and Kopp, A. (2009). Distinct developmental mechanisms underlie
727 the evolutionary diversification of *Drosophila* sex combs. Proceedings of the National
728 Academy of Sciences *106*, 4764-4769.
729 Tanaka, K., Barmina, O., Sanders, L.E., Arbeitman, M.N., and Kopp, A. (2011). Evolution of
730 Sex-Specific Traits through Changes in HOX-Dependent doublesex Expression. PLoS Biol *9*,
731 e1001131.
732 Throckmorton, L.H. (1975). The phylogeny, ecology, and geography of Drosophila. In
733 Handbook of Genetics, R.C. King, ed. (Plenum Press: Plenum Press), pp. 421–469.
734 Tsacas, L. (2002). Le nouveau complexe africain Drosophila loiciana et l'espèce apparentée
735 D. matileana n. sp. (Diptera : Drosophilidae). Ann Soc entomol Fr *38*, 57-70.
736 Tsacas, L., and Chassagnard, M.-T. (2000). Drosophila loiciana, nouvelle espèce africaine et
737 redescription de son epèce affine D. pruinosa Dudas (Diptera, Drosophilidae). Revue
738 française d'entomologie *22*, 213-222.
739 Tsacas, L., and Chassagnard, M.T. (1990). The Species of the Genus Zaprionus with the Fore
740 Femora Bearing Spines (Diptera, Drosophilidae). Annales De La Societe Entomologique De
741 France *26*, 461-487.
742 Wake, D.B. (1991). Homoplasy - the Result of Natural-Selection, or Evidence of Design
743 Limitations. American Naturalist *138*, 543-567.
744 Wake, D.B., Wake, M.H., and Specht, C.D. (2011). Homoplasy: from detecting pattern to
745 determining process and mechanism of evolution. Science *331*, 1032-1035.
746 Watts, J.C., Flynn, A., Tenhumberg, B., and Hebets, E.A. (2019). Contemporary sexual
747 selection does not explain variation in male display traits among populations. Evolution.
748 Wheeler, M.R. (1981). The Drosophilidae. In The Genetics and Biology of Drosophila, M.
749 Ashburner, H.L. Carson, and J.N. Thompson, eds. (Academic Press), pp. 1-97.
750 Wittkopp, P.J., and Beldade, P. (2009). Development and evolution of insect pigmentation:
751 genetic mechanisms and the potential consequences of pleiotropy. Semin Cell Dev Biol *20*,
752 65-71.
753 Wittkopp, P.J., Carroll, S.B., and Kopp, A. (2003a). Evolution in black and white: genetic
754 control of pigment patterns in Drosophila. Trends Genet *19*, 495-504.
755 Wittkopp, P.J., Williams, B.L., Selegue, J.E., and Carroll, S.B. (2003b). Drosophila
756 pigmentation evolution: divergent genotypes underlying convergent phenotypes. Proc Natl
757 Acad Sci U S A *100*, 1808-1813.
758 Wray, G.A. (2002). Do convergent developmental mechanisms underlie convergent
759 phenotypes? Brain Behav Evolut *59*, 327-336.
760 Wynn, S., and Toda, M.J. (1988). Drosophilidae (Diptera) in Burma. IV. The genus Zaprionus.
761 Kontyu *56*, 843-851.
762 Xie, K.T., Wang, G., Thompson, A.C., Wucherpfennig, J.I., Reimchen, T.E., MacColl, A.D.C.,
763 Schluter, D., Bell, M.A., Vasquez, K.M., and Kingsley, D.M. (2019). DNA fragility in the parallel
764 evolution of pelvic reduction in stickleback fish. Science *363*, 81-84.
765 Yassin, A. (2007). A revision of the tumiditarsus group of the subgenus Drosophila and its
766 relation to the genus Zaprionus. Drosophila Information Service *90*, 20-22.
767 Yassin, A. (2013). Phylogenetic classification of the Drosophilidae Rondani (Diptera): the
768 role of morphology in the postgenomic era. Systematic Entomology *38*, 349-364.
769 Yassin, A., Araripe, L.O., Capy, P., Da Lage, J.L., Klaczko, L.B., Maisonhaute, C., Ogereau, D.,
770 and David, J.R. (2008). Grafting the molecular phylogenetic tree with morphological

771 branches to reconstruct the evolutionary history of the genus Zaprionus (Diptera:
772 Drosophilidae). Mol Phylogenet Evol *47*, 903-915.
773 Yassin, A., Da Lage, J.L., David, J.R., Kondo, M., Madi-Ravazzi, L., Prigent, S.R., and Toda, M.J.
774 (2010). Polyphyly of the Zaprionus genus group (Diptera: Drosophilidae). Mol Phylogenet
775 Evol *55*, 335-339.
776 Yassin, A., and David, J.R. (2010). Revision of the Afrotropical species of Zaprionus (Diptera,
777 Drosophilidae), with descriptions of two new species and notes on internal reproductive
778 structures and immature stages. ZooKeys *51*, 33-72.
779 Yassin, A., Delaney, E.K., Reddiex, A.J., Seher, T.D., Bastide, H., Appleton, N.C., Lack, J.B., David,
780 J.R., Chenoweth, S.F., Pool, J.E.*, et al.* (2016). The pdm3 Locus Is a Hotspot for Recurrent
781 Evolution of Female-Limited Color Dimorphism in Drosophila. Curr Biol *26*, 2412-2422.
782 Yokoyama, S. (2002). Molecular evolution of color vision in vertebrates. Gene *300*, 69-78.
783 Zhang, W.-X., and Toda, M.J. (1988). The Drosophila immigrans species-group of the
784 subgenus Drosophila (Diptera: Drosophilidae) in Yunnan, China. Zool Sci *5*, 1095-1103.
785 Zhang, W.-X., and Toda, M.J. (1992). A new species-subgroup of the Drosophila immigrans
786 species-group (Diptera, Drosophilidae), with descriptions of two new species from China
787 and revision of taxonomic terminology. Jpn J Genet *60*, 839-850.
788 Zhang, W.-X., Toda, M.J., and Watabe, H. (1995). Fourteen new species of the Drosophila
789 (Drosophila) immigrans species group from the Oriental region. Jpn J Genet *63*, 25-51.
790 Zwaan, B.J., Azevedo, R.B., James, A.C., Van 't Land, J., and Partridge, L. (2000). Cellular basis
791 of wing size variation in Drosophila melanogaster: a comparison of latitudinal clines on two
792 continents. Heredity *84 (Pt 3)*, 338-347.
793

794 **Figure Legends**

795

796 **Figure 1. Sex brush morphology in distantly related species.** (A, C, E, G, I, K)
797 Brightfield images of the first and second tarsal segments (ta1 and ta2) of the prothoracic
798 leg in males of six species. (B, D, F, H, J, L) SEM images of the ta1 sex brush. A-B) *D.*
799 *pruinosa*. A) The brush occupies the distal ~80% of ta1, replacing most of the transverse
800 bristle rows (TBRs, shown by brackets in this and other panels), which in females covers
801 the entire anterior-ventral surface. The ta1 segment is slightly widened at the distal end.
802 B) The tips of brush hairs are flattened, pointed, and form hooks that curve toward the base
803 of the leg (see inset). C-D) *D. immigrans.* C) The brush covers the distal ~60% of ta1 and
804 most of ta2. The shape of the segments is not modified. D) Similar to *D. pruinosa*, the brush
805 hairs are flattened, pointed, and form proximally curving hooks at the tips (inset). E-F) *D.*
806 *repletoides.* E) The brush covers ~70% of ta1 and most of ta2. Both segments are
807 shortened and have a bulbous shape. F) The tips of brush hairs are flattened but thick and
808 blunt. They form hooks that curve away from the leg base (inset). G-H) *Z. tuberculatus.* G)
809 The brush covers the distal ~60% of ta1. H) The tips of brush hairs curve distally and have
810 a pointed paddle-like shape with a slight depression (inset). I-J) *Z. vittiger.* K-L) *Z.*
811 *bogoriensis.* These species have distally curving tips of brush hairs, similar to *Z.*
812 *tuberculatus*.

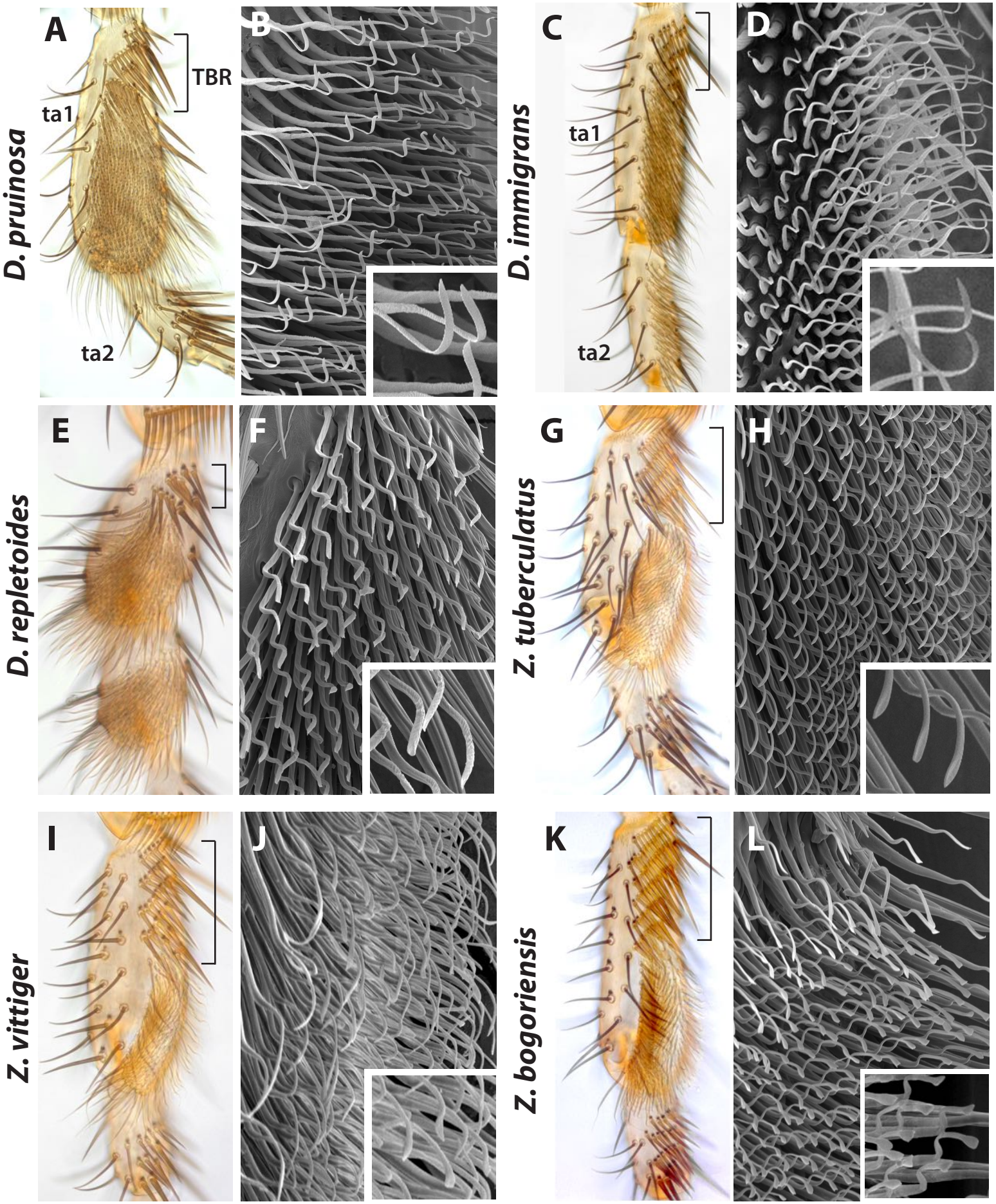
813

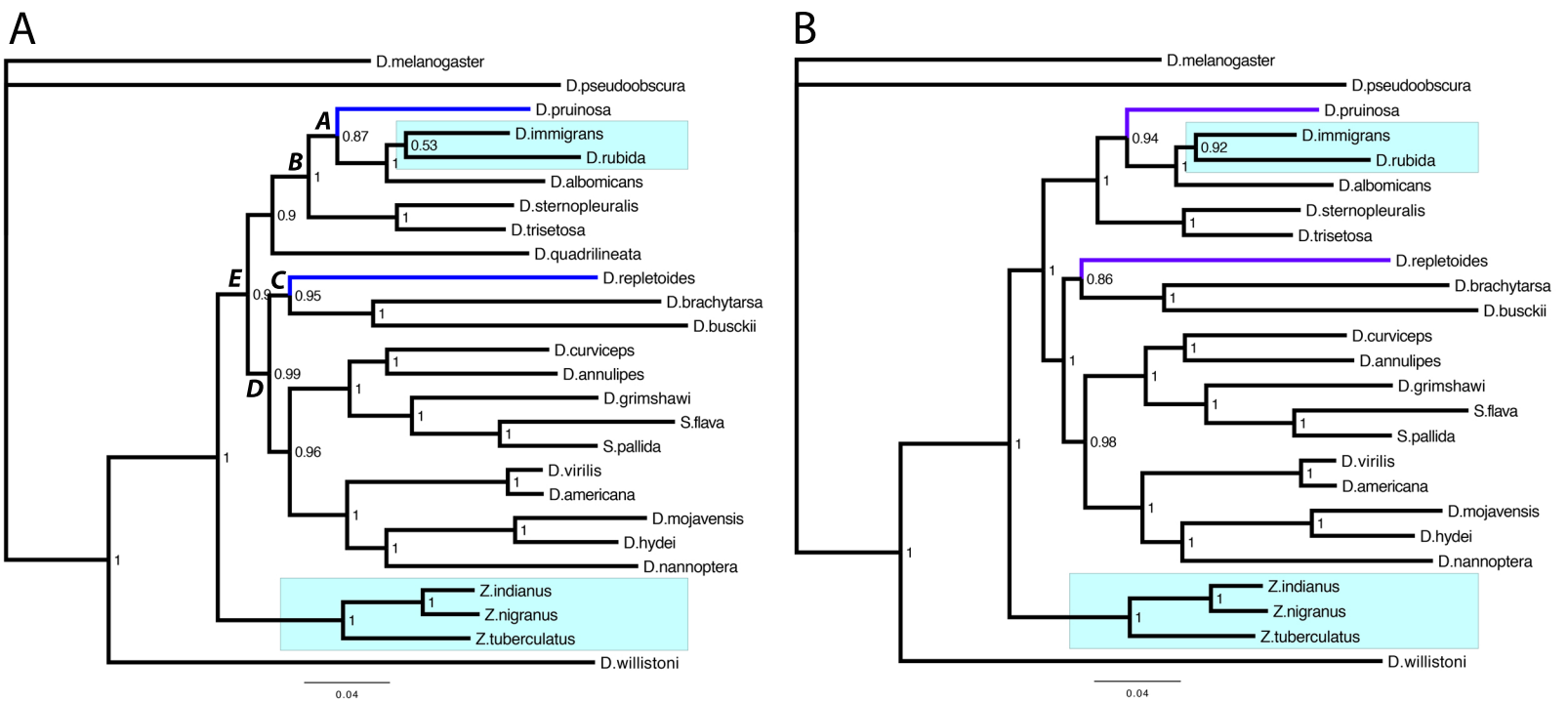
814 **Figure 2. Sex brushes evolved independently at least three times.** Species and clades
815 with male sex brushes are highlighted in blue. A) Bayesian phylogeny of the species
816 represented in this study, based on the combined dataset. Numbers at each node indicate
817 the posterior probabilities of the respective taxon bipartitions. B) Bayesian phylogeny
818 based on the same dataset but excluding *D. quadrilineata*. Posterior probabilities of nodes
819 A-E in different analyses can be found in Supplement Table 3.

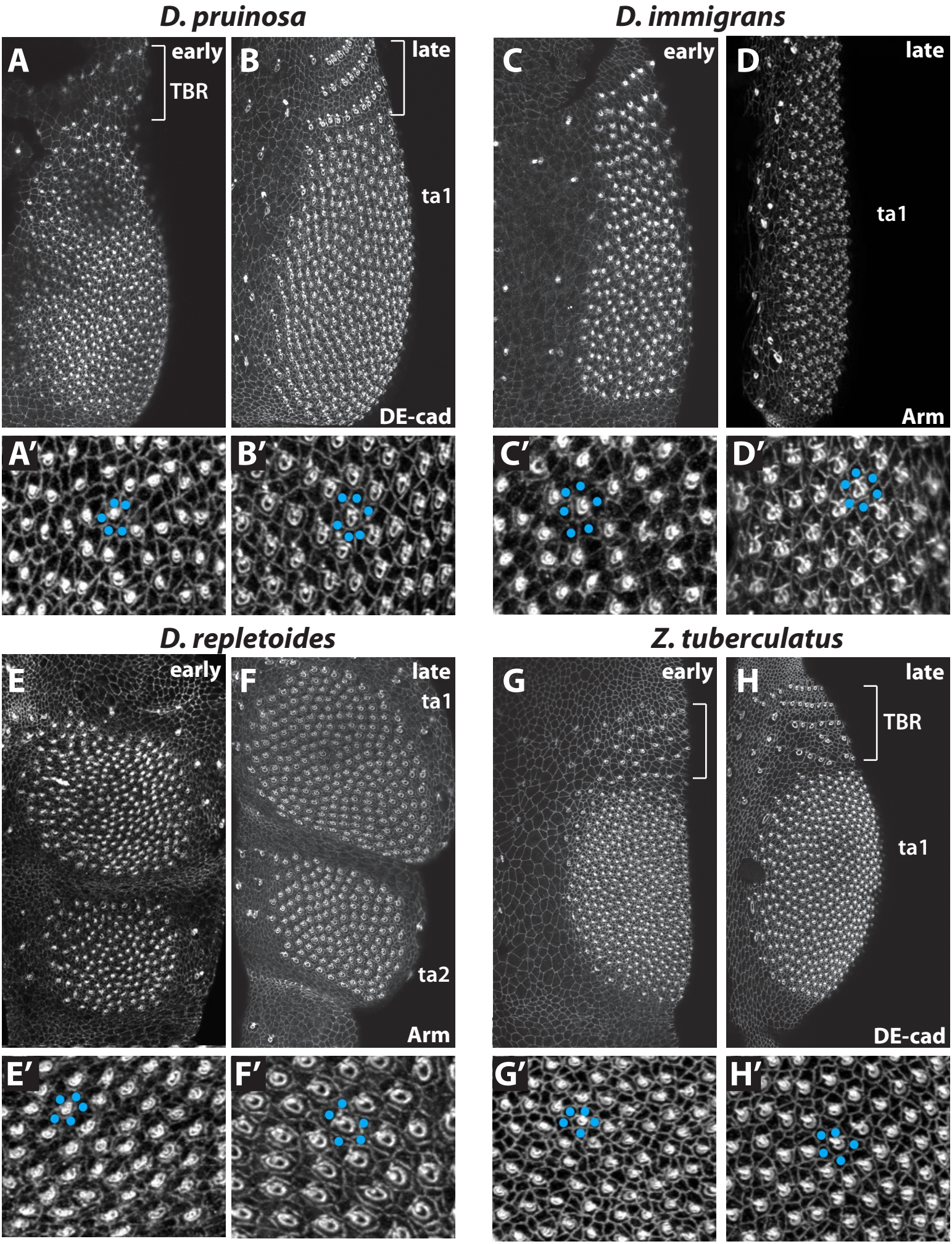
820

821 **Figure 3. Sex brush development shows strong similarities across species.**
822 Developing brush hair cells were visualized by immunostaining for membrane markers E-
823 cadherin (DE-cad) or Armadillo (Arm). For each species, the upper panels show the
824 confocal projections of ta1 segment (A-D, G-H) or both ta1 and ta2 (E-F). The bottom

825 panels (A'-H') show close-up views. For each species, early developmental stages are
826 shown on the left and later stages on the right. A-B') *D. pruinosa*, 27 and 42 hrs after
827 pupariation (AP). C-D') *D. immigrans,* 24 and 43 hrs AP. E-F') *D. repletoides,* 28 and 43 hrs
828 AP. G-H') *Z. tuberculatus,* 34 and 48 hrs AP. Hair progenitor cells can be distinguished from
829 epithelial cells by a ring of bright staining with a dense punctum in the middle. In all four
830 species, each hair cell is surrounded by 4-6 epithelial cells (blue dots), so that neighboring
831 hair cells are separated from each other by 1-2 cells, at both the early and the late stages.
832 The proximal TBRs (square brackets) form by expelling the intervening epithelial cells,
833 with the bristle progenitor cells migrating closer together to make straight rows (Tanaka et
834 al 2009) (e.g., compare A vs B, and G vs H). In contrast, no cell rearrangement is observed
835 in the brush.
836
837 **Supplement Figure 1.** Strict consensus of 11 trees with the cumulative posterior
838 probability of 95% for the complete taxon sample, labeled as in Figure 1. Numbers at each
839 node indicate the posterior probabilities of the respective taxon bipartitions.
840
841 **Supplement Figure 2.** Strict consensus of 11 trees with the cumulative posterior
842 probability of 99% for the taxon sample without *D. quadrilineata*.
843
844 **Supplement Figure 3.** Bayesian phylogenetic tree for the complete taxon sample, with the
845 dataset partitioned by gene and codon.
846
847 **Supplement Figure 4.** Phylogenetic tree for the taxon sample without *D. quadrilineata*,
848 with the dataset partitioned by gene and codon.


A
TBR
ta1
ta2
D. pruinosa
B
C
ta1
ta2
D. immigrans
D
E
D. repletoides
F
G
Z. tuberculatus
H
I
Z. vittiger
J
K
Z. bogoriensis
L


A
D.melanogaster
D.pseudoobscura
D.pruinosa
A
0.87
D.immigrans
0.53
D.rubida
B
1
D.albomicans
0.9
D.sternopleuralis
1
D.trisetosa
D.quadrilineata
E
C
D.repletoides
0.9
0.95
D.brachytarsa
1
D.busckii
D.curviceps
1
D.annulipes
0.99
D
1
D.grimshawi
1
S.flava
1
S.pallida
0.96
D.virilis
1
D.americana
1
D.mojavensis
1
D.hydei
1
D.nannoptera
Z.indianus
1
Z.nigranus
1
Z.tuberculatus
1
D.willistoni
0.04
B
D.melanogaster
D.pseudoobscura
D.pruinosa
0.94
D.immigrans
0.92
D.rubida
1
1
D.albomicans
D.sternopleuralis
1
D.trisetosa
1
D.repletoides
0.86
D.brachytarsa
1
D.busckii
D.curviceps
1
1
D.annulipes
1
D.grimshawi
1
S.flava
1
S.pallida
0.98
D.virilis
1
D.americana
1
D.mojavensis
1
D.hydei
1
D.nannoptera
1
Z.indianus
1
Z.nigranus
1
Z.tuberculatus
D.willistoni
0.04


D. pruinosa
D. immigrans
D. repletoides
Z. tuberculatus
A
early
TBR
B
late
ta1
DE-cad
C
early
D
late
ta1
Arm
A′
B′
C′
D′
E
early
F
late
ta1
ta2
Arm
G
early
H
late
TBR
ta1
DE-cad
E′
F′
G′
H′