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

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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*,

but did not support a sister-group relationship between *D. sternopleuralis* and the *immigrans* species group.

In this study, we used a larger multilocus dataset to test whether the male leg brush evolved independently in each of these four clades, or whether its distribution could be better explained by shared origin in some of these lineages. To facilitate this analysis, we included one or more representatives of each clade, as well as several brush-less species that have been suggested by previous studies to be closely related to the brush-bearing clades. In parallel, we compared the cellular mechanisms that produce the male leg brushes in different species, as well as the role of these ornaments in mating behavior. Our results strongly suggest that the sex brushes evolved convergently in several distantly related lineages, but develop through virtually identical mechanisms.

Materials and Methods

Leg imaging

For brightfield imaging, male front legs were dissected, mounted in Hoyers media between two coverslips, and photographed on a Leica DM500B microscope with a Leica DC500 camera. For electron microscopy, adult legs were dehydrated in 100% ethanol, critical point dried, and coated with gold. Scanning electron micrographs were taken on Thermo Fisher Quattro S and Philips XL30 TMP.

Sequence data collection

The sources of live *Drosophila* strains, fixed specimens, and unpublished genome assemblies used in this study are listed in Table S1. DNA was extracted from live or alcohol-fixed flies using an affinity resin based protocol (Hi Yield® Genomic DNA Mini Kit, Süd-Laborbedarf Gauting, Germany). PCR was carried out using DreamTaq polymerase (Thermo Fisher) and the following cycling conditions: 95° 5’ => (95° 30” => 55° 30” => 72° 80”)x35 => 72° 5’ =>12°; the loci and primer sequences are listed in Table S2. In some

cases, two rounds of PCR with nested primers were needed to obtain amplicons from fixed specimens. Amplified fragments were gel-purified and sequenced from both ends using amplification primers. Sequence chromatograms were trimmed in SnapGene Viewer, and the two end reads were aligned and edited in Geneious. Heterozygous nucleotide positions, if present, were represented by IUPAC ambiguity codes. All new sequences were deposited in Genbank under accession number listed in Table S1. Additional sequences were obtained from Genbank or extracted from whole-genome assemblies using Blast v2.2.23 (Table S1).

Sequence analysis

The sequences of each locus were aligned using the MUSCLE algorithm (Edgar, 2004) implemented in Geneious (Kearse et al., 2012). The alignments were trimmed at the ends, and poorly aligning intronic regions were removed. The alignments of all eight loci were then concatenated for combined analysis. Combined Bayesian analysis was carried out in MrBayes v3.2.6 (Ronquist et al., 2012). Two sets of analyses were conducted. In the first, the dataset was partitioned by gene, and each locus was allowed to follow a different nucleotide substitution model with empirically estimated parameters. In the second, we partitioned the dataset by gene and by codon position, and used PartitionFinder with the PhyML algorithm (Guindon et al., 2010; Lanfear et al., 2012; Lanfear et al., 2017) to identify the appropriate partitioning scheme; this resulted in a total of 21 character subsets, each of which was subsequently allowed to follow its own substitution model with empirically estimated parameters. For each analysis, two parallel runs of 1,500,000 generations, each starting from a different random tree, were carried out, and convergence was confirmed by comparing tree likelihoods and model parameters between the two runs. *D. melanogaster* was used as outgroup. Trees were sampled every 1000 generations and summarized after a 20% relative burn-in. Each analysis was also repeated after excluding *D. quadrilineata* (see Results). Samples of probable trees were extracted from the .tprobs file, and a strict consensus of most probable trees with combined posterior probabilities of 95% or 99% was constructed from these sets of trees in Geneious. Consensus trees were then formatted using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Immunocytochemistry and microscopy

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

Analysis of mating behavior

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

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).

Male leg brushes evolved independently at least three times

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

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

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

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

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

The same cellular processes underlie sex brush development in all species

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

migrate toward each other to form a straight, contiguous row, while the intervening epithelial cells are expelled distally and proximally from the TBRs (Atallah et al. 2009; Tanaka et al. 2009). This mechanism suggests two potential explanations for the tight packing of brush hairs. One possibility is that the hair progenitor cells are specified with minimal spacing; in this case, the initial spacing between hair cells is expected to be much denser than the spacing of TBR progenitors. Alternatively, hair progenitors may first be specified similarly to TBR bristles, with wide separation by epithelial cells, but expel the epithelial cells (either laterally or basally) at later stages to form a densely packed brush. In principle, independently evolved brushes in different species could utilize different cellular mechanisms to produce adult structures that are essentially indistinguishable.

In order to characterize and compare brush development in different species, we used antibodies against membrane-localized proteins to visualize cell arrangement in pupal legs. When labeled with antibodies against the beta-catenin Armadillo (Arm) or the E-cadherin Shotgun (DE-cad), bristle cells can be distinguished from the surrounding epithelial cells by their unique membrane shape (Figure 3). We examined brush development at two timepoints: an early stage roughly corresponding to ~16-21 hr AP in *D. melanogaster*, when the bristle cells of the future TBRs begin to migrate toward each other and expel the intervening epithelial cells, and a later stage when cell migration is completed. We found that at the early stage, most bristle cells in the developing brush are each surrounded by four to six epithelial cells in all species. In effect, the bristle cells are separated from one another by one to two epithelial cells (Figure 3). At the late stage, this spacing remains virtually invariant, although the cells appear more organized compared to the early stage (Figure 3). We did not see evidence of cell migration in any of the examined species. These observations indicate that despite the packed appearance similar to TBRs, the brush cells are not directly adjacent to each other, are specified at high density, and undergo minimal if any migration during development. Importantly, the cellular mechanism of brush development is very similar in all species.

Leg brushes are used in male mating behavior

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

A more systematic analysis, including many lineages that lack male-specific leg modifications, will be needed to test whether morphological evolution correlates with the evolution of behavior. At this point, we can only speculate that male leg brushes, which 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

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

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

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

phenotypic outcomes in different populations or species (Gross et al., 2009; Kingsley et al., 2009; Manceau et al., 2010; Protas et al., 2006; Rosenblum et al., 2009; Yassin et al., 2016).

The sex brush represents a clear case of developmental convergence, but the genetic control of its development remains to be determined. Specification and positioning of the sex brush are likely to involve the HOX gene *Scr* and the sex determination gene *doublesex* (Rice et al., 2018; Tanaka et al., 2011). However, the identity of the downstream genes that translate the regulatory information provided by *dsx* and *Scr* into the final 3-dimensional structure is unknown. In principle, these genes need not be the same in different lineages. Future work may show whether the convergence we observe at the level of cell behavior extends to the genetic and molecular level.

Convergent innovation and the failure to innovate

The evolution of similar structures in response to similar functional demands is perhaps not surprising; should we instead be surprised when organisms faced with similar demands *fail to evolve* convergent phenotypes (Blount et al., 2018)? Males grasp female abdomen with their front legs in many *Drosophila* species, including those that primitively lack any male-specific leg ornaments (Massey et al., 2019; Spieth, 1952). Why don't all *Drosophila* species evolve sex combs, brushes, or other grasping structures? Development does not provide any clues. At the level of cell behavior, both the ancestral/female condition (cell migration that produces tightly packed bristle rows from sparsely spaced precursors) and the derived/male condition (specification of bristle precursors at the maximum density permitted by lateral inhibition) are the same in all lineages where the sex brush is present. There is no a priori reason to think that the transition between these modes of development is easier in some species than others.

The answer may lie instead in either behavior or population genetics. Although males of different species use their sex brushes in at least superficially similar ways, we don't know the female side of the story. If females of different species vary in their responses to male grasping, the evolution of specialized leg structures in males may not be universally favored. This may also explain why both the sex brushes and the sex combs (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
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493
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Supplemental text - The *immigrans* species group

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

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

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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*.

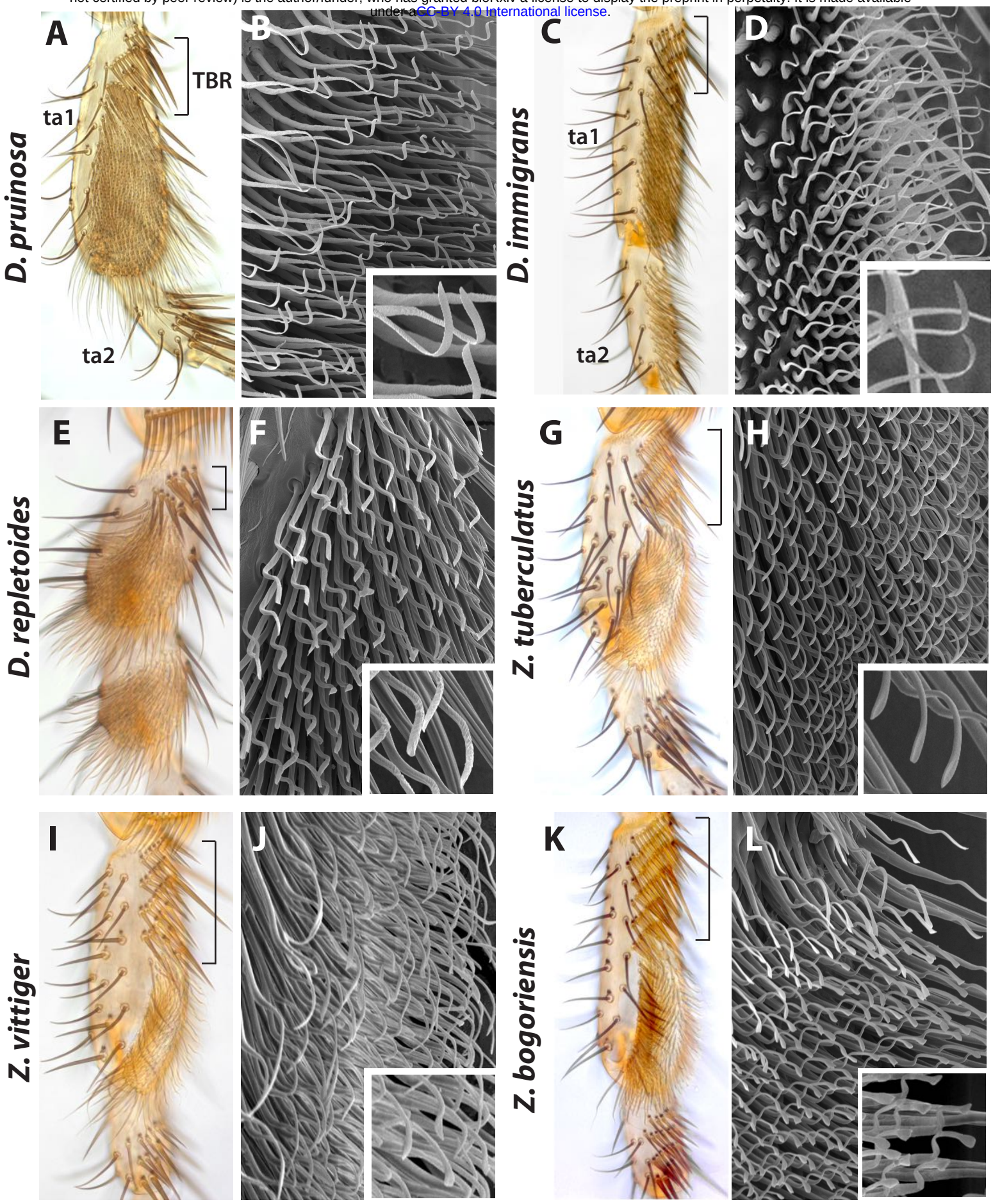
813

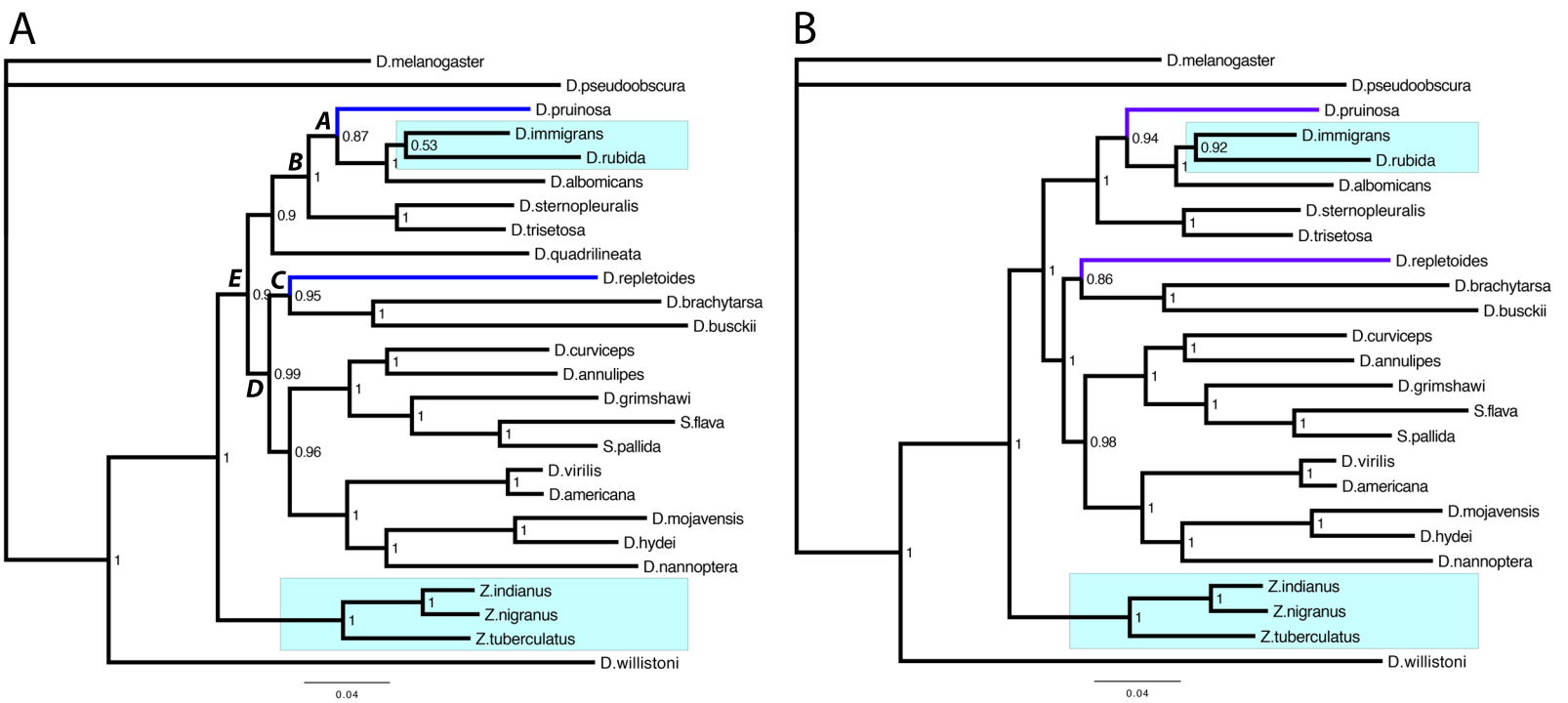
814 **Figure 2. Sex brushes evolved independently at least three times.** Species and clades
815 with male sex brushes are highlighted in blue. A) Bayesian phylogeny of the species
816 represented in this study, based on the combined dataset. Numbers at each node indicate
817 the posterior probabilities 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
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
D.albomicans
1
D.sternopleuralis
1
D.trisetosa
1
D.repletoides
0.86
D.brachytarsa
1
D.busckii
1
D.curviceps
1
D.annulipes
1
D.grimshawi
1
S.flava
1
S.pallida
1
0.98
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