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Many ways to make a novel structure: a new mode of sex comb development in Drosophilidae

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SUMMARY On macroevolutionary time scales, the same genes can regulate the development of homologous structures through strikingly different cellular processes. Comparing the development of similar morphological traits in closely related species may help elucidate the evolutionary dissociation between pattern formation and morphogenesis. We address this question by focusing on the interspecific differences in sex comb development in Drosophilids. The sex comb is a recently evolved, male-specific structure composed of modified bristles. Previous work in the *obscura* and *melanogaster* species groups (Old World *Sophophora*) has identified two distinct cellular mechanisms that give rise to nearly identical adult morphologies. Here, we describe sex comb development in a species from a more distantly related lineage, the genus *Lordiphosa*. Although the expression of key regulatory genes is largely conserved in both clades, the cell behaviors responsible for sex comb formation show major differences between Old World *Sophophora* and *Lordiphosa*. We suggest that the many-to-one mapping between development and adult phenotype increases the potential for evolutionary innovations.

INTRODUCTION

Many developmental processes are regulated by homologous genes in organisms separated by vast evolutionary distances (Gerhart and Kirschner 2007). Specification of the anterior–posterior body axis is perhaps the most celebrated case: animals from nematodes to vertebrates use a common set of transcription factors, the HOX genes, to regulate this critical patterning step (Duboule 2007). Yet even a cursory comparison of embryonic development among animal phyla shows that conserved regulatory genes can control fundamentally different cellular processes. The tempo and mode of evolutionary changes leading to such dissociation between patterning and morphogenesis are poorly understood. A key question is the extent to which similar structures in closely related species, which are presumed to be homologous and are regulated by the same genes, differ in the cellular mechanisms underlying their development.

Here, we address this question using *Drosophila* sex combs as a model. Sex combs are a recently evolved, male-specific morphological structure composed of modified bristles ("teeth") on the front legs (Kopp 2011). Species in the *melanogaster* and *obscura* species groups in the subgenus *Sophophora* differ dramatically in the number of sex comb teeth as well as their morphology and orientation (Barmina and Kopp 2007). The most common phenotype is the extended longitudinal sex comb (Fig. 1), which consists of enlarged, darkly pigmented bristles arranged in a single row that follows the proximo-distal axis of the leg and spans all or most of the first two tarsal segments (Atallah et al. 2009b). This type of sex comb, which is believed on phylogenetic (Barmina and Kopp 2007) and developmental grounds (see below) to have originated more than once in evolution, is found in several lineages within *Sophophora*: the *montium* subgroup, the *rhopaloa,* and *ficusphila* subgroups in the Oriental lineage, *Drosophila guanche* and its close relatives in the *obscura* group, and the *dentissima* species group (see Fig. 2 for phylogenetic relationships) (Tsacas 1980; Kopp 2011).

Although the longitudinal sex combs of different species look similar in adult flies, they form through different developmental mechanisms (Atallah et al. 2009b; Tanaka et al. 2009). In *D. guanche*, the sex comb on each segment develops from an elongated distal transverse bristle row (TBR) that rotates approximately 90° (illustrated in Fig. 5B), a mechanism similar to the ontogeny of the much shorter *D. melanogaster* sex comb (Tokunaga 1962). The TBRs themselves follow a complex developmental path, forming initially as separate, isolated bristle precursors that later assemble into contiguous rows (Atallah et al. 2009a). In contrast, in the *montium* and *ficusphila* subgroups, the sex combs develop from two extended longitudinal rows of bristle precursors that converge and intercalate to form a single contiguous comb (Fig. 5A).

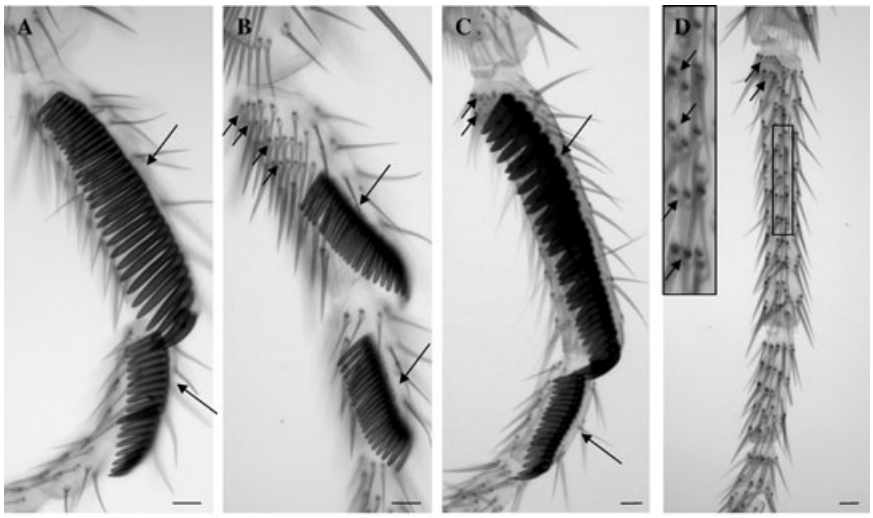

Fig. 1. The extended longitudinal sex comb phenotype. Forelegs are shown in all panels. (A) *Drosophila kikkawai* male. (B) *Drosophila guanche* male. (C) *Lordiphosa magnipectinata* male. Large arrows in (A–C) show the sex combs on ts1 and ts2, whereas small arrows show proximal transverse bristle rows (TBRs). (D) *L. magnipectinata* female, first two tarsal segments. Arrows indicate the TBRs. Note that the two most proximal TBRs have more bristles than the more distal ones in the inset. Distal is down in all panels. Scale bar: 20 μm.

In this case, the comb does not go through an intermediate transverse stage and there is no rotation. Instead, unrotated TBRs often form anterior to the sex comb.

An extensive molecular phylogenetic study of the family Drosophilidae (van der Linde et al. 2010) has provided strong evidence that the *melanogaster* and *obscura* species groups form a monophyletic clade within *Sophophora* (the "Old World *Sophophora*"). The vast majority of species within these groups bear sex combs (Bock 1980; Lakovaara and Saura 1982). Because species in the closest outgroup, the Neotropical *Sophophora*, consisting of the *willistoni* and *saltans* species groups, lack sex combs, a phylogenetic analysis of sex comb evolution using representatives of these taxa points to the conclusion that the structure originated in the ancestor of the Old World species (Barmina and Kopp 2007). However, this simple phylogenetic picture is complicated by the presence of sex combs in several species of *Lordiphosa*, a taxon established by Basden (1961) as a subgenus and later raised to generic status by Grimaldi (1990). Recent work shows that *Lordiphosa* is the sister-group of the Neotropical *Sophophora* (van der Linde et al. 2010; Gao et al. 2011) (Fig. 2). Although most members of this genus lack sex combs, males of the *miki* species group (Lastovka and Maca 1978; Okada 1984) have extended longitudinal sex combs (Fig. 2). Thus, sex combs either evolved independently in *Lordiphosa* and in Old World *Sophophora*, or they evolved once in the common ancestor of *Sophophora* and *Lordiphosa* but were lost in the Neotropical *Sophophora* and much of *Lordiphosa*.

Lordiphosa species, which breed on decaying vegetation, do not lend themselves well to laboratory culture. Here, we examine sex comb development in *Lordiphosa magnipectinata*, a typical member of the *miki* species group, using samples collected in the field (Fig. S1) and maintained in the lab for one generation. We find that although the expression of key genes in the sex comb region of *L. magnipectinata* resembles the *melanogaster* and *obscura* species groups, the cellular mechanisms underlying comb formation clearly differ from the processes seen in *Sophophora*.

MATERIALS AND METHODS

Lordiphosa sample collection

Collections of *L. magnipectinata* were made in Sapporo, northern Japan, in May and June of 2011. Adult flies were captured on the spring-ephemeral plant *Anemone flaccida* F. Schmidt (Fig. S1A) using an insect net, held in glass vials (30 mm in diameter, 100 mm in height), and transferred to the laboratory. *L. magnipectinata* is a leaf-mining species, and cannot be cultured on standard *Drosophila* media. Thus, a new method was adopted in the present study. Leaves of *A. flaccida* collected in the field were frozen to kill the larvae of other insects, then placed in a glass vial with wet filter paper and a small piece of apple for inhibiting mold (Fig. S1B). Female flies were allowed to oviposit in these vials. Mashed leaves of *Spinacia oleracea* L. were added as the larval food became exhausted. Larvae of *L. magnipectinata* developed well in the layer of leaves and filter paper with sap.

Pupal dissection

White prepupae, 0–1 h after pupariation (AP), were collected and aged at 25°C until the desired stage. Since the number of samples was limited, additional pupae were collected at later timepoints and their approximate developmental stages were estimated by comparing the developing tarsal bristle pattern with the timed samples.

An initial dissection was carried out prior to fixation in order to allow sufficient penetration of the fixative into the

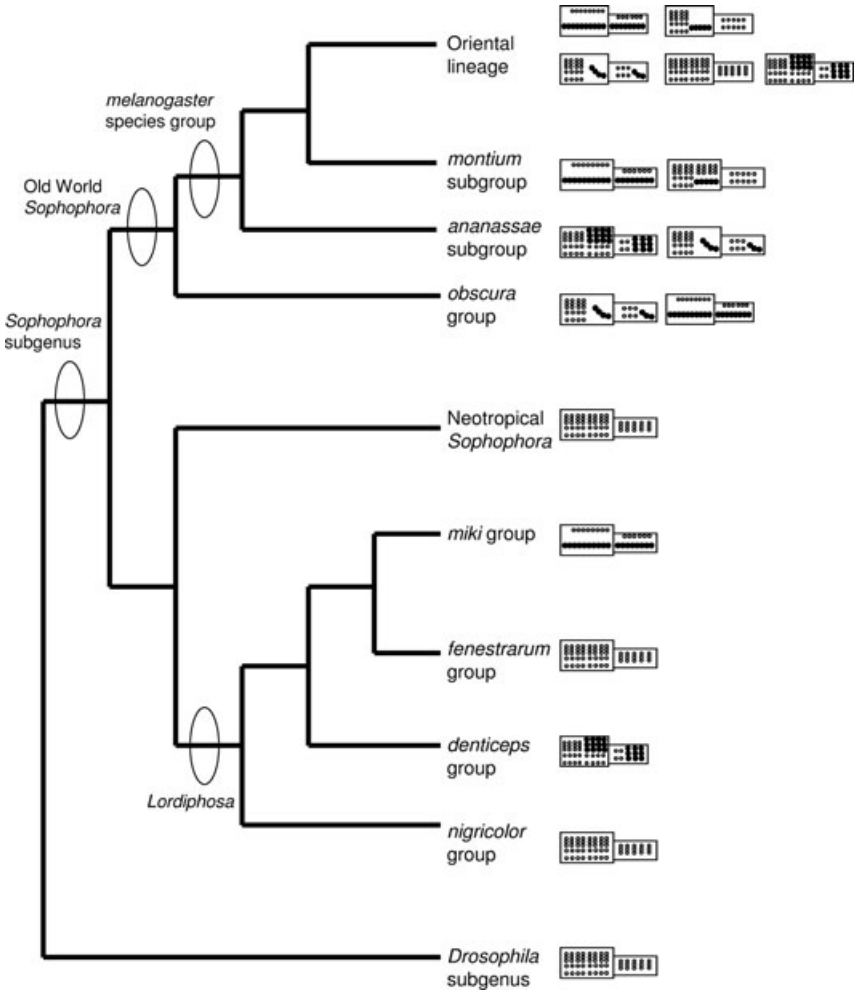

Fig. 2. Phylogeny of sex comb bearing drosophilid lineages (Barmina and Kopp 2007; Gao et al. 2011), with diagrams showing the sex comb phenotypes found in each lineage. Black circles represent sex comb teeth, whereas open circles represent other tarsal bristles. The oriental lineage is a large clade that includes the *melanogaster* subgroup (not to be confused with the *melanogaster* group) in addition to the *ficusphila, rhopaloa,* and other subgroups (Barmina and Kopp 2007). *Sophophora* is paraphyletic, and *Lordiphosa* is a sister-taxon to the Neotropical *Sophophora* (Gao et al. 2011). Although most species in *Lordiphosa* do not bear sex combs, members of the *denticeps* group have small, transverse, sex comb like bristles (Zhang 1993), and species in the *miki* group (which includes *L. magnipectinata*) have large, prominent longitudinal combs (Okada 1984).

leg tissues. This was accomplished either by slicing the pupa in half with a razor blade while immobilized on double-sided tape, or by removing the pupal case in phosphate buffered saline (PBS) with subsequent puncturing of the abdomen. Fixation was carried out in 4% paraformaldehyde at room temperature for 1 h. The tissue was washed three times in PBS followed by three washes in a solution of 10 mM Tris-Cl, 150 mM NaCl, and 0.05% Tween-20 buffer (TNT). All washes, during this step and during antibody staining (described below), lasted at least 15 min. The washes were followed by a second dissection in which most of the tissue around the legs was removed, and the pupal cuticle in the proximal and distal regions of the forelegs was punctured with fine forceps to allow greater antibody access.

Immunofluorescence

Antibody staining was carried out using standard protocols with a few modifications. Briefly, fixed, dissected pupal tissue was blocked for half an hour at room temperature in Image-iT® FX signal enhancer (Life Technologies, Carlsbad, CA, USA), followed by three washes in TNT. Incubation in the primary antibodies was carried out overnight at 4°C, followed by six washes in TNT. The tissue was then transferred to secondary antibodies in TNT. In cases where phalloidin staining was used to label F-actin, the phalloidin, conjugated to a fluorophore (Alexa 488-Phalloidin or Alexa 649-Phalloidin; Life Technologies), was added to the secondary antibodies at a concentration of 1:20. After an overnight incubation, the tissue was washed six times in TNT and mounted in Prolong Gold (Life Technologies). The following primary antibodies were obtained from the Developmental Studies Hybridoma Bank and used at a dilution of 1:2: mouse αScr (4H4.1; Glicksman and Brower 1988), mouse αFlamingo (Flamingo #74; Usui et al. 1999), mouse αDac (mAbdac-1-1; Mardon et al. 1994), mouse αEcadherin (Dcad2; Oda et al. 1994). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and were used at a concentration of 1:200.

Confocal microscopy and image processing

Z-stacks of stained pupal legs were obtained using an Olympus FV1000 confocal microscope. Three-dimensional projections of the stacks were generated using ImageJ software with an opacity setting of 70%.

RESULTS

Similar patterns of gene expression in *Sophophora* and *Lordiphosa*

Sex comb development in *Sophophora* depends on precise spatial regulation of the HOX gene *Sex combs reduced* (*Scr*) (Barmina and Kopp 2007). In *D. melanogaster*, *Scr* is necessary for most aspects of sex comb formation, from the specification of bristle precursors to tissue rotation, and high levels of *Scr* are sufficient to induce sex combs at ectopic locations (Barmina and Kopp 2007; Held 2010; Tanaka et al. 2011). Across species, *Scr* expression is strongest in the cells surrounding the sex comb. In species that bear a sex comb on only the first tarsal segment (ts1), high *Scr* expression is also limited to ts1, whereas in species that also have a prominent sex comb on ts2, strong *Scr* expression extends to this segment as well (Barmina and Kopp 2007). In *L. magnipectinata* males, which have sex combs on both ts1 and ts2 (Fig. 1C), we find that *Scr* is expressed in both segments and is stronger in the region of the developing sex comb teeth (Fig. 3, A–D). Thus, *Scr* expression in this species is comparable to that seen in Old World *Sophophora*. However, a subtle difference between the distribution of Scr in *L. magnipectinata* and the *Sophophora* species previously analyzed is apparent. In the former, we see strong *Scr* expression in sex comb teeth themselves (Fig. 3, A–D), whereas in Old World *Sophophora*, *Scr* appears strong in the region around the teeth, but not the cells that make up the comb (Barmina and Kopp 2007; Tanaka et al. 2011).

The transcription factor *dachshund* (*dac*) is a key patterning gene that defines the medial portion of the leg, including the region that bears sex combs (Kojima 2004). In *D. melanogaster* and other species, *dac* is expressed strongly in ts1, weakly in ts2, and is all but absent from the epithelium of the more distal tarsal segments (Mardon et al. 1994; Atallah et al. 2009b). Although *dac* also plays a general role in the regulation of sensory organ development (reviewed in Angelini and Kaufman 2005), we have found that it is strikingly reduced in the developing sex comb teeth and can represses sex comb formation when expressed ectopically (Atallah et al. 2009b and unpublished data). During sex comb development in *L. magnipectinata*, we observe widespread expression of *dac* in ts1 (Fig. 3, E and F), with much weaker expression in ts2. The expression of *dac* is upregulated in chemosensory bristles, but shows a notable reduction in the sex comb region. This pattern is virtually identical to what we see in Old World *Sophophora* (Atallah et al. 2009b).

Sex comb development in *Sophophora* involves complex movements of epithelial cells that depend on cell adhesion molecules including *Drosophila* E-cadherin, also referred to as *shotgun* (*shg*), and the noncanonical cadherin and planar polarity gene *flamingo* (*fmi*). Both genes are strongly expressed around the developing sex comb in *D. melanogaster*, and perturbing their expression disrupts sex comb development (Atallah et al. 2009a and unpublished data). We see a similar pattern in *L. magnipectinata* (Fig. 4, A–C and F–H), with strong expression of Flamingo and E-cadherin in the sex comb and surrounding cells, suggesting that the same downstream genes are involved in the mechanics of sex comb development in this species.

In *L. magnipectinata*, multiple TBRs rotate and align to form the sex comb

In *Sophophora,* contiguous bristle arrays on the tarsus—namely, the sex comb and TBRs—are formed from bristle precursors that are initially separated by epithelial cells. Both structures later assemble into aligned formations without any intervening cells (illustrated in Fig. 5) as the bristle precursors locate one another and come into contact (Atallah et al. 2009a). In species such as *D. vulcana* in the *montium* subgroup, where sex combs develop from two longitudinal rows, bristle precursors first converge into a single noncontiguous longitudinal row, then come together to form a tightly packed comb (Atallah et al. 2009b; see Fig. 5A). In species where sex combs develop from a rotating distal transverse row (e.g., *D. guanche*), bristle precursors assemble into a contiguous array prior to or during the early stages of rotation (Fig. 5B).

Figure 4 shows what appears to be three stages in the development of the *L. magnipectinata* sex comb. In a young pupal leg, the bristle precursor cells in the region that will form the sex comb are organized into short transverse rows of two to three bristles each, and are still separated by intervening epithelial cells (Fig. 4, A–C and F–H). At a later stage, sex combs on the first and second tarsal segments consist of several short, contiguous rows of bristles, with each row aligned at a slightly different angle relative to the proximo-distal leg axis (Fig. 4, D and I), as would be expected if these rows were in the process of rotation. The rows align and join together into a straight, contiguous sex comb (Fig. 4, E and J; see Fig. 3E for an image of a comb that is entirely contiguous).

Based on these observations, sex comb development in *L. magnipectinata* involves the formation, rotation, and alignment of multiple bristle configurations. The male starts out with short, female-like arrangements (see Fig. 1D) of approximately transverse rows (Fig. 4, A–C), which join together and assemble into progressively longer configurations (Fig. 4, D and E), rotating to a more longitudinal orientation

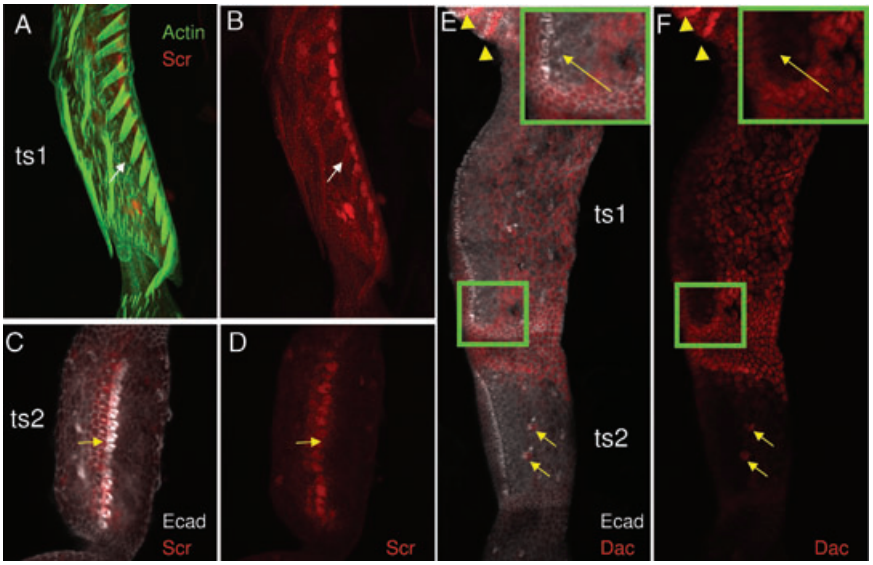

Fig. 3. Expression patterns of *Sex combs reduced (Scr)* and *dachshund (dac)* in *L. magnipectinata* forelegs. (A, B) The first tarsal segment of a developing leg is shown. *Scr* expression (red) is strong in the sex comb. Phalloidin staining (green) outlines bristle shafts, including the sex comb teeth (arrow). (C, D) A similar pattern is observed on the second tarsal segment. E-cadherin staining (white) outlines the bristle and epithelial cells. (E, F) In a 50-h after pupariation (AP) leg stained with antibodies against E-cadherin (white) and Dac (red), we see strong *dac* expression in the tibial TBRs (arrowheads) and in most of ts1, whereas Dac is absent from the sex comb region (large arrow, inset). In ts2, *dac* expression is weak in the distal region of the segment but stronger in chemosensory bristles (arrows). Distal is down in all panels. Scale bar: 10μm.

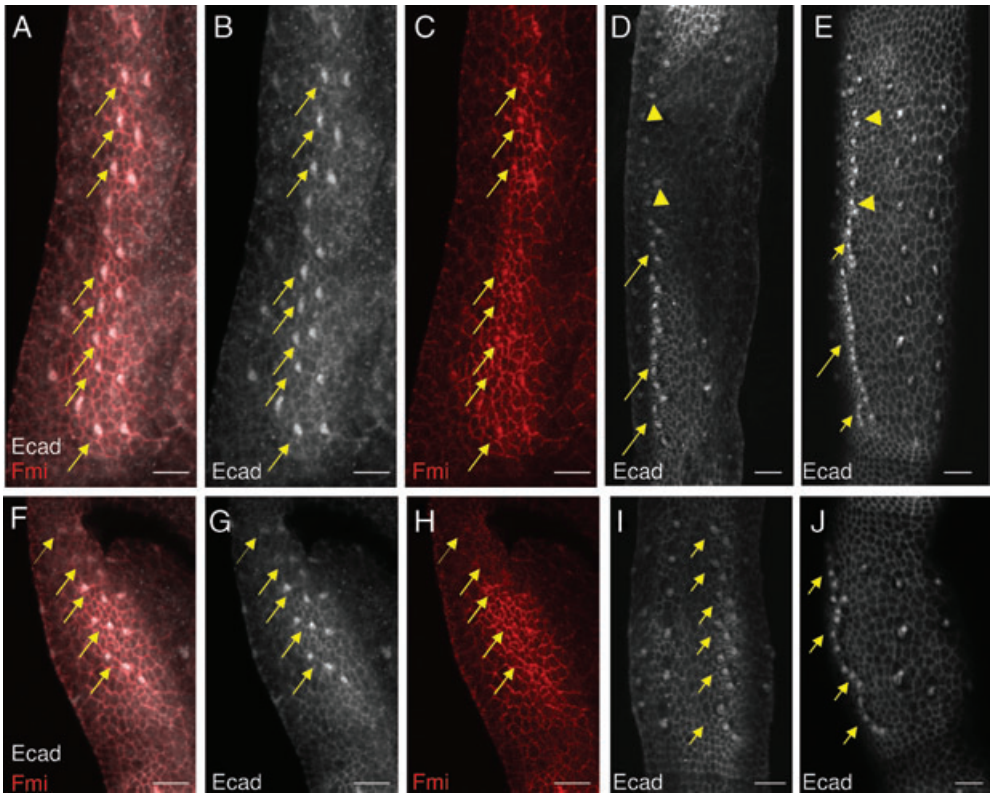

Fig. 4. Morphogenesis of the *L. magnipectinata* sex comb. Panels (A–E) show ts1 and panels (F–J) show ts2. (A–C) A 42-h after pupariation (AP) male pupal foreleg, showing an array of sex comb bristle precursors on ts1 in the sex comb region, with strong expression of E-cadherin (white) and Flamingo (red). The transverse rows (arrows) are still noncontiguous. (D) In a 47-h AP foreleg ts1, the bristle precursors are arranged in short contiguous formations at various degrees of orientation (arrows), with a few noncontiguous bristles still present in the proximal region of the segment (arrowheads). (E) On the ts1 of an older foreleg (>50-h AP), a long contiguous array of bristles is seen in the distal ts1 (large arrow). This array has yet to join up with two shorter rows (small arrows), one proximal and the other distal to it. Configurations of two bristles (arrowheads) and a few isolated bristles are still present in the proximal part of the segment. (F–H) Ts2 of the same leg shown in panels (A–C). (I) An example of the ts2 of a leg showing an intermediate state, with five contiguous TBRs at various angles to the proximodistal axis (arrows). (J) Ts2 of the same leg in (E), showing TBRs in the process of aligning into a contiguous comb. Distal is down in all panels. Scale bar: 10μm.

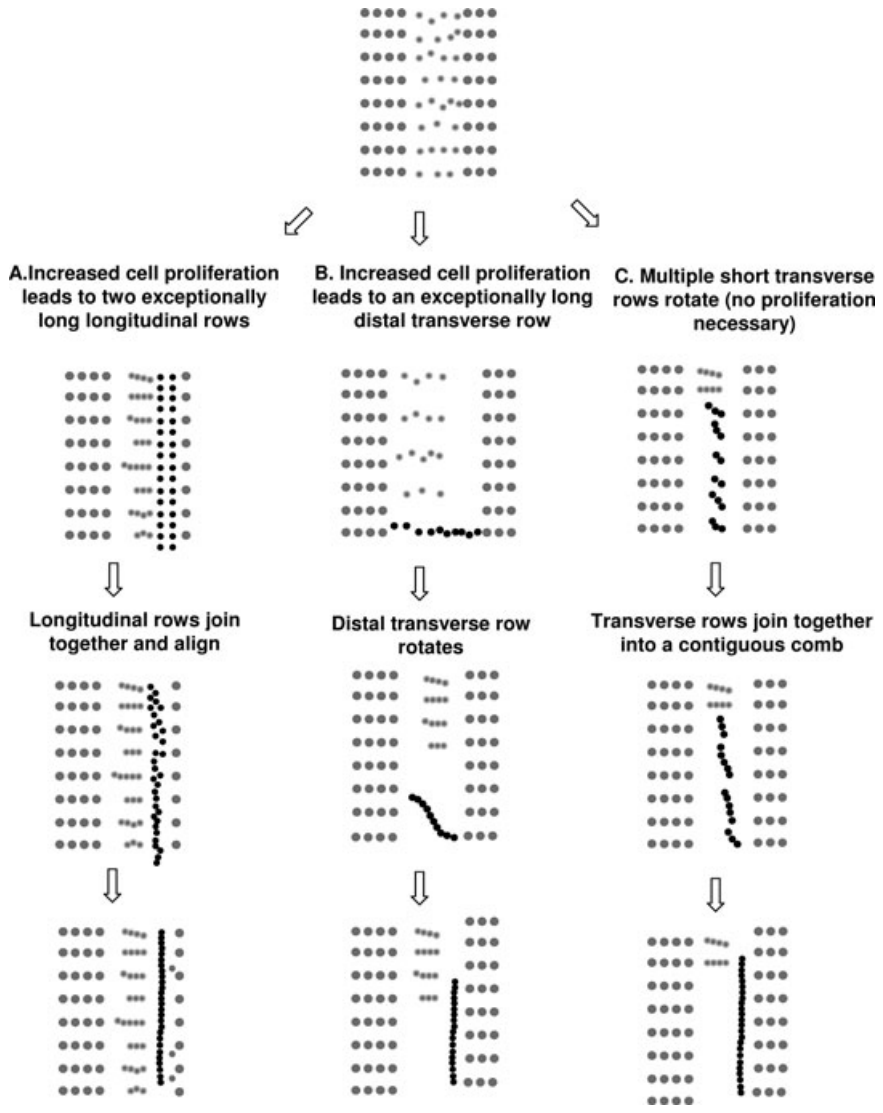

Fig. 5. Extended longitudinal sex combs develop by three different mechanisms. Early in leg development (top), TBRs (small gray circles) develop on the first leg along with seven longitudinal rows (large gray circles). Like the longitudinal row bristles, the TBRs are initially noncontiguous, separated by one or more epithelial cells. Subsequent development of an extended longitudinal sex comb follows one of three processes (bristle precursors that will form the sex comb are shown in black). (A) In species in the *montium* and *ficusphila* subgroups, male-specific cell proliferation causes two of the longitudinal rows, just anterior to the TBRs, to become unusually long. These longitudinal row bristles assemble into a contiguous sex comb. Meanwhile, the TBRs join together into contiguous rows. (B) In *Drosophila guanche*, a comparable wave of male-specific cell proliferation occurs in a distal transverse row. This row self-assembles, then rotates and assumes a longitudinal orientation; the other TBRs self-assemble but do not rotate. (C) In *L. magnipectinata*, multiple TBRs rotate, self-assemble, and join together into a contiguous sex comb, without the male-specific proliferation step involved in the other two mechanisms.

in the process. The *L. magnipectinata* sex comb, therefore is probably homologous to all of the transverse rows on the first two tarsal segments of the adult female (Fig. 1D), with the possible exception of the most proximal one or two rows on ts1, which are sometimes present in both sexes (top of Fig. 1, C and D).

DISCUSSION

The sex comb is a recent evolutionary innovation. As often happens in the evolution of sexual dimorphism, the novel male-specific trait underwent rapid and dramatic diversification, whereas the ancestral morphology was preserved in females with very little change. These features make sex combs an excellent model for examining the plasticity of cellular processes during the evolution of new morphological structures. Are different sex comb morphologies underpinned by the same morphogenetic mechanisms? Conversely, can similar structures be produced in different ways?

Our results argue strongly in favor of the second hypothesis. Clearly, different cellular events give rise to very similar adult structures in different *drosophilid* species. In the *montium* and *ficusphila* subgroups, sex combs form by intercalation of two longitudinal bristle rows. In *D. guanche* and on the first tarsal segment of *D. rhopaloa*, a similar longitudinal sex comb develops by a 90° rotation of a single, greatly enlarged TBR. In both cases, the number of sex comb teeth in males greatly exceeds the number of homologous bristles in females, so that sex comb development requires extensive sex-specific cell proliferation in the presumptive sex comb region. In this report, we document a third cellular mechanism that produces essentially the same adult structure. In *L. magnipectinata,* an extended longitudinal sex comb develops through

what is arguably the simplest evolutionary adjustment, a mechanism that does not require male-specific cell proliferation. Starting from the ancestral, sexually monomorphic arrangement of precursor bristles, multiple short TBRs rotate independently of each other and assemble into a contiguous formation. This mechanism is somewhat reminiscent of the second, but not the first, tarsal segment in *D. rhopaloa* (Tanaka et al. 2009), where two transverse rows join together into a single sex comb. The progressive self-assembly of a sex comb from multiple rotating rows, however, as seen in *L. magnipectinata,* has not been previously observed. Such precise alignment of separate configurations may seem complex, but it likely involves the same cell adhesion mechanisms that drive the assembly of individual TBRs from noncontiguous precursors—a process that occurs in both sexes and long predates the origin of sex combs.

There are other cases where similar and in some cases clearly homologous traits develop by different cellular mechanisms. Insect segments, for example, are homologous as morphological structures and the genetic control of segmentation is strongly conserved, yet segments can form through a variety of processes ranging from subdivision of a fully formed blastoderm to budding from a posterior growth zone (Peel 2004). What is interesting about the sex combs is that a structure that evolved so recently shows such radical disparities in its cellular foundations among closely related species.

The finding that both upstream factors that promote and repress sex comb development in *D. melanogaster*, and downstream adhesion molecules involved in the cell dynamics of sex comb formation, are expressed in comparable patterns in *L. magnipectinata* suggests that this structure has a similar genetic architecture in Old World *Sophophora* and *Lordiphosa*. However, given the conserved functions of these genes in development, this observation is as consistent with their independent co-option in the two lineages as it is with a single origin of the sex comb. It could be argued that it is natural to expect that a trait that evolved on the medial region of first legs would co-opt both the homeotic gene *Scr*, responsible for the development of this body segment, and a leg patterning gene, *dac*, that is critical for the formation of this portion of the appendage. Furthermore, the conserved role of cadherins in cell sorting and cell rearrangement (reviewed in Niessen et al. 2011) ensures that they are likely to be involved in the development of a structure such as the sex comb that forms through dynamic cell rearrangement. Live imaging of these molecules in the developing leg of a variety of species, at a high level of temporal resolution, combined with functional tests, will be necessary to determine whether subtle changes in their distribution underlie the differences in cell dynamics that lead to the formation of sex combs through contrasting mechanisms in divergent lineages.

Future experiments will also be necessary to understand the significance of the only difference we observed in gene expression in *Lordiposa* and Old World *Sophophora*: the strong presence of *Scr* in both the sex comb teeth themselves and the surrounding cells in *L. magnipectinata*, in contrast to the upregulation of *Scr* in the region around the comb, but not the teeth, in *Sophophora* species previously analyzed. In *D. melanogaster*, *Scr* regulates every aspect of comb formation, from morphological development of the teeth to the cell rearrangement in the epithelium responsible for the rotation (Held 2010; Tanaka et al. 2011), but it is not yet understood how the precise spatial distribution of the gene product coordinates these different processes separately.

Neither phylogeny nor comparative analysis of development distinguish between a single or multiple origins of the sex comb, but both scenarios highlight a complex relationship between morphological structures, regulatory genes, and cellular processes. If the sex comb evolved in the common ancestor of *Sophophora* and *Lordiphosa*, conservation at the levels of upstream patterning genes and adult morphology clearly did not preclude major divergence of the cellular processes that connect these levels of biological organization. The other possibility—that sex combs evolved independently in the two lineages—is even more intriguing, as it would indicate that a novel, complex morphological structure can evolve through different changes in development. Such plasticity could considerably lower the bar for evolutionary innovations. The more ways there are to satisfy the same functional requirement by co-opting different cellular processes, the greater the proportion of standing or de novo genetic variation that can be exploited by selection to evolve a novel trait. In this sense, complexity may facilitate rather than impair the origin of evolutionary innovations.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. (A) Vegetation of *Lordiphosa* collection sites, with white flowers of *Anemone flaccida*. (B) Glass vials containing small pieces of leaves and apples for rearing *Lordiphosa magnipectinata*.