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***Drosophila* Sex Combs as a Model of Evolutionary Innovations**

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Abstract

The diversity of animal and plant forms is shaped by nested evolutionary innovations. Understanding the genetic and molecular changes responsible for these innovations is therefore one of the key goals of evolutionary biology. From the genetic point of view, the origin of novel traits implies the origin of new regulatory pathways to control their development. To understand how these new pathways are assembled in the course of evolution, we need model systems that combine relatively recent innovations with a powerful set of genetic and molecular tools. One such model is provided by the *Drosophila* sex comb – a male-specific morphological structure that evolved in a relatively small lineage related to the model species *D. melanogaster*. Our extensive knowledge of sex comb development in *D. melanogaster* provides the basis for investigating the genetic changes responsible for sex comb origin and diversification. At the same time, sex combs can change on microevolutionary timescales and differ spectacularly among closely related species, providing opportunities for direct genetic analysis and for integrating developmental and population-genetic approaches. Sex comb evolution is associated with the origin of novel interactions between HOX and sex determination genes. Activity of the sex determination pathway was brought under the control of the HOX code to become segment-specific, while HOX gene expression became sexually dimorphic. At the same time, both HOX and sex determination genes were integrated into the intrasegmental spatial patterning network, and acquired new joint downstream targets. Phylogenetic analysis shows that similar sex comb morphologies evolved independently in different lineages. Convergent evolution at the phenotypic level reflects convergent changes in the expression of HOX and sex determination genes, involving both independent gains and losses of regulatory interactions. However, the downstream cell differentiation programs have diverged between species, and in some lineages similar adult morphologies are produced by different morphogenetic mechanisms. These features make the sex comb an excellent model for examining not only the genetic changes responsible for its evolution, but also the cellular processes that translate DNA sequence changes into morphological diversity. The origin and diversification of sex combs provides insights into the roles of modularity, cooption, and regulatory changes in evolutionary innovations, and can serve as a model for understanding the origin of the more drastic novelties that define higher-order taxa.

Keywords

Evolution of development; evolutionary innovations; sexual dimorphism; convergent evolution

The origin of new morphological traits implies the origin of new regulatory pathways to control their development. In many cases, evolutionary innovations reflect the emergence of novel regulatory interactions among previously unconnected genes (Wilkins 2002; Carroll 2005; Carroll 2008). Identifying these changes remains a significant challenge. A fundamental constraint is that innovations are not very common, and rarely occur in

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convenient model systems. As a result, most developmental-genetic studies of morphological evolution have focused on secondary losses of traits (Swalla and Jeffery 1996; Sucena and Stern 2000; Abouheif and Wray 2002; Cresko et al. 2004; Shapiro et al. 2004; Yamamoto et al. 2004; Protas et al. 2006), while studies addressing the gain of new characters have been less common (Keys et al. 1999; Kopp et al. 2000; Wang and Chamberlin 2004; Moczek et al. 2006; Prud'homme et al. 2006). Intuitively, however, it seems that the genetic basis of innovation should be considerably more complex than the genetic basis of evolutionary loss.

A truly comprehensive understanding of evolutionary innovations must also include the cellular processes that link the evolution of genes to the evolution of phenotypes. What are the downstream consequences of changes in gene expression or protein activity? How do changes in DNA sequences affect cell differentiation and morphogenesis to produce novel adult structures? The sculpting of three-dimensional body parts involves precisely coordinated patterns of cell behavior, so that the origin and diversification of new structures requires a variety of cellular processes to be modified and brought under the control of new regulators.

The *Drosophila* sex comb, a recently evolved male-specific morphological structure, provides an excellent model for understanding evolutionary innovations. In this review, I describe the genetic control of sex comb development in *D. melanogaster*, and examine changes in the regulatory pathway that correlate with sex comb diversity. I then focus on the cellular mechanisms of sex comb formation to consider potential proximate causes of phenotypic divergence, and examine the microevolution on sex combs in an effort to understand the intraspecific origin of developmental variation and the evolutionary forces that maintain this variation in nature. Finally, I consider the general lessons that *Drosophila* sex combs can teach us about the processes responsible for evolutionary innovations.

Sex comb ontogeny and function

The sex comb of *D. melanogaster* is a male-specific array of modified bristles that develops at a precise position on the prothoracic (T1) leg from a set of precursor bristles present in both sexes (Hannah-Alavah 1958; Tokunaga 1962) (Figure 1). Bristles on the ventral-anterior surface of the distal tibia and the most proximal tarsal segment of the T1 leg are arranged in tightly packed rows perpendicular to the proximo-distal axis of the leg. These transverse bristle rows (TBRs) form a brush that the flies use to clean their head and eyes (Szebenyi 1969), and are present in many Dipterans (McAlpine 1981). In *D. melanogaster*, the sex comb develops from the most distal TBR on the first tarsal segment (ta1). In males, this TBR rotates 90° clockwise (as viewed from the dorsal side), so that the bristles become oriented from anterior/dorsal to posterior/ventral and point away from the leg (Tokunaga 1962; Held et al. 2004; Atallah et al. 2009a) (Figure 1 B, D, F). In females, all TBRs retain normal, proximal to distal orientation (Figure 1 A, C, E). In addition, the bristles ("teeth") recruited into the sex comb undergo a number of morphological modifications. Sex comb teeth are curved and blunt rather than straight and pointed like other mechanosensory bristles, heavily melanized, and thicker than regular tarsal bristles (Figure 1 B, D).

The sex comb is a recently evolved structure; in fact, the vast majority of *Drosophila* species lack sex combs. It is present only in the *melanogaster*, *obscura*, *fima*, and *dentissima* species groups of the subgenus *Sophophora*, and (possibly independently) in the genus *Lordiphosa* (Tsacas 1980; Tsacas 1981; Lakovaara and Saura 1982; Lemeunier et al. 1986; Hu and Toda 2000; Lachaise and Chassagnard 2002). Dramatic differences exist among closely related species in the orientation of the sex comb, the number of sex comb teeth, and the degree of their modification (Figure 2) (Kopp and True 2002; Barmina and Kopp 2007). In species

that lack sex combs, mechanosensory bristle pattern on the T1 leg is identical in males and females, and the female pattern is conserved throughout *Drosophilidae*. This suggests that the sex comb evolved through sex-specific modification of a pre-existing developmental pathway. *Drosophila* males use their sex combs as part of stereotypical mating behavior but their exact function varies among species (Spieth 1952), suggesting that tactile interactions between males and females through the male's sex comb are an important part of the mating ritual and offering a likely explanation for the rapid diversification of this structure.

Genetic control of sex comb development: integration of multiple regulatory inputs

Segment specificity

Sex comb development in the T1 leg is promoted by the HOX gene *Sex combs reduced* (*Scr*). Sex comb is lost entirely in homozygous *Scr* null clones (Struhl 1982) and in trans-heterozygous combinations of strong hypomorphic mutations (Lewis et al. 1980; Pattatucci et al. 1991). A strong reduction in the number of sex comb teeth is seen in weaker hypomorphic combinations and in flies heterozygous for *Scr* nulls or deficiencies (Kaufman et al. 1980; Lewis et al. 1980; Pattatucci et al. 1991). Conversely, *Scr* duplications increase the number of sex comb teeth (Capdevila et al. 1986; Kennison and Russell 1987; Boube et al. 1997). Moreover, gain-of-function mutations that cause ectopic expression of *Scr* in T2 and T3 legs are sufficient to induce ectopic sex combs in these legs (Hannah-Alava 1964; Kaufman et al. 1980; Kennison and Tamkun 1988; Pattatucci and Kaufman 1991). Analysis of temperature-sensitive *Scr* alleles indicates that *Scr* is required for sex comb development during late third larval instar and early pupal development (Pattatucci et al. 1991). HOX proteins often bind DNA and regulate target genes in heterodimeric complexes with another transcription factor, Extradenticle (*Exd*) (Mann and Chan 1996). However, *exd* is not required for sex comb development, although it is essential for other *Scr* functions (Percival-Smith and Hayden 1998).

Sex specificity

Sexually dimorphic differentiation of most somatic tissues in *Drosophila* is controlled by a splicing cascade that culminates in the production of sex-specific isoforms of the transcription factor *doublesex* (*dsx*) (Baker and Ridge 1980; Baker and Wolfner 1988; Bell et al. 1988; McKeown 1992). The male-specific isoform (*dsxM*) promotes the development of male-specific structures, including the sex comb, and represses female-specific structures, while the female-specific isoform (*dsxF*) promotes female-specific and represses male-specific structures (Baker and Ridge 1980; Jursnich and Burtis 1993; Li and Baker 1998; Waterbury et al. 1999). *DsxM* and *DsxF* proteins share a common N-terminal DNA-binding domain, but have different C-terminal domains that have different effects on target gene expression (Burtis et al. 1991; Coschigano and Wensink 1993; Erdman and Burtis 1993; Narendra et al. 2002).

In *dsx* null mutants, both males and females develop a vestigial sex comb: the bristles of the most distal TBR assume morphology intermediate between sex comb teeth and regular bristles, and undergo a partial rotation (Hildreth 1965; Baker and Ridge 1980). A similar intermediate sex comb develops in dominant *dsx* mutants that express both male- and female-specific *dsx* isoforms (Baker and Ridge 1980; Nagoshi and Baker 1990). Overexpression of *dsxM* results in the development of ectopic sex comb teeth (Jursnich and Burtis 1993; Tanaka et al. submitted), whereas *dsxF* can reduce the number of sex comb teeth when expressed in males (Waterbury et al. 1999). *dsx* mutant clones induced at the end of larval development produce intermediate sex combs typical of *dsx* homozygotes,

indicating that *dsx* function is cell-autonomous and required during the pupal stage (Baker and Ridge 1980).

Anterior-Posterior and Dorsal-Ventral axes

Each leg imaginal disc is subdivided into anterior and posterior compartments by the selector gene *engrailed* (*en*) and the Hedgehog (Hh) signaling pathway (Morata and Lawrence 1975; Tabata et al. 1995; Zecca et al. 1995; Lawrence and Struhl 1996). Two other signaling pathways, Wingless (Wg) and Decapentaplegic (Dpp), pattern the leg along the dorso-ventral axis, with Wg promoting ventral and Dpp dorsal pattern elements (Baker 1988; Couso et al. 1993; Brook and Cohen 1996; Jiang and Struhl 1996; Penton and Hoffmann 1996). The sex comb and TBRs develop in the ventro-lateral sector of the anterior compartment, in the lateral portion of the Wg-dependent domain. *en* acts cell-autonomously to restrict sex comb development to the anterior compartment (Tokunaga 1961). In *en* mutants, an ectopic sex comb and a set of TBRs develop in the posterior compartment in mirror-image to the normal structures; the ectopic sex comb also rotates in the opposite direction (Tokunaga 1961; Mukherjee 1965). Along the DV axis, sex comb development is promoted by *wg* and repressed by *dpp*. Sex comb teeth are lost, and tissue rotation fails, in *wg* hypomorphic mutants and in clones that are deficient for Wg signal transduction (Sharma and Chopra 1976; Theisen et al. 1994). Conversely, ectopic *wg* expression can induce sex comb development in lateral and dorsal positions in the leg (Struhl and Basler 1993; Wilder and Perrimon 1995; Maves and Schubiger 1998). Ectopic dorsal sex combs also develop in *dpp* hypomorphs (Spencer et al. 1982; Held et al. 1994) and in mutants that are deficient for Dpp signal transduction (Jiang and Struhl 1996; Penton and Hoffmann 1996; Theisen et al. 1996).

Proximo-distal axis

The patterning of *Drosophila* leg along the proximo-distal (PD) axis is coordinated by the Wg, Dpp, and EGF signaling pathways, which induce the expression of several transcription factors in concentric domains that correspond to future PD regions of the leg (Diaz-Benjumea et al. 1994; Lecuit and Cohen 1997; Campbell 2002; Galindo et al. 2002). Different combinations of these transcription factors define the identity of specific leg segments (Cohen et al. 1989; Godt et al. 1993; Mardon et al. 1994; Lecuit and Cohen 1997; Duncan et al. 1998; Galindo and Couso 2000; Kojima 2004). The distal first tarsal segment (t1), where the sex comb develops in *D. melanogaster*, is characterized by coexpression of *Distal-less* (*Dll*), *dachshund* (*dac*), *rotund* (*rn*), and a low level of *bric a brac* (*bab*) (Agnel et al. 1992; Godt et al. 1993; Campbell and Tomlinson 1998; Couderc et al. 2002; Galindo et al. 2002). This combination of transcription factors is unique within the developing leg. The tibia, the leg segment just proximal to the tarsus, lacks *rn* and *bab* expression, while the second tarsal segment has much lower levels of *dac* but higher levels of *bab* (Godt et al. 1993; Abu-Shaar and Mann 1998; Chu et al. 2002; Couderc et al. 2002).

Dll and *dac* promote sex comb development, whereas *bab* exerts an inhibitory influence. Hypomorphic *Dll* alleles reduce the number of sex comb teeth (Cohen et al. 1989) and suppress the extra sex comb phenotype of *Polycomb* mutants (Kennison and Tamkun 1988). Gain-of-function *dac* alleles that cause ectopic expression of *dac* in the more distal tarsal joints induce the development of additional sex combs in these joints (Docquier et al. 1997). Loss or reduction of *bab* function in the leg leads to the development of ectopic sex combs on t2, t3, and sometimes even on t4 (Godt et al. 1993; Couderc et al. 2002). Conversely, overexpression of *bab* reduces the number of sex comb teeth (Couderc et al. 2002).

Joint-dependent positioning

In addition to its position along the global PD axis of the leg, the developing sex comb seems to “know” its position within each individual leg segment. In *D. melanogaster*, the sex comb normally forms only at the distal end of t1. However, mutations or ectopic expression of a variety of genes, including *bab*, *dac*, *bowl*, and *sex combs distal* lead to the development of ectopic sex combs on t2, and sometimes also t3 and t4 (Kennison 1992; Godt et al. 1993; Boube et al. 1997; Couderc et al. 2002; de Celis Ibeas and Bray 2003). In each case, the ectopic sex combs develop at the distal end of each tarsal segment, skipping its proximal part. Thus, the sex comb becomes discontinuous along the global PD axis of the leg, yet occupies correct PD position within each segment. This rule is only violated in some mutants that disrupt joint formation, leading to the fusion of neighboring leg segments. In such mutants, which include *combgap* (Datta and Mukherjee 1971), *four-jointed (fj)* (Tokunaga and Gerhart 1976), and components of the Jak/Stat and Rho/Rac signaling pathways (Kopp, unpublished), sex comb teeth sometimes “spill over” into the proximal t2.

Leg segmentation in *Drosophila* is controlled by the Notch signaling pathway (de Celis et al. 1998; Bishop et al. 1999; Rauskolb 2001), with an additional role played by a less characterized pathway that involves the secreted Fj protein and the *abelson* tyrosine kinase (Zeidler et al. 2000; Buckles et al. 2001). Notch signaling is activated in a series of rings located just proximal to each future joint (Bishop et al. 1999; de Celis Ibeas and Bray 2003; Hao et al. 2003). It is possible that Notch controls sex comb development by modifying the expression of PD patterning genes, many of which are modulated in a series of peaks and troughs that depend on Notch signaling (Godt et al. 1993; de Celis et al. 1998; Duncan et al. 1998; Bishop et al. 1999; Rauskolb and Irvine 1999). In particular, *bab* expression in the leg is partially regulated by *bowl*, a downstream target of Notch signaling (de Celis Ibeas and Bray 2003).

Integration of sex-specific, homeotic, and intrasegmental patterning cues

As this overview shows, the sex comb must integrate a wide variety of regulatory inputs to develop at the appropriate position. A central role in this integration is played by *Scr* and *dsx* (Barmina and Kopp 2007; Tanaka et al. submitted). *Scr* is expressed at a low level in most of the developing pupal leg, but is strongly upregulated in a ventral-anterior sector of the first tarsal segment and distal tibia, coincident with the future TBR and sex comb region. This expression domain is controlled by *dac* and *bab* along the PD axis, and by Wg signaling around the leg circumference (Barmina and Kopp 2007; Shroff et al. 2007; Randsholt and Santamaria 2008). Overexpression of *Scr* is sufficient to induce sex comb development in distal tarsal segments (Barmina and Kopp 2007). This indicates that quantitative modulation of *Scr* within the T1 leg defines the proximo-distal extent of the sex comb, and that the main function of *dac*, *bab*, and other PD patterning genes in sex comb development is to define the domain of high-level *Scr* expression. In contrast, even very high levels of *Scr* can only induce ectopic sex combs on the anterior-ventral leg surface, suggesting that Wg signaling, in addition to regulating *Scr* expression along the DV axis, is required in parallel with *Scr* to promote sex comb development (Barmina and Kopp 2007; Shroff et al. 2007).

In *D. melanogaster*, *Scr* expression in the T1 leg is sexually dimorphic. In males, *Scr* protein levels are highest in the distal part of t1 around the developing sex comb, whereas no such upregulation is observed in females (Barmina and Kopp 2007). This modulation is controlled by *dsx*, and, given the importance of precise quantitative control of *Scr* expression, plays an important role in determining sex comb morphology and position (Tanaka et al. submitted). *dsx* itself is expressed in the T1, but not in T2 or T3 legs, and is tightly localized to the presumptive sex comb region in the distal, anterior-ventral part of t1 (Robinet et al., 2010; Tanaka et al., submitted). This expression is activated by *Scr* during

late third larval instar, and is further restricted along the PD axis so that *dsx* is expressed only in the distal-most portion of the high-level *Scr* domain. Ectopic expression of *dsx* in the more proximal parts of t1 is sufficient to induce ectopic sex comb teeth, indicating that sex comb position within the high *Scr* domain is defined by localized *dsx* expression (Tanaka et al. submitted).

Thus, *Scr* and *dsx* form a positive feedback loop: *Scr* activates *dsx* expression during late larval stage, and *dsx* modulates *Scr* expression quantitatively in the pupa. This autoregulatory module plays a key role in integrating the homeotic, sex-specific, proximo-distal, and circumferential regulatory inputs to induce sex comb development at a precisely defined position within the leg. The molecular mechanisms of this cross-regulation are not yet clear, and may be different in different cell types. *Scr* is required in the leg epidermis to delimit sex comb position and in the bristle precursor cells to determine the number of sex comb teeth. Similarly, *dsx* functions in the epidermis to position the sex comb and in the bristle cells to specify the male-specific morphology of sex comb teeth (Tanaka et al. submitted). *Scr* protein becomes undetectable in the differentiating sex comb teeth by the end of sex comb rotation, but is strongly upregulated in the adjacent epidermal cells. At the same stage, *Dsx* protein is present at a high level in the differentiating teeth, but disappears from most of the surrounding epithelium (Tanaka et al. submitted). This suggests that the regulatory interaction between *dsx* and *Scr* may be at least partly indirect and involve cell-cell signaling.

A key conclusion emerging from these studies is that the HOX and sex determination genes are not acting like “master regulatory genes” that modulate the output of the spatial patterning network from the outside. Rather, they are deeply integrated inside this network, where they coordinate multiple regulatory inputs and translate them into cell-type-specific differentiation.

Quantitative control of gene expression

An interesting aspect of sex comb development is that many of the regulatory genes including *Scr*, *Dll*, and *bab* (Kaufman et al. 1980; Kennison and Tamkun 1988; Cohen et al. 1989; Godt et al. 1993; Dworkin 2005) affect sex comb size and position in a dosage-sensitive manner. For example, males heterozygous for *Scr* null alleles have only half of the normal number of sex comb teeth (Kaufman et al. 1980), while males heterozygous for deficiencies that delete both *bab* paralogs have larger than normal sex combs on t1 as well as ectopic sex comb teeth on t2 (Godt et al. 1993; Couderc et al. 2002). These phenotypes are consistent with the observation that differences in *Scr* expression between distal and proximal t1, and between distal t1 and t2, are quantitative rather than absolute (Barmina and Kopp 2007; Tanaka et al. submitted). The positive feedback loop between *Scr* and *dsx* may be acting as an amplification mechanism that makes *Scr* and *dsx* expression levels, and thus sex comb development, particularly sensitive to subtle quantitative changes. This feature may have important implications for understanding the rapid diversification and convergent evolution of sex comb morphology (see below).

Evolution of sex comb morphology: phenotypic patterns and regulatory mechanisms

Rapid diversification and convergent evolution

In the *melanogaster* and *obscura* species groups, sex combs vary dramatically in size (the number of teeth), orientation (along or perpendicular to the proximo-distal leg axis), and tooth morphology (size, shape, color, and the presence of bracts) (Lemeunier et al. 1986; Kopp and True 2002). Phylogenetic analysis reveals many instances of rapid divergence and

convergent evolution (Barmina and Kopp 2007) (Figure 2). Some of these changes have occurred on short evolutionary time scales. For example, *D. bipectinata* and *D. malerkotliana* are closely related, inter-fertile species that diverged well under one million years ago (Kopp and Barmina 2005). However, *D. malerkotliana* has a transverse sex comb consisting of only 5-6 teeth whose morphology is barely distinguishable from regular mechanosensory bristles, while *D. bipectinata* has a longitudinal sex comb containing up to 24 teeth that are greatly enlarged, curved, and melanized compared to other bristles (Figure 2). Conversely, the sex comb has been secondarily lost in *D. prolongata*, whose nearest relatives have some of the largest sex combs found in any species (Figure 2). Pervasive convergent changes affect the size, position, and orientation of the sex comb, as well as the morphology of individual teeth. In particular, large longitudinal sex combs occupying most of t1 and t2 evolved independently in the *montium*, *ficuspila*, and *rhopaloea* subgroups of the *melanogaster* species group and in some species of the *obscura* species group (Figure 2). This suggests that some sex-specific regulatory pathways have been reinvented, or at least reestablished, several times.

Evolving expression of *Scr* and *dsx* is associated with sex comb diversity

There is a strong correlation between the expression of *Scr* and *dsx* and the presence, size, and morphology of the sex comb. In species that primitively lack sex combs, *Scr* expression is only weakly modulated in pupal T1 legs, and does not differ between males and females (Barmina and Kopp 2007). A similar pattern is seen in species with transverse sex combs, and in species that have secondarily lost sex combs. On the other hand, in species with longitudinal sex combs, *Scr* is upregulated in the presumptive sex comb region. The distal boundary of high *Scr* expression corresponds to sex comb position: in species that have sex combs on both t1 and t2, *Scr* is also upregulated in both segments, while in species that have sex combs only on t1 *Scr* is upregulated only in t1. The presence of sex combs and high *Scr* expression in t2 is the ancestral condition for the *melanogaster* and *obscura* species groups, and was lost independently in five different clades (Barmina and Kopp, 2007).

dsx shows an equally close correlation with sex comb diversity. In flies that primitively lack sex combs, *dsx* is not expressed in the homologous region in either sex, while in the *melanogaster* and *obscura* species groups the domains of *dsx* expression in males coincide with sex comb position (Tanaka et al. submitted). For example, in species that have longitudinal sex combs along the entire t1 and t2 segments, *dsx* expression is expanded to the full length of these segments so that all sex comb teeth express Dsx. In all species, there is a close correspondence between *dsx* and high *Scr* expression (Tanaka et al. submitted). Moreover, in species with longitudinal sex combs *Scr* expression is sexually dimorphic so that both the spatial extent and the cell-by-cell levels of *Scr* are greater in males than in females (Barmina and Kopp, 2007). On the other hand, flies with transverse sex combs have sexually monomorphic *Scr* expression. Sex-specific expression of *Scr* has been gained and lost multiple times within the *melanogaster* species group, correlating with changes in *dsx* expression and with the gains and losses of longitudinal sex combs (Barmina and Kopp 2007; Tanaka et al. submitted).

This correlation, combined with the experimental analysis of *Scr* and *dsx* functions in *D. melanogaster*, suggests that the cross-regulation between the HOX and sex determination genes is a general feature of sex comb development, and that changes in this interaction have played a key role in sex comb evolution (Figure 3). *dsx* expression was absent, and *Scr* expression was sexually monomorphic, in the ancestral condition. The origin of sex combs required the evolution of *Scr*-dependent *dsx* expression in the T1 leg, while the subsequent diversification of sex comb morphology involved repeated gains and losses of *dsx*-dependent modulation of *Scr* expression, as well as changes in the regulation of both genes by the PD patterning cues.

It is conceivable that the positive cross-regulation between *Scr* and *dsx* facilitated the rapid divergence and convergence of sex combs. Any mutation affecting the expression of either gene would be amplified by this feedback loop to produce a noticeable phenotypic change in the size and orientation of the sex comb and become “visible” to selection. Male-specific traits involved in courtship and mating are often gained and lost on short evolutionary timescales (Wiens 1999; Kimball et al. 2001; Wiens 2001). In many cases, the rapid evolution of male sexual characters reflects equally rapid changes in female preferences for these traits (Wiens 2001; Wong and Rosenthal 2006). It is tempting to speculate that selection for larger or smaller sex combs has often reversed direction in the *melanogaster* and *obscura* species groups, and that the positive cross-regulation between *Scr* and *dsx* has allowed these species to respond rapidly to changes in selective pressure.

Did sex combs evolve more than once?

Although sex combs are best described in a monophyletic lineage composed of the *melanogaster* and *obscura* species groups in the subgenus *Sophophora*, these structures are also found in the *fima* and *dentissima* species groups, whose phylogenetic positions within *Sophophora* are not entirely clear (Tsacas 1980; Tsacas 1981; Tsacas and Lachaise 1981b; Tsacas and Lachaise 1981a; Lachaise and Chassagnard 2002), as well as in some species of the little-studied genus *Lordiphosa* (Gao et al., submitted; Hu and Toda, 2000). In particular, males in the *L. miki* species group have large longitudinal sex combs that are remarkably similar to those found in the *montium* and *hopaloe* subgroups in *Sophophora* (Figure 4 B-D, compare with Figure 2), while species in the *L. denticeps* group have weakly developed transverse sex combs. Phylogenetic analyses using molecular and morphological data suggest that *Lordiphosa* are the sister group of the Neotropical *Sophophora* lineage, which includes the *willistoni* and *saltans* species groups that lack sex combs (Figure 4 A) (Gao et al., submitted; Hu and Toda, 2000; Katoh et al., 2000). Interestingly, the *miki* group occupies a clearly derived position within *Lordiphosa* (Gao et al., submitted). This raises an intriguing possibility that sex combs evolved independently two or three times – once in the common ancestor of the *melanogaster* and *obscura* species groups, and once or twice in *Lordiphosa* (Figure 4 A). Alternatively, sex combs may have originated once in the common ancestor of *Sophophora* and *Lordiphosa*, and subsequently lost in several lineages: once in the Neotropical *Sophophora*, and two or more times within *Lordiphosa*. The rapid evolution of developmental mechanisms underlying sex comb morphology in the *melanogaster* and *obscura* groups makes both of these scenarios plausible. Analysis of sex comb development in the *L. miki* species group could help resolve this question, but unfortunately no *Lordiphosa* species are currently available in culture.

Cellular processes underlying sex comb diversity

Different sex combs develop from homologous precursors

The most obvious interspecific differences in sex comb morphology concern their orientation: longitudinal (along the PD axis) *vs* transverse (perpendicular to the PD axis). Due to this distinction, the sex combs of closely related species can differ dramatically despite developing from homologous TBRs. For example, the t1 sex combs of two close relatives, *D. mimetica* and *D. biarmipes*, develop from the two most distal TBRs in both species, and have the same number of teeth (9-10 in *D. biarmipes* *vs* 9-11 in *D. mimetica*) (Figure 5). However, in *D. mimetica* the bristles recruited into the sex comb remain organized into TBRs, so that the only differences between males and females concern the morphology of individual bristles (Figure 5 A, C, E). In *D. biarmipes*, bristle rows that make up the sex comb are turned 90 degrees relative to the remaining TBRs, while the area normally occupied by these rows is denuded of bristles (Figure 5 B, D, F). This difference is produced by cell rearrangement in the epithelial region surrounding the sex comb in *D.*

biarmipes (Atallah et al. 2009b; Tanaka et al. 2009). Initially, bristle precursor cells at the distal end of t1 are arranged into two TBRs in both males and females of this species. In males, these TBRs undergo a coordinated migration in the anterior-dorsal direction, rotating 90° and coming to rest along the PD leg axis. In contrast, no such movement takes place in *D. mimetica*. Thus, the sex comb of *D. biarmipes* is homologous to female TBR bristles and to the transverse sex combs of other *Drosophila* species. A similar mechanism produces longitudinal sex combs in distantly related species belonging to the *obscura* species group and to the *melanogaster*, *ananassae*, and *rhopaloea* subgroups of the *melanogaster* species group (Figure 2) (Atallah et al. 2009b; Tanaka et al. 2009).

Similar sex combs develop from non-homologous precursors

In other species, the presence of longitudinal sex combs is due to a completely different mechanism. *D. ficusphila* and most species in the *montium* subgroup have sex comb teeth arranged in a single row along the PD leg axis (Figure 6 B, D-G). The entire ventral-anterior surface of the t1 and t2 segments, which is occupied by 8-10 TBRs in females, is devoid of bristles in males, leaving only the vestiges of the two most proximal TBRs. This difference is not produced by bristle migration, which does not occur in these species. Instead, bristle precursor cells are specified at different positions in males vs females (Atallah et al. 2009b; Tanaka et al. 2009). From this point of view, the sex combs of *D. ficusphila* and the *montium* subgroup species are not homologous to the female TBRs, or to the transverse and actively rotating sex combs of other species.

Thus, longitudinal sex combs can develop through either sex-specific patterning of bristle precursor cells, or through male-specific migration of sexually monomorphic precursors. These two modes of development must be based on different molecular changes. Coordinated epithelial movement in species with rotating sex combs must require sex-specific regulation of cytoskeletal and cells adhesion molecules. On the other hand, sex comb development in *D. ficusphila* and the *montium* subgroup most likely involves changes in the spatial patterning of bristle precursors by proneural genes (Orenic et al. 1993; Joshi 2006). Despite these fundamental differences, the two mechanisms can produce similar adult structures, for example in *D. kikkawai* and *D. rhopaloea*, or in *D. nikananu* and *D. melanogaster* (Figure 2). Moreover, each mechanism has evolved repeatedly in different clades (Tanaka et al. 2009), suggesting that selection can recruit not only different genes, but completely distinct developmental pathways to meet similar functional requirements in different species.

Multiple mechanisms contribute to variation in sex comb size

The number of sex comb teeth varies from two in *D. eugracilis* and *D. dispar* to over 40 in *D. ficusphila* and most species of the *montium* subgroup. In species with transverse sex combs, interspecific variation in sex comb size is due solely to the different number of TBR bristles that undergo male-specific modification. In fact, the arrangement of TBRs is so regular and invariant that it is possible to establish the homology of individual sex comb teeth across species (Figures 5, 6). The recruitment of teeth always proceeds from anterior-distal to posterior-proximal bristles. For example, in *D. eugracilis* only the most anterior bristles in the two most distal TBRs on t1 are enlarged to produce sex comb teeth (Figure 6 A, C). On the other hand, in *D. mimetica* all bristles in these two TBRs are recruited into the sex comb (Figure 5 A, C, E), while in *D. ananassae* the sex comb occupies parts of the four most distal TBRs (Figure 2).

In species with rotating sex combs, differences in sex comb size also reflect sex-specific patterning of bristle precursors. In these species, males have fewer TBRs but more bristles per TBR in the presumptive sex comb region. For example, *D. melanogaster* males have an

average of seven TBRs in t1 compared to eight rows in females, but the most distal TBR contains 10-12 bristles in males compared to 3-5 bristles in females. This difference is more extreme in species with larger sex combs. In *D. guanche* males, the most distal t1 TBR has over 20 bristles, while the more proximal part of the segment carries 3-4 TBRs. In contrast, females have twice as many TBRs in t1 but the distal-most TBR contains only 2-4 bristles. Sexual dimorphism in the number and pattern of bristle precursors is even more pronounced in t2. Males of *D. guanche* have 20-22 sex comb teeth in this segment that originate as a single TBR (Tanaka et al. 2009), whereas females have only 6-7 bristles arranged in three TBRs. Similar levels of sexual dimorphism are found in species with non-rotating longitudinal sex combs. In *D. ficusphila*, the number of sex comb teeth in t2 greatly exceeds the number of TBR bristles in females (18-21 vs 9-10) (Figure 6 F, G). In other species with large rotated sex combs, such as *D. kikkawai* or *D. rhopaloea*, the number of sex comb teeth on t2 is also 1.5 – 2 fold greater than the number of female bristles.

This dimorphism suggests that *Scr* and *dsx* act in conjunction with proximo-distal patterning genes to regulate the specification of bristle precursor cells. In *D. melanogaster*, overexpression of *Scr* in t2 induces the development of sex combs on that segment in males, and of supernumerary bristles arranged into small TBRs in females. Comparative evidence also suggests that the development of large rotated sex combs on t2 in some species is caused, at least in part, by distal expansion of *Scr* expression. In some sense, this can be viewed as a male-specific homeotic transformation of the t2 towards t1 segment identity.

Sculpting the bristles

Sex comb rotation shows a strong correlation with the modification of individual teeth. In species that have transverse sex combs, the teeth do not differ greatly from their homologous female bristles. In such species, which include most members of the *ananassae*, *takahashii*, and *elegans* subgroups, sex comb teeth are somewhat enlarged and thickened, and are usually slightly blunter and darker than other t1 bristles. However, they remain completely or nearly straight and are only weakly melanized, so that the difference between sex comb teeth and “normal” mechanosensory bristles is one of degree rather than kind. On the other hand, in species with longitudinal sex combs the difference between sex comb teeth and regular bristles is qualitative and unmistakable. In such species, which include all members of the *montium* and *melanogaster* subgroups, most members of the *rhopaloea* subgroup, *D. ficusphila*, *D. bipectinata*, and several other species, the teeth are usually completely black. They are also enlarged and curved, sometimes dramatically, and in contrast to normal mechanosensory bristles are often rounded at the tips. In some species of the *montium* subgroup, sex comb teeth have a peculiar flattened or “figure 8” – shaped cross-section (Figure 7).

We know enough about bristle development to hazard some tentative guesses about the cellular processes that may shape the evolution of sex comb teeth. Bristle size, shape, and curvature are determined by directed deposition and crosslinking of actin filaments (Tilney et al. 1996; Tilney et al. 2004; Tilney and DeRosier 2005). The bristle shaft is a cytoplasmic extension of a single cell (trichogen), and is supported by massive actin bundles positioned around its circumference. The number and arrangement of these bundles can be determined from the regular ridges they form on the bristle surface. Typical mechanosensory bristles on *Drosophila* legs are composed of ~12-20 such bundles. However, sex comb teeth of many species contain a much greater number of bundles – well over 40 in the case of *D. ficusphila* and most *montium* subgroup species. Bristle elongation occurs at the tip, where actin filaments form numerous dynamic microvilli that eventually merge into larger actin bundles that become stabilized by crossbridging and binding to the cell membrane. Variation in the thickness of sex comb teeth is presumably due to differences in the number of actin microvilli and the pattern of their merger into larger, stable bundles.

Differences in actin filament dynamics at the tip of the bristle shaft may also be responsible for interspecific variation in the shape of sex comb teeth. In species with sharply tapered and pointed teeth, as for example in *D. suzukii* (Figure 7), the rate at which microvilli are initiated may decline steadily as the shaft elongates. In its close relative *D. biarmipes*, which has rounded sex comb teeth (Figure 5 B), microvilli initiation may occur at a constant rate until the very end of tooth elongation, and then cease abruptly.

In many species, a major distinction between sex comb teeth and homologous female bristles is their strong curvature. The actin bundles that surround the bristle shaft are not contiguous. Rather, they are composed of shorter modules stacked end to end. These modules are arranged in register with each other, so that all the bundles around the circumference of the bristle are interrupted at roughly the same locations along the proximo-distal axis of the bristle shaft. The curvature of the bristle is determined by the relative lengths of the modules on different shaft surfaces (Tilney et al. 1996; Tilney et al. 2004; Tilney and DeRosier 2005). If actin bundles form at the same rate on all bristle surfaces, the bristle will be straight. If, however, bundle elongation occurs at a higher rate on one of the surfaces, the bristle shaft will curve in the opposite direction. The curvature of sex comb teeth must then be determined by the relative rate of actin bundle formation on the superior and inferior shaft surfaces. This rate appears to be controlled by the capping and crosslinking of actin filaments, which stabilizes the bundles against disassembly. If so, the shape of sex comb teeth may be controlled by the expression of capping and crosslinking proteins associated with the actin cytoskeleton.

The nuclei of trichogen cells are polyploid. This is probably necessary for producing the large amounts of actin and actin-associated cytoskeletal proteins that comprise the bristle shaft. It is reasonable to suppose that the size of the bristle is determined, at least in part, by the degree of polyploidization of the trichogen nucleus. If this is the case, interspecific differences in the size of sex comb teeth may reflect the control of endoreplication by *Scr*, *dsx*, and other spatial patterning mechanisms. HOX genes have been shown to control both cell size and cell proliferation (Hombria and Lovegrove, 2003; Pavlopoulos and Akam, 2011; Roch and Akam, 2000), so it is not much of a stretch to imagine that one of the functions of *Scr* and *dsx* is to control the endoreplication of trichogen cells that make up the sex comb. In contrast to thoracic macrochaetes (Skaer et al., 2002), differences in the timing of precursor formation are unlikely to account for the increased size of sex comb teeth, since they are specified at the same time as most other mechanosensory leg bristles

Linking pattern formation to cell differentiation and morphogenesis

As this overview shows, evolutionary diversification of sex comb morphology is due to changes in a wide range of cellular processes, including cell proliferation, specification of bristle precursor cells, cytoskeleton remodeling and differential cell adhesion required for cell migration, endoreplication, actin cytoskeleton dynamics, and cell-cell signaling. All of these processes show instances of rapid divergence and convergent evolution in the *melanogaster* and *obscura* species groups (Figure 2). This implies that the HOX and sex determination genes must regulate, directly or indirectly, a large number of target genes, and that the sets of these target genes are subject to rapid evolutionary turnover. Evolutionary changes may occur both in pattern formation (i.e., the interactions between *Scr*, *dsx*, and intrasegmental spatial cues), and in the linkages between pattern formation and morphogenesis (the regulation of downstream target genes by *Scr* and *dsx*). Unraveling of these evolutionary changes must necessarily await the identification and comparative analysis of target genes jointly regulated by *dsx* and *Scr* (Barmina et al. 2005).

Microevolution of sex combs: genetic architecture and selective forces

As the preceding sections show, the evolution of sex combs involved a number of radical changes in morphology, mode of development, and even the presence of these structures. An obvious question is how these transitions occurred in the course of species divergence. Interspecific differences, no matter how drastic, must originate as intraspecific variation. Although no extant species varies in such fundamental traits as the presence/absence or orientation of the sex combs, some species or complexes of closely related species show more subtle variation in sex comb size and the degree of sexual dimorphism. Analyzing the genetic and developmental basis of these differences may shed some light on the early stages of sex comb divergence.

Developmental basis of variation in sex comb size

Are differences in sex comb size due to changes in a male-specific developmental pathway, or do they simply reflect the formation of a greater number of bristle precursor cells in both sexes? *D. bipectinata*, a species widespread in Southeast Asia, has extensive heritable variation in sex comb size, with the mean number of teeth varying >1.5-fold among populations (13 – 20 teeth). The t1 sex comb in this species consists of two rows, which develop by active rotation and correspond to the two most distal TBRs in females. The number of sex comb teeth in both proximal and distal rows is higher in the *Taiwan* strain than in the *Samoa* strain ($P = 10^{-14} - 10^{-17}$) (Figure 8), but the number of bristles in the homologous TBRs in females is also significantly greater in *Taiwan* than in *Samoa* ($P = 0.0005 - 10^{-7}$). However, the number of sex comb teeth in the proximal row in *Samoa* males is not significantly greater than the number of bristles in the corresponding TBR in *Samoa* females ($P = 0.082$), whereas in the *Taiwan* strain this difference is highly significant ($P = 10^{-10}$). Thus, variation in sex comb size in *D. bipectinata* is due to a combined effect of male-specific and non-sex-specific developmental mechanisms. A similar dual mechanism seems to operate in *D. melanogaster*, where artificial selection for increased or decreased sex comb size leads to a correlated but weaker change in the number of bristles in the homologous female TBR (Ahuja and Singh 2008).

Genetics of interspecific differences

Several pairs of closely related, partially inter-fertile *Drosophila* species differ in the number of sex comb teeth, providing an opportunity to investigate the genetic basis of interspecific divergence. A number of studies have shown that these differences are controlled by multiple loci. In *D. simulans* and *D. mauritiana*, the mean sex comb size is 10.12 vs 13.93 teeth, respectively. In an F₂ backcross, most of this difference mapped to the 3rd chromosome, where at least two major QTL intervals were detected, while the X chromosome had little or no effect (True et al. 1997). A more detailed dissection has shown that one of these regions, which had the strongest phenotypic effect, contains at least two separate QTLs (Graze et al. 2007). In *D. yakuba* and *D. santomea*, in contrast, the X chromosome had the strongest effect on the interspecific difference in sex comb size (Coyne et al. 2004), although that study lacked sufficient power to estimate the number of contributing QTLs. In crosses between *D. simulans* and *D. sechellia*, at least 4 QTLs influencing the number of sex comb teeth were detected (Macdonald and Goldstein 1999). In this case, most of the interspecific difference maps to the 2nd chromosome, while the 3rd has no detectable effect. Interestingly, the X chromosome has an effect in the opposite direction to the overall species difference. Similarly, two major 3rd-chromosomal QTLs in the *D. simulans* – *D. mauritiana* cross also affect the phenotype in opposite directions (True et al. 1997). These observations suggest that the differences in sex comb size do not necessarily result from directional selection.

A major weakness of all these studies is that the differences between the parental strains for interspecific crosses are on the same order as among-strain variation within each species, and it is likely that most genetic variation segregates within species. Thus, the results of QTL mapping in interspecific crosses depend strongly on the choice of parental strains, and at least some of the QTLs identified in these crosses may in fact reflect intraspecific standing variation. Identification of these genes would tell us less about the evolution of developmental pathways than about the maintenance of (potentially deleterious or neutral) variation in these pathways.

Genetics of intraspecific variation

The genetic basis of sex comb size variation has also been studied within species. Using recombinant inbred lines in *D. melanogaster*, Nuzhdin and Reiwitch (Nuzhdin and Reiwitch 2000) found 2 significant QTLs, both located on the X chromosome and acting in opposite directions. However, these QTLs accounted for only 8% of the difference between parental strains, pointing to the existence of other, even weaker QTLs. Using a similar approach but different parental strains of *D. melanogaster*, Kopp et al. detected multiple QTLs on all chromosomes, all of them weak and many acting in opposite directions (Kopp et al. 2003). In crosses between two strains of *D. simulans*, several QTLs were found on the 2nd and 3rd chromosomes (Tatsuta and Takano-Shimizu 2006). Most of the difference was explained by two QTLs on the 3rd chromosome, both acting in same direction with large additive effects (44% and 48%); however, weaker QTLs acted in both directions. As in interspecific crosses, QTLs acting in the opposite direction to the overall strain difference appear to be common, suggesting that directional selection is unlikely to be the main cause of variation. The main conclusion emerging from these studies is that intraspecific variation in sex comb size is shaped by the segregation of a large number of small-effect QTLs. It is far from certain that these QTLs alleles provide the material for interspecific divergence, or even affect the same loci that are responsible for macroevolutionary changes in sex comb morphology.

Sex combs and mating behavior

An obvious hypothesis is that the rapid diversification of sex comb morphology is driven by sexual selection. Even in species that primitively lack sex combs, males use their front legs to grasp the base of the female's wings or the abdomen during mounting and copulation. In species with particularly exaggerated sex combs, such as those in the *montium* subgroup, males aggressively grasp the female with their front legs, and the T1 tarsi with the sex combs are used to maintain the male's hold on the female's abdomen (Spieth, 1952). In contrast, in the *obscura* species group and in *D. melanogaster* and *D. simulans*, sex combs are not in contact with the female's body once the male assumes the final copulatory position. In the *obscura* group, male grasps the dorsolateral surfaces of the female abdomen, but this contact is brief; afterwards, the male uses the tarsi to spread the female's wings. In *D. melanogaster* and *D. simulans*, sex combs are used transiently to grasp the female genitalia. Overall, the functions of sex combs are quite different in different species, suggesting that sex comb morphology may coevolve with male and female mating behavior. During courtship and mating, male sex combs come into contact with mechanosensory bristles that cover the female abdomen and genitalia. It is possible that females use these mechanoreceptors to evaluate the size and shape of the male sex combs. Unfortunately, *Drosophila* courtship has not been videorecorded with sufficient resolution and speed to identify the female's receptors that interact with sex combs, or to determine when this interaction first occurs during courtship.

The behavioral functions of sex combs have been tested in several species by surgical (Spieth 1952; Cook 1977; Coyne 1985) or genetic (Ng and Kopp 2008) removal. For example, in *D. pseudoobscura* and *D. persimilis*, surgical removal of the males' front tarsi

above the sex comb greatly reduces mating success, whereas removal below the sex comb has no effect. Direct observations showed that operated males court vigorously and attempt copulation repeatedly, but are unable to achieve it (Spieth 1952). More recently, Ng and Kopp (Ng and Kopp 2008) ablated sex combs genetically by expressing the female isoform of *transformer* in the tarsal segments. This treatment had no effect on the males' vigor and courtship activity, but led to their rejection by females and a severe reduction in mating success.

Both surgical and genetic ablation experiments suggest that *Drosophila* females are capable of mate choice based partly on the presence of sex combs. However, it remains to be determined whether females can perceive subtle differences in sex comb size and morphology. Some clues are provided by field observations. In *D. bipectinata*, studies in a wild population have shown that males caught copulating with females had significantly more sex comb teeth than non-copulating males captured on the same fruit, suggesting that the sex comb is under sexual selection for increased size in this population (Polak et al. 2004). Similar experiments were performed in *D. simulans* and *D. pseudoobscura*. In *D. simulans*, copulating males had fewer sex comb teeth than non-copulating ones, while in *D. pseudoobscura* sex comb size did not differ significantly between copulating and non-copulating males (Markow et al. 1996). Comparison across species is difficult since each species was studied only once in a single population. It appears, however, that selective pressures may be different in different species, explaining the rapid diversification of sex combs. The role of sexual selection in sex comb evolution is still poorly understood and requires closer investigation.

Conclusions: lessons from sex comb evolution

As evolutionary innovations go, the sex comb is a modest one – no more than a set of modified bristles that have clear homologues in the ancestral condition. The very simplicity of this structure raises an exciting prospect of reconstructing the entire developmental pathway responsible for sex comb origin and diversification, from the upstream changes in pattern formation and cell fate to the downstream cellular processes involved in terminal differentiation. By following the assembly of this pathway through the *Drosophila* phylogeny, we may be able to trace how the gain of novel gene interactions led to the formation of a new structure. There is good reason to expect that the sex comb will provide a useful paradigm for understanding the genetic mechanisms of evolutionary innovations in general.

Innovation through cooption?

At the same time, this simplicity is relative. Sex comb development requires integration of diverse cellular processes, suggesting that the origin of this structure required evolutionary changes in the expression or function of many genes. An obvious question is how such changes can be achieved in a sufficiently coordinated fashion to yield a coherent developmental program. At least part of the solution may lie in the modular organization of development (von Dassow and Munro 1999; Schlosser and Wagner 2004; Monteiro and Podlaha 2009). Cell differentiation programs often exist as genetic modules where a small number of regulator genes control the expression of larger batteries of effector genes that are necessary and sufficient for cell-type-specific differentiation. In sex comb development, tissue rotation and the modification of bristle shafts are entirely different cellular processes that presumably involve different sets of genes, and it is easy to imagine that they evolved separately in other developmental contexts before being integrated into a common pathway. For instance, sex combs could have co-opted a pre-existing genetic module for making thick, blunt, highly melanized bristles such as those found on the genitalia and anal plates of many *Drosophila* species (Kopp and True 2002; Ashburner et al. 2005). Sex comb rotation

occurs by a process of directional cell intercalation (Atallah et al. 2009a), which is a common and highly conserved cellular program deployed, for example, during germband extension in the *Drosophila* embryo (Zallen and Blankenship 2008). Thus, the novel developmental pathway responsible for the origin of sex combs could have evolved by bringing several older but previously unconnected developmental modules under the control of a common set of upstream regulators. A key advantage of such modules is that they come fully integrated so that innovation does not require a major increase in genetic complexity but is simply a matter of applying previously optimized cellular machinery for new purposes.

Diversification of morphological structures may also be facilitated by serial homology (Hall 1995; Ruvinsky and Gibson-Brown 2000; Monteiro 2008). In the sex comb, serial homology operates at three levels. First, each bristle represents a discrete unit yet all bristles share the same developmental program. Second, the TBRs are serially homologous and develop in similar ways. Finally, the first and second tarsal segments are also serially homologous units. Thus, sex comb morphology can be changed by applying the same developmental modifications to more or fewer of the available precursor modules. In other words, diversity can arise through individualization of previously identical serial homologs.

Innovation and homology

If evolving a sex comb requires nothing more than wiring together pre-existing genetic and cellular modules, is it truly “an innovation”? Evolutionary innovations are sometimes defined as traits that are “not homologous to any structure in the ancestral species” (Muller and Wagner 1991). However, this definition is easier to apply to morphological structures than to developmental processes that produce them. Homology relationships are often different depending on the level of biological organization one chooses to look at. We have seen, for example, that morphologically similar sex combs can develop from different precursor cells in different species. Such sex combs are homologous as adult structures, in the sense that the last common ancestor of these species had a sex comb, but not homologous as cell populations. Conversely, any morphological structure, no matter how novel, must necessarily evolve from a pre-existing cell population and thus will have a homolog at the cellular level. By focusing on cell populations or cell fates defined by the expression of conserved genetic modules (Wagner 2007), we can trace the probable evolutionary precursors even for such dramatic innovations as Lepidopteran wing scales (Galant et al. 1998; Zhou et al. 2009), insect wings (Averof and Cohen 1997; Damen et al. 2002), or the vertebrate neural crest (Sauka-Spengler and Bronner-Fraser, 2008; Yu, 2010). In this view, it is difficult to draw a sharp boundary between “innovation” and “modification” since many apparent innovations will occur through the cooption, modification, and integration of pre-existing developmental modules into novel pathways. Signatures of such assembly can be seen not only in genetic networks, but also in morphologies preserved in the fossil record (Vermeij 2006). The sex comb offers a good example of this process and may provide new insights into the mechanisms of the more dramatic innovations that occurred deeper in evolutionary time.

Master regulatory genes can evolve rapidly

In *Drosophila* and other insects, the HOX and sex determination genes are among the most upstream regulators of development, yet both have clearly evolved among closely related species and have played key roles in the origin and diversification of a structure that is absent even in most *Drosophila* species. In this context, both *Scr* and *dsx* can be viewed not so much as master regulators as “micromanagers” (Akam 1998). The involvement of these genes in sex comb evolution is made possible by the precise spatial control of their expression. Independent regulation of genes in different tissues or at different stages of

development is one of the defining metazoan features that facilitates phenotypic evolution (Carroll 2008). Thus, any transcription factor can be regulated independently, and control different batteries of downstream genes, in different cell types. Since most if not all regulatory genes are re-used multiple times during development, the concept of a “master regulator” can only be applied to a particular cell type and stage of development, not to the organism as a whole. Thus, there is little reason to expect that any particular class of regulatory genes will be immune from rapid evolutionary change.

Functional shifts and morphological diversity

In lineages that primitively lack sex combs, the arrangement of bristles is virtually identical in all species, likely reflecting their common ancestral function as cleaning utensils. Yet many species in the *melanogaster* and *obscura* groups can be distinguished by their sex combs alone, suggesting that morphological diversification is linked to the acquisition of a new function. The same is likely true for all innovations, such as beetle horns (Emlen et al. 2007; Moczek et al. 2007), Lepidopteran wing scales (Nijhout 1991), or the abdominal appendages of Sepsid flies (Eberhard 2001; Bowsher and Nijhout 2007). An evolutionary innovation can be reasonably defined as “a newly evolved structure or condition that enables its phylogenetically derived bearer to perform a new function” (Vermeij 2006), so the study of evolutionary novelties should be undertaken together with the ecological and behavioral context in which these innovations originate and diversify.

The *Drosophila* sex comb achieves a happy balance – complex enough to be interesting, yet simple enough to be genetically tractable; raising important macroevolutionary questions yet also providing microevolutionary models; displaying spectacular interspecific diversity yet offering the advantages of the *Drosophila* genetic and molecular toolkit. Understanding the development, origin, and diversification of this structure can make an important contribution to our knowledge of the genetic mechanisms of evolutionary innovations.

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Figure 1.

Sex comb structure and ontogeny in *D. melanogaster*. **A.** Female T1 leg showing TBRs (arrows). **B.** Male T1 leg showing the sex comb. **C.** SEM of a female leg showing the most distal TBR on t1. **D.** SEM of a male leg showing the sex comb and the area it came from. White arrow points to the “central bristle”, which develops from the same TBR as the sex comb (see also F). **E.** Schematic drawing of the female t1 showing the arrangement of TBRs. **F.** Schematic drawing of the male t1 showing sex comb migration and final position. Sex comb teeth are shown in solid grey, and the locations of their precursors in lighter grey. The central bristle develops from the ventral-most bristle precursor of the sex comb-forming TBR (compare with D).

Figure 2. Phylogenetic analysis of sex comb evolution in the *obscura* and *melanogaster* species groups. The phylogeny, simplified from (Barmina and Kopp 2007), is based on a combined analysis of 14 loci and is strongly supported. Note the differences in sex comb size and orientation between closely related species, and the convergent origin of similar sex combs in distantly related species.

Figure 3.

A model of developmental changes involved in the origin of sex combs. Ancestral regulatory interactions are indicated in black, and newly evolved interactions in red. In the ancestral condition (left), leg patterning genes lay down the basic bristle pattern and establish a domain of high *Scr* expression on the ventral-anterior surface of the distal Ti and ta1. High levels of *Scr* organize the ventral-anterior bristles into TBRs. *dsx* is not expressed in the TBRs so they develop in a sexually monomorphic manner. In the *melanogaster-obscura* clade (right), *dsx* was recruited into the TBR development pathway under the control of both *Scr* and leg patterning genes. *Scr* activates *dsx* in T1 at the late larval stage, while *dsx* modulates *Scr* at the pupal stage to make its expression sexually dimorphic in some species. Both genes have acquired new downstream targets involved in bristle patterning and morphogenesis.

Figure 4.

Sex comb morphology and evolution in *Lordiphosa*. **A.** Phylogenetic relationships among *Lordiphosa* and *Sophophora* lineages (simplified from (Gao et al., submitted; Hu and Toda, 2000; Katoh et al., 2000)). On branches marked with long oblique lines, at least some species have well-developed longitudinal sex combs. On the branch marked with shorter transverse lines, only simple transverse sex combs are present in some species. All other lineages lack sex combs. **(B-D)** Sex combs in the *Lordiphosa miki* species group. **B.** *L. clarofinis*. **C.** *L. stackelbergi*. **D.** *L. magnipectinata*.

Figure 5.

Differences in sex comb development between *D. mimetica* and *D. biarmipes* (see Fig. 2 for phylogenetic relationships). **A.** SEM of *D. mimetica* male T1 leg. **B.** SEM of *D. biarmipes* T1 leg. Arrows in A and B point to the sex combs; note the absence of the t2 comb in *D. biarmipes* (arrowhead). **C, D.** Arrangement of TBRs in *D. mimetica* (C) and *D. biarmipes* (D) females, shown in orthogonal coordinates. Bristles that develop into sex comb teeth in males are highlighted in black. The orthogonal arrangement of TBR bristles is highly conserved, allowing the homology of sex comb teeth to be established across species. **E, F.** Schematic representation of adult sex comb morphology in *D. mimetica* (E) and *D. biarmipes* (F). Sex comb teeth are shown as solid grey circles. Arrows in F show the path of migrating sex comb precursors. Letters and numbers denote bristle identity according to the orthogonal coordinate system defined in C and D.

Figure 6.

Differences in sex comb morphology between *D. eugracilis* and *D. ficusphila* (see Fig. 2 for phylogenetic relationships). **A.** SEM of *D. eugracilis* male T1 leg. Arrows point to sex comb teeth, which are always two in number in this species. **B.** SEM of *D. ficusphila* male T1 leg: anterior, ventral, and dorsal views. Thick arrows point to the main sex comb row; longer and thinner arrows point to the sparser secondary row; and arrowheads indicate chemosensory bristles. **C, D.** Arrangement of TBRs in *D. eugracilis* males (C) and *D. ficusphila* females (D), shown in orthogonal coordinates. Black bristles in C correspond to the sex comb teeth of *D. eugracilis*. **E.** Arrangement of TBRs (grey) and sex comb teeth (black) on the t1 segment in males of *D. ficusphila*. In this species, sex comb teeth are not homologous to TBR bristles (compare D and E). **F, G.** Bristle arrangements on the t2 segment in females (F) and males (G) of *D. ficusphila*. Note that the number of sex comb teeth in males (black circles in G) is greater than the number of bristles in the homologous region in females (grey circles in F).

Figure 7.

The morphology of sex comb teeth in *D. paralutea* (*takahashii* species subgroup), *D. suzukii* (*suzukii* subgroup), and *D. truncata* (*montium* subgroup). Note the differences in the shape of the bristle cross-section, the number of actin bundles composing the base of each tooth (seen by the grooves along the bristle shaft), the sharpness of bristle tips, and the presence or absence of bracts at the base of sex comb teeth.

Figure 8.
The numbers of sex comb teeth and bristles in homologous TBRs in two strains of *D. bipectinata*.