THE ORIGINATION AND ALTERATION OF A NOVEL ORGAN

by

Rachel M. Pileggi

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This thesis was presented

by

Rachel M. Pileggi

It was defended on

May 23, 2012

and approved by

Dr. Karen M. Arndt, Professor, Department of Biological Sciences

Dr. Gerard L. Campbell, Associate Professor, Department of Biological Sciences

Dr. Jeffrey D. Hildebrand, Associate Professor, Department of Biological Sciences

Thesis Advisor: Dr. Mark R. Rebeiz, Assistant Professor, Department of Biological Sciences

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Rachel M. Pileggi, M.S.

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The living world is filled with limitless three-dimensional variation in size, shape, and the presence of novel structures that only appear in one or a few species. Although there are several examples of genetic alterations that cause the loss of morphological structures, the questions remain as to how novel structures form and how shape changes. Using the highly divergent *Drosophila* genitalia as a model of recent shape evolution, we can begin to uncover how relevant pathways that pattern and control growth are modified to create these diverse morphological forms. Specifically examining the posterior lobe, a recently evolved novelty of the *melanogaster* clade that is rapidly diverging in shape and required for male fertility, we are taking a candidate gene approach to investigate the origination and modification of this organ. After characterization of the development of this tissue, we have begun investigating the *Pox neuro* (*Poxn*) gene, a transcription factor that has been implicated in male genital development. *Poxn* is required for proper posterior lobe development and growth, which raises the question of how it obtained this role in a novel setting, and stimulates the hypothesis that *Poxn* contributes to posterior lobe shape variation. Investigations of *Poxn*, in combination with several other candidate genes (morphogens, signaling molecules, cell cycle control genes), will allow us to understand the flexible points in growth control pathways as well as how nascent genetic programs are established. This will ultimately lead to insights into how novel structures form and how shape evolves.

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PREFACE

I would like to thank Dr. Mark Rebeiz for his mentorship during this project. I would also like to thank Chas Elliott, Winslow Johnson, and Kelsey Stayer for technical assistance. The Noll Lab and Campbell Lab were invaluable resources for fly stocks and reagents. A ntonis Rokas generously performed the character state reconstructions.

1.0 CHAPTER 1: INTRODUCTION

In nature, there exists endless morphological variation. The most extreme examples of such morphological diversity are novelties – structures that have no homologues. Understanding how these unique structures arose and how they change has been the focus of exhaustive research efforts. For example, how did the turtle get its shell or the swordfish get its sword? Here, I present my efforts to understand the origins and modifications of a novel, rapidly evolving morphological structure of *Drosophila melanogaster*.

1.1 EVOLUTIONARY DEVELOPMENT

Since the beginning of time, organisms have evolved many complex morphological, physiological, and behavioral adaptations to increase their survival and fecundity (*1*). The study of evolutionary developmental biology molecularly characterizes the evolutionary changes that result in these adaptations.

Decades of research on the molecular basis of organismal development have revealed many of the intricacies of genes controlling animal development. However, it is unclear to what extent phenotypic variation can arise from genetic variation. Evolutionary developmental biology studies how development itself evolves and how the dynamics of development determine

phenotypic variation resulting from genetic variation, which in turn affect the evolution of form (*2*).

A few defining principles make up the foundation of this discipline. The first of which is modularity (*3*). It has long been appreciated that plants and animals are modular, meaning they are organized into developmentally and anatomically distinct parts. One area of interest in this field is to understand the genetic and evolutionary basis for this division into modules and how partly independent development of such modules arises (*3*).

An idea central to molecular biology and development that holds strong implications for the study of evolutionary developmental biology is that some proteins function as switches whereas others function as diffusible signals. In 1961, the lac operon was discovered within *E. coli*, and it functioned only when "switched on" by an external stimulus (*4*). Researchers later discovered a subgroup of conserved genes in animals that contain the homeobox DNA motif, called Hox genes (*5*). Hox genes function as switches for other genes, and could be induced by morphogens that act analogously to the environmental stimulus in *E. coli*. These discoveries led to the notion that genes can be selectively turned on and off, and that organisms from fruit flies to humans may use the same genes for their development, just by regulating them in different ways. These genes comprise the developmental-genetic toolkit, consisting of the highly conserved genes whose products control development.

The majority of toolkit genes encode for the production of signaling molecules, transcription factors, and secreted morphogens, among others, all participating in forming the body plan of the organism. Differences in the deployment of toolkit genes affect the body plan and the number, identity, and pattern of body parts. Among the most important of the toolkit genes are those of the aforementioned Hox gene cluster. Hox genes function in patterning the

body axis in all creatures in the animal kingdom, determining where limbs and other body segments will develop (*6-9*). Another landmark example of a toolkit gene is *Pax6/eyeless*, which controls eye formation in all animals (*10, 11*). *Drosophila* eyes are rescued when mouse *Pax6/eyeless* is expressed, indicating conservation of protein function (*12*).

Because a large proportion of distinct animals use the developmental genetic toolkit, toolkit genes are excellent candidates for evolution. This leads to the idea that a huge contributor to morphological evolution is driven by variation in the toolkit, either by toolkit genes changing their expression patterns or acquiring new functions. An excellent example of a toolkit gene changing its expression pattern is in the enlargement of the beak in Darwin's large ground-finch (*13*). In this case, increases in the levels of Bmp4 are correlated with the larger beak of this finch, relative to its sister species. Also, the loss of legs in snakes corresponds well with the lack of Distalless expression in the regions where limbs would form in other tetrapods (*14*). Distalless also determines the spot pattern in butterfly wings, indicating that toolkit genes can evolve to control an astoundingly diverse set of developmental decisions (*15*).

Morphological evolution can be influenced by mutations in noncoding regions of such developmental control genes. This suggests that distinctions between different species may be due to a greater extent to differences in spatial and temporal expression of conserved genes and to a lesser extent to differences in the content of gene products. The implication of the assertion that macroevolutionary changes in body morphology are associated with changes in gene regulation suggests that Hox genes and other toolkit genes may play a major role in evolution.

By applying information about genes and their regulation, evolutionary developmental biologists can now readily make predictions about the path of genetic evolution (*16*). In summary, nearly all proteins regulating development are coordinated to participate in many

independent processes in a variety of tissues at distinct times (*17, 18*). And although an ancient toolkit of regulatory pathways that shape animal development is conserved from flies to humans, a vast amount of morphological variation exists in nature (*19-22*). Sequencing of multiple genomes has revealed a surprising lack of disparity among protein coding genes between species, suggesting that the wide variety of complex morphological traits is due to changes in gene regulation (*23*). Learning how these conserved genes and their regulatory regions are modified is key to understanding the generation of organismal diversity.

1.1.1 *Cis*-regulatory evolution

Although first proposed over fifty years ago (*24*), it is only recently that claims surrounding the evolutionary significance of *cis*-regulatory mutations have been empirically supported by numerous studies (*25, 26*). *Cis*-regulatory sequences, such as enhancers, promoters, and insulators, regulate gene expression (*25, 26*). Changes within *cis*-regulatory regions have been confirmed to be the source of a variety of interesting and ecologically important phenotypic differences in morphology, physiology, and behavior (*25, 27-30*). The modular nature of many *cis*-regulatory regions allows for each module to affect a single part of the overall transcription profile of a gene, underscoring the importance of modularity as a key feature of pleiotropic toolkit genes (*1, 16, 25, 31-33*). This means that one *cis*-regulatory mutation could be restricted to a particular developmental stage or tissue, resulting in a specific effect not seen in other stages or tissues where the gene is normally expressed. This is in contrast to coding mutations that change the resulting protein in every place and at every time.

Transcription factors that regulate gene expression can usually bind many target *cis*-regulatory elements. For example, Stark, *et al*., found 124 target genes associated with each of

the 67 *Drosophila* transcription factors they examined (*34*). Specifically, the *Drosophila* transcription factor Twist has nearly 500 target *cis*-regulatory elements required during embryogenesis for a number of distinct cell processes (*35*). The implication in this example is that almost 500 linkages between Twist and different *cis*-regulatory elements have evolved in just one stage of life via a multitude of *cis*-regulatory mutations while simultaneously conserving Twist protein function for normal fly embryogenesis. These examples also suggest that transcription factor-*cis*-regulatory linkages within gene regulatory networks are added or subtracted by modifying *cis*-regulatory elements, directly altering the expression of only one gene and in turn affecting individual morphological features (*16*).

These observations support the *cis*-regulatory hypothesis, whereby mutations in *cis*-regulatory elements are proposed to constitute the predominant genetic path of morphological evolution (*16*). While it is understood that other genetic mechanisms besides *cis*-regulatory mutations contribute to morphological change, it has been shown time and again that *cis*-regulatory sequence changes are sufficient to account for the evolutionary divergence of several traits, and they are necessary for gene regulatory network rewiring for new developmental programs.

To determine the role of cis-regulatory changes in the diversification of morphological traits, recent changes in form must be studied as opposed to changes that have occurred too long ago to dissect the fine-scale mutations that caused them. Possibly the three most widely studied examples of recent morphological changes include *Drosophila* larval trichome density (*30, 36-38*), stickleback pelvic spines (*39*), and *Drosophila* wing pigmentation (*27, 40, 41*). All of these modifications consequently result from *cis*-regulatory mutations in key developmental control genes.

Drosophila larvae are covered in epidermal hairs called trichomes that aid in locomotion. The absence of these structures in *D. sechellia* raises the fascinating question of how something so complex and intricate can be lost (*30, 36, 37, 42*). The genetic program to create these trichomes is incredibly complex, as morphogens provide spatial cues for transcription factors, which in turn regulate the expression of a downstream gene, known as *shavenbaby*, leading to terminal differentiation genes selectively getting turned on. Genetic mapping and interspecific complementation assays pointed to evolution at the *shavenbaby* locus as being entirely responsible for the trichome loss seen in *D. sechellia* (*36*). *Shavenbaby* expression is also correlated with this morphological modification (*36*). Multiple *shavenbaby* enhancers were identified, and functional analyses uncovered a role for mutations in all of these enhancers in *D. sechellia* trichome pattern generation (*37*).

Threespine stickleback fish exhibit vast differences in their pelvic skeletons. Whereas marine sticklebacks maintain a prominent pelvic skeleton, freshwater stickleback populations display complete or partial loss of their pelvic skeleton (*39*). Genome-wide linkage mapping was carried out to understand the genetic basis underlying the evolution of pelvic reduction (*39*). These studies showed that pelvic reduction is controlled by site-specific regulatory mutations altering *Pitx1* expression (*39*). Freshwater sticklebacks display reduced or absent expression of this gene in pelvic precursors (*39*).

The wings of *Drosophila biarmipes* are adorned with a large pigmented spot (*27*). Gompel, *et al*., concluded that expression of the *yellow* pigmentation gene presages adult wing pigmentation, and evolution of these spots involved modifications of a *cis*-regulatory element of *yellow*. This element has gained multiple binding sites for transcription factors, including the

toolkit gene *engrailed*, involved in the development of wings and other parts of the body plan (*27*).

In each of the aforementioned cases, the more recently diverged species exhibited a trait loss or color change (trichomes, pelvic spines, wing spots) attributed to mutations in the *cis*-regulatory regions of developmental control genes. These few examples, as is the case with many developmental traits, can be attributed to changes in well-known high-level regulators. Multiple cases involve morphogens, and many instances involve transcription factors (e.g., *shavenbaby* in trichome density and *Pitx1* in sticklebacks). But the questions of how novel traits are acquired or how shape forms remain less well studied.

1.1.2 Evolution of Shape

One of the more striking concepts that remains enigmatic in the field of evolutionary developmental biology is how evolution of three-dimensional shape proceeds. There are relatively few examples in which the molecular genetic basis of morphological changes at the level of shape have been elucidated. Researchers have looked to the beaks of Darwin's finches and very recently to the wings of *Nasonia* wasps in an attempt to better understand the evolution of tissue shape.

The fourteen closely related species of Darwin's finches display huge variations in their beak morphology (*13, 43, 44*). While these differences in beak size and shape are associated with adaptation to various ecological niches, its developmental and molecular basis has been the focus of much research. Differential levels of Bmp4 and Calmodulin expression corresponding to differences in beak shape were discovered (*13, 43*). It is perhaps not surprising that this relays yet another example of morphogens and signaling molecules underlying morphological

adaptations. However, a correlation between beak size and Bmp4 and Calmodulin signaling cannot definitively point to causation. In other words, the fact that these genes are differentially expressed in this varied morphological feature does not necessarily indicate that these genes are the ones responsible for beak shape divergence. It is hard to discern if expression changes truly cause morphological changes in these species that lack experimental tools to empirically test the function of regulatory sequences or clearly show the phenotypic repercussions of such sequence changes.

In an attempt to understand the genetic changes underlying morphological shape differences, the differing wing size of closely related species of *Nasonia* wasps were investigated (*45*). QTL mapping allowed for the identification of the gene *unpaired-like* (*upd-like*) that induces these shape differences. Subsequent fine-scale mapping and *in situ* hybridizations revealed mutations in the *cis*-regulatory regions of this gene that resulted in changes in the spatiotemporal expression of *upd-like* corresponding to wing shape changes (*45*). Using this microevolutionary approach to study a distinct morphological change in closely related species of wasps allowed for the identification of the genetic basis of this change, which incidentally is in the *cis*-regulatory region of a gene that codes for a signaling protein regulating cell proliferation and differentiation.

1.1.3 Origins of Novelty

Much effort in the field of evolutionary biology is currently focused on revealing not only the means of modifying preexisting traits, but also on the appearance of novel features that lack any obvious homology with other known traits (*46-48*). Many successful studies have been

performed that show evidence of adaptive morphological change, but they have been limited to studies of trait loss or color change, as previously described.

At this juncture, exhaustive research efforts have been focused on finding and validating genes/mutations that contribute to morphological divergence, with a great deal of emphasis on wholesale loss. Delving into an analysis of completely novel structures will revisit the overarching question of where novelty and morphological variation originate.

Investigations of the beetle's horn, a genuine novelty, have revealed the deployment of key developmental regulators known to pattern the proximodistal axis of vertebrate limbs (*1, 46, 47, 49-51*). Another bonafide morphological novelty is the feather. Sonic Hedgehog signaling presages feather development by exhibiting expression in the primordial feather buds (*52, 53*). However, the mere presence of limb patterning gene expression in the beetle's horn and Sonic Hedgehog signaling in feather buds cannot definitively indicate that these genes are the ones responsible for the initial appearance of the horn or feather. It is hard to discern if expression changes truly cause a novelty to appear, as many traits such as these are too long diverged to track the genes and mutations responsible.

The macroevolutionary conundrum of how novel structures appear and how shape changes in three dimensions remains an elusive challenge in the field. Investigating what genes contribute to the development of unique structures and how these genes are modified will allow us to appreciate how new genetic programs are forged.

1.2 *DROSOPHILA* MALE GENITALIA AS A MODEL FOR THE EVOLUTION OF MORPHOLOGICAL NOVELTY AND SHAPE

Macroevolutionary studies, such as those of the origins of feathers and beetle horns, leave us questioning whether the known correlative genes are in fact causative for such novelties. A recent trend in evolutionary developmental biology has been to use a microevolutionary approach, i.e. study closely related species. This approach facilitates the identification of relevant changes that gave rise to a novel trait, as opposed to secondary changes that came after its specification, allowing us to see the initial stages of novelty and better determine the phenotypic consequences of changes we identify. This prompts investigations into the genetic program of a new structure, the genes that are expressed in the new structure, and the history of gene expression in the new structure, among other areas of study. The enormously divergent genitalia of closely related species of *Drosophila* represent a nearly ideal system in which to study morphological novelties from a microevolutionary perspective.

There are hundreds of species of *Drosophila*, all of which diverged from each other very recently (*28*). Studying the evolution of any morphological differences between them, such as their highly divergent genitalia, provides us with the capability to identify the genes that are directly responsible for such changes. One structure of particular interest is the posterior lobe, a hook-shaped outgrowth of the external male genitalia (*54-58*). The posterior lobe is a novelty, and it provides the only reliable morphological trait for distinguishing the four closely related *Drosophila* species of the *melanogaster* clade, *D. melanogaster*, *simulans*, *mauritiana*, and *sechellia*, as the size and shape of this organ wildly differs between them (*54-58*).

This structure is used to grasp the female oviscape during copulation. These shape modifications have a functional consequence in that they enable males to achieve and maintain copulatory position to ultimately acquire successful genital coupling and ensure efficient transfer of sperm (*54*). It is thought that the posterior lobe is rapidly evolving due to intense sexual selection to overcome female resistance to copulation (*54*). The goal of this thesis is to characterize this structure as a microevolutionary example of novelty and shape change.

1.3 SIGNIFICANCE

In each case that has previously been described in the literature, gene expression differences have been discovered in morphological novelties that have diverged too long ago to track the mutations responsible for their appearance. Alternatively, *cis*-regulatory mutations have been demonstrated to account for microevolutionary differences that resulted in trait loss. However, the mechanisms by which novel structures form and diversify shape remain a mystery. Using the highly divergent posterior lobe of *Drosophila* male genitalia, insights can be made into the evolutionary mechanisms responsible for the appearance and subsequent shape changes of entirely novel tissues. It is likely that *cis*-regulatory mutations in high-level regulators such as morphogens or transcription factors will be key in determining how three-dimensional shape evolves.

2.0 CHAPTER 2: EXAMINING THE EVOLUTIONARY ORIGINS AND DIVERSIFICATION OF THE NOVEL POSTERIOR LOBE OF *DROSOPHILA* MALE GENITALIA

2.1 BACKGROUND AND SIGNIFICANCE

A major focus of evolutionary development studies is to elucidate how novel structures appear and the means of subsequently modifying such preexisting traits. Specifically, the molecular genetic basis underlying morphological changes at the level of three-dimensional shape remains a question of high priority in the field. In an attempt to address this matter, a microevolutionary approach investigating the highly divergent *Drosophila* male genitalia was taken. The posterior lobe of male genitalia is unique to species of the recently diverged *Drosophila melanogaster* clade (*54-58*). It is also the only reliable morphological trait for distinguishing between the four species of this clade, as the shape and size of the posterior lobe drastically differs between them (*54-58*). This makes the posterior lobe an excellent model system of morphological evolution, specifically examining three-dimensional shape. The development of this tissue and the genes responsible for its divergence have yet to be elucidated. Studies investigating this organ will begin to answer the question of how novel structures form and subsequently change shape.

2.1.1 Hypothesis

Macroevolutionary novelties have been repeatedly traced back to patterned expression of morphogens and signaling molecules. We hypothesize that more recent evolutionary events will involve the similar deployment of high-level regulators.

2.2 CHARACTERIZATION OF POSTERIOR LOBE DEVELOPMENT IN *DROSOPHILA* SPECIES

In the genital imaginal disc of *Drosophila* larvae, signaling molecules such as Hedgehog, Engrailed, Wingless, and Decapentaplegic are deployed to specify positional information of anterior/posterior compartments and segmental boundaries in the genital primordia (*59-64*). Also, the sex determination pathway allows for the proper formation of sexually dimorphic genitalia and analia. The integration of these several pathways allows for normal growth of the genital disc into male or female derivatives (*63, 65-68*). While adult male genital morphology has previously been described at length, relatively little is known about its development in stages subsequent to the specification of these broad territories during larval development (*59-61*).

In order to find genes involved in the evolution of the posterior lobe, we first characterized the development of the posterior lobe in several *Drosophila* species of the *melanogaster* clade. We hypothesized that such an analysis would contribute to our understanding of the species-specific details of this tissue, and it would hone in on the relevant stages and locations by which to measure effects of candidate genes responsible for its divergence.

2.2.1 Scanning Electron Microscopy Survey of *Drosophila* Genital Morphology

The evolutionary history of the posterior lobe has generated some debate among researchers. Jagadeeshan, et al., claim that the posterior lobe is a true evolutionary novelty of the *D. melanogaster* clade, whereas Kopp, et al., assert that the evolutionary origins of the posterior lobe are not as clear (*54, 58*). They state that while the genitalia are rapidly evolving, secondary losses of male sexual characters are frequent (*58*). Our goal was to evaluate whether the posterior lobe is a genuine novelty by obtaining high-resolution photographs of genitalia in their native conformations, which had not previously been presented for these structures. Scanning electron microscopy (SEM) was initially utilized to capture these high-resolution images of the genitalia at various pupal and adult stages. Figure 1 shows a scanning electron micrograph of *Drosophila sechellia* male genitalia with the various structures labeled and the posterior lobe boxed. The presence of posterior lobes was confirmed in all species of the *melanogaster* clade (*D. melanogaster*, *D. mauritiana*, *D. simulans*, and *D. sechellia*) (Fig. 2).

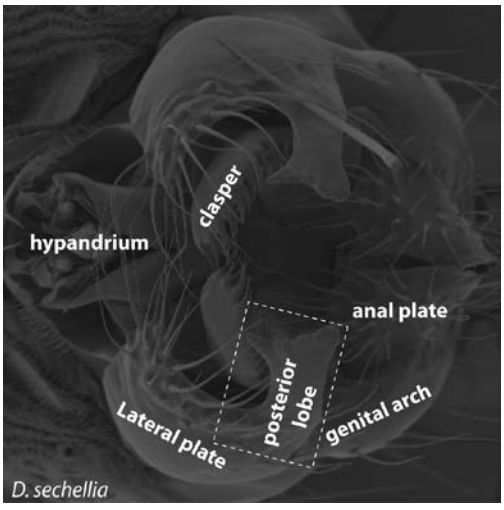


Figure 1. Scanning Electron Micrograph of *Drosophila sechellia* male genitalia. All structures are labeled. The posterior lobe is boxed in white dotted lines.

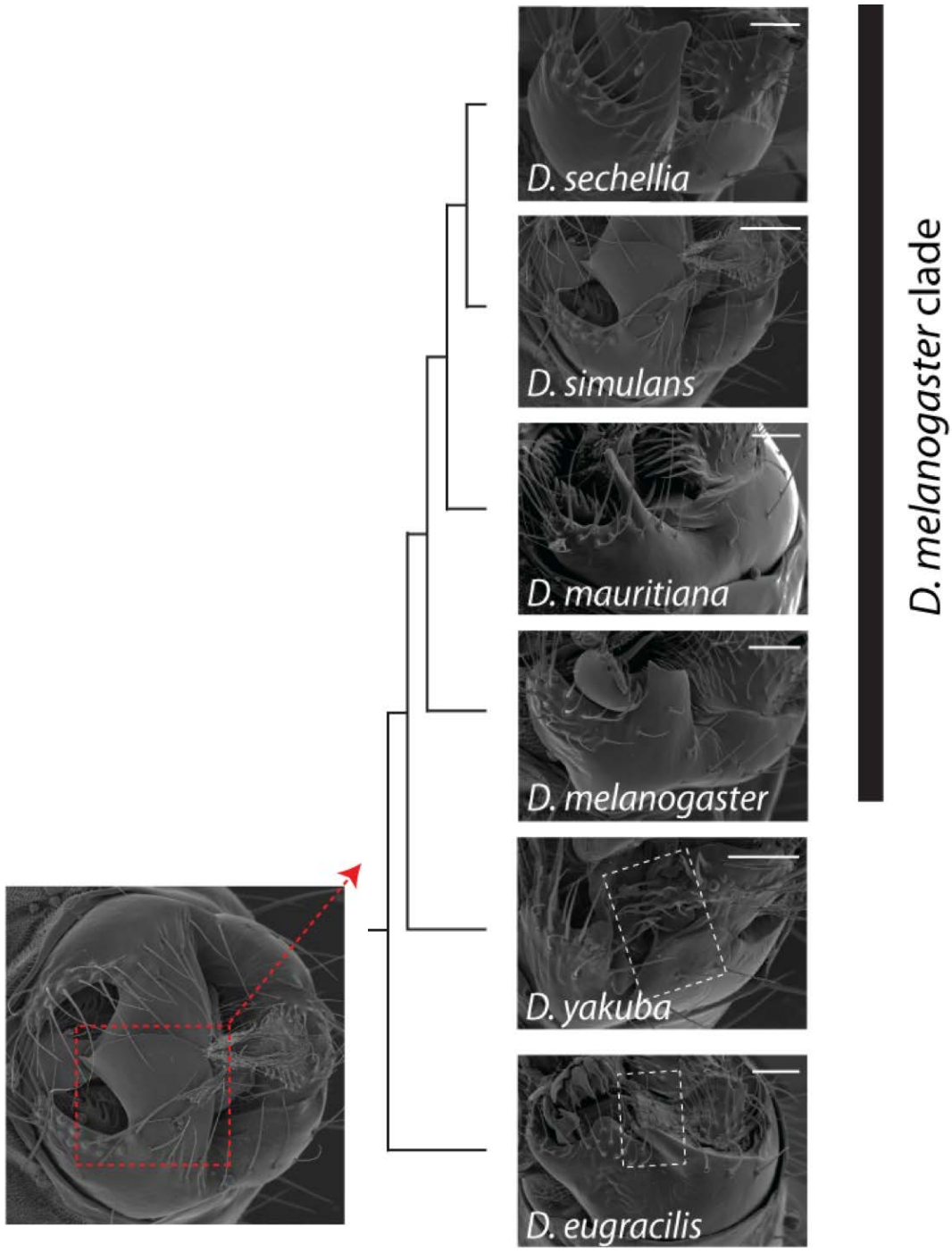


Figure 2. Scanning Electron Micrographs of *Drosophila* male genitalia from the *melanogaster* subgroup. *D. simulans* with posterior lobe boxed in red. Species of the *melanogaster* clade (*D. melanogaster*, *D. mauritiana*, *D. simulans*, and *D. sechellia*) are the only species that have posterior lobes. Outgroup species *D. yakuba* and *D. eugracilis* that lack posterior lobes are shown for comparison. White boxes indicate the expected location of a posterior lobe in the non-lobed species depicted. The white scale bars measure 50 μm.

Species of the *melanogaster* clade are the only species reported to have posterior lobes (*54-58*). To determine when this structure evolved, we must confirm that other species outside of this clade do not possess posterior lobes. A more comprehensive assessment of the lack of posterior lobes in outgroup species is necessary to confirm that this structure is indeed novel to the *D. melanogaster* clade and has not alternatively been lost in some species but retained in those that presently possess the organ.

SEM data corroborate published observations that *D. melanogaster*, *mauritiana*, *simulans* and *sechellia* have posterior lobes, while other species outside of this clade do not. A broader survey of the genitalia of dozens of available *Drosophila* species of the *D. melanogaster* species group by SEM confirmed the lack of posterior lobes in species outside of the *D. melanogaster* clade (Fig. 3).

The genitalia of every species examined were classified by choosing one of two character states – "present" (i.e. "lobed") or "absent" (i.e. "non-lobed"). Three-character-state assessments were also performed by classifying each species as "absent" (i.e. "non-lobed"), "slight protuberance" (i.e. "non-lobed", but possessing a slight bump on the genital arch), or "large protuberance" (i.e. "lobed"). Character state reconstructions were performed for these categorizations using parsimony on the *Drosophila* phylogeny (Figs. 4 & 5). These reconstructions indicate that there was a single origin of the posterior lobe, progressing from absence to slight protuberance to large protuberance as the most parsimonious history of events. These results support the hypothesis that the posterior lobe is a recently evolved morphological novelty.

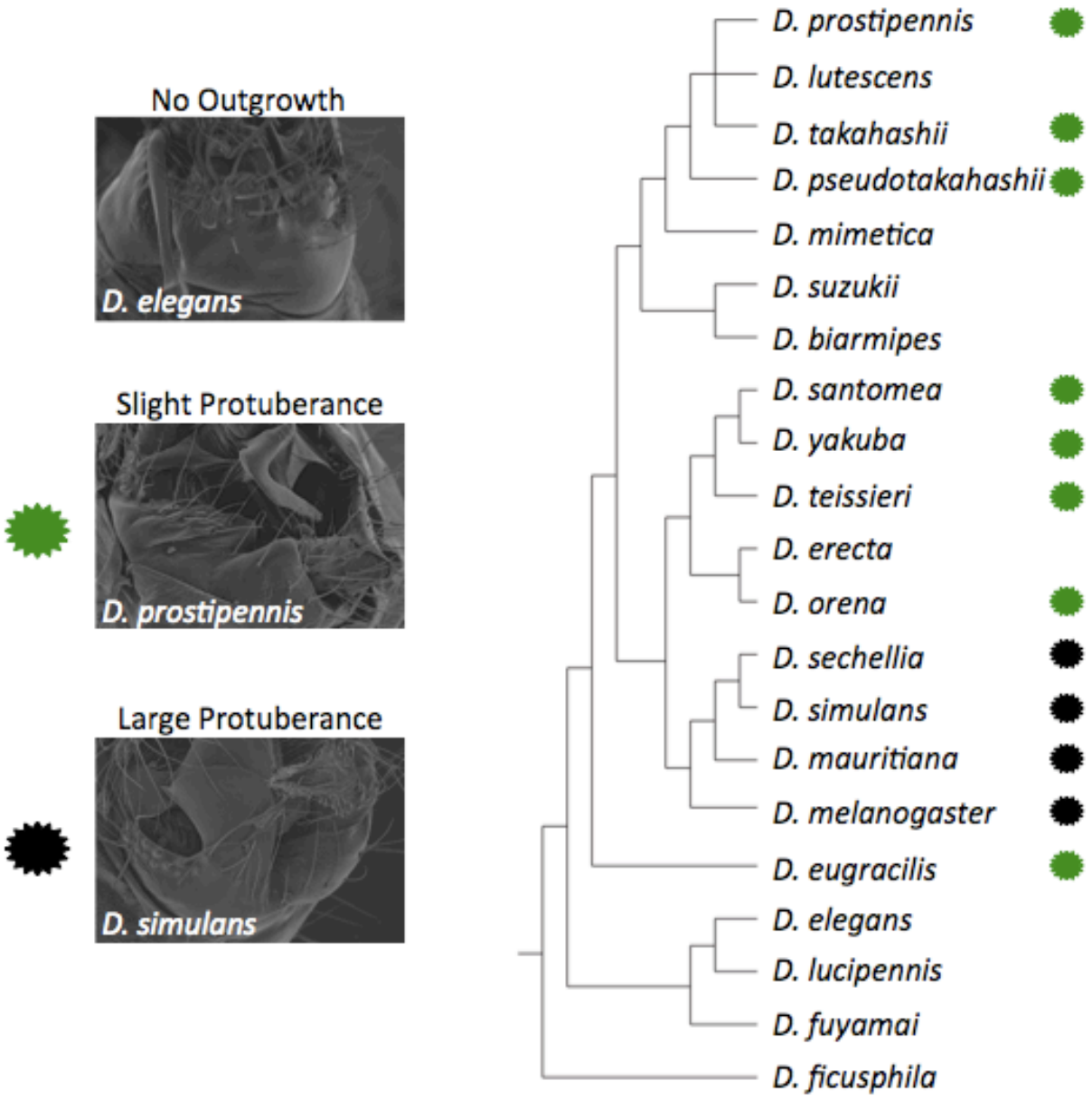


Figure 3. SEM survey of *Drosophila* genital morphology. Green stars indicate the presence of a slight protuberance. Black stars indicate the presence of a large protuberance (posterior lobe). Example images from each category are shown on the left. All images are presented in Appendix D.

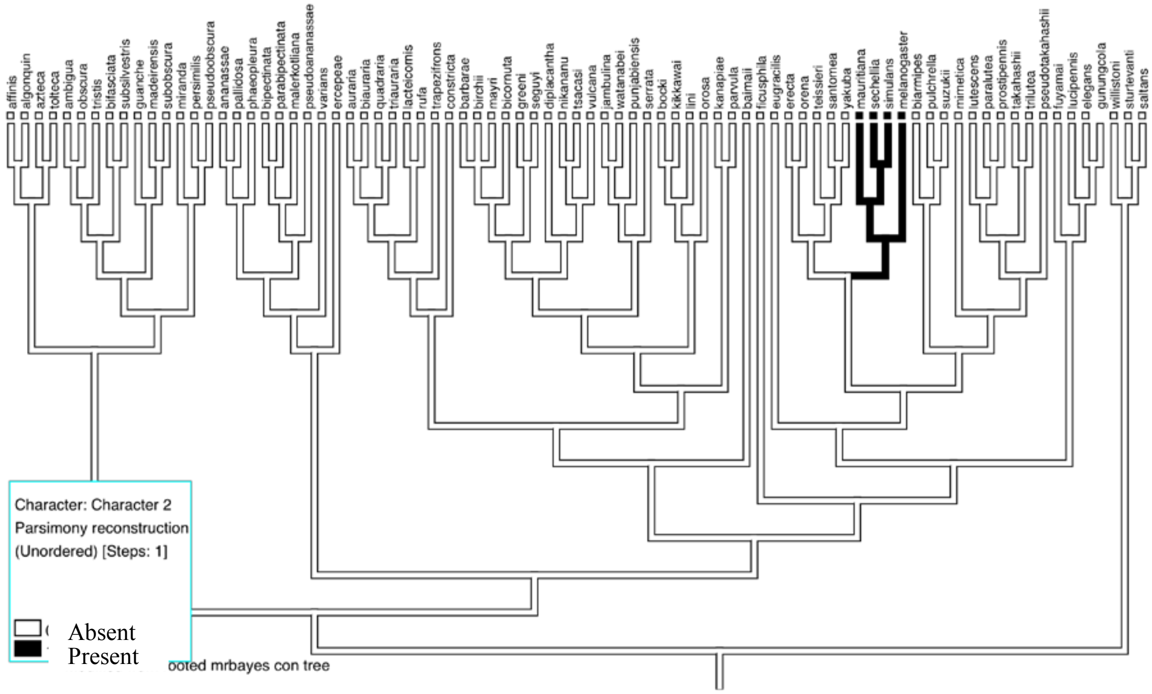


Figure 4. Tw o-state coding character state reconstruction. SEM images were evaluated and each species was classified by choosing one of two character states – "present" (i.e. "lobed") or "absent" (i.e. "non-lobed"). A character state reconstruction was performed using parsimony on the *Drosophila* phylogeny. T his reconstruction indicates that there was a single origin of the posterior lobe at the *melanogaster* clade.

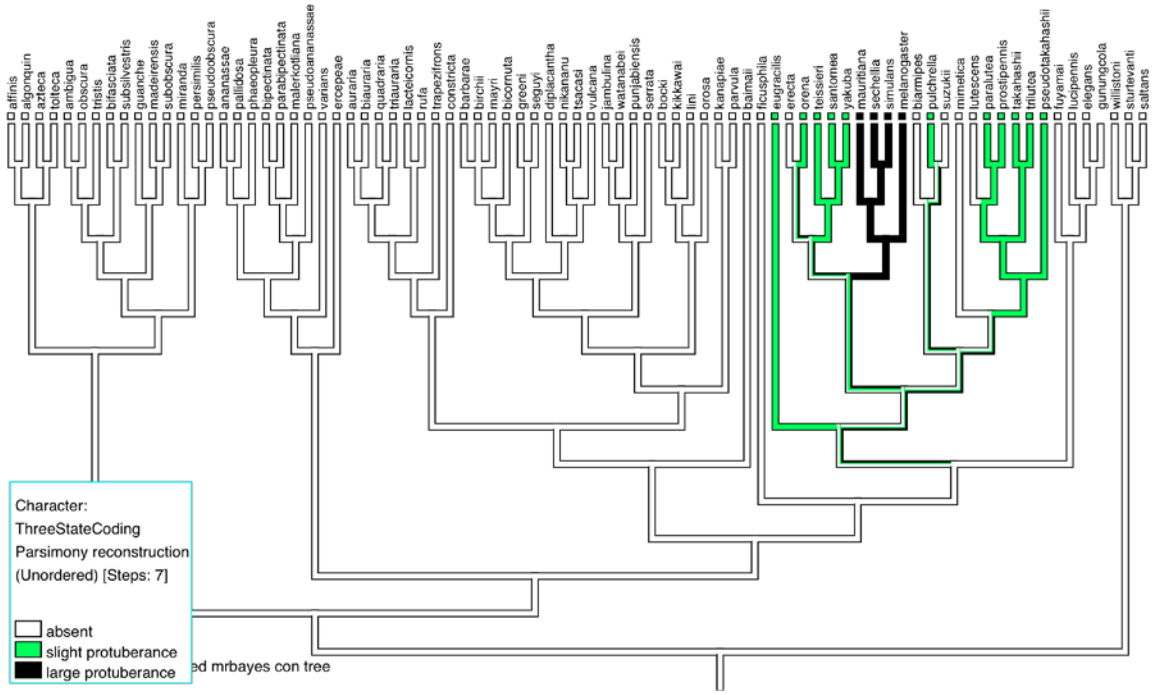


Figure 5. Three-state coding character state reconstruction. SEM images were evaluated and each species was classified by choosing one of three character states – "absent" (i.e. "non-lobed"), "slight protuberance" (i.e. "non-lobed", but possessing a slight bump on the genital arch), or "large protuberance" (i.e. "lobed"). A character state reconstruction was performed for these categorizations using parsimony on the *Drosophila* phylogeny. This reconstruction indicates that there was a single origin of the posterior lobe, progressing from absence to slight protuberance to large protuberance.

2.2.2 Confocal Microscopy of the Posterior Lobes of *Drosophila melanogaster* Clade Species

To study the development of the posterior lobe, we sought to visualize the initial stages of posterior lobe formation. We utilized an Arm-GFP stock of *D. melanogaster* flies that express GFP-tagged Armadillo protein. Armadillo is *Drosophila* β-catenin, a protein that labels cell junctions (*69*). This stock was used to image males at various time points throughout development using the confocal microscope. To the same effect, an antibody against Armadillo

is available to perform this analysis in other species for which the transgenics do not exist. The timing of genital (specifically, posterior lobe) development has been determined using these techniques. We have concluded that while there tends to be quite a bit of variability, the *D. melanogaster* posterior lobe is specified and has adopted a near final shape by approximately 52 hours after puparium formation (APF) (Fig. 6).

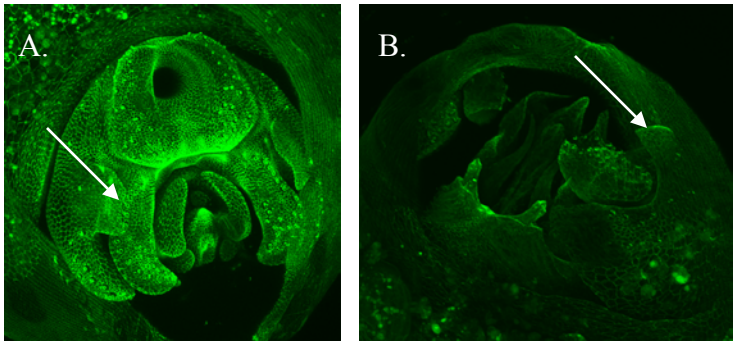


Figure 6. The posterior lobe is specified by 52 hours APF. *D. melanogaster* Arm-GFP pupal genitalia at 52 (A.) and 65 hours APF (B.). Arrows mark posterior lobes.

To further investigate the intricacies of the development of the posterior lobe, a more detailed account of morphology was recorded by monitoring the course of posterior lobe development in live organisms. *D. melanogaster* Arm-GFP males were embedded in agarose with their posterior ends sticking up out of the gel. They were then live-imaged on the confocal microscope using a water immersion lens. This analysis has allowed us to conclude the location of where the posterior lobe initially forms and that it continues to take shape and grow in size until it reaches its final adult form (Fig. 7).

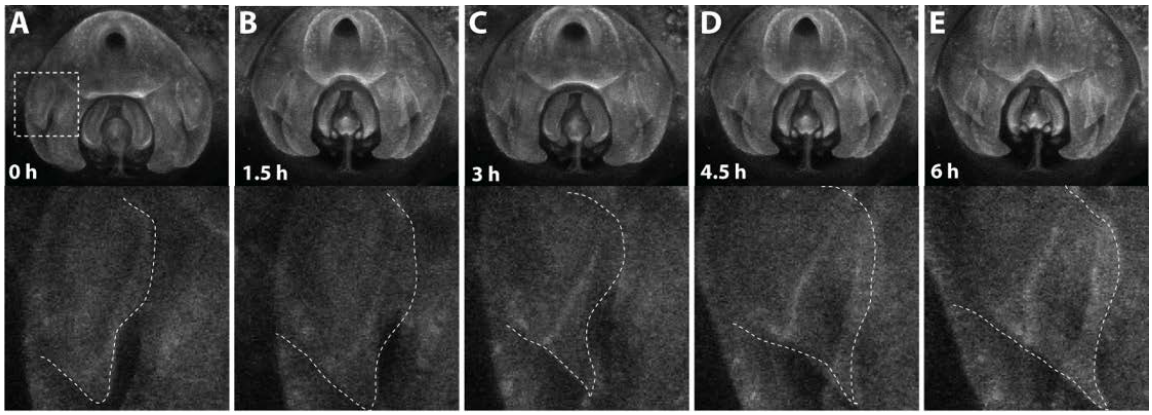


Figure 7. Posterior lobe developmental timecourse. Over a six hour period of development, drastic changes in shape and size of the growing posterior lobe are observed in an *armadillo::GFP* animal. Insets: close-up view of the leftward posterior lobe (white box in A).

2.3 ANALYSIS OF THE DEPLOYMENT OF KEY DEVELOPMENTAL REGULATORS

Morphogens and transcription factors maintain the critical role of directing the pattern of tissue development, raising the hypothesis that (1) these molecules contribute to posterior lobe patterning and (2) that shifts in their expression underlie species-specific differences in the developing posterior lobe. We have taken a candidate gene approach to search for the genes responsible for changes in posterior lobe morphology.

Immunostaining with antibodies against each of these proteins in pupal male genitalia at various developmental stages is a straightforward way to determine whether we should pursue a more rigorous characterization of their potential roles in the morphology of the posterior lobe. Determining the expression patterns of these morphogens and transcription factors will

illuminate the general developmental cues that may control posterior lobe ontology, and it will likely provide genes and pathways that have changed during posterior lobe diversification.

If these candidates are expressed in the posterior lobe, and are differentially expressed between species, we can begin to dissect the enhancers of these genes and determine if any *cis* changes exist and how they are contributing to posterior lobe development. Furthermore, any candidate expressed in the posterior lobe may provide helpful insights into the origin of this structure.

2.3.1 *Pox neuro* (*Poxn*) as a Candidate for Shaping Posterior Lobe Morphology

By performing a literature and database search for relevant signaling molecules with posterior lobe phenotypes, we encountered a gene known as *Pox neuro* (*Poxn*). *Poxn* has been described in the literature as having roles in peripheral nervous system development, adult appendage formation, and fertility (*70, 71*).

In particular, Boll and Noll (2002) found an interesting role for *Poxn* in the development of the genitalia. Male flies that are null for *Poxn* completely lack posterior lobes (*72*). They further examined the expression of *UAS-GFP* driven by a *Poxn-Gal4* and discovered a sexually dimorphic pattern expressed in males but absent in females. They methodically dissected the *Poxn* enhancer functions and found a posterior lobe-specific enhancer. When this enhancer is used to drive *Poxn* expression in *Poxn* null flies lacking posterior lobes, it completely rescues posterior lobe morphology (*72*). The decreased fertility exhibited by *Poxn* null males is restored only when the enhancer is intact (*72*). This implies that male fertility depends on the integrity of the posterior lobe. However, this gene has never been implicated in the evolution of the male

genitalia. We hypothesize that *Poxn* contributed to posterior lobe origination and morphological shape differences between *Drosophila* species.

To confirm the necessary role of *Poxn* in the proper development of the posterior lobe, *Poxn* deficient flies were examined and imaged using a dissecting microscope. Posterior lobes are completely absent in flies that are deficient for *Poxn* (Fig. 8). This corroborates published results implicating *Poxn* in the proper development of posterior lobes, and it supports further investigations into the role of this gene and its posterior lobe enhancer region in the diversification of this tissue.

Figure 8. *Poxn*-deficient flies lack posterior lobes. These males are of the following genotype: $poxn^{\Delta M22\text{-}B5}$ / Df(2)WMG. White asterisks denote where posterior lobes are located in wild type flies.

2.3.1.1 Poxn Expression

We obtained an antibody against Poxn (*72*), and performed an analysis of Poxn expression in larval imaginal discs and pupal genitalia. We confirmed that the antibody is working properly by

the detection of Poxn in the sensory mother cells (SMCs) in white prepupal wing imaginal discs as well as the wing hinge area of larval wing discs (*70, 71*) (Fig. 9). We have also demonstrated that this antibody is effective in other species as well due to its pattern in the equivalent regions (Fig. 9).

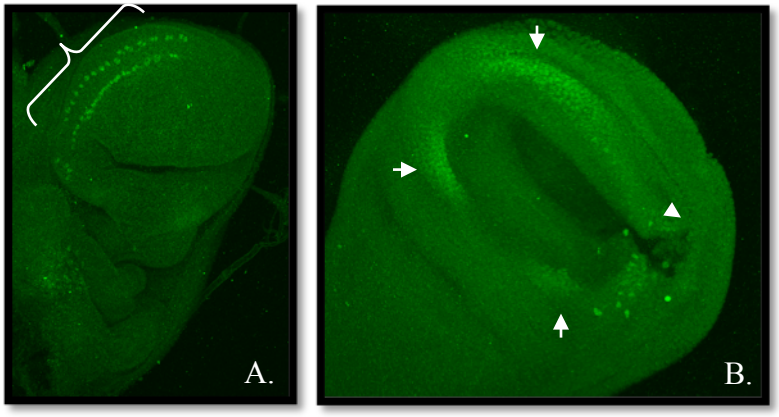


Figure 9. Poxn antibody staining in wing imaginal discs. *D. melanogaster* white pre-pupal wing disc (A.), *D. yakuba* third instar larval wing disc shows antibody is cross-reactive in outgroup species (B.). Bracket denotes Poxn expression in SMCs. Arrowheads denote Poxn expression in the prospective wing hinge area in a quadrant pattern.

Immunostaining in *D. melanogaster*, *mauritiana*, and *simulans* genitalia have allowed us to confirm posterior lobe expression of Poxn once the posterior lobe has begun development (Fig. 10). The presence of Poxn in the posterior lobe provides us with the confidence to continue our investigation of the role of Poxn in this tissue. Poxn is required for proper posterior lobe morphology and may be an interesting member of the developmental program of this organ after its specification.

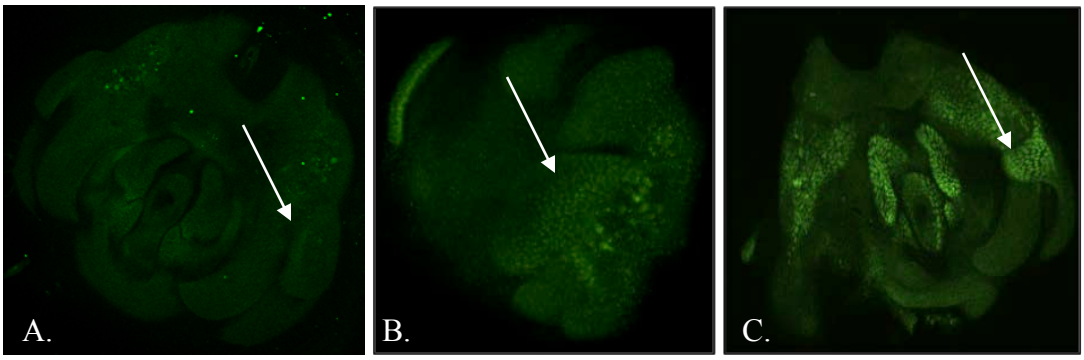


Figure 10. Poxn antibody stains in the pupal genitalia reveal expression in the developing hypandrium, lateral plates, and posterior lobes (arrows) in *D. melanogaster* (A.), *D. simulans* (B.), and *D. mauritiana* (C.).

Complementary to the immunofluorescence assays, we have performed *in situ* hybridizations in larval imaginal discs with a probe for *D. melanogaster Poxn*. *Poxn* transcript was successfully detected in the expected regions, just as Poxn protein was visualized with the antibody (Fig. 11).

We subsequently performed *in situ* hybridizations in the pupal genitalia to determine if *Poxn* transcript expression is present in the suggested regions based on the immunofluorescence data (Fig. 11). Indeed, *Poxn* transcript was visualized in the developing genitalia. This analysis allowed us to establish the *in situ* protocol for other candidate genes that possibly lack available antibodies, and it enabled us to better assess the timing of when *Poxn* is initially specified.

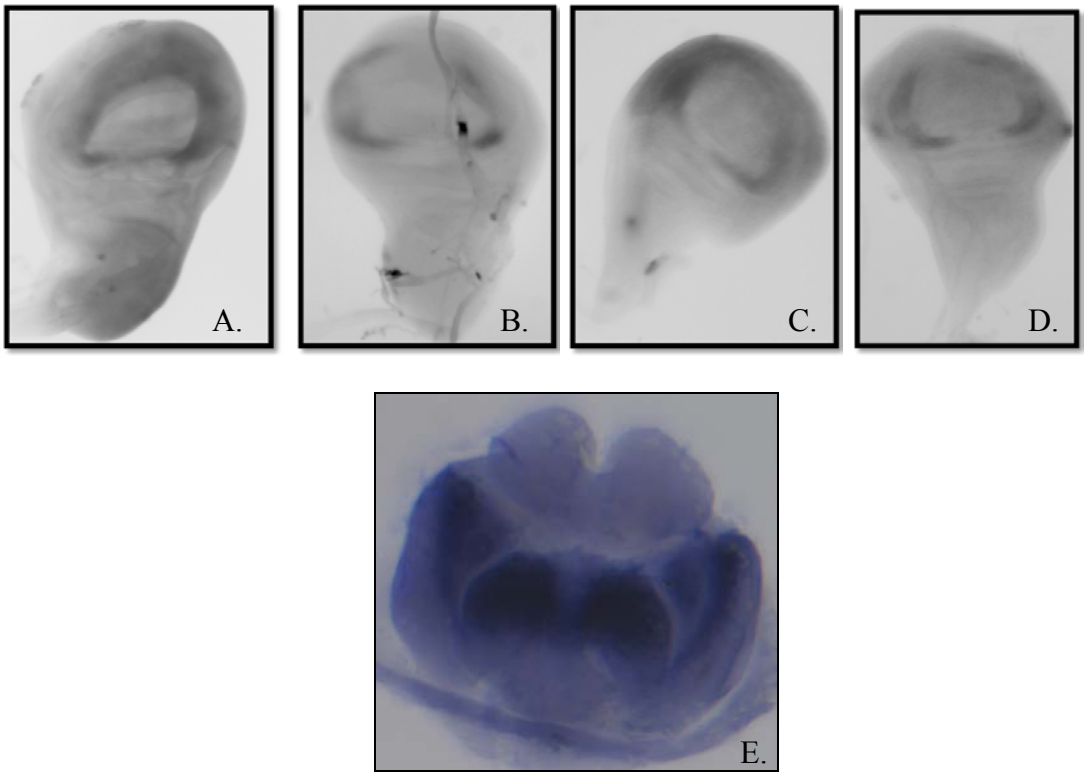


Figure 11. *Poxn in situ* hybridizations with *D. melanogaster Poxn* probe is effective in all species and can be performed in the genitalia. *D. melanogaster* (A.), *D. simulans* (B.), *D. sechellia* (C.), and *D. mauritiana* (D.) all show *Poxn* expression in the wing hinge region in third instar wing discs. *Poxn* transcript is also detectable in the developing genitalia, as seen in *D. mauritiana* (E.). The data in this figure was generated by Kelsey Stayer.

2.3.1.2 Poxn Enhancers

The posterior lobe enhancer for *D. melanogaster Poxn* has been identified (*72*). To test the hypothesis that changes to this enhancer have contributed to the emergence and variation in shape and size of the posterior lobe, we directly compared the activity of this enhancer from the different species of the *melanogaster* clade as well as the outgroup species *D. yakuba*. *Poxn* enhancer sequences specific to *D. melanogaster*, *simulans*, *mauritiana*, *sechellia*, and *yakuba* were cloned into a reporter vector containing GFP and a naïve promoter (Fig. 12). T hese

constructs were then used to make transgenic *D. melanogaster* animals by insertion into the same genomic location to protect against positional effects (*73*).

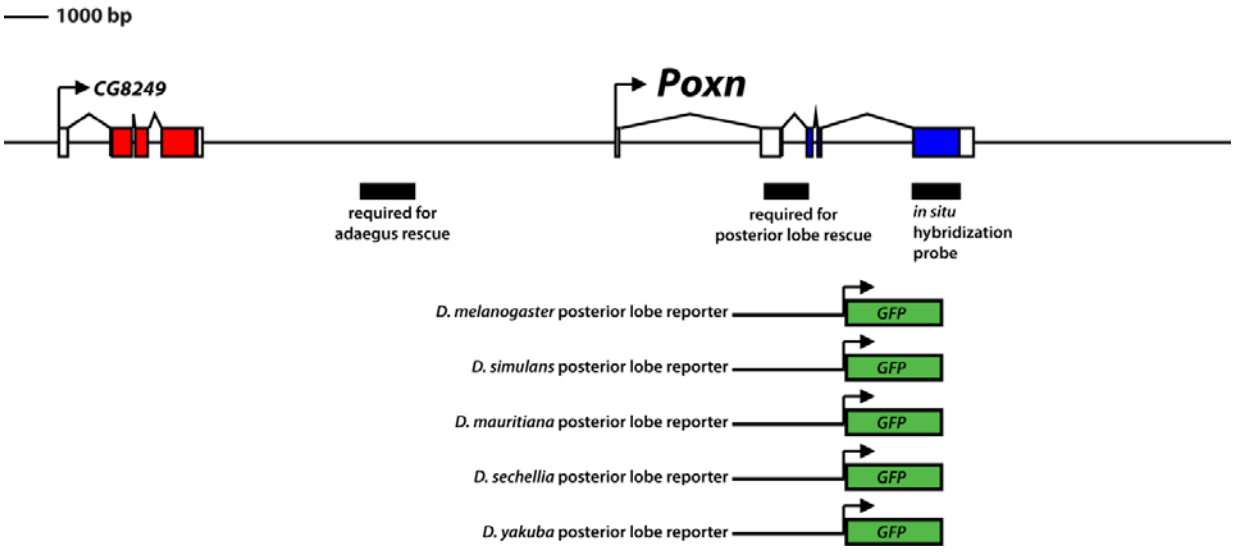


Figure 12. Schematic of the *Poxn* gene. The relevant enhancers with associated functions are depicted in black boxes. The *in situ* hybridization probe for *Poxn* is also depicted in a black box. The species-specific enhancer sequences required for posterior lobe rescue were fused to GFP and subsequently used as a reporter in *D. melanogaster*.

This approach aims to further our understanding of the Poxn expression data by discriminating between whether any observed differences in expression are due to a *cis* change or a *trans* change. In other words, the immunofluorescence assays were performed in each individual species, and therefore in different genetic backgrounds. Given that the *Poxn-GFP* transgenes express the species-specific Poxn protein in the *D. melanogaster* background, this eliminates *trans* effects.

Genitalia from homozygous species-specific *Poxn* PLE reporter lines were imaged, and GFP expression in posterior lobes were quantified and analyzed (Fig. 13). Fascinatingly, *D.*

mauritiana, *D. simulans*, *D. sechellia*, and *D. yakuba* all exhibit higher reporter activity than *D. melanogaster*, with slight variations in activity between each of these species. There are several possible explanations for this. Perhaps the *D. mauritiana* lineage has evolved higher levels of *Poxn* activity from this enhancer. On the other hand, since *D. yakuba* is more distantly related, the *D. melanogaster Poxn* gene could have evolved lower levels of activity. Despite the means of these differences in expression, these data certainly suggest that *D. yakuba*, *D. simulans*, *D. sechellia*, and *D. mauritiana* enhancers are stronger than that of *D. melanogaster*, and these differences could underlie posterior lobe shape distinctions (Fig. 13). The observation that the *D. yakuba Poxn* enhancer is active in the posterior lobe of *D. melanogaster* is especially striking. This suggests that its activity predated the formation of this organ.

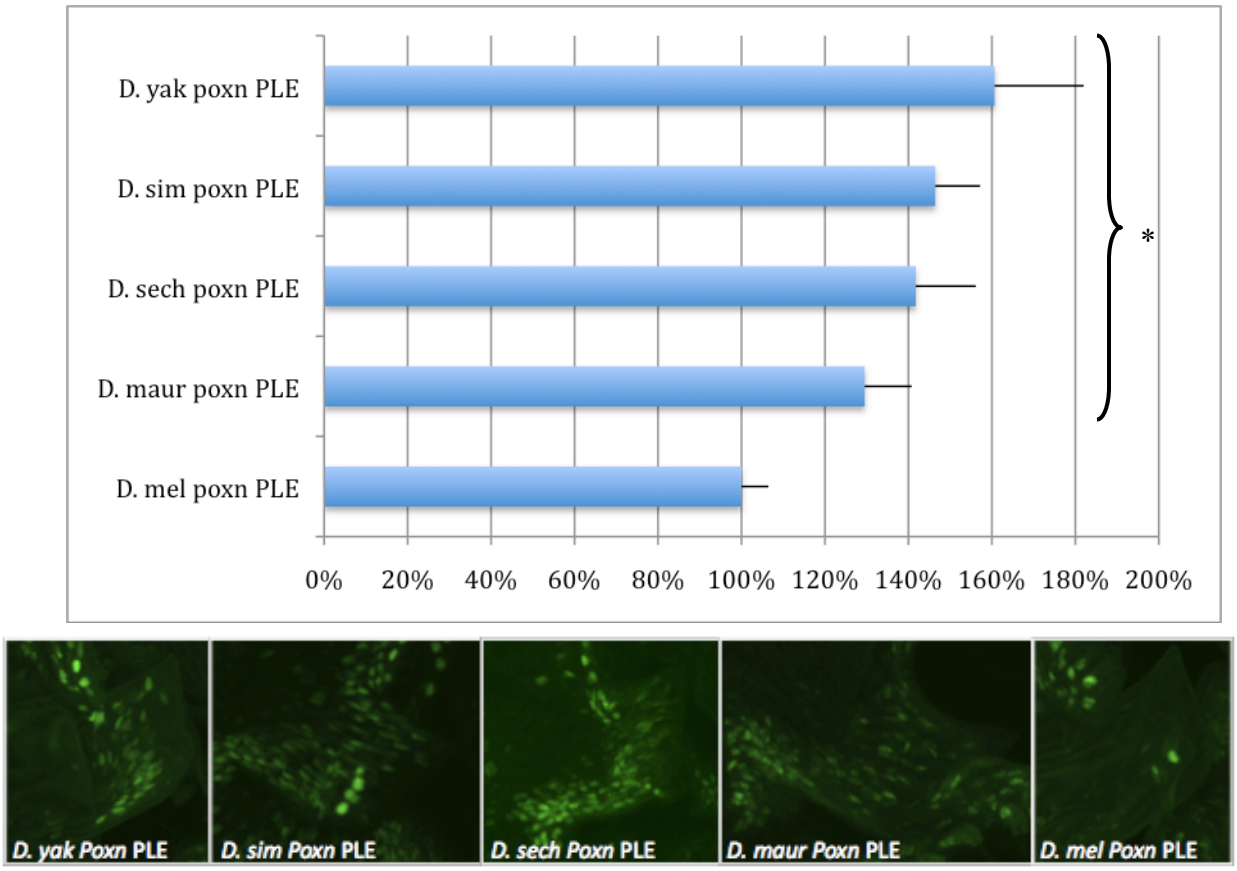


Figure 13. Species-specific *Poxn* posterior lobe enhancer (PLE) reporter GFP values relative to *D. melanogaster*. *D. mauritiana*, *D. simulans*, *D. sechellia*, and *D. yakuba* all exhibit higher reporter activity than *D. melanogaster*, with slight variations in activity between each of these species. Unpaired t-tests were performed to determine statistical significance. *$p < 0.05$. Representative posterior lobes whose GFP was quantified are shown below the graph.

Two *Poxn* Gal4 drivers were used to drive expression of GFP in an effort to analyze their expression domains. *Poxn-Gal4-13* contains the *Poxn* coding exons and upstream region, but it lacks all *Poxn* introns (*72*). Alternatively, *Poxn-Gal4-14* only differs from the other Gal4 construct in that it contains all introns, the most notable of which is the second intron housing the posterior lobe enhancer (*72*). GFP expression being driven by these two Gal4 constructs in the

early genitalia is indistinguishable from one another, which indicates that the upstream region of *Poxn* is responsible for driving expression in broader areas during earlier stages leading to the perdurance of GFP (Fig. 14).

We can draw some conclusions by comparing these data to the *Poxn* posterior lobe enhancer data described previously. The *Poxn* PLE reporters appeared to be active in later stages of posterior lobe development. However, the upstream region of *Poxn* drove broader expression in early stages of genital development leading to the perdurance of GFP. Because both of these *Poxn* Gal4 drivers are clearly driving expression in the posterior lobes, they will be useful in overexpressing other potential candidates or knocking down these candidates using RNAi in this tissue. In spite of this, species-specific *Poxn* posterior lobe enhancer Gal4 drivers will more specifically target experiments in the posterior lobe and will allow for the further characterization of this critical *Poxn* enhancer for proper posterior lobe morphology.

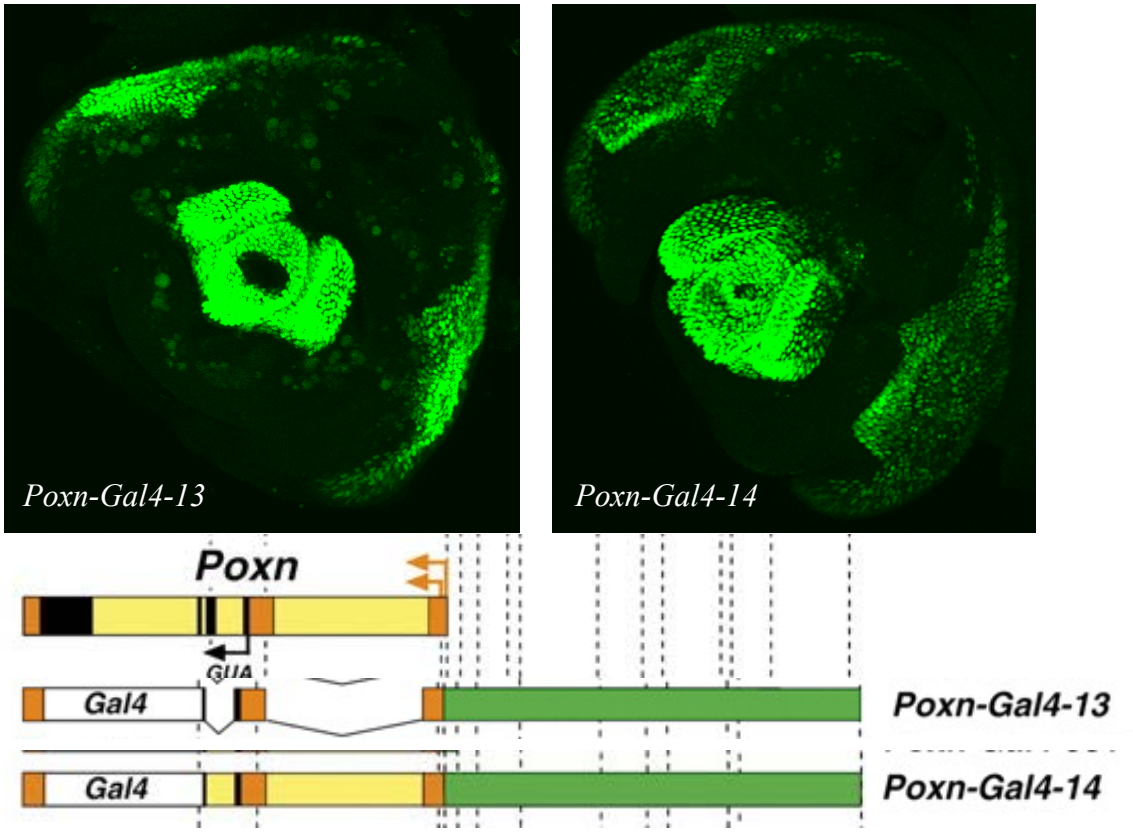


Figure 14. Poxn Gal4 constructs drive GFP expression in the developing genitalia. *Poxn-Gal4-13* contains *Poxn* and its upstream region, but lacks associated introns. *Poxn-Gal4-14* contains *Poxn*, its upstream region, and all associated introns, including the posterior lobe enhancer. These constructs were donated by the Noll Lab (*72*).

2.3.2 Other Candidates

There are still other candidates that must be considered. An excellent candidate to investigate for a potential role in shaping this tissue is *Decapentaplegic* (*Dpp*). *Dpp* is required for the correct patterning of all fifteen imaginal discs, and it regulates tissue growth and size (*74*). *Hedgehog*

(*Hh*) and *Wingless* (*Wg*) are two morphogens that have roles in appendage formation and coordinate the development of several tissues (*41, 68, 75*). They are also practical candidates to investigate potential roles in posterior lobe morphology, and they have never been studied during genital development beyond the genital imaginal disc.

Genes that have been implicated in cell cycle regulation are important contenders for contributing to the distinct morphologies of posterior lobes between species. For example, c-myc is a transcription factor that regulates cell proliferation, cell growth, and apoptosis (*76, 77*). In addition, components of the hippo pathway controlling tissue growth are likely to participate in posterior lobe growth (*78, 79*). These genes were explored for their roles in posterior lobe morphology by originally looking for their expression in the genitalia by way of immunostaining with antibodies specific to these candidates.

An initial screen was performed using antibodies against known signaling molecules, morphogens, and cell cycle regulators (Fig. 15). Any expression profiles that proved to be interesting were further characterized.

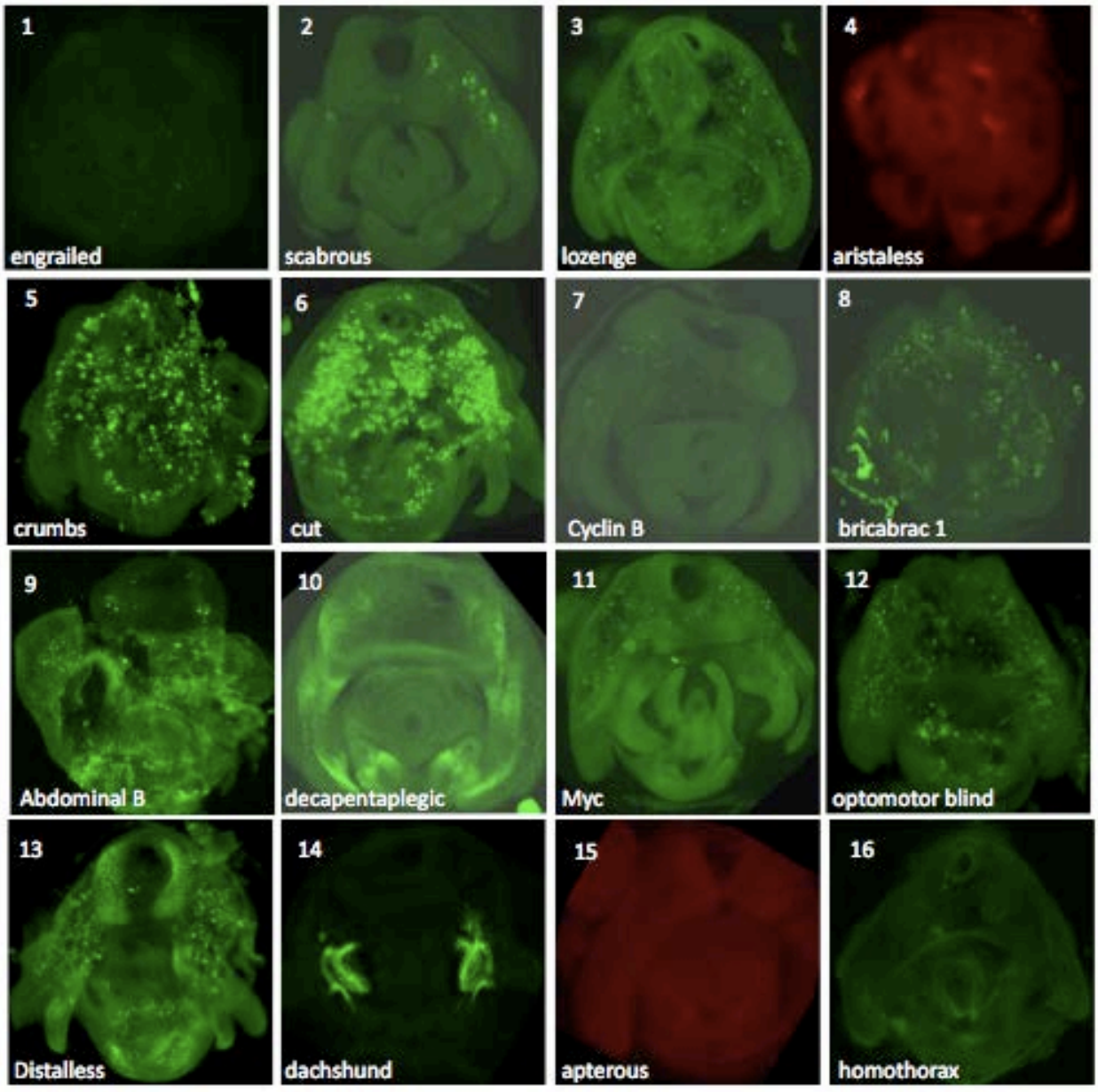


Figure 15. Immunostaining survey of candidates in the developing genitalia. No/minimal genital expression in 1, 2, 3, 5, 6, 7, 8, 11, 12, 15. Uniform/non-specific genital expression in 9 & 16. Al (panel 4) expression in lateral plate. Dpp (panel 10) expression in lateral plate and genital arch. Dll (panel 13) expression in hypandrium, clasper, and anal plate. Dac (panel 14) expression in lateral plate and clasper. The data in this figure was generated by Chas Elliott.

The antibody screen proved to be a valuable endeavor in finding some interesting candidates that would be worth further investigation. While some candidates were determined not to have a role in genital development or morphology due to a lack of expression in the genitalia, others revealed relevant expression patterns. Aristaless (Al), decapentaplegic (Dpp), and dachsund (Dac) are all expressed in the tissue that gives rise to the posterior lobe (Fig. 15). These genes may hold a potentially important role in the development of this organ and should be further characterized in the future.

2.3.2.1 Notch Signaling

Perhaps the most promising candidate to come out of the antibody survey with a relevant expression pattern is Delta (Dl), the Notch pathway ligand (*80-83*). Notch signaling is a key developmental signaling cascade that specifies and restricts cell fates and communication and regulates pattern formation by creating boundaries (*80-83*). Like many high-level regulators, Notch signaling controls several developmental processes in a variety of species from worms to flies to vertebrates.

Notch signaling can be used in different settings to elicit different cellular responses (*80*). For example, Notch can inhibit, delay, or induce cellular differentiation. This signaling pathway can also promote apoptosis, cell division, or keep cells in a static state depending on the setting. This suggests that the redeployment of this signaling pathway throughout evolution could result in varied outcomes.

Performing immunofluorescence assays on pupal genitalia with an antibody against Dl revealed expression in the epithelial zone that will give rise to the posterior lobe (Fig. 16a). Dl

appears to be dynamically deployed in that it is no longer expressed in the posterior lobes after they have formed (Fig. 16b).

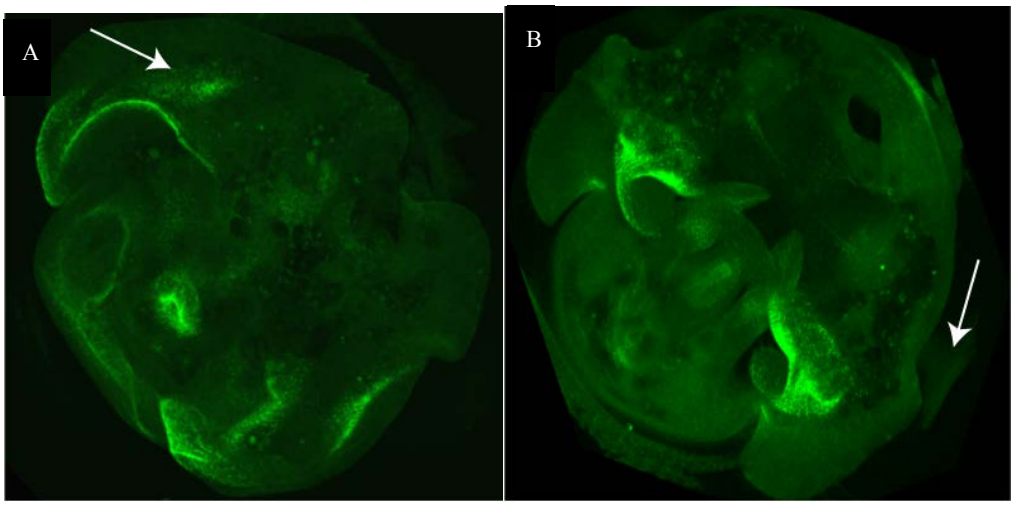


Figure 16. Delta expression presages posterior lobe formation. (A) In a mid-stage pupa, Delta expression is present in the claspers and in the epithelial zone that will give rise to the posterior lobe (white arrow). (B) Later during posterior lobe development, the formed posterior lobe lacks Delta (white arrow), while the claspers continue to express it at high levels. The data in this figure was generated by Chas Elliott.

This relevant spatiotemporal expression of Dl suggests that the Notch pathway is a likely player in the appearance of the novel posterior lobe, as it is expressed in the correct place and at the correct time for posterior lobe emergence.

2.3.2.2 Hippo Signaling

The Hippo signaling pathway is composed of a highly conserved kinase cascade and is necessary for the proper regulation of organ growth and regeneration in *Drosophila* and vertebrates (*78, 79, 84*). Its function in controlling tissue growth makes it a likely candidate to participate in posterior lobe growth. Hippo mutants show tumor and tissue overgrowth phenotypes, raising the

hypothesis that expression differences in Hippo pathway members in *Drosophila* species may underlie differences in posterior lobe morphology.

Dachsous (Ds) is an upstream modulator of the Hippo pathway, whereby its binding to Fat, the Hippo pathway receptor, can elicit a phosphorylation cascade ultimately controlling target gene expression (*78, 79, 84*). There are many *Ds* mutant fly stocks available, and we initially imaged these *Ds* homozygous mutant males by SEM to examine any potential posterior lobe phenotypes. Loss-of-function *Ds* mutants display aberrant posterior lobe phenotypes (Fig. 17). The posterior lobes of these animals are clearly misshaped and appear to be expanded. This confirms that a role for the Hippo pathway exists in the development and possible diversification of this organ.

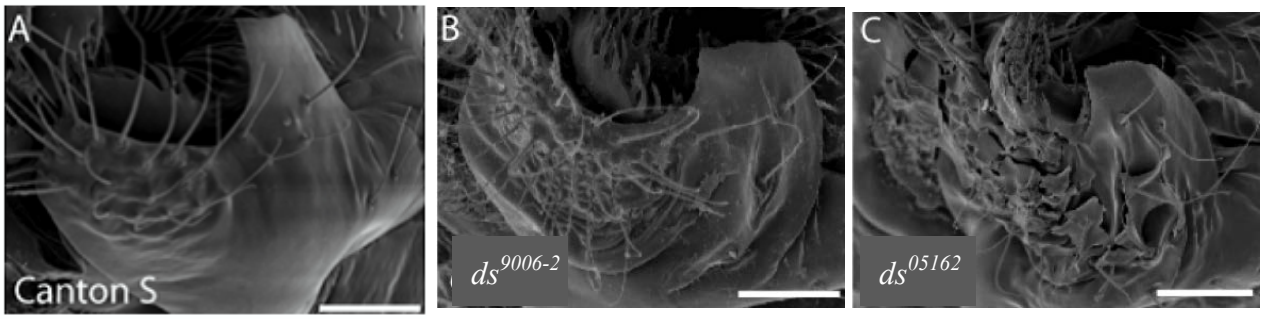


Figure 17. *Dachsous* mutant males exhibit abnormal posterior lobes. (A) Posterior lobe of a wild-type *D. melanogaster* male. (B,C) Loss of function mutations in the *dachsous* gene, an upstream modulator of the Hippo pathway, result in an expanded posterior lobe.

2.4 CONCLUSIONS

These studies probe the macroevolutionary question of novelty and shape change, and our initial results using the posterior lobe as a model indicate that this is a fruitful area of research. We have been able to make several conclusions that will pave the way for future investigations. The SEM survey of *Drosophila* male genitalia confirmed the unique presence of the posterior lobe in *D. melanogaster* clade species, and imaging of live and fixed pupal male genitalia from this clade have localized the time and place of posterior lobe development. Analysis of the *Poxn* gene confirmed its specific expression in the posterior lobe, and transgenic evaluation of the regulatory region required for the posterior lobe function of *Poxn* revealed subtle differences in gene activity, which may be relevant for the evolution of posterior lobe shape differences. Interestingly, the *D. yakuba Poxn* posterior lobe enhancer is active in the posterior lobe, suggesting that this activity predated the formation of this organ. In total, these findings have uncovered the developmental intricacies of this novel tissue, and they shed light on some of the players involved in proper morphology and potential diversification of the posterior lobe.

3.0 CHAPTER 3: CONCLUSIONS

While investigations into the evolution of the posterior lobe are only in their initial stages, many recent findings have been promising in guiding the future directions of this project. Importantly, several protocols have been established to study the developing fly genital system. Scanning electron microscopy, live imaging, immunofluorescence assays, and *in situ* hybridizations have all been successfully established in this relatively uncharted system. This will allow us to study the expression profiles of other candidate genes (those with or without available antibodies).

Some such candidates are *aristaless* (*Al*), *decapentaplegic* (*Dpp*), and *dachsund* (*Dac*), as they are all expressed in the tissue that gives rise to the posterior lobe. These genes may hold a potentially important role in the development of this structure and will be further characterized in the future.

Subsequent molecular characterization of lobe patterning genes will contribute to our knowledge of the initial development of this tissue. Poxn serves as a marker of *melanogaster* clade posterior lobes. Once the expression of Poxn has been thoroughly described in these species that have posterior lobes, we can begin to assess the role of Poxn in non-lobed species. The slight protuberances that these non-lobed species possess may or may not be related to the posterior lobe, and examination of Poxn expression in this tissue will be informative. This also applies to other candidate genes that we find to be associated with the development of the posterior lobe.

Our hypothesis is that the outgrowths observed in species of the *yakuba* clade are of an independent origin from the posterior lobes of the *melanogaster* clade. If this holds true, any confirmed candidates would positively mark the posterior lobe, and they would not be similarly expressed in outgroup species. Alternatively, if these structures share a common origin, the markers would be shared.

Poxn has proven to be an interesting player in the development of the posterior lobe. We know that Poxn is required in this tissue, lobe-bearing species' *Poxn* enhancer activity is present in the posterior lobe, and the *Poxn* posterior lobe enhancer of outgroup species *D. yakuba*, which lacks a posterior lobe, also has activity when placed in *D. melanogaster*. So the question remains as to what function the *D. yakuba Poxn* posterior lobe enhancer is serving in *D. yakuba* itself. Experiments in which the *D. melanogaster* and *D. yakuba Poxn* posterior lobe enhancer reporters will be incorporated into the *D. yakuba* genome are currently underway to address this question. Utilizing the time-lapse protocol to observe the developmental time course of *Poxn* posterior lobe enhancer activity will also reveal the trajectory of expression in a spatiotemporal context. In addition, we have recently made species-specific *Poxn* posterior lobe enhancer Gal4 lines. We can drive expression of *UAS-Poxn* using these Gal4s and determine if the variation in activity of these enhancers between species results in differing posterior lobe phenotypes.

Ultimately, we can analyze the effects of introducing genital expression of Poxn (and other candidates) in non-lobed species. Once we confirm the earliest posterior lobe markers that are expressed as this tissue is being specified, we can introduce them as transgenes or overexpression constructs in *D. yakuba* and determine if they are sufficient to drive posterior lobe-like outgrowths. Any gene that is sufficient to induce posterior lobe growth will be investigated for how their downstream targets are deployed.

Delta, the Notch pathway ligand, and *Dachsous*, the Hippo pathway upstream modulator, have also provided data worth further exploration. Dl and Ds immunofluorescence assays need to be performed in other lobe-bearing species as well as outgroup species. We also obtained a Notch temperature-sensitive mutant stock of flies to examine. Preliminary results show that these flies, when placed at the non-permissive temperature, develop severely stunted posterior lobes. This provides a drastic phenotype to underscore the antibody analysis that simply revealed *Dl* expression in the tissue that gives rise to the posterior lobe.

Due to the result that the *D. yakuba Poxn* posterior lobe enhancer is active in *D. melanogaster*, we revised a working hypothesis for the potential coordination of Poxn and Notch. Perhaps the novel function of Poxn arose as a result of changes upstream. In other words, the *D. yakuba* enhancer is active in *D. melanogaster* because of *D. melanogaster*'s translandscape, raising the possibility that Poxn could be a Notch target. To address this, we must examine *Poxn* enhancer activity in flies that have depleted levels of Dl by way of Dl-RNAi. All of these investigations in combination will reveal the coordination of the many presumed players involved in the development and diversification of the novel posterior lobe.

In a search for candidates that are expressed in the developing genitalia, qRT-PCR or RNA-sequencing of isolated whole genitalia may be fruitful approaches. This will uncover transcripts that are present in the genitalia, but they will need to be further investigated for expression at the right place and time to be explicitly involved in posterior lobe development.

Every gene that is specifically deployed in the posterior lobe tells a part of the history of how posterior lobe expression evolved. By delving into the history of each gene, we have the potential to find a posterior lobe regulatory element and determine its ancestral function. We can then piece together a picture of how this novel structure came into being.

These studies delve into the macroevolutionary question of how novel structures arise and how three-dimensional shape changes. Using a microevolutionary approach to investigate the diversification of a novel organ in closely related species of *Drosophila* allows us to get at this challenging question. As is typical in the development of any tissue, it is becoming clear that many genes are likely being coordinated for the proper development and diversification of the posterior lobe. Exploring what genes contribute to the development of this unique structure and how these genes are modified will allow us to appreciate how new genetic programs are pioneered to make the vast array of distinctive shapes we see in nature.

3.1 MATERIALS AND METHODS

Fly Stocks:

Species from the *Drosophila melanogaster* species group were obtained from the UCSD Stock Center (App. C). *Poxn* mutant and Gal4 lines were obtained from the Noll Lab at the University of Zürich (*72*). Other candidate lines were obtained from the Campbell Lab at the University of Pittsburgh.

Scanning Electron Microscopy:

Samples were prepared for SEM in isoamyl acetate overnight at room temperature. Once dry, samples were adhered to a stub with double-sided conductive carbon tape and coated with gold palladium. Samples were then imaged on a scanning electron microscope.

Developmental Time Lapse:

Pupae were embedded in 2% agarose with the posterior end of the fly pointing up. Using the 40X water immersion lens, pupae were imaged over a six-hour time course on an Olympus Fluoview confocal microscope with z-stacks being taken every fifteen minutes.

Immunofluorescence Assays:

The Poxn antibody was obtained from the Noll Lab at the University of Zürich (*72*). Antibodies against other candidates were acquired from the Carroll Lab at the University of Wisconsin-Madison and the Campbell Lab at the University of Pittsburgh.

Dissected samples were fixed for 30 minutes in 4% paraformaldehyde. Primary antibody was applied for two hours at room temperature or overnight at 4°C. After six ten-minute washes in PBT, Alexa fluor secondary antibody at a concentration of 1:500 in PBT was applied for two hours at room temperature. After another six ten-minute washes, samples were mounted in glycerol on slides and imaged using an Olympus Fluoview confocal microscope.

In situ *Hybridizations*:

Poxn primers were designed using the GenePalette software program. Probe templates were amplified using these primers and later synthesized with DIG RNA labeling mix and T7 RNA polymerase. The transcription reaction was stopped by ethanol precipitation, and the probe was resuspended and stored in hybridization solution.

Dissected samples underwent washes, hybridization, and overnight probe incubation at 65°C. On the second day, the samples were incubated with hybridization solution, washed with PBT, and incubated with Roche anti-DIG AP Fab fragments 1:6000 in PBT overnight at 4°C. Samples were then washed with PBT and staining buffer. Staining solution was added, and

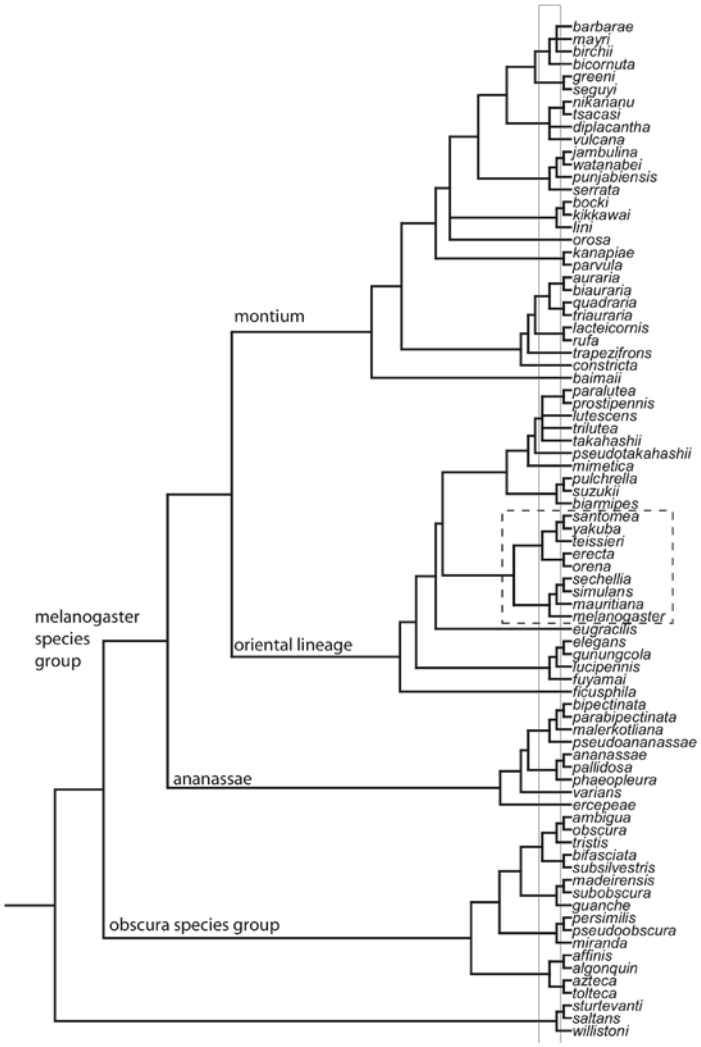
patterns were left to develop in the dark. Once stained, samples were washed, mounted onto slides, and imaged using a high-power Leica dissecting microscope.

Poxn PLE Reporters:

The Poxn posterior lobe enhancer was cloned into the pS3aG vector downstream of an Hsp70 naïve promoter and upstream of a GFP cassette (*85*). This construct was sent for injection into embryos using the PhiC31 integration system performed by Rainbow Transgenic Flies, Inc.

APPENDIX A

MELANOGASTER SPECIES GROUP PHYLOGENY



Adapted from Jeong, et al. 2006 (*28*).

Figure 18. Melanogaster species group phylogeny.

APPENDIX B

PRIMERS USED FOR CLONING AND PROBE GENERATION

Name	Sequence	Orientation	T7 Promoter?	Restriction Site?
poxn in situ F	ACCGTGGTGAAGAAGGATCATCC	Forward	no	no
poxn in situ R	CAGATCAAAACTGGGTCAGTGG	Reverse	yes	no
poxn PLE F	TCGGTGGCTTAACACGCGCATT	Forward	no	AscI
poxn PLE R	ATCGCTGATTCCATGGCCCAGT	Reverse	no	SbfI
dpp in situ F	TCGACGATCGATTTCCGCACCA	Forward	no	no
dpp in situ R	GCATTTGATGCTCTCTCGCTC	Reverse	yes	no
ds in situ F	CCAACGATGTGGACACTTACCC	Forward	no	no
ds in situ R	TCCTGATCGGCATCAATGGCCT	Reverse	yes	no
fj in situ F	AGCAACAGCAACTCACCTGCAG	Forward	no	no
fj in situ R	ATCGCGTTTGATTGATGAGCGG	Reverse	yes	no
dpp in situ F2	CTGCACTTCGACGTGAAGAGCA	Forward	no	no
dpp in situ R2	GACTCTGCGCTCTCAAATCTGC	Reverse	yes	no
fj in situ F2	CCATGAGCGGCTTGAACTTTGAGC	Forward	no	no
fj in situ R2	AGTAGCTGAATATCTCGCCCTG	Reverse	yes	no
dpp in situ F3	CGATTGTTCGAGTTGCTAGCAC	Forward	no	no
dpp in situ R3	CGTAGAGCTTCTTCATCGGCTC	Reverse	yes	no
dpp in situ F4	GCTATCTGCTGTTGGACACCAA	Forward	no	no
dpp in situ R4	CTCCTGGTAGTTCTTCAGCACC	Reverse	yes	no
ds in situ F2	CGATGACGAGGGTAACATTCAC	Forward	no	no
ds in situ R2	CAGCTCCTTAGTCTCGAAATCC	Reverse	yes	no
ds in situ F3	ACGGACGGAGGACGTTATGAAG	Forward	no	no
ds in situ R3	CCGCATCCGAATCGAATGCATG	Reverse	yes	no

Table 1. Primers used for cloning and probe generation.

APPENDIX C

UCSD *DROSOPHILA* SPECIES AND ACCESSION NUMBERS

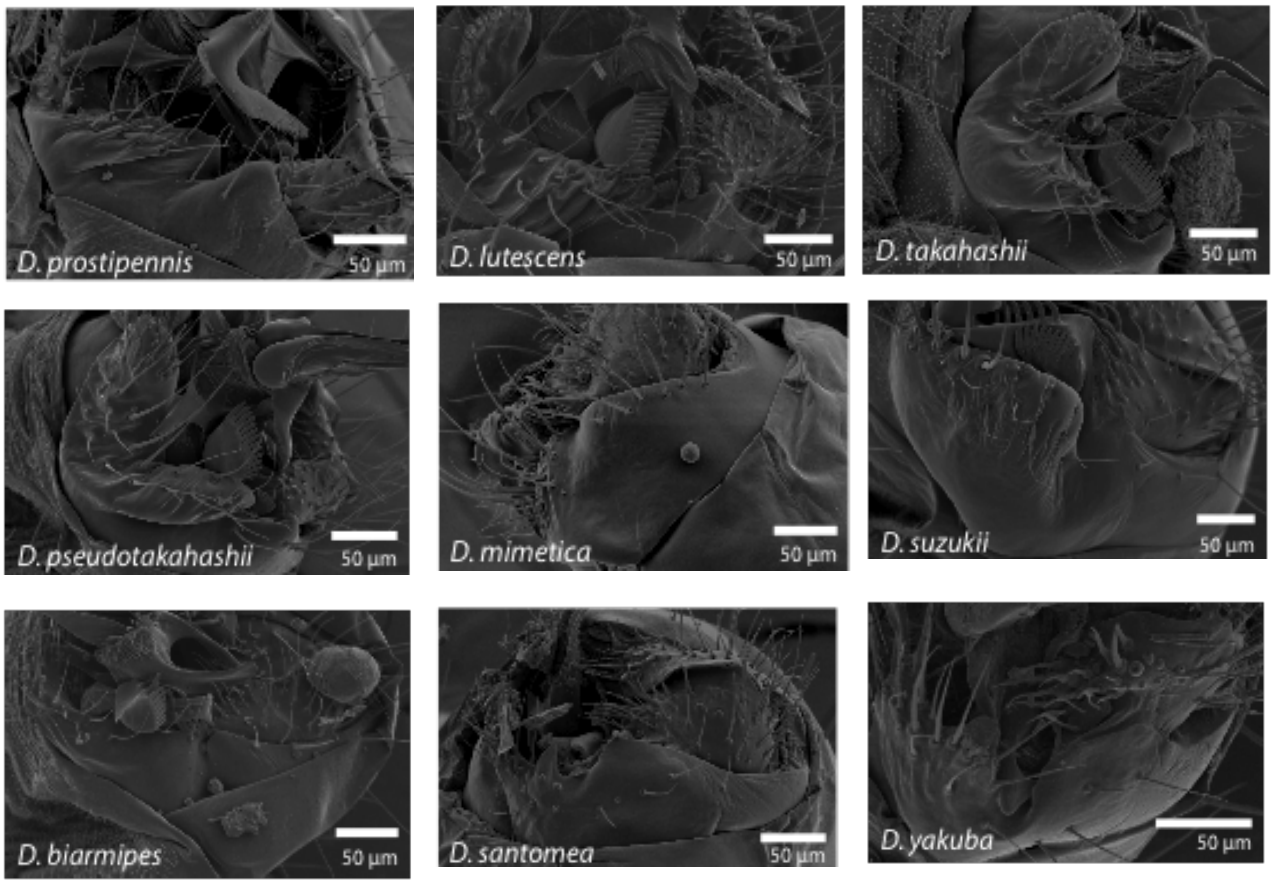
Accession Number	Species Name
14023-0361.09	*Drosophila biarmipes*
14027-0461.00	*Drosophila elegans*
14021-0224.00	*Drosophila erecta*
14026-0451.02	*Drosophila eugracilis*
14025-0441.05	*Drosophila ficusphila*
14029-0011.00	*Drosophila fuyamai*
14023-0331.02	*Drosophila lucipennis*
14022-0271.00	*Drosophila lutescens*
14021-0241.01	*Drosophila mauritiana*
14023-0381.00	*Drosophila mimetica*
14021-0245.01	*Drosophila orena*
14022-0291.00	*Drosophila prostipennis*
14022-0301.01	*Drosophila pseudotakahashii*

14021-0271.00	*Drosophila santomea*
14021-0248.03	*Drosophila sechellia*
14021-0251.001	*Drosophila simulans*
14023-0311.00	*Drosophila suzukii*
14022-0311.13	*Drosophila takahashii*
14021-0257.01	*Drosophila teissieri*
14021-0261.00	*Drosophila yakuba*

Table 2. UCSD Drosophila species and accession numbers.

APPENDIX D

SEM IMAGES OF *DROSOPHILA* MALE GENITALIA



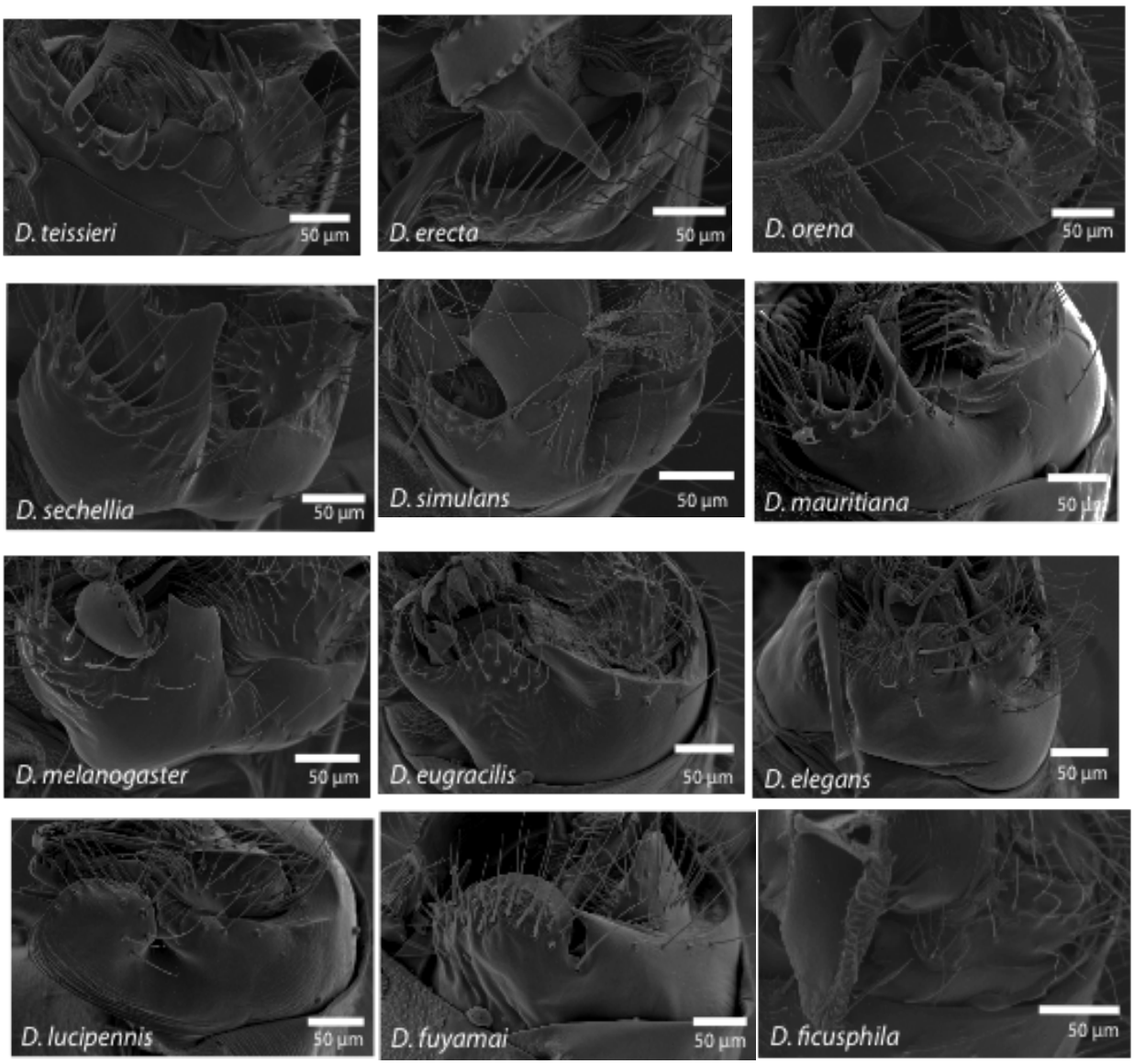


Figure 19. SEM images of Drosophila male genitalia.

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