Molecular Divergence with Major Morphological Consequences: Development and Evolution of Organ Size and Shape

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Abbreviations:

Bmp: bone morphogenic protein

CaM: calmodulin

CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II

ePL: epandrial posterior lobe

CL: clasper

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Abstract

Understanding the causes of the morphological diversity among organisms is a topic of great interest to evolutionary developmental biologists. Although developmental biologists have had great success in identifying the developmental mechanisms and molecular processes that specify organ size and shape within species, only relatively recently have the molecular tools become available to study how variation in these mechanisms gives rise to the phenotypic differences that are observed among closely related species. In addition to these technological advances, researchers interested in understanding how molecular variation gives rise to phenotypic variation have used three primary strategies to identify the molecular differences underlying species-specific traits: the candidate gene approach, differential gene expression screens, and between-species genetic mapping experiments. In this review, we discuss how these approaches have been successful in identifying the genes and the cellular mechanisms by which they specify variation in one of the most recognizable examples of the evolution of organ size, the adaptive variation in beak morphology among Darwin's finches. We also discuss insect reproductive structures as a model with great potential to advance our understanding of the specification and evolution of organ size and shape differences among species. The results from these two examples, and those from other species, show that species-specific variation in organ size and shape typically evolves via changes in the timing, location, and amount of gene/protein expression that act on tissue growth processes.

Introduction

One of the most striking patterns in the natural world is the remarkable diversity of organismal form. Understandably, biologists have long been fascinated with morphological variation among organisms— beginning with cataloging the breadth of organismal variation, to understanding the natural pressures that shape form and function, and with the advent of modern molecular techniques, understanding the mechanistic bases that generate organismal diversity. Developmental biologists have focused on understanding the process by which a single-cell zygote gives rise to a functioning adult organism, by addressing fundamental questions such as, How does a single cell gives rise to multiple differentiated cell types?; How do cells form their correct shapes and organize into tissues and organs?; How do cells initiate and terminate proliferation at the correct times?; and, What is the relative importance of intrinsic or autonomous factors versus extrinsic factors (*e.g.* temperature, nutrition) in directing developmental events? For more than a century, developmental biologists have enjoyed great success in answering these questions and identifying and characterizing the molecular mechanisms and processes by which organisms achieve their adult form.

The results of this large body of work have revealed the several principles governing development, including the importance of inductive interactions between cells involving signal gradients [1,2], the importance of gene regulatory networks that specify cell fates and determination [3,4], the importance of differential gene expression to direct cell differentiation events [5,6] and how cell movement contributes to the formation of complex tissues [7]. Among the major molecular cell signaling pathways, there are several that have been well-studied for their importance in directing cell and tissue growth including the insulin/PI3 kinase pathway [8,9], the Rheb/Tor pathway [10,11], the receptor tyrosine kinase/Ras pathway [12], the Myc

family of transcriptional regulators [13-15], the JAK/STAT pathway [16-18], and the Hippo pathway [19-21]. These molecular pathways specify tissue and organ morphology via directing cellular processes such as cell proliferation, cell competition, and apoptosis or programmed cell death.

In addition to their contributions to our understanding of fundamental developmental processes, these findings have also laid the foundation for addressing questions about how variation in these mechanisms could give rise to phenotypic differences among species. In particular, cell signaling pathways like those described above offer obvious inroads to investigate how variation in the function of those pathways or the genes within them could direct developmental variation. The possibility of studying these mechanisms across species has been bolstered largely by technological advances that have made available a suite of molecular tools that can be deployed in "non-canonical" model organisms, and by a variety of methodological approaches to dissect species differences. These approaches broadly fall into three categories. The first is a candidate gene approach, whereby known developmental regulators are the target of between-species investigation. Here, known genes within signaling pathways that have been characterized for their importance in cell and tissue growth are the focus of dissecting the morphological differences between species. The second approach is a differential gene expression screen, whereby genes that are differentially-expressed between species are identified from whole genome screens to pursue functionally. This type of screen often allows investigators an unbiased approach to identify novel genes that direct developmental differences between species. Finally, a genetic mapping approach can be used when different species can be mated to one another and produce fertile hybrids, which can be used to generate large numbers of recombinant genotypes between species. The advantage to this so-called introgression

mapping approach is that investigators can identify small regions of the genome that have large effects on specifying species-specific morphological variation. We discuss how these approaches have been used to reveal that species differences in organ size and shape typically involve changes in the location of gene/protein expression (heterotopy), changes in the developmental timing of gene/protein expression (heterochrony), and/or changes in the relative amount of gene/protein expression (heterometry).

Adaptive variation in beak morphology: developmental evolution in Darwin's finches

One of the most recognizable and thoroughly studied examples of organ morphological differences between species is the great variation in beak morphology observed among "Darwin's finches"— bird species in the genus *Geospiza* that are endemic to the Galapagos archipelago. These species are a classic example of an adaptive radiation driven by natural selection, where an original founder species colonizes a new habitat and "radiates" out into the available ecological niche space, which ultimately gives rise to new species. In the case of Darwin's finches, decades of studies show that these birds differ in several aspects of their morphology, the most prominent of which are differences in beak size and shape. Beak morphology is an adaptation to the available food sources on these volcanic islands [22]: species that possess larger, broader beaks (the ground finches) feed on large tough seeds, and species with slender, shallower beaks (the cactus and warbler finches) feed on cactus flower parts and the insects that inhabit and forage on those plants. These beak morphologies are the target of natural selection in the harsh environment of the Galapagos islands, which experience frequent climatic change that affects the abundance and quality of the finches' food sources [22]. This

collection of species is closely related, last having shared a common ancestor approximately two and a half million years ago [23].

The development of avian beaks has been well-studied, primarily using the chicken *Gallus gallus* as a model system, and much is known about the molecular players and developmental events that occur to construct a chicken beak [24,25]. In vertebrates, all structures of the head and upper neck derive from a specialized group of cells called craniofacial neural crest [26]. These cells are specified at the dorsal axis of the developing embryo during neural tube closure and migrate ventrally to specify several embryonic cell types. During their migration, neural crest cells encounter a variety of different inductive signals, which direct their cell fate specification. Many of the factors involved in specifying neural crest-derived cell types have been identified, including several that are important for beak development. At the tissue level, the embryonic beak primordium consists of two tissues: the frontonasal mesenchyme, which gives rise to pre-nasal cartilage, and the premaxillary bone. Comparative developmental work performed by Schneider and Helms [27] showed that the variation in overall beak size observed among *Geospiza* species is strongly correlated with the amount of mesenchyme that makes up the frontonasal process at early stages of embryonic development.

To investigate the molecular control of variation in beak morphology, Abzhanov and colleagues [28] screened for variation in the known beak inductive growth factors (the candidate gene approach) during the early stages of development using *in situ* hybridization. In particular, they sought to identify any factors expressed in the frontonasal mesenchyme that showed substantial gene expression differences among ground finch species and cactus finch species that correlate with the adult differences in beak depth and width. Among the growth factors they surveyed, they found that gene expression levels of *Bone morphogenic protein 4* (*Bmp4*) showed

a strong positive correlation with the increasing beak width and beak depth observed in the large ground finch species. (Two other Bmps, *Bmp2* and *Bmp7*, both showed a correlation with overall adult beak size, but showed no correlation with any aspect of beak shape.) Two aspects of *Bmp4* expression were particularly striking— in the largest finch species the authors studied (*G. magnirostris*), *Bmp4* expression appeared to occur earlier during beak development, and it appeared to be expressed at higher levels in the frontonasal mesenchyme compared to other species (Figure 1A). The authors tested the hypothesis that variation in *Bmp4* expression level directs species variation in beak width and depth using chicken as an experimental system. They took advantage of a retroviral vector, the RCAS virus, to engineer two different transgenic viruses: one capable of expressing *Bmp4* (*RCAS:Bmp4*) and another capable of expressing *Noggin* (*RCAS:Noggin*), an inhibitor of Bmp4 function. When the authors infected the frontonasal mesenchyme of chick embryos with the *RCAS:Bmp4* virus, which effectively increases the level of *Bmp4* expression, they observed an increase in beak width and depth compared to controls (Figure 1B). In contrast, when they infected the frontonasal mesenchyme of chick embryos with the *RCAS:Noggin* virus, which effectively reduces the level of Bmp4 activity, they observed an decrease in beak width and depth compared to controls. (Additional work by another group of researchers showed an effect of expression timing of *Bmp4* on beak morphology [29].) The authors also investigated the mechanism by which *Bmp4* contributes to species differences in beak morphology and found that increased *Bmp4* expression gives rise to increased cell proliferation early in development to produce a greater amount of mesenchyme in the frontonasal process [28].

A candidate gene approach like the one used to identify *Bmp4* enables investigators to focus on known developmental genes and proteins, but this approach can sometimes be limited

in its scope to identify many of the relevant genes that specify variation in organ size among species, including those that have been the target(s) of evolutionary forces like natural selection. Although *Bmp4* was identified as correlated with beak depth and width among the known developmentally important genes, none of the other candidates that were studied were correlated with variation in beak length, another characteristic morphological difference among Darwin's finches. To identify additional factors that shape variation in beak morphology, researchers took a broader approach by performing a genome-wide gene expression screen. Abzhanov *et al*. [30] constructed DNA microarrays using libraries obtained from RNA isolated from the frontonasal process of embryos of the medium ground finch, *G. fortis*. Each microarray contained over 21,000 individual probes corresponding to RNA transcripts expressed in the frontonasal mesenchyme and enabled the researchers to identify relative gene expression differences among species of ground and cactus finches. The goal was to perform an unbiased screen to identify differentially expressed genes that were correlated with beak morphological differences. Among the genes that showed some of the highest expression differences between ground and cactus finches (~15-fold expression level difference) was a gene that encodes the protein calmodulin (CaM), a major regulator of protein function that responds to calcium signaling. CaM functions by binding to other proteins (typically enzymes), which makes their activity dependent on the concentration of calcium ions within the cell. The microarray results showed that gene expression levels are much higher in the frontonasal mesenchyme of finch species that possess more pointed, shallower beaks than gene expression levels in species that possess shorter more robust beak morphologies (Figure 1A).

Using *in situ* hybridization, the authors found that in addition to higher expression levels, *CaM* also shows substantial differences in spatial localization among species during embryonic

development in cactus finches. In particular, *CaM* is expressed at the distal end of the frontonasal mesenchyme primarily in the ventral domain of the upper beak. To test the importance of *CaM* expression levels on beak length, the authors again took advantage of the RCAS virus to express *Ca^{2+}/calmodulin-dependent protein kinase II* (*CaMKII*), a constitutively active downstream effector of *CaM*. Their results showed that increasing *CaMKII* expression levels gives rise to elongation of the upper beak (Figure 1B). However, in contrast to the mechanistic basis of cell proliferation that is a consequence of increased *Bmp4* expression, assays for cell proliferation differences between the *CaMKII* overexpression treatment and controls were inconclusive, and thus *CaM* likely directs elongation of the frontonasal mesenchyme via other cellular mechanisms.

A subsequent study focused on characterizing the gene regulatory network that directs development of the premaxillary bone, and the results of this work identified a separate developmental module— the TGFβ signaling pathway— that contributes to specifying species variation in three-dimensional beak morphology [31]. Interestingly, differential expression of genes within the TGFβ pathway occurs in similar domains of the developing beak primordia as those that show differential expression in *Bmp4* and *CaM* among species. These results suggest that multiple pathways functioning in the same tissue can be modified, and even combined, to specify variation in distinct morphological phenotypes produced from a highly coordinated developmental process like beak formation. The modular nature of how growth pathways can be deployed may prove to be a key feature of how complex morphologies can evolve in a single organ over short evolutionary time scales to give rise to dramatic differences among species.

Insect reproductive structures: a powerful model to dissect the molecular bases of organ development and evolution

Among animal external organs, one of the most rapidly evolving are the external reproductive structures in species with internal fertilization [32]. The evolutionary forces responsible for driving these differences in morphology have been studied in many species, and divergence both within and among species in the morphology of these structures is often a consequence of sexual selection and/or sexual conflict— evolutionary forces that act on traits directly related to an individual's ability to obtain mates and maximize their reproductive success [32-36]. Given the remarkable morphological diversity observed among male external genital structures, quantitative geneticists have long been interested in characterizing the genetic architecture of these complex traits. In the past decade there has been a resurgence of interest in the genetics and development of species variation in male genital morphology, driven in part by the availability of molecular tools and whole genome/transcriptome sequencing technologies that make identifying and characterizing the mechanistic bases of species differences possible.

The four species of the *Drosophila melanogaster* species complex (*D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia*) last shared a common ancestor approximately 1-3 million years ago [37], and three of these species (*D. simulans*, *D. mauritiana*, and *D. sechellia*) diverged from each other only 240,000 years ago [38]. Males of these four species possess substantial differences in the morphology of external genital structures that are crucial for mating success (ref. 39; Figure 2A). Two sets of structures are particularly striking with respect to their divergence in size and shape among these four species. The first are the epandrial posterior lobes (ePLs), which are bilaterally symmetric outgrowths of cuticle of the male genitalia [40] that are necessary for proper copulation [41,42] and can have significant effects on female

reproductive processes [42,43]. The ePLs are newly-evolved structures unique to the four species in this group [44], and they evolved via co-option of a gene regulatory network that specifies a larval breathing structure that is present prior to metamorphosis [45]. (Co-option of this network to produce the ePLs includes expression of genes that are part of the JAK/STAT signaling pathway [45].) Most of the *D. melanogaster* complex species can be mated to one another to produce fertile hybrid offspring that can be used to replace genomic segments from one species with the homologous segments from another species. This genetic mapping approach (*i.e.*, introgression mapping) has been successful in identifying regions with large effects on specifying species-specific ePL morphology [46-51], and in combination with genome-wide gene expression studies in the developing genitalia [50,52], have quickly narrowed down the list of potential gene candidates for specifying developmental differences in ePL morphology.

The second set of structures are the surstyli (ref. 40; also referred to as claspers, hereafter abbreviated "CLs"), which are bilaterally symmetric structures that function during mating by grasping and widening the female oviscape to secure a tight genital coupling during copulation [53-55]. *Drosophila mauritiana* has significantly larger CLs than those of its three sibling species, and their CLs also possess significantly more, and morphologically distinct bristles compared to the CLs of their sibling species (ref. 39; Figure 2A). To begin dissecting the molecular bases of developmental differences in CL morphology between *D. mauritiana* and *D. simulans*, an introgression mapping approach was used to replace regions of *D. simulans* genome (smaller CLs) with the homologous regions of the *D. mauritiana* genome (larger CLs). The introgression mapping results identified two regions on the left arm of chromosome 3 with large effects on specifying CL size and bristle number [56]. Hagen *et al.* [52] further narrowed one of

these genomic regions using a large collection of genetic recombinants to ~180 kilobase pairs (kb), which includes 8 protein-coding genes. To help identify potential candidates for morphological differences, the authors also performed a genome-wide gene expression analysis using high-throughput sequencing technology on the very tips of the abdomen from pure species *D. mauritiana* and *D. simulans* males at two different stages during pupal development. Among the eight genes in this region, only one appeared to show expression in the developing male terminal structures, the gene *tartan* (*trn*), which encodes a transmembrane protein thought to mediate cell-cell interactions by modulating cell affinity [57-59]. The sequencing results also revealed that at early developmental stages, the expression level for *trn* in *D. simulans* is significantly higher than that of *D. mauritiana*, but expression levels in *D. mauritiana* become significantly higher than those in *D. simulans* as development proceeds, particularly during the developmental stages when the CLs form. The authors confirmed these expression timing differences using *in situ* hybridization, and the results of those experiments also revealed a broader domain of expression in the base of the CLs of *D. mauritiana* compared to *D. simulans* at later stages of development (Figure 2B).

To test the functional importance of *trn* expression level in specifying CL size, the authors used two complementary experimental approaches. First, they took advantage of the arsenal of genetic tools in *D. melanogaster* to reduce the expression level of *trn* in the developing CLs. Reducing *trn* expression during development produced significantly smaller CLs compared to controls. Second, the authors compared CL size in reciprocal hemizygous null mutants. Specifically, they generated two different genotypes of male flies that were both heterozygous for *D. mauritiana* and *D. simulans* genes within the 180 kb introgressed region, but differed only in whether they possessed a functional copy of *trn* from either *D. mauritiana* or

from *D. simulans*. The results of this comparison were consistent with the expected effect: individuals who possess the function copy of *trn* from *D. mauritiana* have significantly larger CLs than those who possess *trn* from *D. simulans.* These two results thus confirmed the functional importance of *trn* in specifying species-specific CL size. Subsequent studies of CL size between *D. mauritiana* and *D. simulans* have identified several additional candidate genes for specifying species differences, including genes that regulate *trn* expression in the developing genitalia [51]. As the regulatory relationships among these candidate genes is characterized, researchers will be able to begin constructing the gene regulatory network(s) that direct species-specific CL development.

Conclusion

The beaks of Darwin's finches and the external reproductive structures of *Drosophila* are independently evolved traits with different evolutionary histories and have been subject to different selective pressures. Yet, both share a common molecular driver of morphological divergence among species: variation in gene regulation. Indeed, heterotopy, heterochrony, and heterometry are often the mechanisms that direct developmental differences to give rise to morphological evolution among species [60,61]. Another common feature of evolution in organ morphology may be the modular nature of how growth pathways are deployed differently in developing tissues and diverge to specify species differences. This certainly appears to be the case for beak morphology in Darwin's finches, and it may also prove true for genital morphology among the *D. melanogaster* complex species: introgression mapping results between *D. sechellia* and *D. mauritiana* show that variation in ePL size and shape are genetically separable [50]. As advances in molecular techniques continue to present opportunities to dissect the molecular bases

of differences in organ size and shape in a broader range of organisms, this is truly an exciting time for studying the evolution and development of organ morphology.

Summary points

- Technological advances have enabled studying the evolution of organ development across non-canonical model species.
- Species differences in organ development are often shaped by variation in timing, location, and/or amount of gene/protein expression.
- Genes that are functionally important in specifying species differences act via directing variation in cell and tissue growth pathways.
- Identification of genes affecting species differences will enable the reconstruction of gene regulatory networks that have evolved to give rise to species differences in organ development.

Declaration of Interests

The authors have no conflicts of interest to declare.

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Text box 1. Some definitions of phenomena, concepts, and techniques in development and evolution

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Craniofacial neural crest cells: A cell type in vertebrate animals that forms during the developmental process of neural tube closure, and subsequently migrate to embryonic regions of the head and upper neck where they differentiate into a variety of cell types including bone, and cartilage.

Inductive factors: Cell signals that originate from an inducing cell that direct changes in a responding cell. These are often either secreted proteins that bind to cell surface receptors of the responding cell, or components of the extracellular matrix.

***in situ* hybridization:** A molecular technique used to identify the location of nucleic acids (most commonly RNA transcripts) in developing tissues. It is performed by generating a labeled nucleic acid with a sequence complementary to the sequence of interest and allowing the probe to bind within a tissue or cell. It can also be used to provide qualitative information about relative transcript abundance in a sample.

Gene regulatory network: The relationships among genes that encode transcription factors that active or repress other genes, and the enhancers or those genes to which they bind, which direct specific developmental events.

Heterochrony: A change in the timing of a developmental process that gives rise to a change in phenotype.

Heterometry: A change in the amount of a molecule, usually RNA transcripts or proteins, that gives rise to a change in phenotype.

Heterotopy: A change in the spatial localization of a molecule, usually RNA transcripts or proteins, that gives rise to a change in phenotype.

Mesenchyme: Tissues composed of either loosely connected or unconnected cells, that allow for cells to move more easily within the tissue compared to cells in epithelial tissues.

RCAS virus: A retrovirus that can be engineered to include non-endogenous genes used for genetic transfection experiments to in some vertebrate systems. The RCAS virus does not infect cells across basement membranes, which allows for the possibility of targeted transfection of specific cells and tissues.

Reciprocal hemizygosity test: A genetic test used to identify the effect of species-specific alleles on a phenotypic difference observed between species. Two distinct hybrid genotypes are generated, where both hybrid genotypes share identical genomes, except for the gene that is being tested. The focal gene is heterozygous for each species allele, but one of the genotypes possesses a null allele for one species, whereas the other genotype possesses a null allele for the other species (*i.e.*, hemizygosity at that locus). If these reciprocal hemizygotes exhibit

phenotypes that are consistent with the phenotype associated with the species identity of the functional allele, this result provides evidence that the alleles contribute to the species difference in phenotype.

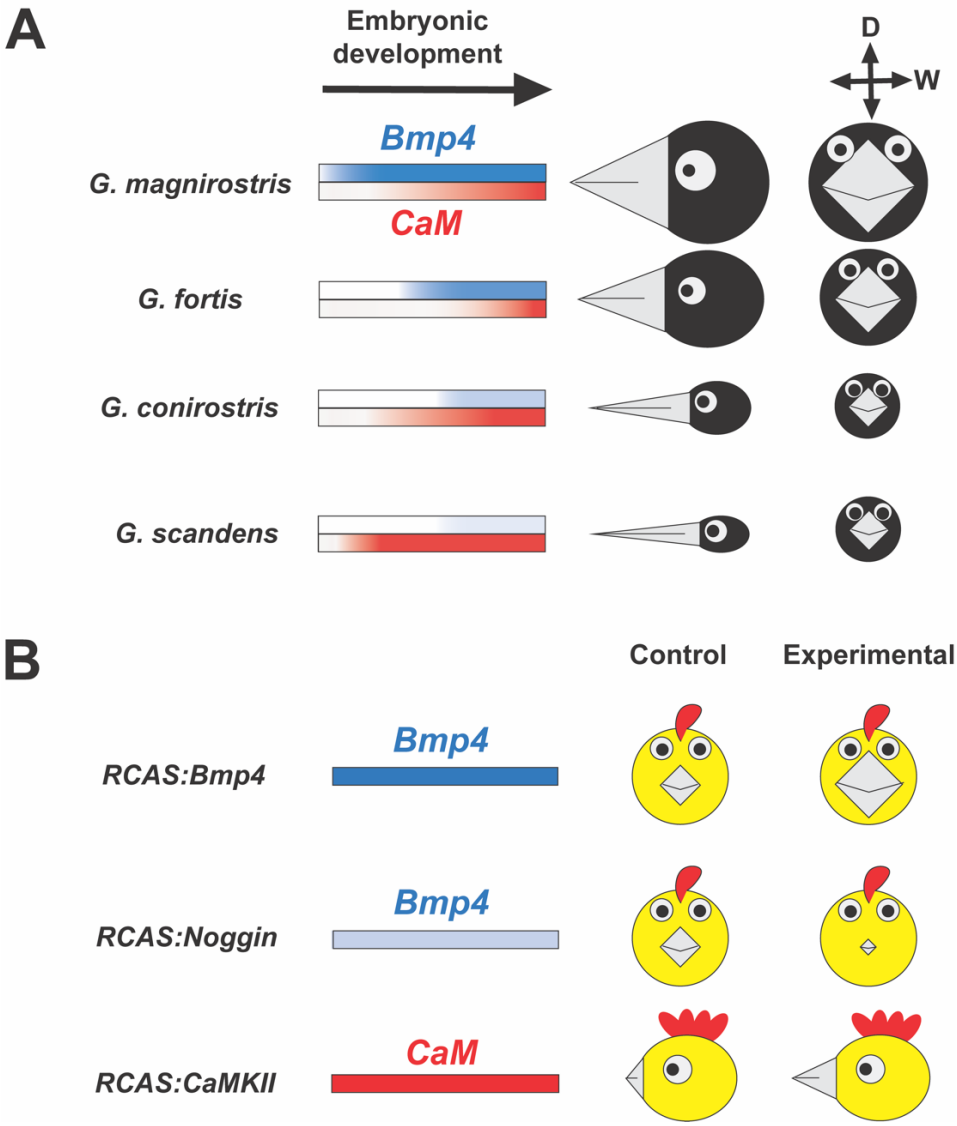

Figure 1. Variation in *Bone morphogenic protein 4* and *calmodulin* specify variation in beak morphology. (A) Variation in beak width (W) and depth (D) is correlated with *Bone morphogenic protein 4* (*Bmp4*) expression level and expression timing (blue), and variation in beak length is correlated with *calmodulin* (*CaM*) expression level and timing (red) during embryonic development in Darwin's finches. Development of the beak mesenchyme is shown from earlier stages to later stages (left to right) and increasing color intensities indicate higher gene expression levels. **(B)** Functional experiments that manipulate *Bmp4* (blue) and *CaM* (red) expression levels during development in chicken produce beak morphologies that support the hypothesis that expression levels of these two genes contribute to the species-specific beak morphologies that are observed in Darwin's finches. Development of the beak mesenchyme is shown from earlier stages to later stages (left to right) and increasing color intensities indicate higher gene expression levels. *RCAS:Bmp4* = overexpression of *Bmp4* in the beak mesenchyme; *RCAS:Noggin* = reduction of Bmp4 activity in the beak mesenchyme; *RCAS:CaMKII* = overexpression of *CaM* in the beak mesenchyme.

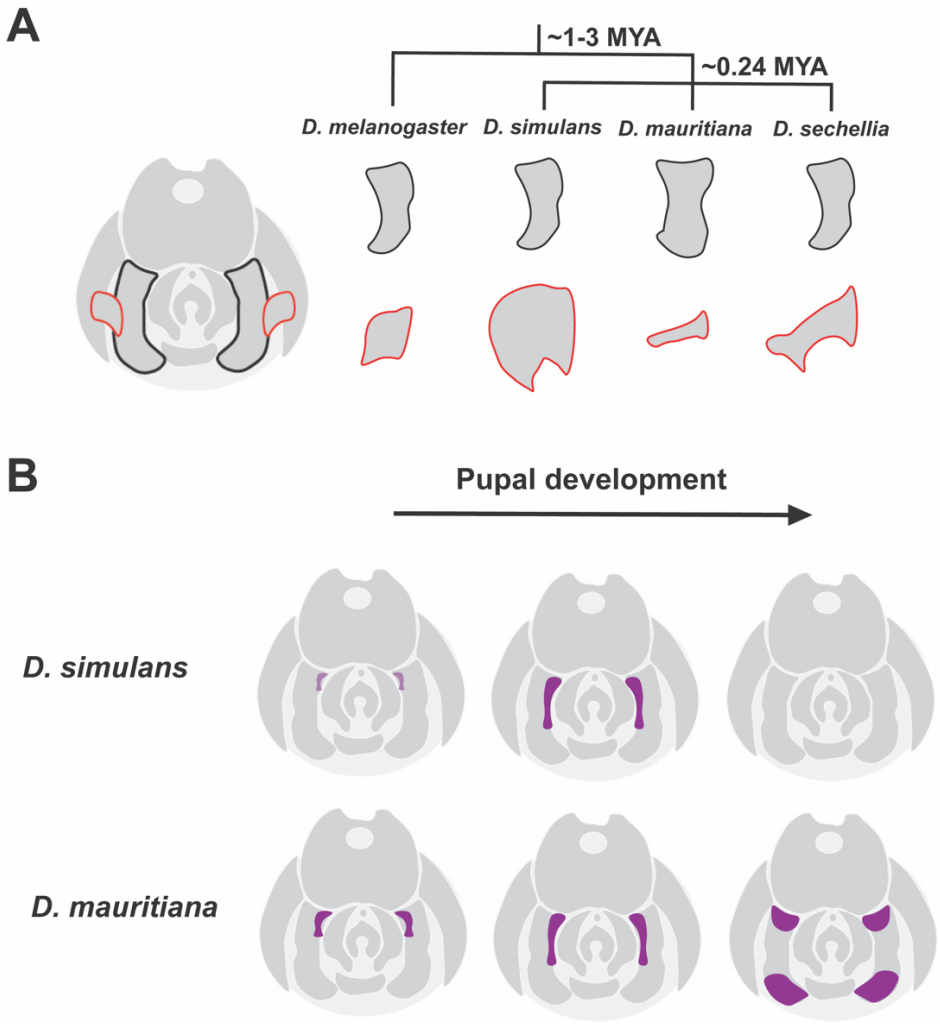

Figure 2. Variation in *tartan* expression level, timing, and localization specifies variation in clasper morphology. (A) The four species of the *Drosophila melanogaster* species complex possess species-specific differences in the size and shape of male genital structures. Left: A representation of the terminal structures of a male at a late stage of pupal development when the claspers (CLs, outlined in black) and the epandrial posterior lobes (ePLs, outlined in red) have formed. Right: Representations of the morphological variation in CLs (black) and ePLs (red) among the species. The phylogenetic tree at the top shows the evolutionary relationships among the species, including their estimated divergence times from a common ancestor. **(B)** Variation in CL size is specified by *tartan* (*trn*, purple) expression level, timing, and localization differences during pupal development between *D. simulans* and *D. mauritiana*. Development of the male genitalia is shown from earlier stages to later stages (left to right) and increasing color intensities indicate higher gene expression levels. For clarity of representation, only *trn* expression in the developing CL regions is shown on the representations of the male terminal structures and *trn* expression in other terminal domains is not shown. A representation of late-stage male terminal structures has also been used in each of the three developmental time points for ease of illustrating the comparisons across time points and between species.