EVOLUTION OF DEVELOPMENT IN CLOSELY RELATED SPECIES OF FLIES AND WORMS

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One of the main challenges in evolutionary biology is to identify the molecular changes that underlie phenotypic differences that are of evolutionary significance. Comparative studies of early development have shown that changes in the spatio-temporal use of regulatory genes, as well as changes in the specificity of regulatory proteins, are correlated with important differences in morphology between phylogenetically distant species. However, it is not known how such changes take place in natural populations, and whether they result from a single, or many small, additive events. Extending this approach to the study of development of closely related species promises to enrich this debate.

SATELLITE SPECIES
Species that are sufficiently closely related to the well-known model species that the underlying genetic regulation of homologous cellular processes can be compared.

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doi:10.1038/nrg947

Evolutionary genetics seeks to understand the genetic changes that underlie differences in phenotype. One way to address this problem is to use direct, classical genetics to identify the genes that contribute to a character that varies between selected populations of a single species or between species. Quantitative trait analyses have been particularly useful and have shown that, although the activities of numerous genes sometimes contribute in an additive fashion to a particular trait, individual loci might account for a large proportion of the total variation[1–3]. Frequently, these are the genes that are required during embryogenesis for the development of the trait in question[1,4,5] (for reviews, see REFS 6,7). A recent trend has been to determine how developmental mechanisms affect phenotypic variation. Instead of starting from the phenotype, this approach focuses on specific genes that are known to have key functions during development. We now know that embryonic development relies on a toolkit of regulatory genes that have been conserved throughout the animal kingdom. Most comparative studies have focused on slowly evolving traits between distantly related animals and have revealed that major changes in the function of regulatory genes have accompanied phenotypic evolution. However, these studies do not address the origin of such changes in natural populations. In particular, they do not address the problem of variation — that is, what is the source of the molecular variation that can potentially become fixed and give rise to phenotypic change[8]. Studies of the development of closely related species, covering a smaller evolutionary timescale, could be a way to identify the nature of the functional variants that underlie small phenotypic differences.

I discuss recent advances using related species of worms and flies to approach these problems. Using the model species *Drosophila melanogaster* and *Caenorhabditis elegans*, decades of intensive research have led to the accumulation of a vast amount of information on the genetic regulation of their development. Some authors have begun to separate out the changes in gene regulation that underlie small morphological differences by focusing on homologous, well-defined processes, such as fly segmentation or nematode vulva development, in SATELLITE SPECIES that are closely related to *D. melanogaster* and *C. elegans*. These studies benefit from the availability of the complete genome sequences of both model species and from information derived from studying distantly related organisms. This should help to bridge the gap between evolutionary genetics and comparative embryology.

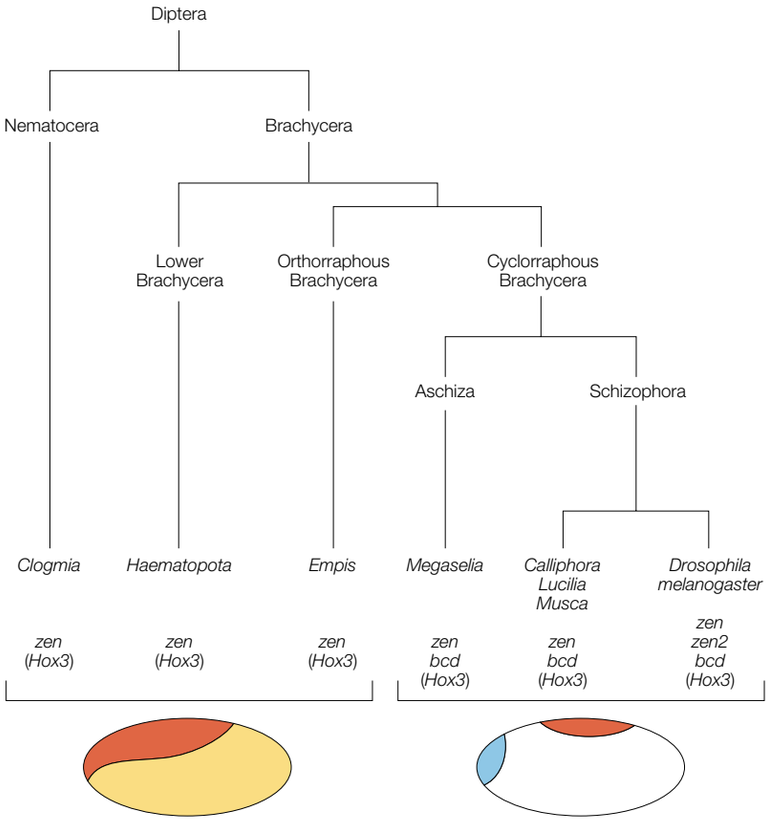


Figure 1 | **Evolution of the *bicoid* gene in Diptera.** A simplified phylogenetic tree of the Diptera is shown. Species from basal taxa, such as *Clogmia albipunctata*, *Haematopota pluvialis* and *Empis livida* have a single *Hox3* gene. The ancestral form of this gene is thought to have been maternally expressed throughout the embryo (yellow) and zygotically in the extra-embryonic tissue ANLAGE (red). A duplication event, presumably in the stem lineage of CYCLORRAPHOUS FLIES, gave rise to the two genes *zerknullt* (*zen*) and *bicoid* (*bcd*), as indicated for *Megaselia abdita*, *Calliphora vicina*, *Lucilia sericata* and *Musca domestica*. An extra duplication has resulted in two *zen* genes in *Drosophila melanogaster*. The functions of *zen*, which lost its maternal expression, and *bcd*, which lost its zygotic expression, then diverged. The anterior localization of Bcd (blue) is associated with the reduction of the extra-embryonic tissue in these species; modified with permission from REF. 28 © (2002) National Academy of Sciences, USA.

Lessons from phylogenetically distant species

Development proceeds through the orderly deployment of genes at the correct times and places, which relies on the *cis*-regulatory information that is associated with each gene and that ensures its appropriate expression. In this sense, development is hard-wired into the genome[9]. Of paramount importance, therefore, are the transcription factors that are encoded by many conserved regulatory genes and their binding sequences that lie in the *cis*-regulatory regions of their target genes. It is generally assumed that evolutionary differences in gene expression will ultimately be reduced to specific changes in *cis*- and *trans*-acting factors[10]. There are many examples of correlation between morphological transitions and changes in expression of the genes that encode transcription factors. For example, changes in Hox gene expression underlie modification of the thoracic appendages in Crustacea and the expanded number of thoracic segments in snakes[11–13]. Other proteins that have developmentally conserved roles are those that participate in signalling pathways — the outcome of which also alters the transcriptional activity of target cells and hence their ultimate fate. In some cases, entire regulatory networks — which usually involve a combination of transcription factors, signalling pathways and feedback loops — have been shown to be conserved between species[10]. For example, cell-fate specification along the dorsoventral axis of embryos by the *short gastrulation*/*chordin* genes and the signalling pathways Decapentaplegic (Dpp)/transforming growth factor-β (TGF-β) are conserved between insects and vertebrates[14]. An example of co-option of a regulatory circuit for a new patterning role is illustrated by the development of eyespots on butterfly wings, which requires the recruitment of a modified *hedgehog*-dependent regulatory circuit that is normally used in early wing development[15].

Transcription factors often regulate several downstream genes, so any change in their expression patterns or binding specificity can have important knock-on effects. This can be further amplified if genes that encode other transcription factors are among their targets. Furthermore, many genes are regulated by more than one transcription factor and therefore have complex regulatory regions that contain binding sites for several proteins[16,17]. Regulatory regions can be grouped into specific modules (or enhancers) that act independently and can reside at considerable distances from the transcription start site. For example, the enhancers that regulate the expression of *Ultrabithorax* (*Ubx*, one of the *Drosophila* Hox genes) are scattered over 70 kb (REFS 18,19). Given that each module has a specific function, the mutation of one module only affects one function of the gene, whereas others remain unchanged. In addition, each module is free to evolve independently by the loss or gain of binding sites. Therefore, the complexity of gene function arises from the gradual recruitment of different activities. In view of the strong functional conservation of many proteins, it has been suggested that changes in *cis*-regulatory sequences are more important during evolution[10,16,20]. Nevertheless, three recent studies provide evidence that naturally selected coding-sequence changes in *Ubx* and *Antennapedia* (*Antp*, another *Drosophila* Hox gene) correlate with morphological transitions in limb development between arthropod groups[21–23].

A good example of a change in both expression and protein specificity, at a smaller evolutionary timescale, is that of *bicoid* (*bcd*) in flies. Bicoid specifies anteroposterior polarity of the *Drosophila* embryo in a concentration-dependent manner[24], whereas *zerknüllt* (*zen*) is required for development of the extra-embryonic tissue[25]. *bcd* and *zen* are thought to be derived from a common *Hox3*/*zen* ancestral gene that was probably duplicated after radiation of the DIPTERA (FIG. 1). Basal groups of Diptera have a single *Hox3*/*zen* gene and are separated by about 250 million years (Myr) from higher, cyclorraphous flies, which have both *bcd* and *zen* genes[26–31]. In the lower flies, the single *Hox3*/*zen*

DIPTERA
The true flies, an order of insects with a single pair of wings.

ANLAGE
A group of cells that are destined to become a specific structure or tissue in the adult, but have not yet differentiated.

CYCLORRAPHOUS FLIES
Highly derived Diptera in which pupal development and metamorphosis take place in a puparium, a modified form of the last larval cuticle.

gene is maternally and zygotically expressed in the anlage of the extra-embryonic tissue that extends to the anterior tip. This comprises two epithelia — the amnion and the serosa — that enclose the embryo but do not contribute to the embryo proper. In cyclorraphous flies, expression of *zen* is exclusively zygotic and that of *bcd* is exclusively maternal (FIG. 1). Loss of the maternal expression of *zen* correlates with the reduction of extra-embryonic tissues to a transient thin band of dorsal epithelium called the amnioserosa[32]. At the same time, in higher flies, Bcd seems to have taken on the function of a maternal coordinator and organizes the segmental pattern of the anterior region of the embryo. In these species, this includes the anterior blastoderm, which is no longer destined to become extra-embryonic tissue[24]. Modifications in the specificity of the Bcd protein have accompanied the new activities. It has acquired the DNA-binding specificity of Orthodenticle, a transcription factor that regulates head development, and has acquired RNA-binding properties, which are required for translational repression of *caudal* mRNA (*caudal* regulates posterior development), both of which are atypical for a Hox protein[33–36].

Bcd is just one example that illustrates the important consequences of a change at a single regulatory locus. However, these observations do not rule out the possibility that the phenotypic change actually results from the sum of numerous small changes at one specific locus[4].

Change over a small evolutionary timescale

If evolution proceeds in small steps, and large changes in gene activity result from several smaller changes at the same locus, then it is important to identify the small changes that might have been individually selected. To do this, a small evolutionary timescale needs to be looked at, by studying closely related species. This approach has the advantage that subtle differences, such as those that might arise from one or a small number of evolutionary steps, can be compared between species. It also allows the design of cross-species functional assays that are likely to be less susceptible to experimental artefacts, which is a concern when comparing species with more-diverged embryonic morphologies. The use of the satellite species of *D. melanogaster* and *C. elegans* offers the potential to use the huge amount of genetic information that is available for these model organisms. As for comparisons between distantly related organisms, candidate genes can be selected for analysis. However, in cases where hybrids are viable, empirical genetic analysis can help to identify the underlying variation. Here, I discuss four studies that use either a candidate-gene approach or classical genetics.

Cis-regulatory change at a fly Hox locus. In *Drosophila*, *Ubx* is responsible for the morphological difference between the second and third thoracic segments. To achieve this, *Ubx* regulates many traits through many different targets[37]. High levels of Ubx at the proximal end of the femur of the second leg of *D. melanogaster* repress TRICHOME development to give a small hairless patch[38]. *D. virilis*, which has no naked patch, has correspondingly lower levels of Ubx. Levels of Ubx in *D. simulans* are similar to those in *D. melanogaster*, although *D. simulans* has a larger naked patch. By exploiting the fact that hybrids between *D. simulans* and *D. melanogaster* are viable, Stern showed that the trichome phenotype of hybrids, which result from the activity of a single wild-type *Ubx* allele, differed depending on which species the allele had come from — the *D. melanogaster* allele causes a smaller patch than that of *D. simulans*[38]. As the proteins from the two species are identical, this variation is presumed to have arisen from differences in the *cis*-regulatory control of gene expression[38].

Change at a nematode Hox locus. This example concerns the gene *lin-39*, the nematode homologue of *Deformed*, a Hox gene that is involved in the development of the head in *Drosophila*[39]. Free-living nematodes, such as *C. elegans*, have a defined number of cells and develop from invariant cell lineages[40] (BOX 1). This simplicity means that various developmental processes can be studied at a cellular resolution that is not possible in most other metazoa and that homologous cells can be recognized between species. Development of the nematode vulva is a well-defined process. Three specific cells, P5.p–P7.p, form the vulva of *C. elegans,* and are singled out from the 12 ventral epidermal precursor cells by a process of induction (BOX 2; for a review, see REF. 41). The vulva of *Pristionchus pacificus* — an established satellite species that is separated from *C. elegans* by ~100 Myr — also forms from P5.p–P7.p. However, whereas the vulval fate in *C. elegans* is induced by a short burst of signal from a specific cell in the gonad, in *P. pacificus* it occurs in response to a continuous signal from many cells of the somatic gonad[42] (BOX 2; for a review, see REF. 43).

In *C. elegans*, *lin-39* is expressed in cells of the vulval equivalence group P3.p–P8.p[44,45] (BOX 2). *lin-39* is required early to prevent these cells from fusing with the hypodermis and later, during vulval induction, it is upregulated in P5.p–P7.p to specify vulval fates. In *P. pacificus*, the vulval equivalence group comprises P5.p–P8.p, and *lin-39* is only required once — at an early stage — to prevent cell death (BOX 2). LIN-39 proteins of *C. elegans* and *P. pacificus* are highly conserved in the hexapeptide and homeodomain regions, which are required for DNA binding, but have diverged in other regions[46]. Nonetheless, when expressed from the *C. elegans lin-39* promoter, LIN-39 protein from *P. pacificus* can rescue *lin-39* functions in *C. elegans* which it does not usually carry out[46]. This indicates that the difference in function of the LIN-39 protein between species is attributable to the different cellular contexts of the species in which they operate. In turn, this indicates that the differences between the two species reside in regulatory, rather than coding sequence[46]. Therefore, as in other organisms, changes in the function of nematode Hox genes can underlie evolutionary changes in cell behaviour.

TRICHOME
A thin, cuticular, non-sensory process that is secreted by an individual cell.

MONOMORPHIC SPECIES
A species in which males and females are structurally identical for the trait that is under consideration.

Integration of pathways by a newly evolved gene. A study by Kopp and colleagues combines the knowledge of a genetic network in *D. melanogaster* with the expression patterns of candidate genes in other drosophilid species to uncover the molecular basis for sex-specific differences in fly pigmentation[47]. In *D. melanogaster*, the abdominal segments A5 and A6 are strongly pigmented in males but not in females; this is a recently evolved trait[48]. Mutation of the Hox gene Abdominal-B (*AbdB*) causes a loss of pigmentation in males, whereas mutations in *bric à brac* (*bab*) and *doublesex* (*dsx*) lead to a pigmentation of female abdomens. Using transgenic flies, the authors showed that *AbdB* represses *bab* in both sexes, but in females this repression is prevented by the female-specific form of *dsx*, dsx^F (FIG. 2). Therefore, the transcription factor that is encoded by *bab* is only expressed in females where it represses pigmentation. A cross-reacting antibody was used to show that, in the *D. melanogaster* species group, *bab* is expressed in females of species with male-specific pigmentation, but not in segments A5 and A6 of males. By contrast, in all MONOMORPHIC SPECIES, Bab is present in both sexes, so its role in antagonizing *AbdB* function and repressing pigmentation is ancestral. However, because there is evidence that, ancestrally, *bab* expression was independent of dsx^F and *AbdB*, this gene must have only recently evolved to integrate input from these two distinct genetic pathways[47,48]. The authors propose that this is attributable to changes in the *cis*-regulatory region of *bab*, and point out that this circuit is flexible and highly evolveable[47]. The phenotype depends on the levels of *bab* expression, which, in turn, depends on the balance between the inputs from *AbdB* and *dsx*.

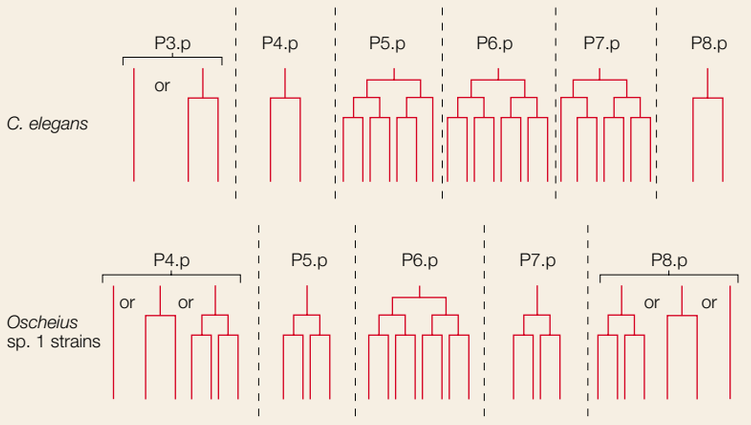
Change in a newly identified gene. Hybrids between closely related species of drosophilids can be used to identify the genes that are responsible for small phenotypic differences. The dorsal cuticle of larvae of the *melanogaster* subgroup of *Drosophila* has an anterior lawn of fine hairs in all species except *D. sechellia*[49]. From interspecific crosses, Sucena and Stern determined that a single X-linked locus was responsible for this trait. By using an overlapping set of X-chromosomal deletions from *D. melanogaster* and recombination mapping, they were able to map the position of this gene to a small chromosomal interval. This interval contains the *D. melanogaster* gene *ovo*/*shavenbaby* (*ovo*/*svb*), which, when mutated, causes a patterned loss of dorsal hairs[50,51]. The mutant failed to COMPLEMENT the phenotype of *D. sechellia* in *melanogaster*/*sechellia* hybrids. Different levels of *ovo*/*svb* transcripts correlated with the patterns in the two species, indicating that the phenotypic differences between *D. sechellia* and the other species are caused by changes in the way that their *cis*-regulatory regions function[49]. This study illustrates how decades of accumulated knowledge of the genetics of this model organism can be harnessed to identify rapidly the genes that are responsible for a morphological difference.

These examples confirm the importance of *cis*-regulatory sequences, which are also the basis for evolutionary change between closely related species. To understand the nature and consequences of sequence changes and their possible evolutionary relevance, it is necessary to identify regulatory elements and upstream regulators, and to acquire an in-depth knowledge about the role of a specific gene.

Box 1 | **Cell lineage in nematodes**

In most animals, the pattern of cell divisions that takes place during development is not linked to the ultimate fate of each cell. The fate of a cell is specified by the position that it occupies in the embryo and its interactions with neighbouring cells, regardless of its origin in terms of descent. *Caenorhabditis elegans* is remarkable in having an invariant cell lineage and a fixed number of cells in the adult[40]. It has been suggested that the lineage allows cell fate to be specified in the correct order and directs cells to the right place, such that there is a link between the pattern of divisions and the fate of a cell[95]. A more recent view indicates that the developmental mechanisms that are used by this organism are fundamentally similar to those of other animals[96]. FATE MAPS constructed at the 80-cell stage indicate that the precursor cells that contribute to any specific tissue or organ are grouped together, despite the fact that the precursors are derived from unrelated lineages and dispersed BLAST CELLS. Furthermore, they express an identical discrete gene that confers tissue specificity. There is, therefore, a process of regionalization that leads to polyclonally derived tissues as in other embryos. Nevertheless, although the processes of cell division and cell fate can be separated in nematodes, the fact that they are often tightly linked provides a useful tool for developmental studies. In the absence of alternative cellular markers, the number and orientation of divisions that are undergone by a specific cell can often be used as a marker of cell fate. Furthermore, as it is possible to recognize the exact cells in different individuals, techniques such as cell ablation that are used to ascertain the regulative properties of intact neighbouring cells are very powerful.

Lineages of the cells that form the vulva have been useful for micro-evolutionary studies. In *C. elegans*, the vulva is derived from an equivalence group of P3.p–P8.p cells that have defined, invariant lineages. P6.p undergoes three divisions to produce eight cells that form the inner vulva. P5.p and P7.p each produce seven cells that form the outer vulva. P4.p and P8.p divide once and then fuse with the hypodermis. Only P3.p has a variable lineage and might or might not divide. This property, however, is not common to all nematodes, some of which have more variable lineages[97]. In *Oscheius* sp. a satellite species of *C. elegans*, P5.p–P7.p have defined lineages, and P6.p divides three times, whereas P5.p and P7.p divide twice. However, the lineages of P4.p and P8.p are variable: these cells can divide once, twice or not at all. Mutations have been isolated in this species that alter the number of divisions of P5.p–P7.p and mimic the natural variation in other cells, as shown here[91]. Adapted with permission from REF. 90 © (2001) Cell Press.



Compensatory molecular evolution

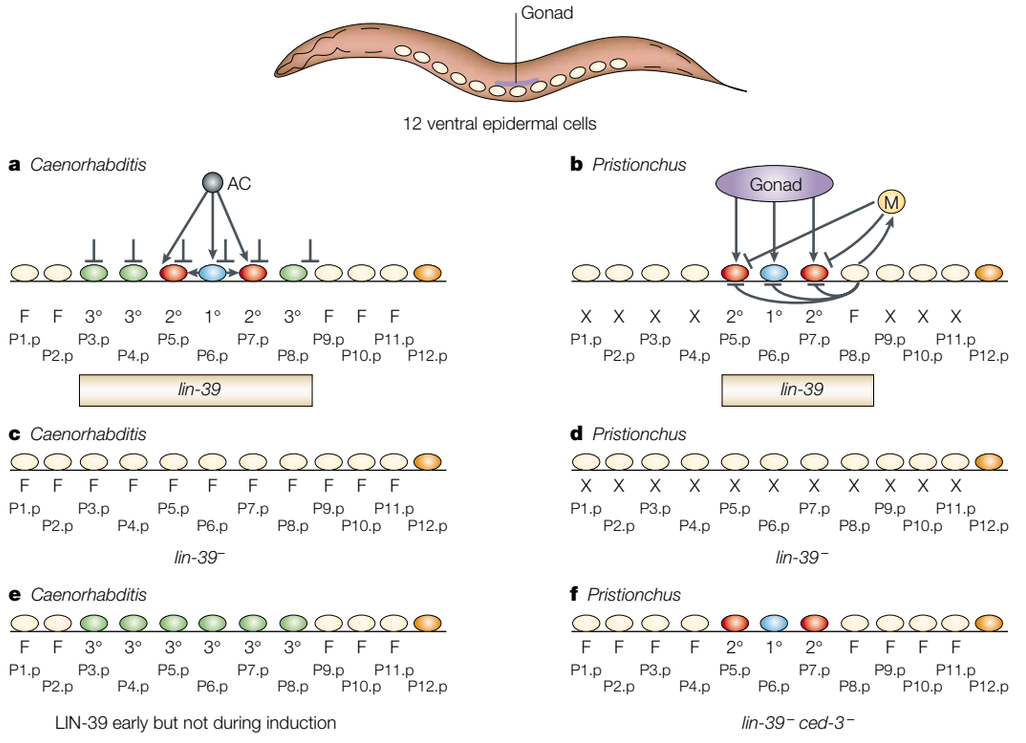
Little phenotypic variation is evident in a population that is well adapted to its environment. Developmental processes are under strong selective pressure to reproduce faithfully a stable adult phenotype, and, in theory, variation between members of a population should tend to decrease. This raises a long-standing problem in evolutionary biology. Where does the molecular variation that

Box 2 | Nematode vulval patterning and its evolution

Of the 12 ventral epidermal cells, P1.p–P12.p, the six central cells, P3.p–P8.p, form the vulval equivalence group of vulval precursor cells (VPCs) because they are all competent to form the vulva[98–100]. The vulval EQUIVALENCE GROUP is also defined by the expression of a Hox gene, *lin-39* (shown in figure part **a**). In the wild type, only P5.p–P7.p form the vulva in response to an inductive signal (arrows) from the anchor cell (AC) that is situated in the gonad[99,100] (mauve, shown in **b**). High levels of the signal induce the primary (1°) fate in P6.p — the cell that lies closest to the AC — the descendants of which form the inner structure of the vulva. Lower levels induce the secondary (2°) fate in P5.p and P7.p cells, the descendants of which form the outer vulva[78]. Descendants of the remaining cells, P3.p, P4.p and P8.p, take up the tertiary (3°) fate and fuse with the other ventral epidermal cells to form the hypodermis (F).

The inductive signal is mediated by the conserved EGF/RAS/MAPK pathway[78,101]. The fates of P5.p–P7.p are reinforced by lateral signalling that is mediated through the LIN-12/NOTCH pathway[79] (arrowheads). P3.p, P4.p and P8.p, which do not receive the inductive signal, adopt the 3° fate (green) and, like other epidermal cells, they fuse to form the hypodermis. In addition, negative signals (lines) from the surrounding hypodermis prevent inappropriate vulval development. In *Pristionchus pacificus*, a satellite species of *C. elegans*, the vulval competence group is smaller than in *C. elegans* and comprises only P5.p–P8.p[84–86]. The inductive signalling from the gonad is a continuous process and involves many gonadal cells (mauve). P8.p inhibits P5.p and P7.p through a lateral signal that is mediated by mesoblast M (yellow) and that causes them to adopt the 2° fate (see panel **b**). P8.p also provides a negative signal (black lines) that affects the vulva versus the non-vulva fate choice. In *P. pacificus*, non-competent ventral epidermal cells (excluding P8.p) undergo programmed cell death (X), instead of fusing with the hypodermis, so these homologous cells have different intrinsic properties in these two nematode species[102]. Expression of *lin-39* in *C. elegans* is required to prevent the VPCs from fusing with the syncytial hypodermis — in *lin-39* mutants, they fuse with the other ventral epidermal cells[44,45,99] (see panel **c**). In *lin-39 P. pacificus* mutants, VPCs undergo cell death (see panel **d**).

As *lin-39* prevents fusion or death in the two species, respectively, its role is different in these two species. If *lin-39* activity is withdrawn from *C. elegans* after formation of the vulval equivalence group, the cells no longer respond to the inductive signal from the anchor cell and adopt the 3° fate (green, see panel **e**). This is consistent with the upregulation of *lin-39* after induction. Removing *lin-39* and the cell-death activity (by removing *ced-3* function) in *P. pacificus* does not prevent P5.p–P7.p from adopting their appropriate fates, indicating that *lin-39* is not a target of inductive signalling in this species[103] (see panel **f**). Whether the same molecules are involved in the signalling processes in *P. pacificus* is not known. Adapted with permission from REF. 87 © (2000) The Company of Biologists.



FATE MAP
The description of the cell divisions from fertilized egg to adult, which are linked to the eventual anatomical position of the cell in the animal and the differentiated state, or fate, of the cell.

BLAST CELL
An undifferentiated precursor cell.

EQUIVALENCE GROUP
A group of cells with the same developmental or genetic potential. Any subsequent differences between them generally result from extrinsic signals.

COMPLEMENT
Two alleles that each give a mutant phenotype when homozygous, are said to complement if they give a wild-type phenotype when combined in the same individual .

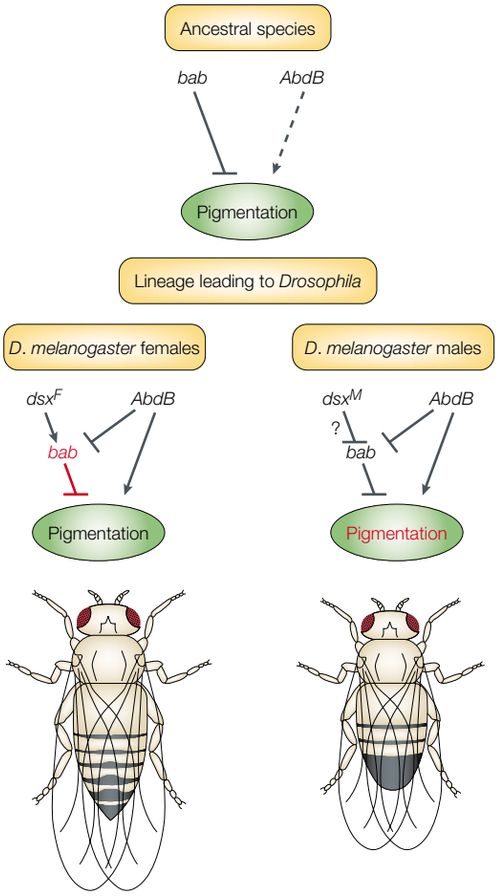


Figure 2 | **Evolution of sexually dimorphic pigmentation in *Drosophila*.** Abdominal pigmentation in *Drosophila* is prevented by the expression of the *bric à brac* (*bab*) gene, which antagonizes the pigment-promoting role of AbdB in both sexes of ancestral species. In *D. melanogaster*, male abdomens are pigmented because *bab* is repressed in males by AbdB. In females, repression of *bab* by AbdB is overcome by the female form of Dsx, Dsx^F, so the females remain unpigmented. It is likely that *bab* has recently evolved to be under the control of AbdB and Dsx^F. It is possible that Dsx proteins regulated *bab* expression in ancestral flies, in which case, the loss of *bab* expression in *D. melanogaster* could have evolved simply by the loss of responsiveness to the male version of Dsx. Adapted with permission from REF. 47 © (2000) Nature Publishing Group.

is relevant to evolution come from? Does evolution rely on new mutational events, or can it draw on variation that already exists in a species? This is particularly important because molecular evolutionary theory predicts that most changes that survive in nature confer neither a selective disadvantage nor an advantage, but are neutral[52]. However, it is clear that, in any population, there is much more genetic variation present than is expressed, as can be seen after artificial selection or under conditions of stress[53–56]. Here, I review data from worms and flies that illustrates the existence of hidden variation in the genes and processes that regulate development. Indeed, the genetic pathways that are responsible for development can diverge considerably without causing any corresponding change at the phenotypic level, raising the question of the evolutionary relevance of such variation. Two processes are considered here: compensatory changes that are associated with developmental homeostasis and the redundancy of developmental mechanisms.

Developmental homeostasis. The genome is subject to continuous change that results from various mechanisms of turnover, including GENE CONVERSION, unequal crossing-over, slippage and transposition, and mutations[57,58]. The rate at which these processes occur, the size of the population and the degree of selective pressure will affect the spread of variant sequences throughout a population. Regulatory sequences are more versatile than coding sequences as they are not constrained by the need to maintain the triplet code and therefore are a much richer source of variation. Developmental homeostasis requires a way of ensuring that the function is maintained by compensating for such sequence divergence. Therefore, any subsequent secondary change that restores the function to its original state is likely to be selected for.

An example of compensatory molecular evolution in *cis*-regulatory sequences came from a study of the stripe 2 enhancer of the *even-skipped* (*eve*) gene of *Drosophila*[59]. *Eve*, like other PRIMARY PAIR-RULE GENES, is expressed in seven transverse stripes in precellular embryos — this expression is the first evidence of the metameric body plan in flies. Individual stripes are regulated by separate enhancers and a minimal 480-bp sequence is sufficient to drive stripe 2 (REFS 60,61). Stripe 2 is activated by the homeoprotein Bcd and the zinc-finger protein Hunchback (Hb), and is repressed at the borders by the zinc-finger and the bZip proteins Kruppel and Giant[60,61]. Several binding sites for all of these factors are found throughout the *cis*-regulatory DNA at the *eve* locus.

The corresponding *eve* enhancers from four species — *D. melanogaster*, *D. yakuba*, *D. erecta* and *D. pseudoobscura* — drive expression of a stripe with sharp boundaries at the correct time and place in *D. melanogaster*, indicating conservation of function[62]. However, sequence comparisons revealed that, although similar binding sites are present in the enhancers from these four species, they vary in number and spacing, and have many nucleotide substitutions[62–64]. The presence of several redundant binding sites for each *trans*-acting protein is presumably the result of selection and contributes to the robustness of the enhancer. Therefore, mutations with small deleterious effects might be tolerated and become fixed by GENETIC DRIFT. Any adaptive, compensatory changes would then be selected for, and the molecular divergence can be explained as a consequence of such STABILIZING SELECTION. Evidence for stabilizing selection came from a study of chimeric enhancers that combine the 5′ and 3′ halves of the *eve* stripe 2 element from *D. melanogaster* and *D. pseudoobscura,* respectively, which no longer drive correct reporter-gene expression[59]. The stripe was expanded and/or shifted at one or both edges, and the authors suggest that most species will be found to differ by many such compensatory substitutions[59].

GENE CONVERSION
The non-reciprocal transfer of genetic information between homologous genes, as a consequence of mismatch repair after heteroduplex formation.

PRIMARY PAIR-RULE GENES
Pair-rule genes are expressed in alternating stripes and function to allocate cells to the different segments of the body. The primary pair-rule genes respond to several upstream factors by means of complex modular promoters.

GENETIC DRIFT
The random fluctuations in allele frequencies over time that are due to chance alone.

STABILIZING SELECTION
Selection that favours intermediate phenotypes over extreme phenotypes.

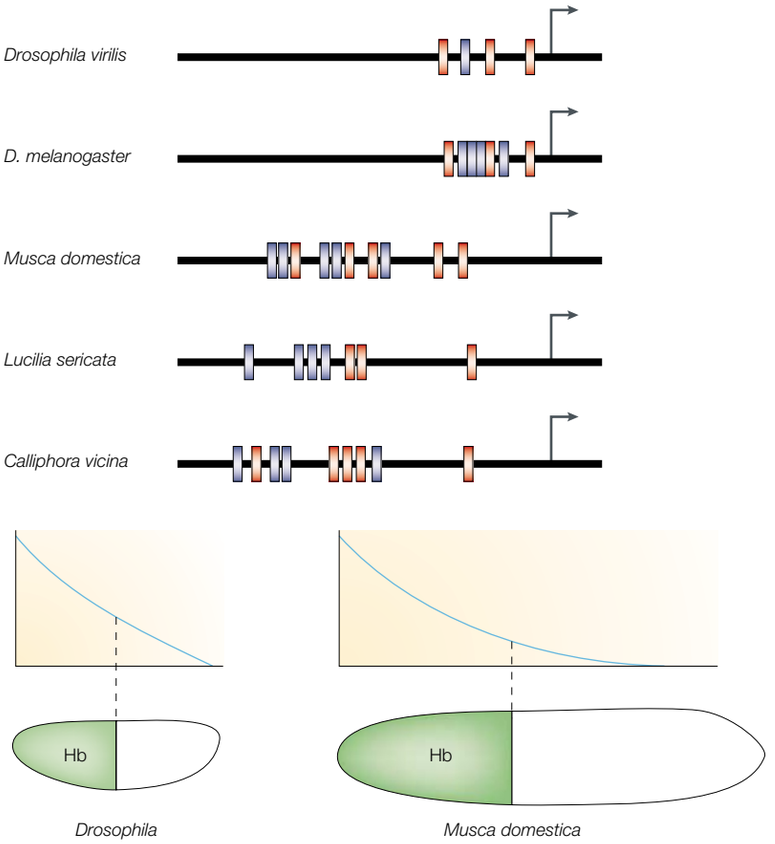


Figure 3 | **Conservation of the *hunchback* promoter in Diptera.** A comparison of the number and distribution of Bicoid-binding sites in the P2 promoter of *hunchback* (*hb*) of five species of flies. *hb* is a gap gene that regulates the early processes that lead to segmentation. Red boxes represent strong binding sites and blue boxes represent weak binding sites. The P2 of *Drosophila melanogaster* has seven Bcd-binding sites, four of which are conserved in *D. virilis* and have been shown by footprinting analysis to mediate strong binding[68,69,72,75]. They are spread over 280 bp. By contrast, the P2 of *Musca domestica* has 10 binding sites that are spread over 700 bp, *Lucilia sericata* has 7 and *Calliphora vicina* has 9, that are spread over 560 and 504 bp respectively[71,73]. Bcd is present in a gradient in the eggs of these flies, and activation of *hb* takes place at certain threshold levels (below). The eggs of the three Muscoidea species are larger than those of *Drosophila* species (a comparison with the egg of *M. domestica* is shown). It is possible that the configuration of binding sites in these species has evolved in reponse to the shallower gradient of Bcd that is present in these eggs. The changes in configuration have been accompanied by sequence changes in the coding region of Bcd (not shown). Similar patterns of substitution in different *Drosophila* spp. have been described in the regulatory regions of *fushi tarazu*, *hairy*, *vestigial* and *period*[104–108]. Adapted with permission from REF. 73 © (2001) Blackwell Publishing.

Molecular co-evolution. Co-evolution at the molecular level involves selection of compensatory changes that restore the functional interaction between two components[65,66]. For example, the spread of a new promoter configuration would lead to the selection of variants of the upstream transcriptional regulator that have an increased ability to interact with the new promoters. This has been proposed to explain how divergence at *cis*-regulatory regions of the *achaete-scute* genes of *D. simulans* and *D. melanogaster* has resulted in the inability of transcription factors from one species to regulate correctly transcription at the *cis*-regulatory sites of the other species[67]. The example discussed below is that of the Bcd–*hb* interaction in different fly species[29].

In *D. melanogaster*, the maternally deposited homeoprotein Bcd regulates expression of the gap gene *hb* in a concentration-dependent manner[68,69] (FIG. 3). This interaction is functionally conserved in other species of the Muscoidea superfamily, which diverged from *Drosophila* ~100 Mya, providing sufficient time for the accumulation of changes[66,70]. Although the *hb* P2 promoters of *D. melanogaster* and *D. virilis* can be aligned, those of Muscoidea species cannot[58,71,72] (FIG. 3). The number of binding sites, the spacing between them, and their orientation and distance from the transcription start site are different for each species[71,73] (FIG. 3). The distance that separates binding sites allows cooperative interactions between Bcd molecules and, together with the number of binding sites, determines the extent (or the width) of the threshold at which Bcd can activate *hb*[68,69,72,74,75]. Experiments with transgenic *Drosophila* indicate that *Drosophila* Bcd can activate the *Musca* P2 promoter at low levels that are insufficient to activate the *Drosophila* P2 (REF. 71). The eggs of the Muscoidea species are large; for example, those of *Musca* are twice the size of those of *Drosophila* (FIG. 3). Perhaps the *Musca* P2 has evolved a more sensitive configuration, allowing it to respond to the shallower gradients of Bcd that are found in the larger eggs[73,76]. The divergence in *hb* promoters has been accompanied by changes in Bcd itself. Bcd homeodomains of *Musca* and *Drosophila* differ by 6 out of 44 amino acids (13.6%) — a large difference for a homeodomain[70]. A serine-rich domain seems to be specific to the Muscoidea[76]. The differences in the amino-acid sequence of Bcd might reflect a co-evolutionary response to changes in P2 (REFS 30,76). The fact that Bcd from one species interacts less efficiently with the *hb* promoter of another is interpreted to be a consequence of the co-evolution of the two interacting components in a species that acts to maintain a strong interaction within each species, but, at the same time, leads to the accumulation of differences between species[77].

Redundancy and evolution of development. After experimental perturbation, many embryos show extensive properties of regulation. This developmental plasticity illustrates that there is often more than one way of making a structure or specifying a cell fate, and the development of the nematode vulva is an example of this. In *C. elegans*, the inductive signal from the anchor cell of the gonad specifies primary (1°) and secondary (2°) fates among vulval precursor cells (VPCs) in a concentration-dependent manner[78] (BOX 2). Descendants of the cell with the 1° fate form the inner vulva, whereas those of cells with the 2° fate form the outer vulva. The choice between 1° and 2° fate is reinforced by lateral signalling between the P5.p–P7.p cells[79]. Inductive and lateral signalling act redundantly[80]. In addition, two other completely redundant genetic pathways that involve proteins that are similar to retinoblastoma (Rb) and to its binding protein RbAp48 — mediate negative signals that antagonize the inductive signal to prevent inappropriate vulval differentiation[81,82].

Vulval development in nematodes has proved a useful model to study the relationship between intraspecific polymorphism and evolutionary variations between species. Although the vulva always forms from homologous precursor cells, the cellular interactions that specify the fates of vulval cells vary remarkably between species. Indeed, many changes in cell–cell signalling processes have been described for several nematode genera[83], and it seems that the configuration of these interactions is constantly being remodelled. Here, I give the example of *P. pacificus*, in which, as previously mentioned, the vulval competence group is composed only of cells P5.p–P8.p[84–86] (BOX 2). Although P8.p is part of the vulval competence group, it loses this competence during larval development and fuses with the hypodermis[87]. In the first few hours after hatching, P8.p can replace P7.p to form the vulva, but only in response to a signal from P6.p. In the absence of P5.p–P7.p, P8.p becomes epidermal, indicating that it cannot respond to the induction signal from the gonad. Furthermore, P5.p and P7.p can adopt a 1° fate in the absence of P8.p, but only a 2° fate when P8.p is present, showing that P8.p signals to these two cells. P6.p is not influenced by this signal and adopts a 1° fate and signals P8.p to adopt a 2° fate. P8.p can only inhibit P5.p and P7.p by acting through another cell, the mesoblast (M), which lies laterally to P8.p. Importantly, in *C. elegans*, this cell has no role in vulval development. Therefore, P8.p represents a new cell type that has not been found in any other nematode genus so far.

The competence of P8.p seems to have evolved in the genus *Pristionchus.* P8.p can adopt the vulval fate in the absence of P5.p–P7.p in some species, but not in others[88]. The lateral inhibitory signal of P8.p on P5.p and P7.p is only present in species in which these cells are competent to form a vulva. So, divergence of the special properties of P8.p seem to affect the signals that are redundant for vulval formation. In an attempt to find the genes that are responsible for these differences, one study examined the molecular variation between 13 strains that belong to four species of *Pristionchus*, which had been sampled from different parts of the world[88]. Interestingly, amplified restriction-fragment length polymorphisms indicated that differences between some strains stem from variation at a small number of loci.

Naturally occurring lineage variation in nematodes. Another fast-evolving trait in vulval development is the variability of the VPC lineages. In *C. elegans*, the number of divisions that vulval precursors undergo does not vary between individuals: P6.p divides three times producing eight cells, P5.p and P7.p each generate seven cells, and P4.p and P8.p each divide once (BOX 1). The lineages were thought to be a consequence of the fate assigned to these cells, as any change in fate is accompanied by a change in division pattern. In the absence of *lin-39*, no cells are competent and none divide (BOX 2). It is difficult to obtain mutants in which cell lineage, but not cell fate, is affected. The recent isolation of rare mutants, such as *cye-1*, in which only the rate of cell division is affected, has shown that normal differentiation of the vulva can take place after too many or too few cell cycles[89]. Although the two processes are separable, they are very tightly coupled in this species.

The coupling between cell fate and a fixed lineage for the P5.p–P7.p cells that form the vulva seems to extend to all other species that have been examined so far. Although in *C. elegans* this also extends to P4.p and P8.p, in other species lineages these VPCs can be variable. In the genus *Oscheius*, P4.p and P8.p lineage is variable both within and between species — they divide once, twice or not at all[90] (BOX 1). This variation is independent of fate because they remain part of the competence group. However, as they do not contribute to the vulva — their descendants fuse with the syncytial hypodermis — the number of divisions they undergo might not be under the same selective pressure as that of cells that do contribute to the vulva. It is worth noting that P3.p is the only cell in *C. elegans* with a variable lineage and is part of the competence group in only 50% of the animals. Fate and lineage are uncoupled in this one cell because this cell divides once in about half of the cases, but the division is not correlated with competence. In *C. briggsae*, P3.p is not part of the competence group and divides once in less than 15% of the animals. So, this polymorphism affects a cell that seems to be evolving in the genus *Caenorhabditis*. Similarly, the range of variation that is seen in P4.p and P8.p differs between species of the genus *Oscheius*, indicating that, here too, this character is evolving.

Two other observations support the idea that the link between cell cycle and cell fate is not stabilized in *Oscheius*. First, vulval development in *Oscheius* is sensitive to environmental stress, such as temperature[90]. Second, in contrast to *Caenorhabditis* spp., mutants that affect cell-division cycles of VPCs, but not their fate, are easily obtained in *Oscheius* spp., indicating a clear dissociation between these two processes[91]. So, interestingly, the mutability correlates with the state of variability of this character. The mutants seem to define genes that act downstream of the programme that specifies competence, to regulate cell lineage *per se*, and might have a role in the coupling of cell identity and lineage[91]. As these mutants mimic the natural variation found in some strains, allelic variation at one or more of these loci might be responsible for the natural variation. Classical-genetics approaches to identifying the genes that are responsible for the variation between strains indicate that several loci might be involved, although one locus had a relatively strong individual effect[90].

Can redundant pathways contribute to evolution?

Early studies in *Drosophila* have provided evidence for the homeostasis concept. This postulates that developmental mechanisms must be sufficiently stable to produce a uniform morphology, despite unpredictable environmental influences and the stochastic nature of developmental processes[92]. The buffering that protects normal development is called CANALIZATION and leads to

CANALIZATION
The buffering or stabilization of developmental pathways against mutational or environmental perturbations, by several genetic factors.

the build-up of cryptic genetic variation[93]. For example, redundancy in the number of binding sites in an enhancer and their co-evolution with the *trans*-acting factors probably contribute to the robustness of these elements, reducing the likelihood of functional error. Perturbation in the wild-type state can lead to a breakdown of this buffering system and result in a phenotypic manifestation of such hidden variance. Vulval development in nematodes illustrates canalization. The multiple, redundant signalling pathways, and the tight coupling between fate and lineage, provide a mechanism for buffering of vulva development and together they ensure its robustness in *C. elegans*. In *Oscheius*, the pattern of division of P4.p and P8.p is not well buffered against temperature variations and the divisions of all P4.p–P8.p cells are easily disrupted by mutation, indicating that some aspects of vulval development are less well canalized in this genus. Interestingly, in *Oscheius*, VPCs with a 2° fate undergo only two cell divisions, whereas in all other species they divide three times. It is possible that this characteristic has only recently evolved in this phylogenetic lineage, leading to the speculation that no redundant buffering system has yet been built up to prevent the isolation of mutants that only affect cell division[94].

In addition to the redundancy *per se*, the data from worms and flies also show that genetic pathways can diverge without any corresponding change at the phenotypic level. Furthermore, the divergence seems to be a continuing process. The configuration of cellular interactions during vulval development seems to be constantly evolving, and the sequence of enhancer elements of *eve* and *hb* seem to be in a permanent state of flux. It is not clear how redundancy arises and how it becomes fixed, but it is worth noting that the cellular interactions that are in the process of evolving seem to to be precisely those that are driven by redundant signals. This indicates that redundancy itself might provide conditions that allow continuous remodelling. The overall balance between the various components that contribute to the buffering process is presumably the result of stabilizing selection. Any changes are likely to be continuously stabilized by selection, because the final outcome — for example, the domain/stripe of expression of *hb*/*eve* or the correct specification of P5.p–P7.p — remains morphologically the same. Selection operates at the level of the functional unit, for example, at the enhancer module itself or at the three cells that make the vulva, not at the level of a specific binding sequence[59]. Therefore, the buffering of developmental processes favours the introduction of redundant elements and pathways, which, in turn, allows reconfiguration of these factors. The build-up of such redundant genetic networks is not associated with morphological change. However, as the original function would be assured by one pathway, a redundant pathway might be free to evolve. One intriguing question is whether a redundant pathway could, under certain circumstances, adopt a new function leading to micro-evolutionary change.

Conclusions

The evolutionary studies of rapidly evolving traits reviewed here indicate that a relatively small number of developmentally important genes seem to underlie many such traits. These studies highlight the use of satellite species of model organisms, such as *D. melanogaster* and *C. elegans*, which promises to be a powerful tool with which to study the evolution of developmental mechanisms. It is suggested that the process of canalization might favour the introduction of redundant mechanisms and pathways during development, which are characterized by high sequence turnover in the regulatory DNA sequence and by rapidly changing configurations in cell-interaction networks. Such redundancy could potentially provide material for evolutionary change.

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Acknowledgements
Discussions with M. Labouesse and comments on the manuscript from J.-M. Gibert and the three anonymous reviewers are gratefully acknowledged. Work in the author's laboratory is funded by The Wellcome Trust.

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