Arthropod Hox genes: insights on the evolutionary forces that shape gene functions

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Comparative studies suggest that gene duplication, changes in *cis*-regulatory elements and changes in protein sequence all contribute to the evolution of Hox gene functions, but the evolutionary dynamics of these changes are probably different. It seems likely that gene duplications arise as neutral changes and acquire an adaptive significance later on. By contrast, some changes in regulatory and protein-coding sequences can have immediate consequences in morphological evolution.

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Introduction

Molecular evolutionary theory tells us that most genetic changes that survive in nature are neutral; thus, these changes confer no significant selective advantage on the individuals that carry them [1]. Yet it is clear that the morphology, physiology and behaviour of organisms evolve continuously and that much of this evolution is adaptive, responding to the particular requirements and conditions of life of each organism. This apparent contradiction poses challenges for evolutionary biologists: to determine the extent to which different types of genetic change are subject to neutral or selective forces, to identify those few changes that have a significant role in adaptive evolution and, ultimately, to provide concrete examples of how adaptive evolution takes place (reconstructing the sequence of events from the generation of a specific genetic change, to its phenotypic consequences, to the selective forces that act on it in populations). In this review, I focus on what we have learnt from comparative studies of Hox genes while searching for the genetic changes that lie behind the evolution of arthropod body plans.

Different types of genetic change, including gene duplication, changes *in cis*-regulatory elements, and changes in protein-coding sequences, have contributed to the evolution of Hox gene functions. Each of these types of change has very different effects on gene function, takes place over different evolutionary timescales (ranging from millions to hundreds of millions of years) and may be subject to different evolutionary forces (stabilizing selection, directional selection or random drift). I will argue that some changes that have an important role in evolution, such as gene duplications, are likely to arise as neutral changes whose fate is determined by random drift. Such changes, however, may acquire a functional significance later on, by enabling other types of genetic change that have clear adaptive effects.

Changes with more direct effects on morphology are likely to include changes in *cis*-regulatory elements and in the coding sequences of Hox genes. In the past, such changes have been difficult to identify, owing to a lack of specific assays for functional comparisons among different species. Some recent studies have begun to address this issue, however, providing examples of how specific genetic changes contribute to the evolution of gene functions and body plans.

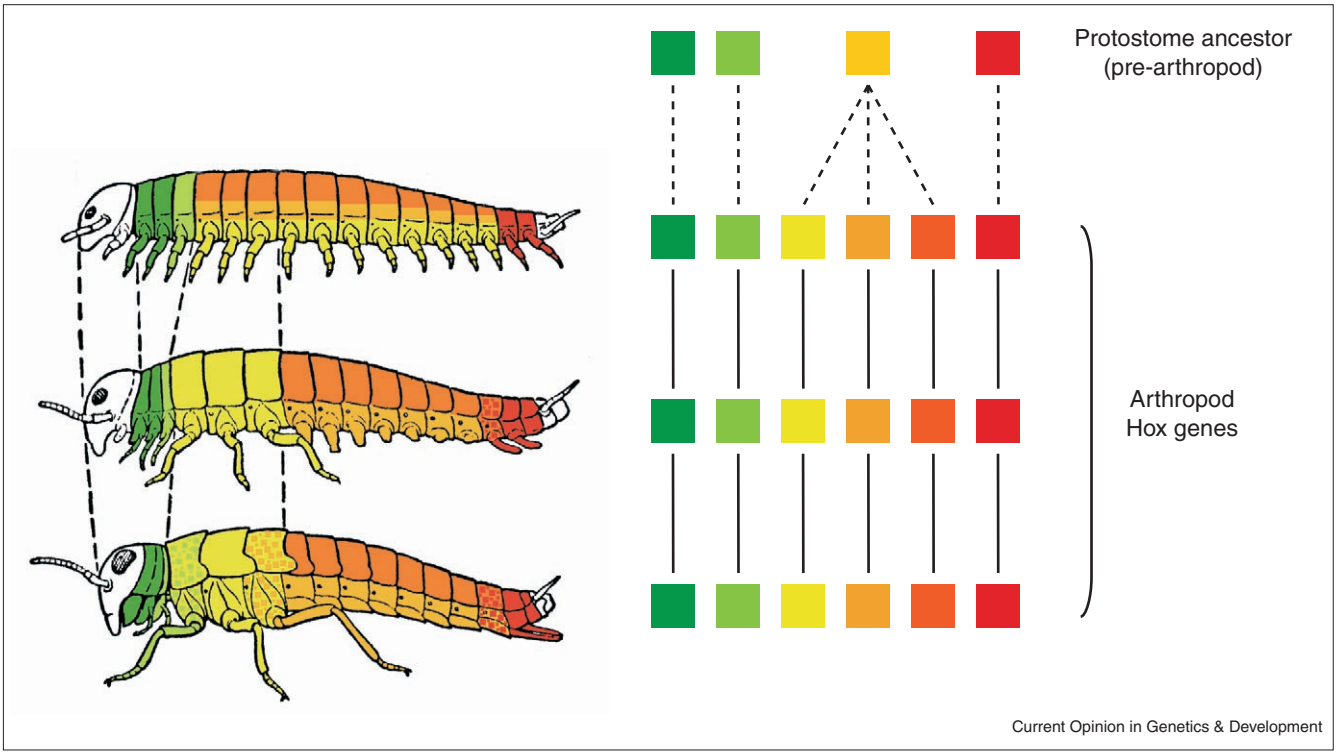
Gene duplications

Most genes are born through the duplication of pre-existing genes. Following the ideas of Ohno, gene duplications are thought to be crucial in the evolution of new gene functions: a duplication creates two gene copies, of which one can retain the original function, while the other is free to evolve and acquire new ones [2]. For Hox genes, in particular, it has been proposed that the creation of new Hox genes by gene duplication may have led to important morphological innovations in arthropod and vertebrate body plans [3–5]. But the evidence from arthropods has been disappointing. All of the major arthropod groups share the same basic set of Hox genes, regardless of the complexity of their body plans (Figure 1) [6–11,12•]: the uniformly segmented trunk of a centipede is specified by the same numbers and types of Hox gene as the trunk of an insect or crayfish, with more than five distinct segment types in that body region [11,12•]. In the few cases where gene duplications have given rise to additional Hox genes within the arthropods, it does not seem that these duplications have contributed much to morphological evolution [8,9,12•].

A comparison of posterior-acting AbdB class Hox genes among flies and vertebrates suggests the same. *Drosophila* has a single *AbdB* gene, whereas gene duplication has produced several AbdB class genes in vertebrates. Yet in both cases, these genes specify a comparable number of regional (or segmental) identities in the posterior part of the body. The *Drosophila AbdB* gene seems to have achieved similar levels of functional complexity as its vertebrate homologues, without gene duplication, by alternative splicing and by evolving unique patterns of gene expression within individual segments [13–15]. As discussed below, such diversification in the functions of individual Hox genes — through the elaboration of patterns of gene expression — is common among arthropod Hox genes [16].

To understand the consequences of gene duplication it is useful to examine in more detail what happens when genes are duplicated. First there is the duplication event

Figure 1



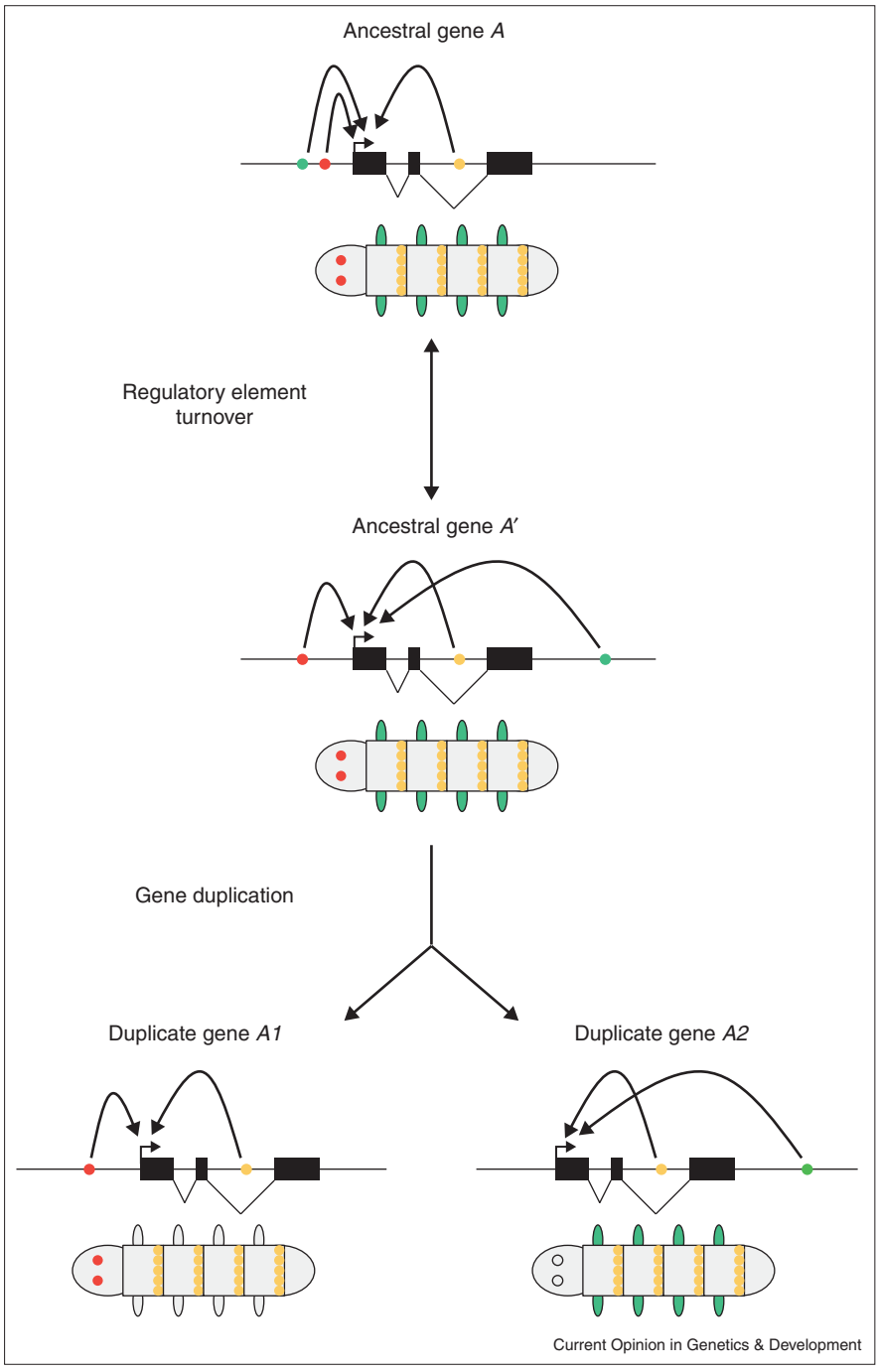
Diversification of Hox gene functions and the evolution of segmental specialization. In this hypothetical example, different Hox genes and their expression patterns are indicated by colors. All arthropods share the same basic set of Hox genes, so the complexity of a body plan is not directly associated with changes in Hox gene numbers or types. Specialization of segments is associated with changes in the expression patterns of Hox genes and in the regulation of their downstream targets. Changes in their expression patterns include large shifts in their regional domains of expression and the evolution of finer differences in their expression within individual segments.

itself, which is likely to occur by some type of illegitimate recombination or chromosomal rearrangement. This is likely to produce two identical copies of the original gene, in other words, two genes with qualitatively indistinguishable functions. What then are the forces that are likely to determine the survival of these identical copies? Certainly these copies will be subject to mutations, and if there is no selective pressure to maintain both (i.e. if their functions are completely redundant), then one of them will sooner or later be hit by a detrimental mutation. It is usually assumed that duplicated genes are preserved owing to the acquisition of novel gene functions by one of their gene products, which leads to positive selection for the maintenance of both copies. Yet this acquisition of novel functions is highly improbable when compared to the frequency of detrimental mutations.

A much more likely process, which is based on the partitioning or differential inactivation of *cis*-regulatory elements in the two gene copies, has become apparent in recent years [17,18]. Most higher eukaryotic genes, particularly developmental genes, are regulated by several separable and functionally independent *cis*-regulatory elements or 'enhancers', which are distributed over long stretches of DNA around the coding sequences of a gene. If the two copies of a duplicated gene were to inherit different subsets of these regulatory elements — either by the partitioning of these elements by the duplication event itself, or by the subsequent mutational loss of some regulatory elements — then each of the duplicates would carry out distinct subsets of the ancestral gene's functions in different developmental contexts, tissues or cell types (Figure 2). This partitioning of the ancestral genes' functions, or 'subfunctionalisation' [17–20], would provide a selective pressure for the preservation of both gene copies.

In contrast to other models of gene duplication, where the coding sequences of gene duplicates have to evolve new functions in order to survive, subfunctionalisation predicts that recent gene duplicates should encode functionally equivalent proteins that are expressed in nonidentical patterns. Several examples of this are now apparent, even for duplicates that arose a long time ago and have acquired very different roles in development [21–23,24••,25•]. An elegant example is the mouse *Hoxa3* and *Hoxd3* genes, which arose by gene duplications that occurred near the origin of vertebrates, around 500 million years ago [24••]. In mice, *Hoxa3* is required for the development of neural-crest-derived pharyngeal tissues, whereas *Hoxd3* is required in mesodermally-derived tissues of the axial

Figure 2



Neutral events that can have long-term consequences for functional evolution. A developmental gene can have multiple roles in development, dependent on expression driven by separate *cis*-regulatory elements. The dispersal of *cis*-regulatory elements around the coding sequences of a gene may have no immediate consequences for gene function (compare expression of genes *A* and *A′*). Similarly, gene duplication has no immediate effects as the duplicate copies carry out, collectively, the same functions as their ancestral gene (compare expression of genes *A′* and *A1* + *A2*). However, in the long term, modular and dispersed *cis*-regulatory elements will provide a better substrate for the partitioning of gene functions by gene duplication (hence for the survival of the gene duplicates by 'sub-functionalization' [17,18]), and gene duplications will allow changes in the two coding sequences to be selected independently, for different gene functions.

skeleton. In spite of extensive sequence divergence, the coding sequences of the two genes are able to complement perfectly each other's functions when reciprocally exchanged between the two genes. The functional differences between *Hoxa3* and *Hoxd3* are exclusively due to differences in their *cis*-regulatory sequences.

Interestingly, the mechanism of subfunctionalisation implies that newly duplicated genes will, collectively, carry out the same functions as their ancestral gene (Figure 2). Thus, such gene duplications are unlikely to have any immediate phenotypic effects and should behave as neutral genetic changes, whose fate in natural populations is governed by random drift. Consistent with this idea, the establishment of gene duplications does not seem to be associated with strong positive selection in their coding sequences. Analyses of genomic sequences from several organisms, including *Drosophila* and *Caenorhabditis elegans* [26•], suggest that gene duplications occur at an average rate of about one per gene per hundred million years. Most

gene duplicates do not survive for more than a few million years, however, which lowers the rate for established gene duplications to one in several hundreds of million years. The duplicates that do survive are largely subject to purifying selection [26•].

Even if duplications are established as neutral changes, they could have an important adaptive role in the longer term. By dissociating gene functions and partitioning them to different genes, gene duplications are likely to release coding sequences from the constraints that are associated with multiple gene functions, allowing them to respond more specifically to the adaptive requirements of individual activities. Thus, gene duplication events may serve as 'pre-adaptations', having no immediate phenotypic effect, but allowing the functional diversification of coding sequences in the long run. The coding sequences of duplicate genes may or may not evolve new functions eventually, depending on the particular evolutionary circumstances. There are now numerous examples of duplicate genes that have evolved for long periods of time without any significant change in the properties of their coding sequences [22,23,24••,25•]. Equally, in other cases gene duplication events have lead to significant functional changes, as in the series of Hox gene duplications that took place in the ancestors of the arthropods and gave rise to distinct *Antp*, *Ubx* and *abdA* genes [27]. These duplications had little impact on the morphology of the arthropods' ancestors, but they were necessary for the functional diversification of the Antp, Ubx and AbdA proteins that evidently took place in the arthropods.

Another example of gene duplications having a role in releasing coding sequences from multiple functional constraints and catalysing the evolution of new gene functions is found in the birth of the rapidly evolving *fushi-tarazu (ftz)* gene. This gene originated by duplication from Hox genes, but gradually lost its homeotic functions and acquired new functions in segmentation during the evolution of insects [12•,28,29,30•,31•]. Gene duplication does not seem to have been necessary for the evolution of the *bicoid/zerknullt* (*zen*) progenitor gene, whose novel functions replaced those of its ancestral Hox gene [12•,32–35,36•]. But the subsequent duplication that gave rise to distinct *bicoid* and *zen* genes must have been necessary to allow the evolution of distinct DNA- and RNA-binding properties in the Bicoid and Zen proteins [37,38].

Changes in *cis*-regulatory elements

Development requires the generation of precise spatial and temporal patterns of gene expression, therefore much of morphological evolution is thought to be mediated by changes in gene regulation. Over the past decade, comparative studies of expression patterns have shown that morphological evolution goes hand in hand with changes in the expression of developmental genes (e.g. see [39,40]). But what have been difficult to pinpoint are the actual genetic changes that lie behind these changes in gene expression. Most of them are thought to lie in the *cis*-regulatory elements of individual genes, although changes in regulatory proteins could also be involved (see below). The identification of the actual changes has been difficult because we are largely ignorant of the 'logic' by which *cis*-regulatory elements operate [41] and we are usually unable to relate their activity to their sequence.

Cis-regulatory elements consist of relatively simple clusters of binding sites for transcription factors with various, context-dependent activating or repressive effects. The individual binding sites are relatively short and loosely defined stretches of sequence (typically 4–10 nucleotides long), and their relative arrangement within an enhancer can be very flexible. This suggests that individual *cis*-regulatory elements should be relatively easy to create or to modify in the course of evolution. This expectation is borne out by studies that have compared homologous *cis*-regulatory elements among closely related species [42–44,45••]: *cis*-regulatory elements can change rapidly — in a timeframe of tens of million years or less. What is particularly interesting to note is that these rapid changes do not occur only in response to selection for functional change. A recent study suggests that rapid turnover of regulatory elements will take place even under conditions of stabilizing selection, when the activity of an enhancer is constrained to remain unchanged [44,45••].

This capacity for evolutionary change is also reflected in changes that have occurred in the expression patterns of Hox genes [46]. Large shifts in their expression domains seem to have been involved in the evolution of body plans among the different arthropod classes. Different patterns of tagmosis (subdivisions of the trunk into distinct subregions) in crustaceans, insects, myriapods and chelicerates seem to result from restrictions in the expression of different Hox genes to particular body regions [8–11,12•,47] (Figure 1). Smaller changes over individual segments have contributed to morphological changes in different orders of arthropods, such as in the evolution or suppression of abdominal prolegs in insects [48] or in the functional transformation of legs to feeding appendages in crustaceans [39,49]. And more subtle changes that modulate the expression of Hox genes within individual segments have occurred on a micro-evolutionary timescale, giving rise to fine morphological changes among closely related species [50].

It is not always appreciated that such changes in the detailed spatio-temporal expression of Hox genes can be important in the evolution of Hox gene functions, even in the long term. Detailed functional studies in *Drosophila* show that these modulations can make all the difference in the specification of strikingly different segmental identities — for example, the last thoracic and first abdominal segments are specified by different patterns of *Ubx* expression [16]. Similarly, the modulation of *AbdB* expression combined with alternative splicing can give rise to several distinct segmental identities in the posterior abdomen of

Drosophila [14,15]. Multiple homeotic functions are thus able to evolve and diversify as distinct *cis*-regulatory elements of a single Hox gene. Notably, these *cis*-regulatory elements are often spread over long distances around the coding sequences of Hox genes, which provides the ideal substrate for the dissociation of these gene functions by gene duplication and 'subfuntionalisation'. As with gene duplication events, the modularity and dispersal of these *cis*-regulatory elements may be neutral in the short term (Figure 2) but they could be important for the evolution of gene functions in the long term.

Changes in protein sequences

It is believed that regulatory proteins with multiple target genes are unlikely to change much during evolution, as any change in their properties would affect many of their targets and thus have multiple nonspecific (pleiotropic) effects. Hox and related proteins, in particular, are believed to regulate the expression of hundreds of target genes [51–53], so changes in their activity would be unlikely to have specific phenotypic effects. Instead, it would seem easy to achieve specific effects through changes in the *cis*-regulatory elements of individual target genes. This belief in the functional conservation of Hox proteins is supported by several studies in which homologues from *Drosophila* and vertebrates have been mis-expressed and compared for their effects in *Drosophila* embryos [54–58]. But the results in many of these experiments have been a mixture of pleiotropic phenotypes, and the effects on individual target genes have not been studied.

In spite of the emphasis that is usually placed on the conservation of Hox proteins, conservation in their sequences is usually limited to the homeodomain, a few amino acids on either side of it and the short hexapeptide motif. The greatest portion of these proteins diverges so fast that it is even difficult to align homologues from different arthropod classes. For the reasons mentioned above, however, most of this divergence was thought to be due to functionally irrelevant, neutral changes. Recent studies, looking at the effects of a Hox protein on a particular target gene, are now challenging this view [59•,60•,61••]. These studies have focused on the ability of the *Drosophila* Ubx protein to repress an early enhancer of the *Distal-less (Dll)* gene — a repression that contributes to the suppression of limbs in the abdominal segments of insects [62]. Studies from two laboratories show that, unlike insect Ubx proteins, crustacean and onychophoran Ubx do not have the ability to repress this enhancer when mis-expressed in flies. Through elegant domain-swapping experiments, these studies have been able to map this functional difference to specific regions of Ubx that are different in insect and crustacean or onychophoran Ubx proteins. These differences include the creation a novel repression domain in the insect Ubx protein [60•] and the loss of several putative serine/threonine phosphorylation sites that have an inhibitory effect on repression [61••]. These changes must have occurred during the early evolution of insects and had a direct effect in shaping the insect body plan by suppressing the development appendages in the abdominal segments.

The effects of these changes seem to be specific for the particular *Dll* enhancer and have no effect on the regulation of other targets by Ubx. For example, the *SRF* and the *Antp* P1 promoters are similarly repressed by insect and crustacean or onychophoran Ubx proteins [59•,61••]. Moreover, the strength of repression of the *Dll* enhancer depends on the additive effects of many changes (the creation of a new repression domain and successive loss of several phosphorylation sites [60•,61••]), which suggests that these changes could have happened gradually during evolution. These observations show that Hox proteins can be changed in ways that achieve very specific quantitative and qualitative effects on their activity (e.g. on the strength of repression or the spectrum of targets being affected).

Changes at the protein level have also been documented for the products of the *ftz* and *bicoid* genes. These rapidly evolving genes derive from Hox genes that are thought to have had canonical homeotic functions in their ancestral state [12•,28,29,30•,31•,32–35]. It is clear that in *Drosophila* these proteins have lost these ancestral functions and acquired new ones, which are mediated by changes in their ability to interact with cofactors [31•] and in their DNA- and RNA-binding activities [35,36•,37,38]. In the case of Bicoid, it is known that a single amino acid substitution in the homeodomain is responsible for a significant change in the DNA-binding specificity of this protein [37].

Conclusions

The evolution of Hox genes has had an important role during arthropod evolution, mediating significant changes in body architecture and segmental specialisation. Comparative studies suggest that this evolution takes place through different types of genetic change (gene duplication, changes *in cis*-regulatory elements and changes in protein-coding sequences) that have very different evolutionary dynamics. *Cis*-regulatory elements are able to change significantly over a period of tens of million years or less, functionally significant changes in protein sequences take place on a scale of tens to hundreds of million years, while productive gene duplications are even more rare, giving rise to new genes in a timeframe of hundreds of million years.

The phenotypic consequences and evolutionary forces that act on these changes are also different. Gene duplications can give rise to new genes, but there is no evidence yet that this is directly linked to the evolution of new gene functions. It seems, instead, that duplications simply partition the ancestral gene functions to two new genes. This change is neutral, but it provides an opportunity for subsequent changes in coding sequences to be selected independently for each of these functions.

Changes in *cis*-regulatory elements and in coding sequences are likely to have more immediate phenotypic

effects. Changes in *cis*-regulatory elements are probably responsible for most adaptive morphological changes because they are able to change rapidly, flexibly, and they can have relatively specific phenotypic effects.

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