Genome Analysis

HOM-C evolution in *Drosophila*: is there a need for *Hox* gene clustering?

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The conservation of Homeotic (*Hox*) gene clustering and colinearity in many metazoans indicates that functional constraints operate on this genome organization. However, several studies have questioned its relevance in *Drosophila*. Here, we analyse the genomic organization of *Hox* and *Hox*-derived genes in 13 fruitfly species and the mosquito *Anopheles gambiae*. We found that at least seven different Homeotic complex (HOM-C) arrangements exist among *Drosophila* species, produced by three major splits, five microinversions and six gene transpositions. This dynamism contrasts with the stable organization of the complex in many other taxa. Although there is no evidence of an absolute requirement for *Hox* gene clustering in *Drosophila*, we found that strong functional constraints act on the individual genes.

Introduction

Homeotic (*Hox*) genes (see Glossary) encode transcription factors involved in the specification of segment identity along the anteroposterior body axis of metazoans. These genes were discovered in the early twentieth century in the fruitfly *Drosophila melanogaster* through mutations that transform one body part into another [1]. Strikingly, Lewis [2] and Kaufman *et al.* [3] found them to be clustered in two separate complexes (see below) and also arranged in the same genomic order as their domains of function along the anteroposterior body axis (i.e. colinearity). *Hox* genes were subsequently determined in vertebrates, and their structural and functional organization suggested that vertebrate *Hox* clusters were homologous to fruitfly Homeotic gene complexes (HOM-C) [4–6]. By the early 1990s, *Hox* genes had been found in all metazoans, including humans, and the clustered arrangement and colinearity were shown to be the general rule [7–9]. The conservation of *Hox* gene clustering and colinearity between vertebrates and invertebrates has suggested that this genomic organization is an essential functional requirement for proper embryonic development. However, the precise reasons are unclear [10], and 'disorganized complexes' have been described in several species, including fruitflies, nematodes and tunicates. The evolutionary analysis of these exceptions is important because it can shed light on the functional constraints operating on the HOM-C and on the ultimate reasons for the unusual organization of these genes.

Here, we reconstruct the evolutionary history of the HOM-C in the genus *Drosophila*, for which three splits have been previously described [11–13]. We annotated the regions including the *Hox* genes in the genomes of ten *Drosophila* species whose genomes have recently been sequenced (http://rana.lbl.gov/drosophila/) and *Anopheles gambiae* [14] (see the supplementary material online) and compared their organization with that of *D. melanogaster*, *Drosophila pseudoobscura* [15] and *Drosophila buzzatii* [16]. Regions of interest were identified by BLAST searches and analysed by comparative gene annotation. The species investigated represent a substantial portion of the evolutionary history of *Drosophila* (between 35% and 48%, assuming 2 000 species in the genus [17]).

Structural evolution of the HOM-C in the genus *Drosophila*

In *D. melanogaster*, *Hox* genes are arranged in two clusters, the Antennapedia complex (ANT-C) and the Bithorax complex (BX-C), separated by ~7.5 Mb on chromosomal arm 3R (Muller's element E [18]). The ANT-C includes five *Hox* genes, *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*) and *Antennapedia* (*Antp*) [19]; whereas the BX-C consists of three *Hox* genes, *Ultrabithorax* (*Ubx*), *abdominalA* (*abdA*) and *AbdominalB* (*AbdB*) [20,21].

Glossary

Colinearity rule: describes the observation that *Hox* genes are arranged in the chromosome in the same order as they are expressed along the anteroposterior body axis of metazoans (spatial colinearity) and/or in the same order as their temporal expression in development (temporal colinearity).
Gene transposition: movement of a relatively small genomic segment, containing usually one or a few genes, from one chromosomal position to another. Genes can transpose by several mechanisms including retroposition (which implies reverse transcription of RNA and insertion of the resultant cDNA into a different genome site) and transposon-mediated excision and insertion of genomic segments.
HOM-C (Homeotic complex): cluster of *Hox* genes located (usually) in a single chromosomal site.
Homeotic (*Hox*) genes: genes that determine the identity of individual segments or body regions in early embryos of metazoans.
Homeotic mutations: those that cause body regions to develop structures appropriate to other regions.
***Hox*-derived genes**: *Hox* genes that have lost their homeotic function or are derived (by duplication) from such a gene.
Microinversion: a small inversion containing at most a few genes that cannot be cytologically detected.
Paracentric inversion: a chromosomal inversion that does not include the centromere. This seems to be the most common type of chromosomal alteration in the evolution of the genus *Drosophila*.
Phylogenetic inertia: refers to the transmission of unchanged traits from ancestor to descendant species (i.e. the fact that a trait can persist in a lineage for a long time after the cessation of the selective forces that have produced or maintained it).

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Although the ancestral HOM-C of arthropods comprised ten genes, in winged insects, two genes – *Hox3* and *fushi tarazu* (*ftz*) – lost their homeotic function, so only eight bona fide homeotic genes remain [22,23]. The ancestral *Hox3* gene underwent two duplications before the *Drosophila* radiation, giving rise to the genes *bcd*, *zen* and *zen2* (which, in addition to *ftz*, we call *Hox*-derived genes).

The organization of *Hox* gene regions in the 13 *Drosophila* species analysed here is shown in Figure 1. We found seven different gene arrangements. None of the species conserves the single integral complex that must have existed in the ancestor of the genus and is now seen in other Diptera, such as *A. gambiae* (Figure 1). All of them possess one or two of the three major splits of the complex already known. The split between the genes *Antp* and *Ubx* that defines the ANT-C and BX-C complexes is present in all species of the *Sophophora* subgenus, which includes *D. melanogaster* and its close relatives, *Drosophila ananassae*, *D. pseudoobscura*, *Drosophila persimilis* and *Drosophila willistoni* (Figure 1, see A). The species of the subgenus *Drosophila* – *Drosophila virilis*, *Drosophila mojavensis* and *D. buzzatii* – and the Hawaiian species *Drosophila grimshawi* (subgenus *Idiomya* [18]) possess a different split, between *Ubx* and *abdA* (Figure 1, see B). These two splits took place in a relatively short period of time between 63 and 43 million years (Myr) ago. The species of the *repleta* group (subgenus *Drosophila*), which includes *D. mojavensis* and *D. buzzatii*, have an additional split, between *lab* and *pb*, that occurred between 20 and 30 Myr ago [13] (Figure 1, see C).

In addition to the three gross paracentric inversions that produced these three splits [12,16], at least five microinversions have occurred within the *Drosophila* HOM-C. Most *Hox* genes share the same orientation, with the 3′-end facing the anterior genes of the complex and the 5′-end towards the posterior genes. One *Hox* gene, *Dfd*, has been inverted and thus breaks this rule in the species of the *melanogaster* subgroup (*D. melanogaster*, *Drosophila simulans*, *Drosophila sechellia*, *Drosophila yakuba* and *Drosophila erecta*) and in *D. willistoni*. Some *Hox*-derived genes have also changed their orientation: for example, *zen2* is inverted in all *Drosophila* species except *D. ananassae* and the species of the *melanogaster* subgroup. The orientation of *zen2* in different species and its position between *pb* and its 5′ regulatory regions seems to be the result of two inversion events (B. Negre, PhD thesis, Universitat Autònoma de Barcelona, 2005). The first inversion event took place in the ancestor of the *Drosophila* genus and involved *zen2* plus the *pb* regulatory regions, whereas the second event took place in the ancestor of the *melanogaster* group and involved only *zen2*. The orientation of *ftz*

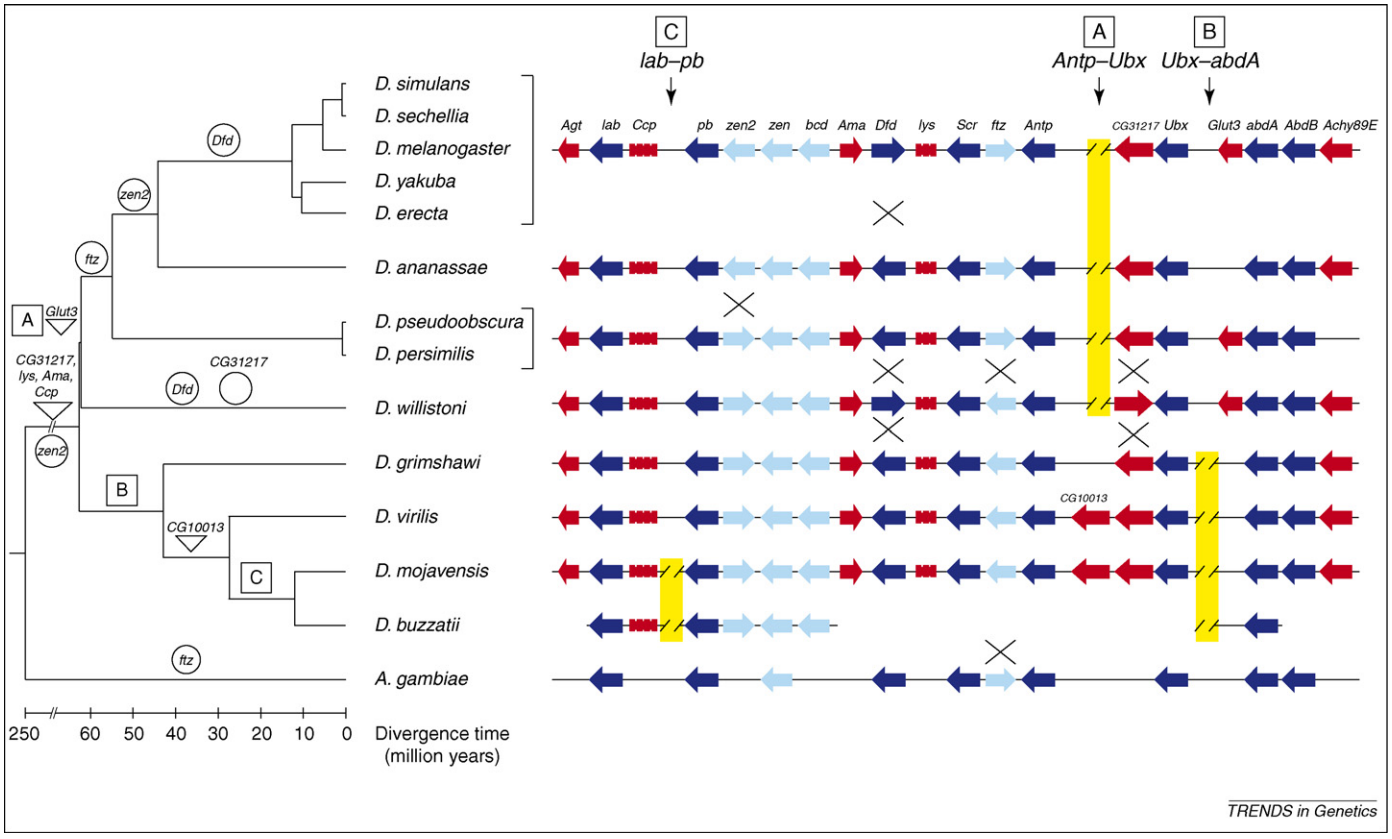

Figure 1. HOM-C structural evolution in the *Drosophila* genus. The *Hox* gene complex has suffered a high number of structural changes during the evolution of the *Drosophila* genus. Three major rearrangements (shown as squares: A, *Antp–Ubx*; B, *Ubx–abdA*; and C *lab–pb*), seven microinversions (circles) and six gene transpositions (inverted triangles) have been identified and mapped to the phylogenetic tree by comparative analysis. The structure of the HOM-C in each species has been analysed from its complete genome sequence, except *Drosophila buzzatii* [16]. Coloured arrows represent genes and their orientation; *Hox* genes are in dark blue, *Hox*-derived genes in light blue and non-*Hox* genes in red. The *Ccp* gene cluster and the *tRNAlys* (denoted *lys*) cluster are not depicted in detail. (Subdivisions indicate the presence of several genes, although not the exact number, which varies between species.) Double diagonal lines in the cluster diagrams represent discontinuities in the sequence, and yellow shadows indicate equivalent breaks. The different segments are drawn in the order of the ancestral HOM-C and do not represent the actual order, orientation or distance between chromosome segments. Crosses indicate different gene orientation in adjacent diagrams and have no phylogenetic meaning. Divergence times are taken from Refs [36] and [37].

and its position, between *Scr* and its 5′ regulatory sequences, resembles that of *zen2* and *pb*; thus, a similar scenario could also explain this structure. In addition, *A. gambiae* also has an inverted *ftz* gene. Thus, either the first *ftz* inversion occurred early in dipteran evolution or the *ftz* gene has undergone repeated inversions in different lineages. Other, yet-smaller inversions (1–2 kb), involving regulatory sequences only, have also been observed in the complex [16] (data not shown). Those microinversions seem to invert individual regulatory elements and thus might have no effect on expression.

Several gene transposition events have occurred within the *Drosophila* HOM-C. Four transpositions, involving the Cuticular genes cluster (*Ccp*), Amalgam (*Ama*), the *tRNAlys* cluster and *CG31217*, are shared by all *Drosophila* species and thus must have occurred before the diversification of the genus. Two other transposition events occurred after the divergence of the two main lineages. The gene *Glut3* transposed between *Ubx* and *abdA* in the ancestor of the *Sophophora* subgenus, and *CG10013* transposed between *Antp* and *Ubx* before the *virilis/repleta* radiation. In addition, there are changes in the number and orientation of genes in the *Ccp* and *tRNAlys* clusters (data not shown). A detailed analysis of the HOM-C sequence from *A. gambiae* shows that despite its longer sequence and numerous insertions of transposable elements, no new genes have been transposed into the complex. Therefore, the six transpositions observed in the *Drosophila* species occurred after the divergence of the *Anopheles* and *Drosophila* lineages.

Functional constraints acting on the *Drosophila* HOM-C

The presence of three major splits, five microinversions and six gene transpositions within the *Drosophila* HOM-C contrasts with the stable organization of the complex described in vertebrates and many other taxa [7–9]. *Hox* gene clustering is thought to be a functional requirement for proper gene expression, although the mechanistic reasons have remained elusive. In vertebrates, temporal rather than spatial colinearity is responsible for keeping the complex together [10]. Additionally, the presence of global control regions, which are located at one side of the cluster and regulate several *Hox* genes at once, precludes breakage of the complexes [24].

Is there a functional requirement for *Hox* gene clustering in *Drosophila*? Experimental observations made in *D. melanogaster* are consistent with the HOM-C evolution in the genus *Drosophila* (Figure 1). Breaks in the ANT-C do not affect more than one gene, suggesting the absence of shared regulatory elements and global enhancers [25–28], which agrees with the conserved expression patterns after the split between *lab* and *pb* of the *repleta* group [16]. However, two of the *Hox*-derived genes, *zen2* and *ftz*, are inserted between a *Hox* gene (*pb* and *Scr*, respectively) and its 5′ regulatory regions. This organization prevents a separation of the *Hox* and the *Hox*-derived genes by an inversion (because it would distance the regulatory sequences of the *Hox* gene from its promoter), although it would be possible for the *Hox*-derived gene to change its position through a transposition event.

In the BX-C, not only is there colinearity of the genes but also of their regulatory regions [29]. The independence of the Ultrabithorax region (*Ubx* and its regulatory regions, which include the noncoding RNA *bxd* [30]) is confirmed by the split between *Ubx* and *abdA* present in the *Drosophila* subgenus and Hawaiian species. This breakpoint is located precisely between the *bxd* and *iab2* regulatory regions. The Abdominal region, which contains *abdA*, *AbdB* and the 100 kb of *cis*-regulatory regions *iab2* to *iab9* [30], shows additional peculiarities. *AbdB* is the only gene that bears most of its regulatory sequences 3′ of the transcription unit instead of 5′, so that the regulatory regions of both *abdA* and *AbdB* are located between the two genes. This is also the only intergenic region within the HOM-C without transposed genes or inversion breakpoints (although there is an annotated gene in the *D. melanogaster* genome, our analysis suggests it is a false positive, Figure 1). A recent study [31] suggests a highly structured and modular organization of this region. Each *cis*-regulatory domain (formed by a set of initiator, maintenance elements and tissue-specific enhancers) directs expression in one parasegment and is separated from the neighbouring domains and competing influences by boundary elements [31]. Although the colinearity of *abdA* and *AbdB* is not essential for proper function [32], these observations suggest the absence of breakable 'intergenic' space between the two genes. Their separation would probably require the duplication of the Mcp boundary element (located between their regulatory regions *iab4* and *iab5*) to ensure the proper function of each regulatory module and to avoid positional effects.

To gain further insight into the functional constraints operating on the *Drosophila* HOM-C, we have estimated the degree of functional constraint ($0 \leq \delta \leq 1$) (Box 1). When the total size of the complex or the amount of intergenic sequence is considered, a significant value, $\delta = 0.84$ or 0.88,

Box 1. Degree of functional constraint operating on the *Drosophila* HOM-C

The degree of functional constraint (δ) is defined here as the fraction of breaks in a given chromosomal region that are unviable (i.e. that natural selection does not allow to become fixed). It can be estimated, relative to the chromosome average, as $\delta = 1 - (No/Ne)$, where *No* is the observed number of breaks in that region and *Ne* the expected number of breaks under a random break distribution. A value of $\delta = 1$ would indicate a fully constrained region, whereas a value of $\delta = 0$ would mean there are no constraints.

A rate of 0.065 disruptions per Mb and Myr (SD = 0.008) has been estimated for the entire Muller's element E in *Drosophila* [38]. The *Drosophila* species analysed here (Figure 1) harbour a total evolutionary history of 402 Myr [36,37], and the size of the HOM-C in *Drosophila melanogaster* amounts to 392 kb (ANT-C) + 320 kb (BX-C) = 712 kb. Thus, the expected number of breaks in the HOM-C is $0.065 \times 0.712 \times 402 = 18.6$. The observed number of breaks is three, about sixfold fewer, hence $\delta = 0.84$ (SD = 0.09).

We can consider that breakpoints of successful inversions will only occur in intergenic regions because inversions with one breakpoint inside a gene will almost invariably be deleterious. If only intergenic regions are considered (Table 1), the disruption rate per Mb and Myr for the entire element E is 0.169 (SD = 0.021), and the expected number of breaks in the HOM-C is $0.169 \times 0.356 \times 402 = 24.2$. In this case, the observed number of breaks is eightfold fewer than expected, and $\delta = 0.88$ (SD = 0.07).

Finally, if we take into account only the number of intergenic regions (Table 1), but not their size, a rate of 0.00055 breaks per intergenic region and Myr (SD = 0.00008) can be estimated for the entire element E. The expected number of breaks in the HOM-C is now $0.00055 \times 23 \times 402 = 5.1$, and $\delta = 0.41$ (SD = 0.34) (not significantly different from 0).

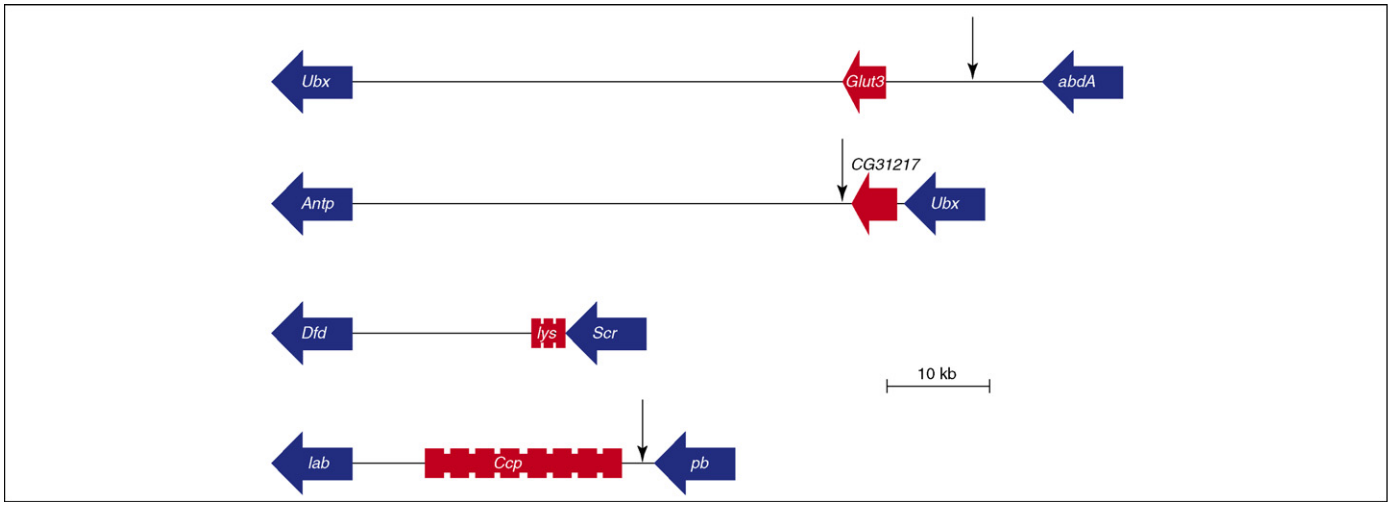

Figure 2. Relative position of gene transpositions and cluster splits in relation to adjacent *Hox* genes. Both transposed genes (red) and inversion breakpoints (black arrows) are located close to the 3′-end of one *Hox* gene and far from the 5′-end of the next *Hox* gene. Intergenic distances are drawn to scale following *Drosophila melanogaster* sizes. Coloured arrows represent genes and their orientation; *Hox* genes are in dark blue, and non-*Hox* genes in red. Black arrows indicate the position of splits. The orientation of *Dfd* is that of the ancestral complex.

is obtained. This indicates that the observed number of splits is reduced to ~1/6 or ~1/8 compared with the average rate for the whole chromosome; thus, there are strong functional constraints acting on the HOM-C. However, when only the number of intergenic regions is taken into account, δ is not significant (Box 1). This means that the probability of finding a break between two adjacent *Hox* genes is similar to that of any other pair of adjacent genes. Therefore, the reduced split rate observed in the *Drosophila* HOM-C results from functional constraints acting on the numerous and complex regulatory sequences surrounding each gene, and not from a need to keep *Hox* genes clustered.

As previously shown [16] and confirmed in this analysis, all splits and transposition events have occurred close to the 3′-end of one transcription unit and far from the 5′-end of the next one (Figure 2). These reorganizations have not altered the regulatory regions of the adjacent genes as they are mostly located in the 5′-region (except for *AbdB*) and in the introns, thereby keeping each gene and its regulatory sequences as an independent module. The larger gene size (Table 1) and the presence of these large regulatory regions diminish the regions where a split can be produced without detrimental functional consequences, and thus reduce the observed split rate per Mb. *Drosophila Hox* genes do not need to be clustered for proper expression, but they do need to keep their regulatory regions intact. Thus, we can conclude that functional constraints in the *Drosophila* HOM-C are not acting on the complex as a whole but on each gene (including the transcription unit and regulatory sequences) independently.

Table 1. General statistics of the *Drosophila melanogaster* genome and HOM-C region

Property	Whole genome[a]	HOM-C region
Size (kb)	116 800	712
Number of genes	13 987	23
Gene density (genes/kb)	0.118	0.032
Average gene size (bp)	5 231	42 115 (*Hox* genes only) 15 466 (all genes in region)
Gene portion (%)	61.80	49.93
Intergenic content (%)	38.20	50.07

[a]Release 4 Notes: updated 15 September 2005 (http://flybase.net/annot/dmel-release4-notes.html).

Concluding remarks

It has been proposed several times that the rapid mode of *Drosophila* embryogenesis might have resulted in release from the selective pressures acting on the ancestor HOM-C [16,33]. Although there are no major morphological or timing differences in development between *Drosophila* and *Anopheles* [34] that would suggest differences in functional constraints on *Hox* genes, the systematic comparison of segmentation regulatory genes suggests that the segmentation gene network has undergone considerable evolutionary change among Diptera [35]. However, little is known about the developmental network of other Diptera, making it difficult to predict when, in the lineage leading to *Drosophila*, those changes arose. Moreover, the release of functional constraints on a given trait is not followed by an immediate change on it (e.g. the Abdominal region). It takes time to change a trait by neutral evolution: this delay results in the phenomenon of phylogenetic inertia. More data are needed to assess how representative the structure of the *A. gambiae* HOM-C is and whether the structural differences seen between *Anopheles* and *Drosophila* are functionally significant or only the result of different rates of chromosomal reorganization between lineages.

In *Drosophila*, the lower split rate in the HOM-C regions suggests the existence of significant functional constraints acting on these regions. However, these constraints do not seem to involve the tight clustering of the genes, as seen in vertebrates, but act on each gene and its own regulatory sequences. Therefore, *Hox* gene clustering seems to be the

result of phylogenetic inertia compounded by a complex regulatory architecture. It is ironic that *Hox* gene colinearity was discovered in *Drosophila* [33], a species in which *Hox* gene clustering is only an ancestral vestige.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tig.2006.12.001.

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