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Anterior Hox genes: Evolution by birth and death with developmental constraints

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Abstract

Hox genes represent an important gene family that is involved in the segmentation pattern and identity of the segments during the formation of the body plan in metazoans. For many years, several studies have sought to establish a correlation between the evolution of these genes and the evolution of large groups of metazoans. Here, we use publicly available sequences of Hox gene clusters to reconstruct the evolutionary history of anterior Hox genes. We show that information harbored by these genes, in part, reflects the evolution and diversification of most animal archetypes, but in many cases, there were conflicts between the evolutionary history of some genes and the history of large groups, these cases may have occurred due to specific and similar selective pressures in relatively distant groups, which may have led to evolutionary convergences. Our findings also reveal that evolution of Hox genes (and clusters) as a multigene family is consistent to a birth-and-death model constrained by development, where there is a trade-off between a relatively fast gene turnover and their central developmental roles.

Keywords: Metazoans, gene family, evolution, development.

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Introduction

The Hox proteins are a group of transcription factors that contain a homeodomain that play a role in patterning animal body and are evolutionary conserved. The homeodomain proteins are characterized by the presence of conservative DNA-binding region known as homeodomain and these proteins are encoded by homeobox genes [1].

Hox genes are a set of structural regulatory genes that are involved the segmentation pattern and segment identity of the segments during the formation of the body plan of metazoans, imprinted through a complex profile of expression across the anteroposterior axis in Bilateria – the '*Hox* code' [2-4]. The *Hox* genes are a stereotypical example of how the understanding of genetic basis of animal development can illuminate aspects of morphological evolution. Changes on the correct local expression of these genes result in homeotic transformations of the body regions involved (*i.e.* misplaced development of morphological structures) [2, 5].

Hox genes are already present in Cnidaria [6], however, the minimal *ProtoHox* cluster must have emerged before the Cnidaria-Bilateria split, composed by only two anterior genes [7]. Further events of gene duplication have originated an ancestral arthropod *Hox* cluster consisting of 10 genes [8], followed by two events of tetraploidization in Deuterostomes: one in Craniata and the second one in teleosts [9-11]. Events of gene duplication and loss, together with analysis of sequence information harbored by *Hox* genes, has been proved useful to understand several aspects of animal phylogeny In fact, it has been hypothesized that evolution of *Hox* clusters is consistent to a birth-and-death model of evolution of multigene families, where a few duplicated gene copies remain in the genome for a long time, whereas others are inactivated, deleted or go through a process of pseudogenization [12]. Kappen et al. [13] using character state analyses of human and mouse *Hox* sequence show two scenarios to the evolution of this gene family. In the first scenario, there was an ancestral cluster containing 13 genes and, in the second scenario, *Hox-b* cluster is considered a direct representation of the ancestral cluster and was required a gain at least 3 genes before any cluster duplication [13]. Ravi et al [14] present the same result of the fist scenario using sequences of elephant shark *Hox* genes from paralogous groups 1, 3, 4, 5, 9, 10, and 13 that contain all of the 4 members (A, B, C, and D) using amphioxus *Hox* as out group [14]. Tracing these events of gene duplication and loss, together with analysis of the information borne by *Hox* genes has been proved useful to understand several aspects of animal phylogeny, such as the support of Ecdisozoa and Lophotrococza lineages within Protostomes [15], relationships

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among the four extant arthropod subphyla (Chelicerates, Crustaceans, Hexapods, and Myriapods) [16] and Vertebrate groups [17].

Here, we analyzed the evolutionary history of anterior *Hox* genes through metazoa and propose a model to explain *Hox* evolution as a multigene family.

Materials and Methods

Homologous *Hox* protein sequences involved with body patterning of the anterior (*Hox1-Hox4*),were retrieved from NCBI (http://ncbi.nlm.nih.gov/) in the refseq using the BLASTp algorithm [18]. The sequences to *Hox3* included in our dataset comprise only vertebrates, since evolution of this gene is characterized by gene duplication and neofunctionalization to *bicoid*, *zerknüllt*, and *z2* in some protostomes [8, 19]. all *Hox* sequences were analyzed individually, but grouping together genes from different clusters (A, B, C and D, when applicable). This approach resulted in four independent sets of data, which were aligned using MAFFT v7 webserver [20] and a combination of available parameters to refine the alignment: iterative refinement methods (E-INS-i, L-INS-i and G-INS-i) and scoring matrix (BLOSUM45 and BLOSUM62) for amino acid sequences. The best evolutionary model for each *Hox* protein/alignment was JTT+I+G+F and was inferred using ProtTest 3.2 [21] considering the Akaike information criterion (AIC). Unrooted maximum likelihood trees of each *Hox* was inferred using PhyML 3.0 webserver [22]. FigTree v1.4.3 was used to visualize the phylogenetic trees.

Results and Discussion

Hox phylogeny: Similarities and inconsistencies with the evolution of animals

We used publicly available protein sequences from Hox genes to reconstruct the evolutionary history of this multigene family. Each gene (Hox1, Hox2, Hox3 and Hox4) was analyzed separately but including all copies of the same gene in a single alignment whenever they were present, resulted in 4 phylogenetic trees (one per Hox gene). The resulting trees show clear shared features. First, they have overall high support values, with slight (non-significant) differences among them (One-way ANOVA: $F(1.631)$; $P < 0.077$). Second, support values are higher for basal nodes – supporting previous findings that Hox gene

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sequences may be a powerful source of information to resolve deep evolutionary relationships [23]. Finally, Hox genes from Protostomes and non-Chordata Deuterostomes (Echinodermata) form separate groups from Vertebrate clusters. Our phylogenies are consistent with Meyer and Zardoya's [24] hypothesis of vertebrate evolution and provide further support to previous hypothesis that Testudines is a sister group of Archosauria [25, 26].

In Vertebrates, anterior *Hox* genes are associated with several features: branchial arch artery development, which are transitional embryo blood vessels [27]; control of basal and alar plates of the hindbrain rhombomeres [28]; differentiation of the first cervical vertebra (atlas) [29]; ability of the neural crest cell population to differentiate and/ or induce adequate differentiation of the surrounding pharyngeal arch and tissue sacs [30]; definition of the boundaries between rhombencephalon and somites [31]; activation and proliferation of lymphocytes [32]; differentiation of keratinocytes [33]; development of the epidermis [34] and/or formation of cervical vertebrae [35]; and determine the thoracic-cervical boundary [36]. In Arthropoda, anterior *Hox* genes are associated with development of the salivary gland and cephalopharyngeal skeleton [8]; development of labial palps and maxillary and buccal parts [8]; maintenance of the boundary between maxilla and mandible, activating apoptosis located in the head lobe [37]; and control of cell fate in the body central region [38] and epidermal cells in nematodes [39].

Our phylogenetic analyses show that Mammalia *Hox1* might be either a sister group of Lepidosauria or more closely related to a branch formed by Crocodylia and Aves (Figure 1). Despite these two different hypotheses, Mammalia is placed within Diapsida in both *HoxA1* and *HoxB1* subtrees (Figure 1A). A second important finding emerging from *Hox1* phylogeny (or *labial*, following the nomenclature for *Drosophila melanogaster*), is a strongly supported split between derived Deuterostomes and a group formed by Protostomes (Onychophora, Tardigrada, Insect, Crustacea, Mollusca and Chelicerata) and non-Chordata Deuterostomes (Echinodermata). Phylogenetic relationships within this group (Protostomes + non-Chordata Deuterostomes) are not fully resolved, which can be observed by the grouping of Echinodermata within Protostomes as a sister group of Chelicerata, and the formation of polyphyletic groups (*e.g.* Chelicerata is observed multiple times across the *labial* subtree). This can be potentially explained by stronger selective pressures in *labial*/*Hox1* in Protostomes and non-Chordata Deuterostomes, which is present as a single copy, in contrast to the presence of up to three *Hox1* copies in derived Deuterostomes (diluting selective pressures in each copy). Strong conservation in Protostome *labial* may

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increase gene similarity across Protostomes – resulting in low phylogenetic resolution –, whereas each gene copy can accumulate more neutral or nearly-neutral mutations in derived Deuterostomes, increasing phylogenetic resolution within this group.

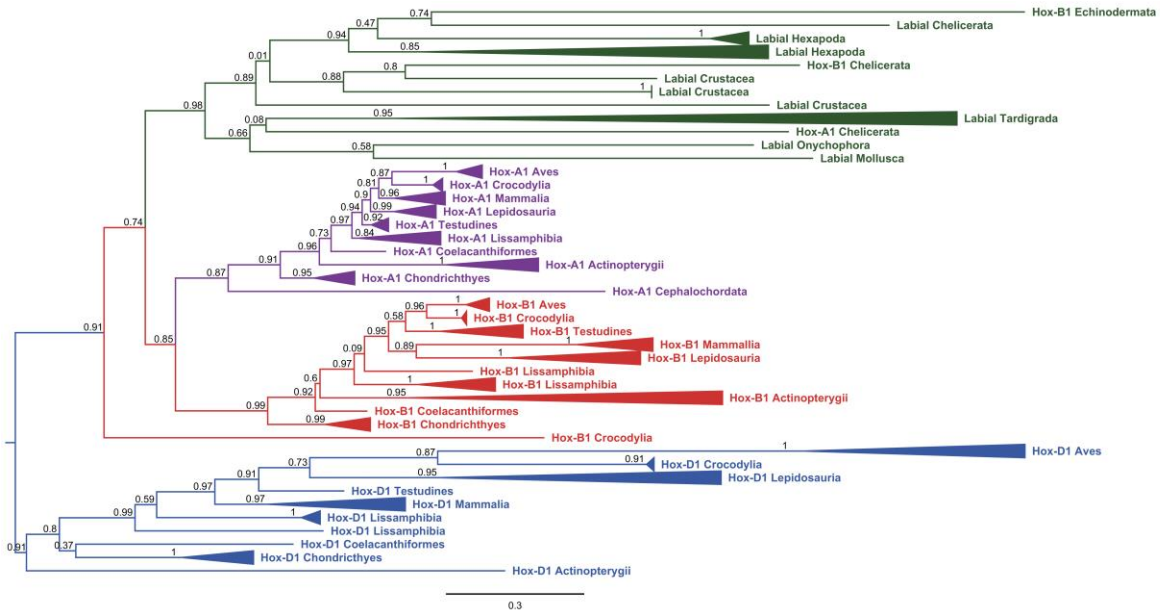

Figure 1. Hox1 derived phylogeny. The green branches show the Labial gene, while purple ones represent the Hox-A1, the red as Hox-B1, and in blue Hox-D1. The support values are represented by Shimodaira-Hasegawa- like.

In *HoxA2*, the position of Mammalia as a sister group of Lepidosauria may be an evolutionary convergence or just an incongruence, since this group has a low support value (0.68) (Figure 2). Similarly, there is an incongruence on the *HoxB2* tree, placing ray-finned fish *HoxB2* closer to the *HoxA2* branch, with low support.

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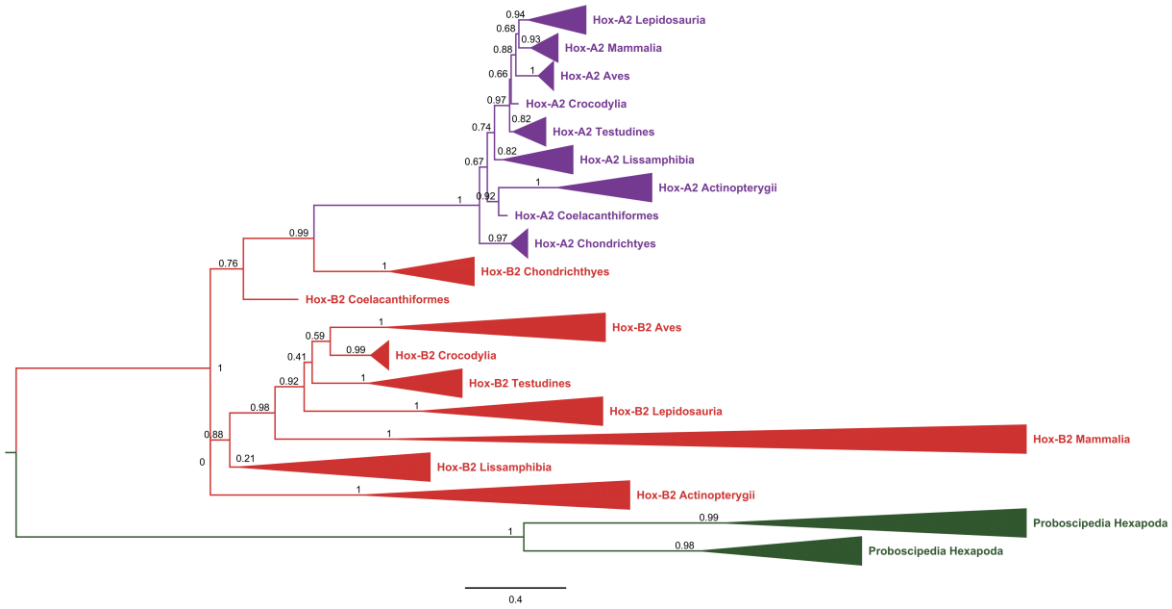

Figure 2. Hox2 phylogeny in green we have the Proboscipedia, in purple have the Hox-A2 and in red Hox-B2. The support values are represented by Shimodaira-Hasegawa-like.

In *HoxA3*, the phylogenetic relationships within Amniota show Aves, instead of Mammalia, as the first group to diverge within Amniota (Figure 3). Gnathostomata phylogenies from *Hox* genes do not always support the hypothesis of the Craniata group proposed by Meyer and Zardoya [24], possibly because our phylogeny reconstruct the relation of Hox genes present in Gnathostomata and differences can be explained with future experimental tests like in the Hox-D3 phylogeny that sequences of Aves and Mammalia (Figure 3) are sibling and the function of Hox-D3 associated with differentiation of the first cervical vertebra Atlas may explain the possible convergence of the sequences between Mammalia and Aves because the atlas vertebra within Amniota provides vertical movement of the head that are made by the articulation of the skull with the Atlas.

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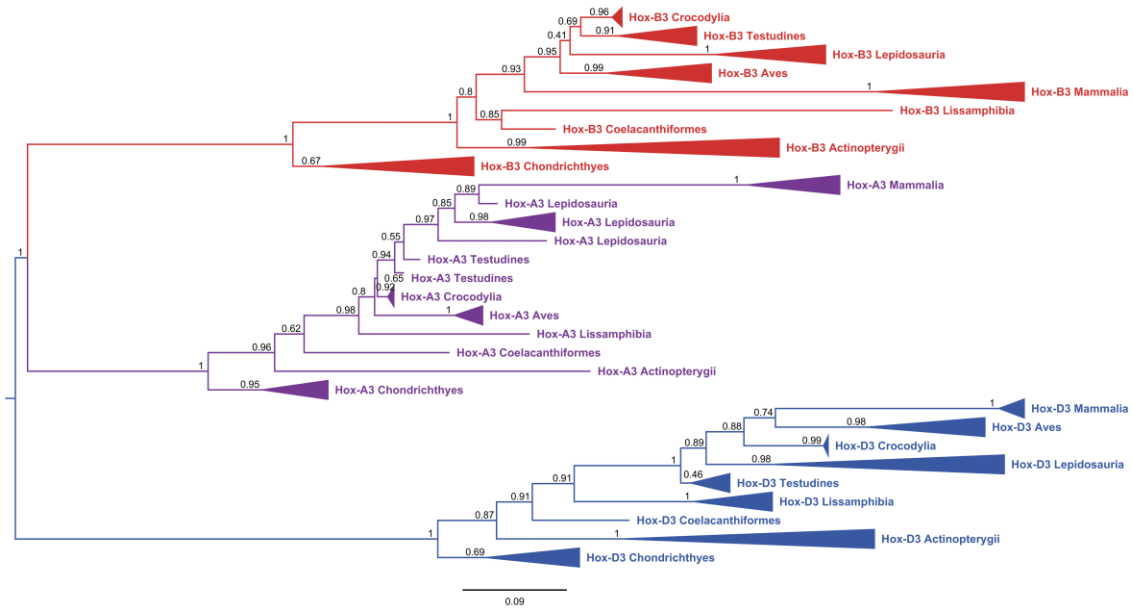

Figure 3. Hox3 phylogeny, in purple have the Hox-A3 and in red Hox-B3 and in blue Hox-D3. The support values are represented by Shimodaira-Hasegawa-like.

The tree generated from *Deformed* successfully divided Protostomes into Ecdysozoa and Platytrochozoa (Platyhelmintes + Lophotrochozoa), supporting previous findings [23] (Figure 4). The *HoxC4* gene uncovered a topology in which Coelacanthiformes is a sister taxon of Amniota and such an incongruous result may be due to poor annotation and/or low support values. Lissamphibia sequences closer to Actinopterygii but also not well supported and Mammalia sibling groups of Lepidosauria. The group of *HoxB4* the sequence of Coelacanthiformes, as a Diapsida sibling group.

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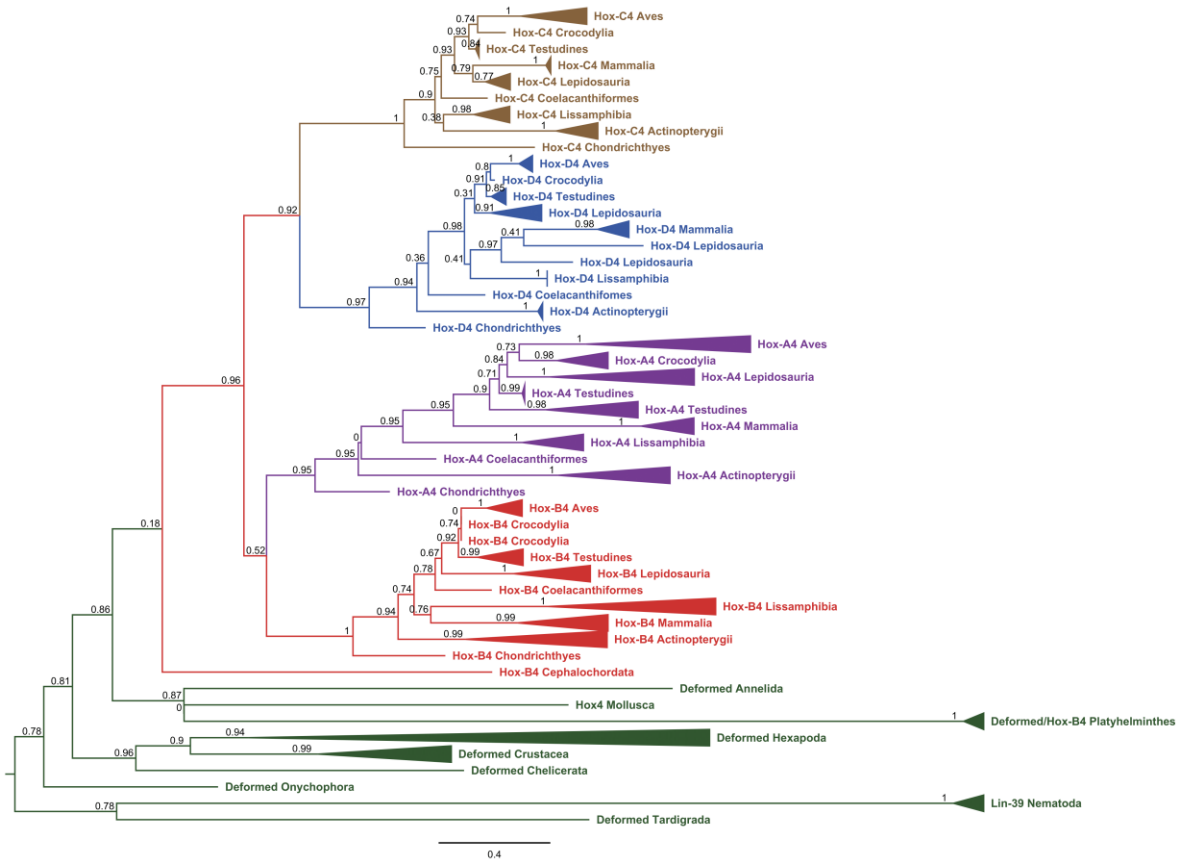

Figure 4. Hox4 phylogeny in green we have the Deformed, in purple have the Hox-A4, in red Hox-B2, inblue Hox-D1 and in brown Hox-C4. The support values are represented by Shimodaira-Hasegawa-like.

Anterior *Hox* genes evolution as part of a multigene family

Hox genes are part of a multigene family, and as such, their inclusion in phylogenetic studies should also regard potential relationships between gene copies within the same genome. By analyzing paralogs for each gene aligned altogether (*A*, *B*, *C*, *D)* it is possible to infer the relative time of gene duplication and the evolutionary dynamics shaping the multigene family. For example, observation that gene clusters are shared by many clades suggests that the duplication event took place in ancient times, before diversification of more recent groups. Moreover, the resulting tree topologies and relationship among gene copies can provide information to distinguish between three alternative scenarios for evolution of multigene families [12]: i) divergent evolution, where genes are phylogenetically related and have diverged gradually as the duplicated genes have gone through neofunctionalization; ii)

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concerted evolution, where all members of a gene family are assumed to evolve in a concerted manner (not independently), such that sequences from all copies are roughly homogeneous (*i.e.* a mutation occurring in a gene copy spreads to all gene copies in the family) by unequal crossover or gene conversion; iii) birth-and-death evolution, where new genes are created by gene duplication, and some duplicated genes are maintained in the genome for a long time, whereas others are lost either by selection (for a compact genome or to regulate gene dosage) or pseudogenization (accumulation of deleterious mutations under relaxed selection) [12].

Orthologue search of *Hox* genes show that most vertebrate groups carry more than one cluster, whereas protostomes and lower deuterostomes (Echinodermata and Cephalochordata) carry only one. Namely, vertebrates harbor three copies of *Hox1* (Figure 1), two copies of *Hox2* (Figure 2), three copies of *Hox3* (A, B and D; Figure 3), and four copies of *Hox4* (A, B, C and D; Figure 4). Because all known vertebrate species share the same gene content in each cluster (ie.:all four anterior genes in clusters A and B, only *Hox*-4 in cluster C and all genes but *Hox2* in cluster D), the establishment of gene content in the four *Hox* clusters seem to have emerged after Urochordata and before cyclostomes (hagfish and lampreys) [40]. For all *Hox*-based phylogenies, each gene copy is closer to orthologues in other species than to paralogs within the same genome (Figures 1-4). For instance, Avian *HoxA1* protein sequences share more similarities to Crocodilian *Hox A1* than to Avian *Hox B1* or *D1* (Figure 1). Moreover, in cases where more than two clusters are present in vertebrate species, sequences from clusters A and B are more closely related to each other than sequences from clusters C and/or D, and vice-versa. These new and interesting findings suggest a tree topology in which clusters A and B are more closely related to each other, as well as C and D are to each other (Figure 5) and corroborates with the first scenario of vertebrate Hox evolution proposed by Kappen et al. [13] and with the work of Ravi et al. [14] but our work have more date since we try to represent we try to represent the largest amount of vertebrate taxa.

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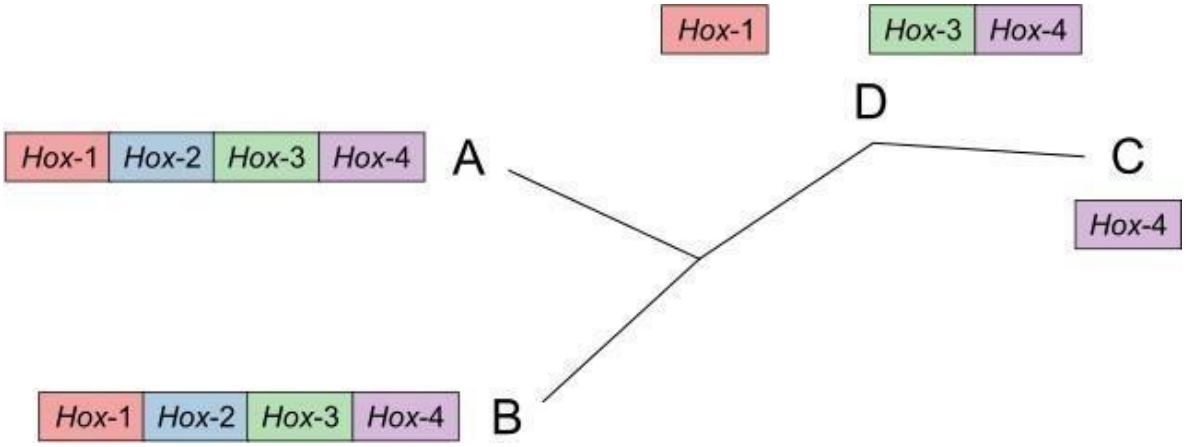

Figure 5. Inferred relationships between vertebrate Hox clusters. Unrooted tree topology was inferred from gene content in each of the four vertebrate clades.

Among the three models for multigene family evolution, our results are more consistent to the birth-and-death model, characterized by a fast gene turnover across groups, and consistent with previous work of Holland [41]. However, the pattern of gene gain/loss in *Hox* gene clusters appears to be restricted by their developmental roles and relationships with other genes associated with the establishment of each bauplan [42]. Thus, because evolutionary rates of *Hox* genes seem to be dependent and constrained by evolutionary rates of other developmental genes, we name this process a 'birth-and-death model constrained by development'.

Conclusion

Our data lead us to re-evaluate the direct relationship between the coordinated expression of the *Hox* genes in the development of the animals' body plan and their relationship with the evolutionary history of large groups. Although we observe that in several evolutionary scenarios for the *Hox* genes we find an overlap between the evolutionary history of the genes and the animal body plan, several scenarios also show a conflicting evolutionary history. The conflicts observed between the evolutionary history of some genes and the history of large groups may have occurred due to specific and similar selective pressures in relatively distant groups, which may have led to evolutionary convergences. However, we can observe that although there is no collinearity between the evolutionary history of large groups and some groups of *Hox* genes, the behavior of *Hox* genes as a multigenic family obey a pattern, where the diversity found must be in line with the

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development of the body plan as a whole. Thus, we propose that we can understand the evolutionary history of the family of *Hox* genes following an evolutionary history of the type of birth-and-death model constrained by development.

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Author contributions

V.M.M. ran the evolutionary analyses. V.M.M., G.H.C.V. and S.T.F. conceived the project and analyses. P.E.T.S. contributed to data processing & bioinformatics analyses, V.M.M., J.L.O., and S.T.F. analyzed the results and wrote the paper.

Competing interests

The authors declare no competing interests.

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