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The rapid variation of *Hox* clusters reveals a clear evolutionary path in a crucian carp-like homodiploid fish lineage

Shi Wang [a,b,1], Qilong Liu [a,b,1], Xuexue Huang [a,b,1], Conghui Yang [a,b], Lan Chen [a,b], Mei Han [a,b], Yuqin Shu [a,b], Min Wang [a,b], Wuhui Li [a,b], Fangzhou Hu [a,b], Ming Wen [a,b], Kaikun Luo [a,b], Yude Wang [a,b], Rong Zhou [a,b], Chun Zhang [a,b], Min Tao [a,b], Rurong Zhao [a,b], Chenchen Tang [a,b], Shaojun Liu [a,b,*]

[a] State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University, Changsha, 410081, Hunan, PR China
[b] College of Life Sciences, Hunan Normal University, Changsha, 410081, Hunan, PR China

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ABSTRACT

The surprising variation in the number of *Hox* clusters and genome structure in ray-finned fish lineages reflects the history of duplications and subsequent lineage-specific gene loss. However, there are few studies on whether *Hox* clusters in the early generations of hybrid lineages show more significant variation due to the continuous genomic oscillation caused by distant hybridization. We sequenced and analyzed *Hox* gene clusters from a crucian carp-like homodiploid fish (NCRC) lineage (a new hybrid lineage derived from common carp (*Cyprinus carpio*) (♀) × blunt snout bream (*Megalobrama amblycephala*) (♂)). In the NCRC lineage, we reconstructed seven *Hox* clusters consisting of 48 *Hox* genes, ten of which were pseudogenes. The number of putative *Hox* clusters generated in NCRC-F_1 was increased greatly by distant hybridization to an average number almost twice that in the maternal parent. This increasing trend continued in the subsequent self-mating generations of NCRC-F_1. In contrast, the number of *Hox* cluster fragments inherited from the original parents gradually decreased as the number of NCRC lineage generations increased. This pattern was also found in the inheritance of recombinant *Hox* clusters. In terms of base composition, some genetic rules for the inheritance of these *Hox* clusters between different generations of the NCRC lineage were identified. Furthermore, the newly derived mutated *Hox* clusters in the NCRC lineage showed phylogenetic relationships that were closer to either crucian carp or silver crucian carp, revealing a clear evolutionary path. This study deepens our understanding of the evolution of *Hox* genes in the ray-finned fish clade.

1. Introduction

Duplications of genes and entire genomes are considered to be important genetic mechanisms giving rise to morphological variation and functional innovation [1–3]. A large number of comparative genomic studies have confirmed the hypothesis that gnathostomes have undergone two rounds of genome duplication (2R) [4–6]. A third round (3R) of genome duplication, the so-called "fish-specific genome duplication" (FSGD), occurred in the ancestral lineage of teleost fishes approximately 320 mya [3–5,7–10]. More than 32,700 fish species have been identified in nature (http://fishdb.sinica.edu.tw/AjaxTree/tree.php), which is greater than the total number of extant species in other vertebrate groups. Teleost fishes are the most abundant aquatic vertebrates living today, with more than 30,000 named species [11], accounting for more than 95% of all extant fishes [12]. Teleost fishes possess chromosomes that display flexibility and exhibit remarkable variation in terms of morphological, behavioral, and physiological adaptations [13–19]. Several authors have suggested that the FSGD is at least partially responsible for the species diversity of teleost fishes [10,20,21].

Hox genes encode homeodomain-containing transcription factors that perform functions essential for the development of various morphological features. *Hox* genes are typically considered to be under strong evolutionary constraints because large changes in body plan are generally detrimental to survival. Nevertheless, a great diversity of body plans

* Corresponding author. State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University, Changsha 410081, Hunan, PR China.
E-mail address: China.lsj@hunnu.edu.cn (S. Liu).
[1] These authors contributed equally to this work.

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exists in nature, and many of the mechanisms underlying this diversity have been attributed to changes in *Hox* genes [22]. Thus, *Hox* genes are of particular interest for understanding the genetic basis of the morphologic diversity of metazoans. While each *Hox* cluster contains the same genes in different mammalian species, the situation is not the same in the extant species of ray-finned fishes, in which both the numbers and organization of *Hox* genes and even *Hox* clusters are variable [23–28]. Most teleost fishes exhibit seven *Hox* clusters owing to the FSGD event in the ray-finned fish lineage, followed by the loss of one duplicate cluster. For example, a duplicate *HoxD* (*HoxDb*) cluster has been lost in zebrafish, and acanthopterygians such as medaka, fugu, and cichlids have lost a duplicate *HoxC* (*HoxCb*) cluster. In addition, some *Hox* genes have experienced lineage-specific secondary losses, resulting in each of these groups of teleost fishes possessing a unique set of *Hox* genes [7,29,30]. Changes in the number and genomic organization of *Hox* genes are important for the evolution of the metazoan animal body plan. It has been hypothesized that genome duplication events have contributed to the extensive radiation of ray-finned fishes [7]. The fact that different types of fish possess different sets of *Hox* genes makes these gene sets the most suitable system for understanding the mechanisms underlying the unequal conservation of duplicated copies [31]. Such highly variable *Hox* gene clusters provide a good starting point for the study of genetic evolution at the genomic level resulting from post-FSGD events.

Hybridization would then be a catalyst not only for speciation but also for major evolutionary innovations [32]. Unlike mutations, hybridization provides an effective means of providing genetic variation in hundreds or thousands of genes in a single generation. Hybridization may accelerate speciation via adaptive introgression or cause near-instantaneous speciation [33]. This near-instantaneous hybrid speciation is accompanied by rapid genomic changes, including chromosomal rearrangements, genome expansion, differential gene expression, and gene silencing [34]. Moreover, rapid genomic changes caused by hybridization provide a practically instantaneous mechanism for recombining the adaptive traits of two species and generating novel phenotypes [35]. *Hox* genes perform functions essential for the development of various morphological features. Whether hybridization promotes the instantaneous evolution of *Hox* gene clusters is a biological issue that still needs to be further explored. As mentioned above, the FSGD is at least partially responsible for the species diversity of teleost fishes. Because of the FSGD, the frequency of genome duplications in fish is higher than that in other vertebrates [36]. Hybridization plays an important role as a powerful promoter of evolutionary adaptation at the level of genome duplications. However, the short-term impact of genome duplications is still not well understood.

In our previous study, we reported the spontaneous occurrence of a crucian carp-like homodiploid fish (2n = 100, abbreviated as NCRC) that originated from a cross of common carp (*Cyprinus carpio*, Cyprininae, 2n = 100, abbreviated as COC) (♀) × blunt snout bream (*Megalobrama amblycephala*, Cultrinae, 2n = 48, abbreviated as BSB) (♂) [37]. Through continuous self-crossing passage, we successfully obtained a fertile NCRC lineage (F_1–F_7). The phenotypes and genotypes (determined by fluorescence *in situ* hybridization and 5S rDNA) of NCRC differ from those of its parents but are closely related to those of existing wild crucian carp [37]. Moreover, the mitochondrial DNA organization and nucleotide composition of NCRC are more similar to those of existing wild crucian carp than those of the parents. Specifically, we first revealed the instability of the mitochondrial DNA of F_1 of NCRC resulting from distant hybridization but eventually established a relatively genetically stable hybrid fish lineage (F_1–F_3) [34]. To further explore genetic evolution at the genomic level in the early stages of the formation of the NCRC lineage, we isolated and sequenced *Hox* genes in different generations (F_1, F_2, and F_5) of the NCRC lineage. It was shown that each generation of NCRC possesses a different set of *Hox* genes, making these fish genes a highly suitable system for understanding the mechanisms underlying such unequal conservation of duplicated copies.

2. Materials and methods

2.1. Ethics statement

The guidelines established by the Administration of Affairs Concerning Animal Experimentation state that approval from the Science and Technology Bureau of China and the Department of Wildlife Administration is not necessary when the fish in question are neither rare nor near extinction (first- or second-class state protection level). Therefore, approval was not required for the experiments conducted in this study.

2.2. Animals and crossing procedure

The natural materials, including COC (2n = 100) and BSB (2n = 48), and the hybrid materials, NCRC lineage (F_1, F_2, and F_5; 2n = 100) from COC (♀) × BSB (♂) were fed in a pool with suitable illumination, water temperatures, dissolved oxygen contents, and forage at the Center for Polyploidy Fish Genetics Breeding of Hunan Province, located at Hunan Normal University, Changsha, Hunan, China. The protocols for crossing and culturing were described previously [37]. All fish were deeply anaesthetized with 100 mg/L MS-222 (Sigma-Aldrich, St. Louis, MO, USA) prior to dissection.

2.3. DNA extraction, amplification and sequencing of Hox genes

Total genomic DNA extracted from the peripheral blood cells of three COC, three BSB, three NCRC-F_1, three NCRC-F_2, and three NCRC-F_5 by routine approaches was used as the template, respectively. Degenerate primers for the amplification of *Hox* genes included the posterior *Hox* forward primers for paralogous groups 9–13 [CGAAAGAAG(C/A) G(N/C)GT(N/C)CC(N/C)TA(T/C)AC], the anterior *Hox* forward primer for paralogous groups 1–9 [GAATTCCACTTCAAC(C/A)(G/A)(C/G)TACCT], and the universal reverse primer [CATCCTGCGGTTTTGGAACCANAT], as described by Amores et al. [26]. PCR was performed in a total volume of 50 μL with approximately 10–30 ng of genomic DNA, 1.5 mM $MgCl_2$, 250 μM dNTPs, each primer at 0.4 μM, and 1.25 U of Taq polymerase (TaKaRa, Dalian, China). The thermal program consisted of an initial denaturation step of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 50–60 °C for 30 s, and 72 °C for 1–3 min, with a final extension step of 72 °C for 7 min. A majority of the PCR products were directly sequenced, and some fragments that were difficult to sequence using the PCR products were cloned into the pMD18-T vector (TaKaRa, Dalian, China). The plasmids were transformed into *E. coli* DH5a cells and purified. To increase the probability of detecting duplicated paralogs and to circumvent errors owing to PCR, 24 clones of each gene from each sample were sequenced with vector-specific primers using the primer walking method on an ABI 3730XL automatic sequencer (ABI PRISM 3730, Applied Biosystems, CA, USA). The obtained sequences were screened for *Hox* gene fragments using BLAST (http://www.ncbi.nlm.nih.gov) searches, and the ClustalW (http://www.ebi.ac.uk/) and MEGA 4.0 programs were used to determine identity. Furthermore, based on the Poisson distribution, alleles and duplicated *Hox* clusters were distinguished according to the methods of Misof and Wagner [38] and Moghadam et al. [39]. The recombinant *Hox* clusters were identified according to the methods of Liu et al. [40].

2.4. Phylogenetic analysis

To further understand the similarity of *Hox* gene sequences between the NCRC lineage and crucian carp, we downloaded *Hox* gene sequences related to crucian carp and silver crucian carp from the NCBI website (https://www.ncbi.nlm.nih.gov/) to further analyze their phylogenetic relationships at the genomic DNA level. The conserved regions of derived amino acid sequences were aligned by using Clustal X 1.81 [41]. Regions of sequences that were difficult to align were removed from the alignment. Gaps were also removed from the alignment. After alignment, we

selected the conserved regions of the amino acid sequences of 17 *Hox* genes for phylogenetic tree analysis. The phylogenetic tree was inferred by using the maximum likelihood method based on the Tamura-Nei model [42]. The tree with the highest log likelihood (−2982.70) is shown. The maximum composite likelihood (MCL) approach was used to estimate a matrix of pairwise distances, the initial trees for the heuristic search were obtained automatically by applying the neighbor-join and BioNJ algorithms, and the topology with the superior log likelihood value was then selected. The tree was drawn to scale, with branch lengths proportional to the number of substitutions per site. The analysis involved 238 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 [43]. The final trees were visualized in FIGTREE 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/. 2018).

2.5. Base composition analyses

We calculated the guanine-cytosine (GC) percentage in the total coding regions and the GC percentage at the third position of the gene codons. Then, further analyses were carried out by using the program CodonW available on the website http://bioweb.pasteur.fr/seqanal/interfaces/codonw.html.

3. Results

3.1. Identification and reconstruction of crucian carp-like homodiploid fish lineage Hox clusters

PCR amplification of genomic DNA of three COC, three BSB, three

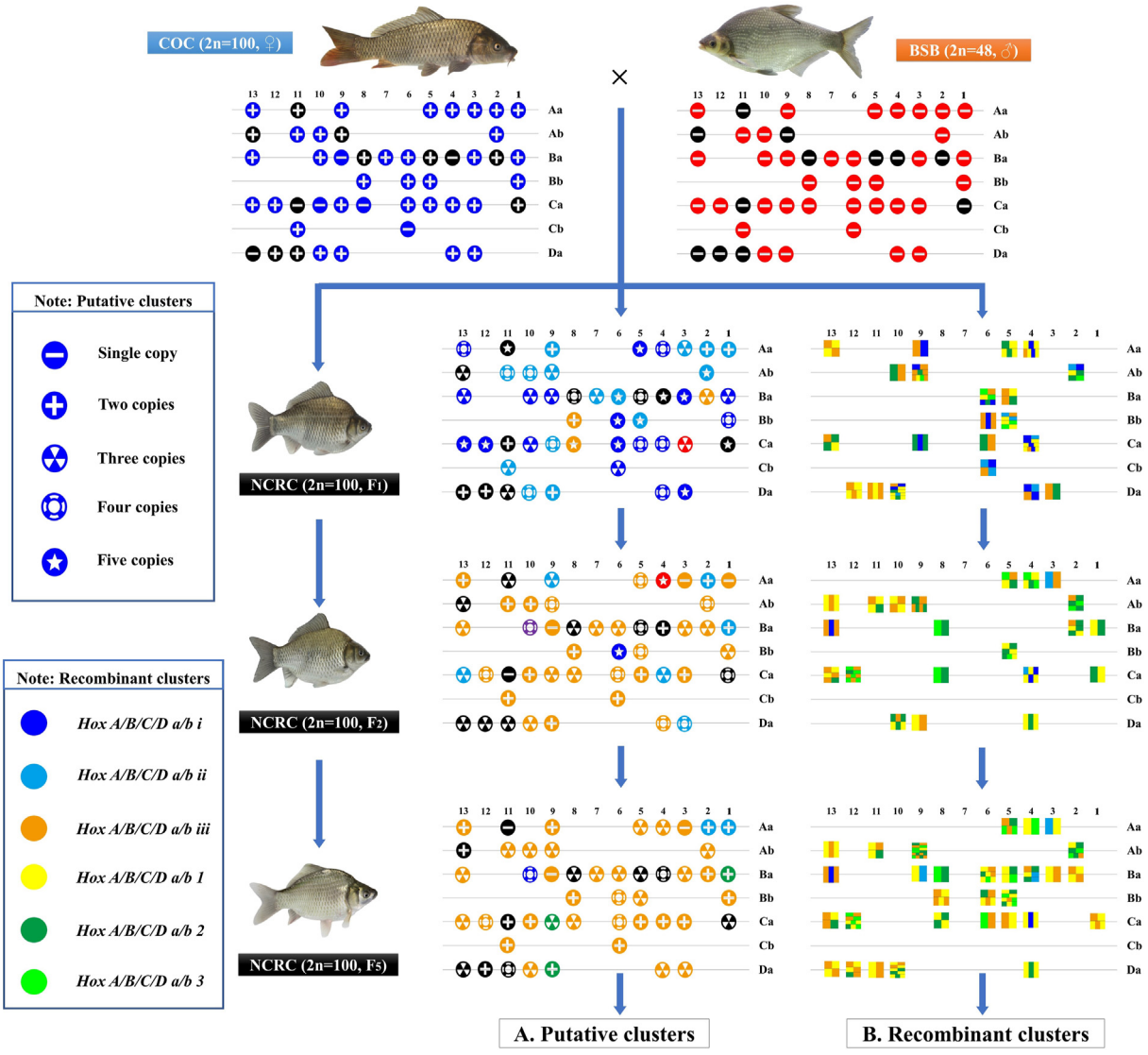

Fig. 1. The crossing procedure, the appearance of COC, BSB, NCRC-F_1, NCRC-F_2, and NCRC-F_5, and the genomic organization of the *Hox* clusters of these species. A. Putative clusters. Each thick horizontal line represents a *Hox* cluster. Each circle represents a gene. Each graph represents the number of copies. Black circles represent pseudogenes. In NCRC (F_1, F_2, and F_5), blue circles denote the genes with two types of *Hox* cluster structures derived from COC and other newly derived cluster structures; light blue circles denote the genes with one type of *Hox* cluster structure derived from COC and other newly derived cluster structures; red circles denote the genes with one type of *Hox* cluster structure derived from BSB and other newly derived cluster structures; purple circles denote the genes with one type of *Hox* cluster structure derived from COC, one type of *Hox* cluster structure derived from BSB, and other newly derived cluster structures; orange circles denote the genes with only newly derived *Hox* cluster structures; green circles indicate that from an existing *Hox* cluster structure in NCRC-F_2, a newly derived *Hox* cluster structure is formed in NCRC-F_5. **B. Recombinant clusters.** Each square represents a gene. Pseudogenes are not marked in panel B. Each color represents a fragment of the *Hox* cluster structure. The composition of modules on the same horizontal line represents a recombinant *Hox* cluster structure. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

NCRC-F_1, three NCRC-F_2, and three NCRC-F_5 individuals using degenerate *Hox* gene-specific primers yielded 60 different homeobox sequences, which were classified into 48 *Hox* and 12 non-*Hox* genes. The 48 *Hox* genes were distributed among seven clusters named *HoxAa*, *Ab*, *Ba*, *Bb*, *Ca*, *Cb*, and *Da* (Fig. 1). To consider the complex patterns between *Hox* clusters, only sequences aligned unambiguously were included in our analyses, whereas indels in sequences were excluded. To avoid the biased amplification of only one copy of the characterized *Hox* genes, 24 clones were sequenced so that 2–24 replicate sequences of each locus were obtained for each detected gene. Based on the full-length nucleotide and deduced amino acid sequences, each gene was identified by BLAST searches in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/).

The organization of the *Hox* clusters in COC, BSB, NCRC-F_1, NCRC-F_2, and NCRC-F_5 is presented in Supplementary Tables 1-2. We obtained partial sequence information for 89 putative *Hox* clusters from COC, 48 putative *Hox* clusters from BSB, 176 putative and 45 recombinant *Hox* clusters from NCRC-F_1, 138 putative and 39 recombinant *Hox* clusters from NCRC-F_2, and 126 putative and 63 recombinant *Hox* clusters from NCRC-F_5 (Fig. 2). In addition to *HoxB4a*, *HoxB9a*, *HoxC6b*, *HoxC8a*, *HoxC10a*, *HoxC11a*, and *HoxD13a*, there were two duplicates of each *Hox* gene in COC. There was only one duplicate of these 48 *Hox* genes in BSB. Moreover, the number of putative *Hox* clusters (except for recombinant *Hox* clusters) generated in NCRC-F_1 was greatly increased by distant hybridization, resulting in 2–5 duplicates of each *Hox* gene and approximately twice the average number of putative *Hox* clusters found in COC ($P > 0.05$) (Fig. 1). For example, NCRC-F_1 had five putative clusters of *HoxA5a*, *HoxA2b*, *HoxB3a*, *HoxB6a*, *HoxB5b*, *HoxB6b*, *HoxC6a*, *HoxC12a*, and *HoxD3a* (Fig. 1 and Supplementary Table 1). This explosive growth trend in the number of putative *Hox* clusters (except for recombinant *Hox* clusters) relative to that in the parents extended into the subsequent self-mating generations of NCRC-F_1. For instance, NCRC-F_2 had five putative clusters of *HoxB6b* and four putative clusters of *HoxA5a*, *HoxA2b*, *HoxB5b*, *HoxC6a*, *HoxC12a*, and *HoxD3a* (Fig. 1 and Supplementary Table 1); NCRC-F_5 had four putative clusters of *HoxB6b* and three putative clusters of *HoxA5a*, *HoxA2b*, *HoxB5b*, and *HoxD3a* (Fig. 1 and Supplementary Table 1).

The most important finding of this study was recombinant *Hox* gene clusters, which may be an inevitable result of the hybridization process of cyprinid fishes or even vertebrates [18,19,44–46]. In the different generations of NCRC, the number of recombinant *Hox* clusters showed a trend of first declining and then increasing (Fig. 2). For instance, *HoxD10a* showed five recombinant *Hox* clusters in NCRC-F_1, two recombinant *Hox* clusters in NCRC-F_2, and five recombinant *Hox* clusters in NCRC-F_5 (Fig. 1 and Supplementary Table 2). In addition, some recombinant *Hox* gene clusters did not appear in NCRC-F_2 and appeared only in NCRC-F_1 and NCRC-F_5, such as clusters of *HoxB5a*, *HoxB6a*, *HoxB6b*, *HoxC6a*, *HoxD11a*, and *HoxD12a* (Fig. 1 and Supplementary Table 2). Furthermore, the recombinant cluster types of six *Hox* genes, namely, including *HoxB3a*, *HoxB4a*, *HoxB8b*, *HoxB9a*, *HoxC5a*, and *HoxD13a*, appeared only in NCRC-F_5 (Fig. 1 and Supplementary Table 2).

3.2. Genetic variation analyses

Fig. 1 illustrates the genetic variation of *Hox* gene clusters in these fish samples in an intuitive manner. By comparing and analyzing the genetic variation of the 48 *Hox* genes of NCRC-F_1, it was found that 10 *Hox* genes had become pseudogenes, 19 *Hox* genes had inherited the complete cluster structures of the maternal parent (COC), 15 *Hox* genes had inherited one of the cluster structures of the maternal parent, one *Hox* gene had inherited the complete cluster structures of the paternal parent (BSB), and all of the cluster structures of the other three *Hox* genes were mutant types (Fig. 1 and Supplementary Table 1). On the basis of inheriting the *Hox* gene cluster structures of the parents, these 48 *Hox* genes of NCRC-F_1 had newly derived mutant cluster structures, which showed greater variability relative to those of the parents. Moreover, in both the COC and BSB parents, *HoxA9b* and *HoxB2a* were pseudogenes, while the newly derived cluster structures of these two genes in NCRC-F_1 were unexpectedly complete and were not pseudogene structures (Fig. 1 and Supplementary Table 1). This interesting phenomenon continued in the self-crossing offspring of NCRC-F_1. To maintain the balance between the internal stability of the species and the continuous concussion

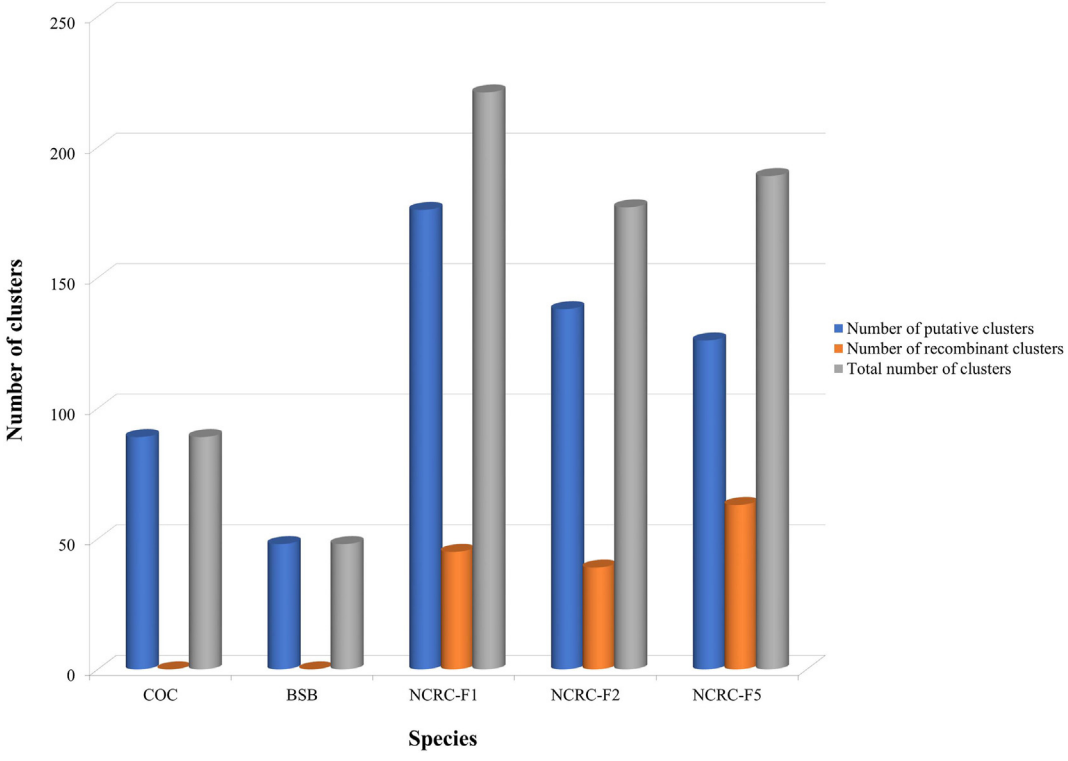

Fig. 2. Statistics of the numbers of *Hox* clusters in COC, BSB, NCRC-F_1, NCRC-F_2, and NCRC-F_5.

impacts of different parental genomes, most of the *Hox* gene clusters in NCRC-F_1 were not stably inherited in NCRC-F_2. In NCRC-F_2, these cluster structures from the original COC and BSB parents were largely lost; the complete cluster structure types of the original maternal parent, COC, were retained only in *HoxB6b*, and one of the cluster structure types of the original maternal parent was retained in the seven *Hox* genes *HoxA2a*, *HoxA9a*, *HoxB1a*, *HoxB10a*, *HoxC4a*, *HoxC13a*, and *HoxD3a* (Fig. 1 and Supplementary Table 1). Accompanying the continuous concussion impacts from different parental genomes, the cluster structure type derived from the original paternal parent, BSB, was found in two of the *Hox* genes (*HoxA4a* and *HoxB10a*) of NCRC-F_2 (Fig. 1 and Supplementary Table 1). In NCRC-F_2, in addition to the presence of 10 *Hox* pseudogenes (similar to the situation in NCRC-F_1), mutation was the main theme of the generation, in which new mutation types arose in 29 (60.42%) *Hox* genes, and greater variability was observed relative to that in both NCRC-F_1 and the original parents. With the continuous self-crossing within NCRC, the internal stability mechanism of this species gradually began to play an increasingly prominent role. Thus, the variability within NCRC-F_5 was not as great as that in the preceding generations; 19 *Hox* genes showed cluster structure types consistent with those of NCRC-F_2, and only three *Hox* genes, *HoxB1a*, *HoxC9a*, and *HoxD9a*, developed new variant types in relation to the cluster structure types of NCRC-F_2 (Fig. 1 and Supplementary Table 1). In NCRC-F_5, the cluster structures of the original parents, COC and BSB, were further lost; the complete cluster structure types of the original maternal parent, COC, were retained only in *HoxB10a*, one of the cluster structure types of the original maternal parent was retained only in the two *Hox* genes *HoxA1a* and *HoxA2a*, and the cluster structure types of the original paternal parent, BSB, were all lost (Fig. 1 and Supplementary Table 1). Furthermore, two *Hox* genes, *HoxA2a* and *HoxB8b*, showed the same cluster structure types in NCRC-F_1, NCRC-F_2, and NCRC-F_5, and they were more conserved than other *Hox* genes in the NCRC population (Supplementary Table 1). In addition, since pseudogenes are obviously not subject to any functional constraints, all mutations within them are selectively neutral and show an equal probability of becoming fixed in the population [31]. In this study, we found that the cluster structure types of six *Hox* pseudogenes, *HoxA11a*, *HoxA13b*, *HoxB4a*, *HoxB8a*, *HoxC11a*, and *HoxD11a*, showed flexible genetic variability in different generations of the NCRC population (Supplementary Table 1).

Among the recombinant *Hox* gene clusters observed between different generations of NCRC, some recombinant cluster types produced by 12 *Hox* genes could be inherited by the next generation. For example, type *HoxA11b-1* + *HoxA11b-2* in *HoxA11b* could be passed from NCRC-F_2 to NCRC-F_5; and type *HoxB13aiii* + *HoxB13ai* + *HoxB13aiii* in *HoxB13a* could be passed from NCRC-F_2 to NCRC-F_5 (Fig. 1 and Supplementary Table 2). Furthermore, some recombinant cluster types produced by four *Hox* genes could be stably inherited from NCRC-F_1 to NCRC-F_5, such as type *HoxA2b-2* + *HoxA2b-3* in *HoxA2b*, which could be passed from NCRC-F_1 to NCRC-F_5, and type *HoxD10a-1* + *HoxD10a-2* + *HoxD10a-1* in *HoxD10a*, which could be passed from NCRC-F_1 to NCRC-F_5 (Fig. 1 and Supplementary Table 2). Similar to the observed genetic variation of putative *Hox* cluster structures, while the different generations of the NCRC lineage stably inherited the partial recombinant cluster types of *Hox* genes, large mutation events were also taking place. As the number of generations increased, the number of genetic fragments derived from the original parents gradually decreased (Fig. 1 and Supplementary Table 2).

3.3. Base composition analyses

We calculated the GC levels in *Hox* gene coding sequences (CDS) in COC, BSB, NCRC-F_1, NCRC-F_2, and NCRC-F_5 (Supplementary Table 3). Some genetic rules governing GC levels in the inheritance of these *Hox* gene clusters were observed between different generations of the NCRC lineage. The *Hox* gene cluster types of the maternal parent, COC, with higher GC levels were more likely to be inherited in NCRC-F_1 and could even be stably inherited in the subsequent self-crossing offspring of F_1. These cluster types were designated *HoxA1ai*, *HoxA2aii*, *HoxB10ai*, and *HoxD9ai* (Supplementary Table 3). Some *Hox* gene cluster types of the maternal parent, COC, could be inherited from NCRC-F_1 to NCRC-F_2, but their GC levels were lower than those of the variant types and could not be stably inherited by the subsequent self-crossing progeny. These cluster types were designated *HoxA9ai*, *HoxB6bi*, *HoxB6bii*, *HoxC13ai*, and *HoxD3aii* (Supplementary Table 3). The variant types with higher GC levels in the *Hox* gene clusters in NCRC-F_1 and those with increasing GC contents in subsequent self-crossing offspring were more likely to be stably inherited in the NCRC lineage. There were 46 variant types that conformed to this rule, such as *HoxA2b-3*, *HoxA4a-1*, *HoxA5aiii*, *HoxB1biii*, *HoxB3a-1*, *HoxB6aiii*, *HoxC4a-1*, *HoxC5aiii*, *HoxC12aiii*, *HoxD3aiii*, *HoxD4aiii*, and *HoxD10aiii*, accounting for 54.12% of all variant types (except for pseudogene and *HoxC3a* cluster types); these variant types were distributed among 27 *Hox* genes, excluding pseudogenes and *HoxC3a* (in which only intron sequences were amplified), accounting for 77.14% of all *Hox* genes (Supplementary Table 3). In the genome of the NCRC lineage, along with the influence of the continuous oscillation of the genomes from different parents, the newly derived variant types with higher GC levels in the *Hox* gene clusters from the self-crossing offspring (F_2) of NCRC-F_1 could be stably inherited by generations up to F_5, as observed for *HoxA4a-2*, *HoxA5a-3*, *HoxA9a-1*, *HoxB7a-2*, *HoxB9a-1*, *HoxB10a-1*, *HoxC6a-3*, *HoxC12a-3*, and *HoxD4a-2* (Supplementary Table 3). The above genetic rules governing *Hox* gene clusters are not applicable to the pseudogenes identified in this study. However, there were also special cases of the inheritance of these pseudogene cluster types. For example, *HoxB2aiii*, which was a newly derived variant cluster type with normal functional structure in the NCRC lineage, could be stably inherited in subsequent self-crossing offspring because of the higher GC level in its CDS region (Supplementary Table 3).

3.4. Phylogenetic analyses

To understand the cluster affiliation and orthology of the *Hox* genes of COC, BSB, NCRC-F_1, NCRC-F_2, NCRC-F_5, crucian carp (*Carassius auratus*), silver crucian carp (*Carassius auratus gibelio*), and zebrafish (*Danio rerio*), we generated a phylogenetic tree based on the alignments of the conserved regions of the derived amino acid sequences encoded by the *Hox* gene family (Fig. 3). This phylogenetic tree was generated for conserved regions by using the *Hox* sequences from zebrafish as an outgroup. The overall phylogenetic tree was divided into 17 well-conserved clades. In our previous study, we revealed that the phenotypes and genotypes of the NCRC lineage differed from those of its parents but were closely related to those of existing wild crucian carp [34, 37]. In this study, to further understand the similarity of *Hox* gene sequences between the NCRC lineage and crucian carp, we downloaded *Hox* gene sequences related to crucian carp and silver crucian carp from the NCBI website to further analyze their phylogenetic relationships at the genomic DNA level (Supplementary Table 4). As shown in Fig. 3, the newly derived *Hox* gene mutation clusters in the NCRC lineage showed phylogenetic relationships that were closest to either crucian carp or silver crucian carp. These *Hox* mutation cluster types clustered first with crucian carp or silver crucian carp and then with the parental cluster types (refer to *HoxA2b*, *HoxA4a*, *HoxD4a*, *HoxA9a*, and *HoxA13b* in the figure for details) (Fig. 3). We also analyzed the percent nucleotide identity and the percent amino acid identity between duplicated *Hox* coding regions in COC, BSB, NCRC-F_1, NCRC-F_2, and NCRC-F_5 (Supplementary Table 5). To evaluate the speciation of the NCRC lineage, the percentages of nucleotide (amino acid) identity among the 48 *Hox* gene groups in COC, BSB, NCRC-F_1, NCRC-F_2, and NCRC-F_5 were determined (Supplementary Table 5). The identities of the orthologous *Hox* genes between the NCRC lineage and COC were much higher than those between NCRC and BSB, except for those of the gene clusters inherited from BSB. Among these 48 *Hox* genes (except for *HoxC3a* and 12 pseudogenes), both the nucleotide and amino acid sequences of 12 (34.29%)

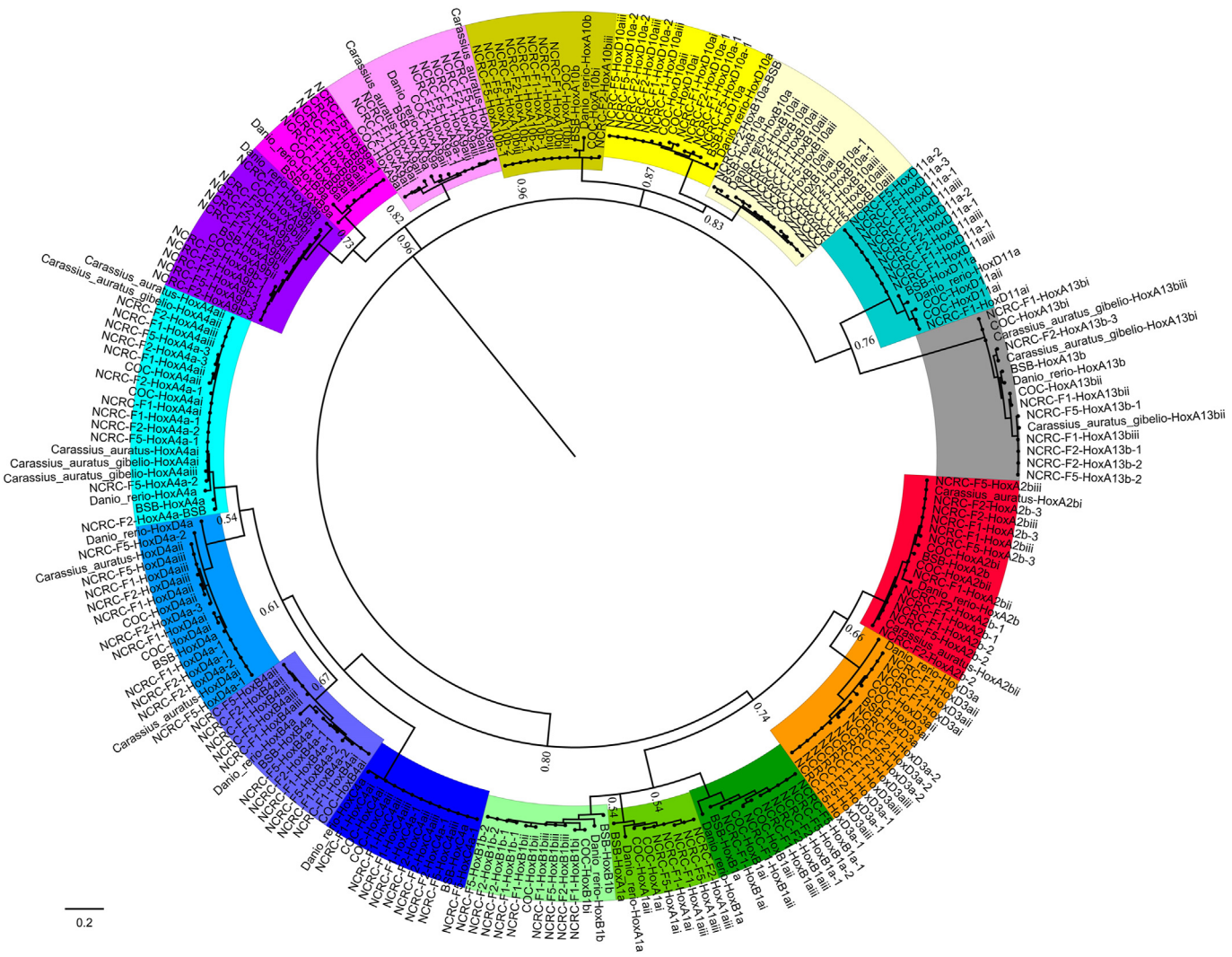

Fig. 3. Phylogenetic analyses of the conserved regions of amino acid sequences of 17 selected *Hox* genes (*HoxA2b*, *HoxD3a*, *HoxB1a*, *HoxA1a*, *HoxB1b*, *HoxC4a*, *HoxB4a*, *HoxD4a*, *HoxA4a*, *HoxA9b*, *HoxB9a*, *HoxA9a*, *HoxA10b*, *HoxD10a*, *HoxB10a*, *HoxD11a*, and *HoxA13b*) in COC, BSB, NCRC-F_1, NCRC-F_2, NCRC-F_5, crucian carp (*Carassius auratus*), silver crucian carp (*Carassius auratus gibelio*), and zebrafish (*Danio rerio*). Phylogenetic tree constructed using the maximum likelihood method based on the Tamura-Nei model [42]. The phylogenetic tree for each *Hox* gene is indicated by a different color, as shown in the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Hox genes (such as *HoxA3a*, *HoxB7a*, *HoxC9a*, and *HoxC13a*) in the NCRC lineage showed a high degree of identity with those in COC and BSB. In contrast, the nucleotide sequences of 22 (62.86%) *Hox* genes (such as *HoxA2b*, *HoxA11b*, *HoxB6b*, *HoxB9a*, *HoxC8a*, and *HoxD4a*) showed lower identities between the NCRC lineage and COC or BSB, but they showed higher amino acid sequence identities, which suggested that most mutations were synonymous. Both the nucleotide and amino acid sequences of one (2.85%) *Hox* gene (*HoxD9a*) in the NCRC lineage presented a low degree of identity with those in COC and BSB (Supplementary Table 5).

4. Discussion

The surprising variation in *Hox* cluster numbers and genome structure among vertebrate lineages, especially in ray-finned fishes, reflects the history of duplications and subsequent lineage-specific gene losses [9]. In mammals, each *Hox* cluster contains the same genes among different species, but this situation is not found in the extant species of ray-finned fishes, in which both the number and organization of *Hox* genes and even *Hox* clusters are variable [23–28]. Since some *Hox* genes have undergone lineage-specific secondary losses, each group of teleost fish possesses a unique set of *Hox* genes [7,29,30]. Hybridization plays an important role as a powerful promoter of evolutionary adaptation at the level of genome duplications. Does hybridization promote the rapid evolution of *Hox* gene clusters in vertebrates, including fish? This biological mechanism is not yet well understood.

In the NCRC lineage, we reconstructed seven *Hox* clusters consisting of 48 *Hox* genes, ten of which were pseudogenes. By comparing and analyzing the cluster structures of these 48 *Hox* genes of the NCRC lineage and its parents, it was revealed that each generation of NCRC possessed a different set of *Hox* gene clusters. In the early stages of distant hybridization to form new species, to maintain the balance between the internal stability of the species and the continuous concussion impacts from different parental genomes, the number of putative *Hox* clusters generated in NCRC-F_1 was increased greatly by distant hybridization, and the average number of these clusters reached almost twice ($P > 0.05$) that in the maternal parent, COC. This increasing trend continued in the subsequent self-mating generations of NCRC-F_1. However, under continuous self-crossing passages, the self-protection mechanism of the species gradually became a dominant force, and the explosive growth trend of putative *Hox* clusters in the NCRC lineage gradually weakened among subsequent generations. This study also revealed recombinant

Hox gene clusters in the NCRC lineage, which might be an inevitable effect of the hybridization process in cyprinid fishes or even vertebrates [18,19,44–46]. Partial recombinant clusters of *Hox* genes were stably inherited from F_1/F_2 to the subsequent self-mating generations in the NCRC lineage. Each generation of NCRC clearly possessed a different set of *Hox* gene clusters, making NCRC a highly suitable system for understanding the mechanisms underlying such unequal conservation of duplicated copies. According to the *Hox* gene clusters observed in the NCRC lineage, hybridization could promote the rapid evolution of *Hox* gene clusters in fish. Such highly variable *Hox* gene clusters provide a good starting point for the study of genetic evolution at the genomic level after hybridization events.

A growing body of research shows that the gene content of *Hox* clusters in teleost fishes is more variable than expected, with each species studied thus far having a different cluster set [3,7,13,18,19,26,31,45,46]. However, there are few studies on whether the gene content of *Hox* clusters in the early generations of hybrid lineages shows more significant variation due to the continuous genomic oscillation caused by the distant hybridization process. In this study, we revealed the genetic variation of 48 *Hox* genes in different generations of the NCRC lineage. Although the highest loss rate of *Hox* gene clusters occurred in the early generations of the hybrid lineage, our analyses showed that the loss of gene clusters continued in subsequent generations of the NCRC lineage. In addition to pseudogenes, 35 (92.11%) *Hox* genes in NCRC-F_1 inherited all or part of the parental cluster structures. All of the *Hox* genes of NCRC-F_1 had newly derived mutant cluster structures, which showed greater variability than in the parents. In NCRC-F_2, mutation was the main theme of the generation; 29 *Hox* genes of this generation showed new mutation types relative to the *Hox* genes of NCRC-F_1, and only nine (23.68%) *Hox* genes in NCRC-F_2 inherited all or part of the original parental cluster structures. Following continuous self-crossing within NCRC, extreme variability was not observed in NCRC-F_5; 19 *Hox* genes in this generation showed cluster structure types consistent with those in NCRC-F_2, but only three (7.89%) *Hox* genes in NCRC-F_5 inherited all or part of the original maternal parent cluster structures. In summary, as the number of generations of the NCRC lineage increased, the number of *Hox* gene cluster fragments inherited from the original parents (COC and BSB) gradually decreased, and this rule also applied to the inheritance of recombinant *Hox* gene clusters.

All of the fish studied to date have shown differences in gene content among their *Hox* clusters [3,7,13,19,26,39,46]. Our results revealed that in the early generations of the hybrid fish lineage, the degree of variation in the gene content of *Hox* clusters was extraordinary, corresponding to an evolutionary path of rapid gene cluster loss. The new NCRC lineage in the family Cyprinidae can provide insight into this dynamic *Hox* evolution process because the duplicated genes are in an early period of gene degeneration. Is it possible that a regular route of genetic variation is being followed in this dynamic *Hox* evolutionary process? In terms of the base composition, it appeared that some genetic rules were followed in the inheritance of these *Hox* gene clusters between different generations of the NCRC lineage. The *Hox* gene cluster types with higher GC levels in the maternal parent, COC, were more likely to be inherited in NCRC-F_1 and even in the subsequent self-crossing offspring of F_1. In these *Hox* gene clusters derived from the maternal parent, the GC level in the subsequent self-crossing offspring decreased with the influence of the unstable state of the NCRC-F_1 genome, or the original GC level was lower than that in the new variant clusters of the corresponding NCRC-F_1 genes. In this genetic background, the *Hox* gene clusters from the maternal parent underwent rapid loss in the subsequent self-crossing offspring of NCRC-F_1. The *Hox* gene cluster variant types with higher GC levels in NCRC-F_1-F_2 and those with increasing GC contents in subsequent self-crossing offspring were more likely to be stably inherited in the NCRC lineage. Since pseudogenes are obviously subject to no functional constraints, all variations within them are selectively neutral and show the same probability of becoming fixed in the population [31]. The abovementioned genetic rules governing *Hox* gene clusters were not applicable to pseudogenes. In the study of GC level changes in the CDS regions of *Hox* gene clusters, Santini and Bernardi elaborated the viewpoint that a reduction in the GC levels of functional *Hox* genes relative to paralogous genes can be an indicator of the potential for non-functionalized genes [31]. This study revealed that a reduction of the GC levels of functional *Hox* gene clusters may help identify gene clusters that cannot be inherited by hybrid offspring.

Our previous study revealed that the phenotypes and genotypes of the NCRC lineage differed from those of its parents but were closely related to those of existing wild crucian carp [34,37]. In this study, a phylogenetic tree analysis revealed that the newly derived *Hox* gene mutation clusters in the NCRC lineage showed phylogenetic relationships that were closest to either crucian carp or silver crucian carp. The NCRC lineage of the family Cyprinidae provides valuable clues for understanding the dynamic process of *Hox* evolution. With the rapid loss of the *Hox* clusters of the original parents (COC and BSB), the newly derived mutant *Hox* gene clusters provide clear clues regarding the evolutionary path of the NCRC lineage. According to the *Hox* gene clusters observed in the NCRC lineage, hybridization could promote the rapid evolution of *Hox* gene clusters in ray-finned fishes. Our data clearly demonstrate that the loss of *Hox* gene clusters in ray-finned fish is an ongoing process, indicating that the loss of *Hox* clusters in the early stages of hybrid fish lineage formation is a rapid process. This study deepens our understanding of the evolution of *Hox* genes in the ray-finned fish clade.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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